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Mary Hunt
Technological University Dublin, mary.hunt@tudublin.ie

Marina Siponen
Technological University Dublin

Stefan Alexson
Karolinska Institutet, Karolinska University Hospital at Huddinge, SE-141 86 Stockholm, Sweden

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Review

The emerging role of acyl-CoA thioesterases and acyltransferases in regulating peroxisomal lipid metabolism ☆

Mary C. Hunt [a],*, Marina I. Siponen [b,1], Stefan E.H. Alexson [c]

[a] Dublin Institute of Technology, School of Biological Sciences, College of Sciences & Health, Kevin Street, Dublin 8, Ireland
[b] Department of Medical Biochemistry and Biophysics, Structural Genomics Consortium, Karolinska Institutet, SE-171 77 Stockholm, Sweden
[c] Karolinska Institutet, Department of Laboratory Medicine, Division of Clinical Chemistry, Karolinska University Hospital at Huddinge, SE-141 86 Stockholm, Sweden

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ABSTRACT

The importance of peroxisomes in lipid metabolism is now well established and peroxisomes contain approximately 60 enzymes involved in these lipid metabolic pathways. Several acyl-CoA thioesterase enzymes (ACOTs) have been identified in peroxisomes that catalyze the hydrolysis of acyl-CoAs (short-, medium-, long- and very long-chain), bile acid-CoAs, and methyl branched-CoAs, to the free fatty acid and coenzyme A. A number of acyltransferase enzymes, which are structurally and functionally related to ACOTs, have also been identified in peroxisomes, which conjugate (or amidate) bile acid-CoAs and acyl-CoAs to amino acids, resulting in the production of amidated bile acids and fatty acids. The function of ACOTs is to act as auxiliary enzymes in the α- and β-oxidation of various lipids in peroxisomes. Human peroxisomes contain at least two ACOTs (ACOT4 and ACOT8) whereas mouse peroxisomes contain six ACOTs (ACOT3, 4, 5, 6, 8 and 12). Similarly, human peroxisomes contain one bile acid-CoA:amino acid N-acyltransferase (BAAT), whereas mouse peroxisomes contain three acyltransferases (BAAT and acyl-CoA:amino acid N-acyltransferases 1 and 2: ACNAT1 and ACNAT2). This review will focus on the human and mouse peroxisomal ACOT and acyltransferase enzymes identified to date and discuss their cellular localizations, emerging structural information and functions as auxiliary enzymes in peroxisomal metabolic pathways. This article is part of a Special Issue entitled: Metabolic Functions and Biogenesis of Peroxisomes in Health and Disease.

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1. Introduction

Peroxisomes are nearly ubiquitous organelles present in yeast, fungi, plants and animals and in the last number of years there has been a huge increase in research into peroxisome biogenesis, peroxisomal lipid metabolism and the role of peroxisomes in human diseases. The involvement of peroxins in peroxisome biogenesis disorders has been widely studied, together with diseases associated with individual enzyme deficiencies and has provided new insights into the functions of peroxisomes in health and disease. There are numerous excellent reviews on peroxisome biogenesis disorders and peroxisomal fatty acid oxidation defects ([1–3] and including two chapters in this current Special Issue). Research including a combination of biochemical techniques, molecular biology and proteomics over the last number of years, has been instrumental in the elucidation of the enzymatic pathways in peroxisomes for the β-oxidation and α-oxidation of acyl-CoAs, glyoxylate metabolism, ether-phospholipid synthesis, cholesterol and isoprenoid metabolism and bile-acid synthesis.

In recent years, a number of enzymes called acyl-CoA thioesterases (ACOTs) and acyltransferases have been identified and characterized in peroxisomes and distinct roles for these enzymes as auxiliary enzymes in peroxisomal lipid metabolism have now been established. This review will focus on these ACOTs and acyltransferases identified to date and their roles in peroxisomal lipid metabolism.

2. Identification and characterization of acyl-CoA thioesterases and acyltransferases in peroxisomes

In the 1950s, the first identification of acyl-CoA thioesterase (ACOT) activity was published describing the partial purification of a succinyl-CoA thioesterase from pig heart [4]. In the intervening years, advances in techniques led to the identification and

characterization of several different families of ACOTs as reviewed in [5–8]. A nomenclature system was introduced in 2005, which is now followed by the scientific community and new ACOT genes identified are assigned the next available number [9]. Within the peroxisome, several ACOTs have been identified and characterized that cleave the thioester bond of acyl groups attached to coenzyme A (CoASH), to release the free acid and CoASH (Fig. 1). The acyl groups have been identified as long-, medium- and short-chain fatty acids, dicarboxylic acids, methyl-branched chain fatty acids and bile acids, depending on the ACOT enzyme involved.

ACOTs are divided into two families, named the Type-I and Type-II thioesterases, where the Type-I thioesterases, together with the acyltransferases, show a high degree of sequence similarity and structurally belong to the α/β-hydrolase superfamily, which is one of the largest superfamilies of proteins. The Type-II thioesterases show a low degree of sequence similarity to each other, however they are structurally related and were found to belong to the HotDog fold family of proteins [10]. While the Type-I thioesterases are only found in (some) bacteria and in the animal kingdom (not in yeast, insects or plants), the Type-II thioesterases are found in all three branches of life. Numerous HotDog domain-containing proteins are fusion proteins in which two separate genes have been fused to give a protein with two functional domains (as is the case for ACOT12 discussed below).

The second group of enzymes identified in peroxisomes is the acyl-CoA:amino acid *N*-acyltransferases, which catalyze the transfer of carboxylic acids from the CoA ester to an amino acid, usually taurine or glycine (Fig. 1). The final step in bile-acid synthesis is a conjugation (or amidation) of the bile acids, producing taurine or glycine conjugated bile acids that are excreted into the bile. This reaction is catalyzed by the well-characterized bile acid-CoA:amino acid *N*-acyltransferase (BAAT). More recently, two further genes have been identified in mouse that code for proteins that are sequence-related to BAAT, called *Acnat1* and *Acnat2* (acyl-CoA:amino acid *N*-acyltransferase 1 and 2), which function in the conjugation of fatty acids to taurine (and possibly other amino acids). The structures and functions of peroxisomal acyl-CoA thioesterases and acyltransferases are the focus of this review.

3. Type-I peroxisomal acyl-CoA thioesterases

In the late 1980s and early 1990s the biochemical analysis of highly purified peroxisomes revealed ACOT activity with a wide range of acyl-CoAs, including long-, medium- and short-chain acyl-CoAs (from C_2–C_{20}-CoA) [11,12] and the partial purification of a peroxisomal ACOT identified a protein with long-chain acyl-CoA thioesterase activity [11]. Gene cloning subsequently identified several *Acots* in mouse and human that contained peroxisomal type 1 targeting signals (PTS1) at their carboxyterminal end. These proteins comprise the Type-I family of ACOTs, and mouse contains six closely related genes (that show 66–93% sequence identity to each other), all localized in a gene cluster on mouse chromosome 12 D3, and are named *Acot1* to *Acot6* [13–16]. The human *ACOT* genes (4 genes in total) are all localized in a gene cluster on human chromosome 14q24.3 [17], named *ACOT1, ACOT2, ACOT4* and *ACOT6*.

In mouse the products of four of the six genes in the gene cluster (ACOT3–6) are localized in peroxisomes, while mouse ACOT1 localizes to cytosol and ACOT2 to mitochondria. ACOT3, ACOT4, ACOT5 and ACOT6 are proteins of about 47 kDa that end with the amino acid sequences -AKL (ACOT3 and ACOT5), -CRL (ACOT4) and -SKL (ACOT6) (see Table 1) and peroxisomal localization was confirmed using green fluorescent fusion protein studies [14–16] and proteomic studies (ACOT3, ACOT4 and ACOT6) in purified mouse kidney peroxisomes [18].

