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CHARACTERIZATION OF 3-HYDROXYACYL-ACP DEHYDRATASE OF MITOCHONDRIAL FATTY ACID SYNTHESIS IN YEAST, HUMANS AND TRYPANOSOMES

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Abstract

In eukaryotic cells, fatty acids are mainly synthesized in the cytoplasm, but recently, in yeast and in humans, the ability to synthesize fatty acids has been characterized in mitochondria. This mitochondrial pathway is similar to bacterial type II fatty acid synthesis (FAS). The main feature of mitochondrial FAS in yeast is the respiratory deficient phenotype and loss of cytochromes when any of genes encoding enzymes for mitochondrial FAS is deleted. Mitochondrial FAS has been demonstrated to have an important role in lipoic acid production, namely it synthesizes octanoyl-ACP, which is used as a precursor for lipoic acid. However, the role and function of mitochondrial FAS is not yet fully understood. Many components of the mitochondrial FAS pathway in yeast have been identified according to their similarity to bacterial counterparts, but 3-hydroxyacyl-ACP dehydratase does not show any easily recognizable similarity to bacterial dehydratases and thus remained unidentified.

In this study 3-hydroxyacyl-ACP dehydratases of mitochondrial FAS were characterized from the yeast *Saccharomyces cerevisiae*, humans, and the human pathogen *Trypanosoma brucei*. The yeast 3-hydroxyacyl-ACP dehydratase (Htd2p) was identified by using a genetic screen, and this protein was shown to be encoded by open reading frame (ORF) *YHR067w*. The product of this gene shows mitochondrial localization and exhibits hydratase 2 activity. The deletion of *HTD2* leads to a respiratory deficient phenotype, loss of cytochromes, reduced lipoic acids levels and changes in mitochondrial morphology.

The ORF encoding human 3-hydroxyacyl-ACP hydratase (HsHTD2) was identified by functional complementation of the respiratory deficient phenotype of the yeast *htd2* mutant with a human cDNA library. The complementing cDNA was previously identified as the *RPP14* transcript encoding the 14 kDa subunit of the human RNase P complex. It was found that this transcript contains another 3' ORF, which encodes a protein that displays hydratase 2 activity and has mitochondrial localization. The bicistronic nature of the transcript is conserved in vertebrates and indicates a genetic link between mitochondrial FAS and RNA processing. The mitochondrial 3-hydroxyacyl-ACP hydratase in *T. brucei* is homologous to human HTD2, can complement the yeast respiratory deficient phenotype, exhibits hydratase 2 activity and localizes to the *T. brucei* mitochondrion.

Keywords: 3-hydroxyacyl-ACP dehydratase, bicistronic transcript, fatty acid synthesis, mitochondria

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Oulu, November 2007 Kaija Autio

Abbreviations

ACP	acyl carrier protein
ATP	adenosine triphosphate
BSF	bloodstream form of *T. brucei*
cDNA	complementary deoxyribonucleic acid
CoA	coenzyme A
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
EMS	ethyl methanesulfonate
ER	endoplasmic reticulum
FAS	fatty acid synthesis
FeS protein	iron-sulfur protein
5' FOA	5-fluoro-orotic acid
GFP	green fluorescent protein
GPI	glycosylphosphatidylinositol
GSM	glycerol sectoring medium
HsHTD2	human hydroxyacyl thioester dehydratase 2
Htd2	yeast hydroxyacyl thioester dehydratase 2
kb	kilobase
kDa	kilodalton
MaoC	monoaminoxidase C
MBP	maltose binding protein
MFE	peroxisomal multifunctional enzyme
mtDNA	mitochondrial deoxyribonucleic acid
NAD(P)H	nicotinamide adenine dinucleotide (phosphate), reduced form
NAD	nicotinamine adenine dinucleotide, oxidized form
NMR	nuclear magnetic resonance
ORF	open reading frame
PBS	phosphate buffered saline
PCF	procyclic form of *T. brucei*
PCR	polymerase chain reaction
RNA	ribonucleic acid
RPP14	14 kDa subunit of the human RNase P complex
rRNA	ribosomal ribonucleic acid
SCD	synthetic complete dextrose
TbHTD2	*T. brucei* hydroxyacyl thioester dehydratase 2

TED1	thioesterase / thiol ester dehydratase / isomerase superfamily
tRNA	transfer ribonucleic acid
VSG	variant surface glycoprotein
YPD	yeast extract peptone dextrose
Å	angstrom, 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 Kastaniotis AJ, Autio KJ, Sormunen RT & Hiltunen JK (2004) Htd2p/Yhr067p is a yeast 3-hydroxyacyl-ACP dehydratase essential for mitochondrial function and morphology. Mol Microbiol 53(5): 1407-1421.

II Autio KJ*, Kastaniotis AJ*, Pospiech H, Miinalainen IJ, Schonauer MS, Dieckmann CL & Hiltunen JK (2008) An ancient genetic link between vertebrate mitochondrial fatty acid synthesis and RNA processing. FASEB J. In press.

III Autio KJ, Guler JL, Kastaniotis AJ, Englund PT & Hiltunen JK (2007) 3-Hydroxyacyl-ACP dehydratase of mitochondrial fatty acid synthesis in *Trypanosoma brucei*. Manuscript.

* Equal contribution

Contents

1 Introduction

Fatty acids have a central role in many cellular functions such as energy storage and as building blocks of biological membranes. In addition, many hormones and intracellular messengers are derived from fatty acids, and fatty acids are also used to modify proteins post-translationally. Fatty acids are synthesized in cells when surplus nutrients are available, phospholipid components of membranes are needed or the synthesis of specialized molecules is required. Disturbances in fatty acid synthesis or breakdown affect many cellular processes and frequently lead to disease.

In eukaryotic cells, fatty acids are produced in the cytoplasm by the type I fatty acid synthase (FAS), a protein di(oligo)mer with multiple enzymatic functions. In contrast, prokaryotic cells synthesize their fatty acids in a type II manner where individual reactions are carried out by separate enzymes. For a long time it was thought that in eukaryotic cells fatty acid synthesis was solely a cytoplasmic process, but the discovery of mitochondrial proteins showing similarities to bacterial type II FAS enzymes revised this view. The existence of a mitochondrial fatty acid synthesis pathway in eukaryotic cells has raised questions regarding its role and biological function in cells and in the body.

In the present study, the 3-hydroxyacyl-thioester dehydratase enzyme of mitochondrial FAS was characterized in the yeast *Saccharomyces cerevisiae*, humans and the human pathogen *Trypanosoma brucei*. Their localization in mitochondria was confirmed and enzymatic hydratase 2 activity was analyzed. In yeast, mitochondrial dehydratase was shown to play an important role in respiratory competence and mitochondrial morphology. The finding of human mitochondrial dehydratase in the same bicistronic transcript as RPP14 of the human RNase P complex reveals a genetic link between mitochondrial fatty acid synthesis and RNA processing.

2 Review of the literature

2.1 Mitochondria

Mitochondria are dynamic cellular organelles that play a central role in energy and intermediary metabolism. The primary metabolic function of mitochondria is oxidative phosphorylation, an energy-transforming process that couples the oxidation of respiratory substrates to the synthesis of ATP (Saraste 1999). Tissues with high aerobic activity, e.g., brain, skeletal and cardiac muscle, have a particularly high number of mitochondria. However, mitochondria are also sites for several additional key cellular processes such as the oxidative decarboxylation of pyruvate, the tricarboxylic acid cycle, and fatty acid oxidation. Certain enzymes of the urea cycle and gluconeogenesis are located in the mitochondrial matrix and the assembly of FeS centers of mitochondrial and cytosolic FeS proteins occurs in mitochondria. Mitochondria are also involved in the regeneration of cytosolic NAD^+, which is essential for glycolysis, and in the intracellular homeostasis of inorganic ions such as calcium and phosphate (Modica-Napolitano & Singh 2004). More recently, mitochondria have been shown to play an integral role in the cascade of intracellular events that lead to programmed cell death, apoptosis (Zamzami *et al.* 1996).

Mitochondria evolved from a symbiotic relationship between aerobic bacteria and primordial eukaryotic cells (Lang *et al.* 1999). Mitochondria contain their own genome, mitochondrial DNA (mtDNA), which was discovered in the 1960's by Nass & Nass (1963). Mitochondrial DNA has a high copy number in most mammalian cells thus one cell contains hundreds of mitochondria, and in turn, one mitochondrion contains several copies of mtDNA (Fernandez-Silva *et al.* 2003). In humans, mtDNA is a 16.5 kilobase circular double-stranded molecule that consists of a heavy (H) and a light (L) chain without any histone coat. Human mitochondrial DNA encodes 22 transfer RNAs (tRNAs) and 12S and 16S ribosomal RNA, as well as 13 proteins. Each of these polypeptides is a highly hydrophobic subunit of one of four respiratory enzyme complexes localized to the inner mitochondrial membrane. They include seven subunits of respiratory enzyme complex I, one subunit of complex III, three subunits of complex IV, and two subunits of complex V (Garesse & Vallejo 2001). All other mitochondrial proteins, including those involved in the replication, transcription and translation of mtDNA, are encoded by nuclear genes and are imported into the

mitochondrion by a specific transport system (Neupert & Herrmann 2007). Unlike nuclear DNA, mammalian mtDNA contains no introns and it is exposed to deleterious reactive oxygen species generated by oxidative phosphorylation. Also genetic code usage in mitochondria differs to some extent from the universal genetic code and mtDNA is inherited maternally (Fernandez-Silva *et al.* 2003).

The mitochondrion is surrounded by two membranes: the outer and inner mitochondrial membranes. The intermembrane space is between the outer and inner membrane, and the region inside the inner membrane is called the matrix. The outer membrane contains transmembrane channel proteins called porins that permit the passage of ions and small molecules to the intermembrane space. In contrast, the inner membrane is impermeable to most solutes. Mitochondrial DNA and many enzymes of pyvuvate oxidation, the TCA cycle and β-oxidation of fatty acids are found in the mitochondrial matrix, while electron transport and oxidative phosphorylation occur through the inner membrane (Becker *et al.* 2006).

Recently, numerous studies have revealed that mitochondrial morphology is both complex and plastic. In many cell types, mitochondria form elongated tubules that are often interconnected (Nunnari *et al.* 1997). Mitochondria are also dynamic, frequently changing in size and shape and traveling long distances on cytoskeletal tracks (Boldogh *et al.* 2001). The connectivity of the mitochondrial network is regulated by two dynamically opposed processes, fusion and fission (Bleazard *et al.* 1999, Sesaki & Jensen 1999). A correct balance between mitochondrial fusion and fission is an important factor in many cellular processes like apoptosis, inheritance of mitochondrial DNA, defense against oxidative stress or development through spermatogenesis (Chen & Chan 2005). In the early 1990s, the first proteins required for mitochondrial distribution and morphology were identified in the yeast *S. cerevisiae* (McConnell *et al.* 1990), and tubulation (Burgess *et al.* 1994, Sogo & Yaffe 1994). Importantly, most proteins mediating yeast mitochondrial fusion and fission are conserved in flies, worms, plants, mice, and humans, indicating that the mechanisms controlling mitochondrial behavior have been maintained during evolution (Okamoto & Shaw 2005).

2.2 Fatty acids

A fatty acid is composed of a hydrocarbon chain of varying length and a terminal carboxyl group. Based on the chain-length of the hydrocarbon chain, fatty acids can be divided into short-chain (< C8), medium-chain (C8-C12), long-chain

(C14-C20) and very long-chain (> C20) fatty acids. In nature, most of the fatty acids have an even number of carbon atoms. A saturated fatty acid does not contain any double bonds in its hydrocarbon chain and fatty acids with one or more double bonds in their hydrocarbon chain are called unsaturated. Monounsaturated fatty acids contain a single double bond, while polyunsaturated fatty acids have two or more double bonds. Under physiological conditions the concentration of cellular free fatty acids is very low, and free fatty acids are not able to act as substrates for biochemical reactions without activation. Activation occurs by linking the fatty acid to a thiol moiety of coenzyme A (in fatty acid breakdown) and/or acyl carrier protein (in fatty acid synthesis). Another typical fate of free fatty acid is to be esterified to glycerol as oxyester.

Fatty acids have two major roles in the body: they act as energy storage molecules and as building blocks of biological membranes. Fatty acids are excellent for energy storage because their oxidation to carbon dioxide and water releases over six times more energy than oxidation of an equal mass of glycogen (Alberts *et al.* 1994). Phospholipids and glycolipids, which contain fatty acids as constituents, form the basic bilayer of biological membranes. Eicosanoid hormones like prostaglandins, thromboxanes and leukotrienes are derived from fatty acids. Triacylglycerols, which are made from fatty acids and glycerol, provide physical protection for the organs of the body and also function as a heat insulator in the form of brown adipose tissue. Specialized lipids derived from fatty acids serve also as pigments (retinal, carotene), cofactors (vitamin K), detergents (bile salts), transporters (dolichols), hormones (vitamin D derivatives, sex hormones), extracellular and intracellular messengers (eicosanoids and derivatives of phosphaditylinositol) and anchors of membrane proteins (covalently attached fatty acids, prenyl groups and phosphaditylinositol) (Nelson 2000). Fatty acids are synthesized when excess nutrients are stored as triacylglycerols, when they are required to maintain phospholipid components of membranes or for synthesis of specialized molecules (Dow *et al.* 1996).

2.3 Synthesis of fatty acids

In eukaryotic cells fatty acids are mainly synthesized in the cytosol by type I fatty acid synthase, where one or two multifunctional polypeptide(s) harbor the enzymatic activities required for all the reaction steps (Smith 1994). In contrast, bacterial cells use the type II fatty acid synthesis pathway, in which individual reactions are carried out by separate polypeptides (Rock & Jackowski 2002). In

the late 1980's, the first component of a mitochondrial FAS pathway, acyl carrier protein (ACP), was discovered in mitochondria of *Neurospora crassa* (Brody & Mikolajczyk 1988). To date, the mitochondrial fatty acid synthesis pathway has been characterized in yeast, plants and mammals. This mitochondrial fatty acid production system resembles bacterial type II FAS. For a long time it was thought that human parasitic pathogens like *T. brucei* were not capable of *de novo* fatty acid synthesis (Dixon *et al.* 1971), but recently it has been shown that fatty acids are synthesized in *T. brucei* by microsomal elongases using butyryl-CoA for priming (Lee *et al.* 2006). Normally those elongases are used in elongation of type I FAS products to very long chain fatty acids in the endoplasmic reticulum. The chemistry of this elongation pathway is similar to that of the type I and II FAS systems, except that the growing acyl chain is esterified to CoA instead of ACP.

2.3.1 Type I FAS

Cytoplasmic type I fatty acid synthase is found in eukaryotic cells and catalyzes the formation of saturated fatty acids like myristate, stearate and as the main product palmitate (C-16). In this pathway, acetyl-CoA is used as a primer, malonyl-CoA as a two-carbon donor and NADPH as a reductant for intermediates. During one reaction cycle, the acyl group is elongated by two carbon units, and hence the synthesis of palmitate requires altogether seven reaction cycles. During the process, the acyl carrier protein (ACP), a domain in the multifunctional enzyme, serves as a carrier of acyl intermediates. The malonyl-CoA needed for fatty acid production is generated by carboxylation of acetyl-CoA by acetyl-CoA carboxylase (ACC). Acetyl- and malonyl-CoA are transferred to ACP by acetyl- and malonyl-CoA transacylases to form acetyl- and malonyl-ACP. The cycles of fatty acid synthesis are initiated by the condensation of acetyl-CoA with malonyl-ACP by β-ketoacyl synthase. In the next step β-ketoacyl-ACP is reduced to β-hydroxyacyl-ACP by ketoacyl reductase in a NADPH-dependent reaction. The β-hydroxy intermediate is subsequently dehydrated by 3-hydroxyacyl dehydratase. In the last step, enoyl reductase reduces the double bond of *trans*-2-enoyl-ACP to form acyl-ACP in a NADPH-dependent manner. The cycles during which two carbon atoms are added to the nascent acyl chain continue until the fatty acid reaches the required length. The completed fatty acid is cleaved from ACP by thioesterase. The enzymatic steps

and reaction intermediates in the first cycle of fatty acid synthesis are represented in figure 1.

