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ENOYL THIOESTER REDUCTASES—ENZYMES OF FATTY ACID SYNTHESIS AND DEGRADATION IN MITOCHONDRIA

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ILKKA MIINALAINEN

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Abstract

Fatty acids are one of the most essential categories of biological lipids and their synthesis and degradation are vital for all organisms. Severely compromised phenotypes of yeast mutants and human patients, which have defective components in their degradative or synthetic processes for fatty acid metabolism, have highlighted the importance of these processes for overall metabolism. Most fatty acids are degraded by β-oxidation, which occurs in mitochondria and peroxisomes in mammals, whereas synthesis is catalyzed by cytosolic multifunctional peptides, although a synthesis system involving individual enzymes in mitochondria has been also proposed.

In this study a novel mitochondrial 2-enoyl thioester reductase Etr1p from the yeast *Candida tropicalis*, its homolog Mrf1p from *Saccharomyces cerevisiae*, and their mammalian ortholog were identified and characterized. Observations indicating that mitochondrial localization as well as enzymatic activity is needed to complement the respiratory-deficient phenotype of the *mrf1*Δ strain from *S. cerevisiae* suggests that Etr1p and Mrf1p might act as a part of the mitochondrial fatty acid synthesis machinery, the proper function of which is essential for respiration and the maintenance of mitochondrial morphology in yeast. The mammalian enzyme, denoted Nrbf-1p, showed similar localization, enzymatic activity, and ability to rescue the growth of the *mrf1*Δ strain suggesting that mammals are also likely to possess the ability and required machinery for mitochondrial fatty acid synthesis.

This study further included the characterization of another mitochondrial thioester reductase, 2,4-dienoyl-CoA reductase, which acts as an auxiliary enzyme in the β-oxidation of unsaturated fatty acids. The function of this gene was analyzed by creating a knock-out mouse model. While unstressed mice deficient in 2,4-dienoyl-CoA reductase were asymptomatic, metabolically challenged mice showed symptoms including hypoglycemia, hepatic steatosis, accumulation of acylcarnitines, and severe intolerance to acute cold exposure. Although the oxidation of saturated fatty acids proceeds normally, the phenotype was in many ways similar to mouse models of the disrupted classical β-oxidation pathway, except that an altered ketogenic response was not observed. This mouse model shows that a proper oxidative metabolism for unsaturated fatty acids is important for balanced fatty acid and energy metabolism.

Keywords: β-oxidation, 2-enoyl thioester reductase, 2,4-dienoyl-CoA reductase, energy metabolism, knock-out mice

To Marjut

"*Mulder, look. There has to be a scientific explanation for this*."

Dana Scully, "Detour"

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Oulu, September 2006 Ilkka Miinalainen

Abbreviations

ABC	ATP-binding cassette
ACAD	acyl-CoA dehydrogenase
ACOX	acyl-CoA oxidase
ACP	acyl carrier protein
ACSL	long-chain acyl-CoA synthetase
ATP	adenosine triphosphate
cDNA	complementary deoxyribonucleic acid
CoA	coenzyme A
CPT	carnitine-palmitoyl transferase
DECR	2,4-dienoyl-CoA reductase protein
Decr	mouse 2,4-dienoyl-CoA reductase gene
DNA	deoxyribonucleic acid
ER	endoplasmic reticulum
ETF	electron-transferring flavoprotein
Etr1p	enoyl thioester reductase 1 protein
FAD	flavine-adenine dinucleotide
FAO	fatty acid oxidation
FAS	fatty acid synthesis
FMN	flavine mononucleotide
GFP	green fluorescent protein
HPLC	high performance liquid chromatography
kb	kilobase
kDa	kilodalton
MFE	peroxisomal multifunctional enzyme
MUFA	monounsaturated fatty acids
NAD(P)H	nicotinamide adenine dinucleotide (phosphate), reduced form
NAD+	nicotinamide adenine dinucleotide, oxidized form
NEFA	nonesterified fatty acids
Nrbf-1p	mammalian ortholog of yeast Ert1p
ORF	open reading frame
PCR	polymerase chain reaction

PUFA	polyunsaturated fatty acid
RNA	ribonucleic acid
SDR	short-chain dehydrogenase/reductase family
TFP	mitochondrial trifunctional protein
Å	ångström, 10^{-10} m

List of original articles

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

I Torkko JM, Koivuranta KT, Miinalainen IJ, Yagi AI, Schmitz W, Kastaniotis AJ, Airanne TT, Gurvitz A & Hiltunen KJ (2001) Candida tropicalis Etr1p and *Saccharomyces cerevisiae* Ybr026p (Mrf1´p), 2-enoyl thioester reductases essential for mitochondrial respiratory competence. Mol Cell Biol 21: 6243-6253.

II Miinalainen IJ, Chen Z-J, Torkko JM, Pirilä PL, Sormunen RT, Bergmann U, Qin Y-M & Hiltunen JK (2003) Characterization of 2-enoyl thioester reductase from mammals – an ortholog of Ybr026p/Mrf1´p of the yeast mitochondrial fatty acid synthesis type II. J Biol Chem 278: 20154-20161.

III Miinalainen IJ, Schmitz W, Soininen R, Conzelmann E & Hiltunen JK. Mitochondrial 2,4-dienoyl-CoA reductase-deficiency in mice results in hypoglycemia with stress intolerance and unimpaired ketogenesis. Manuscript.

Contents

1 Introduction

Due to the essential role of fatty acids as structural components of cellular membranes and in various processes of the cell, including energy metabolism, protein modification, and modulation of gene expression, their de novo synthesis, as well as degradation, are vital for all organisms. The importance of fatty acid metabolism for eukaryotes is underscored by severe phenotypes of yeast mutants and human patients, which have defective components in either their degradative or synthetic pathways.
For a long time fatty acid synthesis was thought to be a solely cytosolic process in eukaryotes, where multifunctional enzyme complexes carry out the required reactions. The discovery of mitochondrial enzymes that showed similarity to soluble enzymes operating in prokaryotic fatty acid synthesis suggested that another system for synthesizing fatty acids might exist in eukaryotic mitochondria.

Metabolic compartmentalization, where certain processes are localized to different cellular organelles, also occurs in fatty acid degradation. β-Oxidation, which degrades most fatty acids via a cyclic process, is found in peroxisomes in yeast, whereas in mammals it is confined to both mitochondria and peroxisomes. Mitochondrial β-oxidation of fatty acids is a predominant source of energy for heart and skeletal muscle at all times. During fasting, or at times when other energy sources are restricted, mitochondria can provide up to 90% of the energy requirement of mammals by degradation of fatty acids. The oxidative degradation of unsaturated fatty acids, which are common constituents in natural lipids, requires, in addition to enzymes of the classical β-oxidation cycle, auxiliary enzymes that act on pre-existing double bonds. Auxiliary enzymes have been identified from eukaryotes, as well as from prokaryotes and different pathways for processing double bonds at even- and odd-numbered positions are known to exist.

In the present study 2-enoyl thioester reductases from the yeast Candida tropicalis and Saccharomyces cerevisiae were characterized and linked to mitochondrial fatty acid synthesis and respiratory competence. A protein with similar activity was also identified and characterized from human and bovine. In addition, the physiological function of another thioester reductase, 2,4-dienoyl-CoA reductase, which participates in the mitochondrial β-oxidation of unsaturated fatty acids, was studied using a knock-out

mouse model to elucidate the importance of this auxiliary enzyme for balanced fatty acid metabolism.

2 Review of the literature

2.1 Fatty acids and their thioesters

Fatty acids are defined as a class of hydrophobic compounds consisting of a long hydrocarbon chain and terminal carboxyl group. Because of their major physiological roles as the building blocks of phospholipids and glycolipids, which are important components of biological membranes, for energy storage in form of triacylglycerols, and as modifiers of membrane proteins, fatty acids are considered to be one of the most essential category of biological lipids.

Based on the number of carbon atoms in the hydrophobic chain, fatty acids can be categorized into short-chain ($<C_8$), medium-chain (C_8-C_{12}), long-chain (C_{14}-C_{20}), and very-long-chain ($>C_{20}$) fatty acids. In its simplest form a fatty acid contains no modifications in the methylene units of the chain and is considered a straight-chain saturated fatty acid. Most naturally occurring saturated fatty acids have an even number (14, 16 or 18) of carbon atoms, of which hexadecenoic acid ($C_{16:0}$, palmitic acid) is considered most abundant. If the alkyl chain contains a double bond, the fatty acid is termed unsaturated and the number of double bonds determines the degree of unsaturation. Monounsaturated fatty acids (MUFA), such as 9Z-octadecenoic acid ($C_{18:1}$, oleic acid), have a single double bond usually in *cis*-configuration (Z), whereas polyunsaturated fatty acids (PUFA) contain multiple double bonds separated by at least one methylene group.

Chain length and the degree of unsaturation have an important effect on the properties of fatty acids and lipids derived thereof. Consequently, fatty acids significantly influence the stability, fluidity and permeability of biological membranes. The double bond in the *cis*-configuration produces a bend in the acyl chain (Figure 1.) that disrupts the ordered packing of chains in membranes and increases their fluidity. This is an important function especially for prokaryotes that can, by regulating the amount of unsaturated fatty acids in membrane phospholipids, maintain a balance between flexibility and rigidity and thus adapt to changing environmental conditions (Weber *et al.* 2001).

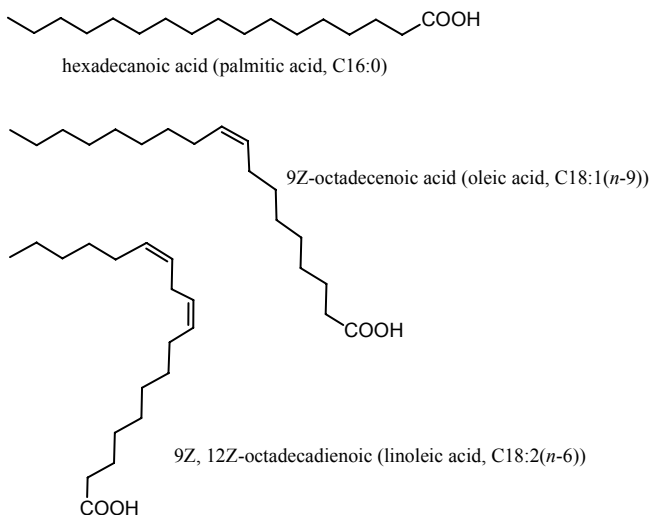


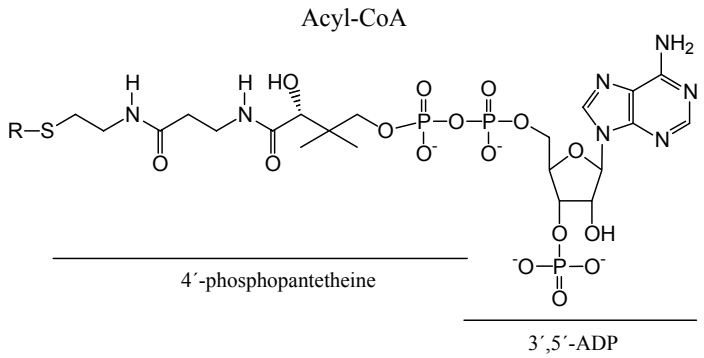
Fig. 1. Structures of saturated and mono- and polyunsaturated fatty acids

Some members of PUFA, such as 9Z, 12Z-octadecadienoic acid ($C_{18:2}$, linoleic acid), can not be synthesized by eukaryotes and must be acquired from the diet. These so called "essential fatty acids" and their longer-chain derivatives are important constituents of mammalian membrane phospholipids and are also precursors of eicosanoids such as prostaglandins and leukotrienes. These are important biological signalling molecules required especially in regulation of activities related to immune and inflammatory responses, pain, and fever (Austin and Funk 1999). Fatty acids and PUFA are also involved in signal transduction and amplify or modify signals controlling the activities of certain enzymes, e.g. protein kinases and phospholipases. Furthermore, it has been shown that PUFA can regulate the transcription of genes involved in fatty acid transport and metabolism (Wahle *et al.* 2003, Sampath and Ntambi 2004) through their effects on transcription factors called peroxisomal proliferator activated receptors (PPAR) and sterol regulatory element binding proteins (SREBP).

The structural and stereochemical properties of specific fatty acids, such as the length of the acyl chain, the position of double bonds (even- or odd-numbered) and *cis/trans*-isomerism, which are needed to modulate the functions mentioned above, have an effect on their processing in the cell. These properties determine the cellular compartment in which they are synthesized, elongated or degraded, and the route of transportation to various cellular locations. The set of enzymes needed for processing and whether auxiliary enzymes are needed in addition to the classical set of enzymes, as in the case of β-oxidation of MUFA and PUFA (see section 2.5), is also determined by the properties of an individual fatty acid.

Before intracellular free fatty acids, arising from *de novo* synthesis, from stored reserves in adipose tissue or obtained directly from the diet, can be subjected to different metabolic processes, such as oxidation, esterification or synthetic modifications, they must be activated. This activation is carried out by linking the fatty acid to a thiol moiety from Coenzyme A (CoA) to form acyl-CoA (Mishra and Drueckhammer 2000). The

highly energetic thioester bond formed in this process facilitates the subsequent transfer of acyl groups to different receptor molecules. In addition to degradative processes of acyl-CoAs (β-oxidation) that occur both in mitochondria and peroxisomes, CoA linked acyl thioesters are utilized in the synthesis of triacylglycerols and complex lipids, and in the modifications of proteins by myristoylation or palmitoylation. Acyl-CoA thioesters also act as intermediates in the elongation and desaturation of fatty acids, which occurs predominantly in the membranes of the endoplasmic reticulum (ER) (Leonardi *et al.* 2005). In fatty acid synthesis the highly energetic thioester bond is formed by linking acyl intermediates to the thiol moiety of acyl carrier protein (ACP) instead of CoA (Prescott and Vagelos 1972). Common to both CoA linked and ACP linked acyl thioesters is that the acyl moiety is coupled to the terminal sulfhydryl of the phosphopantetheine group (Figure 2.). In CoA this group is attached to 3´-5´-diphosphoadenosine moiety, whereas in ACP phosphopantetheine is attached to the protein via a phosphodiester linkage. Interestingly, the phosphopantetheine prosthetic group that is essential for the activity of ACP is transferred from CoA to ACP posttranslationally (Lambalot *et al.* 1996). In addition to fatty acid synthesis systems, acyl-ACP thioesters also have other physiological functions, such as roles in the synthesis of lipoic acid, lipopolysaccharides, antibiotic polypeptides, and also quorum sensing in bacteria.



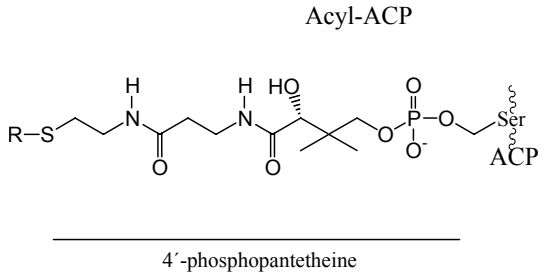


Fig. 2. Schematic drawing of acyl-CoA and acyl-ACP thioesters. Acyl groups are indicated by R.

2.2 Compartmentalization of fatty acid metabolism

Fatty acid metabolism can be considered to be a compartmentalized process with organ-specific and cell type-specific differences. Furthermore, different subcellular organelles have unique roles in the synthetic and degradative processes of fatty acid metabolism. The organization required by compartmentalization is elicited by co-ordinated and targeted transport of enzymes and substrate molecules to distinct localizations across the biomembranes of cellular organelles. In lower eukaryotes the enzymatic steps of β-oxidation are confined to the peroxisomes (Hiltunen *et al.* 2003), whereas in mammals they occur both in peroxisomes and mitochondria (Van Veldhoven and Mannaerts 1999, Bartlett and Eaton 2004). Fatty acid synthesis (FAS), which was previously thought to be a solely cytosolic process in eukaryotes, appears to be divided into two subcellular compartments similar to mammalian β-oxidation as recent evidence also points to its occurance in mitochondria (see sections 2.3.3 and 2.3.4). Different organellar systems serve special functions and although the same sets of oxidative or synthetic reactions are catalyzed in certain organelles they can not complement each other.

Similar chemical conversions can be found both in degradative and synthetic pathways and they also share common thioester intermediates as *trans*-2-enoyl thioesters, 3-hydroxyacyl thioesters, and 3-ketoacyl thioesters are observed in both β-oxidation and FAS pathways. The existence of opposing pathways sharing common intermediates in the same cellular compartment is not common as it might lead to futile cycling, and in general compartmentalization is used to keep catabolic and anabolic reactions physically separated. However, since degradation occurs via substrates that are linked to CoA and synthesis via ACP linked substrates, these pivotal fatty acid metabolism pathways can be kept chemically separate.

2.3 Fatty acid synthesis

Fatty acids are considered to be essential building blocks with important roles in a variety of biological functions. They are important, not only as constituents of energy storing compounds and as neutral and polar lipids that ensure the integrity and functionality of biological membranes, but they can also act as post-translational protein modifiers and control cellular metabolism by modulating gene expression. Due to the various important functions of fatty acids, their *de novo* synthesis is an elementary capability in all organisms. The reaction mechanism of fatty acid synthesis (FAS) is basically the same in all organisms, although different molecular structures for FAS have evolved. Multifunctional polypeptides are responsible for the bulk of the FAS in yeast and mammals (Schweizer *et al.* 1978, Smith 1994) and are referred to as eukaryotic FAS type I (FAS I). In contrast, bacterial and plant fatty acid synthesis is carried out by individual monofunctional enzymes termed FAS type II (FAS II). Recent evidence suggests that, in addition to well-characterized cytosolic FAS I, eukaryotic cells contain another FAS system in mitochondria, which is structurally and functionally different from FAS I. This has discrete soluble enzymes that catalyze each step of the reaction cycle and thus resemble prokaryotic FAS II (Rock and Jackowski 2002).

2.3.1 FAS I

Fatty acid synthesis proceeds through a set of chemical reactions that result in the cyclic stepwise elongation of activated precursor molecules by two carbon units per cycle (Figure 3.). The enzymatic steps of the pathway were initially characterized from *E. coli* and yeast systems (Lynen 1961, Wakil *et al.* 1964). The synthesis is initiated by transfer of acyl moieties from acetyl-CoA and malonyl-CoA to acyl carrier protein (ACP) by malonyl-CoA-/acetyl-CoA:ACP transacylase (MAT) in mammals and by acetyl transferase (AT) and malonyl/palmitoyl transferase (MPT) activities in fungi (Figure 3.). In the condensation reaction of the first cycle, β-ketoacyl synthase (KS) catalyzes the formation of acetoacyl-ACP by decarboxylative condensation of the acyl group with malonyl-ACP. This β-ketoacyl intermediate is further processed by β-ketoacyl reductase (KR) to form β-hydroxyacyl-ACP, which is subsequently dehydrated and reduced by the activities of β-hydroxyacyl dehydratase (DH) and β-enoyl reductase (ER), respectively. The resulting saturated acyl substrate is condensed with the malonyl moiety in each of the following cycles until a length of C_{16} is reached. The growing chain remains attached to ACP throughout its synthesis and in mammals is released from ACP by thioesterase (TE) activity which generates the free acid, whereas in fungi that lack a thioesterase domain in FAS the fatty acids are transferred to CoA by MPT and released as CoA thioesters (Bloch and Vance 1977, Smith 1994).

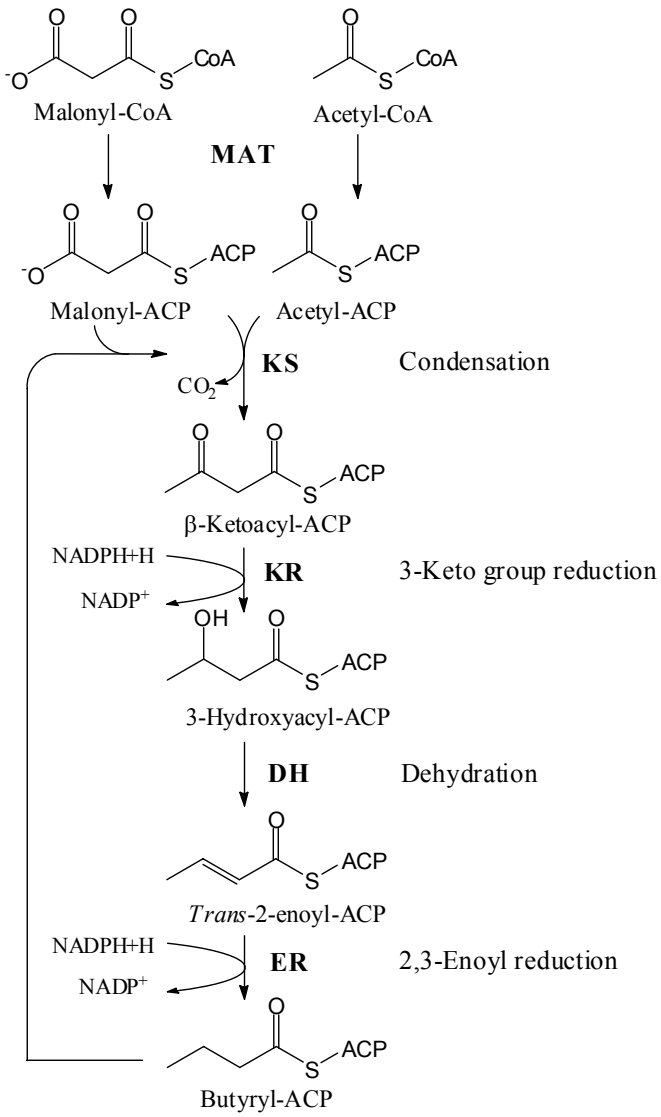


Fig. 3. Enzymatic steps and reaction intermediates in the first cycle of mammalian FAS I. MAT, malonyl-CoA-/acetyl-CoA:ACP transacylase; KS, β-ketoacyl synthase; KR, β-ketoacyl reductase; DH, β-hydroxyacyl dehydratase; ER, β-enoyl reductase. See the text for further details.

2.3.1.1 Domain organization and structure

Mammalian FAS I is a dimeric protein consisting of two identical 270 kDa multifunctional polypeptides (α2) over which the enzymatic activities and ACP are distributed (Wakil *et al.* 1983). The order of the functional domains was confirmed by molecular cloning, which revealed that the polypeptide has the following domain sequence: KS-MAT-DH-ER-KR-ACP-TE (Amy *et al.* 1989, Witkowski *et al.* 1991). The absence of high resolution crystal structures sustained the existence of two different models for the structural organization of FAS I. Originally it was proposed that the polypeptides were orientated in an extended head to tail antiparallel manner (Stoops and Wakil 1981). Later studies suggested a coiled head to head orientation based on mutant complementation studies, cross-linking studies and electron micrography (Witkowski *et al.* 1999, Rangan *et al.* 2001, Asturias *et al.* 2005). Very recently a 4.5 Å crystal structure of mammalian FAS I was presented, which confirmed the head to head orientation and showed that the coiled subunits form an asymmetric X-shaped structure with two reaction chambers (Maier *et al.* 2006).

Although catalyzing essentially the same set of reactions, the molecular and structural architecture of fungal FAS I differs markedly from that of its mammalian counterpart. In fungi the individual enzymatic activities of FAS I are distributed over two non-identical polypeptides that form a dodecamer (α6β6) (Stoops *et al.* 1978). The 210 kDa α-polypeptide contains, in addition to ACP, ketoacyl reductase and ketoacyl synthase activities and also phosphopantetheine transferase (PT) activity in the following sequence: ACP-KR-KS-PT (Mohamed *et al.* 1988, Fichtlscherer *et al.* 2000). In the 230 kDa β-polypeptide the rest of the activities are in the sequence AT-ER-DH-MPT (Schweizer *et al.* 1986). The overall structure of fungal FAS I has been determined by electron microscopy studies and defined as a barrel-shaped structure with six sites of fatty acid synthesis (Stoops *et al.* 1992). The accurate positioning and orientation of catalytic domains in this multienzyme complex was made possible by recent determination of its crystal structure at a resolution of 5 Å. The barrel-shaped structure showed two identical reaction chambers, each harbouring three copies of a full set of the domains needed for FAS, separated by a central wheel (Jenni *et al.* 2006).

It has been generally accepted that multifunctional proteins are formed by fusion of genes encoding individual polypeptides (Hardie *et al.* 1986). As both mammalian and fungal FAS I domains share sequence similarity with their monofunctional counterparts, it is probable that chromosomal rearrangements have led to grouping of the genes of the biosynthetic pathway into multidomain-coding sequences. The fact that in *E. coli* several of the FAS genes are clustered and some of them are cotranscribed (Rawlings and Cronan 1992) provides further support for the gene fusion hypothesis and suggests that fusion of genes originally began in prokaryotes (Smith 1994).