The two first peroxisomal Type-I thioesterases characterized in detail were the mouse ACOT3 and ACOT5. Expression of the recombinant proteins revealed that mouse ACOT3 is a long chain acyl-CoA thioesterase (highest activity with C_{12}–C_{18}-CoA), whereas mouse ACOT5 is a medium chain acyl-CoA thioesterase (highest activity with C_{10}-CoA). Interestingly, ACOT3 and ACOT5 had little or no activity toward 3-hydroxy-palmitoyl-CoA, an intermediate in peroxisomal β-oxidation, suggesting that these enzymes hydrolyze the substrates/products at the beginning and the end of individual β-oxidation cycles, but not the intermediates within the individual cycles [15]. Neither enzyme was active on CoA-esters of bile-acid intermediates (choloyl-CoA or chenodeoxycholoyl-CoA), but both ACOT3 and
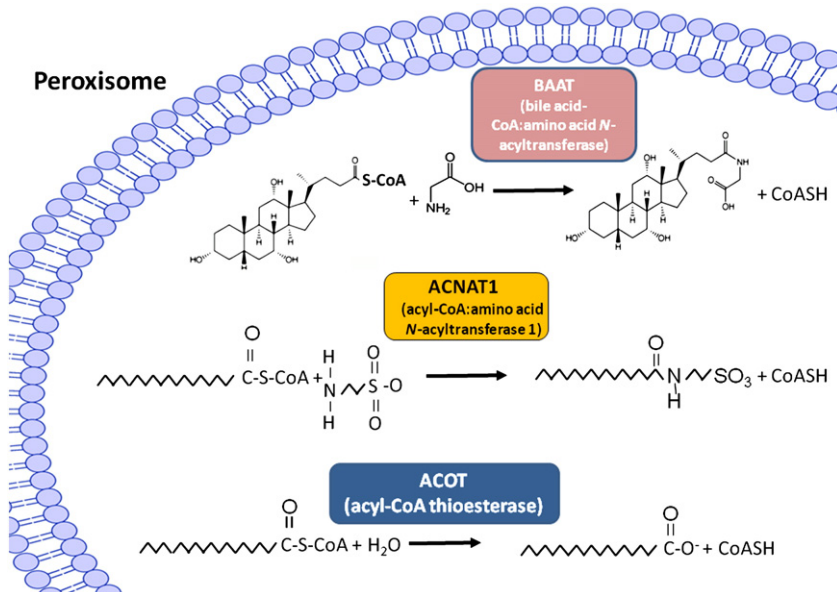
Fig. 1. Reactions catalyzed by peroxisomal acyl-CoA thioesterases and acyltransferases. The bile acid-CoA:amino acid *N*-acyltransferase (BAAT) catalyzes the conjugation (or amidation) of the CoA esters of bile acids to glycine or taurine, resulting in the production of glycine or taurine conjugated bile acids (the figure shows choloyl-CoA with glycine as the acceptor, producing choloylglycine and coenzyme A (CoASH)). The acyl-CoA:amino acid *N*-acyltransferase 1 (ACNAT1) enzyme catalyzes the conjugation of fatty acids to taurine, resulting in the production of *N*-acyl taurines and CoASH (the figure shows stearoyl-CoA ($C_{18:0}$-CoA) and taurine, resulting in the production of *N*-stearoyl taurine). (BAAT can also conjugate fatty acids to taurine and glycine in a similar reaction catalyzed by ACNAT1 but for simplicity only ACNAT1 is shown). Acyl-CoA thioesterases (ACOTs) catalyze the hydrolysis of acyl-CoA esters to produce the free fatty acid and CoASH (the figure shows stearoyl-CoA, resulting in the production of stearic acid and CoASH).

ACOT5 were active on 4,8-dimethylnonanoyl-CoA (DMN-CoA, the end product of β-oxidation of pristanic acid in peroxisomes), with ACOT3 being more efficient. *Acot3* mRNA is expressed as two splice variants, which show a tissue-specific expression in kidney and liver [15]. In contrast, *Acot5* is mainly expressed in white adipose tissue and brain, followed by intestine, albeit at low levels [19]. However, ACOT3 and ACOT5 are only present in rodents, as discussed below.

The mouse ACOT4 was identified as a 421 amino acid protein ending in -CRL [13,14], which was shown to be targeted to peroxisomes when expressed in human skin fibroblasts [14]. Kinetic characterization showed that the mouse ACOT4 was only active with succinyl-CoA ($V_{max} \approx 4$ μmol/min/mg protein, $K_m \approx 13$ μM) and glutaryl-CoA ($V_{max} \approx 1.1$ μmol/min/mg protein, $K_m \approx 37$ μM) [14], which are dicarboxylic acids produced as a result of β-oxidation of even and odd chain dicarboxylic acids, and glutaryl-CoA is also an intermediate in lysine and tryptophan metabolism. Expression of mouse *Acot4* mRNA was identified mainly in liver, kidney and proximal intestine, with at least the former two organs being involved in dicarboxylic acid formation and metabolism. As stated above, the human Type-I *ACOT* gene cluster revealed only one functional peroxisomal gene, *ACOT4*, which was a surprising finding, given that the mouse genome contained four genes coding for peroxisomal Type-I ACOTs. The human *ACOT4* protein contains a variant PTS1 of -PKL, which efficiently targets the protein to peroxisomes [17]. However, functional analysis revealed that the human ACOT4 protein in fact catalyzes the hydrolysis of the same substrates as the combined activities of mouse ACOT3, ACOT4 and ACOT5 (i.e. long-chain acyl-CoAs, succinyl-CoA/glutaryl-CoA and medium-chain acyl-CoAs), and it was proposed that convergent evolution of the human genome resulted in an enzyme that apparently adopted the activities of three distinct mouse enzymes [17]. The ACOT4 gene is found only in mammals (from dolphin (*Tursiops truncatus*) to human) and the protein seems to be peroxisomal in all species with -PKL being the most common PTS1, followed by -SKL, -CRL and -AKL. In human, analysis of the genome also revealed the presence of a further gene on chromosome 19q13.12, which showed 91% sequence identity to the human *ACOT4*

gene and contains a peroxisomal targeting signal of -PKL. However, this gene on chromosome 19 lacks introns and contains several stop codons, and is therefore apparently a non-functional pseudogene [17].

As pointed out in a recent review [20], ACOT3 and ACOT5 are only found in the mouse and the rat, suggesting that ACOT3 and ACOT5 are products of gene duplications after the rat–mouse split and are therefore the result of a divergent evolution. Evolution of new activities is often driven by a despecialization step followed by gene duplications and subsequent specialization of the new activities [21,22]. It therefore appears that ACOT4 represents an ancestor protein with 'promiscuous activity' that after gene duplications results in enzymes with higher specificity. Thus, the advantage of the promiscuous activity of human ACOT4 to catalyze the hydrolysis of a wider range of substrates may be at the cost of catalytic efficiency since the separate mouse ACOTs appear to be more efficient with higher V_{max} (with ≈ 7–20-fold higher specific activity in the mouse proteins compared to the activity with the corresponding substrates by human ACOT4) and similar or lower $K_{m's}$ [14,15,17].

This raises the question as to whether the apparent lack of long-chain acyl-CoA thioesterase activity in humans catalyzed by Type-I ACOTs is compensated for by other thioesterase enzymes. Notably, human ACOT1 (which has been identified as a cytosolic enzyme) and ACOT2 (identified as a mitochondrial enzyme) [17] contain a variant PTS1 (-SKV), which may function as a 'weak' peroxisomal targeting signal resulting in dual peroxisomal/cytosolic localization [23] or peroxisomal/mitochondrial localization. ACOT1 and ACOT2 catalyze the hydrolysis of acyl-CoAs of chain length C_{12}–C_{20} saturated acyl-CoAs, together with $C_{16:1}$ and $C_{18:1}$ with K_m values between 2 and 5 μM and V_{max} values ranging from 250 to 700 nmol/min/mg [17]. Therefore it is possible that both ACOT1 and ACOT2 may be partially peroxisomal and thus contribute long-chain acyl-CoA thioesterase activity in human peroxisomes.

The final peroxisomal enzyme encoded for by the Type-I gene cluster in mouse was recently identified as *Acot6* [16]. ACOT6 is a 419 amino acid polypeptide that is targeted to peroxisomes by a

Table 1
Characteristics of mouse peroxisomal acyl-CoA thioesterases and acyltransferases.

| Gene	Other aliases	Chromosome, accession number and protein family	C-terminal signal and confirmed peroxisomal localization [reference]	Acyl-CoA substrates
Acot3	PTE-Ia	12 D3 NP_599007 α/β hydrolase	-AKL [15,18]	Long-chain acyl-CoAs [15]
Acot4	PTE-Ib, Pte2b	12 D3 NP_599008 α/β hydrolase	-CRL [14,18]	Succinyl-CoA, glutaryl-CoA [14]
Acot5	PTE-Ic	12 D3 NP_663419 α/β hydrolase	-AKL [15]	Medium-chain acyl-CoAs [15]
Acot6	PTE-Id	12 D3 NP_766168 α/β hydrolase	-SKL [16,18]	Phytanoyl-CoA, pristanoyl-CoA [16]
Acot8	PTE-2, PTE1	2 H3 NP_573503 HotDog fold protein family	-SKL [18,29]	Long-, medium- and short-chain acyl-CoAs, bile acid-CoAs, branched chain acyl-CoAs [29,30].
Acot12	CACH-I, MGC105114, mCACH-1, CACH	13 C3 NP_083066 HotDog fold protein family	-SVL [18]	Short-chain acyl-CoAs (in rat) [49]
Baat	BACAT, BAT	4 B3 NP_031545 α/β hydrolase	-SQL [88]	Bile acid-CoAs [119], long-chain acyl-CoAs[a]
Acnat1		4 B3 ABE98441 α/β hydrolase	-SKL [93]	Long-chain and very long-chain acyl-CoAs [93]
Acnat2		4 B3 EU564338 α/β hydrolase	-SKL (Fig. 6)	Unknown

[a] Activity of the recombinantly expressed human BAAT enzyme.

carboxyterminal PTS1 -SKL. Mouse ACOT6 is active on phytanoyl-CoA and pristanoyl-CoA, suggesting a role in regulation of the metabolism of methyl-branched fatty acids [16]. Phytol, which is an important source of methyl branched fatty acids in mammals, enters the body via the diet and is metabolized to phytanic acid and subsequently is α-oxidized in peroxisomes to produce pristanoyl-CoA [24,25]. The identification of ACOT6 that hydrolyzes phytanoyl-CoA and pristanoyl-CoA to phytanic acid/pristanic acid and CoASH, raised questions as to the physiological function of ACOT6 in peroxisomes. *Acot6* mRNA in mouse showed highest expression in white adipose tissue and kidney, similar to the expression of acyl-CoA oxidase 3 (ACOX3), the suggested rate-limiting enzyme in the β-oxidation of methyl branched fatty acids (although ACOX2 may contribute significantly in kidney and particularly in liver). Although liver and kidney are involved in α-oxidation and β-oxidation of methyl branched fatty acids [26,27] the expression of ACOT6 and ACOX3 in white adipose tissue identi-fied a possible pathway for metabolism of phytanic acid and pristanic acid in peroxisomes in this tissue. The α-methyl group in pristanic acid is present in two stereoisomers, 2R and 2S, however only the 2S isomer undergoes β-oxidation [28], and therefore racemization of 2R pristanoyl-CoA to 2S pristanoyl-CoA is required before it can undergo three cycles of β-oxidation in peroxisomes, resulting in the production of DMN-CoA. DMN-CoA, in contrast to pristanoyl-CoA, is a substrate for ACOT8 [29,30] (as discussed below) or ACOT5 [15], or alternatively DMN-CoA can be transferred to carni-tine by the carnitine octanoyltransferase (CROT) for transport to the mitochondria and further oxidation [31]. Expression of the 2-methylacyl-CoA racemase (AMACR), which has a dual localization in peroxisomes and mitochondria, is low in white adipose tissue [16] and therefore the function of ACOT6 may be to hydrolyze excess

