Hiroshima Journal of Medical Sciences
Vol.36, No.2, 227~231, June, 1987
HIJM 36-30

Partial Purification and Characterization of Cholesterol 7α-Hydroxylase from Guinea Pig

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(Received march 24, 1987)

Key words: Cholesterol 7α-hydroxylase, Guinea pig, Cholesterol

ABSTRACT

Cholesterol 7α-hydroxylase (cholesterol, NADPH: oxygen oxidoreductase, 7α-hydroxylating, EC 1.14.13.17), was partially purified from liver microsomes of guinea pig. The purified sample showed a specific activity of 1.76 nmol/min/mg of protein and a turnover number of 2.3 nmol/min/nmol of cytochrome P-450, which were 100 times as high as respective values of microsomes. Cholesterol 7α-hydroxylase activity was reconstituted from the partially purified cytochrome P-450, NADPH-cytochrome P-450 reductase, dilauroylglycery1-3-phosphorylcholine and the NADPH generating system. The reconstituted system showed an absolute requirement for cytochrome P-450, NADPH-cytochrome P-450 reductase and NADPH. The apparent Km value for cholesterol in the reconstituted system was 33 μM and Vmax was 3.4 nmol/min/mg of protein. Cholesterol 7α-hydroxylase activity was significantly inactivated by iodoacetamide and *p*-chloromercuribenzoate, but not either by aminoglutethimide or by metyrapone.

Hydroxylation at 7α position of cholesterol is the initial and rate-limiting step in the conversion of cholesterol into bile acid and the enzyme which catalyzes this reaction, cholesterol 7α-hydroxylase, seemed to be regulated by a variety of factors[6]. Namely, the enzyme is controlled by bile acids returning to the liver via the enterohepatic circulation and by hormones such as thyroid hormone, adrenal cortex hormone and insulin. Recently, phosphorylation-dephosphorylation as mediated by cyclic AMP was suggested to be functioning in the regulation of the enzyme activity. The enzyme is also known to reveal a marked diurnal rhythm in rat.

A CO-inhibition study proved that the enzyme was a member of cytochrome P-450[11]. Despite of its importance in the catabolism of cholesterol, the enzyme has not been purified to homogeneous state. Although some preparations have been reported to date[1,2,4], they revealed lower turnover numbers than those of microsomes and catalyzed hydroxylation not only at 7α position of cholesterol but also at 12α and 25 positions of 5β-cholestane-3α, 7α-diol. However, it is difficult to understand how a strict control of bile acid metabolism could be accomplished with such a versatile enzyme.

Guinea pig is unique in its bile acid composition since it contained little if any trihydroxycholanoic acid (cholic acid or hyocholic acid)[3], and liver microsomes of the animal do not reveal 12α-hydroxylase activity. It is therefore surmised that the bile acid regulation mechanism in this animal might be simpler compared with rat and other mammals. A number of works on ontogeny of cholesterol 7α-hydroxylase and bile acid regulation mechanism have been carried out with this animal species[10]. Yet, no report has

Abbreviations: DTT, dithiothreitol; PEG, polyethylene glycol; cytochrome P-450, P-450.

so far been published about the purification and characterization of cholesterol 7α-hydroxylase of this animal species.

In the present experiment cholesterol 7α-hydroxylase was partially purified from liver microsomes of guinea pig and its properties were studied.

MATERIALS AND METHODS

Materials-Cholesterol oxidase was supplied from Toyo Jozo Co. Ltd. (Shizuoka, Japan), and Emulgen 911 from Kao Chemicals Co. Ltd. (Tokyo, Japan). Metyrapone and aminoglutethimide were the gift of Ciba Geigy Co. (Basel, Switzerland).

Purification of NADPH-cytochrome P-450 reductase-NADPH-cytochrome P-450 reductase was purified from microsomes of phenobarbital treated Hartley guinea pigs as described by Yaskochi and Masters[13].

Enzyme Assay-Microsomal cholesterol 7α-hydroxylase activity was measured according to the method described by Ogishima and Okuda[7]. The method was, however, slightly modified with the solubilized enzyme as follows. One mM of cholesterol emulsified with 1% Emulgen 911 (10 μl), dilauloylglyceryl-3-phosphorylcholine (10 μg) and NADPH-cytochrome P-450 reductase (0.5 U) were added to the incubation mixture. The reaction was carried out for 20 min. The analysis was performed by using a TSK gel SILICA-60 (TOYO SODA Co. Ltd., Tokyo, Japan). The column was eluted with a mixture of *n*-hexane and isopropanol (87:13) at a flow rate of 1.0 ml/min. The HPLC system used consisted of a pump (HLC-803D) equipped with a UV spectrophotometer (UV-8 model-II, TOYO SODA Co. Ltd.) and an integrator (CHROMATOPAC C-R1B, Shimadzu Co., Kyoto, Japan).

