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Review

Mitochondrial fatty acid synthesis – An adopted set of enzymes making a pathway of major importance for the cellular metabolism

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ABSTRACT

The highly conserved fatty acid *de novo* synthesis pathway in mitochondria proceeds in an acyl carrier protein-dependent manner through a discrete set of enzymes. *Saccharomyces cerevisiae* has served as a model for studies of mitochondrial fatty acid synthesis type II (FAS II) and as a template for identification of mammalian components. Inactivation of mitochondrial FAS II in yeast results in respiratory deficiency and loss of cytochromes. The pathway produces the octanoyl-ACP substrate for lipoic acid synthesis, but several pieces of evidence indicate that it is capable of the generation of longer fatty acids. A number of structures of mitochondrial FAS II enzymes have been published in the past few years, allowing for a comparison with their prokaryotic counterparts, several of which have been described as promising targets for antibiotics. Recently, novel links between mitochondrial FAS and RNA processing in yeast and vertebrates have been reported. In *S. cerevisiae*, deficiency in mitochondrial FAS results in failure of maturation of mitochondrial RNAse P, while, in mammals, mitochondrial 3-hydroxyacyl thioester dehydratase and the RPP14 subunit of RNase P are encoded by the same bicistronic transcript. The first publications linking mitochondrial FAS II to disease states in mammals are emerging.

Contents

Abbreviations: ACC, acetyl-CoA carboxylase; ACP, acyl carrier protein; FAS, fatty acid synthesis; BC, biotin carboxylase; BCC, biotin-carboxy carrier protein; BPL, biotin:protein ligase; CT, carboxytransferase; ETR, enoyl thioester reductase; HTD, 3-hydroxyacyl thioester dehydratase; MCAT, malonyl-CoA:ACP transacylase; KAS/CEM1, 3-ketoacyl synthetase/condenzing enzyme; KAR, 3-ketoacyl reductase; PKS, polyketide synthase; PPTase, phosphopantetheine:protein transferase.

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1. Introduction

The research interest in mitochondria has been drifting on tides. This is exemplified by an anecdote told by Michael Yaffe during a Gordon conference on mitochondria and chloroplasts in 2006, when he recounted a conversation he had had, early in his career as a researcher in the 1980s, with a senior scientist. He had been asked about his future research plans, and his reply – indicating an interest in mitochondria- earned him a reaction of surprise and disbelief, as "everything important was already known" about this organelle – or so it was thought. Since then, major discoveries on many previously unknown or neglected aspects of mitochondrial physiology and biochemistry have brought these organelles into the spotlight of research interest in the field of life sciences. These observations include aspects of mitochondrial morphology maintenance, fusion and fission events, mitochondrial-nuclear crosstalk, mitochondrial DNA replication, transcription and translation and their regulation, iron-sulfur cluster biogenesis, the role of mitochondria in apoptosis, human disease, and mitochondrial inheritance as tool for tracking maternal lineages. This repertoire of processes illustrates the fact that the mitochondrial contribution to cell physiology goes far beyond the function of a cellular power plant that it was regarded as until the late 1980s. In light of the progress made during the last two decades in our understanding of mitochondrial roles within the cell, this organelle now rather appears like a cellular conglomerate involved in a multitude of processes and churning out a large number of essential products.

Among the recently recognized features of mitochondrial functions, in yeast as well as humans, is their ability to synthesize fatty acids in an acyl carrier protein (ACP)-dependent manner. This mitochondrial fatty acid synthesis (FAS) pathway follows the bacterial type II FAS (FAS II) mode (Fig. 1), with separate polypeptides carrying out the individual reactions [1]. In contrast, eukaryotic cytosolic type I FAS is performed by multifunctional proteins. Occurrence of FAS in two subcellular compartments in eukaryotic cells raises the question why eukaryotes have maintained a bacterial type FAS in their mitochondria in addition to the "classic" cytoplasmic FAS. The mitochondrial pathway is best described in the yeast *Saccharomyces cerevisiae* [2], where deletion of any member of the mitochondrial FAS pathway leads to a respiratory deficient phenotype, lack of cytochromes, and a decrease in lipoic acid, indicating that this pathway is essential for mitochondrial function [3].

