New insights in peroxisomal beta-oxidation
Ferdinandusse, S.

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New insights in peroxisomal β-oxidation

Sacha Ferdinandusse
New insights in peroxisomal β-oxidation

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit van Amsterdam, op gezag van de Rector Magnificus prof. mr. P.F. van der Heijden, ten overstaan van een door het college voor promoties ingestelde commissie, in het openbaar te verdedigen in de Aula der Universiteit op dinsdag 14 mei 2002, te 12.00 uur

doors

Sacha Ferdinandusse

egeboren te Amstelveen
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## Abbreviations

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>24(E)-ene-THC-CoA</td>
<td>3α,7α,12α-trihydroxy-5β-cholest-24-en-26-oyl-CoA</td>
</tr>
<tr>
<td>24-hydroxy-THC-CoA</td>
<td>3α,7α,12α,24-tetrahydroxy-5β-cholestan-26-oyl-CoA</td>
</tr>
<tr>
<td>24-keto-THC-CoA</td>
<td>3α,7α,12α-trihydroxy-24-keto-5β-cholestanoyl-CoA</td>
</tr>
<tr>
<td>ALD P</td>
<td>adrenoleukodystrophy protein</td>
</tr>
<tr>
<td>BCOX</td>
<td>branched-chain acyl-CoA oxidase</td>
</tr>
<tr>
<td>CACT</td>
<td>carnitine acylcarnitine translocase</td>
</tr>
<tr>
<td>CAT</td>
<td>carnitine acetyltransferase</td>
</tr>
<tr>
<td>COT</td>
<td>carnitine octanoyltransferase</td>
</tr>
<tr>
<td>CPT (I/II)</td>
<td>carnitine palmitoyltransferase (I/II)</td>
</tr>
<tr>
<td>DBP</td>
<td>D-bifunctional protein</td>
</tr>
<tr>
<td>DHA</td>
<td>docosahexaenoic acid</td>
</tr>
<tr>
<td>DHCA</td>
<td>dihydroxycholestanolic acid</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionization</td>
</tr>
<tr>
<td>EST</td>
<td>expressed sequence tag</td>
</tr>
<tr>
<td>GC</td>
<td>gas chromatography</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>LBP</td>
<td>L-bifunctional protein</td>
</tr>
<tr>
<td>LC</td>
<td>liquid chromatography</td>
</tr>
<tr>
<td>LCAD</td>
<td>long-chain acyl-CoA dehydrogenase</td>
</tr>
<tr>
<td>MBP</td>
<td>maltose-binding protein</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>NALD</td>
<td>neonatal adrenoleukodystrophy</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PEX</td>
<td>peroxin</td>
</tr>
<tr>
<td>PTS</td>
<td>peroxisome targeting signal</td>
</tr>
<tr>
<td>RCDP</td>
<td>rhizomelic chondrodysplasia punctata</td>
</tr>
<tr>
<td>SCOX</td>
<td>straight-chain acyl-CoA oxidase</td>
</tr>
<tr>
<td>SCP2</td>
<td>sterol carrier protein-2</td>
</tr>
<tr>
<td>SCPx</td>
<td>sterol carrier protein X</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecylsulfate</td>
</tr>
<tr>
<td>THCA</td>
<td>trihydroxycholestanoic acid</td>
</tr>
<tr>
<td>THC-CoA</td>
<td>trihydroxycholestanoyl-CoA</td>
</tr>
<tr>
<td>VLCAD</td>
<td>very long-chain acyl-CoA dehydrogenase</td>
</tr>
<tr>
<td>VLCFA</td>
<td>very long-chain fatty acids</td>
</tr>
<tr>
<td>XALD</td>
<td>X-linked adrenoleukodystrophy</td>
</tr>
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</table>
Chapter 1

Introduction
Chapter 1

Introduction

Peroxisomes play a major role in whole cell fatty acid β-oxidation by catalyzing the oxidative chain-shortening of a range of fatty acids and fatty acid derivatives, which cannot be broken down by mitochondria. Substrates of the peroxisomal β-oxidation system include both straight-chain fatty acids, like the very long-chain fatty acids C26:0 and C24:0, and 2-methyl-branched-chain fatty acids, like pristanic acid and the bile acid intermediates di- and trihydroxycholestanoic acid (DHCA and THCA). The importance of the peroxisomal β-oxidation system is stressed by the existence of a variety of different diseases in which peroxisomal β-oxidation is impaired. Extensive research has been performed on the peroxisomal β-oxidation system and in the past years the knowledge has expanded rapidly, especially with the discovery of a second set of peroxisomal β-oxidation enzymes and the identification of patients with a deficiency of one of these enzymes, D-bifunctional protein. Many questions remained, however, and the purpose of the studies described in this thesis was to resolve at least some of these questions.

The main focus of this thesis is on the β-oxidation of branched-chain fatty acids and the bile acid intermediates and on patients in whom β-oxidation of these substrates is affected. In chapter two a short review of the current knowledge of the peroxisomal β-oxidation system is given. Chapter three describes the molecular cloning and expression of human carnitine octanoyltransferase (COT) and evidence is presented which shows that COT is involved in the peroxisomal β-oxidation of branched-chain fatty acids. In chapter four patients described in literature with an unresolved defect in peroxisomal β-oxidation are investigated for a deficiency of sterol carrier protein X (SCPx), one of the peroxisomal thiolases, using a newly developed method to measure SCPx activity. In chapter five the only patient ever reported with peroxisomal 3-ketoacyl-CoA thiolase deficiency is reinvestigated. Chapter six describes the identification of patients with a deficiency of α-methylacyl-CoA racemase and in chapter seven the physiological role and the subcellular localization of this enzyme is further investigated. In chapter eight and nine the stereochemistry of the peroxisomal fatty acid oxidation systems is studied and in particular the role of α-methylacyl-CoA racemase therein. In plasma from patients with different peroxisomal fatty acid oxidation disorders the diastereomers of DHCA, THCA and of phytanic acid, pristanic acid and the metabolites of pristanic acid were analyzed. In chapter ten, cell lines of many different patients with an established deficiency of mitochondrial or peroxisomal fatty acid oxidation are used to investigate the subcellular localization of the last step of the biosynthesis of docosahexaenoic acid (DHA), an important polyunsaturated fatty acid. In addition, it is studied which of the peroxisomal β-oxidation enzymes are involved in this process.
Chapter 2

Peroxisomal β-oxidation: a review
Chapter 2

Peroxisomal β-oxidation: a review

Peroxisomes are subcellular organelles present in virtually all eukaryotic cells and are involved in numerous metabolic processes. Only in the 1980s the importance of peroxisomes in cellular metabolism in man became clear, when two key observations were made on a rare inherited disorder called Zellweger syndrome. Zellweger syndrome, also called the cerebro-hepato-renal syndrome, is characterized by the absence of morphologically distinguishable peroxisomes in all cell types, due to mutations in different genes involved in peroxisome biogenesis (so called PEX-genes). First, Brown et al. (1) reported that the levels of the very long-chain fatty acids (VLCFAs) C26:0 and C24:0 were markedly elevated in plasma from patients with Zellweger syndrome. This finding suggested that these VLCFAs are broken down in the peroxisome, which was known to contain a fatty acid β-oxidation system. This has now been firmly established. One year later, Heymans et al. (2) discovered a deficiency of plasmalogens, a special type of phospholipids, in tissues from patients with Zellweger syndrome, indicating that peroxisomes play a central role in the formation of plasmalogens. Since that time, many functions of peroxisomes have been identified, most of which have to do with lipid metabolism. Besides their role in fatty acid β-oxidation and ether-phospholipid formation, peroxisomes are involved in fatty acid α-oxidation, bile acid formation, isoprenoid biosynthesis and the biosynthesis of polyunsaturated fatty acids (PUFAs). Along with the elucidation of the peroxisomal functions, many inherited peroxisomal disorders have been identified.

In this chapter the current knowledge of the peroxisomal fatty acid β-oxidation system will be discussed, in particular in relation to human disorders and mouse models in which peroxisomal β-oxidation is impaired.

Peroxisomal fatty acid β-oxidation

In 1976, Lazarow and De Duve discovered that peroxisomes contain a fatty acid β-oxidation system similar to that present in mitochondria (3). Over the years the significance of this additional β-oxidation system has become clear. One important difference between the mitochondrial and the peroxisomal β-oxidation machinery is the difference in substrate specificity. Mitochondria catalyze the β-oxidation of most of the short-, medium- and long-chain fatty acids derived from the diet, while peroxisomes are responsible for the β-oxidation of VLCFAs, pristanic acid (2,6,10,14-tetramethylpentadecanoic acid bile acid intermediates, long-chain dicarboxylic acids, eicosanoids, certain mono- and polyunsaturated fatty acids and side chains of some xenobiotics.

Another major difference is that in the peroxisome, fatty acids are not degraded completely into acetyl-CoA units. Since the acyl-CoA oxidases present in the peroxisome do not, or hardly, react with short-chain acyl-CoAs (butyryl-CoA, hexanoyl-CoA) (4,5), fatty acids are only chain-shortened in the peroxisome. The chain-shortened products are then transported to the mitochondrion as a carnitine ester, where they are oxidized to completion. For saturated fatty acids such as C26:0, it is not known how many cycles of β-oxidation occur in the peroxisomes, but for pristanic acid it has been established that it
undergoes three cycles of β-oxidation in the peroxisome (6). This will be discussed in more detail later.

**Enzymology of the peroxisomal fatty acid β-oxidation system**
At first, it was believed that a single set of β-oxidation enzymes was responsible for the chain-shortening of fatty acids in the peroxisome. These enzymes were characterized and purified by Hashimoto and coworkers and included an acyl-CoA oxidase, bifunctional protein and peroxisomal thiolase (reviewed in (7)). At this moment, it is well established that in man peroxisomes contain two sets of β-oxidation enzymes (Fig. 1), which will be described below.

![Enzymology of the fatty acid β-oxidation machinery in human peroxisomes.](image)

**Peroxisomal acyl-CoA oxidases**
The first step of peroxisomal β-oxidation is the desaturation of an acyl-CoA to a 2-trans-enoyl-CoA. This reaction is catalyzed by flavin adenine dinucleotide (FAD)-dependent acyl-CoA oxidases, which transfer electrons directly to molecular oxygen, resulting in the production of hydrogen peroxide. In man, two acyl-CoA oxidases are present in the peroxisome, while rat peroxisomes contain three distinct acyl-CoA oxidases (8), which differ in substrate specificity. The first peroxisomal acyl-CoA oxidase isolated from rat liver is inducible by peroxisome proliferators and accepts CoA esters of VLCFAs, dicarboxylic fatty acids, prostaglandins and glutaric acid as substrates (4,9). The human and mouse counterpart of this enzyme, with regard to substrate specificity and molecular characteristics, is the straight-chain acyl-CoA oxidase (SCOX). The second acyl-CoA oxidase is pristanoyl-CoA oxidase, which is expressed in multiple rat tissues and is not inducible by peroxisome proliferators. This enzyme is active with 2-methyl-branched-chain fatty acyl-CoAs such as pristanoyl-CoA, but can also handle straight-chain acyl-CoAs (10,11). The third oxidase in rat, trihydroxycoprostanoyl-CoA oxidase, is only expressed in liver and reacts with the CoA esters of the bile acid intermediates, di- and trihydroxycholestanolic acid (DHCA and THCA, respectively) (9,12). Remarkably, humans have only one additional oxidase next to SCOX, called branched-chain acyl-CoA oxidase (BCOX), which is active with both pristanoyl-CoA and DHCA-CoA and THC-CoA (5). All oxidases in rat and man have been characterized at the molecular level (reviewed in (13)).
**Peroxisomal bifunctional proteins**

Human, rat and mouse peroxisomes contain two distinct bifunctional proteins with both enoyl-CoA hydratase and nicotinamide adenine dinucleotide (NAD\(^+\))-dependent 3-hydroxyacyl-CoA dehydrogenase activities, which catalyze the conversion of a 2-trans-enoyl-CoA to a 3-ketoacyl-CoA. The first bifunctional protein identified is now called L-bifunctional protein (LBP), because it forms and dehydrogenates L-3-hydroxyacyl-CoAs, while D-3-hydroxyacyl-CoAs are formed as intermediates of the reaction catalyzed by the second bifunctional protein, D-bifunctional protein (DBP). Alternative names are multifunctional enzymes I and II (MFE I and II), multifunctional proteins 1 and 2 (MFP1 and 2) and L- and D-peroxisomal bifunctional enzyme (L-PDE and D-PDE). Despite the fact that DBP was identified many years after the first identification of LBP (14-19), it is now well established that DBP is the main, if not exclusive enzyme involved in the β-oxidation of VLCFAs, pristanic acid, DHCA and THCA. Substrate specificity studies have shown that both enzymes react with straight-chain enoyl-CoAs, whereas only DBP is active with the enoyl-CoA esters of pristanic acid and DHCA and THCA (15,17-22). With a number of elegant experiments, Xu and Cuevas showed that LBP cannot be involved in bile acid formation (23). They found that upon incubation of the enoyl-CoA ester of THCA with purified rat LBP (24S,25S)-3α,7α,12α,24-tetrahydroxy-5β-cholestanoyl-CoA was formed, but that the dehydrogenase component of LBP was virtually inactive towards this product and only catalyzed the dehydrogenation of the (24S,25R)-diastereomer. Identification of patients with a deficiency of DBP (24-27) and the generation of a DBP knockout mouse (28) has provided unequivocal evidence for the major role of DBP in the oxidation of VLCFAs, pristanic acid and bile acid formation. In contrast, the physiological role of LBP is still unknown.

Both bifunctional proteins have been characterized at the molecular level (reviewed in (13)). They have very little sequence homology and are structurally very different. The N-terminal part of LBP contains enoyl-CoA hydratase activity and the C-terminal part 3-hydroxyacyl-CoA dehydrogenase activity. Interestingly, LBP also harbors Δ\(^3\), Δ\(^2\)-enoyl-CoA isomerase activity (29). In contrast, the N-terminal domain of DBP is responsible for the 3-hydroxyacyl-CoA dehydrogenase activity, the central part contains enoyl-CoA hydratase activity and the C-terminal domain sterol carrier protein (SCP) 2 activity.

**Peroxisomal thiolases**

The final reaction of the β-oxidation process is catalyzed by a thiolase, which thiolytically cleaves 3-ketoacyl-CoAs into chain-shortened acyl-CoAs and acetyl-CoA or propionyl-CoA. The first peroxisomal thiolase identified (30,31), often referred to as the classic peroxisomal 3-ketoacyl-CoA thiolase, is synthesized as a 44 kDa precursor and undergoes proteolytic processing to a 41 kDa mature protein after import into the peroxisome. A second peroxisomal thiolase was discovered many years later by Seedorf and coworkers (32) and is called peroxisomal thiolase 2 or sterol carrier protein X (SCPx). Extensive studies on the substrate specificity of these two enzymes performed by multiple groups have shown that straight-chain fatty acids are handled by both thiolases, while SCPx is the only thiolase reactive with the 3-ketoacyl-CoAs of pristanic acid and THCA (33-36).
Both peroxisomal thiolases also have been characterized at the molecular level (reviewed in (13)). In rat, two genes (A and B) have been identified for peroxisomal 3-ketocyl-CoA thiolase. Gene A is constitutively expressed at a low level, whereas the transcript of gene B is hardly detectable in normal rat liver but is markedly induced by peroxisome proliferators. In man there is only a single gene coding for peroxisomal 3-ketocyl-CoA thiolase. From the gene encoding SCPx two different transcripts are produced. The larger transcript codes for a 58 kDa protein which contains a thiolase domain and an SCP2 domain. The second transcript codes for pre-SCP2 which undergoes proteolytic processing inside the peroxisome to mature SCP2.

SCP2 is involved in lipid metabolism, however, its true physiological function remains unclear. Initially, SCP2 was found to transfer cholesterol and phospholipids between membranes. Recently, SCP2 was also shown to be able to bind fatty acids and fatty acyl-CoAs and it was suggested that this protein is involved in presenting fatty acyl-CoAs to the enzymes of the peroxisomal \( \beta \)-oxidation system (see for review (37)).

Physiological role of the \( \beta \)-oxidation enzymes in the oxidation of straight-chain and 2-methyl-branched-chain fatty acids

Together with the \textit{in vitro} experiments described above, studies performed in patients and knockout mice with an impaired peroxisomal \( \beta \)-oxidation have been indispensable for the elucidation of the physiological role of the \( \beta \)-oxidation enzymes in the oxidation of VLCFAs, pristanic acid and DHCA/THCA. Several human disorders have been identified with an isolated deficiency of peroxisomal \( \beta \)-oxidation, including SCOX deficiency, DBP deficiency and \( \alpha \)-methylacyl-CoA racemase deficiency. These disorders will be discussed later in more detail. In addition, knockout mice have been generated in which the genes coding for SCOX, LBP, DBP and SCPx have been disrupted. Biochemical analyses have been performed in these different knockout mice and in plasma from patients with the various disorders mentioned above. In addition, \( \beta \)-oxidation measurements have been performed in cultured skin fibroblasts from these patients and mutant mice. An overview of the results is given in Table 1.

\begin{table}[h]
\centering
\begin{tabular}{ |l|l|l|l| } 
\hline
Species & Enzyme deficiency & C26:0 & Pristanic acid & DHCA/THCA \\
\hline
Humans & SCOX & $\uparrow$ & N & N \\
& DBP & $\uparrow$ & $\uparrow$ & $\uparrow^a$ \\
& \( \alpha \)-methylacyl-CoA racemase & N & $\uparrow$ & $\uparrow$ \\
Mice & SCOX & $\uparrow$ & N & N \\
& DBP & $\uparrow$ & $\uparrow$ & $\uparrow$ \\
& LBP & N & N & N \\
& SCPx & N & $\uparrow$ & $\uparrow$ \\
\hline
\end{tabular}
\caption{Plasma levels of VLCFAs, pristanic acid, DHCA/THCA in patients and knockout mice with a defect in peroxisomal \( \beta \)-oxidation}
\end{table}

\textsuperscript{a}DHCA and THCA may be normal in isolated DBP enoyl-CoA hydratase deficiency

In humans and mice with SCOX-deficiency, VLCFAs accumulate and the rate of C26:0 \( \beta \)-oxidation is strongly reduced, while \( \beta \)-oxidation of pristanic acid and THCA is
normal (38-40). In Zellweger syndrome, where there is a deficiency of both peroxisomal oxidases, also pristanic acid, DHCA and THCA accumulate (38). From these observations it can be concluded that SCOX is responsible for the oxidation of straight-chain fatty acids and BCOX for the oxidation of 2-methyl-branched-chain fatty acids (Fig. 2).

![Diagram of fatty acid oxidation machinery](image)

Fig. 2 Schematic representation of the fatty acid β-oxidation machinery in human peroxisomes catalyzing the oxidation of very long-chain fatty acyl-CoAs (VLCFA-CoA) and branched-chain fatty acyl-CoAs (pristanoyl-CoA and THC-CoA). Oxidation of VLCFA-CoAs (C24:0 and C26:0) involves straight-chain acyl-CoA oxidase, D-bifunctional protein (DBP) and both 3-ketoacyl-CoA thiolase and sterol carrier protein X (SCPx), while oxidation of branched-chain fatty acyl-CoAs involves branched-chain acyl-CoA oxidase, DBP and SCPx.

Based on in vitro studies performed with purified LBP and DBP it was believed that LBP was involved in the degradation of the VLCFAs and that DBP was responsible for the oxidation of the 2-methyl-branched-chain fatty acids (15,17-22). This view was completely altered by the identification of patients with a deficiency of DBP. It was found that not only pristanic acid, DHCA and THCA accumulate in plasma from DBP-deficient patients, but also VLCFAs (25-27,40). This clearly shows that DBP is the main enzyme involved in β-oxidation of both straight-chain and 2-methyl-branched-chain fatty acids (Fig. 2), which has been confirmed by β-oxidation studies in fibroblasts from patients with a deficiency of DBP. These conclusions are supported by studies performed in LBP- (41) and DBP-deficient (28) mice. In DBP(-/-) mice the same abnormalities were found as in DBP-deficient patients, whereas in LBP(-/-) mice no abnormalities were found in the fatty acid profiles in plasma (Table 1). The true function of LBP therefore remains elusive.

The situation is less clear for the peroxisomal thiolases, since no deficiency of peroxisomal 3-ketoacyl-CoA thiolase or SCPx has been identified. One case of presumed peroxisomal 3-ketoacyl-CoA thiolase deficiency has been described in literature (42,43), but recent studies have shown that this is not the true defect in this patient (S. Ferdinandusse et al., submitted for publication, Chapter 5). In cells from patients with
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rizomelic chondrodysplasia punctata (RCDP) type 1 (38,44), another peroxisomal disorder, there is a secondary deficiency of peroxisomal 3-ketoacyl-CoA thiolase. This is caused by a functionally inactive peroxisomal targeting signal (PTS) 2 receptor (PTS2R/PEX7p) in this disease due to mutation(s) in the PEX7 gene. As a consequence all peroxisomal PTS2 proteins including phytanoyl-CoA hydroxylase, alkylidihydroxyacetone phosphate synthase and peroxisomal 3-ketoacyl-CoA thiolase are mislocalized in the cytosol where they are rapidly degraded. Remarkably, there is no accumulation of VLCFAs, pristanic acid or DHCA/THCA in these patients, and β-oxidation of C26:0 and pristanic acid in fibroblasts of these patients is completely normal. These findings suggest that SCPx is the main enzyme involved in C26:0 and pristanic acid β-oxidation. However, studies in plasma and fibroblasts from the SCPx(-/-) mouse generated by Seedorf et al. (45,46) have shown that although pristanic acid and DHCA/THCA β-oxidation are indeed deficient in these mice, C26:0 β-oxidation is completely normal (47). These results suggest that SCPx is the key enzyme in the degradation of pristanic acid and bile acid formation, but that both peroxisomal thiolases are involved in C26:0 β-oxidation (Fig. 2). It should be noted, however, that these conclusions are based on the assumption that the physiological role of the β-oxidation enzymes is similar in man and mice.

![Fig. 3 Pathway of DHA biosynthesis.](image)

DHA is synthesized from dietary linolenic acid (C18:3n-3) in a series of microsomal elongation and desaturation reactions, followed by retroconversion of C24:6n-3 to C22:6n-3 in the peroxisome via one round of β-oxidation.

**Role of the peroxisomal β-oxidation enzymes in the biosynthesis of docosahexaenoic acid (DHA)**

Very recently, the peroxisomal β-oxidation enzymes involved in the biosynthesis of DHA have been identified (Chapter 10; (48,49)). DHA (C22:6n-3) is the major PUFA in adult mammalian brain and retina. For a long time, the exact mechanism of DHA formation has remained unclear, but now it is known that it involves the production of C24:6n-3 from dietary linolenic acid (C18:3n-3) via a series of microsomal elongation and desaturation reactions, followed by β-oxidation of C24:6n-3 to C22:6n-3 (Fig. 3) (50,51).
The intracellular site of retroconversion of C24:6n-3 has been the subject of discussion (52,53), but recent studies have firmly established that the β-oxidation step in the biosynthesis of DHA is performed in peroxisomes (48,49,54). An important observation in this respect has been the finding that fibroblasts of patients with a peroxisome biogenesis disorder, who lack functional peroxisomes, did not form any labeled DHA upon incubation with [1-14C]-C18:3n-3, [1-14C]-C20:5n-3, [1-14C]-C22:5n-3 or [3-14C]-C24:6n-3, whereas fibroblasts of patients with a mitochondrial fatty acid oxidation disorder synthesized normal amounts of labeled DHA compared to fibroblasts of control subjects (see (48,49,54) and Chapter 10). As described in detail in Chapter 10, C24:6n-3 is β-oxidized by the same set of enzymes as used for the β-oxidation of the VLCFAs C26:0 and C24:0 (see Fig. 4) (48,49). In SCOX- and DBP-deficient fibroblasts a strongly reduced rate of C24:6n-3 β-oxidation was found, whereas the production of DHA was normal in fibroblasts from LBP(-/-) and SCPx(-/-) mice and in fibroblasts from patients with RCDP type 1, characterized by the absence of 3-ketoacyl-CoA thiolase in their peroxisomes. These results show that SCOX and DBP are the major enzymes involved in the first three steps of the β-oxidation of C24:6n-3 and that both peroxisomal thiolases are able to perform the last step of C24:6n-3 β-oxidation (Fig. 4).

C24:6 (n-3)-CoA

![Diagram](https://via.placeholder.com/150)

**Fig. 4** Schematic representation of the fatty acid β-oxidation machinery in human peroxisomes involved in the retroconversion of C24:6n-3 to C22:6n-3. Our results showed that C24:6n-3 is β-oxidized by the same set of enzymes involved in the β-oxidation of the very long-chain fatty acids C26:0 and C24:0 (see Fig. 2). Oxidation of C24:6n-3 involves straight-chain acyl-CoA oxidase (SCOX), D-bifunctional protein (DBP) and both 3-ketoacyl-CoA thiolase and sterol carrier protein X (SCPx). Branched-chain acyl-CoA oxidase and L-bifunctional protein, however, are also both able to handle this substrate, but cannot maintain normal C22:6n-3 production without SCOX and DBP activity, respectively.

**Stereochemistry of peroxisomal fatty acid β-oxidation**

*α-Methylacyl-CoA racemase*

Both the peroxisomal and mitochondrial β-oxidation system are stereospecific (55). Only (2S)-methyl-branched-chain fatty acids can be degraded, because the (peroxisomal)
Peroxisomal β-oxidation: a review

acyl-CoA oxidases (56-58) and (mitochondrial) acyl-CoA dehydrogenases act exclusively on (S)-stereoisomers (59). This implies that for the β-oxidation of (2R)-methyl-branched-chain fatty acids a racemase is needed to convert them to their corresponding (2S)-isomer. Such a racemase, called α-methylacyl-CoA racemase was identified by Schmitz and Conzelmann (60,61). The enzyme was purified from rat and human liver, and was found to accept CoA esters of a range of 2-methyl-branched-chain fatty acids, including pristanoyl-CoA and THC-CoA, as substrates. Subsequently, they cloned the corresponding rat and mouse cDNAs (62), and recently we cloned the human cDNA encoding α-methylacyl-CoA racemase (Chapter 6; (63)). It was found that the amino acid sequence of the rat α-methylacyl-CoA racemase is identical to the sequence of 2-arylpropionyl-CoA epimerase. This enzyme was already purified in 1993 (64), but was cloned in the same year as the racemase (65). 2-Arylpropionyl-CoA epimerase catalyzes the chiral inversion of a number of nonsteroidal anti-inflammatory drugs such as Ibuprofen. Studies on the reaction mechanism of 2-arylpropionyl-CoA epimerase have shown that the α-proton is abstracted from the substrate by a basic moiety in the active site of the enzyme followed by stereospecific rehydration (Fig. 5). In this proposed mechanism, the thioester bond of CoA esters is required, since it makes the α-carbon atom acidic, thereby facilitating proton abstraction. The resulting carbanion tautomerizes into its enolate ion, which is rehydrated resulting in chiral inversion. Experiments with (R)-2-deuterium labeled Ibuprofenyl-CoA have demonstrated that the hydrogen atom of the new C-H bond is derived from the solvent (66,67).

