Comparison of side chain oxidation of potential C_{27}-bile acid intermediates between mitochondria and peroxisomes of the rat liver: presence of β-oxidation activity for bile acid biosynthesis in mitochondria

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Abstract The oxidation of the side chains of two potential bile acid intermediates, 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA) and 3α,7α-dihydroxy-5β-cholestanoic acid (DHCA), were investigated in rat liver mitochondria and peroxisomes. Both THCA and DHCA were efficiently oxidized to yield cholic acid and chenodeoxycholic acid, along with 3α,7α,12α-trihydroxy-5β-cholest-24-enoic acid and 3α,7α-dihydroxy-5β-cholest-24-enoic acid, respectively, in both the mitochondria and peroxisomes. However, the spectrum of the metabolites in the mitochondria differed greatly from those in the peroxisomes. The major products from THCA and DHCA in the mitochondria were 3α,7α,12α-trihydroxy-5β-chol-22-enoic acid and 3α,7α-trihydroxy-5β-chol-22-enoic acid, respectively, which were tentatively identified from the mass spectral data. However, the formation of these C_{24}-unsaturated bile acids was not observed in the peroxisomes. These results strongly suggest that the cleavage of the side chain of the C_{27}-intermediates for bile acid biosynthesis also occurs independently in the mitochondria, not due to the contamination of peroxisomes.—**Une, M., M. Konishi, M. Yoshii, T. Kuramoto, and T. Hoshita.** Comparison of side chain oxidation of potential C_{27}-bile acid intermediates between mitochondria and peroxisomes of rat liver: presence of β-oxidation activity for bile acid biosynthesis in mitochondria. *J. Lipid Res.* 1996. **37**: 2550–2556.

Supplementary key words Δ^{22}-bile acids • C_{27}-bile acids • cholic acid • chenodeoxycholic acid

Primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA), are formed, via several steps, from cholesterol in the mammalian liver (1). It is also known that 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA) (2–5) and 3α,7α-dihydroxy-5β-cholestanoic acid (DHCA) (6–9) are obligatory intermediates in the major pathway for the biosynthesis of CA and CDCA, respectively. It has been considered that some intracellular organelles are involved in the oxidation of the side chains of these C_{27}-intermediates (3, 4, 7, 8).

Interestingly, significant amounts of C_{27}-bile acids have been found in biological fluids from patients with peroxisomal disorders, such as Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile Refsum's disease (10–16). Furthermore, it has also been shown that the peroxisomal fraction of human (17) and rat (8, 18, 19) liver homogenates has the highest capacity of all subcellular fractions to convert THCA and DHCA to CA and CDCA, respectively. Thus, it has been found that peroxisomes have a major role in the cleavage of the side chain of C_{27}-bile acid intermediates; however, the role of other organelle fractions needs to be clarified.

Recently, C_{27}-intermediates for cholic acid formation were detected along with CA in patients with partially defective β-oxidation enzymes, but CDCA intermediates were not detected, probably because they were efficiently converted to CDCA (20, and M. Une, M. Konishi, and T. Hoshita unpublished results). This results led us to examine the side chain cleavage of THCA and DHCA in an organelle other than the peroxisomes. It is most likely that mitochondria may participate in the oxidation, because β-oxidation enzyme system of fatty acid is known to exist in this organelle.

This report examines the oxidation of the side chains of the C_{27} intermediates, THCA and DHCA, during bile

Abbreviations: THCA, 3α,7α,12α-trihydroxy-5β-cholestanoic acid; DHCA, 3α,7α-dihydroxy-5β-cholestanoic acid; Δ^{24}-THCA, 3α,7α,12α-trihydroxy-5β-cholest-24-enoic acid; Δ^{24}-DHCA, 3α,7α-dihydroxy-5β-cholest-24-enoic acid; CA, 3α,7α,12α-trihydroxy-5β-cholanoic acid (cholic acid); CDCA, 3α,7α-dihydroxy-5β-cholanoic acid (chenodeoxycholic acid); Δ^{22}-CA, 3α,7α,12α-trihydroxy-5β-chol-22-enoic acid; Δ^{22}-CDCA, 3α,7α-dihydroxy-5β-chol-22-enoic acid; β-muricholic acid, 3α,6β,7β-trihydroxy-5β-cholanoic acid; Δ^{22}-β-muricholic acid, 3α,6β,7β-trihydroxy-5β-chol-22-enoic acid; TMS, trimethylsilyl.