Preparation of Microsomes-Eight male Hartley guinea pigs were killed by cervical dislocation. The livers were quickly removed and perfused with ice-cold 0.9% (w/v) NaCl, then homogenized with 9 vol. of 0.25M sucrose containing 20 mM Tris-HCl (pH 7.4), 1 mM EDTA and 1 mM DTT. The homogenate was centrifuged at 10,000 × *g* for 15 min. The supernatant was further centrifuged at 100,000 × *g* for 60 min. Microsomal pellets were washed in 20 mM Tris-HCl buffer (pH 7.4) containing 0.15 M KCl and 1 mM EDTA, then suspended in 0.1 M Tris-HCl buffer (pH 7.4) containing 1 mM EDTA, 1 mM DTT, 1μg/ml each of leupeptin and pepstatin.

Purification of Cholesterol 7α-Hydroxylase-All buffer solutions contained 20% glycerol, 0.1 mM EDTA, 0.1 mM DTT, and 1 μg/ml each of leupeptin and pepstatin unless otherwise stated.

Microsomes were solubilized with sodium cholate (3%), and the supernatant was fractionated with 7—17% polyethylene glycol. After dialyzing against 100 mM potassium phosphate buffer (pH 7.4), the fraction was re-solubilized with sodium cholate (0.7%), and applied to an ω-aminohexyl-Sepharose 4B column (3.1 × 14 cm) equilibrated with 100 mM potassium phosphate buffer (pH 7.4) containing 0.5% sodium cholate. After washed with the equilibrating buffer, the column was eluted with 100 mM potassium phosphate buffer (pH 7.4) containing 0.4% sodium cholate and 0.06% Lubrol PX. Fractions showing cholesterol 7α-hydroxylase activity were combined and dialyzed against 25 mM potassium phosphate buffer (pH 7.4) containing 0.2% sodium cholate and 0.2% Lubrol PX. The dialyzed fraction was then applied to a hydroxylapatite column (2.5 × 10 cm) equilibrated with the dialyzing buffer. The column was washed with 50 mM potassium phosphate buffer (pH 7.4) containing 0.2% sodium cholate and 0.2% Lubrol PX and eluted with 250 mM potassium phosphate buffer (pH 7.4) containing the same detergents. Fractions rich in the enzyme activity were dialyzed against 10 mM Tris-acetate buffer (pH 7.4) containing 0.4% Emulgen 911. After this step, the protease inhibitors were omitted. One ml of the dialyzed fraction was injected into a TSK gel DEAE-5PW column (7.5 × 75 mm, TOYO SODA Co. Ltd.) equilibrated with 10 mM Tris-acetate buffer (pH 7.4) containing 0.4% Emulgen 911. The column was eluted with linear gradients of sodium acetate (10—60 min, 0—0.15 M; 60—90 min, 0.15—1.0 M). The flow rate was 0.4 ml/min and the effluent was monitored at 417 nm. This chromatography was performed at room temperature using a pump, TRI ROTOR SR, equipped with a controller, TRI ROTOR SR2 and a UV spectrophotometer, UVIDEC-100-IV (Jasco Co. Ltd., Tokyo, Japan). The fractions showing the enzyme activity were collected and stored as a final preparation.

Table 1. Purification of Cholesterol 7α-Hydroxylase

Step	Total Protein	Total P-450	Specific Content	Total Activity	Specific Activity	Turnover Number
	(mg)	(nmol)	(nmol/mg)	(nmol/min)	(nmol/min/ mg protein)	(nmol/min/ nmol P-450)
Microsomes	2520	1950	0.77	42.8	0.017	0.022
PEG	1320	1536	1.16	15.8	0.012	0.010
ω-Aminohexyl-Sepharose 4B	399	729	1.83	13.2	0.033	0.018
Hydroxylapatite	88.4	218	2.47	11.0	0.124	0.050
DEAE-5PW[a]	0.254	0.194	0.76	0.447	1.76	2.32

[a]A portion (1 ml) of the eluate from the hydroxylapatite column (10.0 ml) was applied.

