CONVERSION OF 3α,7α,12α-TRIHYDROXY-5β-CHOLESTANOIC ACID INTO CHOLIC ACID BY RAT LIVER PEROXISOMES

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Received 9 October 1980

1. Introduction

3α,7α,12α-Trihydroxy-5β-cholestanoic acid (THCA) is considered to be a precursor for cholic acid formation [1]. 3α,7α,12α,24-Tetrahydroxy-5β-cholestanoic acid is a probable intermediate in the reaction [2–4]. 24-Hydroxylation of THCA has been shown to be catalyzed by the mitochondrial fraction of rat liver in combination with the 100 000 × *g* supernatant fluid [4,5]. The microsomal fraction in combination with the 100 000 × *g* supernatant and ATP also catalyzes 24-hydroxylation [4] and the reaction was shown to be due to the combined action of a desaturase and a hydratase [4]. The final thiolytic cleavage of the side chain in cholic acid formation [6] has been shown to be catalyzed by the mitochondrial fraction [3,5,7], the soluble fraction [3,5], or by a combination of the microsomal fraction and the 100 000 × *g* supernatant [7].

The over-all formation of cholic acid from THCA in the mitochondrial fraction is dependent on the presence of ATP, coenzyme A, NAD and Mg^{2+} in the reaction medium [5,7]. It thus appears that this reaction has certain features in common with the β-oxidation of fatty acids.

Recently, a fatty acid oxidizing system different from the one present in the mitochondria has been identified in the peroxisomal fraction of both rat [8,9] and human [10] liver. On the basis of these findings the subcellular localization of cholic acid formation has been further investigated. This work shows that the peroxisomal fraction of rat liver is able to catalyze the conversion of THCA into cholic acid.

2. Materials and methods

3α,7α,12α-Trihydroxy-5β-[7β-^{3}H]cholestanoic acid (0.2 mCi/μmol) was prepared as in [4]. The compound was purified on high pressure liquid chromatography (HPLC) (see below) immediately before use. The compound was a mixture of the two C-25 stereoisomers, but since both forms are converted equally well into cholic acid by rat liver subcellular fractions [7] no attempt was made to separate the 25*R* from the 25*S* form. 3α,7α,12α-Trihydroxy-5β-cholestanoic acid was isolated from a sample of bile of a Caiman species [4]. 3α,7α,12α,24-Tetrahydroxy-5β-[7β-^{3}H]cholestanoic acid (0.2 mCi/μmol) was prepared biosynthetically as in [7]. ^{14}C-Labeled cholic acid used as a reference substance was from the Radiochemical Centre (Amersham). Percoll was the product of Pharmacia (Uppsala). NAD, ATP and coenzyme A were from Sigma (St Louis, MO). All solvents were analytical or HPLC grade. Other chemicals were standard commercial high purity materials.

Male Wistar rats (~200 g body wt) and given a commercial pellet diet were used.

2.1. *Preparation of liver subcellular fractions*

From a 10% liver homogenate in 0.25 M sucrose, 15 mM Hepes buffer (pH 7.4), 1 mM EGTA, a postnuclear supernatant was prepared by centrifugation at 2500 rev./min for 10 min in the HB 4 rotor in a Sorvall RC2-B centrifuge. The pellet was rehomogenized and the suspension recentrifuged. The combined supernatants were centrifuged at 7500 rev./min (5200 × g_{av}) for 10 min in the SS 34 rotor. The resulting pellet was washed 2 times and finally resuspended in the homogenization buffer. This fraction is called the heavy mitochondrial fraction. The 5200 × *g* supernatant was centrifuged at 16 200 rev./min (24 200 × g_{av}) for 10 min in the SS 34 rotor. The pellet was washed twice, resuspended in

the homogenization buffer and termed light mitochondrial fraction. The supernatant was centrifuged at 29 000 rev./min for 1 h in the 30 rotor of a Beckman ultracentrifuge. The resulting microsomal pellet was resuspended in the buffer. The supernatant was used as cytosolic fraction.