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acid biosynthesis in rat liver mitochondria and peroxisomes.

EXPERIMENTAL

General

Gas–liquid chromatography (GLC) was carried out on a Shimadzu GC-14A gas chromatograph using a fused silica capillary column (30 m × 0.25 mm i.d.), coated (0.15 μm film) with DB-17HT (J & W Scientific, CA). The column temperature was 220–300°C, 2°C/min.

Gas–liquid chromatography–mass spectrometry (GC–MS) was carried out on a Hewlett-Packard 5890 gas chromatograph and a JEOL JMS-SX 102 mass spectrometer under the following conditions: column, a fused silica capillary column (15 m × 0.32 mm i.d.) coated (0.15 μm film) with DB-17HT (J & W Scientific); column oven temperature, 200–280°C at a rate of 2°C/min; injection port temperature, 280°C; ion source temperature, 250°C, the flow rate of helium carrier gas, 2.0 ml/min; ionizing energy, 70eV; ionizing current, 300 μA.

Materials

Cholic acid (CA) and chenodeoxycholic acid (CDCA) were purchased from Sigma Co. 3α,7α,12α-Trihydroxy-5β-cholest-24-enoic acid (Δ^{24}-THCA) (21), 3α,7α-dihydroxy-5β-cholest-24-enoic acid (Δ^{24}-DHCA) (22), 3α,7α,12α-trihydroxy-5β-chol-22-enoic acid (Δ^{22}-CA) (23), and 3α,7α-dihydroxy-5β-chol-22-enoic acid (Δ^{22}-CDCA) (23) were synthesized according to the method reported previously. (25RS)-3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA) and (25RS)-3α,7α-dihydroxy-5β-cholestanoic acid (DHCA) were synthesized from Δ^{24}-THCA and Δ^{24}-DHCA, respectively, by catalytic hydrogenation.

Preparation of rat liver mitochondria and peroxisomes

Male Wistar rats (180–200 g) were used. The liver was minced and put into 5 volumes of 0.25M sucrose, 1 mM EDTA, 0.1% (v/v) ethanol, and 10 mM HEPES-NaOH buffer (pH 7.4) and homogenized by one stroke in a Potter-Elvehjem homogenizer. The homogenates were centrifuged at 800 *g* for 12 min. The pellet was rehomogenized and the suspension was recentrifuged under the same conditions. The combined supernatants were centrifuged at 2500 *g* for 13 min (heavy mitochondrial fraction). The 2500 *g* supernatant was centrifuged at 20000 *g* for 22 min. The resulting pellet (light mitochondrial fraction) was washed once and resuspended in the homogenizing medium. The light mitochondrial and the heavy mitochondrial fractions were separately layered on top of a sucrose gradient (1.15–1.25), centrifuged at 74700 g_{av} for 3 h, and fractionated into 1.5 ml each from the bottom. The fractions with the highest amounts of mitochondrial or peroxisomal enzyme markers were pooled and diluted 1:8 with 0.25 M sucrose/10 mM HEPES (pH 7.4). The peroxisomes and mitochondria were subsequently sedimented at 20000 *g* for 30 min. Each pellet was resuspended in 0.2 ml of 0.25 M sucrose/10 mM HEPES (pH 7.4).

Incubation, extraction, and the analytical procedures

The incubation mixture contained the following in 1 ml of 0.1 M Tris-HCl buffer (pH 8.0); 7.5 μmol ATP, 7.5 nmol FAD, 1.5 μmol NAD, 0.1 μmol CoASH, 10 μmol $MgCl_2$, and 1.0 mg of mitochondrial protein or 0.2 mg of peroxisomal protein. After preincubation at 37°C for 10 min, the reaction was started by the addition of THCA or DHCA (50–300 μg), and the incubation was continued for 30–120 min. The reaction was terminated by the addition of 10% KOH (1.0 ml), and the reaction mixture was heated at 70°C for 120 min. Then, the reaction mixture was extracted with a Sep-Pak C_{18} cartridge (Waters) after neutralizing with diluted HCl. An aliquot of the each extract was converted to methyl ester–trimethylsilyl ether (TMS) derivatives. The methyl ester–TMS derivatives of the biological samples were analyzed by GLC and GLC–MS.

