Substrate Specificities of 3-Oxoacyl-CoA Thiolase A and Sterol Carrier Protein 2/3-Oxoacyl-CoA Thiolase Purified from Normal Rat Liver Peroxisomes

STEROL CARRIER PROTEIN 2/3-OXOACYL-CoA THIOLASE IS INVOLVED IN THE METABOLISM OF 2-METHYL-BRANCHED FATTY ACIDS AND BILE ACID INTERMEDIATES

(Received for publication, May 20, 1997, and in revised form, July 30, 1997)

Vasily D. Antonenkova, Paul P. Van Veldhovenb, Etienne Waelkens, and Guy P. Mannaaertsa

From the Katholieke Universiteit Leuven, Departement Moleculaire Celbiologie, Afdeling Farmacologie en Biochemie, Campus Gasthuisberg (O & N), Herestraat 49, B-3000 Leuven, Belgium

The two main thiolase activities present in isolated peroxisomes from normal rat liver were purified to near homogeneity. On sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the first enzyme preparation displayed a single band of 41 kDa that was identified as 3-oxoacyl-CoA thiolase A (thiolase A) by N-terminal amino acid sequencing. The second enzyme preparation consisted of a 58- and a 46-kDa band. The 58-kDa polypeptide reacted with antibodies raised against either sterol carrier protein 2 or the thiolase domain of sterol carrier protein 2/3-oxoacyl-CoA thiolase (SCP-2/thiolase), formerly also called sterol carrier protein X, whereas the 46-kDa polypeptide reacted only with the antibodies raised against the thiolase domain. Internal peptide sequencing confirmed that the 58-kDa polypeptide is SCP-2/thiolase and that the 46-kDa polypeptide is the thiolase domain of SCP-2/thiolase. Thiolase A catalyzed the cleavage of short, medium, and long straight chain 3-oxoacyl-CoAs, medium chain 3-oxoacyl-CoAs being the best substrates. The enzyme was inactive with the 2-methyl-branched 3-oxo-2-methylpalmitoyl-CoA and with the bile acid intermediate 24-oxo-trihydroxycoprostanoyl-CoA. SCP-2/thiolase was active with medium and long straight chain 3-oxoacyl-CoAs as well as medium chain 3-oxoacyl-CoAs being the best substrates. The enzyme was inactive with the 2-methyl-branched 3-oxo-2-methylpalmitoyl-CoA and with the bile acid intermediate 24-oxo-trihydroxy-coprostanoyl-CoA. SCP-2/thiolase was active with medium and long straight chain 3-oxoacyl-CoAs but also with the 2-methyl-branched 3-oxoacyl-CoA and the bile acid intermediate. In peroxisomal extracts, more than 90% of the thiolase activity toward straight chain 3-oxoacyl-CoAs was associated with thiolase A. Kinetic parameters (Km and Vmax) were determined for each enzyme with the different substrates.

Our results indicate the following: 1) the two (main) thiolases present in peroxisomes from normal rat liver are thiolase A and SCP-2/thiolase; 2) thiolase A is responsible for the thiolytic cleavage of straight chain 3-oxoacyl-CoAs; and 3) SCP-2/thiolase is responsible for the thiolytic cleavage of the 3-oxoacyl-CoA derivatives of 2-methyl-branched fatty acids and the side chain of cholesterol.

Thiolases catalyze the thiolytic cleavage of 3-oxoacyl-CoAs in an acyl-CoA shortened by two carbon atoms and acetyl-CoA (propionyl-CoA in the case of 2-methyl-branched acyl-CoAs). Although reversible, the reaction is thermodynamically unfavorable in the direction of condensation. Nevertheless, condensation occurs in vivo in such biosynthetic processes as cholesterol synthesis, ketone body synthesis, and fatty acid elongation. Usually, thiolases are divided into two groups: acetocetyl-CoA thiolases and 3-oxoacyl-CoA thiolases (1). Acetoacetyl-CoA thiolases cleave only acetoyctyl-CoA, and in vivo they seem to work preferably in the direction of condensation, catalyzing, for example, the first reaction in cholesterol and ketone body synthesis (2–6). 3-Oxoacyl-CoA thiolases act on 3-oxoacyl-CoAs of different chain length and catalyze the last step in mitochondrial and peroxisomal β-oxidation (4, 7–12).

A first peroxisomal 3-oxoacyl-CoA thiolase was purified from livers of rats treated with the peroxisome proliferator di(2-ethylhexyl)phthalate by Hashimoto and co-workers (10). It is active with short, medium, and long straight chain 3-oxoacyl-CoAs, medium chain CoA esters being the best substrates (11). The enzyme, which was subsequently cloned, consists of two identical subunits of 41 kDa. The subunit is synthesized as a 44-kDa precursor containing a 26-amino acid N-terminal leader sequence that functions as a peroxisome targeting signal and that is cleaved off after import of the polypeptide in the peroxisome (13). Some years later Hashimoto and co-workers (14) and independently Bodnar and Rachubinski (15) found that the rat genome contains not one but two closely related thiolase genes, gene B encoding the precursor of the previously purified peroxisomal thiolase (thiolase B)1 and gene A encoding a thiolase (thiolase A) precursor with a 10-residue longer N-terminal presequence. In their mature form thiolases A and B differ in only 6 (14) or 9 (15) amino acid residues. mRNA analysis revealed that the gene is constitutively transcribed albeit at a low level and that transcription of the B gene is strictly dependent on activation of the gene by a peroxisome proliferator (14, 15). Thiolase A has not been purified yet, so it is not known how far its properties differ from those of the inducible thiolase B.