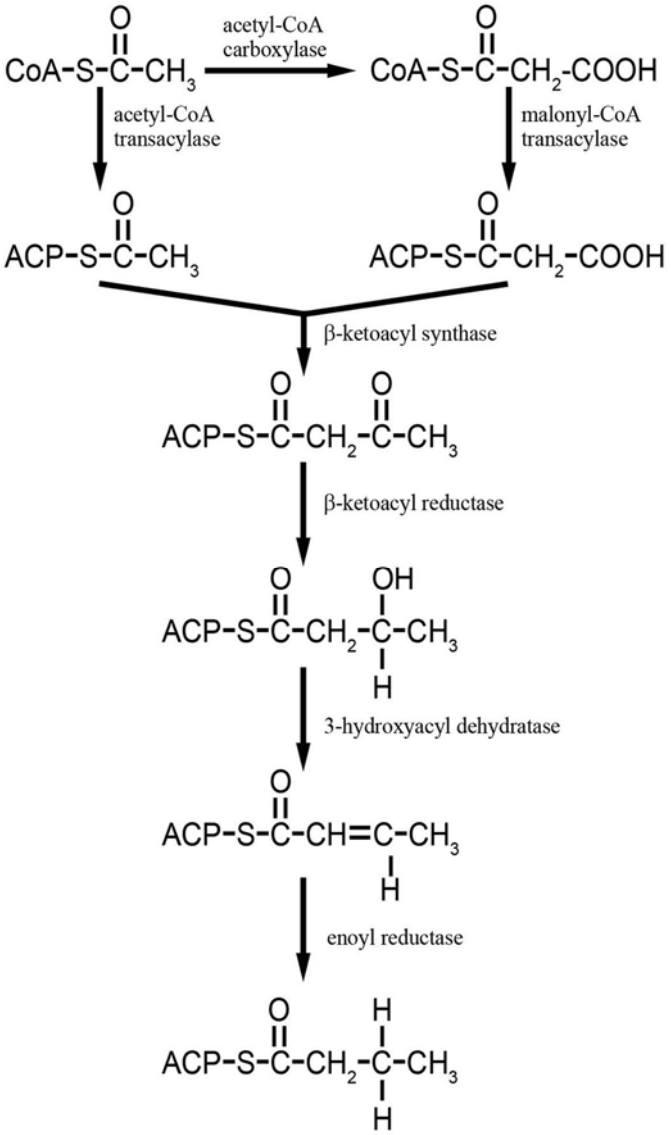

Fig. 1. The reaction intermediates and enzymes involving in the first cycle of fatty acid synthesis.

At the beginning of the fatty acid synthesis pathway, acetyl-CoA carboxylase (ACC) converts acetyl-CoA to malonyl-CoA. In fungi and mammals, the ACC enzyme is one multifunctional polypeptide with distinct domains. The biotin carboxylase (BC) domain is at the N-terminus of the polypeptide, followed by the biotin carboxyl carrier protein (BCCP) domain immediately downstream, and the carboxyltransferase (CT) domain at the C-terminus of polypeptide (Tanabe *et al.* 1975). Biotin is covalently attached to a lysine residue within the BCCP domain and mediates the capture and subsequent transfer of a carboxyl group. The ACC reaction occurs in two separate steps. First, the BC domain catalyzes the ATP-dependent carboxylation of biotin to form carboxybiotin. Then the CT domain mediates the transfer of the carboxyl group from biotin to acetyl-CoA to form malonyl-CoA. The net reaction is the production of malonyl-CoA from acetyl-CoA with ATP and bicarbonate as cofactors (Brownsey *et al.* 2006). The acetyl-CoA carboxylase gene *ACC1* is highly expressed in liver, mammary gland and adipose tissue (Bianchi *et al.* 1990) and the expression of *ACC1* can be inhibited by starvation or insulin deficiency (Iritani 1992). The enzyme itself is controlled either in an allosteric manner by activating or inhibitory ligands, or by covalent modification via phosphorylation (Brownsey *et al.* 2006).

In the mammalian cytoplasm all the other enzyme activities needed for fatty acid production are found in one polypeptide. This type I fatty acid synthase (FAS I) is encoded by one gene that produces a multifunctional protein with a size of ~272 kDa. The mammalian FAS I is a homodimer; the monomeric protein is not active. The organization of the catalytic activities along the polypeptide chain from the N-terminus to the C-terminus is as follows: β-ketoacyl synthase, acetyl- and malonyl-CoA transacylases, 3-hydroxyacyl dehydratase, enoyl reductase, ketoacyl reductase, ACP and thioesterase. Between the dehydratase and enoyl-reductase domains there is a core domain, which is essential for the function although it has no known catalytic activity (Chirala *et al.* 2001). This domain organization was established based on proteolytic mapping of the subunits and confirmed by the predicted amino acid sequences based on the cloning and nucleotide sequences of the cDNA of the FAS component enzymes in rat (Amy *et al.* 1989, Schweizer *et al.* 1989) and chicken (Chirala *et al.* 1989). Later the multifunctional human fatty acid synthase and its subdomains were cloned and expressed in *E. coli*. The recombinant proteins were catalytically active and thus the order of catalytic domains in human FAS I was established (Jayakumar *et al.* 1995, Jayakumar *et al.* 1996).

For a long time, the model for the organization of FAS I was based on the assumption that the two fully extended polypeptides of FAS I were oriented in a head-to-tail manner. More recent studies concerning the formation of the FAS I complex have led to a complete revision of the model (Witkowski *et al.* 2004, Asturias *et al.* 2005) and this was confirmed by the crystallization of full-length porcine FAS I at 4.5 Å resolution (Maier *et al.* 2006). The ACP and thioesterase domains could not be placed on this structure, presumably because of their inherent flexibility. The human thioesterase (Chakravarty *et al.* 2004) and rat ACP (Reed *et al.* 2003) domains have also been crystallized separately, and both structures support the idea of considerable intradomain flexibility in the type I FAS structure.

Type I FAS is highly expressed in liver, adipose tissue and lactating mammary gland, but nearly every tissue in the human body has at least basal levels of FAS expression (Jayakumar *et al.* 1995). FAS I plays an important role in energy homeostasis and membrane biogenesis, and is present at abnormally elevated levels in various cancer types like breast (Alo' *et al.* 1996), prostate (Shurbaji *et al.* 1996), colon (Rashid *et al.* 1997), ovary (Gansler *et al.* 1997), thyroid (Sekiguchi *et al.* 2001) and skin (Innocenzi *et al.* 2003) cancer. Increased FAS levels in cancer tissues generally indicate a poor prognosis for cancer treatment. Fatty acid synthesis is also essential in embryonic development, because FAS I null mutants and most of the heterozygote mice died *in utero* (Chirala *et al.* 2003).

In yeast the *FAS1* and *FAS2* genes encode proteins that form a multifunctional complex for fatty acid synthesis. The *FAS1* gene encodes subunit β, a 230 kDa polypeptide with acetyltransferase, enoyl reductase, dehydratase and malonyl/palmitoyl transferase activities. Accordingly, the *FAS2* gene encodes subunit α, a 210 kDa protein harboring ACP, ketoacyl reductase and ketoacyl synthase activities. In addition, the C-terminus of the α-chain contains also an apoFAS activating phosphopantetheine transferase (PPT) activity. Yeast FAS is a large $\alpha_6\beta_6$ heterododecameric complex with a molecular weight of 2.6 MD. The reaction mechanism of yeast FAS is identical to animal FAS I, with the exception that in yeast the palmitoyl end product is transferred from ACP to CoA by malonyl/palmitoyl transferase, whereas in mammals the fatty acid is released from ACP by thioesterase.

The crystal structure of the FAS I of the filamentous fungus *Thermomyces lanuginosus* has been solved first at 5 Å resolution (Jenni *et al.* 2006) and then improved to 3.1 Å resolution (Jenni *et al.* 2007). This huge assembly contains two

separated reaction chambers, each equipped with three sets of active sites separated by distances of up to ~130 Å, across which ACP shuttles substrates during the reaction cycle. Very recently, the crystal structure of *S. cerevisiae* type I fatty acid synthase was determined at 3.1 Å and 4 Å resolution simultaneously by two different groups (Leibundgut *et al.* 2007, Lomakin *et al.* 2007). The barrel-shape FAS particle is separated into two halves by a wheel-like structure at the equator, made up of six α subunits. Two trimers of the β subunit lie on both sides of the wheel. These trimers are not connected to each other and are linked only via interactions with the central wheel. Six reaction chambers are formed in the structure, and the individual active sites for the synthesis reaction are embedded in the chamber walls facing inward. ACP is located inside the hollow particle and presumably moves from one active site to the next one delivering the substrate. The PPT domain lies outside the particle and does not have access to ACP for activation. Potentially, PPT first activates the ACP and then the final structure of FAS is assembled for fatty acid synthesis. (Lomakin *et al.* 2007)

2.3.2 Type II FAS

Escherichia coli has been the widely used model organism to study type II fatty acid synthesis (FAS II), but currently studies are expanding to other microorganisms, especially human pathogens. All the genes and their products contributing to type II FAS in *E. coli* are known and also the high resolution X-ray and/or NMR structures of those proteins are available. Type II FAS can produce saturated fatty acids with different chain lengths and, in addition, unsaturated, branched-chain and hydroxy fatty acids for a variety of needs in cellular metabolism. The genes and their products involved in type II FAS are listed in Table 1.

Table 1. Genes and enzymes of type II fatty acid synthesis in bacteria.

Gene	protein	Enzyme activity / function	organism
acpP	ACP	Acyl carrier protein	*E.coli*
acpS	AcpS	ACP synthase	*E.coli*
accABCD	AccABCD	Acetyl-CoA carboxylase	*E.coli*
accA	AccA	Acetyl-CoA carboxylase, carboxyltransferase α-subunit	*E.coli*
accB	AccB	Acetyl-CoA carboxylase, carboxybiotin carrier protein	*E.coli*
accC	AccC	Acetyl-CoA carboxylase, biotin carboxylase	*E.coli*
accD	AccD	Acetyl-CoA carboxylase, carboxyltransferase β-subunit	*E.coli*
fabD	FabD	Malonyl-CoA:ACP transacylase	*E.coli*
fabH	FabH	β-Ketoacyl-ACP synthase III	*E.coli*
fabB	FabG	β-Ketoacyl-ACP synthase I	*E.coli*
fabF	FabF	β-Ketoacyl-ACP synthase II	*E.coli*
fabG	FabG	β-Ketoacyl-ACP reductase	*E.coli*
fabA	FabA	3-Hydxoxyacyl-ACP dehydratase / isomerase	*E.coli*
fabZ	FabZ	3-Hydxoxyacyl-ACP dehydratase	*E.coli*
fabI	FabI	*trans*-2-Enoyl-ACP reductase I	*E.coli*
fabK	FabK	*trans*-2-Enoyl-ACP reductase II	*S.pneumoniae*
fabL	FabL	*trans*-2-Enoyl-ACP reductase III	*B.subtilis*

The central player in the FAS II pathway is the acyl carrier protein (ACP), which is one of most abundant proteins in *E. coli*, constituting 0.25% of the total soluble protein. ACP is a small, acidic polypeptide that carries all of the fatty acid synthesis intermediates as thioesters attached to the terminus of its 4'phosphopantetheine prosthetic group. The prosthetic group sulfhydryl is the only thiol group of ACP, and it is attached to the protein via a phosphodiester linkage to Ser36 (in *E. coli*) of the AspSerLeu motif (Rock & Cronan 1979). This AspSerLeu motif is conserved in all ACP sequences. Apo-ACP is encoded by the *acpP* gene, and apo-ACP is activated to holo-ACP through addition of the 4'-phosphopantetheine prosthetic group from CoA by [ACP]Synthase AcpS.

The first step in type II fatty acid biosynthesis is the formation of malonyl-CoA from acetyl-CoA catalyzed by acetyl-CoA carboxylase (Acc). In bacteria, Acc is a heterotetrameric protein complex, in which the AccBC and AccAD complexes interact, in contrast to FAS I where ACC is one multifunctional polypeptide. The type II FAS Acc protein complex is encoded by four genes, *accA, accB, accC* and *accD* (Cronan & Waldrop 2002). In bacterial cells, the only

known metabolic use of malonyl-CoA is fatty acid production, and thus Acc is a key regulatory enzyme during this process. Indeed, the expression of the *accA*, *accB*, *accC* and *accD* genes is correlated with the growth rate of the cell (Li & Cronan 1993), where increased levels of gene expression lead to elevated rates of fatty acid production (Davis *et al.* 2000) and conversely Acc is inhibited by acyl-ACPs (Davis & Cronan 2001).

The malonyl-CoA:ACP transacylase (FabD) transfers the malonyl group from CoA to ACP. This step is essential not only to deliver malonyl-ACP for initiation, but also to supply each round of fatty acid elongation. Only a single isoform of FabD is known in *E. coli*, and temperature-sensitive *fabD* mutants revealed that this enzyme is essential for the operation of FASII (Harder *et al.* 1974).

β-Ketoacyl-ACP synthase III (FabH) catalyzes the condensation of acetyl-CoA with malonyl-ACP to initiate cycles of fatty acid elongation and it is also an essential enzyme (Revill *et al.* 2001, Lai & Cronan 2003). FabH utilizes CoA thioesters as primers rather than acyl-ACPs and its overexpression results in overall shortening of fatty acid chain-lengths (Jackowski & Rock 1987). β-Ketoacyl-ACP synthase I (FabB) and β-ketoacyl-ACP synthase II (FabF) are responsible for the condensation reaction in chain-elongation steps of fatty acid synthesis. They use acyl-ACP as primer with malonyl-ACP, and their catalytic efficiency is higher compared to FabH.

The overall tripartite reaction mechanism of condensing enzymes is the same: (i) transfer of a phosphopantetheine-bound primer to the cysteine residue of the condensing enzyme; (ii) decarboxylation of an acyl carrier bound donor unit to produce a carbanion; and (iii) condensation of the carbanion with the carbonyl carbon of the enzyme-bound primer. The most important difference between the FabH and FabB/F condensing enzymes is in the active site: the FabH proteins have a Cys-Asn-His catalytic triad whereas the FabB/F class has a Cys-His-His configuration in the active site. All the condensing enzymes belong to the thiolase superfamily of proteins. Two antibiotic compounds cerulenin and thiolactomycin inhibit the condensing reaction by binding to the active site cysteine.

As initiator of the pathway, FabH determines the number of fatty acids produced, and it is subject to stringent feedback regulation of long-chain acyl-ACPs. When the degree of inhibition of FabH increases, the chain-length of the fatty acids produced will increase (Heath & Rock 1996a). FabH substrate specificity is also important in determining fatty acid structure. In *E. coli*, which synthesizes straight-chain saturated and unsaturated fatty acids, FabH uses only

acetyl- and propionyl-CoA as substrates (Heath & Rock 1996a). In contrast, in bacteria that produce branched-chain fatty acids, the FabH component most efficiently uses branched-chain acyl-CoA derived from amino acid catabolism (Choi *et al.* 2000, Han *et al.* 1998). Whether FabB and FabF have differences in their substrate specificities is not yet known, although the crystal structures of these proteins are solved.

In the next stage of fatty acid formation, the β-ketoacyl-ACP is reduced to β-hydroxyacyl-ACP by β-ketoacyl-ACP reductase (FabG) in an NADPH-dependent manner. FabG belongs to the short-chain reductase/dehydrogenase superfamily and it has a Ser-Lys-Tyr catalytic triad. There is only a single isozyme of FabG known in nature, and thus it is an essential protein (Lai & Cronan 2004).