ACP from *Neurospora crassa* was isolated as a first component of mitochondrial FAS pathway [4]. Since then, a mitochondrial form of ACP has been demonstrated to be present in other eukaryotes and most of the other FAS components have been characterized in yeast and humans. Most of eukaryotic FAS II enzymes show amino acid sequence similarities to their prokaryotic counterparts, and hence data base mining has been a frequently used technique in the quest for the identification of mitochondrial equivalents in eukaryotes. Furthermore, several prokaryotic homologs have been shown to rescue the respiratory deficient phenotype if expressed as mitochondrially targeted variants in corresponding yeast null mutants. To date, ACP, phosphopantetheine transferase (PPTase) [5], malonyl transferase (MCAT) [6], β-ketoacyl-ACP synthase (KAS) [7], 3-hydroxyacyl-thioester dehydratase (HTD) [8], enoyl thioester reductase (ETR) [9] of the human mitochondrial FAS II pathway, and very recently also the last enzyme of the mammalian pathway, 3-ketoacyl reductase (KAR) have been identified [10].

This review aims to summarize the current knowledge on proteins involved with mitochondrial FAS II and to provide a glimpse into its role in the physiology of the cell. We will focus on properties and structural aspects of the enzyme components of this pathway (Table 1) in comparison to known prokaryotic structures. We will also put emphasis on the mitochondrial FAS pathway in yeast because most of our current understanding has been obtained through this model organism.

2. Enzymology

2.1. Phosphopantetheinyl transferase (PPTase)

Both fatty acid synthesis and breakdown in β-oxidation proceed with acyl substrates attached via a thioester bond to phosphopantetheine (Fig. 2). The CoA molecule which contains the phosphopantetheine also serves as the donor for phosphopantetheine when holo-ACP is generated in a reaction catalysed by phosphopantetheine:protein transferase (PPTase). PPTases comprise an enzyme superfamily in which the proteins are sorted into three separate subgroups based on amino acid sequence similarities and structural characteristics of the polypeptides [11,12]. Group I contains enzymes most similar to *Escherichia coli* AcpS PPTase/holo-ACP synthase, typically 120 amino acids long and essential in most prokaryotes for ACP-mediated FAS. The N-termini of these proteins form mainly α-helices, while the C-termini folds into β-strands that arrange into a β-sheet. These α/β domain proteins assemble into trimers to form the active AcpS-type enzyme [13,14]. Group II is made up of proteins that are commonly found to be associated with nonribosomal peptide synthesis systems. These enzymes are approximately twice as long as Group I PPTases and exemplified by the *Bacillus subtilis* Sfb protein [15], which is required for the production of the peptide antibiotic surfactin, and accepts a wide range of substrate proteins for phosphopantetheine transfer [11]. The crystal structure of the enzyme revealed a monomeric protein folding into two individual domains of nearly identical structure and pseudo-2-fold symmetry. The individual domains display a strong similarity to the AcpS monomers [16]. The third enzymatic group encompasses PPTase domains covalently attached to multifunctional enzymes such as the *S. cerevisiae* Fas2p subunit of the cytosolic fatty acid synthase, which are specific for the associated ACP domain [17–19]. The yeast genome also encodes two additional proteins that display sequence similarity to bacterial PPTases. The *LYS5* gene codes for a PPTase required for activation of the Lys2p alpha-aminoadipate reductase involved in lysine biosynthesis [20]. The yeast candidate gene for mitochondrial PPTase (*PPT2*/*YPL148c*) was identified based on sequence similarity to the Ppt1p *Brevibacterium ammoniagenes* and *E. coli* AcpS PPTases [21]. Deletion of the