2.3.1.2 The ACP domain

The phosphopantetheine arm of ACP to which the growing acyl chain is linked during synthesis has been indentified as a key factor that transfers intermediates between catalytic domains. It was originally proposed that the mobility of this arm facilitates interaction with different domains (Lynen 1967). However, later studies showed that estimated distances between active sites are markedly greater than 18 Å, which is the length of the phosphopantetheine arm, and thus ruled out the "swinging arm" hypothesis (Foster *et al.* 1985, Yuan and Hammes 1986). Crystal structures of mammalian and fungal FAS I, although not providing details at the atomic level, provide further information on the mechanism of substrate movement between catalytic domains. In mammalian FAS I considerable movement of ACP is needed to enable the access of ACP to various domains. When suitable proximity is reached the phosphopantetheine arm of ACP is needed for the insertion of ACP-bound substrates into deep-set active centers. This movement possibly results from the internal flexibility of ACP, as well as from the flexibility of the linker region, together with slight conformational changes in domains, as observed in the asymmetrical reaction chambers and in electron microscopy studies (Asturias *et al.* 2005, Maier *et al.* 2006). In the fungal model ACPs are confined to the reaction chambers and as in the mammalian system movement is needed in order to reach the proximity of the catalytic centers. This movement is not facilitated by large conformational changes, but rather by movement of the ACP domain itself after which the phosphopantetheine arm is responsible for the delivery of substrates to hydrophobic catalytic pockets (Jenni *et al.* 2006).

2.3.1.3 Alternative substrates and product specificity

In most organisms palmitic acid (C_{16}) is the main product of FAS I. The specificities of enzymes responsible for substrate loading, chain elongation and termination ensure that once elongation has started it proceeds efficiently and terminates when a chain length of C_{16} is reached. In mammals the key regulator appears to be the thioesterase domain of FAS I that shows maximum activity for a C_{16} acyl chain length with greatly declined activities for substrates shorter than C_{16} or longer than C_{18} (Mattick *et al.* 1983, Pazirandeh *et al.* 1989, Chakravarty *et al.* 2004). FAS I also normally produces small amounts of lauric acid (C_{12}), as well as myristic acid (C_{14}), which have important role as acyl moieties in several proteins (Farazi *et al.* 2001).

An important characteristic of FAS I is that depending on the organism or particular tissue, initiation and termination reactions may vary producing products other than palmitic acid. The use of propionyl-CoA as a priming substrate instead of acetyl-CoA results in the generation of odd-numbered saturated fatty acids, mainly C_{15} and C_{17} (Jones *et al.* 1978, Seyama *et al.* 1981). When extender substrate, malonyl-CoA, is replaced by methylmalonyl-CoA, which in mammals can happen only in certain tissues, such as sebaceous glands, (multi)methyl-branched chain fatty acids will be synthesized. Although the synthesis rate of methyl-branched chain fatty acids is significantly lower than that of straight-chain fatty acids, in harderian and meibomian glands and avian uropygial glands

methyl-branched fatty acids are the dominant products of FAS I. In the tissues mentioned above specific FAS-independent malonyl-CoA decarboxylase lowers the level of malonyl-CoA and thus restricts the availability of extender substrates to methylmalonyl-CoA (Buckner *et al.* 1978, Kim and Kolattukudy 1978, Seyama *et al.* 1981).

Use of alternative substrates accounts for the synthesis of odd-numbered and methyl-branched fatty acids but changes in the chain length of products require the use of a special chain terminating enzyme. In the mammary glands of nonruminant animals a monofunctional thioesterase, termed thioesterase II, that is not part of the multifunctional FAS I complex, is responsible for the premature termination of the growing chain and production of medium chain-length fatty acids (C_8-C_{14}), which are essential compounds of milk fat triacylglycerols (Libertini and Smith 1978, Smith 1981). A similar thioesterase also operates in the preen glands of aquatic birds producing lipids needed for the water-proofing of feathers (De Renobales *et al.* 1980). In the mammary glands of ruminants (e.g. cows, goats and sheep) a special thioesterase is not needed since malonyl/acetyl transferase has relaxed substrate specificity and is able to translocate medium chain-length substrates from the synthesis pathway by transacylation. This results in medium chain-length acyl-CoA thioesters, which are subsequently used in the production of milk fat (Hansen and Knudsen 1980, Knudsen and Grunnet 1982).

2.3.2 FAS II

In type II fatty acid synthesis a series of individual enzymes encoded by separate genes is responsible for initiation of acyl chain synthesis and subsequent chain elongation and termination. A model organism for this system that operates in bacteria, as well as in plant plastids and parasites, is *E. coli* from which all of the individual genes have been cloned and characterized. The protein structures of all the members of the pathway have also been resolved, which has greatly helped in understanding the catalytic mechanism, substrate recognition and chain length specificity of individual proteins. The basic steps of the fatty acid synthesis are common to all bacteria and the genes are generally well conserved. Figure 4 depicts the fatty acid biosynthesis pathway in *E. coli* showing the catalytic steps and participating enzymes.

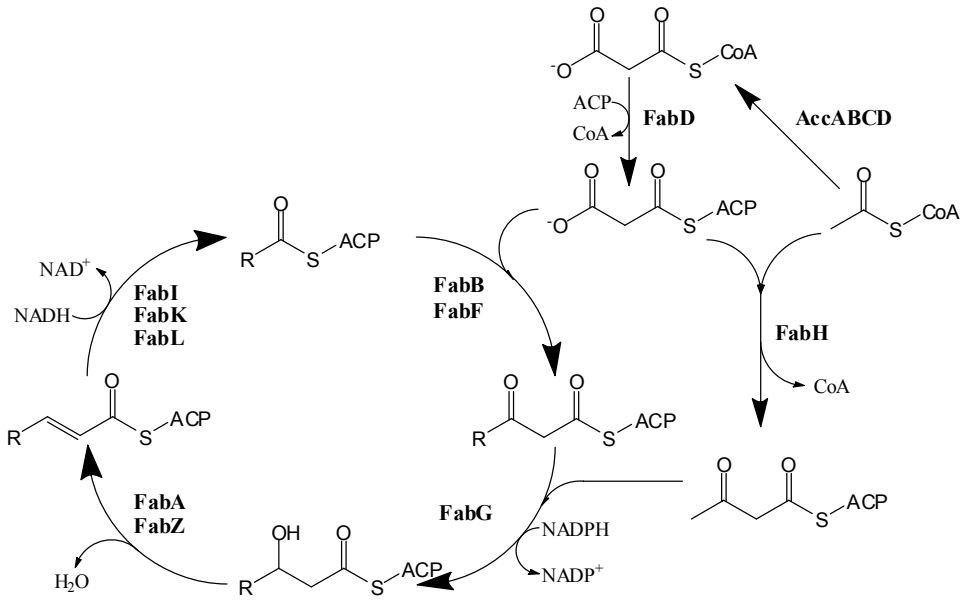


Fig. 4. Type II fatty acid biosynthetic pathway of prokaryotes. In the first committed step heterotetrameric acetyl-CoA carboxylase (AccABCD) coverts acetyl-CoA to malonyl-CoA, which is then transferred to ACP by malonyl-CoA:ACP transacylase (FabD). The chain elongation is initiated by condensation of acetyl-CoA with malonyl-ACP catalyzed by β-ketoacyl-ACP synthase III (FabH). The cycle continues with reactions catalyzed by β-ketoacyl-ACP reductase (FabG), β-hydroxyacyl-ACP dehydratase (FabA, FabZ), and *trans*-2-enoyl-ACP reductase (FabI, FabK, FabL). In the subsequent cycles the condensation reaction is catalyzed by β-ketoacyl-ACP synthase I/II (FabB, FabF).

2.3.2.1 Soluble ACP of FAS II

In contrast to FAS I, where ACP is part of the multifunctional polypeptide, the ACP in FAS II is present as a soluble protein. All the acyl intermediates in the synthesis pathway are carried as thioesters from enzyme to enzyme by ACP making it indispensable for FAS II. ACP contains a 4′-phosphopantetheine prosthetic group attached to a serine residue in the conserved AspSerLeu motif of the protein and the thioester linkage is formed between the acyl group and terminal sulfhydryl of the prosthetic group (Figure 2.).

The ACP in *E. coli* is the most extensively studied and since its structural characterization (Holak *et al.* 1988), structures of several other ACPs have been analyzed (White *et al.* 2005). ACPs form a family of highly related small (~ 9 kDa) acidic proteins with very similar structural elements and overall folding consisting of four α-helices grouped into a bundle. It has been shown that ACPs from other bacteria, plant, and even the ACP domain of rat FAS I can act as an acyl carrier with the FAS enzymes of *E. coli* (Ohlrogge *et al.* 1995, Tropf *et al.* 1998).

With the exception of acetyl-CoA carboxylase, an ACP carrying an acyl group interacts transiently with all the enzymes of FAS II. It has been suggested that this interaction occurs between conserved exposed negative residues on ACP and a positively charged ACP-binding motif on the surface of FAS II enzymes (Zhang *et al.* 2003). Based on the structural homology between FAS I and FAS II enzymes it has also been proposed that a guiding mechanism based on the similar interactions facilitates the entry of substrates into the different active sites of mammalian FAS (Maier *et al.* 2006). Structural studies also suggest that the prosthetic group in ACP can be bound in the hydrophobic pocket adjacent to the AspSerLeu motif or as solvent exposed (Wong *et al.* 2002) and that the ACP interacts with the attached acyl intermediates using the same hydrophobic region (Roujeinikova *et al.* 2002). Furthermore it has been hypothesized that acyl intermediates with a longer chain length are more favoured in interactions with ACP, whereas with short chain length intermediates the hydrophobic interactions are not favoured and the acyl chains are exposed to the solvent (White *et al.* 2005).

2.3.2.2 Initiation of synthesis

The first committed step of fatty acid synthesis, the conversion of acetyl-CoA to malonyl-CoA, is catalyzed by acetyl-CoA carboxylase (Acc) (Guchhait *et al.* 1974). The reaction is composed of two half reactions and requires the coordinated action of four different genes (*AccA, AccB, AccC, AccD*) encoding interacting AccBC and AccAD complexes that catalyze the ATP-dependent formation of carboxybiotin and the transfer of a carboxyl group to acetyl-CoA, respectively (Cronan and Waldrop 2002). In bacteria the malonyl-CoA formed is exclusively used for fatty acid synthesis and thus the role of the reaction catalyzed by Acc as a regulatory step has been studied. The expression of Acc correlates with the growth rate of the cell (Li and Cronan 1993) and it has been shown that overexpression leads to elevated fatty acid synthesis (Davis *et al.* 2000). Feedback inhibition of Acc activity by acyl-ACP has also been observed, illustrating the role of Acc as a key regulatory enzyme in FAS (Davis and Cronan 2001).

Before the malonyl moiety is used in FAS it is transferred from malonyl-CoA to ACP. This readily reversible step is catalyzed by monomeric malonyl-CoA:ACP transacylase (FabD) that exists as a single isoform, which has been identified from all known bacterial genomes. FabD is highly specific for malonyl-CoA and its activity is needed not only for delivery of malonyl-ACP for initiation of FAS, but also to supply the subsequent rounds of elongation, as has been shown with temperature-sensitive mutants (Harder *et al.* 1974). This enzyme is present in excess and has no apparent regulatory function in the FAS pathway.

β-Ketoacyl-ACP synthase III (FabH) initiates chain elongation by catalyzing the condensation of acetyl-CoA with malonyl-ACP producing β-ketobutyryl-ACP with concomitant loss of CO_2 (Jackowski *et al.* 1989). Since FabH is essential for initiation of FAS (Lai and Cronan 2003), it has a role in determining the amount of fatty acids produced by the pathway. Consequently, the activity of FabH is controlled by feedback inhibition by long-chain acyl-ACPs, which coordinate the rate of initiation of new acyl chains with the rate of utilization of the chains formed (Heath and Rock 1996). The

substrate specificity of FabH has a major role in determining the structure of the fatty acids formed and thus the fatty acid composition of membrane phospholipids. In *E. coli* FabH uses only acetyl- and propionyl-CoA as substrates and only straight-chain saturated and unsaturated fatty acids can be formed (Heath and Rock 1996), whereas in bacteria, such as *Bacillus subtilis*, that produce branched-chain fatty acids, FabH prefers branched-chain acyl-CoA substrates derived from amino acid catabolism (Choi *et al.* 2000).

2.3.2.3 Elongation of acyl chains

After the initial condensation reaction four enzymes are responsible for further cycles of chain elongation, wherein a two-carbon unit from malonyl-ACP is added to the growing acyl-chain in each cycle until a chain length of C_{16} - C_{18} is reached.

The first of the two reductive steps of the cycle, the NADPH-dependent reduction of β-ketoacyl-ACP intermediates to β-hydroxyacyl-ACP, is catalyzed by β-ketoacyl-ACP reductase (FabG). FabG is essential for the growth and viability of *E. coli* and is needed for all of the elongation cycles required to synthesize long-chain fatty acids (Zhang and Cronan 1998, Lai and Cronan 2004). It can utilize β-ketoacyl-ACPs of all chain lengths as a substrate and is a highly conserved component of FAS for which no alternative isoforms have been described among bacteria (Heath and Rock 1995, Heath 2001). FabG belongs to the short-chain dehydrogenase/reductase (SDR) family of enzymes, whose members catalyze a broad range of reduction and dehydrogenase reactions using a nucleotide cofactor (Jornvall *et al.* 1995, Oppermann *et al.* 2003). Unlike most of the SDR family members, FabG undergoes a rearrangement of the active site residues upon cofactor binding as evidenced by crystal structures (Price *et al.* 2004).

The next step in the cycle is the dehydration of β-hydroxyacyl-ACP to *trans*-2-enoyl-ACP. In bacteria there are two known isoforms of β-hydroxyacyl-ACP hydratase, FabA and FabZ. FabZ can process both saturated and unsaturated β-hydroxyacyl-ACPs of all chain lengths, whereas FabA dehydrates only saturated intermediates being most active towards 10-carbon substrates (Mohan *et al.* 1994, Heath and Rock 1996). In addition to dehydratase activity FabA also catalyzes the isomerization reaction that introduces the double bond into the Δ^3 position of the 10-carbon intermediate and routes the synthesis from saturated fatty acids towards the formation of unsaturated fatty acids. In this reaction *trans*-2-decenoyl-ACP formed after dehydration stays bound to FabA and is isomerised to *cis*-3-decenoyl-ACP instead of being reduced by enoyl-ACP reductase (FabI). The crystal structure of FabA revealed that the length of the active site tunnel is suitable for a 10-carbon substrate, which explains the specificity of the isomerization reaction (Leesong *et al.* 1996). In addition to FabA, the synthesis of unsaturated fatty acids in *E. coli* requires β-ketoacyl-ACP synthase I (FabB) activity for further elongation of *cis*-3-decenoyl-ACP and this activity determines the rate of unsaturated fatty acid synthesis (Clark *et al.* 1983, Zhang *et al.* 2002). Unlike FabZ, which is ubiquitously expressed in bacteria, FabA is not as widely distributed and is lacking in gram-positive bacteria (Campbell and Cronan 2001). In these bacteria FabZ is the only dehydratase and double bonds are introduced either into the growing acyl chain by a special isomerase (FabM in *Streptococcus pneumoniae*) (Marrakchi *et al.* 2002) or into pre-existing acyl

chains in membrane phospholipids by desaturation (Δ5 desaturase in *B. subtilis*) (Aguilar *et al.* 1998, Mansilla *et al.* 2003).

The second reductive step and the last step in each round of the cycle is the NADH-dependent reduction of *trans*-2-enoyl-ACP to acyl-ACP. This step is catalyzed in *E.coli* by a single enoyl-ACP reductase called FabI. Similar to FabG, FabI belongs to the SDR family of proteins, which have a conserved active site motif Ser-X10-Tyr-Xn-Lys, although FabI differs from typical members of the SDR family in that n=6 and Ser is replaced by Tyr. FabI is considered a key regulator of FAS since it has a determinant role in completing elongation cycles (Heath and Rock 1995) and its activity is regulated by long-chain acyl-ACP inhibition. This product inhibition ensures that new chains are formed according to requirement (Heath and Rock 1996). FabI is a highly conserved protein within FAS II and can be identified from most bacteria. However, genes related to FabI can not be found in the genomes of *Streptococci* or *Clostridia* even though they contain genes encoding all the other members of FAS II. *Streptococcus pneumonia* uses *trans*-2-enoyl-ACP reductase II, termed FabK, which can be identified from several Gram-positive bacteria and *Pseudomonas*. FabK is a FAD containing, NADH-dependent protein that has a similar function in the elongation cycle as FabI but shows no similarity to it (Heath and Rock 2000). *B. subtilis* contains, in addition to FabI, another enoyl-ACP reductase, named FabL, which is NADPH-dependent. This protein has some degree of homology to FabI and contains a motif that resembles the active site motif of FabI, and these two protein share overlapping functions as has been shown by mutation studies (Heath *et al.* 2000).

An elongation class of condensation enzymes, β-ketoacyl-ACP synthase I (FabB) and β-ketoacyl-ACP synthase II (FabF), acts in the subsequent rounds of elongation. These enzymes operate by adding a two-carbon unit from malonyl-ACP to the growing acyl-ACP in a condensation reaction where CO_2 is released (Garwin *et al.* 1980). FabB is a homodimeric enzyme that elongates C_6 - C_{12} acyl-ACP substrates efficiently while showing minimal activity with C_{14} and C_{16} substrates (Edwards *et al.* 1997). Importantly, FabB is essential for unsaturated fatty acid synthesis as it can utilize *cis*-3-decenoyl-ACP produced by FabA and thus initiate the cycle leading to production of long-chain unsaturated fatty acids (Clark *et al.* 1983). Similar to FabB, FabF is a homodimeric protein and accepts a wide range of acyl-ACP molecules as a substrate. FabF can also utilize palmitoleic acid ($C_{16:1}$) required for the synthesis of *cis*-vaccenic acid ($C_{18:1}$) and thus has an essential role in the regulation of fatty acid composition in response to temperature changes (Gelmann and Cronan 1972, Garwin *et al.* 1980, Edwards *et al.* 1997). FabB and FabF share substantial similarity at the amino acid level and their crystal structures are also very similar. An extended hydrophobic pocket near their active sites can accommodate acyl chains up to C_{16} and thus the substrate binding site limits the length of fatty acids that can be produced by FAS II systems (Huang *et al.* 1998, Olsen *et al.* 1999, Olsen *et al.* 2001). Although both enzymes share a conserved catalytic triad and their active site architecture is very similar, significant differences exist in their substrate binding. Only FabB can utilize *cis*-3-decenoyl-ACP ($C_{10:1}$) as a primer, whereas elongation of *cis*-hexadecenoyl-ACP ($C_{16:1}$) is carried out exclusively by FabF. It has been proposed that small differences in their substrate binding hydrophobic pockets are responsible for these observed substrate preferences (Moche *et al.* 2001).

2.3.3 Mitochondrial FAS in eukaryotes

Similarity searches from genome databases, as well as genetic screening studies, have revealed several genes from fungi and mammals that share similarity with the members of bacterial FAS II, and this has led to the theory that a FAS II system operates in the mitochondria of eukaryotes. This system is best characterized from *Saccharomyces cerevisiae*, which has been shown to encode proteins comprising an essentially complete set of fatty acid synthesis type II proteins localized to the mitochondria. The mammalian system for mitochondrial fatty acid synthesis is less well characterized, although recent isolation and functional characterization of several of its members greatly strengthens the evidence for its existence. The enzymatic steps of FAS in eukaryotic mitochondria and participating enzymes in fungi are shown in figure 5.

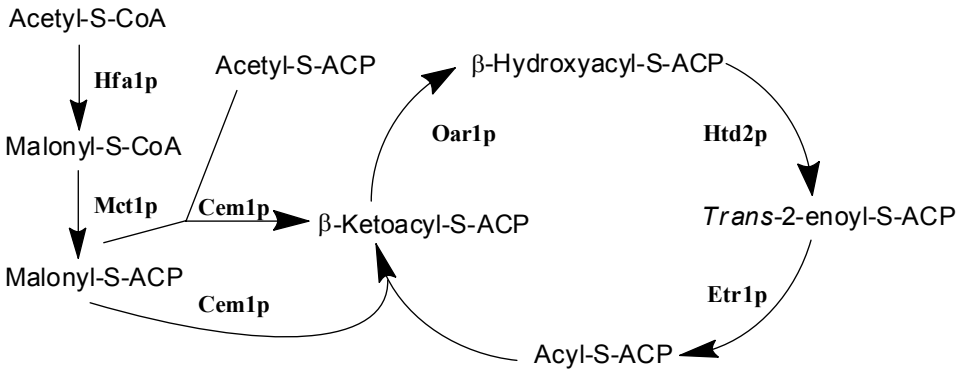


Fig. 5. Catalytic steps and participating enzymes of mitochondrial FAS II in *S. cerevisiae*. Hfa1p, mitochondrial acetyl-CoA carboxylase; Mct1p, malonyl-CoA:ACP transferase; Cem1p, β-ketoacyl-ACP synthase (condensing enzyme 1); Oar1p, β-ketoacyl-ACP reductase (3-oxoacyl-ACP reductase); Htd2p, 3-hydroxyacyl-ACP dehydratase; Etr1p, 2-enoyl-ACP reductase.

2.3.3.1 The fungal model

The first finding suggesting the existence of a fatty acid synthesis system in the mitochondria of eukaryotes was the discovery of ACP in the mitochondria of the fungus *Neurospora crassa* (Brody and Mikolajczyk 1988) and later in *S. cerevisiae* (Schneider *et al.* 1995). The ACPs from these organisms closely resembled the ACPs of the prokaryotic FAS type II system and in *N. crassa* ACP was found to be associated with respiratory complex I (Sackmann *et al.* 1991), whereas in *S. cerevisiae*, which lacks complex I, ACP exists as an independent mitochondrial protein. Disruption of the gene coding for mitochondrial ACP was shown to produce a respiratory-deficient phenotype in both organisms as in *N. crassa* it resulted in loss of complex I, and in *S. cerevisiae* complexes III and IV of the respiratory chain were absent (Schneider *et al.* 1995).

Subsequently several genes associated with mitochondrial FAS – β-ketoacyl-ACP synthase (named CEM1), malonyl-CoA:ACP transferase (named MCT1), and β-ketoacyl-ACP reductase (named OAR1) – were identified from *S. cerevisiae* based on their similarity with bacterial FAS II genes. Similar to the disruption of *ACP1*, inactivation of any of these genes resulted in respiratory-deficient phenotypes (Harington *et al.* 1993, Schneider *et al.* 1997).

The identification of fungal counterparts to bacterial β-hydroxyacyl-ACP dehydratase and 2-enoyl-ACP reductase, enzymes responsible for the last steps of the FAS cycle, was hindered by the fact that candidates bearing similarity to the bacterial enzymes could not be located from available databases. The gene encoding mitochondrial 3-hydroxyacyl-ACP dehydratase was identified recently by genetic screening from *S. cerevisiae* and named *HTD2* (Kastaniotis *et al.* 2004). It was shown that disruption of *HTD2* caused respiratory deficiency and loss of cytochromes in a manner identical to other mitochondrial FAS mutants. Furthermore, the mutant phenotype could be complemented by mitochondrially localized *E. coli* FabA and FabZ proteins, which are functional analogues of Htd2p. It was also shown that the protein bore low similarity to the hydroxyacyl dehydratases of fatty acid synthase complexes and resembled the 2-enoyl-CoA hydratase part of peroxisomal multifunctional protein type 2 (MFE II) in having a hydratase-2 motif (Kastaniotis *et al.* 2004).

The initial characterization of mitochondrial 2-enoyl thioester reductase from *Candida tropicalis* and *S. cerevisiae* based on protein purification and molecular cloning is presented in original article I included in this thesis and discussed in more detail below (see results and discussion sections).

Like other FAS systems, mitochondrial FAS is dependent on malonyl-CoA as a chain extender substrate. In fungi this substrate for cytosolic FAS is provided by carboxylation of acetyl-CoA to malonyl-CoA in the reaction catalyzed by acetyl-CoA carboxylase (ACC1). The finding that an open reading frame *HFA1* encodes the putative protein with a high degree of similarity to ACC1 suggested the existence of functionally differentiated organellar acetyl-CoA carboxylase in yeast (Kearsey 1993). However, the biochemical function of this gene remained unclear and it was proposed that malonyl-CoA produced by ACC1 is also used in mitochondria (Hoja *et al.* 1998), although it is likely that this molecule can not penetrate mitochondrial membranes. Recent studies with *HFA1* showed that gene disruption results in a phenotype very similar to other mitochondrial FAS mutants. It was also shown that gene product Hfa1p is localized to the mitochondria and that mitochondrial localization is required for complementation of *HFA1* null mutants. From these results it was concluded that *HFA1* encodes mitochondrial acetyl-CoA carboxylase providing malonyl-CoA for mitochondrial FAS (Hoja *et al.* 2004).