Interestingly, Schmitz and Conzelmann found that α-methylacyl-CoA racemase activity was present in both mitochondria and peroxisomes in man and mouse, whereas in rat racemase activity was strictly mitochondrial (61). Studies by ourselves (Chapter 7; (68)) and others (69,70) have shown that irrespective of the species (man, rat or mouse) α-methylacyl-CoA racemase activity is present in both mitochondria and peroxisomes. In addition, we showed that both the mitochondrial and peroxisomal enzyme are derived from the same gene, because fibroblasts from patients with an established α-methylacyl-CoA racemase deficiency caused by missense mutations in the encoding gene were deficient for both mitochondrial and peroxisomal racemase activity (68). The same was also demonstrated for mouse α-methylacyl-CoA racemase by Schmitz and coworkers (70) with combined Northern and Southern blot analyses. These studies

![Fig. 5 Proposed mechanism of the chiral inversion of 2-methyl-branched-chain fatty acyl-CoAs by α-methylacyl-CoA racemase. The α-proton is abstracted from the substrate by a basic moiety in the active site of the enzyme followed by stereospecific rehydration. The hydrogen atom of the new C-H bond is derived from the solvent.](image-url)
suggested differential targeting of the same gene product, and, indeed, subsequent studies revealed the presence of a mitochondrial targeting signal at the N-terminus and a peroxisomal targeting sequence at the C-terminus of human α-methylacyl-CoA racemase (71).

**Pristanic acid β-oxidation**

Pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) is derived from phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) via α-oxidation in the peroxisomes, but also directly from dietary sources. In 1967, Ackman and Hansen (72) studied the stereochemical composition of phytanic and pristanic acid in ruminant fats and fish oils. They found that there are two diastereomers of these fatty acids present, namely the (S,R,R)- and (R,R,R)-isomer. Phytanic acid, which is synthesized from phytol of plant origin, consists also of these two isomers. Croes and coworkers (73) have shown that peroxisomal α-oxidation is not a stereospecific process so that after α-oxidation of phytanic acid both (2R,6R,10R,14)- and (2S,6R,10R,14)-pristanic acid are formed. Because β-oxidation, in contrast to α-oxidation, is stereospecific, α-methylacyl-CoA racemase activity is needed to convert (2R)-pristanoyl-CoA to its (2S)-isomer before it can be degraded. For this reason, racemase-deficient patients have elevated plasma levels of pristanic acid (63). Recent studies have shown that (2R)-pristanic acid is the predominant isomer which accumulates in these patients (Chapter 9). After two cycles of β-oxidation, however, another (2R)-methyl-branched-chain fatty acyl-CoA is formed, which is called (2R,6R,10)-trimethylundecanoyl-CoA (Fig. 6). This substrate also requires racemase activity before it can be further broken down. Recently, we have shown that trimethylundecanoic acid and trimethylundecanoyl-carnitine accumulate in plasma from patients with a deficiency of α-methylacyl-CoA racemase, strongly suggesting that this racemase is responsible for the chiral conversion of this compound as well (Chapter 9). After yet another cycle of β-oxidation (3 cycles in total) (4R,8)-dimethylnonanoyl-CoA is formed, which is subsequently transported from the peroxisome to the mitochondrion as a carnitine ester for further oxidation (Fig. 6) (6). We have shown (Chapter 3) that carnitine octanoyltransferase (COT) is responsible for the peroxisomal conversion of dimethylnonanoyl-CoA to its corresponding carnitine ester (74). Dimethylnonanoyl-carnitine is taken up into the mitochondrion via carnitine acylcarnitine translocase (CACT) and reconverted into a CoA ester via carnitine palmitoyl transferase II (6). Dimethylnonanoyl-CoA will then be broken down via the mitochondrial β-oxidation system. After one cycle (2R,6)-dimethylheptanoyl-CoA is formed. Since the mitochondrial β-oxidation system, like the peroxisomal system, is stereospecific, racemase activity is required for further oxidation of this substrate (55,59). We showed that the mitochondrial α-methylacyl-CoA racemase is responsible for the conversion of this substrate, because fibroblasts from α-methylacyl-CoA racemase-deficient patients were not able to racemize dimethylheptanoyl-CoA. After conversion of (2R,6)-dimethylheptanoyl-CoA to its (2S)-isomer by α-methylacyl-CoA racemase, it can be broken down to completion (68). From studies described above performed in patients with a deficiency of α-methylacyl-CoA racemase it can be concluded that α-methylacyl-CoA racemase is
Fig. 6 Schematic representation of the pristanic acid β-oxidation and the involvement of racemase activity in mitochondria and peroxisomes. (2R,6R,10R,14)-pristanoyl-CoA (I), which is the configuration of half of the naturally occurring pristanoyl-CoA, needs to be converted to its (S)-stereoisomer before it can enter the β-oxidation spiral because the peroxisomal oxidases, the first enzymes of the β-oxidation system, can only handle (S)-stereoisomers. The resulting product, (4R,8R,12)-trimethyltridecanoyl-CoA (II) can be β-oxidized without any problem, but the next intermediate in the breakdown process of pristanic acid is again a 2-methyl-branched fatty acyl-CoA ((2R,6R,10)-trimethylundecanoyl-CoA (III)) with the (R)-configuration and requires therefore a racemase to convert it to its (S)-isomer. After another cycle of β-oxidation (4R,8)-dimethylnonanoyl-CoA (IV) is transported from the peroxisome to the mitochondrion as carnitine ester for further oxidation. One cycle of mitochondrial β-oxidation results in the production of (2R,6)-dimethylheptanoyl-CoA (V) and a racemase is needed to form the (S)-isomer, which can be β-oxidized to completion.

required for the complete β-oxidation of pristanic acid both in the peroxisome and the mitochondrion (Fig. 6).

**DHCA and THCA β-oxidation**

DHCA and THCA are obligatory intermediates in the major biosynthesis route of the primary bile acids chenodeoxycholic acid and cholic acid, respectively. They are formed in
the liver from cholesterol via a complicated set of reactions. After activation at the endoplasmic reticulum membrane (75), DHC-CoA and THC-CoA are transported across the peroxisomal membrane via a mechanism yet unknown and undergo one cycle of \( \beta \)-oxidation in the peroxisome. However, since the synthesis of DHCA and THCA is stereospecific and leads exclusively to the formation of the (25R)-stereoisomer (76-79), (25R)-DHC-CoA and (25R)-THC-CoA first have to be converted to their (25S)-isomer before they can enter the \( \beta \)-oxidation spiral. \( \alpha \)-Methylacyl-CoA racemase is responsible for this conversion, as concluded from the observation of the exclusive accumulation of the (25R)-isomer of both free and taurine-conjugated DHCA and THCA in plasma from patients with a deficiency of \( \alpha \)-methylacyl-CoA racemase (Chapter 8; (80)). After chain-shortening via \( \beta \)-oxidation, chenodeoxycholoyl-CoA and choloyl-CoA are converted into their corresponding taurine or glycine conjugates via bile acid-CoA:amino acid N-acyltransferase, which is localized in the peroxisome (81). The conjugates are then exported from the peroxisome and finally excreted into bile after transport across the canalicular membrane.

Disorders of peroxisomal fatty acid \( \beta \)-oxidation

The following peroxisomal fatty acid \( \beta \)-oxidation disorders have been identified: 1) X-linked adrenoleukodystrophy (XALD) (MIM 300100) 2) acyl-CoA oxidase deficiency (SCOX) (MIM 264470) 3) DBP deficiency (MIM 261515) and 4) \( \alpha \)-methylacyl-CoA racemase deficiency (MIM 604489). In addition, peroxisomal \( \beta \)-oxidation is deficient in patients with a peroxisome biogenesis disorder. Because of a defect in peroxisome assembly these patients have a generalized loss of peroxisomal functions. Due to their peroxisomal \( \beta \)-oxidation deficiency they accumulate VLCFAs, pristanic acid and DHCA/THCA (38).

**XALD**

XALD is the most common peroxisomal disorder (see for review (82,83)). The clinical presentation is very diverse, at least six phenotypic variants can be distinguished ranging from a severe lethal childhood cerebral form to an Addison-only form with no neurological dysfunction. Patients with XALD accumulate VLCFAs due to an impaired peroxisomal \( \beta \)-oxidation of these fatty acids. This is, however, not caused by a deficiency of one of the enzymes of the \( \beta \)-oxidation system, but by a defect in the peroxisomal membrane protein ALDP (adrenoleukodystrophy protein), which is believed to be involved in the transport of VLCFAs into the peroxisome.

**Acyl-CoA oxidase deficiency**

Only a few cases of acyl-CoA oxidase deficiency have been described (reviewed in (40)). The main clinical symptoms in these patients are severe neurological abnormalities including early-onset seizures, hypotonia, hearing impairment and visual loss due to retinopathy. In these patients there is an accumulation of VLCFAs, because they cannot be oxidized due to a deficiency of SCOX. The levels of pristanic acid and DHCA/THCA are normal in these patients.
**DBP deficiency**

Although DBP deficiency is a rare disorder, more patients with DBP deficiency have been described than patients with acyl-CoA oxidase deficiency (reviewed in (40)). The clinical presentation of DBP deficiency is severe and resembles that of Zellweger syndrome in many respects. Patients with this disorder have severe neurological abnormalities including seizures, hypotonia and craniofacial dysmorphia (macrocephaly, high forehead, flat nasal bridge, low-set ears, large open fontanelle). They have a severe developmental delay and usually die very young. Interestingly, in most cases neuronal migration is disturbed as described for Zellweger syndrome. DBP deficiency can be divided in three subgroups. In the first group, the patients have a complete DBP deficiency (26), in the second group there is an isolated DBP enoyl-CoA hydratase deficiency (27) and in the third group an isolated DBP 3-hydroxyacyl-CoA dehydrogenase deficiency (25). Plasma analysis in these patients reveal accumulation of VLCFAs, pristanic acid and in most cases there are also elevated levels of DHCA/THCA. In some patients with an isolated DBP enoyl-CoA hydratase deficiency, however, no bile acid intermediates are found (27).

**α-Methylacyl-CoA racemase deficiency**

At this moment only a few patients with a deficiency of α-methylacyl-CoA racemase have been identified. Based on the clinical presentation of the first patients described (63), it was suggested that there is an adult-onset of the clinical symptoms in these patients, and that racemase deficiency is associated with neuropathy. Three out of the four patients suffered from sensory motor neuropathy and three patients had eye problems. Two of these patients had retinitis pigmentosa accompanied by visual loss and in two patients there was optic atrophy. In addition, two patients had a tremor. Other symptoms seen in at least one of these patients were cerebellar dysarthria, spastic paraparesis and epileptic seizures. In contrast to the clinical symptoms, the biochemical abnormalities found in plasma are the same in all these patients. They have normal levels of the VLCFAs and elevated levels of the branched-chain fatty acids, pristanic acid and phytanic acid. The level of phytanic acid is only marginally elevated, whereas the level of pristanic acid is strongly increased. In addition, they accumulate DHCA and THCA. This abnormal profile in plasma of these patients clearly shows that α-methylacyl-CoA racemase deficiency affects the oxidation of 2-methyl-branched-chain fatty acids and the bile acid intermediates. Indeed, in fibroblasts of these patients a complete deficiency of α-methylacyl-CoA racemase activity was found (63).

Very recently, a patient was diagnosed with α-methylacyl-CoA racemase deficiency shortly after birth (84). This patient had blood streaked mucus in the stool and a liver biopsy revealed giant-cell neonatal hepatitis. Analysis of the urine revealed the presence of the taurine conjugates of THCA and reduced primary bile acid levels. Plasma levels of VLCFAs and pristanic acid/phytanic acid were normal. The latter can be explained because there is no dietary intake of the branched-chain fatty acids shortly after birth.

The patients diagnosed at our laboratory have all been characterized at the molecular level (63). All patients had a missense mutation in the cDNA encoding α-methylacyl-CoA racemase, leading to an amino acid change. These amino acid changes were shown to abolish the enzyme activity completely, by expression studies in E. coli.
Disorders of peroxisomal fatty acid α-oxidation

Refsum disease
Refsum disease (MIM 266500) is the only disorder identified of the phytanic acid α-oxidation and is caused by a defect of phytanoyl-CoA hydroxylase (85,86), the first enzyme of the α-oxidation system. As a result, these patients accumulate phytanic acid. The main symptoms include retinitis pigmentosa, peripheral neuropathy and cerebellar ataxia. In most patients the onset of the first clinical symptoms is before the age of 20.

Concluding remarks and future prospects
Currently, all the enzymes of the peroxisomal β-oxidation system involved in the degradation of VLCFAs and branched-chain fatty acids have been identified. Studies performed in patients and knockout mice with an impaired peroxisomal β-oxidation, in addition to in vitro studies performed with the purified enzymes, have played a major role in the elucidation of the physiological role of the β-oxidation enzymes in the oxidation of VLCFAs, pristanic acid and DHCA/THCA and in the biosynthesis of DHA. At this moment the only peroxisomal β-oxidation enzyme whose function remains unclear, is LBP. Surprisingly, generation of an LBP knockout mouse did not provide new insights in this matter. Although it is generally believed that peroxisomal 3-ketoacyl-CoA thiolase is involved in the β-oxidation of VLCFAs, the findings in patients with RCDP type 1 (normal levels of VLCFAs and normal C26:0 β-oxidation) are hard to reconcile with this belief unless SCPx and 3-ketoacyl-CoA thiolase can take over each other’s function with regard to VLCFAs oxidation. Since the only patient described with a deficiency of 3-ketoacyl-CoA thiolase turned out to be a DBP-deficient patient when reinvestigated, generation of a peroxisomal 3-ketoacyl-CoA thiolase knockout mouse would be of great help to establish the precise physiological function of this thiolase. Apart from the substrates discussed above, many other compounds undergo β-oxidation in the peroxisome including eicosanoids, long-chain dicarboxylic acids and certain xenobiotics. For most of these substrates it remains to be established which enzymes are involved in their oxidation.
References


Chapter 2


Molecular cloning and expression of human carnitine octanoyltransferase (COT): evidence for its role in the peroxisomal β-oxidation of branched-chain fatty acids

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Abstract
To study the putative role of human carnitine octanoyltransferase (COT) in the β-oxidation of branched-chain fatty acids, we identified and cloned the cDNA encoding human COT and expressed it in the yeast Saccharomyces cerevisiae. Enzyme activity measurements showed that COT efficiently converts one of the end products of the peroxisomal β-oxidation of pristanic acid, 4,8-dimethylnonanoyl-CoA, to its corresponding carnitine ester. Production of the carnitine ester of this branched/medium-chain acyl-CoA within the peroxisome is required for its transport to the mitochondrion where further β-oxidation occurs. In contrast, 4,8-dimethylnonanoyl-CoA is not a substrate for carnitine acetyltransferase, another acyltransferase localized in peroxisomes, which catalyses the formation of carnitine esters of the other products of pristanic acid β-oxidation, namely acetyl-CoA and propionyl-CoA. Our results shed new light on the function of COT in fatty acid metabolism and point to a crucial role of COT in the β-oxidation of branched-chain fatty acids.

Introduction
In recent years the involvement of carnitine in the peroxisomal β-oxidation of fatty acids has been clearly established in both higher (1,2) and lower (Saccharomyces cerevisiae) eukaryotes (3). The exact mechanism and the nature of the enzymes involved, however, have not been completely elucidated (see (4) for review). There is growing evidence that the end products of the peroxisomal β-oxidation system, including acetyl-CoA, propionyl-CoA and medium-chain acyl-CoA esters, are converted into carnitine esters by carnitine acyltransferases before they are exported from the peroxisomes to the mitochondria where further β-oxidation occurs (2,5). Mammalian peroxisomes contain at least three distinct carnitine acyltransferases, namely carnitine acetyltransferase (CAT) (6), carnitine octanoyltransferase (COT) (7,8) and a less well characterized medium/long-chain acyltransferase (9). While all three carnitine acyltransferases catalyse the reversible transfer of fatty acyl groups between CoA and carnitine, the individual enzymes differ in their particular substrate specificities. CAT has been reported to have a preference for short-chain acyl-CoAs (C2-C4) (6), whereas COT is most active with medium-chain length substrates (C6-C10) (7,8).

Thus far, all the substrate specificity studies for CAT and COT have been performed with straight-chain fatty acids. In addition to the β-oxidation of straight-chain fatty acids,
however, peroxisomes also play a crucial role in the β-oxidation of branched-chain fatty acids, including pristanic acid (2,6,10,14-tetramethylpentadecanoic acid). Recent studies by Verhoeven et al. (2) indicated that peroxisomal β-oxidation of pristanic acid proceeds efficiently for three cycles yielding 4,8-dimethylnonanoyl-CoA (C11-CoA), which is then converted to its corresponding carnitine ester and exported from the peroxisome. Further β-oxidation occurs in the mitochondrion after import of the C11-carnitine ester by the carnitine-acylcarnitine translocase (CACT) localized in the mitochondrial inner membrane, followed by reconversion into C11-CoA by the mitochondrial carnitine palmitoyltransferase II (CPT II) (2). At the onset of this study, however, it was unknown which peroxisomal carnitine acyltransferase is responsible for the conversion of C11-CoA to its carnitine ester. We now report that this conversion is catalyzed by COT. To study this, we identified and cloned the human cDNA encoding COT and expressed it in the yeast S. cerevisiae followed by enzyme activity measurements.

Materials and Methods

Materials
Acetyl-CoA, octanoyl-CoA and L-carnitine were purchased from Sigma Chemicals (St. Louis, MO). [1-14C]carnitine was obtained from NEN ('s Hertogenbosch, The Netherlands). CAT purified from pigeon breast (80 U/mg) and Complete protease inhibitor were purchased from Boehringer Mannheim (Mannheim, Germany). Yeast nitrogen base and amino acids were obtained from Difco Laboratories Inc. (Detroit, MI), Profitar from Nutricia (Zoetermeer, The Netherlands) and AG 1-X8 and goat anti-rabbit IgG antibodies conjugated with alkaline phosphatase from Bio-Rad Laboratories (Richmond, CA). Antibodies raised against COT were a kind gift from Prof. Dr. T. Hashimoto (Shinshu University, Matsumoto, Japan).

Identification of the cDNA encoding human COT
The expressed sequence tags database (dbEST) of the National Center of Biotechnology Information was screened with the amino acid sequence of rat and bovine COT, and several partial human EST clones with high homology were identified. Based on the EST sequences two sets of primers with −21M13 or M13rev extensions were designed (Table 1) and used to amplify the entire open reading frame of COT cDNA in two overlapping fragments by RT-PCR. First strand cDNA was prepared from total RNA isolated from cultured human skin fibroblasts as described before (10) and used as template. PCR fragments were sequenced in both directions by means of −21M13 and M13rev fluorescent primers on an ABI 377A automated DNA sequencer according to the manufacturer’s protocol (Perkin-Elmer).

Expression of COT cDNA in S. cerevisiae
The coding sequence of human COT cDNA was amplified by PCR using the cloning primers described in Table 1 and subsequently cloned into the yeast expression vector pEL26 under transcriptional control of the oleate-inducible CTA1 promoter (11).
Chapter 3

S. cerevisiae strain BJ1991 in which the YCAT gene was disrupted (BJ1991 Δycat::LEU2) was transformed with the expression plasmid using the lithium acetate method (12). Transformants were selected and grown at 28°C on minimal medium containing 6.7 g/L yeast nitrogen base without amino acids, 30 g/L glucose and 20 mg/L of the appropriate amino acids. Induction was initiated by shifting the cells to a rich medium containing oleic acid (5 g/L potassium phosphate buffer, pH 6, 3 g/L yeast extract, 5 g/L peptone and 1 g/L oleic acid + 2 g/L Tween-40). The cells were harvested and resuspended in phosphate buffered saline (PBS) containing Complete protease inhibitor cocktail (1 tablet in 25 ml H2O). To prepare cell lysates, 200 µl glass beads were added and the suspension was vortexed 11 times for 15 sec with a 45 sec interval at 4°C. The lysates were subsequently homogenized by sonication and cell debris was removed by centrifugation at 10,000 × g for 30 sec. The supernatant was used for immunoblot analysis and enzyme activity measurements.

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<sup>a</sup>21M13 extension: tgtaaaacagccgcat; <sup>b</sup>M13rev extension: caggaacagcttgacc.<sup>c,d</sup>The restriction sites for SalI and SphI are underlined.

Immunoblot analysis

40 µg of protein was subjected to electrophoresis on a 10% (w/v) SDS-polyacrylamide gel essentially as described by Laemmli (13) and transferred to a nitrocellulose sheet. After blocking of non-specific binding sites with 50 g/L Protifar and 10 g/L BSA in PBS + 1 g/L Tween-20 for 1 h, the blot was incubated for 2 h with rabbit polyclonal antibodies raised against COT (prepared as described (7)) and diluted (1:2,000) in 40 g/L normal goat serum. Goat anti-rabbit IgG antibodies conjugated with alkaline phosphatase were used for detection according to the manufacturer's instructions (Bio-Rad).

Carnitine acyltransferase activity measurements

Carnitine acyltransferase activity was assayed in the direction of acylcarnitine formation. The incubations consisted of 1.3 µg lysate of yeast expressing COT or 5 ng purified CAT, 50 mM HEPES, pH 7.4, 1 mM dithiothreitol, 1 mM EDTA, 1.3 mg/ml bovine serum albumine, 150 mM potassium chloride, and 0.5 mM L-carnitine (including [1-<sup>14</sup>C]carnitine, 4 kBq) in a final volume of 250 µl. Reactions were initiated by the addition of 100 µM acyl-CoA. After an incubation period of 10 min at 37°C reactions were terminated by the addition of 250 µl of 1.2 M HCl. Acylcarnitines were extracted essentially as described by Solberg (14). In experiments where acetyl-CoA was used as substrate the incubations consisted of 50 mM HEPES, pH 7.4, 5 mM L-carnitine and 300
µM acetyl-CoA (including [1-14C]acetyl-CoA, 4 kBq) in a final volume of 100 µl. Reactions were initiated by the addition of 0.1 ng purified CAT and terminated after an incubation period of 60 min at 37°C by the addition of 100 µl ice cold 99% ethanol. Assay mixtures were applied to an AG 1-X8 (200-400 mesh, chloride form) column. Upon washing with ethanol, acetylcarnitine passed through the column, whereas acetyl-CoA remained bound. Radioactivity was determined by scintillation counting.

**Synthesis of 2,6-dimethylheptanoyl-CoA and 4,8-dimethylnonanoyl-CoA**

2,6-Dimethylheptanoyl-CoA was synthesized as described before (15). 4,8-Dimethylnonanoic acid was prepared from 3,7-dimethyloctanol by a one carbon chain elongation. 3,7-Dimethyloctanol was first reacted with methanesulfonylchloride and triethylamine to form the methanesulfonate which was purified by a silica gel chromatography with hexane-diethyl ether (8:2 v/v). The methylsulfonate was subsequently converted to 4,8-dimethylnonanitrile with potassium cyanide in dry dimethyl sulfoxide. The 4,8-dimethylnonanitrile was extracted from the reaction mixture with hexane and further purified on a silica gel column with hexane-ethylacetate (9:1 v/v). Finally, the nitrile was hydrolyzed with 1 M sodium hydroxide in ethanol-H2O to yield 4,8-dimethylnonanoic acid. The acid was purified by column chromatography on silica gel with hexane-diethyl ether 98:2 and 95:5 (v/v). Gas liquid chromatography-mass spectrometry of the methylester showed one homogeneous peak with a molecular ion at m/z 200. Mass spectrometry of the unesterified acid: m/z 186 (M+ 4.3%), 171 (8.7%), 143 (15.0%), 115 (38.0%), 110 (18.0%), 74 (100%), 55 (22.0%), 43 (25.0%). Overall molar yield from 3,7-dimethyloctanol to 4,8-dimethylnonanoic acid was 58%. The CoA ester of 4,8-dimethylnonanoic acid was prepared by the method of Rasmussen et al. (16).

**GenBank accession numbers**

Rat COT U26033; bovine COT U65745; human COT AF168793.

**Results**

**Cloning of human COT cDNA and its expression in S. cerevisiae**

The EST database at the NCBI was searched with the amino acid sequences of rat and bovine COT for cDNA sequences encoding the human homologue. Several partial human cDNA clones with high homology were found. Primers were designed based on the cDNA sequences to amplify the complete coding sequence from cDNA prepared from human skin fibroblasts. The nucleotide sequence was determined and revealed an open reading frame of 1839 bp encoding a polypeptide of 612 amino acids with a calculated molecular weight of 70 kDa. The deduced amino acid sequence showed 85% identity with both the bovine (17) and rat (18) COT sequence (Fig. 1). As in the bovine and rat sequences, a putative peroxisomal targeting signal type 1 was identified at the carboxy terminus (-THL). Subsequently, the entire coding sequence of human COT was expressed in an S. cerevisiae strain with a targeted disruption of the YCAT gene encoding the yeast carnitine acetyltransferase. Expression of the protein was confirmed by
immunoblot analysis (data not shown). In yeast cells transformed with the expression vector containing the coding sequence for COT, a protein with an estimated size of 66 kDa was expressed after induction with oleic acid. This is in accordance with the molecular weight of COT determined by SDS gel electrophoresis as reported in literature (7). No cross-reactive material with α-COT antibody was observed in yeast cells transformed with the expression vector without insert.

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**Fig. 1** Alignment of the amino acid sequences of human, bovine and rat COT. The black boxes represent identical amino acids and gray boxes represent similar residues. The human amino acid sequence shares 85% identity with both the bovine (17) and rat (18) COT sequence. As in the bovine and rat sequences, the human sequence contains a putative peroxisomal targeting signal type 1 at the carboxy terminus (-THL).
Carnitine octanoyltransferase

Enzyme activity measurements with human COT expressed in S. cerevisiae

Activity measurements showed that human COT expressed in S. cerevisiae was active with C8-CoA as substrate, which was in line with literature data showing that COT is most active with medium-chain acyl-CoAs (C6-C10) (Table 2). The aim of this study, however, was to investigate whether COT is responsible for the conversion of C11-CoA, one of the main end products of the peroxisomal β-oxidation of pristanic acid, into its carnitine ester. Activity measurements in yeast lysates with C11-CoA as substrate showed a high rate of C11-carnitine formation, comparable to the activity measured with C8-CoA. Remarkably, 2,6-dimethylheptanoyl-CoA (C9-CoA), which is derived from C11-CoA after one cycle of β-oxidation, was not handled by COT. This is in agreement with the earlier observation by Verhoeven et al. (2) that peroxisomal β-oxidation does not proceed beyond C11-CoA. No activity could be measured with any of these substrates in wild-type yeast transformed with the expression vector without insert.

