Sterol carrier protein X (SCPx) is a peroxisomal branched-chain beta-ketothiolase specifically reacting with 3-oxo-pristanoyl CoA: e newe, unique role for SCPx in branched-chain fatty acid metabolism in peroxisomes

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Sterol Carrier Protein X (SCPx) Is a Peroxisomal Branched-Chain β-Ketothiolase Specifically Reacting with 3-Oxo-pristanoyl-CoA: A New, Unique Role for SCPx in Branched-Chain Fatty Acid Metabolism in Peroxisomes

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One of the most important functions of peroxisomes, at least in humans, is the β-oxidation of a range of different fatty acids and fatty acid derivatives. Recent studies have shown that the enzymatic machinery required for the β-oxidations of these substrates, may be much more complex as originally thought. We now report that the conventional peroxisomal thiolase which has sofar been thought to catalyze the thiolytic cleavage of the 3-oxoacyl-CoA esters of all fatty acids oxidized in peroxisomes, shows poor reactivity towards the 3-oxoacyl-CoA esters of 2-methyl branched-chain fatty acids such as pristanic acid. Our data further show, that SCPx, a 58 kDa protein with both thiolase and sterol carrier protein activity but unknown function sofar, readily reacts with 3-oxopristanoyl-CoA. Taken together, our data show that SCPx plays a central role in branched chain fatty acid β-oxidation in peroxisomes. This finding has major implications not only for the functional organization of the peroxisomal β-oxidation system but also for studies dealing with the resolution of the underlying defect in patients with some defect in peroxisomal β-oxidation.

Sterol carrier protein 2 (SCP2), alternatively called non-specific lipid transfer protein (nsLTP) is a small basic protein assumed to participate in the intracellular transport of sterols and other lipids [1]. Indeed, SCP2 has been found to promote the exchange of various lipids and sterols between membranes, stimulates the enzymatic conversion of 7-dehydrocholesterol to cholesterol in microsomes and also the acyltransferase mediated esterification of intracellular cholesterol [1]. Moreover, in steroid hormone producing cells SCP2 is thought to facilitate the transport of cholesterol to mitochondria, where the first committed step in the formation of pregnenolone takes place [2-5].

SCP2 has been purified from a variety of sources and its structure appears to be highly conserved across mammalian species [1]. SCP2 is synthesized on free polysomes as a larger precursor polypeptide of 14.5 kDa [6,7] with a Type 1 Peroxisome Targeting Sequence (PTS1) at its carboxy-terminus [8-13]. This ensures uptake of pre-SCP2 into peroxisomes followed by processing to the mature 13.2 kDa SCP2 inside peroxisomes which explains its predominant if not exclusive localization in peroxisomes [14-17]. Several lines of evidence pointed to the existence of a SCP2-related protein. Firstly, the antibody raised against SCP2 was found to crossreact with a higher molecular mass protein of 58 kDa [6,7,14-16]. In addition, the livers of all mammalian species studied contain SCP2-related transcripts encoding for larger proteins that were found to be identical to pre-SCP2 at their C-terminal domains [8-13; 18-21]. It is now clear that the 58 kDa protein called SCPx [1] and the 14.5 kDa pre-SCP2 are the products of *different* mRNAs produced from the *same* gene which has two promoters [22]. Interestingly, significant sequence homology was found between the first 400 amino acid residues of SCPx and both mitochondrial and peroxisomal 3-oxoacyl-CoA thiolase [11,18,23]. Direct activity measurements showed that SCPx, indeed, possesses 3-oxoacyl-CoA thiolase activity next to its intrinsic sterol carrier protein and lipid transfer activity [24]. The substrate specificity of SCPx, however, was found not to differ much from that of the

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conventional peroxisomal thiolase characterized by Hashimoto and coworkers [25].

