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ACYL-CoA THIOESTERASES- AUXILIARY ENZYMES IN PEROXISOMAL LIPID METABOLISM

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Dedicated to the future reader

‘En enda droppe gör havet större’

-Anonym

ABSTRACT

Peroxisomes are small organelles essential for life, which carry out a variety of functions mostly related to lipid metabolism, including α- and β-oxidation as well as the first steps of plasmalogen biosynthesis. Peroxisomal β-oxidation metabolizes many different lipids including very long-chain fatty acids, dicarboxylic fatty acids, branched-chain fatty acids, eicosanoids and certain xenobiotic fatty acids.
This PhD project began with the identification of four novel putative peroxisomal acyl-CoA thioesterase genes, named acyl-CoA thioesterase 3-6 (*Acot3-Acot6*). The acyl-CoA thioesterases are a group of enzymes that catalyze the hydrolysis of acyl-CoAs to free fatty acids and coenzyme A. The identification of these novel acyl-CoA thioesterases raised questions as to their function(s) and what the need is for so many peroxisomal acyl-CoA thioesterases. Answering these questions was therefore the aim of this project. Biochemical characterization showed that expression of all four enzymes is upregulated by peroxisome proliferators in a peroxisome proliferator-activated receptor α (PPARα) dependent manner, suggesting that they function in fatty acid metabolism. Further characterization revealed that ACOT3 and ACOT5 are a long- and a medium-chain acyl-CoA thioesterase, respectively, while ACOT4 and ACOT6 were shown to be specific enzymes catalyzing the hydrolysis of succinyl-CoA and phytanoyl-CoA/pristanoyl-CoA, respectively. ACOT3-ACOT6 were also shown to have distinct tissue expression patterns, suggesting that these enzymes carry out specific functions in different tissues. The peroxisomal acyl-CoA thioesterases not only have the potential to terminate oxidation by hydrolysis of the acyl-CoAs, but the free fatty acids produced may also be able to exit peroxisomes, suggesting a function for these enzymes in transport of fatty acids out of peroxisomes. A wide variety of lipids are oxidized in peroxisomes producing a diversity of products that require to exit peroxisomes, which demonstrates the need for multiple acyl-CoA thioesterase activities in peroxisomal lipid metabolism.

LIST OF PUBLICATIONS

I. **Westin, M. A. K.**, Alexson, S. E. H., and Hunt, M. C.
Molecular cloning and characterization of two mouse peroxisome proliferator activated receptor α (PPARα) -regulated peroxisomal acyl-CoA thioesterases
J. Biol. Chem. (2004) 279 (21): 21841-21848

II. **Westin, M. A. K.**, Alexson, S. E. H., and Hunt, M. C.
The identification of a succinyl-CoA thioesterase suggests a novel pathway for succinate production in peroxisomes
J. Biol. Chem. (2005) 280 (46): 38125-38132

III. **Westin, M. A. K.**, Hunt, M. C., and Alexson, S. E. H.
Peroxisomes contain a specific phytanoyl-CoA/pristanoyl-CoA thioesterase acting as a novel auxiliary enzyme in alpha- and beta-oxidation of methyl-branched fatty acids
Submitted

IV. **Westin, M. A. K.**, Hunt, M. C., and Alexson, S. E. H.
Tissue expression studies of enzymes involved in peroxisomal lipid metabolism- new insights into the functions of acyl-CoA thioesterases and carnitine acyltransferases
Submitted

Related work:
Hunt, M. C., Rautanen, A., **Westin, M. A. K.**, Svensson, L. T., and Alexson, S. E. H.
Analysis of the mouse and human acyl-CoA thioesterase (ACOT) gene clusters shows that convergent, functional evolution results in a reduced number of human peroxisomal ACOTs
FASEB J. (2006) 20: 1855-1864

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LIST OF ABBREVIATIONS

Acnat	Acyl-CoA:amino acid *N*-acyltransferase
Acot	Acyl-CoA thioesterase
Acox	Acyl-CoA oxidase
ACS	Acyl-CoA synthetase
AGT	Alanine:glyoxylate aminotransferase
Amacr	2-methylacyl-CoA racemase
AMN	adrenomyeloneuropathy
Bacat	Bile acid-CoA:amino acid *N*-acyltransferase
CCALD	Childhood cerebral adrenoleukodystrophy
CoASH	Coenzyme A
Crat	Carnitine acetyltransferase
Crot	Carnitine octanoyltransferase
DHCA	Dihydroxycholestanoic acid
ER	Endoplasmic reticulum
GFP	Green fluorescent protein
Mfp	Multi-functional protein
mPTS	Peroxisomal membrane protein targeting signal
Nudt	Nudix hydrolase
PBD	Peroxisome biogenesis disorder
Pex	Peroxin
PMP	Peroxisomal membrane protein
PPAR	Peroxisome proliferator-activated receptor
PPRE	Peroxisome proliferator response element
PTL	Peroxisomal thiolase
PTS	Peroxisomal targeting signal
PUFA	Polyunsaturated fatty acid
RCDP	Rhizomelic chondrodysplasia punctata
RXR	Retinoid x receptor
THCA	Trihydroxycholestanoic acid
VLCFA	Very long chain fatty acid
X-ALD	X-linked adrenoleukodystrophy

INTRODUCTION

Peroxisomes are present in virtually all eukaryotic cells and were first described in 1954 in a thesis from Karolinska Institutet, but since there was no known function for this novel organelle at that time, it was simply called microbody (1). The name peroxisome was coined in 1966 when it was discovered that this organelle harbours hydrogen peroxide producing oxidases and catalase that degrades hydrogen peroxide into molecular oxygen and water (2). Ten years later in 1976 it was shown that peroxisomes are capable of fatty acid oxidation (3), and subsequently that they harbor the complete set of enzymes for β-oxidation (4). Today, over half a century later, peroxisomes have been shown to exhibit an array of functions that varies not only between species but also between tissues and cell types, showing the great diversity exhibited by this organelle (5). Lipid metabolism is considered to be one of its most essential functions, which includes oxidation of a variety of lipids that are poorly oxidized by mitochondrial β-oxidation, as well as biosynthesis of plasmalogens (6). The importance of peroxisomes has also been clearly demonstrated by its involvement in a number of severe genetic diseases (for review see (7)). There are, however, still many questions to be answered with respect to functions of novel enzymes, membrane permeability and metabolite transport. The research presented in this thesis has been focused on the characterization of four novel peroxisomal enzymes that belong to a family of acyl-CoA thioesterases (8). Acyl-CoA thioesterases are a group of enzymes that catalyze the hydrolysis of acyl-CoAs to the corresponding free fatty acids and coenzyme A, which are localized in various compartments of the cell (9). The identification of four novel peroxisomal acyl-CoA thioesterases in the beginning of this project raised the question of their function(s) in peroxisomal lipid metabolism. This thesis will provide an introduction to peroxisomes and acyl-CoA thioesterases, present the research undertaken on these four enzymes in particular, and finally discuss their roles in lipid metabolism. This research has also been part of a project launched by the European Commission, called “Peroxisomes in Health and Disease”, with research not only focused on the identification and characterization of novel peroxisomal proteins, but also research on peroxisome biogenesis and the role of peroxisomes as modulators in diseases of complex inheritance such as atherosclerosis, cancer and Alzheimer’s disease, further demonstrating the importance of this organelle.

Evolution of peroxisomes and the peroxisomal proteome

Peroxisomes are small single membrane bound organelles that vary in size from 0.1-1μm and in abundance from less than one hundred to more than one thousand per cell (10, 11). In contrast to their morphological simplicity they harbour a variety of functions that vary between species, tissues and even between different cell type. Most pathways catalyzed in peroxisomes are specific to peroxisomes, however some reactions are shared with other compartments such as cytosol and mitochondria. For example, in higher eukaryotes, β-oxidation is shared between mitochondria and peroxisomes whereas in yeast peroxisomes are the sole site of β-oxidation.
Peroxisomes are evolutionarily linked to glyoxysomes and glycosomes and all belong to the microbody family. Glyoxysomes are mainly found in plant seedlings where the glyoxylate cycle, a modified version of the citric acid cycle take place, producing succinate that can then be used for carbohydrate synthesis. Leaf peroxisomes instead take part in the glycolate and glycerate pathways of photorespiration. Plant peroxisomes also have an important function in synthesis of hormones and other signalling molecules, such as indole acid, jasmonate, nitric oxide and some reactive oxygen species. Glycosomes are found in trypanosomes where they contain the enzymes for glycolysis. Mammalian peroxisomes instead have a central role in lipid metabolism, which will be described in detail below.

Peroxisome biogenesis and import of peroxisomal proteins

There has been much controversy regarding the origin of peroxisomes, which were first suggested to originate from the endoplasmic reticulum (ER), but later to be an autonomous organelles (for review see (12)). The latter was proposed since most peroxisomal proteins are synthesized on free polyribosomes in the cytosol and imported to peroxisomes posttranslationally, together with the fact that peroxisomes had their own import machinery and were shown to be able to divide (10). Although this is still true, a number of papers were recently published showing that the ER is involved in *de novo* synthesis of peroxisomes, where its primary function is to donate lipid material for the peroxisomal membrane (13-15).
Peroxisomal proteins required for peroxisome biogenesis are called peroxins, which are encoded by so called PEX-genes. To date 32 different peroxins have been identified with functions in membrane biogenesis, import of matrix proteins and peroxisome proliferation (16, 17). Peroxisomal membrane protein (PMP) targeting signals (mPTS)

have been identified for several PMPs, which consists of a basic amino acid sequence in conjunction with at least one transmembrane region. Some PMPs even have multiple targeting signals, which is suggested to be of importance in distinguishing targeting to different peroxisome populations. Only 3 peroxins have been shown to have a function in import of newly synthesized PMPs, namely Pex3p, Pex16p and Pex19p. Pex19p is predominantly a cytosolic protein and is known to bind multiple PMPs and might therefore function as an import receptor recognizing the mPTS containing PMPs in the cytosol and directing them to the peroxisomal membrane. Pex3p is instead located in the peroxisomal membrane and functions as a docking protein for Pex19p. Pex16p binds to Pex3p in the membrane and has also an essential function in import of newly synthesized PMPs, which was demonstrated by the lack of detectable peroxisomal membrane structures in pex16p deficient cells, but how this PMP participates is still unknown. How PMPs are inserted into the peroxisomal membrane also remains an unresolved issue. Since peroxisomes are dynamic organelles that might vary in size and abundance in different tissues, there are also peroxins that control size and abundance of peroxisomes. These include Pex11p, which promotes division of peroxisomes, and Pex16p, which is involved in inhibition of division by complex-formation with acyl-CoA oxidase (18). Peroxisomal size and abundance is also under metabolic control (11), where peroxisomal β-oxidation mutants show a reduced number of enlarged peroxisomes. Also Pex25p and Pex27p are thought to have a function in peroxisomal size and abundance, where gene knockouts in *S. cerevisae* led to enlarged peroxisomes and overexpression lead to many small peroxisomes.

Import of peroxisomal matrix proteins

Peroxisomes are unusual in that they can import folded proteins and oligomeric protein complexes (19). This import is mediated via an evolutionary conserved import pathways, which utilize different targeting signals and receptor proteins but largely the same import machinery. The most common targeting signal, which is responsible for import of over 90% of the peroxisomal matrix proteins is called the peroxisomal type 1 targeting signal (PTS1). This signal constitutes a carboxyterminal tripeptide with a consensus sequence of serine, lysine, leucine (-SKL) and its variants (S/A/C)(K/H/R)(L/M) (20). However it has lately been shown that other variants of this conserved tripeptide can target proteins to peroxisomes, for example if the preceding amino acid to a non-consensus targeting signal is a basic residue, it may still act as a

functional targeting signal (21-23). The second targeting signal is the so called peroxisomal type 2 targeting signal (PTS2), which is instead located close to the aminoterminus of the protein and is a nonapeptide with a general consensus sequence of (R/K)(L/V/I)X_5(H/Q)(L/A) (24-26).

A diversity of peroxins have functions in the peroxisomal matrix protein import machinery, but the individual roles have only been elucidated for some of the peroxins (**Fig. 1**) (for review see (27-29)). The receptor for proteins containing a PTS1 targeting signal is Pex5p, which is composed of two main domains, a PTS1 binding site and a site with the peroxisomal targeting information. There are two variants of Pex5p, a long and a short variant, however both variants are fully functional PTS1 receptors. The receptor responsible for PTS2 import is instead Pex7p, but targeting of PTS2 proteins also requires the long variant of Pex5p (30). Pex13p and Pex14p have a function in docking of the Pex5p/PTS1 protein (31, 32). Pex8p, Pex10p, and Pex12p also bind this complex, but instead seem to be required for translocation of the cargo (33, 34). For receptor recycling the ubiquitin conjugating enzymes Pex4p and Pex22p are probably required (35). Pex1p and Pex6p are two AAA ATPases that function as a dimer and are suggested to provide the ATP needed in the import process (36). This however does not describe the mechanism for how oligomeric enzymes can be transported over the peroxisomal membrane, nor in which process ATP is required. To gain insight into the mechanistic aspects of peroxisomal matrix protein import the preimplex hypothesis was put forward (27). This hypothesis suggests that since every Pex5p monomer can bind one PTS1 containing protein and both Pex5p and most peroxisomal proteins are oligomers (37), this results in large complexes of Pex5p and newly synthesized PTS1 proteins, which are called pre-import complexes or preimplexes. In this model, Pex14p is suggested to be the primary docking site, which binds Pex5p with high affinity, resulting in localization of the preimplexes to the peroxisomal surface. These large preimplexes do not exist later inside the peroxisome so therefore they must dissociate either before or during translocation, and this process is shown to be ATP dependent. There are several models for how Pex5p is recycled, for example in the extended shuttle model Pex5p is suggested to be translocated into the peroxisomal matrix and then recycled back out into the cytoplasm. Instead in the trap-door model Pex5p is suggested only to face the lumen during translocation, but never to be released into the matrix.