(3R)-Hydroxyacyl-ACP dehydratase catalyzes the removal of water from β-hydroxyacyl-ACP to form *trans*-2 enoyl-ACP. There are two known isoforms of this enzyme in *E. coli*, FabA and FabZ. FabZ solely catalyzes the dehydration of β-hydroxyacyl-ACPs. In contrast, FabA can also isomerize the double bond in the 10-carbon intermediate from the *trans*-2 position to the *cis*-3 position in addition to carrying out the dehydration reaction. Concerning the dehydratase reaction, both FabA and FabZ have been shown to have broad, overlapping chain length specificity. The FabZ dehydratase efficiently catalyzes the dehydration of short chain β-hydroxyacyl-ACPs and long chain saturated and unsaturated β-hydroxyacyl-ACPs. FabA is most active on intermediate chain length β-hydroxyacyl-ACPs and also possesses significant activity toward both short and long chain saturated β-hydroxyacyl-ACPs. Significantly, FabA is virtually inactive in the dehydration of long chain unsaturated 3-hydroxyacyl-ACP (Heath & Rock 1996c). Both dehydratases possess a “hot dog” fold in their structures, which is typical of the superfamily of thioesterases and dehydratases (Dillon & Bateman 2004). Although their structural fold is the same, the active site residues differ: an Asp in FabA and a Glu in FabZ. Another critical residue is a His in both isoforms.

In the production of unsaturated fatty acids, the isomerization reaction catalyzed by FabA is an essential step in *E. coli*. Further on in this process FabB is used as a condensing enzyme in the elongation of *cis*-3-decenoyl-ACP. Thus FabA and FabB are always found together in gram-negative bacteria, in *Pseudomonas aeruginosa fabA* and *fabB* are even co-transcribed in a *fabA-fabB* operon (Hoang & Schweizer 1997). *fabA* and *fabB* homologues are not present in gram positive bacteria, but these bacteria can still produce unsaturated fatty acids by using FabM, a *trans*-2-*cis*-3-enoyl-ACP isomerase (Marrakchi *et al.* 2002). As

FabB is absent in the organisms, FabF carries out the condensing reaction of both saturated and unsaturated fatty acids. The second dehydratase FabZ is ubiquitously expressed in all FAS II systems, where it catalyzes only the dehydratase, not the isomerase reaction.

The final step in the elongation cycle is the NADH-dependent reduction of enoyl-ACP carried out by the enoyl reductase FabI in *E. coli*. Because the equilibrium for the dehydratase reaction lies on the side of the 3-hydroxy intermediate, cycles of elongation are pulled to completion by FabI (Heath & Rock 1995). FabI is a single, essential enzyme and is regulated by long-chain acyl-ACP product inhibition (Heath & Rock 1996b). Isoniazid, which is used in tuberculosis treatment, and triclosan, a commonly used anti-bacterial agent for example in toothpastes and deodorants, inhibit enoyl reductase activity (Banerjee *et al.* 1994, Levy *et al.* 1999, Heath *et al.* 1998). The bacterial enoyl reductase FabI, like the β-ketoacyl-ACP reductase FabG, belongs to the short-chain reductase/dehydrogenase superfamily.

Although enoyl reductases are highly conserved within the type II FAS pathway, a gene that could encode a protein similar to *E. coli* FabI is not found in the genome of *Streptococci* or *Clostridia*. Instead of FabI, *Streptococcus pneumoniae* uses a completely unrelated flavoprotein as a *trans*-2-enoyl-ACP reductase II (FabK) (Heath & Rock 2000). FabK is an NADH-dependent, FMN containing protein that has no similarity to FabI in the primary sequence. Enoyl-ACP reductase III (FabL) was identified in *Bacillus subtilis* and is related to FabI, but is nevertheless classified as a separate group (Heath *et al.* 2000). *B. subtilis* also possesses FabI, and it is not known why the FabL protein is necessary.

2.3.3 Mitochondrial FAS

Yeast mitochondrial FAS

Currently the mitochondrial FAS pathway is best characterized in yeast *S. cerevisiae* (for review see Hiltunen *et al.* 2005). Mitochondrial fatty acid synthesis, the enzymes involved and the intermediate products are illustrated in figure 2.

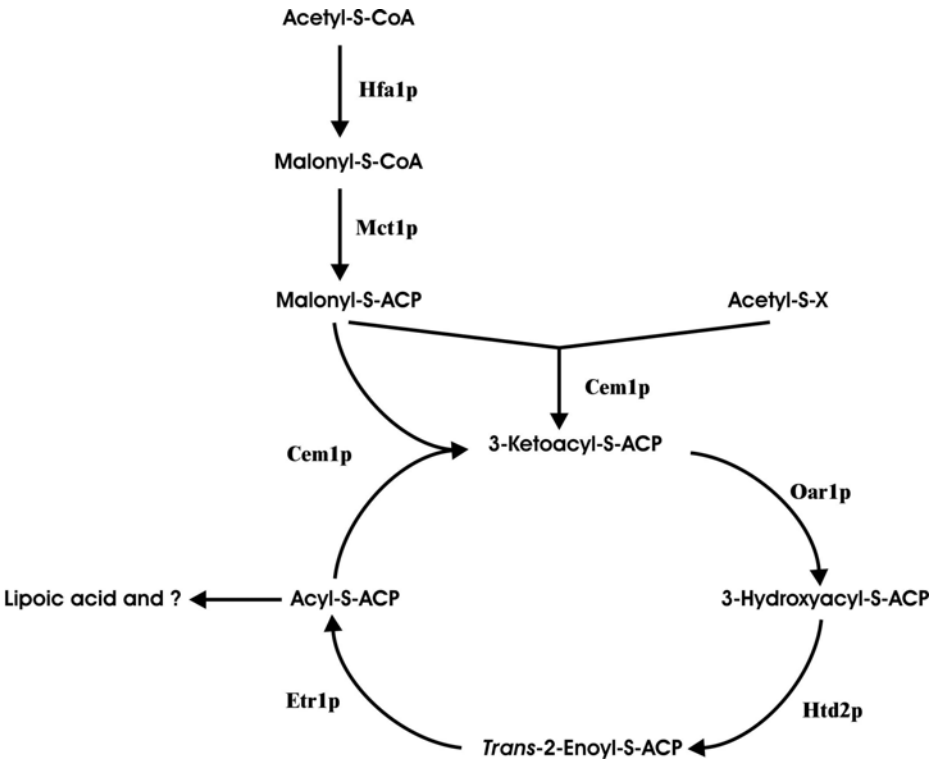

Fig. 2. Reactions of mitochondrial fatty acid synthesis in *S. cerevisiae* (from Hiltunen *et al.* 2005).

The first enzyme of mitochondrial FAS to be characterized was ACP in *N. crassa* (Brody & Mikolajczyk 1988), bovine heart (Runswick *et al.* 1991) and later in the yeast *S. cerevisiae* (Schneider *et al.* 1995). Originally ACP was identified as a subunit of the respiratory NADH:ubiquinone oxidireductase (complex I) in *N. crassa* and bovine heart (Runswick *et al.* 1991), but *S. cerevisiae* lacks complex I and thus ACP lacks this association. In *S. cerevisiae*, the *Δacp1* mutant showed a respiratory deficient phenotype with complex III and IV being absent (Schneider *et al.* 1995). The *ACP1* gene encodes Acp1p in its apo-form, and Acp1p is converted to the holo-form by adding the 4'-phosphopantetheine in a reaction catalyzed by the mitochondrial phosphopantetheine:protein transferase (Ppt2). *ppt2* null mutants were also

respiratory deficient and their cellular lipoic acid content was almost undetectable (Stuible *et al.* 1998).

The genes encoding β-ketoacyl-ACP synthase (Cem1p) (Harington *et al.* 1993), malonyl-CoA:ACP transferase (Mct1p) and β-ketoacyl-ACP reductase (Oar1p) (Schneider *et al.* 1997a) were identified in *S. cerevisiae* based on their amino acid sequence similarity to bacterial counterparts. Both the 3-hydroxyacyl-ACP dehydratase and the enoyl reductase remained unidentified, because they lack similarity to the corresponding bacterial FAS members. As noticed for the first time with the *Δacp1* strain, deletion of any genes in mitochondrial FAS leads to a respiratory-deficient phenotype. Loss of cytochromes has also been demonstrated in many other mitochondrial FAS deletion strains (Harington *et al.* 1993, Harington *et al.* 1994, Schneider *et al.* 1997a, Yamazoe *et al.* 1994). Yeast mitochondrial acetyl-CoA carboxylase (Hfa1p) was also characterized according to its sequence similarity to the cytoplasmic yeast acetyl-CoA carboxylase (Acc1p). Deletion of the *HFA1* gene causes a respiratory deficient phenotype with reduced (< 10%) lipoic acid content (Hoja *et al.* 2004) similar to the other members of mitochondrial FAS.

The initial identification of enoyl-ACP reductase (Etr1p) from *Candida tropicalis* and *S. cerevisiae* was achieved by purifying the protein with this activity from a mitochondrial extract (Torkko *et al.* 2001). Deletion or overexpression of *ETR1* affects mitochondrial morphology in yeast cells. Etr1p overexpression caused enlarged mitochondria and conversely, deletion of *ETR1* led to small, rudimentary organelles. During the characterization of *ETR1* it was demonstrated that a deletion of the *ETR1* gene could be complemented by mitochondrially targeted bacterial reductase FabI. Thus far the only known crystal structure of a yeast mitochondrial FAS member is from *Candida tropicalis* mitochondrial Etr1p. The structure shows similarity to that of the medium chain dehydrogenases / reductases (MDR) superfamily, but not to other published structures of 2-enoyl thioester reductases. The tyrosine residue in the NADPH-binding cleft of Etr1p was shown to be critical for enzyme activity (Airenne *et al.* 2003).

The idea for an alternative way to identify yeast 3-hydroxyacyl-ACP dehydratase was inspired by the observation that *Δetr1* can be complemented by the mitochondrially targeted bacterial reductase FabI. This finding prompted us to generate a yeast mutant strain that is unable to lose a plasmid with mitochondrially targeted bacterial FabA dehydratase on a non-fermentable carbon source. Further characterization revealed that the mutation was in the *YHR067w*

gene, encoding the 3-hydroxyacyl-thioester dehydratase 2 (Htd2p) described in detailed in the original article I.

The respiratory deficient phenotype *CEM1* deletion strain can be rescued by the suppressor *FAM1-1* (Harington *et al.* 1994). The Fam1-1 protein is an acyl-CoA synthetase with a mitochondrial targeting signal that was introduced by a mutation that generates an additional ATG initiation codon upstream of the *FAM1/FAA2* gene translation initiation site. The authors suggested that Fam1-1p might produce a CoA-activated fatty acid or fatty acid-like molecule in mitochondria that could be utilized in yeast strains with disrupted mitochondrial FAS or in a pathway downstream of it. In the original article I it is shown that *FAM1-1* can suppress all the individual deletions of mitochondrial FAS genes.

Mammalian mitochondrial FAS

Characterization of mitochondrial FAS members in yeast and the availability of the human genome sequence have facilitated the characterization of human mitochondrial FAS genes and proteins. Thus far, malonyltransferase, acyl carrier protein (Zhang *et al.* 2003a), enoyl reductase (Miinalainen *et al.* 2003) and β-ketoacyl-ACP synthase (Zhang *et al.* 2005) in humans are known. ACP was originally identified as a subunit of complex I according to its homology to bovine mitochondrial ACP (Triepels *et al.* 1999) and later on its linkage to mitochondrial FAS was recognized (Zhang *et al.* 2003a). Recently it was shown that in bovine heart, the bulk of ACP is soluble and only a small fraction is in the inner membrane complex I form. This finding supports the idea that mitochondrial FAS is also present in mammals (Cronan *et al.* 2005). To date only one human 4'-phosphopantetheinyl transferase is known and it also uses mitochondrial ACP as substrate *in vitro*. However, this enzyme has no similarity to yeast Ppt2p and is localized in the cytosol; hence it is not clear if there is another phosphopantetheine transferase for mitochondrial FAS or if the mitochondrial ACP is converted to the holo-form in the cytosol and imported subsequently into the mitochondria (Joshi *et al.* 2003).

The mitochondrial localization of malonyltransferase, acyl carrier protein, β-ketoacyl-ACP synthase and enoyl reductase has been shown as fusion proteins to a fluorescent protein in HeLa cells. These enzymes were also expressed and purified as recombinant proteins and their enzymatic activities were shown. The expression level of human *CEM1* and *ETR1* is highest in heart and skeletal muscle and these enzymes can also rescue the growth of the corresponding yeast

deletion strain on a non-fermentable carbon source. The crystal structure of CEM1 was solved, revealing that the enzyme is a dimer with a typical α-β-α-β-α-thiolase fold (Christensen *et al.* 2007). Kinetic assays of human β-ketoacyl-ACP synthase have revealed bimodal (C6 and C10-12) substrate preferences (Zhang *et al.* 2005) and the structure of this enzyme shows two different potential acyl-binding-pocket extensions, explaining how this bimodal distribution is achieved (Christensen *et al.* 2007).

Mitochondrial FAS in other organisms

In plants, mitochondrial FAS was first characterized in pea leaves where ACP is involved in fatty acid synthesis, and most of the fatty acids synthesized serve as lipoic acid precursors (Wada *et al.* 1997). The mitochondria of *Arabidopsis thaliana* have three predicted ACPs, two of which dominate the ACP composition and are present in the mitochondrial matrix in a soluble form rather than bound to the membrane with complex I (Meyer *et al.* 2007). The β-ketoacyl-ACP synthase of *A. thaliana* mitochondria was also characterized (Yasuno *et al.* 2004) and studies with the *Arabidopsis* mutant *mtkas-1* showed that mitochondrial β-ketoacyl-ACP synthase is important, but not obligatory, for lipoylation of mitochondrial proteins (Ewald *et al.* 2007). End product analyses in plants showed the bimodal distribution to be C8 and C14-16 with *A. thaliana* β-ketoacyl-ACP synthase, which is similar to that of human β-ketoacyl-ACP synthase (Yasuno *et al.* 2004). This can be explained by the shape of acyl binding pocket seen in the crystal structure of the plant enzyme (Olsen *et al.* 2004).

The parasite *T. brucei* uses a unique microsomal elongation pathway to synthesize the majority of its fatty acids. In addition, *T. brucei* has a type II fatty acid synthesis system in mitochondria. The candidate proteins for β-ketoacyl synthase, three β-ketoacyl reductases, two enoyl reductases and ACP encoded by the *T. brucei* genome were shown to be mitochondrial (Stephens *et al.* 2007). Also the gene encoding mitochondrial malonyl-CoA:ACP transacylase was found (van Weelden *et al.* 2005). RNAi or genomic deletion of ACP or β-ketoacyl synthase indicates that mitochondrial FAS is essential for the parasite, and a lack of ACP causes reduced lipoic acid levels (Stephens *et al.* 2007). The characterization of the mitochondrial 3-hydroxyacyl-ACP dehydratase of *T. brucei* is described in original article III.

Function of mitochondrial FAS

The role of mitochondrial FAS has been presumed to be the production of octanoyl-ACP for lipoic acid synthesis. Lipoic acid is a cofactor needed for proper function of several mitochondrial enzymes like the pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, the branched chain α-keto acid dehydrogenase and glycine cleavage complexes (Perham 2000). The chemical structure of lipoic acid is presented in figure 3.

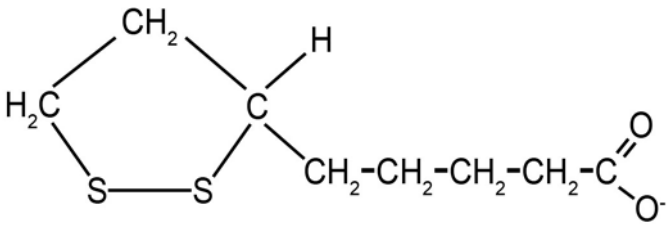

Fig. 3. Lipoic acid.