Until recently it was believed that one set of peroxisomal β-oxidation enzymes would be responsible for the peroxisomal β-oxidation of the large variety of fatty acids and fatty acid derivatives known to be β-oxidized in peroxisomes. Recent studies, however, have shed new light on the pathway of fatty acid oxidation in peroxisomes and have shown that at least the first step in the β-oxidation of straight-chain and 2-methyl branched-chain fatty acids is catalyzed by *distinct* acyl-CoA oxidases (see [26] for review). Elaborating on this work we now provide strong evidence against the concept of a single peroxisomal thiolase catalyzing the thiolytic cleavage of all kinds of different 3-ketoacyl-CoA esters in peroxisomes. We show that 3-ketoacyl-CoA esters of 2-methyl branched-chain fatty acids can not be handled by the conventional, clofibrate-inducible thiolase [25] but instead by SCPx (SCP2/3-oxoacyl-CoA thiolase). These results provide further evidence for the existence of two distinct β-oxidation systems in peroxisomes.

MATERIALS AND METHODS

Preparation of peroxisomes from control and di-(2-ethylhexyl) phthalate (DEHP) treated rats. Male Wistar rats (200-250 g) were fed a standard laboratory diet with or without 1% (w/v) DEHP for 9 days as described before [27]. Livers were subsequently removed, finely minced and homogenized using a standard isolation medium containing 250 mM sucrose, 2 mM EDTA and 5 mM Mops-NaOH (final pH 7.4). The resulting homogenate was subjected to differential centrifugation to prepare a light-mitochondrial fraction which was then applied to a Nycodenz density gradient prepared as described elsewhere [28]. After centrifugation the gradient was unloaded and marker enzyme activities (catalase for peroxisomes, glutamate dehydrogenase for mitochondria, esterase for microsomes) were measured in the fractions obtained. The fractions highest in catalase activity contain peroxisomes of high purity (>98%) [27] and were used as such.

Enzyme activity measurements. Catalase was measured spectrophotometrically using a newly developed method based on the peroxidative action of catalase at high ethanol concentrations. The assay medium contained the following standard components: 100 mM potassium phosphate (pH 7.4), 0.05% (w/v) Triton X-100, 10% (v/v) ethanol, 10 mM pyrazol, 1 mM NAD and 1 U/ml aldehyde dehydrogenase. Reactions were started by the addition of 5 mM H_2O_2 and the absorbance at 340 nm was followed in time.

Acyl-CoA oxidase activities were measured as described before [29] using palmitoyl-CoA and pristanoyl-CoA as substrates and the homovanillic acid/peroxidase system to monitor formation of H_2O_2. See [29] for full details.

Thiolase activity measurements were performed as described below using 3-oxopalmitoyl-CoA and 3-oxopristanoyl-CoA as substrates. The reaction mixture contained the following standard components: 100 mM Tris/HCl pH 8.0, 70μM CoASH, 25 μM 3-oxopalmitoyl-CoA or 10 μM 3-oxopristanoyl-CoA (unless otherwise indicated) with or without 4 μM defatted bovine serum albumin (Sigma Co., St. Louis, USA). Reactions were started by the addition of enzyme preparation (purified peroxisomes, purified peroxisomal thiolase I [25] or purified SCPx [24]). Appropriate dilutions were made in 20 mM Mops/50%(v/v)glycerol (final pH 7.4). Incubation volume: 100 μl. Reactions were terminated at appropriate time points (usually 5 min.) by the addition of 10μl of a 50 mM N-ethylmaleimide (NEM) solution followed by acetonitrile (final concentration: 40% (v/v). After centrifugation at 4°C the supernatant was applied to a reverse-phase C18-column (Supelcosil SPLC-18-DB, 250 mm × 10 mm, Supelco). Resolution between the different CoA-esters was achieved by elution with a gradient of acetonitrile (40 → 58% (v/v)) in 16.9 mM sodium phosphate buffer (pH 6.9) at a flow rate of 3 ml/min. This procedure allows good resolution between the substrates (3-oxopalmitoyl-CoA and 3-oxopristanoyl-CoA) and the respective products (myristoyl-CoA and 4,8,12-trimethyltridecanoyl-CoA) of the thiolase reactions. Absorbance at 254 nm was measured continuously.

