

Peroxisomal fatty acid oxidation disorders and 58 kDa sterol carrier protein X (SCPx): activity measurements in liver and fibroblasts using a newly developed method

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Abstract Sterol carrier protein X (SCPx) plays a crucial role in the peroxisomal oxidation of branched-chain fatty acids. To investigate whether patients with an unresolved defect in peroxisomal β -oxidation are deficient for SCPx, we developed a novel and specific assay to measure the activity of SCPx in both liver and fibroblast homogenates. The substrate used in the assay, $3\alpha,7\alpha,12\alpha$ -trihydroxy-24-keto-5 β -cholestanoyl-CoA (24-keto-THC-CoA), is produced by preincubating the enoyl-CoA of the bile acid intermediate THCA with a lysate from the yeast *Saccharomyces cerevisiae* expressing human D-bifunctional protein. After the preincubation period, liver or fibroblast homogenate is added plus CoASH, and the production of choloyl-CoA is determined by HPLC. The specificity of the assay was demonstrated by the finding of a full deficiency in fibroblasts from an SCPx knock-out mouse. In addition to SCPx activity measurements in fibroblasts from patients with a defect in peroxisomal β -oxidation of unresolved etiology, we studied the stability and activity of SCPx in fibroblasts from patients with Zellweger syndrome, which lack functional peroxisomes. We found that SCPx is not only stable in the cytosol, but displays a higher activity in fibroblasts from patients with Zellweger syndrome than in control fibroblasts. Furthermore, in all patients studied with a defect in peroxisomal β -oxidation of unknown origin, SCPx was found to be normally active, indicating that human SCPx deficiency remains to be identified.—Ferdinandusse, S., S. Denis, E. van Berkel, G. Dacremont, and R. J. A. Wanders. **Peroxisomal fatty acid oxidation disorders and 58 kDa sterol carrier protein X (SCPx): activity measurements in liver and fibroblasts using a newly developed method.** *J. Lipid Res.* 2000. 41: 336–342

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It is currently well established that peroxisomes contain two distinct pathways involved in the β -oxidation of various fatty acids and fatty acid derivatives. In humans, the CoA esters of straight-chain fatty acids are first desatu-

rated by the acyl-CoA oxidase identified by Osumi, Hashimoto, and Ui (1), now called straight-chain acyl-CoA oxidase. The enoyl-CoAs produced are subsequently converted to 3-ketoacyl-CoAs by I-bifunctional protein (2), which first hydrates *trans*-enoyl-CoAs to their 1-hydroxy form and then dehydrogenates the 3-hydroxyacyl-CoAs to the corresponding 3-ketoacyl-CoAs. In contrast, 2-methyl-branched-chain acyl-CoA esters, including pristanoyl-CoA and the bile acid intermediates di- and trihydroxycholestanoyl-CoA (DHC-CoA and THC-CoA), are handled by the branched-chain acyl-CoA oxidase (3, 4) and then converted to 3-keto-2-methylacyl-CoAs by d-bifunctional protein (D-BP) via the d-hydroxy stereoisomer (5–8). Recent studies have also shown that the two known peroxisomal thiolases that catalyze the last step of the peroxisomal β -oxidation spiral, namely, 3-ketoacyl-CoA thiolase identified by Miyazawa and coworkers (9), and sterol carrier protein X (SCPx) identified by Seedorf and Assmann (10) have different roles. Indeed, it has been demonstrated that the 3-ketoacyl-CoA esters of pristanic acid, DHCA, and THCA, are handled by SCPx but not by the classic 3-ketoacyl-CoA thiolase (11, 12), which implies that SCPx plays a unique role in the peroxisomal β -oxidation of branched-chain fatty acids (12) and in bile acid formation (13, 14).

