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ATP-Dependent Transport of Bile Acid Intermediates across Rat Liver Peroxisomal Membranes

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The bile acid intermediate 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA) is converted to cholic acid exclusively in peroxisomes by the oxidative cleavage of the side chain. To investigate the mechanism by which the biosynthetic intermediates of bile acids are transported into peroxisomes, we incubated THCA or its CoA ester (THC-CoA) with isolated intact rat liver peroxisomes and analyzed their oxidation products, cholic acid and 3α,7α,12α-trihydroxy-5β-cholest-24-enoic acid. The oxidation of both THCA and THC-CoA was dependent on incubation time and peroxisomal proteins, and was stimulated by ATP. THC-CoA was efficiently oxidized to cholic acid and 3α,7α,12α-trihydroxy-5β-cholest-24-enoic acid as compared with THCA, suggesting that THC-CoA is the preferred substrate for transport into peroxisomes. The oxidation of THC-CoA was significantly inhibited by sodium azide, verapamile, and N-ethylmaleimide. Furthermore, the stimulatory effect of ATP on the oxidation was not replaced by GTP or AMP. In addition, the ATP-dependent oxidation of THC-CoA was markedly inhibited by pretreatment of peroxisomes with proteinase K when peroxisomal matrix proteins were not degraded. These results suggest that an ATP-dependent transport system for THC-CoA exists on peroxisomal membranes.

Key words: ABC protein, ATP, bile acids, β-oxidation, membrane transport, peroxisomes.

Abbreviations: CA, cholic acid; THCA, 3α,7α,12α-trihydroxy-5β-cholestanoic acid; GLC, gas-liquid chromatography; GC-MS, gas-liquid chromatography and mass spectrometry; NEM, *N*-ethylmaleimide, PMP70, 70 kDa peroxisomal membrane protein; ALDP, adrenoluekodystrophy protein; P70R, PMP70 related protein; ALDRP, ALDP related protein.

Peroxisomes are involved in a variety of metabolic processes including the oxidative degradation of purine and fatty acids, and the synthesis of plasmalogen and cholesterol (*1*). Oxidative cleavage of the bile acid biosynthetic intermediates 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA) and 3α,7α-dihydroxy-5β-cholestanoic acid also occurs to yield cholic acid (CA) and chenodeoxycholic acid (CDCA), respectively, in peroxisomes in a similar manner to fatty acid β-oxidation (*2–5*). Although numerous studies dealing with the catalytic enzymes and the chemical structures of intermediates in the sequential reactions involved in the conversion of THCA to CA have been carried out (*6–12*), there has been no study focusing on the transport of these substrates across peroxisomal membranes.

Recently, the exchange of metabolites between peroxisomes and cytosol has been suggested to require specific transporters (*13*). At least one of the substrates of acyl-CoA: dihydroxyacetonephosphate acyltransferase has been shown not to be freely permeable to the peroxisomal membrane in human fibroblasts unless ATP is present (*14*). Peroxisomal ATP-binding cassette (ABC) proteins seem to catalyze the transport of fatty acids into peroxisomes. Verleur *et al.* have suggested that Pxa1p and Pxa2p of *S. cerevisiae* are involved in the transport of long chain acyl-CoA into peroxisomes (*15*). We also demonstrated that the overexpression of a 70 kDa peroxisomal membrane protein (PMP70) in Chinese hamster ovary cells increased the rate of palmitic acid β-oxidation in peroxisomes, and that peroxisomes prepared from the cells stimulated palmitoyl-CoA β-oxidation (*16*). The adrenoleukodystrophy protein (ALDP) defect results in impaired peroxisomal β-oxidation and the accumulation of very long chain fatty acids, strongly suggesting that ALDP is involved in the ATP-dependent import of very long chain fatty acids into peroxisomes (*17*, *18*). Thus, it is likely that a transporter(s) associates with the transport of the intermediates of bile acid biosynthesis into peroxisomes.

In this study we incubated isolated peroxisomes with THCA and THC-CoA under several conditions and analyzed their oxidation products. We found that THC-CoA is transported into peroxisomes by an ATP-dependent and protein-facilitated mechanism.