2R-pristanoyl-CoA to 2R-pristanic acid and CoASH. ACOT6 activity is insensitive to CoASH, suggesting that ACOT6 regulates intraperoxi-somal levels of phytanoyl-CoA and pristanoyl-CoA independent of CoASH levels. The released pristanic acid may exit the peroxisome to become esterified into triacylglycerols or alternatively trans-ported from white adipose tissue to liver or kidney for further oxida-tion (for review see [8]). Interestingly, ACOT6 does not show any preference for either the 2R or 2S isomer of pristanoyl-CoA, which would allow for the hydrolysis of accumulating 2R-pristanoyl-CoA. It is also possible that accumulated 2R-pristanic acid is reactivated via the per-oxisomal very long chain acyl-CoA synthetase inside peroxisomes to allow for β-oxidation [32], or to remove it from the β-oxidation pathway, and the resultant 2R-pristanic acid can be esterified into triacylglycerols or undergo β-oxidization in liver after racemization (for overview see Fig. 2).

In human, there is an ortholog of the mouse *Acot6* and searches in databases show that an ACOT6 gene is apparently found in all mam-mals, but not in non-mammalian species. The human *Acot6* is howev-er transcribed from the second exon, and translated from an ATG at the end of exon 2, therefore resulting in a 207 amino acid protein that contains a variant of the PTS1, -SKI, at its C-terminal end [17]. It is not clear whether this shorter protein is active since it only con-tains 15 amino acids N-terminal of the so-called nucleophilic elbow (located in exon 3 of ACOT proteins), which is a key structural compo-nent of the catalytic machinery in α/β-hydrolases, and therefore the shorter protein lacks the N-terminal domain that may be involved in the formation of the active site as discussed in Section 6.1. Although the mRNA is expressed in humans [17], the protein has not yet been detected in human peroxisomes. The carboxyterminal tripeptide -SKI present in human ACOT6 does not target the protein
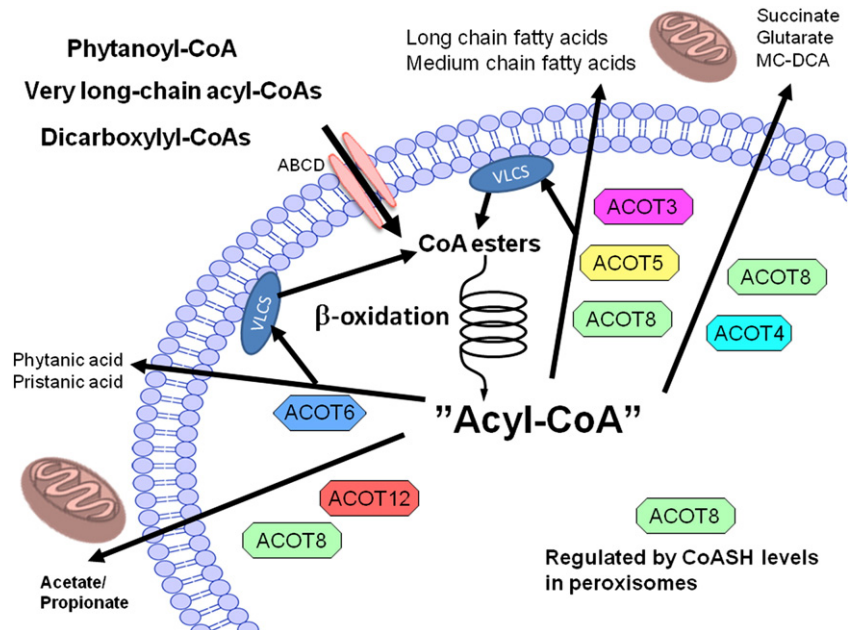
Fig. 2. Functions of acyl-CoA thioesterases in peroxisomes. Various acyl-CoA esters are transported into the peroxisome via ABCD transporters and undergo β-oxidation. The resultant products (shown as 'acyl-CoAs') are then substrates for ACOTs. Phytanoyl-CoA and pristanoyl-CoA can be hydrolyzed by ACOT6 resulting in the production of phytanic acid or pristanic acid, which can either be re-esterified to CoASH inside peroxisomes by very long chain acyl-CoA synthetase (VLCS), or are exported from peroxisomes and can be further esterified into triacylglycerols or be transported from white adipose tissue to liver for further β-oxidation in mitochondria. Short chain acyl-CoAs such as acetyl-CoA and propionyl-CoA are produced in peroxisomes by β-oxidation. Acetyl-CoA is released following each cycle of β-oxidation, whereas propionyl-CoA is produced by the β-oxidation of bile acid intermediates and methyl branched fatty acids, and in every second β-oxidation cycle of pristanic acid. Acetyl-CoA and propionyl-CoA are both substrates for ACOT12 and ACOT8, which following hydrolysis by ACOTs would be exported as acetate or propionate to cytosol or mitochondria. Long- and medium-chain acyl-CoAs produced by β-oxidation of very long-chain acyl-CoAs in peroxisomes can be hydrolyzed by ACOT3, ACOT5 or ACOT8 to release CoASH. These non-esterified long- and medium-chain fatty acids can either be re-esterified to CoASH inside peroxisomes by very long-chain acyl-CoA synthetase (VLCS) or exported from peroxisomes, and medium-chain fatty acids may be transported out of the peroxisome. Finally, ACOT4 and ACOT8 can hydrolyze succinyl-CoA and glutaryl-CoA to produce succinate and glutarate, which can be transported to mitochondria. Similarly ACOT8 can hydrolyze medium chain dicarboxylic acids (such as adipyl-CoA, suberyl-CoA, sebacyl-CoA and dodecanedioyl-CoA — MC-DCA) to free fatty acids, which can be exported from peroxisomes. ACOT8 is the only ACOT identified in peroxisomes that is regulated by CoASH and therefore the activity of ACOT8 will depend on the intra-peroxisomal CoASH levels, which will change under different physiological conditions.

to peroxisomes, but results in a cytosolic localization in human skin fibroblasts, when fused to green fluorescent protein (GFP) [17]. However, -SKI is conserved in ACOT6 in more than half of the compared mammalian ACOT6 genes (17/33 different mammalian genomes), followed by -SKL/-SRL (8/33), -SKM (6/33) and -NKM (2/33). The PTS1 variant -SKM was shown to function as a partial PTS1 signal, resulting in dual peroxisomal/cytosolic localizations in human skin fibroblasts [33], suggesting that ACOT6 would be at least partially localized to peroxisomes in several species. In spite of the apparent lack of peroxisomal localization of human ACOT6 in transfection experiments, it is still possible that ACOT6, as well as other proteins carrying the -SKI variant, may be at least partially peroxisomal. Other examples of functional non-consensus PTS1 are the bile-acid conjugating enzyme BAAT (carrying -SQL in the C-terminal end), which in proteomic analysis is present in rat liver peroxisomes [34], and hydroxyacid oxidase 1 (HAO1), which also ends -SKI [35]. In spite of weak interaction between -SKI-containing peptides and PEX5 [36], HAO1 efficiently localizes to peroxisomes in human skin fibroblasts [35] and is found in rat liver peroxisomes as analyzed by proteomic analysis [34]. Thus, in spite of the conflicting data regarding the functionality of -SKI as a PTS1, it cannot yet be excluded that human ACOT6 may be at least partially localized in peroxisomes. Whether the short form of ACOT6 folds into an active enzyme in humans and the effect on substrate specificity remains an open question as discussed below.

4. Characterization of peroxisomal Type-II acyl-CoA thioesterases

Peroxisomes contain two Type-II acyl-CoA thioesterases, ACOT8 and ACOT12. ACOT8 (which has been characterized under various aliases, see Tables 1 and 2) was originally identified in 1997 as a protein that binds to the HIV-1 Nef protein [37,38]. Cellular localization experiments demonstrated that the identified Nef-binding protein (ACOT8) is peroxisomal due to a C-terminal PTS1 (-SKL), and that co-expression of the Nef-binding protein with Nef resulted in a co-localization in peroxisomes (as further discussed below). Based on its homology to *Escherichia coli* thioesterase II (tesB) it was identified as a novel mammalian acyl-CoA thioesterase with a broad acyl-CoA chain-length specificity, which was further characterized in human and yeast [39], mouse and rat [29,30]. ACOT8 is a highly conserved protein that is expressed from bacteria to man, including yeast and plants but apparently not in insects. The bacterial homolog TEII, which shows about 40% sequence identity to human ACOT8, hydrolyzes medium- to long-chain acyl-CoAs [40].