Peroxisomes also contain a 58-kDa protein that cross-reacts...
with antibodies raised against SCP-2. Molecular cloning of the protein revealed that it consists of an N-terminal 404 amino acid domain that shows homology with a number of thiolases and a C-terminal 143 amino acid domain that is identical to the N-terminal presence (20 amino acids) and mature sequence (123 amino acids) of SCP-2(16). The 58-kDa protein, which was called SCP-X, and SCP-2 are transcribed from a single gene that contains two independent promoters (17). SCP-X has not been purified in its active form but the recombinant protein posses 3-oxoacyl-CoA thiolase activity and displayed a straight chain length specificity that is roughly similar to that of thiolase B (18). In view of its thiolase activity, Seedorf et al. (18) proposed to rename the protein SCP-2/thiolase. Related to SCP-2/thiolase is a peroxisomal 46-kDa protein that cross-reacts with antibodies raised against various epitopes of the thiolase domain of SCP-2/thiolase but not with antibodies raised against SCP-2 (19). The data suggest that the 46-kDa protein may originate from SCP-2/thiolase by intraperoxisomal proteolytic cleavage, possibly at the SCP-2/presence-mature sequence transition (19).

Peroxisomes β-oxidize a wide variety of substrates including straight chain fatty acids, 2-methyl-branched fatty acids (e.g. pristanic acid), and the side chain of the bile acid intermediates di- and trihydroxycoprostanic acids (for reviews, see Refs. 20–22). We have recently found that peroxisomes contain several β-oxidation pathways with different substrate specificities. In rat liver, for example, straight chain acyl-CoAs are desaturated by palmitoyl-CoA oxidase (23), and their enoyl-CoAs are then converted to 3-oxoacyl-CoAs by MFP-1, which forms (hydration) and dehydrogenates 1,3(3S)-hydroxoyacyl-CoAs (24, 25). In contrast, 2-methyl-branched fatty acyl-CoAs and the CoA esters of the bile acid intermediates di- and trihydroxycoprostanic acids are desaturated by pristanoyl-CoA oxidase and trihydroxycoprostanoyl-CoA oxidase, respectively (23), and their enoyl-CoAs are then converted to the corresponding 3-oxoacyl-CoAs by MFP-2, which forms and dehydrogenates 1,3(3R)-hydroxoyacyl-CoAs (24–26).

The present study aimed at elucidating the functional role of the different peroxisomal 3-oxoacyl-CoA thiolases catalyzing the last step in β-oxidation. We purified the two main thiolases present in peroxisomal fractions from normal rat liver and comparatively investigated their substrate specificity. The enzymes proved to be thiolase A and SCP-2/thiolase. Using various substrates, we could demonstrate that thiolase A cleaves straight chain 3-oxoacyl-CoAs, whereas SCP-2/thiolase is responsible for the cleavage of the 3-oxoacyl-CoAs of 2-methyl-branched fatty acids and the bile acid intermediates.

**EXPERIMENTAL PROCEDURES**

**Materials**—Acetyl-CoA, acetoacetyl-CoA, medium chain acyl-CoAs, acyl-CoA oxidase (from *Arthrobacter sp.*), 3-hydroxyacyl-CoA dehydrogenase (from porcine heart), crotonase (from bovine liver), and bovine serum albumin were obtained from Sigma. Palmitoyl-CoA, CoA, DEAE-Sephacel (fast flow), and Blue Sepharose CL-6B were obtained from Pharmacia Biotech Inc. Oxaloacetate, pyruvate, malate dehydrogenase (from pig liver), lactate dehydrogenase (from pig heart), NAD, NADH, and conjugated antibodies were from Boehringer Mannheim. Cellulose phosphate, type P-11, was from Whatman. Econo-Pac hydroxyapatite cartridges were from Bio-Rad. DTNB and benzamidine were purchased from Janssen Chimica, and C18 silica Bondelut (500 mg; 3 ml) cartridges were from Varian Benelux. Ultrafiltration devices (Centriprep) with a molecular mass cutoff of 10,000 were obtained from Amicon. Methyl-coenzyme A (CoA) (10 μM, final concentration) and applied to a phosphocellulose column eluted with a