SCPx is a 58 kDa protein that consists of an amino-terminal thiolase domain and a carboxy-terminal sterol carrier protein-2 (SCP2) domain (15–17). After import into peroxisomes the domains are cleaved giving rise to a 46 kDa thiolase and a 13 kDa SCP2 (18). In vitro studies revealed that SCPx displays two activities: a 3-ketoacyl-CoA

Abbreviations: DHCA, dihydroxycholestanic acid; THCA, trihydroxycholestanic acid; 24(*E*)-ene-THC-CoA, $3\alpha,7\alpha,12\alpha$ -trihydroxy-5 β -cholest-24-en-26-oyl-CoA; 24-hydroxy-THC-CoA, $3\alpha,7\alpha,12\alpha,24$ -tetrahydroxy-5 β -cholestan-26-oyl-CoA; 24-keto-THC-CoA, $3\alpha,7\alpha,12\alpha$ -trihydroxy-24-keto-5 β -cholestanoyl-CoA; d-BP, d-bifunctional protein; SCPx, sterol carrier protein X; SCP2, sterol carrier protein-2.

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thiolase activity and a sterol carrier protein lipid transfer activity (19). The thiolase domain shares significant sequence homology with both the mitochondrial and peroxisomal 3-ketoacyl-CoA thiolases (17, 20, 21), but differs in substrate specificity as already mentioned.

The identification of SCPx as one of the major enzymes involved in branched-chain fatty acid oxidation is of great importance especially since many patients have been described with an unresolved defect in peroxisomal β -oxidation. Most of these patients show a range of fatty acid abnormalities in plasma including elevated levels of pristanic acid, DHCA, and THCA (see ref. 22 for details). As such abnormalities would also be predicted for patients with SCPx deficiency, we developed an assay for SCPx applicable to both liver and skin fibroblast homogenates and used the assay to determine the activity in several of these patients. In addition, we studied whether SCPx is active in cells of patients with Zellweger syndrome which lack peroxisomes and, as a consequence, have peroxisomal matrix proteins mislocalized in the cytosol.

MATERIALS AND METHODS

Materials

24(*E*)-ene-THC-CoA was synthesized as described (23). Oxaloacetate, malate dehydrogenase (from pig heart), NAD⁺, and CoASH were purchased from Boehringer Mannheim. Protifar was obtained from Nutricia and goat anti-rabbit IgG antibodies conjugated to alkaline phosphatase were from Bio-Rad Laboratories. Antibodies raised against the thiolase domain of SCPx were a kind gift from Prof. Dr. K. Wirtz (Utrecht, The Netherlands).

Methods

Cloning and expression of D-BP in yeast. The cloning of D-BP and its expression in *Saccharomyces cerevisiae* was described by Van Grunsven et al. (24).

Cell lines of patients with Zellweger syndrome. The Zellweger fibroblasts studied in this paper were from four patients with all the clinical and biochemical abnormalities described for Zellweger syndrome, including the full set of peroxisomal abnormalities in fibroblasts (deficient plasmalogen synthesis, deficient C26:0 and pristanic acid β -oxidation, deficient phytanic acid α -oxidation and the complete absence of peroxisomes as shown by catalase immunofluorescence microscopy (22)). Informed consent was obtained from parents or guardians of the patients whose fibroblasts were studied in this paper and the studies were approved by the Institutional Review Board of the Academic Medical Center, University of Amsterdam.

SCPx assay. SCPx activity was assayed in two successive steps. First, the substrate for SCPx 3 α ,7 α ,12 α -trihydroxy-24-keto-5 β -cholestanoyl-CoA (24-keto-THC-CoA) was synthesized enzymatically by incubation of 3 α ,7 α ,12 α -trihydroxy-5 β -cholest-24-en-26-oyl-CoA (24(*E*)-ene-THC-CoA) with human D-BP expressed in yeast in the presence of oxaloacetate plus malate dehydrogenase to regenerate NAD⁺ during the assay. The composition of the preincubation medium was as follows: 50 mM bis-tris-propane (BTP) pH 9.0, 150 mM KCl, 1 mM NAD⁺, 0.5 mM oxaloacetate, 0.5 U/ml malate dehydrogenase, 100 μ M 24(*E*)-ene-THC-CoA, and 4 mU/ml D-BP, which was added as a crude yeast lysate. After a preincubation of 15 min at 37°C, 200 μ M CoASH was added followed by the addition of liver or fibroblast homogenates prepared in PBS by sonication under continuous cooling with ice