RESULTS

Purification of both the peroxisomes and mitochondria was carried out separately by a sucrose gradient centrifugation of the heavy mitochondrial and light mitochondrial fractions obtained from the rat liver homogenates. As seen from the patterns of the marker enzymes (cytochrome C oxidase and catalase), the contamination between fractions was less than a few percent (**Fig. 1** and **Fig. 2**). Both fractions also contained only small amounts of microsomes, estimated from the activity of the microsomal marker enzyme, glucose-6-phosphatase. Fraction number 7 in Fig. 1 and fraction numbers 10 and 11 in Fig. 2 were used in the following experiments as the peroxisomal and mitochondrial fractions, respectively.

3α,7α,12α-Trihydroxy-5β-cholestanoic acid (THCA) and 3α,7α-dihydroxy-5β-cholestanoic acid (DHCA) were incubated with mitochondria and peroxisomes fortified with cofactors, NAD, FAD, CoA, ATP, and Mg^{2+}. The products were extracted using a Sep-Pak C_{18} cartridge. The extracts were dissolved in 10% KOH so-

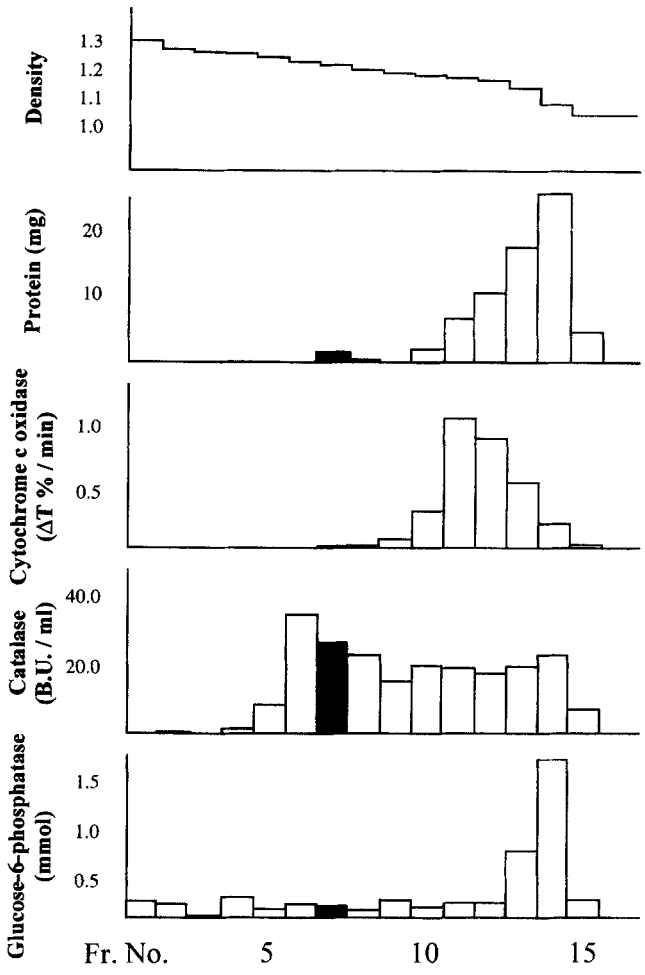

Fig. 1. Density, protein concentration, and enzymatic activity profiles obtained after sucrose gradient centrifugation of the light mitochondrial fraction.