In *E. coli* two sulfur atoms are added to positions C6 and C8 of the octanoyl-precursor to form lipoic acid. This reaction is catalyzed by lipoic acid synthase LipA (Miller *et al.* 2000). Lipoyl transferase LipB transfers the lipoic acid to the acceptor protein before or after addition of sulfur atoms (Jordan & Cronan 1997). In eukaryotes, homologues of LipA and LipB were also identified (Yasuno & Wada 1998, Wada *et al.* 2001, Fujiwara *et al.* 1997, Morikawa *et al.* 2001). In yeast it has been shown that mutants with lesions in mitochondrial FAS contain less than 10% of the wild-type lipoic acid level (Brody *et al.* 1997, Stuible *et al.* 1998). Data obtained from plant mitochondria indicate that the major products of mitochondrial FAS are octanoyl- and long chain-length acyl-ACPs (Gueguen *et al.* 2000). The substrate specificities of plant and human mitochondrial β-ketoacyl synthase support a model of a bimodal distribution with maxima at C8 and C14-16 chain lengths (Zhang *et al.* 2005, Yasuno *et al.* 2004). A recent study of Smith and co-workers shows that in bovine heart mitochondria the major product of FAS is octanoyl-ACP, which is used for protein lipoylation (Witkowski *et al.* 2007). In yeast, addition of exogenous lipoic acid to the medium cannot correct the respiratory deficient phenotype, indicating that the *de novo* pathway for the production of lipoyl moieties is critical for mitochondrial function (Brody *et al.* 1997). Mice lacking the lipoic acid synthase gene died *in utero*, and supplementation of lipoate in the maternal diet could not prevent the prenatal

death (Yi & Maeda 2005). All these studies strongly support the idea that mitochondrial FAS produces octanoyl-ACP that is used in the production of lipoyl moieties needed for mitochondrial function.

Several open questions remain. It is not clear why mitochondrial FAS produces fatty acids longer that C8 and what is their physiological role. Furthermore, a role in lipoic acid production cannot explain all the features seen in mitochondrial FAS, unless a role of lipoic acid is postulated in proteins that are not yet known. The increased amount of lysophospholipids in the *N. crassa* mutant lacking mitochondrial ACP points to a role of mitochondrial FAS in providing ACP-bound fatty acids as substrates for the repair or remodel of mitochondrial phospholipids (Schneider *et al.* 1995, Schneider *et al.* 1997b). To provide acyl-moieties for synthesis of cardiolipin is one suggested function of mitochondrial FAS (Zensen *et al.* 1992). Due to the fact that deletion or overexpression of enoyl reductase or dehydratase genes affects mitochondrial morphology in yeast cells, mitochondrial fatty acid synthesis must have a role in maintenance of mitochondrial morphology. The original article II describes a genetic link between mitochondrial protein translation and mitochondrial FAS based on the finding that RPP14, the 14 kDa subunit of the RNase P complex, and mitochondrial dehydratase HsHTD2 are in the same transcript. Bicistronic transcripts in eukaryotes, by analogy to bacteria, are thought to encode proteins of related function (Blumenthal 2004). Unpublished data in our laboratory and that of our collaborator also support the linkage between mitochondrial protein translation and mitochondrial FAS, although we do not yet know the molecular link between these processes. Already 15 years ago mutations in the yeast *LIP5* gene encoding lipoic acid synthase, were shown to cause defects in 5’ processing of mitochondrially encoded tRNAs (Sulo & Martin 1993).

2.4 RNase P complex

Transfer RNAs (tRNAs) carry amino acids to the ribosome for protein synthesis, and each of the 20 common amino acids has at least one unique tRNA to serve as a carrier. In eukaryotes there are discrete sets of tRNA molecules for protein synthesis in the cytoplasm, mitochondria or chloroplasts. Functional tRNAs are generated from their precursor tRNAs by the removal of extra sequences from the 5’ and 3’ termini in a complex series of processing steps. The final maturation of the 5’ terminus is undertaken by the RNase P complex (Gopalan *et al.* 2002). RNase P catalyzes the cleavage of the leader sequence of precursor tRNAs to

generate the mature 5’ end by hydrolyzing a specific phosphodiester bond in pre-tRNA, leaving a phosphate group at the 5’ end of the mature tRNA and a hydroxyl group at the 3’ end of the leader. RNase P activity is found in Bacteria, Archaea and Eucarya. All forms of RNase P act as ribonucleoproteins consisting of an essential RNA subunit and one or more protein subunits. Several metal ions are also important for RNase P function (Kirsebom 2002).

Bacterial RNase P is composed of a large RNA subunit (350-400 nt) and one small (12-14 kDa), highly basic protein subunit. The RNA subunit of bacterial RNase P can act as a ribozyme by recognizing and cleaving substrates of precursor tRNAs in the absence of the protein subunit under high ionic strength *in vitro* (Pace & Brown 1995). However, the protein component is considered to be essential for RNase P function *in vivo* by interacting directly with the substrate and positioning it near the catalytic core of RNA (Crary *et al.* 1998). The protein subunit of bacterial RNase P may also broaden the substrate specificity and thus bacterial RNase P can recognize and cleave non-tRNA substrates like the precursor of 4.5S rRNA (Peck-Miller & Altman 1991), 10Sa RNA (Komine *et al.* 1994) and the polycistronic *his* operon mRNA (Alifano *et al.* 1994).

In eukaryotic cells, the nucleus, mitochondria and chloroplasts harbor their own forms of RNase P. In the yeast *S. cerevisiae*, the nuclear RNAse P complex consists of a RNA subunit *RPR1* and nine protein subunits: Pop1p, Pop3p, Pop4p, Pop5p, Pop6p, Pop7p, Pop8p, Rpp1p and Rpr2p. The proteins range in the size from 15.5 to 100 kDa and with the exception of Pop8p and Pop5p, they are highly basic (Houser-Scott *et al.* 2002). Very little is known about the specific functions of the individual proteins, but it has been shown that depletion of a single protein of the complex results in a drastic reduction of mature *RPR1* RNA (Chu *et al.* 1997, Stolc & Altman 1997, Stolc *et al.* 1998). The *RPR1* gene is transcribed as a 486-nucleotide primary transcript and converted to a 369 nt mature *RPR1* RNA by trimming the 5’ and 3’ termini (Lee *et al.* 1991). The available data suggest that *RPR1* maturation occurs during or after the assembly of the protein complex.

The human nuclear RNase P is composed of an H1 RNA molecule and at least 10 protein subunits: RPP14, RPP20, RPP21, RPP25, RPP29, RPP30, RPP38, RPP40, hPOP1, hPOP5 (Jarrous 2002). RPP14 is the smallest (14kDa) subunit of RNase P (Jarrous *et al.* 1999) and its nucleolar localization might be facilitated by a piggyback process because it does not contain any nuclear targeting signals in its sequence (Jarrous *et al.* 1999). RPP14 and RPP21 bind to pre-tRNAs *in vitro* and thus may be similar to the protein component of bacterial RNase P in substrate binding (Jarrous *et al.* 2001). Due to the fact that RNase P

ribonucleoproteins are not restricted to a specific nuclear compartment, the RNase P complex is dynamically assembled and recruited to discrete sites of active gene transcription and RNA processing (Jarrous & Altman 2001).

The mitochondrial RNase P is best characterized in *S. cerevisiae*, where it consists of an essential RNA subunit Rpm1r (490 nt) encoded by the mitochondrial genome (Miller & Martin 1983, Underbrink-Lyon *et al.* 1983) and the protein subunit Rpm2p (105 kDa) encoded by a nuclear gene (Dang & Martin 1993). The Rpm2p protein subunit is required for enzymatic activity, and mutant alleles of Rpm2p cause accumulation of mitochondrial pre-tRNAs with an unprocessed 5' end (Morales *et al.* 1992). Rpm2p is also needed for Rpm1r RNA processing (Stribinskis *et al.* 1996, Stribinskis *et al.* 2001). Deletion of the *RPM2* gene is lethal in yeast. Since mitochondrial protein synthesis is needed only for respiratory growth, but not for fermentative growth, this finding has led to the conclusion that Rpm2p is essential for viability, but its specific function is not yet known. (Kassenbrock *et al.* 1995).

Human mitochondrial RNase P is not yet well characterized. In earlier studies, RNase P activity in mitochondria of HeLa cells appeared to be entirely protein based without an RNA component with substrate specificity for mitochondrial RNAs (Rossmanith & Karwan 1998, Rossmanith *et al.* 1995). A few years later Puranam and Attardi (Puranam & Attardi 2001) reported that human mitochondrial RNase P contains an essential RNA component which is identical to human nuclear RNase P RNA H1. The human mitochondrial genome does not encode a mitochondrial RNase P RNA similar to that of yeast mitochondria.

The RNase MRP (mitochondrial RNA processing) complex is structurally closely related to nuclear RNase P, but is found only in eukaryotes. Originally, the RNase MRP complex was identified as an RNA-containing endoribonuclease that cleaves the mitochondrial RNA primers for DNA replication *in vitro* (Chang & Clayton 1987a, Chang & Clayton 1987b). The RNase MRP complex is found primarily in the nucleolus where it cleaves a specific site (A_3) in the internal transcribed spacer 1 region of precursor rRNA leading to generation of the mature 5.8S rRNA (Chu *et al.* 1994, Clayton 1994). A third function of RNase MRP is cleavage of *CLB2* mRNA (Gill *et al.* 2004).

In *S. cerevisiae* the RNase MRP complex consists of the RNA subunit *NME1* and 10 protein subunits. Eight of them are the same as those in nuclear RNase P, but Snm1p and Rmp1p are unique to RNase MRP (Chamberlain *et al.* 1998, Salinas *et al.* 2005, Schmitt & Clayton 1994). Both the RNA and protein subunits

of RNase MRP are essential for yeast viability, even though neither cleavage of pre-rRNA at the A3 site nor mitochondrial DNA replication are required for viability, indicating that RNase MRP may have some important yet uncharacterized function not involved in pre-rRNA modification.

The human RNase MRP complex consists of the RNA subunit 7-2 (RMRP) and some protein subunits, which are shared with nuclear RNase P, like hPOP1, RPP40, RPP38, RPP30 and possibly hPOP5. RPP20 and RPP25 appear to be associated with only a subset of RNase MRP, while hPOP4, RPP21 and RPP14 do not associate with RNase MRP (Welting *et al*. 2006). Mutations in the RMRP RNA subunit cause a recessively inherited disease called cartilage-hair hypoplasia. These patients have short limbs, hair hypoplasia, a defective immune system and hematological abnormalities (Ridanpaa *et al*. 2001). The subunit composition of nuclear RNase P and RNase MRP from *S. cerevisiae* and human are represented in Table 2.

Table 2. Subunit composition of nuclear RNase P and RNase MRP from *S. cerevisiae* and human.

Yeast gene		Subunit type	Molecular mass (kDa)	Isoelectric point (pI)	Human gene	
RNase P	RNase MRP				RNase P	RNase MRP
RPR1	-	RNA			*H1*	
-	*NME1*	RNA				*7-2*
POP1	*POP1*	protein	100.5	9.84	*hPOP1*	*hPOP1*
POP3	*POP3*	protein	22.6	9.57		
POP4	*POP4*	protein	32.9	9.26	*RPP29 / hPOP4*	
POP5	*POP5*	protein	19.6	7.79	*hPOP5*	*hPOP5*
POP6	*POP6*	protein	18.2	9.28		
POP7 / RPR2	*POP7 / RPR2*	protein	15.8	9.34	*RPP20*	*(RPP20)*
POP8	*POP8*	protein	15.5	4.57		
RPP1	*RPP1*	protein	32.2	9.76	*RPP30*	*RPP30*
RPR2		protein	16.3	9.99	*RPP21*	
	RMP1	protein	23.6	10.53		
	SNM1	protein	22.5	9.81		
		protein	38	9.6	*RPP38*	*RPP38*
		protein	40	5.2	*RPP40*	*RPP40*
		protein	25	9.7	*RPP25*	*(RPP25)*
		protein	13.8	7.62	*RPP14*	

2.5 The organisms used in this study

2.5.1 The yeast Saccharomyces cerevisiae

The yeast *S. cerevisiae* is a unicellular eukaryote, which humans have used in baking and brewing for millennia. During the last decades, *S. cerevisiae* has become a favorite organism of many molecular biologists who study the biology of eukaryotes. *S. cerevisiae* is an excellent model organism because it grows fast, is easy to maintain and manipulate, and is not a human pathogen. It was the first eukaryote transformed by a plasmid (Beggs 1978) and also the first eukaryote for which precise gene knock-outs were generated (Rothstein 1983). The *S. cerevisiae* genome was the first eukaryotic genome to be completely sequenced in 1996 (Goffeau *et al*. 1996). The low amount of introns in yeast genes made gene to protein predictions simple. Yeast cells resemble in many respects human cells; cell organelles are almost the same and many genes are conserved from yeast to humans, although the size of the yeast cell is smaller and also the genome is smaller compared to humans. Knock-out strains, where each of 5916 yeast genes are deleted, are available in complete collections, and can be obtained at low cost (Scherens & Goffeau 2004). In addition, yeast was the first organism of which the complete mitochondrial proteome was analyzed by mass spectrometry (Sickmann *et al.* 2003).

2.5.2 The human pathogen Trypanosoma brucei

T. brucei is a unicellular eukaryote that causes sleeping sickness in humans and nagana in cattle. According to World Health Organization statistics, *T. brucei* threatens 60 million people in sub-Saharan Africa and in 2002 caused 48 000 deaths. *T. brucei* lives exclusively as an extra-cellular parasite in the mammalian host blood and in the advanced stage of human sleeping sickness, it transverses the blood-brain barrier and invades the cerebrospinal fluid, causing the neurological symptoms of sleeping sickness disease (Grab *et al.* 2004).

T. brucei parasites undergo a complex life cycle through the bloodstream of their mammalian host and the blood-feeding insect vector, the tsetse fly, and adapt to multiple environments. To transfer the infection to a new host, the long, slender bloodstream form (BSF) of the parasite differentiates into a short, stumpy form that is then ingested by the tsetse fly vector during a blood meal. Within the fly, the parasite progresses through the procyclic form (PCF) and other stages while

moving from the gut to the salivary glands. Finally, to complete the life cycle, the parasite is transmitted as a metacyclic form to a new mammalian host in the saliva when the fly bites a human. Both BSF and PCF forms of *T. brucei* can be cultured in the laboratory. (Hee Lee *et al.* 2007)

The generation of ATP in the BSF form depends entirely on glycolysis in peroxisome-like organelles called glycosomes. There glucose is degraded to 3-phosphoglycerate, which is then further broken down in the cytosol to pyruvate and excreted into the bloodstream of the host. In the PCF form, on the other hand, pyruvate is not excreted, but is further metabolized to acetate mainly in the mitochondria. During the production of acetate, surplus ATP is generated. In addition to carbohydrates, amino acids, mainly proline and threonine, are used as substrates for ATP synthesis in the PCF form. (Michels *et al.* 2006) The mitochondrion of *T. brucei* differs from classical eukaryotic mitochondria, because the *T. brucei* cell contains only a single, large mitochondrion. That mitochondrion contains a special structure called a kinetoplast, which is a giant network of concatenated circular DNAs. Kinetoplast DNA consists of thousands of minicircles (0.5-10 kb) that are heterogeneous in sequence and 40-50 maxicircles (20-40 kb) encoding typical mitochondrial gene products like ribosomal RNA and respiratory chain subunits. tRNAs in *T. brucei* mitochondria are nuclear encoded and imported from the cytosol. The replication of kinetoplast DNA and the division of the mitochondrion are directly linked to cell division. (van Hellemond *et al.* 2005)

As described above, fatty acids are not a significant source of energy in *T. brucei*, and the rate of β-oxidation is minimal. It was long thought that *T. brucei* was not capable of synthesizing fatty acids *de novo*, but relied on uptake of most lipids from host blood. (van Hellemond & Tielens 2006) The discovery of the trypanosome variant surface glycoprotein (VSG) covering each BSF cell revised this view, because VSG is attached to the lipid bilayer of the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor containing exclusively two myristate molecules (Ferguson & Cross 1984). The VSG is an important protein for the parasite, because it enables the parasite to evade the host's immune response (Donelson 2003). Since VSG is an abundant protein (constituting 10% of the total protein of the cell) and the BSF reaches high densities in blood, the demand of myristate cannot be met solely by uptake from the environment, as myristate is relatively rare in the host blood circulation.