Synthesis and purification of 3-oxopalmitoyl-CoA and 3-oxopristanoyl-CoA. 3-Oxopalmitoyl-CoA was synthesized enzymatically from palmitoyl-CoA (Sigma, St. Louis, USA) using acyl-CoA oxidase, crotonase and 3-hydroxy-acyl-CoA dehydrogenase which are all commercially available (Sigma, St. Louis, USA). Apart from the enzymes the reaction medium had the following composition: 50mM Tris/HCl, 100 μM FAD, 5 mM pyruvate, 1 mM NAD, 2.5 U/ml lactate dehydrogenase, 150 mM KCl. Final pH 8.5. 3-Oxopristanoyl-CoA was also synthesized enzymatically. Pristanoyl-CoA was used as starting substrate as prepared from pristanic acid following the method of Rasmussen et al [30]. A purified peroxisomal fraction (0.25 mg/ml) was used to convert pristanoyl-CoA into 3-oxopristanoyl-CoA. The 3-oxoacyl-CoA esters were purified by HPLC using the chromatographic conditions described above and stored at −20°C in a buffer containing 20 mM MES, final pH 6.0 in which they were found to be stable for extended periods of time.

Purification of the peroxisome proliferator-inducible thiolase [*25*] *and SCPx* [*24*]. The peroxisome proliferator-inducible thiolase was purified to apparent homogeneity on SDS/PAGE from clofibrate-treated rats using the procedure described by Hashimoto and coworkers [25]. Rat liver 58kDa SCPx (SCP2/3-oxoacyl-CoA thiolase) was prepared by expressing the full-length cDNA coding for the entire SCPx (547 amino acids) in E. Coli followed by purification of the protein (see [24] for full details).

RESULTS

We and others recently discovered that straight-chain fatty acyl-CoA esters like palmitoyl-CoA and 2-methyl branched-chain acyl-CoA esters like pristanoyl-CoA are oxidized by two *distinct* peroxisomal acyl-CoA oxidases. The first indication for the existence of two distinct oxidases came from experiments in which we studied the effect of clofibrate, a known inducer of peroxisomes [26], on the peroxisomal oxidase activities with palmitoyl-CoA and pristanoyl-CoA as substrates in rat liver and kidney [29]. Palmitoyl-CoA oxidase activity was found to be stimulated manyfold by clofibrate whereas pristanoyl-CoA oxidase activity remained unchanged [29]. These studies were soon followed by the identification of rat pristanoyl-CoA oxidase as a distinct enzyme following its purification and subsequent cDNA cloning (see [26] for review).

We have now used the same approach to resolve whether the 3-oxoacyl-CoA esters of straight-chain and branched-chain fatty acids are handled by the conventional peroxisome proliferator-inducible peroxisomal thiolase as believed until now or not. To this end peroxisomes were isolated from livers of control and DEHP-treated rats using density-gradient centrifugation on Nycodenz which procedure results in peroxisomes of

TABLE 1

Activity Measurements of 3-Oxopristanoyl-CoA Thiolase, 3-OxopalmitoylCoA Thiolase, and Other Enzymes in Peroxisomes from Normal-Fed and Diethylhexylphthalate (DEHP)-Fed Rats

Enzyme activity measured	Control	DEHP-fed
• Pristanoyl-CoA oxidase[a]	4.2 ± 0.8 (3)	3.4 ± 1.1 (3)
• Palmitoyl-CoA oxidase[a]	48 ± 4 (4)	206 ± 45 (4)
• Catalase[b]	15.8 ± 1.2 (4)	5.3 ± 0.9 (4)
• 3-Oxopalmitoyl-CoA thiolase[a]	604 ± 120 (3)	5295 ± 840 (3)
• 3-Oxopristanoyl-CoA thiolase[a]	41 ± 8.2 (3)	10 ± 1.5 (3)

Results are in [a]nmol/min.mg protein or [b]μmol/min.mg protein and represent the mean ± S.D. with the number of different preparations analyzed between parentheses.

high purity [28]. In accordance with literature data peroxisomes from peroxisome proliferator- treated rats have increased palmitoyl-CoA oxidase activity whereas the specific activity of catalase is lower in these peroxisomes (Table I).

