J. Biochem. 123, 347-352 (1998)

Thiolase Involved in Bile Acid Formation[1]

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Received for publication, October 17, 1997

The formation of cholic acid and chenodeoxycholic acid through cleavage of the side chains of CoA esters of 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid and 3α,7α-dihydroxy-5β-cholestan-26-oic acid is believed to occur in peroxisomes. Recently, we found a new peroxisomal enzyme, D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase bifunctional protein, and suggested that this bifunctional protein is responsible for the conversion of 3α,7α,12α-trihydroxy-5β-cholest-24-en-26-oyl-CoA and 3α,7α-dihydroxy-5β-cholest-24-en-26-oyl-CoA to their 24-oxo-forms. In the present study, the products of this bifunctional protein reaction were analyzed by gas chromatography-mass spectrometry, and the formation of 24-oxo-27-*nor*-cholestanes was confirmed. Previously, we found a new thiolase in *Caenorhabditis elegans*, P-44, and suggested that P-44 and sterol carrier protein x, a peroxisomal protein, constitute a second group of 3-oxoacyl-CoA thiolases. The production of cholic acid and chenodeoxycholic acid from the precursors on incubation with the bifunctional protein and sterol carrier protein x or P-44 was confirmed by gas chromatography.

Key words: bifunctional protein, bile acid, peroxisome, sterol carrier protein x, thiolase.

The formation of cholic acid (CA) and chenodeoxycholic acid (CDCA) from 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid (THCA) and 3α,7α-dihydroxy-5β-cholestan-26-oic acid (DHCA), respectively, was shown to be confined to peroxisomes (*1, 2*), after the demonstration of the peroxisomal fatty acid oxidation system (*3*). The dependency of bile acid formation on CoA, ATP, and NAD^+ suggests that this chemical process must be similar to fatty acid oxidation. At that time, the peroxisomal fatty acid oxidation system was shown to be composed of acyl-CoA oxidase, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional protein (L-bifunctional protein), and 3-oxoacyl-CoA thiolase (*4*). Later, the dehydrogenation of the CoA esters of THCA and DHCA was found to be catalyzed by a specific oxidase, coprostanoyl-CoA oxidase (*5, 6*), but not acyl-CoA oxidase acting on straight chain fatty acyl-CoAs. The products of the oxidase, the enoyl-forms of these C_{27}-bile acid CoA esters, were thought to be converted to the oxo-forms by L-bifunctional protein, and then the oxo-forms were cleaved by 3-oxoacyl-CoA thiolase. Recently, the presence of a new bifunctional protein, D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase bifunctional protein (D-bifunctional protein), was confirmed (*7-10*). This enzyme converts the enoyl-forms of intermediates in bile acid formation and 2-methyl-branched fatty enoyl-CoAs to oxo-forms as well as straight chain enoyl-CoAs to 3-oxoacyl-CoAs (*11*).

In the present study, we examined the thiolytic cleavage of the oxo-form intermediates in bile acid formation. Peroxisomal and mitochondrial 3-oxoacyl-CoA thiolases and mitochondrial enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase/3-oxoacyl-CoA thiolase trifunctional protein (trifunctional protein) were inactive in this reaction. We investigated the thiolytic cleavage of the oxo-forms by sterol carrier protein x (SCPx), because molecular cloning of the cDNA of this protein revealed that the carboxyl terminal part was identical to that of sterol carrier protein 2, and the amino terminal part was similar to that of 3-oxoacyl-CoA thiolases (*12*), and the recombinant SCPx expressed in *Escherichia coli* was reported to cata-