2 J. Adamski, F. Leenders, D. Novikov, P. P. Van Veldhoven, and G. P. Mannaerts, unpublished work.
linear gradient of KCl. Peroxisomal thiolases were not retained under these conditions, whereas traces of contaminating mitochondrial thiolases (3-oxoacyl-CoA thiolase and acetoacetyl-CoA thiolase) were bound to the column. Separation of peroxisomal thiolases was achieved by DEAE-Sepharose chromatography. Thiolase A was eluted with the flow-through fractions, which were intensively dialyzed for the removal of CoA before reloading onto a phosphocellulose column. In the absence of CoA thiolase A bound to the column. The enzyme was eluted with a KCl gradient and passed through a Sephacryl-S-100-HR column for final purification. SCP-2/thiolase was bound to the DEAE-Sepharose column and was eluted with increasing KCl concentrations. The enzyme preparation was then subjected to hydroxylapatite chromatography with KCl as the eluent. In the final purification step SCP-2/thiolase was passed through a Blue-Sepharose column, and the bound activity was eluted with a linear gradient of CoA (0–100 μM).

Enzyme purification was monitored by measuring the thiolase activities (acetoacetyl-CoA and 3-oxoactoacyl-CoA as the substrates) and by analyzing the eluates by SDS-PAGE followed by immunoblotting with antibodies raised against SCP-2 or the thiolase domain of SCP-2/thiolase (kindly provided by Prof. K. Wirtz). Final identification of the thiolases was performed by peptide sequencing of the purified enzymes. The complete purification procedure for peroxisomal thiolases, subunit structure of SCP-2/thiolase, and the effect of CoA at low concentrations on the chromatographic behavior of the thiolases will be discussed in full detail in a separate publication.

MFP-1 and MFP-2 were purified from rat liver peroxisomes as described previously (24).

**Enzyme Assays**—Two different methods were used for the detection of thiolase activity in the direction of 3-oxoacyl-CoA cleavage. First, the enzymes were assayed by measuring the increase in absorption at 233 nm due to the formation of new thioester bonds (ε, 4,500 M⁻¹ cm⁻¹) (9). The reaction mixture (25 °C) routinely consisted of 50 mM Tris-Cl, pH 8.0, 60 μM CoA, and 10–60 μM oxo compound. Second, thiolase activity was also determined in terms of Mg²⁺-enolate complex disappearance (303 nm) as a result of the thiolytic cleavage (4, 7). The assay mixture (25 °C) contained 50 mM Tris-Cl, pH 8.0, 5 mM MgCl₂, albumin, CoA, and 3-oxoacyl-CoA as indicated. The extinction coefficients for the Mg²⁺-enolate complexes were determined by measuring the increase in absorbance at 303 nm after addition of acetyl-CoA oxidase to the incubation mixture for the synthesis of the oxo compounds (see above) and by measuring the concentration of the 3-oxoacyl-CoA in the same samples by means of the hydroxylamine/DTNB test. Since measurements of the Mg²⁺-enolate complex disappearance resulted in an underestimation of the thiolytic cleavage rates when short and medium chain substrates were used⁴ and since 2-methyl-branched oxo compounds such as 3-oxo-2-methylpalmitoyl-CoA and 24-oxo-THC-CoA do not form Mg²⁺-enolate complexes (28), the Mg²⁺-enolate methad was used in only a limited number of experiments (see Fig. 7) with 3-oxoalmitoyl-CoA (ε, 8,900 M⁻¹ cm⁻¹) and 3-oxoheptadecanoyl-CoA (ε, 10,600 M⁻¹ cm⁻¹), 3-Hydroxyacyl-CoA dehydrogenase (24), crotonase (24), and acyl-CoA oxidase (30) activities were assayed as described.

Units of enzyme activity are expressed as μmol of substrate utilized or product formed per min.

**Analysis of Acyl-CoA Thioesters by HPLC**—Products of the thiolytic cleavage of 3-oxo-2-methylpalmitoyl-CoA and 24-oxo-THC-CoA were analyzed at 25 °C on a Waters dual-pump gradient system by using an Econosphere C₁₈ reverse-phase column (150 × 4.6 mm). Separation of the acyl-CoA thioesters was achieved by linearly increasing the acetonitrile content of the 50 mM potassium phosphate, pH 5.5, elution buffer (see legends to figures for more details).

**Preparation of Antibodies**—Antibodies against thiolase A were prepared as follows: enzyme samples were subjected to preparative SDS-PAGE, and after transient visualization of the proteins (31), the 41-kDa protein was excised and injected into rabbits according to standard procedures (32).

**Amino Acid Sequencing**—For N-terminal sequencing of the purified thiolase A, the enzyme was digested with 0.5% trypsin in 20 mM potassium phosphate, pH 7.8, and concentrated. The protein sample was then collected on a ProSorb membrane (Perkin-Elmer) and subjected to automated Edman degradation using a Procise 492 sequenator (Applied Biosystems). Diffi-

---

3 V. D. Antonenkov, P. P. Van Veldenhoven, and G. P. Mannaerts, unpublished work (see also Ref. 10).  
4 V. D. Antonenkov, P. P. Van Veldenhoven, and G. P. Mannaerts, manuscript in preparation.  
5 V. D. Antonenkov, P. P. Van Veldenhoven, and G. P. Mannaerts, unpublished work.  
6 V. D. Antonenkov, P. P. Van Veldenhoven, and G. P. Mannaerts, unpublished work.
during maturation thiolase A is not cleaved at the same site as thiolase B but three amino acids downstream, or 2) the mature thiolase A loses its first three amino acids by proteolysis during purification. The purified thiolase A preparation was also used for the production of polyclonal antibodies (see Fig. 1, panel B).

