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EVIDENCE FOR A SPECIFIC CYTOCHROME P-450 WITH SHORT HALF-LIFE CATALYZING 7α-HYDROXYLATION OF CHOLESTEROL

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SUMMARY

Newly synthesized cytochrome P-450 was labeled by administration of radioactive δ-aminolevulinic acid to the rats prior to killing. Cytochrome P-450 fractions were isolated by solubilization of microsomes with sodium cholate followed by chromatography on octylamine-Sepharose and hydroxylapatite. The cholesterol 7α-hydroxylase activity was separated from the 5β-cholestane-3α,7α-diol 12α, 25- and 26-hydroxylase activities. Cholesterol 7α-hydroxylase activity was found in a minor cytochrome P-450 fraction with low specific radioactivity. On the other hand, the 12α, 25- and 26-hydroxylase activities were found in a major cytochrome P-450 fraction with higher specific radioactivity.

The results show that cholesterol 7α-hydroxylation is catalyzed by a cytochrome P-450 with short half-life and provide further evidence for the presence of a specific cytochrome P-450 species catalyzing the reaction.

Cholesterol 7α-hydroxylation is the first and rate-limiting step in the formation of bile acids in the liver (1). The reaction is catalyzed by reconstituted systems from rat and rabbit liver microsomes containing purified cytochrome P-450 and NADPH-cytochrome P-450 reductase (2,3). In previous work, attempts have been made to separate cholesterol 7α-hydroxylase activity from other hydroxylase activities in various cytochrome P-450 fractions. Recently, it has been possible to achieve at least a partial separation of cholesterol 7α-hydroxylase activity from other hydroxylase activities in cytochrome P-450 fractions from both rat and rabbit liver microsomes (4,5). These findings and previous findings concerning properties of the cholesterol 7α-hydroxylation reaction in microsomal preparations indicate the existence of a specific cholesterol 7α-hydroxylating species of cytochrome P-450 (1,6). Another approach to providing evidence for the concept of a specific cytochrome P-450 species catalyzing 7α-hydroxylation of cholesterol would be to make use of an early finding that the half-life time of cholesterol 7α-hydroxylase activity is much shorter than that of

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another hydroxylase activity involved in bile acid biosynthesis, the 12α-hydroxylase (7). By administering labeled δ-aminolevulinic acid, isolating cytochrome P-450 fractions and comparing their specific radioactivities and catalytic properties it might be possible to obtain results bearing on the question of the presence of a specific cholesterol 7α-hydroxylating cytochrome P-450.

In the present report, this approach has been used and further evidence has been obtained for a specific species of cytochrome P-450 catalyzing 7α-hydroxylation of cholesterol.

EXPERIMENTAL PROCEDURE

Materials. [4-^{14}C]-Cholesterol was obtained from the Radiochemical Centre, Amersham, England. 5β[7β-^{3}H]-Cholestane-3α,7α-diol (500 Ci/mol) was prepared as described previously (3). [3,5-^{3}H] δ-Aminolevulinic acid (480 Ci/mol) was obtained from NEN, Dreiech, F.R.G.

Methods. Male rats of the Sprague- Dawley strain, weighing about 200 g, were treated with cholestyramine, 3% (w/w) in the diet, for one week (3). Labeled δ-aminolevulinic acid, 0.24 mg/kg (8), was injected intraperitoneally and the animals were killed 3 to 4 hours after the injection.

Cytochrome P-450 was prepared from liver microsomes as described previouly (4,9) with the following modifications. After elution of cytochrome P-450 from the octylamine-Scpharose column with 0.06% (w/v) Emulgen 913, additional cytochrome P-450 was eluted with 0.2% Emulgen in the buffer (10). Cytochrome P-450-containing fractions were pooled as shown in Fig. 1 and subjected to hydroxylapatite chromatography. The hydroxylapatite columns were first washed with 35 mM phosphate buffer. No cholesterol 7α-hydroxylase activity was eluted with this concentration of phosphate. Cytochrome P-450 was then eluted from the columns with 300 mM phosphate in the buffer. The fractions were treated with Amberlite XAD-2 (4) and dialyzed against 50 mM Tris-acetate buffer, pH 7.4, containing 20% (v/v) glycerol and 0.1 mM EDTA. Cytochrome P-450 was measured as described by Omura and Sato (11). The amount of radioactivity in the fractions was measured with a liquid scintillation spectrometer (Packard, Model 3950) using Lumagel (Lumac systems, Switzerland) as scintillation liquid.

