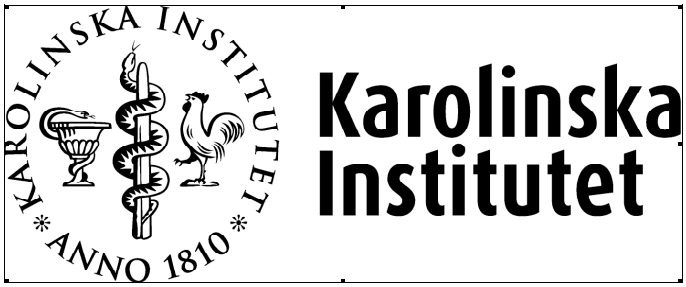
From the Department of Laboratory Medicine,
Division of Clinical Chemistry,
Karolinska Institutet, Stockholm, Sweden

FUNCTIONAL CHARACTERIZATION OF A PEROXISOMAL ACYLTRANSFERASE GENE FAMILY

Sarah-Jayne Reilly



Stockholm 2008

Published by Karolinska University
Printed by Larserics Digital Print AB

ISBN 978-91-7409-205-9

Dedicated to my family

"There's no point in crying about what you did laughing"

- *My Mum*

ABSTRACT

Peroxisomes are cell organelles that play an important role in the metabolism of a variety of lipids including very long chain fatty acids, dicarboxylic acids, bile acids, and xenobiotic carboxylic acids. The metabolism of lipids in peroxisomes requires the concerted efforts of many different enzymes, which includes members of the type I acyl-CoA thioesterase/acyltransferase protein family and the nudix hydrolases.

We have identified two new peroxisomal members of the thioesterase/acyltransferase protein family, which we have named acyl-CoA:amino acid *N*-acyltransferase 1 (ACNAT1) and 2 (ACNAT2). ACNAT1 acts as an acyltransferase that efficiently conjugates long-chain and very long-chain fatty acids to taurine; these novel metabolites are known as *N*-acyltaurines (NATs) and can allow the excretion of fatty acids as taurine conjugates, or alternatively these NAT may have roles in cell signaling. While we have not yet established an activity for ACNAT2 we hypothesize that it also functions as an acyltransferase, but with different substrate specificity to ACNAT1. The type I acyl-CoA thioesterase/acyltransferase family contains nine members in total that display two different types of activities, one is hydrolysis of fatty acyl-CoAs and the other is the conjugation of bile acids or fatty acids to glycine and/or taurine. These proteins belong to the α/β hydrolase family in which a highly conserved structure, called the nucleophilic elbow is very important for activity. By using site directed mutagenesis we demonstrate the importance of the amino acids in the nucleophilic elbow for activity, and propose a hypothesis for the evolution of this gene family.

Coenzyme A (CoASH) is an important cofactor in peroxisomal metabolic pathways. Peroxisomes contain their own pool of CoASH and an unresolved issue is how this pool is regulated. It has been suggested that nudix hydrolase 7α (NUDT7α) regulates the peroxisomal CoASH pool by metabolizing CoASH. However, our study shows that NUDT7α is mainly active towards a wide range of acyl-CoAs, with highest activity towards medium-chain acyl-CoAs and lower activity with CoASH. This suggests that NUDT7α, together with the acyl-CoA thioesterases, has a function in the regulation of peroxisomal acyl-CoA/CoASH levels.

LIST OF PUBLICATIONS

I. **Reilly S.J.**, O'Shea E.M., Andersson U., O'Byrne J., Alexson S.E.H. and Hunt M.C.
A peroxisomal acyltransferase in mouse identifies a novel pathway for taurine conjugation of fatty acids.

FASEB J. (2007) 21 (1): 99-107.

II. **Reilly S.J.**, Buch C., Alexson S.E.H. and Hunt M.C.
Characterization and metabolic regulation of a novel peroxisomal acyltransferase in mouse.

Manuscript.

III. O'Byrne J., **Reilly S.J.**, Hunt M.C. and Alexson S.E.H.
Divergent evolution of the nucleophilic elbow in an acyl-CoA thioesterase gene family defines esterase and acyltransferase activity.

Manuscript.

IV. **Reilly S.J.***, Tillander V.*, Ofman R., Alexson S.E.H. and Hunt M.C.
The nudix hydrolase 7 is an acyl-CoA diphosphatase involved in regulating peroxisomal coenzyme A homeostasis.

* Shared first authorship

J. Biochem. In press. Epub 2008 Sep 17. doi:10-1093/jb/MVN 114

Other publications not included in this thesis:

Glantz A., **Reilly S.J.**, Benthin L., Lammert F., Mattsson L.A. and Marschall H.U.

Intrahepatic cholestasis of pregnancy: Amelioration of pruritus by UDCA is associated with decreased progesterone disulphates in urine.

Hepatology (2008) 47(2): 376-9.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ACOT	Acyl-CoA thioesterase
ACGNAT	Acyl-CoA:glycine *N*-acyltransferase
ACNAT	Acyl-CoA:amino acid *N*-acyltransferase
ACOX	Acyl-CoA oxidase
ACS	Acyl-CoA synthetase
BAAT	Bile acid-CoA:amino acid *N*-acyltransferase
BACS	Bile acid-CoA synthetase
CDCA	Chenodeoxycholic acid
CoASH	Coenzyme A
DBP	D-specific bifunctional protein
DHCA	Dihydroxycholestanoic acid
DMP	Discovery metabolite profiling
FA	Fatty acid
FAA	Fatty acid amide
FAAH	Fatty acid amide hydrolase
HNF4α	Hepatocyte nuclear factor 4 alpha
LCFA	Long chain fatty acid
MCFA	Medium chain fatty acid
NAG	*N*-acylglycine
NAT	*N*-acyltaurine
NUDT	Nudix hydrolase
PEX	Peroxin
PPARα	Peroxiome proliferator activated receptor alpha
PTS	Peroxisomal targeting signal
THCA	Trihydroxycholestanoic acid
VLCFA	Very long-chain fatty acid
X-ALD	X-linked adrenoleukodystrophy
ZS	Zellweger syndrome

INTRODUCTION

Peroxisomes

Peroxisomes are cell organelles that belong to the microbody family, together with glyoxysomes and glycosomes. They were first described in 1954 in a thesis from the Karolinska Institutet by a Swedish graduate student, Johannes Rhodin, in which he identified small single membrane-bound organelles in rat renal tissue (1). It was more than 10 years later before they received their name "peroxisomes" from the work of Christian de Duve and co-workers. The name arose due to the fact that these organelles contain hydrogen peroxide producing oxidases and catalase that degrade hydrogen peroxide into molecular oxygen and water (2). Since then the list of functions associated with peroxisomes has grown quite extensively. One of the more important discoveries in peroxisome research was the identification of a fatty acid β-oxidation system in mammalian peroxisomes (3). Lipid metabolism is considered to be one of most essential functions of mammalian peroxisomes and more than half of the 50 plus enzyme activities that have been described in mammalian peroxisomes play a role in lipid metabolism. It is now well established that peroxisomes have many metabolic roles that include not only the β-oxidation of fatty acids but also α-oxidation, glyoxylate metabolism, ether-phospholipid synthesis, isoprenoid metabolism and bile acid synthesis (for review see (4)). It has even been suggested that peroxisomes are also involved in cholesterol biosynthesis but this has recently been under debate (5-8). However, it was recently shown that enzymes involved in the presqualene segment of cholesterol synthesis are localized in peroxisomes (9).

Peroxisomal disorders

The importance of peroxisomes is underlined by the existence of a group of genetic diseases in humans, which were first described by Sidney Goldfischer in 1973 (10, 11). They are low in occurrence but are usually very severe and often without any treatment. Peroxisomal disorders can be divided into two groups – the peroxisomal biogenesis disorders and the single peroxisomal enzyme deficiencies.

The peroxisomal biogenesis disorders are characterized by the absence of functional peroxisomes and are comprised of the Zellweger syndrome (ZS), neonatal adrenoleukodystrophy (NALD), infantile Refsum disease (IRD) and rhizomelic

chondrodysplasia punctata (RCDP) type 1 (for review see (12)). Symptoms common to all three are liver disease, neurodevelopmental delay, retinopathy and deafness. Patients with ZS usually die within the first year of life, while those with NALD have less severe clinical phenotypes than the ZS and normally die in late infancy. IRD is considered to have the mildest phenotype and is characterized by hearing impairment, retinal degeneration and mild psychomotor retardation. These patients can survive longer than the second decade of life. In RCDP type 1, there is a defect in import of peroxisomal proteins containing a peroxisomal targeting signal type 2 (PTS2), which results in calcific stippling of multiple joints and severe mental and growth retardation. Most patients die in the first year or two of life but some survive into the second decade. The range of disease abnormalities may be a result of a corresponding range of peroxisome failures; that is, in severe cases of ZS, the failure is nearly complete, while in IRD, there is some degree of peroxisome activity.

The second group of disorders are the single peroxisomal enzyme deficiencies, and these are grouped according to the peroxisomal metabolic pathway they affect. The pathways affected include α and β-oxidation, ether phospholipid synthesis, glyoxylate detoxification and hydrogen peroxide metabolism (for review see (13)). This group includes X-linked adrenoleukodystrophy (X-ALD), and Refsum's disease. Refsum's disease was first described in the 1940s by Sigvald Refsum (14) and is biochemically identifiable by the accumulation of phytanic acid in plasma and tissues (15). Refsum's disease can be treated by restricting the intake of phytanic acid and its precursors in the diet. The most common single peroxisomal disorder however is X-ALD with an incidence rate of 1:21,000 males in the USA (16). It is caused by a mutation in ABCD1 gene (ATP-binding cassette, sub-family D (ALD), member 1) (17), which codes for the ABC half transporter ALDP (Adrenoleukodystrophy protein). Mutation of this tranporter leads to a deficiency in the uptake of very long-chain fatty acids (VLCFAs) into peroxisomes. The consequence of this is that the breakdown of VLCFAs via peroxisomal β-oxidation cannot occur (18). X-ALD is biochemically characterized by elevated levels of VLCFAs in plasma and tissues, and these are believed to be disruptive to the structure and stability of certain cells, especially those associated with the central nervous system and the myelin sheath (the fatty covering of nerve fibres).

Biogenesis of peroxisomes and peroxisomal protein import

Peroxisomes are single membrane bound organelles that vary in size from 0.1-1μm and in cells their numbers range from less than one hundred to more than one thousand per cell (19, 20). The origin of peroxisomes is a controversial issue. The discovery that peroxisomal proteins are synthesized on free polyribosomes in the cytoplasm and then imported into peroxisomes led to the "growth and division model", with the biogenesis of new peroxisomes coming from pre-existing organelles (19). This has been the prevailing concept for the past two decades. In peroxisomal biogenesis, 32 proteins have been identified and these are known as peroxins (PEXs). PEXs are involved in the recruitment of lipids for peroxisomal membrane formation, insertion of proteins into the peroxisomal membrane, import of matrix proteins and the fission and inheritance of peroxisomes (for review see (21)).

Studies in yeast and mammals have recently challenged the "growth and division" model. The peroxins Pex3p, Pex16p and Pex19p are required to maintain the peroxisomal membrane and loss of function results in the absence of peroxisomes in yeast cells. However, when the wild-type genes were reintroduced into yeast strains lacking these PEX proteins, peroxisomes reappeared (22-27). This raised the question as to where did these new peroxisomes come from? The dictum 'omnis membrane e membrana' (Günther Blobel, Nobel Prize 1999) says that a membrane system must be present that can regenerate the peroxisomes. The above studies and others implicate the ER, since Pex3p and Pex19p were observed to initially localize to the ER before maturing into import competent peroxisomes, indicating that the ER is the source of the newly synthesized organelle (25). Peroxisomes can rapidly adapt to environmental changes and cellular demands and this can result in an increase in peroxisome number and size.

Fibrates, a group of synthetic ligands that activate the peroxisome proliferator activated receptor α (PPARα), result in an increase in size and number of peroxisomes and the transcriptional induction of enzymes involved in β-Oxidation (28). The PPARα belongs to the nuclear receptor superfamily. Three isoforms have been described so far, PPARα, PPAR-δ/β and PPARγ. They were identified in the 1990s in rodents and named after their property as peroxisome proliferators (29). The PPARs bind to DNA as a heterodimer with the retinoid x receptor (RXR) at peroxisome proliferator response

elements (PPREs) (30). PPARα has been shown to be a key regulator of lipid metabolism. PPARα is expressed in various tissues in rodents and humans such as liver, heart, kidney, small intestine and brown adipose tissue (31-33). It is activated by both natural and synthetic ligands, and also by conditions such as fasting (34). The natural ligands include various free fatty acids (35) and acyl-CoAs (36), whose levels can be regulated by the action of the various members of the ACOT family (enzymes that hydrolyze acyl-CoAs to free fatty acids and coenzyme A).

Import of peroxisomal matrix proteins

Peroxisomes do not contain DNA, which means that peroxisomal matrix proteins are encoded for by nuclear genes, and are synthesised on free cytosolic ribosomes. A striking feature of peroxisomes is their ability to post translationally import these fully folded matrix proteins (37). The import of the proteins is based on pathways that involve various targeting signals, receptor proteins and import machinery. The whole process can be broken down into 4 simple steps – proteins destined for the peroxisomal matrix are firstly recognised in the cytosol by a receptor, this then results in the docking of the receptor-protein complex on the peroxisomal membrane. The complex is then translocated across to the luminal site of the membrane, where it is disassembled and the protein is released into the peroxisomal lumen. Finally, the receptor is recycled back to the cytosol. The most common targeting signal used is the peroxisomal targeting signal 1 (PTS1), which is used by over 90% of peroxisomal matrix proteins. It is a carboxyterminal tripeptide sequence of serine-lysine-leucine (-SKL) or variants thereof [(S/C/A)(K/R/H)L] (38-40). The second targeting signal is known as the peroxisomal targeting signal 2 (PTS2), and although it is mainly found at the N-terminal, it can function internally. The PTS2 is a bi-partite signal of (R/K)(L/V/I) X5 (H/Q)(L/A) (41) and variants thereof (42).

Functions of peroxisomes

As mentioned above, peroxisomes play an important role in lipid metabolism and one of the central features of this organelle is fatty acid oxidation. This thesis will focus on the metabolism of fatty acids and bile acids in peroxisomes. It should be noted however, that peroxisomes are involved in a number of other pathways, including oxygen metabolism, amino acid metabolism and glyoxylate metabolism.

β-Oxidation

The major pathway for fatty acyl-CoA breakdown is via β-oxidation. In yeast and plants this pathway is exclusively peroxisomal but in mammals it is peroxisomal and mitochondrial. β-Oxidation of fatty acids in both organelles occurs via four recurring steps; [1] dehydrogenation, [2] hydration, [3] a second dehydrogenation, and [4] thiolysis, yielding as products a 2 carbon chain-shortened acyl-CoA and acetyl-CoA (see Fig. 1 for an overview). Both mitochondria and peroxisomes are involved in the degradation of medium and long chain saturated and unsaturated fatty acids but the two systems differ regarding substrate specificity, enzymology and also energy production. The peroxisomal β-oxidation pathway is solely responsible for the oxidation of very long-chain fatty acids, long-chain dicarboxylic acids, leukotrienes, prostaglandins, carboxylic derivatives of some xenobiotics, isoprenoid-derived fat soluble vitamins, bile acid intermediates and pristanic acid (which comes from α-oxidation of phytanic acid) (for review see (4)). In mitochondria fatty acids are completely oxidized, however in peroxisomes, β-oxidation is only partial and most substrates only undergo two to three rounds of β-oxidation, resulting in chain shortening (43).

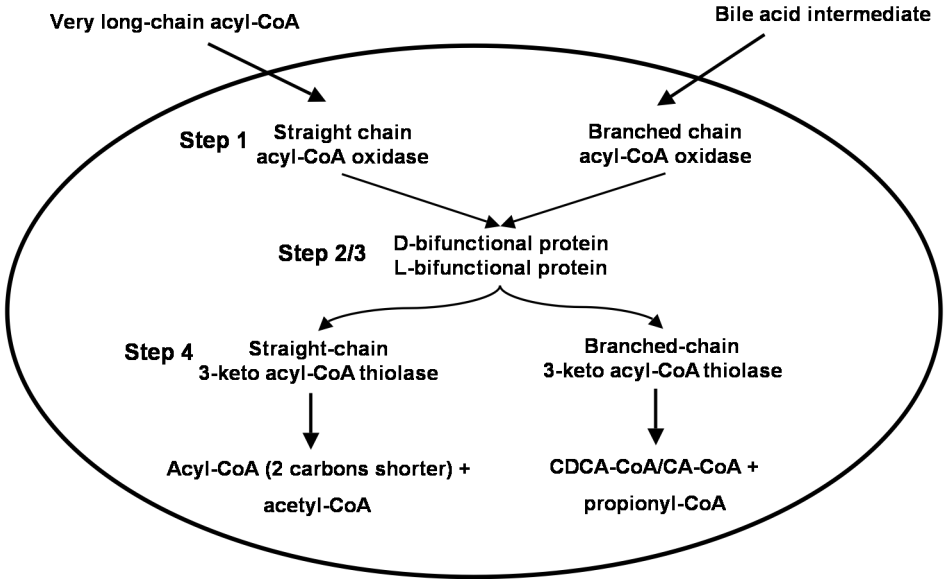


Fig. 1. β-Oxidation of fatty acids and bile acid intermediates in peroxisomes. Fatty acids and bile acid intermediates are activated to their CoA esters by acyl-CoA synthetases before undergoing β-oxidation in peroxisomes. One round of fatty acid β-oxidation, results in an acyl-CoA that is 2 carbons shorter plus acetyl-CoA, while in bile acid β-oxidation, the products are CDCA-CoA or CA-CoA and propionyl-CoA. CDCA-CoA, chenodeoxycholoyl-CoA; CA-CoA, choloyl-CoA.

The first step in peroxisomal β-oxidation is catalyzed by acyl-CoA oxidases (ACOX). In mouse and rat peroxisomes there are three ACOXs with different substrate specificities. ACOX1 is active on straight chain acyl-CoAs of varying chain length (44, 45), ACOX2 is active towards bile acid intermediates and ACOX3 is active on 2-methyl-branched chain fatty acids (46). In humans there are two ACOXs, ACOX1 is active towards a range of straight chain fatty acyl-CoA esters, while the human ACOX2 catalyzes the same reactions as mouse ACOX2 and ACOX3 (47). The next reactions in the peroxisomal system are a hydration and a second oxidation step, mediated by two multifunctional enzymes (MFE), which catalyze the same reaction but with different stereospecificity. Two MFEs exist in mammals, an L-specific bifunctional protein (LBP), also known as MFE-1 (48) and a D-specific bifunctional protein (DBP) also known as MFE-2 (49-51). MFE-2 is important not only for fatty acid metabolism but also bile acid synthesis (see below).

The final reaction of β-oxidation is the thiolytic cleavage of 3-ketoacyl-CoA to acetyl-CoA and an acyl-CoA shortened by two carbons, catalyzed by one of three ketoacyl-CoA thiolases - thiolase A, thiolase B or sterol carrier protein X (SCPx). Thiolase A and B are active on similar substrates - straight chain acyl-CoAs, with the main difference being that thiolase A is constitutively expressed, while thiolase B expression can be induced by peroxisome proliferators (52-54). SCPx is involved in the cleavage of 2-methyl branched chain substrates and is involved in the breakdown of pristanic acid and the synthesis of bile acids (55). Humans have two thiolases, one is a straight chain thiolase and the other is an ortholog of SCPx. As mentioned earlier, peroxisomal β-oxidation acts as a chain shortening system, but what happens to these chain shortened acyl-CoAs? It is believed that they may be transferred to carnitine and transported to the mitochondria for complete oxidation (56), or that they are substrates for other enzymes such as the acyl-CoA thioesterases and nudix hydrolases (see below).