When thiolase activities were measured in the two types of peroxisomes, different results were found depending upon the type of substrate used. Indeed, 3-oxopalmitoyl-CoA thiolase activity was found to be much higher in peroxisomes from DEHP-treated rats whereas with 3-oxopristanoyl-CoA as substrate a lower thiolase activity was found in peroxisomes from DEHP-fed rats (Table I).

The finding that DEHP-treatment had a differential effect on the 3-oxopalmitoyl-CoA and 3-oxopristanoyl-CoA thiolase activities in peroxisomes, strongly suggested that these two 3-oxoacyl-CoA esters are *not* handled by a single thiolase. The data on the effect of DEHP on the 3-oxopristanoyl-CoA thiolase activity in peroxisomes implies that 3-oxopristanoyl-CoA might be a poor substrate for the peroxisome proliferator-inducible peroxisomal thiolase identified by Hashimoto and coworkers [25]. In order to investigate this we purified rat-liver peroxisomal thiolase from clofibrate-induced rat livers using published procedures [25] and tested its reactivity with 3-oxopalmitoyl-CoA and 3-oxopristanoyl-CoA. These experiments performed in the presence and absence of BSA (4 μM) revealed that this thiolase shows virtually no activity with 3-oxopristanoyl-CoA (Fig.1). The results show that BSA does not only have an effect on the apparent affinities but also eliminates the substrate inhibition found at higher substrate concentrations.

The results obtained raised the question which peroxisomal thiolase would be responsible for the thiolytic cleavage of 3-oxopristanoyl-CoA. The recently identified SCPx with sterol carrier protein *and* thiolase activity [24] is a likely candidate especially since it is known that peroxisome proliferators have no effect on SCP2/SCPx mRNA and protein levels [31]. The results of Fig.2 show that SCPx is not only reactive with 3-oxopalmitoyl-CoA in line with data described before [24] but also with 3-oxopristanoyl-CoA. The results further show that SCPx has high affinity for both types of 3-oxoacyl-CoA esters especially in the absence of BSA. Clearly, SCPx showed highest activity with 3-oxopristanoyl-CoA.

DISCUSSION

The 56 kDa SCPx, alternatively called SCP2/3-oxoacyl-CoA thiolase [24], and SCP2 are produced from different mRNAs derived from a single gene having two promoters. SCP2 has been implicated in the intracellular trafficking of a variety of lipids which conclusion is largely based on *in vitro* experiments, however.

The function of the 56 kDa SCPx with 3-oxoacyl-CoA thiolase activity and intrinsic sterol carrier protein ac-

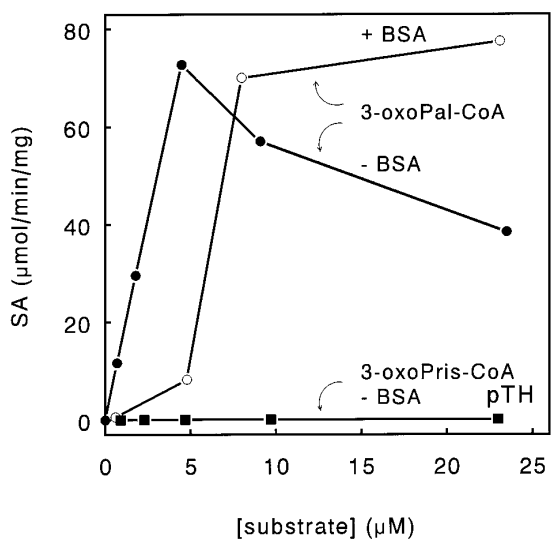

FIG. 1. Reactivity of the conventional peroxisome proliferator-inducible peroxisomal thiolase with 3-oxopalmitoyl-CoA and 3-oxopristanoyl-CoA. Peroxisomal thiolase was purified from rat livers taken from rats fed 2% (w/v) di-(2-ethylhexyl) phthalate according to Hashimoto and coworkers [25]and thiolase activity measurements were performed as described in Materials and Methods using 3-oxopalmitoyl-CoA (○,●) and 3-oxopristanoyl-CoA (■) as substrates at the concentrations as indicated in the Figure. ●,■ = minus BSA (4μM); ○ = plus BSA (4μM).