2.2. *Preparation of peroxisomes*

A peroxisomal-enriched fraction was separated on a Percoll gradient in a vertical rotor (Beckman VTi 50) by minor modifications of the procedure in [11]. The subcellular liver fraction L layered on top of the gradient was obtained by centrifugation of the post-nuclear supernatant above at 16 300 rev./min (24 500 X g_{av}) for 10 min in the Sorvall SS 34 rotor. Fractions of 2.5 ml were collected from the bottom of the gradient.

The following marker enzymes were measured in the collected fractions: cytochrome *c* oxidase [12]; monoamine oxidase [13]; rotenone-insensitive NADPH-cytochrome *c* reductase [14]; urate oxidase [15]; catalase [16]; β-*N*-acetyl-D-glucosaminidase [17]. Protein was determined by the Lowry method [18].

2.3. *Incubation, extraction and chromatographic procedures*

Under standard conditions the incubation mixture contained the following in 1.5 ml 0.1 M Tris–HCl buffer (pH 8.5): 1.5 μmol NAD, 3.75 μmol ATP, 1.3 μmol coenzyme A, 15 μmol $MgCl_2$ and subcellular fraction corresponding to 1–2 mg protein. The reaction was started by the addition of 20 μg unlabeled THCA together with ~100 000 cpm of labeled THCA in a total of <15 μl ethanol, and continued for 60 min at 37°C. The reaction was terminated by the addition of 30 μl 6 N KOH. After 30 min of hydrolysis at 50°C the mixture was acidified by HCl and extracted twice with ethylacetate. The ethylacetate was washed twice with H_2O, evaporated under nitrogen and the residue was redissolved in 100 μl methanol. Omission of the hydrolysis step resulted in the loss of an appreciable proportion of the radioactivity, especially after incubation with the microsomal fraction. Aliquotes of the extract were injected into a Spectra-Physics HPLC instrument fitted with a Zorbax ODS column (4.6 X 250 mm) and eluted with a solvent consisting of 10 mM acetate buffer (pH 4.37), 18.5% in methanol. The flow rate was 1 ml/min and 1 ml fractions were collected. Counting solution (Dilusolve, Packard Instruments) 4 ml, was added and the fractions were counted in a Packard Tricarb liquid scintillation spectrometer. To locate the cholic acid formed, ^{14}C-labeled cholic acid was used as a reference. Recovery of radioactivity from the HPLC column was essentially complete.

3. Results

In consonance with [3,5,7] it was confirmed that both the microsomal fraction fortified with the 100 000 X *g* supernatant and a conventionally prepared mitochondrial fraction of rat liver (9200 X g_{av} for 10 min) catalyzed the formation of cholic acid from THCA in the presence of NAD, CoA, ATP and Mg^{2+}. By further fractionation of the mitochondria into a heavy (a 5200 X g_{av} pellet) and a light (24 000 X g_{av} pellet) fraction it was observed that the latter was the most active. A typical HPLC profile of the incubation extract of such a fraction is shown in fig.1. The main product peak had the same retention time as cholic acid. The identity of the product was verified by gas chromatography–mass spectrometry [19]. After conversion of the material into methyl ester trimethylsilyl derivative, peaks were observed in the mass spectrum at *m/e* 638 (M), *m/e* 623 (M-15),