Bile acid metabolism

Bile acid production is the predominant mechanism to remove cholesterol from the body. Approximately 500 mg of cholesterol is converted to bile acids each day in the human liver. Bile acid synthesis involves modifications of the cholesterol ring structure, β-oxidative cleavage of the side chain and finally conjugation (amidation) to

an amino acid. The 17 enzymes involved in this pathway are predominantly expressed in liver within different subcellular compartments. The initial steps in the classical pathway of bile acid synthesis are involved in the conversion of cholesterol into the bile acid intermediates 3α,7α-dihydroxycholestanoic acid (DHCA) and 3α,7α,12α-trihydroxycholestanoic acid (THCA) (for review see (57)). To continue along the pathway both DHCA and THCA need to be activated to their CoA ester. Two enzymes can activate these compounds, the bile acyl-CoA synthetase (BACS), also known as FATP5, which is localized in the endoplasmic reticulum and is believed to be responsible for activating primary and secondary bile acids (58). The second enzyme is the very long-chain acyl-CoA synthetase, which is localized in both the ER and peroxisomal membrane (58, 59). The activated DHCA-CoA and THCA-CoA are then transported into the peroxisome and here the first step is the conversion of the (25R)-stereoisomer into the (25S)-isomer by α-methylacyl-CoA racemase (AMACR) (60), before undergoing one round of peroxisomal β-oxidation (see Fig.1 for an overview).

In humans, the β-oxidation of bile acid intermediates commences with the branched chain acyl-CoA oxidase (BCOX, also known as *ACOX2*) (47), while in mice and rat, it is THCA-CoA oxidase (encoded for by *Acox2*) that is responible (61, 62). The next two steps are catalyzed by the DBP, which contains hydratase and dehydrogenase activities and results in the production of 24-keto-THCA-CoA and 24-keto-DHCA-CoA (50). These products are cleaved to choloyl-CoA or chenodeoxycholoyl-CoA by SCPx, with the release of propionyl-CoA (see Fig. 1). The final step is the conjugation of the bile acid to taurine or glycine by another peroxisomal enzyme, the bile acid-CoA:amino acid *N*-acyltransferase (BAAT), which allows for secretion of the conjugated bile acid into bile.

Entry of substrates into peroxisomes

Cell organelles such as mitochondria and peroxisomes allow the cell to compartmentalize specific chemical reactions. As mentioned earlier peroxisomes are involved in a number of metabolic pathways, and this requires the transport of molecules across the peroxisomal membrane, including substrates, metabolites and co-factors.

Fatty acid activation

Fatty acids are "activated" by thioesterification to coenzyme A (CoASH) to form acyl-CoAs. The formation of acyl-CoAs allows the otherwise "non-reactive" fatty acid to participate in various metabolic pathways, such as β-oxidation. This reaction is catalyzed by a group of enzymes known as acyl-CoA synthetases (ACS) (for review see (63)). The ACSs can be divided into five sub-families and this is based on the chain length of their preferred acyl group – they are short-chain acyl-CoA synthetase (ACSS), which activates C_2 to C_4; medium-chain (ACSM), C_4 to C_{12}; long-chain (ACSL), C_{12} to C_{20}; and very long-chain (ACSVL), C_{18}-C_{26}. Members of the ACSVL family are also involved in bile acid synthesis (58).

With regard to the substrates of peroxisomal metabolic pathways, it seems that the peroxisomal membrane contains a long-chain and very long-chain ACS. Out of five mammalian ACSLs (long chain), ACSL1 is the only member of this family that is believed to be peroxisomal but it does not seem to be an intrinsic membrane protein (64) and it's active site faces the cytosol. This suggests that substrates of ACSL1 are activated to CoA esters outside the peroxisome and subsequently transported in e.g. via the ABC transporters (see below).

In humans and rodents the ACSVL (very long-chain) family has six members and all have been shown to be capable of activating C_{24} fatty acids but differ in tissue expression, subcellular localization and substrate specificities (for review see (63)). ACSVL1 is mainly expressed in liver and kidney and has been localized to the ER and peroxisomes, and was shown to activate C_{16} and C_{24} fatty acids and the branched chain fatty acids phytanic and pristanic acid (59). In peroxisomes of HepG2 cells, human ACSVL1 was shown to face the peroxisomal matrix suggesting it is involved in intraperoxisomal activation of substrates (59). It can also activate trihydroxycholestanoic acid (THCA), which implicates it in *de novo* bile acid synthesis (58), however, it is unknown whether this occurs in the peroxisome or ER. Another member that should be mentioned is the ACSVL6 (also known as BACS, VLCS-H2 and FATP5) that is a bile acid-CoA ligase (synthetase) whose expression is liver specific. It is found primarily in the ER and its preferred substrates are the primary (cholic and chenodeoxycholic acids) and secondary (deoxycholic and lithocholic acids) bile acids and the bile acid precursor THCA. This suggests that *de*

novo bile acid synthesis precursors are activated to the CoA ester before being transported into the peroxisome.

The peroxisomal membrane and metabolite transport

The permeability of the peroxisomal membrane has been a subject of controversy for more than four decades. It has been speculated that peroxisomes contain non-selective channels that are freely permeable to solutes or/and they have selective membrane transporters (65). Both points of view have experimental support, and it was recently shown that that the peroxisomal membrane is permeable to solutes that have a molecular mass less than 300 Da (66). This means that the membrane seems to restrict the access of larger compounds, such as co-factors which are required for the various metabolic processes within the peroxisome e.g. NAD/H, NADP/H and CoASH (see below), which suggests that peroxisomes contain their own pool of cofactors (66). One peroxisomal transporter that has been widely studied is the yeast Ant1p. This protein was first identified in a proteomic screen and showed amino acid similarity with a mitochondrial ADP/ATP translocator. Ant1p has been studied extensively *in vitro* and has been shown to be an adenine nucleotide transporter, involved in the transport of ATP and AMP in peroxisomes (67, 68). The human orthologue of the yeast Ant1p is PMP34 and was shown to have similar catalytic activities (68).

The ABC transporters

A group of proteins that are implicated in peroxisomal metabolite transport are the adenosine-triphosphate-binding cassette (ABC) transporters (for review see (69)). These are integral membrane proteins that transport a wide range of substrates from ions to proteins. The peroxisomal ABC transporters are ABC-half transporters, which means that they dimerize into fully functional complexes. Four ABCs have been detected in mammalian peroxisomes, although functional roles for all have yet to be established. They are ALDP (ABCD1), ALDR (ABCD2), PMP70 (ABCD3) and PMP69 (ABCD4), and it is believed that different combinations of these transporters may transport different substrates into peroxisomes (69).

Co-immunoprecipitation studies indicate that there is homodimerization of ALDP, heterodimerization of ALDP with PMP70 or ALDRP, and heterodimerization of

ALDRP with PMP70 (70). Human ALDP was first identified in 1993 (18) and was recently shown to function as a homodimer that is involved in the import of various long- and very long-chain acyl-CoAs (including C_{16}, $C_{18:1}$, C_{22}, $C_{24:6}$) into the peroxisome (71). As mentioned earlier, mutations in the ABCD1 gene, which codes for ALDP, are associated with the human disorder X-ALD (17). The function of the remaining three transporters remains largely unresolved but it is believed that ALDR function overlaps with that of ALDP (72), since overexpression of ALDR in cells lacking ALDP results in an increase in the oxidation of C_{24} and C_{26} fatty acids (73). PMP70 has been implicated in the transport of long-chain acyl-CoAs into peroxisomes (74), and it was recently proposed that it is also involved in the transport of the bile acid precursors THCA-CoA and DHCA-CoA, and branched-chain fatty acids into peroxisomes (75) (see Fig. 2 for an overview). Although PMP69 was identified in 1997 there is still no information regarding its physiological role in peroxisomes (76, 77).

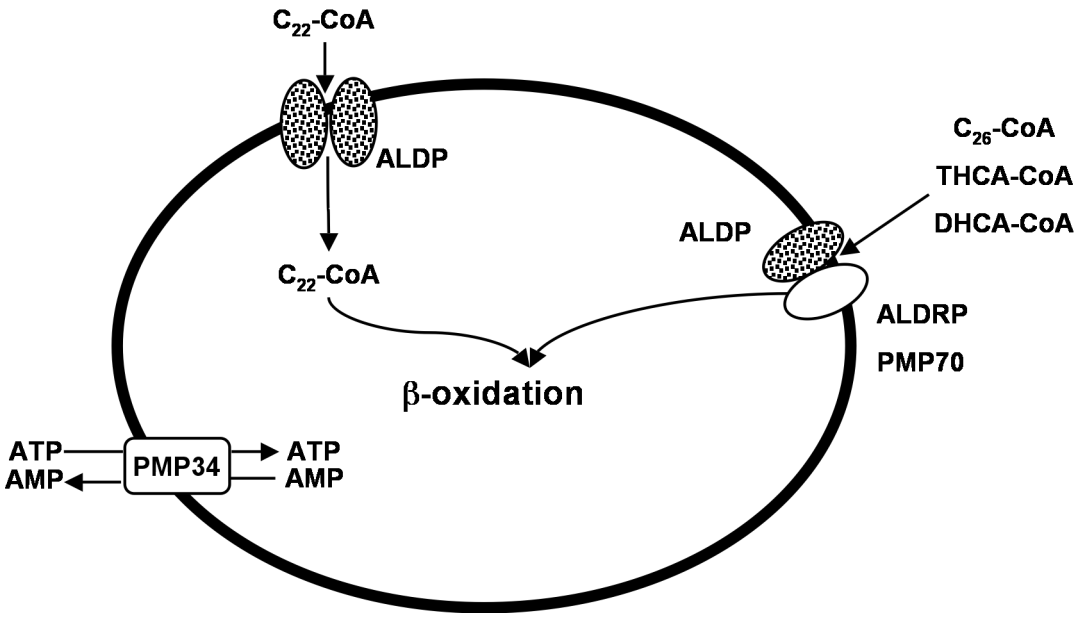


Fig. 2. Possible transport mechanisms for entry of substrates and cofactors into peroxisomes. Fatty acids and bile acid intermediates are activated to their CoA esters by acyl-CoA synthetases. The CoA ester may then be transported into the peroxisome via ABC transporters. ATP and AMP can be translocated via PMP34. ATP, adenosine triphosphate; AMP, adenosine monophosphate; THCA-CoA, trihydroxycholestanoyl-CoA; DHCA-CoA, dihydroxycholestanoyl-CoA.

Peroxisomal β-oxidation products: their possible fates

As mentioned earlier peroxisomal β-oxidation acts as a chain shortening system and one question raised is what happens to the chain shortened products? It has been suggested that they are substrates for various enzyme families, which includes the acyl-CoA thioesterases (ACOTs), nudix hydrolases (NUDTs), carnitine acyltransferases and the bile acid conjugating enzyme, BAAT.

The Acyl-CoA Thioesterases

The major work of our group for the past decade has focused on the characterization of the type I acyl-CoA thioesterase gene family (ACOTs). This gene family contains enzymes that are involved in various aspects of lipid metabolism within different cell compartments, including the peroxisome, mitochondria and cytosol.
Acyl-CoA thioesterase activity was first identified in the 1950s (78), and they catalyze the cleavage of the thioester bond of acyl groups that are attached to CoASH, to release the free fatty acid and CoASH (see Fig. 3).

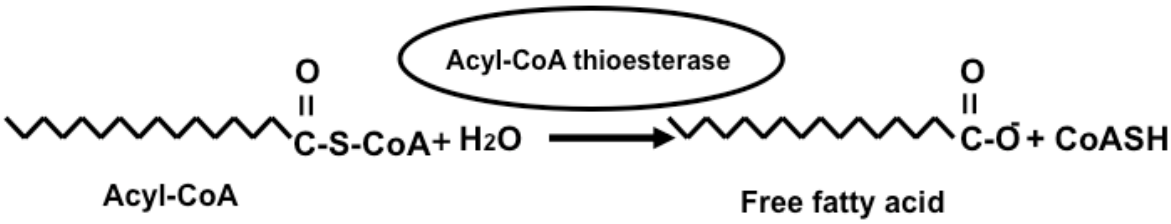


Fig. 3. Reaction catalyzed by the acyl-CoA thioesterases. ACOTs hydrolyse the CoA ester of various fatty acids into the free fatty acid and coenzyme A (CoASH).

The type I acyl-CoA thioesterases are a gene family of six mouse and four human ACOTs (79-86). ACOT1 is localized in the cytosol and is active mainly on long chain acyl-CoAs of C_{12}-C_{18} (81, 82), ACOT2 is localized in mitochondria and also hydrolyses long-chain acyl-CoAs (81, 83). In mouse peroxisomes there are four ACOTs, ACOT3 which is active towards long chain acyl-CoAs (84), ACOT4 which catalyzes the hydrolysis of succinyl-CoA to succinate and CoASH (85), ACOT5, a medium chain acyl-CoA thioesterase (84), and finally ACOT6, which hydrolyzes pristanoyl-CoA and phytanoyl-CoA to pristanic acid and phytanic acid respectively (86) (see Fig. 4).

In the corresponding gene cluster in human, there are four genes coding for thioesterase proteins, these are ACOT1, ACOT2, ACOT4 and ACOT6. Although, it is not clear if ACOT6 translates into a functional protein since it is coded for by a methionine at the end of the second exon, resulting in translation of only the third exon. Human ACOT1 and ACOT2 carry out similar functions to their mouse counterparts, while ACOT4, the only human peroxisomal enzyme, catalyzes the reactions of three of the mouse enzymes (ACOT3, ACOT4 and ACOT5) (81).

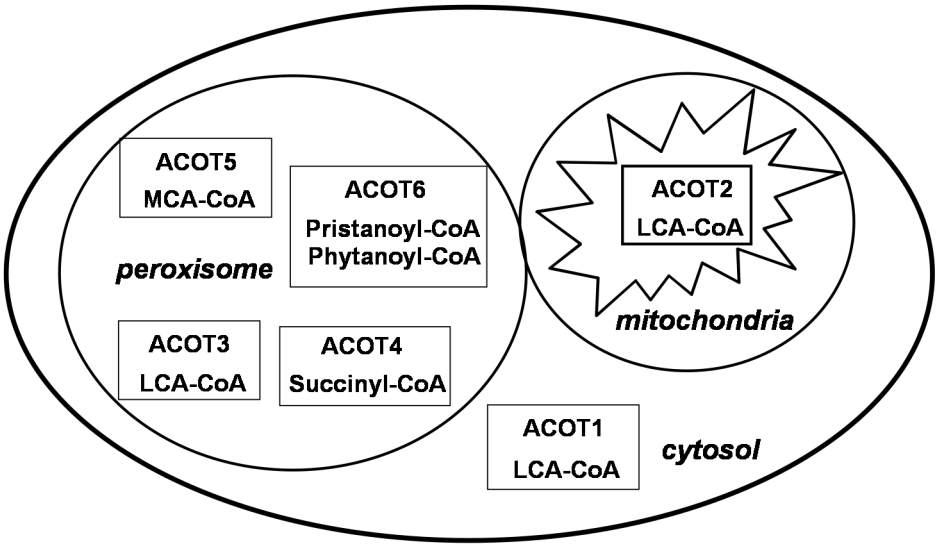


Fig. 4. Localization and substrate specificities of the type I acyl-CoA thioesterases (ACOTs) in the cell. The different cellular localizations of members of the ACOTs are shown, with their various substrate specificities. MCA-CoA, medium-chain acyl-CoA; LCA-CoA, long-chain acyl-CoA; ACOT, acyl-CoA thioesterase.

The roles postulated for the peroxisomal ACOTs are as auxiliary enzymes in peroxisomal α- and β-oxidation, in the removal of metabolites from peroxisomes as free acids and/or the regulation of coenzyme A (CoASH) levels in peroxisomes (for review see (87)). Also peroxisomes contain two further acyl-CoA thioesterases that are not related to the type-I gene family. ACOT8, hydrolyzes a wide variety of acyl-CoAs (88-90) and is one of only two peroxisomal thioesterases regulated by CoASH (88). The second thioesterase ACOT12, is an acetyl-CoA thioesterase (short chain) that is inhibited by CoASH and ADP (91, 92).

Coenzyme A and peroxisomes

Coenzyme (CoASH) is an indispensable co-factor in living organisms as it is involved in over one hundred reactions in intermediary metabolism and required by approximately 4% of all cellular enzymes (93). Levels of CoASH in the cell depend on its metabolic state and within the cell itself CoASH is compartmentalized in the cytosol, mitochondria and peroxisomes. As discussed above, fatty acids are esterified to CoASH in order for β-oxidation to proceed. In addition, CoASH is required for the final step of β-oxidation, catalyzed by the 3-ketoacyl-CoA thiolases or SCPx to produce the chain shortened acyl-CoA and acetyl-CoA or propionyl-CoA. Peroxisomes are estimated to contain between 0.23-0.7 mM CoASH (94, 95). As mentioned previously, the peroxisomal membrane is impermeable to so called 'bulky' solutes i.e. over 300 Da, such as CoASH (66), and therefore acyl-CoAs transported into the peroxisomal lumen are believed be the source of peroxisomal CoASH. It is important in peroxisomes to control the levels of CoASH, especially under times of high β-oxidation where there is a change in intra-peroxisomal levels of acyl-CoAs and a demand for CoASH, such as during starvation (96) and high fat feeding (97). The extent of CoASH sequestration may therefore depend on the size of the CoASH pool, the amount and types of lipids in the peroxisomal β-oxidation system, as well as certain peroxisomal enzyme activities such as the ACOTs. Another enzyme family that has recently been implicated in the regulation of peroxisomal CoASH levels is the nudix hydrolases.

The Nudix Hydrolases

The nudix hydrolases (NUDTs) are a group of widely distributed enzymes whose proposed functions include the elimination of potentially toxic endogenous metabolites from the cell as well as the regulation of the availability of many different nucleotide substrates, co-factors and signaling molecules (98). The majority are nucleotide diphosphatases that hydrolyze the diphosphate linkage in a variety of nucleoside triphosphates, dinucleoside polyphosphates, nucleotide sugars and related compounds, with substrates having the general structure of a nucleotide diphosphate linked to another moiety (98).

In mammalian peroxisomes two new members of this family have been identified that are active on CoASH or CoA derivatives. These are NUDT7α (99) and NUDT19 (RP2p) (100). These enzymes function as diphosphatases that can cleave CoASH or CoA esterified to a fatty acid, with the products being 4'-phosphopantetheine or acyl-phosphopantetheine and 3'5'-ADP (see Fig. 5). Mouse NUDT7α was first identified in 2001 and was shown to be active on CoASH, 3'-dephospho-CoA, oxidized CoA, acetyl-CoA and succinyl-CoA (99). NUDT19 was found to be active on CoASH and oxidized CoA. However, it was also shown that it is active on a range of acyl-CoA esters including straight chain acyl-CoAs, methyl branched chain acyl-CoAs and bile acid-CoAs (100).