The second enzyme preparation contained two bands on SDS-PAGE, a 58- and a 46-kDa band (Fig. 1, panel A). Both bands cross-reacted with antibodies raised against the thiolase domain of SCP-2/thiolase (Fig. 1, panel B). Only the 58-kDa polypeptide reacted with antibodies raised against SCP-2 (data not shown). The sequences of two tryptic peptides of the 58-kDa protein (Phe-Met-Lys-Pro-Gly-Glu-Asn-Ser-Arg and Ile-Ala-Gly-Asn-Met-Gly-Leu-Ala-Thr-Gly-Leu-Ala, which starts at Gly-354 of the thiolase domain of SCP-2/thiolase, respectively). Peptide sequencing of the 46-kDa protein yielded the sequence Gly-His-Pro-Leu-Gly-Ala-Thr-Gly-Leu-Ala-Thr-Gly-Leu-Ala, which starts at Gly-354 of the thiolase domain of SCP-2/thiolase. These data confirm that the 58-kDa polypeptide is SCP-2/thiolase and that the 46-kDa polypeptide is its N-terminal thiolase part. Molecular sieving experiments indicated that the enzyme may be present in the cell as a mixture of three dimeric isoforms consisting of homo- and heterodimeric combinations of the 58- and 46-kDa subunits.

The 58-kDa polypeptide has been purified from rat liver also by others but under denaturing conditions (16). The recombinant 58-kDa protein expressed in Escherichia coli possessed thiolase activity with straight chain 3-oxoacyl-CoAs (18). Like the recombinant protein, our purified enzyme was fully active as a thiolase (see below).

Thiolase Activities with Straight Chain 3-Oxoacyl-CoAs—The short chain acetoacetyl-CoA was a good substrate for the purified thiolase A (see Table I) but a poor substrate for the purified SCP-2/thiolase (1.3 μmol/min·mg protein; n = 3). Further measurements with substrates of increasing chain length showed that the two thiolases display optimum activity with medium chain 3-oxoacyl-CoAs (C10 for thiolase A; C8 for SCP-2/thiolase; Fig. 2). At chain lengths of C12 and longer, substrate inhibition occurred at elevated 3-oxoacyl-CoA concentrations with both enzymes. The substrate inhibition, which was particularly marked with 3-oxopalmityl-CoA, could be abolished by the addition of albumin. The more polar 3-oxohexadecanediyl-CoA did not induce substrate inhibition of either of the thiolases (data not shown).

Thiolase Activity with 2-Methyl-branched 3-Oxoacyl-CoA—In rat peroxisomes straight chain acyl-CoAs are oxidized via palmityl-CoA oxidase and MFP-1, which displays enoyl-CoA hydratase and L-3(3S)-hydroxyacyl-CoA dehydrogenase activities (23, 24), whereas 2-methyl-branched acyl-CoAs are oxidized via pristanoyl-CoA oxidase (23) and MFP-2, which displays enoyl-CoA hydratase and δ-3(3R)-hydroxyacyl-CoA dehydrogenases activities (26). One of the aims of this study was to investigate whether straight chain acyl-CoAs and 2-methyl-branched acyl-CoAs would be metabolized also by separate thiolases. The main obstacle for revealing the thiolase involved in branched chain fatty acid breakdown is the chemical synthesis of the appropriate substrates. However, we found that commercially available mitochondrial 3-hydroxyacyl-CoA dehydrogenase is capable of catalyzing the synthesis of 3-oxo-2-methylpalmityl-CoA from racemic 3-hydroxy-2-methylpalmityl-CoA. During incubation of the 3-hydroxyacyl-CoA with the enzyme, NADH reduction went hand in hand with the appearance of a new CoA derivative as revealed by reverse phase HPLC analysis (Fig. 3, panel A). The compound was rapidly cleaved by low concentrations (25 mM) of neutral hydroxylamine, whereas other CoA derivatives were unaffected.

| Substrate                  | Apparent Kₘ (μM) | Apparent Vₘₐₓ (μmol/min·mg protein) |
|----------------------------|------------------|-------------------------------------|
| 3-Oxoacyl-CoA               | CoA              |
| Acetoacetyl-CoA             | 7.7              | 7.8                                 |
| 3-Oxooctanoyl-CoA           | 9.1              | 9.0                                 |
| 3-Oxopalmitoyl-CoA          | 7.8*             | 4.5                                 |
| 3-Oxoheptadecanediyl-CoA    | 3.4              | 8.5                                 |
| 3-Oxo-2-methylpalmitoyl-CoA | No detectable activity |
| 24-Oxo-THC-CoA              | No detectable activity |
| SCP-2/thiolase              |                  |
| 3-Oxooctanoyl-CoA           | 4.6              | 2.7                                 |
| 3-Oxopalmitoyl-CoA          | 4.0*             | 11.2                                |
| 3-Oxoheptadecanediyl-CoA    | 2.9              | 7.1                                 |
| 3-Oxo-2-methylpalmitoyl-CoA | 3.0*             | 1.7                                 |
| 24-Oxo-THC-CoA              | 2.8*             | 18.0                                |