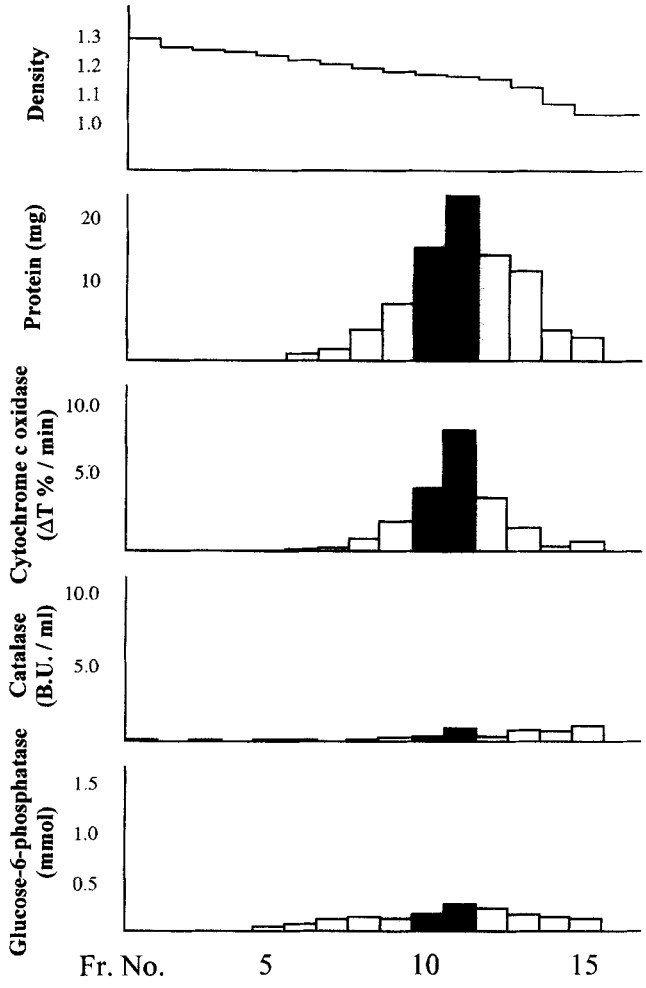

Fig. 2. Density, protein concentration, and enzymatic activity profiles obtained after sucrose gradient centrifugation of the heavy mitochondrial fraction.

lution, and heated at 70°C for 120 min in order to hydrolyze the CoA esters. The hydrolysates were analyzed by GLC and GLC–MS after their conversion to methyl ester–trimethylsilyl (TMS) derivatives. As shown in **Fig. 3,** as the formation of metabolites in the incubation of THCA with mitochondria was at least linear up to 120 min, the incubation period of 120 min was used. **Table 1** shows the composition of metabolites in the incubation of THCA or DHCA with mitochondria and peroxisomes. The major metabolite of THCA in the peroxisomal fraction was (24E)-3α,7α,12α-trihydroxy-5β-cholest-24-enoic acid ((24 E)-Δ^{24}-THCA), which is an intermediate of β-oxidation, and cholic acid (CA) was also detected as a minor component. These bile acids were identified by the direct comparison of their retention times on GLC and mass spectra with authentic samples. DHCA incubated with the peroxisomes was also metabolized to chenodeoxycholic acid (CDCA) and (24E)-3α,7α-dihydroxy-5β-cholest-24-enoic acid

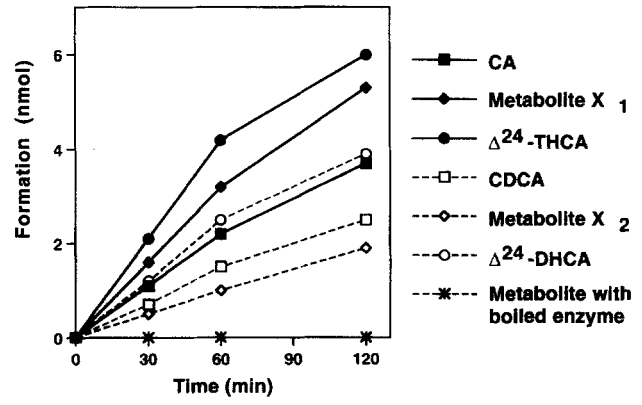

Fig. 3. Effect of time on the formation of metabolites in the incubation of THCA and DHCA with rat liver mitochondria. Closed and open symbols show metabolites of THCA and DHCA, respectively. The symbol (X) shows the formation of metabolites in the incubation of THCA with boiled enzyme.

TABLE 1. Composition of metabolites in the incubation of THCA and DHCA with rat liver mitochondria and peroxisomes

Substrates	Metabolites	Retention Times[a]	Mitochondria	Peroxisomes
		min	%	
THCA				
	CA	26.94	24.7	6.7
	Metabolite X_1	28.96	35.1	n.d.
	Δ^{24}-THCA	36.64	40.2	93.3
DHCA				
	CDCA	28.21	31.1	5.0
	Metabolite X_2	30.32	23.0	n.d.
	Δ^{24}-DHCA	38.60	45.9	95.0

Retention times of (22E)-3α,7α,12α-trihydroxy-5β-chol-22-enoic acid and (22E)-3α,7α-dihydroxy-5β-chol-22-enoic acid were 28.02 min and 30.01 min, respectively. THCA, 3α,7α,12α-trihydroxy-5β-cholestanoic acid; DHCA, 3α,7α-dihydroxy-5β-cholestanoic acid; CA, cholic acid, Δ^{24}-THCA, (24E)-3α,7α,12α-trihydroxy-5β-cholest-24-enoic acid; CDCA, chenodeoxycholic acid; Δ^{24}-DHCA, (24E)-3α,7α-dihydroxy-5β-cholest-24-enoic acid.