Table 2 Enzyme activity of human COT expressed in S. cerevisiae and CAT purified from pigeon breast

<table>
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<tr>
<th>Substrate</th>
<th>COT (nmol/min/mg)</th>
<th>CAT (μmol/min/mg)</th>
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<tr>
<td>C8-CoA</td>
<td>118*</td>
<td>6*</td>
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<tr>
<td>C2-CoA</td>
<td>-</td>
<td>90*</td>
</tr>
<tr>
<td>C11-CoA</td>
<td>99*</td>
<td>ND</td>
</tr>
<tr>
<td>C9-CoA</td>
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*, not measured. ND, not detectable. Each value represents the mean of four experiments. Each value represents the mean of two experiments.

Next, we compared the activities of COT and CAT, which is also localized in peroxisomes, with these branched-chain substrates. Initial experiments to clone and express human CAT in S. cerevisiae with a targeted disruption of the YCAT gene were not successful because a second CAT protein, responsible for about 5% of the cellular CAT activity of wild-type yeast (19), interfered with activity measurements when acetyl-CoA was used as substrate. Since it has been demonstrated that CATs from different tissues and species have comparable enzymatic properties (20), we decided to use commercially available CAT purified from pigeon breast for this study. As shown in Table 2, CAT was active with C2-CoA and slightly active with C8-CoA, but not able to convert the branched-chain substrates C11-CoA and C9-CoA to their corresponding carnitine esters.

Discussion

In 1998, Verhoeven et al. (2) showed that peroxisomal β-oxidation of pristanic acid proceeds for three cycles, after which C11-CoA is exported from the peroxisome to the mitochondrion for further oxidation. In addition, they showed that this transport occurs as carnitine ester. They performed tandem mass spectrometric analysis of acylcarnitine intermediates in intact human fibroblasts from control subjects and from patients with established deficiencies of either carnitine palmitoyltransferase I (CPT I), CPT II, or CACT. In CACT-deficient cell lines and CPT II deficient cell lines they observed an
increased amount of C11-carnitine and either a decreased amount of C9-carnitine or a total absence of C9-carnitine, respectively. These results showed that CACT and CPT II are indispensable for further oxidation of C11-CoA to C9-CoA. Hence, it was concluded that C11-CoA is formed in peroxisomes, after which it is converted to its carnitine ester. C11-carnitine is then imported into the mitochondrion by CACT and reactivated by CPT II to C11-CoA, which can be further degraded by the mitochondrial β-oxidation system. The results obtained with the CPT I-deficient cell lines indicated that CPT I is not involved in the conversion of C11-CoA to its carnitine ester, but it remained unclear which carnitine acyltransferase did catalyze this reaction (2). In this paper we showed that COT is responsible for the peroxisomal conversion of C11-CoA to its corresponding carnitine ester. In order to determine whether the activity was specific for C11-CoA, activity was also measured using C9-CoA as substrate. C9-CoA is produced from C11-CoA after one cycle of β-oxidation and is also a branched/medium-chain fatty acyl-CoA. COT was found to be inactive with this substrate. This finding stresses the functional significance of the measured COT activity towards C11-CoA.

\[
\text{Peroxisome} \quad \text{Mitochondrion}
\]

In contrast to COT, CAT was not able to convert either of these two branched-chain substrates. It is clear, however, that CAT is also indispensable for the peroxisomal β-oxidation of pristanic acid because it is responsible for the transport of acetyl-CoA and propionyl-CoA to the mitochondrion as carnitine esters (21). In Fig. 2 the proposed organization of the β-oxidation of pristanic acid, including the interaction between peroxisomes and mitochondria, is depicted. This figure demonstrates the crucial role of COT in the β-oxidation of pristanic acid as indicated by the results presented in this paper.
Acknowledgments
We are grateful to Prof. Dr. T. Hashimoto (Shinshu University, Matsumoto, Japan) for the antibodies raised against COT. This work was supported by the Princess Beatrix Fund (The Hague, The Netherlands).

References


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

Chapter 4

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, 3a,7a,12a-trihydroxy-24-keto-5β-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. Following the preincubation period, liver or fibroblast homogenate is added plus CoA, and the production of choholyl-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.

Introduction
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 man, the CoA esters of straight-chain fatty acids are first desaturated by the acyl-CoA oxidase identified by Osumi and coworkers (1), now called straight-chain acyl-CoA oxidase. The enoyl-CoAs produced are subsequently converted to 3-ketoacyl-CoAs by L-bifunctional protein (2), which first hydrates trans-enoyl-CoAs to their L-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 (DBP) via the D-hydroxy stereoisomer (5-8). Recent studies have also shown that the two known peroxisomal thiolases that catalyse the last step of the
Sterol carrier protein X

peroxisomal β-oxidation spiral, namely, 3-ketoacyl-CoA thiolase identified by Hashimoto and coworkers (9), and sterol carrier protein X (SCPx) identified by Seedorf and coworkers (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 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 (16,20,21), 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 (22) for details). Since 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 CoA were purchased from Boehringer Mannheim. Protifar was obtained from Nutricia and goat anti-rabbit IgG antibodies conjugated to alkaline phosphatase from Bio-Rad Laboratories. Antibodies raised against the thiolase domain of SCPx were a kind gift from Prof. Dr. K. Wirtz (Utrecht, The Netherlands).

Cloning and expression of DBP in yeast
The cloning of DBP 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 3α,7α,12α-trihydroxy-24-keto-5β-cholestanoyl-CoA (24-keto-THC-CoA) was synthesized enzymatically by incubation of 3α,7α,12α-trihydroxy-5β-cholest-24-en-26-oyl-CoA (24(E)-ene-THC-CoA) with human DBP 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 μM 24(E)-ene-THC-CoA and 4 mU/ml DBP, which was added as a crude yeast lysate. After a preincubation of 15 min at 37°C, 200 μM CoA 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 20,000 × g at 4°C, the supernatant was applied to a reversed-phase C₁₈-column (Supelcosil SPLC-18-DB, 250 mm × 10 mm, Supelco). Resolution between the different CoA esters was achieved by elution with a linear gradient of acetonitrile (25 → 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 DBP reaction, i.e. 3α,7α,12α,24α-tetrahydroxy-5β-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 thiolysic 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 μg 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 1:2,000 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 coworkers (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 in 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.

Since 24-keto-THC-CoA is not commercially available, we studied whether it could be produced using DBP 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 DBP (in a crude yeast lysate), and oxaloacetate and malate dehydrogenase for regeneration of NAD⁺. We found that DBP at a concentration of 4 mM 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 μU/ml DBP. The actual thiolase reaction is started after 15 min at 37 ºC by adding CoA, 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 CoA at pH 9.0 and an incubation time of 15 min as standard assay conditions. Since 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.
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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 CoA on the production of choloyl-CoA was determined. The $K_m$ of the reaction for CoA was 37.7 μM. In subsequent experiments 200 μM CoA was used.

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).

**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

Table 1 Activity measurements of SCPx in homogenates of cultured skin fibroblasts

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<td>Zellweger patients [n = 4]</td>
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<td>(33)</td>
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<td>DBP patient 2</td>
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Results represent the mean ± SD; n represents the number of measurements. References to case reports described in literature are given. Detection limit < 0.01 nmol.

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(E)-ene-THC-CoA, whereas peak 2 and 3 are the products of the 15 min preincubation with DBP, 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.
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 \( \beta \)-oxidation

Several patients have been described in literature with an unresolved defect in the peroxisomal \( \beta \)-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 DBP (24,36), one of the other enzymes of branched-chain fatty acid \( \beta \)-oxidation. In one of these DBP patients SCPx activity was increased, while the activity in the other patient was within the normal range (Table 1).

![Image](Fig. 3 Immunoblot analysis of SCPx in fibroblasts from 6 control subjects, 4 patients with Zellweger syndrome (ZS1-4), 6 patients with an unresolved \( \beta \)-oxidation defect (patient 1-6) and DBP 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 \( \beta \)-oxidation most of the cross-reacting material is the 46 kDa thiolase domain.)

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 \( \beta \)-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-
Sterol carrier protein X

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 since many peroxisomal proteins, including dihydroxyacetone phosphate acyltransferase (37,38), alkyldihydroxyacetone 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 DBP, 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.

Acknowledgments
We thank H.R. Waterham and L. IJlst for helpful discussion and critical reading of the manuscript, and C. Dekker for technical assistance. We are grateful to Dr. K. Wirtz (Utrecht, The Netherlands) for kindly providing the antibodies against SCPx. This work was supported by a grant from the Princess Beatrix Fund, The Hague, The Netherlands.

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Chapter 5

Reinvestigation of peroxisomal 3-ketoacyl-CoA thiolase deficiency: identification of the true defect at the level of D-bifunctional protein.

Reinvestigation of peroxisomal 3-ketoacyl-CoA thiolase deficiency: identification of the true defect at the level of D-bifunctional protein

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Abstract
So far only one single patient with a deficiency of peroxisomal 3-ketoacyl-CoA thiolase has been reported. The patient accumulated very long-chain fatty acids and the bile acid intermediate trihydroxycholestanoic acid in body fluids. At the time, these abnormalities were believed to be the logical consequence of the assumption that 3-ketoacyl-CoA thiolase was the only thiolase involved in the peroxisomal β-oxidation of all fatty acyl-CoAs. Recent studies have shown, however, that peroxisomes contain two sets of β-oxidation enzymes, including a second peroxisomal thiolase, i.e. sterol carrier protein X, responsible for the β-oxidation of branched-chain fatty acids but also of bile acid intermediates. Since the reported biochemical aberrations could no longer be explained by a deficiency of 3-ketoacyl-CoA thiolase, we reinvestigated the previously reported patient. In this paper, we show that the true defect in this patient is at the level of D-bifunctional protein (DBP) and not at the level of 3-ketoacyl-CoA thiolase. Immunoblot analysis revealed the absence of DBP in post-mortem brain of the patient, whereas 3-ketoacyl-CoA thiolase was normally present. In addition, we found that the patient had a homozygous deletion of part of exon 3 and intron 3 of the DBP gene, resulting in skipping of exon 3 at the cDNA level. Our findings have great implications, since they imply that the group of identified single peroxisomal β-oxidation enzyme deficiencies is limited to straight-chain acyl-CoA oxidase, DBP and α-methylacyl-CoA racemase deficiency and that there is no longer evidence for the existence of 3-ketoacyl-CoA thiolase deficiency as a distinct clinical entity.

Introduction
In 1986, Goldfischer et al. (1) described a patient with clinical features similar to those of patients with Zellweger syndrome. In contrast to patients with Zellweger syndrome who lack functional peroxisomes, however, this patient had apparently normal peroxisomes in liver and kidney. There was an accumulation of very long-chain fatty acids (VLCFAs) in plasma and of 3α,7α,12α-trihydroxycholestanoic acid (THCA) in duodenal aspirate of the patient (1). Later studies by Clayton et al. (2) showed that 3α,7α,12α,24-tetrahydroxycholestanolic acid (varanic acid), an intermediate in the formation of cholic acid from THCA, was present in body fluids of the patient. Immunoblot experiments by Schram et al. (3) revealed the absence of 3-ketoacyl-CoA thiolase in post-mortem liver of the patient, whereas normal levels were found for other peroxisomal matrix enzymes.
Reinvestigation of peroxisomal 3-ketoacyl-CoA thiolase deficiency

(acyl-CoA oxidase, bifunctional protein and catalase). These results led to the conclusion that the strongly reduced rate of peroxisomal β-oxidation measured in liver of the patient and the accumulation of VLCFAs and THCA in body fluids were caused by a deficiency of 3-ketoacyl-CoA thiolase. Following the identification of the gene encoding human 3-ketoacyl-CoA thiolase in 1991, molecular studies in this patient were performed but no large DNA rearrangements involving the thiolase gene were observed in Southern blot experiments (4).

At the time the patient was described, it was believed that the peroxisomal β-oxidation system consisted of only a single set of enzymes: an acyl-CoA oxidase catalyzing the first step, a bifunctional protein catalyzing the second and third step, and a thiolase responsible for the last step of the β-oxidation process. In the last few years, however, a number of studies have shed new light on the enzymology of the peroxisomal β-oxidation system (see for recent reviews (5-7)). These studies have shown that peroxisomes contain two sets of β-oxidation enzymes which differ in substrate specificity (Fig. 1). In addition to the original acyl-CoA oxidase, which is now called straight-chain acyl-CoA oxidase (SCOX), a second oxidase was identified, called branched-chain acyl-CoA oxidase (BCOX). SCOX is responsible for the oxidation of VLCFAs such as C26:0 and C24:0, whereas BCOX is involved in the β-oxidation of pristanic acid and the bile acid intermediates THCA and dihydroxycholestanolic acid (DHCA) (Fig. 1). The second bifunctional protein that was identified, has been named D-bifunctional protein (DBP) because it forms and dehydrogenates D-3-hydroxyacyl-CoAs, in contrast to the original

\[ \text{VLCFA-CoA} \rightarrow \text{Straight-chain acyl-CoA oxidase} \]

\[ \text{Pristanoyl-CoA} \rightarrow \text{Branched-chain acyl-CoA oxidase} \]

\[ \text{L-Bifunctional protein} \]

\[ \text{D-Bifunctional protein} \]

\[ \text{3-Ketoacyl-CoA thiolase} \]

\[ \text{Sterol carrier protein X} \]

\[ \text{VLCFA-CoA n-2} \rightarrow \text{Trimethyltridecanoyl-CoA} \rightarrow \text{Choloyl-CoA} \]

Fig. 1 Schematic representation of the fatty acid β-oxidation machinery in human peroxisomes catalyzing the oxidation of very long-chain fatty acyl-CoAs (VLCFA-CoA) and branched-chain fatty acyl-CoAs (pristanoyl-CoA and THC-CoA). Oxidation of VLCFA-CoAs (C24:0 and C26:0) involves straight-chain acyl-CoA oxidase, D-bifunctional protein (DBP) and both 3-ketoacyl-CoA thiolase and sterol carrier protein X (SCPx), while oxidation of branched-chain fatty acyl-CoAs involves branched-chain acyl-CoA oxidase, DBP and SCPx (see (6) for review).
protein, L-bifunctional protein (LBP), which produces L-hydroxy intermediates. Both in vitro studies performed with the purified bifunctional proteins and the identification of patients with a deficiency of DBP (8-11) has provided unequivocal evidence that DBP is involved in the degradation of VLCFAs as well as the branched-chain fatty acids, pristanic acid and DHCA/THCA. The physiological function of LBP remains elusive at this moment. Both peroxisomal thiolases are believed to be involved in VLCFAs degradation. In addition, sterol carrier protein X (SCPx), the second peroxisomal thiolase that was identified, which contains both a thiolase domain and a sterol carrier protein domain, is the key enzyme in the β-oxidation of pristanic acid and DHCA/THCA.

Since the new insights into the peroxisomal β-oxidation system and the physiological function of the different β-oxidation enzymes no longer provided an explanation for the biochemical findings in the reported patient, we reinvestigated this unique case. In this paper, we describe the unraveling of the true enzymatic and genetic defect in this patient.

Materials and Methods

Patient L.C.
The patient’s clinical and biochemical characteristics have been described in (1). Skin fibroblasts were obtained from the patient’s parents, who were first cousins.

Immunoblot analysis
Homogenates of post-mortem brain and kidney material (100 and 5-50 µg of protein, respectively) were subjected to electrophoresis on a 10% (w/v) SDS-polyacrylamide gel essentially as described by Laemmli (12) and transferred to a nitrocellulose sheet. After blocking of non-specific binding sites with 50 g/L Profitar and 10 g/L BSA in 1 g/L Tween-20/PBS for 1 h, the blot was incubated for 2 h with different antibodies against peroxisomal matrix enzymes. The antibodies used were: anti-3-ketoacyl-CoA thiolase (diluted 1:2,000 in 3 g/L BSA) (13), anti-SCPx (diluted 1:1,000) (14), anti-SCOX (diluted 1:3,000) (13), anti-LBP (diluted 1:5,000) (13) and anti-DBP (diluted 1:10,000) (15). Goat anti-rabbit IgG antibodies conjugated to alkaline phosphatase were used for detection, according to the manufacturer’s instructions (Bio-Rad, CA).

DNA isolation
DNA was isolated from post-mortem brain and kidney material from patient L.C. and from fibroblasts of the patient’s parents using the Wizard® Genomic DNA purification kit, according to the manufacturer’s instructions (Promega, WI).

RNA isolation and cDNA synthesis
Total RNA was isolated from brain and kidney material from patient L.C. and from fibroblasts of the patient’s mother by the acid guanidium thiocyanate-phenol-chloroform extraction procedure described by Chomczynski and Sacchi (16) and subsequently used to prepare cDNA (17).
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**PCR**

### 3-ketoacyl-CoA thiolase

The cDNA encoding 3-ketoacyl-CoA thiolase was amplified by PCR in two overlapping fragments. The first fragment (bases -58 to 484) was amplified with the primers THIOF-58 (5'-TGT TAA CTC CGC GGT CAG TTC CCG GAC TGG-3') and THIOR 484 (5'-CCA GGG TTC CCT CTG TCA GCC AGG GAC ATG-3'), and the second fragment (403-1326) was amplified with the primers THIOF 403 (5'-GTG GCA TCA GAA ATG GGT CTT ATG ACA TTG-3') and THIOR 1326 (5'-GCT GCT AGA GCA GCA GGA CTG TCT GCG TAG-3').

### DBP

The cDNA encoding DBP was amplified by PCR in three overlapping fragments by means of three primer sets tagged with either -21M13 (5'-tgt aaa acg acg gcc agt-3') or universal M13rev (5'-cag gaa aca gct atg acc-3') extensions. The first fragment (bases -48 to 806) was amplified with the primers -21MDBP -48 (5'-[-21M13]-GGC CAG CGC GTC TGC TTG TTC-3') and M13RDBP 806 (5'-[M13rev]-ACT GCC TCA GGA GTC ATT GG-3'), the second fragment (bases 675 to 1543) was amplified with the primers -21MDBP 675 (5'-[-21M13]-TTG TCA CGA GAG TTG TGA GG-3') and M13RDBP 1543 (5'-[M13rev]-GTA AGG GAT TCC AGT CTC CAC-3') and the third fragment (bases 1489 to 2313) was amplified with the primers -21MDBP 1489 (5'-[-21M13]-ACC TCT CTT AAT CAG GCT GC-3') and M13RDBP 2313 (5'-[M13rev]-CCC TGC ATC TTA GTT CTA ATC AC-3').

For sequence analysis, the 3' end of intron 2, exon 3 and intron 3 were amplified by PCR with the primers -21MDBPIVS2 -55F (5'-[-21M13]-CAC ATT TTG AAA GTC TAG AA-3') and M13DBPIVS3+E4 (5'-[M13rev]-CAC CTA TTC TTC CAA AAG CAT CC-3').

**Sequencing**

PCR fragments were sequenced in both directions either by means of -21M13 and M13rev fluorescent primers or by means of big dye-deoxy terminators (Applied Biosystems, CA) on an Applied Biosystems 377A automated DNA sequencer according to the manufacturer's protocol (Perkin Elmer, CA).

**Enzyme activity measurements**

The activity of DBP in cultured skin fibroblasts of the patient's parents were measured as described in (9).

**Results**

**Molecular analysis of peroxisomal 3-ketoacyl-CoA thiolase**

Patient L.C. is the only patient reported to suffer from a deficiency of peroxisomal 3-ketoacyl-CoA thiolase (3). This was concluded from immunoblot experiments which revealed the normal presence of SCOX and LBP, but no 3-ketoacyl-CoA thiolase in post-
mortem liver material from the patient. To determine whether this thiolase deficiency is caused by mutations in the gene encoding 3-ketoacyl-CoA thiolase, we sequenced the cDNA amplified by RT-PCR from RNA isolated from post-mortem brain and kidney material from the patient and a control subject. Unfortunately no liver material from the patient could be used for this study, because the liver samples used for the studies described in Schram et al. (3) were not available anymore. No mutations were identified by sequence analysis of the cDNA encoding the thiolase from both brain and kidney in the patient.

![Immunoblot analysis in post-mortem kidney and brain of a control subject (indicated by C), a patient suffering from Zellweger syndrome (indicated by Z) and patient L.C. (indicated by P). Antibodies were used against (A) peroxisomal 3-ketoacyl-CoA thiolase (THIO), (B) D-bifunctional protein (DBP), (C) L-bifunctional protein (LBP), (D) straight-chain acyl-CoA oxidase (SCOX), and (E) sterol carrier protein X (SCPx). In (A) the arrowheads indicate the 44 kDa precursor form and the 41 kDa mature form of 3-ketoacyl-CoA thiolase. In (B) the arrowheads indicate the 79 kDa full-length protein, the 45 kDa enoyl-CoA hydratase component of DBP and the 35 kDa 3-hydroxyacyl-CoA dehydrogenase component of DBP. In (C) the arrowhead indicates the 79 kDa full-length LBP. In (D) the arrowheads indicate the 70, 50 and 20 kDa components of SCOX. In (E) the arrowheads indicate the 58 kDa full-length protein and the 46 kDa thiolase component of SCPx.](image)

**Biochemical reinvestigation**

The absence of mutations in the cDNA encoding the thiolase in conjunction with the current view that peroxisomes contain two sets of β-oxidation enzymes, prompted us to reinvestigate the patient at the biochemical level. To this end, we performed immunoblot experiments using antibodies against the different enzymes, except BCOX since no antibody against this enzyme was available. The results are shown in Fig. 2. In contrast to the previous data in liver, the mature 41 kDa form of 3-ketoacyl-CoA thiolase was normally present in both brain and kidney from patient L.C. (Fig. 2A). Also the other peroxisomal thiolase, SCPx, as well as the 70, 50 and 20 kDa components of SCOX and
Reinvestigation of peroxisomal 3-ketoacyl-CoA thiolase deficiency

LBP were normally present. DBP, however, was deficient in brain from patient L.C., while it was normally present in brain of the control subject. The full-length protein of 79 kDa was not detectable, as well as the two proteolytically processed polypeptides: the 45 kDa band corresponding to the enoyl-CoA hydratase component of DBP and the 35 kDa band corresponding to the 3-hydroxyacyl-CoA dehydrogenase component of DBP. In kidney, no DBP could be detected in both the control subject and patient L.C. (Fig. 2B).

Since no skin fibroblasts of patient L.C. were available, we measured DBP activity in fibroblasts of the patient’s parents and found a partially reduced activity (Table 1), which is in agreement with heterozygosity for DBP deficiency.

Table 1 Activity measurements of the enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase component of D-bifunctional protein in fibroblasts of the patient’s parents and control subjects.

<table>
<thead>
<tr>
<th>Activity measured</th>
<th>Mother of patient L.C.</th>
<th>Father of patient L.C.</th>
<th>Controls (n = 24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydratase (formation of 24-OH-THC-CoA)</td>
<td>86</td>
<td>127</td>
<td>240 ± 65&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dehydrogenase (formation of 24-keto-THC-CoA)</td>
<td>10</td>
<td>26</td>
<td>73 ± 33&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>mean value ± SD

Resolution of the molecular basis of DBP deficiency

To confirm the apparent DBP deficiency in patient L.C. at the molecular level, we amplified the cDNA encoding DBP from brain and kidney by PCR in three overlapping fragments and subsequently sequenced the PCR products. We found a homozygous deletion of base pair 113 through base pair 220, corresponding to exon 3 of the DBP gene (18). We also analyzed the cDNA encoding DBP in fibroblasts of the patient’s mother and found a heterozygous deletion of exon 3. To determine the cause of skipping of exon 3 in patient L.C., the 3’ end of intron 2, exon 3 and intron 3 of the DBP gene was amplified from brain and kidney DNA and the PCR products subsequently sequenced. This revealed a deletion of 138 base pairs, encompassing base pair 145 through base pair 220 of exon 3 and the first 63 base pairs of intron 3 (Fig. 3). In fibroblasts from the parents of the patient the same deletion was identified in heterozygous form.

Discussion

The data presented in this paper show that the true defect in the only patient documented with a deficiency of 3-ketoacyl-CoA thiolase is at the level of DBP. No DBP protein could be detected by immunoblot analysis in brain of the patient, whereas 3-ketoacyl-CoA thiolase was normally present. These results were confirmed by cDNA analysis in brain and kidney. The cDNA encoding 3-ketoacyl-CoA thiolase was completely normal, whereas the patient had a homozygous deletion of exon 3 in DBP cDNA. Studies at the genomic level revealed that skipping of exon 3 in this patient is caused by a deletion of part of exon 3 and the 5’ end of intron 3. The parents of the patient, who were
consanguineous, are heterozygous for this deletion, which results in a partially reduced DBP activity as measured in their fibroblasts. Exon 3 consists of 108 base pairs and skipping of this exon leads to an in-frame deletion of 36 amino acids. Since neither the full-length 79 kDa band nor the 45 and 35 kDa bands, corresponding to the enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase components of DBP respectively, were present in brain material from the patient, the mutated protein is probably unstable and rapidly degraded.

![Diagram](image)

**Fig. 3** (A) Schematic representation of exon 2-4 of D-bifunctional protein (DBP) and the intervening intron sequences. The deletion in the DBP gene in patient L.C. is indicated (from bp 145 in exon 3 through the first 63 bps of intron 3). This was determined by amplifying part of the DBP gene with the primers -21MDBPIVS2 -55F and M13DBPIVS3+E4, which are depicted, and subsequent sequencing of the PCR products. The deletion on the genomic level results in skipping of exon 3 at the cDNA level. (B) Products of amplification of the DBP gene with the primers -21MDBPIVS2 -55F and M13DBPIVS3+E4 in brain of a control subject (C), the patient’s father (F), the patient’s mother (M) and patient L.C. (P).

Our immunoblot experiments showed the normal presence of 3-ketoacyl-CoA thiolase in kidney and brain from patient L.C., which is in contrast with the earlier data from Schram et al. (3) showing the absence of thiolase in liver. The most likely explanation for these discrepant results is that the quality of the liver material used by Schram et al. (3), which was obtained post-mortem, was very poor. Unfortunately, this possibility cannot be investigated, since this liver material is no longer available.