[1] This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.
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Abbreviations: D-bifunctional protein, D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase bifunctional protein; L-bifunctional protein, enoyl-CoA hydratase/L-3-hydroxyacyl-CoA dehydrogenase bifunctional protein; trifunctional protein, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase/3-oxoacyl-CoA thiolase trifunctional protein; SCPx, sterol carrier protein x; CA (cholic acid), 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid; CDCA (chenodeoxycholic acid), 3α,7α-dihydroxy-5β-cholan-24-oic acid; *homo*-cholic acid, 3α,7α,12α-trihydroxy-24α-*homo*-5β-cholan-24α-oic acid; *nor*-cholic acid, 3α,7α,12α-trihydroxy-24-*nor*-5β-cholan-23-oic acid; DHCA, 3α,7α-dihydroxy-5β-cholestan-26-oic acid; 24*E*-DHCA, 3α,7α-dihydroxy-5β-cholest-24-en-26-oic acid; 24-oxo-DHCA, 3α,7α-dihydroxy-24-oxo-5β-cholestan-26-oic acid; 24-oxo-27-*nor*-DHC, 3α,7α-dihydroxy-24-oxo-27-*nor*-5β-cholestane; DVA, 3α,7α,24-trihydroxy-5β-cholestan-26-oic acid; THCA, 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid; 24*E*-THCA, 3α,7α,12α-trihydroxy-5β-cholest-24-en-26-oic acid; 24-oxo-THCA, 3α,7α,12α-trihydroxy-24-oxo-5β-cholestan-26-oic acid; 24-oxo-27-*nor*-THC, 3α,7α,12α-trihydroxy-24-oxo-27-*nor*-5β-cholestane; VA (varanic acid), 3α,7α,12α,24-tetrahydroxy-5β-cholestan-26-oic acid. The CoA esters of the above bile acids are indicated by the abbreviation, CoA.

lyze the thiolytic cleavage of straight chain 3-oxoacyl-CoAs including 3-oxopalmitoyl-CoA (*13*). In addition, we used P-44, because we recently found this new protein in *Caenorhabditis elegans*, and demonstrated that its structural and catalytic properties were similar to those of SCPx (*14*). In this study, we found that these proteins exhibit thiolase activity toward the presumed precursors of bile acids.

MATERIALS AND METHODS

Materials—Hexadecenoyl-CoA, 2-methylhexadecenoyl-CoA (*11*), and the CoA esters of stereoisomeric 3α,7α,12α,24-tetrahydroxy-5β-cholestan-26-oic acid (varanic acid, VA), 3α,7α,24-trihydroxy-5β-cholestan-26-oic acid (DVA) (24*R*,25*R*-, 24*R*,25*S*-, 24*S*,25*R*-, and 24*S*,25*S*-isomers), 3α,7α,12α-trihydroxy-5β-cholest-24-en-26-oyl-CoA (24*E*-THCA-CoA), and 3α,7α-dihydroxy-5β-cholest-24-en-26-oyl-CoA (24*E*-DHCA-CoA) (*15, 16*) were prepared as described. D-Bifunctional protein (*8*), L-bifunctional protein (*17*), peroxisomal and mitochondrial 3-oxoacyl-CoA thiolases (*18*), and trifunctional protein (*19*) were purified from rat liver.

The following compounds were also synthesized in our laboratory by means of condensation of 3α,7α,12α-triformyloxy- and 3α,7α-diformyloxy-5β-cholan-24-al with ethyl bromopropionate, oxidation with chromic acid, and subsequent alkaline hydrolysis to give decarboxylated products. 3α,7α,12α-Trihydroxy-24-oxo-27-*nor*-5β-cholestane (24-oxo-27-*nor*-THC): mp 192-193°C, ^{1}H-NMR ($CDCl_3$): 0.67 (3H, s, 18-Me), 0.89 (3H, s, 19-Me), 0.96 (3H, d, J=6.4 Hz, 21-Me), 1.06 (3H, t, J=7.3 Hz, 26-Me), 2.17 (2H, q, J=7.3 Hz, 25-H), 3.45 (1H, m, 3-H), 3.85 (1H, bs, 7-H), 3.97 (1H, bs, 12-H), Anal. Calcd for $C_{26}H_{44}O_4$: C, 74.27; H, 10.54. Found: C, 74.00; H, 10.56. 3α,7α-Dihydroxy-24-oxo-27-*nor*-5β-cholestane (24-oxo-27-*nor*-DHC): amorphous powder: mp 76-81°C, ^{1}H-NMR ($CDCl_3$): 0.66 (3H, s, 18-H), 0.91 (3H, s, 19-H), 0.91 (3H, d, J=6.0 Hz, 21-H), 1.05 (3H, t, J=7.3 Hz, 26-H), 2.17 (2H, q, J=7.3 Hz, 25-H), 3.46 (1H, m, 3-H), 3.85 (1H, bs, 7-H), Anal. Calcd for $C_{26}H_{44}O_3$: C, 77.17; H, 10.96. Found: C, 77.30; H, 10.88. The CoA esters of *homo*-cholic acid (3α,7α,12α-trihydroxy-24*a*-*homo*-5β-cholan-24*a*-oic acid) and *nor*-cholic acid (3α,7α,12α-trihydroxy-24-*nor*-5β-cholan-23-oic acid), used as internal standards, were synthesized according to the reported method (*16*).