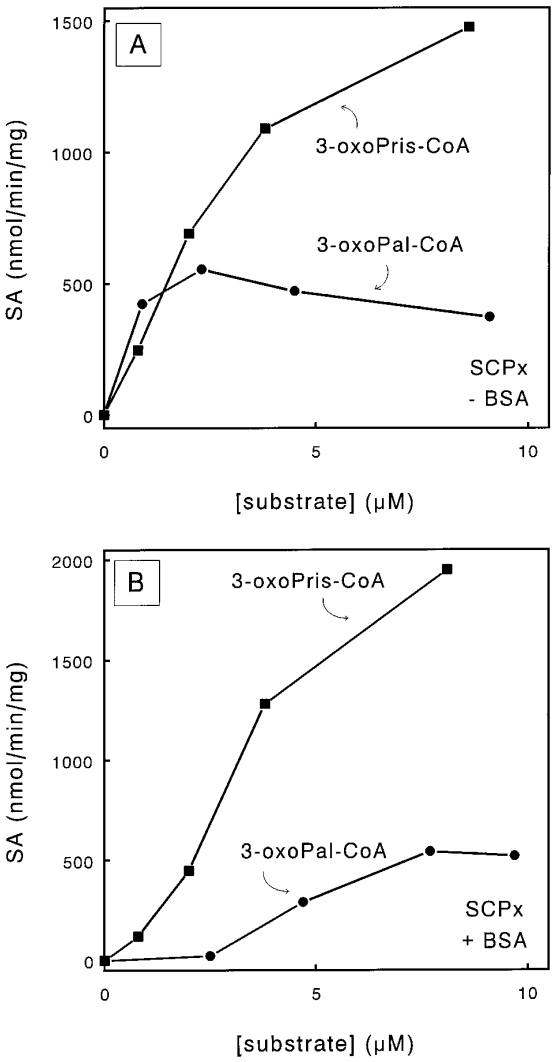

FIG. 2. Reactivity of rat liver SCPx (SCP2/3-oxoacyl-CoA thiolase) with 3-oxopalmitoyl-CoA and 3-oxopristanoyl-CoA. Rat liver SCPx was prepared by expressing the full-length cDNA coding for the entire SCPx (547 amino acids) in E.Coli followed by purification of the protein as described before [24]. Thiolase activity measurements were performed as described in Materials and Methods using 3-oxopalmitoyl-CoA (●) and 3-oxopristanoyl-CoA (■) in the absence (Fig. 2A) or presence (Fig. 2B) of 4 μM BSA.

tivity remained obscure especially since its substrate specificity was found to correspond closely with that of the conventional peroxisomal thiolase identified by Hashimoto and coworkers [25]. The results described in this paper indicate that SCPx fulfils a unique role in peroxisomal fatty acid oxidation catalyzing the thiolytic cleavage of the 3-oxoacyl-CoA ester of pristanic acid, a 2-methyl branched-chain fatty acid. The lack of activity of the conventional peroxisomal thiolase [25] with 3-oxopristanoyl-CoA suggests that SCPx is essential for the β-oxidation of pristanic acid and possibly other 2-methyl branched-chain fatty acids. In this respect it is important to mention di- and trihydroxycholestanoic acid which also possess a methyl-group at the 2-position. Both cholestanoic acids are produced from cholesterol, undergo activation to their CoA-esters and are then β-oxidized in peroxisomes with the CoA-esters of chenodeoxycholate and cholate as endproducts, respectively [26]. Preliminary studies show that the 3-oxoacyl-CoA esters of these cholestanoic acids are also handled exclusively by SCPx and are not accepted as substrate by the conventional thiolase.