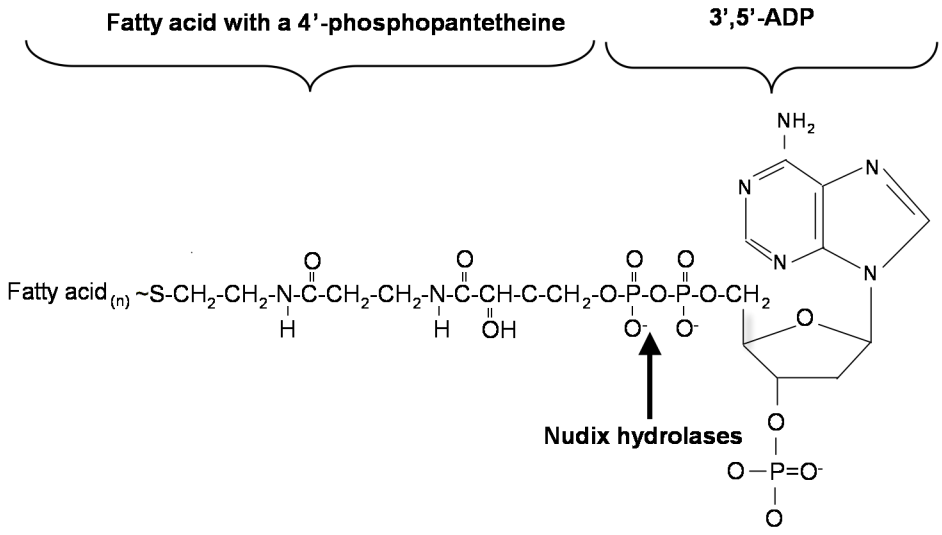


Fig. 5. Reaction catalyzed by the nudix hydrolases. The nudix hydrolases (NUDTs) can produce 4'-phosphopantetheine or a fatty acid with phosphopantetheine attached (acyl-phosphopantetheine) and 3'5'-ADP. The arrow indicates the site of hydrolysis.

In *Sacchraromyces cerevisiae*, the PCD1 nudix hydrolase gene was speculated to have a role in the removal of potentially toxic oxidized CoA from peroxisomes in order to maintain β-oxidation (101). It has a low K_m (24 μM) for oxidized CoA (CoASSCoA), which is more than ten times lower than the K_m for CoA ($K_m \approx 280$ μM), suggesting that it removes potentially toxic or non-functional oxidized CoA in peroxisomes (101). The corresponding NUDT enzyme in *Caenorhabditis elegans* (*C. elegans*) has a K_m for free CoA of 220 μM, which is similar to that for oxidized CoA at 320 μM, showing that this enzyme has no preference for free or oxidized CoA (102). The mouse homolog of the

Pcd1p gene was later identified and named NUDT7α and it was believed that it would have a similar role to that of the yeast and *C. elegans* proteins and indeed both NUDT7α and NUDT19 were identified as CoASH metabolizing enzymes (99, 100).

The Carnitine Acyltransferases

A third peroxisomal enzyme family involved in CoASH regulation are the carnitine acyltransferases. These catalyze the reversible esterification of fatty acids to CoASH and carnitine. In peroxisomes there are two carnitine acyltransferases - carnitine acetyltransferase (CRAT), active on short-chain acyl-CoAs, and carnitine octanoyltransferase (CROT) active on medium-chain acyl-CoAs (103, 104). CRAT, has highest activity towards butyryl-CoA, propionyl-CoA and acetyl-CoA (105), while CROT is active towards straight chain and branched medium-chain acyl-CoAs (105, 106). Through the production of carntitine esters, the carnitine acyltransferases have been implicated in the removal of metabolites of peroxisomal β-oxidation for transport to mitochondria for further metabolism.

Bile acid-CoA:amino acid N-acyltransferase

So far the fate of the fatty acyl-CoAs has been discussed, but as mentioned earlier another important pathway in peroxisomes is bile acid synthesis. As previously stated, the bile acid precursors undergo one round of β-oxidation with the final step being the conjugation (or amidation) of the C_{24} bile acids, choloyl-CoA and chenodeoxycholoyl-CoA to the amino acids taurine or glycine (depending on the species). This reaction is carried out by the bile acid-CoA:amino acid *N*-acyltransferase (BAAT) (see Fig. 6) (107-111). The conjugation of bile acids plays several important roles, not only does it remove cholesterol from the body, it also increases the detergent properties of bile acids, which aids lipid and vitamin absorption.

A homozygous mutation has been reported in the BAAT gene in the Amish community and the affected patients have familial hypercholanemia, which is characterized by high levels of unconjugated bile acids in serum, itching and fat malabsorption (112). Three single nucleotide polymorphisms (SNPs) were recently identified in Japanese individuals, however, it is not known if these SNPs affect the function of BAAT (113).

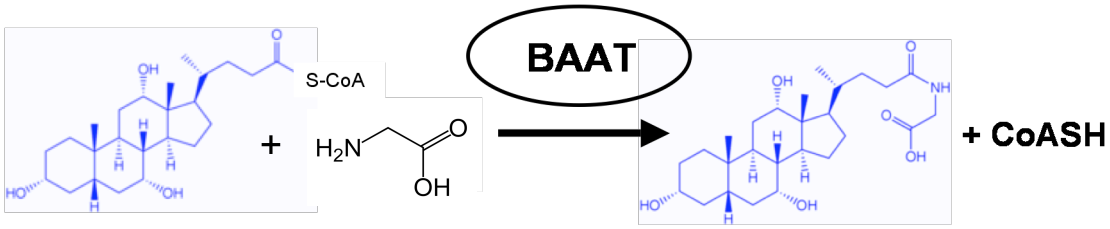


Fig. 6. Reaction catalyzed by bile acid-CoA:amino acid *N*-acyltransferase. The bile acid-CoA:amino acid *N*-acyltransferase (BAAT) catalyzes the conjugation (amidation) of bile acids to glycine or taurine, resulting in a conjugated bile acid. The above reaction shows the conjugation of choloyl-CoA to glycine to produce glycocholate and coenzyme A (CoASH).

The enterohepatic circulation of bile acids means that the primary bile acids can be deamidated by bacteria in the intestine and converted into various secondary bile acids e.g. lithocholic acid and deoxycholic acid. Both primary and secondary bile acids are returned to the liver. It is speculated that BAAT is involved in two conjugation pathways, one where it is responsible for the conjugation of *de novo* synthesized bile acids in the peroxisome, and a second where it is responsible for the reconjugation of deconjugated bile acids recycled from the intestine to the liver, suggesting a dual localization in peroxisomes and cytosol (114). However, the localization of BAAT still remains controversial and raises the possibility of the existence of more than one BAAT enzyme. Human, rat and mouse BAAT all contain conserved C-terminal amino acid sequences of –SQL (serine, glutamine, leucine), which is a non-consensus variant of the peroxisomal targeting signal 1 (PTS1) and could function in the targeting of BAAT to the peroxisome. BAAT has been purified and characterized from human liver cytosol (115) but activity and protein have also been localized in rat liver peroxisomes (109, 116). Studies using green fluorescent fusion showed that in human skin fibroblasts BAAT was cytosolic (110, 117), while in studies using primary hepatocytes from rat and human, BAAT was localized in peroxisomes (117).

HNF-4α null mice show undetectable expression of BAAT mRNA, yet they still have high levels of conjugated bile acids. Even more interesting is that there is also an increase in the levels of glycine conjugated bile acids in the gallbladder (118). However, mouse BAAT has no glycine conjugating activity (107), suggesting that there may be another enzyme that can glycine conjugate bile acids in mouse. It was also

recently shown that BAAT conjugates fatty acids to glycine (110) and taurine (O'Byrne et al, unpublished data).

Amino acid conjugation and lipoaminoacids

So far, conjugation has only been discussed in respect to bile acids, however, it is now well established that a wide variety of endogenous molecules including carboxylic acids and xenobiotics are also conjugated with amino acids in reactions catalyzed by various acyltransferase enzymes (119). Amino acid conjugation is a two-step process; the first step requires the activation of the carboxylic acid to the CoA ester, which is catalyzed by the acyl-CoA synthetases. As mentioned previously, the second step, the conjugation reaction, is catalyzed by an acyl-CoA:amino acid *N*-acyltransferase (EC 2.3.1). While the conjugation of bile acids and carboxylic acids to taurine and glycine is the most common, conjugation to L-asparagine and L-glutamine can also occur (120). The conjugation of xenobiotics is a way to remove these compounds from the body. Other molecules that can be conjugated to amino acids are lipids, which are known as lipoaminoacids and includes the *N*-acylglycines (NAGs) and the *N*-acyltaurines (NATs).

N-acylglycines (NAGs)

N-acylglycines (NAGs) are produced *in vivo* from activated fatty acyl-CoA esters and glycine by the mitochondrial enzymes acyl-CoA:glycine *N*-acyltransferase (ACGNAT) (see Fig. 7). This enzyme activity was first identified by Schachter and Taggart in pig kidney and bovine liver mitochondria in the 1950s (121, 122), and then in human liver in 1986 (123). NAGs can be further metabolized into another class of signaling molecules, the fatty acid amides (FAAs). The enzyme peptidyl glycine α-amidating monooxygenase (PAM) has been implicated in the production of FAAs via oxidative cleavage of NAGs (124). Like many other chemical transmitters, these lipid messengers need to be regulated to ensure tight control over their signaling activity and the inactivation of NAGs and FAAs is mediated primarily by the enzyme fatty acid amide hydrolase (FAAH) (125, 126).

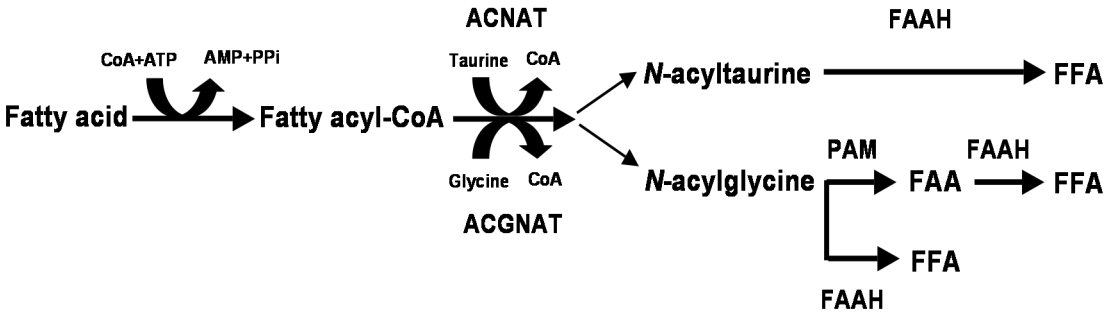


Fig. 7. Synthesis and metabolism of *N*-acyltaurines and *N*-acylglycines. Fatty acids are first activated to their CoA ester by the action of acyl-CoA synthetase (ACS). Then the fatty acyl-CoA is conjugated to an amino acid such as taurine by an acyl-CoA:amino acid *N*-acyltransferase (ACNAT) or to glycine by acyl-CoA:glycine *N*-acyltransferase (ACGNAT). NAGs can be further metabolized to fatty acid amides (FAA) by peptidyl glycine α-amidating monooxygenase (PAM). FAAs, NAGs and NATs are inactivated by the enzyme fatty acid amide hydrolase (FAAH), which hydrolyzes them to the free fatty acid (FFA).

N-acyltaurines (NATs)

While the existence of *N*-acylglycines has been known for a long time, fatty acids conjugated to taurine i.e. NATs, were only recently identified. Saghatelain et al used an LC-MS based analytical method termed discovery metabolite profiling (DMP) to look at the effects of enzyme inactivation in vivo. In this first study they examined the metabolite profiles from central nervous tissues (CNS) of wild-type mice and mice lacking the enzyme fatty acid amide hydrolase [FAAH (-/-) mice]. They detected a group of unknown metabolites elevated in the brain and spinal cord of these FAAH (-/-) mice, long-chain fatty acids conjugated to taurine i.e. NATs (127). A second study looked further at NAT tissue distribution and levels, and NATs of chain length C_{16}-C_{24} were also detected in liver, kidney and brain (128).

Polyunsaturated NATs such as arachidonoyltaurine ($C_{20:4}$) have also been identified in liver and kidney at levels of 20 pmol/g and 157 pmol/g respectively (128). In mice treated with a chemical inhibitor of FAAH, the highest NAT changes in liver and kidney were mainly those with polyunsaturated acyl chains, while in brain only saturated and monosaturated NATs were increased. The differences in the distribution of NATs between liver, kidney and brain suggests two different mechanisms for the production of NATs, one in the liver and kidney producing polyunsaturated NATs and one in brain producing saturated and monounsaturated NATs. A question raised upon

the discovery of NATs was what enzyme is responsible for their production? It was suggested that they would be produced in a similar way to the NAGs but by an enzyme capable of using taurine as the acceptor molecule instead of glycine (see Fig. 7). One candidate enzyme that was put forward was BAAT (128). However, within mouse peroxisomes there are two newly identified genes that are related to *Baat* that were also possible candidates, which have been named acyl-CoA:amino acid *N*-acyltransferase 1 and 2 (*Acnat1* and *Acnat2*) (Paper I and II).

The Acyl-CoA:amino acid N-acyltransferases

Using blast searches we have identified two new peroxisomal proteins and named them acyl-CoA:amino acid *N*-acyltransferase 1 and 2 (ACNAT1 and ACNAT2). These enzymes show 92% sequence identity to each other, 55% to BAAT and approximately 43% to the type I ACOTs at amino acid level (see Fig. 8). *Baat*, *Acnat1* and *Acnat2* are located within 100 kb on mouse chromosome 4 B3. Studies into the gene organisation of these three acyltransferases show that they all have a similar genomic organisation. The open reading frame (ORF) of each gene is encoded for by three exons, with the catalytic site located in the third exon.

The similarity between the BAAT, the ACOTs and the newly identified ACNATs, suggested that they are acyltransferases whose substrates could be acyl-CoAs or bile acid-CoAs. A similar acyltransferase "cluster", containing genes for *BAAT* and *ACNAT1* can be found in humans on chromosome 9q31.1. However, the *ACNAT1* gene does not appear to encode a functional protein, and contains several stop codons.

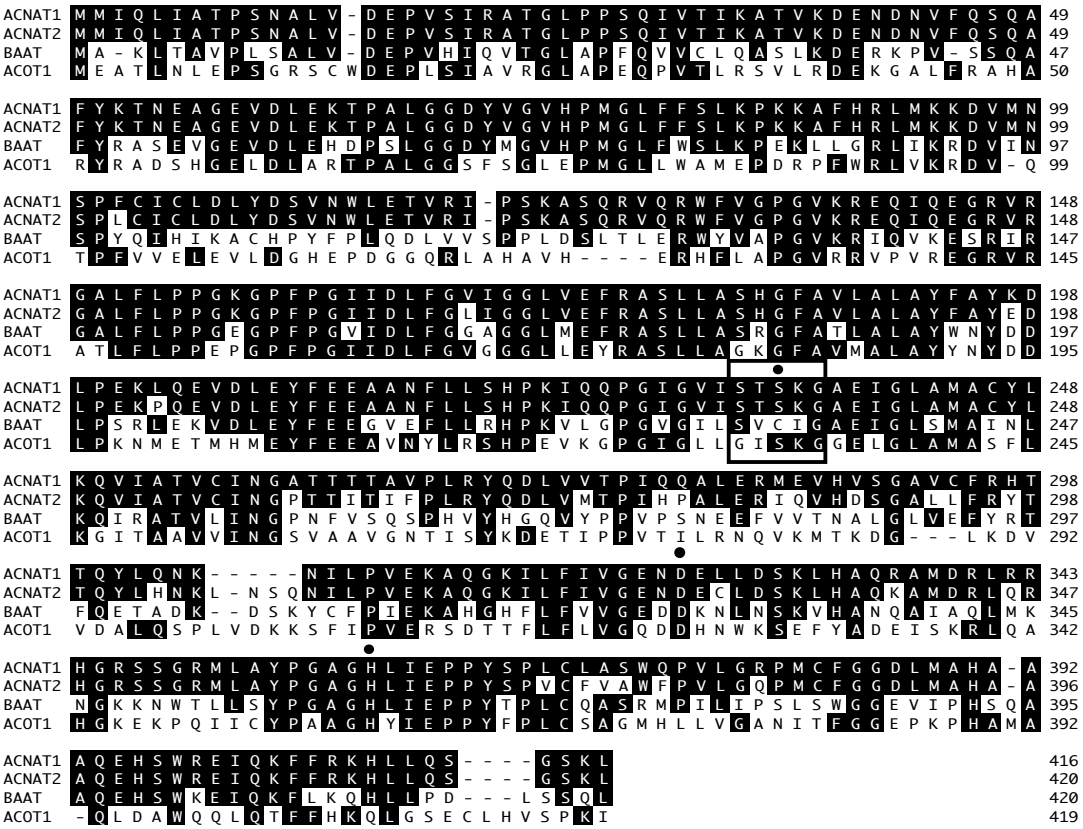


Fig. 8. Sequence alignment of ACNAT1, ACNAT2, BAAT and ACOT1. Alignment of the amino acid sequences was performed using Clustal X method. ACNAT1 and ACNAT2 have a sequence identity of 92% at amino acid level, while they share 55% identity with BAAT an approximately 43% identity with ACOT1. ACNAT, acyl-CoA:amino acid *N*-acyltransferase; BAAT, bile acid-coenzyme A:amino acid *N*-acyltransferase; ACOT, acyl-CoA thioesterase. The amino acids of the nucleophilic elbow are boxed and the amino acids of the catalytic triad are indicated by circles.

The thioesterases and acyltransferases belong to the α/β hydrolase protein family

The work in this thesis has focused on various members of the type I acyl-CoA thioesterase/acyltransferase gene family. Initial sequence analysis of four ACOTs, ACOT1, ACOT2, ACOT3 and ACOT5 identified these as members of a larger family of proteins known as the α/β hydrolase superfamily (80).

The α/β hydrolases are one of the largest families of proteins and have various catalytic functions which includes lipid hydrolases, thioester hydrolases and peptide hydrolases to name a few. These have all been classified together based on their similar structure – an α/β hydrolase fold which contains the catalytic machinery of the enzyme (see Fig.

9). The catalytic triad consists of a nucleophile, a histidine and an acidic residue. The nucleophile can either be a serine, a cysteine, an aspartic acid or an asparagine. The acidic residue can be glutamic acid or aspartic acid, while the histidine is completely conserved. The α/β hydrolase fold is an eight β-stranded mostly parallel α/β structure. It has one strand (β2) that is antiparallel to the rest. One of the most notable structural features of the α/β hydrolase superfamily is the nucleophilic elbow. This nucleophilic elbow, in most cases, contains a serine in a highly conserved motif of Gly-Xaa-Ser-Xaa-Gly (GxSxG), where Xaa are variable amino acids. As the α/β hydrolase family expands, it has become apparent that there are many variations of this motif.

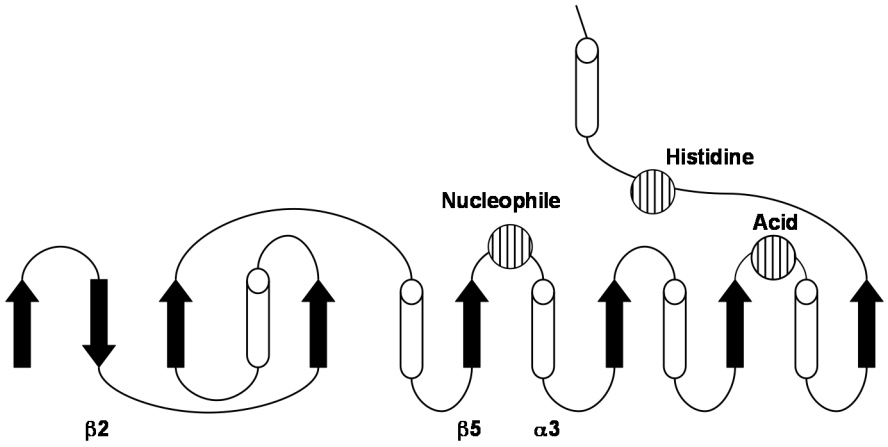


Fig. 9. The α/β hydrolase fold. It has eight β strands (shown as arrows) connected by six α-helices (shown by cylinders). The positions of the nucleophile, the acid and histidine are shown in circles. The nucleophilic elbow is located between β strand 5 and α helix 3.

The members of the thioesterase/acyltransferase family are located in two clusters in the mouse and human genomes. ACOT 1-6 are located on mouse chromosome 12 D3 in a cluster within 120 kb of each other, with a similar human gene cluster of four ACOTs on chromosome 14q24.3 (81). The acyltransferase cluster of ACNAT1, ACNAT2 and BAAT are located in close proximity (within 100 kb) on chromosome 4 B3 in mouse (see Fig. 10).