The first patient with a deficiency of DBP was described in 1997, 10 years after the reported thiolase deficiency in patient L.C. (8). Since then several other cases of DBP deficiency have been reported in literature (reviewed in (5)) and to date DBP deficiency constitutes one of the most frequently occurring single peroxisomal enzyme deficiency disorders. The clinical as well as the biochemical abnormalities in patient L.C. were similar to those reported in patients with an established DBP deficiency. The mutation identified
in patient L.C. has not been reported before. Our findings have great implications, since they imply that the group of single peroxisomal β-oxidation enzyme deficiencies is limited to SCOX (19), DBP (8-11) and α-methylacyl-CoA racemase deficiency (20), and that 3-ketoacyl-CoA thiolase deficiency is no longer a distinct disease entity. To conclude, this study stresses the importance of reinvestigation of patients that have been described in literature with an unknown defect of peroxisomal β-oxidation now that the knowledge of the peroxisomal β-oxidation system and the enzymes involved has improved greatly in recent years. The elucidation of the true defect in these patients will further increase our understanding of the peroxisomal β-oxidation system and its substrates, and will be important for prenatal diagnosis in this group of patients.

Acknowledgments
We are grateful to Prof. Hashimoto (Shinshu University, Matsumoto, Japan) for kindly providing the antibodies raised against SCOX, DBP, LBP and 3-ketoacyl-CoA thiolase, and to Prof. Wirtz (Utrecht University, Utrecht, the Netherlands) for kindly providing the antibodies raised against SCPx. This work was supported by the Princess Beatrix Fund (The Hague, The Netherlands).

References


Chapter 6

Mutations in the gene encoding peroxisomal $\alpha$-methylacyl-CoA racemase cause adult-onset sensory motor neuropathy.

Chapter 6

Mutations in the gene encoding peroxisomal α-methylacyl-CoA racemase cause adult-onset sensory motor neuropathy


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Abstract

Sensory motor neuropathy is associated with various inherited disorders including Charcot-Marie-Tooth disease (1,2), X-linked adrenoleukodystrophy/adrenomyeloneuropathy (3) and Refsum disease (4). In the latter two, the neuropathy is thought to result from the accumulation of specific fatty acids. We describe here three patients with elevated plasma concentrations of pristanic acid (a branched-chain fatty acid) and C27-bile acid intermediates. Two of the patients suffered from adult-onset sensory motor neuropathy. One patient also had pigmentary retinopathy, suggesting Refsum disease, whereas the other patient had upper motor neuron signs in the legs, suggesting adrenomyeloneuropathy. The third patient was a child without neuropathy. In all three patients we discovered a deficiency of α-methylacyl-CoA racemase. This enzyme is responsible for the conversion of pristanoyl-CoA and C27-bile acyl-CoAs to their (S)-stereoisomers (5,6), which are the only stereoisomers that can be degraded via peroxisomal β-oxidation (7,8). Sequence analysis of the α-methylacyl-CoA racemase cDNA from the patients identified two different mutations that are likely to cause disease, based on analysis in Escherichia coli. Our findings have implications for the diagnosis of adult-onset neuropathies of unknown etiology.

We analyzed the plasma of two patients with adult-onset sensory motor neuropathy and additional clinical signs suggesting Refsum disease (patient 1) or X-linked adrenoleukodystrophy/adrenomyeloneuropathy (patient 2), and found a similar profile. Very long-chain fatty acids (VLCFAs) were not elevated, which excluded X-linked adrenoleukodystrophy/adrenomyeloneuropathy (Table 1). Phytanic acid was marginally elevated, but, in contrast to patients with Refsum disease, the levels of pristanic acid and the C27-bile acid intermediates di- and trihydroxycholestanolic acid (DHCA and THCA) were markedly increased (Table 1). This suggested a specific defect in the peroxisomal β-oxidation of branched-chain fatty acids and not a defect in the α-oxidation system, the first enzyme step of which is defective in Refsum disease (9,10) (Fig. 1). This was confirmed by a reduced pristanic acid β-oxidation activity in cultured skin fibroblasts of the patients (Table 2). When we subsequently measured the activities of the enzymes directly involved in the β-oxidation of branched-chain fatty acids (namely, branched-chain...
### Table 2: Biochemical data and mutations in patients with X-linked adrenoleukodystrophy

<table>
<thead>
<tr>
<th>Alleles</th>
<th>Change in activity</th>
<th>Phenotypic and metabolic alterations</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0/0.05</td>
<td>&lt; 0.0.05</td>
<td>ND</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>&gt; 0.0.05</td>
<td>&gt; 0.05</td>
<td>ND</td>
<td>0.7</td>
<td>2.9</td>
<td>0.05</td>
</tr>
<tr>
<td>0.05-0.4</td>
<td>0.0-0.03</td>
<td>ND</td>
<td>1.3</td>
<td>2.9</td>
<td>0.05</td>
</tr>
<tr>
<td>0-2</td>
<td>2.0</td>
<td>ND</td>
<td>105</td>
<td>105</td>
<td>0.05</td>
</tr>
<tr>
<td>&gt; 0.15</td>
<td>&gt; 0.2</td>
<td>ND</td>
<td>22</td>
<td>22</td>
<td>0.05</td>
</tr>
</tbody>
</table>

**All concentrations are given in mmol/L. X-linked adrenoleukodystrophy.**

<table>
<thead>
<tr>
<th>Control subjects</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>THCα</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>DHCα</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>0.77-2.41</td>
<td>0.0-0.3</td>
<td>0.0-0.22</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>0.3-1.25</td>
<td>0.4-0.55</td>
<td>0.15-1.25</td>
</tr>
<tr>
<td>Palmitoleic acid</td>
<td>0.77-2.41</td>
<td>0.0-0.3</td>
<td>0.0-0.22</td>
</tr>
<tr>
<td>VLCFAα</td>
<td>0.00-0.05</td>
<td>0.00-0.024</td>
<td>0.00-0.022</td>
</tr>
</tbody>
</table>

**Table 1: Analysis of plasma VLCFA's, branched-chain fatty acids and bile acids.**
acyl-CoA oxidase, D-bifunctional protein and sterol carrier protein X), however, we found them all to be normal.

![Diagram](Fig. 1 Schematic representation of the steps involved in the oxidation of (3R)- and (3S)-phytanic acid as derived from dietary sources and (25R)-THCA produced from cholesterol in the liver. After the activation of (3R)- and (3S)-phytanic acid to their corresponding CoA esters, they both become substrates for the peroxisomal \( \alpha \)-oxidation system, which produces (2R)- and (2S)-pristanoyl-CoA. Because branched-chain acyl-CoA oxidase, the first enzyme of the \( \beta \)-oxidation system, can only handle (S)-stereoisomers, (2R)-pristanoyl-CoA needs to be converted by \( \alpha \)-methylacyl-CoA racemase into its (2S)-isomer. The bile acid intermediates DHC and THCA are exclusively produced as (25R)-stereoisomers. To be \( \beta \)-oxidized, the CoA esters of the (25R)-stereoisomer also need to be converted by \( \alpha \)-methylacyl-CoA racemase into their (25S)-isomers.

Because pristanic acid \( \beta \)-oxidation activity was reduced but not fully deficient, we next examined whether the patients were deficient in \( \alpha \)-methylacyl-CoA racemase activity. \( \alpha \)-methylacyl-CoA racemase is not directly involved in the \( \beta \)-oxidation itself, but it is important in the \( \beta \)-oxidation of branched-chain fatty acids and \( \text{C}_{27} \)-bile acids. This peroxisomal enzyme catalyzes the interconversion of (R)- and (S)-stereoisomers of \( \alpha \)-methyl-branched-chain fatty acyl-CoA esters (5,6,11), including pristanoyl-CoA, which naturally occurs as a mixture of two different stereoisomers ((2R)- and (2S)-pristanoyl-CoA, see Fig. 1) (12,13). In addition it catalyzes the interconversion of the CoA esters of DHCA and THCA (DHC-CoA and THC-CoA, respectively), which are exclusively produced as (25R)-stereoisomers (14), into their respective (25S)-stereoisomers. Although \( \alpha \)-methylacyl-CoA racemase is able to convert both the (R)- and (S)-stereoisomers, its physiological function is to produce the (S)-stereoisomers, because only these serve as substrate for branched-chain acyl-CoA oxidase (7,8), the first enzyme of the peroxisomal \( \beta \)-oxidation system of branched-chain fatty acids (Fig. 1). We therefore predicted that a deficiency of \( \alpha \)-methylacyl-CoA racemase would result in a partially reduced pristanic acid...
α-Methylacyl-CoA racemase deficiency

β-oxidation in cultured skin fibroblasts because the pristanic acid used in the assay is a racemic mixture.

Furthermore, we predicted that only the (R)-stereoisomers of pristanic acid, DHCA and THCA would accumulate in plasma from these patients. Therefore we further analyzed the plasma of patient 1 and 2 by liquid chromatography/tandem mass spectrometry (LC/MS/MS) and detected an accumulation of only (25R)-THCA (data not shown). In addition we measured α-methylacyl-CoA racemase activity in fibroblasts from both patients and found it to be fully deficient (Fig. 2 and Table 2).

![Fig. 2 Measurement of α-methylacyl-CoA racemase activity in fibroblast homogenates from a control subject (A,B) and patient 1 (C). Activity was measured by monitoring the production of (25R)-THC-CoA (peak 2) from (25S)-THC-CoA (peak 1) using HPLC. Homogenates were incubated with (25S)-THC-CoA at 37°C for 60 min (B,C) or, as a control, for 0 min (A). α-Methylacyl-CoA racemase activity was detectable in the fibroblast homogenate of the control (B), but no activity was measured for patient 1 (C) or patients 2 and 3 (data not shown).](image)

During the course of this study a third patient, diagnosed with Niemann-Pick type C (NPC) (15), was identified. This patient, in addition to typical NPC features, showed biochemical abnormalities similar to those of the other two patients, suggesting a second genetic defect (Table 1). α-Methylacyl-CoA racemase activity was also fully deficient in fibroblasts from this patient (Table 2).

A search of the EST database of the National Center of Biotechnology Information with the amino acid sequences of mouse and rat α-methylacyl-CoA racemase identified one human EST clone that was mapped at chromosome 5p13.2–5q11.1 and predicted to contain the entire ORF. On the basis of the DNA sequence of this EST clone, we amplified the human α-methylacyl-CoA racemase cDNA by RT-PCR. Human α-methylacyl-CoA racemase showed 81% and 77% identity, respectively, with the amino acid sequence of rat and mouse α-methylacyl-CoA racemase (16) (Fig. 3).
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![Alignment of the amino acid sequences of mouse, rat, and human α-methylacyl-CoA racemase. Black boxes indicate identical amino acids and gray boxes represent similar amino acids. Amino acid changes frequently observed in human control alleles are indicated below the human sequence. The human sequence contains an additional 21 aa at its amino terminus compared with the published sequences of the mouse and rat α-methylacyl-CoA racemase (the arrowhead indicates the initiation methionine of the published mouse and rat α-methylacyl-CoA racemase sequence (16)). Translation of the presumed 5’ noncoding regions of the rat and mouse cDNA sequence, however, indicated that the latter two must represent 5’ truncated cDNA species. The human α-methylacyl-CoA racemase contains a putative carboxy-terminal peroxisomal targeting signal type 1 (ASL), like the rat and the mouse proteins (ANL).

Sequence analysis of the α-methylacyl-CoA racemase cDNA amplified by RT–PCR identified three homozygous nucleotide differences in patients 1 and 2, resulting in the amino acid changes V9M, S52P and G175D. In patient 3 we found three other homozygous nucleotide differences leading to the amino acid changes L107P, S210L and K277E. We found high frequencies in 114 control alleles of the amino acid changes V9M and G175D, both identified in patients 1 and 2, and S201L and K277E, identified in patient 3, suggesting that they represent polymorphisms (Table 2). In contrast, the remaining two amino acid changes, S52P (patients 1 and 2) and L107P (patient 3), were not detected among the controls. This observation, in conjunction with the complete absence of α-methylacyl-CoA racemase activity in fibroblasts of the patients, suggested...
that these two amino acid changes are disease causing. We confirmed this by expression of the two mutant and corresponding wild-type proteins as fusions to maltose binding protein (MBP) in *E. coli*. Enzyme measurements after affinity purification of the fusion proteins from *E. coli* lysates showed that both the S52P and the L107P amino acid changes resulted in inactive proteins (Fig. 4).

![Graph showing racemase activity](image)

**Fig. 4** Expression of S52P and L107P mutant and corresponding control α-methylacyl-CoA racemase cDNAs in *E. coli*. Allele 1 contains the methionine at position 9 and the aspartic acid at position 175 (both present in patients 1 and 2). Allele 2 contains the leucine at position 201 and the glutamic acid at position 277 (both present in patient 3). The coding sequences of the various α-methylacyl-CoA racemase cDNAs were amplified by RT-PCR and expressed as a fusion with maltose binding protein (MBP) in *E. coli*. α-Methylacyl-CoA racemase enzyme activities of α-methylacyl-CoA racemase-MBP fusion proteins were measured after affinity purification from *E. coli* lysates and normalized for the amount of protein to correct for differences in expression. The results are the mean of four independent measurements.

Our results indicate that α-methylacyl-CoA racemase deficiency is associated with neurological disease in adult life. The common feature in the two adults was sensory motor neuropathy, although in one case the electrophysiology suggested an axonal neuropathy and in the other, a demyelinating neuropathy. Patient 3 contributes little to our understanding about the neurology of α-methylacyl-CoA racemase deficiency, because all of the child's symptoms could be accounted for by NPC. The similar clinical signs associated with α-methylacyl-CoA racemase deficiency and Refsum disease (which is caused by phytanoyl-CoA hydroxylase deficiency (10,17)) indicate that sustained elevated levels of branched-chain fatty acids are progressively deleterious and result in adult-onset neuropathies.

Clinical data indicate that the symptoms associated with α-methylacyl-CoA racemase deficiency are relatively mild. This, together with the fact that routine plasma analysis in adults usually does not include analysis of bile acids and branched-chain fatty acids (18,19), implies that thus far many patients with α-methylacyl-CoA racemase deficiency may have remained undiagnosed. This stresses the importance of undertaking multiple analyses when investigating adult patients suffering from motor and sensory neuropathies.
of unknown etiology. Especially because a dietary regimen reduced in phytanic and pristanic acid may alleviate the progression of the neuropathy in \( \alpha \)-methylacyl-CoA racemase deficiency, as in Refsum disease (20). Identification of additional patients with \( \alpha \)-methylacyl-CoA racemase deficiency is required to appreciate the full spectrum of neurological abnormalities that can result from this enzyme deficiency.

Materials and Methods

Patients

Patient 1 was a male of European descent who exhibited a typical retinitis pigmentosa with restriction of his visual field and acuity, and primary hypogonadism when examined at 44 years of age. He also suffered from epileptic seizures and conduction studies showed a widespread axonal sensory motor neuropathy affecting the legs more severely than the arms. In childhood he showed mild developmental delay, and at the age of 18 an encephalitic illness left him temporarily blind. During the following two years his vision partially recovered, but has slowly deteriorated since then.

Patient 2 was a female of European descent who was completely well until the age of 48, when she began to tire easily and was found to be hypothyroid. She then developed heaviness of her legs on exercise, with dragging of both feet on walking. She had a spastic paraparesis, but the MRI scan of the cervical spine showed no abnormality. Nerve conduction studies showed a demyelinating sensory motor polyneuropathy. Analysis of plasma VLCFAs was undertaken to determine whether the patient had adrenomyeloneuropathy (as a symptomatic heterozygote). Phytanic acid and pristanic acid levels were analyzed simultaneously and found to be elevated.

Patient 3 was the second child born to doubly consanguineous Asian parents (15). At the age of 18 months he was diagnosed with NPC (complementation group 1). In addition to the typical biochemical features of NPC, an accumulation of pristanic acid, \( C_{27} \)-bile acid intermediates and, to a lesser extent, phytanic acid was detected in plasma from this patient, but not in other NPC patients studied, suggesting a second genetic defect. He has shown progressive neurological signs consistent with NPC. With this background, detection of a subtle neuropathy is not possible.

Pristanic acid \( \beta \)-oxidation

We measured pristanic acid \( \beta \)-oxidation as described (21).

Synthesis of (25S)- and (25R)-THC-CoA

The CoA thioester of THCA (22) was chemically synthesized as described (23). We purified the two stereoisomers by high-performance liquid chromatography (HPLC) using a reversed-phase \( C_{18} \)-column (Supelcosil SPLC-18-DB, 250 mm \( \times \) 10 mm) and determined the stereospecificity of the two isomers of THC-CoA after mild alkaline hydrolysis of the CoA thioesters and analysis of the free acids by LC-MS as described (24).
Enzyme assays
We measured α-methylacyl-CoA racemase activity in fibroblast homogenates with (25S)-THC-CoA (50 μM) as substrate and monitored the production of (25R)-THC-CoA with HPLC. The incubation mixture consisted of sodium phosphate buffer (6.4 mM, pH 7.4), NaCl (70 mM), ATP (10 mM), MgCl₂ (10 mM) and CoA (100 μM). Reactions were allowed to proceed for 60 min at 37°C and terminated by the addition of HCl (0.18 M), followed by resolution of the (25S)- and (25R)-THC-CoA by HPLC. We carried out HPLC with a reversed-phase C₁₈-column (Alltima 250 mm × 4.6 mm, Alltech) and achieved optimal resolution by elution with a linear gradient of methanol in potassium phosphate buffer (50 mM, pH 5.3). We performed activity measurements of α-methylacyl-CoA racemase-MBP fusion proteins as described for the α-methylacyl-CoA racemase activity measurements in fibroblast homogenates.

Mutation analysis of the human α-methylacyl-CoA racemase cDNA
We prepared first-strand cDNA from total RNA isolated from cultured skin fibroblasts as described (25). Two sets of α-methylacyl-CoA racemase-specific primers with –21M13 or M13rev extensions were used to amplify the α-methylacyl-CoA racemase cDNA in two overlapping fragments by RT-PCR. We sequenced the PCR fragments in both directions by means of –21M13 and M13rev fluorescent primers on an ABI 377A automated DNA sequencer according to the manufacturer’s protocol (Perkin-Elmer).

Expression of the α-methylacyl-CoA racemase cDNA in E. coli
We amplified by PCR the coding sequence of wild-type and mutant α-methylacyl-CoA racemase cDNAs, cloned it in-frame with the coding sequence of MBP in pMALc2 (New England BioLabs) and sequenced to exclude Taq polymerase-introduced errors. E. coli DH5α cells were transformed with the resulting expression plasmids and induced for 2 h with isopropyl-β-D-thiogalactoside (2 mM) at 37°C. Subsequently we purified α-methylacyl-CoA racemase-MBP fusion protein from the E. coli lysate by one-step affinity chromatography according to the manufacturer’s protocol (New England BioLabs).

GenBank accession numbers
Human ESTs, H19271 (STS WI-16117), H19272; mouse α-methylacyl-CoA racemase, U89906; rat α-methylacyl-CoA racemase, U89905; human α-methylacyl-CoA racemase, AF158378.

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References

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Chapter 7

Subcellular localization and physiological role of α-methylacyl-CoA racemase.

Subcellular localization and physiological role of α-methylacyl-CoA racemase

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Abstract

α-Methylacyl-CoA racemase plays an important role in the β-oxidation of branched-chain fatty acids and fatty acid derivatives because it catalyzes the conversion of several (2R)-methyl-branched-chain fatty acyl-CoAs to their (S)-stereoisomers. Only stereoisomers with the 2-methyl group in the (S)-configuration can be degraded via β-oxidation. Patients with a deficiency of α-methylacyl-CoA racemase accumulate in their plasma pristanic acid and the bile acid intermediates di- and trihydroxycholestanoic acid, which are all substrates of the peroxisomal β-oxidation system. Subcellular fractionation experiments, however, revealed that both in humans and rats α-methylacyl-CoA racemase is bimodally distributed to both the peroxisome and the mitochondrion. Our findings show that the peroxisomal and mitochondrial enzymes are produced from the same gene and that, as a consequence, the bimodal distribution pattern must be the result of differential targeting of the same gene product. In addition, we investigated the physiological role of the enzyme in the mitochondrion. Both in vitro studies with purified heterologously expressed protein and in vivo studies in fibroblasts of patients with an α-methylacyl-CoA racemase deficiency revealed that the mitochondrial enzyme plays a crucial role in the mitochondrial β-oxidation of the breakdown products of pristanic acid by converting (2R,6)-dimethylheptanoyl-CoA to its (S)-stereoisomer.

Introduction

Peroxisomes in mammals harbor two distinct pathways for fatty acid β-oxidation. The first pathway catalyzes the β-oxidation of very long-chain fatty acids, such as C26:0, and the second pathway catalyzes the β-oxidation of branched-chain fatty acids and fatty acid derivatives, such as pristanic acid and the bile acid intermediates di- and trihydroxycholestanoic acid (DHCA and THCA, respectively). The central role of peroxisomes in the oxidation of branched-chain fatty acids and fatty acid derivatives is clearly demonstrated by studies in patients with Zellweger syndrome, who lack functional peroxisomes. Analysis of plasma from these patients reveals a series of abnormalities including the accumulation of DHCA, THCA, phytanic acid, and pristanic acid, which is derived from phytanic acid after one cycle of α-oxidation in the peroxisome (1). Previous studies have shown that the peroxisomal β-oxidation system is stereospecific (2-4), because the peroxisomal oxidases (branched-chain acyl-coenzyme A (CoA) oxidase in humans and trihydroxycholestanoyl-CoA (THC-CoA) oxidase and pristanoyl-CoA oxidase in rat) can handle only the (S)-isomer of 2-methyl-branched acyl-CoAs (2,3). Because both phytanic
Physiological role of α-methylacyl-CoA racemase

Acid (3,7,11,15-tetramethylhexadecanoic acid) and pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) naturally occur as a mixture of two different diastereomers ((2S,6R,10R) and (2R,6R,10R) in the case of pristanic acid) (5), (2R)-pristanic acid first needs to be converted to its (S)-isomer to become substrate for the peroxisomal β-oxidation (Fig. 1). This conversion is catalyzed by a racemase called α-methylacyl-CoA racemase, which catalyzes the interconversion of a large variety of (R)- and (S)-2-methyl-branched-chain fatty acyl-CoAs (6-9). The same racemase is also essential for the degradation of DHCA and THCA (7,9), of which only the (25R)-stereoisomers are produced via (R)-specific mitochondrial 27-hydroxylation (10) (Fig. 1).

Fig. 1 Schematic representation of the steps involved in the oxidation of (3R)- and (3S)-phytanic acid as derived from dietary sources and (25R)-THCA produced from cholestrol in the liver. After the activation of (3R)- and (3S)-phytanic acid to their corresponding CoA esters, they both become substrates for the peroxisomal α-oxidation system, which produces (2R)- and (2S)-pristanoyl-CoA. Because branched-chain acyl-CoA oxidase, the first enzyme of the β-oxidation system, can handle only (S)-stereoisomers, (2R)-pristanoyl-CoA needs to be converted by α-methylacyl-CoA racemase into its (2S)-isomer. The bile acid intermediates DHCA and THCA are exclusively produced as (25R)-stereoisomers. To be β-oxidized, the CoA esters of the (25R)-stereoisomer also need to be converted by α-methylacyl-CoA racemase into their (25S)-isomers.

Studies on the subcellular localization of α-methylacyl-CoA racemase revealed that the enzyme activity is not only localized in peroxisomes but is also present in mitochondria, at least in humans (7,8). In rat, however, the localization is controversial. Conzelmann and co-workers, who purified the enzyme from rat liver, reported that it is exclusively localized in mitochondria (7), while Van Veldhoven and co-workers also detected racemase activity in rat liver peroxisomes although the distribution among the two organelles was quite different from that in human liver (8).
The peroxisomal localization of this enzyme is obvious in view of the importance of peroxisomes in the degradation of branched-chain fatty acids. It is less clear why mitochondria would need α-methylacyl-CoA racemase activity. It has been hypothesized, however, that this is necessary for the further oxidation of the breakdown products of pristanic acid (8,11) because pristanic acid contains three chiral carbon atoms. The methyl groups at positions 6 and 10 of naturally occurring pristanic acid have the
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(R)-configuration. Therefore, (2R,6R,10)-trimethylundecanoyl-CoA, which is formed from pristanic acid after two β-oxidation cycles, requires racemization before it can be further degraded (see Fig. 2). After three cycles of β-oxidation (4R,8)-dimethylnonanoyl-CoA is exported from the peroxisome as a carnitine ester (12,13) and subsequently further β-oxidized in the mitochondrion. As in peroxisomes, the dehydrogenating enzymes in the mitochondrion have been shown to be absolutely specific for the (2S)-isomer (4,14). As a consequence, (2R,6)-dimethylheptanoyl-CoA, which is formed after four β-oxidation cycles (Fig. 2), first needs to be converted to its (S)-isomer before further degradation is possible. It is unknown which racemase is responsible for this conversion, but α-methylacyl-CoA racemase is a good candidate.

In this article, we have studied the subcellular localization of α-methylacyl-CoA racemase in both human and rat, and show that α-methylacyl-CoA racemase is the enzyme that is responsible for the racemase activity measured in both peroxisomes and mitochondria. Furthermore, we have studied the physiological role of α-methylacyl-CoA racemase in the mitochondrion and demonstrate that this enzyme is the main if not the only racemase that converts (2R,6)-dimethylheptanoyl-CoA into its (S)-isomer.

Materials and Methods

Subcellular fractionation of liver homogenates

Livers obtained from male Wistar rats that had been fed a standard laboratory diet supplemented with 1% (w/w) di-(2-ethylhexyl)-phthalate for 7 days, were homogenized in 250 mM sucrose, 5 mM morpholinepropan e sulfonic acid (MOPS), and 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (final pH 7.4). A postnuclear supernatant was produced by centrifugation of the homogenate at 600 × g for 10 min at 4°C and subjected to differential centrifugation as described previously (15). The light mitochondrial fraction, enriched in peroxisomes and lysosomes, was subfractionated by equilibrium density gradient centrifugation in a linear Nycode nz gradient as described (16). Pieces of human liver were obtained from patients undergoing liver resection. The tissue was homogenized in 250 mM sucrose, 2 mM MOPS, and 0.5 mM ethylenediaminetetraacetic acid (EDTA) (final pH 7.4), and subcellular fractionation was carried out as described for rat liver. Catalase (17) and glutamate dehydrogenase (18) were used as marker enzymes for peroxisomes and mitochondria, respectively.

Expression of α-methylacyl-CoA racemase in Escherichia coli

The cDNA encoding human α-methylacyl-CoA racemase (GenBank accession number AF158378) was expressed as a fusion protein with maltose-binding protein (MBP) as described previously (9). The fusion protein was purified from Escherichia coli lysate by one-step affinity chromatography according to the manufacturer protocol (New England Biolabs, Beverly, MA).

Synthesis of the substrates for the enzyme assays

The CoA thioesters of THCA (19) and 2,6-dimethylheptanoic acid (20) were chemically synthesized by the method described by Rasmussen et al. (21). The two stereoisomers of
THCA were purified by high performance liquid chromatography (HPLC) as described previously (9).

**Patient cell lines**

The cell lines used in this study were from two patients with a defined deficiency of α-methylacyl-CoA racemase caused by mutations in the encoding gene. Racemase activity in fibroblasts of these patients as measured with THC-CoA as substrate was completely deficient (9).