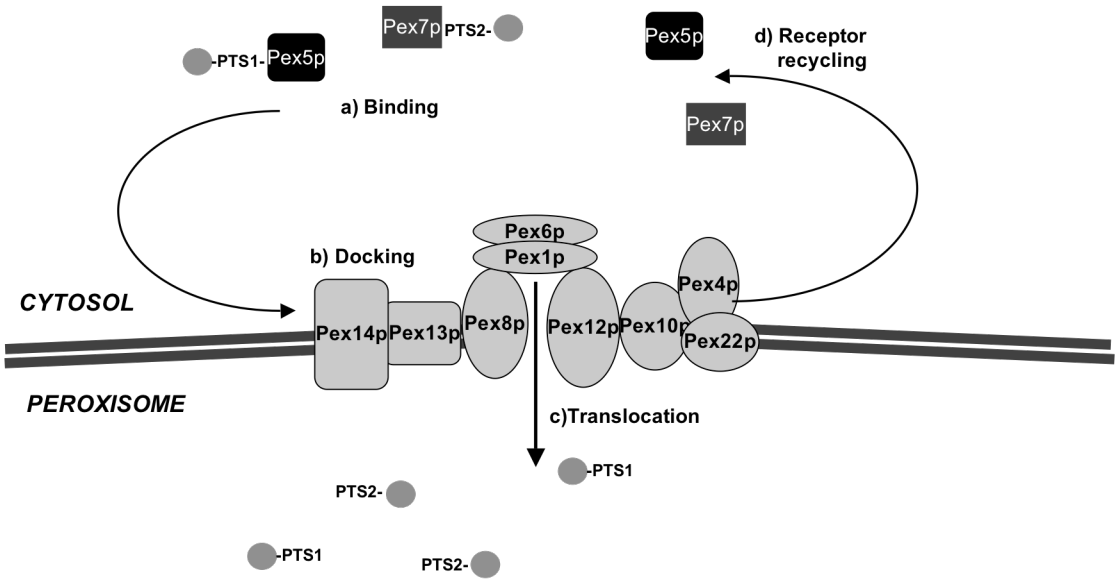


Fig. 1. **Import of peroxisomal matrix proteins.** Proteins destined for the peroxisomal matrix contains one of the two peroxisomal targeting signals, PTS1 or PTS2. The import of matrix proteins are mediated via a number of peroxisome biogenesis proteins, also called peroxins (pex). Proteins containing PTS1 and PTS2 signals bind their receptors called pex5p and pex7p, respectively, which are then targeted to a docking complex on the peroxisomal membrane. This results in an ATP- dependent translocation of the peroxisomal matrix proteins and the receptors can then be recycled back to the cytosol for another round of import.

Peroxisomal membrane permeability and cofactor regeneration

There have been many conflicting ideas on the permeability of the peroxisomal membrane over the years. These conflicting data has mostly arisen from the fact that peroxisomes isolated *in vitro* by subcellular isolation techniques are fragile and tend to leak, suggesting that the peroxisomal membrane is permeable to most metabolites (38). However more recent data suggest that peroxisomes are freely permeable only to small metabolites (<300 Da) (39), while they are not permeable to larger metabolites, such as cofactors including NAD(H), NADP(H) and CoASH (40, 41), suggesting that peroxisomes have their own pool of cofactors. To explain this unusual behaviour the two-channel concept was put forward, suggesting the existence of pore forming channels for transport of small metabolites together with transporters for bulky solutes such as cofactors and acyl-CoAs (39, 42). One example of such a transporter is the ATP antiporter, which seems to be able to exchange ATP for ADP as well as AMP (43), which differs from the substrate specificity of the mitochondrial exchangers. This two-channel concept enables peroxisomes to have both their own set of cofactors as

well as being able to transport the many substrates and products of peroxisomal metabolism. However even though this concept has many supporters, there is also much conflicting data, such as recent identification of a number of specific transporters including two monocarboxylate transporters (44), phosphate transporter (45), a carnitine transporter (46) as well as a bile acid transporter (47).

It has been suggested that a pH gradient exists over the peroxisomal membrane both in humans and yeast. There is, however, no agreement of the orientation of the gradient since some groups suggests lower pH in peroxisomes than the cytosol while others have found the opposite (48, 49). Also, it is not known whether such a proton gradient would be due to an active proton translocating protein or a consequence of a high intraperoxisomal metabolic activity, or both. If it is the result of metabolic activity, different metabolic status could explain the conflicting results obtained.

Since the peroxisomal membrane is impermeable to large cofactors like NAD(H) and NADP(H), and the peroxisomal β-oxidation system leads to reduction of NAD+ and oxidation of NADPH, peroxisomes require shuttle mechanisms or transport systems for regeneration of cofactors. In peroxisomes a number of shuttle systems have been suggested to function in the reconversion of peroxisomal cofactors without transporting them over the membrane, similar to systems also found in the mitochondria. In mammals, oxidation of NADH proceeds via the lactate/pyruvate and glycerophosphate shuttles, which have been shown to be partially located in peroxisomes (50, 51). Transfer of electrons from NADH forms lactate and glycerol-3 phosphate, which can then easily pass the peroxisomal membrane for reconversion in the cytosol and transport of NADH to mitochondria and the electron transport chain for ATP production. NADPH instead uses glucose-6 phosphate dehydrogenase or isocitrate dehydrogenase, which are both partially localized in peroxisomes (52, 53).

Fatty acid activation

Fatty acids need to be activated into their corresponding CoA esters to undergo oxidation, including α- and β-oxidation, or esterification into complex lipids, such as triglycerides, phospholipids and cholesterol esters. This is carried out by a group of enzymes called acyl-CoA synthetases (ACS) in an ATP dependent two-step reaction (54). These ACS's were first identified over half a decade ago (55) and since then many ACS activities have been described in different species including bacteria, protozoa, yeast, higher plants and animals. ACS's are classified according to their substrate

specificity, such as short- (56, 57), medium- (58) long- (55) and very long-chain ACS's (59). These substrate specificities also overlap somewhat with the long-chain ACS also active on phytanic acid (60), and the very long-chain acyl-CoA synthetase active also with long-chain and methyl-branched fatty acids (61-63). It should also be noted that there are several different acyl-CoA synthetases with similar activities, for example in rat, five different long-chain acyl-CoA synthetases have been identified. The acyl-CoA synthetases also show different subcellular localizations, where the ER harbours a short-, long- and very long-chain ACS. The mitochondrial membrane harbours a short-, medium- and a long-chain ACS, while the peroxisomal membrane contains a long- and a very long-chain ACS.

In addition to the different subcellular localizations, these enzymes also show different topography, with the peroxisomal long-chain ACS facing the cytosol and the very long-chain ACS facing the peroxisomal matrix (64, 65). In addition, both the subcellular localization and tissue distribution differs between enzymes (66, 67). The short-chain ACS's are present in two forms where one is an acetate specific enzyme, while the other is a general short-chain acyl-CoA synthetase (56, 57). The acetyl-CoA synthetase is basically only expressed in liver, kidney and heart, whereas the short-chain ACS is present in most tissues, but is very low in adipose tissue and muscle. The medium-chain ACS's are expressed in all tissues. Expression of long-chain ACS is highest in liver, adipose tissue and heart, and the very long-chain ACS is most highly expressed in heart, adrenal, testis, brain and muscle. Since these enzymes show both different subcellular localization and tissue distribution, it has been suggested that these enzymes, especially the long-chain ACS's, may play a role in channelling of fatty acids towards different routes, such as oxidation or synthesis of complex lipids. The activity of the ACS's is first dependent on substrate availability, including CoASH, fatty acids and ATP. Since levels of CoASH are tightly regulated this is an important regulator of ACS activity. CoASH is synthesized in the cytosol from panthotenic acid, ATP and cysteine. In the synthesis panthoteine kinase is the rate-limiting enzyme, which is regulated by cytosolic levels of CoASH and acetyl-CoA. However nutritional state and hormonal status also affects ACS activity.

Import of acyl-CoAs into peroxisomes

Short- and medium-chain fatty acids can enter the mitochondria directly, whereas long-chain fatty acids are imported in the form of carnitine esters formed by carnitine

palmitoyl transferase 1 (CPT1). The acylcarnitine is imported via a translocase, and then the acyl-CoA is regenerated by carnitine palmitoyltransferase 2 (CPT2) in the mitochondrial matrix. In contrast import of fatty acids into peroxisomes is not carnitine dependent, but is instead believed to occur in the form of acyl-CoAs. For this purpose mammalian peroxisomes contain four different ABC half transporters, including the adrenoleukodystrophy protein (ALDP) encoded by the ABCD1 gene, the adrenoleukodystrophy related protein (ALDRP) encoded by ABCD2, the 70 kDa peroxisomal membrane protein (PMP70) encoded by ABCD3 and the PMP70 related protein (PMP70R) (68) encoded by ABCD4 (for review see (69)). These ABC half transporters belong to a large family of structurally related proteins that couple ATP hydrolysis to substrate transport. The functional ABC transporter is composed of two similar hydrophobic halves, each with six transmembrane α-helices and one conserved hydrophilic nucleotide-binding domain. To form a functional transporter the half transporters need to homo- or heterodimerize (70-72). The four peroxisomal half transporters show individual, but partially overlapping, tissue expression, which might suggest that they form different dimeric combinations in specific tissues to transport different substrates. One example of heterodimerization of peroxisomal ABC transporters is the yeast pxa1 and pxa2 that imports oleoyl-CoA into peroxisomes (70). The role of the mammalian peroxisomal ABC transporters is however not yet fully understood, but it is well known that ALDP has a function in transport of very long-chain fatty acids, since fibroblasts from X-ALD patients deficient in ALDP are defective in import of very long-chain fatty acids (73). It has also been shown that VLCFAs are poorly activated in these patients, which suggests a physical interaction between the very long-chain acyl-CoA synthetase and ALDP. ALDPR has been suggested to have overlapping substrate specificity with ALDP since overexpression of this enzyme in X-ALD fibroblasts restores the import of very long-chain fatty acids. Overexpression of PMP70 leads to an increase in palmitic acid β-oxidation (74), while the PMP70 knockout mouse model has been shown to have a decreased oxidation of very long-chain fatty acids, suggesting a function in transport of long- and very long-chain acyl-CoAs. So far there is no data regarding the function of PMP70R.

Functions of mammalian peroxisomes

In mammals, the peroxisomal proteome is comprised of more than sixty different enzymes, involved in a variety of pathways, which were recently compiled in a

database located at http://www.peroxisomeDB.org (75). These pathways include for example lipid metabolism and defence against reactive oxygen species, but also a variety of other reactions (5, 6, 76). β-Oxidation of straight-chain fatty acids and hydrogen peroxide based respiration are two of the most central functions for peroxisomes, which occur in all mammalian peroxisomes, independent of tissue and cell type. Other aspects of peroxisomal lipid metabolism include α-oxidation of 3-methyl-branched fatty acids, β-oxidation of a variety of compounds other than straight-chain acyl-CoAs as well as biosynthesis of etherphospholipids and possibly also part of the cholesterol synthesis pathway. Peroxisomes also carry out a number of reactions not involved in lipid metabolism however these functions will only be described briefly.

β-Oxidation

As stated above both mitochondria and peroxisomes catalyze the four cyclic reactions of β-oxidation in mammals (77-79). The four steps of peroxisomal β-oxidation involves 1) oxidation to form a 2-enoyl-CoA, 2) hydration to form a 3-hydroxyacyl-CoA, 3) a second oxidation forming 3-ketoacyl-CoA and 4) a thiolytic cleavage resulting in production of a two carbon chain-shortened fatty acid with concomitant release of acetyl-CoA or propionyl-CoA depending on the substrate oxidized. There are, however, several differences between peroxisomal and mitochondrial β-oxidation, such as substrate specificity, enzymology and energy production. Mitochondria oxidize the bulk of dietary lipids, including stearic (C18:0), oleic (C18:1) and linoleic acid (C18:2) as well as short- and medium-chain mono- and dicarboxylic fatty acids. Instead peroxisomes show a broader substrate spectrum, oxidizing fatty acids or fatty acid derivatives that are not good substrates for the mitochondria (78, 80). This includes very long straight-chain fatty acids, long-chain dicarboxylic fatty acids (81), 2-methyl-branched fatty acids such as pristanic acid (82) and some fatty acid derivatives such as eicosanoids and xenobiotic fatty acids (83, 84). Peroxisomal β-oxidation is also involved in bile acid synthesis by catalyzing one round of β-oxidation of the bile acid precursors di- and trihydroxycholestanoic acid (DHCA and THCA) producing the primary bile acids chenodeoxycholic acid and cholic acid, respectively (85). Another important difference is that peroxisomal β-oxidation is only partial, in which most substrates only undergo 2-3 rounds of β-oxidation (86). The products of peroxisomal β-oxidation are then transported out of the peroxisome either for further β-oxidation in the mitochondria, or for excretion out of the body via urine or bile. A third difference

between peroxisomal and mitochondrial β-oxidation is the enzymes and coenzymes involved; peroxisomes utilize oxidases instead of dehydrogenases and this results in a transfer of electrons from FAD directly to oxygen, generating hydrogen peroxide, which in turn can be broken down to molecular oxygen and water by catalase and results in the energy being dissipated as heat. Therefore peroxisomal β-oxidation only produces less than half of the energy produced by mitochondrial β-oxidation.