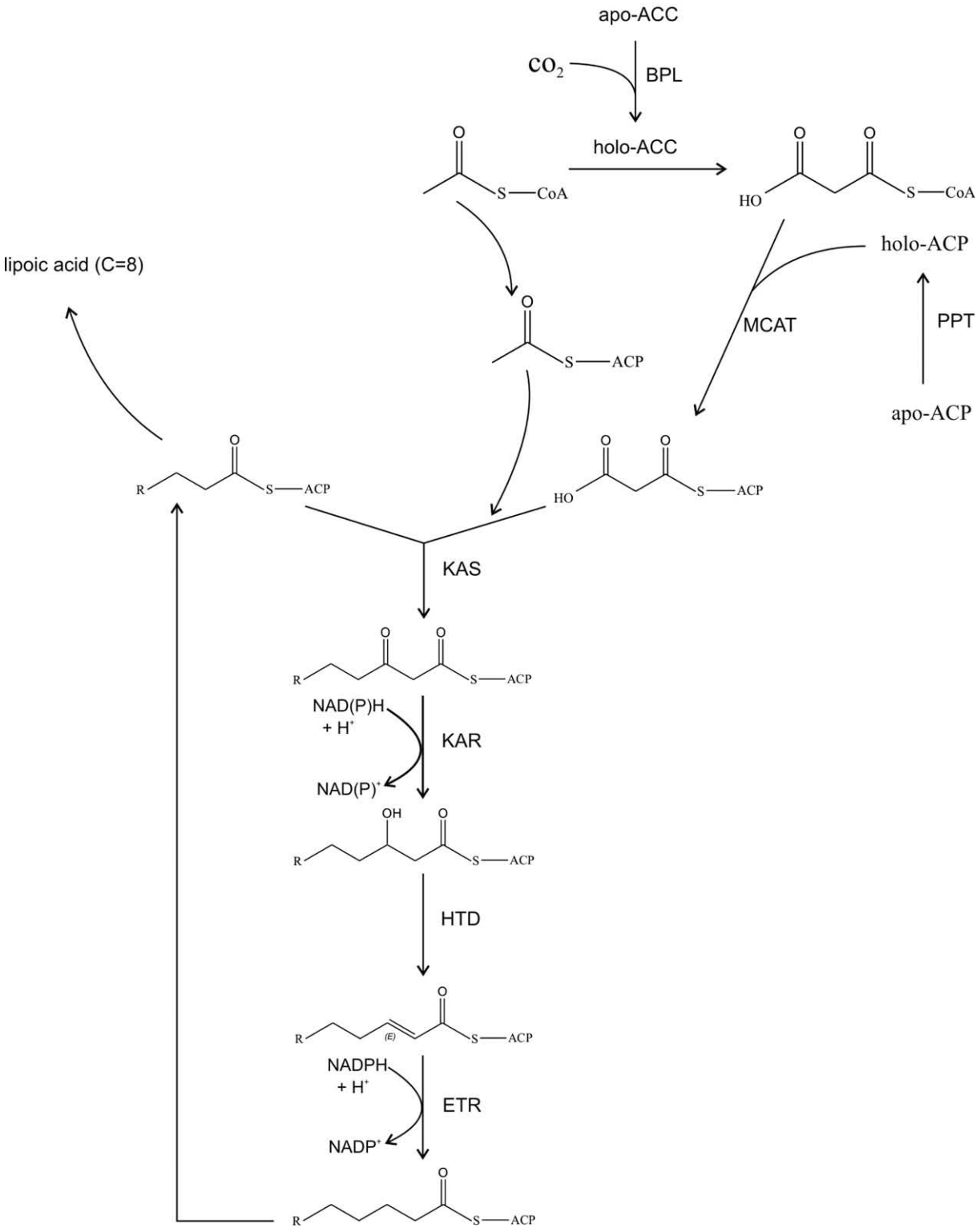

Fig. 1. The mitochondrial fatty acid synthesis pathway. The octanoyl group (C8) is one product of FAS II used for the synthesis of lipoic acid. The source of mammalian mitochondrial malonyl-CoA has not yet been confirmed. The BPL enzyme, which converts apo-ACC to holo-ACC, has not been identified in human. The properties and cofactor preferences for mitochondrial KAR can be either NADH or NADPH, dependent on the species. For abbreviations, see text and list of abbreviations.