**Enzyme assays**

α-Methylacyl-CoA racemase activity in the subcellular fractions obtained by differential centrifugation of rat and human liver homogenates was measured with (25R)-THC-CoA as substrate. The production of (25S)-THC-CoA was monitored by HPLC essentially as described previously (9) with one minor modification: 100 mM Bis-Tris-Propane (pH 7.5) was used as buffer in the incubation. Racemase activity measurements of the purified human α-methylacyl-CoA racemase-MBP fusion protein with (25S)-THC-CoA as substrate were performed as described (9).

Racemase activity of the purified human α-methylacyl-CoA racemase-MBP fusion protein was also determined with 2,6-dimethylheptanoyl-CoA as substrate. Because the two stereoisomers of 2,6-dimethylheptanoyl-CoA could not be separated by our HPLC method, we developed a coupled assay with purified long-chain acyl-CoA dehydrogenase (LCAD) to measure the activity. Purified LCAD (2.6 μU, determined with C8-CoA as substrate) was incubated with a racemic mixture of 2,6-dimethylheptanoyl-CoA in the absence or the presence of 3 μg of purified α-methylacyl-CoA racemase-MBP fusion protein. The incubation mixture consisted of 100 mM sodium phosphate-0.1 mM EDTA (pH 7.2), 0.4 mM hexafluorophosphate, 20 μM FAD, and 50 μM 2,6-dimethylheptanoyl-CoA. Reactions were allowed to proceed for 15, 30, or 60 min at 37°C and terminated by the addition of 0.18 M HCl. Production of 2,6-dimethylheptenoyl-CoA was followed by HPLC. This was done with a reversed-phase C18-column (Altima 250 x 4.6 mm; Alltech, Deerfield, IL) and optimal resolution was achieved by elution with a linear gradient of methanol in 50 mM potassium phosphate buffer (pH 5.3).

The coupled assay was also used to determine racemase activity for 2,6-dimethylheptanoyl-CoA in fibroblast homogenates. Instead of a racemic mixture, however, 50 μM purified (2R,6)-dimethylheptanoyl-CoA was used as substrate in the assay. The protein concentration was 0.5 mg/ml and the reactions were allowed to proceed for 30 min at 37°C.

**Purification of LCAD**

Purified LCAD was a generous gift from T. Hashimoto (Shinshu University School of Medicine, Matsumoto, Japan). Purification was performed as described (22).

**Preparation of antibodies**

α-Methylacyl-CoA racemase-MBP fusion protein expressed in E. coli was purified from the lysate, subjected to preparative sodium dodecylsulfate (SDS)-polyacrylamide gel
electrophoresis, isolated from the gel, and used to raise antibodies. To this end, a female New Zealand White rabbit was injected subcutaneously with 250 µg of the antigen mixed with an equal volume of Freund’s complete adjuvant. After 1 month, the immunization was continued by booster injections (each containing 250 µg of antigen in Freund’s incomplete adjuvant) until a satisfactory antibody titer was obtained.

**Immunoblot analysis**

Thirty microliters of each fraction of the Nycodenz density gradients from rat or human liver was subjected to electrophoresis on a 10% (w/v) SDS-polyacrylamide gel essentially as described by Laemmli (23) and transferred to a nitrocellulose sheet. After blocking of nonspecific binding sites with Protifar (50 g/L; Nutricia, Zoetermeer, The Netherlands) in Tween 20 at 1 g/L in phosphate-buffered saline (Tween 20-PBS) for 1 h, the blot was incubated for 2 h with rabbit polyclonal antibodies raised against human α-methylacyl-CoA racemase diluted 1:5,000 in Protifar (10 g/L). Goat anti-rabbit IgG antibodies conjugated to alkaline phosphatase and CDP-star were used for detection according to the manufacturer instructions (Boehringer Mannheim Biochemicals, Indianapolis, IN).

**Results**

**Subcellular localization of α-methylacyl-CoA racemase in rat liver**

Thus far, the subcellular localization of α-methylacyl-CoA racemase in rat has been controversial. To resolve this, we have performed both differential and density gradient centrifugation experiments. A peroxisome-enriched fraction was first prepared by differential centrifugation and subsequently further fractionated by Nycodenz equilibrium density gradient centrifugation. The distinct activity patterns for the marker enzymes catalase (peroxisomes) and glutamate dehydrogenase (mitochondria) demonstrate a good separation between the various subcellular organelles (Fig. 3A). When racemase activity was measured in the gradient fractions with (25R)-THC-CoA as substrate, most of the activity was associated with the mitochondrial fractions and some activity was found in the peroxisomal fractions (Fig. 3B).

To study the possibility that the racemase activities in peroxisomes and mitochondria are derived from the same enzyme, we used a specific antiserum raised against recombinant human α-methylacyl-CoA racemase expressed in and purified from *E. coli*. Immunoblotting experiments revealed that this antiserum cross-reacts with the rat liver enzyme and specifically recognizes a single protein species of ~44 kDa, which is in good agreement with the predicted molecular mass of rat α-methylacyl-CoA racemase (data not shown). Immunoblot analysis of the fractions from the density gradient revealed a similar distribution pattern for the 44 kDa protein as for the racemase activity (Fig. 3B–D), suggesting that α-methylacyl-CoA racemase could be responsible for both the mitochondrial and the peroxisomal racemase activity measured.
Subcellular localization of α-methylacyl-CoA racemase in human liver

Measurement of racemase activity in the different fractions of the human gradient also revealed a bimodal activity profile as observed for rat liver. In contrast to the situation in rat, however, the activity associated with peroxisomes was higher than the mitochondrial activity (Fig. 4B). In addition, some racemase activity was measured in the upper part of the gradient. This activity is most likely due to α-methylacyl-CoA racemase released from peroxisomes broken during the homogenization process because these fractions also contain catalase, a peroxisomal matrix protein. The pattern of distribution obtained by immunoblot experiments using the antiserum against α-methylacyl-CoA racemase was similar to the distribution of the activity measured in the gradient fractions (Fig. 4B–D). Furthermore, the antiserum recognized only one protein species of ~44 kDa in the various fractions, which is the predicted molecular mass of human α-methylacyl-CoA racemase.
Physiological role of α-methylacyl-CoA racemase

**Fig. 4** Human liver subcellular fractions were obtained by equilibrium density gradient centrifugation as described in Materials and Methods. Fractions were analyzed for the activity of the peroxisomal marker enzyme catalase (solid squares) and the mitochondrial marker enzyme glutamate dehydrogenase (solid triangles) (A), and α-methylacyl-CoA racemase (solid circles) measured with THC-CoA as substrate (B). Relative activities are expressed as a percentage of total gradient activity present in each fraction. (C) Immunoblot analysis with an antibody raised against human α-methylacyl-CoA racemase. (D) Densitometric analysis of the immunoblot (solid diamonds). The pattern of distribution of racemase activity and the mean density of the cross-reactive immunological material were similar.

**In vitro study of the mitochondrial function of α-methylacyl-CoA racemase**

A possible physiological function of α-methylacyl-CoA racemase in the mitochondrion is that it is involved in the mitochondrial β-oxidation of the breakdown products of pristanic acid, notably 2,6-dimethylheptanoyl-CoA (Fig. 2). We first tested this hypothesis by measuring the activity of purified human α-methylacyl-CoA racemase expressed as a fusion protein with MBP in *E. coli*, using 2,6-dimethylheptanoyl-CoA as substrate. To this end, we developed a coupled assay making use of purified rat LCAD, which was previously shown to dehydrogenate 2,6-dimethylheptanoyl-CoA into its corresponding enoyl-CoA ester (20) and to be stereospecific for (S)-stereoisomers (4,14). Indeed, on incubation of a chemically synthesized racemic mixture of 2,6-dimethylheptanoyl-CoA with LCAD, only half the mixture was converted to the enoyl-CoA ester (Fig. 5). When the mixture was incubated with LCAD in the presence of purified α-methylacyl-CoA racemase, however, an increasing amount of 2,6-dimethylheptenoyl-CoA was formed over
Fig. 5 2,6-Dimethylheptanoyl-CoA was incubated with purified rat LCAD in the absence or in the presence of purified human α-methylacyl-CoA racemase-MBP fusion protein. The production of the enoyl-CoA esters, 2,6-dimethylheptenoyl-CoA (solid squares), and the consumption of the substrate, 2,6-dimethylheptanoyl-CoA (solid triangles), was monitored over time by HPLC analysis.

time (Fig. 5). These results show that α-methylacyl-CoA racemase is able to convert (2R,6)-dimethylheptanoyl-CoA into its (S)-isomer, which can then be desaturated by LCAD. The calculated activity of the purified α-methylacyl-CoA racemase-MBP fusion protein with 2,6-dimethylheptanoyl-CoA was comparable to the activity measured with THC-CoA as substrate (17.9 and 15.4 nmol/min/mg, respectively).

In vivo study of the mitochondrial function of α-methylacyl-CoA racemase
To examine the putative mitochondrial function of α-methylacyl-CoA racemase in vivo, we first measured the racemase activity using 2,6-dimethylheptanoyl-CoA as substrate in fibroblast lysates from control subjects and from patients with an established α-methylacyl-CoA racemase deficiency. Previously, we already showed that fibroblast lysates of these patients were no longer able to convert (25R)-THC-CoA into the (25S)-form (9) (Table 1). Using 2,6-dimethylheptanoyl-CoA as substrate, racemase activity was also fully deficient in fibroblasts of the patients, in contrast to control fibroblasts, in which we measured ample activity with this substrate (Table 1). This confirms that 2,6-dimethylheptanoyl-CoA is a physiological substrate of α-methylacyl-CoA racemase and that there is no other racemase involved in racemization of 2,6-dimethylheptanoyl-CoA.

Table 1 Activity measurements of α-methylacyl-CoA racemase in homogenates of cultured skin fibroblasts using (25S)-THC-CoA and (2R,6)-dimethylheptanoyl-CoA as substrates.

<table>
<thead>
<tr>
<th></th>
<th>(25S)-THC-CoA</th>
<th>(2R,6)-dimethylheptanoyl-CoA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>92 ± 30 (n=11)</td>
<td>85 ± 18 (n=5)</td>
</tr>
<tr>
<td>Patient 1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Patient 2</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*expressed in pmol/min/mg, patients 1 and 2 correspond to patients 2 and 3 described before (9). Results represent the mean ± SD, n represents the number of measurements. ND, not detectable.
Next, we measured racemase activity in the mitochondrial and peroxisomal peak fractions of the density gradients from rat and human liver using 2,6-dimethylheptanoyl-CoA as substrate to determine the subcellular localization of the activity. We found a similar bimodal distribution to both peroxisomes and mitochondria as observed with the substrate THC-CoA, suggesting that the same enzyme is involved in the racemization of the two substrates. This is supported by the similar ratios of racemase activities measured with THC-CoA and 2,6-dimethylheptanoyl-CoA in the different organelle fractions. In the rat gradient this ratio was 1.3 in the mitochondrial fraction and 1.0 in the peroxisomal fraction. In the human gradients the ratios were 2.0 and 2.2 in the mitochondrial and peroxisomal fractions, respectively.

Discussion

In this study we investigated the localization of α-methylacyl-CoA racemase in subcellular fractions of human and rat liver obtained by equilibrium density gradient centrifugation. In both organisms we found a bimodal distribution pattern, in contrast to the results obtained by Conzelmann and co-workers, who reported that in rat α-methylacyl-CoA racemase is exclusively localized in the mitochondrion (7). There was, however, a considerable difference in distribution between humans and rats. In rat liver the enzyme activity was mainly associated with mitochondria, while in human liver the highest racemase activity was measured in the peroxisomal fractions. This species-dependent difference in distribution is remarkable and may be related to a different contribution of peroxisomes and mitochondria to branched-chain fatty acid oxidation in humans and rats. Indeed, according to Schmitz and Conzelmann (4) mitochondria contribute much more to whole cell branched-chain oxidation in the rat as compared with humans. In this respect it is also important to mention the data from Vanhove et al. (24), who studied the oxidation of 2-methylpalmitate in rat liver. According to these authors oxidation of this branched-chain fatty acid is shared between peroxisomes and mitochondria. Furthermore, they showed that the contribution of peroxisomes and mitochondria to whole cell 2-methylpalmitate oxidation is dependent on the nutritional status of the animal because clofibrate was found to induce mitochondrial much more than peroxisomal 2-methylpalmitate oxidation.

The similar distribution of racemase activity with only one immuno cross-reactive 44 kDa racemase protein in density gradients of both rat and human liver suggested that both the mitochondrial and the peroxisomal racemase activities are produced by the same enzyme, namely α-methylacyl-CoA racemase. Unequivocal evidence that both the mitochondrial and peroxisomal enzyme activity is derived from a single gene was provided by our findings in fibroblasts from patients with an established α-methylacyl-CoA racemase deficiency caused by missense mutations in the encoding gene (9). In homogenates of these fibroblasts we found a complete absence of racemase activity both for THC-CoA and for 2,6-dimethylheptanoyl-CoA.

The finding that α-methylacyl-CoA racemase is encoded by one gene but localized in two different subcellular compartments implies differential targeting of the same gene product. This phenomenon is not unprecedented: for example, the cDNAs encoding
mitochondrial and peroxisomal carnitine acetyltransferase originate from alternative splicing of one single gene (25). Another example is \( \Delta^{3,5} \Delta^{2,4} \)-dienoyl-CoA isomerase, first identified by Luo and co-workers (26), which has both a mitochondrial targeting signal at the amino terminus and a peroxisomal targeting signal at the carboxy terminus (27). The molecular basis of the differential targeting of \( \alpha \)-methylacyl-CoA racemase, however, is still unknown. The human and rat enzyme both contain a carboxy-terminal peroxisomal targeting signal type 1 (–KASL in humans, –KANL in rats). Inspection of the amino terminus of both rat and human racemase does not reveal an obvious mitochondrial targeting signal. By using software predicting cleavage site motifs in mitochondrion-targeting peptides (28), however, a weak potential mitochondrial transit peptide was predicted at positions 1–34 of human \( \alpha \)-methylacyl-CoA racemase.

Patients with a deficiency of \( \alpha \)-methylacyl-CoA racemase accumulate pristanic acid and the bile acid intermediates DHCA and THCA in plasma (9). This clearly demonstrates that racemase activity is needed in the peroxisome for \( \beta \)-oxidation of pristanic acid, DHCA, and THCA. To elucidate the putative mitochondrial function of \( \alpha \)-methylacyl-CoA racemase, we first studied in vitro whether the purified enzyme is able to convert (2R,6)-dimethylheptanoyl-CoA to its (S)-isomer, the only stereoisomer that can be \( \beta \)-oxidized in the mitochondrion (4,14), and found that this was indeed the case. Subsequently, we studied whether this is the true physiological function of the enzyme. We found that fibroblasts from patients with an established \( \alpha \)-methylacyl-CoA racemase deficiency were not able to convert (2R,6)-dimethylheptanoyl-CoA to its (S)-isomer, which confirms that \( \alpha \)-methylacyl-CoA racemase activity is essential at several steps in the degradation of pristanic acid to \( \text{CO}_2 \) and \( \text{H}_2\text{O} \) in the peroxisome as well as in the mitochondrion.

To obtain additional \textit{in vivo} evidence of the role of mitochondrial racemase in the oxidation of 2,6-dimethylheptanoyl-CoA, we performed acylcarnitine analysis in \( \alpha \)-methylacyl-CoA racemase-deficient patients. These studies did not reveal accumulation of 2,6-dimethylheptanoyl-carnitine (data not shown). The reason for this is most probably that even though half the pristanic acid can enter the \( \beta \)-oxidation spiral in these patients, as it naturally occurs as a racemic mixture (see Fig. 2), it cannot proceed beyond 2,6,10-trimethylundecanoyl-CoA, of which all methyl groups have the (R)-configuration. For this compound to be further \( \beta \)-oxidized, the (2R)-methyl group needs to be converted to the (S)-configuration, which is most likely also catalyzed by \( \alpha \)-methylacyl-CoA racemase (see Fig. 2). This is supported by the finding of 2,6,10-trimethylundecanoyl-carnitine in the plasma of one of the racemase-deficient patients.

Acknowledgments

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Physiological role of α-methylacyl-CoA racemase

References


Plasma analysis of di- and trihydroxycholestanoic acid diastereomers in peroxisomal α-methylacyl-CoA racemase deficiency

Chapter 8

Plasma analysis of di- and trihydroxycholestanoic acid diastereomers in peroxisomal α-methylacyl-CoA racemase deficiency

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Abstract

Recently, we identified a new peroxisomal disorder caused by a deficiency of the enzyme α-methylacyl-CoA racemase. Patients with this disorder show elevated plasma levels of pristanic acid and the bile acid intermediates di- and trihydroxycholestanoic acid (DHCA and THCA), which are all substrates for the peroxisomal β-oxidation system. α-Methylacyl-CoA racemase plays an important role in the β-oxidation of branched-chain fatty acids and fatty acid derivatives since it catalyzes the conversion of several (2R)-methyl-branched-chain fatty acyl-CoAs to their (2S)-isomers. Only stereoisomers with the 2-methyl group in the (S)-configuration can be degraded via β-oxidation. In this study we used liquid chromatography/tandem mass spectrometry (LC/MS/MS) to analyze the bile acid intermediates which accumulate in plasma from patients with a deficiency of α-methylacyl-CoA racemase and, for comparison, in plasma from patients with Zellweger syndrome and patients suffering from cholestatic liver disease. We found that racemase-deficient patients accumulate exclusively the (R)-isomer of free and taurine-conjugated DHCA and THCA, whereas in plasma of patients with Zellweger syndrome and patients suffering from cholestatic liver disease both isomers were present. Based on these results we describe an easy and reliable method to diagnose α-methylacyl-CoA racemase-deficient patients by plasma analysis. Our results also show that α-methylacyl-CoA racemase plays a unique role in bile acid formation.

Introduction

Peroxisomes play an important role in the biosynthesis of bile acids from cholesterol since the peroxisomal β-oxidation is responsible for chain shortening of the C₂₇-bile acid intermediates di- and trihydroxycholestanoic acid (DHCA and THCA), which results in formation of the primary bile acids chenodeoxycholic acid and cholic acid respectively. The enzymes involved in this process do not only handle DHCA and THCA as substrates but also other 2-methyl branched-chain fatty acids, like pristanic acid (see Fig. 1). The first step of the β-oxidation is catalyzed by branched-chain acyl-CoA oxidase (1,2), which converts the 2-methyl branched-chain acyl-CoAs into their enoyl-CoA ester. These are subsequently hydrated into a hydroxyacyl-CoA and then dehydrogenated into a β-ketoacyl-CoA. Both these steps are catalyzed by D-bifunctional protein (3-6). Finally, sterol carrier protein X (SCPx) is responsible for the thiolytic cleavage of the β-ketoacyl-CoA esters of pristanic acid as well as DHCA and THCA (7-11).

It has been demonstrated that the peroxisomal β-oxidation system is stereospecific, because the first enzyme, branched-chain acyl-CoA oxidase, can only handle (2S)-isomers
DHCA and THCA diastereomers in α-methylacyl-CoA racemase deficiency

(12,13). For this reason, a racemase called α-methylacyl-CoA racemase, identified by Conzelmann and coworkers (14,15), is also involved in the β-oxidation of branched-chain fatty acids. This enzyme is able to convert (2R)-pristanoyl-CoA, (25R)-DHC-CoA and (25R)-THC-CoA into their (S)-isomers (14,15) (Fig. 1). This conversion is essential for degradation of these substrates, because naturally occurring pristanic acid is a mixture of two diastereomers, (2R,6R,10R) and (2S,6R,10R) (16), whereas in case of DHCA and THCA only the (25R)-isomers are produced from cholesterol (17-20). As a consequence, patients who are unable to convert the (R)-isomer of pristanoyl-CoA and the C27-bile acyl-CoAs to their respective (S)-isomers, which are the true substrates for the β-oxidation system, are predicted to accumulate these compounds in their plasma. We have recently identified three patients with a complete α-methylacyl-CoA racemase deficiency due to mutations in the encoding gene as shown by expression studies in E. coli (21). The main clinical symptom in these patients was an adult-onset sensory motor neuropathy. As expected, plasma analysis in these patients revealed an accumulation of both pristanic acid and the bile acid intermediates DHCA and THCA.

In the present study we further analyzed the C27-bile acid intermediates accumulating in plasma from these patients and, for comparison, from patients with Zellweger disease.

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**Fig. 1** Schematic representation of the steps involved in the oxidation of (3R)- and (3S)-phytanic acid as derived from dietary sources and (25R)-THCA produced from cholesterol in the liver. After the activation of (3R)- and (3S)-phytanic acid to their corresponding CoA esters, they both become substrates for the peroxisomal α-oxidation system, which produces (2R)- and (2S)-pristanoyl-CoA. Since branched-chain acyl-CoA oxidase, the first enzyme of the β-oxidation system, can only handle (S)-stereoisomers, (2R)-pristanoyl-CoA needs to be converted by α-methylacyl-CoA racemase into its (2S)-isomer. The bile acid intermediates DHCA and THCA are exclusively produced as (25R)-stereoisomers. In order to be β-oxidized, the CoA esters of the (25R)-stereoisomer also need to be converted by α-methylacyl-CoA racemase into their (25S)-isomers.
syndrome and patients suffering from cholestatic liver disease using liquid chromatography/tandem mass spectrometry (LC/MS/MS) to discriminate between the different diastereomers of DHCA and THCA. The results obtained indicate that α-methylacyl-CoA racemase is, indeed, indispensable for the oxidation of the bile acid intermediates and that there is no other racemase which takes over the role of the deficient enzyme. Furthermore, the plasma analysis we describe in this paper provides an easy and reliable method to diagnose α-methylacyl-CoA racemase-deficient patients.

Materials and Methods

Materials

The two diastereomers of THCA were obtained as described before (21). Taurine was purchased from Serva (Heidelberg, Germany), 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide.HCl (EDC) from Sigma (St. Louis, MO) and [2,2,4,4-2H₄]cholic acid was from J.H. Ritmeester BV (Utrecht, The Netherlands).

Patients

Plasma samples were obtained from three patients with a deficiency of α-methylacyl-CoA racemase, four patients with Zellweger syndrome and five patients suffering from cholestatic liver disease. The ages of the patients with cholestatic liver disease (3 males and 2 females) and Zellweger syndrome (2 males and 2 females) varied between 1 month and 3 years. The α-methylacyl-CoA racemase-deficient patients all had distinct mutations in the encoding gene and racemase activity in fibroblasts of these patients as measured with THC-CoA as substrate was completely deficient (21). Patient 1, a boy, is now 7 years old, patient 2 is a man with the age of 49 and patient 3 is a 48-year-old woman. The patients with Zellweger syndrome all had the clinical and biochemical abnormalities described for Zellweger syndrome (22). Informed consent was obtained for all patients whose plasma was studied and the studies were approved by the Institutional Review Board of the Academic Medical Center, University of Amsterdam.

Derivatization of THCA with taurine

The two diastereomers of THCA were derivatized with taurine to be able to determine the stereospecificity of the different isomers of taurine-conjugated THCA in plasma of the patients. Derivatization of THCA was performed essentially as described by Zhang et al. (23). Briefly, 0.37 μmoles (25R)- or (25S)-THCA was dissolved in 0.2 ml 0.1 M pyridine hydrochloride (pH 5.0). Fifty μmoles EDC and 100 μmoles of taurine were added and the mixture was left for 16 h at room temperature. It was then passed through a SPE-C₁₈-column (1.5 x 0.8 cm) (J.T. Baker, Phillipsburg, NJ). After washing the column with water, taurine-conjugated THCA was eluted with methanol. The yield was approximately 70%.

Plasma sample preparation

Fifty μl of the internal standards (IS) [2,2,4,4-²H₄]cholic acid or [2,2,4,4-²H₄]-taurocholic acid was added to 50 μl plasma. The mixture was deproteinized by addition of 500 μl acetonitrile followed by subsequent centrifugation for 15 min at 20,000 x g at 4°C. The
supernatant was then evaporated under a stream of N₂ gas and the residue redissolved in 100 µl methanol/water (40/60). Twentyfive µl was injected into an LC/MS/MS system.

**Liquid Chromatography/Tandem Mass Spectrometry (LC/MS/MS)**

LC/MS/MS was carried out using a Hewlett-Packard (Palo Alto, CA) HP 1100 binary pump and a Micromass (Manchester, UK) Quattro II tandem Mass Spectrometer equipped with electrospray ionization (ESI). The LC separation was performed on an Altima C₁₈ reversed-phase (5 µm) column (250 mm × 2.1 mm) (Alltech, Deerfield, IL) and optimal resolution was achieved by elution with a linear gradient of methanol (70%→100%) in 5 mM ammoniumformiate buffer (pH 5.0) at a flow rate of 0.3 ml/min. MS/MS parameters were as follows: negative ion mode, capillary voltage 3.1 kV, cone voltage 70 V, collision energy 60 eV, collision pressure 0.003 mBar. Argon was used as collision gas. Taurine-conjugates were analyzed by MRM using the following transitions (IS 518.3 → 79.8; tauro-DHCA 540.3 → 79.8; tauro-THCA 556.3 → 79.8), the free compounds by SIR (IS 411.3; DHCA 433.3; THCA 449.3). The limit of detection of the bile acid intermediates was 0.05 µM.

**Results and discussion**

DHCA and THCA are obligatory intermediates in the major biosynthesis route of the primary bile acids chenodeoxycholic acid and cholic acid from cholesterol. They are produced from 5β-cholestane-3α,7α-diol and 5β-cholestane-3α,7α,12α-triol, respectively. The mitochondrial 27-hydroxylase involved in this pathway has been shown to be stereospecific, which exclusively leads to the formation of the (25R)-isomer of DHCA and THCA (17-20). Activation of DHCA and THCA occurs at the membrane of the endoplasmic reticulum followed by transport of DHC-CoA and THC-CoA into the peroxisome via a mechanism yet unknown. In the peroxisome (25R)-DHC-CoA and (25R)-THC-CoA are rapidly converted by α-methylacyl-CoA racemase (14,15) into their (25S)-isomers, that can enter the β-oxidation spiral.