**TABLE 1** Apparent kinetic constants of peroxisomal thiolase A and SCP-2/thiolase

Activity of the purified enzymes was measured at 233 nm without albumin. For the determination of the Kₘ and Vₘₐₓ values for the 3-oxoacyl-CoAs, the concentration of CoA was fixed at 60 μM except for thiolase A and 3-oxopalmityl-CoA, where the CoA concentration was 20 μM due to its significant inhibiting effect at higher concentrations. The apparent Kₘ values for CoA were determined at 10 μM fixed concentrations of the oxocompounds (25 μM for acetoacetyl-CoA). Kₘ and Vₘₐₓ were calculated from Lineweaver-Burk plots. In the case of substrate inhibition, the linear parts of the plots were used. Asterisks indicate the occurrence of substrate inhibition. SCP-2/thiolase was only weakly active with acetoacetyl-CoA (see text).

**FIG. 2.** Chain length specificities of thiolase A (panel A) and SCP-2/thiolase (panel B) with medium straight chain 3-oxoacyl-CoAs. Enzyme activity was measured at 233 nm with 0.02% (w/v) albumin (●) or without (○). Concentrations of 3-oxoacyl-CoAs and CoA were fixed at 15 and 60 μM, respectively. In choosing the 3-oxoacyl-CoA concentrations, a compromise was made between the saturation of the enzyme by the substrates and the inhibiting effect of C12–C14 oxocompounds at higher concentrations.
50 mM Tris-Cl, pH 8.0, at 37 °C. Reactions were terminated by placing the samples on ice and adjusting them to pH 5.5 with 1 M KH₂PO₄. Proteins were removed by means of Centriprep concentrators (Amicon).

**Panel A**

UV-absorbing compound was evident (Fig. 6, panel A) followed by reverse phase HPLC, the appearance of a new compound by HPLC, it was identified as 24-oxo-THC-CoA on the basis of its sensitivity to hydroxylamine (see also Fig. 6, panel A) and the other lines of evidence given above for 3-oxo-2-methylpalmitoyl-CoA. Addition of NAD⁺ resulted in the appearance of a broad absorbance peak that partially coincided with the peaks of the varanoyl-CoA stereoisomers. After further purification of the newly formed compound by HPLC, it was identified as 24-oxo-THC-CoA on the basis of its sensitivity to hydroxylamine (see also Fig. 6, panel A) and the other lines of evidence given above for 3-oxo-2-methylpalmitoyl-CoA (data not shown). Addition of thiolase A and CoA to the above mentioned reaction mixtures containing the newly synthesized oxocompound did not result in any changes in the HPLC pattern (Fig. 6, panel A). However, addi-

**Panel B**

**FIG. 3.** HPLC analysis of the products formed after incubation of enzymatically synthesized 3-oxo-2-methylpalmitoyl-CoA with thiolase A or SCP-2/thiolase. Panel A: a, chemically synthesized racemic 3-hydroxy-2-methylpalmitoyl-CoA; b, same as a but after incubation for 30 min with NAD⁺, an NAD⁺-regenerating system, and 3-hydroxyacyl-CoA dehydrogenase (see “Experimental Procedures”); c, same as b plus 25 mM hydroxylamine; d and e, samples after enzymatic synthesis of the oxocompound were incubated for 10 min in the presence of 60 μM CoA and thiolase A (d) or SCP-2/thiolase (e). Thiolase activity of each enzyme was 0.03 μmol of 3-oxoctanoyl-CoA cleaved per min per sample (1.0 ml). *Asterisk,* 3-oxo-2-methylpalmitoyl-CoA; *arrow,* myristoyl-CoA. HPLC conditions: 0.05 M potassium phosphate, pH 5.5, as an eluent with a linear gradient of 20–45% (v/v) acetonitrile over 30 min at a flow rate of 1 ml/min. Panel B, products formed from purified 3-oxo-2-methylpalmitoyl-CoA. a, sample (1.0 ml) containing 10 μM 3-oxo-2-methylpalmitoyl-CoA and 30 μM CoA; b, same as a after incubation for 10 min in the presence of SCP-2/thiolase (activity: 0.03 μmol per min per sample with 3-oxoctanoyl-CoA as the substrate). Peaks are numbered as follows: 1, CoA; 2, 3-oxo-2-methylpalmitoyl-CoA; 3, myristoyl-CoA; 4, propionyl-CoA. HPLC conditions: 0.05 M potassium phosphate, pH 5.5, as an eluent with a linear gradient of 0–45% (v/v) acetonitrile over 50 min at a flow rate of 1 ml/min. All enzymatic incubations (panels A and B) were carried out in 50 mM Tris-Cl, pH 8.0, at 37 °C. Reactions were terminated by placing the samples on ice and adjusting them to pH 5.5 with 1 M KH₂PO₄. Proteins were removed by means of Centriprep concentrators (Amicon).

