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RAT LIVER MITOCHONDRIAL CYTOCHROME P-450—A C_{27}-STEROID 26-HYDROXYLASE

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SUMMARY

A cytochrome P-450 has recently been identified and solubilized from rat liver mitochondria [3]. In the presence of ferredoxin, NADPH-ferredoxin reductase and NADPH this heme protein will catalyze the transformation of cholesterol into more polar steroids. By gas chromatography, radio-gas chromatography and combined gas chromatography–mass spectrometry the main product of the reaction was identified as 26-hydroxycholesterol. Less than 10% of the product was identified as 25-hydroxycholesterol. The involvement of this hydroxylase in the formation of bile acids is briefly discussed.

INTRODUCTION

Recently a cytochrome P-450 has been identified and solubilized from rat liver mitochondria [1–3]. In a reconstituted system composed of the heme preparation, liver ferredoxin, NADPH-ferredoxin reductase and NADPH it was found that cholesterol was transformed into a more polar product [1, 3]. The product was not identified but, based on the chromatographic behaviour on thin layer chromatography and on previous studies with intact rat liver mitochondria [4], it was assumed to be 26-hydroxycholesterol. This assumption has now been confirmed and the present communication describes the identification of the product as being mainly 26-hydroxycholesterol, with a small amount of 25-hydroxycholesterol.

METHODS

Materials

Cytochrome P-450 was solubilized from rat liver mitochondria as previously reported [1]. The specific content was 0.14 nmol per mg protein. The endogenous content of cholesterol in the preparation was determined by gas chromatography after chloroform/methanol extraction and found to be 13 mol per mol cytochrome P-450. Adrenal ferredoxin and ferredoxin reductase were purified as described [1]. 25-hydroxycholesterol and 26-hydroxycholesterol, synthesized as described [4] were gifts from Dr I. Björkhem, Huddinge University Hospital, Huddinge, Sweden. [1α, 2α (*n*) – ^{3}H] cholesterol (43 Ci/mmol) from The Radiochemical Center, Amersham, England, was purified by thin layer chromatography [5] prior to use.

26-hydroxy [1α, 2α (*n*)–^{3}H] cholesterol was the generous gift of Dr J. Gustafsson, Institute for Pharmaceutical Biochemistry, University of Uppsala, Sweden. The compound had been biosynthetically produced by incubating mitochondria in the presence of labelled cholesterol and NADPH as described [6], and had previously been identified [6]. Other chemicals and biochemicals were standard commercial high purity materials.

Incubation procedure and analysis of incubation extracts

Incubations containing the soluble cytochrome P-450 preparation, adrenodoxin, adrenal NADPH-ferredoxin reductase, a NADPH generating system and labelled cholesterol were preformed as described [1, 3]. To be able to trace the radioactive products of the reaction, a high specific activity of the added cholesterol was found essential, presumably because of incomplete equilibration between added and endogenous cholesterol. For this reason no cold cholesterol was added. Approximately 200,000 c.p.m. of labelled cholesterol were added in 10 μl of aceton to each incubation flask (assuming full equilibration with the endogenous cholesterol this would correspond to 200,000 c.p.m. per nmol cholesterol). After incubation for 40 min at 30°C the samples were extracted with chloroform:methanol 1:2 [1]. A trace amount of the chloroform extract was subjected to thin layer chromatography with benzene–ethylacetate as the solvent [6]. The larger part of the extract was subjected to high pressure liquid chromatography (HPCL) [3]. The fractions corresponding to the products of the reaction (10–14% of the total radioactivity) were collected and without further treatment aliquotes were analysed by gas chromatography [5].

Other aliquotes were, after conversion to trimethylsilyl ethers [7], subjected to radio-gas chromatography on an instrument [8] equipped with a 1.5% SE-30 column. Aliquotes of the trimethylsilyl ethers were furthermore analyzed by combined gas chromatography–mass spectrometry using the LKB 9000 instrument equipped with a 1.5% SE-30 column (2 mm i.d. × 1 m) and a multiple ion detector (MID).