NADPH-cytochrome P-450 reductase was prepared from liver microsomes of phenobarbital-treated rats as described by Yasukochi and Masters (12).

Incubations with cholesterol and 5β-cholestane-3α,7α-diol were performed and analyzed as described previously (13).

RESULTS

Chromatography of solubilized microsomal protein on octylamine-Sepharose resulted in two peaks of cytochrome P-450 both containing radioactivity (Fig. 1). The first and the major peak was eluted with 0.06% Emulgen. The second peak was eluted with 0.2% Emulgen. As shown in Fig. 1, the ratio between radioactivity and absorbance at 416 nm was above 1 in the first peak but lower than 1 in the second peak. The fractions corresponding to the first peak and the second peak were pooled to give pools I and III, respectively. The fractions eluted between the two peaks were pooled to

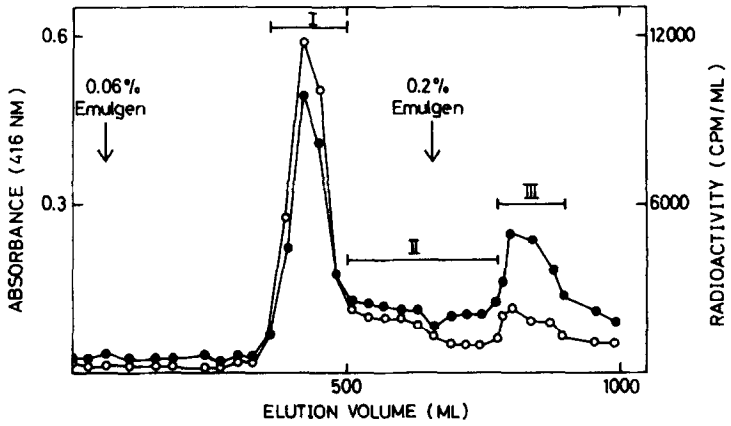

Fig. 1. Octylamine-Sepharose chromatography in the purification of cytochrome P-450 from rat liver microsomes. The chromatography and the analysis of the fractions were performed as described in the Experimental Procedure. ●——●, Absorbance at 416 nm; o——o, Radioactivity.

give pool II. The three pools of cytochrome P-450 were further purified by hydroxylapatite chromatography. The 300 mM phosphate eluates from hydroxylapatite chromatography were analyzed for cytochrome P450, radioactivity and hydroxylase activities as summarized in Table 1. The hydroxylations studied were the 7α-hydroxylation of cholesterol and the 12α, 25 and 26-hydroxylations of 5β-cholestane-3α,7α-diol. The specific cytochrome P-450 content in pools I, II and III was 5.7, 2.6 and 3.0 nmol/mg of protein, respectively. The specific radioactivity - cpm/nmol of cytochrome P-450 - was highest in pool I and lowest in pool III. Cholesterol 7α-hydroxylase activity was lowest in pool I. Pool III had the highest 7α-hydroxylase activity. On the other hand, the 12α, 25- and 26-hydroxylase activities were highest in pool I and lowest in pool III. Pool II showed intermediate values with respect to specific radioactivity and hydroxylase activities.

DISCUSSION

The cholesterol 7α-hydroxylase system has several properties which are different from those of other cytochrome P-450 dependent hydroxylase systems in liver microsomes (1,6). The 7α-hydroxylase activity is not stimulated by treatment with the common monooxygenase inducers but is stimulated severalfold by biliary drainage or by feeding cholestyramine - a bile acid binding anion exhanger. It exhibits a circadian rhythm and it is influenced by certain hormones (1,14). Another property, also indicating the involve-

Table 1.