water. Reactions were allowed to proceed for 15 and 30 min for liver and fibroblast homogenates, respectively, and were terminated by the addition of 2 M HCl to a final concentration of 0.18 M. The reaction mixture was then neutralized using 0.6 M MES plus 2 M KOH, followed by the addition of acetonitrile (final concentration: 28% (v/v)). After centrifugation for 10 min at 20000 *g*_{av} at 4°C, the supernatant was applied to a reverse-phase C18-column (Supelcosil SPLC-18-DB, 250 mm \times 10 mm, Supelco). Resolution between the different CoA esters was achieved by elution with a linear gradient of acetonitrile (25 \rightarrow 37% (v/v)) in 16.9 mM sodium phosphate buffer (pH 6.9) at a flow rate of 3 ml/min under continuous monitoring of the absorbance at 254 nm. This procedure allows good resolution of the substrate 24(*E*)-ene-THC-CoA, the products of the D-BP reaction, i.e., 3 α ,7 α ,12 α ,24-tetrahydroxy-5 β -cholestan-26-oyl-CoA (24-hydroxy-THC-CoA) and 24-keto-THC-CoA, and choloyl-CoA, which is one of the products of the reaction catalyzed by SCPx. Propionyl-CoA, the other product of the thiolytic cleavage of the side chain of 24-keto-THC-CoA, elutes in the void volume and cannot be detected using this method. The amount of choloyl-CoA formed was calculated from the ratio of choloyl-CoA over the total amount of substrate and products, e.g., 24(*E*)-ene-THC-CoA, 24-hydroxy-THC-CoA, 24-keto-THC-CoA and choloyl-CoA, and was used to calculate the enzyme activity. This method of quantification corrects for the hydrolysis of the CoA esters by thioesterases present in the homogenate.

Immunoblot analysis. Fibroblast homogenates (50 μ g of protein) were subjected to electrophoresis on a 10% (w/v) SDS-polyacrylamide gel essentially as described by Laemmli (25) and transferred to a nitrocellulose sheet. After blocking of non-specific binding sites with 50 g/L Protifar and 10 g/L BSA in 1 g/L Tween-20/PBS for 1 h, the blot was incubated for 2 h with rabbit polyclonal antibodies raised against SCPx (prepared as described in Ossendorp et al., 26) and diluted 1:2000 in 3 g/L BSA. Goat anti-rabbit IgG antibodies conjugated to alkaline phosphatase were used for detection, according to the manufacturer's instructions (Bio-Rad).

RESULTS

Development and optimization of the enzyme assay

In our initial experiments designed to measure SCPx activity in crude liver and fibroblast homogenates, we used 3-ketopristanoyl-CoA as a substrate. This was based on earlier findings (11, 12) which showed that 3-ketopristanoyl-CoA is a good substrate for purified SCPx but not for the other peroxisomal thiolase identified by Miyazawa and co-workers (9). As expected, the 3-ketopristanoyl-CoA readily underwent thiolytic cleavage to 4,8,12-trimethyltridecanoyl-CoA and propionyl-CoA in both types of homogenates. The availability of cultured skin fibroblasts from an SCPx knock-out mouse, described by Seedorf et al. (27), allowed us to assess the specificity of this assay in homogenates. Surprisingly, when thiolase activity was measured in fibroblast homogenates of the SCPx knock-out mouse using 3-ketopristanoyl-CoA as substrate, a relatively high residual activity was found (19% of the mean control value). This indicated that 3-ketopristanoyl-CoA is not an exclusive substrate for SCPx and, as a consequence, cannot be used for accurate SCPx activity measurements in crude tissue homogenates. We therefore developed a novel method which is suitable for this purpose, based on the use of another substrate for SCPx, namely 24-keto-THC-CoA.