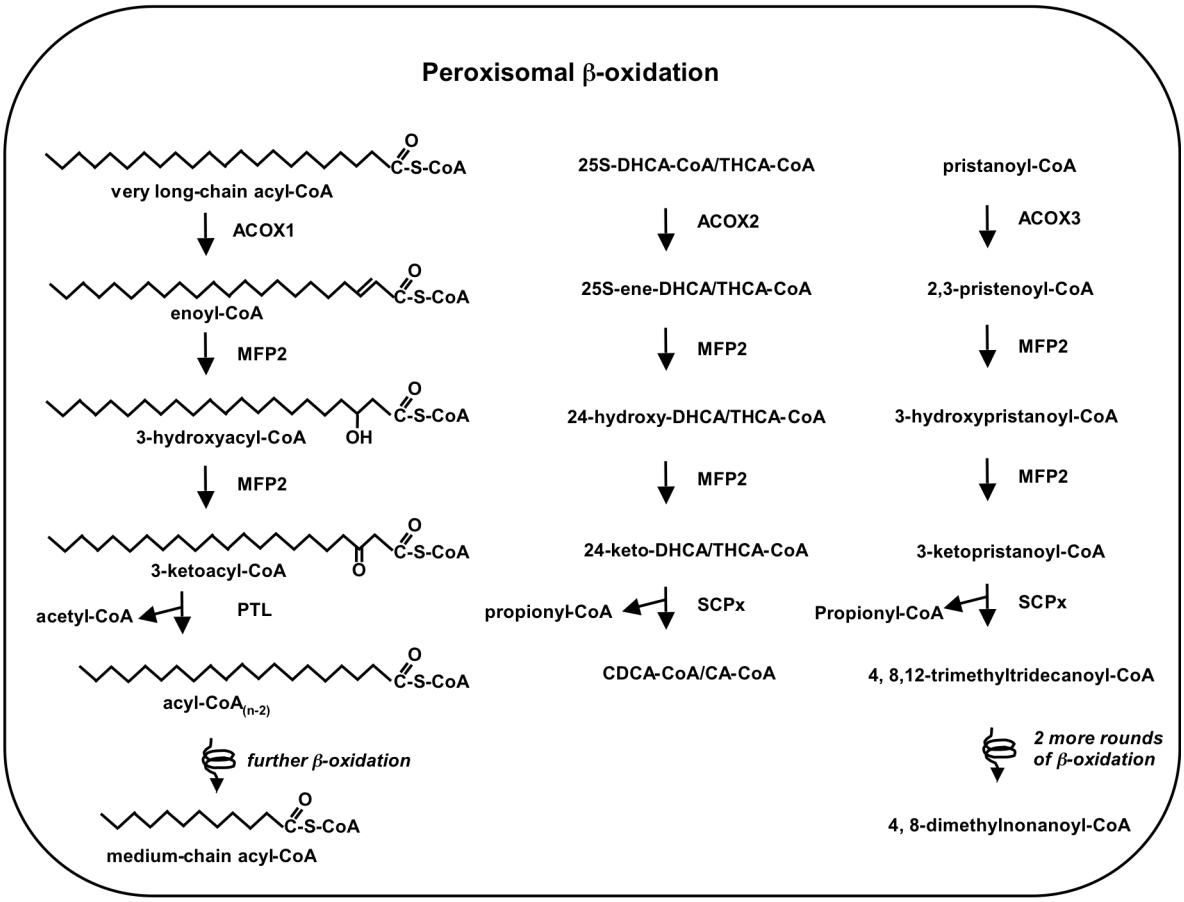


Fig. 2. **Peroxisomal β-oxidation**. Peroxisomes are involved in β-oxidation of a wide variety of lipids and to handle these different substrates peroxisomes contain three different acyl-CoA oxidases (ACOXs), where ACOX1 is active on straight-chain mono and dicarboxylic acyl-CoAs of varying chain-length. ACOX2 is active on the bile acid intermediates and ACOX3 on methyl-branched fatty acids. The second and third step are catalyzed by multifunctional protein 2 (MFP2) for all substrates, and the fourth and final step is catalyzed by the peroxisomal thiolase (PTL) for straight-chain fatty acids, while the sterol carrier protein x (SCPx) catalyzes the fourth step for methyl-branched fatty acids as well as for the bile acid intermediates. Abbreviations: DHCA-CoA; dihydroxycholestanoyl-CoA, THCA-CoA, trihydroxycholestanoyl-CoA, CA-CoA; choloyl-CoA, CDCA-CoA; chenodeoxycholoyl-CoA.

The peroxisomal β-oxidation pathways are outlined in **Fig. 2**, where the first step in peroxisomal β-oxidation is catalyzed by one of the three acyl-CoA oxidases with different substrate specificities. Acyl-CoA oxidase 1 (ACOX1) (87, 88) is active on

straight-chain acyl-CoAs of varying chain-lengths down to short-chain acyl-CoAs, whereas acyl-CoA oxidase 2 (ACOX2) is instead active on the bile acid intermediates, DHCA-CoA and THCA-CoA, and acyl-CoA oxidase 3 (ACOX3) is active on 2-methyl-branched fatty acids such as pristanoyl-CoA (89). In humans ACOX2 catalyzes the activities of both mouse ACOX2 and ACOX3 (90), however in human there is also a homologue of ACOX3, it is not whether it is expressed.

The second and third step of peroxisomal β-oxidation is catalyzed by one of the two multifunctional proteins, multifunctional protein 1 (MFP1) or multifunctional protein 2 (MFP2), where both enzymes catalyze the same reaction only with mirror imaging stereochemistry (L- and D- specificity). Surprisingly MFP1 and MFP2 are not sequence related and even though both MFP1 and MFP2 seem to have broad substrate spectrums, the function for MFP1 is still unclear since MFP1 is only partially redundant in the severely affected MFP2 knockout mouse. Interestingly there is no recognizable phenotype in the MFP1 knockout mouse (91). These data suggest that MFP2 catalyses the second and third steps of β-oxidation for almost all substrates, but since MFP1 is one of the most abundant peroxisomal enzymes it might have another presently unknown specific function. The last reaction of peroxisomal β-oxidation is catalyzed by three ketoacyl-CoA thiolases including thiolase A (PTLA), thiolase B (PTLB), and sterol carrier protein x (SCPx). PTLA and PTLB are active on straight-chain fatty acids of varying chain-lengths with the difference being that PTLA seems to be constitutively expressed, while PTLB is inducible by peroxisome proliferators. SCPx instead catalyzes the thiolytic cleavage of branched substrates such as pristanic acid and bile acid intermediates. The enzymes catalyzing β-oxidation of straight-chain fatty acids, including ACOX1, MFP2 and PTLB are all upregulated by peroxisome proliferators in a PPARα-dependent manner (79).

Fatty acids containing *cis* or *trans* double bonds at odd number positions or *cis* double bonds at even number positions require additional auxiliary enzymes to enabling them undergo β-oxidation, including the $\Delta^{3,2}$-enoyl-CoA isomerase, $\Delta^{3,5}\Delta^{2,4}$-dienoyl-CoA isomerase and 2,4-dienoyl-CoA reductase (80). Also fatty acids and bile acids with 2-methyl groups in the R-configuration cannot undergo β-oxidation immediately, but must first be converted to the S-configuration by the 2-methylacyl-CoA racemase (AMACR) (92). Deficiency of the AMACR enzyme indeed leads to accumulation of 2R-methyl-branched substrates, including 2R-pristanic acid, DHCA and THCA (93). Fatty acids with a methyl branch in the third position are blocked for immediate β-

oxidation and must therefore first be α-oxidized into a 2-methyl-branched fatty acid as described in the next section, which can then undergo β-oxidation.

α-Oxidation

Fatty acids with a methyl branch at the β-carbon (carbon 3) such as the chlorophyll derivative phytanoyl-CoA cannot undergo immediate β-oxidation but instead must first undergo α-oxidation into a 2-methyl-branched fatty acid (94, 95). α-Oxidation consists of three steps and results in a fatty acid shortened by one carbon with the concomitant release of formyl-CoA as presented in **Fig. 3**. In the first step of α-oxidation phytanoyl-CoA is hydroxylated into 2-hydroxyphytanoyl-CoA by the phytanoyl-CoA 2-hydroxylase, and in the second step, formyl-CoA is cleaved off by the 2-hydroxyphytanoyl-CoA lyase, forming pristanal. The formyl-CoA produced in this step is subsequently hydrolyzed into formate and oxidized to CO_2 in the cytosol. In the third and last step of α-oxidation, pristanal is converted into pristanic acid by an aldehyde dehydrogenase. It is still not completely clear where the last step in α-oxidation is carried out and by what enzyme since both a microsomal and a peroxisomal aldehyde dehydrogenase have been suggested (96, 97).

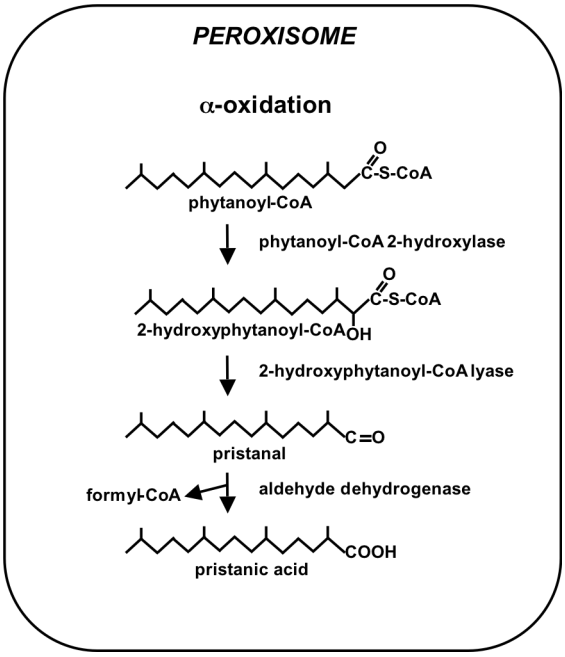


Fig. 3. **Peroxisomal α-oxidation**. 3-methyl-branched fatty acids, e.g. phytanic acid are blocked for immediate β-oxidation and must therefore first undergo α-oxidation. This is a 3-step process, which shortens the fatty acid by one carbon, with the concomitant release of formyl-CoA, creating a fatty acid that can then undergo β-oxidation.

Other peroxisomal pathways

Etherphospholipid synthesis

Etherphospholipids are a group of phospholipids that contain an ether bond at the *sn*-1 position of the glycerol backbone. This ether bond can occur in two forms, either as a regular ether bond giving rise to, for example, platelet activating factor (PAF), or as a vinyl-ether bond giving rise to plasmalogens (98, 99). In plasmalogens the *sn*-1 position is occupied by a long-chain alkyl moiety such as palmitate (C16:0), stearate (C18:0) and oleate (C18:1), whereas the *sn*-2 position is instead occupied by a polyunsaturated fatty acid (PUFA). The head group can either be choline or ethanolamine, giving rise to ethanolamine (PE) plasmalogens and choline (PC) plasmalogens, respectively. The levels of PC and PE plasmalogens differs between tissues, where brain myelin contains the highest amount of PE plasmalogens and heart muscle the highest amount of PC plasmalogens. The fraction of glycerophospholipids in different tissues that are plasmalogens varies widely from about 2% in liver to about 70% in myelin sheaths. The first 2-3 steps of etherphospholipid biosynthesis are located in peroxisomes and the first step involves esterification of dihydroxyacetone phosphate (DHAP) with a long-chain acyl group, by the DHAP acyltransferase (DHAPAT). In the second step the characteristic ether bond is formed by exchange of the long-chain fatty acid in the *sn*-1 position by a long-chain fatty alcohol, which is carried out by alkyl-DHAP synthase (ADHAPS). This fatty alcohol can either be derived from the diet or by reduction of the corresponding acyl-CoA by an acyl-CoA reductase. It has also been suggested that peroxisomes can form these fatty alcohols by chain-elongation of primarily lauroyl-CoA (C12) resulting in formation of hexadecanol (C16) (100). The third step is localized in peroxisomes, and also in the ER, while the remaining steps are exclusively localized in the ER. In the third step the ketone group at the *sn*-2 position is reduced by the acyl/alkyl-DHAP reductase (AADHAPR) forming 1-alkyl-sn-glycero-3 phosphate. The remaining steps localized in the ER involves acylation by the alkyl/acyl-glycero-3-phosphate acyltransferases (AAG3PAT) and removal of the phosphate group by the phosphohydratase (PH). In the formation of PE plasmalogens cytidine-diphosphate-ethanolamine (CDP-ethanolamine) is incorporated by ethanolamine phosphotransferase (E-PT), which is then desaturated by a Δ1-desaturase. Instead PC plasmalogens are primarily formed by polar head group modifications.

Cholesterol synthesis

There has long been an ongoing debate whether peroxisomes are involved in cholesterol biosynthesis (101-104) and the subcellular localization of the enzymes involved in the presqualene segment of cholesterol synthesis was lately reinvestigated which clearly showed that phosphomevalonate kinase, isopentyl-PP isomerase, mevalonate kinase and farnesyl-PP synthase are localized in peroxisomes (105). It should however be noted that in the PEX5 knock-out mouse these enzymes are still equally active (106), but mislocalization of the enzymes does not necessarily have to disrupt the enzymatic activity.