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EXPERIMENTAL PROCEDURES

Materials—CA, *N*-ethylmaleinimide, verapamile, sodium azide, and proteinase K were purchased from Sigma. THCA, THC-CoA, and 3α,7α,12α-trihydroxy-5β-cholest-24-enoic acid were prepared as described previously (*19*). Rabbit anti-PMP70 antibody was raised against the COOH-terminal 15 amino acids of rat PMP70 (*20*). Rabbit anti-ALD related protein (ALDRP) antibody was raised against the COOH-terminal 15 amino acids of human ALDRP. Rabbit anti-PMP70 related protein (P70R) was raised against amino acids of 413 to 606 of human P70R that was fused to the COOH-terminal of the maltose-binding protein. Rabbit anti-ALDP raised against the COOH-terminal 24 amino acids of human ALDP (*21*) was kindly provided by Dr. T. Yamada (Kyushu University). Rabbit anti-rat acyl-CoA oxidase, anti-rat L-bifunctional protein, and anti-human D-bifunctional protein were kindly provided by Dr. T. Hashimoto (Shinshu University).

Isolation of peroxisomes—Peroxisomes were purified from the livers of male Wister rats by differential centrifugation followed by isopycnic centrifugation in sucrose (*22*), with some modifications. About 3 ml of light mitochondrial fraction was layered onto a 20 ml linear sucrose gradient (density span, 1.15–1.25 g/ml) in a Hitachi P28S rotor, and centrifuged at 74,700 ×*g* for 3 h at 4°C. Fractions of ~1.5 ml were collected from the bottom and the fractions with the highest amounts of catalase, peroxisomal marker enzyme, were pooled. The pooled fraction was diluted 8-fold with 0.25 M sucrose containing 10 mM HEPES (pH 7.4). The peroxisomes were subsequently sedimented at 20,000 ×*g* for 30 min. The resulting pellet was resuspended in 0.2 ml of 0.25 M sucrose containing 10 mM HEPES (pH 7.4) and used for the oxidation of THCA and THC-CoA.

Oxidation of THCA and THC-CoA in Peroxisomes—To measure the oxidation of THCA and THC-CoA in peroxisomes, an aliquot of 100 μl of peroxisomal fraction (0.5 mg protein) was mixed with a final volume of 1.5 ml of solution containing 5 μM FAD, 1 mM NAD, 67 μM CoASH, 6.7 mM $MgCl_2$, 0.1% bovine serum albumin, and 0.1 M Tris-HCl (pH 8.0), and the mixture was preincubated in the presence or absence of 5.0 mM ATP at 37°C for 15 min. Then, the reaction was started by the addition of THCA or THC-CoA (50 μg), and the incubation was continued for 30 or 60 min. In some experiments, sodium azide (final concentration 10 mM), verapamile (100 μM), and *N*-ethylmaleimide (50 μM) were added to the incubation mixture. 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 neutralized with diluted HCl and extracted with a Sep-pak C_{18} cartridge (Waters). An aliquot of each extract was converted to the methyl ester-trimethylsilyl ether (TMS) derivative. The methyl ester-TMS derivatives were analyzed by gas-liquid chromatography (GLC) and GLC–mass spectrometry (GC-MS) (*22*).

Protease treatment of peroxisomes—Proteinase K treatments were carried out as described previously (*23*). Isolated peroxisomes (~2.0 mg) in 0.25 M sucrose and 10 mM HEPES (pH 7.4) were incubated with proteinase K (10 μg/ml) for 30 min at 0°C, and the reaction was terminated by the addition of phenylmethansulfonylfluorode (0.5 mg/ml). The samples were centrifuged at 20,000 ×*g* for 22 min and the resulting pellet was resuspended in 10 mM HEPES (pH 7.4) containing 0.25 M sucrose, 0.1% ethanol, and 1 mM EDTA, and used for the oxidation of THC-CoA.

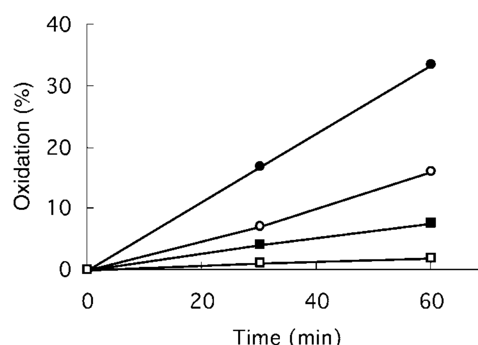

Fig. 1. **The effect of time on the oxidation of THCA and THC-CoA in isolated peroxisomes.** THCA or THC-CoA was incubated with peroxisomes with or without ATP and the oxidized products were analyzed by GLC and CLC-MS. To calculate % oxidation, the amount of oxidized products, including CA and 3α,7α,12α-trihydroxy-5β-cholest-24-enoic acid, was divided by the amount of the oxidized products and remaining substrate. The oxidation of THCA and THC-CoA increased with time in an ATP-dependent manner. Solid circles, THC-CoA (+ATP); open circles, THC-CoA (–ATP); solid squares, THCA (+ATP); open squares, THCA(-ATP).