As 24-keto-THC-CoA is not commercially available, we studied whether it could be produced using D-BP expressed in yeast. To this end, 100 μM 24(*E*)-ene-THC-CoA was incubated for 15 min in the presence of different concentrations of D-BP (in a crude yeast lysate), and oxaloacetate and malate dehydrogenase for regeneration of NAD^+ . We found that D-BP at a concentration of 4 mU/ml catalyzed rapid formation of 24-keto-THC-CoA, a steady state being reached after 15 min. Based on these findings we adopted the following preincubation conditions for the activity measurements of SCPx (see Materials and Methods): 50 mM BTP (pH 9.0), 150 mM KCl, 1 mM NAD^+ , 0.5 mM oxaloacetate, 0.5 U/ml malate dehydrogenase, 100 μM 24(*E*)-ene-THC-CoA, and 4 mU/ml D-BP. The actual thiolase reaction is started after 15 min at 37°C by adding CoASH, followed by the addition of homogenate.

Next, we determined the optimal conditions for the assay in rat liver homogenates, the results of which are detailed in Fig. 1. Based on these results we selected a

protein concentration of 0.3 mg/ml in the presence of 200 μM CoASH at pH 9.0 and an incubation time of 15 min as standard assay conditions. Because, for humans, liver samples for activity measurements are difficult to obtain, we studied whether the same assay could also be used for cultured skin fibroblast homogenates. Using the same assay conditions as used for activity measurements in rat liver homogenates, except for the protein concentration which was increased to 0.5 mg/ml, the reaction was found to proceed linearly for up to 60 min (data not shown). As a standard, a 30-min incubation time was chosen for measurements in fibroblasts, because sufficient choloyl-CoA is formed to be readily detectable.

The specificity of this newly developed method for the measurement of SCPx was again determined in cultured skin fibroblasts from the SCPx knock-out mouse. In these mutant cells no choloyl-CoA was formed, while abundant activity was found in control cells indicating that the assay is indeed specific for SCPx (Fig. 2).