The *Arabidopsis thaliana* genome contains two genes coding for peroxisomal ACOT8-like thioesterases (with C-terminal PTS1 signals of -AKL and -SKL, respectively), which show about 68% sequence identity to each other. One of these ACOTs was characterized in detail and shown to hydrolyze C_{12}–C_{20} saturated and unsaturated acyl-CoAs, but based on the expression pattern in plant tissues it was suggested that the function of this ACOT is probably not linked to fatty acid oxidation in plants [41].

A more in-depth kinetic characterization of the mouse ACOT8 showed two interesting features, namely an unusually broad acyl-CoA substrate specificity with ACOT8 being active on all tested acyl-CoAs ranging from two to twenty carbon atoms, saturated as well as unsaturated fatty acids, methyl-branched fatty acids and bile-acid intermediates, with the highest activity seen with the methyl-branched fatty acids and bile-acid intermediates [29,30]. The second feature is a strong inhibition of activity by CoASH [29], which is not seen with the Type-I ACOTs. The sensitivity to CoASH and the very broad substrate specificity suggests a role for this enzyme in regulation of intraperoxisomal acyl-CoA/CoASH levels (by sensing CoASH levels) in order to optimize the flux of fatty acids through the β-oxidation system (Fig. 2). In contrast to the peroxisomal Type-I ACOTs, ACOT8 show a broad tissue expression in mouse and human [29,37], and expression is regulated by fibrates and fasting via the peroxisome proliferator-activated receptor alpha (PPARα), suggesting a role for ACOT8 in regulation of fatty acid oxidation [29]. The human *ACOT8* gene is located on chromosome 20 (20q13.12), contains 6 exons and a putative peroxisome proliferator response element (PPRE) at −438 bp, suggesting that expression of the human gene is also under control of PPARα [29]. However, experimental or clinical data are sparse to unambiguously clarify the importance of this enzyme in lipid metabolism. So far there is no report on gene deletion or deficiency of *ACOT8* in mice or humans that is linked to impaired lipid metabolism. Also, the effect of deletion of the ACOT8 ortholog (Pte1) in yeast is not clear since it may cause a partial (about 50%) growth defect on oleate [39] and in another report, yeast cells grew on oleate without any obvious phenotype [42]. However, over-expression of human ACOT8 in human and murine cell lines as well as in transgenic mice was associated with peroxisome proliferation and increased lipid accumulation (determined as number of lipid droplets) [43]. The peroxisome proliferation was associated with increased amounts of catalase protein and increased levels of acyl-CoA oxidase 1 (*Acox1*) mRNA. All these effects required active ACOT8 protein and also that the protein was correctly targeted to peroxisomes, as the effects were abolished in cells transfected with ACOT8ΔSKL or an ACOT8 mutant with strongly reduced enzymatic activity. Interestingly, the over-expression was also associated with increased PPARγ expression, suggesting that very high ACOT8 activity causes lipid accumulation and possibly a more adipogenic phenotype. Taken together, these data indicate that products of ACOT8 activity mediate peroxisome proliferation, possibly via PPARα, and that

Table 2
Characteristics of human peroxisomal acyl-CoA thioesterases and acyltransferases.

Gene	Other aliases	Chromosome, accession number and protein family	C-terminal signal and confirmed peroxisomal localization [reference]	Acyl-CoA substrates
ACOT4	PTE-Ib, PTe-2b, hPTE-I	14q24.3 NP_689544 α/β hydrolase	-PKL [17]	Succinyl-CoA, glutaryl-CoA, long-chain acyl-CoAs [17]
ACOT8	PTE-2, hTE, hACTEIII, PTE1	20q12–q13.1 NP_005460 HotDog fold protein family	-SKL [39]	Medium- and long-chain acyl-CoAs[a] [39]
ACOT12	CACH-1, MGC105114, CACH	5q14.1 NP_570123 HotDog fold protein family	-SKF[b]	Short-chain acyl-CoAs [48]
BAAT	BACAT, BAT	9q22.3 NP_001692 α/β hydrolase	-SQL [88]	Bile acid-CoAs, long- and very long-chain acyl-CoAs [89] (Fig. 5)

[a] Only tested with medium and long-chain acyl-CoAs but given the broad substrate specificity of the mouse and rat ACOT8, it is likely that human ACOT8 can hydrolyze a broad range of acyl-CoAs, including bile acid-CoAs, branched chain acyl-CoAs and short-, medium- and long-chain acyl-CoAs.
[b] Not confirmed as peroxisomal in human. Mouse ACOT12 contains a -KSVL at its carboxyterminal and has been identified in peroxisomes (see Table 1).

strong over-expression of ACOT8 inhibits peroxisomal β-oxidation to such an extent that lipids accumulate in droplets. In the future it would be of interest to challenge ACOT8 over-expressing cells with various fatty acids/lipids that are substrates for peroxisomal β-oxidation to explore the lipid specificity of the observed phenotypes and to delete the gene in e.g. mouse, and study the possible phenotypes in various tissues since the other peroxisomal ACOTs show highly tissue-specific expression patterns.

A somewhat unexpected possible function of ACOT8 (Pte1) in *Saccharomyces cerevisiae* is the apparent involvement in the efficient metabolism of straight and methyl-branched short- to medium-chain fatty acids [44]. This study showed that polyhydroxyalkanoate synthesis and cell growth was somewhat impaired in *pte1Δ* strain cultured in the presence of 10-cis-heptadecenoic acid, and severely impaired when 8-methyl-nonanoic acid was added to the medium. Translation of these findings to the mammalian ACOT8 may suggest a role in regulation of methyl-branched acyl-CoA levels, in line with the high activity of ACOT8 with methyl-branched fatty acids and bile acid intermediates [29,30].

4.1. Possible functions for ACOT8 in interaction with other proteins

As discussed above, ACOT8 was originally identified in 1997 as a protein that interacted with the HIV-1 Nef [37,38]. Nef is a 27-kDa myristoylated cytosolic protein that associates with the plasma membrane and other intracellular vesicle surfaces and is important for high virus load and disease progression. Nef has multiple functions as it down-regulates CD4 (cluster of differentiation), which is one of the receptors for HIV, and the cell surface expression of major histocompatibility complex class I (MHC-I). Nef also alters the state of T-cell activation and macrophage signal transduction pathways and impairs the formation of immunological synapses. The interaction with ACOT8 relocalizes Nef to peroxisomes and mediates CD4 down-regulation. There was a strong correlation between the interaction with ACOT8 and the ability of Nef alleles to induce CD4 down-regulation [38], and abolishment of the protein–protein interaction by point mutations in the core region of Nef abrogates the ability of Nef to down-regulate CD4. However, not all CD4 down-regulating Nef alleles interacted with ACOT8, leaving the role of ACOT8 in HIV infection uncertain [45]. If Nef is targeted to peroxisomes via its interaction with ACOT8, it is unlikely that Nef will exit peroxisomes since it appears that 'once a protein is inside the peroxisome it remains there' [46]. In a recent review ('Viruses exploiting peroxisomes'), Lazarow proposes some possible mechanisms by which ACOT8 may participate in the down-regulation of CD4 [47]. While N-terminal myristoylation is irreversible, palmitoylation of cysteine residues is reversible and can be deacylated by acyl protein thioesterases. It is possible that Nef-ACOT8 oligomers formed when Nef is expressed during viral infection may not be completely targeted to peroxisomes, but have multiple cellular locations and alternatively Nef may target ACOT8 to the plasma membrane. If ACOT8 can function as a protein thioesterase (which has apparently not been tested yet), ACOT8 may deacylate Lck (lymphocyte-specific protein tyrosine kinase p56[lck]), which contains an N-terminal myristoylation and is palmitoylated on two cysteines that is essential for its association with the plasma membrane. Lck binds to the cytoplasmic tail of CD4 and thereby preventing CD4 internalization. Removal of Lck would thus promote endocytosis of CD4, which would be further accelerated by Nef, however, the possible role of ACOT8 (or some other thioesterase) in CD4 down-regulation needs to be further substantiated experimentally.

4.2. The ACOT12 is a peroxisomal Type-II thioesterase in mouse that hydrolyzes short chain acyl-CoAs

ACOT12 was first isolated from rat liver as an extra-mitochondrial, predominantly cytosolic, cold labile acetyl-CoA thioesterase that is active on short-chain acyl-CoAs [48]. The enzyme shows unusual physicochemical properties as ATP and ADP affects oligomerization (monomers/dimers/tetramers/hexamers) and activity (ATP-stimulated and ADP-inhibited) of this enzyme [49,50]. Further subcellular fractionation experiments showed that the enzyme is partially localized to peroxisomes, at least in rat liver, and treatment with clofibrate increases the activity about 4-fold [51]. Further evidence for a peroxisomal localization was obtained in mouse kidney by proteomic characterization [18]. Molecular cloning of the mouse, rat and human ACOT12 enzymes revealed a novel structure with no sequence similarity to the Type-I enzymes, but shows 46–50% sequence identity to mitochondrial ACOT11 [52]. ACOT11 and ACOT12 consist of three domains, two (duplicated) thioesterase domains, referred to as Hot-Dog domains, and a C-terminal Steroidogenic acute regulatory related lipid transfer (Start) domain. Structurally these proteins are referred to as double HotDog fold proteins and the acyl-CoA thioesterases represent the largest subfamily within the HotDog superfamily [10]. In spite of the very low sequence similarity of ACOT8 to ACOT11 and ACOT12, ACOT8 is also a double HotDog fold protein, but lacking the Start domain. Structural analysis of the Start domain of ACOT11 (STARD14) suggests that it likely binds fatty acids, compatible with the function of ACOT11 in hydrolyzing medium- to long-chain acyl-CoAs [53]. Sequence analysis of the rat and mouse ACOT12 enzymes reveals a variant C-terminal PTS1 signal of -KSVL that is likely responsible for the partial peroxisomal localization in these species. However, the human ACOT12 protein ends -STF (as in all primates) and of all species compared it appears that ACOT12 is only localized to peroxisomes in rat and mouse. Thus, ACOT8 seems to be the only short-chain acyl-CoA thioesterase activity in human peroxisomes, although the human ACOT8 appears to have lower short-chain acyl-CoA thioesterase activity compared to the mouse enzyme [29,39]. As ACOT12 is localized in peroxisomes in mouse and shows a different tissue expression pattern compared to peroxisomal carnitine acetyltransferase (*Crat*), the latter which results in the production of acetyl/propionylcarnitine esters (C_2/C_3-carnitine esters), it was proposed that ACOT12 and CRAT provide alternative/complementary systems for transport of short-chain products of β-oxidation out of peroxisomes [19] (Fig. 2). It is therefore likely that transport of short-chain β-oxidation products is different in mouse and human peroxisomes.