Recent studies from different laboratories [32-35] have led to the identification of a new peroxisomal Multifunctional β-Oxidation Protein (pMOP2) in peroxisomes with 3 functional units including enoyl-CoA hydratase activity, 3-hydroxyacyl-CoA dehydrogenase and remarkably, sterol carrier protein activity. This enzyme was first discovered by Adamski and coworkers [36] as a distinct 17β-hydroxysteroid dehydrogenase. Convincing evidence has been brought forward suggesting that this enzyme (pMOP2) and *not* the long-

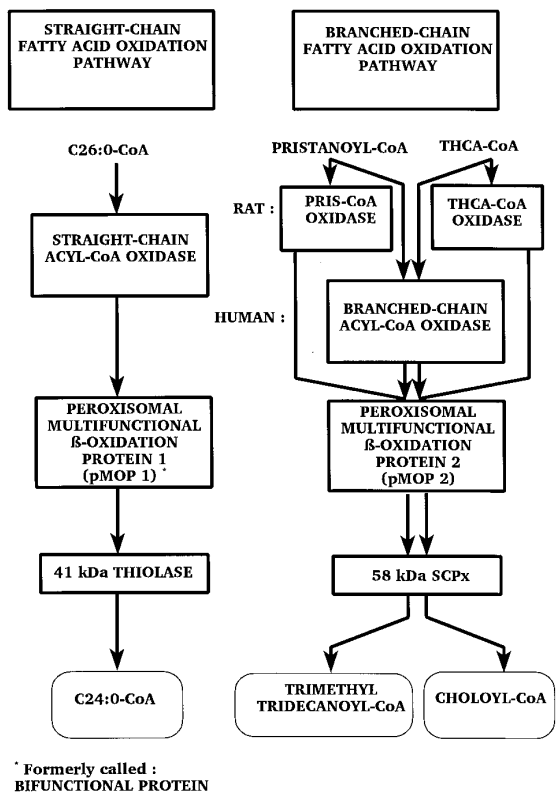

FIG. 3. Proposed structure of the peroxisomal fatty acid β-oxidation machinery. Based on the results described in this paper and data in literature we propose separate systems for the oxidation of straight-chain and branched-chain fatty acids. Abbreviations used: PRIS-CoA oxidase = pristanoyl-CoA oxidase; THCA-CoA = trihydroxycholestanoyl-CoA; pMOP1 = peroxisomal multifunctional β-oxidation protein 1, also called bifunctional protein (see [37]); pMOP2 = peroxisomal multifunctional β-oxidation protein 2, also known as: 17β-hydroxysteroid dehydrogenase IV [32], multifunctional protein 2 (MFP2) [33], peroxisomal multifunctional enzyme II (per MFE-II) [34]) and D-specific bifunctional protein [35].

known bifunctional protein with hydratase and 3-hydroxyacyl-CoA dehydrogenase activity [37] (here named: pMOP1) catalyzes the hydration and subsequent dehydrogenation of the enoyl-CoA esters of 2-methyl branched-chain fatty acids such as pristanic acid [34] and trihydroxycholestanoic acid [33]. Earlier studies had already shown the existence of separate oxidases for straight-chain and branched-chain acyl-CoA esters (see [26] for details). Taken together, these data suggest the existence of two distinct pathways in peroxisomes for straight-chain and 2-methyl branched-chain fatty acids as depicted schematically in Fig. 3.

The finding that SCPx plays a unique role in 2-methyl branched-chain fatty acid oxidation in peroxisomes, will also have implications for human diseases. Indeed, a great number of patients has been identified with an unknown defect in peroxisomal β-oxidation. These patients almost invariably show a range of fatty acid abnormalities in plasma including elevated pristanic acid and di- and trihydroxycholestanoic acid levels (see [38] for details), which results from some defect in peroxisomal β-oxidation. Our results suggest that SCPx deficiency may well be one of the underlying defects in these patients. Such studies are underway.

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