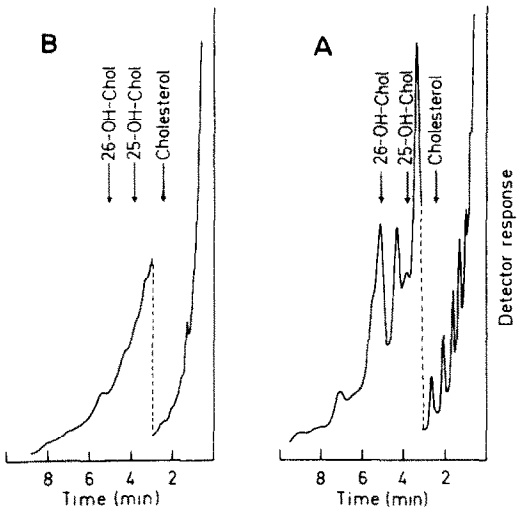

Fig. 1. Gas chromatogram of material recovered by high pressure liquid chromatography and corresponding to the product of the reconstituted steroid hydroxylation reaction. Trace A corresponds to the complete reconstituted system (0.33 nmol cytochrome P-450). Trace B corresponds to an identical system (same amount of cytochrome P-450) except that no ferredoxin was present in the incubation mixture. The 2 samples were treated identically (see Methods) and equal aliquotes were injected into the instrument. The arrows indicate the position of external standards, cholesterol, 25-hydroxycholesterol and 26-hydroxycholesterol, respectively. The attenuator of the instrument (Varian 1400) was switched from 16 to 2 at the broken line.

RESULTS

Thin layer chromatography of the incubation extracts showed radioactivity appearing in a position corresponding to a more polar compound than cholesterol with mobility identical to that of 25-hydroxycholesterol and 26-hydroxycholesterol. It has previously been shown that in the absence of ferredoxin in the incubation mixture no product formation is detected [3].

After partial purification of the product on HPLC the subsequent gas-chromatographic analysis showed the appearance of several peaks more polar than cholesterol (Fig. 1A). In particular peaks with retention times identical to both 25-hydroxy- and 26- hydroxycholesterol were detected. Extracts of the blank incubations containing the cytochrome P-450 preparation with no ferredoxin, but otherwise treated exactly as the samples, did not disclose any such peaks (Fig. 1B).

Radio-gas chromatography of the partially purified product of the reaction showed that approximately 90% of the radioactivity was detected in a peak with a retention time identical to 26-hydroxycholesterol. Less than 10% of the activity had a retention time identical to 25-hydroxycholesterol.

The presence of both 25-hydroxy- and 26-hydroxycholesterol among the products was confirmed by combined gas chromatography–mass spectrometry. Typical mass fragmentation patterns of the trimethylsilyl ethers of 25-hydroxycholesterol and 26-hydroxycholesterol were observed both by MID detection and by running the complete spectra of the respective peaks (Fig. 2). As previously reported [4] the mass spectrum of the trimethylsilyl ether of synthetic 25-hydroxycholesterol showed a prominent base peak at *m/e* 131 (cleavage between C_{24} and C_{25}), and peaks at *m/e* 129 (3β-Δ5-structure), 456 (M-90), 531 (M-15), 546 (M) and unexplained peaks at *m/e* 271 and 327. The same peaks with approximately the same relative intensity were detected in the sample (Fig. 2A). The trimethylsilyl ether of synthetic 26-hydroxycholesterol showed peaks at *m/e* 546 (M), 531 (M-15), 456 (M-90), 441 (M-90-15), 417 (M-129) and 129 [6]. The same main peaks could be identified in the sample (Fig. 2B). The presence of other peaks not found in the standard (e.g. *m/e* 353, 281, 225, 207) shows that the preparation is not pure as would be expected from the gas chromatographic picture in Fig. 1.

DISCUSSION

Hydroxylation of cholesterol in the 25 and the 26 position is known to take place in liver mitochondria [4, 9]. Thus, in incubations of mouse liver mitochondria with cholesterol, 60% of the product was accounted for as 26-hydroxycholesterol and 40% as 25-hydroxycholesterol [9]. A more thorough study of this reaction in rat liver mitochondria identified 26-hydroxycholesterol as the predominant product [4]. The enzymatic properties of the reaction as revealed by using intact rat liver mitochondria have suggested the involvement of a cytochrome P-450. Thus, the reaction was NADPH dependent and the oxygen incorporated was derived from molecular oxygen [4]. Furthermore, the 26-hydroxylation of both cholesterol [6] and 5β-cholestane-3α, 7α, 12α-triol [10] was effectively inhibited by CO and this inhibition could be relieved by light at 450 nm [11]. Recently, both spectrophotometric [12] and isolation procedure [3, 13] have shown the presence of cytochrome P-450 in rat liver mitochondria. A soluble form of cytochrome P-450 from rat liver mitochondria was found to catalyze the conversion of cholesterol into a more polar product [3] in the presence of a ferredoxin [1], NADPH-ferredoxin reductase [14] and NADPH. Based on indirect evidence the product was tentatively identified as 26-hydroxycholesterol [3]. From the results presented in this communication it is evident that the cytochrome P-450 in fact catalyzes the hydroxylation of cholesterol mainly on carbon 26 in accordance with previous findings with intact rat liver mitochondria [4].