Taq DNA polymerase, *Sph*I restriction endonuclease, and bacteriophage T4 DNA ligase were obtained from Takara. The oligonucleotides used as primers in PCR amplification were products of Sawady Technology.

Preparation of SCPx and P-44—The primers, 5′-CGACCGCATGCCTTCAGTCGCGTTGAACTC and 5′-TCGACGCATGCTCACAGCTTAGCTTTGTCCGGCTG, were used to amplify the entire region of the ORF of SCPx with a rat cDNA library, which was constructed with poly(A)-rich RNA from normal rat liver as the template. The PCR product was cloned into the expression vector, pQE32 (Qiagen), at the *Sph*I site; the resulting plasmid was designated as pEMB770. Hexahistidine-tagged SCPx and P-44 were expressed in cells of *Escherichia coli* JM109 carrying plasmids pEMB770 and pEMB728 (*14*), respectively, and purified as described (*14*).

Thiolase Assay—Thiolase activity was assayed after coupling with the D- or L-bifunctional protein, or both. The reaction mixture comprised 100 mM Tris-Cl, pH 7.5, 100 mM KCl, 20 μM enoyl-CoA, 200 μM NAD^+, 10 μM CoA, and 5 μg of D- and/or L-bifunctional protein, in a total volume of 1 ml. The increase in absorbance at 340 nm was followed after the addition of one of the bifunctional proteins or both, followed by standing for a few minutes until equilibrium of the reaction of the bifunctional protein was attained. Then the thiolase preparation was added and the rate of increase in absorbance at 340 nm was determined. One unit of enzyme was defined as the amount of the enzyme producing 1 μmol of NADH per min at 30°C.

Analysis of the Products of the Thiolase Reaction—The

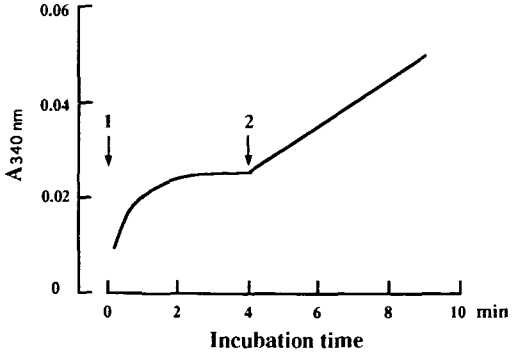


Fig. 1. **Time course of the reaction.** The assay conditions are given under "MATERIALS AND METHODS," and 24*E*-THCA-CoA was used as a substrate. When the D-bifunctional protein was added (arrow 1), the absorbance at 340 nm increased to an equilibrium level in a few minutes. Then, the absorbance started to increase steadily on the addition of SCPx (0.5 μg, arrow 2).