Hydroxylase activities in cytochrome P-450 fractions from rat liver microsomes

Cholesterol, 25 nmol, and 5β-cholestane-3α,7α-diol, 100 nmol, were incubated for 20 min at 37°C with 0.5 nmol cytochrome P-450, 1.5 unit NADPH-cytochrome P-450 reductase, 25 μg dilauroylglycero-3-phosphorylcholine and 1.0 μmol NADPH in a total volume of 1 ml buffer. Incubations with cholesterol contained in addition 0.6 mg Triton X-100 and 6 μmol dithiothreitol (5,13). The buffer used was 50 mM Tris-acetate, pH 7.4, containing 20% glycerol and 0.1 mM EDTA for incubations with cholesterol and 150 mM potassium phosphate, pH 7.4, containing 20% glycerol and 0.1 mM EDTA for incubations with 5β-cholestane-3α,7α-diol. Radioactivity was measured as described under Experimental Procedure.

Fraction	Cytochrome P-450		Hydroxylation of			
	Specific content	Specific radioactivity	Cholesterol 7α	5β-Cholestane-3α,7α-diol 12α	25	26
	nmol/mg protein	cpm/nmol	pmol/nmol cytochrome P-450/min			
Pool I	5.7	4485	20	80	60	60
Pool II	2.6	3280	100	40	4	3
Pool III	3.0	2800	150		10[a]	

[a]Sum of hydroxylated products

ment of a specific cytochrome P-450 in the 7α-hydroxylation of cholesterol, is the short half-life of the hydroxylase activity. The half-life has been calculated to be 2 to 3 hours in rat liver microsomes whereas that of drug-induced cytochrome P-450 is much longer - about 40 hours (7,8,15).

In agreement with a previous report (16) the present results show a separation of the cholesterol 7α-hydroxylase activity from the 12α, 25- and 26-hydroxylase activities by octylamine-Sepharose chromatography. The 12α, 25- and 26-hydroxylase activities were found in a major cytochrome P-450 fraction whereas the cholesterol 7α-hydroxylase activity was found in a minor cytochrome P-450 fraction. In some experiments, separations of the hydroxylase activities were obtained first after rechromatography of the pools on octylamine-Sepharose.

In the present investigation, the heme groups of newly synthesized cytochrome P-450 were labeled by administration of labeled δ-aminolevu-

linic acid to the rats (8). This approach should make it possible to analyze isolated cytochrome P-450 fractions with respect to their turnover. Thus, a cytochrome P-450 fraction with a short half-life time should have a lower specific radioactivity, as calculated from the amount of radioactivity per nmol of cytochrome P-450, than a fraction with a longer half-life time. Assuming that the short half-life time of cholesterol 7α-hydroxylase activity observed in experiments with microsomal preparation depends on the reaction being catalyzed by a short-lived cytochrome P-450 species, the cholesterol 7α-hydroxylase activity should be found in the present experiments in a cytochrome P-450 fraction with low specific radioactivity. Indeed, the present results show that cholesterol 7α-hydroxylation was most efficient with the cytochrome P-450 fraction of lowest specific radioactivity whereas the other hydroxylase activities were most efficient in the fraction of highest specific radioactivity. In fact, the cholesterol 7α-hydroxylase activity gradually increased and the specific radioactivity gradually decreased when measured in pools I, II and III, respectively. The other hydroxylase activities behaved in the opposite way.

In order to recover reasonable amounts of radioactivity the purification of the cytochrome P-450 was not carried out as far as described previously (4,9) and consequently the specific cytochrome P-450 content of the fractions was not so high. However, the cytochrome P-450 content of the fractions is irrelevant since the specific radioactivity - cpm per nmol cytochrome P-450 - was measured and compared to specific hydroxylase activity. The results of the present study strongly indicate the presence of a specific cytochrome P-450 with short half-life in rat liver microsomes catalyzing 7α-hydroxylation of cholesterol.

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