The physiological substrates of liver mitochondrial cytochrome P-450 are most probably 5β-cholestane-3α, 7α-diol and 5β-cholestane-3α, 7α, 12α-triol hydroxylated in the 26 position prior to degradation of the side chain and formation of chenodeoxycholic and cholic acid, respectively [6, 10]. Preliminary ex-

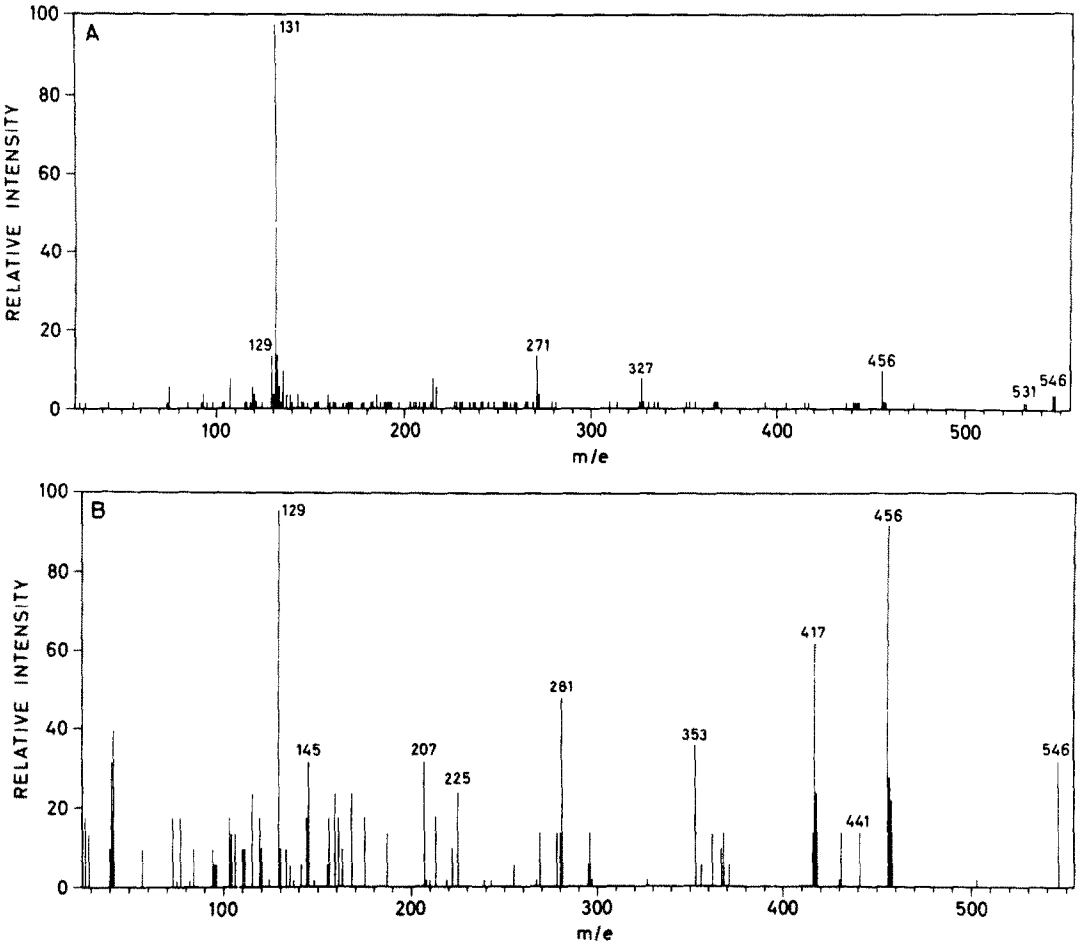

Fig. 2. Mass spectra of material recovered by high pressure liquid chromatography and corresponding to the product of the reconstituted steroid hydroxylation reaction. The gas chromatographic retention times correspond to 25-hydroxycholesterol (spectrum A) and to 26-hydroxycholesterol (spectrum B).

periments with 5β-cholestane-3α, 7α, 12α-triol as substrate for soluble liver mitochondrial cytochrome P-450 have revealed that this compound is transformed much more efficiently into more polar products than is cholesterol (J. I. Pedersen, unpublished).

A more detailed study of these reactions is under way.

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