YPL148c ORF in *S. cerevisiae* resulted in respiratory deficient cells unable to produce the pantetheinylated holo-form of ACP, and the purified yeast Ppt2p was sufficient to pantetheinylate yeast mitochondrial ACP *in vitro*. Surprisingly, the human genome only appears to encode a PPTase homologous to the yeast Lys5p, a protein required to activate alpha-aminoadipate reductase to its phosphopantetheinylated holo-form [22]. The human enzyme displayed broad substrate specificity and the ability to transfer the phosphopantetheine group to human mitochondrial ACP [5].

2.1.1. The crystal structure of human PPTase

The crystal structure of human PPTase was solved for the apo-form as well as for co-crystals with CoA and Mg^{2+} and also with CoA and a mutated form of the substrate ACP domain of the human

Table 1
FAS type II and related proteins. The table lists all the structures of eukaryotic FAS II and related proteins found in PDB by the end of 2008.

PDB code	Protein	Origin	Subcellular location	Related PDB entries
2qnw	Acyl carrier protein	*Toxoplasma gondii rh*	Apicoplast	
3ce7	Acyl carrier protein	*Toxoplasma gondii rh*	Mitochondrion	
2bdd	Acyl carrier protein synthase	*Plasmodium yoelii yoelii*	Unknown	2qg8
2byd	Phosphopantetheine transferase	*Homo sapiens*	Cytoplasm	2c43, 2cg5
1w93	Biotin carboxylase domain of ACC	*Saccharomyces cerevisiae*		1w96
1od2	Carboxyltransferase domain of ACC	*Saccharomyces cerevisiae*	Cytoplasm	1od4, 1uyr, 1uys, 1uyt, 1uyv, 1w2x
2hjw	Biotin carboxylase domain of ACC2	*Homo sapiens*	Intracytoplasmic membranes	
2c2n	Malonyl-CoA:ACP transacylase	*Homo sapiens*	Mitochondrion	
2c9h	β-Ketoacyl-ACP synthase	*Homo sapiens*	Mitochondrion	2iwy, 2iwz
1w0i	β-Ketoacyl-ACP synthase II	*Arabidopsis thaliana*	Mitochondrion	2ix4
2c07	β-Ketoacyl-ACP reductase	*Plasmodium falciparum*	Apicoplast	
1edo	β-Ketoacyl-ACP reductase	*Brassica napus*	Chloroplast	
1z6b	β-Hydroxyacyl-ACP dehydratase	*Plasmodium falciparum*	Apicoplast	1zhg, 2okh, 2oki
2vcy	2-Enoyl thioester reductase	*Homo sapiens*	Mitochondrion	1zsy
1gu7	2-Enoyl thioester reductase (Etr1p)	*Candida tropicalis*	Mitochondrion	1guf, 1gyr
1h0k	2-Enoyl thioester reductase (Etr2p)	*Candida tropicalis*	Mitochondrion	
1v35	Enoyl-ACP reductase	*Plasmodium falciparum*	Apicoplast	1uh5, 2nq8, 1zxb, 1zxl, 2ol4, 2oos,
1vrw	Enoyl-ACP reductase	*Plasmodium falciparum*	Apicoplast	1nhg, 1nnu, 1nhw, 1zw1, 2op0, 2op1,
2o2y	Enoyl-ACP reductase	*Plasmodium falciparum*	Apicoplast	2foi
2ptg	Enoyl-ACP reductase	*Eimeria tenella*	Apicoplast	
2o50	Enoyl-ACP reductase	*Toxoplasma gondii*	Apicoplast	2o2s
1eno	Enoyl-ACP reductase	*Brassica napus*	Chloroplast	1enp, 1d7o, 1cwu