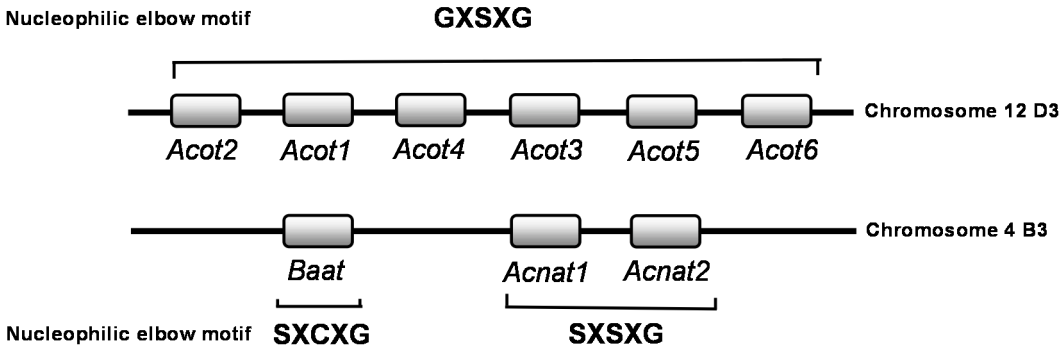


Fig. 10. Chromosomal localization of mouse acyl-CoA thioesterases and acyltransferases. Type I acyl-CoA thioesterases/acyltransferases are located in two gene clusters. The amino acid sequence of the nucleophilic elbow is shown. One of the main features of the α/β hydrolase family is the highly conserved nucleophilic elbow motif; in most cases it contains a nucleophilic serine in a GxSxG motif. *Baat* and the *Acnats* contain variations of this motif (SxCxG and SxSxG respectively). *Acot*, acyl-CoA thioesterase; *Baat*, bile acid-CoA:amino acid *N*-acyltransferase; *Acnat*, acyl-CoA:amino acid *N*-acyltransferase; G, glycine; S, serine; C, cysteine; x, variable amino acid.

Sequence alignment of the ACOTs, BAAT and ACNATs has identified three variations of the α/β hydrolase nucleophilic motif within this gene family (see Fig. 8 and Fig. 10). The ACOTs all contain the highly conserved common motif of GxSxG (79-82, 84-86), while ACNAT 1 and 2 contain SxSxG (**Paper I**), and BAAT contains SxCxG (110, 129). Based on the variations in the nucleophilic elbow in ACOTs, ACNATs and BAAT, we hypothesized that subtle changes in this elbow may be important in mediating their different activities. Furthermore, the overall structural similarities between the ACOTs, BAAT and the ACNATs, and the clustering of the genes in the genome suggest a strong evolutionary link between these enzymes.

AIMS

The identification of two new members of the acyl-CoA thioesterase/acyltransferase gene family in peroxisomes naturally led to the question of the roles of these proteins in peroxisomal lipid metabolism. One aim of this project was to characterize ACNAT1 and ACNAT2 regarding localization, substrate specificity and the regulation of their expression.

One area in which the sequence of the acyl-CoA thioesterase/acyltransferase family differs is in the nucleophilic elbow motif of their active sites, which is normally highly conserved. This study set out to see how important the amino acids in this motif are for the various activities of each protein. Mutation studies were employed to identify the "optimal" residues in the active sites that are necessary for the respective activities of three members of the acyl-CoA thioesterase/acyltransferase family.

Coenzyme A (CoASH) is an obligate cofactor for lipids undergoing β-oxidation in peroxisomes but it is not known how the peroxisomal CoASH pool is regulated. The acyl-CoA thioesterases have been implicated in controlling peroxisomal CoASH levels, together with a second family of enzymes the nudix hydrolases. In this study we set out to further chracterize a peroxisomal member of this family, NUDT7α, to elucidate its possible role in regulation of CoASH/acyl-CoA metabolism in peroxisomes.

RESULTS AND DISCUSSION

Characterization of ACNAT1 and ACNAT2 – Paper I & Paper II

Blast searches identified two new members of the type I acyl-CoA thioesterase/acyltransferase family, ACNAT1 and ACNAT2. Both ACNAT1 and ACNAT2 contain the consensus peroxisomal targeting signal 1 (PTS1) of –SKL at their C-terminal ends. To test if this PTS1 does indeed target both proteins to peroxisomes, ACNAT1 and ACNAT2 were expressed as fusion proteins with green fluorescent protein (GFP), leaving the carboxyterminal –SKL accessible. The localization studies showed that ACNAT1 and ACNAT2 are both peroxisomal proteins (**Paper I** and **Paper II**).

Roles for ACNATs in NAT production

Characterization of recombinant ACNAT1 identified it as an acyltransferase that can efficiently conjugate acyl-CoAs of chain lengths C_{12} to C_{24} to taurine (see Fig. 11). No activity was seen with glycine. The identification of ACNAT1 is the first description of a specific fatty acid taurine conjugating enzyme that is a long-chain acyl-CoA:amino acid *N*-acyltransferase. The identification of this activity raised the question as to the physiological role of this enzyme in peroxisomal lipid metabolism.

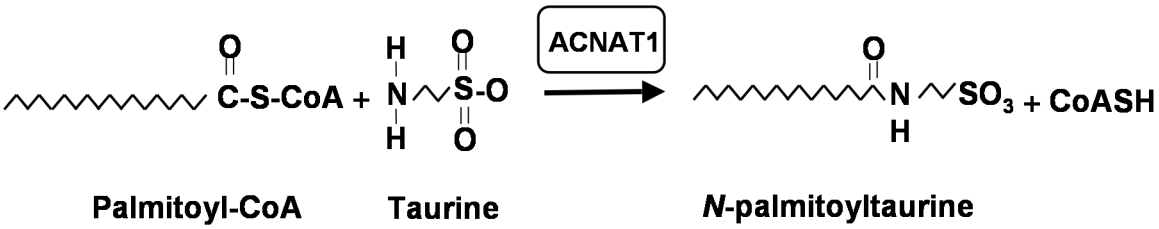


Fig. 11. Reaction catalyzed by ACNAT1. ACNAT1 conjugates the CoA ester of various fatty acids to taurine to produce *N*-acyltaurines and coenzyme A (CoASH). Conjugation of palmitoyl-CoA (C_{16}) to taurine is shown above.

At the time when we discovered the activity of ACNAT1, Saghatelian et al reported the first identification of the NATs in the brain and spinal cord of mice lacking the enzyme fatty acid amide hydrolase [FAAH (-/-)] (127). FAAH is involved in the inactivation of the signaling molecules NAGs and FAAs (125, 126). The main NAT species identified

were of chain lengths C_{22}, $C_{24:1}$ and C_{24} and these were elevated 4-40 fold in the FAAH (-/-) mice (127). FAAH also has high expression in rodents in liver, kidney and testes (125), and after the initial studies in the central nervous system, the focus turned to these tissues. NATs with *N*-acyl chains of C_{16} to C_{24} were found in liver and kidney, however, the two most elevated NATs in liver were the polyunsaturated NATs of arachidonoyltaurine ($C_{20:4}$) and docosahexanoyltaurine ($C_{22:6}$) (128). BAAT, the bile acid conjugating enzyme, was put forward as the enzyme responsible for NAT production. However, we recently identified ACNAT1 as an enzyme that conjugates long-chain and very long-chain fatty acids to taurine (C_{12}-C_{24}) (**Paper I**). ACNAT1 has highest tissue expression in liver and kidney and could be responsible in part for the production of some of the NAT species produced, however, ACNAT1 has no activity towards mono- or polyunsaturated fatty acids (**Paper I**).

Since the most abundant species of NATs observed in liver and kidney were polyunsaturated NATs e.g. $C_{20:4}$ and $C_{22:6.}$, and since ACNAT1 has no activity with polyunsaturated fatty acyl-CoAs, we speculate that ACNAT2 may be responsible for the conjugation of these acyl-CoAs. The sequence identity between ACNAT1 and ACNAT2 is 92%, which suggests that ACNAT2 may also have a similar enzymatic activity to ACNAT1. *Acnat2* mRNA is highest in kidney and is also detectable in liver. One of the main differences between ACNAT2 and ACNAT1 is that in ACNAT1 there is a deletion of four amino acids (LNSQ) in the region between the nucleophilic serine and the aspartic acid of the catalytic triad (**Paper II**). It should also be noted that most of the amino acid differences between ACNAT1 and ACNAT2 occur in the third exon, which contains the catalytic machinery (**Paper II**). Currently, there is no information regarding the crystal structures of the members of this acyltransferase family, so we are unable to perform modelling studies for ACNAT2. However, it is possible that these amino acid differences could result in a change in the structure of the substrate binding pocket of ACNAT2 that would allow it to accept more "bulky" substrates than ACNAT1, such as unsaturated fatty acids (see Fig. 12). However, we have as yet been unable to produce functional ACNAT2 protein in several expression systems, so we can only speculate as to its function as an acyltransferase.

The NAGs are normally minor components of blood and urine, however, under certain conditions and diseases such as medium chain acyl-CoA dehydrogenase deficiency, their levels increase (130). A similar situation could exist regarding fatty acids within

peroxisomes, since it appears that the levels are low under normal conditions and it may be that ACNAT1 and ACNAT2 are involved in the removal of fatty acids in bile under conditions when levels could become toxic. Preliminary data from our group identified NATs of chain length C_{14} to C_{24} in mouse serum; this is also a relevant finding regarding the possible transport of these NAT molecules between various tissues.

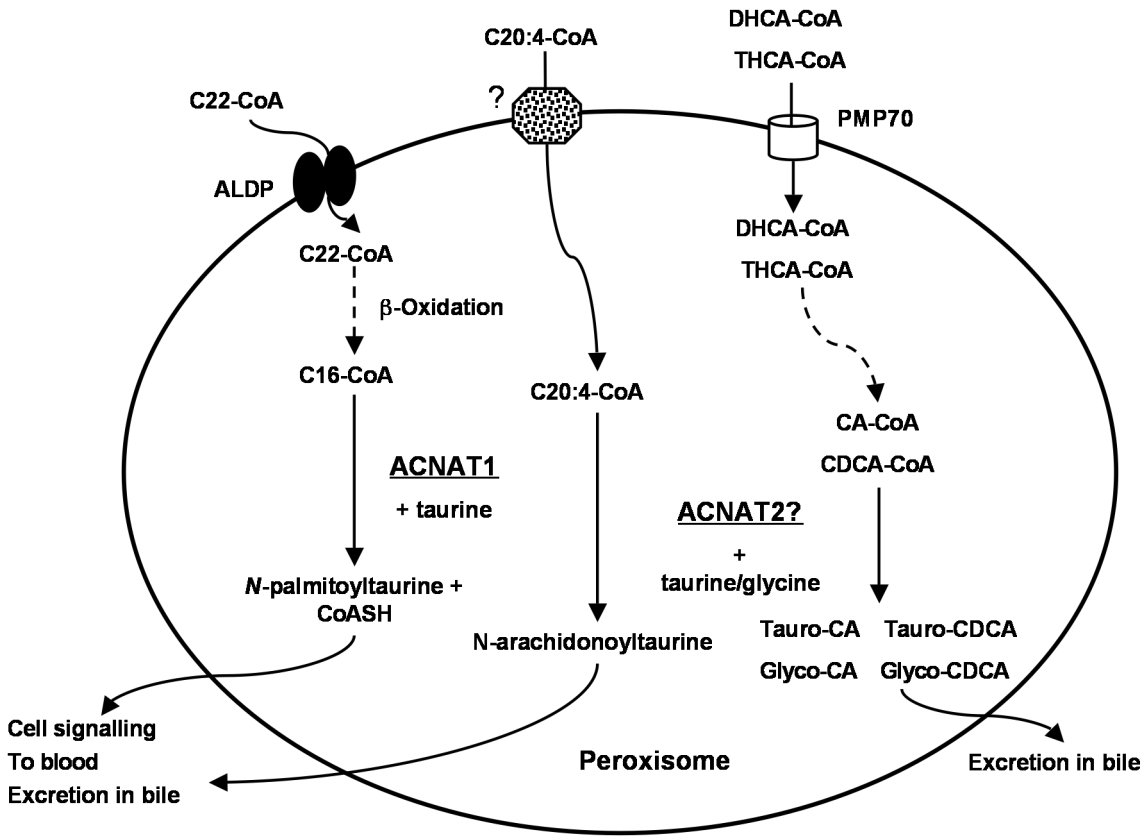


Fig. 12. Roles for ACNATs in peroxisomes. ACNAT1 conjugates saturated long-chain fatty acids to taurine to produce *N*-acyltaurines (NATs). These NATs could then be removed from peroxisomes for excretion in bile or transported in blood as signaling molecules. ACNAT2 may have a role in the conjugation of mono- and polyunsaturated fatty acids to taurine, which may also represent an excretory pathway for these molecules or they could also be involved in cell signaling. ACNAT2 may also conjugate *de novo* synthesized bile acids to taurine and/or glycine for excretion in bile. ACNAT, acyl-CoA:amino acid *N*-acyltransferase; THCA-CoA, trihydroxycholestanoyl-CoA; DHCA-CoA, dihydroxycholestanoyl-CoA; CA, cholic acid; CDCA, chenodeoxycholic acid; CoASH, coenzyme A.

Since, NAGs and their derivatives, the fatty acid amides, function as signaling molecules it was hypothesized that perhaps NATs served a similar function. Fatty acid amides have been identified as ligands for various receptors including cannabinoid receptors (131), PPARα (132) and members of the transient receptor potential (TRP)

family of cation channels (133, 134). It is also well known that various fatty acids serve as natural ligands for the PPARs (35, 135, 136). Studies with NATs did not reveal any activation with cannabinoid receptors or PPARα, however, they did serve as ligands for two members of the TRP family, the transient receptor potential vanilloid 1 (TRPV1) and TRPV4 (128). TRPV1 has roles in various functions in the body such as inflammation, thermoregulation, satiety (137), hearing modulation (138) and gastrointestinal motility (139). TRPV1 has also recently been implicated in the prevention of adipogenesis and obesity (140). TRPV4 is believed to be involved in the regulation of thermogenesis (141), pain signaling (142), osmoregulation (143) and hearing (144). Arachidonoyltaurine was also identified as a substrate of the lipoxygenases (LOX), 12S-LOX and 15S-LOX, and was shown to be rapidly taken up by murine resident peritoneal cells and enter the lipoxygenase pathway. This may represent another pathway to terminate the action of NATs, together with the actions of FAAH, or it may represent a new pathway to synthesize signaling molecules (145). 12/15-LOX are expressed in mammals in monocytes and macrophages and have important roles in inflammatory diseases, including atherosclerosis, cancer and diabetes (for review see (146)). ACNAT1 and ACNAT2 could be involved in the production of ligands for the TRPV receptors or substrates for the lipoxygenases, and therefore be indirectly involved in the regulation of various cellular processes.

A peroxisomal BAAT?

As mentioned earlier, the localization of BAAT is still controversial, with activity identified in cytosol and peroxisomes in humans (114, 115), rats (116) and mouse (147). The chromosomal localization of *Acnat1* and *Acnat2,* together with their similar amino acid sequence identities, led to the hypothesis that one of these gene products may be a novel peroxisomal BAAT. ACNAT1 can catalyse the conjugation of bile acids to taurine (see Fig. 13), which suggests that it may have a role in de novo bile acid biosynthesis, however, its ability to conjugate bile acids occurs at a very low level, which implies that ACNAT1 may only have a minor role in bile acid biosynthesis. ACNAT2 is however also a candidate enzyme for the conjugation of bile acids. Hepatocyte nuclear factor 4α (HNF4α) is a member of the nuclear receptor hormone superfamily that is mainly expressed in liver, kidney, intestine and pancreas (148, 149) and is involved in the regulation of many genes involved in lipid homeostasis (150). In a recent study, mice lacking hepatic HNF4α had an increase in unconjugated bile acids

but they also had increased levels of glycine conjugated bile acids in gallbladder bile, even though there was a strong decrease in the expression of BAAT mRNA (118). This is interesting considering the fact that mouse bile normally contains only taurine conjugated bile acids and mouse BAAT has no glycine conjugating abilities (107). In HNF4α knockout mice there is an increase in the mRNA levels of a "BAAT homolog" that corresponds to ACNAT2 (118), which implies that it could be involved in bile acid conjugation to glycine.

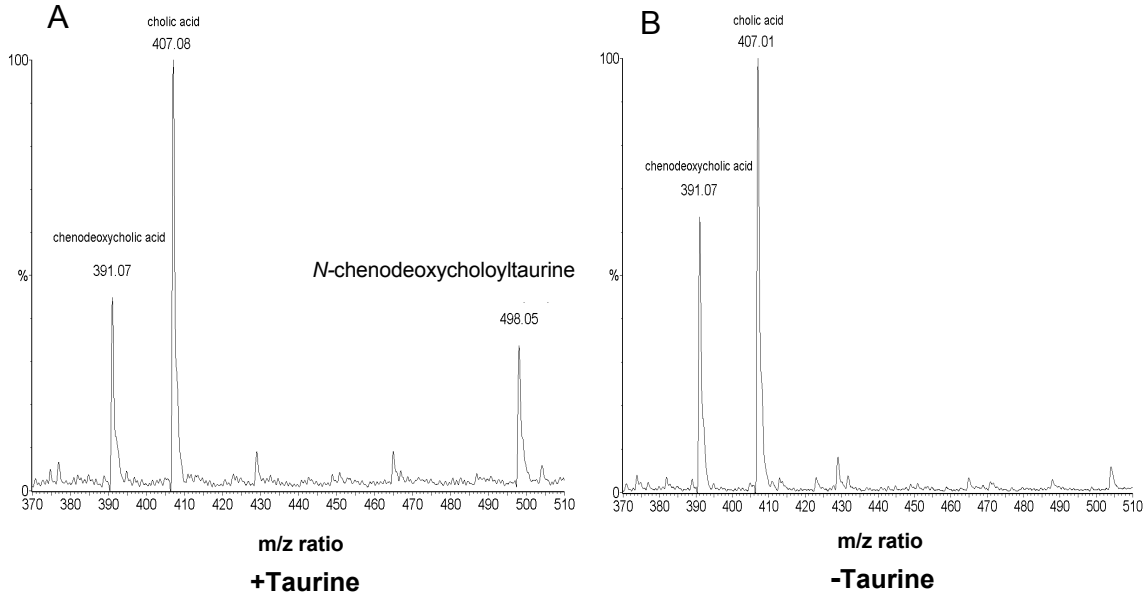


Fig. 13. ACNAT1 conjugates bile acids. Recombinant ACNAT1 (20 μg) was incubated with 50 μM chenodeoxycholoyl-CoA in the presence (A) or absence (B) of 50 mM taurine. The peak at 498.05 corresponds to *N*-chenodeoxycholoyltaurine, the peak at 391.07 is chenodeoxycholic acid and the peak at 407 is the internal standard cholic acid.

Another study in mice involving deletion of the fatty acid transport protein 5 (FATP5) helps to strengthen this hypothesis. FATP5 null mice have an impaired activation of recycled bile acids to the CoA ester but still produce primarily conjugated bile acids, presumably in peroxisomes (151). However, unlike mice lacking HNF4α, these animals had no glycine conjugated bile acids, and examination of the "BAAT homolog" (i.e. ACNAT2) that is upregulated in the HNF4α studies showed that it is downregulated in these animals (151). Together the results from these two studies suggest a possible role for ACNAT2 in the conjugation of the primary bile acids to glycine and/or taurine.