Other methods—Protein and catalase were assayed as described previously (*16*). Immunoblot analysis was done using ECL+Plus, a Western blotting detection system (Amersham Biosciences).

RESULTS

Oxidation of THCA and THC-CoA in Isolated Peroxisomes—Isolated peroxisomes were incubated with THCA and THC-CoA, and the oxidized products, including CA and 3α,7α,12α-trihydroxy-5β-cholest-24-enoic acid, were analyzed by GLC and GC-MS. The import of THCA and THC-CoA into the peroxisomes was evaluated by the oxidation activity of these substrates. As shown in Fig. 1, THCA was not oxidized to CA or 3α,7α,12α-trihydroxy-5β-cholest-24-enoic acid without ATP, but was oxidized in the presence of ATP and the oxidation increased with time up to 60 min. However, we could not evaluate whether the transport of THCA into peroxisomes requires ATP, since the oxidation of THCA depended on at least two steps: the transport of THCA into the peroxisomes, and the activation of THCA to THC-CoA by ATP. Therefore, we examined the effects of ATP on the oxidation of THC-CoA. Although THC-CoA was oxidized without ATP, THC-CoA oxidation was increased 2-fold in the presence of ATP. These results suggest that THCA is imported through the peroxisomal membrane as a CoA ester in both an ATP-dependent and -independent manner. The β-oxidation of THC-CoA increased linearly with the amount of peroxisomal proteins up to 0.2 mg in the presence or absence of ATP (data not shown).

ATP-dependent transport of THC-CoA into peroxisomes— The above finding suggests the existence of a protein on peroxisomal membranes that mediates the

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Table 1. **Effects of ATPase inhibitors on the ATP-dependent and independent oxidation of THC-CoA.**

	Oxidation activity (% of control)	
	+ATP	–ATP
None (Control)	100	45 ± 3
Sodium Azide	63 ± 4	58 ± 1
Verapamil	83 ± 4	52 ± 2
NEM	29 ± 3	8 ± 2

The oxidation of THC-CoA was examined in the presence of inhibitors with or without ATP. Each value (n = 4–5) is expressed as % of control (the presence of ATP, but no inhibitor). NEM, *N*-ethylmaleimide.

ATP-dependent transport of THC-CoA. We examined the effects of sodium azide and N-ethylmaleimide (NEM) on the oxidation of THC-CoA. As shown in Table 1, sodium azide, which is known to inhibit the ATPase activity of several ABC proteins (*24*, *25*), diminished the oxidation of THC-CoA by 63%. NEM remarkably decreased the ATP-dependent oxidation activity, but it also exhausted the ATP-independent oxidation of THC-CoA. Verapamile, which inhibits the substrate transport of MDR1 (P-glycoprotein) (*26*), also diminished the oxidation by 83%. However, sodium azide and verapamile did not affect the ATP-independent oxidation of THC-CoA. Next, we investigated the effects of various nucleotides on the oxidation of THC-CoA. As shown in Fig. 2, ATP increased the oxidation of THC-CoA by 2-fold. In contrast, AMP and GTP increased the oxidation of THC-CoA by ~20%. This effect might be non-specific since similar observations have been reported for other ATP-dependent transport systems (*27*, *28*). In addition, ADP increased the oxidation by ~60% and the effect of ADP seems to be significant (see “DISCUSSION”).

These results based on the effect of inhibitors on and the nucleotide specificity of the oxidation of THC-CoA support the existence of an ATP-dependent transport system for THC-CoA on peroxisomal membranes.

Effect of Protease Pretreatment of Peroxisomes on the Oxidation of THC-CoA—To confirm the existence of a transporter, isolated peroxisomes were treated with proteinase K and re-isolated in the presence of a protease inhibitor. The β-oxidation of THC-CoA was strongly inhibited by mild protease treatment (Table 2). Under these conditions, peroxisomal ABC proteins, PMP70, ALDP, and ALDRP detected by immunoblotting, disappeared after mild proteolysis (Fig. 3A), although the P70R band remained. After proteinase K treatment, a PMP70 fragment of ~25 kDa was detected as shown in Ref. *23*, but no fragments of ALDP or ALDRP were observed. This is probably because the carboxyl-terminal regions of ALDP and ALDRP, which are recognized by the antibodies, were digested into small fragments by proteinase K. In the case of matrix proteins, β-oxidation enzymes such as acyl-CoA oxidase (subunits a and b) and D- and L-bifunctional proteins existing in the peroxisomes were not degraded, as judged by immunoblotting (Fig. 3B). These results suggest the existence of a transporter that mediates the import of THC-CoA into peroxisomes.