Recently, three patients have been identified with a deficiency of α-methylacyl-CoA racemase due to mutations in the encoding gene. Plasma analysis revealed a marked increase in the levels of pristanic acid and of the C₂₇-bile acid intermediates DHCA and THCA (21). These compounds, however, are known to accumulate in several other peroxisomal disorders, including isolated defects in the peroxisomal β-oxidation system and defects in peroxisomal biogenesis (22,24-26). To examine the plasma C₂₇-bile acids in closer detail, we developed a method to study the different diastereomers of DHCA and THCA in plasma from patients with Zellweger syndrome and patients with an isolated α-methylacyl-CoA racemase deficiency. In addition, we studied plasma from patients suffering from cholestatic liver disease, who also accumulate bile acid intermediates in plasma but do not have a metabolic disorder affecting the oxidation of branched-chain fatty acids and fatty acid derivatives per se. The diastereomers of both free and taurine-conjugated C₂₇-bile acids could be studied with our LC/MS/MS method. To determine the elution pattern of the diastereomers of taurine-conjugated THCA, (25R)- and (25S)-THCA were derivatized with taurine. Both free and taurine-conjugated (25S)-THCA eluted at a lower concentration of methanol than the (25R)-isomer (Fig. 2). Unfortunately,
Fig. 2 Separation of the diastereomers of free and taurine-conjugated DHCA and THCA by LC/MS/MS. Analysis of the standards for (25S)- and (25R)-THCA in the free acid form and taurine-conjugated are shown (A and B, respectively). Plasma analysis in patients with Zellweger syndrome (C) revealed the presence of both diastereomers of free and taurine-conjugated THCA, whereas patients with a deficiency of α-methylacyl-CoA racemase (D) accumulate only the (25R)-isomer. No standards were available of the separate diastereomers of free and taurine-conjugated DHCA, but the exclusive accumulation of peak 2 for both compounds in racemase-deficient patients strongly suggests that peak 2 represents the (25R)-isomer.

no standards were available for DHCA. Therefore, we can only speculate about the identification of the diastereomers of free and taurine-conjugated DHCA.

Examination of plasma from four different patients with Zellweger syndrome revealed the presence of two diastereomers of both free and taurine-conjugated DHCA and THCA

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DHCA and THCA diastereomers in α-methylacyl-CoA racemase deficiency

(Table 1 and Fig. 2). DHCA was mainly present as free acid, whereas in most patients more THCA was taurine-conjugated than unconjugated. The predominant peak of both free and taurine-conjugated THCA corresponded to the (25R)-isomer. The mean values (± SD) for the (25S/25R)-isomer ratios in these four patients were 0.23 (± 0.05) and 0.26 (± 0.03) for free and taurine-conjugated THCA, respectively (Table 1). These results are in agreement with the ratio (25S/25R)-THCA found in urine from an infant with Zellweger syndrome by Une and coworkers (27). The presence of both isomers indicates that α-methylacyl-CoA racemase is enzymatically active in patients with Zellweger syndrome. A residual racemase activity of 10% for pristanoyl-CoA in fibroblasts from patients with Zellweger syndrome compared to controls has indeed been reported (15), and corresponds to the results we obtained with THC-CoA as substrate in fibroblasts from patients with Zellweger syndrome (controls 97 ± 28 pmol/min/mg [n = 13]; patients with Zellweger syndrome 17 ± 5 pmol/min/mg [n = 3]). For free and taurine-conjugated DHCA, respectively, the mean values (± SD) for the ratios peak 1/peak 2 in the four patients with Zellweger syndrome were 0.19 (± 0.02) and 0.43 (± 0.08) (Table 1).

Table 1 Analysis of the diastereomers of free and taurine-conjugated DHCA and THCA in plasma from five patients with cholestatic liver disease, four patients with Zellweger syndrome and three patients with an α-methylacyl-CoA racemase deficiency.

<table>
<thead>
<tr>
<th>THCA free acid</th>
<th>Cholestatic</th>
<th>Zellweger</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>(S)-a</td>
<td>ND</td>
<td>1.9-13.6</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>(R)-a</td>
<td>ND</td>
<td>9.7-75.5</td>
<td>2.2</td>
<td>2.2</td>
<td>0.1</td>
</tr>
<tr>
<td>(S/R)-b</td>
<td>0.23 ± 0.05</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHCA free acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(peak 1)-a</td>
<td>ND</td>
<td>4.4-14.3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>(peak 2)-a</td>
<td>ND</td>
<td>22.0-76.8</td>
<td>30.9</td>
<td>21.3</td>
<td>4.4</td>
</tr>
<tr>
<td>(1/2)-b</td>
<td>0.19 ± 0.02</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>THC conjugated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(S)-a</td>
<td>0.06-0.43</td>
<td>3.8-10.5</td>
<td>ND</td>
<td>ND</td>
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</tr>
<tr>
<td>(R)-a</td>
<td>0.23-2.39</td>
<td>16.1-34.8</td>
<td>0.6</td>
<td>9.9</td>
<td>3.8</td>
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<tr>
<td>(S/R)-b</td>
<td>0.25 ± 0.08</td>
<td>0.26 ± 0.03</td>
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<td>DHCA conjugated</td>
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<td></td>
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</tr>
<tr>
<td>(peak 1)-a</td>
<td>ND</td>
<td>0.8-3.2</td>
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<td>1.7-7.0</td>
<td>ND</td>
<td>2.0</td>
<td>0.6</td>
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<tr>
<td>(1/2)-b</td>
<td>0.43 ± 0.08</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Cholic acidb,c</td>
<td>14.1-74.8</td>
<td>0.3-13.0</td>
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<td>0.2</td>
<td>0.2</td>
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<tr>
<td>Chenodeoxycholic acidb,c</td>
<td>32.2 - 100.9</td>
<td>4.3 - 37.0</td>
<td>0.2</td>
<td>0.7</td>
<td>0.5</td>
</tr>
</tbody>
</table>

*range in μM. b ratio mean ± SD. c sum of glycine and taurine conjugated species (normal range 0.7-10 μM chenodeoxycholic acid; 0.1-4.7 mM cholic acid). ND, not detectable. Cholestatic, cholestatic liver disease patients (n=5); Zellweger, patients with Zellweger syndrome (n=4); patient 1-3, α-methylacyl-CoA racemase deficient patients.

In the patients with cholestatic liver disease the mean value (± SD) for the (25S/25R)-isomer ratios for taurine-conjugated THCA was 0.25 (± 0.08), which is similar to the ratio found in patients with Zellweger syndrome (0.26 ± 0.03; p>0.05; t-test). These results confirm that plasma from Zellweger patients can be used as a control in this assay, even though the biogenesis of peroxisomes, where the racemase is localized, is disturbed in these patients. The amount of free THCA and free and taurine-conjugated DHCA in
plasma of patients with cholestatic liver disease was too low to draw any conclusions about the distribution of the different diastereomers.

Plasma analysis of C27-bile acid intermediates in the three patients with a defined α-methylacyl-CoA racemase deficiency revealed the exclusive accumulation of the (25R)-isomer of both free and taurine-conjugated THCA (Table 1 and Fig. 2). Only one diastereomer of DHCA was present in both free acid form and in taurine-conjugated form. This strongly suggests that, as for THCA, peak 2 of free and taurine-conjugated DHCA, which elutes at a higher methanol concentration than peak 1, represents the (25R)-isomer (Fig. 2). The concentrations of the normal C24-bile acids cholic acid and chenodeoxycholic acid were in the lower part of the normal range. These bile acids could be synthesized using the alternative 25-hydroxylation pathway (28), but the lack of 25-hydroxylated bile alcohols (data not shown) in plasma of racemase-deficient patients suggest that other pathways might be responsible for the residual C24-bile acid biosynthesis.

Routine plasma analysis in adult patients with sensory motor neuropathy usually does not include analysis of bile acids and branched-chain fatty acids. This, together with the fact that the clinical symptoms associated with α-methylacyl-CoA racemase deficiency are relatively mild, implies that thus far many patients with α-methylacyl-CoA racemase deficiency may have remained undiagnosed. The method described in this paper provides a unique diagnostic tool for this disorder. Only a small amount of plasma is needed, the analysis takes little time and the exclusive accumulation of the (25R)-isomer of free and taurine-conjugated DHCA and THCA indisputably reveals a deficiency of α-methylacyl-CoA racemase in the patient. Finally, our data indicate that α-methylacyl-CoA racemase plays an indispensable role in bile acid formation.

Acknowledgments
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DHCA and THCA diastereomers in α-methylacyl-CoA racemase deficiency

References


Chapter 9

Stereochemistry of the peroxisomal branched-chain fatty acid α- and β-oxidation systems in patients suffering from different peroxisomal disorders.

Stereochernistry of the peroxisomai branched-chain fatty acid α- and β-oxidation systems in patients suffering from different peroxisomal disorders

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Abstract
Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) is a branched-chain fatty acid derived from dietary sources and broken down in the peroxisome to pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) via α-oxidation. Pristanic acid then undergoes β-oxidation in peroxisomes. Phytanic acid naturally occurs as a mixture of (3S,7R,11R)- and (3R,7R,11R)-diastereomers. In contrast to the α-oxidation system, peroxisomal β-oxidation is stereospecific and only accepts (2S)-isomers. Therefore, a racemase called α-methylacyl-CoA racemase, is required to convert (2R)-pristanic acid into its (2S)-isomer. To further investigate the stereochernistry of the peroxisomal oxidation systems and their substrates, we have developed a method using gas chromatography/mass spectrometry to analyze the isomers of phytanic, pristanic and trimethylundecanoic acid in plasma from patients with various peroxisomal fatty acid oxidation defects. In this study, we show that in plasma of patients with a peroxisomal β-oxidation deficiency the relative amounts of the two diastereomers of pristanic acid are almost equal, while in patients with a defect of α-methylacyl-CoA racemase (2R)-pristanic acid is the predominant isomer. Furthermore, we show that in α-methylacyl-CoA racemase deficiency not only pristanic acid accumulates, but also one of the metabolites of pristanic acid, 2,6,10-trimethylundecanoic acid, providing direct in vivo evidence for the requirement of this racemase for the complete degradation of pristanic acid.

Introduction
Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) is a branched-chain fatty acid derived from the chlorophyll component phytol and is degraded in the peroxisome. Phytanic acid first undergoes α-oxidation which leads to shortening of the chain by one carbon atom yielding pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) and carbon dioxide (see (1) for review). Pristanic acid is then further degraded in the peroxisome via 3 cycles of β-oxidation, followed by transport of the pristanic acid metabolite, 4,8-dimethylnonanoic acid, to the mitochondrion where it is β-oxidized to completion (2). The β-oxidation process in peroxisomes consists of four sequential enzymatic steps. After activation of pristanic acid to its CoA ester, pristanoyl-CoA is converted into 2,3-pristenoyl-CoA by branched-chain acyl-CoA oxidase (BCOX). This compound is then hydrated to 3-hydroxy-pristanoyl-CoA and subsequently dehydrogenated to 3-keto-pristanoyl-CoA. These reactions are catalyzed by D-bifunctional protein (DBP), which
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harbors both enoyl-CoA hydratase and 3-hydroxy-acyl-CoA dehydrogenase activity. Finally, thiolytic cleavage occurs via sterol carrier protein X (SCPx), yielding propionyl-CoA and 4,8,12-trimethyltridecanoyl-CoA, which reenters the β-oxidation spiral (for review of peroxisomal β-oxidation see (1,3)).

In 1967, Ackman and Hansen studied the stereochemical composition of phytanic and pristanic acid in ruminant fats and fish oils. They found that there are two diastereomers of these fatty acids present, namely the (S,R,R)- and (R,R,R)-isomers. Phytanic acid synthesized from phytol of plant origin also consists of these two isomers (4). Recent studies have shown that peroxisomal α-oxidation is not a stereospecific process (5), so that after α-oxidation of phytanic acid both (2R,6R,10R,14)- and (2S,6R,10R,14)-pristanic acid are formed. In contrast to the α-oxidation system, however, the peroxisomal β-oxidation system is stereospecific because only (2S)-pristanoyl-CoA is accepted as substrate by BCOX, the first enzyme of the β-oxidation system (6-8). For (2R)-pristanoyl-CoA to be degraded, it first needs to be converted to its (2S)-isomer by the enzyme α-methylacyl-CoA racemase (9,10) (Fig. 1). After 2 cycles of β-oxidation

![Diagram](image-url)

Fig. 1 Schematic representation of the oxidation of phytanic and pristanic acid in the peroxisome. Both (3R,7R,11R,15)- and (3S,7R,11R,15)-phytanoyl-CoA (I) undergo one round of α-oxidation, producing a mixture of (2R,6R,10R,14)- and (2S,6R,10R,14)-pristanoyl-CoA (II). The (2R)-isomer needs to be converted to its (S)-isomer before it can enter the β-oxidation spiral, because the peroxisomal oxidase, the first enzyme of the β-oxidation system, can only handle (S)-isomers. The resulting product, (4R,8R,12)-trimethyltridecanoyl-CoA (III) can be β-oxidized without any problem, but the next intermediate in the breakdown process of pristanic acid is again a 2-methyl-branched fatty acyl-CoA ((2R,6R,10)-trimethylundecanoyl-CoA (IV)) with the (R)-configuration and therefore requires a racemase to convert it to its (S)-isomer. After another cycle of β-oxidation (4R,8)-dimethylnonanoyl-CoA (V) is transported from the peroxisome to the mitochondrion as carnitine ester for further degradation.
(2R,6R,10)-trimethylundecanoyl-CoA is formed. Before this substrate can be degraded further, it needs to be converted to the (2S)-isomer. α-Methylacyl-CoA racemase is most likely the enzyme responsible for this racemization, although this has not yet been demonstrated experimentally.

A variety of different genetic diseases in man have been identified in which there is a defect in the peroxisomal α- and/or β-oxidation of fatty acids, resulting in the accumulation of certain fatty acids in plasma of these patients. Two groups can be distinguished. In the first group both fatty acid α- and β-oxidation are impaired. These patients, who suffer from a peroxisome biogenesis disorder, lack functional peroxisomes and, as a consequence, are deficient for many processes taking place in the peroxisome, including the degradation of very long-chain fatty acids and branched-chain fatty acids via β-oxidation. In the second group either the fatty acid α-oxidation or β-oxidation is deficient, because in this group of patients only a single enzyme is deficient. Patients suffering from Refsum disease have a deficiency of the first enzyme of the α-oxidation system (phytanoyl-CoA hydroxylase) (11,12) and, as a consequence, accumulate phytic acid in their plasma. Patients with a deficiency of DBP are deficient in peroxisomal β-oxidation of both very long-chain fatty acids and the branched-chain fatty acids including the bile acid intermediates (13-16). Recently, we identified a new disorder, α-methylacyl-CoA racemase deficiency, which affects the peroxisomal oxidation of 2-methyl branched-chain fatty acids and the bile acid intermediates (17).

To obtain more insight in the stereochemistry of the peroxisomal oxidation systems and their substrates, we developed a method to determine the relative amounts of phytanic, pristanic, and trimethylundecanoic acid diastereomers in plasma samples of patients suffering from the various peroxisomal disorders described above.

Materials and Methods

Patient material

All samples used in this study were obtained from patients with a confirmed deficiency of α-methylacyl-CoA racemase (17), DBP (13-16) or phytanoyl-CoA hydroxylase (11,12) due to mutations in the encoding genes, or from patients affected by a peroxisome biogenesis disorder as demonstrated by biochemical studies performed in fibroblasts (18). Informed consent was obtained from parents or guardians of the patients whose plasma was studied in this paper and the studies were approved by the Institutional Review Board of the Academic Medical Center, University of Amsterdam.

Synthesis of 2,6,10-trimethylundecanoic acid

2,6,10-Trimethylundecanoic acid was synthesized from 2,6,10-trimethyl-5,9-undecadien-1-ol (Acros Organics, Geel, Belgium). First, 2,6,10-trimethyl-5,9-undecadien-1-ol (a mixture of diastereomers) was hydrogenated in ethanol in the presence of 5 mol% PtO2 as a catalyst, under a H2 pressure of 3 bars for 16 h to 2,6,10-trimethylundecan-1-ol with a yield of 95%. To form the corresponding aldehyde, 2,6,10-trimethylundecan-1-ol was dissolved in dichloromethane/acetonitrile (9:1, v/v, 2 ml/mmol) and reacted with 1.5 molar equivalents of N-methylmorpholine-N-oxide in the presence of 500 mg/mmol 4A
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Molecular sieve and 5 mol% tetrapropylammonium perruthenate at room temperature for 2 h. The aldehyde was purified on a silica gel column using dichloromethane/ethyl acetate (8:2, v/v) as eluent. Subsequently, the eluent was taken to dryness with a rotary evaporator and the residue was purified by silica gel chromatography using hexane/ethyl acetate (95:5, v/v) as eluent. The yield of the aldehyde was 85%. For oxidation of 2,6,10-trimethylundecan-1-ol to the corresponding carboxylic acid, the aldehyde was dissolved in acetonitrile (1 ml/mmol) and mixed with 5 molar equivalents of H2O2 (added as a 35% aqueous solution) in the presence of 0.66 M NaH2PO4 buffer, pH 2. Subsequently, 1.4 molar equivalents of 1 M aqueous NaClO2 was slowly added over a period of 1 h at 10°C. The reaction was allowed to continue for 4 h at room temperature. After addition of a small amount of Na2SO3 to destroy unreacted HOCl and H2O2, the product was extracted from the reaction mixture with hexane. The hexane was removed under a stream of N2 and the residue purified by chromatography on a silica gel column with a discontinuous gradient of hexane/ethyl acetate (98:2 - 95:5, v/v) as solvent. The yield of 2,6,10-trimethylundecanoic acid was 80%. Gas-liquid chromatography/mass spectrometry of the methyl ester showed one homogeneous peak with a molecular ion at m/z 242. Mass spectrometry of the unesterified acid gave the following results: m/z 228 (M+ 2.5%), 152 (9.2%), 115 (5.7%), 97 (20.7%), 87 (31.4%), 74 (100%), 55 (50%), 41 (65%). The overall yield from 2,6,10-trimethyl-5,9-undecadien-1-ol to 2,6,10-trimethylundecanoic acid was 60%.

Quantification and analysis of plasma phytanic, pristanic and trimethylundecanoic acid diastereomers

Fatty acids were extracted from plasma as described (19). After extraction, the hexane phase was used for both quantification of the branched-chain fatty acids and analysis of the diastereomers of the branched-chain fatty acids. Phytanic and pristanic acid were quantified as described (19). Trimethylundecanoic acid was quantified using standard gas chromatography-analysis of methylated essential fatty acids, essentially as described (20). The isomers of the different branched-chain fatty acids were separated essentially as described by Schmitz et al. (9). One hundred microliters of the hexane-phase, containing the branched-chain fatty acids, were evaporated under a stream of N2. Five hundred microliters of 30 mM carbonyldiimidazole (Sigma, St. Louis, MO) dissolved in toluene were added and, after 10 min at room temperature, the sample was acidified with 10 µl glacial acetic acid. Subsequently, 50 µl (R)-1-phenylethylamine (Sigma, St. Louis, MO) was added. After 30 min at room temperature, the sample was mixed with 5 ml 50 mM sodium/potassium phosphate pH 7.5, the reaction products were extracted with 1 ml ethyl acetate which was dried under a stream of N2 and the residue dissolved in 75 µl hexane. Finally, the different branched-chain fatty acids were analyzed by GC/MS. Capillary column: 25 m x 0.25 mm i.d. CP-sil 19 CB (Chrompack, Middelburg, the Netherlands); column temperature: 50°C for 2 min; 50-240°C at a rate of 30°C/min; 240°C for 5 min; 240-285°C at a rate of 2.5°C/min; 285°C for 2 min; injection port and GC-MS interface at 250 and 300°C, respectively; ionization energy: 70 eV; 2 µl splitless injection; carrier gas...
helium, pressure 0.7 bar, 1.5 ml/min constant flow. Single ion monitoring was used for the respective M$^+$ ions (m/z 331, 401 and 415; masses of the molecular ions of the phenylethylamide derivatives of trimethylundecanoic, pristanic and phytanic acid, respectively). Racemic mixtures of trimethylundecanoic acid (synthesized as described above), pristanic acid (purchased from Dr. H. ten Brink, Amsterdam, The Netherlands) and phytanic acid (Sigma, St. Louis, MO) were used to set up the analysis.

**Identification of the (2R)- and (2S)-isomers of pristanic and trimethylundecanoic acid**

To assess the configuration of the isomers of pristanic and trimethylundecanoic acid, the racemic mixtures of these fatty acids were converted into their CoA esters as described by Rasmussen et al. (21) and incubated with purified long-chain acyl-CoA dehydrogenase (LCAD), which was a generous gift from Prof. Dr. T. Hashimoto (Shinshu University School of Medicine, Matsumoto, Japan) (22). Because only (2S)-isomers are substrate for LCAD (23), incubation with and without LCAD allows discrimination between the (2S)-and (2R)-isomers (Fig. 2). The incubation mixture consisted of purified LCAD (2.6 µU, determined with C8-CoA as substrate), 100 mM sodium phosphate/0.1 mM EDTA (pH 7.2), 0.4 mM hexafluorophosphate, 20 µM FAD and 50 µM trimethylundecanoyl-CoA or pristanoyl-CoA, in a final volume of 100 µl. Reactions were allowed to proceed for 60 min at 37°C. After termination of the reaction, the CoA esters were hydrolyzed by
addition of 10 µl 5 N NaOH followed by an incubation period of 2 h at 60°C. The isomers of the branched-chain fatty acids were then analyzed as described above (Fig. 2).

Results

GC/MS analysis of chemically synthesized standards of phytanic, pristanic and trimethylundecanoic acid as their (R)-1-phenylethylamine derivatives resulted in two clusters of peaks for all three branched-chain fatty acids (see Fig. 3A). For both pristanic and trimethylundecanoic acid the clusters clearly consisted of two peaks each (numbered 1 through 4 in Fig. 3A). As described in the Materials and Methods section, we incubated the CoA esters of these fatty acids with purified LCAD, to discriminate between the (2S)- and the (2R)-isomers because LCAD only dehydrogenates (2S)- but not (2R)-isomers (23). After an incubation period of 60 min, there was a strong reduction of the abundance of the second peak cluster both for trimethylundecanoyl-CoA (Fig. 2) and pristanoyl-CoA (data not shown), while the abundance of the first peak cluster remained unchanged, indicating that the second cluster consists of the (2S)-isomers. These results are in agreement with the results obtained for the (R)-1-phenylethylamine derivatives of 2-methylmyristic acid (9) and for 2-methylpentadecanoic acid (5), where the second cluster corresponded to the (2S)-isomers. Fig. 3 B-E show representative chromatograms of the GC/MS analysis of phytanic, pristanic and trimethylundecanoic acid in plasma from a patient with Refsum disease (panel B), Zellweger syndrome (panel C), DBP deficiency (panel D) and α-methylacyl-CoA racemase deficiency (panel E). In plasma from all patients affected by a peroxisomal disorder only peak 2 and 3 could be detected (Fig. 3C-E).

Plasma analysis in all the patients studied showed that the ratio (3S/3R)-phytanic acid did not differ significantly between the different peroxisomal disorders (see Table 1). The mean value (± SD) for the (3S/3R)-phytanic acid ratio was 0.46 (± 0.10), which is similar to the ratio found by Ackman and Hansen (0.39) in patients suffering from Refsum disease (4). In plasma samples from patients with a deficiency of α-methylacyl-CoA racemase (17), the ratio (2S/2R)-pristanic acid was significantly lower than in plasma from patients with Zellweger syndrome (p<0.005, t-test) and in most patients with a deficiency of DBP (see Table 1). The mean value (± SD) for the (2S/2R)-pristanic acid ratio was 1.06 (± 0.24) in patients with Zellweger syndrome and 0.89 (± 0.47) in patients with a DBP deficiency, while the mean value (± SD) in patients with a deficiency of α-methylacyl-CoA racemase was 0.24 (± 0.06).

Next to phytanic and pristanic acid, trimethylundecanoic acid accumulated in plasma from patients with a deficiency of α-methylacyl-CoA racemase (Table 1, Fig. 3E). Although the (2R)-isomer was the predominant isomer, also a small amount of (2S)-isomer was found. In addition, one patient suffering from Zellweger syndrome accumulated this fatty acid in his plasma (Table 1).
Fig. 3 Representative chromatograms of the GC/MS analysis of phytanic, pristanic and trimethylundecanoic acid isomers in a chemically synthesized standard mixture (panel A), and in plasma from a patient with Refsum disease (panel B), Zellweger syndrome (panel C), DBP deficiency (panel D) and α-methylacyl-CoA racemase deficiency (panel E). The stereochemical configuration of the first methyl group (R/S) is indicated for all the branched-chain fatty acids. GC/MS analysis of (R)-1-phenylethylamine derivatives of both pristanic and trimethylundecanoic acid revealed four peaks for each fatty acid (numbered 1-4). In plasma from patients with a deficiency of the peroxisomal β-oxidation, however, only peak 2 and 3 were present. This strongly suggests that peak 2 and 3 correspond to (2R,6R,10R,14)- and (2S,6R,10R,14)-pristanic acid, respectively, and in case of trimethylundecanoic acid to the (2R,6R,10)- and (2S,6R,10)-isomer, respectively. The identity of peak X is unknown.
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Table 1 Concentrations and relative amounts of branched-chain fatty acid isomers in different peroxisomal disorders

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<th>Trimethylundecanoic acid</th>
<th>Pristanic acid</th>
<th>Phytic acid</th>
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<td></td>
<td>(µM)</td>
<td>(S/R)-isomer</td>
<td>(S/R)-isomer</td>
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<td>ND</td>
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</tr>
<tr>
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<td>-</td>
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<tr>
<td>Refsum 4</td>
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<td>-</td>
<td>0.3</td>
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*a ratio; *b range; *c ND, not detected; *d Refsum, patients with Refsum disease; *e Zellweger, patients with Zellweger syndrome; *f DBP, patients with a deficiency of DBP; *g Racemase, patients with a deficiency of α-methylacyl-CoA racemase

Discussion

GC/MS analysis of (R)-1-phenylethylamine derivatives of both pristanic and trimethylundecanoic acid revealed four peaks for each fatty acid. In plasma from patients with a deficiency of the peroxisomal β-oxidation, however, only peak 2 and 3 were present. In combination with the fact that the sixth and tenth carbon atom of naturally occurring pristanic acid have the (R)-configuration (4), this strongly suggests that peak 2 and 3 correspond to (2R,6R,10R)- and (2S,6R,10R)-pristanic acid, respectively, and in case of trimethylundecanoic acid to the (2R,6R)- and (2S,6R)-isomer, respectively. When these branched-chain fatty acids are synthesized chemically they can exist in several different stereoisomeric configurations. Only four different stereoisomers of trimethylundecanoic acid exist, suggesting that peak 1 and 4 are the (2R,6S)- and (2S,6S)-isomer, respectively. Pristanic acid, however, when synthesized chemically, can exist in eight different stereoisomeric configurations. Therefore, the exact identity of peak 1 and 4 of pristanic acid is not known.