Mouse *Acnat2* is upregulated at mRNA level by various treatments including the peroxisome proliferator Wy-14,643, the bile acid CDCA, cholesterol and the cholesterol lowering drug atorvastatin (**Paper II**). Liver X receptor α (LXRα) acts as a cholesterol sensor and feeding mice with elevated levels of cholesterol results in an upregulation of CYP7A1 expression, the rate-limiting enzyme in the classic pathway of bile acid synthesis (152). This results in an increase in bile acid synthesis and therefore a need to increase the rate of bile acid conjugation. The farnesoid X receptor (FXR), however, functions as a nuclear receptor for bile acids and when bound to bile acids inhibits their synthesis by repressing CYP7A1 synthesis (153-155). However, studies in rat liver show that both the bile acid CoA synthetase (BACS) and BAAT are induced by FXR in response to natural and synthetic FXR ligands (156). *Acnat2* mRNA expression is increased by treatment with cholesterol and CDCA, and both of these treatments would result in a need to remove excess cholesterol and bile acids. If ACNAT2 is indeed a peroxisomal bile acid conjugating enzyme then its upregulation would aid in cholesterol and bile acid clearance, however, this remains to be determined.

Statins are a group of compounds that are used in the treatment of hypercholesterolemia and they exert their effects by inhibiting the rate-limiting enzyme in cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase). Atorvastatin treatment resulted in a nine-fold increase in *Acnat2* mRNA expression levels in liver, however this effect was seen only in male mice. In female mice *Acnat2* mRNA levels remain unchanged. An increase in *Acnat2* mRNA upon atorvastatin treatment could result in an increase in the conjugation of bile acids and help in the removal and lowering of cholesterol. Atorvastatin has been shown to activate transcription of PPARα target genes via inhibition of the RhoA signaling pathway (157, 158). It is therefore possible that statins inhibit this signaling pathway in a gender-specific manner. In humans the cytochrome P450 3A4 (CYP3A4) was shown to be the major P450 responsible for atorvastatin metabolism (159). CYP3A4 is expressed predominantly in female mouse liver (160, 161) which suggests that in female mice, atorvastatin may be metabolized more quickly than in males, and could have an effect on the regulation of *Acnat2* expression.

The active sites of the acyl-CoA thioesterases and acyltransferases defines their activity – Paper III

Divergent evolution is the development of proteins with different functions from a common ancestor and members of a family often share a characteristic functional feature, such as a common binding property or mechanistic strategy in catalysis. Nature it seems has opted for stable protein cores and folds and has "fine tuned" enzyme function through amino acid substitutions or deletions, which leads to enzymes with diverse catalytic functions with apparently only a few changes, such as the α/β hydrolase superfamily (162). Enzymes with an α/β hydrolase fold provide an example of a superfamily that has conserved mechanistic features but that can also catalyse an array of functions, including amongst others carboxylic acid ester hydrolase, lipid hydrolase, thioester hydrolase, peptide hydrolase and haloperoxidase (163). The α/β hydrolase fold has been preserved because it is a simple, stable and effective way of building a variety of different catalytic triads which can catalyze hydrolysis reactions (164). The conservation of structural and catalytic features in the acyl-CoA thioesterase/acyltransferase family strongly suggests that the enzymes arose via divergent evolution from a common ancestor to accept different substrates and to catalyze different reactions.

Importance of the active site residues in the thioesterase and acyltransferase family

The nucleophilic elbow in α/β hydrolases is usually made up of the highly conserved motif of GxSxG, although as the family expands more variations of this motif have been identified. Sequence analysis of the six ACOTs revealed that all contain the "consensus" GxSxG motif and in this study ACOT1 was chosen as the representative thioesterase. ACOT1 is active towards acyl-CoAs of chain length C_{12}-C_{20} and also unsaturated acyl-CoAs e.g. $C_{14:1}$ and $C_{18:2}$. Mutation of its active site motif to the motifs seen in the acyltransferases (i.e. ACNATs SxSxG or BACATs SxCxG) resulted in a decrease of thioesterase activity when incubated with C_{14}-CoA. There was a 60% decrease in thioesterase activity when the GxSxG motif was mutated to the ACNAT1 motif of SxSxG and a 98% decrease with the BAAT motif (SxCxG) (**Paper III**). Wild-type ACOT1 is unable to conjugate fatty acids or bile acids to glycine or taurine and

mutation of the active site to those seen in the acyltransferases did not result in an enzyme that could conjugate fatty acids. However, the SxSXG and SxCxG mutants resulted in enzymes that could conjugate the bile acid CDCA-CoA to glycine (**Paper III**). The fact that the SxSxG motif can conjugate CDCA-CoA to glycine is interesting, since this finding in a way suggests that ACNAT2, which has the SxSxG motif in its active site, could function as a glycine conjugating enzyme.

The active site of human wild-type BAAT allows the protein to conjugate not only bile acids to taurine and glycine, but also fatty acids, although at about 20% of the bile acid conjugating activity. In the absence of glycine and taurine, BAAT can also hydrolyze the CoA esters of these substrates to the free bile acid or free fatty acid and CoASH, although with a much lower activity. Mutation of the BAAT nucleophilic elbow (SxCxG) to that in ACNAT1 (SxSxG) and ACOT1 (GxSxG) generates proteins that have higher thioesterase activity and lower conjugation activity than the wild type enzyme (**Paper III**). Both of these mutants lose the ability to conjugate CDCA to glycine but can still conjugate CDCA to taurine. This is intriguing considering that ACNAT1 contains the SxSxG motif and can only conjugate bile acids to taurine (**Paper I**).

ACNAT1 (SxSxG) shares characteristics of both the ACOT1 and BAAT active sites, it has a serine nucleophile like ACOT1 and, similar to BAAT, it has a serine in place of the first glycine in the nucleophilic elbow. ACNAT1 is an efficient acyltransferase that can conjugate fatty acids to taurine. Mutation of its active site (SxSxG) to the ACOT1 (GxSxG) and BAAT (SxCxG) active sites resulted in proteins that showed a decreased ability to conjugate C_{16}-CoA to taurine, showing that the SxSxG active site is optimal for fatty acid conjugation to taurine (**Paper III**). Generation of the ACOT1 motif in ACNAT1 had no effect on its bile acid conjugating ability, however, mutation to that seen in BAAT resulted in a protein that conjugated bile acids approximately three times more efficiently than the wild-type protein (**Paper III**). This suggests that the SxCXG motif is optimal for bile acid conjugation.

It can be seen from these protein engineering studies that each of the three chosen members of the thioesterase/acyltransferase family- ACOT1, ACNAT1 and BAAT have evolved in a way that small differences in the amino acids of their active sites have resulted in proteins that are optimal for the reactions that they catalyse and also mediate substrate specificity.

Evolution of the acyl-CoA thioesterase/acyltransferase gene family

Based on the sequence identity and chromosomal localization it can be assumed that the members of the type-I acyl-CoA thioesterase/acyltransferase gene family have evolved by gene duplications, possibly from a common ancestor. Jensen said that new enzyme functions are established most easily and most commonly by recruitment of proteins already catalyzing analogous reactions (165). One question raised by having three such motifs in a small gene family is which one of these motifs was the common ancestor?

To answer this question we turned to the codon usage of the amino acids in the nucleophilic elbow, mainly those used for the nucleophilic residues, which are cysteine in BAAT and serine in ACOT1 and ACNAT1. Sydney Brenner in his 1988 paper, "The molecular evolution of gene proteins: a tale of two serines", said that essential serine residues within proteases show that there have been two lines of descent for the codons used and that the codons used for serine (UCN and AGY) are connected by two other codons, ACN for threonine and TGY for cysteine. Brenner concludes by saying that modern serine enzymes are likely to have arisen from cysteine precursors (166). If we use the hypothesis put forward by Brenner and apply it to our family of enzymes, this would suggest that BAAT is the common ancestor from which the ACOTs and the ACNATs evolved. The two sets of serines codons used by the ACOTs and ACNATs could have arisen from single mutations in the BAAT cysteine codon (see Table I).

However, if we look through the codons used by various orthologs in different species it would seem that in the acyl-CoA thioesterase/acyltransferase family that Brenners' hypothesis does not apply. If we look in bacteria, the elbow motif in the "putative" bacterial thioesterase ortholog is GxSxG. This suggests that the ancesteral gene is bacterial and codes for an esterase with a GxSxG motif, such as that seen in ACOT1, therefore suggesting ACOT1 is the common ancestor that evolved to give rise to the acyltransferases. Several amino acids have multiple codons but serine is unique in that it has two sets, UCN and AGY, and these cannot be interconverted into each other by single nucleotide changes, as was seen in mammalian mitochondrial genomes (167). This means that the serine in ACNAT1 coded for by AGC could not have arisen from a single mutation of the serine codon in ACOT1 (UCC). Another study that analyzed sequence databanks for *Escherichia coli*, *Sacchromyces cerevisiae* and *Bacillus subtilis*

revealed a codon bias for some amino acids at conserved positions, and that the primordial codon for serine is TCN and that the AGY codon appeared later (168), such as serine used in the ACOTs (UCC). Therefore, taken together this data suggests that the thioesterase/acyltransferase gene family evolved from ACOTs, to a cysteine nucleophile such as that seen in BAAT and further to the serine nucleophile seen in the ACNATs (see Table 1). This is further supported by our finding that the AGY codon appears late in evolution (see Table III in **Paper III**).

NUCLEOPHILE	CODON
ACOT1 (Serine)	UCC
BAAT (Cysteine)	UGC
ACNAT1 (Serine)	AGC

Table 1. The codon usage of the nucleophilic residues of the mouse Type I acyl-CoA thioesterases and acyltransferases. The codons used for the nucleophilic residues in ACOT1, BAAT and ACNAT1 are shown. The arrows show the single mutations that could have occurred in the serine codon in ACOTs to give rise to the cysteine used by BACAT, which then could have resulted in the serine seen in ACNAT1.

NUDT7α and peroxisomal coenzyme A homeostasis – Paper IV

Although much of this project was mainly focused on novel acyltransferase enzymes in peroxisomes that conjugate the CoA esters of fatty acids or bile acids to glycine or taurine, we were also interested in the fate of the CoASH in peroxisomes. The current dogma is that CoASH enters peroxisomes as CoA esters but it is still unknown how CoASH exits the organelle. To date three enzyme families have been identified in peroxisomes that release CoASH from acyl-CoAs, namely the ACOTs, the carnitine acyltransferases (CRAT and CROT) and the acyltransferases (BAAT, ACNAT1 and ACNAT2). In this regard we therefore studied the nudix hydrolase, NUDT7α, which has been proposed to metabolize CoASH into smaller molecules that may exit the peroxisome. The mouse NUDT7α was first characterized in 2001 and expression of recombinant protein showed that this enzyme hydrolyzed CoASH, 3'-dephospho-CoA, oxidized CoA (CoASSCoA), acetyl-CoA and succinyl-CoA (99). NUDT19 is a further peroxisomal nudix hydrolase with activity towards CoASH, oxidized CoA and a wide

range of CoA esters, including bile acid-CoAs and branched chain acyl-CoAs (100). In the initial studies of NUDT7α, the enzyme was only characterized with CoASH and a limited set of acyl-CoAs, and since NUDT19 showed activity towards a large range of CoA esters, it was decided to perform a more in-depth study on the activity of NUDT7α.

Nudix Hydrolases and CoASH regulation

Nudt7α shows highest tissue expression in liver, followed by brown adipose tissue, heart and white adipose tissue. Its co-expression with straight-chain β-oxidation enzymes in liver and brown adipose tissue points to a role for this enzyme as an auxiliary enzyme in β-oxidation. Activity measurements with CoASH and various acyl-CoAs at a fixed concentration of 200 μM showed a very broad specificity, with lower enzyme activity with longer chain acyl-CoAs, with the best substrates being medium chain acyl-CoAs, CA-CoA and THCA-CoA (**Paper IV)**. Kinetic studies show that the K_m values are highest with CoASH and short-chain acyl-CoAs, with substantially lower K_m for medium to long chain acyl-CoAs. Also, the V_{max} values are much higher with medium-chain acyl-CoAs (C_6-C_{12}-CoA), suggesting that these acyl-CoAs are indeed the best substrates for NUDT7α (**Paper IV**).

The identification of NUDT7 and NUDT19 seemed to provide a simple explanation of what happens to CoASH in peroxisomes i.e. that it is broken down into two smaller molecules, 4'-phosphopantetheine and 3',5'-ADP (see Fig. 5), which could be transported out of the peroxisome. It is possible that the 4'-phosphopantetheine is transported out of peroxisomes and may be reused in CoASH synthesis. CoASH synthesis occurs in five enzymatic steps and the final two steps involve the transfer of an adenyl group from ATP by phosphopantetheine adenylyltransferase to 4'-phosphopantetheine to form dephospho-CoA followed by phosphorylation by dephospho-CoA kinase to obtain CoASH. In mammalian cells, proteins located in the cytosol catalyze the first three steps of the CoASH synthesis pathway (169-171), while the last two take place on the outer mitochondrial membrane (172). The fate of the 3',5'-ADP produced is unknown - it may be that it could be reused within the peroxisome, or it could be transported out of the peroxisome to be reused within the cell. In yeast and human two adenine nucleotide transporters Ant1p and PMP34, respectively, have been identified that could transport 3',5'-ADP out of the peroxisome

(67, 68). The 3',5'-ADP produced by the NUDTs could also result in the inhibition of the acetyl-CoA thioesterase ACOT12 (92), which suggests a role for the ADP produced in peroxisomes in the regulation of acetate formation.

So many enzymes, so few substrates?

In peroxisomes various CoA esters, such as the chain shortened products of β-oxidation, are substrates for not only the NUDTs, but also the ACOTs, the carnitine acyltransferases and even the newly identified ACNATs (see Fig. 14). The presence of so many enzymes within peroxisomes that are active towards the same substrates raises the question of competition for substrates and which enzyme system prevails at any one time? This is especially relevant when considering the metabolic status of a cell at a given time and the affinity of the various members of these protein families for their substrates. The K_m of the peroxisomal ACOTs for acyl-CoAs is lower than that of the NUDTs, NUDT19 and NUDT7α have K_m-values of ≈80 μM and 22 μM for lauroyl-CoA respectively (100) and **Paper IV**, while ACOT3 and ACOT8 have K_ms of 7.6 μM and 2.8 μM respectively (84, 88), which suggests that the ACOTs may preferentially hydrolyze acyl-CoAs over the NUDTs. It is important to remember though that the ACOTs cannot cleave CoASH (only acyl-CoAs), and that NUDT activity on acyl-CoAs will result instead in formation of acyl-phosphopantetheine and 3',5'-ADP, which could be produced in parallel with the products of ACOT activity.

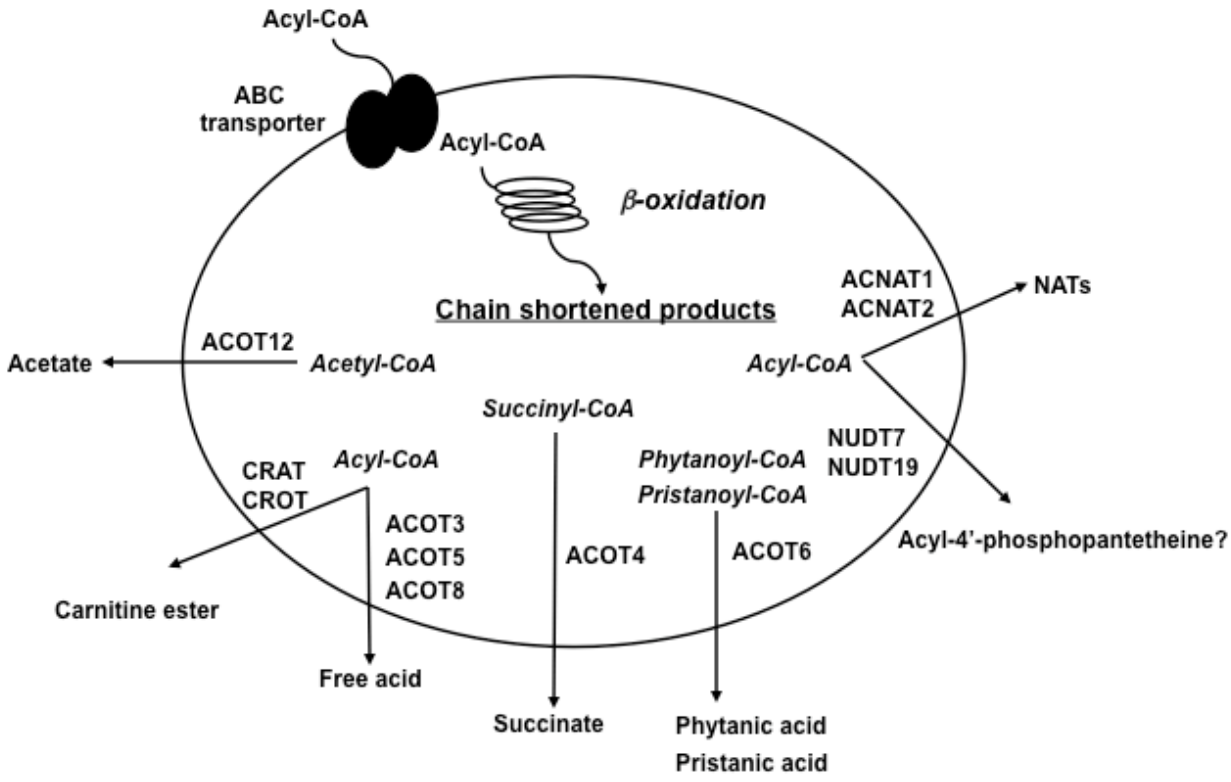


Fig. 14. Routes for excretion of fatty acids from peroxisomes. Very long-chain fatty acid CoA esters undergo β-oxidation in peroxisomes, which results in a chain shortened CoA ester plus acetyl-CoA. There are a number of potential pathways for these chain shortened products. These include esterification to carnitine by CRAT and CROT, conjugation to taurine by the ACNATs and hydrolysis to the free fatty acids, acetate or succinate by the ACOTs or acyl-4'-phosphopantetheine by the NUDTs. CRAT, carnitine acetyltransferase; CROT, carnitine octanoyltransferase; ACNAT, acyl-CoA:amino acid *N*-acyltransferase; ACOT, acyl-CoA thioesterase; NUDT, nudix hydrolase.

The question of which enzyme activity prevails at any one time in peroxisomes can also be partly explained by a study performed by Westin et al, which looked at the tissue expression of various enzymes involved in peroxisomal lipid metabolism (173). The tissue expression of mRNAs for the carnitine acyltransferases *Crat* and *Crot*, and the acyl-CoA thioesterases, *Acot12* and *Acot5* were examined. This study showed that the enzymes, which have similar substrate specificities i.e CRAT and ACOT12 (active on short chain acyl-CoAs), CROT and ACOT5 (active on medium chain acyl-CoAs), actually have different tissue expression profiles (see Fig. 15).
This suggests that that carnitine acyltransferases and acyl-CoA thioesterases do not compete with each other for acyl-CoA substrates in peroxisomes, but that they actually represent complementary systems for the production of metabolites to be transported across the peroxisomal membrane (173).