[a]GLC conditions were described in Methods.

((24E)-Δ^{24}-DHCA). On the other hand, the spectrum of the incubation products in mitochondria was quite different from those of peroxisomes. In the mitochondrial fraction, the presence of an additional metabolite (metabolite X_1) of THCA was observed along with CA and Δ^{24}-THCA. The mass spectrum of the metabolite X_1 is shown in **Fig. 4B.** The mass spectrum shows the base peak at *m/z* 253, a fragment ion characteristic of bile acids carrying a cholic acid type nucleus. There was a molecular ion at *m/z* 636, and a series of fragment ions at *m/z* 546, 456, and 366, which arose from the consecutive loss of one to three TMS-OH groups from the molecule. This series of fragment ions were at *m/z* values two mass units lower than those of cholic acid, *m/z* 548, 458, and 368 (Fig. 4A). These data suggested that the metabolite X_1 has a double bond in the side chain of cholic acid, and appears to be 3α,7α,12α-trihydroxy-5β-cholan-22-enoic acid (Δ^{22}-CA). To confirm this structural assignment, (22E)-Δ^{22}-CA was chemically synthesized from cholic acid as described previously (23). Although the mass spectrum of the metabolite X_1 remarkably resembled that of the synthetic (22E)-Δ^{22}-CA (Fig. 4C), its retention time was not identical with that of (22E)-Δ^{22}-CA. Thus, metabolite X_1 was tentatively identified as the geometric isomer of the double bond at C-22 and C-23, (22Z)-3α,7α,12α-trihydroxy-5β-cholan-22-enoic acid at this time.

The mass fragmentation pattern of the metabolite X_2, a metabolite of DHCA in the mitochondria, was similar to that of CDCA except for the shift of two mass units downfield, and closely resembled the mass spectrum of (22E)-Δ^{22}-CDCA. However, its retention time was not