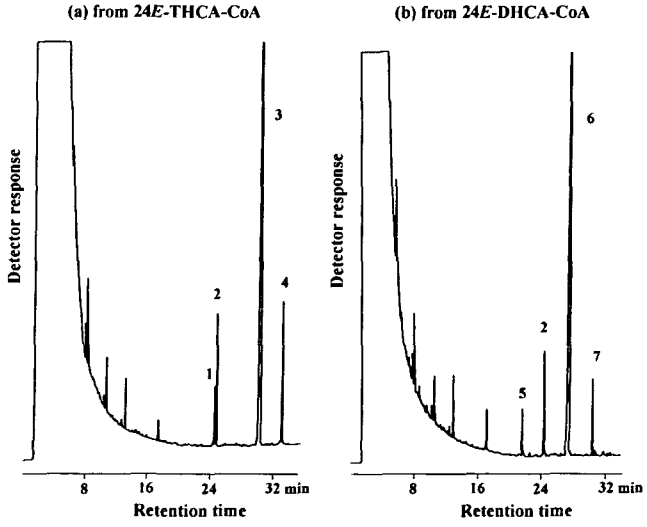


Fig. 2. **Gas chromatograms of methyl ester-dimethylethylsilyl ether derivatives of products obtained on incubation of 24*E*-THCA-CoA (a) and 24*E*-DHCA-CoA (b) with the D-bifunctional protein in the presence of NAD^+.** Column: DB-1 (30 m×0.25 mm; J & W Scientific, Folsom, CA, USA), temperature: 210°C (3 min)→270°C (10°C/min)→300°C (2°C/min), carrier gas: He (45 cm/min). 1: 24-oxo-27-*nor*-THC, 2: *homo*-cholic acid (internal standard), 3: 24*E*-THCA (substrate), 4: VA, 5: 24-oxo-27-*nor*-DHC, 6: 24*E*-DHCA (substrate), 7: DVA. Other peaks were due to contamination by derivatization reagents and the incubation medium.

enzyme reaction was performed with 2.5–8 μg of either or both of the bifunctional proteins and 1.5–2.0 μg of SCPx or P-44 in the presence of 160 μM of a substrate, 2 mM NAD^+, and 1 mM CoA in 100 mM Tris-Cl, pH 8.0, in a total volume of 250 μl, at 30°C for 10 min.

Homo-cholic acid CoA ester or *nor*-cholic acid CoA ester (5 μg), as an internal standard, was then added to the ice-cooled reaction solution and the mixture was subjected to centrifugal ultrafiltration at 4°C. Two molar KOH (100 μl) was added to the filtrate, followed by warming at 60°C for 30 min. The solution was ice-cooled and then acidified by the addition of 2 M HCl (150 μl). The mixture was extracted with ethyl acetate (3 ml), and the extract was washed with H_2O (1 ml) and then evaporated. The residue was treated with CH_2N_2 and dimethylethylsilylimidazole to give methyl ester-dimethylethylsilyl ether derivatives, and then an aliquot was passed through a short silica gel column (35×8 mm) eluted with hexane/ethyl acetate (3 : 1, v/v, 3 ml). After evaporation, the residue was dissolved in hexane (50 μl) and 1 μl was injected for gas chromatography-mass spectrometry (GC-MS) according to the reported method (*20*). Throughout the analytical procedure, the relative recovery of the enzymatic product as to the internal standard was over 96%.

RESULTS AND DISCUSSION

Production of 24-Oxo-Intermediates through the Bifunctional Protein Reaction—The thiolase activity of the fatty acid oxidation system has been usually measured as the decrease in absorbance at 303 nm due to the enolate-form of the Mg^{2+}-3-oxoacyl-CoA complex (*18*). In this study, the thiolase reaction was assayed after coupling to the bifunctional protein reaction using the enoyl- or 3-hydroxyacyl-form of the substrate, because (1) chemically synthesized oxo-forms of bile acid precursors were not available, and (2) the association constant of the Mg^{2+}-2-methylbranched oxoacyl-CoA is very low (*21*).

As shown in Fig. 1, the increase in the absorbance at 340 nm on the addition of the D-bifunctional protein in the presence of 24*E*-THCA-CoA was suggested to be due to the production of an oxo-intermediate (*11*). The absorbance at 340 nm further increased on the addition of the thiolase preparation after the equilibrium of the bifunctional protein reaction was attained.