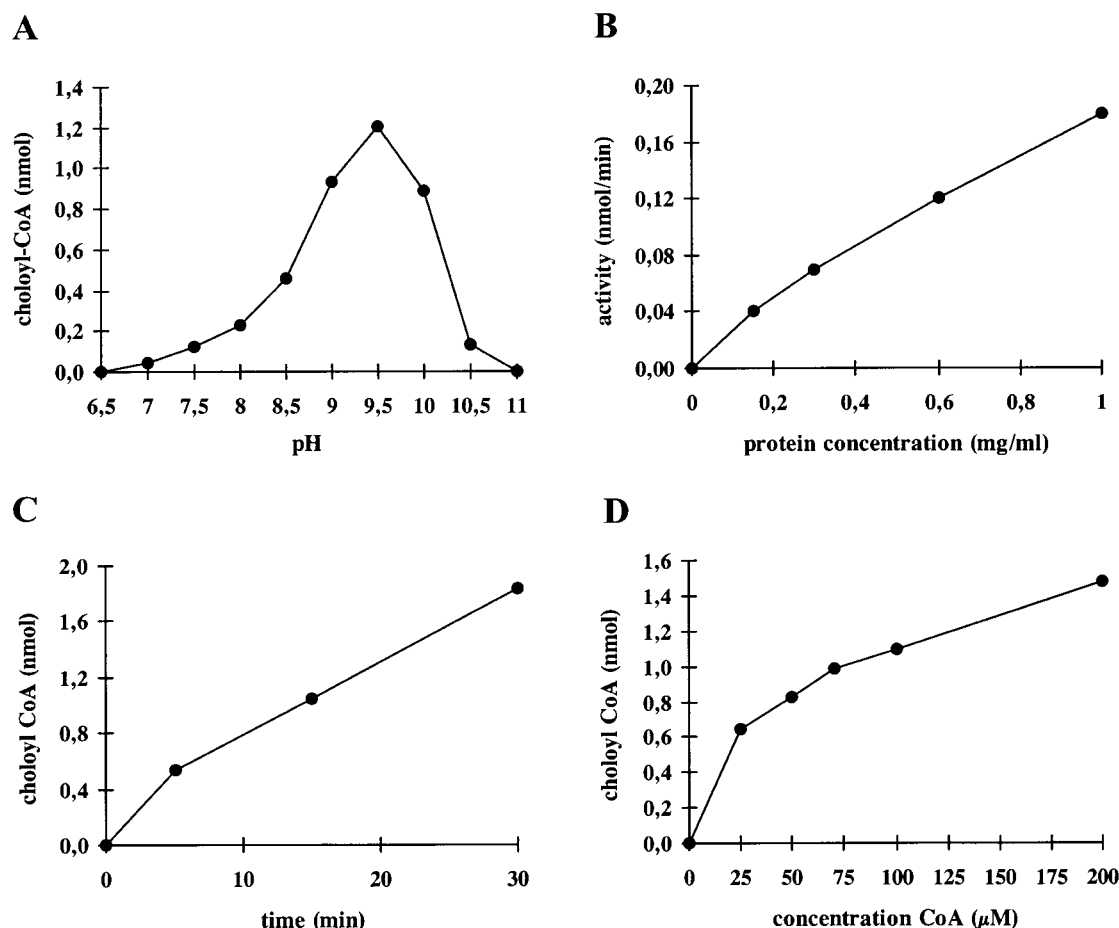


Fig. 1. Optimization of the SCPx activity assay in rat liver homogenate. A: Shows the effect of the pH of the incubation mixture on the production of choloyl-CoA. Activity was optimal at pH 9.5, but to minimize the risk of hydrolysis of CoA esters, the incubations were performed at pH 9 in further experiments. B: Shows the effect of the amount of homogenate present in the assay medium on the reaction rate. A linear increase was observed up to 30 μg protein. At this protein concentration (0.3 mg/ml), the production of choloyl-CoA was followed in time (C) and found to be linear for up to 30 min. Fifteen minutes was chosen as standard incubation time. Finally (D), the effect of the concentration of CoASH on the production of choloyl-CoA was determined. The K_m of the reaction for CoASH was 37.7 μM . In subsequent experiments 200 μM CoASH was used.

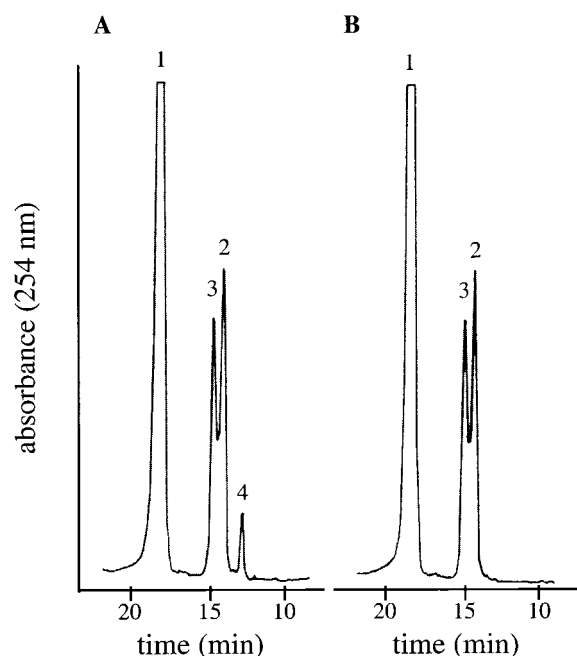


Fig. 2. HPLC analysis of SCPx activity measurements in fibroblasts from a wild-type mouse (A) and an SCPx knock-out mouse (B). Peak 1 is the substrate 24(B)-ene-THC-CoA, whereas peak 2 and 3 are the products of the 15-min preincubation with D-BP, 24-hydroxy-THC-CoA, and 24-keto-THC-CoA, respectively, and peak 4 is the product of the SCPx reaction, choloyl-CoA. In the SCPx knock-out mouse no SCPx activity could be measured, demonstrating the specificity of the assay.

SCPx activity measurements in patients with Zellweger syndrome

Earlier studies showed that the 58 kDa SCPx is processed inside peroxisomes to produce a 13 kDa SCP2 and a 46 kDa thiolase (28). Due to the lack of a specific assay for SCPx activity, however, it could never be determined whether this processing is required for the activation of the thiolase. We therefore studied the activity of SCPx in cells from patients lacking functional peroxisomes using the newly developed specific assay. First, we examined lysates of these cells for the presence of unprocessed 58 kDa protein by immunoblot analysis using antibodies against