2.3.3.2 The mammalian model

Since the discovery of an ACP-like protein in animal mitochondria (Runswick *et al.* 1991, Triepels *et al.* 1999), the existence of a FAS system in mammalian mitochondria has been suspected. This protein was found to be a subunit of a tightly membrane bound complex, called complex I, in the respiratory chain. Since all the characterized proteins

from FAS II are soluble and act on intermediates bound to ACP, the question remained as to whether complex I containing mitochondria have a distinct ACP species that is targeted to the matrix, or whether complex I-bound ACP can participate in FAS reactions. Data from complete genome sequences, as well as biochemical data, support the existence of multiple ACPs in several eukaryotes, including *Caenorhabditis elegans* and *N. crassa*, however only one gene coding for mitochondrial ACP is known in mammalian species (Schulte 2001). Very recently it was reported that most of the ACP-like protein in bovine mitochondria is actually present as soluble matrix protein available to carry the intermediates of mitochondrial FAS (Cronan *et al.* 2005). As it is known that the ACP-like protein in complex I contains phosphopantetheine and is acylated (Runswick *et al.* 1991, Hirst *et al.* 2003) it was suggested that this protein interacts with phosphopantetheinyl transferase and the proteins of mitochondrial FAS before it is recruited to complex I during its assembly (Cronan *et al.* 2005).

The cloning and characterization of 2-enoyl thioester reductase from human and bovine paved the way for characterization of other mammalian enzymes participating in mitochondrial fatty acid synthesis. This study is presented in original article II included in this thesis and discussed in more detail below (see results and discussion sections).

At the same time as our work on mammalian 2-enoyl thioester reductase was conducted, phosphopantetheinyl transferase and mitochondrial malonyl-CoA:ACP transacylase (malonyl transferase) were cloned and characterized from human. Human phosphopantetheinyl transferase was found to be a monomeric protein capable of transferring a phosphopantetheine moiety from CoA to both the ACP domain of cytosolic multifunctional FAS and ACP associated with mitochondria. It was also shown that the transferase can phosphopantetheinylate peptidyl carrier proteins and ACPs from prokaryotes (Joshi *et al.* 2003). In contrast to the transferase domains of mammalian cytosolic FAS I, which can transfer malonyl and acetyl moieties, human malonyl transferase is specific for malonyl-CoA and in that resembles *E. coli* malonyl-CoA:ACP transacylase (FabD). Furthermore, malonyl transferase can also transfer a malonyl group to prokaryotic ACPs in addition to mitochondrial ACP, but not to the ACP domain of human cytosolic FAS. Thus the functional properties of human malonyl transferase are closer to prokaryotic FabD proteins than to the transferase domain of cytosolic FAS I and this relationship is also evident at the amino acid sequence level (Zhang *et al.* 2003).

Recently a characterization of mitochondrial β-ketoacyl synthase was reported. This enzyme shows significant similarity to *S. cerevisiae* and *N. crassa* β-ketoacyl synthases, as well as to prokaryotic synthases I (FabB) and II (FabF), at the amino acid level, and can effectively utilize acyl chains containing 2 - 14 carbon atoms as substrates, elongating them with malonyl-ACP. Like other characterized mitochondrial FAS enzymes mammalian ketoacyl synthases appear to be more closely related to their prokaryotic and fungal counterparts than to cytosolic FAS I. The substrate specificity of human β-ketoacyl synthase is quite similar to that of prokaryotic FabB and FabF with the exception that it can also catalyze the initial condensation reaction using acetyl-ACP as a primer. The ability to complement a *S. cerevisiae* mutant lacking ketoacyl synthase (CEM1) activity indicates that the kinetic properties, as well as the substrate specificity of the human enzyme correspond to those of CEM1 (Zhang *et al.* 2005).

2.3.4 The physiological significance of mitochondrial FAS

Although the components involved in mitochondrial FAS have been reasonably well characterized, especially in yeast, the physiological significance of this system is not clear. The majority of mitochondrial fatty acids are synthesized by cytosolic FAS and imported to the mitochondria. Therefore, significant changes in mitochondrial fatty acid composition can not be observed in cells where components of mitochondrial FAS have been disrupted (Harington *et al.* 1993). However, the importance of the FAS system in mitochondria is clearly shown in yeast by respiratory-deficient phenotypes unable to sustain growth on nonfermentable carbon sources, if any of the components are defective.

The major products of prokaryotic FAS II are long chain acyl-ACPs with 16 or 18 carbon atoms and a C_8 intermediate (octanoyl-ACP) is routed off for the synthesis of lipoic acid. Lipoic acid is an important cofactor for at least pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, the glycine cleavage system and branched-chain α-ketoacid dehydrogenases, and in eukaryotes these enzymes localize to the mitochondria. The products of mitochondrial FAS in yeast and plants have been reported to contain 8 - 18 carbon atoms, the major acyl chains in *N. crassa* being C_8 and C_{14} (Mikolajczyk and Brody 1990) and in plants C_8, C_{16}, and C_{18} (Gueguen *et al.* 2000, Yasuno *et al.* 2004). While the role of long chain fatty acids produced in mitochondria is not clear, it is generally accepted that the octanoic acid (C_8) produced serves as a precursor in lipoic acid synthesis. In *S. cerevisiae* this has been shown by the disruption of *ACP1*and *HFA1* genes, which resulted in respiratory-deficient mutants with greatly reduced lipoic acid content. The addition of lipoic acid to growth media did not rescue the phenotype indicating that yeast cells can not incorporate lipoic acid into mitochondria or that it is not activated (Brody *et al.* 1997, Hoja *et al.* 2004). Decreased lipoic acid content by itself does not explain all the phenotypes seen in yeast mutants deficient in mitochondrial FAS, as it has been shown that disruption of the lipoic acid synthesis pathway, in contrast to mitochondrial FAS, does not produce a cytochrome-deficient phenotype (Sulo and Martin 1993).

It is usually thought that the lipoic acid requirement of mammals is met by dietary intake. However, recently, a lipoic acid synthase that converts octanoyl-ACP to lipoyl-ACP was characterized from mammalian mitochondria (Morikawa *et al.* 2001). This finding and the close relationship between several components of prokaryotic FAS II and eukaryotic mitochondrial FAS suggests the possibility that, similar to plants and fungi, one role of mitochondrial FAS may be to supply octanoyl-ACP for lipoic acid production and to ensure a constant supply of this essential cofactor regardless of its availability through the diet.

The capability of mitochondria to produce long chain fatty acids would indicate partial functional redundancy between cytosolic and mitochondrial FAS systems. However, mitochondrial FAS is clearly unable to complement cells with inactivated cytosolic FAS as it has been shown that the growth media of such cells needs to be supplemented with fatty acids to prime the synthesis of very long chain fatty acids (Rossler *et al.* 2003). It has been suggested that mitochondrial FAS provides ACP-bound substrates for the repair of damaged phospholipids in mitochondrial inner membranes and/or for the remodelling of membrane phospholipids (Schneider *et al.* 1995, Schneider *et al.* 1997). It has also

been proposed that mitochondrial FAS has a role in the production of fatty acids required for the membrane insertion of proteins and a role in the maintenance of mitochondrial morphology (Harington *et al.* 1993, Kastaniotis *et al.* 2004). Since the experimental evidence validating these theories is not unequivocal, the role of the long chain fatty acids produced by mitochondrial FAS and the overall importance of this system for mitochondrial function remains to be determined.

2.4 Fatty acid degradation

In addition to having an essential role in several cellular processes such as signal transduction and the synthesis of membrane lipids, fatty acids serve as energy storage in the form of triacylglycerols. Oxidative degradation of fatty acids released from triacylglycerols or obtained from the diet is a main energy provider for heart and skeletal muscle, and during starvation or prolonged fasting, when carbohydrates are scarce, fatty acids provide energy for the whole organism. Degradation of fatty acids and their derivatives is not only important for energy metabolism and homeostasis but also for the degradation of metabolites that would otherwise accumulate and cause deleterious effects when present in abnormal quantities inside the cells. Fatty acids are oxidized in three different cellular organelles, with β-oxidation confined solely to peroxisomes in yeast and to mitochondria and peroxisomes in mammals, and with ω-oxidation occurring in the endoplasmic reticulum. Though β-oxidation occurs both in mitochondria and peroxisomes, the enzymatic steps are catalyzed by different enzymes encoded by different genes and the roles of β-oxidation in different compartments are different but functionally complementary.

2.4.1 Mitochondrial β-oxidation

The majority of short-, medium-, and long-chain fatty acids are oxidized by the mitochondrial β-oxidation system. The abundance of long-chain fatty acids in dietary fat and thus also in the triacylglycerol stores of adipose tissues makes them the predominant source of energy under normal dietary conditions for heart and skeletal muscle. During prolonged fasting or strenuous activity, when mobilized from triacylglycerols, oxidation of fatty acids is crucial for energy homeostasis providing 80 - 90% of the required energy (Mitchell *et al.* 1995). Energy in the form of ATP is generated directly when the reduced cofactors are fed into the respiratory chain located in the inner mitochondrial membrane and when the product of β-oxidation, acetyl-CoA, is further oxidized to CO_2 and H_2O in the citric acid cycle. Acetyl-CoA is also used as a precursor in the synthesis of ketone bodies (acetoacetate and 3-hydroxybutyrate), which can act as an alternative energy source for the brain and muscles, helping to decrease the need for glucose.

2.4.1.1 Activation and transport of fatty acids into the mitochondrial matrix

Before fatty acids can be subjected to degradation they must be transported into mitochondria and activated to their corresponding acyl-CoA thioesters (Figure 6.). Short- and medium-chain fatty acids (C_4 - C_{14}) can readily cross the outer and inner mitochondrial membranes and are activated in the matrix by ATP-dependent chain length specific acyl-CoA synthetases (ACS) (Aas 1971, Barth *et al.* 1971). Longer-chain fatty acids must be transported by specific means since they can not directly enter mitochondria and cross the inner mitochondrial membrane. Long-chain fatty acids are activated by members of the long-chain acyl-CoA synthetase family (ACSL), which has 5 different isoforms (ACSL1, ACSL3-6) in humans and rodents bound either to the outer mitochondrial membrane, the peroxisomal membrane or the membrane of the endoplasmic reticulum (Lewin *et al.* 2001, Mashek *et al.* 2004). In addition to having acyl-CoA synthase activity, certain mammalian ACSL isoforms also seem to function in fatty acid transport by vectorial acylation in the plasma membrane (Tong *et al.* 2006). Since each of the known isoforms has a distinct tissue distribution, subcellular location, and is regulated independently by diverse hormones and nuclear transcription factors, it has been suggested that the fate of a particular acyl-CoA depends on which isoform of the ACSL catalyzes its synthesis. However, it is not yet firmly established which isoforms are responsible for channelling fatty acids towards oxidative or synthetic pathways, although it has been suggested that ACSL5 and ACSL4 provide activated fatty acids for mitochondrial and peroxisomal β-oxidation, respectively (Coleman *et al.* 2002, Lewin *et al.* 2002). It should be noted that certain mammalian members of the fatty acid transport protein family can also activate various fatty acid substrates to acyl-CoA esters (DiRusso *et al.* 2005).

After activation long-chain acyl-CoAs are transported across the outer mitochondrial membrane by the activity of carnitine-palmitoyl transferase I (CPT I), which catalyzes the conversion of acyl-CoA to an acyl-carnitine derivative and subsequently transports it into transmembrane space. This step limits the rate of fatty acid entry into the mitochondria and is considered to be the rate-limiting reaction in mitochondrial β-oxidation. CPT I has been characterized as different isoforms from liver and muscle (Kolodziej *et al.* 1992). Although named liver and muscle isoforms, both isoforms are expressed in the heart (Weis *et al.* 1994) and, in addition, a skeletal isoform can be found in testis and both white and brown adipose tissue, whereas liver isoform can also be found in the kidney, lung, ovary, spleen, intestine, brain and pancreatic islets (Esser *et al.* 1996, Brown *et al.* 1997). CPT I resides within the outer mitochondrial membrane and has two membrane-spanning domains and C- and N-terminal parts facing the cytosol (Fraser *et al.* 1997). A third CPT I isoform that is expressed predominantly in the brain has also been described recently (Price *et al.* 2002). This isoform differs from other CPT I isoforms as it has not been shown to exhibit acyl transferase activity with any of the tested acyl-CoA substrates and the reaction catalyzed by it remains undetermined. It was, however, suggested that it participates in the transport of a special class of fatty acids required for brain function. Very recent studies with a mutant mouse deficient in this isoform also suggest that it has a function in the regulation of energy homeostasis (Wolfgang *et al.* 2006).

Transport of acyl-carnitines produced by CPT I across the inner mitochondrial membrane is facilitated by carnitine acylcarnitine translocase (CACT) (Pande 1975). In addition to translocating acyl-carnitines into the mitochondrial matrix, CACT transports free carnitine from the matrix back to the transmembrane space in unidirectional fashion (Indiveri *et al.* 1991). At the molecular level CACT has been characterized from rodents and human. There seems to be only a single isoform, whose localization in mitochondria or peroxisomes is determined by alternative splicing (Huizing *et al.* 1997, Indiveri *et al.* 1997). CACT resides in the inner mitochondrial membrane with the N-terminus, two small hydrophilic loops and the C-terminus facing the transmembrane space and three larger hydrophilic loops facing the matrix side (Ramsay *et al.* 2001). The crystal structure of human and mouse CACT has been recently solved (Lian *et al.* 2002, Jogl and Tong 2003).

Reconversion of acyl-carnitines back to acyl-CoAs in the matrix is catalyzed by carnitine palmitoyl transferase II (CPT II), which resides in the matrix side of the inner mitochondrial membrane. Characterization of CPT II at the molecular level indicated that it has no isoforms and it is expressed ubiquitously and in variable quantities in most mammalian tissues (Woeltje *et al.* 1990, Finocchiaro *et al.* 1991, Brown *et al.* 1997). The crystal structure of human and rat CPT II was recently solved and based on this structural information it was proposed that mutations observed in CPT II deficiency (see section 2.6.1) affect substrate recognition and structural integrity. Furthermore, it was suggested that a highly hydrophobic area on its surface facilitates the association of CPT II with the inner membrane of the mitochondria (Hsiao *et al.* 2006, Rufer *et al.* 2006).

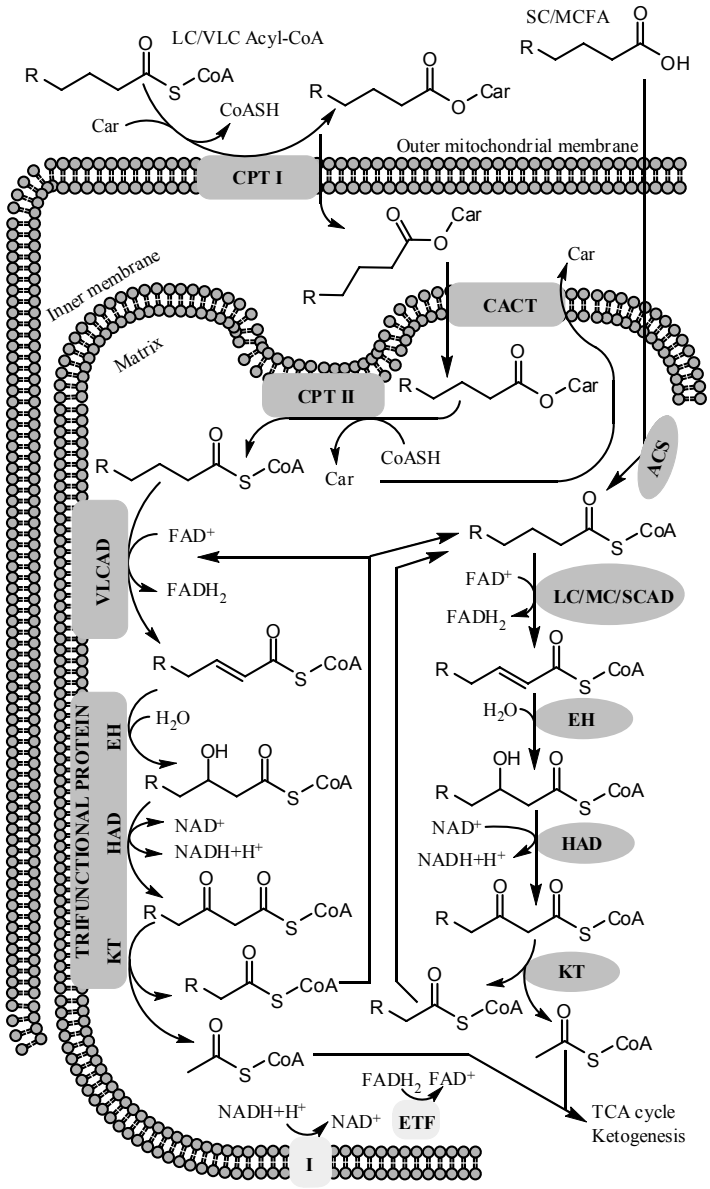


Fig. 6. Mitochondrial β-oxidation of saturated fatty acids. Activation and transport of fatty acids into the mitochondrial matrix, as well as enzymatic steps catalyzed by membrane-bound and soluble enzymes are shown. CPT I, carnitine-palmitoyl transferase I; CACT, carnitine acylcarnitine translocase; CPT II, carnitine-palmitoyl transferase II; ACS, acyl-CoA synthetase; VLCAD, very long chain acyl-CoA dehydrogenase; EH, enoyl-CoA hydratase; HAD, 3-hydroxyacyl-CoA dehydrogenase; KT, 3-ketoacyl-CoA thiolase; I, complex I of the respiratory chain; ETF, electron-transferring flavoprotein. See text for further details.

2.4.1.2 Membrane-bound and soluble enzymes of the β-oxidation spiral

The chain shortening of acyl-CoAs occurs via repetition of four consecutive steps that are catalyzed by acyl-CoA dehydrogenase, 2-enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase, which remove one molecule of acetyl-CoA per cycle from the initial fatty acid substrate (Eaton *et al.* 1996). There are multiple enzymes with variable chain length specificities for each of the steps and furthermore the β-oxidation system has two distinct components that function as bound to the inner side of the inner mitochondrial membrane or as free enzymes in the matrix (Figure 6.). Before the membrane-bound system was discovered it was generally assumed that fatty acids are oxidized by the concerted action of several individual matrix enzymes.

The first reaction in the membrane-bound system, the dehydrogenation of fatty acid to *trans*-2-CoA, is catalyzed by very-long-chain acyl-CoA dehydrogenase (VLCAD), which shows activity towards acyl-CoAs with chain lengths C_{12} - C_{24}, with palmitoyl-CoA (C_{16}) being the favoured substrate. VLCAD is a homodimeric protein with a molecular mass of 150 kDa and it contains flavine-adenine dinucleotide (FAD), which is needed in the dehydrogenation process as a cofactor (Izai *et al.* 1992). At the molecular level this enzyme has been characterized from various mammalian species (Aoyama *et al.* 1994, Strauss *et al.* 1995, Orii *et al.* 1997). Recently another homodimeric membrane-bound acyl-CoA dehydrogenase was characterized and named ACAD-9. The properties and tissue distribution of ACAD-9 are very similar to VLCAD but unlike other acyl-CoA dehydrogenases ACAD-9 is also expressed in the brain (Zhang *et al.* 2002). Although originally ACAD-9 was shown to have optimal activity with palmitoyl-CoA and thought to have a role in the oxidation of long-chain saturated fatty acids, recent studies indicated maximal activity with long-chain unsaturated acyl-CoAs as substrates, suggesting a role in the mitochondrial β-oxidation of long-chain unsaturated fatty acids (Ensenauer *et al.* 2005).

The last three steps of the membrane-bound system are catalyzed by the long-chain enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase trifunctional protein complex (TFP). This multifunctional hetero-octomeric polypeptide has a mass of 460 kDa and has been purified from different sources, and it shows optimal activity with acyl-CoA substrates with a chain length of C_{12} - C_{16} (Carpenter *et al.* 1992, Uchida *et al.* 1992, Luo *et al.* 1993). The enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities in this trifunctional protein are carried by the α-subunit, which is also required for membrane binding (Weinberger *et al.* 1995), whereas the thiolase activity resides in the β-subunit. This enzyme has also been characterized at the molecular level and the location and the structure of genes coding for the α- and β-subunits have been resolved (Yang *et al.* 1996, Orii *et al.* 1997).

Fatty acyl-CoAs chain-shortened in the membrane-bound system are subsequently completely oxidized by individual enzymes residing in the matrix. In the first step the dehydrogenation is carried out in a manner dependent on the chain length of the acyl-CoA either by long-chain acyl-CoA dehydrogenase (LCAD, C_{14} - C_{20}), medium-chain acyl-CoA dehydrogenase (MCAD, C_8 - C_{12}) or by short-chain acyl-CoA dehydrogenase (SCAD, C_4 - C_6). These enzymes, like VLCAD, carry FAD as a cofactor but unlike VLCAD, which is homodimer, have a homotetrameric structure. They have been purified

from various sources including rat, human and bovine liver (Furuta *et al.* 1981, Davidson and Schulz 1982, Finocchiaro *et al.* 1987) and molecular characterization has also been carried out from rat and human (Matsubara *et al.* 1986, Matsubara *et al.* 1987, Matsubara *et al.* 1989, Naito *et al.* 1989) and more recently from mice (Kelly and Wood 1996, Tolwani *et al.* 1996, Kurtz *et al.* 1998). The crystal structures of SCAD and MCAD have also been solved (Kim and Wu 1988, Battaile *et al.* 2002).

In the second step 2-enoyl-CoA hydratase, also known as crotonase, is responsible for the hydratation of *trans*-2-acyl-CoAs to their corresponding 3-hydroxyacyl-CoAs (Stern and Del Campillo 1956). The enzyme accepts a wide range of short- and medium-chain length (C_4 - C_{16}) substrates, being most active towards crotonyl-CoA (C_4) (Waterson and Hill 1972). The crystal structure of rat 2-enoyl-CoA hydratase has been solved and it showed that the enzyme is a hexamer that consists of six identical subunits, which form a dimer of trimers, and has a molecular mass of 161 kDa (Engel *et al.* 1996). Although purification of a second matrix-associated mitochondrial 2-enoyl-hydratase with a preference for medium-chain substrates was reported (Jackson *et al.* 1995), it was later identified as a peroxisomal protein (Jiang *et al.* 1996).

The third step of the cycle is carried out by 3-hydroxyacyl-CoA dehydrogenase (SCHAD), which catalyzes the dehydrogenation of 3-hydroxyacyl-CoAs to the corresponding 3-ketoacyl-CoAs and is a 68 kDa dimer formed from two identical subunits. This NAD^+-dependent enzyme has been purified from various mammalian sources (Wakil *et al.* 1954, Noyes and Bradshaw 1973, Osumi and Hashimoto 1980) and has broad substrate specificity with short- and medium-chain substrates from C_4 to C_{16}, although like short-chain 2-enoyl-CoA hydratase it favours substrates with shorter chain length (He *et al.* 1989). From the crystal structure of the rat protein it has been shown that the binding capacity of the NAD^+ cofactor resides in the aminoterminal domain (Birktoft *et al.* 1987). The crystal structure of the human heart 3-hydroxyacyl-CoA dehydrogenase has also been solved as an apoenzyme and with a bound substrate and NAD^+ cofactor (Barycki *et al.* 1999, Barycki *et al.* 2000). Characterization of this enzyme has also been performed at the molecular level (Amaya *et al.* 1986, Nomura *et al.* 1995, Vredendaal *et al.* 1996). Another monofunctional enzyme with 3-hydroxyacyl-CoA dehydrogenase activity and a homotetrameric structure has been purified from bovine liver (Kobayashi *et al.* 1996, Furuta *et al.* 1997). This enzyme has no homology with SCHAD or LCHAD from the trifunctional protein and it is not clear what its function in β-oxidation is. Kinetic as well as crystallographic studies revealed that it has broad substrate specificity encompassing, in addition to 3-hydroxyacyl-CoA derivatives, hydroxysteroids, alcohols and β-hydroxybutyrate, and the capacity to bind amyloid-β peptides (He *et al.* 1999, Powell *et al.* 2000). It has been suggested that it has an important metabolic role via its utilization of other substrates, such as β-hydroxybutyrate, potentially contributing to ATP production in response to stress (Yan *et al.* 2000).