Biochemical studies soon revealed that trypanosome membrane preparations, from the BSF and the PCF, robustly synthesize fatty acids in a cell-free assay

(Morita *et al.* 2000). Trypanosomes produce the bulk of their fatty acids via an elongase based mechanism (Lee *et al.* 2006). In other eukaryotes, elongases are used to extend long chain fatty acids on the cytosolic side of the endoplasmic reticulum. The reaction steps needed for elongation are the same as in type I or II FAS, but the growing acyl chain is attached to CoA instead of ACP. There are three different elongases in *T. brucei*: ELO1 converts C4 to C10, ELO2 extends the chain length from C10 to myristate and ELO3 from C14 to C18. The source of the butyryl-CoA primer is not yet known. ELOs are clearly essential to PCF trypanosomes and potentially also to the BSF. (Hee Lee *et al.* 2007, Lee *et al.* 2006)

3 Aims of the study

The thioesterase / thiol ester dehydratase / isomerase (TED1) superfamily includes a wide variety of proteins with a hot dog fold in their structure for different functions in cells. MaoC dehydratases are one subfamily in TED1, and include, for example, dehydratases involved in fatty acid synthesis. Most of the components of the mitochondrial fatty acid synthesis pathway in *S. cerevisiae* were identified based on their similarity to the corresponding enzymes in bacteria. When this study was initiated, the 3-hydroxyacyl-ACP dehydratase component of mitochondrial FAS in *S. cerevisiae* was uncharacterized due to lack of or low similarity to bacterial dehydratases. Hence, the following aims were set for this study:

1. To identify and characterize the gene encoding mitochondrial 3-hydroxyacyl-ACP dehydratase in *S. cerevisiae.* To study the effects on respiration and mitochondrial morphology when this gene was deleted or inactivated by mutation.

Despite identification of the yeast mitochondrial dehydratase, the corresponding human enzyme remained unknown due to the lack of homology to the yeast protein. Consequently, the further studies were established with the following aims:

1. To identify the gene encoding human mitochondrial 3-hydroxyacyl-ACP dehydratase from human cDNA libraries by transformation into a yeast dehydratase mutant strain and screening for rescue of the respiratory-deficient phenotype of this strain. To characterize the enzymatic activity of this protein product and its localization in the cell. To carry out a phylogenetic analysis of the MaoC family of dehydratases. Because this protein was expressed from a bicistronic transcript, distribution of this bicistronic structure in other eukaryotes was studied.
2. To characterize the mitochondrial 3-hydroxyacyl-ACP dehydratase of a human pathogen *T. brucei*, which was identified based on its similarity to human mitochondrial dehydratase.

4 Materials and methods

Detailed descriptions of materials and method can be found in the original articles (I-III).

4.1 Plasmids, strains, culture media and growth conditions

Descriptions of the construction of the plasmids for localization studies, protein expression and purification, complementation assays and mutation analysis, as well as primer sequences, bacterial and yeast strains used and the composition of different culture media and growth conditions are given in the original articles (I-III).

4.2 Mutagenesis and sectoring screen for yeast dehydratase (I)

Wild type W1536 8B yeast cells were subjected to ethyl methanesulfonate (EMS) mutagenesis then plated on several Petri dishes containing glycerol sectoring medium (GSM) (Kastaniotis *et al.* 2004) and incubated at 30^{o}C for 7-15 days. Colonies unable to lose the plasmid construct were identified by their uniform red color. To confirm the phenotype, non-sectoring red colonies were picked and streaked on GSM media plates for single colonies. Next, the isolates were streaked onto glucose plates to test their ability to generate sectored colonies and thus to assess their dependency on the plasmid expressing mitochondrially targeted FabA only on a non-fermentable carbon source. The mutants that passed this test were genetically analyzed and subsequently transformed with the yeast multicopy HeAl library (Kastaniotis *et al.* 2004) to clone the wild type gene.

4.3 Library complementation screen for human dehydratase (II)

The mutant yeast strain *htd2-1* was transformed with human cerebellum and/or kidney cDNA libraries in the pMETtPGK3-1/2/3 vector (Zhang *et al.* 2003b) using the Liac/SS carrier DNA/PEG method (Gietz & Woods 2002). After transformation the cells were plated on SC-Met^{-}Ura^{-} plates with 3% glycerol as the sole carbon source and grown for 6-7 days at +30°C. The transformants were treated with 5-fluoro-orotic acid (5'FOA), on which only the cells without a plasmid carrying a Ura-marker, can survive. These transformants that were not able to grow on glycerol after 5'FOA-treatment were selected for further tests,

because their growth was dependent on the library plasmid. The plasmid DNA was isolated from selected cells and transformed into *E. coli* TOP10 cells (Invitrogen, Carlsbad, CA, USA) on LB-plates with ampicillin to multiply the plasmid DNA. Plasmid DNA was isolated from the *E. coli* cells with the NucleoSpin Plasmid-kit (Macherey-Nagel, Düren, Germany) and transformed back into the yeast strains *htd2-1* and *Δhtd2* to confirm their ability to complement the *htd2-1* and *Δhtd2* strain on glycerol. Isolated plasmid-DNAs were digested with *Bst*XI and *Not*I restriction enzymes and analyzed on an 0.8% agarose gel. Finally the isolated plasmids were sequenced.

4.4 Human and *T. brucei* dehydratase complementation in yeast (II, III)

Yeast strains *htd2-1* and *Δhtd2* were used to transform these plasmids pYE352-*HsHTD2*, pYE352-*HsHTD2*D62A, pYE352-*HsHTD2*H67A, pYE352-*HsHTD2*D62AH67A, pYE352-*TbHTD2*, pYE352-*HTD2* and pYE352-*CTA1* and grown on SC-Ura$^-$plates with 2% glucose. The transformants were moved to synthetic complete plates with 3% glycerol and grown at 30°C for 4 days for *T. brucei* dehydratase or 6-7 days for human dehydratase.

To test if the bicistronic *RPP14/HsHTD2* transcript without a promoter can complement the yeast strains *htd2-1* and *Δhtd2*, plasmids *HsRpp14cDNA1A*+YCplac33, the original human cerebellum cDNA library plasmid 1A, and the YCplac33 vector were transformed into these yeast strains and plated on SC-Ura$^-$ with 2% glucose. The transformants were transferred to synthetic complete plates with 2% glucose or 3% glycerol and grown at +30°C for 7 days.

4.5 Overexpression of yeast dehydratase and isolation of mitochondrial proteins for hydratase-2 activity measurements (I)

For enzymatic activity assays, the yeast strain BY4741 was transformed with plasmid pYE352-*HTD2*, and Htd2p was overexpressed in medium containing oleic acid. The mitochondria were isolated as described (Meisinger *et al.* 2000), but sucrose gradient purification was omitted. The mitochondria were suspended in breaking buffer (20 mM Tris-HCl, 1mM EDTA, 10 mM benzamidine hydrochloride (BA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.4 M NaCl,

0.01% Triton X-100, pH 7.8, containing 5% glycerol) and broken by sonication. The mixture was centrifuged at 100 000 × g for 1 hour at + 4ºC. The supernatants were collected and their protein concentrations were measured by Bradford assay. As a control, the fumarase activity was measured as described earlier (Remes *et al.* 1992).

4.6 Production of recombinant protein HsHTD2 and TbHTD2 (II, III)

HsHTD2 was produced as a recombinant protein fused to maltose binding protein and lacking the mitochondrial targeting signal (mature HsHTD2). The recombinant protein was expressed in the *E. coli* BL21(DE3)pLysS strain containing a RARE plasmid in LB medium with 50 μg/ml carbenicillin and 34 μg/ml chloramphenicol. A 10 ml portion of overnight culture of *E. coli* cells containing the pMAL-c2x-pET23a-mat*HsHTD2* plasmid was used to inoculate one liter of culture. The cells were grown at 37 ºC until an OD_{600} of 0.4 was reached and the expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 0.1 mM. The induction occurred at 18 ºC for 20 hours and the cells were harvested, washed with PBS and stored at -70 ºC until further use.

Tbhtd2p was produced as a recombinant protein fused to maltose binding protein by cloning the *TbHTD2* gene into expression vector pETMBP-1b (a kind gift from Gunter Stier) resulting in plasmid pETMBP_1b-*Tbhtd2*. For protein expression, the *E. coli* BL21(DE3)pLysS strain was transformed with the pETMBP_1b-*Tbhtd2* plasmid and the bacterial cells were grown overnight in LB-medium supplemented with 30 μg/ml kanamycin, 34 μg/ml chloramphenicol and 2% glucose at 37 ºC. A 10 ml portion of overnight culture of *E. coli* cells was used to inoculate one liter of culture in ZYM-5052 medium (Studier 2005). The cells were allowed to grow at 37 ºC under aerobic conditions until an OD_{600} of 0.4 was reached. The expression of the recombinant protein was induced by autoinduction at 20 ºC for 24 hours. The cells were harvested, washed with PBS and stored at -70 ºC until used.

4.7 Recombinant protein purification (II, III)

A bacterial cell pellet was suspended in 20 mM Tris, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM BA, 0.1 mM PMSF pH 7.4-solution and the cells were broken by sonication. The suspension was centrifuged at 9000 × g for 30 minutes

at 4 ºC and the supernatant was applied to an amylose resin column (New England Biolabs, Beverly, MA, USA) equilibrated with column buffer (20 mM Tris, 200 mM NaCl, 1 mM EDTA pH 7.4). The column was washed with column buffer and the bound protein was eluted with column buffer containing 10 mM maltose. The pooled fractions containing the recombinant protein were concentrated and applied to a size exclusion chromatography column Superdex 200 HR 10/30 (Amersham Pharmacia Biotech AB, Uppsala, Sweden) equilibrated with 30 mM HEPES, 200 mM NaCl, 1 mM NaN_3 pH 7.4. Proteins were eluted at a flow rate of 500 µl/min and the protein purification was monitored by SDS-PAGE.

The maltose binding protein was cleaved off from TbHTD2 by Tobacco etch virus NIa Proteinase (TEV-protease) (van den Berg *et al.* 2006) at 4 ºC overnight in a solution containing 1 mM DTT. Because both maltose binding protein and TEV-protease were His-tagged, the cleavage mixture was mixed with Ni-NTA (Qiagen) to separate them from TbHTD2. Finally the purified TbHTD2 was applied to a size exclusion chromatography column Superdex 200 HR 10/30 which was equilibrated with 30 mM HEPES, 200 mM NaCl, 1 mM sodium azide (NaN_3) pH 7.4.

4.8 Hydratase-2 activity assay (I, II, III)

Hydratase 2 activity was measured from the purified sample as the complex formation of ketoacyl-CoA with Mg^{2+} (Hiltunen *et al.* 1989) using *trans*-2-butenoyl-CoA, *trans*-2-hexenoyl-CoA, *trans*-2-octenoyl-CoA or *trans*-2-decenoyl-CoA as a substrate and recombinant (*3R*)-hydroxyacyl-CoA dehydrogenase (consisting of both domains A and B) from *C. tropicalis* as an auxiliary enzyme (Qin *et al.* 1999). Alternatively hydratase 2 activity was measured in the direction of hydration of *trans*-2-$C_{12,14,16}$-CoA to D-3-OH-$C_{12,14,16}$-CoA followed by NADH formation catalyzed by recombinant (*3R*)-hydroxyacyl-CoA dehydrogenase. The NADH formation was monitored spectrophotometrically in 50 mM Tris-Cl, 50 mM KCl, pH 8.0 buffer with an equal molar ratio of fatty acid free BSA. The kinetic data were calculated with GraFit5 computer software (Sigma).

4.9 Cytochrome spectra (I)

Wild-type BY4741 yeast cells, which were transformed with a pHAP1 plasmid (Pfeifer *et al.* 1987) or *Δhtd2* with pHAP1 and pTSV30A, or pTSV30mtFabA plasmid, were grown overnight on SCD medium lacking leucine and uracil. Similarly, wild-type W1536 8B and the *htd2-1* mutant were grown on YPD medium. The mutant *htd2-1* complemented with the YCplac33/ΔKpnI plasmid was grown overnight on SCD-Ura$^-$ medium. All the cell cultures were diluted to an OD_{600} of 0.3 and grown overnight. Cytochrome spectra were analyzed from yeast extracts frozen in liquid nitrogen (Lindenmayer & Estabrook 1958).

4.10 Electron microscopy (I)

For transmission electron microscopy the yeast strain W1563-8B was transformed with plasmids pYES2-*Htd2* and pYES2-*CtEtr1*. Untransformed W1536 8B and *htd2-1* mutant cells were grown on YPD medium and transformed strains were grown on SC-Ura medium overnight at 30°C. The cultures were diluted 1:50 with the same media and grown for 6 hours. The cultures were then diluted to an OD_{600} of 0.01 with YP-medium or SC-Ura containing 2% raffinose and grown to an OD_{600} = 0.5. The cells were collected by centrifugation at 1500×g for 5 minutes and resuspended in 50 ml of YP-medium or SC-Ura containing 2% galactose. After induction at +30 °C for 24 hours, 1.5 ml of the culture was harvested at 1500 × g for 5 minutes. After fixation in 2 × fixation solution, the cells were centrifuged as before and covered with 200 µl of fixation solution (0.2 M PIPES, 2.5% glutaraldehyde, pH 6.8).

For immunoelectron microscopy, untransformed W1536 8B and htd2-1 mutant cells were grown as described above for transmission electron microscopy. The cells were fixed in a 4% paraformaldehyde, 0.2 M PIPES, 2.5% sucrose pH 6.8 solution for an hour. The fixed cells were collected by centrifugation and immersed in 2% agarose in 0.2 M PIPES pH 6.8, after which the blocks were immersed in 2.3 M sucrose in phosphate buffered saline (PBS) and frozen in liquid nitrogen. For the immunolabeling, thin sections were first incubated in 0.05 M glycine in PBS followed by incubation in 5% BSA with 0.1% CWFS (cold water fish skin) gelatin (Aurion, Wageningen, The Netherlands) in PBS. The antibodies and the gold conjugate were diluted in 0.1% BSA-C (Aurion) in PBS. All washes were performed in 0.1% BSA-C in PBS. The sections were incubated with rabbit anti-porin1 antibody for an hour followed by

incubation with protein A-gold complex (size 10 nm, (Slot & Geuze 1985)) for 30 minutes. The sections were embedded in methylcellulose and examined in a Philips CM100 transmission electron microscope (FEI company, Eindhoven, The Netherlands). Images were captured with a CCD camera equipped with the TCL-EM-Menu version 3 from Tietz Video and Image Processing Systems (Gaunting, Germany).

4.11 Fluorescence microscopy of yeast cells (I)

BJ1991 cells transformed with plasmids pYE352-proA and pYE352-*Htd2*-proA were grown to mid-log phase in SC-Ura medium with 2% glucose and then in the same medium with 3% glycerol overnight to an OD_{600} of about 1. MitoTracker Red dye (Molecular Probes, Eugene, OR, USA) was added to a final concentration of 200 nM 45 minutes before harvesting. The cells were treated as described earlier (Lee *et al.* 1996), probed first with rabbit-anti-goat/HRP conjugated as primary antibody and then goat-anti-rabbit/FITC conjugated as secondary antibody (Jackson Laboratories, West Grove, PA, USA).

For the mtGFP-experiments, W1536 wild type or *htd2-1* mutant cells were transformed with plasmid pYX232-mtGFP, grown to mid-log phase and processed as described above without the antibody incubation steps. Cells were studied with an Olympus BX51 fluorescence microscope, and the pictures were captured by using analySIS (Soft Imaging System, Münster, Germany). The fluorescence microscopy pictures of cells carrying the pYX232-mtGFP plasmid were examined by three different individuals to quantitate the differences in mitochondrial phenotypes of the *htd2-1* mutant versus wild type. Four basic mitochondrial morphology phenotypes were found in both wild type and mutant.

4.12 Subcellular localization studies (II, III)

For determination of the subcellular localization of HsHTD2 or RPP14, HeLa cells (ATCC CCL-2) cultured on glass coverslips were transfected with plasmid encoding either HsHTD2 with or without a mitochondrial targeting signal or RPP14, with EGFP fused to the C-termini of all of these proteins. 28 h after transfection the cells were treated with the 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 fluorescence microscopy.