SCPx. As is clear from **Fig. 3**, in all cells the 58 kDa protein is present while the 46 kDa thiolase was not detected, in contrast to control cells in which the 46 kDa thiolase is readily observed. Subsequent activity measurements in the cell lysates of the patients with Zellweger syndrome showed that the unprocessed SCPx is catalytically active (**Table 1**). This demonstrates that processing of the 58 kDa protein is not required for the activation of the thiolase. In fact, the activity was even higher in fibroblast homogenates of most patients with Zellweger syndrome as compared to control fibroblasts. To examine whether this is caused by a difference in SCPx protein levels, we determined the amount of 58 kDa SCPx protein plus the amount of 46 kDa protein because in control cells most of the 58 kDa SCPx is processed to the 46 kDa thiolase plus 13 kDa SCP2. Densitometric analysis of the immunoblot with six control cell lines and the four Zellweger cell lines shown in **Fig. 3** revealed the following: in control cells the mean density of the 58 kDa plus 46 kDa bands was 587 ± 147 (arbitrary units) whereas a value of 451 ± 59 was found in the Zellweger cells. The corresponding thiolase activities were 111 ± 20 and 214 ± 65 pmol/min/mg, respectively. These data indicate that the increased thiolase activity in fibroblasts from patients with Zellweger syndrome is not due to an increased protein level (this will be discussed in more detail in the discussion).

Analysis of SCPx activity in patients with a defect in the peroxisomal β -oxidation

Several patients have been described in literature with an unresolved defect in the peroxisomal β -oxidation. Many of these patients show a range of fatty acid abnormalities in plasma including elevated levels of pristanic acid, DHCA, and THCA. We examined whether SCPx is the defective enzyme in six of these candidate patients (29–35). Immunoblot analysis showed that SCPx was present and normally processed in these patients (**Fig. 3**). In addition, activity measurements revealed that SCPx was normally active in fibroblast homogenates from all patients studied (**Table 1**). In fact, in some patients, SCPx activity was increased compared to control values. For comparison, we also determined the activity of SCPx in two patients with an established deficiency of D-BP (24, 36), one of the

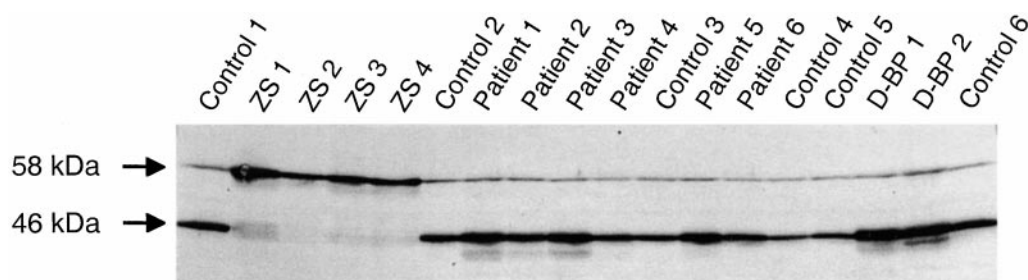


Fig. 3. Immunoblot analysis of SCPx in fibroblasts from 6 control subjects, 4 patients with Zellweger syndrome (ZS1–4), 6 patients with an unresolved β -oxidation defect (patients 1–6), and D-BP patients 1 and 2 using an antibody directed against the thiolase domain of SCPx. In patients with Zellweger syndrome, only full-length SCPx (58 kDa) is present, while in controls and patients with a defect of the peroxisomal β -oxidation most of the cross-reacting material is the 46 kDa thiolase domain.

TABLE 1. Activity measurements of SCPx in homogenates of cultured skin fibroblasts

Subjects	n	Ref.	Specific activity (<i>pmol/min/mg</i>)
Controls	10		74 ± 24
Zellweger patients	4		214 ± 65
Patient 1		29	172
Patient 2		30	85
Patient 3		31	254
Patient 4		32,33	82
Patient 5		34	143
Patient 6		35	104
D-BP patient 1		24	87
D-BP patient 2		36	271

Results represent the mean ± SD; n represents the number of measurements. References to case reports described in the literature are given. Detection limit < 0.01 nmol.

other enzymes of branched-chain fatty acid β -oxidation. In one of these D-BP patients SCPx activity was increased, while the activity in the other patient was within the normal range (Table 1).