The thiolytic cleavage of 3-ketoacyl-CoAs in the last step of the cycle is catalyzed by ketoacyl thiolase and produces acetyl-CoA and the corresponding chain-shortened acyl-CoA ester. Two soluble activities with different substrate specificities have been characterized from mitochondria (Middleton 1973, Staack *et al.* 1978). Short-chain 3-ketoacyl-CoA thiolase, also referred to as general thiolase, is reactive with all substrates from C_4 to C_{12} and thus has overlapping substrate specificity with the ketoacyl-CoA thiolase activity of the membrane-bound trifunctional protein. However, thiolytic

cleavage of C_4 substrates is exclusively carried out by the general thiolase, whereas C_{16} substrates are processed by the trifunctional protein (Uchida *et al.* 1992). This enzyme has been purified from various mammalian sources and has a homotetrameric structure with a mass of 169 kDa (Seubert *et al.* 1968, Staack *et al.* 1978, Miyazawa *et al.* 1980). From mammalian species molecular characterization has been carried out with rat and human enzymes (Miura *et al.* 1986, Arakawa *et al.* 1987). There is also a second individual thiolase in the matrix but this enzyme has a significant function only in the ketone body synthesis and utilization, as well as in the metabolism of branched-chain amino acids. This enzyme is specific for acetoacetyl-CoA and 2-methylacetoacyl-CoA and has been named accordingly acetoacetyl-CoA thiolase (Middleton and Bartlett 1983).

As mentioned earlier, the main function of mitochondrial β-oxidation is to provide reducing equivalents for the mitochondrial respiratory chain for the production of ATP. Reducing equivalents are generated at two different stages in β-oxidation. FAD, which is a required cofactor of acyl-CoA dehydrogenases is reduced in the dehydrogenation process and reoxidized by the electron-transferring flavoprotein (ETF) located in the mitochondrial matrix. From ETF electrons are passed to ETF:ubiquinone oxidoreductase located in the inner membrane and further to the respiratory chain by ubiquinone in Complex III. A second connection to the respiratory chain is at the level of 3-hydroxyacyl-CoA dehydrogenases that require NAD^+ as a cofactor. In the process dehydrogenating 3-hydroxyacyl-CoAs to 3-ketoacyl-CoAs NAD^+ is reduced to NADH, which is subsequently reoxidized when electrons are transferred to ubiquinone in Complex I of the respiratory chain.

2.4.2 Peroxisomal β-oxidation

Early studies with rat liver peroxisomes established the ability of these organelles to degrade fatty acids through β-oxidation. It was found that the mechanism of the peroxisomal degradation system is similar to that of the mitochondrial one, and comprises four subsequent steps: dehydrogenation, hydration, a second dehydrogenation and thiolytic cleavage. As in the mitochondrial β-oxidation system, fatty acyl-CoA is chain-shortened by two carbon atoms and one acetyl-CoA molecule is produced per cycle (Lazarow and De Duve 1976). The physiological meaning of two mechanistically similar β-oxidation systems in mammals has become apparent since their different functions and distinct roles in whole-cell fatty acid metabolism has been studied and uncovered. Differing from the mitochondrial system that is responsible for the oxidation of the majority of saturated and unsaturated fatty acids, peroxisomes can degrade, in addition to those same substrates, a different set of fatty acids and their derivatives, which can not be processed by the mitochondrial system. The substrates for peroxisomal β-oxidation include very long-chain fatty acids, 2-methyl branched-chain fatty acids, di- and trihydroxycholestanoic acid, long-chain dicarboxylic acids, very long-chain monocarboxylic acids, eicosanoids and certain xenobiotics. Another important difference is that mammalian peroxisomes are not able to completely degrade their substrates through β-oxidation - only to chain-shorten them. Shortened substrates that can not be further oxidized can be converted to the corresponding carnitine esters and transferred to

the mitochondria for full oxidation. Alternatively, shortened acyl-CoA esters may be hydrolyzed to the free acid and CoA by peroxisomal thioesterases. Furthermore, peroxisomes lack a citric acid cycle and respiratory chain, hence acetyl-CoAs can not be oxidized in peroxisomes. The NADH-generated reducing equivalents created in the β-oxidation cycle can not be used for production of ATP as in mitochondria either. The involvement of different enzymes in peroxisomal β-oxidation and the degradation of various substrates is depicted in figure 7.

2.4.2.1 Activation and transport of fatty acids into peroxisomes

As in the mitochondrial pathway, in peroxisomal β-oxidation fatty acids must be activated to their CoA-esters before they can be used as substrates. It has been suggested that this activation is carried out by long-chain acyl-CoA synthetase 4 (ACSL4) or very-long-chain acyl-CoA synthetase (VLCAS), which is located in peroxisomes in addition to ER (Steinberg *et al.* 1999). Of the known acyl-CoA synthetase isoforms (see section 1.4.1.1), only ACSL4 is associated with, in addition to the mitochondrial-associated membrane, the peroxisomal membrane (Coleman *et al.* 2002). The catalytic and molecular properties of ACSL4 and VLCAS differ from each other (Uchida *et al.* 1996, Uchiyama *et al.* 1996). The catalytic site of ACSL4 is exposed to the cytosol, whereas it has been shown that the catalytic residues of very-long-chain acyl-CoA synthetase face the peroxisomal matrix (Smith *et al.* 2000). It had previously been proposed that branched-chain fatty acids were activated by specific synthetases present in different subcellular compartments (Singh *et al.* 1992), however it was later found that VLCAS shows high activity with branched-chain fatty acids, in addition to straight-chain substrates (Steinberg *et al.* 1999). According to the current view, bile acids returning to the liver via enterohepatic circulation are reactivated for recycling to their CoA esters in the ER by VLCAS homolog termed bile acyl-CoA synthetase, while VLCAS activates precursors of bile acid *de novo* synthesis as well as very-long-chain fatty acids (Mihalik *et al.* 2002). Experiments, which have mainly been carried out with yeast, have shown that medium chain fatty acids can also be activated within peroxisomes (van Roermund *et al.* 2000).

The transport of fatty acids across the peroxisomal membrane to the matrix where β-oxidation occurs differs from the mitochondrial carnitine-dependent system. The peroxisomal membrane does not contain CPT I and carnitine translocase and thus another system must facilitate the transport of activated fatty acids into peroxisomes. Members of the ATP-binding cassette transporter (ABC transporter) superfamily are involved in transport of a wide variety of molecules across biological membranes and it has also been indicated that they play a role in peroxisomal β-oxidation (Hettema and Tabak 2000, Pohl *et al.* 2005). Peroxisomal ABC transporters belong to subfamily D and are half-transporters, which dimerize as homo- or heterodimers to form functional transporters. In mammals four members of this family have been identified: Adrenoleukodystrophy protein (ALDP)(Mosser *et al.* 1993), adrenoleukodystrophy related protein (ALDRP)(Lombard-Platet *et al.* 1996), 70 kDa peroxisomal membrane protein (PMP

70)(Kamijo *et al.* 1990) and 69 kDa peroxisomal membrane protein (PMP 69)(Shani *et al.* 1997).

The discovery that adrenoleukodystrophy (X-ALD), which is an inborn error in the peroxisomal β-oxidation of very long-chain fatty acids, is caused by mutations in the gene coding for ALDP, led to the suggestion that ALDP catalyzes the transport of fatty acids into peroxisomes. It has been suggested that ALDP transports long-chain and very long-chain acyl-CoAs directly across the peroxisomal membrane (Guimaraes *et al.* 2005). However, previous contradictory findings with a mouse model for X-ALD showed that the peroxisomal β-oxidation of very long-chain fatty acids proceeds normally (McGuinness *et al.* 2003), leading to the suggestion of alternative roles for ALDP.

ALDRP is highly similar to ALDP at the amino acid level and it has been proposed that these two proteins, which have a distinct but overlapping expression pattern, are functionally redundant (Pujol *et al.* 2004, Ferrer *et al.* 2005).

The function of PMP 70 has been strongly linked to transport of long-chain fatty acids by overexpression studies (Imanaka *et al.* 1999) and the importance of ATP binding and hydrolysis for the function of PMP 70 has also been shown (Tanaka *et al.* 2002). PMP 69 has not been as well characterized as the other identified ABC transporter family members but based on the sequence similarity with PMP 70 and ubiquitous expression it is thought to have similar function to PMP 70 (Holzinger *et al.* 1997, Shani *et al.* 1997).

Despite much effort, it is still unclear whether all peroxisomal ABC transporters function in the direct ATP-dependent transport of fatty acids and their metabolites, or whether they mediate transport via another mechanism. Yeast homologs of ALDP, Pax1 and Pax2, have been shown to be responsible for direct ATP-dependent transport of long-chain acyl-CoA esters into the peroxisomal matrix (Shani *et al.* 1995, Swartzman *et al.* 1996). Further studies have revealed that Pax1 and Pax2 act as a heterodimer to form a functional transporter (Hettema *et al.* 1996, Shani and Valle 1996). Based on the yeast studies it was proposed that mammalian ABC transporters also form functional heterodimers and that heterodimerisation contributes to functional diversity (Valle and Gartner 1993). Although dimerisation of ALDP with ALDRP and PMP 70 has been shown *in vitro* (Liu *et al.* 1999) and native complexes of ALDP and PMP 70 have been isolated (Tanaka *et al.* 2002), the fact that X-ALD is not associated with mutations in ABC transporters other than ALDP and the distinct, although partially overlapping, expression patterns of ALDP, ALDRP and PMP 70 (Troffer-Charlier *et al.* 1998) suggest that mammalian ABC transporters act as homodimers. Additionally, a preference for the homodimeric existence of ALDP and PMP 70 *in vivo* has been recently indicated (Guimaraes *et al.* 2004).

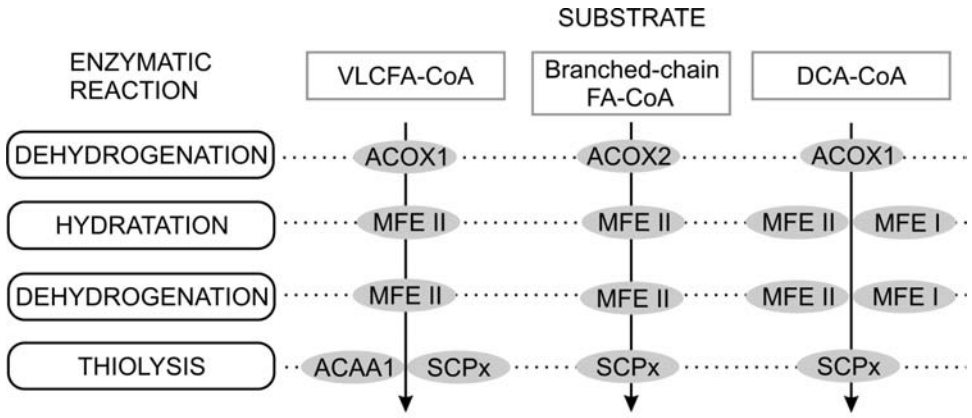


Fig. 7. Involvement of different peroxisomal β-oxidation enzymes in the degradation of various substrates. VLCFA, very long chain fatty acid; DCA, long chain dicarboxylic acid; ACOX1, palmitoyl-CoA oxidase; ACOX2, branched-chain acyl-CoA oxidase; MFE II, multifunctional enzyme II; MFE I, multifunctional enzyme I; ACAA1, 3-ketoacyl-CoA thiolase; SCPx, SCP-2/3-ketoacyl-CoA thiolase. See the text for details (modified from Wanders and Waterham 2006).

2.4.2.2 The peroxisomal β-oxidation spiral

Differing from the first step of mitochondrial β-oxidation catalyzed by acyl-CoA dehydrogenases, the dehydrogenation of acyl-CoA esters to their corresponding *trans*-2-enoyl-CoA esters in peroxisomes is catalyzed by acyl-CoA oxidase enzymes (ACOX). Mammalian peroxisomes contain several acyl-CoA oxidases, which are FAD-dependent and donate electrons directly to molecular oxygen thereby generating H_2O_2 (Osumi and Hashimoto 1978). Palmitoyl-CoA oxidase (ACOX1) in rat and human catalyzes the reaction with CoA esters of straight-chain fatty acids, dicarboxylic acids and prostaglandins (Osumi *et al.* 1980, Casteels *et al.* 1990). Both enzymes are homodimeric with a mass of 140 kDa and are highly active towards substrates with longer chain lengths but practically inactive towards substrates with eight or fewer carbon atoms. In humans, branched-chain acyl-CoA oxidase (ACOX2) is responsible for catalyzing the first step with 2-methyl-branched fatty acids, such as activated pristanic acid, and also with bile acid intermediates containing a 2-methyl substitution, such as di- and trihydroxycoprostanoic acid (Casteels *et al.* 1990, Vanhove *et al.* 1993). In rat however, these substrates are processed by two separate enzymes; pristanoyl-CoA oxidase (ACOX3) acts mainly on the CoA esters of 2-methyl-branched fatty acids and also shows some activity towards straight-chain substrates, whereas bile acid intermediates are processed by trihydroxyprostanoyl-CoA oxidase (ACOX2) (Schepers *et al.* 1990, Van Veldhoven *et al.* 1991).

The second step of the peroxisomal β-oxidation pathway, hydration of *trans*-2-enoyl-CoA to 3-hydroxyacyl-CoA and the third step, dehydrogenation of 3-hydroxyacyl-CoA to

3-ketoacyl-CoA, can be catalyzed by two multifunctional enzymes (MFE). Multifunctional enzyme I (MFE I) was first described as a bifunctional enzyme (LBP) with enoyl-CoA hydratase and L-3-hydroxyacyl-CoA dehydrogenase activities (Osumi and Hashimoto 1979) but it was later found to also contain Δ^3,Δ^2-isomerase activity (Palosaari and Hiltunen 1990). From mammalian species, this monomeric 80 kDa protein has been purified from rat and human (Osumi *et al.* 1985, Reddy *et al.* 1987) and it has been shown that its hydratase and isomerase activities locate in the N-terminal half of the polypeptide, whereas its dehydrogenase activity locates in the C-terminal half (Kiema *et al.* 2002). MFE I has broad substrate specificity and it shows activity towards short- and long-chain (C_4 - C_{16}) enoyl-CoA esters, CoA esters of hydroxylated C_{27} bile acid synthesis intermediates and dicarboxylic acids (Kurosawa *et al.* 2001, Ferdinandusse *et al.* 2004).

Multifunctional enzyme II (MFE II) was first characterized from yeast (Hiltunen *et al.* 1992) and when characterized from mammals it was initially known as 17β-hydroxysteroid dehydrogenase (Leenders *et al.* 1994). Later this homodimeric 154 kDa enzyme was found to have 2-enoyl-hydratase and D-3-hydroxyacyl-CoA dehydrogenase activities (Dieuaide-Noubhani *et al.* 1996, Jiang *et al.* 1996) and it was also shown that MFE II is cleaved *in vivo* to an enoyl-hydratase component (45 kDa) and to a component with dehydrogenase activity (Dieuaide-Noubhani *et al.* 1996). The D-3-hydroxyacyl-CoA dehydrogenase activity of MFE II is localized in the N-terminal part of the protein, whereas the 2-enoyl-hydratase activity resides in the central domain and is followed by the C-terminal domain in having similarity to sterol carrier protein 2 (Adamski *et al.* 1995, Leenders *et al.* 1996, Qin *et al.* 1997). In addition to enoyl-CoA esters of straight-chain fatty acids, MFE II reacts with pristanoyl-CoA, di- and trihydroxyprostanoyl-CoA esters and CoA esters of leukotrienes and dicarboxylic acids (Ferdinandusse *et al.* 2002, Ferdinandusse *et al.* 2004).

MFE I and MFE II have very little sequence homology and they differ from each other in stereochemical properties, substrate specificities and have different interdomain organization. It was previously thought that MFE I was responsible for processing straight-chain substrates (Schulz 1991) and that MFE II processed the side chain of cholesterol and methyl-branched fatty acids (Dieuaide-Noubhani *et al.* 1997). However, studies with MFE I-deficient mice showed that MFE I is dispensable for peroxisomal lipid metabolism and MFE II can replace the functions of MFE I (Qi *et al.* 1999). The first indication of the special function of MFE I came recently from the studies of Ferdinandusse and co-workers (Ferdinandusse *et al.* 2004), where they found that both MFE I and MFE II are involved in the processing of CoA esters of dicarboxylic acids and that in the case of MFE II deficiency MFE I can efficiently process these substrates. It was suggested that contribution of these enzymes to the oxidation of dicarboxylic acids depends on their content in different tissues, which has been shown to vary (Jiang *et al.* 1997).

The final reaction in the peroxisomal β-oxidation spiral, the thiolytic cleavage of 3-ketoacyl-CoA, is catalyzed by 3-ketoacyl-CoA thiolase as in mitochondria. In concert with the preceding steps of the spiral the final step is also catalyzed by multiple enzymes. Early studies with rat peroxisomes revealed two 3-ketoacyl-CoA thiolases both of which are active with straight-chain substrates of varying chain lengths (Miyazawa *et al.* 1981). Thiolase A is constitutively expressed while thiolase B is highly induced by peroxisome

proliferators (Hijikata *et al.* 1990). Both of these thiolases are homodimers and synthesized as precursors, which are proteolytically cleaved inside peroxisomes to mature size (Swinkels *et al.* 1991). In humans, a single gene homologous with rat genes encodes 3-ketoacyl-CoA thiolase (ACAA1) that is equivalent to rat inducible thiolase (Bout *et al.* 1991). Rat and human peroxisomes were later found to contain a 58 kDa protein with 3-ketoacyl-CoA thiolase activity in the N-terminal domain and a sterol carrier protein 2 (SCP-2) domain at the C-terminus. This enzyme was named accordingly SCP-2/3-ketoacyl-CoA thiolase (SCPx) and it was shown to be reactive with 2-methyl branched-chain 3-ketoacyl-CoA esters in addition to straight-chain substrates (Seedorf *et al.* 1994, Antonenkov *et al.* 1997, Wanders *et al.* 1997).

2.5 Degradation of unsaturated fatty acids

Saturated straight-chain fatty acids with 14, 16, and 18 carbon atoms are the most abundant saturated fatty acids found in animal and plant tissues. However, monounsaturated fatty acids (oleic acid being the most abundant of the monounsaturated fatty acids) and polyunsaturated fatty acids (linoleic acid being the most abundant polyunsaturated fatty acid in animal tissues) constitute a high proportion of the total fatty acids in most natural lipids. While saturated acyl-CoA esters are readily degraded in the β-oxidation pathway, mono- and polyunsaturated acyl-CoA esters are problematic for the following reasons:

1. In most unsaturated fatty acids double bonds in even- or odd-numbered positions exist in the *cis*-configuration
2. The only unsaturated intermediate in the β-oxidation pathway is *trans*-2-enoyl-CoA

The problems that degradation of unsaturated fatty acids create for the β-oxidation pathway can be overcome by the use of auxiliary enzymes that act on pre-existing double bonds and thus produce carbon chain conformations suitable for further oxidation by the main pathway. Like the classical enzymes of the β-oxidation pathway that operates both in mitochondria and peroxisomes, the enzymes needed for degradation of PUFA can be found in both cellular locations.

2.5.1 The auxiliary enzymes of β-oxidation

Pre-existing double bonds in fatty acids can be located at odd- and even-numbered positions along the carbon chain of polyunsaturated fatty acids and several auxiliary enzymes are involved in their removal. Figure 8 summarizes the three known auxiliary enzymes and the reactions they catalyze in the oxidation of PUFA.

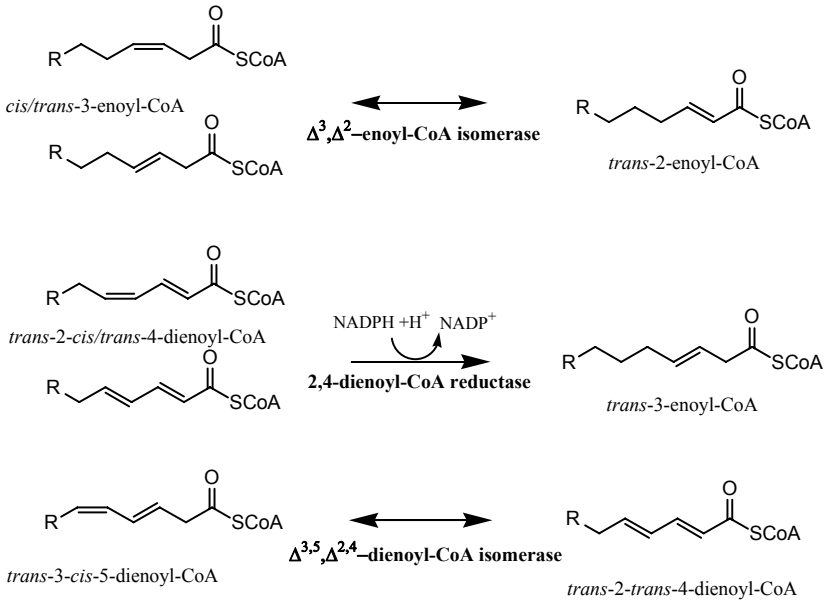


Fig. 8. The auxiliary enzymes of β-oxidation.

When even-numbered double bonds are encountered, they are removed by the activities of 2,4-dienoyl-CoA reductase (DECR) and Δ^3, Δ^2-enoyl-CoA isomerase (ECI) (Figure 9, left). Odd-numbered double bonds exist in many unsaturated dietary fatty acids, such as oleic acid, and can also be encountered in the metabolic intermediates of PUFA, such as linolenic acid. Earlier it was thought that odd-numbered double bonds were removed solely by ECI through an isomerase-dependent pathway (Figure 9, middle). However, the additional observation of NADPH-dependent reduction once the double bond is in the Δ^5 position led to the discovery of a reaction sequence where the double bond is shifted to the Δ^2 position (Tserng and Jin 1991, Smeland *et al.* 1992). This alternative pathway was termed the reductase-dependent pathway, which requires, in addition to ECI and DECR activities, also $\Delta^{3,5}$, $\Delta^{2,4}$-dienoyl-CoA isomerase (DECI) activity (Figure 9, right). When the contribution of these two different pathways to the degradation of odd-numbered double bonds was studied, it was shown that approximately 80% of 2,5-dienoyl-CoAs are processed via the isomerase-dependent pathway (Shoukry and Schulz 1998). It has also been shown that although entry to the reductase-dependent route is limited by a competitive route, the flux through it is further limited by the rate-limiting activity of DECR. The main reason for greater channelling towards the isomerase-dependent pathway is the low activity of ECI towards 2,5-dienoyl-CoAs. However, when 3,5-dienoyl-CoAs are formed they can be effectively degraded only via the reductase-dependent pathway (Shoukry and Schulz 1998, Ren and Schulz 2003). Both pathways are essential for the degradation of unsaturated fatty acids with odd-numbered double bonds. As the isomerase-dependent route is responsible for the major flux, the reductase-dependent route ensures that otherwise undegradable intermediates do not accumulate.

Figure 10 shows the schematic representation of the participation of auxiliary enzymes in the β-oxidation of (poly)unsaturated fatty acids.

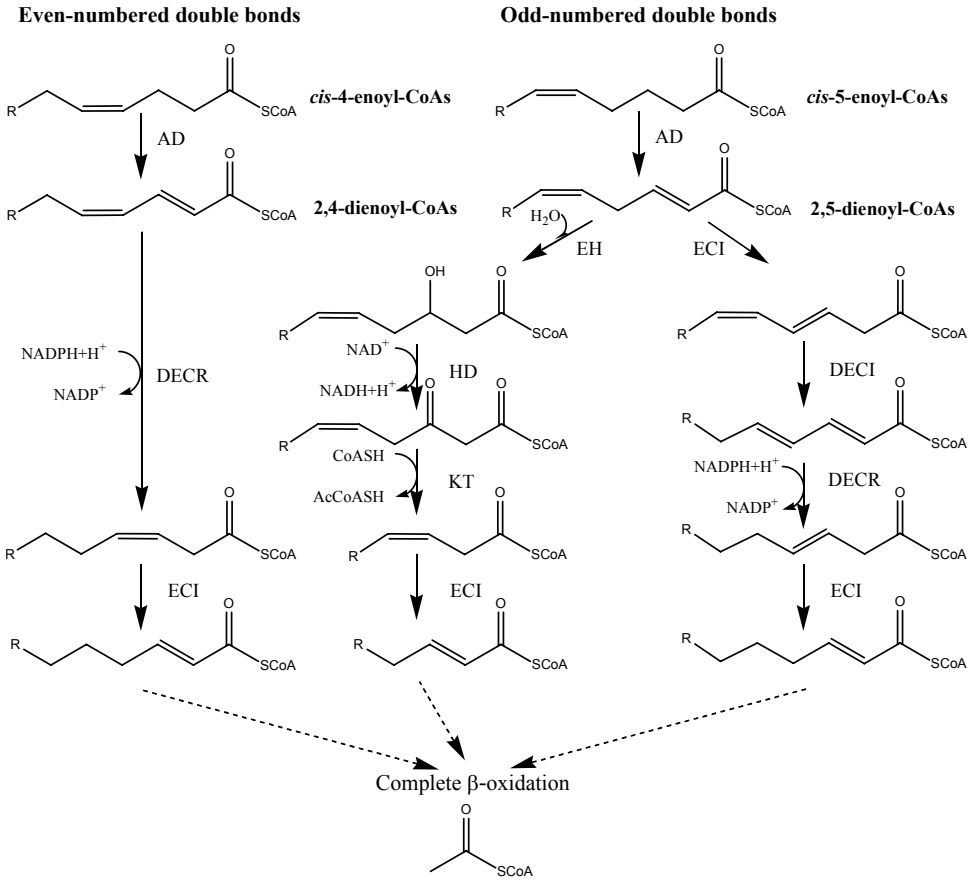


Fig. 9. Enzymatic steps required for β-oxidation of fatty acids with double bonds at even- or odd -numbered positions. Degradation of fatty acids with even-numbered double bonds results in 2,4-dienoyl-CoA esters, which are oxidized as shown on the left. 2,5-dienoyl-CoA esters arising from odd-numbered double bonds can be oxidized either via an isomerase-dependent pathway (middle) or via a reductase-dependent pathway (right). AD, acyl-CoA dehydrogenase; EH, enoyl-CoA hydratase; HD, 3-hydroxyacyl-CoA dehydrogenase; KT, 3-ketoacyl thiolase; ECI, Δ^3,Δ^2-enoyl isomerase; DECI, $\Delta^{3,5}$,$\Delta^{2,4}$-dienoyl-CoA isomerase; DECR, 2,4-dienoyl-CoA reductase.

