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CONVERSION OF 5β-CHOLESTANE-3α,7α,12α,26-TETROL INTO 3α,7α,12α-TRIHYDROXY-5β-CHOLESTANOIC ACID BY RABBIT LIVER MITOCHONDRIA

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Rabbit liver mitochondria in the presence of NAD^+ were found to catalyze the conversion of 5β-cholestane-3α,7α,12α,26-tetrol into 3α,7α,12α-trihydroxy-5β-cholestanoic acid. The peroxisomal fraction did not catalyze the reaction. Sonication of the mitochondria or dialysis overnight against a hypotonic buffer increased the rate of oxidation twofold. Most of the enzyme activity was recovered in the supernatant fraction after centrifugation at 100,000xg of sonicated mitochondria. 4-Heptylpyrazole, an inhibitor of cytosolic ethanol dehydrogenase, inhibited the mitochondrial formation of 3α,7α,12α-trihydroxy-5β-cholestanoic acid by 70%. Disulfiram, an inhibitor of cytosolic acetaldehyde dehydrogenase, did not inhibit the reaction. The role of the mitochondrial dehydrogenase system in bile acid biosynthesis is discussed.

The oxidation of 5β-cholestane-3α,7α,12α,26-tetrol into 3α,7α,12α-trihydroxy-5β-cholestanoic acid is a step required for degradation of the C_{27}-steroid side chain in the biosynthesis of cholic acid (1,2). The reaction is catalyzed *in vitro* by ethanol (alcohol) dehydrogenase and acetaldehyde dehydrogenase in the cytosolic fraction of the liver (3-8). It has been proposed that oxidation of 26-hydroxylated C_{27}-steroids in bile acid biosynthesis is one of the physiological functions of liver ethanol dehydrogenase (8). The role of liver ethanol dehydrogenase in bile acid biosynthesis has been recently questioned. Sjövall *et al.* (9) analyzed the composition and size of the bile acid pool in deermice, genetically lacking ethanol dehydrogenase. The study showed that this strain had a normal pool of bile acids

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with a composition like that of animals with a normal activity of ethanol dehydrogenase. The authors concluded that ethanol dehydrogenase is not obligatory for normal bile acid biosynthesis.

The findings by Sjövall et al. (9) indicate that subcellular fractions of liver other than the cytosolic fraction could be involved in the oxidation of 26-hydroxylated C_{27}-steroids. Dean and Whitehouse (10) found that the mitochondrial fraction of mouse and guinea pig liver catalyzed the oxidative removal of $^{14}CO_2$ from 26-hydroxycholesterol more efficiently than any other subcellular fraction. Krisans et al. (11) reported that peroxisomes from rat liver catalyzed the formation of 3β-hydroxy-5-cholenoic acid from 26-hydroxycholesterol. Since 26-hydroxycholesterol is not considered to be an intermediate in the major pathways for bile acid formation, the relevance of this finding with respect to normal bile acid biosynthesis can not be assessed. In the major pathways, the 26-hydroxylated derivatives are 5β-cholestane-3α,7α,26-triol and 5β-cholestane-3α,7α,12α,26-tetrol (1,2). The possibility that these compounds can be oxidized by mitochondrial and/or peroxisomal fractions has not been studied.

The present communication shows the presence of an enzyme system in rabbit liver mitochondria that catalyzes the conversion of 5β-cholestane-3α,7α,12α,26-tetrol into 3α,7α,12α-trihydroxy-5β-cholestanoic acid.

EXPERIMENTAL PROCEDURE

Materials. 5β-[7β-^{3}H]-Cholestane-3α,7α,12α,26-tetrol (500 Ci/mol) was prepared biosynthetically from 5β-[7β-^{3}H]-cholestane-3α,7α,12α-triol using purified 26-hydroxylating cytochrome P-450 from rabbit liver mitochondria (12). The product was purified with HPLC (high performance liquid chromatography) using the system described previously (13). 25R- and 25S-3α,7α,12α-Trihydroxy-5β-[7β-^{3}H]cholestanoic acid (0.2 mCi/μmol) were generous gifts from Dr Jan Gustafsson, Uppsala, Sweden (14). Unlabeled 3α,7α,12α-trihydroxy-5β-cholestanoic acid was isolated from a sample of alligator bile as described previously (14,15).