Oxygen metabolism

Since peroxisomes have many oxidases that reduce oxygen into hydrogen peroxide, there is a need for enzymes that metabolizes hydrogen peroxide. These enzymes not only include catalase (CAT), but also glutathione peroxidase and peroxiredoxin V (PMP20). Peroxisomes, however, also produce other reactive oxygen species such as superoxide anions, which can be metabolized by several superoxide dismutases. Other peroxisomal activities towards reactive oxygen species include peroxynitrate reductase activity, epoxide hydrolase activity and glutathione S-transferase activity.

Amino acid metabolism

Peroxisomes contain enzymes for degradation of D-amino acids including D-amino acid oxidase and D-aspartate oxidase (107). This oxidation produces the corresponding keto acid, ammonia and hydrogen peroxide. Peroxisomes are also involved in the oxidation of some L-amino acids such as L-lysine which is degraded via the sacharopine or L-pipecolate pathways, where L-pipecolate oxidase is a peroxisomal enzyme. Lysine, hydoxylysine and tryptophane can all be converted to glutaryl-CoA by a peroxisomal glutaryl-CoA oxidase activity which co purifies with the palmitoyl-CoA oxidase, suggesting one enzyme with dual activities.

Glyoxylate metabolism

Glyoxylate generated in peroxisomes is converted into glycine using alanine as an amino group donor by the peroxisomal liver specific alanine:glyoxylate aminotransferase (AGT), preventing glyoxylate from being converted into the toxic metabolite oxalate. AGT deficiency causes a very severe hyperoxaluria type 1, where

glyoxylate in patients is instead converted to oxalate which precipitates in liver and other organs leading to kidney failure (108).

Export of β-oxidation products out of peroxisomes

Since peroxisomes oxidize a wide variety of substrates and since this oxidation is only partial, a diversity of chain-shortened acyl-CoA derivatives need to be transported out of peroxisomes. However, acyl-CoAs cannot freely diffuse out of peroxisomes and since no transporter for acyl-CoAs has been identified, the acyl-CoAs produced need to be converted into other metabolites to allow exit. For this purpose peroxisomes contain two carnitine acyltransferases (109-111) that catalyze the conversion of acyl-CoAs into carnitine esters, namely carntine acetyltransferase (CRAT) and carnitine octanoyltransferase (CROT) (109, 112, 113), which are active on short- and medium-chain acyl-CoAs, respectively. Carnitine esters are in comparison to CoA esters much smaller solutes, which have been suggested to be able to cross the peroxisomal membrane without any transporter (114). However a mammalian carnitine transporter was lately identified in peroxisomes, suggesting that this transporter might be utilized for export of carnitine esters from the peroxisome (46). This system is likely to work well for the most common β-oxidation products, including acetyl-CoA, propionyl-CoA, medium-chain acyl-CoAs and branched medium-chain acyl-CoAs (113) since the carnitine acyltransferases show activity towards these acyl-CoAs. These carnitine acyltransferases are also upregulated via peroxisome proliferators (115), which is in line with a general upregulation of peroxisomal lipid metabolism by treatment with peroxisome proliferators. However, for other β-oxidation products this pathway cannot be utilized, suggesting that other routes for excretion of acyl-CoAs out of peroxisomes exists (see below).

Peroxisomal disorders

Peroxisomal disorders is a group of genetic diseases with low prevalence, however since they are usually very severe and without any treatment, they represent an enormous burden on the affected individuals and their families (116). Peroxisomal disorders are generally divided into two groups, where one group comprises the peroxisome biogenesis disorders (PBDs) (117), where the affected individuals typically either completely lack peroxisomes or have residual peroxisomal membranes (also called peroxisomal ghosts), with no or almost no peroxisomal functions. If peroxisomal

proteins are mistargeted to the cytosol they are usually rapidly degraded, however, a few peroxisomal enzymes such as catalase and AGT are assembled correctly and are still stable and active in the cytosol. The prototype for the PBDs is the Zellweger syndrome, also called the cerebro-hepato-renal syndrome, a disease that was first described in the 1960s, although its association with peroxisomes was not described until 1973. Zellweger patients lack functional peroxisomes and are characterized by an array of abnormalities, such as liver disease, severe developmental delay, hypotonia and seizures and these patients usually die within the first year of life. The Zellweger syndrome is a multi-genetic disease, which can be caused by mutations in at least twelve different PEX genes (118). Biochemically PDBs are characterized by an accumulation of a wide range of peroxisomal substrates, including VLCFAs, pristanic and phytanic acid, DHCA and THCA and pipecolic acid, leading to a shortage of end products of peroxisomal metabolism such as plasmalogens, cholic and chenodeoxycholic acid (the primary bile acids), as well as docosahexanoic acid. Other PBDs include neonatal adrenoleukodystrophy (NALD) and infantile Refsum disease (IRD), which have many of the characteristics of Zellweger syndrome but are not as severe. Instead rhizomelic chondrodysplasia punctata (RCDP) type 1, which also belongs to the PBDs, and is caused by a mutation in PEX7 abolishing import of PTS2 containing proteins, is considerably different to the other PBDs. These patients have short stature, mostly affecting proximal parts of extremities, typical facial abnormalities, congenital contractures, severe growth deficiency and mental retardation. Biochemically RCDP type 1 is characterized by increased levels of phytanic acid as well as low levels of plasmalogens, since enzymes involved in α-oxidation and plasmalogen biosynthesis are targeted by a PTS2 targeting signal.
The second group of peroxisomal disorders is the single enzyme deficiencies (119), where only one enzyme in a peroxisomal pathway is affected. This group of peroxisomal disorders includes Refsum's disease (120), AMACR deficiency (121), MFP2 deficiency, X-ALD (73), RCDP type 2 and type 3, hyperoxaluria type 1 and acatalasemia. Refsum's disease is caused by a deficiency in phytanoyl-CoA hydroxylase, resulting in accumulation of phytanic acid in plasma and tissues. However, this disease can be treated by dietary restriction of foods containing phytanic acid and its precursors. If the dietary restriction is not followed patients will develop cerebellar ataxia, peripheral polyneuropathy, retinitis pigmentosa and anosmia. AMACR deficiency resembles a mild Refsum's-like disease, while MFP2 deficiency, which disrupts peroxisomal β-oxidation, clinically resembles a PBD. X-linked

adrenoleukodystrophy or X-ALD is caused by disruption in the ABCD1 gene and leads to a deficiency in oxidation of very long chain-fatty acids. X-ALD presents in two forms, childhood cerebral adrenoleukodystrophy (CCALD), where the affected boys have a normal development for the first years of life, which is then followed by rapid deterioration, and death or as adrenomyeloneuropathy (AMN). The second is a milder form of X-ALD than CCALD. The occurrence of peroxisomal diseases not only shows the importance of peroxisomes in general, but also emphasizes the importance of specific peroxisomal pathways, especially peroxisomal α-, β-oxidation, and plasmalogen biosynthesis.

Mouse models of peroxisomal disorders

Early research on peroxisomal disorders has been focused on the biochemical hallmarks and clinical aberrations, and subsequently on detecting the cause of the disease. However, lately much emphasis has instead been placed on the patophysiology of a particular phenotype and how that develops, through the creation of knockout mouse models of various peroxisomal genes involved in disorders as well as of other genes to investigate their function in peroxisomes (5). It should however be noted that the mouse models in general develop a milder phenotype, suggesting that mice are metabolically very robust. Also deteriorating diseases might not have time to develop since mice have a considerably shorter life span than humans. For example X-ALD mice only show mild neurological and behavioral abnormalities later in life, which more resembles the milder AMN form. Also SCPx and AMACR knockout mice only present with symptoms when they are challenged with phytanic acid feeding. The MFP1, PTLB and catalase knockout mice do not show any particular phenotype.

Free fatty acids and acyl-CoAs in the cell

Fatty acids derived from the diet or by lipolysis of triglycerides, enter the cell from the circulation either by passive diffusion or facilitated transport via a number of membrane associated proteins, including the fatty acid transport proteins (FATPs) (122, 123), fatty acid translocase (FAT/CD36) (124) and the plasma membrane fatty acid binding protein (FABP (pm)) (125). Upon entering the cell, fatty acids become rapidly esterified into acyl-CoAs either as an event coupled to uptake or inside the cell by one of the acyl-CoA synthetases (126, 127). These acyl-CoAs can then be hydrolyzed back into free fatty acids by a group of enzymes called acyl-CoA thioesterases (9). Acyl-

CoAs constitute a major source of energy and degradation of acyl-CoAs takes place at various locations in the cell, with β-oxidation localized to both mitochondria and peroxisomes and α-oxidation solely localized in peroxisomes. ω-Oxidation instead does not require activation of the fatty acid, but instead utilizes free fatty acids and this oxidation occurs partially in the ER and partially in the cytosol. The site of oxidation is first dependent on the nature of the fatty acid, but also on the metabolic status of the cell, where most energy is produced by mitochondrial β-oxidation. However, acyl-CoAs and free fatty acids are not only used for energy production, but also have many other functions in the cell such as in synthesis of complex lipids and also in regulation of a number of cellular events (128). Long-chain acyl-CoAs for example regulate insulin secretion (129) by regulating opening of ATP-sensitive K^+-ion channels (130), and activation of Ca^{2+} ATPases (131). They also regulate signal transduction through protein kinase C (132), as well as gene transcription of many enzymes in lipid metabolism by functioning as ligands for various nuclear receptors. Long-chain acyl-CoAs are antagonists for PPARα (133) and are ligands for HNF-4α (134). Long-chain free fatty acids instead function as ligands for all PPARs (135-137). Since both acyl-CoAs and free fatty acids regulate a number of cellular events it is essential that the ratio between acyl-CoAs and free fatty acids is tightly regulated.
The levels of total CoA, which is the sum of free CoASH and all CoA esters, varies between different cellular compartments where mitochondria has the highest concentrations, followed by peroxisomes, with the lowest concentration found in the cytosol (138). Also the concentrations of total CoA changes markedly in response to different conditions such as high-fat diets, fasting, diabetes and clofibrate treatment (138). The peroxisomal levels of total CoA changes the most upon different treatments, where levels of total CoA in rat liver peroxisomes increase from 0.7 mM up to 5 mM after high-fat feeding. However, it is difficult to measure levels of specific acyl-CoAs and CoASH due to the high acyl-CoA thioesterase activity exhibited by peroxisomes in vitro, creating artificially high levels of free fatty acids and CoASH.

Gene regulation of lipid metabolism

As described above there are several nuclear receptors controlling lipid metabolism, and one of the most important nuclear receptors is the PPARα. This nuclear receptor was identified by it's ability to be activated by peroxisome proliferators (139), which are a diverse group of compounds, including fibrates, that lower circulating triglyceride

levels in man. However feeding rodents with peroxisome proliferators leads to peroxisome proliferation, hepatomegaly and to hepatocarcinogenesis after long term treatment (140-142), which does not occur in humans. Free fatty acids have been shown to be natural endogenous ligands for this receptor as described above (135, 136, 143). The importance of PPARα in lipid metabolism was shown by targeted disruption of this gene in mouse (144), and many of the enzymes involved in both mitochondrial and peroxisomal β-oxidation as well as ω-oxidation are upregulated by treatment with peroxisome proliferators in a PPARα-dependent manner (145-147). Functionally, PPARα binds as a heterodimer with the retinoid x receptor (RXR) to a peroxisome proliferator response element (PPRE) that is usually located upstream in the promoter region of the target gene (**Fig. 4**). The PPRE is composed of a direct repeat spaced by one nucleotide (DR1), consisting of a hexanucleotide consensus sequence of AGGTCAnAGGTCA. Induction of transcription occurs when ligands bind to the heterodimer, which recruits cofactors such as the steroid receptor coactivator1 (SRC-1), PPAR-binding protein (PBP), PPARγ coactivator-1 (PGC-1) and PPAR interaction protein (PRIP) to form a large transcriptional complex. PPARα is highly expressed in metabolically active tissues such as brown adipose tissue, heart, liver and kidney (148, 149).

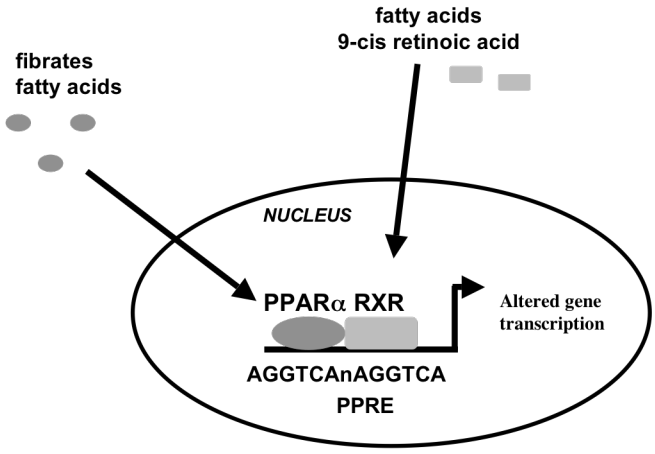


Fig. 4. **PPARα regulation of gene expression**. The peroxisome proliferator-activated receptor α (PPARα) is a nuclear receptor that controls lipid metabolism. Upon activation by a PPARα ligand, this nuclear receptor hetrodimerizes with the retenoid X receptor (RXR) and can then bind to a peroxisome proliferator response element (PPRE) upstream in the target gene to alter transcription. Fibrates and fatty acids are ligands for PPARα while fatty acids and 9-cis retinoic acid are ligands for RXR.