5. Role of acyl-CoA thioesterases and acyltransferases in metabolite transport

Peroxisomes have important functions in the degradation of a broad spectrum of fatty acids, in particular fatty acids that are poorly oxidized by mitochondria. Medium-chain fatty acids may be transported across the peroxisomal membrane as free acids and are activated to the corresponding CoA ester inside the peroxisome [54]. In contrast, long-chain fatty acids are activated outside the peroxisome, followed by transport of the CoA esters across the peroxisomal membrane via ABCD1 or ABCD2 (for review, see [55]). The peroxisomal β-oxidation system mainly produces chain-shortened acyl-CoAs and acetyl/propionyl-CoA, which are then transported out of the peroxisome. It is hypothesized that the combined activities of ACOT3, ACOT4, ACOT5, ACOT6 and ACOT8 result in the hydrolysis of a wide range of fatty acyl-CoA esters, allowing transport of shorter fatty acids out of the peroxisome, while long-chain β-oxidation substrates/products hydrolyzed by ACOT5 or ACOT8 may be re-activated inside peroxisomes by the very long chain acyl-CoA synthetase oriented to the peroxisomal matrix [32] (as outlined in Fig. 2). While ACOT3–6 have specialized functions (acyl-CoA specificities), ACOT8 is a highly promiscuous thioesterase that hydrolyzes 'all' acyl-CoAs. However, the activity of ACOT8 is under strong regulation by CoASH, suggesting a role in sensing CoASH levels. So how are these metabolites transported out of the peroxisome? Peroxisomes contain carnitine acetyl and carnitine octanoyltransferases (CRAT and CROT) that can convert short- to medium-chain acyl-CoAs to the corresponding carnitine ester for transport to mitochondria [56]. Recent work

has shown that the peroxisomal membrane is permeable to small solute molecules while larger, bulky molecules require membrane transporters ([57,58], as reviewed in [59] and in this current Special Edition [60]), which has implications for the transport of both substrates and metabolites 'in-and-out' of peroxisomes. The peroxisomal membrane contains three ABCD transporters that mediate the uptake of various acyl-CoA esters (reviewed in [61]) and an ATP/ADP/AMP transporter [62]. It was recently shown that Pxmp2 forms a membrane channel that enables free diffusion of small solutes (<300 Da), and partially restricted diffusion of solutes of >300 Da<500–600 Da [58]. This implies that many, if not most, of the products of peroxisomal ACOTs, ACNAT1 and the nudix hydrolase enzymes (NUDT7 and NUDT19 acyl-CoA diphosphatase enzymes regulating acyl-CoA/CoASH homeostasis in peroxisomes [63,64]) may be 'freely' transported out of peroxisomes via Pxmp2. For a detailed review on this subject, see [60] in this Special Issue.

6. Relationship of the crystal structure of human ACOTs to their functions

6.1. Crystallization of Type-I acyl-CoA thioesterases

Sequence analysis, secondary structure predictions and site-directed mutagenesis experiments identified the Type-I ACOTs as members of the α/β-hydrolase protein superfamily, members of which contain a lipase/esterase active site motif that is characterized by a conserved serine residue (acting as a nucleophile) located in a GlyXaaSerXaaGly motif (GxSxG) [65]. In addition these α/β-hydrolases contain a conserved histidine residue and an aspartic/glutamic acid residue, which together with the nucleophile constitute a catalytic triad in this protein family. The first crystal structure of a Type-I ACOT, human ACOT2 that was published in 2009 [66] revealed an N-terminal and a C-terminal domain. The N-terminal domain contains a seven-stranded β-sandwich and the C-terminal domain having the characteristic α/β-hydrolase fold of this Type-I thioesterase family. The Type-I ACOTs are encoded for by three exons [13] of which the first exon contributes the N-terminal domain and the second and third exons the C-terminal domain. In spite of the fact that the function of the N-terminal domain is unknown, the first and the (short) second exons are highly conserved suggesting important functions for the N-terminal domain.

The X-ray crystallographic structure of human ACOT4 was determined in 2009 by the Structural Genomics Consortium at Karolinska Institutet, Stockholm, Sweden. While coordinates are available in the PDB (PDB ID: 3K2I), the detailed analysis of this structure will be published elsewhere (Siponen et al., manuscript in preparation) and a preliminary analysis of this structure is described. The structure of human ACOT4 exhibits the same two-domain organization as other Type-I ACOT family members and the high degree of sequence conservation within this subfamily has revealed a high degree of structural similarity within the Type-I ACOTs. Resembling the cytosolic human ACOT1 (Siponen et al., unpublished results) and mitochondrial human ACOT2 [66], human ACOT4 is structured into an N- and a C-terminal domain (Fig. 3). Similar to other family members, the N-terminal domain of human ACOT4 contains a seven-stranded β-sandwich, with one sheet having three short anti-parallel β-strands and the other sheet having four longer anti-parallel β-strands. This domain was previously reported in the crystallographic structure of human ACOT2 as having limited structural similarity with other proteins [66].

The C-terminal domain of human ACOT4 has a central, mostly-parallel (with the exception of one strand), eight-stranded β-sheet that is surrounded by five α-helices. This domain also contains two short, three-stranded β-sheets, the first being an anti-parallel sheet on the surface, and the second partially covering the active site pocket. This domain also contains several protruding loops, notably in the vicinity of the active site pocket. The C-terminal domain also contains the catalytic triad Ser232-His360-Asp326 (as highlighted in Fig. 3). These triad residues are located near the C-terminal end of the

parallel β-strands in the central β-sheet. While the catalytic triad is contained within this domain, the active site is actually located in a large pocket at the interface between the N- and C-terminal domains and several long loops from both domains contribute to the formation of this active site. Although still very little is known concerning the molecular level details of substrate specificity, it is likely that one or more of these loops is involved in substrate recognition and/or binding. The structural data does not yet however explain the 'dual' substrate specificity of human ACOT4 (i.e. the long chain acyl-CoA activity, and the succinyl-CoA/glutaryl-CoA activity) [17].

The human ACOT4 protein forms a dimer and analysis of the dimeric interface present in the crystallographic structure of human ACOT4 shows that the buried surface area is rather big, more than 1400 Å, which is within the sizes observed for physiological protein–protein interactions. Inspection of the interface revealed that several amino acids specific to human ACOT4 participate in the dimerization, forming a hydrophobic pocket that is solvent exposed in other Type-I family members. This pocket is made up of residues Phe90, Phe286 and Pro371 in the human ACOT4. This multimerization has also been confirmed in solution by analytical size exclusion chromatography and appears to be specific to ACOT4 (Siponen and Berglund, unpublished results).

Based on the structure of ACOT2 and ACOT4, human ACOT6 would be missing the N-terminal domain as well as the first 4 strands of the C-terminal domain but it was suggested that the remaining C-terminal domain could still form a stable structure [66]. However, in the absence of the N-terminal domain the active site would be 'open' and thus the substrate specificity may be quite different from the full-length (mouse) ACOT6 [66]. It would be of interest to solve this issue, however attempts to express human ACOT6 as a recombinant fusion protein have so far failed to produce soluble protein (Hunt and Alexson, unpublished results).