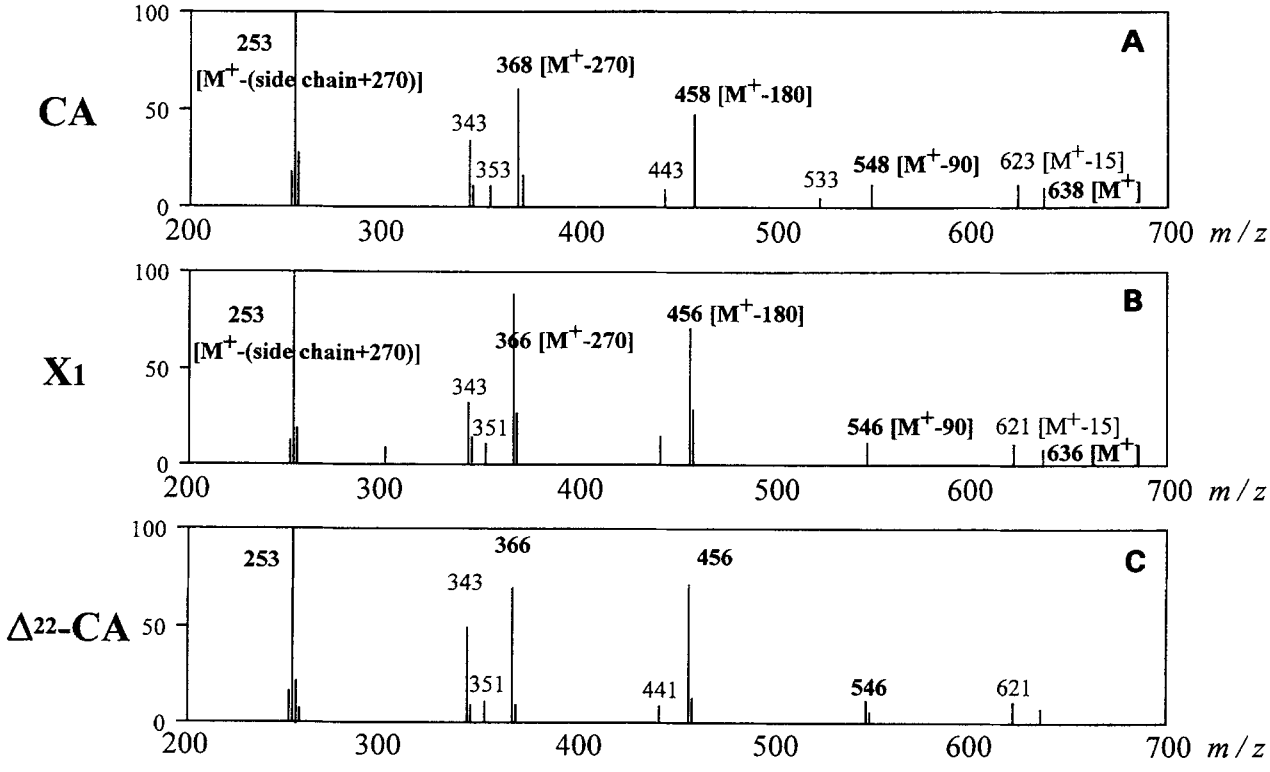

Fig. 4. Mass spectra of cholic acid (CA), the unidentified metabolite of THCA(X_1), and chemically synthesized (22E)-3α,7α,12α-trihydroxy-5β-chol-22-enoic acid (Δ^{22}-CA).

TABLE 2. β-Oxidation rates of the side chains of THCA and DHCA by rat liver mitochondria and peroxisomes

	Mitochondria[a]	Peroxisomes
	nmol/h/mg	
THCA	7.5	186.0
DHCA	4.2	22.5

THCA, 3α,7α,12α-trihydroxy-5β-cholestanoic acid; DHCA, 3α,7α-dihydroxy-5β-cholestanoic acid.

[a]Including unsaturated C_{24}-bile acid.

identical with that of the synthetic (22E)-Δ^{22}-CDCA. Thus, the metabolite X_2 was tentatively identified as (22Z)-3α,7α-dihydroxy-5β-cholan-22-enoic acid (Δ^{22}-CDCA).

The oxidative activities, including Δ^{22}-CA and Δ^{22}-CDCA, of the side chains of THCA and DHCA in the mitochondria and the peroxisomes are summarized in **Table 2**. These metabolites were undetectable in the incubation with the boiled enzyme. The oxidative activity was significantly lower in mitochondria than in the peroxisomes; however, both THCA and DHCA could be oxidized to the same extent in the mitochondria.

DISCUSSION

It is well known that C_{27}-bile acids accumulate in several peroxisomal disorders (10–16, 20). Accordingly, it is evident that peroxisomes play an important role in oxidative cleavage of the side chains of C_{27}-intermediates during the production of bile acids. However, C_{24}-bile acids, cholic acid (CA), and chenodeoxycholic acid (CDCA), still exist in patients with peroxisomal deficiencies such as Zellweger syndrome. Furthermore, in individuals with a thiolase deficiency and a partial enzyme deficiency of peroxisomal β-oxidation system, the C_{27}-intermediates for cholic acid formation were found, whereas the CDCA intermediates were not detectable, probably because they almost completely cleaved to yield CDCA (20, M. Une, M. Konishi, and T. Hoshita unpublished results). From these results we suspected that an organelle, probably the mitochondria, exhibiting β-oxidation activity of fatty acid as peroxisomes, may also participate in the cleavage of the side chains of C_{27}-bile acid.

In this study, we have shown that the side chains of THCA and DHCA are also oxidatively cleaved in mitochondria of the rat liver, even though their activity is significantly low compared to that of peroxisomes. Although Prydz et al. (8) previously observed this β-oxidative activity in the mitochondrial fraction, it was concluded that this activity was due to peroxisomal contamination, as the activity was not inhibited by KCN, and was removed by washing. Our results exclude the possibility of peroxisomal contamination, as additional metabolites of THCA and DHCA, which were not detected in peroxisomes, were formed only in the mitochondria. In addition, we demonstrated that the formation of the metabolites was diminished to about 50% of the control by the addition of KCN (5 mM) in the mitochondrial incubation mixture, even though could not be exclusively depleted (data not shown). From these findings it has been confirmed that rat liver mitochondria can β-oxidatively cleave the side chains of C_{27}-bile acid intermediates. However, the physiological importance as well as the association with the enzyme system of fatty acid β-oxidation still remain to be elucidated.