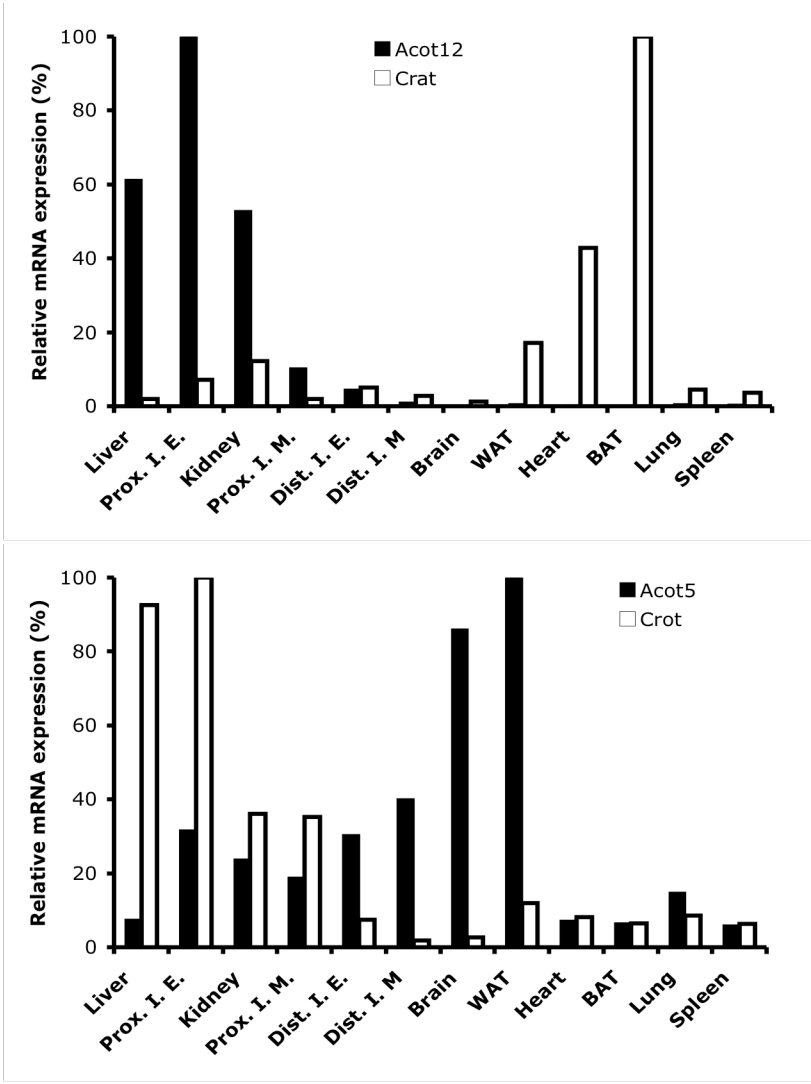


Fig. 15. Tissue expression of the acyl-CoA thioesterases and carnitine acyltransferases in mouse. The mRNA expression levels are shown for *Acot5*, *Acot12*, *Crat* and *Crot*. Prox. I. E.; proximal intestine epithelium, Prox. I. M.; proximal intestine muscle, Dist. I. E.; distal intestine epithelium, Dist. I. M; distal intestine muscle, BAT; brown adipose tissue, WAT; white adipose tissue. **Westin et al 2008, Cell Mol Life Sci.**

Nudt7α has highest tissue expression in liver, brown adipose tissue and heart (**Paper IV**), while Nudt19 has highest tissue expression in kidney with lower levels in brown adipose tissue and heart (see Fig. 16). It would be interesting to see if a similar system exists regarding the NUDTs, i.e do they compete for acyl-CoAs with other enzyme families or represent an alternative pathway for acyl-CoA and/or CoASH metabolism in other tissues. It would be of interest to compare the levels of each of the various members of the NUDT, ACOT, ACNAT and carnitine acyltransferase families in various tissues under various metabolic states relative to each other.

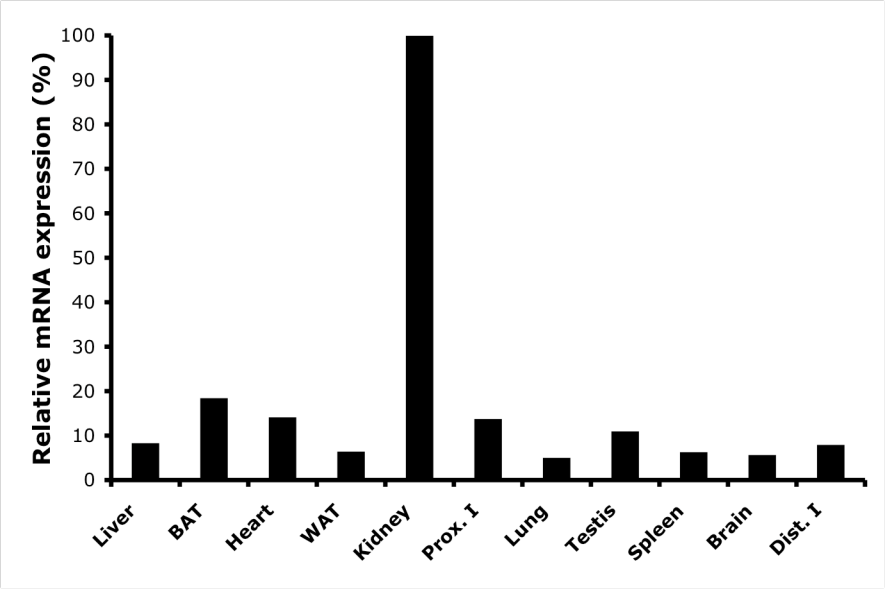


Fig. 16. Tissue expression of NUDT19. The mRNA expression levels are shown for *Nudt19* in various mouse tissues. Prox. I; proximal intestine, Dist. I; distal intestine, BAT; brown adipose tissue, WAT; white adipose tissue.

As mentioned before the PPARα is a key regulator of lipid and conditions such as fasting and fibrate treatment activate the PPARα. It has been reported that these conditions result in an increase of peroxisomal total and free CoASH levels, perhaps through the upregulation of the ACOTs. Nudt7α is downregulated by Wy-14,643 treatment at mRNA level in a PPARα-dependent manner, and activity is decreased by clofibrate treatment in purified peroxisomes (**Paper IV**). This suggests that during conditions of high requirement for CoASH in peroxisomes e.g. when β-oxidation activity is high, NUDT7 activity is decreased. This decrease in activity would result in an increase of peroxisomal CoASH as the downregulation of Nudt7α would preserve the CoASH in peroxisomes, which can then be used in the thiolase reaction of β-oxidation.

FUTURE PERSPECTIVES

The acyl-CoA thioesterase/acyltransferase gene family has nine members (seven peroxisomal) and work by our group has answered many questions as to the roles of each of the various members in peroxisomes. However, in science, as is the case with many things in life, we tend to answer questions with even more questions, and with the acyl-CoA thioesterase/acyltransferase family there are still a lot of questions to be answered.

One of the most important stories to complete in this project is to identify the activity of ACNAT2 - we have speculated as to what that could be but it is necessary to obtain functioning soluble protein so that its role in peroxisomal lipid metabolism can be established. The presence of so many enzymes in one organelle that are active towards the same substrates is interesting and raises the question of why do we need so many enzymes? A study has been planned that will look at the mRNA expression of not only peroxisomal enzymes but also mitochondrial enzymes in various mouse tissues under various metabolic conditions, and it is hoped that this study will give more insight into their physiological functions. To establish the *in vivo* physiological roles of the thioesterases and acyltransferases, the creation of knockout mouse models would provide valuable insights.

It would be really interesting to perform an in-depth study on the evolution of the acyl-CoA thioesterases and acyltransferases, especially since the human ACNAT1 appears to be a pseudogene and in human there is one peroxisomal thioesterase, ACOT4, which can catalyze the reactions of three mouse proteins (ACOT3, ACOT4 and ACOT5). Evolutionary studies would provide insights into when these “extra” activities were lost. The crystallization of any of the proteins in this gene family would give structural and functional information and allow modelling studies to be performed.

POPULAR SCIENCE

The human body contains many different organs, such as the heart, lung, and kidney, with each organ performing a different function. By the time a human reaches adulthood, his or her body consists of close to 100 trillion cells. The majority of processes of life take place within these cells, which is why they are often called the building blocks of life. Cells also have their own set of "little organs," called organelles, which are specialized in carrying out specific and vital functions. One such organelle is the peroxisome.

The human body can be considered or thought of as a machine that needs energy. Some of this energy comes from fats that we eat in food. The peroxisome together with another cell organelle, the mitochondria, is responsible for helping to get the energy the body requires from these fats. Peroxisomes are very important for the body to function normally. They are so important that people who have no peroxisomes or peroxisomes that are defective, suffer from developmental delay, mental retardation and vision and hearing problems. In the more severe cases, patients will usually not survive beyond their first year. It is therefore important that research is done to increase our understanding of peroxisome functions and these disorders so that we can find ways to prevent, treat, and cure them.

The work of the peroxisome is carried out by proteins, many of which are known as enzymes. Without enzymes, many of the important processes of life could not happen. In peroxisomes, over half of the fifty enzymes are involved in fat metabolism (these are the chemical reactions that occur in order to maintain life). Some of these enzymes are indirectly involved in helping to extract the energy from fat. Our group found a new family of enzymes in peroxisomes and our work has focused on finding out what the functions of these enzymes are. This thesis has focused mainly on two of these new enzymes, known as acyltransferases. They take fats (acyl) that are in peroxisomes and attach them (transferase) to another compound - an amino acid. By doing this they allow fats to be transported out of peroxisomes for use elsewhere in the cell e.g. for energy production in mitochondria or they may act as signaling molecules in the body, with roles in pain signaling and hearing.

ACKNOWLEDGEMENTS

Silent gratitude isn't much use to anyone ~ G.B. Stern

So in saying that I would first of all like to thank my supervisor, **Mary Hunt**. You really have been the most fantastic supervisor! Thank you so much for all the support, encouragement and guidance over the past few years!

My co-supervisor **Stefan Alexson**, thank you for inviting me to be a part of your group all those years ago and for always having your door open for all things science and non-science related.

Thank you to **Ingemar Björkhem** for giving me the opportunity to work in the Clinical Chemistry department.

To current and past group members – **James O'Byrne**, my "conjugating" hero, thank you for taking care of me all those years ago when I was just known as "Jimmy's student". **Maria Westin**, thesis writing just hasn't been the same without your "godis" support, nor has the group! To the two newest members - **Tomas Lundåsen**, the king of "ordspråk" and **Vera Tillander**, thank you for all the help over the past year but more importantly for never ever questioning my music taste! Lycka till! **Elisabet Nordström,** you were only in the group for a short time but I really enjoyed having you and surprisingly your bump as my office neighbours. Good luck in the States!

To my collaborators for all their help – **Ulla Andersson, Ethna O'Shea, Charlotta Buch -** good luck with your own thesis, **Vera Tillander, Hans Ulrich Marschall** and **James O'Byrne.**

Jenny Bernström, "my Swedish Mum", for help with all things administrative and for never getting annoyed with me when you remind me about my time sheets and get a response of "oh yeah them" followed my quick disappearance.

To everyone at work for office distractions (which are always welcome!), fikas and fun times…**Ulla Andersson**, tack så mycket för allt! För din hjälp med all svenska och allt vetenskapligt. **Lisa-Mari Nilsson,** thanks to you my Swedish vocabulary has vastly increased, as has my waistline from all your fantastic cakes! **Lillian Larsson,** for laughing at my jokes "på svenska" even when I am 100% sure that my direct

translation from English to Swedish means they are not funny at all!!! **Matteo Pedrelli**, I hope you enjoy your time in Sweden as much as I have. The two "Tinas" **Katharina Slatis** and **Kristina Kannisto** and also **Zhao Yan Jang,** for making me see colon scraping in a whole new, strange and disturbing light!

To other members of the department present and past, who have helped and made things just that little bit easier along the way **Anita**, **Ann, Deirdre**, **Gertrud**, **Gösta**, **Hanna**, **Johan**, **Magnus**, **Maria O**, **Mats**, **Padideh**, **Paolo**, **Paul**, **Per**, **Petra**, **Riona**, **Ulf** and **Valerio.**

Charlotte Murphy, **Maura Heverin** and **Steve Meaney**, you guys may have left us but we certainly haven't forgotten ye! I can't count the amount of times Marjan and myself have sighed and said, "Awwww, I really miss _____ (Enter name as appropriate)"

To the people here in Stockholm, who I don't think themselves or anybody else will ever know how much they have meant to me over the past five years...
Firstly, **Pádraig D'Arcy** and **Emma Patterson**. **Pádraig** you are without doubt the most optimistic and dedicated scientist I know…many would have given up on my "moderate lush" theory years ago. I love you for the fact that you want to keep on testing it out with me. I am looking forward to many more failures! **Emma**, you really have been and are the most amazing friend, thank you so much for everything and for always being there. It really amazes me that in the 4+ years that we have known each other that you have only told me to shut up once (I have to admit though that I am still in shock!).
Paula Cunnea, they may have said in Galway you were the source of all things evil, but I think you are a source of some seriously good fun! Thanks for all the good times both here in Sweden and Ireland. **Elie Järnmark**, "my Swedish equivalent", for being equally as fond of "holes" as I am. I cannot think of anyone else that I would rather go digging with! Love you! **Marjan Shafaati,** for the fun times in Malmö and for making me feel like a part of her fantastic family, which includes some good old fashion sibling rivalry…but ours takes place on the dance floor! People and poles, Beware!
Oliver O'Hehir, for some of the weirdest and funniest conversations that I have ever had the pleasure of being a part of! They really gave me ☺

Agaristi Lamprokostopoulou, even though it may mean waiting 3 more hours than I have planned to, your company is always well worth the wait! **Emmanouella Chatzidaki-Trinks,** for always having time for a 3pm fika and also for all the developing help, the dark room has never been so much fun! **Wessen Maruwge**, thank you for spending all those hours in various cafés around Stockholm with me.

To all those people who have had the fortunate and sometimes unfortunate pleasure of my company on the train journeys to and from work and at various social events – firstly, to two purveyors of some of the finest dancing I have ever witnessed, **Tomas L** and **Tomas S**. **John**, **Steve**, **Steph**, **Mina**, **"Swedish" Nina**, **"Russian" Nina**, **Stephen**, **Hanna**, **Torbjörn**, **Christian**, **Inga**, **Lydia**, **Neli**, **Rona**, **Anders, Ivan, Joelle** and the best next door neighbour/worst guitar player ever, **Lumni.** Thanks for all the great memories!!!

Gail, **Fergal**, **Jill**, **Helen**, **Joacim**, **Oonagh**, **Daniel**, **Seán** and **Máire**. Ye guys always helped to make Stockholm feel like home when it was needed!

My best friends at home, who always make me feel like it was only days since we last spoke or met. **Jenny O'Sullivan**, you are one of the coolest and craziest people that I know! Don't ever change! I will miss our phone battles over who actually really did do the stupidest or weirdest thing over the weekend. Hopefully we will soon get to do them together! **Judith Horgan,** thanks for always being there, no matter how random my phone calls are! For being a source of good advice... especially regarding fashion! (Although the neon pink eel skin bag has raised a few eyebrows!)

Finally, to my family, my **Mum** and **Dad**, and my sister, **Anne-Marie**, for being the most fantastic family imaginable! Thank you so much for supporting me and believing in me all these years. I don't think I will ever be able to say how much it has meant to me. I love ye guys! This also of course includes **Paul**, currently an "honorary" member of the Reilly family but soon to become a permanent one! Oh and lastly, but just because I think I might get in trouble if I don't mention them, my "other" sisters, **Callie** and **Roo**! Woof!

REFERENCES

1. Rhodin, J. (1954) Correlation of ultrastructural organization and function in normal and experimentally changed proximal convoluted tubule cells of the mouse kidney. Karolinska Institutet, Stockholm
2. De Duve, C., and Baudhuin, P. (1966) Peroxisomes (microbodies and related particles). *Physiol Rev* 46, 323-357
3. Lazarow, P. B. (1978) Rat liver peroxisomes catalyze the beta oxidation of fatty acids. *J Biol Chem* 253, 1522-1528
4. Wanders, R. J., and Waterham, H. R. (2006) Biochemistry of mammalian peroxisomes revisited. *Annu Rev Biochem* 75, 295-332
5. Hogenboom, S., Tuyp, J. J., Espeel, M., Koster, J., Wanders, R. J., and Waterham, H. R. (2004) Human mevalonate pyrophosphate decarboxylase is localized in the cytosol. *Mol Genet Metab* 81, 216-224
6. Hogenboom, S., Tuyp, J. J., Espeel, M., Koster, J., Wanders, R. J., and Waterham, H. R. (2004) Mevalonate kinase is a cytosolic enzyme in humans. *J Cell Sci* 117, 631-639
7. Hogenboom, S., Tuyp, J. J., Espeel, M., Koster, J., Wanders, R. J., and Waterham, H. R. (2004) Phosphomevalonate kinase is a cytosolic protein in humans. *J Lipid Res* 45, 697-705
8. Kovacs, W. J., Olivier, L. M., and Krisans, S. K. (2002) Central role of peroxisomes in isoprenoid biosynthesis. *Prog Lipid Res* 41, 369-391
9. Kovacs, W. J., Tape, K. N., Shackelford, J. E., Duan, X., Kasumov, T., Kelleher, J. K., Brunengraber, H., and Krisans, S. K. (2007) Localization of the pre-squalene segment of the isoprenoid biosynthetic pathway in mammalian peroxisomes. *Histochem Cell Biol* 127, 273-290
10. Goldfischer, S., Johnson, A. B., Essner, E., Moore, C., and Ritch, R. H. (1973) Peroxisomal abnormalities in metabolic diseases. *J Histochem Cytochem* 21, 972-977
11. Goldfischer, S., Moore, C. L., Johnson, A. B., Spiro, A. J., Valsamis, M. P., Wisniewski, H. K., Ritch, R. H., Norton, W. T., Rapin, I., and Gartner, L. M. (1973) Peroxisomal and mitochondrial defects in the cerebro-hepato-renal syndrome. *Science* 182, 62-64
12. Wanders, R. J., and Waterham, H. R. (2005) Peroxisomal disorders I: biochemistry and genetics of peroxisome biogenesis disorders. *Clin Genet* 67, 107-133
13. Wanders, R. J., and Waterham, H. R. (2006) Peroxisomal disorders: the single peroxisomal enzyme deficiencies. *Biochim Biophys Acta* 1763, 1707-1720
14. Refsum, S. (1945) Heredoataxia hemeralopica polyneuritiformis. *Nordisk Medicin* 28, 2682-2685
15. Klenk, E., and Kahike, W. (1963) On The Presence Of 3,7,11,15-Tetramethylhexadecanoic Acid (Phytanic Acid) In The Cholesterol Esters And Other Lipoid Fractions Of The Organs In A Case Of A Disease Of Unknown Origin (Possibly Heredopathia Atactica Polyneuritiformis, Refsum's Syndrome). *Hoppe Seylers Z Physiol Chem* 333, 133-139
16. Bezman, L., Moser, A. B., Raymond, G. V., Rinaldo, P., Watkins, P. A., Smith, K. D., Kass, N. E., and Moser, H. W. (2001) Adrenoleukodystrophy: incidence, new mutation rate, and results of extended family screening. *Ann Neurol* 49, 512-517
17. Contreras, M., Mosser, J., Mandel, J. L., Aubourg, P., and Singh, I. (1994) The protein coded by the X-adrenoleukodystrophy gene is a peroxisomal integral membrane protein. *FEBS Lett* 344, 211-215
18. Mosser, J., Douar, A. M., Sarde, C. O., Kioschis, P., Feil, R., Moser, H., Poustka, A. M., Mandel, J. L., and Aubourg, P. (1993) Putative X-linked adrenoleukodystrophy gene shares unexpected homology with ABC transporters. *Nature* 361, 726-730