**FIG. 4.** The effect of pH on the absorbance spectrum of HPLC-purified 3-oxo-2-methylpalmitoyl-CoA. Curve A, 50 mM Tris/NaOH, pH 12.0; curve B, 50 mM potassium phosphate, pH 5.5.
Substrate Specificities of Peroxisomal Thiolases from Rat Liver

Fig. 5. Velocity of the SCP-2/thiolase reaction as a function of the 3-oxo-2-methylpalmitoyl-CoA concentration. Assays were performed at 293 nm in the presence (○) or absence (□) of 0.02% (w/v) albumin. The CoA concentration was fixed at 60 μM.

Substrate Specificity of Thiolase A and SCP-2/Thiolase—Table I summarizes the kinetic constants obtained for the purified enzymes with the different substrates. As shown in the table both enzymes are optimally active with medium chain 3-oxo-compounds. Interestingly, 3-oxohexadecanoyl-CoA is a better substrate for both thiolas than its monocarboxylic counterpart 3-oxopalmitoyl-CoA. Acetoacetyl-CoA is a good substrate for thiolase A but only a poor substrate for SCP-2/thiolase. More importantly, the 2-methyl-branched compounds 3-oxo-2-methylpalmitoyl-CoA and 24-oxo-THC-CoA are good substrates for SCP-2/thiolase but no substrate for thiolase A, implying that SCP-2/thiolase is responsible for the thiolytic cleavage of 2-methyl-branched fatty acids and the side chain of the bile acid intermediates di- and trihydroxycoprostanic acids. The role of thiolase A and SCP-2/thiolase in the cleavage of straight chain mono- and dicarboxylates will be addressed in the next paragraph.

Thiolase A displayed a pH optimum of 8.0 when acetoacetyl-CoA and 3-oxoctanoyl-CoA were used as the substrates. The pH optimum for SCP-2/thiolase was near 7.6 with 3-oxoctanoyl-CoA as substrate.

The experiments described in the text were carried out with 24-oxo-TC-CoA synthesized by means of MFP-1. MFP-1 hydrates trihydroxycoprostanoyl-CoA to 24S,25S-varanoyl-CoA, but dehydrogenates 24R,25R-varanoyl-CoA, the naturally occurring intermediate in bile acid synthesis (see Ref. 25). We, therefore, repeated a number of key experiments with 24-oxo-THC-CoA, synthesized by means of MFP-2. Results were similar to those obtained with the oxocompound synthesized by MFP-1 (data not shown). The exact configuration of the 25-methyl group of 24-oxo-THC-CoA is not known. Although one would expect the formation of 24-oxo-(25R)-THC-CoA from racemic varanoyl-CoA by both MFP-1 and MFP-2, preliminary evidence obtained by us7 and others (47) indicates that a spontaneous (25R) ↔ (S) conversion of 24-oxo-THC-CoA may occur, probably via a planar enol tautomer formation. CoA and 3-oxo-2-methylpalmitoyl-CoA. The pH profile with 24-oxo-THC-CoA was complex, showing a shoulder at pH 7.2–8.2 but increasing further at higher pH.

Contribution of Thiolase A and SCP-2/Thiolase to the Peroxisomal Cleavage of Straight Chain 3-Oxoaoyl-CoAs—Since both thiolase A and SCP-2/thiolase catalyze the cleavage of straight chain 3-oxoaoyl-CoAs, we wanted to determine the contribution of each enzyme to the overall reaction. Therefore, peroxisomal matrix proteins released by sonication of a purified peroxisomal fraction were passed through a phosphocellu-
lose column that binds traces of contaminating mitochondrial thiolas,
and the flow-through fraction containing the peroxi-
somal thiolas was separated on a DEAE-Sepharose column.
As illustrated by immunoblot analysis, thiolase A was eluted
first with the flow-through volume, whereas SCP-2/thiolase,
consisting of the 58- and 46-kDa polypeptides, was eluted with
increasing KCl concentrations (Fig. 7, upper panel). As could be
expected, thiolytic activity with 3-oxo-2-methylpalmitoyl-CoA
was recovered exclusively in the fractions containing SCP-2/
thiolase, whereas the activity with acetoacetyl-CoA was con-
fined to the fractions containing thiolase A (Fig. 7, lower panel).
Thiolytic activity with 3-oxo-octanoyl-CoA, 3-oxopalmitoyl-CoA,
and 3-oxohexadecanediyl-CoA displayed a bimodal distri-
bution. By far the major (>90%) portion of the activity was eluted
with the thiolase A peak, whereas the small remaining part
was eluted with the SCP-2/thiolase peak. Since recoveries ver-
sus the original whole peroxisome preparation were essentially
the same for both enzymes as illustrated by the comparable
recoveries obtained with 3-oxo-octanoyl-CoA (40%) and 3-oxo-2-
methylpalmitoyl-CoA (44%) and since the two enzymes display
similar Km values for the different substrates (Table I), one can
conclude that the activity profile of the DEAE column reflects
the situation in the intact liver cell and that thiolase A is
responsible for the bulk of the thiolytic cleavage of straight
chain 3-oxoacyl-CoAs.