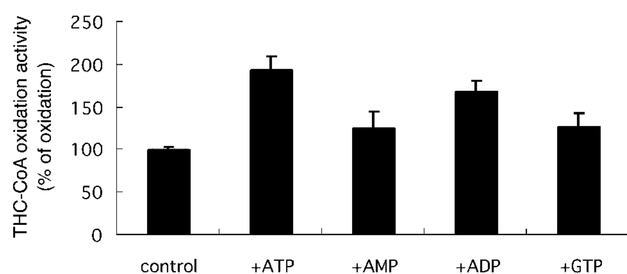

Fig. 2. **The effects of various nucleotides on the oxidation of THC-CoA.** Peroxisomes were incubated with THC-CoA in the presence of the indicated nucleotides (5 mM). Results obtained with nucleotides are plotted as the percent of values obtained without ATP. Each value is the mean ± SE of four experiments.

Table 2. **Effects of Proteinase K on the ATP dependent oxidation.**

	ATP dependent oxidation (%)
Control	100
Proteinase K	19.9

The oxidation of THC-CoA was measured with peroxisomes pretreated or untreated with proteinase K.

DISCUSSION

In the mammalian liver, primary bile acids (CA and CDCA) are synthesized from cholesterol. Numerous studies have described the metabolic sequences involved. Consequently, it has been shown that cholesterol is converted to THCA and 3α,7α-dihydroxy-5β-cholestanoic

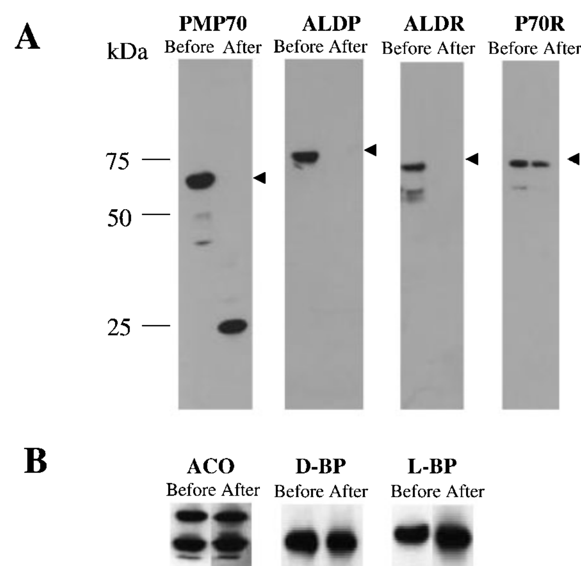

Fig. 3. **Immunoblot analysis of several peroxisomal membrane and matrix proteins before and after treatment with proteinase K.** Peroxisomes (2 mg) were incubated with proteinase K (10 μg) for 30 min at 0°C, and the reaction was terminated with phenylmethansulfonylfluorode. The samples were subjected to SDS-PAGE and immunoblotted with antibodies against the peroxisomal proteins indicated. (A) Peroxisomal membrane proteins. Arrowheads indicate the positions of the peroxisomal ABC proteins. (B) Mtrix enzymes. ACO, acyl-CoA oxidase; D-BP, D-bifuctional protein; L-BP, L-bifunctional protein.

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acid, C_{27}-homologs of CA and CDCA, respectively, by microsomal, cytosolic, and mitochondrial enzyme reactions. Ultimately, the side chain of the C_{27}-homologs is cleaved to yield CA and CDCA in peroxisomes in a manner similar to the β-oxidation of fatty acid (*2*, *29*). However, it is unclear how the C_{27}-intermediates are translocated through peroxisomal membranes. In the present study we used peroxisomes isolated from rat liver to evaluate the incorporation of substrates by analyzing the oxidation products in the incubation mixture by GLC and GC-MS. This assay method allowed us to evaluate the transport of THCA and THC-CoA into peroxisomes without interference by nonspecific adsorption of the substrates by the peroxisomal membranes.

