New insights in peroxisomal beta-oxidation
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Chapter 2

Peroxisomal β-oxidation: a review
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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
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.

<table>
<thead>
<tr>
<th>Straight-chain acyl-CoA oxidase</th>
<th>Branched-chain acyl-CoA oxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Bifunctional protein</td>
<td>D-Bifunctional protein</td>
</tr>
<tr>
<td>3-Ketoacyl-CoA thiolase</td>
<td>Sterol carrier protein X</td>
</tr>
</tbody>
</table>

Fig. 1 Enzymology of the fatty acid β-oxidation machinery in human peroxisomes.

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 trihydroxycholestanoic 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)).

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undergoes three cycles of β-oxidation in the peroxisome (6). This will be discussed in more detail later.
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 \(\beta\)-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 Cueba 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)-3\(\alpha\),7\(\alpha\),12\(\alpha\),24-tetrahydroxy-5\(\beta\)-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 \(\Delta^3\), \(\Delta^2\)-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 \(\beta\)-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 β-oxidation system (see for review (37)).

**Physiological role of the β-oxidation enzymes in the oxidation of straight-chain and 2-methyl-branched-chain fatty acids**

Together with the *in vitro* experiments described above, studies performed in patients and knockout mice with an impaired peroxisomal β-oxidation have been indispensable for the elucidation of the physiological role of the β-oxidation enzymes in the oxidation of VLCFAs, pristanic acid and DHCA/THCA. Several human disorders have been identified with an isolated deficiency of peroxisomal β-oxidation, including SCOX deficiency, DBP deficiency and α-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, β-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**.

<table>
<thead>
<tr>
<th>Species</th>
<th>Enzyme deficiency</th>
<th>C26:0</th>
<th>Pristanic acid</th>
<th>DHCA/THCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Humans</td>
<td>SCOX</td>
<td>↑</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>DBP</td>
<td>↑</td>
<td>↑</td>
<td>↑*</td>
</tr>
<tr>
<td></td>
<td>α-methylacyl-CoA racemase</td>
<td>N</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Mice</td>
<td>SCOX</td>
<td>↑</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>DBP</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
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<tr>
<td></td>
<td>LBP</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>SCPx</td>
<td>N</td>
<td>↑</td>
<td>↑</td>
</tr>
</tbody>
</table>

*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 β-oxidation is strongly reduced, while β-oxidation of pristanic acid and THCA is
normal (38-40). In Zellweger syndrome, where there is a deficiency of both peroxisomal oxidases, also pristamic 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).

Based on \textit{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 pristamic 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 \(\beta\)-oxidation of both straight-chain and 2-methyl-branched-chain fatty acids (Fig. 2), which has been confirmed by \(\beta\)-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 \textit{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 thiolas, 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 \textit{et al.}, submitted for publication, Chapter 5). In cells from patients with

\begin{center}
\textbf{VLCFA-CoA} \hspace{1cm} \textbf{Pristanoyl-CoA} \hspace{1cm} \textbf{THC-CoA}
\end{center}

\begin{center}
\textbf{Straight-chain acyl-CoA oxidase} \hspace{2cm} \textbf{Branched-chain acyl-CoA oxidase}
\end{center}

\begin{center}
\textbf{L-Bifunctional protein} \hspace{3cm} \textbf{D-Bifunctional protein}
\end{center}

\begin{center}
\textbf{3-Ketoacyl-CoA thiolase} \hspace{3cm} \textbf{Sterol carrier protein X}
\end{center}

\begin{center}
\textbf{VLCFA-CoA n-2} \hspace{3cm} \textbf{Trimethyltridecanoyl-CoA} \hspace{3cm} \textbf{Choloyl-CoA}
\end{center}

\begin{center}
\textbf{Fig. 2} Schematic representation of the fatty acid \(\beta\)-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.
\end{center}
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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, alkyldihydroxyacetone 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. 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).

**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)
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).

![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)

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
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 \( \alpha \)-methylacyl-CoA racemase (71).

**Pristanic acid \( \beta \)-oxidation**

Pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) is derived from phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) via \( \alpha \)-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 \( \alpha \)-oxidation is not a stereospecific process so that after \( \alpha \)-oxidation of phytanic acid both (2R,6R,10R,14)- and (2S,6R,10R,14)-pristanic acid are formed. Because \( \beta \)-oxidation, in contrast to \( \alpha \)-oxidation, is stereospecific, \( \alpha \)-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 \( \beta \)-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 \( \alpha \)-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 \( \beta \)-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 \( \beta \)-oxidation system. After one cycle (2R,6)-dimethylheptanoyl-CoA is formed. Since the mitochondrial \( \beta \)-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 \( \alpha \)-methylacyl-CoA racemase is responsible for the conversion of this substrate, because fibroblasts from \( \alpha \)-methylacyl-CoA racemase-deficient patients were not able to racemize dimethylheptanoyl-CoA. After conversion of (2R,6)-dimethylheptanoyl-CoA to its (2S)-isomer by \( \alpha \)-methylacyl-CoA racemase, it can be broken down to completion (68). From studies described above performed in patients with a deficiency of \( \alpha \)-methylacyl-CoA racemase it can be concluded that \( \alpha \)-methylacyl-CoA racemase is
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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-Co A 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 believe 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


Peroxisomal β-oxidation: a review


Chapter 2