19. Lazarow, P. B., and Fujiki, Y. (1985) Biogenesis of peroxisomes. *Annu Rev Cell Biol* 1, 489-530
20. Chang, C. C., South, S., Warren, D., Jones, J., Moser, A. B., Moser, H. W., and Gould, S. J. (1999) Metabolic control of peroxisome abundance. *J Cell Sci* 112 (Pt 10), 1579-1590
21. Platta, H. W., and Erdmann, R. (2007) Peroxisomal dynamics. *Trends Cell Biol* 17, 474-484
22. Matsuzono, Y., Kinoshita, N., Tamura, S., Shimozawa, N., Hamasaki, M., Ghaedi, K., Wanders, R. J., Suzuki, Y., Kondo, N., and Fujiki, Y. (1999) Human PEX19: cDNA cloning by functional complementation, mutation analysis in a patient with Zellweger syndrome, and potential role in peroxisomal membrane assembly. *Proc Natl Acad Sci U S A* 96, 2116-2121
23. South, S. T., and Gould, S. J. (1999) Peroxisome synthesis in the absence of preexisting peroxisomes. *J Cell Biol* 144, 255-266
24. Faber, K. N., Haan, G. J., Baerends, R. J., Kram, A. M., and Veenhuis, M. (2002) Normal peroxisome development from vesicles induced by truncated *Hansenula polymorpha* Pex3p. *J Biol Chem* 277, 11026-11033
25. Hoepfner, D., Schildknegt, D., Braakman, I., Philippsen, P., and Tabak, H. F. (2005) Contribution of the endoplasmic reticulum to peroxisome formation. *Cell* 122, 85-95
26. Kim, P. K., Mullen, R. T., Schumann, U., and Lippincott-Schwartz, J. (2006) The origin and maintenance of mammalian peroxisomes involves a de novo PEX16-dependent pathway from the ER. *J Cell Biol* 173, 521-532
27. Haan, G. J., Baerends, R. J., Krikken, A. M., Otzen, M., Veenhuis, M., and van der Klei, I. J. (2006) Reassembly of peroxisomes in *Hansenula polymorpha* pex3 cells on reintroduction of Pex3p involves the nuclear envelope. *FEMS Yeast Res* 6, 186-194
28. Schoonjans, K., Staels, B., and Auwerx, J. (1996) Role of the peroxisome proliferator-activated receptor (PPAR) in mediating the effects of fibrates and fatty acids on gene expression. *J Lipid Res* 37, 907-925
29. Issemann, I., and Green, S. (1990) Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature* 347, 645-650
30. Kliewer, S. A., Umesono, K., Noonan, D. J., Heyman, R. A., and Evans, R. M. (1992) Convergence of 9-cis retinoic acid and peroxisome proliferator signaling pathways through heterodimer formation of their receptors. *Nature* 358, 771-774
31. Auboeuf, D., Rieusset, J., Fajas, L., Vallier, P., Frering, V., Riou, J. P., Staels, B., Auwerx, J., Laville, M., and Vidal, H. (1997) Tissue distribution and quantification of the expression of mRNAs of peroxisome proliferator-activated receptors and liver X receptor-alpha in humans: no alteration in adipose tissue of obese and NIDDM patients. *Diabetes* 46, 1319-1327
32. Braissant, O., Foufelle, F., Scotto, C., Dauca, M., and Wahli, W. (1996) Differential expression of peroxisome proliferator-activated receptors (PPARs): tissue distribution of PPAR-alpha, -beta, and -gamma in the adult rat. *Endocrinology* 137, 354-366
33. Hunt, M. C., Lindquist, P. J., Peters, J. M., Gonzalez, F. J., Diczfalusy, U., and Alexson, S. E. H. (2000) Involvement of the peroxisome proliferator-activated receptor alpha in regulating long-chain acyl-CoA thioesterases. *J Lipid Res* 41, 814-823
34. Kersten, S., Seydoux, J., Peters, J. M., Gonzalez, F. J., Desvergne, B., and Wahli, W. (1999) Peroxisome proliferator-activated receptor alpha mediates the adaptive response to fasting. *J Clin Invest* 103, 1489-1498
35. Kliewer, S. A., Sundseth, S. S., Jones, S. A., Brown, P. J., Wisely, G. B., Koble, C. S., Devchand, P., Wahli, W., Willson, T. M., Lenhard, J. M., and Lehmann, J. M. (1997) Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma. *Proc Natl Acad Sci U S A* 94, 4318-4323
36. Helledie, T., Jorgensen, C., Antonius, M., Krogsdam, A. M., Kratchmarova, I., Kristiansen, K., and Mandrup, S. (2002) Role of adipocyte lipid-binding protein

(ALBP) and acyl-CoA binding protein (ACBP) in PPAR-mediated transactivation. *Mol Cell Biochem* 239, 157-164
37. Walton, P. A., Hill, P. E., and Subramani, S. (1995) Import of stably folded proteins into peroxisomes. *Mol Biol Cell* 6, 675-683
38. Gould, S. J., Keller, G. A., and Subramani, S. (1988) Identification of peroxisomal targeting signals located at the carboxy terminus of four peroxisomal proteins. *J Cell Biol* 107, 897-905
39. Gould, S. J., Keller, G. A., Schneider, M., Howell, S. H., Garrard, L. J., Goodman, J. M., Distel, B., Tabak, H., and Subramani, S. (1990) Peroxisomal protein import is conserved between yeast, plants, insects and mammals. *EMBO J* 9, 85-90
40. Swinkels, B. W., Gould, S. J., and Subramani, S. (1992) Targeting efficiencies of various permutations of the consensus C-terminal tripeptide peroxisomal targeting signal. *FEBS Lett* 305, 133-136
41. Swinkels, B. W., Gould, S. J., Bodnar, A. G., Rachubinski, R. A., and Subramani, S. (1991) A novel, cleavable peroxisomal targeting signal at the amino-terminus of the rat 3-ketoacyl-CoA thiolase. *EMBO J* 10, 3255-3262
42. Petriv, O. I., Tang, L., Titorenko, V. I., and Rachubinski, R. A. (2004) A new definition for the consensus sequence of the peroxisome targeting signal type 2. *J Mol Biol* 341, 119-134
43. Osmundsen, H., Neat, C. E., and Borrebaek, B. (1980) Fatty acid products of peroxisomal beta-oxidation. *Int J Biochem* 12, 625-630
44. Osumi, T., and Hashimoto, T. (1978) Acyl-CoA oxidase of rat liver: a new enzyme for fatty acid oxidation. *Biochem Biophys Res Commun* 83, 479-485
45. Nohammer, C., El-Shabrawi, Y., Schauer, S., Hiden, M., Berger, J., Forss-Petter, S., Winter, E., Eferl, R., Zechner, R., and Hoefler, G. (2000) cDNA cloning and analysis of tissue-specific expression of mouse peroxisomal straight-chain acyl-CoA oxidase. *Eur J Biochem* 267, 1254-1260
46. Schepers, L., Van Veldhoven, P. P., Casteels, M., Eyssen, H. J., and Mannaerts, G. P. (1990) Presence of three acyl-CoA oxidases in rat liver peroxisomes. An inducible fatty acyl-CoA oxidase, a noninducible fatty acyl-CoA oxidase, and a noninducible trihydroxycoprostanoyl-CoA oxidase. *J Biol Chem* 265, 5242-5246
47. Vanhove, G. F., Van Veldhoven, P. P., Fransen, M., Denis, S., Eyssen, H. J., Wanders, R. J., and Mannaerts, G. P. (1993) The CoA esters of 2-methyl-branched chain fatty acids and of the bile acid intermediates di- and trihydroxycoprostanic acids are oxidized by one single peroxisomal branched chain acyl-CoA oxidase in human liver and kidney. *J Biol Chem* 268, 10335-10344
48. Furuta, S., Miyazawa, S., Osumi, T., Hashimoto, T., and Ui, N. (1980) Properties of mitochondria and peroxisomal enoyl-CoA hydratases from rat liver. *J Biochem (Tokyo)* 88, 1059-1070
49. Jiang, L. L., Kurosawa, T., Sato, M., Suzuki, Y., and Hashimoto, T. (1997) Physiological role of D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase bifunctional protein. *J Biochem (Tokyo)* 121, 506-513
50. Kurosawa, T., Sato, M., Yoshimura, T., Jiang, L. L., Hashimoto, T., and Tohma, M. (1997) Stereospecific formation of (24R,25R)-3 alpha,7 alpha,12 alpha,24-tetrahydroxy-5 beta-cholestan-26-oic acid catalyzed with a peroxisomal bifunctional D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase. *Biol Pharm Bull* 20, 295-297
51. Qin, Y. M., Haapalainen, A. M., Conry, D., Cuebas, D. A., Hiltunen, J. K., and Novikov, D. K. (1997) Recombinant 2-enoyl-CoA hydratase derived from rat peroxisomal multifunctional enzyme 2: role of the hydratase reaction in bile acid synthesis. *Biochem J* 328 (Pt 2), 377-382
52. Antonenkov, V. D., Van Veldhoven, P. P., Waelkens, E., and Mannaerts, G. P. (1999) Comparison of the stability and substrate specificity of purified peroxisomal 3-oxoacyl-CoA thiolases A and B from rat liver. *Biochim Biophys Acta* 1437, 136-141
53. Antonenkov, V. D., Van Veldhoven, P. P., and Mannaerts, G. P. (2000) Isolation and subunit composition of native sterol carrier protein 2/3-oxoacyl-

coenzyme A thiolase from normal rat liver peroxisomes. *Protein Expr Purif* 18, 249-256
54. Chevillard, G., Clemencet, M. C., Etienne, P., Martin, P., Pineau, T., Latruffe, N., and Nicolas-Frances, V. (2004) Molecular cloning, gene structure and expression profile of two mouse peroxisomal 3-ketoacyl-CoA thiolase genes. *BMC Biochem* 5, 3
55. Antonenkov, V. D., Van Veldhoven, P. P., Waelkens, E., and Mannaerts, G. P. (1997) Substrate specificities of 3-oxoacyl-CoA thiolase A and sterol carrier protein 2/3-oxoacyl-CoA thiolase purified from normal rat liver peroxisomes. Sterol carrier protein 2/3-oxoacyl-CoA thiolase is involved in the metabolism of 2-methyl-branched fatty acids and bile acid intermediates. *J Biol Chem* 272, 26023-26031
56. van Roermund, C. W., Hettema, E. H., van den Berg, M., Tabak, H. F., and Wanders, R. J. (1999) Molecular characterization of carnitine-dependent transport of acetyl-CoA from peroxisomes to mitochondria in Saccharomyces cerevisiae and identification of a plasma membrane carnitine transporter, Agp2p. *Embo J* 18, 5843-5852
57. Russell, D. W. (2003) The enzymes, regulation, and genetics of bile acid synthesis. *Annu Rev Biochem* 72, 137-174
58. Mihalik, S. J., Steinberg, S. J., Pei, Z., Park, J., Kim, D. G., Heinzer, A. K., Dacremont, G., Wanders, R. J., Cuebas, D. A., Smith, K. D., and Watkins, P. A. (2002) Participation of two members of the very long-chain acyl-CoA synthetase family in bile acid synthesis and recycling. *J Biol Chem* 277, 24771-24779
59. Steinberg, S. J., Wang, S. J., Kim, D. G., Mihalik, S. J., and Watkins, P. A. (1999) Human very-long-chain acyl-CoA synthetase: cloning, topography, and relevance to branched-chain fatty acid metabolism. *Biochem Biophys Res Commun* 257, 615-621
60. Schmitz, W., Fingerhut, R., and Conzelmann, E. (1994) Purification and properties of an alpha-methylacyl-CoA racemase from rat liver. *Eur J Biochem* 222, 313-323
61. Van Veldhoven, P. P., Vanhove, G., Assselberghs, S., Eyssen, H. J., and Mannaerts, G. P. (1992) Substrate specificities of rat liver peroxisomal acyl-CoA oxidases: palmitoyl-CoA oxidase (inducible acyl-CoA oxidase), pristanoyl-CoA oxidase (non-inducible acyl-CoA oxidase), and trihydroxycoprostanoyl-CoA oxidase. *J Biol Chem* 267, 20065-20074
62. Van Veldhoven, P. P., Van Rompuy, P., Vanhooren, J. C., and Mannaerts, G. P. (1994) Purification and further characterization of peroxisomal trihydroxycoprostanoyl-CoA oxidase from rat liver. *Biochem J* 304 (Pt 1), 195-200
63. Watkins, P. A. (2008) Very-long-chain acyl-CoA synthetases. *J Biol Chem* 283, 1773-1777
64. Lewin, T. M., Van Horn, C. G., Krisans, S. K., and Coleman, R. A. (2002) Rat liver acyl-CoA synthetase 4 is a peripheral-membrane protein located in two distinct subcellular organelles, peroxisomes, and mitochondrial-associated membrane. *Arch Biochem Biophys* 404, 263-270
65. Van Veldhoven, P. P., Just, W. W., and Mannaerts, G. P. (1987) Permeability of the peroxisomal membrane to cofactors of beta-oxidation. Evidence for the presence of a pore-forming protein. *J Biol Chem* 262, 4310-4318
66. Antonenkov, V. D., Sormunen, R. T., and Hiltunen, J. K. (2004) The rat liver peroxisomal membrane forms a permeability barrier for cofactors but not for small metabolites in vitro. *J Cell Sci* 117, 5633-5642
67. Palmieri, L., Rottensteiner, H., Girzalsky, W., Scarcia, P., Palmieri, F., and Erdmann, R. (2001) Identification and functional reconstitution of the yeast peroxisomal adenine nucleotide transporter. *EMBO J* 20, 5049-5059
68. Visser, W. F., van Roermund, C. W., Waterham, H. R., and Wanders, R. J. (2002) Identification of human PMP34 as a peroxisomal ATP transporter. *Biochem Biophys Res Commun* 299, 494-497

69. Wanders, R. J., Visser, W. F., van Roermund, C. W., Kemp, S., and Waterham, H. R. (2006) The peroxisomal ABC transporter family. *Pflugers Arch* 453, 719-734
70. Liu, L. X., Janvier, K., Berteaux-Lecellier, V., Cartier, N., Benarous, R., and Aubourg, P. (1999) Homo- and heterodimerization of peroxisomal ATP-binding cassette half-transporters. *J Biol Chem* 274, 32738-32743
71. van Roermund, C. W., Visser, W. F., Ijlst, L., van Cruchten, A., Boek, M., Kulik, W., Waterham, H. R., and Wanders, R. J. (2008) The human peroxisomal ABC half transporter ALDP functions as a homodimer and accepts acyl-CoA esters. *FASEB J*
72. Pujol, A., Ferrer, I., Camps, C., Metzger, E., Hindelang, C., Callizot, N., Ruiz, M., Pampols, T., Giros, M., and Mandel, J. L. (2004) Functional overlap between ABCD1 (ALD) and ABCD2 (ALDR) transporters: a therapeutic target for X-adrenoleukodystrophy. *Hum Mol Genet* 13, 2997-3006
73. Netik, A., Forss-Petter, S., Holzinger, A., Molzer, B., Unterrainer, G., and Berger, J. (1999) Adrenoleukodystrophy-related protein can compensate functionally for adrenoleukodystrophy protein deficiency (X-ALD): implications for therapy. *Hum Mol Genet* 8, 907-913
74. Imanaka, T., Aihara, K., Takano, T., Yamashita, A., Sato, R., Suzuki, Y., Yokota, S., and Osumi, T. (1999) Characterization of the 70-kDa peroxisomal membrane protein, an ATP binding cassette transporter. *J Biol Chem* 274, 11968-11976
75. Visser, W. F., van Roermund, C. W., Ijlst, L., Waterham, H. R., and Wanders, R. J. (2007) Metabolite transport across the peroxisomal membrane. *Biochem J* 401, 365-375
76. Holzinger, A., Kammerer, S., and Roscher, A. A. (1997) Primary structure of human PMP69, a putative peroxisomal ABC-transporter. *Biochem Biophys Res Commun* 237, 152-157
77. Shani, N., Jimenez-Sanchez, G., Steel, G., Dean, M., and Valle, D. (1997) Identification of a fourth half ABC transporter in the human peroxisomal membrane. *Hum Mol Genet* 6, 1925-1931
78. Gergely, J., Hele, P., and Ramakrishnan, C. V. (1952) Succinyl and acetyl coenzyme a deacylases. *J Biol Chem* 198, 324-334
79. Hunt, M. C., Nousiainen, S. E., Huttunen, M. K., Orii, K. E., Svensson, L. T., and Alexson, S. E. H. (1999) Peroxisome proliferator-induced long chain acyl-CoA thioesterases comprise a highly conserved novel multi-gene family involved in lipid metabolism. *J Biol Chem* 274, 34317-34326
80. Huhtinen, K., O'Byrne, J., Lindquist, P. J., Contreras, J. A., and Alexson, S. E. H. (2002) The peroxisome proliferator-induced cytosolic type I acyl-CoA thioesterase (CTE-I) is a serine-histidine-aspartic acid alpha /beta hydrolase. *J Biol Chem* 277, 3424-3432
81. Hunt, M. C., Rautanen, A., Westin, M. A., Svensson, L. T., and Alexson, S. E. H. (2006) 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* 20, 1855-1864
82. Lindquist, P. J., Svensson, L. T., and Alexson, S. E. H. (1998) Molecular cloning of the peroxisome proliferator-induced 46-kDa cytosolic acyl-CoA thioesterase from mouse and rat liver--recombinant expression in Escherichia coli, tissue expression, and nutritional regulation. *Eur J Biochem* 251, 631-640
83. Svensson, L. T., Engberg, S. T., Aoyama, T., Usuda, N., Alexson, S. E. H., and Hashimoto, T. (1998) Molecular cloning and characterization of a mitochondrial peroxisome proliferator-induced acyl-CoA thioesterase from rat liver. *Biochem J* 329 (Pt 3), 601-608
84. Westin, M. A., Alexson, S. E., and Hunt, M. C. (2004) Molecular cloning and characterization of two mouse peroxisome proliferator-activated receptor alpha (PPARalpha)-regulated peroxisomal acyl-CoA thioesterases. *J Biol Chem* 279, 21841-21848
85. Westin, M. A., Hunt, M. C., and Alexson, S. E. H. (2005) The identification of a succinyl-CoA thioesterase suggests a novel pathway for succinate production in peroxisomes. *J Biol Chem* 280, 38125-38132