THCA must be activated by the formation of its CoA ester prior to β-oxidation. An enzyme catalyzing this reaction has been shown to be located almost completely in the endoplasmic reticulum (*30*). The present study shows that THC-CoA, rather than THCA, is preferentially oxidized in peroxisomes, confirming that THC-CoA produced outside peroxisomes is used for β-oxidation. Since ATP is not required for the sequential oxidation reaction in the peroxisomal matrix, it is supposed that ATP is essential for the transport of THC-CoA across the membranes. The existence of a transporter that mediates the transport of THC-CoA on peroxisomes is suggested from following results. (i) β-Oxidation of THC-CoA is stimulated in the presence of ATP (Fig. 1). (ii) No stimulation of β-oxidation occurs in the presence of GTP or AMP (Fig. 2). (iii) ATPase inhibitors such as sodium azide, NEM (*24*, *25*) and verapamile, an inhibitor of MDR1 (*26*), inhibit β-oxidation (Table 1). (iv) Pretreatment of peroxisomes with proteinase K markedly inhibits β-oxidation under conditions where the matrix proteins are not degraded (Table 2 and Fig. 3).

Peroxisome stability is critically important for the assay of substrate transport. Under our experimental conditions, the latency of catalase was ~85% and did not decrease during incubation at 37°C for 60 min (data not shown). However, THC-CoA was still oxidized even in the absence of ATP. Some THC-CoA might diffuse passively into peroxisomes and be oxidized to its metabolites in the absence of ATP, since early studies demonstrated that isolated peroxisomes are permeable to compounds of low molecular weight (*31*). Recently, we reported similar observations that palmitoyl-CoA is oxidized in isolated peroxisomes of CHO cells overexpressing PMP70 in an ATP-dependent manner, although some palmitoyl-CoA is oxidized in the absence of ATP (*16*). As the concentration of THC-CoA (27.8 μM) under our experimental conditions was much higher than that in liver cells, an ATP-dependent transport system seems to contribute to the transport of THC-CoA into peroxisomes *in vivo*.

Concerning the effect of ADP on the oxidation of THC-CoA, we do not yet know why a partial stimulation of oxidation occurs, although it might be explained as follows. Recently, the structure of the lipid flippase MsbA, a homolog of the multidrug resistance ABC transporters, was determined by X-ray crystallography, and a model for lipid A transport by MsbA was presented (*32*, *33*). This model suggests a general mechanism for the export of lipid substrates in which an association of ADP with a transporter induces a conformational change and allows the transporter to export substrate existing in the outer leaflet of the membrane. Under our experimental conditions, THC-CoA seems to translocate from the outer to the inner leaflet of peroxisomal membranes in the absence of ATP (Fig. 1). If a putative THC-CoA transporter had a similar "flip-flop" mechanism for the export of THC-CoA, the association of ADP with the transporter might stimulate the export of THC-CoA from the inner leaflet of peroxisomal membranes to the inside of peroxisomes.

It is known that four ABC proteins are present in mammalian peroxisomal membranes: PMP70 (*23*, *34*), ALDP (*35*), ALDP-related protein (ALDRP) (*36*, *37*), and PMP70-related protein (P70R) (*38*, *39*). In order to elucidate further the involvement of these proteins in the transport of THC-CoA, we prepared human hepatoma HuH7 cells overexpressing PMP70 and ALDP by about 2–3 fold, and examined the β-oxidation of THCA. However, the β-oxidation of THCA did not increase when the β-oxidation of long chain and very long chain fatty acids increased. (data not shown). Adrenoleukodystrophy (ALD) patients also exhibit normal bile acid metabolism (*40*). Therefore, it is unlikely that PMP70 and ALDP are involved in the transport of bile acid intermediates. ALDRP is inducible by peroxisome proliferators such as clofibrate and fenofibrate (*41*), however, the activity of THC-CoA oxidase does not increase in rat liver following the administration of these drugs. In addition, P70R was not degraded by protease treatment that caused the β-oxidation of THCA to be markedly inhibited (Table 2 and Fig. 2). Therefore, ALDRP and P70R are not candidates for the transport of THC-CoA. Previously, we identified two ATPases on rat liver peroxisomes (*42*, *43*). One of which is NEM-sensitive and was almost completely inhibited by 50 μM NEM (*42*). This ATPase is also sensitive to proteinase K treatment. The activity of this ATPase is decreased by ~80% following incubation with proteinase K for 30 min at 0°C (*43*). The inhibition of the ATPase activity by NEM as well as proteinase K correlates with the effects of these compounds on THC-CoA transport, suggesting this ATPase as a candidate for the transporter. However, the involvement of this ATPase in the transport of THC-CoA remains to be confirmed.

In summary, our findings provide the first experimental evidence for the existence of a protein that mediates the transport of THC-CoA into peroxisomes. The demonstration of the involvement of an ATPase and a detailed characterization of the mechanisms by which THC-CoA is transported into peroxisomal membranes will be the subjects of further research.

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