The production of oxo intermediates, 3α,7α,12α-trihydroxy-24-oxo-5β-cholestan-26-oyl-CoA (24-oxo-THCA-CoA) and 3α,7α-dihydroxy-5β-cholestan-26-oyl-CoA (24-

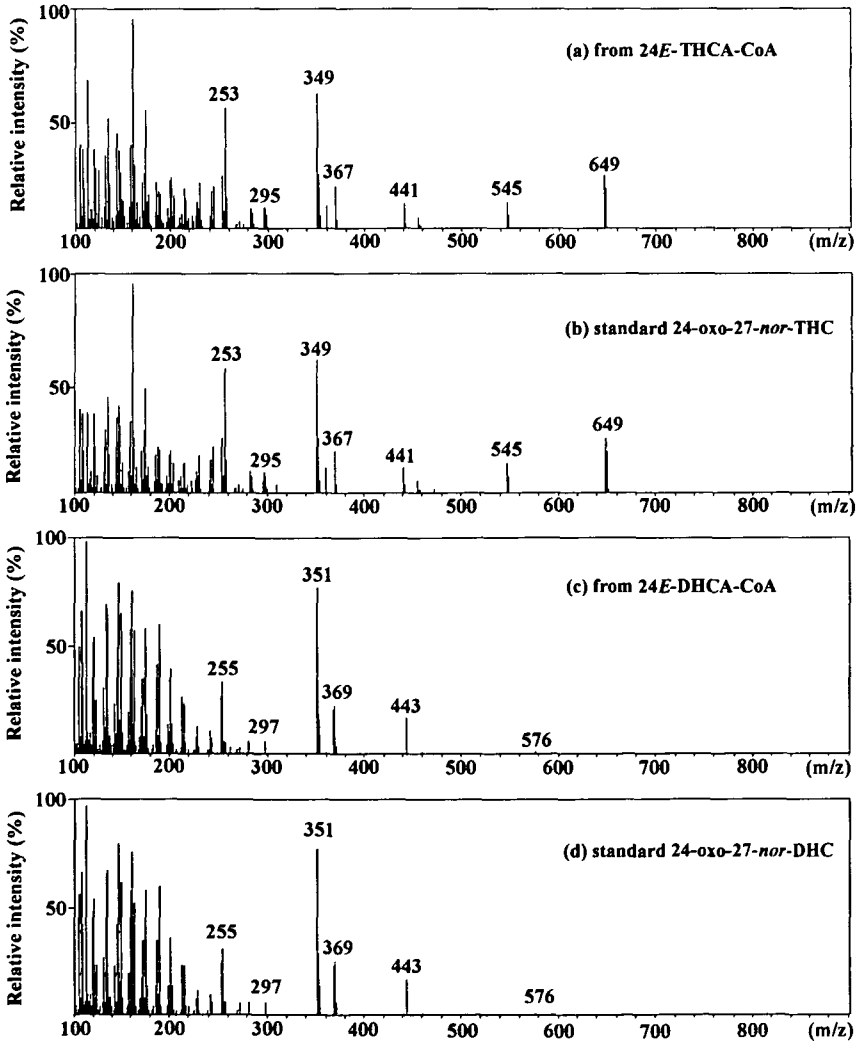


Fig. 3. **Mass spectra of the dimethylethylsilyl ether derivatives of standard 24-oxo-27-*nor*-cholestanes and decarboxylated products obtained on incubation of 24*E*-THCA-CoA and 24*E*-DHCA-CoA with the D-bifunctional protein in the presence of NAD^+.** (a) Product from 24*E*-THCA-CoA (peak 1 in Fig. 2), (b) 24-oxo-27-*nor*-THC (standard), (c) product from 24*E*-DHCA-CoA (peak 5 in Fig. 2), (d) 24-oxo-27-*nor*-DHC (standard).

oxo-DHCA-CoA), by the D-bifunctional protein was confirmed by GC-MS analysis. Figure 2 shows gas chromatograms of the products from 24*E*-THCA- and 24*E*-DHCA-CoA with the D-bifunctional protein in the presence of NAD^+ after hydrolysis of the CoA esters. The formation of 24-oxo-27-*nor*-THC and 24-oxo-27-*nor*-DHC was observed, since 24-oxo-THCA and 24-oxo-DHCA easily underwent decarboxylation to give the above 24-oxo-27-*nor*-cholestanes (*22*). Figure 3 shows mass spectra of the above decarboxylated product (peaks 1 and 5 in Fig. 2). The characteristic fragmentation of these compounds was completely identical with that of synthetic standards.