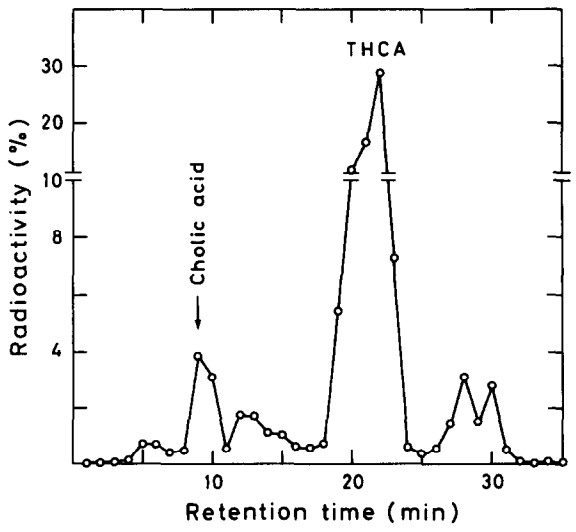

Fig.1. HPLC on Zorbax ODS of the ethylacetate extract of incubation of THCA with the light mitochondrial fraction of rat liver. The incubation conditions as well as extraction and chromatography are described in section 2.

and *m/e* 368 (M-3 X 90). In addition to the product with the same retention time as cholic acid one smaller product peak slightly more polar than the substrate was seen. This material was not identified. The retention time on HPLC was different from that of 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid. Also some material less polar than the product was observed.

When the specific activity of the individual fractions for cholic acid formation were compared, by far the most active was the light mitochondrial fraction (table 1). This finding suggested that this activity was not truly localized to the mitochondria but to some contaminating subcellular particles. After separation of the light mitochondrial fraction (24 500 X g_{av} pellet) on a Percoll gradient it was found that the activity for cholic acid formation from THCA in the different fractions followed the same pattern as the marker enzyme for peroxisomes, i.e., urate oxidase (fig.2), and also catalase (not shown). The peroxisomal fraction was to some extent contaminated by microsomes as detected by the NADPH-cytochrome *c* reductase activity. The data in table 1 exclude the possibility that the cholic acid formation in this fraction is catalyzed by the contaminating microsomes. The lysosomes as detected by β-*N*-acetyl-D-glucosaminidase were well separated from the peroxisomes (not shown).

In order to obtain a reasonable percentage transformation of labeled THCA throughout the gradient only 1 μg substrate was used in the particular experiment shown in fig.2. When near saturating level of substrate was used (20 μg) the specific activity in the fraction containing the highest urate oxidase activity (fraction 11) was ~750 ng cholic acid formed . mg^{-1} . h^{-1}, i.e., an increase of ~50% compared to the light mitochondrial fraction (table 1).

Table 1
Formation of cholic acid from 3α,7α,12α-trihydroxy-cholestanoic acid by rat liver subcellular fractions (ng . mg^{-1} . h^{-1})

Fraction	Formation of cholic acid
Heavy mitochondria	134
Light mitochondria	547
Peroxisomes[a]	752
Microsomes[b]	157
Cytosol	18

[a] Corresponding to fraction no. 11 from the Percoll gradient (section 2)
[b] In the absence of the 100 000 X *g* supernatant fraction
The incubations were performed as in section 2