A

Organisms	Synthesis (*de novo*)			β-Oxidation		
	cytosol	mitochondria	chloroplasts	cytosol	mitochondria	peroxisomes
Bacteria	+			+		
Fungi	+	+		-	-	+
Animals	+	+		-	+	+
Plants	±	+	+	-	-	+

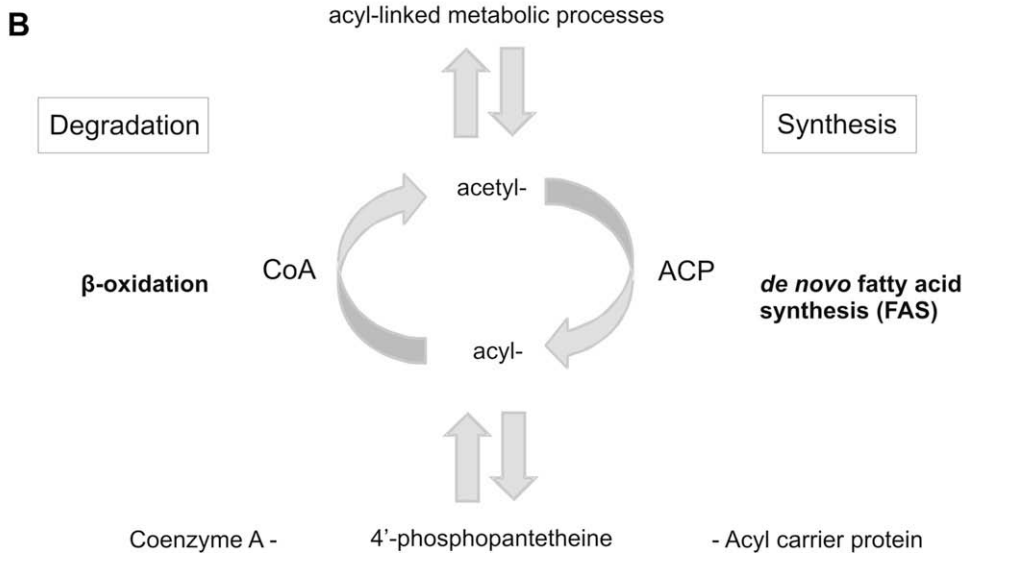

Fig. 2. Subcellular localization and interrelationship of fatty acid breakdown *via* β-oxidation and *de novo* synthesis in different species. Panel A summarizes the localization of the processes in various subcellular compartments of different organisms. Only compartments where fatty acids are known to be synthesized *de novo* starting from an acetyl group are indicated, and therefore, for instance the endoplasmic reticulum harboring the fatty acid elongation system is excluded. As shown in the Panel B, both fatty acid synthesis and β-oxidation require acyl-groups attached by a thioester bond to 4′-phosphopantetheine linked either to CoA (β-oxidation) or ACP (synthesis).

FAS protein, where the phosphopantetheine-accepting serine was replaced by an alanine residue (Ser2156Ala) to allow the establishment of the stable ternary complex [23]. Human PPTase resembles most closely the *B. subtilis* Sfp protein [16]. Two almost identical AcpS-like domains, differing in N/C-terminal extensions of 16 and 42 residues, respectively, are connected by a single residue linker in human PPT [23]. The domain extensions are unique for the human enzyme and may be involved in the proper positioning of the domains for optimal catalysis. The cleft between the two domains serves as ACP binding interface, directing the orientation of ACP by interaction of three hydrophobic patches on the PPTase with hydrophobic "islands" on ACP. The latter is thus positioned to present the phophopantetheine-accepting residue – in the structure an alanine, but a serine in the wild type protein – to the bound CoA. Mg^{2+} and CoA itself gather in a shallow groove at the interface of the two domains, in agreement with the binding of these cofac-

tors in Sfp. Mg^{2+} is coordinated by two acidic amino acid side chains and phosphate groups of CoA, but not required for CoA binding. The pantetheine moiety of the latter apparently points away from the active site in the presence of ACP to prevent collision with this protein. CoA is bound in a bent state, with the pyrophosphate at the apex of the bend. Catalysis of the transfer of the phosphopantetheine moiety is initiated by CoA and Mg^{2+} binding, after which ACP joins the complex.