To analyze the localization of *T. brucei* mitochondrial dehydratase in the procyclic form cells, the full length sequence of the *TbHTD2* gene was expressed with a C-terminal GFP tag. The expression was induced by tetracycline for 1 day and the mitochondria were stained with Mitotracker Red CMX-ROS for 10 minutes. The cells were studied under live cell microscopy as previously described (Motyka *et al.* 2006).

4.13 Lipoic acid analysis (III)

The amount of lipoic acid in yeast cells was analyzed by a biological assay described previously (Brody *et al.* 1997, Hayden *et al.* 1993) using the lipoic acid deficient JRG33 *E. coli* strain. Yeast strains were grown in 50 ml of SCD medium and acid hydrolysis was carried out in 0.5 ml 9 N H_2SO_4. JRG33 cultures were inoculated to an initial OD_{600} of 0.015 in 2 ml of 1 × basal growth medium (Herbert & Guest 1970) containing 50 mM sodium succinate and grown for 36 - 48 hours.

4.14 Northern blots (II)

In northern blot analysis, a human Multiple Tissue Northern Blot (BD Biosciences Clontech, Palo Alto, CA, USA) was hybridized with a ^{32}P-labeled RPP14 or HsHTD2 cDNA. The hybridization was carried out in ExpressHyb (BD Biosciences Clontech, Palo Alto, CA, USA) solution according to the manufacturer's instructions and the blot was analyzed by using a phosphorimager (Molecular Imager FX, Bio-Rad Laboratories, CA, USA).

4.15 Bioinformatics (II)

The BLAST and PSI-BLAST tools (Altschul *et al.* 1997) were used in DNA homology searches and the discovery of identities to the coding region of human mitochondrial dehydratase in the Genbank and TrEMBL/SwissProt databases. Additional vertebrate homologues were retrieved by BLASTX searches of the Genbank redundant and EST databases, followed by identification of open reading frames using the Translate tool at ExPASy. The authenticity of the identified mitochondrial dehydratase candidates was further established by

reciprocal homology searches using every retrieved dehydratase sequence as the query. A multiple alignment was then constructed based on the Pfam MaoC_dehydratase family (Accession number Pf01575). A representative set of sequences chosen from the complete family alignment was biased towards sequences with characterized functions or structures. The sequences of mitochondrial dehydratases homologous to the human sequences as well as the previously identified fungal mitochondrial dehydratases (Kastaniotis *et al.* 2004) were aligned to the MaoC dehydratase using the ClustalX 1.8 program (Thompson *et al.* 1997) with default settings. Secondary structure information from the Protein Structure Database was annotated and the resulting alignment was manually inspected. The final alignment included 60 sequences and 107 positions around the conserved fingerprint of the MaoC dehydratase family. A subset of 31 sequences and 105 positions was used to construct phylogenetic trees. Distance, parsimony and maximum likelihood analysis including 100 bootstrap replicates, respectively, was performed using the programs PROTDIST, NEIGHBOR, FITSCH, PROTPARS, PROML SEQBOOT and CONSENSE of the PHYLIP package (Felsenstein 2006). Information on transcripts, genomic sequences and exon-intron organization of chordate mitochondrial dehydratases was retrieved from the Ensembl database at the EBI and the Genbank and UniGene databases at NCBI. In cases where a *RPP14/HTD2* transcript could not be identified unambiguously by keyword searches, sequences were identified by BLAST searches. Alternative transcripts were sought by mapping of transcripts and genomic sequences to the NCBI EST database, as well as the construction of contigs from retrieved ESTs.

5 Results

5.1 Identification of genes encoding 3-hydroxyacyl-thioester dehydratases of mitochondrial FAS II

5.1.1 A colony-color-sectoring screen for yeast mitochondrial 3-hydroxyacyl-thioester dehydratase

It was shown previously that the respiratory phenotype of the yeast mitochondrial enoyl reductase *ETR1* deletion strain could be rescued by an *E. coli* enoyl reductase FabI when it is targeted to mitochondria (Torkko *et al.* 2001). Thus it was reasoned that a mitochondrially localized *E. coli* 3-hydroxyacyl-ACP-dehydratase FabA could complement the respiratory deficient phenotype caused by mutations in the yeast mitochondrial dehydratase. Wild-type cells readily lost the plasmid encoding bacterial dehydratase with a mitochondrial targeting signal at a certain frequency, resulting in sectored colonies in the Δ*ade2, Δade3* strain background, as the plasmid also carried the *ADE3* gene (Bender & Pringle 1991) (Fig.1A, I). After mutagenesis of strains W1536 8B (α) and W1536 (**a**) carrying pYEmtFabA plasmid, approximately 40,000 colonies were screened for the inability to lose the plasmid on medium containing only a non-fermentable carbon source. 141 initially picked colonies appeared red throughout on the GSM medium, and for 15 of them, the phenotype was confirmed (Fig.1B, I). By streaking the mutant strains on SC medium containing 2% dextrose it was shown that the mutations cause a respiratory defect, because the true candidates can lose plasmid encoding mitochondrially targeted FabA when growth could be supported by fermentation (Fig.1C, I). Four of the fifteen candidates passed this test and all four mutants fell into the same complementation group. A 2:2 segregation ratio (respiratory deficient *vs.* respiratory competent phenotype) after mating mutant W1536 8B *htd2-1* back to wild type, sporulation and tetrad dissection, indicated that the lesion in this particular mutant was in one gene.

The mutants without any plasmid were transformed with a pYE352-plasmid carrying the Fam1-1 suppressor allele, *E. coli* dehydratase FabA and FabZ with the mitochondrial targeting signal or FabA without a mitochondrial targeting signal and the transformants were tested for the ability of these plasmids to rescue the respiratory deficient phenotype. Both FabA and FabZ can complement the mutant better than the Fam1-1 suppressor indicating that the mutations in all of

the four isolates are in the mitochondrial 3-hydroxyacyl-ACP-dehydratase (Fig.3, I). Without the mitochondrial targeting signal FabA does not rescue the respiratory deficient phenotype.

The mutants carrying plasmid encoding mitochondrially targeted FabA were transformed with a yeast multicopy library on GSM–ura medium to select for library plasmid take-up and screened for transformants that were able to lose the plasmid encoding mitochondrially targeted FabA and thus form sectoring colonies. Three colonies were identified (from three independent transformations of two different mutants, W1536 8B *htd2-1* and W1536 8B *htd2-3*) that had lost the plasmid encoding mitochondrially targeted FabA, verified by their inability to grow on medium lacking leucine. From these colonies, the plasmids that were able to complement the respiratory deficiency of the mutants were isolated. All three library plasmids contained the same insert with three complete and one partial open reading frame (Fig.4A, I). Two protein products of these open reading frames (*YHR067w*/*RMD12* and *YHR070w*/*TRM5*) were predicted to be mitochondrial with a probability of 62% and 98%, respectively. Only subclones containing the complete *YHR067w* ORF were able to complement the respiratory deficient phenotype of the *htd2-1* mutant (Fig.4B and C, I). The complementation was also possible by *YHR067w* on a single copy plasmid, which ruled out a multicopy suppressor effect (Fig.4D and E, I). Mating the strain containing the *htd2-1* mutation to a strain harboring the *ssz1(yhr064c)::KANMX* gene replacement and subsequent sporulation and tetrad analysis revealed close linkage of the *htd2-1* mutation to the *ssz1* locus and therefore to *yhr067w.* Sequencing of the *yhr067w* allele in *htd2-1* showed that nucleotide G247 was mutated to A, which causes a change of Gly83Arg. Like the mutants in the screen, the deletion of *YHR067w* in the strain BY4741 is respiratory deficient, which was also previously reported (Dimmer *et al.* 2002).

5.1.2 The screen for human mitochondrial dehydratase

In order to clone the human 3-hydroxyacyl thioester dehydratase, the yeast dehydratase mutant strain described above was transformed with human cerebellum and kidney cDNA libraries, and the candidate clones were selected for their ability to rescue the growth of the respiratory deficient *htd2-1* mutant on glycerol as a sole carbon source. After large-scale transformation with the cerebellum cDNA, representing approximately 1.8 x 10^6 transformants, the library yielded 6 colonies growing after 7 days on glycerol, and transformation

with the kidney library (2.8 x 10^6 transformants) yielded 7 colonies growing after 6 days on glycerol. To confirm that the complementation was due to a plasmid-borne factor, these transformants were then plated on medium containing 5'-FOA to select for loss of the library plasmids and subsequently tested for loss of respiratory competence. Three of the 6 cerebellum library isolates and all 7 of the kidney library isolates passed the 5'FOA test. After isolation of two versions (A and B) from each transformant and re-transformation of the yeast strains, only four (1A, 1B, 5A and 5B) were able to complement both yeast dehydratase knock-out strain, Δ*htd2* in the BY4741 strain, and the mutant *htd2-1* in the W1536 strain. Correspondingly, plasmid isolates 3A, 3B, and 4B from the kidney library were able to complement both deletion and mutant strains. The complementing plasmids were digested with *Bst*XI and *Not*I restriction enzymes to release the cDNA inserts. 1A, 1B, 5A, 5B and 4B had an insert with an approximate size of 1.5 kb, while isolates 3A and 3B displayed a different restriction pattern, with the insert cut into two fragments of around 1 kb and 250 bp. Sequencing of the isolated plasmids revealed that isolates 1A, 1B, 5A, 5B, and 4B contained the complete *RPP14* transcript (Jarrous *et al.* 1999), whereas isolates 3A and 3B contained only the 3´end of the same transcript lacking the *RPP14* coding region. The coding sequence of the human *RPP14* is located in chromosome 3 (3p14.3) and the gene size is over 30 kb with 6 exons. The inserts of 1A, 1B, 4B, 5A, and 5B covered all 6 exons, whereas clones 3A and 3B contained only exon 6 with a short internal deletion and an additional sequence representing an Alu-repeat that is located downstream of the gene.

The exon 6 of the *RPP14* mRNA contained an additional large ORF and only this second 3' cistronic ORF was present in all the isolated clones. Thus it was reasoned that the *RPP14* mRNA might be bicistronic, encoding two proteins on the same transcript, and that the second 3' ORF may encode the protein which complemented the mitochondrial dehydratase deficiency in the yeast screen. This novel ORF was 507 bp in length and encoded a polypeptide of 168 amino acids. The predicted molecular mass for the polypeptide was 18.5 kDa. This protein was predicted to belong to the MaoC dehydratase family and it was homologous to prokaryotic acyl dehydratases, yeast peroxisomal Fox2p and mammalian MFE2 and FAS1. The polypeptide contained also the "hydratase-2" fingerprint. The MITOPROT II, mitochondrial-targeting program, identified the first 17 N-terminal amino acids of this dehydratase candidate as a potential mitochondrial targeting signal, and the protein was predicted to be localized to mitochondria with a 67% probability.

5.1.3 Identification of T. brucei mitochondrial dehydratase

During characterization of human mitochondrial dehydratase, our phylogenic analysis indicated that the *T. brucei* proteome also contained a member of the MaoC family of dehydratases, namely Q580H9_9TRYP. This gene is located in chromosome 8 and the ORF has a length of 489 bp encoding a protein of 163 amino acids. It showed 27% sequence identity and 41% similarity to human mitochondrial dehydratase HsHTD2. This *T. brucei* protein also contains the "hydratase-2" fingerprint (Qin *et al.* 2000). The MITOPROT II mitochondrial prediction program predicted the protein to be localized in mitochondria with 81% probability (Claros & Vincens 1996). The predicted molecular mass for the protein is 17.8 kDa, which is similar to bacterial dehydratases (Heath & Rock 1996c) and human HTD2.

Table 3. represents the predicted properties of yeast, human and *T. brucei* mitochondrial dehydratase.

Table 3. Predicted properties of different mitochondrial dehydratases.

Organism	Gene (nt)	Protein (kDa)	Predicted to be mitochondrial	Hydratase-2 fingerprint
S. cerevisiae	*YHR067w* (843)	33.1	62%	yes
Human	*RPP14/HTD2* (507)	18.5	67%	yes
T. brucei	Q580H9_9TRYP (489)	17.8	81%	yes

5.2 Complementation in yeast

The respiratory deficient phenotype of yeast *Δhtd2* and *htd2-1* can be complemented by the mitochondrially targeted *FabA* and *FabZ* gene products, but not by the *fabA* construct lacking the mitochondrial import sequence (Fig.3, I). The deletion mutant respiratory deficient phenotype can also be rescued by the *FAM1-1* suppressor allele. The pYE352-plasmid harboring human dehydratase is able to rescue the respiratory deficient phenotype of yeast *Δhtd2* and *htd2-1*, but the plasmid expressing human dehydratase without a mitochondrial targeting signal can not complement the knockout and mutant yeast strain on glycerol plates (Fig.1, II). If the conserved active site residues Asp62 and His67 of the human dehydratase are mutated to alanine, the complementation is also prevented (Fig.3B, II). Similarly, the full-length *T. brucei* dehydratase can rescue the respiratory deficient phenotype of yeast *Δhtd2* and *htd2-1* (Fig.2, III).

5.3 Purification and enzyme assays

Because attempts to purify yeast dehydratase failed, the dehydratase was overexpressed in yeast, the mitochondria were isolated and hydratase 2 activity from mitochondrial extracts was analyzed. The hydratase 2 activity in the mitochondrial extracts expressing dehydratase was 69 ± 13 nmol/min×mg protein^{-1} (n = 8) compared to 27 ± 18 nmol/min×mg protein^{-1} (n = 4) measured in the samples from the wild type cells ($p<0.001$). The activity of fumarase, a soluble enzyme of the tricarboxylic acid cycle, did not change being 2.3 ± 0.5 µmol/min×mg protein^{-1} (n = 6) in cells expressing dehydratase and 2.0 ± 0.2 (n = 4) µmol/min×mg protein^{-1} in wild type cells.

Human dehydratase HsHTD2 was expressed as a fusion to maltose binding protein (MBP) and the recombinant protein was purified from the *E. coli* lysate with amylose resin and further purified by gel filtration. The k_{cat}-value of (*3R*)-specific hydratase 2 activity of the recombinant protein was 0.24 ± 0.07 1×s^{-1} (n = 4) when using a *trans*-2-hexenoyl-CoA substrate and 0.36 ± 0.05 1×s^{-1} (n = 3) with a *trans*-2-decenoyl-CoA substrate. When using (*3S)*-specific dehydroxygenase as an auxiliary enzyme the reaction did not occur, indicating that the reaction is *(3R)*-specific. The native molecular mass of the recombinant MBP+HsHTD2 protein was found to be 230 kDa by size exclusion chromatography on a Superdex 200 HR column, whereas based on SDS-polyacrylamide gel electrophoresis analysis the polypeptide was estimated to have a molecular mass of 59 kDa, indicating that the recombinant protein is a tetramer.

The dehydratase of *T. brucei*, TbHtd2, was also expressed as a fusion to maltose binding protein and purified in the same way as the human dehydratase. The MBP was cleaved off with TEV-protease and gel filtration chromatography was used to determine the native molecular mass of the dehydratase. TbHTD2 eluted in three separate peaks with sizes of 70.8, 31.6 and 20.9 kDa, whereas SDS-polyacrylamide gel electrophoresis analysis showed the polypeptide to have a molecular mass of 18 kDa (Fig.4, III). Hence, the peaks contained TbHTD2 as tetramer, dimer and monomer respectively. Only the tetrameric form showed the hydratase 2 activity.

The k_{cat} and k_m values in the (*3R*)-specific hydratase 2 activity of TbHTD2 were measured with *trans*-2-$C_{4,6,8,10,12,14,16}$-CoA substrates (Table III, III) . The TbHTD2 showed the highest catalytic efficiency (k_{cat} / k_m) with *trans*-2-C_{10}-CoA substrate and other substrates were ranked in the following order C8 > C12 > C6

> C4. The k_m value was also the lowest with the *trans*-2-C_{10}-CoA substrate. With the *trans*-2-C_{14}-CoA substrate the enzymatic activity as an increase of $NADH^+$ was detectable, but so low that only a specific activity 0.067 nmol/min×mg $protein^{-1}$ (n = 3) was determined. The activity with the *trans*-2-C_{16}-CoA substrate was below the detection limit of the assay used.