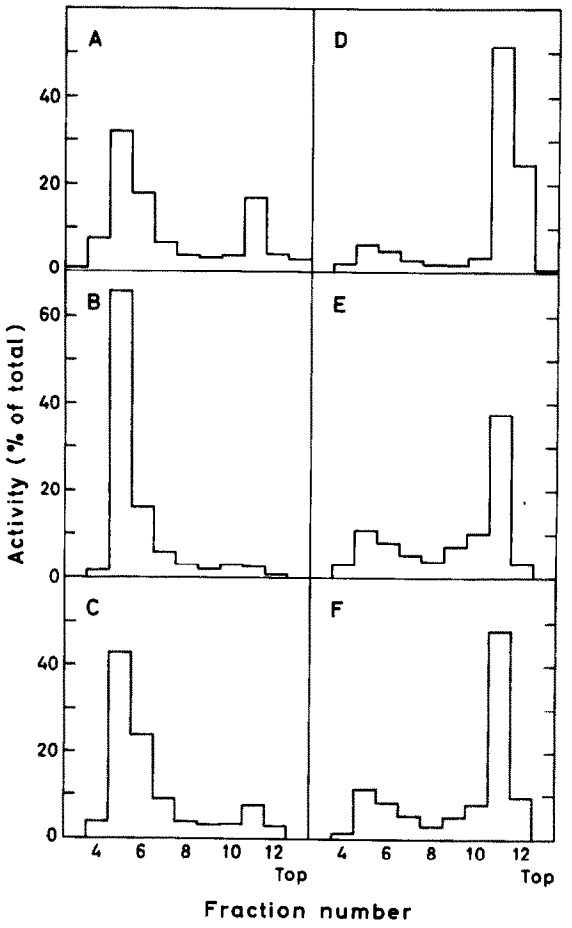

Fig.2. Profiles of protein and enzyme activities from a self-generated Percoll gradient after centrifugation in a vertical rotor. A subcellular fraction L from rat liver was centrifuged in the VTi 50 rotor as in section 2. The gradient was fractioned into 2.5 ml fractions. Protein and enzyme activities are expressed as percentage of the amount in the whole gradient. (A) Protein; (B) cytochrome *c* oxidase; (C) monoamine oxidase; (D) rotenone-insensitive NADPH-cytochrome *c* reductase; (E) urate oxidase; (F) cholic acid formation.

The formation of cholic acid from THCA by the peroxisomal fraction required the presence of NAD, CoA, ATP and Mg^{2+} (table 2).

4. Discussion

Several studies [3,5,7] have shown that the mitochondrial fraction of rat liver catalyzes the formation of cholic acid from THCA. In these studies the mito-

Table 2
Cofactor requirement for the conversion of 3α,7α,12α-trihydroxy-5β-cholestanoic acid to cholic acid by rat liver peroxisomal fraction (%)

Alterations	Conversion
Complete system[a]	22.6
Minus NAD	2.4
Minus ATP	0.6
Minus CoA	0.6
Minus $MgCl_2$	0.9
Minus all cofactors	n.d.

[a] The peroxisomal fraction corresponding to 0.63 mg protein was incubated with 1 μg THCA as in section 2; n.d., not detectable

chondrial fraction was prepared by centrifugation at 8500 × *g* or 6500 × *g* and may have contained other subcellular particles such as peroxisomes and lysosomes in addition to the mitochondria. No attempts had been made to correlate the C_{27}-steroid side chain cleavage activity with that of subcellular marker enzymes.

These results clearly show that the conversion of THCA into cholic acid can be efficiently catalyzed by the peroxisomal fraction of rat liver. The requirement of the reaction for NAD, CoA, ATP and Mg^{2+} lends support to the contention that the reaction may involve the CoA ester. Concerning the assumed intermediate formation of 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid it remains to be established if a similar mechanism prevails as with the combined microsomal and 100 000 × *g* supernatant fractions, i.e., the combined action of a desaturase and a hydratase [4].

Recently a long chain fatty acid oxidizing system was discovered in peroxisomes of rat liver [8] and the activity was increased by treatment of the animals by clofibrate. In purified peroxisomes the presence of crotonase, β-hydroxy-butyryl-CoA dehydrogenase and thiolase was subsequently demonstrated [9]. The involvement of these enzymes would fit into a similar mechanism for oxidation to cholic acid as discussed above.

At the present we can only speculate on the physiological importance of the peroxisomal formation of cholic acid. The role played by this oxidizing system in the pathogenesis in Zellweger's syndrome, a disease among other findings characterized by impaired formation of cholic acid THCA [20] and the absence of liver peroxisomes [21], is one of the interesting questions that emerge from the above findings.

Acknowledgements

We thank Dr Ingemar Björkhem for the performance of the GC-MS analysis. The skillful technical assistance of Signe Nyhuus and Eva Torma Grabner is appreciated. This work was supported by the Norwegian Research Council for Science and the Humanities and by the Swedish Medical Research Council (project 19P-6033-01).

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