2.1.2. The reaction mechanism of PPTase

It was suggested that the catalytic residue Glu181 of PPTase abstracts a proton form the ACP-Ser OH group. In this model, the resulting hydroxylate group subsequently engages in a nucleophilic attack on the β-phosphate of CoA, ultimately leading to the cleavage of the pyrophosphate and attachement of the phosphopantetheine to ACP. This mechanism is similar to one of the proposed mechanisms for Sfp [24], and differs fundamentally from the mode of action proposed for AcpS, where a water molecule has been suggested to act as the catalytic base [13,14].

A puzzling remaining question concerns the compartment in which the PPTase modifies mitochondrial ACP. As the human enzyme is not predicted to be imported into mitochondria, and subcellular localization studies place it in the cytosol [5], ACP maturation may have to occur in the cytosol, and mature ACP would have to be imported into mitochondria, in contrast to the situation in yeast, where Ppt2p is a mitochondrial protein. Alternatively, a yet undetected transcript variant from which a mitochondrially localized PPTase can be expressed may exist in humans. Further investigation of this gene and its product(s) is required to determine in which compartment phosphopantetheinylation of human ACP occurs.

2.2. Malonyl-CoA:ACP transacylase (MCAT)

Malonyl-CoA:ACP transacylase (malonyltransferase, MCAT) catalyses one of the early reactions of fatty acid biosynthesis and was first characterized from *E. coli* [25–27]. The *S. cerevisiae MCT*1 gene encoding the putative yeast mitochondrial malonyl-CoA:ACP transacylase was identified by its homology to the bacterial enzyme [28]. Disruption of *MCT*1 in yeast led to respiratory deficient cells lacking mitochondrially encoded cytochromes. The studies with the human ortholog showed that the *Homo sapiens* MCAT was localized in the mitochondria and was specific for the malonyl-CoA substrate [6]. Human MCAT did not form the acetylated intermediate when the enzyme was incubated with acetyl-CoA. Moreover, the enzyme recognized only the ACP of FAS II but not the ACP domain of the FAS I pathway. Therefore, human MCAT transferred the malonyl group exclusively and only to mitochondrial ACP. The first inhibitor of a bacterial MCAT, corytuberine, which acts in a non-competitive fashion was recently reported [29].

2.2.1. The crystal structure of MCAT

To date, crystal structures of MCAT from various bacteria and human have been obtained. While MCATs from different species share a very similar overall fold, there are some differences in the secondary structure elements [30,31]. MCAT is a monomer with its active site located in a cavity between the two subdomains. The larger of these subdomains has a core similar to α/β hydrolases consisting of central parallel β-sheet surrounded by α-helices; some of the helices of this major subdomain form a flap above the core. The smaller subdomain has a ferredoxin-like fold with a four stranded anti-parallel β-sheet shielded by two α-helices as seen also in acylphosphatases. In the liganded structures, the two subdomains are located closer to each other when compared to their position in the apo enzyme [32,33], suggesting a slight hinge motion upon substrate binding similar to the rearrangement observed with Etr1p of FAS II pathway [34].

There are few interesting fine details in the active site of MCAT. The serine acting as the nucleophile is located at the end of a sharp turn between a β-strand and the N-terminus of the α-helix in a major subdomain. This structure resembles the so called nucleophilic elbow characteristic of α/β hydrolases [35]. The positive electrostatic potential of the N-terminus of the α-helix together with the localization of the serine in the tight turn might favor the deprotonation step of the serine [36,37] needed in initiating the reaction cycle. A second observation concerns the specificity of the enzyme. The structures suggest that either methionine or serine in a distinct position prevents the binding of α-substituents such as (2S)-methylmalonyl-CoA [32].