Nycodenz was from Nyegaard & Co. A/S, Oslo, Norway. 4-Heptylpyrazole was a generous gift from Prof. Richard Dahlbom, Department of Pharmaceutical Organic Chemistry, Uppsala, Sweden (16). Disulfiram, cofactors and reagents were obtained from Sigma.
Methods. Untreated male rabbits of the New Zeeland strain, weighing 2.5 kg were used. Mitochondria, predominantly consisting of heavy mitochondria, were prepared from rabbit liver as described previously (12). The light mitochondrial fraction was prepared from rabbit liver according to the method described by Prydz et al. (17). Peroxisomes were separated from mitochondria on a linear Nycodenz gradient (17). Fractions of 2.5 ml were collected and the concentration of Nycodenz in the fractions was

determined by measuring the refractive index according to the manufacturer. The fractions were dialyzed against 10 mM potassium phosphate buffer, pH 7.4, prior to measurement of enzymatic activities since the Nycodenz disturbed the assay of catalase used as peroxisomal marker enzyme (18). Cytochrome c oxidase was used as the mitochondrial marker enzyme (19). Protein was determined by the Lowry method (20). In the sonication experiments, mitochondria were suspended in 100 mM potassium phosphate buffer, pH 7.4, to a protein concentration of 20 mg/ml and the suspension was sonicated for a total period of 3 min at intervals of 15 sec using a Branson sonifier (cell disrupter B15) at setting 8 of the output control.

Incubations were carried out for 15 min at 37°C. 5β-Cholestane-3α,7α,12α,26-tetrol, 8 nmol in 25 µl acetone, was incubated with 1-3 mg of mitochondrial protein or with 2 ml of each fraction from the gradient centrifugation in 3 ml of 10 mM potassium phosphate buffer, pH 7.4, containing 1.5 mM of NAD^+. In other experiments, appropriate parts to give the same amount of protein were taken from the gradient fractions and were incubated. The incubations were terminated by addition of 5 ml of ethanol. The mixture was acidified with HCl and extracted twice with ether. The organic phases were combined and washed with water until neutral and subjected to thin-layer chromatography. The developing solvent was toluene/isopropanol/acetic acid (75:25:2.5, v:v:v). The radioactivity in the chromatoplates was measured with an LB 2723 Berthold Dünnschicht-Scanner II. Analyses of incubations were also performed by HPLC using an LKB 2150 HPLC pump, LKB 2151 Variable Wavelength Monitor, Berthold LB 503 D HPLC Radioactivity Monitor with a 100 µl flow through cell (solid glass scintillator) and a LiChrosorb RP-18 column (150x3 mm i.d., 5 µm, Merck). N_2-dried incubation extracts were dissolved in 100 µl mobile phase. The analyses were performed with aqueous solutions and the columns were eluted isocratically. Flow rate was 0.5 ml/min. The mobile phase was 75% aqueous methanol and 0.2% acetic acid. The retention time for 25R- and 25S-3α,7α,12α-trihydroxy-5β-cholestanoic acid were 23 and 25 min, respectively. The retention time for 5β-cholestane-3α,7α,12α,26-tetrol was 29 min. The incubations were also separated by ion exchange chromatography and analyzed by capillary column gas-liquid chromatography-mass spectrometry (9).

RESULTS

Labeled 5β-cholestane-3α,7α,12α,26-tetrol was incubated with four times-washed mitochondria from rabbit liver in the presence of NAD^+ and 10 mM potassium phosphate buffer. The incubations resulted in the formation of one major product as analyzed by thin-layer chromatography, ion exchange chromatography and HPLC. The rate of product formation under the conditions used was 110 pmol/ mg of protein per min. The product was an acid and its R_f was identical with that of 3α,7α,12α-trihydroxy-5β-cholestanoic acid. With the HPLC system used it was possible to separate the two C-25 stereoisomers. The product formed in the incubations with rabbit liver mitochondria and 5β-cholestane-3α,7α,12α,26-

tetrol, biosynthesized by rabbit liver mitochondrial cytochrome P-450, was the 25R-form. The gas-chromatographic retention time and mass spectrum of the methyl ester trimethylsilyl ether derivate of the product were identical with those of the same derivative of 3α,7α,12α-trihydroxy-5β-cholestanoic acid, i.e. molecular ion at m/z 680 and prominent fragment ions at m/z 500, 410, 343 and 253.