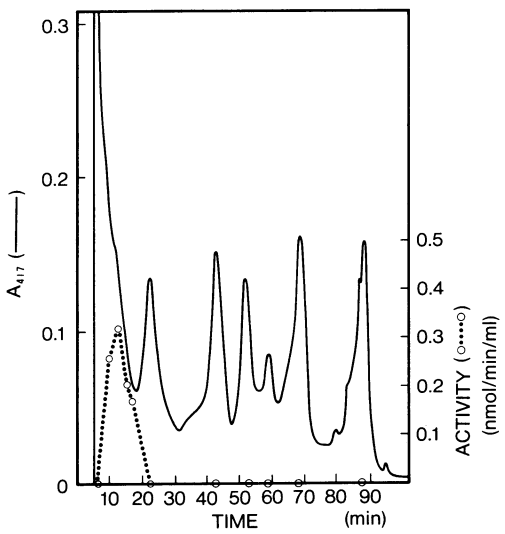

Fig. 1. Purification of cholesterol 7α-hydroxylase by high performance anion-exchange chromatography. A portion (1.0 ml) of the eluate from the hydroxylapatite column (10.0 ml) was applied to a TSK gel DEAE-5PW (7.5 × 75 mm). Equilibration and elution of the column was performed as described under "Materials and Methods".

Other Methods-Proteins were determined by the method of Lowry et al[5] using bovine serum albumin as the standard. Cytochrome P-450 concentration was estimated from a reduced CO difference spectrum using an extinction coefficient of 91 $mM^{-1}cm^{-1}$ [8].

RESULTS

Purification of Cholesterol 7α-hydroxylase-Table 1 shows the overall purification of cholesterol 7α-hydroxylase of guinea pig. Liver microsomes were solubilized by cholate and the resulting solution was fractionated with polyethylene glycol. Then the fraction was subjected to chromatographies on columns of ω-aminohexyl-Sepharose 4B and hydroxylapatite, which was followed by high performance anion exchange column chromatography (Fig. 1). Although the specific cytochrome P-450 content was increased by solubilization and PEG fractionation, there was no net increase of catalytic activity either per mg of protein or per nmol of cytochrome P-450. This seemed to have been due to the inactivation or the inhibition of the enzyme by these treatment. However, after these steps the catalytic activity was substantially increased. Thus, the specific activity of the final sample was 1.76 nmol/min/mg of protein, and the turnover number was 2.3 nmol/min/nmol of cytochrome P-450, which were about 100

Table 2. Reconstitution of Cholesterol 7α-Hydroxylase

		Relative Activity
		(%)
Complete System		100
minus	Cholesterol	0
minus	Cytochrome P-450	0
minus	NADPH-cytochrome P-450 reductase	0
minus	Dilauroylglyceryl-3-phosphorylcholine	83
minus	NADPH generating system	0

Complete system contained 0.1 M potassium phosphate buffer (pH 7.4), 0.1 mM EDTA, 20 mM cysteamine, 5 mM MgCl2, 5 mM sodium isocitrate, 0.075 U of isocitrate dehydrogenase, 0.5 mM NADPH, 40 μM cholesterol, 10 μg of dilauroylglyceryl-3-phosphorylcholine, 0.5 U of NADPH-cytochrome P-450 reductase and the partially purified protein (5 μg) in a final volume of 0.25 ml. The reaction was performed as described under "Materials and Methods".

times as high as those in microsomes, respectively. However, the cytochrome P-450 content of the final preparation was 0.76 nmol/mg of protein. This value was almost the same as that of microsomes. Judging from the spectrum of the eluate from DEAE-5PW a significant fraction of the enzyme seemed to be converted into cytochrome P-420, a denatured form of cytochrome P-450, by high performance liquid chromatography.

Reconstitution of Enzyme Activity-Cholesterol 7α-hydroxylase activity was reconstituted from the partially purified cytochrome P-450, NADPH-cytochrome P-450 reductase, dilauroylglyceryl-3-phosphorylcholine and NADPH generating system (Table 2). The reconstituted system had an absolute requirement for cytochrome P-450, NADPH-cytochrome P-450 reductase and NADPH generating system.

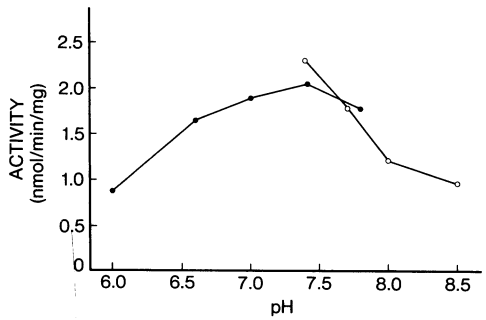

Fig. 2. Effect of pH on cholesterol 7α-hydroxylase. Enzyme activity was assayed as described under "Materials and Methods" except that incubation was performed at varying pH. ●——●, potassium phosphate buffer; ○——○, Tris-HCl buffer.