2.2.2. The reaction mechanism of MCAT

The MCAT reaction cycle consists of two parts and follows the classical ping–pong bi–bi mechanism involving the His-Ser dyad and resembling the serine protease mechanism [1,25,32,38–40].

During the first half of the reaction cycle the thioester carbonyl of malonyl-CoA is located in the oxyanion hole. At the same time, the histidine abstracts a proton from the serine, turning it into an active nucleophile. The deprotonated serine then attacks the thioester carbonyl of malonyl-CoA, resulting in a tetrahedral intermediate, followed by protonation of the CoA leaving group by a histidine. The malonyl moiety remains covalently bound to the serine via an oxo-ester bond. The acyl-enzyme complex is stabilized by the oxyanion hole and NH1 and NH2 of an arginine that locates close to the active site serine. The side chain of the arginine forms a bidante salt bridge with the carboxylate of the malonyl moiety. The acyl-enzyme complex of MCAT is stable and deacylation occurs only in the presence of specific thiol acceptor. In the second half-reaction, holo-ACP forms a complex with MCAT and injects the 4′-phosphopantetheine group into the active site of MCAT. This time the histidine acts as an acid by accepting a proton from the terminal thiol group of 4′-phosphopantetheine, which becomes a nucleophile and attacks the carbonyl ester of the serine-O-malonyl intermediate forming a second tetrahedral intermediate. Finally, histidine reprotonates the active site serine and malonyl-ACP leaves the active site of MCAT.

Li and co-workers have proposed different mechanisms for the activation of the active site serine. The schemes involve an asparagine which polarizes the serine via a water molecule or a histidine residue [31]; this histidine is distinct from the above-mentioned residue. Both reaction models, however, result in the negatively charged serine acting as the nucleophile.

2.3. 3-Ketoacyl-ACP synthase/condensing enzyme (KAS/CEM1)

KAS catalyses the chain elongation step of the fatty acid synthesis cycle, by which acyl-ACP is extended by a two carbon acetyl-unit. The KAS enzymes belong to the thiolase superfamily, which include also the thiolases [41], the polyketide synthases (the PKS enzymes) [42,43] and the KS-domains of the FAS I megacomplexes [44]. These enzymes have approximately 420 residues per subunit constructed from two core domains which are related by a local two fold axis. The catalytic cysteine, which is a key feature among the members of this superfamily, is located at the N-terminus of a buried helix. This cysteine becomes acylated in the priming reaction of the catalytic cycle of these enzymes. Fig. 3 shows the three steps of this catalytic cycle, (i) the acylation of the catalytic cysteine, (ii) the activation of the substrate (acetyl-CoA in thiolases or malonyl-ACP in KAS-enzymes) and (iii) the Claisen condensation reaction, by which the activated substrate is extended by the acyl unit.