To check the possibility of contamination of the mitochondrial fraction with cytosolic ethanol and acetaldehyde dehydrogenases, incubations of the supernatant after the last washing step were carried out. No 5β-cholestane-3α,7α,12α,26-tetrol dehydrogenase activity was detected in this supernatant.

To examine the possibility that peroxisomes present in the mitochondrial fraction could be responsible for the 5β-cholestane-3α,7α,12α,26-tetrol dehydrogenase activity, experiments designed to separate these subcellular components were performed. The light mitochondrial fraction was isolated since this fraction is enriched in peroxisomes (17,21). Mitochondria were then separated from peroxisomes by centrifugation on a linear Nycodenz gradient. The distribution of the 5β-cholestane-3α,7α,12α,26-tetrol dehydrogenase activity through the gradient is shown in Fig.1 together with the profiles of the marker enzymes, the gradient density profile and the protein distribution. 5β-Cholestane-3α,7α,12α,26-tetrol was most efficiently converted into 3α,7α,12α-trihydroxy-5β-cholestanoic acid by the fractions with highest mitochondrial marker enzyme activity. No conversion to 3α,7α,12α-trihydroxy-5β-cholestanoic acid was detected in the fraction with highest peroxisomal marker enzyme activity. The profile was the same also when an equal amount of protein from the different fractions was incubated. Similar results were obtained in experiments with fractions containing predominantly heavy mitochondria (cf. Experimental Procedure) with the exception that much less peroxisomal protein was recovered from this source.

Sonication of the mitochondria or dialysis overnight against a hypotonic buffer, 10 mM potassium phosphate buffer, before incubation increased the formation of 3α,7α,12α-trihydroxy-5β-cholstanoic acid from 5β-cholestane-3α,7α,12α,26-tetrol twofold. These results indicate that 5β-cholestane-3α,7α,12α,26-tetrol dehydrogenase is released from mitochondria upon disruption. Experiments were therefore performed in which the mitochondria were suspended in 100 mM phosphate buffer, a buffer believed to

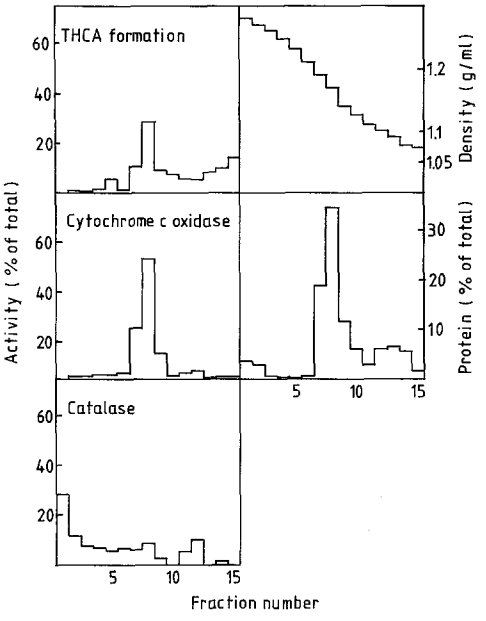

Fig. 1. Distribution of enzyme activities (left hand panels), density profile (upper right hand panel) and protein distribution (lower right hand panel) in fractions after centrifugation of the light mitochondrial fraction of rabbit liver on a Nycodenz density gradient (cf. Experimental Procedure). Enzyme activities and protein distribution are expressed as percentage of the amount in the whole gradient. Cytochrome c oxidase was used as mitochondrial marker enzyme and catalase was used as peroxisomal marker enzyme. THCA, 3α,7α,12α-trihydroxy-5β-cholestanoic acid.

prevent disruption and leakage of intramitochondrial protein. One part of the suspended mitochondria was then centrifuged at 100,000xg for 1 h. The other part was sonicated prior to centrifugation. The two 100,000xg supernatant fractions were incubated with 5β-cholestane-3α,7α,12α,26-tetrol and NAD^+. The supernatant fraction from the sonicated mitochondria catalyzed the oxidation to 3α,7α,12α-trihydroxy-5β-cholestanoic acid six times more effectively than that from the non-sonicated. About 65% of the activity was recovered in the supernatant and 35% remained in the membrane fraction collected after sonication and centrifugation.