Thiolase Activity of SCPx and P-44—As shown in Fig. 1, further NADH production occurred on the addition of SCPx to the reaction mixture after equilibrium of the D-bifunctional protein reaction with 24*E*-THCA-CoA was attained. This NADH production is supposed to be due to the thiolase reaction, because it was dependent on CoA. Similar activity was observed with P-44. But, no thiolase activity was detected when any of peroxisomal 3-oxoacyl-CoA thiolase, mitochondrial 3-oxoacyl-CoA thiolase or trifunctional protein was used.

The formation of CA and CDCA was confirmed by gas chromatographic analysis. A gas chromatogram of the product from an isomer of the CoA ester of varanic acid (24*R*,25*R*-VA-CoA) with SCPx in the presence of the D-bifunctional protein and NAD^+ indicated that CA production is dependent on SCPx (Fig. 4). CDCA production from 24*E*-DHCA-CoA by SCPx was also confirmed (data not shown). Figure 5 shows gas chromatograms of the products from 24*E*-THCA- and 24*E*-DHCA-CoA with the combination of the D-bifunctional protein and P-44. The formation of CA (peak 2) and CDCA (peak 4) was apparently ob-

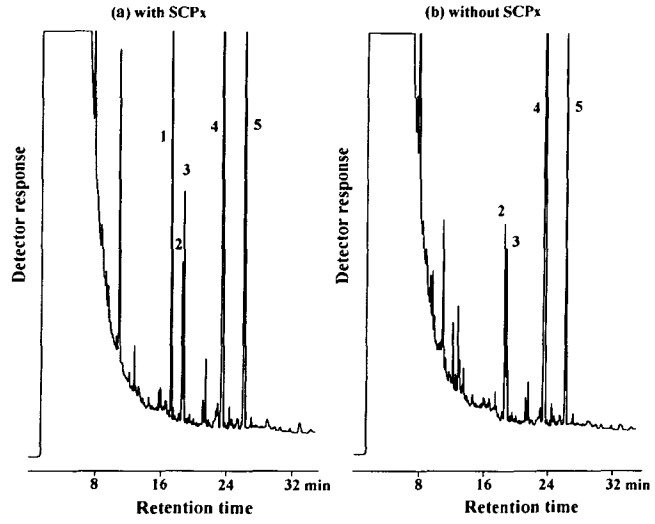


Fig. 4. **Gas chromatograms of methyl ester-dimethylethylsilyl ether derivatives of the products obtained on incubation of 24*R*,25*R*-VA-CoA with (a) or without (b) SCPx.** Column: DB-1 (30 m×0.25 mm), temperature: 230°C (3 min)→270°C (10°C/min)→310°C (2°C/min), carrier gas: He (45 cm/min). 1: CA, 2: *homo*-cholic acid (internal standard), 3: 24-oxo-27-*nor*-THC, 4: 24*E*-THCA, 5: 24*R*,25*R*-VA (substrate).

TABLE I. **Thiolase activities of SCPx and P-44.** Activity is expressed in units/mg.