Acyl-CoA thioesterases

As mentioned above, acyl-CoA thioesterases are a group of enzymes that catalyze the hydrolysis of acyl-CoAs to the corresponding free fatty acids and coenzyme A as shown in **Fig. 5**, thereby harbouring the potential to regulate the intracellular levels of these compounds (9). Since fatty acids and acyl-CoAs are implicated in many cellular processes other than just fatty acid oxidation as described above, it is of great importance that the levels of these compounds are controlled. The first acyl-CoA thioesterase was characterized in the 1950's (150) and since then a variety of acyl-CoA thioesterases have been identified and characterized with localizations throughout the cell including cytosol, ER, mitochondria and peroxisomes (9). These enzymes have many aliases in the literature and therefore recently a new nomenclature was agreed upon, re-naming the twelve acyl-CoA thioesterases identified to date as ACOT1-ACOT12 (151).

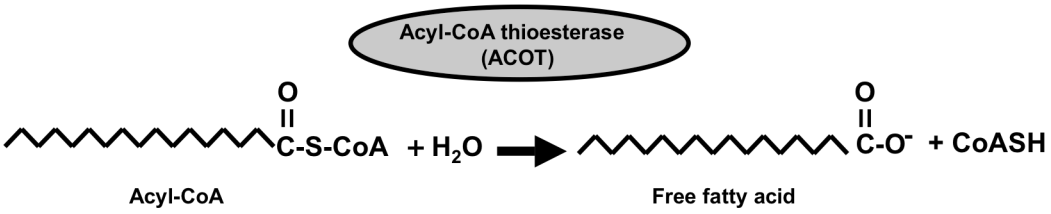


Fig. 5. **The reaction catalyzed by acyl-CoA thioesterases**. Acyl-CoA thioesterases ACOT are enzymes that catalyze the hydrolysis of acyl-CoAs to the corresponding free fatty acids and coenzyme A (CoASH).

The type I acyl-CoA thioesterase gene family

Type I acyl-CoA thioesterases were first identified in 1995 as a peroxisome proliferator inducible 40-kDa protein family with activities in cytosol, mitochondria and peroxisomes (152, 153). Molecular cloning of the cytosolic and mitochondrial activity revealed a family of four homologous genes named *Acot1-Acot4* (previously CTE-I, MTE-I, PTE-Ia and PTE-Ib) (8), and later on two further genes named *Acot5 and Acot6* (previously, PTE-Ic and PTE-Id) were identified in the gene family (**Paper I**). These six genes are all localized in a narrow cluster on chromosome 12 D3 in mouse (**Fig. 6A**), which corresponds to a gene cluster on chromosome 14q24.3 in human (154). Both ACOT2 and ACOT1 were characterized as long-chain acyl-CoA thioesterases with localization in mitochondria and cytosol, respectively (152, 155).

Three further more distantly related genes have also been identified, namely the bile acid-CoA:amino acid *N*-acyltransferase (*Bacat*) (156), acyl-CoA:amino acid *N*-acyltransferase 1 and 2 (*Acnat1 and Acnat2*) ((157) and (Reilly et al unpublished results)), which are localized in a narrow cluster on mouse chromosome 4 B3. BACAT was previously characterized as an acyltransferase that conjugates bile acids and fatty acids to taurine or glycine (158). BACAT contains non-consensus PTS1 a and its subcellular localization is still under debate since it has been shown to be exclusively cytosolic in human skin fibroblasts (158), but lately shown to be peroxisomal in human primary hepatocytes (159). Both ACNAT1 and ACNAT2 have been shown to be exclusively peroxisomal ((157) and (Reilly et al. unpublished results)) and ACNAT1 was recently characterized as an acyltransferase that conjugates long- and very long-chain fatty acids to taurine (157). However, the substrate specificity for ACNAT2 is still not known.

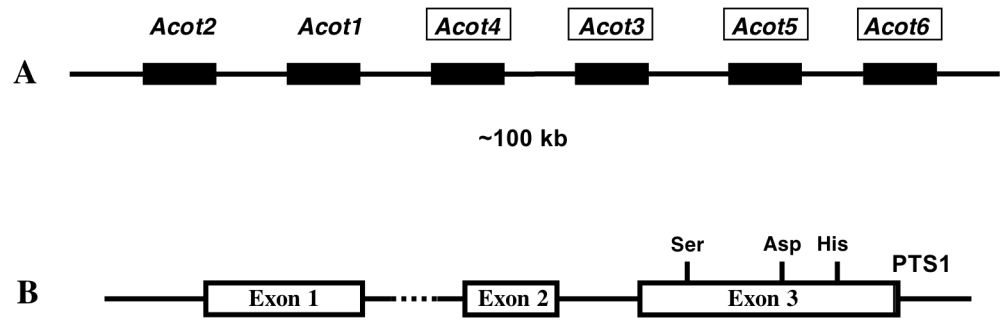


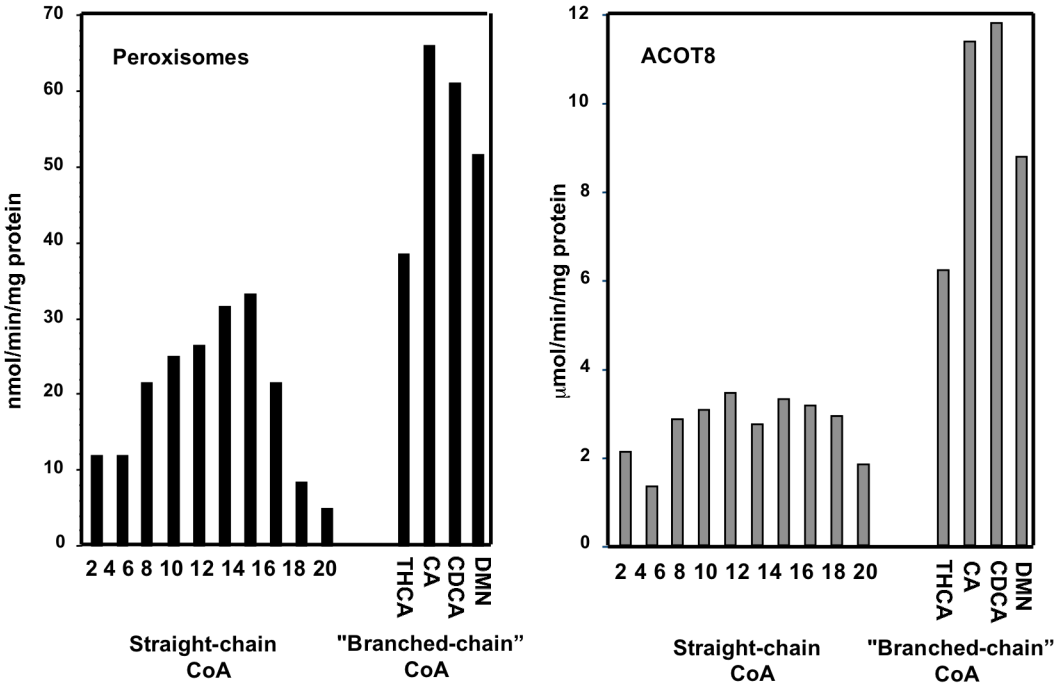
Fig. 6. **The type I acyl-CoA thioesterase gene cluster**. (A) The six members of the type I acyl-CoA thioesterase gene family, named *Acot1-Acot6*, are localized in a narrow cluster on chromosome 12 D3 in mouse. (B) All the open reading frames are encoded by three exons, where the active site catalytic triad residues of a serine, histidine and an aspartic acid are localized in the third exon. At the end of exon 3, *Acot3-Acot6* contain a sequence coding for a possible peroxisomal type 1 targeting signals (PTS1), suggesting a peroxisomal localization for these enzymes.

The open reading frames of the type I acyl-CoA thioesterases and acyltransferases are composed of three exons and show a varying degree of sequence similarity. The type I acyl-CoA thioesterases belong to the large protein family called α/β hydrolases (160). This large protein family contains many different enzymes, including acyl-CoA thioesterases, lipid hydrolases, peptide hydrolases, haloperoxidases and epoxide hydrolases. The active site in all α/β-hydrolases is a catalytic triad composed of a nucleophile, an acidic residue and a histidine. The nucleophile can either be serine, cysteine, aspartic acid or aspargine and the acidic residue can be glutamic or aspartic

acid, while the histidine residue is conserved throughout the whole family of α/β hydrolases. In the type I acyl-CoA thioesterases this catalytic triad is composed of a serine, an aspartic acid and the histidine are located in the C-terminal half of the protein (**Fig. 6B**). The α/β hydrolase fold is composed of 5-11 mostly parallel β-strands and six α-helices. This structure allows the catalytic triad residues into close proximity to generate the catalytic activity. One important feature of the α/β hydrolase fold is the nucleophilic elbow, which holds the nucleophilic serine. This fold is composed of a highly conserved GlyXSerXGly motif where the nucleophile is located in the sharp turn centre of the nucleophilic elbow, keeping the nucleophile in a correct position. Conservation of the active site amino acids and the nucleophilic elbow is vital for optimal activity, which was recently shown by mutation analysis of the active site of ACOT1 (160).

Peroxisomal acyl-CoA thioesterases and thioesterase activities

Peroxisomes contain a wide range of acyl-CoA thioesterase activities as shown in **Fig. 7** (161), and except for the four putative peroxisomal type I acyl-CoA thioesterases, peroxisomes also contain a further acyl-CoA thioesterase, named ACOT8. This enzyme, which is unrelated to the type I acyl-CoA thioesterase gene family, was characterized in 2002 showing that the expression is regulated by peroxisome proliferators, has a quite general tissue expression and was active on all acyl-CoA substrates tested (162). However, the activity was shown to be regulated by free CoASH with an IC_{50} of 10-15 μM, suggesting a possible role for this enzyme in regulation of intraperoxisomal levels of CoASH. The activity pattern for ACOT8 and that of purified peroxisomes does no correlate completely, suggesting that further peroxisomal acyl-CoA thioesterases may exist. The identification of four novel putative peroxisomal acyl-CoA thioesterase genes (Acot3-Acot6) therefore provided excellent candidate genes for further characterization in this PhD thesis.



Hunt, M. C. et al. *J. Biol. Chem.* (2002) 277, 1128-38.

Fig. 7. **Acyl-CoA thioesterase activity in purified peroxisomes and activity of recombinant ACOT8**. Peroxisomes show a broad acyl-CoA thioesterase activity, which is similar to the acyl-CoA thioesterase activity pattern exhibited by recombinantly expressed and purified acyl-CoA thioesterase 8 (ACOT8). Abbreviations: DMN; dimethylnonanoyl-CoA, THCA; trihydroxycholestanoyl-CoA, CA; choloyl-CoA, CDCA, chenodeoxycholoyl-CoA.

AIM

The identification of four novel genes that code for putative peroxisomal acyl-CoA thioesterases, named Acot3-Acot6, raised the question as to what function(s) these enzymes have in peroxisomal lipid metabolism. To address this issue the aim of this PhD project was to characterize these four novel enzymes regarding subcellular localization, substrate specificity, tissue expression and regulation of expression as well as to relate their functions to peroxisomal lipid metabolism.

RESULTS AND DISCUSSION

Bioinformatics

Only five years after acyl-CoA thioesterase activity was measured in purified peroxisomes, computational searches and molecular cloning led to the discovery of four of the genes belonging to the type I acyl-CoA thioesterase gene family (8), with the subsequent discovery of two further genes (**Paper I and III**). Sequence analysis revealed that ACOT3-ACOT6 contain consensus PTS1 sequences at the C-terminal end of the proteins, as indicated in **Fig. 8**. The type I genes are composed of three exons as indicated in **Fig. 6,** with the individual exon boundaries shown in **Fig. 8**.
All the type I acyl-CoA thioesterase genes are highly conserved and show a considerable sequence identity to each other ranging from 65-81% (**Fig. 8**). The sequence identity to the more distantly related enzymes, BACAT, ACNAT1 and ACNAT2 is approximately 41-45%. The type I acyl-CoA thioesterases share a conserved catalytic triad of a serine, an aspartic acid and a histidine that is located at positions 243, 337, and 371 of ACOT3 as indicated in **Fig. 8**. Also note the conserved flanking glycine residues in a GlyXSerXGly motif next to the nucleophilic serine, which together comprise the nucleophilic elbow. Of the three exons that encode these proteins, the third exon containing the catalytic triad is the least conserved, and here the sequence identity drops to 47-72%. These sequence differences around the active site might allow for conformational changes resulting in formation of the substrate binding pockets and therefore may result in different substrate specificities. Since ACOT3 and ACOT5 are the two most similar proteins at the level of primary structure, we hypothesized that these two enzymes might also have the most similar functions and were therefore characterized together in **Paper I**. ACOT3 is however slightly different to the other enzymes at the N-terminal end of the protein as it exists in two splice variants of which one of the variants contains 11 extra amino acids.