**DISCUSSION**

Our present results indicate that in normal rat liver, thiolase
A is responsible for the peroxisomal thiolytic cleavage of
straight chain mono- and dicarboxylic 3-oxoacyl-CoAs, whereas
SCP-2/thiolase cleaves the 3-oxoacyl-CoAs of 2-methyl-
 branched fatty acids (the synthetic 2-methylpalmitic acid and,
as a consequence, most probably also the naturally occurring
pristanic acid) and of the bile acid intermediates di- and trihy-
droxyprostanic acids, which also possess a 2-methyl branch
in their side chain. These data complete our earlier results
which specifically forms (hydration) and dehydrogenatesD-
and 3-hydroxy derivatives (24, 25). Finally, the 3-oxo-2-methylacyl-
CoAs are cleaved by SCP-2/thiolase (present paper), yielding acetyl-CoA and an acyl-
CoA shortened by two carbon atoms.

In rat liver, the bile acid intermediate trihydroxyprostan-
yl-CoA is desaturated by trihydroxyprostanoyl-CoA oxidase,
which specifically dehydrogenates (25S)-trihydroxyprostan-

**FIG. 7. Separation of thiolase A and SCP-2/thiolase by DEAE
anion exchange chromatography.** Upper panel, immunoblot analy-
sis of thiolas after DEAE-Sepharose chromatography. After separa-
tion by SDS-PAGE, proteins contained in 100 μl of every second column
fraction (starting at fraction 5) were transferred to nitrocellulose
and incubated with antibodies raised against the thiolase domain of SCP-
thiolase (panel A) and thiolase A (panel B). The migration of molecular
mass markers (in kDa) is indicated at the left side of the figure.
Lower panel, column eluate fractions were analyzed for thiolase activity
in 50 mM Tris-Cl, pH 8.0, with 60 μM CoA and the following substrates
recoveries (from the column are indicated in parentheses): panel A, 50 μM
3-oxo-octanoyl-CoA (94%); panel B, 15 μM 3-oxo-2-methylpalmitoyl-
CoA (98%); panel C, 50 μM acetacetyl-CoA (107%); panel D, 25 μM
3-oxopalmityl-CoA (115%); panel E, 15 μM 3-oxohexadecanediyl-CoA
(102%). Panel F indicates the absorbance at 280 nm and the concentra-
tion of the KCl gradient. Enzyme activity was measured at 233 nm in
the presence of 0.02% (w/v) albumin (3-oxo-octanoyl-CoA and 3-oxo-2-
methylpalmitoyl-CoA) or without albumin (acetacetyl-CoA). Activity
with 3-oxopalmityl-CoA (in the presence of 0.05% albumin) and 3-
oxohexadecanediyl-CoA (in the absence of albumin) was measured at
303 nm with 5 mM MgCl2. At this concentration, Mg2
ligands did not affect the
activity of thiolase A and SCP-2/thiolase with 3-oxopalmityl-CoA and
3-oxohexadecanediyl-CoA (data not shown).
Substrate Specificities of Peroxisomal Thiolases from Rat Liver

**FIG. 8.** Organization of the β-oxidation of long straight chain fatty acids, 2-methyl-branched fatty acids, and the bile acid intermediates di- and trihydroxycoprostanic acids in rat liver peroxisomes. Varanoyl-CoA is the 3-hydroxyacyl-CoA intermediate of trihydroxycoprostanoyl-CoA, in which the 24-hydroxyl and 25-methyl groups correspond to 3-hydroxy and 2-methyl groups, respectively, in the side chain. In the rat, palmitoyl-CoA oxidase and pristanoyl-CoA oxidase are expressed in liver and extrahepatic tissues; trihydroxycoprostanoyl-CoA oxidase is expressed only in liver and (to a minor extent) in kidney. In the human, branched chain fatty acids and the bile acid intermediates are desaturated by branched chain acyl-CoA oxidase, which is the human homolog of trihydroxycoprostanoyl-CoA oxidase. The branched chain acyl-CoA oxidase is expressed, like the human palmitoyl-CoA oxidase, in liver and extrahepatic tissues (22, 45). Although the pristanoyl-CoA oxidase gene is present in the human genome, its product cannot be detected in adult human tissues (49). Only one peroxisomal thiolase gene is present in the human (46). MFP-1 and MFP-2 and the peroxisomal thiolase(s) are expressed in liver and extrahepatic tissues in the rat and the human (22).