6.2. The crystal structure of peroxisomal Type-II enzymes

The Type-II ACOTs (ACOT7 through ACOT13) all contain a similar HotDog fold domain organization. While ACOT13 is predicted to
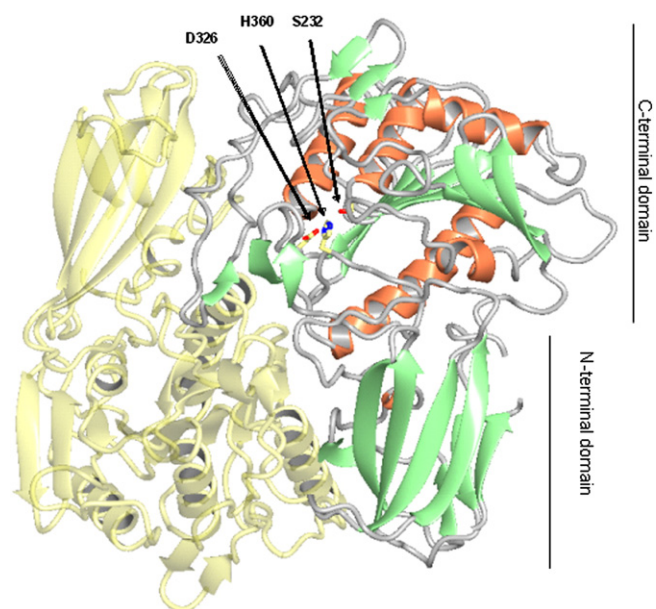
Fig. 3. X-ray crystallographic structure of human ACOT4. The overall structure of human ACOT4 is a dimer (with one dimer labeled in yellow and the second dimer in green/orange) with the monomer being composed of an N-terminal and a C-terminal domain. The N-terminal domain is composed of a seven-stranded β-barrel while the C-terminal domain is a typical α/β hydrolase fold. The catalytic triad of serine–histidine–aspartic acid (S232, H360, D326) present in the C-terminal domain is also indicated.

contain a single HotDog domain, ACOT7 through ACOT12 contain two tandem HotDog domains. To date, structural characterization of Type-II ACOTs by X-ray crystallography has revealed the structures of mouse ACOT7 [67], human ACOT12 (PDB ID: 3B7K; Lehtiö et al., unpublished results) and the START domain of human ACOT11 [53]. So far, no structural data exists for any mammalian ACOT8. However, while the Type-II enzymes show low similarity at the sequence level, they show surprisingly high structural similarity. In this review we therefore provide an overview on the current structural knowledge of Type-II thioesterases and link this knowledge to understanding the structural organization of human ACOT8.

Mouse ACOT7 and human ACOT12 show a sequence identity of only 24.8%. Despite this low identity, the determination of their respective structures by X-ray crystallography revealed the same structural arrangement of the two HotDog domains. Each individual HotDog fold features a central 5-stranded anti-parallel β-sheet "bun" enveloping a five-turn α-helix "sausage", two of which are side by side resulting in a 10 stranded β-sheet core enclosing 2 α-helices. This architecture referred to as the double HotDog fold, then associates into a trimeric quaternary structure (Fig. 4A). Both these structures also contain an additional C-terminal external helix, which packs on the opposite side of the central β-sheet.

The active site architecture appears similar in both mouse ACOT7 and human ACOT12. Although both these enzymes contain tandem HotDog domains of limited sequence similarity, they contain two structurally analogous active sites per protein monomer. Based on mouse ACOT7 studies, only one of the two analogous sites is active, resulting in a single active site per set of fused HotDog domains, suggesting that the second site has more of a regulatory function [67]. Preliminary analysis of the human ACOT12 structure also supports this half-site reactivity hypothesis since only one coenzyme A molecule is present at the junction of the tandem HotDog domains of a human ACOT12 monomer.

Although detailed structural information is available for these Type-II enzymes, only preliminary analysis of catalytic residues has been performed. Although Asn24 and Asp213 have been identified as required for activity in mouse ACOT7, the precise catalytic mechanisms of both mouse ACOT7 and human ACOT12 remain elusive. However, a recent X-ray crystallographic study of *Bacillus halodurans* ACOT (BhACOT) reveals significant ligand-induced conformational changes ([68]; PDB ID: 1VPM, 3SPS). Examination of the structure of BhACOT, with respect to mouse ACOT7 and human ACOT12, reveals a similar hexameric organization. Furthermore, the human ACOT12 bound to coenzyme A reveals the formation of a β-bulge at the junction of both HotDog domains, similar to that observed in BhACOT upon ligand binding. This may suggest a common mechanism of action for the hexameric Type-II enzymes.

The structurally determined *E. coli* thioesterase II (TEII) shares 39.9% identity with human ACOT8, the highest percentage of identity for any structurally determined Type-II enzyme ([69]; PDB ID: 1C8U). Notably, the overall double HotDog fold described in the structures of mouse ACOT7 and human ACOT12 is conserved in TEII. The X-ray crystallographic structure of TEII revealed a similar 10-stranded β-sheet core enclosing 2 α-helices, with an active site formed at the interface of both HotDog repeats (Fig. 4B). The overall structure shows two major differences with the mammalian enzymes described above. Firstly, its overall quaternary structure appears to be dimeric forming a tetramer of HotDog domains as opposed to the hexamer observed in both mouse ACOT7 and human ACOT12. Secondly, while the main core of the protein monomer remains the same, the placement of additional α-helices differs significantly, which results in differential active site placement (Fig. 4B).

In view of the above, some preliminary conclusions based on the crystallographic structures of mouse ACOT7, human ACOT12 and TEII toward the overall architecture of human ACOT8 can be made. Analysis of secondary structure predictions for human ACOT8 using the PSIPRED server [70], indicates a near identical β-sheet and α-helix topology as per TEII. Furthermore, 3D structure predictions performed with MODELLER [71] and 3D-JIGSAW (http://bmm.cancerresearchuk.org/~3djigsaw/), predict a similar organization of human ACOT8 to TEII with the main double HotDog fold being present with minor differences in placement of additional α-helices surrounding the main double HotDog domain. Whether human ACOT8 will form a hexameric or a tetrameric organization of HotDog folds remains difficult to predict. However, the presence of a conserved catalytic triad between TEII (Asp204-Gln278-Thr228) and human ACOT8 (Asp232-Gln306-Ser256), in addition to a near 40% sequence identity suggests that human ACOT8 adopts a tetrameric dimer of the HotDog domain dimer organization as does TEII. A dimeric organization of ACOT8 is further supported by the absence of any higher molecular mass thioesterase activity upon size-exclusion chromatography of matrix proteins from rat liver peroxisomes [72]. Although clear-cut conclusions concerning the precise mode of action of human ACOT8 cannot be drawn from 3D structure predictions, the structural information may explain the CoASH-sensitivity of ACOT8. The binding of CoASH in the structure of ACOT12 suggests that acyl-CoAs bind via the CoASH moiety and that CoASH may compete for this binding and this may be a common feature of Type-II ACOTs.

7. Acyltransferases in peroxisomes involved in bile acid and fatty acid amidation (conjugation)

7.1. The bile acid-CoA:amino acid N-acyltransferase conjugates bile acids in peroxisomes

Bile acids are synthesized in liver and are involved in the intestinal absorption of fats and fat-soluble vitamins and are also a means of excess cholesterol elimination from the body. Bile acids are produced
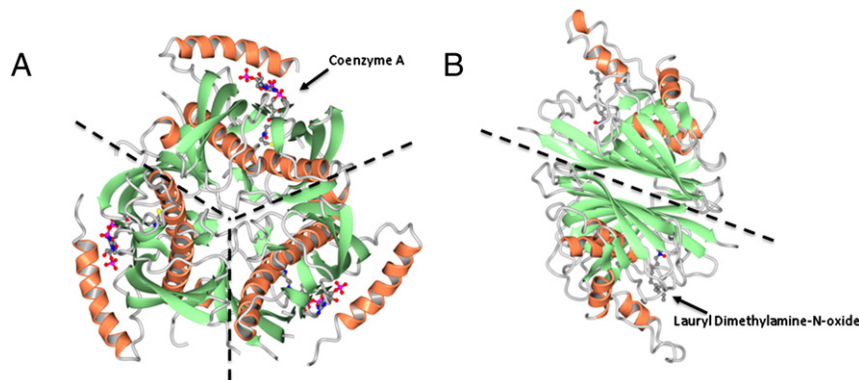
Fig. 4. Structural representation of Type-II thioesterases. (A) Human ACOT12 (PDB ID: 3B7K) is represented as a Type-II thioesterase that hexamerizes forming a trimer of tandem HotDog folds. (B) *Escherichia coli* TEII (PDB ID: 1C8U) is represented as a Type-II thioesterase that tetramerizes forming a dimer of tandem HotDog folds. Ligands used in co-crystallization (coenzyme A in (A) and lauryl dimethylamine-N-oxide in (B)) and observed in X-ray crystallographic structures are marked, indicating active site positions.

from cholesterol by a series of enzymatic steps involving multiple cellular compartments, with the final steps being the β-oxidation of the side-chain of the C27 bile acid precursors trihydroxycoprostanoyl-CoA and dihydroxycoprostanoyl-CoA to choloyl-CoA and chenodeoxycholoyl-CoA (C24 bile acids) and a final conjugation (or amidation) of the C24 primary bile acids to glycine or taurine (for review see [73,74]). This final conjugation step is carried out by BAAT in peroxisomes (Fig. 7). The intracellular localization of BAAT has been widely discussed and early studies in 1967 identified bile acid conjugation activity in the lysosomal fraction (or possibly the peroxisomal fraction) of human liver [75]. Later studies by Kase et al. identified BAAT activity in microsomal and peroxisomal fractions in rat liver [76], with other groups identifying activity in microsomes, cytosol or peroxisomes in rat, mouse and human [77–82]. In view of the apparent disparate data on expression of BAAT, it was hypothesized that two pathways for bile acid conjugation exist, one in peroxisomes which conjugates the CoA esters of the de novo synthesized primary bile acids (choloyl-CoA and chenodeoxycholoyl-CoA), and a second pathway in cytosol involved in the re-conjugation of secondary bile acids (deoxycholic acid, and lithocholic acid) recycled back to the liver via the enterohepatic circulation [77]. Human very long chain acyl-CoA synthetase homolog 2 (VLCS- and also known as BACS, bile acid-CoA synthetase) activates primary bile acids such as cholic acid [83] and secondary bile acids (deoxycholic acid and lithocholic acid) to their CoA esters [84] in the cytosol or endoplasmic reticulum, also supporting two pathways of bile acid conjugation. Rembacz et al. recently showed that cholic acid (a primary bile acid) shuttles through peroxisomes in hepatocytes for taurine (and possibly glycine) conjugation [85]. Since taurocholic acid was detected in isolated peroxisomes and readily accumulated in hepatocytes, apparently transport of conjugated bile acids across the peroxisomal membrane (via a as yet unambiguously identified bile salt transporter, [86]), as well as across the plasma membrane via the bile salt export pump (BSEP), is rate-limiting in this process. It would be of interest to determine if secondary bile acids shuttle through peroxisomes for conjugation by BAAT and the debate as to whether BAAT is also localized in cytosol to conjugate recycled secondary bile acids remains open.