Substrate	Bifunctional protein[a]	SCPx	P-44
$C_{16:1}$-CoA[b]	D,L	8.7	16
2-Me-$C_{16:1}$-CoA[b]	D	2.7	3.5
24*E*-THCA-CoA	D	1.6	2.4
24*R*,25*R*-VA-CoA	D	1.3	2.4
24*R*,25*S*-VA-CoA[c]	D	—	—
24*S*,25*R*-VA-CoA	L	1.5	2.7
24*S*,25*S*-VA-CoA[d]	D+L	1.0	2.1
24*E*-DHCA-CoA	D	1.3	1.1
24*R*,25*R*-DVA-CoA	D	1.3	1.2
24*R*,25*S*-DVA-CoA	D	0.9	1.0
24*S*,25*R*-DVA-CoA	L	2.0	2.0
24*S*,25*S*-DVA-CoA[d]	D+L	1.5	1.2

[a]Bifunctional protein producing NADH: D, D-bifunctional protein; L, L-bifunctional protein. [b]$C_{16:1}$-CoA, hexadenoyl-CoA; 2-Me-$C_{16:1}$-CoA, 2-methylhexadecenoyl-CoA. [c]The thiolase activity was not determined because of the low rate of NADH production with the D-bifunctional protein. [d]No NADH production was observed with either the D- or L-bifunctional protein, but a high rate of NADH production was observed in the presence of both the D- and L-bifunctional proteins.

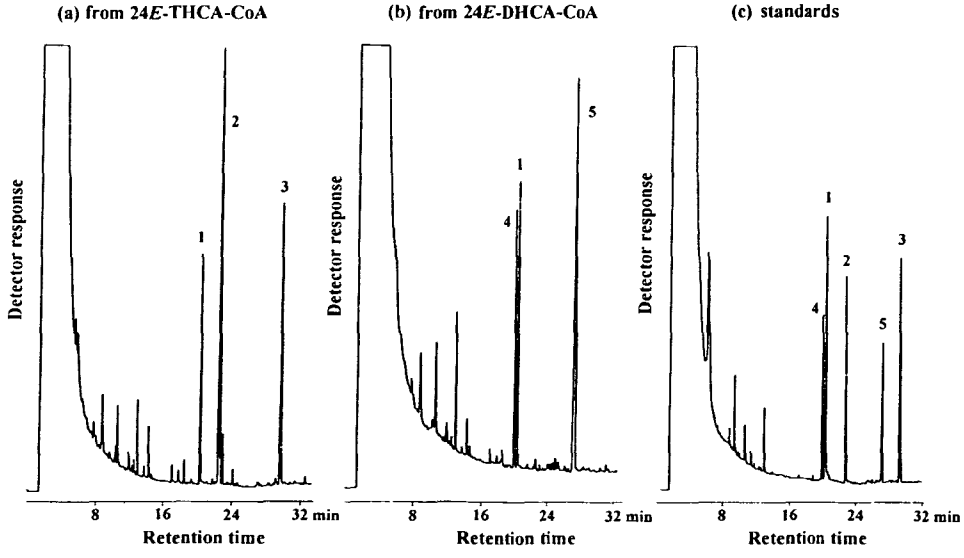
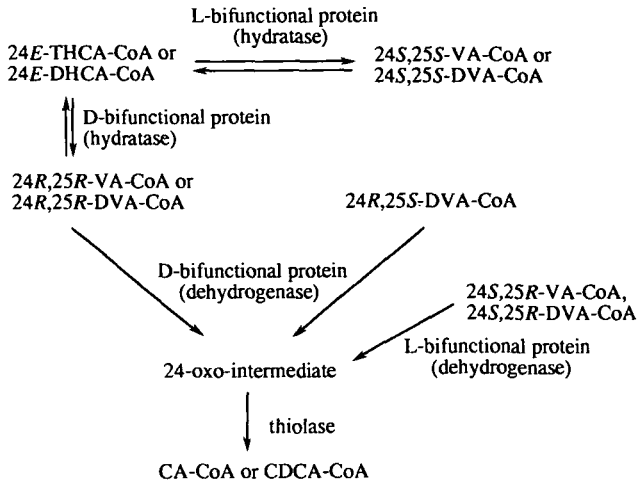


Fig. 5. **Gas chromatograms of the methyl ester-dimethylethylsilyl ether derivatives of products from 24*E*-THCA-CoA (a) and 24*E*-DHCA-CoA (b) obtained on incubation with the D-bifunctional protein and P-44.** Column: DB-1 (30 m×0.25 mm), temperature: 210°C (3 min)→270°C (10°C/min)→300°C (2°C/min), carrier gas: He (45 cm/min). 1: *nor*-cholic acid (internal standard), 2: CA, 3: 24*E*-THCA, 4: CDCA, 5: 24*E*-DHCA. A chromatogram for the standards is shown in (c).