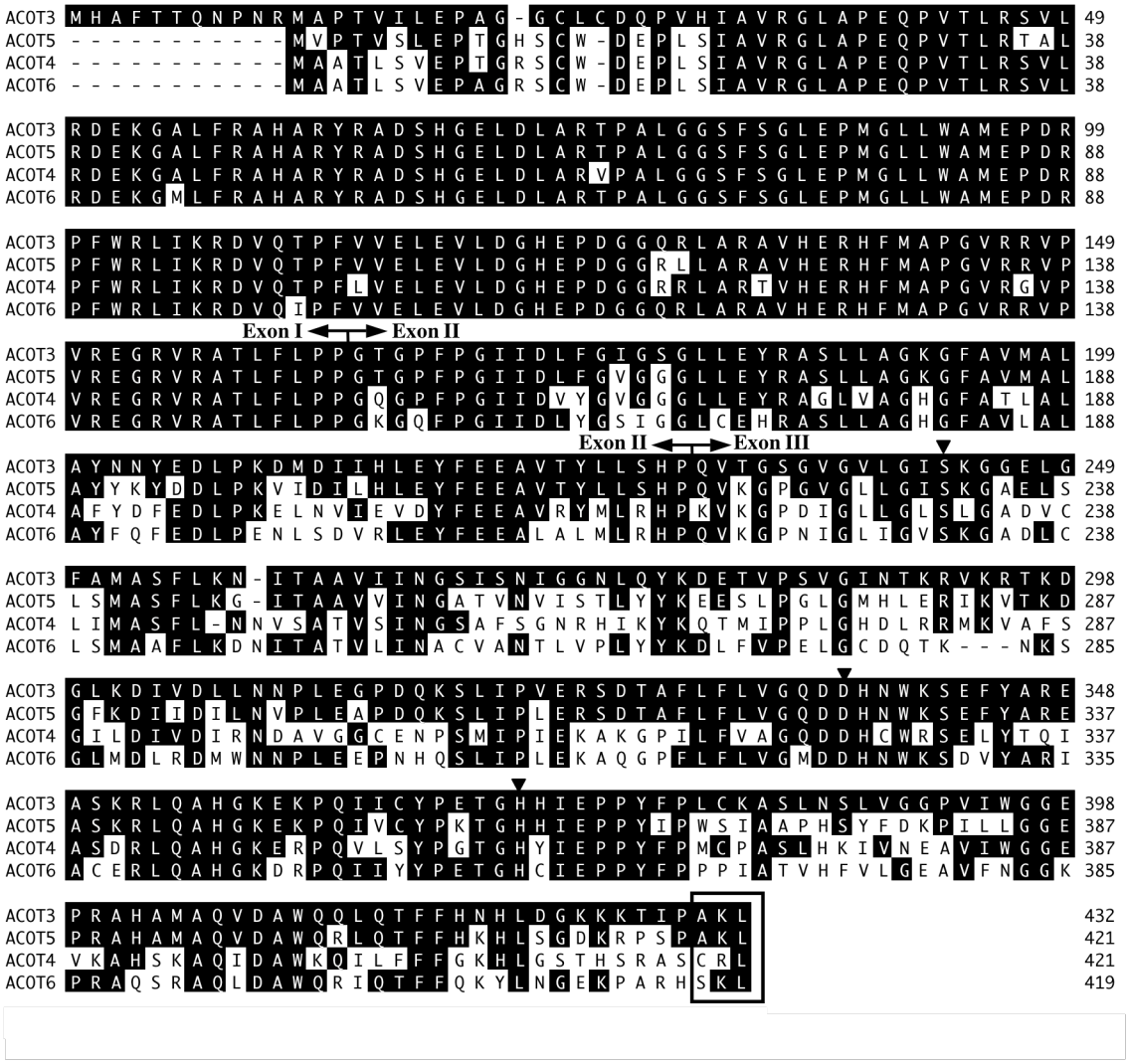


Fig. 8. **The type I peroxisomal acyl-CoA thioesterases show a high degree of sequence identity**. The sequences of peroxisomal Acot3-Acot6 were aligned using the Clustal method. Residues identical to ACOT3 in the sequence alignment are boxed in black, and the three exon boundaries are marked with arrows, while the catalytic triad residues are marked with arrowheads. The C-terminal peroxisomal type 1 targeting signals (PTS1) for all enzymes are marked with a box.

Localization of ACOT3-ACOT6 to peroxisomes

The C-terminal ends of -AKL for ACOT3 and ACOT5, -CRL for ACOT4 and -SKL for ACOT6 conform to consensus PTS1 targeting signals, and were indeed shown to target the four proteins to peroxisomes. For this purpose the proteins were expressed as N-terminal green fluorescent protein fusion proteins and using cell transfection experiments the subcellular localization were detected by immunofluorescence microscopy (**Paper I-III**). We here took advantage of the fact that skin fibroblasts from Zellweger patients are unable to peroxisomal import matrix proteins, resulting in a

diffuse cytosolic staining, while transfection into normal human skin fibroblasts results in a punctate staining.

Regulation of expression via PPARα

It has previously been shown that expression of Acot3 and Acot4, along with Acot1 and Acot2 are upregulated by the peroxisome proliferator clofibrate in a PPARα dependent manner at mRNA level using Northern blot, which initially suggested a function for these enzymes in lipid metabolism (8, 149). In **Paper I-III** we investigated the regulation of Acot3-Acot6 expression in more detail at mRNA and protein level by semi-quantitative PCR or real-time PCR, and Western blotting, using the even more potent peroxisome proliferator Wy-14,643. These experiments confirmed the upregulation of Acot3 and Acot4 at mRNA level by peroxisome proliferators, and also showed that both Acot5 and Acot6 are also highly upregulated at mRNA level by Wy-14,643. This upregulation was also confirmed at protein level for all four enzymes. The potent upregulation via PPARα strongly connects these ACOTs functionally to fatty acid degradation. The regulation of these genes by peroxisome proliferators in a PPARα-dependent manner also suggests that a peroxisome proliferator response element (PPRE) mediating these effects should be localized possibly in the promotor of these genes, and since these genes are so evolutionary conserved this might suggest that also the PPREs are conserved. Recently Acot1 was shown to contain a PPRE, which binds PPARα and hepatocyte nuclear factor 4 alpha (HNF-4α) (163).
The connection of these enzymes to lipid metabolism through their upregulation by peroxisome proliferators may sound like a paradox, considering that these enzymes have the potential to terminate oxidation by hydrolysis of the acyl-CoAs. However our current view is that these ACOTs function as auxiliary enzymes required for efficient oxidation rather than preventing oxidation, which will be further discussed below. It should also be noted that the increase in expression due to treatment with peroxisome proliferators does not result in an increased peroxisomal concentration of these enzymes, but rather that the increased expression follows actual peroxisome proliferation, resulting in an unchanged specific activity within the peroxisome. This is for example demonstrated in **Paper III**, where the ACOT activity in peroxisomes towards medium-chain acyl-CoAs, phytanoyl-CoA and pristanoyl-CoA remains about the same after treatment with peroxisome proliferators.

Tissue expression of the peroxisomal type I ACOTs

In general all peroxisomal ACOTs seem to have specific tissue expression patterns. Tissue expression analysis using real-time PCR shows that Acot3 is almost specifically expressed in kidney (**Fig. 9**), which is also evident from the semi-quantitative PCR data in **Paper I**. For Acot5, however, the real-time PCR data shown in **Paper IV** differs somewhat from the semi-quantitative PCR data seen in **Paper I** in that the high expression in white adipose tissue seen in the real-time PCR data is not evident in the earlier semi-quantitative PCR data, and the high expression seen in spleen using semi-quantitative PCR is not evident in the real-time PCR data. The reason for this discrepancy is not clear, but requires further investigation. Acot4 instead was shown to be most highly expressed in kidney and liver as seen in **Paper II**, and Acot6 was shown to be most highly expressed in white adipose tissue (**Paper III)**.

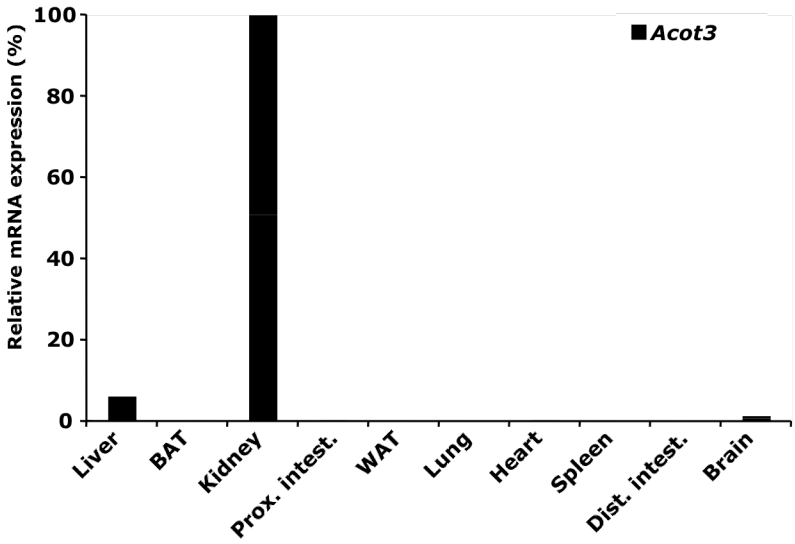


Fig. 9. **Acot3 is highly expressed in kidney**. Tissue expression of Acot3 was measured at mRNA level using real-time PCR. For cDNA synthesis total RNA was pooled from three individual animals and real-time PCR was run in single-plex using mouse hypoxanthine guanine phosphoribosyl transferase (Hprt) as an endogenous control. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Abbreviations: BAT; brown adipose tissue, WAT; white adipose tissue, Prox. intest.; proximal intestine, Dist. intest.; distal intestine.

Substrate specificity of the peroxisomal type I ACOTs

ACOT activity was measured spectrophotometrically with a wide range of substrates for all the enzymes. For ACOT3-ACOT5 activity was measured at 412 nm in a 5,5'-dithiobis 2-nitrobenzoic acid (DTNB) containing medium, however, no activity could be measured for ACOT6 in this medium. This is most likely due to binding of DTNB

to one or more of the nonconserved cysteine residues that may be located near the active site and may thereby block the active site and inhibit the activity. Therefore ACOT6 activity was instead measured at 232 nm as the cleavage of the thioester bond. In **Paper I,** ACOT3 and ACOT5 were identified as a long- and a medium-chain acyl-CoA thioesterase respectively, which fits well with the prediction that ACOT3 and ACOT5 might have similar substrate specificities since they show such a high sequence similarity. In **Paper II** ACOT4 was identified as a highly specific succinyl-CoA thioesterase, and in **Paper III** ACOT6 was identified as a phytanoyl-CoA/pristanoyl-CoA specific thioesterase. A summary of the combined activities of the type I ACOTs is also shown in **Fig. 10**.

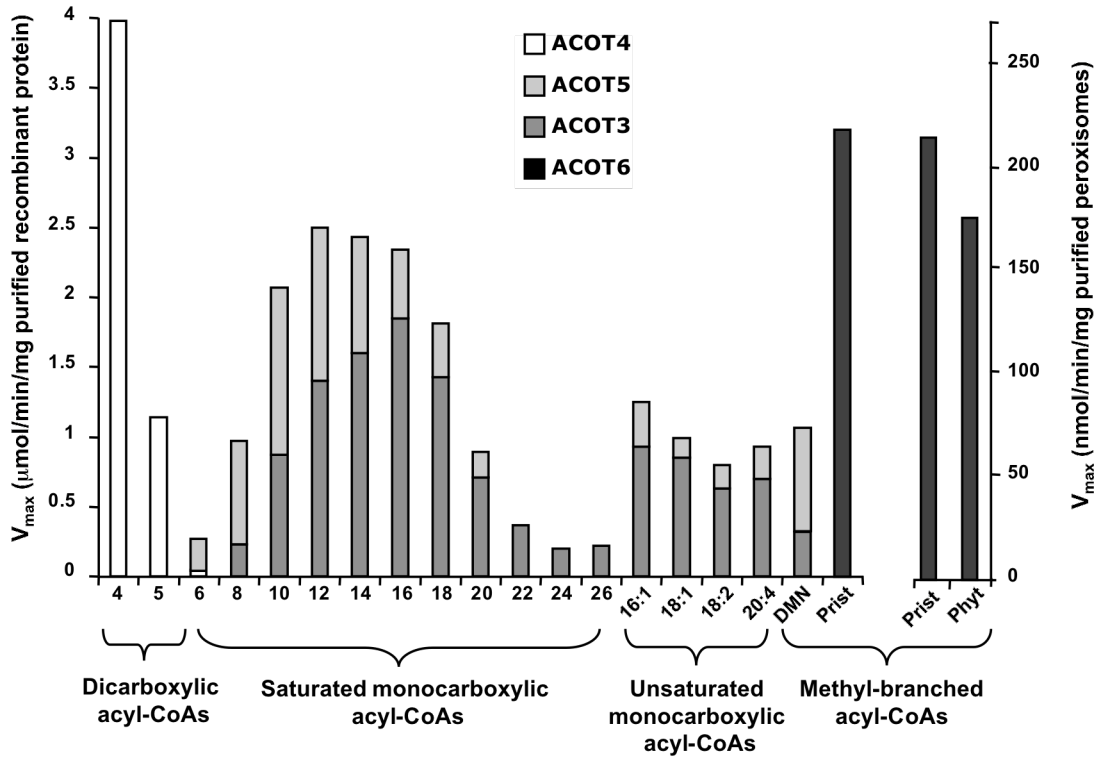


Fig. 10. **Acyl-CoA thioesterase activity of the peroxisomal type I ACOTs**. ACOT activity was measured for each enzyme on a variety of acyl-CoAs and V_{max} was calculated using either the Sigma plot software or the Prism enzyme kinetics software. Activity was measured on purified recombinant protein for ACOT3-ACOT6, and ACOT6 activity was also measured for pristanoyl-CoA and phytanoyl-CoA in purified mouse liver peroxisomes.