2.5.2 2,4-Dienoyl-CoA reductase

The activity of DECR is needed in the NADPH-dependent reduction of *trans*-2-*cis*/*trans*-4-dienoyl-CoA intermediates of PUFA during β-oxidation. In eukaryotes the end product

from this reaction is *trans*-3-enoyl-CoA (Kunau and Dommes 1978). Lack of stereospecificity enables reduction of both natural fatty acids with *cis* double bonds, as well as substrates containing *trans* double bonds (Cuebas and Schulz 1982). *Trans*-3-enoyl-CoA is processed to *trans*-2-enoyl-CoA by ECI before further oxidation by the β-oxidation pathway.

From mammalian species, reductase activity was first characterized from rat liver mitochondria and peroxisomes (Dommes *et al.* 1981) and a protein was purified from bovine mitochondria (Dommes *et al.* 1982). The bovine DECR was found to be a 120 kDa homotetrameric protein with a subunit size of 32 kDa. In addition to the 120 kDa isoform, a novel 60 kDa isoform with a subunit size of 37 kDa was detected in rat liver mitochondria (Hakkola and Hiltunen 1993). The physiological properties of the 60 kDa isoform differ from the 120 kDa isoform and the antibody against 120 kDa does not cross-react with it. After initial characterization, cDNA molecules coding for the rat, human and mouse 120 kDa isoform were cloned (Hirose *et al.* 1990, Koivuranta *et al.* 1994, Miinalainen 1998) and subsequently the gene structure for human and mouse DECR was solved (Helander *et al.* 1997, Miinalainen 1998). The similarity of DECR at the nucleotide level between human, rat and mouse is 80 - 90%, whereas similarity at the amino acid level is 80 - 95%. The highest expression of *Decr* was detected in the heart, liver, skeletal muscle and kidney tissue and the expression pattern was comparable between rat, mouse and human (Hirose *et al.* 1990, Koivuranta *et al.* 1994, Miinalainen 1998). A high resolution structure of human DECR was recently solved as a binary complex with the cofactor NADPH, and as a ternary complex with $NADP^+$ and a substrate. Structural information from different complexes showed that substrate binding and processing does not induce any large conformational changes. The active site pocket was found to be large and lined with a flexible loop. It is believed that the openness of the active site and flexible loop contribute to the broad range of CoA derivatives that can be processed by DECR (Alphey *et al.* 2005).

Although DECR activity was detected in peroxisomes (Dommes *et al.* 1981) and its importance in the peroxisomal degradation of fatty acids with double bonds at odd- or even-numbered positions was shown (Hiltunen *et al.* 1986), its purification from wild type sources has not been successful. An early report concerning the purification of DECR from rat liver peroxisomes (Kimura *et al.* 1984) was questioned by later results showing that an antibody raised against this protein recognized the antigen only in isolated rat liver mitochondria (Hakkola *et al.* 1989). Peroxisomal reductase was successfully characterized at the molecular level from rat and mouse and subsequently from human (Fransen *et al.* 1999, Geisbrecht *et al.* 1999, De Nys *et al.* 2001). Proteins encoded by cloned cDNAs had a molecular mass of 31 kDa and the similarity of the human protein towards rat and mouse protein was 83 and 82%, respectively. It was observed that peroxisomal DECR prefers long and very long fatty acids with double bonds near the middle of the acyl chain as substrates. This finding is consistent with the established role of peroxisomes in the metabolism of complex, very long chain polyunsaturated fatty acids.

DECR activity has also been detected and purified from the yeast *C. lipolytica* and *C. tropicalis* (Dommes *et al.* 1983, Mizugaki *et al.* 1985). In *C. lipolytica* a 360 kDa protein was reported to consist of 10 - 12 identical subunits. In *S. cerevisiae* 2,4-dienoyl-CoA reductase was found to be encoded by the *Sps19* gene. The protein encoded by *Sps19* is a

homodimer with a molecular mass of 69 kDa and is localized to the peroxisomes. Like mammalian DECR proteins, Sps19p catalyzes a reaction with *trans*-3-enoyl-CoA esters as end products. The inability of the *S. cerevisiae sps19Δ* strain to grow on petroselenic acid showed the indispensability of 2,4-dienoyl-CoA reductase activity for the degradation of the *trans*-2,*cis*-4-dienoyl-CoA intermediates (Gurvitz *et al.* 1997).

DECR activity has been characterized and purified from *E. coli* (Mizugaki *et al.* 1982, Mizugaki *et al.* 1982, Dommes and Kunau 1984). The gene encoding *E. coli* DECR, *Fadh*, has also been cloned (He *et al.* 1997) and the crystal structure was also solved recently (Hubbard *et al.* 2003). Bacterial enzymes contain FMN and FAD noncovalently bound to a single polypeptide with a molecular mass of 73 kDa and it has been shown to contain a 4Fe-4S cluster (Liang *et al.* 2000). The enzyme works as a monomer and reduces substrates by a hydride transfer mechanism in which reducing equivalents from NADPH are supplied indirectly to the substrate. In contrast to mammalian and yeast enzymes that produce *trans*-3-enoyl-CoA esters, the end product from the reaction catalyzed by E.coli is 2-*trans*-enoyl-CoA, which can be incorporated directly into the β-oxidation pathway.

2.5.3 Δ^3,Δ^2-Enoyl-CoA isomerase

ECI catalyzes the reaction where the *cis*/*trans* double bond from Δ^3 is isomerised to the *trans*-Δ^2 configuration and it is required in the β-oxidation of fatty acids with double bonds at odd-numbered positions (Stoffel *et al.* 1964). Since DECR produces *trans*-Δ^3-enoyl esters when metabolizing even-numbered double bonds, ECI is also required for further processing of fatty acids where double bonds initially exist at even-numbered positions. In mammals three mitochondrial and two peroxisomal enzymes with enoyl-CoA isomerase activity have been described. One of the rat mitochondrial isoforms is a dimeric 60 kDa protein, which prefers substrates with chain-lengths between C_6- C_{12} (Stoffel and Grol 1978, Palosaari *et al.* 1990). Another ECI activity in mitochondria shows different substrate specificity being most active towards longer-chain substrates (Kilponen *et al.* 1990). In addition to these two ECI activities, low intrinsic isomerase activity was found in mitochondrial enoyl-CoA hydratase (crotonase) (Kiema *et al.* 1999). One mitochondrial ECI has been characterized from humans and it has been suggested that it is a counterpart for rat ECI with a short-chain preference. This enzyme has no clear substrate preference and it has same subunit and native size as its rat counterpart (Kilponen and Hiltunen 1993). The crystal structure of human mitochondrial ECI has been recently solved (Partanen *et al.* 2004). Peroxisomal ECI activity was first found as part of a MFE I (Palosaari and Hiltunen 1990) but later monofunctional peroxisomal ECI was also characterized from mammalian sources (Geisbrecht *et al.* 1999). Further studies with different rat isoforms of ECI revealed that monofunctional peroxisomal ECI is also localized to mitochondria and actually is identical to an enzyme that was earlier characterized as mitochondrial ECI with a long-chain substrate preference (Zhang *et al.* 2002). Enzymological characterization of peroxisomal and mitochondrial isoforms has shown that in mitochondria dually localized ECI contributes significantly to *trans*-3 → *trans*-2 isomerization, whereas *cis*-3 → *trans*-2 and 2,5 → 3,5

isomerization is catalyzed by the mitochondrial isoform. In peroxisomes the contributions of MFE I and ECI to β-oxidation of unsaturated fatty acids is not known although it is suspected that MFE I most likely catalyses the 2,5 → 3,5 isomerization due to its high catalytic efficiency (Zhang *et al.* 2002). Interestingly rat straight-chain acyl-CoA oxidase (ACOX1) was recently found to have intrinsic Δ^3,Δ^2-enoyl-CoA isomerase activity. Catalytic values of ACOX1 for the isomerization reaction are comparable with those of ECI and MFE I and thus it has been suggested that ACOX1 might function as an isomerase in the β-oxidation of unsaturated fatty acids in peroxisomes (Zeng *et al.* 2006).

2.5.4 $\Delta^{3,5}$,$\Delta^{2,4}$-Dienoyl-CoA isomerase

Studies showing that unsaturated fatty acids with a double bond at odd-numbered positions can also be degraded via an NADPH-dependent pathway (Tserng and Jin 1991) led to the discovery of $\Delta^{3,5}$,$\Delta^{2,4}$-dienoyl-CoA isomerase (DECI) (Smeland *et al.* 1992). The activity of this enzyme is needed when some of the *trans*-2-*cis*-5-dienoyl-CoA intermediates from the oxidation of unsaturated fatty acids are converted to the *trans*-3-*cis*-5-dienoyl-CoA ester by ECI. This intermediate is a substrate for DECI, which catalyzes the isomerization of *trans*-3-*cis*-5-dienoyl-CoA to *trans*-2-*trans*-4-dienoyl-CoA, creating a substrate for DECR (Chen *et al.* 1994). DECI activity was first purified from mitochondria and the enzyme was thought to be homotetramer with a subunit size of 32 kDa (Chen *et al.* 1994, Luo *et al.* 1994). When the peroxisomal isoform was purified, immunological studies and its kinetic properties suggested that it was a counterpart for mitochondrial DECI (He *et al.* 1995). Molecular characterization revealed that the deduced amino acid sequence harboured a peroxisomal targeting signal in the C-terminal part and that the N-terminal sequence was consistent with mitochondrial targeting. Dual localization was confirmed with immunoelectron microscopy, which showed that DECI can be localized to both mitochondria and peroxisomes (Filppula *et al.* 1998). The crystal structure of DECI confirmed earlier modelling studies and showed that instead of being a homotetramer DECI is a hexameric protein formed by a dimer of trimers. DECI has been shown to be able to catalyze reactions with unsaturated substrates from C_6 to C_{20} in length and the solved structure indicated that in order to bind to the catalytic site of the protein, long-chain substrates have to be in a bent conformation (Modis *et al.* 1998).

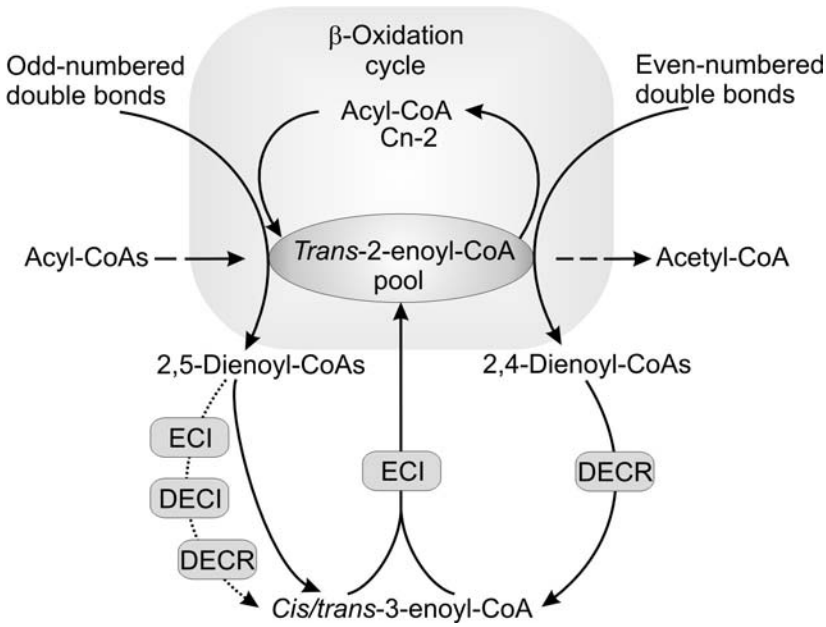


Fig. 10. Participation of auxiliary enzymes in the β-oxidation of (poly)unsaturated fatty acids. Intermediates with odd-numbered double bonds are directed at the level of 2,5-dienoyl-CoA either to an isomerase-dependent pathway or to a reductase-dependent pathway (dotted arrow). 2,4-Dienoyl-CoA intermediates produced from the degradation of fatty acids with double bonds at even-numbered positions are returned to the *trans*-2-enoyl-CoA pool after modification by DECR and ECI. ECI, Δ^3,Δ^2-enoyl-CoA isomerase; DECI, $\Delta^{3,5}$,$\Delta^{2,4}$-dienoyl-CoA isomerase; DECR, 2,4-dienoyl-CoA reductase.

2.6 Disorders of mitochondrial fatty acid β-oxidation

Considering the essential role of mitochondrial β-oxidation in energy metabolism during times of fasting or metabolic stress when higher energy consumption is required, deleterious effects on the well being of individuals caused by β-oxidation deficiencies are not surprising (Wanders *et al.* 1999, Rinaldo *et al.* 2002). Genetic defects with variable mutation types causing these deficiencies have been identified from most of the genes encoding enzymes that catalyze reactions at different steps in this pathway. The clinical features in patients with inherited disorders of mitochondrial β-oxidation are diverse and usually include hypoketotic hypoglycemia, liver disease, and disruption of muscle function (skeletal myopathy, cardiomyopathy). These characteristics are often related to an abnormal acyl-carnitine profile and urinary organic acids resulting from defective oxidation of acyl-CoA thioesters. Symptoms may appear at any age, usually during metabolic stress, and symptoms and their severity vary greatly with different defects and even among individuals with same defect. However, the most severe symptoms are

usually related to the liver, heart, or neuromuscular systems (Vockley and Whiteman 2002).

The most common and best described deficiencies of enzymes responsible for transport of acyl groups into mitochondria and for their subsequent degradation are discussed below. A deficiency in the pathway of unsaturated fatty acid degradation, namely 2,4-dienoyl-CoA reductase deficiency, is also discussed.

2.6.1 Mitochondrial acylcarnitine transport defects

Three enzymes are needed in the transportation of long chain acyl-CoA thioesters into the mitochondrial matrix (see section 2.4.1.1). Carnitine palmitoyl transferase I (CPT I), which has three different isoforms with specific tissue distribution, is responsible for conjugating fatty acids with carnitine. Subsequently carnitine-acylcarnitine translocase (CACT) transports acylcarnitine across the inner membrane in exchange for free carnitine that is produced by carnitine palmitoyl transferase II (CPT II), which is located on the matrix side of the inner membrane where it releases a fatty acid from acylcarnitine.

Deficiency of liver specific CPT I was first described in 1981 (Bougneres *et al.* 1981) and all the CPT I deficiencies demonstrated to date are due to defects in the liver type CPT I (Longo *et al.* 2006). Symptoms are usually triggered by fasting or viral illnesses between birth and 18 months of age and include hypoketotic hypoglycemia or hepatomegaly with subsequent hypoglycemic attacks and mild hyperammonemia (Bonnefont *et al.* 2004). CPT I deficiency, in contrast with other defects in mitochondrial FAO, is almost always associated with an elevated level of plasma carnitine (Stanley *et al.* 1992) and occasionally mild dicarboxylic aciduria with prominent C_{12} dicarboxylic acid is noted (Korman *et al.* 2005). Various gene mutations have been reported in patients suffering from CPT I deficiency and correlation between the degree of enzymatic impairment caused by mutation and the severity of clinical symptoms has been observed (Bonnefont *et al.* 2004).

CACT deficiency was first described in 1992 (Stanley *et al.* 1992). This deficiency is most often diagnosed from neonates, where episodes of seizures and irregular heart beat are triggered by birth stress leading to progressive deterioration of hepatic and cardiac functions. Typical findings include hypoketotic hypoglycemia, hyperammonemia and reduced levels of carnitine. An increase in long chain acylcarnitines can be observed in the acylcarnitine profile and excess levels of unsaturated dicarboxylic acids are commonly associated with severe organic aciduria. In milder cases symptoms with seizures and hypoglycemia caused by infections and fasting are present later in life (Longo *et al.* 2006). Heterogeneous mutations have been identified in different patients and although genotype/phenotype correlations have not been observed, residual enzyme activity has been associated with the milder phenotype, whereas complete deficiency leads to a rapidly progressing disease (Iacobazzi *et al.* 2004).

The CPT II deficiency, which was the first described defect in mitochondrial fatty acid oxidation (DiMauro and DiMauro 1973) has two distinct clinical presentations including a more common mild form present in adolescents and young adults, and a severe form present during the neonatal period and in infancy. Exercise-induced myopathy and

myoglobinuria are common symptoms of the mild form of CPT II deficiency, and reduced carnitine levels and an increase in the long chain carnitine fraction may also be observed. Hypoglycemia or dicarboxylic aciduria is not observed and the only affected organ seems to be skeletal muscle. The severe neonatal form is rapidly fatal and presents itself shortly after birth with hypoglycemia, hepatomegaly, and cardiomyopathy. In the infantile form repeated periods of hypoketotic hypoglycemia and transient hepatomegaly are triggered by fasting, infections, or fever (Longo *et al.* 2006). More than 40 mutations are associated with CPT II deficiency and two of them are considered to be common mutations in the adult form of the disease. Some degree of genotype/phenotype correlation seems to exist between the two different clinical presentations of CPT II deficiency, which also correlates to some extent with the enzymatic and functional data (Bonnefont *et al.* 2004).

2.6.2 Defects in acyl-CoA dehydrogenases

The first step in the cyclic shortening of fatty acids via β-oxidation is catalyzed by acyl-CoA dehydrogenases (ACAD). In mitochondria there are several isoforms either present as soluble matrix proteins or as membrane-bound proteins with different, although partially overlapping, chain length specificities (see section 2.4.1.2).

Short chain acyl-CoA dehydrogenase (SCAD) deficiency was described in 1987 (Amendt *et al.* 1987) and since then over 20 clinical cases have been reported. In most cases variable symptoms including metabolic acidosis, seizures, and myopathy occur during the neonatal period and can be fatal, although asymptomatic adult patients have also been reported. Interestingly, only a few patients have been described with symptoms related to energy deficiency. This is thought to result from the fact that only the last cycle in the degradation of long chain fatty acids is blocked and thus the reducing equivalents needed for ATP production are produced nearly normally (Gregersen *et al.* 2004). Characteristic metabolites include ethylmalonic and methylsuccinic acid, as well as butyrylglycine and butyrylcarnitine, all of which are the result of accumulation of butyryl-CoA in mitochondria. Various mutations have been identified from SCAD-deficient patients and two relatively common gene variants that predispose patients to excessive ethylmalonic acid production have also been identified (Corydon *et al.* 2001). However the importance of these gene variants and additional factors in the clinical presentation of SCAD deficiency remain unclear (Rinaldo *et al.* 2002). Recent findings that ethylmalonic acid compromises energy metabolism in skeletal muscle by inhibiting the electron transport chain and creatine kinase might offer an explanation for the myopathy observed in SCAD-deficient patients (Barschak *et al.* 2006).

Since its initial characterization over 20 years ago, medium chain acyl-CoA dehydrogenase (MCAD) deficiency has become the most common disorder of fatty acid oxidation with over 300 reported cases and a frequency of 1/15000 in northern Europe (Rinaldo *et al.* 2001). Patients suffering from MCAD deficiency are normal at birth and usually present symptoms in the second year of life in response to fasting or common intercurrent infections. Symptoms that usually resolve between episodes include hypoketotic hypoglycemia, dicarboxylic aciduria, muscle weakness, and hepatic steatosis.

Heart-related symptoms, such as arrhythmia, are rare and the severity of commonly encountered symptoms vary from fatal to mild disabilities. Sudden death during the first metabolic decompensation is common in MCAD deficiency and has been observed in infants as well as in adults but patients may also remain completely asymptomatic (Rinaldo *et al.* 2002). MCAD deficiency shows a specific acylcarnitine profile with elevated concentrations of C_8-carnitine, as well as C_6-, C_{10}-, and $C_{10:1}$-carnitine species (Vreken *et al.* 1999). A single common mutation present in the homozygous form has been identified as present in 80% of MCAD-deficient patients. This mutation alters one amino acid and compromises protein folding and renders it unstable.

There have been no confirmed cases of long chain acyl-CoA dehydrogenase (LCAD) deficiency, since all the cases diagnosed as LCAD deficiency were shown to have a defect in membrane-bound very long chain acyl-CoA dehydrogenase (VLCAD) (Yamaguchi *et al.* 1993). Symptoms in VLCAD deficiency can be categorized into different phenotypes according to age of onset. In the first phenotype infants with severe cardiac symptoms and liver disease die within first year of life. The second group consists of older children suffering from hypoketotic hypoglycemia and hepatomegaly but who do not have cardiac symptoms. Skeletal myopathy without liver or cardiac symptoms in adults and young adults defines the third phenotype group (Wanders *et al.* 1999). Metabolic abnormalities seen during episodes in severe cases include increased levels of organic acids in the urine and a specific acylcarnitine profile with elevated levels of long chain saturated and unsaturated fatty acids, usually dominated by $C_{14:1}$. A common mutation has not been found and various mutations in the homozygous form present in severe cases of VLCAD deficiency are responsible for total loss of enzymatic activity. Mutations in the disease with a later onset do not totally eliminate the VLCAD activity and thus severe energy deficiency can be avoided leading to a milder phenotype (Gregersen *et al.* 2004).

2.6.3 Defects in trifunctional protein

The mitochondrial trifunctional protein (TFP), which possesses enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase (LCHAD) and 3-ketoacyl-CoA thiolase activity is localized bound to the matrix side of the inner membrane and is responsible for degradation of long chain fatty acids (see section 2.4.1.2). In TFP deficiency, which was first described in 1992 (Jackson *et al.* 1992, Wanders *et al.* 1992), various mutations in genes coding α- and β-subunits compromise all three catalytic activities, whereas in the more commonly encountered (>100 reported cases) isolated LCHAD deficiency that was described prior to this (Wanders *et al.* 1990) a common mutation in the α-subunit results in the loss of only LCHAD activity. The clinical phenotype in TFP deficiency varies from severe neonatal/infantile cardiomyopathy with early death to milder sensorimotor neuropathy. Symptoms in LCHAD deficiency include fatal neonatal cardiomyopathy, skeletal myopathy, and hypoketotic hypoglycemia. Adult patients have also been described with exercise-induced skeletal myopathy. In most cases of TFP and LCHAD deficiency dicarboxylic aciduria with C_6-C_{14} 3-hydroxydicarboxylic acids is present and the acylcarnitine profile shows characteristic 3-hydroxy $C_{16:0}$-, $C_{16:1}$-, $C_{18:0}$-, and $C_{18:1}$-

acylcarnitines. In isolated LCHAD deficiency patients carry a prevalent mutation on one or both alleles, whereas in TFP-deficient patients various other mutations are found. It has also been observed that heterogeneous women carrying fetuses with LCHAD deficiency may develop AFLP (acute fatty liver of pregnancy) or HELLP (hemolysis, elevated liver enzymes, and low platelets) syndromes (Yang *et al.* 2002) and TFP-deficient individuals have a higher likelihood of suffering from prematurity and IUGR (intrauterine growth retardation) syndrome (Tyni *et al.* 1998)

2.6.4 2,4-Dienoyl-CoA reductase deficiency

Although numerous clinical cases concerning inherited defects of various enzymes in the mitochondrial β-oxidation pathway have been described, there is only a single report describing one neonatal patient with a defect related to degradation of unsaturated fatty acids (Roe *et al.* 1990). The biochemical studies of this hypotonic patient with feeding difficulties revealed hyperlysinemia, carnitine deficiency, a normal organic acid profile, and an unusual acylcarnitine species in the plasma and urine. The diagnosis of a 2,4-dienoyl-CoA reductase defect was based on the identification of the accumulating acyl carnitine as *trans*-2-*cis*-4-decadienoylcarnitine derived from incomplete oxidation of linoleic acid. After dietary therapy with carnitine supplementation and a change of dietary fat to medium chain triglycerides, the acylcarnitine profile returned to normal. However, despite the therapy, the patient died of respiratory acidosis at the age of four months. Post-mortem studies revealed that DECR activity had decreased to 40% of the normal value in the liver and down to 17% of the normal value in the muscle tissue, and it was suggested that the remaining activity was due to the peroxisomal isoform (Roe *et al.* 1990). No other histological or biochemical evidence suggesting significantly altered metabolism were reported.