When 16 first amino acid residues from N-terminus were deleted, the protein did not have hydratase-2 activity *in vitro* and it could not complement the yeast knock-out and mutant strains showing that at least some of those residues are needed for enzymatic activity and possibly also for mitochondrial localization.

5.4 Localization

The mitochondrial localization of yeast dehydratase was confirmed by expressing the dehydratase as a chimeric protein with protein-A. After induction of expression of the fusion protein, the cells were *in situ* probed using FITC-conjugated antibody and analyzed under a microscope. The fluorescent signal localized to tubular structures was only detected in cells transformed with the construct, and this signal was colocalized with the red fluorescence signal from the Mitotracker dye (Fig.6, I). Control cells expressing only protein A showed a diffuse FITC stain throughout the cytosol.

The subcellular localization of the human 3-hydroxyacyl-ACP dehydratase was studied in HeLa cells by fluorescence microscopy. The HeLa cells were transfected with a plasmid expressing an HsHTD2-GFP fusion protein and the mitochondria of the cells were stained with MitoTracker Red, while the nuclear DNA was counterstained with DAPI. The cells showed a green fluorescent signal that colocalized with MitoTracker staining (Fig.4A, II). When the cells were transfected with a plasmid lacking the 51 nucleotides encoding the first 17 N-terminal amino acids of HsHTD2, a diffuse uniform cellular fluorescent signal, not superimposable with MitoTracker staining, was observed (Fig.4B, II). Thus, HsHTD2 is localized to mitochondria and the N-terminal region of the full-length protein is required for mitochondrial targeting. To study the localization of RPP14, the cDNA of *RPP14* was cloned into the pEGFP-N1 vector and HeLa cells were transfected with this plasmid. The fluorescent signal was distributed throughout the transfected cells without any specific punctated pattern in the nucleus (Fig.4C, II).

The localization of *T. brucei* mitochondrial dehydratase was confirmed by fluorescence microscopy. TbHTD2 was expressed as a fusion protein with the

green fluorescent protein (GFP) at the carboxy-terminus, and this protein colocalized with the Mitotracker Red staining in *T. brucei* procyclic form cells (PCF) showing that this protein is indeed mitochondrial (Fig.3, III).

5.5 Effects of yeast dehydratase overexpression or deletion on mitochondrial morphology and cytochromes

The cytochrome spectra of mutant *htd2-1* and the BY4741 *Δhtd2* strain were analyzed and the *htd2-1* mutant exhibited loss of cytochromes characteristic for mitochondrial FAS mutants. This loss of mitochondrial cytochromes was reversed by introduction of a plasmid carrying *YHR067w*. The cytochrome spectra of *Δhtd2* cells, which were transformed with the p*HAP1* plasmid to complement the *hap1* mutant phenotype in this strain (Brachmann *et al.* 1998, Gaisne *et al.* 1999), also showed the loss of cytochromes. Introducing mitochondrially localized dehydratase *fabA* into these cells restored the cytochromes to wild type levels.

The studies with electron microscopy showed that overexpression of mitochondrial dehydratase in yeast cells on a multicopy plasmid leads to mitochondrial enlargement, similar to the phenotype exhibited by cells overexpressing *S. cerevisiae* or *C. tropicalis* Etr1p (Torkko *et al.* 2001) (Fig.7C, I). By contrast, the *htd2-1* mutant cells appeared enlarged and contained severely misshapen mitochondria and a large number of small, dark structures, which were interpreted to be mitochondrial remnants (Fig.7A, D and E, I). Additionally, yeast dehydratase mutant and wild type cells expressing mitochondrially localized GFP (Westermann & Neupert 2000) were studied by fluorescence microscopy (Fig.8, I). A large fraction of the mitochondria of the *htd2-1* mutant (32%, compared to 3% in the wild type) appeared to be elongated, spindly and highly branched. In wild type cells most of the mitochondria (56%, compared to about 9% in the mutant) appeared to be fragmented to a larger degree, and also thicker and more robust. This result suggests a possible deficiency in mitochondrial fission processes in the dehydratase mutant. Similar to the EM results, a slight enlargement of cell size in the *htd2-1* mutant versus wild type cells was observed.

5.6 Lipoic acid analysis

Deletion of the yeast mitochondrial dehydratase gene causes a reduction in lipoic acid level in yeast (Table II, III). In the Δ*htd2* yeast, the amount of lipoic acid is less than 10% of the wild-type values. When the yeast dehydratase deletion strain

is transformed with a plasmid expressing yeast mitochondrial Htd2p, the lipoic acid levels recover to wild type levels. Similarly, complementation of the yeast dehydratase deletion strain by *T. brucei* mitochondrial dehydratase restored the amount of lipoic acid to the level of wild type yeast cells.

5.7 The *FAM1-1* allele suppresses deletion mutations in all known mitochondrial FAS genes

The *FAM1-1* allele of the *FAM1/FAA2* gene, coding for an acyl-CoA synthetase, was identified to act as a suppressor for a mutation in the mitochondrial condensing enzyme CEM1 (Harington *et al.* 1994). *FAM1-1* carries a mutation, which introduces an additional in-frame ATG up-stream of the original initiation codon, generating a mitochondrial targeting signal at the N-terminus of this protein. The *FAM1-1* allele, generated by PCR, was cloned into a multicopy vector, and the strain BY4741 and the corresponding mitochondrial FAS deletion derivatives Δ*mct1*, Δ*cem1*, Δ*oar1* and Δ*etr1* were transformed with this plasmid. The growth of these transformants on medium containing glycerol as the sole carbon source was studied. In all cases, the respiratory deficient phenotype of the deletion mutants was suppressed in these transformants, while the same mutants carrying a control plasmid were not able to grow (Fig.2, I). Contrary to the results of Harington *et al.* (Harington *et al.* 1994), the suppression effect was also observed on synthetic complete medium.

5.8 Transcription of *RPP14* and *HsHTD2* in human tissues

Transcription of *RPP14* and *HsHTD2* in different human tissues was investigated by Northern blot analysis with two parallel blots (Fig.5, II). The probes corresponding to the ORFs *RPP14* and *HsHTD2* hybridized to a major transcript with an approximate size of 1.3 kb. With the *RPP14*-specific probe, a band of about 1.5 kb was also detected and this band was not detected with the *HsHTD2* probe. Any transcripts containing *HsHTD2* alone, which would correspond to isolate 3A/B obtained in the library screen, were not detected. The identity of the 1.5 kb transcript recognized only by the *RPP14* probe is unknown, analysis of human EST databases provided evidence only for bicistronic *RPP14/HsHTD2* transcripts differing slightly in their 5’ ends, but no support for the existence of a transcript encoding only RPP14.

The tissue-specific distribution was the same with both probes. The transcripts were most abundant in heart and liver tissues and were present at relatively low levels in skeletal muscle, spleen, kidney and placenta. The transcription pattern of HsHTD2 in different human tissues correlated well with the transcription patterns reported for human β-ketoacyl synthase and enoyl reductase of the mitochondrial fatty acid synthesis pathway (Miinalainen *et al.* 2003, Zhang *et al.* 2005).

5.9 Linkage of *RPP14* and *HTD2* is highly conserved in vertebrates

The bicistronic arrangement of *RPP14* and *HTD2* in eukaryotes is conserved throughout evolution (Fig.2, II). The linkage between *RPP14* and *HTD2* on a common transcript has remained unchanged from fish and frogs to birds and humans. The exon-intron organization of this gene also appears to be conserved in higher eukaryotes. The ORF coding for RPP14 is interrupted by several introns, whereas the HsHTD2 is encoded by the last exon of the gene without any interruptions by introns. In zebrafish, there is evidence for two alternatively spliced forms of the *RPP14* transcript. One transcript form is shortened due to the removal of an additional intron within the last exon sequence, causing the in-frame fusion of the ORFs of RPP14 and HTD2 and thus this transcript codes for a single fusion protein. Searches of EST databases for zebrafish revealed both transcript forms.

The tunicate *Ciona intestinalis RPP14* transcript is not available (Rosenblad *et al.* 2006), but BLAST searches identified the potential *HTD2* homologue in the genome of this organism. This ORF for HTD2 is predicted to be downstream of another ORF coding for a conserved hypothetical protein that is homologous to human hypothetical protein LOC58493. Further analysis of *C. intestinalis* ESTs supported the presence of the putative bicistronic transcript. In other metazoans such as *Drosophila melanogaster*, homologues of *RPP14* and *HTD2* are encoded by two different genes located on different chromosomes.

5.10 Expression of 3' *HsHTD2* in yeast

When both *in vivo* and *in vitro* studies to determine if HsHTD2 is translated from the bicistronic transcript in human cells failed, the expression of *HsHTD2* from the bicistronic transcript was studied in yeast. The original bicistronic cDNA was cloned into a promoterless single copy YCp*lac*33-vector and yeast strains Δ*htd2*

and *htd2-1* were transformed with this plasmid. The construct was able to very weakly complement the respiratory deficient phenotype of mutants compared the original library plasmid 1A. These results indicate that, while yeast is able to weakly transcribe the *RPP14/HsHTD2* cDNA sequence in the absence of a *bona fide* yeast promoter, a genuine yeast promoter is required to obtain full complementation by the bicistronic cDNA.

5.11 Phylogenetic analysis of the MaoC family of dehydratases

Phylogenetic analysis of the MaoC family of dehydratases identified several subgroups (Fig.6, II). Hydratases of the multifunctional enzymes type 2 and dehydratases of the eukaryotic fatty acid synthases 1 form discrete clades in the phylogenetic tree backed up by solid bootstrap support. Human mitochondrial dehydratases and other homologous eukaryotic enzymes form a branch with bacterial PhaJ hydratases. These bacterial dehydratases are involved in polyhydroxyalkanoate synthesis (Fukui & Doi 1997). The fungal mitochondrial dehydratases were not in the same branch with other eukaryotic mitochondrial dehydratases, but they form a separate clade with bacterial enzymes such as the *Pseudomonas* sp. L1 Ich1 protein (Q14DY6_9PSED). This Ich1 protein is annotated to be an itaconyl-CoA hydratase involved in bio-degradation of the tulip allergen tulipalin A. Many other well-conserved, uncharacterized sequences from proteobacteria, the chloroflexus group, as well as some actinomycetes, can also be found in this branch. BLASTP searches using the yeast Htd2p or the *Pseudomonas* Ich1 sequence as the query retrieved fungal mitochondrial dehydratases as well as bacterial Ich1 homologues, but no non-fungal eukaryotic dehydratases or bacterial PhaJ, MaoC or NodN sequences. This finding explains why it was not possible to identify a human mitochondrial dehydratase with the yeast Htd2 sequence. According to phylogenetic analyses, we suggest that fungal mitochondrial dehydratases have a different bacterial phylogenic origin with respect to the other eukaryotic enzymes.

6 Discussion

6.1 3-Hydroxyacyl-thioester dehydratases from yeast, human and *Trypanosoma brucei* as members of mitochondrial FAS

The fatty acid biosynthesis (FAS) type II pathway typically occurs in bacteria and the components involved in the *E. coli* type II FAS pathway have been extensively characterized (White *et al.* 2005). The mitochondrial type fatty acid synthesis pathway resembles the bacterial type II FAS, and it was first discovered in *S. cerevisiae* and *N. crassa* (Brody & Mikolajczyk 1988, Harington *et al.* 1993, Schneider *et al.* 1997a). Later on the existence of this pathway was also confirmed in plants and mammals, including humans (Joshi *et al.* 2003, Miinalainen *et al.* 2003, Yasuno *et al.* 2004, Zhang *et al.* 2003a, Zhang *et al.* 2005). Several enzymes of this pathway in yeast were identified based on their similarity to known *E. coli* proteins. Functional mitochondrial fatty acid synthesis is needed for proper mitochondrial function in yeast, because deletions of any of genes coding for mitochondrial FAS enzymes result in a respiratory deficient phenotype and lack of cytochromes. Some of these deletion mutants also have been shown to lose mitochondrial DNA at a certain frequency (Brody *et al.* 1997, Schneider *et al.* 1997a).

Because *E.coli* type II FAS dehydratases FabA and FabZ do not have any homologues in *S. cerevisiae*, yeast mitochondrial dehydratase was identified by using a colony-color-screen approach, in which mutants were identified that were unable to lose a mitochondrially localized bacterial dehydratase The mutations created in the colony-color-screen were in ORF *YHR067w* encoding Htd2p, and the mutants exhibited phenotypes similar to the other mitochondrial FAS mutants. These mutant phenotypes were complemented by single and multicopy plasmids harboring *YHR067w.* A *S. cerevisiae* strain carrying a deletion allele of *YHR067w* was respiratory deficient and displayed cytochrome spectra alterations similar to the mutant generated in the screen and other mitochondrial FAS mutants. The respiratory deficient phenotype of the mutants can be rescued by mitochondrially localized bacterial dehydratases FabA and FabZ. The lipoic acid level in the Δ*htd2* strain was less than 10% of the lipoic acid level in wild type yeast cells. The protein Htd2p is predicted to contain a mitochondrial localization sequence, and a proteinA-tagged version of Htd2p has mitochondrial localization. In addition, the genome-wide study of protein localization in yeast (Kumar *et al.*

2002) shows that Htd2p is a mitochondrial protein. Htd2p has also a hydratase-2 finger print motif, and mitochondrial extracts overexpressing *YHR067w* have elevated levels of hydratase-2 activity.

The gene encoding human mitochondrial 3-hydroxyacyl-ACP hydratase was identified from cDNA libraries expressed in yeast. The plasmid that can complement the respiratory deficient phenotype of the yeast *htd2-1* mutant contained a human bicistronic transcript *RPP14*. Previously it has been shown that the 5' ORF of this transcript encodes the RPP14 subunit of the human RNase P complex. The present study revealed that the last exon of the *RPP14* gene encodes a mitochondrial 3-hydroxyacyl-ACP dehydratase. This finding was supported by several pieces of evidence: only library clones from the human cerebellum or kidney cDNA libraries containing the complete bicistronic cDNA of *RPP14* or its last exon alone were able to complement the yeast *htd2-1* mutant strain respiratory deficient phenotype on a non-fermentable carbon source. The ORF encoding only *HSHTD2*, but not the ORF encoding *RPP14* alone, was sufficient to complement both the yeast dehydratase knock-out and the *htd2-1* mutation on non-fermentable medium. The HsHTD2 protein belongs to the MaoC dehydratase family according to an NCBI-conserved domain search, contains the "hydratase-2" fingerprint, and consequently shows similarity to prokaryotic acyl dehydratases and the hydratase-2 domains of Fox2p and mammalian MFE2 and FAS1. The HsHTD2-GFP fusion protein is localized to mitochondria in HeLa cells, indicating that HsHTD2 is a mitochondrial protein. This localization is dependent on the presence of the predicted N-terminal mitochondrial targeting sequence of HsHTD2. Purified recombinant HsHTD2 is enzymatically active, catalyzing the hydratase 2 reaction.

The mitochondrial dehydratase of *T. brucei* was identified according to its homology to human mitochondrial dehydratase. This trypanosomal protein can rescue the respiratory deficient phenotype of the yeast mitochondrial dehydratase knock-out and mutant strains. Additionally, introduction of TbHTD2 into the yeast Δ*htd2* strain on a yeast expression plasmid, results in the recovery of the lipoic acid levels in the yeast mutant to the level of wild type cells. The mitochondrial localization of TbHTD2 was confirmed for the GFP-fusion protein in PCFs. The hydratase-2 activity of TbHTD2 was shown with different chain length substrates and it exhibited the highest catalytic efficiency (k_{cat} / k_m) with the *trans*-2-C_{10}-CoA substrate, when the k_m value was also the lowest.