Total peroxisomal acyl-CoA thioesterases activity

Peroxisomes have been shown to exhibit numerous thioesterase activities towards a range of acyl-CoAs as shown in **Fig. 7.** Before this project started, only one peroxisomal acyl-CoA thioesterase had been characterized, namely ACOT8, which was

shown to be active to a varying extent on all acyl-CoA tested (162). Comparison of the activity patterns for acyl-CoA thioesterase activity in purified peroxisomes with the activity pattern of recombinant ACOT8, suggested that peroxisomes contain some "extra" medium-/long-chain acyl-CoA thioesterase activity that would be due to thioesterases other than ACOT8. This "missing" activity is most likely due to the combined activities of ACOT3 and ACOT5. In a previous study where peroxisomal acyl-CoA thioesterase activity was measured (162), the activity was however not measured with dicarboxylic acyl-CoAs, and therefore there was no prediction for such an ACOT in peroxisomes. ACOT4 is active only with succinyl-CoA and shows some low activity towards glutaryl-CoA, strongly suggesting that either or both CoA-esters are formed in peroxisomes (**Paper II**). In contrast, ACOT8 was shown to be active on longer dicarboxylyl-CoAs and comparison of K_m and V_{max} values suggests that ACOT4 handles succinyl-CoA while ACOT8 hydrolyzes glutaryl-CoA and longer chain dicarboxylyl-CoAs, in line with the proposed function of peroxisomes in chain-shortening of dicarboxylic acids (164). Measurement of phytanoyl-CoA/pristanoyl-CoA thioesterase activity in purified peroxisomes (**Paper III and Fig. 7)** showed that peroxisomes contain this activity at levels comparable to the activity with other branched-chain substrates (~70 nmol/min/mg). Immunoprecipitation experiments shows that most of the activity on phytanoyl-CoA and pristanoyl-CoA is due to ACOT6.

Peroxisomes have also been shown to contain acetyl-CoA thioesterase activity (161, 165, 166), which at least in part is due to ACOT8 (162). In **Paper IV** we suggest that peroxisomes also contain a specific acetyl-CoA thioesterase, ACOT12, an enzyme that was initially characterized as an extra-mitochondrial acetyl-CoA thioesterase (167). However, ACOT12 contains a putative, non-consensus, type 1 peroxisomal targeting signal of –(K)SVL, and previous subcellular fractionation experiments showed that although most of the activity is found in the cytosol, there is also acetyl-CoA thioesterase activity in peroxisomes. This activity is most likely due to ACOT12 based on detection by Western blotting and the fact that purified ACOT12 and the activity in peroxisomes show the same cold-lability and ATP dependence (168). This suggests that either ACOT12 is dually localized in cytosol and peroxisomes, due to its "imperfect" peroxisomal targeting signal, or that the cytosolic localization is an artefact due to leakage from peroxisomes during fractionation. It should also be noted that although acetyl-CoA thioesterase activity was detected in peroxisomes as shown in **Fig. 7**, this activity is most likely due to ACOT8 since ACOT12 is probably inactivated due

to the conditions used to isolate peroxisomes, and the method used for activity measurement shown in **Fig. 7**. Taken together peroxisomes contain at least six ACOTs with varying substrate specificities (ACOT3-ACOT6, ACOT8 and ACOT12), which raises the question of the need for so many peroxisomal ACOT activities and what their functions are in peroxisomal lipid metabolism.

Functions for the peroxisomal acyl-CoA thioesterases

Peroxisomal ACOTs could serve several different functions, such as a) in regulation of intraperoxisomal levels of acyl-CoAs and free CoASH, b) in "termination" of β-oxidation and c) in formation of products that could be transported out of peroxisomes. Previous characterization of ACOT8 as a 'non-specific' acyl-CoA thioesterase that is instead regulated by free CoASH suggests a function for this enzyme in regulation of intraperoxisomal levels of free CoASH. When CoASH levels become limiting, ACOT8 is activated, which suggests that ACOT8 is important during conditions of increased β-oxidation, such as in fasting, diabetes and high fat feeding, to provide free CoASH for efficient β-oxidation. An important function of ACOT8 in lipid metabolism is underscored by the fact that ACOT8 is conserved from bacteria to man, and is the major ACOT in yeast peroxisomes (162, 169, 170). None of the type I ACOTs are regulated by free CoASH, suggesting that these enzymes could instead have functions in "termination" of β-oxidation and/or in the formation of products to be transported out of peroxisomes. As described in the introduction, peroxisomal β-oxidation substrates usually undergoes 2-3 rounds of β-oxidation, creating a multitude of metabolites that need to exit peroxisomes for further metabolism elsewhere, or for excretion in urine or bile. Since the number of cycles of β-oxidation seem to correlate with the chain-length of the fatty acid (171-173), it is likely that ACOT3, ACOT5 and ACOT8, being active on long- and medium-chain acyl-CoAs as described in **Paper I**, do have a function in termination of, or removal of acyl-CoAs from the β-oxidation pathway, which may explain why peroxisomal β-oxidation merely acts as a chain-shortening system. In contrast ACOT4 and ACOT12 are not active on acyl-CoAs that can undergo further β-oxidation. ACOT4 is, as described in **Paper II**, active on succinyl-CoA, which is suggested to result from peroxisomal β-oxidation of longer-chain dicarboxylyl-CoAs (or some other hitherto unknown pathway), however, peroxisomal β-oxidation cannot proceed further than succinyl-CoA (174). ACOT6 is active on both phytanoyl-CoA and pristanoyl-CoA **(Paper III)**, but a specific function

suggested for ACOT6 is in hydrolysis of the 2R-isomer of pristanoyl-CoA, which cannot undergo further β-oxidation unless it is transformed to the S-isomer by AMACR. Also, each round of β-oxidation results in release of either acetyl-CoA or propionyl-CoA, which cannot undergo further oxidation in peroxisomes. It is believed that the acyl-CoAs produced by peroxisomal β-oxidation cannot exit peroxisomes, but that free fatty acids up to a certain chain-length seem to be able cross the peroxisomal membrane. This would suggest a function for the peroxisomal ACOTs not only in termination of β-oxidation, but also in transport of β-oxidation products out of peroxisomes since free fatty acids may exit peroxisomes by flip flop mechanisms. Recently Antonenkov et al showed that the presence of L-FABP in peroxisomes stimulates acyl-CoA thioesterase activity, suggesting that these proteins may create a system for removal of fatty acids for peroxisomes (175).

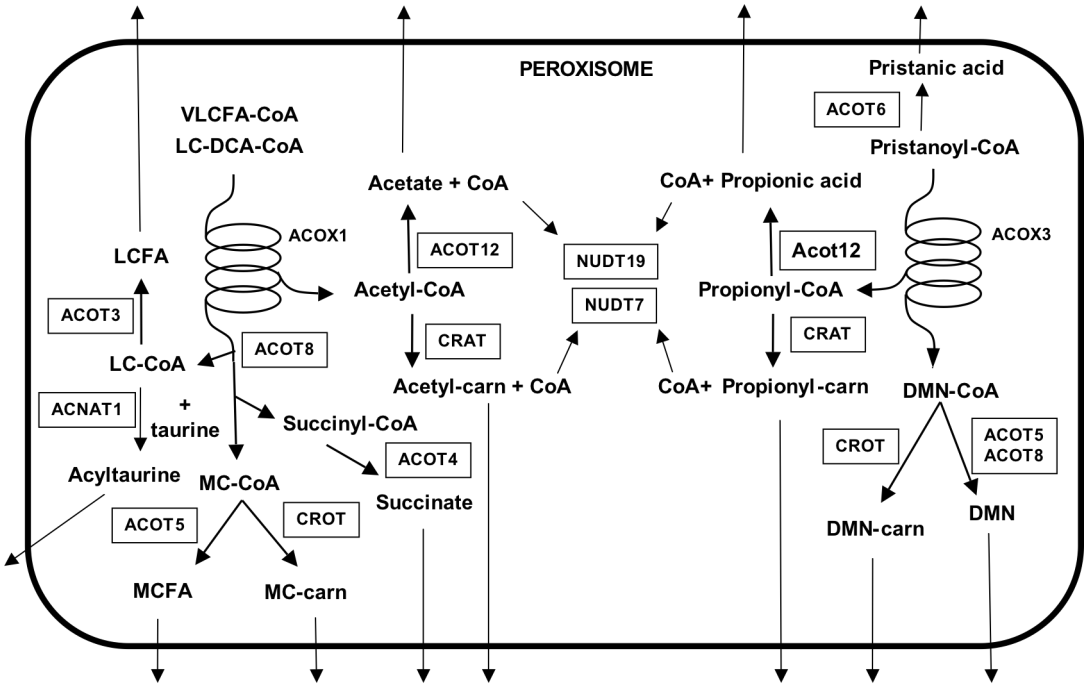


Fig. 11. **Products of peroxisomal β-oxidation**. Peroxisomes are able to β-oxidize a wide variety of lipids, which give rise to a range of products that may either be hydrolyzed to the free fatty acid by the acyl-CoA thioesterases (ACOTs), be conjugated to carnitine by the carnitine acyltransferases (CRAT and CROT), or be conjugated to taurine by the acyl-CoA:amino acid N-acyltransferases (ACNATs)
These enzymes are responsible for producing "small" (chainshortened) products that can then exit peroxisomes. Abbreviations: carn; carnitine, Nudt; nudix hydrolase, MCFA; medium-chain fatty acid, LCFA; long-chain fatty acid, LC-CoA; long-cahin acyl-CoA; MC-CoA; medium-chain acyl-CoA, DMN; dimethylnonanoyl-CoA.

However, there are also other enzymes with the potential to terminate β-oxidation and to direct fatty acids out of peroxisomes. The peroxisomal carnitine acyltransferases CRAT and CROT have previously been characterized as short- and medium-chain carnitine acyltransferases, respectively, and have been implicated in transport of β-oxidation products out of peroxisomes via a peroxisomal membrane transporter for acylcarnitines which was recently identified (46). Interestingly, the recently identified acyl-CoA:amino acid N-acyltransferases (ACNATs) (which are related to peroxisomal type I ACOTs) can conjugate long- and very long-chain fatty acids to taurine and have the potential to form products which can be transported out of peroxisomes (157). Indeed, these taurine conjugated fatty acids may possibly be transported across the peroxisomal membrane via the newly identified bile acid transporter (47). Possible functions for the acyl-CoA thioesterases, carnitine acyltransferases and acyl-CoA:amino acid N-acyltransferases in termination of peroxisomal β-oxidation and transport of products out of peroxisomes is summarized in **Fig. 11**.

Direction of β-oxidation products into different routes

Most of the ACOTs, carnitine acyltransferases and acyl-CoA:amino acid N-acyltransferases (ACNATs) have distinct substrate specificities. However, both ACOT12 and CRAT use short-chain acyl-CoAs as substrates, while both ACOT5 and CROT use medium-chain acyl-CoAs as substrates, suggesting that ACOTs and carnitine acyltransferases may compete for the same substrates. In **Paper IV** we therefore investigated the tissue expression of these enzymes to examine if localization of these enzymes could determine the fate of the acyl-CoA. In this paper it was shown that the complementary enzymes indeed show completely different expression patterns, suggesting that these enzymes does not compete for the same substrates *in vivo* but rather function in different tissues. The carnitine acyltransferases have earlier been implicated in transport of peroxisomal β-oxidation products to the mitochondria for further oxidation. It has for example been shown that oxidation of propionyl-CoA produced in peroxisomes is impaired in human skin fibroblasts deficient in the mitochondrial carnitine/acylcarnitine transporter, which suggests both that propionyl-CoA is transported in the form of a carnitine ester in fibroblasts and that the carnitine ester is destined to the mitochondria for further oxidation (77). Also medium-chain DMN-CoA produced in peroxisomes is apparently transported to mitochondria in the form of a carnitine ester in human skin fibroblasts (113). In contrast, the destiny of free

fatty acids formed in peroxisomes by the ACOTs is more uncertain. Short-chain dicarboxylic acids seem to be excreted in urine as suggested in **Paper II**, while acetate produced in rat heart peroxisomes seems to be incorporated into malonyl-CoA (171). In contrast, free acetate produced in rat liver and intestine contribute to circulating levels of acetate (176), and may be used either for biosynthetic purposes (177), such as cholesterol synthesis, fatty acid synthesis or chain-elongation, or could be used for energy utilization depending on the metabolic status of the cell as discussed in **Paper IV**. The fate of the fatty acyltaurines produced by ACNAT1 is not yet known as they have only been recently identified, but they may to be excreted in urine or bile (157), similar to many other endogenous carboxylic fatty acids as well as xenobiotic fatty acids. Together these results suggest that while the carnitine esters primarily seems to be destined for transport to the mitochondria for further oxidation and the acyltaurines may primarily be destined for excretion in urine or bile, the fate of the free fatty acids produced by ACOTs seems to be more uncertain. The functions for these enzymes in directing the beta-oxidation products into different routes is also outlined in **Fig.12**.