The formation of 3α,7α,12α-trihydroxy-5β-cholestanoic acid from 5β-cholestane-3α,7α,12α,26-tetrol by the mitochondrial system required the presence of NAD^+, 1.5 mM, in the incubation mixture. When NAD^+ was replaced by the same concentration of $NADP^+$ the rate of formation of 3α,7α,12α-trihydroxy-5β-cholestanoic acid was 20-50% lower. The effects of 4-heptylpyrazole, a

potent ethanol dehydrogenase inhibitor (16), and disulfiram, an acetaldehyde dehydrogenase inhibitor (22,23), on the oxidation of 5β-cholestane-3α,7α,12α,26-tetrol by the mitochondrial and the cytosolic fractions of rabbit liver were analyzed. 4-Heptylpyrazole (20 μM) inhibited the mitochondrial system by 70% and the cytosolic system completely. Disulfiram (20 μM) did not inhibit the mitochondrial oxidation but inhibited the cytosolic oxidation by 80%.

DISCUSSION

Several studies have shown that isozymes of ethanol dehydrogenase and acetaldehyde dehydrogenase isolated from the cytosolic fraction of liver catalyze the oxidation of 5β-cholestane-3α,7α,12α,26-tetrol into 3α,7α,12α-trihydroxy-5β-cholestanoic acid (3-8). As mentioned in the introduction, there are only a few earlier reports suggesting that subcellular components other than the cytosolic fraction of the liver cell could be involved in the oxidation of 26-hydroxylated C_{27}-steroids in bile acid biosynthesis (9-11).

The results of the present study demonstrate that the mitochondrial fraction but not the peroxisomal fraction of rabbit liver contains an enzyme system catalyzing the oxidation of 5β-cholestane-3α,7α,12α,26-tetrol into 3α,7α,12α-trihydroxy-5β-cholestanoic acid. Since most of the enzyme activity was found in the supernatant fraction after sonication and centrifugation of the mitochondria, it can be concluded that the enzyme system is either loosely attached to the mitochondrial membrane or located in the mitochondrial matrix.

The inhibition of 5β-cholestane-3α,7α,12α,26-tetrol oxidation observed in both the cytosolic and the mitochondrial system with the potent ethanol dehydrogenase inhibitor 4-heptylpyrazole (16) indicates that the two systems have some common properties. On the other hand, the results with disulfiram indicate that there are also differences. Thus disulfiram, which inhibits cytosolic acetaldehyde dehydrogenase by blocking an essential sulfhydryl group (22,23), did not inhibit mitochondrial oxidation of 5β-cholestane-3α,7α,12α,26-tetrol. In this connection it might be mentioned that two major isozymes of acetaldehyde dehydrogenase, one cytosolic and one mitochondrial, are known to be present in the liver (23,24). A difference in the properties of the two isozymes is that the cytosolic acetaldehyde dehydrogenase

is much more sensitive to inhibition by disulfiram than the mitochondrial (23,24). It is conceivable that mitochondrial acetaldehyde dehydrogenase is involved in side chain oxidation of C_{27}-steroids.

The physiological importance of the mitochondrial system described in the present study relative to cytosolic ethanol dehydrogenase and acetaldehyde dehydrogenase in bile acid biosynthesis can not be directly assessed at present. It has been previously believed that cytosolic ethanol dehydrogenase and acetaldehyde dehydrogenase are responsible for the oxidation of the C_{27}-steroid side chain (1,3,4). This concept has been questioned by two recent reports. Cronholm (25) reported that biliary excretion of sulfated 5β-cholestane-3α,7α,12α,26-tetrol is not increased by administration of ethanol or the ethanol dehydrogenase inhibitor, 4-metylpyrazole, in the rat. Sjövall et al. (9) showed that deermice, genetically lacking ethanol dehydrogenase, have a normal bile acid pool and composition. In view of these findings and the fact that the preceding step in side chain oxidation, the 26-hydroxylation, is catalyzed by a mitochondrial cytochrome P-450 system, it does not seem unlikely that the mitochondrial dehydrogenase system plays a role in bile acid biosynthesis. Since it has been reported that mitochondria are able to convert 3α,7α,12α-trihydroxy-5β-cholestanoic acid into cholic acid (14,26,27) the mitochondria would then be capable of complete side chain oxidation in bile acid biosynthesis.

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