The molecular defect behind this disorder has not been characterized, nor is it known which isoform was defective. Missense mutations causing single amino acid substitutions have been found in pigs in the gene coding mitochondrial DECR (Davoli *et al.* 2002, Amills *et al.* 2005). Although the observed mutations evidently do not have an effect on enzymatic activity, they suggest that a single nucleotide polymorphism in this gene can also occur in humans.

2.7 Mouse models of mitochondrial fatty acid β-oxidation defects

Characterized inherited defects in the mitochondrial β-oxidation pathway are usually limited to a single enzyme and a single enzymatic activity. However, because of the central role of fatty acid degradation in overall metabolism, the disease phenotype is determined by the interaction of several metabolic pathways. This can be seen from the fact that in many cases changes in the environmental conditions and/or diet can result in the conversion of an asymptomatic state to diseased state with symptoms varying from mild to severe. Mouse models offer an accurate tool to study clinical, biochemical, as well as histological responses to environmental and dietary stress and several mouse

models have been generated to study the disease conditions caused by genetic defects in the mitochondrial β-oxidation pathway. These include models for CPT I deficiency, ACAD deficiencies (including a spontaneous mutation model for SCAD deficiency), and TFP deficiency.

In the mouse model for CPT I deficiency, a gene coding for the liver isoform of this enzyme was disrupted (Nyman *et al.* 2005). Gestational lethality was indicated by the absence of homozygous embryos or fetuses. This finding was surprising considering the existence of human patients with homozygous deficiency but it shows the critical role of CPT I in the metabolic function of mouse embryos. Heterozygous mice were viable with decreased CPT I activity in the liver of male mice, whereas no difference in the activity was observed in female mice compared to wild type controls. However, when exposed to metabolic stress (fasting), heterozygous mice showed elevated free fatty acid concentrations and decreased blood glucose concentrations (hypoglycemia). Fasting associated symptoms make this mouse model suitable for studying the role of CPT I in human disease (Nyman *et al.* 2005).

Spontaneous mutation leading to SCAD deficiency has been used as a model for human SCAD deficiency. It was found that mutant mice developed pathological and biochemical features, including the development of fatty liver and hypoglycemia, which resembled the human disease after metabolic challenge (Wood *et al.* 1989, Armstrong *et al.* 1993). Organic aciduria was present even without fasting and was characterized by elevated levels of ethylmalonic acid, methylsuccinic acid, and butyrylglycine. Severe cold intolerance and hypoglycemia have also been observed under cold exposure (Guerra *et al.* 1998). Feeding studies have indicated that a diet rich in long-chain triglycerides significantly increases the survival rate of SCAD-deficient mice during the cold exposure challenge (Schuler *et al.* 2004).

A recently developed mouse model for MCAD deficiency presents a phenotype with biochemical and pathological features resembling the human deficiency (Tolwani *et al.* 2005). Fasted mutant mice showed organic aciduria and an abnormal acylcarnitine profile. In contrast to human patients, the predominant acylcarnitine was $C_{10:1}$ instead of C_8. The hepatic steatosis observed after fasting is consistent with histological findings in the human deficiency, however the cardiac lesions that were occasionally observed have not been reported in human patients, although cardiac dysfunction has been reported (Feillet *et al.* 2003). Fasting mice showed a tendency towards high free fatty acid levels, lowered glucose concentration, and cold intolerance. It was also suggested that the significant neonatal mortality (approximately 40% of pups surviving to weaning) resembled the mortality associated with clinical episodes in MCAD-deficient patients. As with human patients, no mortality due to MCAD deficiency was detected in surviving adult mutant mice unless challenged with metabolic stress (Tolwani *et al.* 2005).

Although there are no confirmed cases of human LCAD deficiency, the LCAD-deficient mouse model has been produced (Kurtz *et al.* 1998). Compared to any other ACAD deficiency model the LCAD mutant mouse exhibits the most severe clinical phenotype and resembles the human VLCAD deficiency. Significant gestational lethality in homozygous and heterozygous pups might offer one explanation for the absence of human patients. A phenotype of surviving mutant mice includes unprovoked sudden death, fasting intolerance with hypoketotic hypoglycemia, cold intolerance, and severe hepatic steatosis and cardiac lipidosis. The organic acid profile in the urine showed the

accumulation of several dicarboxylic acids, of which the accumulation of octenedioic ($C_{8:1}$) acid was most prominent. The acylcarnitine profile indicated the elevation of C_{12}-C_{14} acylcarnitines with a predominance of $C_{14:1}$- and $C_{14:2}$-acylcarnitines (Kurtz *et al.* 1998).

In VLCAD-deficient mice clinical symptoms include mild hepatic steatosis, fatty acid changes in the heart in response to fasting, as well as cold intolerance. Organic aciduria was not observed, but acylcarnitine analysis showed predominant elevation of C_{16} - C_{18}-acylcarnitines. Neither gestational loss nor sudden death was observed (Cox *et al.* 2001). In another study, biochemical changes that predisposed VLCAD-deficient mice to cardiac lipidosis and arrhythmic death were observed without exposure to physiological stress or metabolic challenge. Exposure of this mouse model to fasting or cold triggered hypoglycemia, hypothermia, liver and heart steatosis, as well as severe bradycardia, resembling the symptoms of human VLCAD deficiency. The phenotype of mice homozygous for mutation was most severe but heterozygous mice also showed an intermediate but potentially lethal phenotype (Exil *et al.* 2003, Exil *et al.* 2006).

The mouse model for TFP deficiency was generated by disrupting both α- and β-subunits of TFP resulting in complete loss of TFP (Ibdah *et al.* 2001). Homozygous mice showed clinical symptoms and biochemical changes identical to the human deficiency including organic aciduria, elevated C_{14} - C_{18} acylcarnitines, hypoglycemia, and early neonatal death. Histological analysis indicated rapid development of hepatic steatosis and progressive degeneration of cardiac and diaphragmatic myocytes. Although gestational loss was not observed as in LCAD-deficient mice, homozygous fetuses suffered from the IUGR syndrome that is also identified in human patients (Yang *et al.* 2002). Early placental and fetal expression of TFP and accumulation of long chain fatty acids in TFP-deficient fetuses suffering from intrauterine growth retardation syndrome (IUGR) indicate the importance of mitochondrial fatty acid oxidation as a source of energy for fetal development. Based on the TFP mutant mouse it was proposed that the observed sudden deaths were caused by cardiac lesions causing arrhythmia and diaphragmatic lesions causing respiratory insufficiency (Ibdah *et al.* 2001).

3 Outlines of the present study

Enoyl thioester reductases are essential enzymes for lipid metabolism. These enzymes catalyze the reduction of *trans*-2-enoyl thioesters to their saturated counterparts and can be found in various cellular compartments. They are associated with fatty acid synthesis and degradation, as well as fatty acid elongation. This study focused on two mitochondrial thioester reductases that act in different pathways of fatty acid metabolism. 2-enoyl thioester reductase is an enzyme participating in the type II fatty acid synthesis in mitochondria and 2,4-dienoyl-CoA reductase (DECR) is an auxiliary enzyme in β-oxidation participating in the degradation of (poly)unsaturated fatty acids.

The identification of enoyl thioester reductase activity in the mitochondria of the yeast *C. tropicalis* led to a study with following aims:

1. To clone the gene encoding the *C. tropicalis* reductase (Etr1p), characterize the enzymatic activity of the gene product and identify homologs in other species.

The identification of a functional homolog of Etr1p from *S. cerevisiae* (Mrf1p) and subsequent database search with an *S. cerevisiae* gene (*YBR026c*/*MRF1*) encoding Mrf1p revealed possible candidate human EST clones, and lead to further studies with the following aims:

2. To identify, clone and characterize mitochondrial thioester reductase from mammals.
3. To perform functional analysis of the mammalian reductase gene product in the respiratory-deficient *S. cerevisiae mrf1*Δ strain.

To study the physiological function of DECR and the importance of the functional system for the degradation of unsaturated fatty acids for the well being of animals, the following aim was set:

4. To create a mouse model for DECR-deficiency with the knock-out technique and to characterize the phenotype of the DECR-deficient mouse.

4 Materials and methods

Detailed descriptions of the materials and methods used can be found in the original articles referred to in the text by their Roman numerals I - III.

4.1 Plasmids, strains, culture media and growth conditions

Detailed descriptions of the construction of plasmids for localization studies, protein expression and purification, complementation assays and homologous recombination, as well as primer sequences and the composition of yeast and mammalian cell culture media and growth conditions, are given in original articles I - III.

4.2 Genomic and cDNA cloning (I, II)

To obtain the *ETR1* gene, purified Etr1p from *C. tropicalis* was subjected to trypsin and endoproteinase digestions. Based on the amino acid sequences obtained by sequencing the peptide fragments, degenerate primers were synthesized and PCR was performed using *C. tropicalis* genomic DNA as a template. Ligation-mediated PCR amplification and genomic library screening resulted in a DNA fragment containing an open reading frame encoding a polypeptide that fully matched the sequences of the proteolytic peptides. RT-PCR with *C. tropicalis* mRNA and sequencing of the products verified that the *ETR1* gene contained no introns.

Isolation of human NRBF-1 cDNA was carried out using *S. cerevisiae* gene *YBR026c* to search for human EST clones in the expressed sequence tag database at the National Center for Biotechnology Information. An EST clone, AA393871 (IMAGE ID 504485), was obtained from I.M.A.G.E. Consortium (UK HGMP Resource Centre, Hinxton, Cambridge, UK) and sequenced.

For the cloning of the bovine NRBF-1 cDNA total RNA was isolated from bovine heart tissue using the QuickPrep system (Amersham Pharmacia Biotech, Uppsala, Sweden). cDNA was amplified from isolated RNA with a RobustRT-PCR kit (Finnzymes,

Espoo, Finland). The composition of the 5´-primer was based on the sequence of bovine EST clone BE682523, containing the 5´ end of the cDNA as shown in the database search with the full length human NRBF-1 sequence. The composition of the 3´-primer was based on the consensus sequence obtained from the alignment of human, rat, and mouse sequences.

4.3 Protein purification and analysis (I, II, III)

C. tropicalis cells were used as a starting material for purification of native Etr1p. A sample of cell lysate produced by disruption with glass beads in a Bead-Beater homogenizer (Biospec Products, Bartlesville, OK, USA) was applied to a Phenyl-Sepharose 6 Fast Flow hydrophobic interaction column (Amersham Pharmacia Biotech, Uppsala, Sweden) after ultracentrifugation. Subsequent purification steps were carried out with pooled fractions that contained reductase activity and included a Mono Q HR (5/5) anion-exchange column (Amersham Pharmacia Biotech) and a Superdex-200 HR (10/30) size-exclusion column (Amersham Pharmacia Biotech). For the purification of the Etr1p or Ybr026p produced in *S. cerevisiae*, cells were broken in a French press (Spectronic Instruments, Rochester, NY, USA). Purification was performed as described above, except that the Mono Q HR (5/5) column was replaced by an ADP-Sepharose column (Amersham Pharmacia Biotech). Also, unlike the wild-type Etr1p purification, the Resource S ion-exchange column (Amersham Pharmacia Biotech) preceded the Superdex 200 column in the purification of the overexpressed Etr1p (I).

For the purification of recombinant human and bovine Nrbf-1p, *E. coli* BL21 (DE3) pLysS cells overexpressing said recombinant proteins were disrupted using a French press. Supernatant from the cell lysate was applied to a Q-Sepharose anion-exchange column (Amersham Pharmacia Biotech) and the presence of Nrbf-1p in the fractions was screened for during the purification with *C. tropicalis* anti-Etr1p antibody. Subsequent purification steps included a 2´5´ADP-Sepharose 4B column (Amersham Pharmacia Biotech), a Resource S cation-exchange column (Amersham Pharmacia Biotech) and a Superdex 200 HR 10/30 size exclusion column (Amersham Pharmacia Biotech). In the purification of bovine recombinant protein, an antibody against human Nrbf-1p was used to select fractions containing Nrbf-1p (II).

The concentration of highly purified protein preparations was determined spectrophotometrically at 280 nm (I, II). Otherwise protein concentration was determined with the Bradford method using BIO-RAD chemistry (Hercules, CA, USA) or Roti-Quant chemistry (Karl Roth GmbH, Karsruhe, Germany) at 595 nm. For Western blot – analysis, samples were separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred using electroblotting to a nitrocellulose transfer membrane (PROTRAN®, Schleicher & Schuell, Dassel, Germany). Immunodetection of human and bovine Nrbf-1p in Western blotting (II) was carried out using polyclonal antibodies raised in rabbits against *C. tropicalis* Etr1p or human Nrbf-1p and a horseradish peroxidase (HRP)-conjugated secondary antibody (BIO-RAD). Immunodetection of mouse DECR (III) was carried out using antibodies raised in rabbits against the corresponding rat protein (Hakkola *et al.* 1989).

4.4 Subcellular localization studies (I, II)

Fluorescent microscopy was used to study the subcellular localization of *S. cerevisiae* Mrf1p (I). A plasmid expressing Mrf1p as a fusion protein with GFP was transformed into *S. cerevisiae* cells. For fluorescence analysis, cells were spread on a polylysine-coated slide and dried. The fixation was carried out with cold acetone at -20 °C and subsequently cells were stained with DAPI. Localization of fluorescent signals from GFP and DAPI was determined using an Olympus BX 60 microscope.

For the determination of the subcellular localization of *Hs*Nrbf-1p (II), HeLa cells (ATCC CCL-2) cultured on glass coverslips were transfected with a plasmid encoding *Hs*Nrbf-1p with EGFP fused to either the N- or C-terminus of the protein. The cells were treated 36 h after transfection with mitochondrion selective fluorescent dye MitoTracker Red CMXRos (Molecular Probes Europe BV, Leiden, Netherlands), fixed with paraformaldehyde and permeabilized, after which the nuclear DNA was counterstained with DAPI. The slides were examined by fluorescent microscopy to localize the fluorescent signal from GFP fusion protein.

For the study of the localization of Nrbf-1p in rat heart, a crude mitochondrial fraction was obtained and further purified by fractioning using isopygnic ultracentrifugation on a self-generated Percoll gradient. Marker enzyme activities for mitochondria, microsomes and cytosol were measured from the fractions and immunoblotting with anti-*Hs*ETRAb was used to visualize *Rn*Nrbf-1p.

4.5 Enzyme assays and end product analysis (I, II)

Enoyl thioester reductase activities were measured using a Shimadzu UV 3000 dual-wavelength spectrophotometer monitoring the oxidation of NADPH at room temperature. The assay mixture consisted of 125 μM NADPH and 60 μM *trans*-2-hexenoyl-CoA, *trans*-2-decenoyl-CoA, *trans*-2-hexadecenoyl-CoA or *trans*-2-*trans*-4-hexadienoyl-CoA in 50 mM KP_i, at pH 7.2, supplemented with 50 μg/ml of fatty acid free albumin (I). The kinetic constants for the mammalian enoyl thioesters' reductase activities (II) were determined using substrate concentrations of 1.25-200 μM for the above mentioned *trans*-2-enoyl-CoA substrates. Otherwise the assay mixture contained 125 μM NADPH and 100 μg/ml bovine serum albumin in 50 mM KP_i, at pH 7.5. The K_m-values were calculated from the slopes of the curves using GraFit software (Sigma).

For the characterization of the end product from the reaction catalyzed by recombinant Etr1p or Mrf1p (I) *trans*-2,*trans*-4-hexadienoyl-CoA or *trans*-2-hexenoyl-CoA (10 mM) was incubated with said proteins and 10 mM NADPH in 50 mM KP_i (pH 7.4) at 37 °C. KOH was added to aliquots from the reaction mixture, and free fatty acids were liberated by heating at 80 °C for 3 h. The fatty acids were esterified overnight at 30 °C using acetyl chloride. After a saturated $CuCl_2$ solution was added, the esterified samples were extracted with hexane and a sample of the organic layer was subjected to gas-liquid chromatography using a gas chromatographic system (model 5890, Hewlett-Packard, Bad Homburg, Germany) with a flame ionization detector and an FS-FFAP-CB-0.25 (20 m) column (Cs-Chromatographic Service GmbH, Langerwehe, Germany).

For end product analysis of the reaction catalyzed by Nrbf-1p (II), recombinant tr*Hs*Nrbf-1p was incubated in the presence of 60 µM *trans*-2-hexenoyl-CoA and 300 µM NADPH in 20 mM potassium phosphate, at pH 7.8 at 22 °C. Aliquots taken from the reaction were acidified and samples from the aliquots were applied to a reversed phase column (Waters Novapak C18, Waters Millipore, Millford, MA, USA) connected to an HPLC instrument (Waters 626 pump, Waters 996 photodiode array detector, Waters Millipore). The masses of the metabolites in the eluted peaks were determined by matrix-assisted laser desorption ionization time of flight mass spectroscopy (MALDI-tof ms) (ABI Voyager-DETM STR Biospectrometry Workstation).

4.6 Functional complementation studies (I, II)

In the *S. cerevisiae* genome a single gene, *YBR026c/MRF1*, encodes the mitochondrial reductase, the disruption of which results in a respiration-deficient *mrf1*Δ strain that can not grow on a medium containing nonfermentable carbon sources, such as glycerol, as a sole carbon source (Yamazoe *et al.* 1994). The disruption of *MRF1* was carried out using the short flanking homology procedure (Wach *et al.* 1994). To examine the capability of differentially targeted Etr1p, Mrf1p (I) and human Nrbf-1p (II) to functionally complement the reductase-deficient strain, the deletion strain was separately transformed with plasmid DNA encoding each of these proteins. The complementation of the respiration-deficient phenotype was tested by growing the transformed strains on a synthetic complete glycerol medium.

4.7 Animal care (III)

The use of animals and the research plans were approved by the University of Oulu committee of animal experimentation (License numbers 73/00, 080/03, 066/05). Animal experiments were carried out in the animal facility of the Department of Biochemistry, University of Oulu and in the germ free Barrier of the University of Oulu. Mice were housed in a temperature controlled animal room with a 12-hour lighting period (07:00 – 19:00) and provided unrestricted access to water and standard chow. For fasting experiments mice were housed individually and food was withdrawn for 24 to 48 hours, during which period water was provided *ad lib*. Cold tolerance was tested by exposing individually housed fasted (20 h) or non-fasted mice to +4 °C for a maximum of 4 hours or until body temperature dropped below 25 °C. Temperature was measured using a ThermoScan® thermometer (PRO 4000, Braun GmbH, Germany). Urine samples for amino acid and dicarboxylic acid analysis were collected prior to fasting and after fasting using metabolic cages (Tecniplast, Italy). All experiments were carried out with 4 to 7 month old adult mice, using age- and sex-matched wild type mice as controls.

4.8 Generation of *Decr*$^{-/-}$ mice (III)

The genomic clone (from the 129/SvJ strain) corresponding to the mouse *Decr* locus was obtained from Genome systems Inc. (St Louis, MO, USA). A 3.3 kb fragment upstream of the first exon and a 4.4 kb fragment from the first intron were modified to harbour suitable restriction sites. For the construction of replacement vector, the modified fragments were ligated to the pPGKneo/TK-2 vector, where they flanked the PGKneo cassette. The neomycin resistance (neo) gene was used for positive and thymidine kinase (TK) gene for negative selection. A linearized replacement vector was electroporated into RW4 embryonic stem (ES) cells (129/SvJ, Tyrchp/Tyrcp) that were subsequently grown under selection. Correctly targeted ES cell clones were identified using Southern analysis of genomic DNA. Germline chimeric mice were produced by microinjecting ES cell aggregates from positive clones into C57BL/6 blastocysts using standard procedures. The genotypes of the mice were determined from tail DNA samples by PCR analysis.

4.9 Histological analysis (I, II, III)

For immunoelectron microscopy (I), *S. cerevisiae ybr026c*Δ cells expressing Ybr026p or Etr1p were fixed with paraformaldehyde/glutaraldehyde solution and subjected to further fixation by freeze substitution in absolute methanol at -80 ^{o}C. Thin sections cut with a Reichert Ultracut ultramicrotome from cells embedded in LR White resin (Electron Microscopy Sciences, Fort Washington, PA, USA) were incubated with anti-Etr1p antibodies, followed by a protein A-gold complex. Counterstained sections were examined using a Philips EM 410 microscope (Philips ElectronOptics, Eindhoven, Netherlands).

For the electron microscopy studies *S. cerevisiae ybr026c*Δ cells overexpressing full-length or truncated Nrbf-1p (II) were fixed with 2.5% glutaraldehyde, immersed in Agar, further post fixed in Agar blocks with 1% osmium tetroxide and embedded in Embed 812 (Electron Microscopy Sciences). Thin sections of 80 nm were cut with a Reichert Ultracut ultramicrotome and examined using a Philips CM100 transmission electron microscope.

For light microscopy analysis (III), samples from various tissues were fixed in 4% phosphate-buffered paraformaldehyde, embedded in paraffin, sectioned and stained with hematoxylin and eosin. For staining of lipids, tissue samples were embedded in O.C.T. – compound (Tissue-Tek, Zoeterwoude, Netherlands) and frozen with liquid nitrogen. Cryosections were cut from embedded samples with a Reichert-Jung 2800 Frigocut cryomicrotome and stained with Oil red O and hematoxylin using standard methods.

4.10 Analysis of serum acyl-carnitines and liver total fatty acids (III)

For the determination of serum acyl-carnitines, lipids including the acyl-carnitines were separated from other serum components using an RP-8 matrix. Neutral lipids and acyl-

methyl esters were separated from acyl-carnitines by extraction. For the determination of total fatty acids from liver homogenates, fatty acids were isolated using acid hydrolysis and hexane extraction and derivatized to their methyl-esters. The recording of mass spectral data from the extracted total liver fatty acids and acyl-carnitines isolated from sera was done in positive ion mode by accumulating 256 scans at 256K resolution using an APEX II FTICR mass spectrometer. The spectrometer was equipped with a 7T active shielded magnet and an APOLLO electrospray ion source, and controlled with Xmass 6.0.0 software (Bruker Daltonics, Billerica, MA, USA).

4.11 Expression pattern and mRNA analysis (II, III)

To determine the expression pattern of human Nrbf-1p (II), Multiple Tissue Northern Blot™ (Clontech, Palo Alto, CA, USA) was hybridized with ^{32}P-labeled *HsNRBF-1* cDNA. Hybridization was done in ExpressHyb™ (Clontech) solution according to the manufacturer's instructions and the hybridized fragments were visualized by autoradiography using X-Omat™ AR imaging film (Eastman Kodak, Rochester, NY, USA).

For real-time quantitative PCR analysis (III), cDNA was produced with a First Strand cDNA Synthesis Kit (MBI Fermentas, Heidelberg, Germany) from total RNA isolated with an RNeasy Mini Kit (Qiagen Gmbh, Hilden, Germany). PCR was performed with a 7500 Real Time PCR System (Applied Biosystems, CA, USA) using fluorogenic probe-based TaqMan chemistry with Taqman Universal PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. Primers and 5´ FAM-labelled probes were designed using Primer Express software (Applied Biosystems) and were purchased from Sigma-Genosys (Sigma-Genosys, Haverhill, UK). For the relative quantification of gene expression the results were normalized using GAPDH as an endogenous control for each sample and analyzed with 7500 System Software (Applied Biosystems).

4.12 Blood chemistries (III)

Blood samples were collected from anesthetized mice by orbital bleeding to Multivette® collection tubes (Sarsted, Nümbrecht, Germany) and serum was separated by centrifugation within 15 to 30 minutes. Serum total cholesterol, triglycerides, albumin, alkaline phosphatase, alanine aminotransferase, glutamyl transferase, β-hydroxybutyrate and serum amino acids were analyzed by the clinical laboratory of the University Hospital of Oulu, Finland. Serum glucose (Glucose, Wako Chemicals GmbH, Neuss, Germany) and free fatty acids (NEFA C, Wako Chemicals GmbH) were determined by enzymatic colorimetric methods.

5 Results

Results obtained from the studies concerning the characterization of fungal and mammalian 2-enoyl thioester reductases participating in mitochondrial FAS II (I and II) and analysis of the mutant mice deficient in 2,4-dienoyl-CoA reductase participating in the β-oxidation of PUFA (III) are presented below, separately.