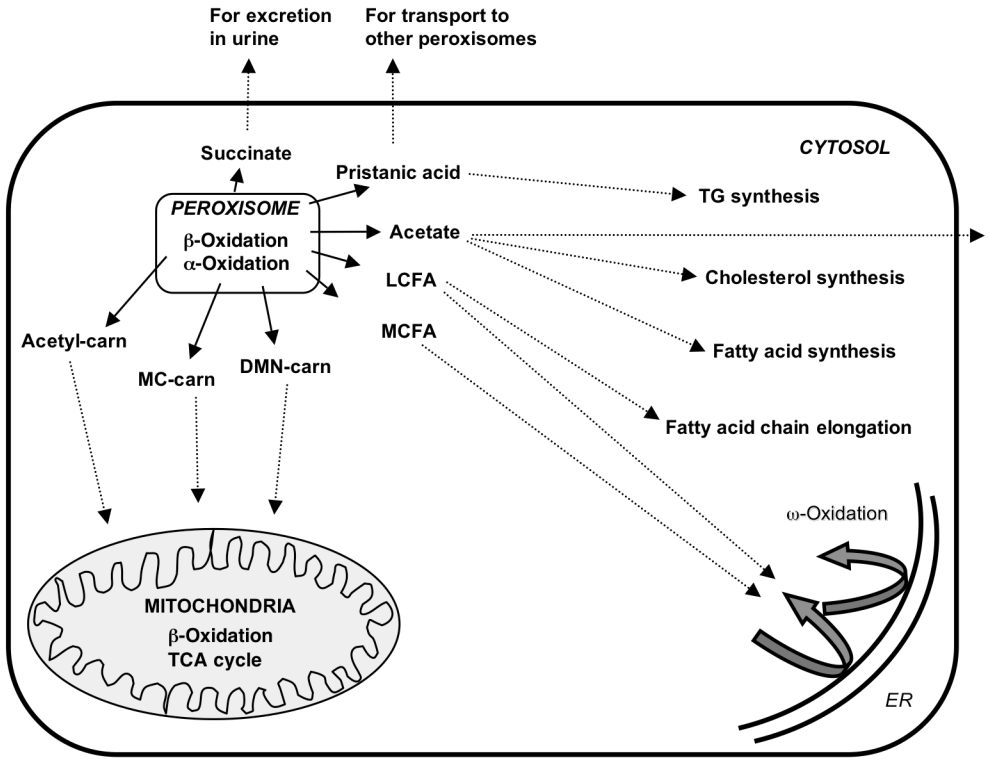


Fig. 12. **Destinies of the peroxisomal oxidation products**. Carnitine esters produced by the carnitine acyltransferases from peroxisomal β-oxidation products are mainly targeted to the mitochondria for further oxidation. Instead the destiny of the free fatty acids produced by the acyl-CoA thioesterases depends on the nature of the particular fatty acid. These may enter elongation or synthesis pathways. Abbreviations: LCFA;long-chain fatty acid, MCFA; medium-chain fatty acid, DMN-carn;

dimethylnonanoyl-carnitine, MC-carn; medium-chain acyl-carnitine, acetyl-carn; acetyl-carnitine

Functions of peroxisomes in different tissues

The peroxisomal β-oxidation system is not primarily a system for energy production, but instead a complement to the mitochondrial β-oxidation system where energy production is the primary function. Instead peroxisomal β-oxidation is considered to be more of a detoxification system for fatty acids and fatty acid derivatives that are poorly metabolized by the mitochondria. The importance of peroxisomes as a detoxification system is also demonstrated by the severe diseases caused by defects in peroxisomes. However, peroxisomal β-oxidation results in products that sometimes can be used as substrates in other pathways, suggesting that this peroxisomal β-oxidation might not only function as a detoxification mechanism but also secondarily to provide substrates for other pathways. Since the different peroxisomal "termination and export routes" in the form of acyl-CoA thioesterases, carnitine acyltransferases and taurine acyltransferases all show distinct tissue expression patterns, these complementary pathways may be at work in different tissues. As discussed above these enzymes may also have specific functions in providing substrates for different subsequent pathways. When tissue expression patterns of ACOT3-ACOT6 as well as CRAT and CROT and a selection of other β-oxidation enzymes were compared in **Paper IV**, it is evident that the different enzymes have different expression patterns. Peroxisomal β-oxidation enzymes as investigated by measuring ACOX1 and MFP2 expression, appear to be co-expressed in liver, proximal intestine, and kidney together with ACOT12 that would produce acetate and propionate. However, ACOX1 and MFP2 are also co-expressed with CRAT in white adipose tissue, brown adipose tissue and heart, which should result in the production of short-chain carnitine esters. Suggesting that acetylcarnitine and propionylcarnitine are the main products of peroxisomal β-oxidation in these tissues, which are transported to the mitochondria. In addition the lack of expression of CROT and other medium-chain thioesterases in brown adipose tissue and heart may suggest that peroxisomal β-oxidation proceeds further in these tissues, resulting in more acetyl-CoA and propionyl-CoA, which can enter directly into the TCA cycle.

Possible involvement of the Nudix hydrolases in regulation of peroxisomal CoASH content

Since fatty acids are mainly transported into peroxisomes in the form of acyl-CoAs but leave the CoASH moiety behind when exiting the peroxisome, it appears that peroxisomes must contain pathways to regulate the intraperoxisomal levels of cofactors such as CoASH. This task is carried out by a group of enzymes called nudix hydrolases, which is a diverse family of proteins that predominantly hydrolyze diphosphate linkages. There are at least three distinct nudix hydrolases located in peroxisomes, where two are involved in CoASH metabolism, namely nudix hydrolase 7 (NUDT7) (178) and nudix hydrolase 19 (NUDT19) (179) also called Rp2p. NUDT7 and NUDT19 catalyze the hydrolysis of free CoASH and its derivatives and show a slight preference towards oxidized CoA, suggesting a role for these enzymes the degradation of damaged CoA as well as in regulation of the intraperoxisomal CoA levels **Fig. 11**.

Is there a role for peroxisomal acyl-CoA thioesterases in gene regulation?

The cytosolic acyl-CoA thioesterases have been suggested to have a function in providing ligands for nuclear receptors for gene regulation (9). For other acyl-CoA thioesterases not localized in the cytosol, a function in gene regulation does not appear as likely. It should, however, be noted that peroxisomes are involved in oxidation of many of the fatty acids that have been shown to have a potential function in gene regulation since ablation of the ACOX1 gene in mouse results in spontaneous PPARα activation (180). Since the acyl-CoA thioesterases have the potential to hydrolyze acyl-CoAs and thereby direct them out of peroxisomes, they could potentially have a function in gene regulation. This could be the case in particular for ACOT6 that catalyzes the hydrolysis of phytanoyl-CoA and pristanoyl-CoA to the corresponding free fatty acids, as these have been shown to be able to activate RXRα and PPARα, respectively, at physiological concentrations (137, 181). ACOT3 being active on long-chain saturated and unsaturated acyl-CoAs could also potentially produce ligands for PPARα (**Paper I**).

Do the peroxisomal acyl-CoA thioesterases have a function in human physiology?

The identification of the six type I acyl-CoA thioesterase genes in mouse led to subsequent identification of a similar cluster of four genes in humans (154). The human cluster contains orthologues to Acot1, Acot2, Acot4 and Acot6, but Acot3 and Acot5 are missing, and the Acot6 orthologue is transcribed from the second exon and results in a much shorter cytosolic protein, which may not be an active protein. This would suggest that human peroxisomes only contain one type I acyl-CoA thioesterase, which of course raises the question of the importance of these enzymes in human physiology. However, characterization of human ACOT4 showed that this enzyme is not only active on succinyl-CoA, as its homologue in mouse, but also exhibits the activity of both mouse ACOT3 and ACOT5 by also hydrolyzing medium- and long-chain acyl-CoAs. Therefore the only "missing" activity in human peroxisomes is the phytanoyl-CoA/pristanoyl-CoA thioesterase activity, which may instead be catalyzed by human ACOT8. There are, however, very few reports on patients with acyl-CoA thioesterase activity deficiencies, which makes it difficult to predict the clinical manifestations of a loss of function. Recent investigations in skin fibroblasts from patients with an uncharacterized oxidation deficiency identified three patients with reduced ACOT7 protein and activity (182). Although ACOT7 is not a peroxisomal acyl-CoA thioesterase, it might still suggest that loss of acyl-CoA thioesterase activity could result in β-oxidation deficiency-like symptoms. Overexpression of human ACOT8 in a murine T-cell line resulted in an increased abundance of peroxisomes and lipid droplet formation (183), which suggests that absence of this enzyme may result in lowered peroxisome number and therefore decreased peroxisomal activities. However, the substrate range for ACOT8 is wider than human ACOT4, and ACOT8 is distinct in its regulation by CoASH, which makes it difficult to hypothesize about the effects of a loss of human ACOT4.

CONCLUDING REMARKS

The diversity of the lipids oxidized in peroxisomes leads to a wide variety of products that need to be transported out of peroxisomes either for further metabolism elsewhere or for excretion in urine or bile. The peroxisomal carnitine acyltransferases have earlier been implicated in this function, and now characterization of four novel acyl-CoA thioesterases in this project suggests that these enzymes provide a complementary system to the carnitine acyltransferases, and have the potential to terminate β-oxidation as well as create metabolites that can exit peroxisomes. The diversity of the products of peroxisomal β-oxidation explains the need for the many acyl-CoA thioesterases together with the carnitine acyltransferases. Also the complementary enzymes, based on chain-length specificity, in these two systems show differential tissue expression, suggesting that the two routes operate in different tissues, and also suggests that these enzymes promote the production of different metabolites. The presence of the ACOTs and the carnitine acyltransferases in peroxisomes in different tissues also shows that these metabolites might be directed into different metabolic pathways depending on the tissue.

FUTURE PERSPECTIVES

This project has answered many of the questions concerning the basic biochemical functions of the peroxisomal type I acyl-CoA thioesterases, which allows us to hypothesize about their functions *in vivo*. However, to further establish their functions *in vivo,* more studies are needed. Such work could for example include:

- Experiments to further investigate which β-oxidation products are produced by peroxisomes in different tissues as well as in which pathways these products "end up", by using radiolabelled fatty acids as performed by the group of Brunengraber (173).
- The tissue expression of these enzymes could also be studied in more detail by for example immunohistochemistry to elucidate where these enzymes are located in certain tissues, such as kidney where most of the type I acyl-CoA thioesterases are expressed. This would give further insights into their physiological function.
- Creation of knockout mouse models for these four enzymes as well as for CRAT, CROT and ACOT8 would give further insights into the *in vivo* function of these enzymes. Such studies are, however, both extensive and expensive and therefore an alternative or complement to such studies may be to use cell systems to either overexpress or knock down expression of these enzymes.
- Studies on when various genes are introduced during evolution and when the convergent evolution into the human genes took place, could give further insights into their functions.
- Crystallization and structural determination of these enzymes is important to gain further insights into the structure/function relationship of these enzymes.

PEROXISOMER?

En populärvetenskaplig sammanfattning

Om vi tittar in i kroppens minsta byggsten, cellen, är även den ett helt nytt universum, med ”planeter”, ”stjärnor” och ”meteoriter”. Dessa himlakroppar eller cellorganeller har olika funktioner som till exempel att se till att tillräckligt mycket energi bildas för att vi ska kunna fungera i vårt dagliga liv. Denna energi som ursprungligen kommer från våran kost omvandlas med hjälp av cellerna och dess olika cellorganeller till kemisk energi, som sedan antingen kan förbrukas direkt eller lagras som fettdepåer. En av dessa cellorganeller kallas peroxisomer, vilka det inte bara finns en av i varje cell utan ibland upp till över tusen. Om man med hjälp av mikroskopi tittar in i en cell där peroxisomerna är märkta med fluorescens kan man få se en sådan bild som finns på framsidan av avhandlingen, som faktiskt påminner lite om himlen en stjärnklar natt. Peroxisomerna har inte som huvuduppgift att bilda energi, utan fungerar istället bland annat som ett avgiftningssystem som bryter ner ovanliga typer av fetter som inte kan brytas ner på vanligt vis. Denna funktion är väldigt viktig, vilket blir tydligt vid sjukdomar där peroxisomerna saknas eller är defekta. Dessa sjukdomar är som tur är väldigt ovanliga, men de barn som föds med peroxisomdefekter lider bland annat av kramper, leverproblem och allvarliga utvecklingsstörningar och dessa barn dör vanligtvis inom det första levnadsåret. Varje år läggs det ner stora resurser på medicinsk forskning och en del av dessa resurser kommer från EU där ett av de många områdena som det nu satsas på handlar om forskning kring peroxisomer vid hälsa och sjukdom. Detta forskningsprojekt är väldigt stort och spänner från att försöka hitta nya funktioner hos peroxisomer till att kunna relatera peroxisomer till vanliga sjukdomar med många underliggande orsaker såsom Alzheimers sjukdom och hjärt- och kärl sjukdomar. Vår forskargrupp identifierade i starten till mitt doktorandprojekt fyra nya proteiner som är lokaliserade till peroxisomerna och därför har det här projektet varit fokuserat på att försöka ta reda på vad dessa proteiner har för funktion i peroxisomerna. Eftersom peroxisomer bryter ner så många olika typer av ovanliga fetter leder det till att många olika typer av produkter bildas som sedan måste transporteras ut ur peroxisomerna. Dessa produkter är dock för stora för att kunna transporteras ut och de data som mitt projekt har lett fram till tyder på att ”mina” proteiner har en funktion i att göra dessa produkter mindre till storleken så att de sedan kan transporteras ut ur peroxisomerna.

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