6.2 Evolution of mitochondrial dehydratases

Dehydratases of mitochondrial FAS belong to the MaoC dehydratase like subfamily of the thioesterase / thiol ester dehydratase / isomerase (TED1) superfamily. This superfamily contains proteins from bacteria, archaea and eukaryotes, with diverse functions from thioester hydrolysis to phenylacetic acid degeneration and transcriptional regulation of fatty acid biosynthesis (Dillon & Bateman 2004). The TED1 superfamily consists of 17 subfamilies, two of which are represented by the FabA and FabZ dehydratases. Originally, the *maoC* gene was found to be part of the same operon with the *maoA* gene, encoding monoamine oxidase in *Klebsiella aerogenes* and *E. coli* (Sugino *et al.* 1992). Park and co-authors showed that the maoC gene encodes an enoyl-CoA dehydratase active in polyhydroxyalkanoate (PHA) synthesis (Park & Lee 2003). The NodN-like subfamily, where the *nod*N gene product is involved in the production of the root hair deformation factor during the interaction of Rhizobia and leguminous plants, is very close to the MaoC-subfamily and it is usually considered to be part of MaoC-subfamily (Dillon & Bateman 2004).

The common feature in all family members is so-called hot dog fold structure. For the first time, the hot dog fold was described in the structure of *E. coli* 3-hydroxydecanoyl thiol ester dehydratase (Leesong *et al.* 1996). Later on, this fold was found in structures of thioesterases (Li *et al.* 2000), the hydratase 2 domain of the peroxisomal multifunctional enzyme type 2 (Koski *et al.* 2004) and FabZ dehydratases (Kimber *et al.* 2004). The hot dog fold consists of a long and hydrophobic α-helix ("sausage") wrapped in an anti-parallel β-sheet ("bun").

The molecular masses of human and *T. brucei* mitochondrial dehydratases correspond to the mass of the FabA or FabZ proteins (18.8 kDa and 17.0 kDa, respectively), indicating potential structural similarities between these enzymes. Interestingly, the predicted molecular mass of yeast mitochondrial dehydratase is 33.1 kDa, approximately twice to that of the human and *T. brucei* mitochondrial dehydratases and is close to the mass of the hydratase 2 part (31.0 kDa) of the peroxisomal multifunctional enzyme type 2 (Qin *et al.* 2000). In addition, our phylogenetic analysis showed that fungal mitochondrial dehydratases form their own clade in phylogenetic tree separately to other eukaryotic mitochondrial dehydratases (Fig.6, II). Mitochondrial dehydratases in yeast, human and *T. brucei* contain a hydratase-2 finger print in the immediate vicinity of the active catalytic site (Fig.1, III). According to data presented previously, it is likely that the structure of the yeast mitochondrial dehydratase resembles the structure of the

hydratase 2 part of the peroxisomal multifunctional enzyme type 2 with double hot dog fold. The structures of the human and *T. brucei* mitochondrial dehydratases might be close to thioesterase II, FabA or FabZ structures.

6.3 Bicistronic transcript

The human mitochondrial dehydratase is encoded as a second, 3' ORF on the *RPP14* bicistronic transcript. The bicistronic nature of this transcript has remained unchanged during the evolution of vertebrates. Commonly, bicistronic transcripts in eukaryotes, by analogy to bacteria, are thought to encode proteins of related function (Blumenthal 2004). In humans, three biscistronic transcripts have been characterized earlier (Gray *et al.* 1999, Lee 1991, Reiss *et al.* 1999) and only one bicistronic transcript described so far, for which the function of both proteins are known (Gray *et al.* 1999).

The *RPP14/HsHTD2* bicistronic transcript encodes proteins for RNA processing and mitochondrial fatty acid synthesis, but the molecular link between these two pathways is unknown. However, there is some evidence in yeast that supports the existence of a functional link between these two pathways. One of the main roles of RNase P is the cleavage of the 5' leader sequence of tRNAs during the process of tRNA maturation. In this context, it is intriguing to note that it was shown nearly 15 years ago that mutations in the yeast *LIP5* gene, encoding lipoic acid synthase, cause defects in 5' processing of mitochondrially encoded tRNAs (Sulo & Martin 1993). Also there is growing evidence obtained in yeast that supports the notion of a functional link between these two pathways (Schonauer and Dieckmann, unpublished).

Thus far, human mitochondrial RNase P has not been purified to homogeneity, and although two groups have characterized this enzyme, they came to different conclusions about its general structure and dependence on an RNA subunit (Puranam & Attardi 2001, Rossmanith *et al.* 1995). According to the results of Puranam *et al.*, human mitochondrial and nuclear RNase P complexes share many components (Puranam & Attardi 2001), one of which may be RPP14. Human RPP14 is mainly localized to the nucleus (Jarrous *et al.* 1999), but these and our results can not exclude the possibility that a small fraction of the total cellular RPP14 may also be present in human mitochondria. The RPP14 amino acid sequence does not contain a clear mitochondrial targeting signal, but there is also no nuclear localization signal sequence.

In light of the potential connection between mitochondrial FAS and RNA processing in yeast, it seems unlikely that *RPP14* and *HsHTD2* are in the same transcript just by coincidence. This arrangement has remained conserved through millions of years of evolution, indicating positive selection. It appears that mitochondrial fatty acid synthesis and RNA processing are linked, but the recognition of the molecular link and the mechanism behind it, require more studies in this field.

A different question is whether RPP14 and HsHTD2 are both translated from the same transcript. The identification of the cDNAs complementing the yeast deletion strain containing only the *HsHTD2* ORF may indicate the presence of alternative transcripts or splicing products lacking the *RPP14* ORF. However, human EST databases did not provide evidence for alternative promoter usage or alternative splicing, the resulting transcript containing only *HsHTD2*. Therefore, the short cDNA variant observed in the screen may present an artifact that has arisen during library preparation. For RPP14, only this bicistronic transcript has been described (Jarrous *et al.* 1999).

If *HsHTD2* is translated from the bicistronic transcript, a leaky translation initiation mechanism (Porras *et al.* 2006) seems very unrealistic, due to the fact that there are nine ATGs between at the *RPP14* start codon and the *HsHTD2* start codon. It is possible that the regulation of *HsHTD2* expression is analogous to *GCN4* in yeast. *GCN4* expression is regulated via modulation of translational initiation at four small ORFs upstream of the *GCN4* ORF (Hinnebusch 2005). After termination of translation of the first upstream ORF (uORF), the 40s ribosomal subunit will frequently stay on the mRNA and scan for further start codons to re-initiate translation. In the *RPP14/HsHTD2* transcript, there are three ATGs between the *RPP14* ORF stop codon and the *HsHTD2* translation initiation site. The first ATG can be found immediately after the *RPP14* translational stop signal in the same reading frame. The other proteins encoded by this mechanism would range from 4 to 49 amino acids and one of the ORFs is overlapping the *HsHTD2* ORF.

An alternative scenario is the use of an internal ribosomal entry site (IRES) to translate the second gene. The poliovirus and encephalomyocarditis virus (EMCV) transcripts were the first to be described that utilize IRES elements (Jang *et al.* 1988, Pelletier & Sonenberg 1988). Later on, it was shown that cellular mRNA also can be translated despite inhibition of cap-dependent translation, potentially through the use of an IRES element (Macejak & Sarnow 1991). On the other hand, the existence of IRESs in higher eukaryotes is still

under debate (Kozak 2005). Our attempts to shed light on the question of translation of *HsHTD2* from the bicistronic transcript by *in vitro* translation or detection of fluorescent reporters *in vivo* were unsuccessful. The results of complementation studies in yeast with *MET3*-driven and promoterless constructs can be interpreted in two ways. It is possible that HsHtd2 is translated from mRNAs that are initiated downstream of the *RPP14* initiator AUG. Yeast initiates transcription often at multiple sites in the area 40-120 bp downstream of the TATA-box (Hampsey 2006). In the *RPP14/HsHTD2* transcript, the *HsHTD2* initiation site is much further downstream than 120 bp from TATA-box and preceded by several out-of-frame initiation codons. Thus it is possible that RPP14 and HsHTD2 are both translated from the same bicistronic mRNAs in yeast. Hence, the mechanism of translation of the dehydratase in mammalian cells, as well as the regulation of this process, remain unknown.

6.4 Mitochondrial FAS function

Thus far, the role of mitochondrial FAS in cells and organisms has been unclear. More and more evidence has been presented to demonstrate that mitochondrial FAS is needed to produce octanoyl-ACP, which is used as a precursor for lipoic acid synthesis. In this study it was shown that in a yeast strain in which the mitochondrial dehydratase gene is deleted, the lipoic acid content of the cells is less than 10% of the lipoic acid amount in wild type cells (Table II, III). The amount of lipoic acid can be restored to wild type levels in the yeast Δ*htd2* strain by transforming these cells with a plasmid expressing yeast endogenous or *T. brucei* mitochondrial dehydratase. This is additional evidence that mitochondrial FAS is required for lipoic acid production in mitochondria.

It appears likely that mitochondrial FAS can also produce fatty acids longer than the C8 that is needed for lipoic acid synthesis. In *T. brucei* cells mitochondrial FAS can produce up to C16 of length (Stephens *et al.* 2007) and enzyme kinetic analysis of mitochondrial dehydratase from *T. brucei* supports this data as this enzyme accepts *trans*-2-C_{14}-CoA as the longest substrate for the hydratase 2 reaction (Table III, III). Likewise, the human mitochondrial β-ketoacyl synthase can utilize substrates up to C14 (Zhang *et al.* 2005) and bovine heart mitochondria produce fatty acids containing up to 14 carbon atoms (Witkowski *et al.* 2007). It has been reported that ACP of complex I in *N. crassa* (Brody & Mikolajczyk 1988) and bovine mitochondria (Runswick *et al.* 1991) carries a long chain fatty acid moiety, and, in addition, complex I does not

assemble correctly in *N. crassa* mutants lacking mitochondrial ACP (Schneider *et al.* 1995). One suggestion based on these results is that the longer fatty acids produced in mitochondrial FAS might be needed for proper assembly of complex I in higher eukaryotes (Witkowski *et al.* 2007).

The morphological changes seen in yeast strains overexpressing or lacking Htd2p and Etr1p have also remained unexplained (Fig.7, I). The similarity of the phenotype of the mitochondrial dehydratase mutant to *gag3* mutants (Fekkes *et al.*, 2000) would imply a defect in mitochondrial fission, but it is not clear if this altered morphology is a primary effect of loss of mitochondrial FAS or a subsequent lesion due to loss of respiratory function.

The discovery of human mitochondrial dehydratase on the same transcript with *RPP14* gives a new potential link between mitochondrial fatty acid synthesis and mitochondrial protein translation via tRNA processing. If these two pathways are really connected, the respiratory deficient phenotype seen in all mitochondrial FAS deletion strains could be explained by failure in mitochondrial protein translation, which would cause defects in the mitochondrial respiratory chain. Although one function of mitochondrial FAS is likely to be lipoic acid production, it does not explain all the features related to mitochondrial FAS satisfactorily and thus more studies are required to understand the role of mitochondrial FAS.

6.5 Future prospects

This thesis describes characterization of dehydratase enzymes involved in mitochondrial FAS in three different organisms: yeast, human and the human pathogen *T. brucei*. The differences in the amino acid sequence between bacterial FabA and FabZ, yeast and human mitochondrial dehydratase prevented the identification of the yeast and human enzymes by bioinformatic approaches. Additionally the yeast mitochondrial dehydratases form a clade in the phylogenetic tree that is separate from other eukaryotic mitochondrial dehydratases. The size of the yeast enzyme is almost double compared to molecular mass of human and *T. brucei* dehydratases. The active residues aspartate and histidine of the hydratase 2 domain of multifunctional enzyme 2 are identical in human and *T. brucei* mitochondrial dehydratases, but in yeast mitochondrial dehydratase there is asparagine in the active site instead of aspartate. In spite of all these differences between fungal and mammalian enzymes, their biological function is identical as indicated by the fact that human

and *T. brucei* mitochondrial dehydratases can fully complement the yeast dehydratase deletion strain. Purified Htd2p and HsHTD2 can be crystallized and their crystal structures, in addition to kinetic analysis with a variety chain-length substrates, can be determined. All this data would shed light on the question of how yeast mitochondrial dehydratase differs from the mammalian enzyme.

The complete biological role of mitochondrial dehydratase and more broadly the whole pathway in the human body is still unknown. Generation and analysis of genetically modified dehydratase mouse lines may give answers to these questions. The deletion of the entire dehydratase gene may lead to a respiratory deficient phenotype similar to a yeast dehydratase knock-out, and may affect *RPP14* transcription and translation. These facts must be taken into account when designing the mouse line. An alternative option may be the generation of a mouse line with mutations for example in the active site of the enzyme.

One question remaining is if human mitochondrial dehydratase is translated from the bicistronic *RPP14* transcript and how this is regulated?

7 Conclusions

In this thesis study, the genes encoding 3-hydroxyacyl-ACP dehydratases of the mitochondrial fatty acid synthesis pathway and their protein products in yeast *S. cerevisiae*, human and the human pathogen *T. brucei* were characterized. A genetic screen in *S. cerevisiae* resulted in mutants, in which the respiratory deficient phenotype was rescued by the *E. coli* dehydratase FabA with a mitochondrial targeting signal. These mutants lacked cytochromes and displayed an abnormal mitochondrial morphology. The mutation was found to be in the yeast *YHR067w* gene that encodes mitochondrial 3-hydroxyacyl-thioester dehydratase 2 (Htd2p). The Htd2p is predicted to be a member of the thioesterase/thioester dehydratase-isomerase superfamily, containing a hydratase 2 finger print. Hydratase-2 activity in mitochondrial extracts from cells overexpressing *YHR067w* was increased, and these overexpressing cells displayed a striking mitochondrial enlargement phenotype. Htd2p was also shown to be localized in mitochondria, which is similar to the protein A-tagged version. The mitochondrial phenotypes of the deletion and overexpression mutants suggest that Htd2p is essential for the maintenance of mitochondrial respiratory competence and morphology in yeast.

The human mitochondrial dehydratase was identified from cerebellum and kidney cDNA libraries as clones that were able to rescue the respiratory deficient phenotype of the yeast mitochondrial dehydratase *htd2-1*mutant strain. These clones contained a biscistronic *RPP14* transcript or only the last exon of this transcript as an insert. A second, 3' ORF on the last exon of the *RPP14* transcript was identified to encode a protein showing similarity to known dehydratases and hydratase 2 enzymes. The protein was localized in mitochondria according to fluorescent microscopy studies. The purified recombinant protein exhibited (*3R)*-specific hydratase 2 activity. Based on these results, the protein was named human 3-hydroxyacyl-thioester dehydratase 2 (HsHTD2) involved in mitochondrial fatty acid synthesis. The bicistronic arrangement of *RPP14* and *HsHTD2*, as well as the general exon structure of the gene, is conserved in vertebrates from fish to humans, indicating a genetic link conserved for 400 million years between RNA processing and mitochondrial fatty acid synthesis.

Phylogenetic analysis of the MaoC family of dehydratases revealed a homologue for human mitochondrial dehydratase in the human pathogen *T. brucei*. When this gene was cloned into a yeast expression plasmid and yeast *htd2-1* cells were transformed with the plasmid, the respiratory deficient

phenotype of yeast was rescued indicating the *T. brucei* gene encoded a protein with the correct enzymatic function. In the yeast dehydratase deletion strain the amount of lipoic acid was less than 10% of wild type cells lipoic acid content. The amount of lipoic acid in Δ*htd2* cells was restored to wild-type levels, when the cells contained a plasmid expressing yeast or *T. brucei* mitochondrial dehydratase. The mitochondrial localization of TbHTD2 was confirmed by fluorescence microscopy with a TbHTD2-GFP fusion protein. The recombinant *T. brucei* protein was purified and shown to exhibit hydratase 2 activity with various chain length substrates. The highest catalytic efficiency (k_{cat} / k_m) was achieved with a *trans*-2-C_{10}-CoA substrate. These data indicate that this gene encodes the mitochondrial 3-hydroxyacyl-ACP dehydratase in *T. brucei*.

Ultimately, the 3-hydroxyacyl-ACP dehydratase of mitochondrial fatty acid synthesis was characterized in three eukaryotes, and although they lack any clear similarity in amino acid sequence, the biological function was the same for all characterized enzymes.

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