DISCUSSION


In this paper we describe a novel and specific method to measure the activity of SCPx in crude tissue homogenates using 24-keto-THC-CoA as a substrate. SCPx catalyzes the last step of the peroxisomal β -oxidation of branched-chain fatty acids and the side chain of the bile acid intermediates DHCA and THCA. The specificity of our method was demonstrated by studies in fibroblast homogenates from mice with a targeted disruption of the SCPx gene, which revealed a fully deficient thiolase activity. In contrast to 24-keto-THC-CoA, 3-ketopristanoyl-CoA, which has been used to measure the activity of purified SCPx in previous studies, was thiolytically cleaved in fibroblast homogenates from the SCPx knock-out mouse, although the measured activity was markedly reduced compared to the activity in fibroblast homogenates from the control mouse. These results show that, at least in mice, 3-ketopristanoyl-CoA is handled by multiple thiolases, while 24-keto-THC-CoA is exclusively thiolytically cleaved by SCPx.

SCPx, a 58 kDa protein, is processed inside the peroxisome to produce a thiolase domain and an SCP2 domain. To study whether SCPx is catalytically active in cells lacking peroxisomes, activity measurements and immunoblot analysis were performed in fibroblasts from patients with Zellweger syndrome. In agreement with previous results by Suzuki et al. (28), immunoblot analysis showed that no processing of SCPx occurs in the absence of peroxisomes. We now demonstrate that the full-length protein is not only stable in the cytosol, but also displays thiolase activity. This is remarkable as many peroxisomal proteins, including dihydroxyacetone phosphate acyltransferase (37, 38), alkyl-dihydroxyacetone phosphate synthase (38, 39), phytanoyl-CoA hydroxylase (40), and the first enzyme of the peroxisomal branched-chain β -oxidation system branched-chain acyl-CoA oxidase (41), are rapidly degraded in the cytosol

because they cannot be imported into the peroxisome in patients with Zellweger syndrome. As a consequence, most peroxisomal enzymes are deficient in cells from patients with Zellweger syndrome. However, SCPx is not the only peroxisomal enzyme that shows normal activity in Zellweger syndrome. Indeed, it is known that several other peroxisomal enzymes also show normal activity in cells from patients with Zellweger syndrome and are apparently stable in the cytosol. These include catalase (42, 43), d-amino acid oxidase (42), glycolate oxidase (42), and alanine glyoxylate aminotransferase (44).

The SCPx activities measured in fibroblasts from patients with Zellweger syndrome were higher than the activities measured in controls. We showed that this is not due to an increased SCPx protein level. It could be the result of a difference in K_m or V_{max} for 24-keto-THC-CoA of the unprocessed SCPx compared to the cleaved 46 kDa thiolase domain of SCPx. For instance, SCP2 may play a role in the presentation of the substrate to the catalytic center or in removing the product from the catalytic site. This hypothesis is supported by the finding that SCP2 binds fatty acyl-CoAs (45) and is associated with fatty acid oxidation enzymes in peroxisomes (46).

Finally, we studied whether a deficiency of SCPx could be the underlying defect in a series of patients with an unresolved defect of the peroxisomal β -oxidation. In theory, a deficiency of SCPx could result in the fatty acid abnormalities observed in the plasma of these patients (29–35). However, SCPx activity was not deficient in any of the patients studied. In contrast, the activity of SCPx was even increased in some of the patients. Although the underlying mechanism remains to be resolved, this might be part of a mechanism to compensate for the loss of function of another enzyme of the peroxisomal β -oxidation system.

It is quite remarkable that so far no patients with an SCPx deficiency have been identified whereas several patients with a defect in D-BP, which is also involved in the peroxisomal oxidation of branched-chain fatty acids, have been described (24, 36, 47). It may be that SCPx deficiency is lethal in utero, although the mutant mice lacking SCPx completely (27) may suggest otherwise. Interestingly, these mice only show minor abnormalities unless they are fed a diet containing phytol, which may imply that the clinical presentation of SCPx deficiency is also mild. 

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