The rodent and human BAAT proteins all contain the variant peroxisome type-1 targeting signals of -SQL and the functionality of this PTS1-variant has been a matter of debate. A peptide ending in -SQL flanked by a serine in the −4 position (-SSQL) was found to interact very weakly with human Pex5p [87], while a peptide containing a basic amino acid residue in the −4 position (-KSQL) interacted strongly with Pex5p, which also targeted green fluorescent protein (GFP) to peroxisomes in HeLa cells [87]. In accordance with these findings, transfection of HeLa cells with human and mouse wild-type BAAT-GFP fusion proteins resulted in a cytosolic localization of the fusion proteins, while mutation to a basic residue in the −1 position (-KSQL) targeted BAAT to peroxisomes [46]. Recent studies using GFP expression of BAAT and advanced microscopy techniques have identified that BAAT is cytosolic in some cell lines and is peroxisomal in other cell lines. In human and rat primary hepatocytes, BAAT is localized in peroxisomes [88], whereas expression studies in human fibroblasts resulted in a cytosolic localization of BAAT [88,89]. Competition studies in rat primary hepatocytes were carried out using the human BAAT-GFP fusion protein and a peroxisomal marker DSRed-SKL, which resulted in a cytosolic localization of BAAT, indicating that the -SQL non-consensus targeting signal (e.g. in BAAT) is not as efficient as the -SKL and that competition between these will occur, resulting in a mislocalization of the non-consensus containing protein. These results suggest that the -SQL non-consensus targeting signal may have different efficiencies in different cell lines or may need interaction with some other protein(s) in addition to Pex5p. It thus appears that the targeting of BAAT to peroxisomes may be more complicated than previously thought and that targeting may depend on the saturation of the import machinery and competition between 'consensus' and 'non-consensus' PTS1s.

There are a number of studies identifying regulation of BAAT at mRNA or protein level in mouse or rat but data on regulation of

gene expression in human is more limited. In 1996, BAAT (then known as kan-1) mRNA was measured by Northern blot in liver samples of resected patients suffering from hepatocellular carcinoma (HCC) and it was suggested that BAAT could be used as a prognostic marker in HCC patients following resection [90]. Human BAAT has recently been identified as being strongly associated with microRNA miR492 expression in hepatoblastoma (HB), the most common childhood malignant liver tumor [91]. Three novel single nucleotide polymorphisms (SNPs) in the BAAT gene have been identified in the Japanese population, two of which are silent mutations and one of which results in the nonsynonymous Arg201Pro mutation [92]. However the functional implications of this latter mutation on BAAT activity have not been determined and Arg201 is not conserved in the related ACOT and ACNAT proteins. Shonsey et al. recently identified that the histidine residue localized in the AGH motif at position 362 in BAAT which is part of the catalytic triad of the protein (this His is also conserved in all ACOT and ACNAT enzymes [89,93]), is modified and forms adducts with 4-hydroxynonenal (4-HNE) at physiological concentrations [94]. 4-HNE is an electrophilic lipid generated by the oxidation of polyunsaturated long-chain fatty acids, linking BAAT to models of oxidative stress. The formation of these 4-HNE adducts with His 362 resulted in a dose-dependent reduction of BAAT activity, with activity reduced by 70% in the presence of 16 μM 4-HNE [94]. O'Byrne et al. also identified that mutation of His 362 to Gln all but abolished human BAAT activity [89].

7.2. The role of peroxisomes in the production of amidated fatty acids

In recent years, several amides of fatty acids that are structurally related to endocannabinoids (for review see [95]), have been isolated from mammalian sources. One group of these fatty acid amides are the N-acyl amino acids, which have generated renewed research interest in part due to their structural relationship to the endocannabinoids [96,97]. N-Acyl amino acids are fatty acids conjugated (or amidated) to amino acids such as glycine, taurine, alanine, serine etc. and fatty acid conjugates with the common 20 amino acids have now been identified in mammalian tissues [96]. N-Acyl amino acids are emerging as putative signaling molecules with a wide range of biological activities (for review see [98]).

Peroxisomes contain enzymatic pathways that produce taurine and glycine conjugated (amidated) fatty acids. Human BAAT can conjugate long- and very-long chain acyl-CoAs ($C_{12:0}$–$C_{26:0}$) to glycine [89] and taurine (Fig. 5), resulting in the production of N-acyl amino acids, although the fatty acid conjugation activity of BAAT represents about 10% of the bile acid conjugating activity of this enzyme. In addition, two acyl-CoA:amino acid N-acyltransferases (ACNAT1 and ACNAT2) have been identified in mouse peroxisomes in 2007 [93]. These two gene products show 55% sequence identity to BAAT and are 95% identical to each other at amino acid level. ACNAT1 and 2 are both targeted to peroxisomes and contain the consensus peroxisomal targeting signal -SKL ([18,93] and Fig. 6). Recombinant ACNAT1 catalyzes the conjugation of long-chain and very-long-chain saturated acyl-CoA esters (C_{16}–C_{24}-CoAs) to taurine, resulting in the production of N-acyl taurines (NATs) [93]. Interestingly, this enzyme had no conjugation activity with the amino acid glycine and very low conjugating activity toward bile acid-CoAs, making it a specific fatty acid taurine conjugating enzyme. The substrate specificity for ACNAT2 has not yet been identified, but given the high sequence identity between ACNAT1 and ACNAT2 (95%), it is speculated that ACNAT2 may conjugate unsaturated fatty acids to taurine.

Supporting the characterization of ACNAT1 in 2007 as a taurine-conjugating enzyme producing NATs, these NATs were identified invivo in the mouse [99,100]. The identification of ACNAT1 in peroxisomes, together with the identification of fatty acyl-CoA taurine conjugating
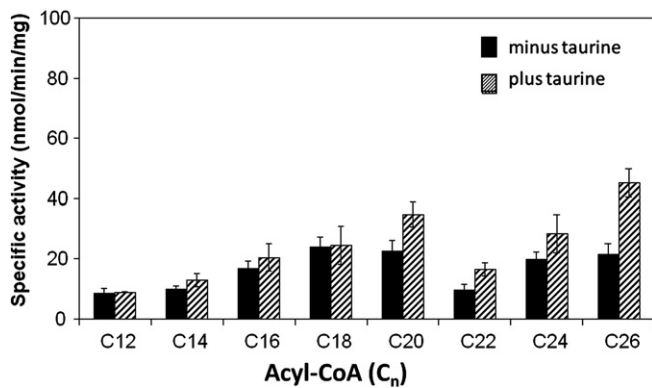
Fig. 5. The bile acid-CoA:amino acid *N*-acyltransferase (BAAT) conjugates fatty acids to taurine. Recombinant human BAAT was expressed in BL21(DE3)pLysS cells and purified as described in [89]. BAAT (2–5 μg) was incubated with 40 μM of various saturated acyl-CoA esters as indicated, plus or minus 50 mM taurine. Reactions were measured spectrophotometrically as described in [89]. Thioesterase activity is shown in black bars and activity in the presence of taurine (thioesterase and taurine conjugation activity) is shown in hatched bars. Data shown are means ± S.E.M. (n = 2/3).

activity for BAAT (Fig. 5, showing the activity ± taurine) suggested that peroxisomes could be involved in the production of NATs. A comprehensive study of the levels of NATs in mouse tissues was recently carried out, showing that NATs (chain lengths $C_{16:0}$, $C_{18:0}$, $C_{18:2}$, $C_{20:4}$, $C_{22:0}$ and $C_{22:6}$) are present in plasma, brown adipose tissue, heart, white adipose tissue, spleen, lung, testis, kidney and liver [101]. *N*-Arachidonoyl taurine has been detected in liver and kidney under normal conditions, with levels of *N*-arachidonoyl taurine and other NATs increasing substantially in the fatty acid amide hydrolase (FAAH) knockout mouse model [102]. The ACNAT1 identified in mouse peroxisomes cannot conjugate arachidonoyl-CoA (or other unsaturated acyl-CoA esters) to taurine and is active only with saturated acyl-CoAs, and it may be that ACNAT2 is involved in the taurine conjugation of unsaturated acyl-CoAs (Fig. 7). Long et al. have suggested the existence of a transport system for NATs that transfers these lipids from liver to other tissues e.g. spleen [101]. Therefore NATs produced at one particular site could potentially be transported by the plasma to other organs and may be transported out of peroxisomes via the yet unidentified bile acid transporter protein, which can transport both glyco- and tauro-conjugated bile acids across the peroxisomal membrane [86]. The physiological functions and effects of increased levels of NATs are not well studied to date. *N*-Arachidonoyl taurine activates the transient receptor potential vanilloid (TRPV) receptor 1 and 4 ion channels [102]. Very recently,

NATs (particularly *N*-oleoyl taurine and *N*-arachidonoyl taurine) have been shown to have anti-proliferative effects in prostate carcinoma PC-3 cells [103] and treatment of pancreatic β-cells with NATs results in insulin release (Waluk et al., manuscript in preparation).