5.1 Characterization of 2-enoyl thioester reductases of mitochondrial FAS II

5.1.1 Identification and purification of 2-enoyl-thioester reductase from C. tropicalis *and mammals (I, II)*

Protein extracts from *C. tropicalis* cells grown either in glucose or oleic acid containing media were analyzed using hydrophobic interaction chromatography. The reductase activity profile measured using *trans*-2,*trans*-4-hexadienoyl-CoA as a substrate revealed two separate activity peaks (Fig. 1C and D in I). Fractions corresponding to the second activity peak cross-reacted with an antibody against *C. cerevisiae* 2,4-dienoyl-reductase (Sps19p) (Gurvitz *et al.* 1997) and were assumed to contain a homolog of Sps19p. Fractions in the first activity peak showed minimal cross-reactivity with the Sps19 antibody and the novel protein, named enoyl-thioester reductase (Etr1p), responsible for reductase activity in those fractions was further purified to apparent homogeneity (Fig. 1E in I). Purified Etr1p had a native molecular mass of 75 kDa as indicated by size-exclusion chromatography, and SDS-PAGE analysis showed a single band with a size of 40 kDa suggesting a homodimeric nature (Fig. 1E in I).

For further analysis the gene (*ETR1*) encoding Etr1p was cloned from a genomic library and the obtained ORF was observed to encode a putative polypeptide of 42 kDa including a presequence absent from mature Etr1p. The sequence of the putative polypeptide also matched the sequence obtained by proteolytic cleavage of purified Etr1p. A database search indicated that the *S. cerevisiae* Ybr026p, also termed

mitochondrial respiratory function protein (Mrf1p)(Yamazoe *et al.* 1994), had a high amino acid identity (42%) with Etr1p. To further characterize and compare the enzymatic activities of Etr1p and Mrf1p, both enzymes were overexpressed in *S. cerevisiae* and purified.

In order to search for a mammalian homolog of 2-enoyl thioester reductase, a BLAST search (Altschul *et al.* 1990) of protein databases was conducted with *S. cerevisiae* Ybr026p/Mrf1p and *C. tropicalis* Etr1p. The search revealed homologs from various eukaryotes, including humans and the EST, AA393871, corresponding to human protein with a predicted mass of 37 kDa was sequenced and termed *Hs*NRBF-1. Based on the sequence information from *Hs*NRBF-1, a cDNA coding for Nrbf-1p was cloned from bovine heart (*BtNRBF-1*). Amino acid sequence alignment (Fig. 1 in II) showed that the identities between *Hs*Nrbf-1p (BtNrbf-1p) and Ybr026p and Etr1p were 40% (35%) and 36% (38%), respectively, and the identity to rat Nrbf-1p (14) was 80% (83%). To characterize the functions of *Hs*Nrbf-1p and *Bt*Nrbf-1p further, the proteins were overexpressed in *E. coli* and purified to homogeneity. Size-exclusion chromatography indicated a native molecular mass of 65 kDa and SDS-PAGE analysis showed a single protein band of 37 kDa (Fig. 3 in II), suggesting that both proteins are, like their yeast counterparts, homodimers.

5.1.2 Enzymatic properties of 2-enoyl thioester reductases (I, II)

To analyze the reaction catalyzed by Etr1p and Mrf1p, *trans*-2,*trans*-4-hexadienoyl-CoA was used as a substrate and incubated with purified proteins in the presence of NADPH. The hydrolyzed and esterified reaction products were subjected to gas chromatography and the accumulation of 4-hexenoic acid ethyl ester was observed (Fig. 2A in I). When *trans*-2-hexenoyl-CoA was used as a substrate, accumulation of hexanoic acid ethyl ester was observed (Fig. 2B in I). This data and the absence of 2- or 3-hexenoic acid ethyl esters when *trans*-2,*trans*-4-hexadienoyl-CoA was used as a substrate indicated that Etr1p and Mrf1p acted as 2-enoyl thioester reductases, specifically reducing *trans*-2 double bonds in 2-monoenic and 2,4-dienic structures. The specific activities for Etr1p and Mrf1p with *trans*-2-hexenoyl-CoA and *trans*-2,*trans*-4-hexadienoyl-CoA as substrates were 20 and 10 μmol/min/mg protein, respectively.

The high sequence similarity of human and bovine Nrbf-1p with Etr1p and Mrf1p suggested similar enzymatic activity. The end product of the reaction catalyzed by *Hs*Nrbf-1p was analyzed using *trans*-2-hexenoyl-CoA as a substrate in the presence of NADPH. HPLC analysis of the samples (Fig. 4 in II) showed that the disappearance of *trans*-2-hexenoyl-CoA was followed by concomitant accumulation of a metabolite, which was identified as hexanoyl-CoA by comparing it to the HPLC profile of pure hexanoyl-CoA and by MALDI-TOF mass spectrometry analysis. These results indicate that *Hs*Nrbf-1p is a 2-enoyl thioester reductase that reduces *trans*-2-enoyl thioesters to their saturated counterparts. *Trans*-2-hexenoyl-CoA and *trans*-2-decenoyl-CoA were used to determine the kinetic parameters for *Hs*Nrbf-1p. K_m-values (k_{cat}-values) of 37.0 ± 5.4 (0.53 ± 0.03 s^{-1}) and 7.1 ± 0.9 μM (0.93 ± 0.03 s^{-1}) were obtained, respectively. These values translated to catalytic efficiencies (k_{cat}/K_m) of 1.44 x 10^4 and 1.31 x 10^5 M^{-1} s^{-1} for

C_6 and C_{10} substrates. Comparable K_m- and k_{cat}-values were obtained for *Bt*Nrbf-1p when *trans*-2-decenoyl-CoA was used as a substrate. *Hs*Nrbf-1p also showed low activity with *trans*-2-hexadecenoyl-CoA, but kinetic parameters were not determined.

5.1.3 Mitochondrial localization of Etr1p and Mrf1p and the requirement of 2-enoyl thioester activity for respiratory competence (I)

It has previously been shown that *S. cerevisiae* strain (*ybr026c*Δ) deficient in Ybr026p can not grow on nonfermentable carbon sources (e.g. glycerol) and exhibits decreased cytochrome content. Due to the effect of Ybr026p on the respiratory growth of *S. cerevisiae*, it was named mitochondrial respiratory function protein (Mrf1p)(Yamazoe *et al.* 1994). To study whether Etr1p and Mrf1p, in addition to sequence similarity and 2-enoyl thioester reductase activity, also have similar physiological function, Etr1p and Mrf1p were overexpressed in the *ybr026c*Δ strain and complementation, as well as cytochrome spectra were examined. The observation that growth could be restored by expression of either Etr1p or Mrf1p (Fig. 4. in I) together with the restoration of mitochondrial cytochrome complexes indicated that the physiological function of Etr1p, similar to Mrf1p, was connected to respiratory competence.

The proposed nuclear localization of Mrpf1p (Yamazoe *et al.* 1994) and the N-terminal sequence of Etr1p, which has characteristics of the mitochondrial targeting sequence, prompted investigation of their subcellular localization. Immuno-electronmicroscopy (IEM) with *C. tropicalis* cells using an anti-Etr1p antibody indicated that native Etr1p was specifically localized to mitochondrial structures (Fig. 5A in I). The localization of Etr1p and Mrf1p was further studied by overexpressing them in the *ybr026c*Δ strain grown on a nonfermentable carbon source. IEM with the anti-Etr1p antibody that cross-reacted strongly with Mrf1p showed that both proteins were predominantly localized to mitochondria. Interestingly, in contrast to the rudimentary mitochondria of the *ybr026c*Δ strain and normal mitochondria of the wild-type strain, mitochondria in the strain complemented by Etr1p or Mrf1p were vastly enlarged (Fig. 5D and E in I).

The N-terminal sequencing of purified and overexpressed proteins had revealed the absence of a 22 amino acid long presequence in mature Etr1p, and an 8 amino acid long presequence in Mrf1p. The significance of these presequences for targeting, as well as for complementation of the *ybr026c*Δ strain, was studied by expressing truncated variants of Etr1p and Mrf1p devoid of presequences in the *ybr026c*Δ strain. IEM showed that truncated Etr1p was primarily extramitochondrial indicating that the presequence acted as a mitochondrial targeting signal (Fig. 5F in I). Deletion of the targeting signal also abolished the ability of Etr1p to rescue the respiratory growth of *ybr026c*Δ cells despite the conserved enzymatic activity. This confirmed the necessity of mitochondrial localization for the physiological function (Fig. 4 in I). In contrast to localization of truncated Etr1p, IEM showed that truncated Mrf1p was, although diffusively localized, also present in mitochondria (Fig. 5G in I). The truncated version of Mrf1p was also able to sustain the growth of *ybr026c*Δ cells on glycerol, probably due to partially retained mitochondrial localization (Fig. 4 in I). In order to challenge the prior suggestion that

Mrf1p acts in the nucleus to maintain respiratory functions by regulating genes responsible for the assembly of respiratory complexes (Yamazoe *et al.* 1994), the nuclear targeting sequence of Simian virus 40 protein large antigen (25) was used to replace the presequence of Mrf1p. Although the protein was localized in the nucleus as indicated by IEM (Fig. 5H in I), it was only poorly able to rescue the growth of the respiratory-deficient *ybr026c*Δ strain, despite fully preserved enzymatic activity (Fig. 4 in I).

To determine whether Etr1p and Mrf1p have a structural role in facilitating the correct assembly of cytochrome complexes or whether their 2-enoyl thioester reductase activity was essential for respiratory competence, the *ybr026c*Δ strain was transformed with a plasmid expressing NADPH-dependent 2-enoyl-ACP reductase (FabI) from *E. coli* (Heath and Rock 1995). This nonhomologous *E. coli* protein, which is part of the prokaryotic fatty acid synthesis pathway (FAS II), was able to rescue the respiratory-deficient Mrf1p deletion strain when extended with the mitochondrial targeting signal (Fig. 4) and the complemented strain further showed restored cytochrome spectra. This observation indicates that the enzymatic activity of Etr1p and Mrf1p is essential for respiratory competence in *S. cerevisiae*. Furthermore, the overexpression of mitochondrially targeted FabI resulted in an enlargement of mitochondria similar to that with overexpressed Etr1p or Mrf1p (Fig. 5I in I).

5.1.4 Localization and physiological function of mammalian Nrbf-1p (II)

Features of a mitochondrial targeting signal (Pfanner 2000) can be observed in the N-terminal amino acid sequence of human, bovine, and rat Nrbf-1p. Subcellular localization predictions programs (Claros and Vincens 1996, Emanuelsson *et al.* 2000) also indicated a high probability of mitochondrial localization. The previously proposed cytosolic/nuclear localization of rat Nrbf-1 and its suggested role as a nuclear receptor binding factor (Masuda *et al.* 1998) contradicted the characterized 2-enoyl thioester reductase activity and predicted mitochondrial localization of mammalian Nrbf-1ps. To reinvestigate the subcellular localization of mammalian Nrbf-1p, *Hs*Nrbf-1p with C-terminal fusion with green fluorescent protein (GFP) was expressed in HeLa cells. Fluorescent microscopy analysis of cells transfected with a plasmid expressing Nrbf-1p-GFP fusion protein revealed a well defined fluorescence pattern that superimposed with the mitochondrial control stain (Fig. 5A, C and D in II) indicating mitochondrial localization. Subcellular fractionation of rat heart organelles by differential and isopycnic centrifugation was used to study the localization of wild-type Nrbf-1p. Immunoblotting with anti-*Hs*ETRAb revealed a 37-kDa band corresponding to the predicted polypeptide size of rat Nrbf-1p in the same fractions that contained the mitochondrial marker enzyme (cytochrome c oxidase) activity (Fig. 5I in II). The detected existence of 2-enoyl thioester reductase activity in the same fractions also suggested mitochondrial localization of Nrbf-1p in rat heart.

To study the physiological function of mammalian Nrbf-1p and whether *Hs*Nrbf-1p can act as the human ortholog of yeast Mrf1p and Etr1p, complementation analysis was performed. Human Nrbf-1p and truncated Nrbf-1p devoid of a mitochondrial targeting

signal were overexpressed in respiratory-deficient *ybr026*Δ cells and their growth on a nonfermentable carbon source was assessed. Human Nrbf-1p was able to rescue the growth of *ybr026*Δ cells almost to a wild type level, whereas the protein variant with the truncated mitochondrial targeting signal did not rescue growth (Fig. 7 in II). Interestingly, transmission electron microscopy revealed enlargement of mitochondria in *ybr026*Δ cells expressing human Nrbf-1p (Fig. 8D in II). These findings were similar to those observed in complementation analysis with the yeast 2-enoyl thioester reductases, Etr1p and Mrf1p (see section 4.1.3 and original article I), indicating that mammalian Nrbf-1ps are mitochondrial 2-enoyl thioester reductases.

5.2 Characterization of the phenotype of DECR-deficient mice

5.2.1 The clinical phenotype of Decr $^{-/-}$ mice under normal conditions (III)

The disruption of *Decr* was analyzed using Southern blotting, where hybridization of a probe to a 4.7 kb genomic fragment confirmed the correct targeting (Fig. 1B in III). Later, different genotypes of mice were distinguished using PCR (Fig. 1C in III). Western blot analysis of mitochondrial homogenates from liver, muscle and heart tissue using antibodies against rat DECR revealed a detectable signal corresponding to DECR from wild type mice, whereas no signal could be detected from *Decr*$^{-/-}$ mice (Fig. 1D in III).

Under normal conditions mice with disrupted *Decr* appeared normal. Crossbreeding analysis with *Decr*$^{+/-}$ mice showed that their progeny followed Mendelian inheritance ratios with no gender bias (Table 2 in III). This observation ruled out the effect of deficiency on embryonic development. *Decr*$^{-/-}$ mice were physiologically and histologically indistinguishable from wild type mice. Both male and female mutant mice were viable and fertile having normal weight gain and life span. Histological analysis of the major organs also failed to show any differences between wild type and mutant mice.

5.2.2 Identification of phenotype using metabolic stress (III)

Although the inability to use fatty acids as an energy source, as in the case of human disorders in mitochondrial β-oxidation, produces various clinical phenotypes, a common feature is that individuals are usually asymptomatic under normal conditions. The same phenomenon can be seen in several animal models for fatty acid oxidation disorders. Clinical symptoms arise only after metabolic stress, such as prolonged physical exercise or fasting, when fatty acids become a primary energy source. In order to study the effect of disrupted mitochondrial β-oxidation of unsaturated fatty acids during metabolic stress, *Decr*$^{-/-}$ mice were subjected to fasting.

5.2.2.1 Altered lipid and glucose homeostatic response to fasting (III)

Hepatic steatosis, defined as abnormal accumulation of lipids in liver hepatocytes, is commonly observed in fatty acid oxidation (FAO) disorders and corresponding animal models. When the organs of the sacrificed animals where studied, it was noted that the livers of the *Decr*$^{-/-}$ mice were markedly pale and weights, when determined as a percentage of the body weight, were significantly higher than those of the wild type (Fig. 2 in III). Although histological analysis of livers in the fed state showed no differences (Fig. 3A and B in III), fasted *Decr*$^{-/-}$ mice showed the presence of abnormal hepatocytes with a foamy appearance and centralized nuclei, characteristics of microvesicular steatosis (Fig. 3C and D in III). Oil red O staining of neutral lipids showed massive and homogeneously distributed micro- and macrovesicular lipid droplet formation, whereas normal minor accumulation was present in age-matched wild type controls (Fig. 3E and F in III). This data indicated that fasting resulted in disturbed hepatic lipid homeostasis in the *Decr*$^{-/-}$ mice.

The amounts of circulating nonesterified fatty acids (NEFA), which are released during fasting from triacylglycerides stored in adipose tissue and can contribute to hepatic lipid accumulation, were analyzed from the sera of *Decr*$^{-/-}$ mice and wild type mice. The fed state mean serum NEFA levels of wild type and *Decr*$^{-/-}$ mice were comparable (0.43 ± 0.11 mmol/l vs. 0.52 ± 0.03 mmol/l). However, after fasting *Decr*$^{-/-}$ mice demonstrated abnormally high serum NEFA levels reaching 1.28 ± 0.12 mmol/l after 48 h compared to wild type levels of 0.68 ± 0.16 mmol/l (Fig. 4A in III).

In order to analyze whether the defect in the mitochondrial oxidation of unsaturated fatty acids generates a hypoglycemic condition, which is a common symptom associated with inherited defects of mitochondrial fatty acid oxidation, serum glucose levels were determined from wild type and *Decr*$^{-/-}$ mice after 24 h and 48 h fasting (Fig. 4B in III). In the fed state glucose levels were comparable, however, 24-hour fasting caused significant decreases in the glucose levels of *Decr*$^{-/-}$ mice compared to wild type mice (6.6 ± 0.2 mmol/l vs. 10.9 ± 0.3 mmol/l). Prolonged fasting (48 h) further decreased glucose levels in *Decr*$^{-/-}$ mice (2.3 ± 0.3 mmol/l) and a decrease was also observed in wild type mice, 5.9 ± 0.9 mmol/l. These data indicate that *Decr*$^{-/-}$ mice have a hypoglycemic response to fasting.

To study the ketogenic response to fasting, which is often impaired in FAO disorders and associated with hypoglycemia, serum β-hydroxybutyrate levels were analyzed (Fig. 4C in III). In the fed state formation of ketone bodies was normally low in wild type and *Decr*$^{-/-}$ mice (0.13 ± 0.01 mmol/l and 0.16 ± 0.06 mmol/l, respectively). Fasting increased serum β-hydroxybutyrate values in the wild type and *Decr*$^{-/-}$ mice to comparable levels (1.04 ± 0.14 mmol/l and 1.11 ± 0.10, respectively) indicating normal ketogenic response.

The hypoglycemic state of the *Decr*$^{-/-}$ mice after fasting was neither due to differences in storage or use of glycogen in the liver or muscle. This was shown by measurement of glycogen content before and after 24 h fasting (Fig. 5A and B in III), wherein comparable values for wild type and *Decr*$^{-/-}$ mice were observed.

5.2.2.2 Altered fatty acid pattern and organic acid and acylcarnitine profile (III)

To further analyze the hepatic lipid accumulation in *Decr*$^{-/-}$ mice, liver lipids were characterized by mass spectrometry. Under the fed state the amount of total liver fatty acids and proportion of saturated fatty acids (SAFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) in wild type and *Decr*$^{-/-}$ mice were comparable (Fig. 6A and C in III). Analysis of total liver fatty acids after fasting for 24 h revealed that the accumulating fatty acid species in *Decr*$^{-/-}$ mice were unsaturated. Compared to wild type mice *Decr*$^{-/-}$ mice had significantly increased (2.5- to 3.8-fold) levels of palmitoleic acid ($C_{16:1}$), oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$) and linolenic acid ($C_{18:3}$) (Fig. 6B in III). Fasting had a relatively minor effect (29% increase) on the lipid content of wild type liver, wherein levels of SAFA and PUFA were slightly increased (Fig. 6C in III). In *Decr*$^{-/-}$ mice however, fasting increased the total concentration of fatty acids by 108%. This increase was solely due to levels of MUFA and PUFA, which were increased 288% and 254%, respectively. The main contributors to increased MUFA concentrations were oleic acid and palmitoleic acid, whereas the increase in the PUFA concentrations was due to increased linoleic acid and linolenic acid concentrations. The abnormal effect of fasting on liver lipid homeostasis in *Decr*$^{-/-}$ mice was most pronounced in the concentrations of linoleic and linolenic acids, which were increased 5.5-fold and 6.9-fold compared to fed state values. The concentration of SAFA in *Decr*$^{-/-}$ mice remained relatively unchanged, although fasting induced a slight increase in the concentration of palmitic acid and a decrease in the concentration of stearic acid.

The initial diagnosis of long-chain fatty acid oxidation disorders is routinely carried out by analyzing serum or plasma acylcarnitines. By profiling serum acylcarnitines it is possible to deduce which acyl-CoA esters are accumulating intramitochondrially and subsequently transesterified with carnitine and released into the blood stream, and therefore to identify the site of the metabolic block in the β-oxidation cycle.

The effect of *Decr* disruption on the acylcarnitine profile was determined from non-fasted and fasted wild type and *Decr*$^{-/-}$ mice by mass spectrometry. In the fed state the total amount of serum acylcarnitines in the wild type and *Decr*$^{-/-}$ mice (263 ± 29 nM and 240 ± 16 nM, respectively) showed no differences. The levels of individual acylcarnitines with chain lengths between 8 to 20 carbon atoms (Fig. 7A in III) were also comparable. In wild type mice fasting increased the total concentration of acylcarnitines 2-fold (567 ± 32 nM), whereas in *Decr*$^{-/-}$ mice a markedly higher 9-fold increase was observed (2150 ± 230 nM). All analyzed acylcarnitine species were highly elevated in *Decr*$^{-/-}$ mice compared to wild type mice but the most distinctive increase was observed at the level of decadienoylcarnitine ($C_{10:2}$), the concentration of which was 44-fold higher in the sera of *Decr*$^{-/-}$ mice than the wild type control (Fig. 7B in III).

When amounts of dicarboxylic acids in the urine of wild type and *Decr*$^{-/-}$ were compared, a prominent accumulation of $C_{7:2}$, $C_{8:2}$, $C_{10:2}$, $C_{10:3}$, and $C_{14:3}$ dicarboxylic acid species was observed in *Decr*$^{-/-}$ mice after fasting. The excretion of these dicarboxylic acids, especially $C_{10:2}$ and $C_{14:3}$, which were not detected from the urine of wild type mice, was also observed in *Decr*$^{-/-}$ mice in the fed state (data not shown).

5.2.2.3 Changes in the expression of genes of mitochondrial and extramitochondrial fatty acid metabolism (III)

In *Decr*$^{-/-}$ mice the normal lipid and glucose homeostatic response to fasting is disturbed. Indications of this include accumulation of unsaturated fatty acids in liver lipids, a highly increased concentration of acylcarnitines in sera, and hypoglycemia. The effect of these disturbances on the expression of several genes from fatty acid and carbohydrate metabolism pathways was studied using the quantitative real-time PCR method.

Comparison of mitochondrial β-oxidation enzymes in liver (Fig. 8 in III) indicated a 2-fold increase in the expression level of the rate-limiting enzyme carnitine palmitoyltransferase (CPT I) in *Decr*$^{-/-}$ mice. The expression of one of the auxiliary enzymes in the oxidation of polyunsaturated fatty acids, enoyl-CoA isomerase (ECI), was also slightly up-regulated (1.5-fold). Peroxisomal β-oxidation genes showed 2.3- and 3.4-fold higher expression of acyl-CoA oxidase (Acox) and peroxisomal multifunctional enzyme 1 (MFE I) in *Decr*$^{-/-}$ mice, respectively. The expression level of acetyl-CoA carboxylase (Acaca), which catalyzes the first step in the fatty acid synthesis pathway, was lower, although not significantly, in *Decr*$^{-/-}$ mice. The messenger RNA level of the enzyme responsible for the synthesis of monounsaturated fatty acids, stearoyl-CoA desaturase 1 (SCD1), however, was significantly lower in *Decr*$^{-/-}$ mice. Marked differences were also observed in the expression level of a key enzyme in microsomal ω-oxidation, cytochrome P450 IVA1 (CYP 4A10), which was 2.1-fold higher compared to wild type mice. Key enzymes in the gluconeogenesis and glyceroneogenesis pathway, phosphoenoylpyruvate carboxykinase (PEPCK-C) and glucose-6-phosphatase (G-6Pc), showed significantly lower expression levels (0.5- and 0.45-fold, respectively) in *Decr*$^{-/-}$ mice, whereas there were no differences in the level of hydroxymethylglutaryl-CoA synthetase (HMGCS), which participates in the synthesis of ketone bodies. Fasting produced no differences in the expression level of peroxisome proliferator activated receptor α (PPARα) between wild type and *Decr*$^{-/-}$ mice, however the expression of sterol regulatory element-binding protein 1 (SREBP1) in *Decr*$^{-/-}$ mice was significantly repressed.

5.2.2.4 Impaired thermogenesis during acute cold exposure (III)

Cold intolerance has been indicated with many animal models of FAO disorders. Mice with FAO disorders are unable to maintain normal body temperature when exposed to a cold atmosphere with or without prior fasting. A cold exposure study showed that metabolically challenged *Decr*$^{-/-}$ mice rapidly develop severe hypothermia. After short exposure to 4 ^{o}C the average body temperature of *Decr*$^{-/-}$ mice was approximately 10 ^{o}C lower than that of wild type mice and experiment was terminated prematurely as temperatures declined below an unrecoverable 25 ^{o}C (Fig. 9A in III). Muscle shivering initially present in *Decr*$^{-/-}$ mice became less evident during the course of the experiment and was totally absent when the mice became lethargic. As expected, wild type mice were able to maintain their body temperature throughout the experiment and shivering was also clearly observed. The metabolic challenge was a requisite for cold intolerance in *Decr*$^{-/-}$

mice, as no differences between wild type and *Decr*$^{-/-}$ mice were observed in the ability to maintain body temperature in non-fasted mice (Fig. 9B in III).

