12α-HYDROXYLATION OF 7α-HYDROXY-4-CHOLESTEN-3-ONE BY A RECONSTITUTED SYSTEM FROM RAT LIVER MICROSOMES

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SUMMARY

A preparation of partially purified cytochrome P-450 from rat liver microsomes was found to catalyze 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one in the presence of NADPH and phosphatidyl choline. The reaction was stimulated two- to four-fold by addition of a preparation of cytochrome P-450 reductase. The reaction was inhibited by carbon monoxide to a considerably less extent than other hydroxylations catalyzed by the reconstituted system. In the presence of optimal concentrations of cytochrome P-450 reductase, cytochrome P-450 prepared from livers of starved rats catalyzed the 12α-hydroxylation more efficiently than cytochrome P-450 prepared from livers of normal rats or rats treated with phenobarbital.

12α-Hydroxylation is an important step in the biosynthesis of bile acids from cholesterol in the liver (1). Together with the 26-hydroxylase, the microsomal 12α-hydroxylase may regulate the relative amounts of cholic acid and chenodeoxycholic acid formed from cholesterol (1,2). The 12α-hydroxylase seems to differ from most other microsomal mixed function oxidases in several respects. It has been reported that the 12α-hydroxylase is insensitive (3) or rather insensitive (4) to carbon monoxide and it has been suggested that the 12α-hydroxylase is independent of cytochrome P-450 (3). Phenobarbital treatment leads to an increase of the bulk of cytochrome P-450 in the liver and an increased activity of most mixed function oxidases in the liver but the activity of the 12α-hydroxylase is decreased after treatment with phenobarbital (3,4). Starvation also

leads to an increase of the bulk of cytochrome P-450 in the liver (cf. ref. 5) and in this case, the activity of the 12α-hydroxylase is increased up to fourfold (5). It was shown recently that the 12α-hydroxylase was considerably more sensitive to treatment with inorganic salts than most other mixed function oxidases (6). It was concluded that if cytochrome P-450 is involved in the 12α-hydroxylation, the cytochrome is probably different from the bulk of cytochrome P-450 in the liver.

To obtain further information concerning the mechanism of 12α-hydroxylation the reaction has now been studied in a reconstituted system (7,8) consisting of partially purified cytochrome P-450 and cytochrome P-450 reductase from rat liver microsomes.

EXPERIMENTAL PROCEDURE

Materials. 6β-^{3}H-7α-Hydroxy-4-cholesten-3-one was prepared as described previously (9) and had a specific radioactivity of 80 μCi/mg. Tritium-labeled lithocholic acid was prepared by exposure of unlabeled lithocholic acid to tritium gas according to the Wilzbach procedure (10). The material was treated with alkali and purified by thin-layer chromatography prior to use giving material with a specific radioactivity of 30 μCi/mg. The mixture of di- and monolauroylglyceryl-3-phosphorylcholine was a generous gift from Dr. W.E.M. Lands and was the same preparation as the one used in a previous report (8). Pyridine nucleotides, D,L-isocitric acid and isocitric acid dehydrogenase (Type IV) were obtained from Sigma Chemical Co., St. Louis, Mo.

Methods. Male rats of the Sprague-Dawley strain weighing about 200 g were used. In some experiments rats were treated by in-

traperitoneal injection of phenobarbital (100 mg/kg body weight) once daily for 3 days or were starved for 72 hours prior to sacrifice. Liver microsomes as well as cytochrome P-450 and cytochrome P-450 reductase were prepared according to Lu et al. (7) and as described in a previous report (8). The cytochrome P-450 preparations from untreated, starved and phenobarbital-treated rats contained about 0.6, 0.9 and 1.9 nmoles of cytochrome P-450 per mg protein respectively when assayed according to Omura and Sato (11). The cytochrome P-450 reductase preparations from untreated, starved and phenobarbital-treated rats contained about 200, 200 and 500 units per mg protein respectively when assayed according to Masters et al. (12). Protein was determined according to Lowry et al. (13). The fresh preparation of cytochrome P-450 from phenobarbital-treated rats contained about 10% cytochrome P-420 whereas the cytochrome P-450 preparations from untreated and starved rats contained 25-35% cytochrome P-420. In the standard incubation procedure used in the experiments shown in Fig. 1, 5 µg of 6β-^{3}H-7α-hydroxy-4-cholesten-3-one, dissolved in 25 µl of acetone, were added to 1.0 nmole of cytochrome P-450, 400 units of cytochrome P-450 reductase, 50 µg of mono- and dilauroylglyceryl-3-phosphorylcholine and an NADPH-generating system (8) in a total volume of 3 ml of modified Bucher medium (14). In the experiments given in Table 1, 8 µg of 6β-^{3}H-7α-hydroxy-4-cholesten-3-one, 1.5 nmoles of cytochrome P-450 and 500 units of cytochrome P-450 reductase were used. Incubations were performed at 37°C for 20 min and were terminated by addition of 20 volumes of chloroform-methanol (2:1, v/v). Extraction, subsequent thin-layer chromatography and determination of radioactivity in different chromatographic zones on the chromato-