Properties of Reconstituted Cholesterol 7α-Hydroxylase System-Optimum pH of this enzyme was 7.4 as shown in Fig. 2. The enzyme reaction proceeded in time-linear fashion up to 20 min. The reaction velocity of cholesterol 7α-hydroxylase was linear with respect to the amount of enzyme at least up to the concentration of 36 μg/ml. Michaelis constant calculated from Lineweaver-Burk plot was 33 μM and Vmax was 3.43 nmol/min/mg of protein (Fig. 3).

Inhibition of Enzyme Activity-As shown in Table 3 the enzyme activity was inhibited by iodoacetamide and *p*-chloromercuribenzoate, suggesting that the enzyme requires sulfhydryl group as rat liver cholesterol 7α-hydroxylase.

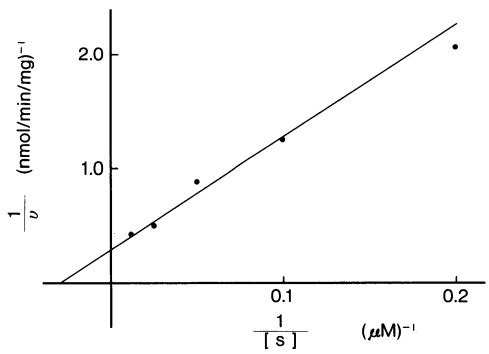

Fig. 3. Effect of substrate concentration on cholesterol 7α-hydroxylase. Enzyme activity was assayed as described under "Materials and Methods" except that incubation was performed at varying concentrations of the substrate.

Table 3. Effect of Inhibitors on Cholesterol 7α-Hydroxylase

Inhibitors	Concentration	Relative Activity
		(%)
Control		100
Aminoglutethimide	40 μM	106
Metyrapone	40 μM	103
SKF-525A	40 μM	84
p-chloromercuribenzoate	80 μM	4
Iodoacetamide	20 mM	14

The reaction was performed as described in Table 2. In cases of iodoacetamide and *p*-chloromercuribenzoate, NADPH was used as an electron donar instead of adding NADPH generating system.

However, the enzyme activity was not inhibited either by aminoglutethimide or by metyrapone, which are well known inhibitors of P-450scc and P-450 11β, respectively.

DISCUSSION

Several reports have been published about the preparation of cholesterol 7α-hydroxylase from rat and rabbit liver microsomes[1,2,4,9]. However, turnover numbers of most preparations were lower than those of microsomes as the starting material. The turnover number of the present preparation was 2.3 nmol/min/nmol of cytochrome P-450 and was much higher than those reported so far. The preparation, therefore, could be useful for the study of catalytic properties of the enzyme.

The enzyme activity could be reconstituted

from the partially purified cytochrome P-450, NADPH-cytochrome P-450 reductase, dilauroylglyceryl-3-phosphorylcholine and NADPH generating system and the omission of either cytochrome P-450 or NADPH-cytochrome P-450 reductase resulted in complete loss of activity, whereas the omission of phospholipid resulted in only 17% loss of activity. These results indicated that cytochrome P-450 and NADPH-cytochrome P-450 reductase were essential for 7α-hydroxylation of cholesterol and other components such as cytochrome b5 were not required. The inhibition of the enzyme activity by iodoacetamide and *p*-chloromercuribenzoate suggested that the requirement of sulfhydryl group in the molecule for activity is a common property of cholesterol 7α-hydroxylase in various species of animals[12]. Furthermore, cholesterol 7α-hydroxylase seemed to be different from the cytochromes P-450 involved in side chain cleavage of cholesterol and 11β-hydroxylation of deoxycorticosterone in adrenal mitochondria, since aminoglutethimide and metyrapone had no inhibitory effect on the enzyme activity.

In conclusion, cholesterol 7α-hydroxylase was partially purified from liver microsomes of guinea pig and its catalytic properties were studied.

ACKNOWLEDGEMENTS

The author is indebted to Professor T. Shimosato, Department of Oral and Maxillofacial Surgery II and Professor K. Okuda, Department of Biochemistry, Hiroshima University School of Dentistry for their instructions during this experiment. The author thanks Dr. M. Noshiro, Department of Biochemistry, Hiroshima University School of Dentistry and Dr. T. Ogishima, Department of Biochemistry, School of Medicine, Keio University for their invaluable advices.

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