The mean value (0.46) for the (3S/3R)-phytanic acid ratio in all patients studied probably reflects the ratio of these isomers in a normal diet. Both isomers occur in animals, but in terrestrial mammals the (3R,7R,11R)-isomer predominates, while in marine life the (3S,7R,11R)-isomer is predominant (4). Different ratios of these isomers in the diet might explain why the values in the patients ranged between 0.18-0.79, although in most patients the ratio was close to the mean.
In plasma samples from patients with a deficiency of α-methylacyl-CoA racemase (17), the ratio (2S/2R)-pristanic acid was significantly lower than in plasma from patients with Zellweger syndrome and in most patients with a deficiency of DBP. In patients with a deficiency of α-methylacyl-CoA racemase, (2S)-pristanic acid can be degraded normally via peroxisomal β-oxidation, but (2R)-pristanic acid cannot be converted to its (2S)-isomer and therefore accumulates. In contrast to the exclusive accumulation of (25R)-DHCA and (25R)-THCA in plasma of patients with an α-methylacyl-CoA racemase deficiency (24), however, no exclusive accumulation of (2R)-pristanic acid was found. This can be explained by the fact that DHCA and THCA are only synthesized in the liver as (R)-isomer, while pristanic and phytanic acid are derived from the diet, consisting of a mixture of isomers. Accumulation of both pristanic acid and the bile acid intermediates probably inhibits β-oxidation of branched-chain fatty acids in the peroxisome, which causes some dietary (2S)-pristanic acid to accumulate. For this reason, the method described in this paper for the analysis of the isomers of pristanic acid, cannot be used for the unequivocal diagnosis of patients with a deficiency of α-methylacyl-CoA racemase. Identification of α-methylacyl-CoA racemase deficiency can best be done using our previously described method in which LC/MS/MS is used to analyse the isomers of DHCA and THCA in plasma (24).

In patients with a deficiency of α-methylacyl-CoA racemase half of the pristanic acid can enter the β-oxidation spiral. The degradation can, however, not proceed beyond 2,6,10-trimethylundecanoyl-CoA, of which all methyl groups have the (R)-configuration. For this compound to be β-oxidized further, the (2R)-methyl group needs to be converted to the (S)-configuration by a racemase. In case of a deficiency of α-methylacyl-CoA racemase trimethylundecanoic acid accumulates in plasma, confirming that this is the racemase responsible for the conversion of this compound. Unexpectedly, however, the (2R)-isomer was not the only isomer which accumulated, since also a small amount of (2S)-isomer was found. This might be explained by the presence of some racemic trimethylundecanoic acid derived from the diet. In this respect it is important to mention that all racemase patients were adults when their plasma was analyzed and could have accumulated some (2S)-trimethylundecanoic acid over the years. A problem with this interpretation remains that (2S)-trimethylundecanoic acid would be expected to undergo normal β-oxidation. In analogy to the situation with (2S)-pristanic acid, we hypothesize that the peroxisomal β-oxidation of both (2S)-pristanic acid and (2S)-trimethylundecanoic acid is compromised due to inhibition by the competing (2R)-compounds.

Since all the patients with a peroxisomal β-oxidation deficiency were still very young when their plasma was analyzed, no accumulation of dietary trimethylundecanoic acid was expected. One patient suffering from Zellweger syndrome, however, did accumulate this fatty acid in his plasma. In addition, this patient had extremely elevated levels of both phytanic and pristanic acid. These high levels of phytanic and pristanic acid, in combination with a low residual pristanic acid β-oxidation activity in fibroblasts from this patient (56 pmol/hr/mg versus 898 ± 223 pmol/hr/mg [\(n=50]\) control fibroblasts), suggest that there might have been some endogenous production of trimethylundecanoic acid. Probably, the (2S)-trimethylundecanoic acid in plasma of this patient is formed by
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the mitochondrial α-methylacyl-CoA racemase, which is the same enzyme as the peroxisomal but is unaffected in Zellweger syndrome (25).

In conclusion, we have shown that in plasma from patients with a peroxisomal fatty acid oxidation defect only two diastereomers of phytanic and/or pristanic acid accumulate, most likely the (S,R,R)- and (R,R,R)-isomers. In patients with a defect of α-methylacyl-CoA racemase almost all pristanic acid was the (2R)-isomer, while in patients with a peroxisomal β-oxidation deficiency the relative amounts of the two diastereomers of pristanic acid were almost equal. Furthermore, we have shown that in α-methylacyl-CoA racemase deficiency not only pristanic acid accumulates, but also one of the metabolites of pristanic acid, 2,6,10-trimethylundecanoic acid, providing direct in vivo evidence for the requirement of this racemase for the complete degradation of pristanic acid.

Acknowledgments

This article is dedicated to the memory of our colleague Dr. Peter Vreken, who played a major role in the studies described in this paper. We thank Drs. H.R. Waterham and M. Duran for critically reading of the manuscript. This work was supported by the Princess Beatrix Fund (The Hague, The Netherlands).

References


Identification of the peroxisomal β-oxidation enzymes involved in the biosynthesis of docosahexaenoic acid.

Identification of the peroxisomal β-oxidation enzymes involved in the biosynthesis of docosahexaenoic acid

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Abstract
Docosahexaenoic acid (DHA, C22:6n-3) is an important polyunsaturated fatty acid (PUFA) implicated in a number of (patho)physiological processes. For a long time, the exact mechanism of DHA formation has remained unclear, but now it is known that it involves the production of C24:6n-3 from dietary linolenic acid (C18:3n-3) via a series of elongation and desaturation reactions, followed by β-oxidation of C24:6n-3 to C22:6n-3. Although DHA is deficient in patients lacking peroxisomes, the intracellular site of retroconversion of C24:6n-3 has remained controversial. Making use of fibroblasts from patients with defined mitochondrial and peroxisomal fatty acid oxidation defects, we show in this paper that peroxisomes, and not mitochondria, are involved in DHA formation by catalyzing the β-oxidation of C24:6n-3 to C22:6n-3. Additional studies in fibroblasts from patients with X-linked adrenoleukodystrophy (XALD), straight-chain acyl-CoA oxidase (SCOX) deficiency, D-bifunctional protein (DBP) deficiency and rizomelic chondrodysplasia punctata (RCDP) type 1, and fibroblasts from L-bifunctional protein (LBP) and sterol carrier protein X (SCPx) knockout mice, show that the main enzymes involved in β-oxidation of C24:6n-3 to C22:6n-3 are SCOX, DBP and both 3-ketoacyl-CoA thiolase and SCPx. These findings are of importance for the treatment of patients suffering from a defect in peroxisomal β-oxidation.

Introduction
For years, it was generally assumed that the biosynthesis of polyunsaturated fatty acids (PUFAs) takes place in the endoplasmic reticulum, which is also the main site for phospholipid biosynthesis (1). Docosahexaenoic acid (DHA) (C22:6n-3), the major PUFA in adult mammalian brain and retina, was believed to be synthesized from dietary linolenic acid (C18:3n-3) in a pathway consisting of a series of elongation and desaturation reactions. This pathway required that C22:5n-3 becomes desaturated at position 4 by a microsomal acyl-CoA dependent Δ4-desaturase to form C22:6n-3. Several studies in the past few years, however, have indicated that such a Δ4-desaturase does not appear to exist (2-5). Instead, it was found that a 24-carbon n-3 fatty acid is synthesized, which is desaturated at position 6 to produce C24:6n-3, followed by one round of β-oxidation with C22:6n-3 as final product. Although still disputed, the peroxisome is the likely site of C24:6n-3 β-oxidation. After its formation, DHA is transported back to the endoplasmic reticulum where it is esterified into membrane lipids (6,7). Fig. 1 shows the
Peroxisomal \( \beta \)-oxidation enzymes involved in DHA biosynthesis

### Revised Pathway for the Biosynthesis of DHA

The synthesis of arachidonic acid (C20:4n-6) and C22:5n-6 from dietary linoleic acid (C18:2n-6) follows a similar pathway (1).

The \( \beta \)-oxidation step in the revised pathway of PUFA biosynthesis requires a considerable exchange of unsaturated fatty acids between different subcellular compartments (6). Several lines of evidence suggest that peroxisomes are the intracellular site of this \( \beta \)-oxidation step. First, patients suffering from Zellweger syndrome (a peroxisome biogenesis disorder), who lack functional peroxisomes, have clearly reduced levels of DHA, especially in brain and retina but also in liver, kidney (8) and blood (9). In addition, in newborn PEX5 knockout mice, a mouse model for Zellweger syndrome, the DHA concentration in the brain is also strongly reduced (40% as compared with levels in normal littermates) (10). In an extensive study on n-3 fatty acid metabolism, Moore et al. reported that control human fibroblasts metabolized \([1-^{14}C]18:3n-3\) to labeled C24:5n-3, C24:6n-3 and C22:6n-3 (5). In contrast, fibroblasts from patients suffering from Zellweger syndrome metabolized \([1,^{14}C]18:3n-3\) to C24:5n-3 and C24:6n-3, but not to C22:6n-3. Likewise, \([3-^{14}C]\)-labeled 22:5n-3, 24:5n-3 and 24:6n-3 were all metabolized to C22:6n-3 in control, but not in Zellweger fibroblasts. Similar results were obtained by Petroni et al., who incubated control and Zellweger fibroblasts with \([1-^{14}C]20:5n-3\) (11). In a recent paper, it was demonstrated that peroxisomes are required for biosynthesis of DHA from linolenic acid in livers from neonatal piglets (12). This was concluded from the observation that isotope-labeled DHA, and all the intermediates of the pathway, were formed only when combined microsomal and peroxisomal fractions were incubated with \([^{13}C-U]18:3n-3\).

In spite of the many experiments which show that peroxisomes are involved in the biosynthesis of PUFA, Infante et al. propose that synthesis of these fatty acid occurs in the outer mitochondrial membrane via a channeled carnitine-dependent pathway (13,14).
Although there is not much direct experimental evidence to support the existence of such a mitochondrial pathway, a role for the mitochondrion in the biosynthesis of DHA cannot be ruled out with absolute certainty. We therefore set out to study the role of peroxisomes and mitochondria, and their fatty acid oxidation systems, in DHA synthesis in more detail.

Fig. 2 shows a schematic representation of the peroxisomal β-oxidation system. There are two complete sets of β-oxidation enzymes present in the peroxisome (15). Straight-chain acyl-CoA oxidase (SCOX) is responsible for the initial oxidation of very long-chain fatty acyl-CoAs, while branched-chain acyl-CoA oxidase (BCOX) oxidizes branched-chain fatty acyl-CoAs. The enoyl-CoA esters of both straight- and branched-chain fatty acids are then hydrated and subsequently dehydrogenated by the same enzyme: D-bifunctional protein (DBP). The function of the second multifunctional protein present in the peroxisome, L-bifunctional protein (LBP), is still unknown. The last step of the β-oxidation process, the thiolylatic cleavage, is performed by sterol carrier protein X (SCPx) in case of the branched-chain substrates, while straight-chain substrates most likely are handled by both SCPx and the classical 3-ketoacyl-CoA thiolase.

![Diagram of fatty acid β-oxidation](image_url)

Fig. 2 Schematic representation of the fatty acid β-oxidation machinery in human peroxisomes catalyzing the oxidation of very long-chain fatty acyl-CoAs (VLCFA-CoA) and branched-chain fatty acyl-CoAs (pristanoyl-CoA and THC-CoA). Oxidation of VLCFA-CoAs (C24:0 and C26:0) involves straight-chain acyl-CoA oxidase, D-bifunctional protein (DBP) and both 3-ketoacyl-CoA thiolase and sterol carrier protein X (SCPx), while oxidation of branched-chain fatty acyl-CoAs involves branched-chain acyl-CoA oxidase, DBP and SCPx (see (15) for review).

Until now, only patients with an isolated defect of SCOX and DBP have been identified. In addition, patients suffering from Rizomelic Chondrodysplasia Punctata (RCDP) type 1 lack 3-ketoacyl-CoA thiolase in their peroxisomes. This is, however, not the only deficiency in these patients. Due to a defect in PEX7, the gene encoding the peroxisome targeting signal 2 (PTS2) receptor, their peroxisomes lack all proteins imported via this receptor, including alkyl-dihydroxyacetonephosphate synthase, an enzyme of the
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Plasmalogen biosynthetic pathway, and phytanoyl-CoA hydroxylase, the first enzyme of the peroxisomal α-oxidation pathway (16-18). No patients have been identified with a deficiency of BCOX, LBP and SCPx, but knockout mice have been created for the latter two enzymes (19,20).

To elucidate the role of both the peroxisome and mitochondrion, we studied the biosynthesis of DHA from [1-14C]18:3n-3, [1-14C]20:5n-3 and [3-14C]24:6n-3 in fibroblasts from patients with a peroxisome biogenesis disorder and from patients with a deficiency of one of the following mitochondrial enzymes: carnitine palmitoyltransferase 1 (CPT I), carnitine acylcarnitine translocase (CACT), carnitine palmitoyltransferase 2 (CPT II) and very long-chain acyl-CoA dehydrogenase (VLCAD). The first three enzymes are necessary for the transport of activated fatty acids across the inner mitochondrial membrane (21) and the last enzyme is part of the mitochondrial β-oxidation system (22). In addition, we investigated the role of the various peroxisomal β-oxidation enzymes in DHA biosynthesis by incubating fibroblasts from patients with a deficiency of SCOX and DBP, patients suffering from RCDP type 1, and from LBP and SCPx knockout mice with 14C-labeled precursors. We also studied DHA synthesis in fibroblasts from a patient suffering from X-linked adrenoleukodystrophy (XALD). These patients accumulate very long-chain fatty acids because of an impaired peroxisomal β-oxidation of these fatty acids. However, this is not caused by a deficiency of one of the enzymes of the β-oxidation system, but by a defect of the peroxisomal membrane protein ALDP (adrenoleukodystrophy protein) (23,24).

Materials and Methods

Materials

Radiolabeled [1-14C]18:3n-3, [1-14C]20:5n-3 and [1-14C]22:6n-3 were purchased from New England Nuclear (DuPont, Boston, MA). [3-14C]24:6n-3 was synthesized as described previously (2). Each radiolabeled fatty acid had a specific activity between 50 and 55 mCi/mmol.

Patient cell lines

Cell lines were used from several patients suffering from various peroxisomal and mitochondrial fatty acid β-oxidation disorders. The fibroblasts from patients with a peroxisome biogenesis disorder studied in this paper were from four patients with Zellweger syndrome and one patient with neonatal adrenoleukodystrophy (NALD), which is a less severe form of a peroxisome biogenesis defect. These patients all had the clinical and biochemical abnormalities described for patients with a peroxisome biogenesis disorder, including deficient C26:0 and pristanic acid β-oxidation and phytanic acid α-oxidation (25). The fibroblasts from the XALD patient had impaired C26:0 β-oxidation, which is caused by a mutation in the gene encoding the peroxisomal membrane protein ALDP (23,24). The SCOX and DBP deficient patients all had mutations in the encoding gene and no enzyme activity could be measured in fibroblasts of these patients (26-28). Peroxisomes from the patients with RCDP type 1 we studied, lack 3-ketoacyl-CoA thiolase due to a mutation in the PEX7 gene encoding the PTS2 receptor. Immunoblot studies
performed with an antibody raised against 3-ketoacyl-CoA thiolase revealed that only the unprocessed protein of 44 kDa is present in fibroblast homogenates. It is known that 3-ketoacyl-CoA thiolase is synthesized as a precursor protein and is proteolytically cleaved to its mature form of 41 kDa inside the peroxisome (29). Cultured skin fibroblasts from an SCPx knockout mouse were obtained from Dr. U. Seedorf (Westphalian Wilhelms-University Münster, Germany) (19) and fibroblasts of an LBP knockout mouse were generated by Qi et al. (20). Both knockout mice have been fully characterized and completely lack SCPx or LBP gene function, respectively. The fibroblasts from patients with a mitochondrial β-oxidation disorder used in this study were from patients with a confirmed deficiency of CPT I, CACT, CPT II or VLCAD due to mutations in the encoding genes (see (22) for review). These mutations result in a deficiency of mitochondrial fatty acid oxidation as established by individual enzyme activity measurements in cultured skin fibroblasts.

All patient cell lines used in this study were taken from the cell repository of the Laboratory for Genetic Metabolic Diseases (Academic Medical Center, University of Amsterdam, the Netherlands) and were derived from patients diagnosed in this center. 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.

**Experimental protocol**

DHA synthesis from [1-14C]18:3n-3, [1-14C]20:5n-3 and [3-14C]24:6n-3 was studied in cultures of fibroblasts grown in tissue culture flasks (25 cm²). Incubations were carried out in minimal essential medium (MEM) supplemented with penicillin/streptomycin, and containing 10% fetal calf serum (fatty acid free), 20 mM HEPES and 14C-labeled fatty acid. In case of [1-14C]18:3n-3 and [1-14C]20:5n-3 the incubation was carried out with 2 µM labeled fatty acid, while [3-14C]24:6n-3 was used at a concentration of 0.2 µM. The fibroblasts were kept in an incubator at 37°C for 96 h except for the incubations with [3-14C]24:6n-3, which were terminated after 24 h. Parallel incubations were performed to determine the amount of protein.

**Lipid analyses**

Lipids were extracted from the incubation medium as described by Moore et al. (5). Briefly, the lipids were extracted with a 2:1 (v/v) mixture of chloroform-methanol containing 1% acetic acid (v/v). The chloroform phase was dried under N2, the residue resuspended in 2 ml 1.5 N HCl/methanol and heated to 90°C for 2 h to produce fatty acid methyl esters. After extraction of the methyl esters in heptane, the heptane phase was dried under N2 and the residue resuspended in 150 µl 70% acetonitrile, which was stored at -20°C until analysis. Seventy microliters of the sample was subjected to high performance liquid chromatography (HPLC) analysis as described below.

To isolate cellular lipids, the incubation medium was removed and the fibroblasts were scraped into 1 ml of methanol and transferred to a screw-top glass vial. The tissue culture flasks was washed with 1 ml 3N HCl/methanol, which also was transferred to the glass vial. Finally, fatty acid methyl esters were produced and extracted as described above.
Peroxisomal β-oxidation enzymes involved in DHA biosynthesis

**HPLC analysis**

Radioactive methyl esters prepared from the cell lipids or incubation medium were separated by reversed-phase HPLC. A reversed-phase C18-column (4.6 x 150 mm; Beckman, Fullerton, CA) with 5 μm spherical packing was used with a mobile phase of water and acetonitrile in a two-step isocratic elution (76% acetonitrile for 50 min, 90% acetonitrile for 10 min), followed by an equilibration period of 10 min at 76% acetonitrile. The effluent was mixed with scintillation solution at a 1:1 ratio, and the radioactivity was measured by passing the mixture through an online Radiomatic Instruments (Packard, Meriden, CT) Flo One–β radioactivity detector. The system was standardized with methyl esters of the following radiolabeled fatty acids: C18:3n-3, C20:5n-3, C24:6n-3 and C22:6n-3.

**Results**

**Peroxisomal versus mitochondrial involvement in DHA biosynthesis**

*Linolenic acid (C18:3n-3) utilization*

After a 96-h incubation of control human skin fibroblasts with [1,14C]18:3n-3, substantial amounts of the radiolabeled fatty acids contained in the cells consisted of C22:6n-3 (mean value in 5 different control fibroblast cell lines was 15.5%) (see Table 1). In addition, radioactivity was detected in almost all intermediates of the proposed pathway of DHA biosynthesis (Fig. 1), including radiolabeled C24:5n-3 and C24:6n-3 (Fig. 3). Similar results were obtained with fibroblasts from patients with a deficiency of mitochondrial fatty acid oxidation. Fibroblasts from patients with a deficiency in one of the steps involved in the mitochondrial carnitine shuttle (CPT I, CACT or CPT II) as well as from a patient with a defect of the first enzyme of the mitochondrial fatty acid oxidation system, VLCAD, revealed normal synthesis of DHA from radiolabeled linolenic acid compared to the synthesis observed in control fibroblasts (Table 1). In contrast, no radiolabeled C22:6n-3 was formed in fibroblasts from patients with Zellweger syndrome, although the [1-14C]18:3n-3 was converted to other intermediates in the biosynthetic pathway. In addition, increased amounts of radiolabeled C24:6n-3, the precursor of DHA, were found. Fibroblasts from a patient with NALD, a milder variant of Zellweger syndrome characterized by a less severe peroxisomal deficiency, synthesized some radiolabeled DHA but only 1% of the radioactivity was converted to C22:6n-3 after the incubation period (Table 1).

Analysis of the radiolabeled fatty acids excreted in the medium revealed a similar pattern as the analysis of the fatty acids contained in the cells. This was true for both control skin fibroblasts as well as fibroblasts from patients with a mitochondrial or peroxisomal defect (data not shown). This is in agreement with the findings by Moore et al. (5), who concluded that these mitochondrial and peroxisomal defects do not cause selective retention or release of certain radiolabeled fatty acids. Therefore, all results shown are obtained by analysis of the cells only.
Table 1 Radiolabeled fatty acids produced by human and mouse skin fibroblasts after a 96 h-incubation with [1-\(^{14}\)C]18:3n-3. Amount of radiolabeled fatty acid detected (counts per min) per mg protein

<table>
<thead>
<tr>
<th>Fibroblast type</th>
<th>C18:3n-3</th>
<th>C20:4n-3</th>
<th>C22:5n-3</th>
<th>C24:5n-3</th>
<th>C24:6n-3</th>
<th>C22:6n-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (n = 5)</td>
<td>3,470 ± 407'</td>
<td>1,269 ± 554</td>
<td>41,264 ± 10,159</td>
<td>39,346 ± 13,977</td>
<td>803 ± 604</td>
<td>1,057 ± 762</td>
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<tr>
<td>Zellweger (n = 4)</td>
<td>7,570 ± 2,055'</td>
<td>3,500 ± 775</td>
<td>33,305 ± 5,612</td>
<td>78,883 ± 20,305</td>
<td>2,696 ± 361</td>
<td>8,081 ± 2,185</td>
</tr>
<tr>
<td>NALD</td>
<td>3,092</td>
<td>1,727</td>
<td>13,255</td>
<td>60,404</td>
<td>755</td>
<td>4,834</td>
</tr>
<tr>
<td>CPT I</td>
<td>8,393</td>
<td>3,786</td>
<td>74,214</td>
<td>79,215</td>
<td>2,557</td>
<td>2,068</td>
</tr>
<tr>
<td>CACT (n = 3)</td>
<td>8,696 ± 2,999'</td>
<td>4,215 ± 1,852</td>
<td>60,030 ± 9,776</td>
<td>99,987 ± 28,947</td>
<td>3,814 ± 818</td>
<td>2,624 ± 800</td>
</tr>
<tr>
<td>CPT II</td>
<td>7,199</td>
<td>2,845</td>
<td>55,798</td>
<td>84,113</td>
<td>3,581</td>
<td>2,049</td>
</tr>
<tr>
<td>VLCAD</td>
<td>6,599</td>
<td>3,319</td>
<td>54,998</td>
<td>60,449</td>
<td>2,781</td>
<td>2,024</td>
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<tr>
<td>XALD</td>
<td>10,560</td>
<td>2,211</td>
<td>61,678</td>
<td>58,828</td>
<td>2,482</td>
<td>1,635</td>
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<tr>
<td>SCOX (n = 3)</td>
<td>7,861 ± 1,706'</td>
<td>4,625 ± 2,104</td>
<td>54,654 ± 15,350</td>
<td>94,991 ± 42,300</td>
<td>3,783 ± 1,124</td>
<td>6,310 ± 2,505</td>
</tr>
<tr>
<td>DBP (n = 3)</td>
<td>4,137 ± 2,590'</td>
<td>2,203 ± 1,199</td>
<td>42,769 ± 21,368</td>
<td>54,065 ± 21,838</td>
<td>1,892 ± 814</td>
<td>3,090 ± 1,028</td>
</tr>
<tr>
<td>RCDP type 1 (n = 3)</td>
<td>3,262 ± 1,093'</td>
<td>2,698 ± 1,013</td>
<td>36,447 ± 10,017</td>
<td>66,120 ± 23,755</td>
<td>2,626 ± 2,670</td>
<td>2,688 ± 137</td>
</tr>
<tr>
<td>Control mouse</td>
<td>1,671</td>
<td>773</td>
<td>56,858</td>
<td>70,228</td>
<td>882</td>
<td>2,024</td>
</tr>
<tr>
<td>LBP -/- mouse</td>
<td>2,669</td>
<td>0</td>
<td>122,890</td>
<td>78,476</td>
<td>1,187</td>
<td>3,065</td>
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<tr>
<td>SCPX -/- mouse</td>
<td>2,005</td>
<td>1,912</td>
<td>88,115</td>
<td>48,912</td>
<td>1,439</td>
<td>1,671</td>
</tr>
</tbody>
</table>

The methyl esters of the radiolabeled fatty acids contained in the cells were separated by HPLC. n = number of different cell lines; all incubations were performed in triplicate; 'mean value ± standard deviation.
Peroxisomal β-oxidation enzymes involved in DHA biosynthesis

Eicosapentaenoic acid (C20:5n-3) utilization

Similar results were obtained after incubation of fibroblasts with [1-14C]20:5n-3 (Table 2). After an incubation of 96 h, control fibroblasts produced radiolabeled C22:5n-3 and C22:6n-3, as well as small amounts of C24:5n-3 and C24:6n-3. Fibroblasts from patients with a deficiency of either CPT I, CACT, CPT II or VLCAD revealed normal synthesis of DHA from [1-14C]20:5n-3. Fibroblasts from patients with Zellweger syndrome, however, produced no radiolabeled DHA. In contrast, they accumulated large amounts of C24:6n-3 (about 10-times more than observed in control fibroblasts). Incubations of fibroblasts from a patient with NALD resulted in intermediate values. These fibroblasts produced 10% of the amount of radiolabeled DHA formed in control fibroblasts and accumulated about 6-times the normal amount of C24:6n-3.

Fig. 3 Radiolabeled fatty acids contained in the cell lipids of fibroblast cultures incubated with 2 μM [1-14C]18:3n-3 for 96 h. After the cell lipids were extracted and methylated, the radiolabeled material was analyzed by high performance liquid chromatography (HPLC) with an online flow liquid scintillation detector. The HPLC traces shown are from a control human skin fibroblast cell line (A), a patient with Zellweger syndrome (B) and a CACT- deficient patient (C).