plates were performed as described previously (4,6). In some experiments the labeled product was extracted with methanol from the chromatoplates, converted into the trimethylsilyl ether (15) and analyzed by radio-gas chromatography using a Barber Colman 5000 instrument equipped with a 3% QF-1 column. In some experiments, the radioactive product was diluted with authentic, unlabeled 7α,12α-dihydroxy-4-cholesten-3-one and crystallized to constant specific radioactivity. Tritium-labeled lithocholic acid was incubated with reconstituted system under the conditions described previously for incubations with taurodeoxycholic acid (8) and the conversion into the 6β-hydroxylated product was assayed also as described previously (16).

RESULTS

Incubation of 6β-^{3}H-7α-hydroxy-4-cholesten-3-one with cytochrome P-450 from starved rats, an NADPH-generating system and a mixture of mono- and di-lauroylglyceryl-3-phosphatidyl choline resulted in a conversion of 3-7% into a product with the thin-layer chromatographic properties of 7α, 12α-dihydroxy-4-cholesten-3-one. The reaction was stimulated two- to fourfold by addition of cytochrome P-450 reductase. The product was converted into the trimethylsilyl ether and was analyzed by radio-gas chromatography. About 90% of the radioactivity had the chromatographic properties of trimethylsilyl ether of 7α,12α-dihydroxy-4-cholesten-3-one and about 5-10% had the chromatographic properties of 7α,26-dihydroxy-4-cholesten-3-one (cf. ref. 17). The identity of most of the product with 7α,12α-dihydroxy-4-cholesten-3-one was confirmed by crystallization to constant specific radioactivity together with authentic 7α,12α-dihydroxy-4-cholesten-3-one. After three

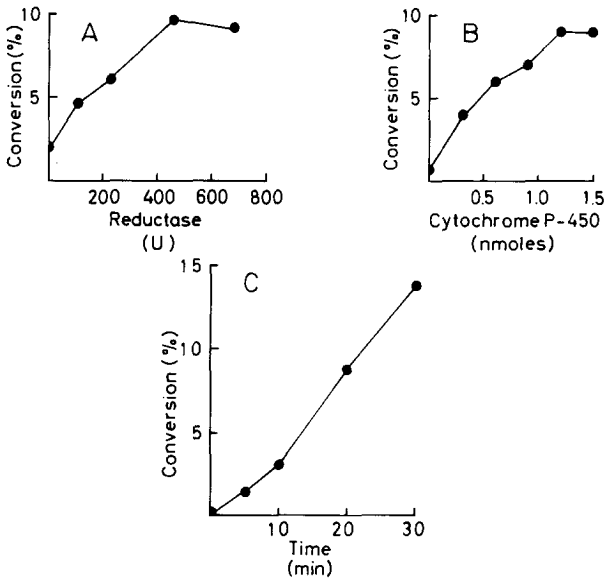

Fig. 1. Effect of cytochrome P-450 reductase concentration (A), cytochrome P-450 concentration (B), and time (C) on 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one by the reconstituted system with cytochrome P-450 from starved rats (cf. Methods). With the exception of the variable factor in each set of experiments the standard incubation conditions were used (cf. Methods).

crystallizations the specific radioactivity of the crystals was about 90% of that of the starting material. Under the conditions employed the rate of 12α-hydroxylation was linear with the amount of cytochrome P-450 up to about 1.2 nmoles and with time up to about 30 min (Fig. 1). There was no saturation of the enzyme with substrate under standard incubation conditions in which 5 μg of substrate were added. Under conditions of saturation with substrate (above 30 μg) the conversion in % of added labeled substrate was very low making reasonably accurate assays difficult. The amount of cytochrome P-450 reductase necessary for optimal conversion was 400-600 units (Fig. 1). 12α-Hydroxylation by the complete system was inhibited by about 30% when the reaction was carried out in an atmosphere containing 4% oxygen, 56% nitrogen and 40% carbon monoxide.