86. Westin, M. A., Hunt, M. C., and Alexson, S. E. (2007) 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 in mouse. *J Biol Chem* 282, 26707-26716
87. Hunt, M. C., and Alexson, S. E. (2008) Novel functions of acyl-CoA thioesterases and acyltransferases as auxiliary enzymes in peroxisomal lipid metabolism. *Prog Lipid Res* 47, 405-421
88. Hunt, M. C., Solaas, K., Kase, B. F., and Alexson, S. E. H. (2002) Characterization of an acyl-CoA thioesterase that functions as a major regulator of peroxisomal lipid metabolism. *J Biol Chem* 277, 1128-1138
89. Ofman, R., el Mrabet, L., Dacremont, G., Spijer, D., and Wanders, R. J. (2002) Demonstration of dimethylnonanoyl-CoA thioesterase activity in rat liver peroxisomes followed by purification and molecular cloning of the thioesterase involved. *Biochem Biophys Res Commun* 290, 629-634
90. Westin, M. A., Hunt, M. C., and Alexson, S. E. (2005) The identification of a succinyl-CoA thioesterase suggests a novel pathway for succinate production in peroxisomes. *J Biol Chem* 280, 38125-38132
91. Prass, R. L., Isohashi, F., and Utter, M. F. (1980) Purification and characterization of an extramitochondrial acetyl coenzyme A hydrolase from rat liver. *J Biol Chem* 255, 5215-5223
92. Söling, H. D., and Rescher, C. (1985) On the regulation of cold-labile cytosolic and of mitochondrial acetyl-CoA hydrolase in rat liver. *Eur J Biochem* 147, 111-117
93. O'Toole, N., and Cygler, M. (2003) The final player in the coenzyme A biosynthetic pathway. *Structure* 11, 899-900
94. Horie, S., Isobe, M., and Suga, T. (1986) Changes in CoA pools in hepatic peroxisomes of the rat under various conditions. *J Biochem (Tokyo)* 99, 1345-1352
95. Van Broekhoven, A., Peeters, M. C., Debeer, L. J., and Mannaerts, G. P. (1981) Subcellular distribution of coenzyme A: evidence for a separate coenzyme A pool in peroxisomes. *Biochem Biophys Res Commun* 100, 305-312
96. Ishii, H., Horie, S., and Suga, T. (1980) Physiological role of peroxisomal beta-oxidation in liver of fasted rats. *J Biochem (Tokyo)* 87, 1855-1858
97. Neat, C. E., Thomassen, M. S., and Osmundsen, H. (1980) Induction of peroxisomal beta-oxidation in rat liver by high-fat diets. *Biochem J* 186, 369-371
98. McLennan, A. G. (2006) The Nudix hydrolase superfamily. *Cell Mol Life Sci* 63, 123-143
99. Gasmi, L., and McLennan, A. G. (2001) The mouse Nudt7 gene encodes a peroxisomal nudix hydrolase specific for coenzyme A and its derivatives. *Biochem J* 357, 33-38
100. Ofman, R., Speijer, D., Leen, R., and Wanders, R. J. (2006) Proteomic analysis of mouse kidney peroxisomes: identification of RP2p as a peroxisomal nudix hydrolase with acyl-CoA diphosphatase activity. *Biochem J* 393, 537-543
101. Cartwright, J. L., Gasmi, L., Spiller, D. G., and McLennan, A. G. (2000) The Saccharomyces cerevisiae PCD1 gene encodes a peroxisomal nudix hydrolase active toward coenzyme A and its derivatives. *J Biol Chem* 275, 32925-32930
102. AbdelRaheim, S. R., and McLennan, A. G. (2002) The Caenorhabditis elegans Y87G2A.14 Nudix hydrolase is a peroxisomal coenzyme A diphosphatase. *BMC Biochem* 3, 5
103. Markwell, M. A., McGroarty, E. J., Bieber, L. L., and Tolbert, N. E. (1973) The subcellular distribution of carnitine acyltransferases in mammalian liver and kidney. A new peroxisomal enzyme. *J Biol Chem* 248, 3426-3432
104. Markwell, M. A., Bieber, L. L., and Tolbert, N. E. (1977) Differential increase of hepatic peroxisomal, mitochondrial and microsomal carnitine acyltransferases in clofibrate-fed rats. *Biochem Pharmacol* 26, 1697-1702
105. Farrell, S. O., Fiol, C. J., Reddy, J. K., and Bieber, L. L. (1984) Properties of purified carnitine acyltransferases of mouse liver peroxisomes. *J Biol Chem* 259, 13089-13095

106. Ferdinandusse, S., Mulders, J., L, I. J., Denis, S., Dacremont, G., Waterham, H. R., and Wanders, R. J. (1999) Molecular cloning and expression of human carnitine octanoyltransferase: evidence for its role in the peroxisomal beta-oxidation of branched-chain fatty acids. *Biochem Biophys Res Commun* 263, 213-218
107. Falany, C. N., Fortinberry, H., Leiter, E. H., and Barnes, S. (1997) Cloning, expression, and chromosomal localization of mouse liver bile acid CoA:amino acid *N*-acyltransferase. *J Lipid Res* 38, 1139-1148
108. Falany, C. N., Johnson, M. R., Barnes, S., and Diasio, R. B. (1994) Glycine and taurine conjugation of bile acids by a single enzyme. Molecular cloning and expression of human liver bile acid CoA:amino acid *N*-acyltransferase. *J Biol Chem* 269, 19375-19379
109. He, D., Barnes, S., and Falany, C. N. (2003) Rat liver bile acid CoA:amino acid *N*-acyltransferase: expression, characterization, and peroxisomal localization. *J Lipid Res* 44, 2242-2249
110. O'Byrne, J., Hunt, M. C., Rai, D. K., Saeki, M., and Alexson, S. E. (2003) The human bile acid-CoA:amino acid N-acyltransferase functions in the conjugation of fatty acids to glycine. *J Biol Chem* 278, 34237-34244
111. Killenberg, P. G., and Jordan, J. T. (1978) Purification and characterization of bile acid-CoA:amino acid *N*-acyltransferase from rat liver. *J Biol Chem* 253, 1005-1010
112. Carlton, V. E., Harris, B. Z., Puffenberger, E. G., Batta, A. K., Knisely, A. S., Robinson, D. L., Strauss, K. A., Shneider, B. L., Lim, W. A., Salen, G., Morton, D. H., and Bull, L. N. (2003) Complex inheritance of familial hypercholanemia with associated mutations in TJP2 and BAAT. *Nat Genet* 34, 91-96
113. Tougou, K., Fukuda, T., Ito, T., Yamazaki, H., Fujio, Y., and Azuma, J. (2007) Genetic polymorphism of bile acid CoA: amino acid *N*-acyltransferase in Japanese individuals. *Drug Metab Pharmacokinet* 22, 125-128
114. Solaas, K., Ulvestad, A., Söreide, O., and Kase, B. F. (2000) Subcellular organization of bile acid amidation in human liver: a key issue in regulating the biosynthesis of bile salts. *J Lipid Res* 41, 1154-1162
115. Johnson, M. R., Barnes, S., Kwakye, J. B., and Diasio, R. B. (1991) Purification and characterization of bile acid-CoA:amino acid *N*-acyltransferase from human liver. *J Biol Chem* 266, 10227-10233
116. Kase, B. F., and Björkhem, I. (1989) Peroxisomal bile acid-CoA:amino-acid *N*-acyltransferase in rat liver. *J Biol Chem* 264, 9220-9223
117. Pellicoro, A., van den Heuvel, F. A., Geuken, M., Moshage, H., Jansen, P. L., and Faber, K. N. (2007) Human and rat bile acid-CoA:amino acid *N*-acyltransferase are liver-specific peroxisomal enzymes: implications for intracellular bile salt transport. *Hepatology* 45, 340-348
118. Inoue, Y., Yu, A. M., Inoue, J., and Gonzalez, F. J. (2004) Hepatocyte nuclear factor 4 alpha is a central regulator of bile acid conjugation. *J Biol Chem* 279, 2480-2489
119. Vessy, D. A. (1997) *Enzymes involved in formation of amide bonds* Vol. 3, Elsevier Science, Oxford, England
120. Nandi, D. L., Lucas, S. V., and Webster, L. T., Jr. (1979) Benzoyl-coenzyme A:glycine N-acyltransferase and phenylacetyl-coenzyme A:glycine *N*-acyltransferase from bovine liver mitochondria. Purification and characterization. *J Biol Chem* 254, 7230-7237
121. Schachter, D., and Taggart, J. V. (1953) Benzoyl coenzyme A and hippurate synthesis. *J Biol Chem* 203, 925-934
122. Schachter, D., and Taggart, J. V. (1954) Glycine *N*-acylase: purification and properties. *J Biol Chem* 208, 263-275
123. Gregersen, N., Kølvraa, S., and Mortensen, P. B. (1986) Acyl-CoA:glycine *N*-acyltransferase: in vitro studies on the glycine conjugation of straight- and branched-chained acyl-CoA esters in human liver. *Biochem Med Metab Biol* 35, 210-218
124. Merkler, D. J., Merkler, K. A., Stern, W., and Fleming, F. F. (1996) Fatty acid amide biosynthesis: a possible new role for peptidylglycine alpha-amidating

enzyme and acyl-coenzyme A:glycine *N*-acyltransferase. *Arch Biochem Biophys* 330, 430-434
125. Cravatt, B. F., Giang, D. K., Mayfield, S. P., Boger, D. L., Lerner, R. A., and Gilula, N. B. (1996) Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. *Nature* 384, 83-87
126. Cravatt, B. F., and Lichtman, A. H. (2002) The enzymatic inactivation of the fatty acid amide class of signaling lipids. *Chem Phys Lipids* 121, 135-148
127. Saghatelian, A., Trauger, S. A., Want, E. J., Hawkins, E. G., Siuzdak, G., and Cravatt, B. F. (2004) Assignment of endogenous substrates to enzymes by global metabolite profiling. *Biochemistry* 43, 14332-14339
128. Saghatelian, A., McKinney, M. K., Bandell, M., Patapoutian, A., and Cravatt, B. F. (2006) A FAAH-regulated class of *N*-acyl taurines that activates TRP ion channels. *Biochemistry* 45, 9007-9015
129. Sfakianos, M. K., Wilson, L., Sakalian, M., Falany, C. N., and Barnes, S. (2002) Conserved residues in the putative catalytic triad of human bile acid Coenzyme A:amino acid N-acyltransferase. *J Biol Chem* 277, 47270-47275
130. Rinaldo, P., O'Shea, J. J., Coates, P. M., Hale, D. E., Stanley, C. A., and Tanaka, K. (1988) Medium-chain acyl-CoA dehydrogenase deficiency. Diagnosis by stable-isotope dilution measurement of urinary n-hexanoylglycine and 3-phenylpropionylglycine. *N Engl J Med* 319, 1308-1313
131. Devane, W. A., Hanus, L., Breuer, A., Pertwee, R. G., Stevenson, L. A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A., and Mechoulam, R. (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* 258, 1946-1949
132. Fu, J., Gaetani, S., Oveisi, F., Lo Verme, J., Serrano, A., Rodriguez De Fonseca, F., Rosengarth, A., Luecke, H., Di Giacomo, B., Tarzia, G., and Piomelli, D. (2003) Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR-alpha. *Nature* 425, 90-93
133. Zygmunt, P. M., Petersson, J., Andersson, D. A., Chuang, H., Sorgard, M., Di Marzo, V., Julius, D., and Hogestatt, E. D. (1999) Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature* 400, 452-457
134. Smart, D., Gunthorpe, M. J., Jerman, J. C., Nasir, S., Gray, J., Muir, A. I., Chambers, J. K., Randall, A. D., and Davis, J. B. (2000) The endogenous lipid anandamide is a full agonist at the human vanilloid receptor (hVR1). *Br J Pharmacol* 129, 227-230
135. Forman, B. M., Chen, J., and Evans, R. M. (1997) Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta. *Proc Natl Acad Sci U S A* 94, 4312-4317
136. Göttlicher, M., Widmark, E., Li, Q., and Gustafsson, J. A. (1992) Fatty acids activate a chimera of the clofibric acid-activated receptor and the glucocorticoid receptor. *Proc Natl Acad Sci U S A* 89, 4653-4657
137. Ahern, G. P. (2003) Activation of TRPV1 by the satiety factor oleoylethanolamide. *J Biol Chem* 278, 30429-30434
138. Zheng, J., Dai, C., Steyger, P. S., Kim, Y., Vass, Z., Ren, T., and Nuttall, A. L. (2003) Vanilloid receptors in hearing: altered cochlear sensitivity by vanilloids and expression of TRPV1 in the organ of corti. *J Neurophysiol* 90, 444-455
139. Geppetti, P., and Trevisani, M. (2004) Activation and sensitisation of the vanilloid receptor: role in gastrointestinal inflammation and function. *Br J Pharmacol* 141, 1313-1320
140. Zhang, L. L., Yan Liu, D., Ma, L. Q., Luo, Z. D., Cao, T. B., Zhong, J., Yan, Z. C., Wang, L. J., Zhao, Z. G., Zhu, S. J., Schrader, M., Thilo, F., Zhu, Z. M., and Tepel, M. (2007) Activation of transient receptor potential vanilloid type-1 channel prevents adipogenesis and obesity. *Circ Res* 100, 1063-1070
141. Lee, H., Iida, T., Mizuno, A., Suzuki, M., and Caterina, M. J. (2005) Altered thermal selection behavior in mice lacking transient receptor potential vanilloid 4. *J Neurosci* 25, 1304-1310

142. Alessandri-Haber, N., Yeh, J. J., Boyd, A. E., Parada, C. A., Chen, X., Reichling, D. B., and Levine, J. D. (2003) Hypotonicity induces TRPV4-mediated nociception in rat. *Neuron* 39, 497-511
143. Mizuno, A., Matsumoto, N., Imai, M., and Suzuki, M. (2003) Impaired osmotic sensation in mice lacking TRPV4. *Am J Physiol Cell Physiol* 285, C96-101
144. Tabuchi, K., Suzuki, M., Mizuno, A., and Hara, A. (2005) Hearing impairment in TRPV4 knockout mice. *Neurosci Lett* 382, 304-308
145. Turman, M. V., Kingsley, P. J., Rouzer, C. A., Cravatt, B. F., and Marnett, L. J. (2008) Oxidative metabolism of a fatty acid amide hydrolase-regulated lipid, arachidonoyltaurine. *Biochemistry* 47, 3917-3925
146. Kuhn, H., and O'Donnell, V. B. (2006) Inflammation and immune regulation by 12/15-lipoxygenases. *Prog Lipid Res* 45, 334-356
147. Solaas, K., Kase, B. F., Pham, V., Bamberg, K., Hunt, M. C., and Alexson, S. E. H. (2004) Differential regulation of cytosolic and peroxisomal bile acid amidation by PPAR alpha activation favors the formation of unconjugated bile acids. *J Lipid Res* 45, 1051-1060
148. Drewes, T., Senkel, S., Holewa, B., and Ryffel, G. U. (1996) Human hepatocyte nuclear factor 4 isoforms are encoded by distinct and differentially expressed genes. *Mol Cell Biol* 16, 925-931
149. Jiang, S., Tanaka, T., Iwanari, H., Hotta, H., Yamashita, H., Kumakura, J., Watanabe, Y., Uchiyama, Y., Aburatani, H., Hamakubo, T., Kodama, T., and Naito, M. (2003) Expression and localization of P1 promoter-driven hepatocyte nuclear factor-4 alpha (HNF4 alpha) isoforms in human and rats. *Nucl Recept* 1, 5
150. Hayhurst, G. P., Lee, Y. H., Lambert, G., Ward, J. M., and Gonzalez, F. J. (2001) Hepatocyte nuclear factor 4alpha (nuclear receptor 2A1) is essential for maintenance of hepatic gene expression and lipid homeostasis. *Mol Cell Biol* 21, 1393-1403
151. Hubbard, B., Doege, H., Punreddy, S., Wu, H., Huang, X., Kaushik, V. K., Mozell, R. L., Byrnes, J. J., Stricker-Krongrad, A., Chou, C. J., Tartaglia, L. A., Lodish, H. F., Stahl, A., and Gimeno, R. E. (2006) Mice deleted for fatty acid transport protein 5 have defective bile acid conjugation and are protected from obesity. *Gastroenterology* 130, 1259-1269
152. Peet, D. J., Turley, S. D., Ma, W., Janowski, B. A., Lobaccaro, J. M., Hammer, R. E., and Mangelsdorf, D. J. (1998) Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXR alpha. *Cell* 93, 693-704
153. Makishima, M., Okamoto, A. Y., Repa, J. J., Tu, H., Learned, R. M., Luk, A., Hull, M. V., Lustig, K. D., Mangelsdorf, D. J., and Shan, B. (1999) Identification of a nuclear receptor for bile acids. *Science* 284, 1362-1365
154. Parks, D. J., Blanchard, S. G., Bledsoe, R. K., Chandra, G., Consler, T. G., Kliewer, S. A., Stimmel, J. B., Willson, T. M., Zavacki, A. M., Moore, D. D., and Lehmann, J. M. (1999) Bile acids: natural ligands for an orphan nuclear receptor. *Science* 284, 1365-1368
155. Wang, H., Chen, J., Hollister, K., Sowers, L. C., and Forman, B. M. (1999) Endogenous bile acids are ligands for the nuclear receptor FXR/BAR. *Mol Cell* 3, 543-553
156. Pircher, P. C., Kitto, J. L., Petrowski, M. L., Tangirala, R. K., Bischoff, E. D., Schulman, I. G., and Westin, S. K. (2003) Farnesoid X receptor regulates bile acid-amino acid conjugation. *J Biol Chem* 278, 27703-27711
157. Martin, G., Duez, H., Blanquart, C., Berezowski, V., Poulain, P., Fruchart, J. C., Najib-Fruchart, J., Glineur, C., and Staels, B. (2001) Statin-induced inhibition of the Rho-signaling pathway activates PPARalpha and induces HDL apoA-I. *J Clin Invest* 107, 1423-1432
158. Landrier, J. F., Thomas, C., Grober, J., Duez, H., Percevault, F., Souidi, M., Linard, C., Staels, B., and Besnard, P. (2004) Statin induction of liver fatty acid-binding protein (L-FABP) gene expression is peroxisome proliferator-activated receptor-alpha-dependent. *J Biol Chem* 279, 45512-45518

159. Park, J. E., Kim, K. B., Bae, S. K., Moon, B. S., Liu, K. H., and Shin, J. G. (2008) Contribution of cytochrome P450 3A4 and 3A5 to the metabolism of atorvastatin. *Xenobiotica* 38, 1240-1251
160. Sakuma, T., Takai, M., Endo, Y., Kuroiwa, M., Ohara, A., Jarukamjorn, K., Honma, R., and Nemoto, N. (2000) A novel female-specific member of the CYP3A gene subfamily in the mouse liver. *Arch Biochem Biophys* 377, 153-162
161. Sakuma, T., Endo, Y., Mashino, M., Kuroiwa, M., Ohara, A., Jarukamjorn, K., and Nemoto, N. (2002) Regulation of the expression of two female-predominant CYP3A mRNAs (CYP3A41 and CYP3A44) in mouse liver by sex and growth hormones. *Arch Biochem Biophys* 404, 234-242
162. O'Brien, P. J., and Herschlag, D. (1999) Catalytic promiscuity and the evolution of new enzymatic activities. *Chem Biol* 6, R91-R105
163. Holmquist, M. (2000) Alpha/Beta-hydrolase fold enzymes: structures, functions and mechanisms. *Curr Protein Pept Sci* 1, 209-235
164. Ollis, D. L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S. M., Harel, M., Remington, S. J., Silman, I., Schrag, J., and et al. (1992) The alpha/beta hydrolase fold. *Protein Eng* 5, 197-211
165. Jensen, R. A. (1976) Enzyme recruitment in evolution of new function. *Annu Rev Microbiol* 30, 409-425
166. Brenner, S. (1988) The molecular evolution of genes and proteins: a tale of two serines. *Nature* 334, 528-530
167. Henaut, A. (1982) The serine codons UCX and AGY are not interchangeable in the mammalian mitochondrial genomes. *Biosci Rep* 2, 515-519
168. Diaz-Lazcoz, Y., Henaut, A., Vigier, P., and Risler, J. L. (1995) Differential codon usage for conserved amino acids: evidence that the serine codons TCN were primordial. *J Mol Biol* 250, 123-127
169. Rock, C. O., Calder, R. B., Karim, M. A., and Jackowski, S. (2000) Pantothenate kinase regulation of the intracellular concentration of coenzyme A. *J Biol Chem* 275, 1377-1383
170. Aberhart, D. J., Ghoshal, P. K., Cotting, J. A., and Russell, D. J. (1985) Coenzyme A biosynthesis: steric course of 4'-phosphopantothenoyl-L-cysteine decarboxylase. *Biochemistry* 24, 7178-7182
171. Scandurra, R., Barboni, E., Granata, F., Pensa, B., and Costa, M. (1974) Pantothenoylcysteine-4' -phosphate decarboxylase from horse liver. *Eur J Biochem* 49, 1-9
172. Zhyvoloup, A., Nemazanyy, I., Panasyuk, G., Valovka, T., Fenton, T., Rebholz, H., Wang, M. L., Foxon, R., Lyzogubov, V., Usenko, V., Kyyamova, R., Gorbenko, O., Matsuka, G., Filonenko, V., and Gout, I. T. (2003) Subcellular localization and regulation of coenzyme A synthase. *J Biol Chem* 278, 50316-50321
173. Westin, M. A., Hunt, M. C., and Alexson, S. E. (2008) Short- and medium-chain carnitine acyltransferases and acyl-CoA thioesterases in mouse provide complementary systems for transport of beta-oxidation products out of peroxisomes. *Cell Mol Life Sci* 65, 982-990