Tetracosahexaenoic acid (C24:6n-3) utilization

The results with [1-14C]18:3n-3 and [1-14C]20:5n-3 support the finding by Moore et al. (5) that the pathway for DHA synthesis from n-3 fatty acid precursors in human fibroblasts involves the formation of 24-carbon polyunsaturated intermediates, followed by


<table>
<thead>
<tr>
<th>Fibroblast type</th>
<th>C20:5n-3</th>
<th>C22:5n-3</th>
<th>C24:5n-3</th>
<th>C24:6n-3</th>
<th>C22:6n-3</th>
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<td>Controls (n = 5)</td>
<td>52,198 ± 8,570</td>
<td>57,325 ± 10,241</td>
<td>113 ± 179</td>
<td>1,782 ± 489</td>
<td>25,740 ± 5,801</td>
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<tr>
<td>Zellweger (n = 4)</td>
<td>35,375 ± 5,460</td>
<td>173,244 ± 47,838</td>
<td>454 ± 114</td>
<td>18,217 ± 6,319</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>NALD</td>
<td>12,434</td>
<td>127,834</td>
<td>254</td>
<td>10,424</td>
<td>2,709</td>
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<tr>
<td>CPT I</td>
<td>75,871</td>
<td>104,389</td>
<td>62</td>
<td>2,395</td>
<td>25,703</td>
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<td>CACT (n = 3)</td>
<td>65,920 ± 3,356</td>
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<td>608 ± 216</td>
<td>2,607 ± 430</td>
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<tr>
<td>CPT II</td>
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<td>753</td>
<td>2,682</td>
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<td>VLCAD</td>
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<td>89,327</td>
<td>824</td>
<td>3,129</td>
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<td>XALD</td>
<td>91,424</td>
<td>120,148</td>
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<td>23,738</td>
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<td>SCOX (n = 3)</td>
<td>54,264 ± 7,102</td>
<td>147,870 ± 19,946</td>
<td>540 ± 208</td>
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<td>DBP (n = 3)</td>
<td>53,579 ± 26,796</td>
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<td>5,544</td>
<td>5,545 ± 2,990</td>
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<tr>
<td>RCDP type 1 (n = 3)</td>
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<td>71,072 ± 10,403</td>
<td>618 ± 105</td>
<td>3,164 ± 588</td>
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<td>Control mouse</td>
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<td>2,398</td>
<td>18,505</td>
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</table>

The methyl esters of the radiolabeled fatty acids contained in the cells were separated by HPLC. n = number of different cell lines; all incubations were performed in triplicate; *mean value ± standard deviation.
Peroxisomal \( \beta \)-oxidation enzymes involved in DHA biosynthesis

retroconversion of C24:6n-3 to C22:6n-3. To study this retroconversion reaction more directly, fibroblasts were incubated with \([3-{\textsuperscript{14}}C]24:6n-3\) (Table 3). Control fibroblasts converted almost all radiolabeled C24:6n-3 to C22:6n-3, which also was observed in fibroblasts from patients with a CACT deficiency. The rate of \( \beta \)-oxidation of this substrate in these cell lines was 10 pmol/h/mg. Fibroblasts from patients with Zellweger syndrome, however, produced no radiolabeled DHA (Fig. 4).

**Table 3** Radiolabeled fatty acids produced by human and mouse skin fibroblasts after a 24-h incubation with \([3-{\textsuperscript{14}}C]24:6n-3\)

<table>
<thead>
<tr>
<th>Fibroblast type</th>
<th>C24:6n-3</th>
<th>C22:6n-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>3,609</td>
<td>15,227</td>
</tr>
<tr>
<td></td>
<td>2,945</td>
<td>13,832</td>
</tr>
<tr>
<td>Zellweger</td>
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<td>0</td>
</tr>
<tr>
<td></td>
<td>10,023</td>
<td>0</td>
</tr>
<tr>
<td>CACT</td>
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</tr>
<tr>
<td>SCOX</td>
<td>11,091</td>
<td>856</td>
</tr>
<tr>
<td></td>
<td>11,068</td>
<td>2,129</td>
</tr>
<tr>
<td>DBP</td>
<td>8,356</td>
<td>839</td>
</tr>
</tbody>
</table>

The methyl esters of the radiolabeled fatty acids contained in the cells were separated by HPLC. All incubations were performed in triplicate.

Role of the peroxisomal fatty acid \( \beta \)-oxidation enzymes in DHA biosynthesis

**Linolenic acid (C18:3n-3) utilization**

The results obtained in fibroblasts from patients lacking functional peroxisomes and patients with a mitochondrial fatty acid oxidation defect confirmed that the peroxisome, and not the mitochondrion, is the site for retroconversion of C24:6n-3 in the pathway for C22:6n-3 synthesis. Since the peroxisomal \( \beta \)-oxidation system consists of two separate sets of enzymes, the question was which enzymes would be responsible for the \( \beta \)-oxidation process in the pathway of DHA synthesis. To investigate this, we studied DHA synthesis from radiolabeled precursors in fibroblasts from patients with a deficiency of one of the \( \beta \)-oxidation enzymes or from knockout mice lacking one of the enzymes. After 96-h incubation of human skin fibroblasts deficient for SCOX activity with \([1-{\textsuperscript{14}}C]18:3n-3\), 1% of the radiolabeled fatty acids contained in the cells consisted of C22:6n-3 compared to 15.5% in control fibroblasts (see Table 1 and Fig. 5). In fibroblasts from patients with a deficiency of DBP 2.6% of the radiolabeled fatty acids was C22:6n-3 (Table 1 and Fig. 5). Both SCOX- and DBP-deficient fibroblasts accumulated relatively large amounts of C24:6n-3 (about 6- and 3-times more than observed in control fibroblasts, respectively). In contrast, DHA synthesis from \([1-{\textsuperscript{14}}C]18:3n-3\) was normal in fibroblasts from patients with RCDP type 1, characterized by the absence 3-ketoacyl-CoA thiolase in their peroxisomes, and from an XALD patient. The results obtained after incubation of skin fibroblasts from a control mouse with \([1-{\textsuperscript{14}}C]18:3n-3\), were comparable with the results in control human
skin fibroblasts. In fibroblasts from both the LBP and SCPx knockout mouse normal synthesis of C22:6n-3 was observed (Table 1).

![Graph A](image)

![Graph B](image)

![Graph C](image)

![Graph D](image)

**Fig. 4** Radiolabeled fatty acids contained in the cell lipids of fibroblast cultures incubated with 0.2 μM [3-14C]24:6n-3 for 24 h. After the cell lipids were extracted and methylated, the radiolabeled material was analyzed by high performance liquid chromatography (HPLC) with an online flow liquid scintillation detector. The HPLC traces shown are from a control human skin fibroblast cell line (A), a patient with Zellweger syndrome (B), a DBP-deficient patient (C), and a CACT-deficient patient (D).

**Eicosapentaenoic acid (C20:5n-3) utilization**

Similar results were obtained after incubation with [1,14C]20:5n-3 (see Table 2). In fibroblasts of patients with SCOX and DBP deficiency about 10% and 18%, respectively, of the amount of radiolabeled DHA synthesized by control fibroblasts was produced. In fibroblasts from patients with RCDP type 1 and XALD, however, no deficiency in DHA synthesis was found. Compared to the C22:6n-3 production from [1-14C]20:5n-3 in control mouse skin fibroblasts, the production in fibroblasts from both the LBP and SCPx knockout mouse was also normal.

**Tetracosahexaenoic acid (C24:6n-3) utilization**

Incubations with [3-14C]24:6n-3 were performed to study directly the retroconversion of this substrate to C22:6n-3 in peroxisomal β-oxidation mutants. Both fibroblasts of patients with an SCOX deficiency and of a patient with a deficiency of DBP formed very little radiolabeled DHA, only 10% and 6% respectively of the amount produced in
Peroxisomal β-oxidation enzymes involved in DHA biosynthesis

control fibroblasts (Table 3, Fig. 4). The β-oxidation rate of [3-14C]24:6n-3 in these fibroblasts was 0.7 and 0.4 pmol/h/mg, respectively, compared to 10 pmol/h/mg in control fibroblasts.

![Graphs A, B, C, D showing radioactivity over time for different conditions.](image)

**Fig. 5** Radiolabeled fatty acids contained in the cell lipids of fibroblast cultures incubated with 2 μM [1-14C]18:3n-3 for 96 h. After the cell lipids were extracted and methylated, the radiolabeled material was analyzed by high performance liquid chromatography (HPLC) with an online flow liquid scintillation detector. The HPLC traces shown are from a control human skin fibroblast cell line (A), an SCOX-deficient patient (B), a DBP-deficient patient (C) and an RCDP type 1 patient (D).

**Discussion**

Although many experimental data have been presented in the last few years which indicate that a peroxisomal β-oxidation step is part of the biosynthetic pathway of DHA, the role of the peroxisome in this pathway has remained subject of discussion. Our studies with control fibroblasts and fibroblasts from patients with Zellweger syndrome show that synthesis of C22:6n-3 in human cells involve peroxisomal retroconversion of C24:6n-3. When fibroblasts from Zellweger patients were incubated with [1-14C]18:3n-3 or [1-14C]20:5n-3 radiolabeled C24:6n-3 was formed, while no radiolabeled C22:6n-3 could be detected. This shows that C24:6n-3 is not an elongation-product from C22:6n-3, but that C24:6n-3 is an intermediate in DHA synthesis. In fibroblasts from patients with a deficiency of one of the mitochondrial enzymes CPT I, CACT, CPT II or VLCAD, the synthesis of radiolabeled C22:6n-3 from either [1-14C]18:3n-3 or [1-14C]20:5n-3 was normal. These results support the pathway proposed by Voss et al. (2), who suggested that instead of a direct conversion of C22:5n-3 to C22:6n-3 by a microsomal A4-desaturase, C22:5n-3 is first elongated to C24:5n-3, which is then desaturated by a Δ6-desaturase to
C24:6n-3, followed by retroconversion of C24:6n-3 to C22:6n-3. This is also in agreement with recent findings in a patient with a Δ6-desaturase deficiency, a newly identified disorder (30). Fibroblasts of this patient hardly formed any C22:6n-3 from C24:5n-3, while the conversion of C24:6n-3 to C22:6n-3 was normal. In this paper, we directly evaluated this last step of DHA synthesis and found that fibroblasts from peroxisome-deficient patients did not convert [3-^{14}C]24:6n-3 to radiolabeled C22:6n-3, while this conversion was normal in fibroblasts from patients with a mitochondrial fatty acid oxidation defect. This confirms the results obtained by Moore et al. (5) that DHA biosynthesis in human fibroblasts is a peroxisome-dependent process.

DHA plays an important role in the structure of cell membranes, particularly of neuronal tissues and retinal photoreceptor cells, which suggests that the DHA deficiency observed in Zellweger patients could very well be involved in the clinical symptomatology of this syndrome (demyelination, psychomotor retardation and retinopathy). It has been claimed that supplementation of DHA might result in, at least, some clinical improvement in Zellweger patients (31,32). Since peroxisomal β-oxidation is an essential step in the biosynthesis of DHA, studies in patients with a deficiency of a single β-oxidation enzyme could shed more light on the role of DHA in the pathology of peroxisomal fatty acid oxidation disorders. Because of these possible clinical implications, it is important to know which of the β-oxidation enzymes are responsible for the oxidation of C24:6n-3.

Fig. 6 Schematic representation of the fatty acid β-oxidation machinery in human peroxisomes involved in the retroconversion of C24:6n-3 to C22:6n-3. Our results showed that C24:6n-3 is β-oxidized by the same set of enzymes involved in the β-oxidation of the very long-chain fatty acids C26:0 and C24:0 (see Fig. 2). Oxidation of C24:6n-3 involves straight-chain acyl-CoA oxidase (SCOX), D-bifunctional protein (DBP) and both 3-ketoacyl-CoA thiolase and sterol carrier protein X (SCPX). Branched-chain acyl-CoA oxidase and L-bifunctional protein, however, are also both able to handle this substrate, but cannot maintain normal C22:6n-3 production without SCOX and DBP activity, respectively.
Peroxisomal β-oxidation enzymes involved in DHA biosynthesis

The results obtained in this study show that C24:6n-3 is β-oxidized by the same set of enzymes as used for the β-oxidation of the very long-chain fatty acids C26:0 and C24:0 (see Fig. 2 and Fig. 6). Current evidence holds that oxidation of C24:0 and C26:0 involves SCOX, DBP and both 3-ketoacyl-CoA thiolase and SCPx (15). Upon incubation of fibroblasts of patients with a deficiency of SCOX with [3-14C]24:6n-3, a strongly reduced rate of C24:6n-3 β-oxidation was found. However, some residual activity was present. It is not likely that the defective SCOX protein is responsible for this residual activity, since no SCOX protein can be detected when performing immunoblot analysis in any of the SCOX-deficient cell lines used in this study and it has been established that one of these patients has a large deletion of the SCOX gene (26). This suggests that the other peroxisomal oxidase, BCOX, can also handle this substrate, but that its activity is not sufficient for normal C22:6n-3 production. It is also unlikely that the mitochondrial β-oxidation system is responsible for the residual activity, because in fibroblasts of Zellweger patients, in which both SCOX and BCOX are lacking, there is no C24:6n-3 β-oxidation activity, while in patients with a deficiency of mitochondrial fatty acid β-oxidation normal activity was measured. In case of the second and third step of the β-oxidation process of C24:6n-3, our studies showed that, as for C26:0, DBP is responsible for these steps. As in SCOX-deficient patients, some residual activity was present in DBP-deficient patients. The fact that no DBP protein can be detected upon immunoblot analysis in virtually all patients studied, argues against a role of DBP in residual C22:6n-3 formation in DBP-deficient cells. The most likely explanation is that the other peroxisomal multifunctional enzyme, LBP, can also act on this substrate. Studies in fibroblasts of an LBP knockout mouse have shown, however, that LBP activity is not essential for DHA production. In the presence of either one of the peroxisomal thiolases normal amounts of DHA were produced from labeled precursors, which shows that both thiolases are able to perform the last step of C24:6n-3 β-oxidation and can maintain normal C22:6n-3 production by themselves, in the absence of the other thiolase. This also has been observed for C26:0 β-oxidation, which is normal both in patients with RCDP type 1 and in SCPx knockout mice, in contrast to pristanic acid β-oxidation which is deficient in mice lacking SCPx function (33). It should be noted, however, that for the interpretation of our results we assume that the pathway of DHA biosynthesis is similar in man and mice.

Our results obtained with fibroblasts from an XALD patient indicate that β-oxidation of C24:6n-3 does not follow the exact same route as C26:0 and C24:0. The peroxisomal membrane protein ALDP, which is mutated in patients suffering from XALD resulting in impaired β-oxidation of very long-chain fatty acids, including C26:0 and C24:0, appears not to be involved in retroconversion of C24:6n-3. We found that DHA synthesis is normal in fibroblasts from an XALD patient, which is in agreement with results obtained by Petroni et al. (11).

Because of our results in fibroblasts from SCOX- and DBP-deficient patients, it would be interesting to investigate DHA levels in these patients. Martinez already showed that one patient with a deficiency of DBP (which was called bifunctional enzyme deficiency at that time) had low brain DHA levels, although they were not so severely reduced as in
patients with a peroxisome biogenesis disorder (8). In addition, a preliminary study performed by our group on PUF A composition in plasma samples from 10 DBP-deficient patients, revealed reduced DHA levels in 5 patients (unpublished data). This might reflect the reduced but not fully deficient DHA synthesis we observed in fibroblasts of these patients, in contrast to the complete deficiency in Zellweger patients. The presence of DHA and its precursors in the diet influences the PUF A composition of membrane lipids, and this influence probably becomes even greater in case of reduced DHA synthesis as in DBP patients.

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Summary & Samenvatting
Summary

Peroxisomes are subcellular organelles with an indispensable role in cellular metabolism. One very important function of the peroxisome is the β-oxidation of a range of fatty acids and fatty acid derivatives, which cannot be broken down in the mitochondrion. These include very long-chain fatty acids (VLCFAs), like C26:0, and 2-methyl-branched-chain fatty acids, like pristanic acid, and the bile acid intermediates di- and trihydroxycholestanolic acid (DHCA and THCA, respectively). The importance of the peroxisomal β-oxidation system is stressed by the existence of a variety of different diseases in which peroxisomal β-oxidation is impaired. Over the years extensive research has been performed on the peroxisomal β-oxidation system. One remarkable finding was that peroxisomes contain two distinct sets of β-oxidation enzymes instead of one, which was believed for a long time. The identification of patients with an isolated deficiency of one of the peroxisomal β-oxidation enzymes and the generation of knockout mice for one of the enzymes have been crucial for the elucidation of the physiological role of the different enzymes in the oxidation of VLCFAs, pristanic acid and DHCA/THCA. Despite the extensive current knowledge, however, there are still many questions about the peroxisomal β-oxidation system that remain unanswered and there are still patients with a peroxisomal β-oxidation deficiency of unknown origin, in whom the underlying defect remains to be established.

In this thesis an answer to some of these questions is presented and the underlying defect in a small group of patients has been identified. Chapter three reports the molecular cloning and expression of human carnitine octanoyltransferase (COT). In addition, it is shown that COT plays an important role in the peroxisomal β-oxidation of branched-chain fatty acids by converting one of the end products of peroxisomal β-oxidation of pristanic acid, 4,8-dimethylnonanoyl-CoA, to its corresponding carnitine ester. Dimethylnonanoyl-carnitine is then transported from the peroxisome to the mitochondrion for further β-oxidation. In chapter four we have investigated the possibility of sterol carrier protein X (SCPx) deficiency in a group of patients with a defect in peroxisomal β-oxidation of unknown origin. SCPx was found to be normally active in fibroblasts of these patients, indicating that human SCPx deficiency remains to be identified. In chapter five peroxisomal 3-ketoacyl-CoA thiolase deficiency, which has been reported in only one patient, is reinvestigated. The studies described in this chapter revealed that the true defect in this patient is at the level of D-bifunctional protein (DBP) and not at the level of 3-ketoacyl-CoA thiolase. Chapter six reports the identification of a new peroxisomal fatty acid β-oxidation disorder: α-methylacyl-CoA racemase deficiency. A deficiency of this enzyme was found to be associated with an adult-onset sensory motor neuropathy. In chapter seven we show that in human and rat α-methylacyl-CoA racemase is localized both in the peroxisome and in the mitochondrion. In addition, it is shown by using both in vitro studies with purified heterologously expressed protein and in vivo studies in fibroblasts of patients with an α-methylacyl-CoA racemase deficiency that the mitochondrial enzyme plays a crucial role in the mitochondrial β-oxidation of the breakdown products of pristanic acid. In chapter eight an easy and reliable method for the diagnosis of α-methylacyl-CoA racemase-deficient patients by plasma analysis of DHCA.
and THCA diastereomers is described. In plasma from racemase-deficient patients the exclusive accumulation of the (R)-isomer of free and taurine-conjugated DHCA and THCA was found. Chapter nine describes the analysis of phytanic, pristanic and trimethylundecanoic acid diastereomers in plasma from patients suffering from different peroxisomal disorders. This study stresses again the importance of α-methylacyl-CoA racemase for the β-oxidation of pristanic acid and its metabolites. In chapter ten the argument concerning the subcellular localization of the last step in the biosynthesis of docosahexaenoic acid (DHA) is finally laid to rest. Unequivocal evidence is provided which show that the retroconversion of C24:6n-3 to DHA takes place in the peroxisome. In addition, studies with patient cell lines revealed that the peroxisomal β-oxidation enzymes involved in this retroconversion are the same enzymes that are responsible for the oxidation of the VLCFAs C24:0 and C26:0.
Samenvatting voor iedereen

Een cel bevat een aantal kleinere compartimenten die organellen worden genoemd. Deze organellen worden door een membraan omgeven en zijn daarom afgescheiden van de rest van de cel. Ze hebben ieder een eigen taak en dragen daarmee bij aan het geheel van chemische processen dat in een cel plaats vindt. Eén van die organellen is het peroxisoom. De processen die in het peroxisoom plaats hebben zijn essentieel voor het goed functioneren van de cel en daardoor dus ook voor het functioneren van het hele menselijk lichaam. Patiënten die geen peroxisomen hebben, zijn ernstig ziek en sterven meestal op zeer jonge leeftijd (tijdens het eerste levensjaar). Een zeer belangrijke functie van het peroxisoom is het afbreken van vetzuren. Vetzuren zijn een bestandsdeel van vet wat je binnen krijgt via het voedsel. Deze vetzuren worden afgebroken door middel van een proces dat β-oxidatie wordt genoemd. Tijdens β-oxidatie wordt er steeds een gedeelte van het vetzuur afgehaald en dit gebeurt net zo lang totdat het hele vetzuur is afgebroken. Echter, in het peroxisoom kan het vetzuur alleen maar tot een bepaalde lengte korter worden gemaakt. Daarna moet het verkorte vetzuur naar een ander organel, het mitochondrion, worden getransporteerd, waar het helemaal wordt afgebroken, ook door middel van β-oxidatie. Vetzuren die alleen in het peroxisoom kunnen worden ge-β-oxideerd zijn zeer lange-keten vetzuren, vertakt-keten vetzuren zoals pristaanzuur en intermediaren in de galzuursynthese. Er zijn patiënten die specifiek een defect hebben in het peroxisomale β-oxidatiesysteem, dat wil zeggen dat één van de eiwitten/enzymen die gewoonlijk samenwerken om de vetzuren te verkorten, niet meer goed functioneert. Als gevolg hiervan hopen vetzuren, die alleen in het peroxisoom kunnen worden afgebroken, zich op in hun weefsen en lichaamsvloeistoffen. Het is nog niet bekend hoe dit precies leidt tot het ontwikkelen van klinische symptomen bij de patiënt, maar wel is bekend dat stapeling van vetzuren membraanstructuren verstoren wat kan leiden tot onder andere problemen in het zenuwstelsel. Bij sommige patiënten is inmiddels bekend welk eiwit niet goed functioneert, maar er is nog een aantal patiënten waarvan wel bekend is dat ze een defect hebben in het peroxisomale β-oxidatie maar waarvan nog niet bekend is welk eiwit dit defect veroorzaakt.

In dit proefschrift beschrijf ik een aantal studies die ik de afgelopen vier jaar in samenwerking met anderen als onderdeel van mijn promotieonderzoek heb uitgevoerd en die allemaal te maken hebben met het peroxisomale β-oxidatiesysteem. Om te beginnen geef ik in hoofdstuk twee een overzicht van de huidige kennis over het peroxisomale β-oxidatiesysteem. In hoofdstuk drie wordt de rol onderzocht van het enzym carnitine octanoyltransferase (COT) in de afbraak van de vertakt-keten vetzuren, met name van pristaanzuur. Er is gevonden dat, nadat pristaanzuur in het peroxisoom is verkort door middel van β-oxidatie, COT betrokken is bij het transport van het verkorte vetzuur naar het mitochondrion, waar het verder wordt afgebroken.

In de hoofdstukken vier, vijf en zes wordt een aantal studies beschreven met patiënten die een defect hebben in het peroxisomale β-oxidatiesysteem, waarbij is gezocht naar de oorzaak van de stapeling van vetzuren in deze patiënten. In hoofdstuk vier is onderzocht of in een aantal patiënten de oorzaak wellicht een deficiëntie van het enzym sterol carrier protein X (SCPx) was, maar dit bleek niet het geval te zijn. In hoofdstuk vijf is onderzoek
Samenvatting

gedaan naar een patiënt die bekend stond als de enige patiënt met een deficiëntie van het enzym 3-ketoacyl-CoA thiase. Omdat de kennis over het peroxisomale β-oxidatiesysteem sterk is toegenomen sinds deze patiënt voor het eerst werd beschreven en omdat met de huidige kennis een deficiëntie van het enzym 3-ketoacyl-CoA thiase eigenlijk niet in overeenstemming is met de biochemische afwijkingen die in deze patiënt zijn gevonden, was een nieuw onderzoek nodig. Inderdaad bleek deze patiënt geen deficiëntie van het enzym 3-ketoacyl-CoA thiase maar een deficiëntie van het enzym D-bifunctional protein (DBP) te hebben. In hoofdstuk zes wordt een aantal patiënten met een sensore motor-neuropathie beschreven, die de vertakt-keten vetzuren, phytaanzuur en pristaanzuur, en intermediairen in de galzuursynthese in hun plasma stapelden. Deze patiënten bleken een deficiëntie van het enzym α-methylacyl-CoA racemase te hebben. Van vertakt-keten vetzuren bestaan verschillende ruimtelijke vormen die stereoisomeren worden genoemd, terwijl het β-oxidatiesysteem alleen één bepaalde ruimtelijke vorm van zo’n vertakt-keten vetzuur kan afbreken. Daarom is er het enzym α-methylacyl-CoA racemase dat ervoor zorgt dat de verkeerde stereoisomeer wordt omgezet in de goede zodat het vetzuur kan worden afgebroken. In de patiënten die we bestudeerden, vonden we dat dit α-methylacyl-CoA racemase niet actief was, hetgeen veroorzaakt werd door mutaties in het coderende gen. Ons onderzoek heeft aangetoond dat het belangrijk is om in het geval van een onbegrepen sensore motor-neuropathie op volwassen leeftijd te denken aan een mogelijke erfelijke stofwisselingsziekte. Een goede diagnose is van groot belang omdat deze patiënten geholpen kunnen worden door middel van een phytaanzuur-vrij dieet en mogelijk door plasmaderese. Deze therapie is al succesvol gebleken bij patiënten met de ziekte van Refsum. Eén van de racemase deficiënte patiënten is reeds op een phytaanzuur-vrij dieet gezet en dit heeft geleid tot het tot stilstand komen van de ontwikkeling van de neuropathie.

In hoofdstuk zeven vonden we dat α-methylacyl-CoA racemase niet alleen in het peroxisoom zit maar ook in het mitochondriën en dat het daar verantwoordelijk is voor de omzetting van de afbraakproducten van pristaanzuur in de juiste ruimtelijke vorm, zodat deze verder kunnen worden afgebroken. In hoofdstuk acht staat de ontwikkeling beschreven van een snelle en nauwkeurig methodes voor de diagnose van patiënten met een deficiëntie van α-methylacyl-CoA racemase. Hierbij wordt gekeken naar de stereoisomeren van de galzuur-intermediairen in plasma van de patiënt. Patiënten met een deficiëntie van α-methylacyl-CoA racemase stapelen slechts één stereoisomeer, terwijl patiënten die helemaal geen peroxisomen hebben beide stereoisomeren van de galzuur-intermediairen stapelen. In hoofdstuk negen staat een methode beschreven om in plasma naar de stereoisomeren van de vertakt-keten vetzuren te kunnen kijken. Dit hebben we gedaan in plasma van patiënten met verschillende defecten in het peroxisomale β-oxidatiesysteem.

In hoofdstuk tien wordt een onderzoek beschreven naar de laatste stap in de aanmaak van een zeer belangrijk meervoudig onverzadigd vetzuur, docosahexaenoic acid (DHA). DHA komt vooral voor in membranen in de hersenen en het netvlies, en hoewel er veel aanwijzingen waren dat de laatste stap in de aanmaak van DHA in het peroxisoom plaats vond, is lange tijd onduidelijk geweest of dit echt zo was. In dit hoofdstuk wordt onomstotelijk vastgesteld dat dit inderdaad het geval is en daarbij wordt aangetoond welke enzymen van het β-oxidatiesysteem hierbij betrokken zijn.
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Sacha
List of publications


Phytanoyl-CoA hydroxylase from rat liver. Protein purification and cDNA cloning with implications for the subcellular localization of phytanic acid α-oxidation. (1999) J. Lipid Res. 40(12), 2244-2254.


New Insights in Peroxidase Oxidation