The role of peroxisomes and BAAT in the production of glycine conjugated bile acids is well established (as described in Section 7.1). However, BAAT also has low glycine conjugating activity toward fatty acids [89]. Several *N*-acyl glycines have been identified in vivo including *N*-arachidonoyl glycine (NAGly — arachidonic acid conjugated to glycine), *N*-oleoyl glycine, and *N*-palmitoyl glycine [104–107]. NAGly was identified in rat brain, spinal cord, small intestine and glabrous skin, with low levels detected in liver [106]. NAGly is synthesized by several pathways, including an enzymatic conjugation of arachidonoyl-CoA with glycine by BAAT [89], enzymatic conjugation of arachinonoyl-CoA with glycine by glycine *N*-acyltransferase like-2 (GLYATL2) localized in the endoplasmic reticulum [108], synthesis by cytochrome c in the presence of arachidonoyl-CoA and hydrogen peroxide [109], and finally by oxidation of anandamide (an endocannabinoid) by alcohol and aldehyde dehydrogenases [110]. A recent review on the identification and functions of *N*-acyl glycines [104] reveals that these lipid molecules act as ligands for a number of G-protein coupled receptors such as GPR18 [111], GPR92 [112] and GPR72 [113].

It is worthwhile noting that the connection between peroxisomes and the production of signaling lipids such as *N*-acyl glycines and NATs has not yet been studied in detail and it would be of interest to examine the levels of *N*-acyl glycines and NATs in patients with Zellweger syndrome or other peroxisomal disorders, to firmly establish the role of peroxisomes in the production of these 'endocannabinoid-like' lipids.

## 8. Human diseases associated with peroxisomal ACOTs and BAAT

To date, there is very limited information on the role of ACOTs in human health. An isoform of ACOT7 (initially characterized as brain cytosolic acyl-CoA hydrolase, BACH, but with mitochondrial and cytosolic isoforms) was found to be deranged in the hippocampus of patients with mesial temporal lobe epilepsy [114] and the mitochondrial isoform of ACOT7 was decreased in mitochondria from patients with mitochondrial fatty acid oxidation defects [115]. A recent mapping of genes associated with human mitochondrial disorders identified ACOT9 (a mitochondrial ACOT) associated with MEHMO syndrome (Mental retardation, Epileptic seizures, Hypogenitalism, Microcephaly and Obesity) [116], an X-linked disorder which includes symptoms such as mental retardation, seizures, obesity, hypogonadism. However, so far, no specific peroxisomal disorders or single enzyme deficiencies have been identified in human for ACOT proteins.

In human, many bile acid abnormalities exist in patients with peroxisomal disorders and these are covered in detail in a recent review
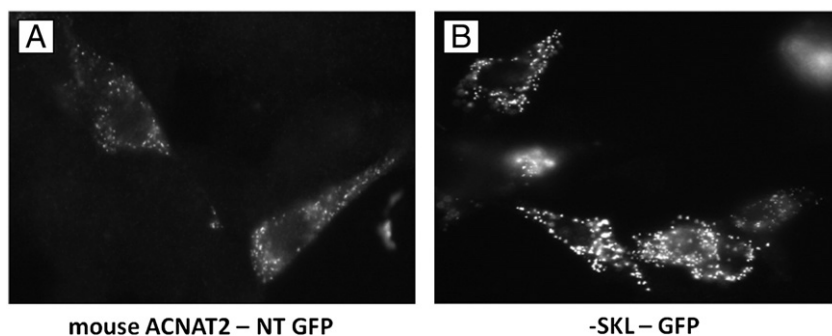
Fig. 6. Mouse ACNAT2 is localized in peroxisomes. The open reading frame of mouse *Acnat2* was cloned in frame with green fluorescent protein (GFP) and expressed in human skin fibroblasts. A GFP fusion protein containing -SKL (the consensus peroxisomal type 1 targeting signal) was also expressed in human skin fibroblasts to label peroxisomes. Immunofluorescence microscopy was carried out using a Tritc-labeled anti-GFP antibody. (A) Mouse ACNAT2 and (B) -SKL-GFP.
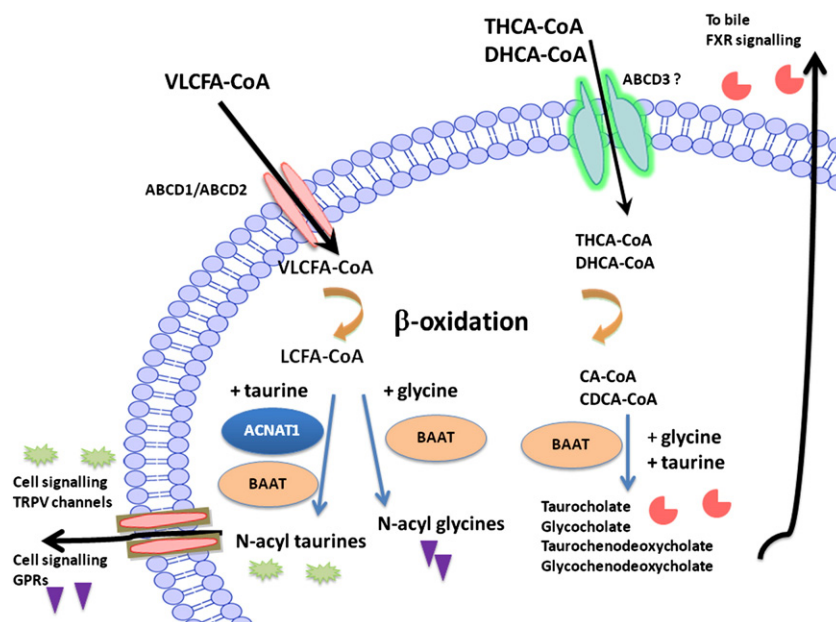
Fig. 7. The role of acyltransferases in the production of *N*-acyl glycines and *N*-acyl taurines in peroxisomes. The precursors to primary bile acids, trihydroxycoprostanoyl-CoA (THCA-CoA) and dihydroxycoprostanoyl-CoA (DHCA-CoA) are transported into peroxisomes, possibly via ABCD3. Once inside the peroxisome, they undergo one cycle of β-oxidation to produce chenodeoxycholoyl-CoA (CDCA-CoA) or choloyl-CoA (CA-CoA) (the CoA esters of the primary bile acids). BAAT then conjugates CDCA-CoA or CA-CoA to glycine or taurine, resulting in the production of glycocholate, taurocholate, glycochenodeoxycholate or taurochenodeoxycholate. These primary bile acids are then exported from the peroxisome (by a yet unidentified transporter) to bile. Primary bile acids can also act as ligands for the farnesoid X receptor (FXR). Saturated and unsaturated very long-chain fatty acids (VLCFA-CoA) are imported into peroxisomes as CoA esters via the ABCD1 and ABCD2 transporters. These fatty acids then undergo chain shortening via the peroxisomal β-oxidation system, producing long or medium-chain acyl-CoAs. Very long-chain, long-chain or medium-chain acyl-CoAs can then be conjugated to glycine (by BAAT) or to taurine (by ACNAT1 or BAAT) (for simplicity, only the conjugation of long-chain acyl-CoAs (LCFA-CoA) is shown in the figure). Taurine conjugated fatty acids can function as cell signaling molecules for e.g. the Transient Receptor Potential Vanilloid (TRPV) receptors TRPV1 and TRPV4. Glycine conjugates function in activation of G-protein coupled receptors (GPRs).

[74]. However, to date only one human disease has been identified where BAAT is directly implicated. BAAT is involved in the inheritance of familial hypercholanemia [117]. Familial hypercholanemia is characterized by increased levels of serum bile acids, itching and fat malabsorption. A study on members of the Amish population identified a mutation in BAAT, 226A-G mutation, resulting in a M76V mutation, which is a highly conserved methionine in all ACOT, ACNAT and BAAT proteins. Individuals with the BAAT M76V mutation had associated lower levels of conjugated bile acids and serum analysis revealed that bile acids were mainly unconjugated [117]. BAAT is a member of the α/β hydrolase protein family and contains an active site with a variant of the α/β hydrolase GlyXaaSerXaa lipase motif (GxSxG motif). In human, rat and mouse BAAT the motif contains a cysteine as the nucleophilic residue in a SerXaaCysXaaGly (SxCxG) motif, together with a histidine and aspartic acid, which form the catalytic triad [89,118]. Mutation of the active site cysteine (SxCxG) to alanine (SxAxG) abolishes BAAT activity [89], however it is not known what effect the M76V mutation will have on the activity of BAAT. This mutation would be located in the first exon of the BAAT/ACOT/ACNAT proteins, which contributes the N-terminal domain, whereas all amino acids in the active site are located in the third exon [13,17]. As discussed above (in Section 6.1) the function of the N-terminal domain is not yet understood, but since this domain contributes to the active site it is conceivable that mutation of the highly conserved Met76 may distort the structure of this domain and thereby affect the active site of BAAT. It would be of interest to create the same mutation to assess the effect on structure and activity of ACOTs and BAAT.

9. Future perspectives

Although our knowledge of ACOT and acyltransferase enzymes in peroxisomes has expanded greatly in the past number of years, there is still a large body of work to be completed in respect of the physiological effects of the lack of ACOT or acyltransferase enzymes in-vivo, in human or animal models, and their interactions with other cellular proteins. Further structural characterization of both Type-I and Type-II ACOTs would help our understanding of their functions with regard to substrate specificity and allosteric regulation. Overexpression, silencing and gene knockouts should provide more insight into the functions of ACOTs in health and disease.

Acknowledgements

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