Under the same conditions, 7α-hydroxylation of taurodeoxycholic acid and 6β-hydroxylation of testosterone were inhibited by about 90% (cf. ref. 8). Table I summarizes the results of experiments in which combinations of cytochrome P-450 and cytochrome P-450 reductase from untreated, starved and phenobarbital-treated rats were used. The rate of 12α-hydroxylation was fastest with cytochrome P-450 from starved rats, regardless of whether cytochrome P-450 reductase from untreated, starved or phenobarbital-treated rats was used. For reasons of comparison the 6β-hydroxylation of tritium-labeled lithocholic acid, which is known to be stimulated by phenobarbital, was assayed in some of the different, reconstituted systems (Table 1). The 6β-hydroxylation was most efficient with cytochrome P-450 from phenobarbital-treated rats. These results indicate that the stimulatory effects of starvation on 12α-hydroxylation and of phenobarbital treatment on 6β-hydroxylation reside in the cytochrome P-450 fractions.

DISCUSSION

The present work shows that a partially purified cytochrome P-450 preparation from rat liver catalyzes 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one in the presence of NADPH and phosphatidyl choline. The reaction was stimulated up to fourfold by addition of a cytochrome P-450 reductase preparation. These results indicate that the cytochrome P-450 fraction contains the terminal oxidase of the 12α-hydroxylase system and that the electrons from NADPH are transferred by cytochrome P-450 reductase. The involvment of a cytochrome reductase in the 12α-hydroxylation has been previously suggested by Suzuki, Mitropoulos and Myant (3) on the basis of the finding that the 12α-hydroxylation is in-

Table I

12α-Hydroxylation of 7α-hydroxy-4-cholesten-3-one and 6β-hydroxylation of lithocholic acid by different reconstituted systems from rat liver microsomes. Irrespective of source, 1.5 nmoles of cytochrome P-450 and 500 U of cytochrome P-450 reductase were used.

System	12α-Hydroxylation of 7α-hydroxy-4-cholesten-3-one	6β-Hydroxylation of lithocholic acid
	%	
Cytochrome P-450 from untreated rats	2.8	1.5
Cytochrome P-450 from untreated rats + cytochrome P-450 reductase from untreated rats	4.6	
Cytochrome P-450 from untreated rats + cytochrome P-450 reductase from starved rats	5.1	
Cytochrome P-450 from untreated rats + cytochrome P-450 reductase from phenobarbital-treated rats	4.9	3.1
Cytochrome P-450 from starved rats	6.8	1.2
Cytochrome P-450 from starved rats + cytochrome P-450 reductase from untreated rats	11.8	
Cytochrome P-450 from starved rats + cytochrome P-450 reductase from starved rats	11.3	
Cytochrome P-450 from starved rats + cytochrome P-450 reductase from phenobarbital-treated rats	15.2	2.8
Cytochrome P-450 from phenobarbital-treated rats	3.2	1.6
Cytochrome P-450 from phenobarbital-treated rats + cytochrome P-450 reductase from untreated rats	3.9	
Cytochrome P-450 from phenobarbital-treated rats + cytochrome P-450 reductase from starved rats	4.6	
Cytochrome P-450 from phenobarbital-treated rats + cytochrome P-450 reductase from phenobarbital-treated rats	5.9	7.2

hibited partially by addition of cytochrome c. The present results do not permit definite conclusions concerning the nature of the terminal oxidase in 12α-hydroxylation. It is conceivable that the partially purified cytochrome P-450 preparation might contain small amounts of as yet unknown oxidases, different from cytochrome P-450. If cytochrome P-450 is involved in the 12α-hydroxylation present and previous results indicate strongly that it must be a species of cytochrome P-450 different from the bulk of cytochrome P-450 in the liver (3-6). In consonance with previous results using "intact" microsomes (3,4) the 12α-hydroxylation by the reconstituted system was rather insensitive towards carbon monoxide (cf. ref. 8). The experiments with different types of reconstituted systems indicate that the cytochrome P-450 fraction containing the terminal oxidase is responsible for the specific properties of the 12α-hydroxylase system.

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