24-trans-trihydroxycoprostenoyl-CoA to 24-trans-trihydroxycoprostenoyl-CoA (37). A peroxisomal racemase activity already mentioned above also catalyzes the (25R) → (S) conversion of trihydroxycoprostanoyl-CoA (48). 24-trans-Trihydroxycoprostenoyl-CoA is then converted to 24-oxo-(25R)-trihydroxycoprostanoyl-CoA\(^7\) by MFP-2, which forms (hydration) and dehydrogenates the intermediate (24R,25R)-varanoyl-CoA (25). (The 24 and 25 positions correspond to the 3 and 2 positions in the side chain of trihydroxycoprostanoyl-CoA, respectively.) Subsequently, 24-oxo-trihydroxycoprostanoyl-CoA is cleaved by SCP-2/thiolase (present paper) yielding propionyl-CoA and choleoyl-CoA.

MFP-2 and SCP-2/thiolase metabolize not only the branched chain fatty acids and the bile acid intermediates but also straight chain fatty acids, which because of their relative excess in concentration might seriously hinder the efficient breakdown of the branched carboxylates. One might speculate, therefore, that the β-oxidation pathways described above are catalyzed by multienzyme complexes, consisting of an association of the appropriate oxidase, MFP and thiolase, and in which the intermediates are channeled from one enzyme to the other without being released into the surrounding matrix. If this picture holds, the substrate specificity of the complex would be determined by the substrate specificity of the oxidase, and the straight chain fatty acid intermediates would be prevented from having access to MFP-2 or SCP-2/thiolase.

Our results also confirm the earlier contention (from mRNA analysis, see Refs. 14 and 15) that in normal rat liver thiolase A is the prevailing enzyme. A very recent comparison of the substrate spectrum of thiolase A with that of the closely related inducible thiolase B, which is the prevailing enzyme in livers from rats treated with peroxisome proliferators, revealed that there are no essential differences.4

Finally, a portion of SCP-2/thiolase appears to be present as cleavage products: the 46-kDa N-terminal thiolase domain and, presumably, the C-terminal domain that is identical to SCP-2. Interestingly, two other peroxisomal β-oxidation enzymes also occur in a partially cleaved form. Palmitoyl-CoA oxidase consists of a mixture of subunits (A\(_1,\) ABC, B\(_2C_2\)), in which the B (52 kDa) and C (20 kDa) subunits originate by intraperoxisomal proteolytic cleavage of the A (72 kDa) subunit (39, 40). Likewise, a portion of MFP-2 (79 kDa) is cleaved in the peroxisome in an N-terminal 34-kDa polypeptide that comprises the n-3-hydroxyacyl-CoA dehydrogenase (and 17-β-hydroxysteroid dehydrogenase) domain and a C-terminal 45-kDa polypeptide that consists in its N-terminal part of the 2-enoyl-CoA hydratase domain and in its C-terminal part of a domain that displays similarity with SCP-2 (41, 42). It is presently unknown whether the parent polypeptides and their cleavage products display differences in substrate specificity. Separation of the cleavage products from the parent molecules or separate expression of the cleavage products and parent molecules will be required to obtain such knowledge. SCP-2 is known to bind sterols (reviewed in Ref. 43) and acyl-CoAs (44). This raises the question as to whether the C-terminal SCP-2 domain of SCP-2/thiolase and the C-terminal domain of MFP-2, which is similar to SCP-2, are involved in the binding of certain substrates or in intermediate channeling from one enzyme to the other.

**Acknowledgments**—We are grateful to Prof. Dr. Karel W. A. Wirtz from the State University of Utrecht, The Netherlands, for providing us with the antibodies against the SCP-2 and the thiolase domain of SCP-2/thiolase. We thank S. Asselberghs, L. Govaert, and C. Brees for expert technical assistance and M. Barel for dedicated secretarial help.

**REFERENCES**

1. Gehring, U., and Lynen, F. (1972) in *The Enzymes* (Boyer, P. D., ed, 3rd Ed, Vol. 7, pp 391–405, Academic Press, New York.
2. Huth, W., Dierich, C., Oeynhausen, V., and Seubert, W. (1973) Hoppe-Seyler's *Z. Physiol. Chem.* 354, 635–649.
3. Clinkenbeard, K. D., Sugiyama, T., Moss, J., Reed, W. D., and Lane, M. D. (1973) *J. Biol. Chem.* 248, 2275–2284.
4. Middleton, B. (1974) *Biochem. J.* 132, 717–730.
5. Middleton, B. (1977) *Biochem. J.* 139, 109–121.
6. Huth, W., Jonas, R., Wunderlich, I., and Seubert, W. (1975) *Eur. J. Biochem.* 59, 475–489.
7. Seubert, W., Lamberts, I., Kramer, R., and Ohy, B. (1968) *Biochim. Biophys. Acta* 164, 498–517.
8. Staal, H., Binstock, J. F., and Schulz, H. (1978) *J. Biol. Chem.* 253, 1827–1831.