The additional metabolites of THCA and DHCA in mitochondria were indicated to possess a double bond in the side chain according to their mass spectra, probably Δ^{22}-CA and Δ^{22}-CDCA, respectively. In order to confirm the structural assignment, the chemical synthesis of Δ^{22}-CA and Δ^{22}-CDCA was performed. However, the chemical synthesis of Δ^{22}-CA and Δ^{22}-CDCA, carried out as described previously, gave exclusively (22E)-isomers, and thus (22Z)-isomers were not available. As the retention times of TMS-derivatives of their metabolites on GLC were not identical to those of authentic (22E)-Δ^{22}-CA and (22E)-Δ^{22}-CDCA, respectively, we tentatively identified those as the (22Z)-isomers of Δ^{22}-CA and Δ^{22}-CDCA, because of the similarity of their mass spectra. However, the possibility of the presence of the double bond at the other positions (e.g., between C-20 and C-21, or between C-20 and C-22), cannot be excluded, though it is very unlikely. In any case, it is evident that the unsaturated C_{24}-bile acids could be formed by the oxidative cleavage between C-24 and C-25 of THCA and DHCA.

One possible mechanism for the formation of the C_{24}-unsaturated bile acids is a subsequent partial β-oxidation of CA and CDCA derived from the oxidative cleavage of C_{27}-bile acid intermediates. When CA and CDCA, however, were incubated with mitochondria under the same conditions, no metabolites were detected (data not shown). Although there exists another possibility that the double bond was introduced before the scission of the side chain, we could not detect the possible intermediates, Δ^{22}-THCA and Δ^{22}-DHCA, in the incubation mixture. Consequently, the mechanism for the enzyme catalyzing mitochondrial dehydrogenation could not be defined at this time.

We expected that mitochondria might prefer the intermediate(s) for CDCA biosynthesis to those of CA as substrates for β-oxidation, based on the fact that only C_{27}-bile acids possessing CA nuclei accumulate along with CDCA in the peroxisomal thiolase deficiency. The present results showed that the oxidation of DHCA in the mitochondria was slightly less than the oxidation of THCA. However, this oxidative activity was significantly

lower in peroxisomes. It has been shown that the cleavage of the side chains of THCA and DHCA occurred almost to the same extent in the rat liver peroxisomal fraction (8, 17). It cannot be excluded that our results may underestimate the oxidative side chain cleavage of DHCA in the mitochondria as well as in the peroxisomes.

It is known that β-muricholic acid is a major and characteristic bile acid in rats and that it is transformed from CDCA. The presence of β-muricholic acid with a double bond in the side chain, Δ^{22}-β-muricholic acid, in rat bile has been reported by several studies (24–28). Kayahara et al. (28) showed that (22E)-Δ^{22}-β-muricholic acid was formed by intestinal bacteria, and its formation is correlated with the deconjugation activity of tauro-β-muricholic acid in feces.

On the other hand, Setchell et al. (29) and Rodrigues et al. (30) have recently shown that ursodeoxycholic acid (UDCA) can be metabolized to Δ^{22}-UDCA in the rat liver peroxisomes, and that the Δ^{22}-bile acid appears to be highly specific toward bile acids possessing a functional 7β-hydroxyl group. Thus, it has been presumed that the Δ^{22}-bile acids are formed by the partial β-oxidation of the corresponding C_{24}-bile acid in the rat peroxisomes. This study confirmed the formation of Δ^{22}-CA and Δ^{22}-CDCA from THCA and DHCA, respectively, in the rat liver. However, the formation occurred only in mitochondria; and the unsaturated C_{24}-bile acids were not derived from CA and CDCA. Therefore, it seems unlikely that the present findings are associated with the formation of Δ^{22}-bile acids in rat bile.

In conclusion, rat liver mitochondria apparently exhibit the activity to convert THCA and DHCA to CA and CDCA, respectively, but in significantly lower amounts compared to that in the peroxisomes. Furthermore, Δ^{22}-CDCA and Δ^{22}-CA, which are produced by the cleavage of the side chains of C_{27}-bile acid intermediates, were formed only in the mitochondria. This finding supports the contention that the oxidative side chain cleavage of C_{27}-bile acid intermediates in the mitochondrial fraction is not due to the peroxisomal contamination.

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