6 Discussion

6.1 2-Enoyl thioester reductases from *C. tropicalis* and *S. cerevisiae* as components of the mitochondrial fatty acid synthesis system

In addition to the cytosolic multifunctional enzyme complexes responsible for fatty acid synthesis (FAS I) in eukaryotes (Smith 1994), a synthesis system comprised of individual soluble enzymes similar to the prokaryotic system (FAS II) has been postulated in fungal mitochondria. It has been indicated that several *S. cerevisiae* genes encode mitochondrial proteins, which are categorized as components of this machinery based on their sequence similarities to prokaryotic enzymes. It has also been observed that these enzymes are necessary for the proper respiratory function of yeast, as gene disruption results in strains that are unable to grow on nonfermentable carbon sources (Harington *et al.* 1993, Schneider *et al.* 1995, Schneider *et al.* 1997).

2-Enoyl thioester reductase can be found as part of multifunctional FAS I in eukaryotes and it is also essential for prokaryotic FAS II (Heath and Rock 1995). However, database searches with bacterial reductase do not reveal any candidates from eukaryotic sources. Chromatographic analysis of the soluble extract from *C. tropicalis* cells indicated two separate reductase activities, one of which was characterized as a homolog of *S. cerevisiae* 2,4-dienoyl-CoA reductase, Sps19p, from the β-oxidation pathway (Gurvitz *et al.* 1997), based on immunological crossreactivity with an Sps19p-antibody. A novel enoyl thioester reductase (Etr1p) with a molecular mass of 75 kDa and subunit size of 40 kDa was purified from chromatographic fractions, which contained reductase activity but did not show the presence of an Sps19p-like protein. For further characterization Etr1p was cloned and overexpressed in *S. cerevisiae*. The possibility that Etr1p might act as a 2,4-dienoyl-CoA reductase was ruled out by reaction end product analysis. Analysis using *trans*-2,*trans*-4-hexadienoyl-CoA and *trans*-2-hexenoyl-CoA as substrates showed that Etr1p acts specifically on *trans*-2 double bonds, as the end products were *trans*-4-enoyl-CoA and hexanoyl-CoA, respectively.

The finding that Etr1p shared sequence homology with Mrf1p (Ybr026p), a *S. cerevisiae* protein indicated to have functions linked to respiration (Yamazoe *et al.* 1994), led to characterization of the enzymatic activity of Mrf1p. Similar to Etr1p, Mrf1p was

found to catalyze NADPH-dependent reduction of *trans*-2 double bonds. The respiratory-deficient phenotype of the *mrf1*Δ strain with reduced cytochrome content is similar to the phenotype obtained by disruption of any of the *S. cerevisiae* genes postulated to encode components involved in mitochondrial FAS II. These findings, together with the localization studies that indicated that Etr1p as well as Mrf1p (in contrast to earlier suggestions of nuclear localization) were mitochondrial proteins, suggest that Etr1p and Mrf1p are 2-enoyl thioester reductases of fungal mitochondrial FAS II. Furthermore, it was shown by the complementation studies that expression of Etr1p or Mrf1p could restore cytochromes as well as the respiratory competence of the *mrf1*Δ strain. When the presequence responsible for mitochondrial targeting was omitted from Etr1p, complementation was not observed. It was also shown for Mrf1p that mitochondrial localization is essential for respiratory growth, as Mrf1p targeted to nucleus failed to complement the phenotype of *mrf1*Δ. The importance of the enzymatic activity of Etr1p and Mrf1p for the observed complementation of *mrf1*Δ became evident when complementation studies were conducted with nonhomologous 2-enoyl-ACP reductase (FabI) from the *E. coli* FAS II system (Bergler *et al.* 1996). Since the ability to use respiration when grown on a nonfermentable carbon source was restored by a structurally non-related protein, it was clear that the 2-enoyl reductase activity rather than structural function of the protein was responsible for the complementation.

The link between respiratory competence and mitochondrial FAS II is not clear, nor is it clear which fatty acids are produced by this system. It has been proposed that disruption of genes encoding proteins involved in mitochondrial FAS II does not affect mitochondrial transcription or translation, as indicated by intact mitochondrial DNA and apocytochromes in *mrf1*Δ cells (Yamazoe *et al.* 1994). Generation of precursors for the synthesis of lipoic acid, which is an important cofactor of several mitochondrial enzymes, has been suggested as the main function of fungal mitochondrial FAS II, and greatly reduced lipoic acid content has been reported in respiratory-deficient yeast strains with disrupted *ACP1* or *HFA1*, which code for acyl carrier protein and acyl-CoA carboxylase, respectively (Brody *et al.* 1997, Hoja *et al.* 2004). A decreased lipoic acid content in the *mrf1*Δ strain has also been observed in our group. However, disruption of the lipoic acid synthesis pathway in yeast, in contrast to mitochondrial FAS II, does not produce a cytochrome-deficient phenotype (Sulo and Martin 1993), and thus the phenotypes seen in yeast mutants deficient in mitochondrial FAS can not be explained solely by lowered lipoic acid content. It is known that various lipids, in addition to numerous proteins, are needed in the assembly and organization of respiratory complexes (Dimmer *et al.* 2002). One such lipid is myristic acid (C_{14}) that has been found covalently linked to subunits of complex I and V in *N. crassa* (Vassilev *et al.* 1995, Plesofsky *et al.* 2000). It has been proposed that mitochondrial FAS II provides acyl groups to enzyme complexes in the respiratory chain and thus facilitates the proper assembly and/or catalytic mechanism. A role in the synthesis and repair of membrane phospholipids has also been proposed (Schneider *et al.* 1997).

Morphological findings in *mrf1*Δ cells as well as in *mrf1*Δ cells overexpressing Etr1p or Mrf1p indicate a possible role for mitochondrial FAS II in the maintenance of mitochondrial structure. In mutant cells only rudimentary organelles with atypical structures could be seen, which might indicate impaired biogenesis with disrupted fission or fusion processes. On the other hand, in complemented strains mitochondria were

significantly enlarged. Similar results were also obtained with the recently identified mitochondrial 3-hydroxyacyl-ACP dehydratase (Kastaniotis *et al.* 2004). However, it is not clear whether the changes in morphology are directly caused by changes in respiratory competence or *vice versa*.

Candidates for all the proteins needed in mitochondrial fatty acid synthesis in fungi have been identified and most of them have also been characterized. Currently, however, the physiological function of this system for purposes other than the production of precursors for lipoic acid synthesis, which is generally accepted, is not clear. In order to identify the products of mitochondrial FAS II and generate experimental evidence to support various proposed functions, careful morphological studies, as well as analysis of the lipid profiles of different FAS II mutant strains, are required.

6.2 Mammalian mitochondrial 2-enoyl thioester reductase

The identification of mitochondrial 2-enoyl thioester reductase from *C. tropicalis* and *S. cerevisiae* prompted us to search for their mammalian counterparts. Despite the fact that such activity was reported to exist in mammalian mitochondria many years ago (Cvetanovic *et al.* 1985), the protein responsible for the activity had not been characterized.

Protein data base searches with Etr1p and Mrf1p revealed homologs from several mammalian sources, including rat Nrbf-1p that was described as nuclear receptor binding factor-1, which interacts with various nuclear receptors (Masuda *et al.* 1998). Following the database searches a human EST clone encoding a homolog for rat Nrbf-1p was obtained and the cDNA of a bovine homolog was also cloned. For further characterization the human and bovine proteins were overexpressed in *E. coli* and the enzymatic properties of the purified proteins were analyzed using different substrates. 2-Enoyl thioester reductase activity, similar to Etr1p and Mrf1p, was confirmed when the end product from the reaction using *trans*-2-hexenoyl-CoA as a substrate was characterized as hexanoyl-CoA. Additional analysis showed that Nrbf-1p can also use 2,4-hexadienoyl-CoA as a substrate and can catalyze the reduction of acyl thioesters with chain lengths between C_6 – C_{16}. The mitochondrial localization of mammalian Nrbf-1p, as predicted by the possible mitochondrial targeting signal in the N-terminal part of the protein, was confirmed with localization studies using fluorescence microscopy, as well as subcellular fractionation. The final evidence that mammalian Nrbf-1p is the ortholog of *C. tropicalis* Etr1p and *S. cerevisiae* Mrf1p and that it has a similar physiological function came from the complementation studies. Heterogeneous expression of full-length human *NRBF1* in the *mrf1*Δ strain restored the growth of mutant cells on glycerol indicating the restoration of a functional respiratory chain. As with fungal 2-enoyl thioester reductases, mitochondrial localization was indispensable for complementation and overexpression also resulted in the enlargement of mitochondria.

The mitochondrial localization of Nrbf-1p and the enzymatic activity contradict earlier findings suggesting cytosolic/nuclear localization and a function as a nuclear coactivator (Masuda *et al.* 1998). The observed differences in localization can be explained by different experimental procedures. In the previous study localization was analyzed using

recombinant Nrbf-1p with an N-terminal marker, which could potentially block the mitochondrial targeting signal. In this study green fluorescent protein was fused either to the C-terminal or N-terminal part of Nrbf-1p. As the results from the protein with N-terminal GFP showed diffused fluorescence in the cytosol as well as in the nucleus, whereas but the protein with GFP attached to the C-terminus gave a well-defined mitochondrial signal, it is probable that large molecules in the vicinity of the mitochondrial targeting signal interfere with proper targeting.

The existence of fatty acid synthesis in mammalian mitochondria has been suspected since the identification of an ACP-like protein covalently bound to complex I in the respiratory chain (Runswick *et al.* 1991). The characterization of 2-enoyl thioester reductase provided molecular and biochemical data in which enzymatic activity was linked to mammalian mitochondrial FAS for the first time. Since then several other components of this pathway have been described. Phosphopantetheinyl transferase, responsible for transferring a phosphopantetheine moiety from CoA to ACP associated with mitochondria, as well as malonyl transferase, which catalyzes the transfer of a malonyl moiety from CoA to ACP, have been characterized from humans (Joshi *et al.* 2003, Zhang *et al.* 2003). Malonyl transferase resembles prokaryotic transacylase (FabD) at the amino acid level and also has similar substrate specificity. Recently mitochondrial β-ketoacyl synthase, which shows significant similarity to fungal (CEM1) and prokaryotic synthases (FabB and FabF), was characterized. Similar to the human 2-enoyl thioester reductase, it was shown that β-ketoacyl synthase can complement the respiratory-deficient phenotype of its mutated yeast counterpart (*cem1*Δ strain), indicating similar physiological properties (Zhang *et al.* 2005). Further characterization of the ACP-like protein recently showed that it is mostly present in a soluble form in mitochondria and thus can carry acyl intermediates in the mitochondrial FAS pathway from one enzyme to another (Cronan *et al.* 2005). Common to all the characterized mammalian mitochondrial FAS enzymes is that they are more closely related to their prokaryotic and/or fungal counterparts than to cytosolic FAS I. This observation strengthens the hypothesis that the mitochondrial FAS system has an ancient prokaryotic origin.

In fungi and in plants the generation of precursors (octanoyl-ACP) for lipoic acid synthesis is considered to be the most significant function of mitochondrial FAS. The close relationship between the components of fungal and mammalian mitochondrial FAS and their physiological compatibility, as indicated by complementation studies, as well as characterization of lipoic acid synthase from mammalian sources (Morikawa *et al.* 2001), necessitate a re-evaluation of the generally accepted idea that the lipoic acid requirement in mammals is solely met by dietary intake. It is possible that in mammals one role of mitochondrial FAS is to provide precursors for lipoic acid synthesis and thus ensure the availability of this essential cofactor at times when it can not be obtained through the diet.

Yeast studies have shown that phenotypes observed in mutant strains with disrupted components in their mitochondrial FAS can not be explained by reduced lipoic acid content. This suggests that other products are essential for respiratory competence. The products of mitochondrial FAS in yeast and plants have been reported to contain 8 - 18 carbon atoms (Mikolajczyk and Brody 1990, Gueguen *et al.* 2000, Yasuno *et al.* 2004). The kinetic properties of human 2-enoyl thioester reductase showed that the mammalian enzyme accepts a wide range of *trans*-2-enoyl substrates with chain lengths between C_6 –

C_{16}, and has a preference for substrates with medium chain length. β-ketoacyl synthase was also shown to effectively utilize substrates containing acyl chains with a length between C_2 – C_{14} (Zhang *et al.* 2005). These observations indicate the possibility that mitochondrial FAS produces long chain fatty acids in mammals as well.

Although components of mitochondrial FAS in mammals have been relatively well characterized, enzymes catalyzing the β-ketoacyl synthase step and the β-hydroxyacyl dehydratase step in mammalian mitochondria have evaded identification so far. The physiological functions, as well as *in vivo* products, are not clear either. One interesting observation is that Nrbf-1p, ACP, lipoic acid synthase, and β-ketoacyl synthase are all highly expressed in heart and skeletal muscle; tissues with a high rate of oxidative phosphorylation and mitochondrial activity. The physiological role of mammalian mitochondrial FAS and whether it is linked to respiration, as in yeast, or whether it has a role in providing certain fatty acid species for the modification of membrane phospholipids or certain proteins, remains to be solved.

6.3 2,4-Dienoyl-CoA reductase-deficient mice

A mouse line deficient in mitochondrial *trans*-2,*cis*/*trans*-4-dienoyl-CoA reductase (DECR) was produced and characterized in this study in order to determine the importance of DECR activity for the auxiliary enzyme system in β-oxidation and for the complete oxidation of (poly)unsaturated fatty acids, and to provide a mouse model for 2,4-dienoyl-CoA reductase deficiency.

Most of the clinical cases of fatty acid oxidation disorders described so far concern deficiencies in the transport of fatty acids into mitochondria and deficiencies of the enzymes needed for the degradation of saturated fatty acids. Common to these disorders is that otherwise asymptomatic patients develop symptoms rapidly under metabolic stress when intact fatty acid oxidation is needed to maintain an energy balance. Clinical features vary but usually include hypoketotic-hypoglycemia, hepatosteatosis, organic aciduria, and cardiac and/or skeletal myopathies (Rinaldo *et al.* 2002). Only a single clinical case with 2,4-dienoyl-CoA reductase deficiency has been reported. The patient, who died at the age of 4 months, had reduced reductase activity and showed hyperlysinemia, hypocarnitinemia, and an abnormal urine acylcarnitine profile (Roe *et al.* 1990). Several mouse models have been developed for fatty acid oxidation disorders to deepen our understanding of them and to help develop suitable supportive care for patients. In many cases, these mouse models exhibit symptoms comparable to those of human patients when exposed to stress, and in addition show cold intolerance (Cox *et al.* 2001, Tolwani *et al.* 2005).

Decr$^{-/-}$ mice were found to be physiologically and histologically indistinguishable from wild type mice under normal conditions. It could also be observed from crossbreeding studies that deficiency did not result in any skewed inheritance or gender bias, indicating normal embryonic development. An asymptomatic phenotype was somewhat expected based on the observations made with mice deficient in Δ^3,Δ^2-enoyl-CoA isomerase (ECI), where mitochondrial β-oxidation of unsaturated fatty acids is halted at the level of their *cis*/*trans*-3-enoyl-CoA intermediates. *Eci*$^{-/-}$ mice showed

symptoms, including hepatic steatosis and dicarboxylic aciduria, only after prolonged fasting (Janssen and Stoffel 2002). When *Decr*$^{-/-}$ mice were tested for their ability to adapt to metabolic stress, several symptoms were observed. These included fasting-induced hepatic steatosis with an altered hepatic fatty acid profile, an elevated free fatty acid concentration in the blood, hypoglycemia, accumulation of acylcarnitines in sera, and severe cold intolerance.

Normally, during fasting fatty acids released from stored triacylglycerols by lipolysis are taken up by hepatocytes as free fatty acids and oxidized in mitochondria to provide energy through ATP formation and acetyl-CoAs for ketone body synthesis. In *Decr*$^{-/-}$ mice impaired oxidation of unsaturated fatty acids leads to their re-esterification and storage as triacylglycerols in hepatocytes. Specific accumulation of mono- and polyunsaturated fatty acids is reflected in the total liver fatty acids, which have an altered fatty acid composition, with oleic and linoleic acids becoming the dominant species.

In the case of polyunsaturated fatty acids with double bonds at odd- and even-numbered positions, their mitochondrial degradation in *Decr*$^{-/-}$ mice is halted either at the level of *trans*-2,*cis*-4-dienoyl-CoA after some of the 2,5-dienoyl-CoA intermediates are channelled to the reductase-dependent pathway (Ren and Schulz 2003) or when an even-numbered double bond at position Δ^4 is produced by successive cycles of β-oxidation. In the reported clinical case recognition of 2,4-dienoyl-CoA reductase deficiency was based upon the detection of *trans*-2,*cis*-4-decadienoylcarnitine from urine and plasma (Roe *et al.* 1990). This carnitine species, derived from incomplete oxidation of linoleic acid, was also highly elevated in *Decr*$^{-/-}$ mice. Despite the elevation of total acylcarnitine concentration, no other highly accumulating species were observed in the acylcarnitine profile, indicating that the major route for oxidation of 2,5-dienoyl-CoAs arising from the β-oxidation of unsaturated fatty acids with double bonds at odd-numbered positions is an Δ^3,Δ^2-enoyl-CoA isomerase-dependent pathway, as previously described (Ren and Schulz 2003).

Microsomal fatty acid ω–hydroxylation can, together with the peroxisomal β-oxidation pathway, provide an alternative pathway to prevent the accumulation of fatty acids or their derivatives in hepatocytes (Okita and Okita 2001) and organic aciduria, including dicarboxylic aciduria has been observed in patients with FAO disorders. Enhanced expression of microsomal cytochrome CYP 4A10, peroxisomal acyl-CoA oxidase (ACOX1) and multifunctional enzyme 1 (MFE1) in *Decr*$^{-/-}$ mice indicated induced ω-oxidation and peroxisomal β-oxidation. The processing of accumulating unsaturated intermediates in Decr$^{-/-}$ mice by microsomes and peroxisomes led to excretion of unsaturated dicarboxylic acids in the urine.

The reason for a hypoglycemic response to fasting in *Decr*$^{-/-}$ mice is not clear. In many fatty acid oxidation disorders and their mouse models fasting induced hypoglycemia is accompanied by rapid depletion of glycogen stores and hypoketonemia. This is due to insufficient energy and acetyl-CoA production from β-oxidation leading to a reduced ketogenic response, as well as inhibited gluconeogenesis. Analysis showed that in *Decr*$^{-/-}$ mice ketone bodies were produced in normal quantities, and glycogen stores and their usage were also similar in wild type and *Decr*$^{-/-}$ mice. The gene expression studies, however, indicated significantly decreased expression levels of enzymes controlling the rate of gluconeogenesis, possibly providing a mechanism for hypoglycemia. Normally, in the fasted state these enzymes, phosphoenoylpyruvate carboxykinase (PEPCK) and

glucose-6-phosphatase (G6Pase), are highly activated. Sterol regulatory element binding protein 1 (SREBP-1) has been shown to inhibit the transcription of gluconeogenic genes, including PEPCK and G6Pase. However, in *Decr*$^{-/-}$ mice the expression level of SREBP-1 is only 1/3 of the normal level after fasting, indicating that factors other than SREBP-1 are responsible for the decreased expression of gluconeogenic genes. As it is known that polyunsaturated fatty acids and their thioesters regulate the expression of numerous genes in metabolic pathways (Wahle *et al.* 2003, Sampath and Ntambi 2004), it is possible that accumulating unsaturated fatty acids or their derivatives in *Decr*$^{-/-}$ mice negatively affect the expression of PEPCK and G6Pase leading to decreased gluconeogenic flux and ultimately to hypoglycemia.

It is generally accepted that the cold intolerance observed in several mouse models of fatty acid oxidation disorders is due to impaired nonshivering thermogenesis (Guerra *et al.* 1998), by which brown adipose tissue normally produces heat when β-oxidation is uncoupled from ATP production. The normal ketogenic response to fasting in *Decr*$^{-/-}$ mice indicated that despite the accumulation of unsaturated intermediates β-oxidation was proceeding, and also that a capacity for nonshivering thermogenesis should exist. In this sense the severe cold intolerance of metabolically stressed *Decr*$^{-/-}$ mice was not expected. However, it has been shown that heat production by muscle shivering is the normal response to acute cold exposure and only after acclimatization to cold can nonshivering thermogenesis be used as the main mechanism for maintaining body temperature (Griggio 1988, Golozoubova *et al.* 2001). As there was no difference in the thermoregulatory capacity between wild type mice and *Decr*$^{-/-}$ mice when cold exposure was not preceded by fasting, the probable cause of cold intolerance in *Decr*$^{-/-}$ mice was their inability to maintain muscle shivering for heat production under acute cold exposure due to their hypoglycemic state. The observation in *Decr*$^{-/-}$ mice that shivering was initially present and then disappeared before the mice became lethargic lends support to the hypothesis mentioned above.

7 Conclusions

This study aimed to characterize fungal 2-enoyl thioester reductases involved in mitochondrial fatty acid synthesis and their mammalian counterparts. The study also consisted of characterization of the physiological function of mitochondrial 2,4-dienoyl-CoA reductase, which participates in the degradation of unsaturated fatty acids.

Observation of two thioester reductase activities from the soluble extract of *C. tropicalis* led to characterization of a novel 2-enoyl thioester reductase, Etr1p, and its homolog form *S. cerevisiae*, Mrf1p (Ybr026p). Immunolocalization and *in vivo* targeting experiments showed these proteins to be predominantly mitochondrial. The respiratory-deficient phenotype of the *S. cerevisiae mrf1*Δ strain with decreased cytochrome content and rudimentary mitochondria could be rescued by overexpressing Etr1p or Mrf1p. Overexpression also led to development of enlarged mitochondria. Mitochondrial localization proved to be essential for respiratory competency, as targeting of Etr1p or Mrf1p to other subcellular locations failed to rescue respiratory growth. It was further observed that the respiratory-deficient phenotype of the *mrf1*Δ strain could also be complemented by mitochondrially targeted nonhomologous 2-enoyl-acyl carrier protein reductase (FabI) from *E. coli* participating in type II fatty acid synthesis. This observation indicated that the enzymatic activity of 2-enoyl thioester reductase was critical for the mitochondrial function. Together these findings suggested that novel 2-enoyl thioester reductases, Etr1p and Mrf1p, can act as part of the mitochondrial fatty acid synthesis machinery, the proper function of which is essential for respiration and the maintenance of mitochondrial morphology.

Data base searches revealed several Mrf1p and Etr1p homologs, termed Nrbf-1p, from mammalian sources. Recombinant human and bovine proteins were purified and characterization of their enzymatic activity indicated an NADPH-dependent reduction of *trans*-2-enoyl thioesters with a chain length of C_6 – C_{16} to the corresponding saturated acyl thioesters. Localization studies using fluorescent microscopy and subcellular fractionation showed that Nrbf-1ps are mitochondrial proteins. In common with the results obtained from overexpression of Etr1p and Mrf1p in the *mrf1*Δ strain, the overexpression of human Nrbf1p resulted in enlarged mitochondria. As the complementation studies with the respiratory-deficient *S. cerevisiae mrf1*Δ strain indicated that mammalian mitochondrial 2-enoyl thioester reductase can rescue the

respiratory growth of a mutant strain on a nonfermentable carbon source only if it is localized to mitochondria, it was concluded that Nrbf-1ps are the orthologs of yeast 2-enoyl thioester reductases. This also suggests that mammals are likely to possess the ability and enzymatic machinery for mitochondrial fatty acid synthesis.

A mouse line deficient in *trans*-2, *cis*/*trans*-4-dienoyl-CoA reductase (DECR) revealed the physiological consequences of the disruption of the mitochondrial β-oxidation of unsaturated fatty acids. Similar to many genetic diseases of fatty acid oxidation and their animal models *Decr*$^{-/-}$ mice remained asymptomatic under normal conditions but rapidly developed symptoms when stressed. Metabolically challenged *Decr*$^{-/-}$ mice suffered from hypoglycemia and microvesicular steatosis with massive accumulation of unsaturated fatty acids in liver triacyglycerols. Accumulation of acylcarnitines in serum, especially decadienoylcarnitine, which is the product of incomplete oxidation of linoleic acid, was also observed. In contrast to many other animal models of fatty acid oxidation disorders, hypoglycemia was not associated with an altered ketogenic response. However, the normal thermogenic response was perturbed as shown by severe intolerance to acute cold exposure. Despite the fact that oxidation of saturated fatty acids is expected to proceed normally, this phenotype of *Decr*$^{-/-}$ mice is in many ways similar to mouse models of the disrupted classical β-oxidation pathway. The observations made in this study underscore the importance of a proper oxidative metabolism for unsaturated fatty acids in ensuring a balanced fatty acid and energy metabolism.

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