Scheme 1. **Metabolic pathway of bile acid production.** The reversibility of the dehydrogenase reaction was not confirmed because of the lack of availability of 24-oxo-intermediates.

served. The mass spectra of these compounds were identical with those of authentic specimens. These data support that both SCPx and P-44 exhibit thiolase activity toward the oxo-forms of the bile acid precursors.

The production of NADH with hexadecenoyl-CoA during incubation was observed with either the D- or L-bifunctional protein, as previously described (*11*). The thiolase activities of SCPx and P-44 with hexadecenoyl-CoA and the D-bifunctional protein were 8.7 and 16 units/mg, respectively, under the conditions given under "MATERIALS AND METHODS" (Table I). Similar thiolase activities were observed with this substrate and the L-bifunctional protein with SCPx and P-44 as well as other 3-oxoacyl-CoA thiolases (data not shown).

With the use of 2-methylhexadecenoyl-CoA, NADH production was observed only when the D-bifunctional protein was used (*11*). Significant thiolase activity was detected with SCPx and P-44 (Table I), but no activity was observed when peroxisomal and mitochondrial 3-oxoacyl-CoA thiolases and the trifunctional protein were use

The thiolase activities of these enzymes with the v
precursors of bile acids are listed in Table I togethe
the names of the active coupling enzymes producii
oxo-forms. The thiolase activities of SCPx ranged from
2 units/mg. Most of the intermediates in bile acid formation were converted to the oxo-forms by the D-bifunctional protein, but there were exceptional intermediates. The production of 24*S*,25*R*-VA-CoA and 24*S*,25*R*-DVA-CoA was catalyzed by the L-bifunctional protein, but not the D-bifunctional protein. The thiolase activity with 24*R*,25*S*-VA-CoA was not determined because the D-bifunctional protein exhibited a low rate of NADH production. The L-bifunctional protein was completely inactive with this substrate. An increase in absorbance at 340 nm on the addition of either the D- or L-bifunctional protein was not observed with 24*S*,25*S*-VA-CoA and 24*S*,25*S*-DVA-CoA. However, a high rate of increase in the absorbance was observed when both these enzymes were added to the reaction mixture. It is supposed that 24*S*,25*S*-VA-CoA and 24*S*,25*S*-DVA-CoA are converted to 24*E*-THCA-CoA and 24*E*-DHCA-CoA, respectively, by the L-bifunctional protein, and then these enoyl-forms are further converted to 24*R*,25*R*-VA-CoA and 24*R*,25*R*-DVA-CoA, respectively, by the D-bifunctional protein, because the hydratase/dehydratase reaction was confirmed in previous studies (*23, 24*) (see Scheme 1).

We previously suggested that SCPx and P-44 constitute a second isoform of thiolases, proposing the term type-II 3-oxoacyl-CoA thiolases, because the structural and catalytic properties of SCPx and P-44 are close but less similar to those of other previously known thiolases (*14*). Here, we showed that the type-II thiolase acted on 2-methyl-branched substrates as well as straight chain substrates, although the type-I thiolase acted only on straight chain substrates.

A method for determining the thiolase activity involved in bile acid formation in rat liver has not been established yet, and there has also been no report of the content of the thiolase domain of SCPx in rat liver, although the content of sterol carrier protein 2, the carboxyl terminal part of SCPx, has been estimated by means of a quantitative immunochemical method with an antibody against sterol carrier protein 2: 0.6–0.8 μg/mg of cytosol protein (*25, 26*). Therefore, further study is needed to reveal whether or not SCPx plays a major physiological role in bile acid formation.

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