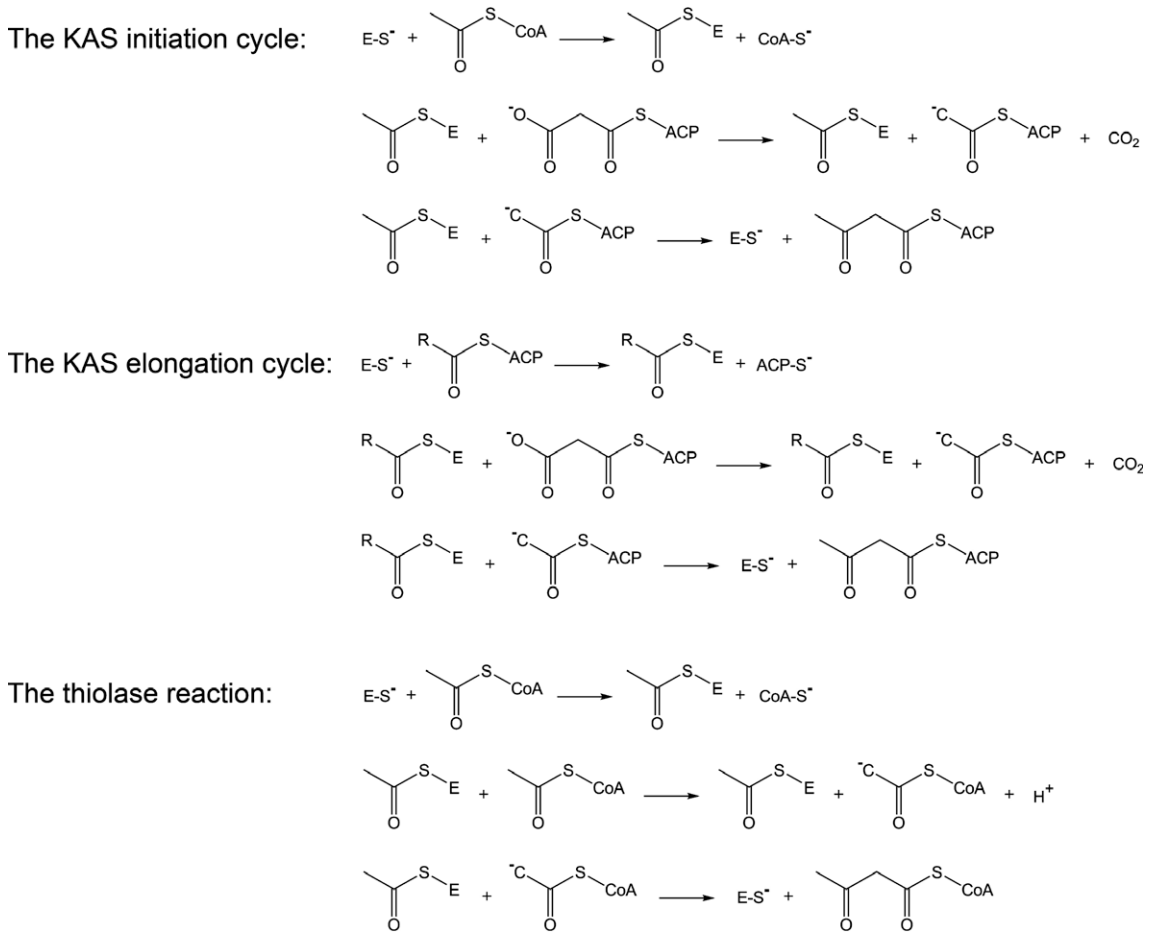

Fig. 3. Reactions catalysed by KAS enzymes and thiolases. The three subsequent reactions catalysed by the KAS enzymes and thiolases are the priming reaction, the activation step and the Claisen condensation reaction. In the priming reaction the catalytic cysteine is acylated. *The KAS initiation reaction*: In *E. coli* the initiation reaction is catalysed by FabH/KASIII. *E. coli* KAS III is primed by acetyl-CoA. The structure of KAS III shows that the *E. coli* KAS III fatty acid binding pocket can only accept short tails. In the acetylated form the enzyme binds malonyl-ACP which is decarboxylated to acetyl-ACP. Subsequently, the activated acetyl-ACP is extended by a Claisen condensation with the acetylated enzyme in the third reaction, resulting in the 3-acetoacetyl-ACP molecule as the product. *The KAS elongation reaction*: In *E. coli* the elongation reaction is catalysed by the FabB/KAS I and FabF/KAS II enzymes. These enzymes are primed with much longer fatty acid tails delivered by acyl-ACP (C4–C14). In the acylated form the enzyme binds malonyl-ACP which is decarboxylated to acetyl-ACP. Subsequently, the activated acetyl-ACP is extended by a Claisen condensation reaction with the acylated enzyme in the third reaction, resulting in the 3-ketoacyl-ACP molecule as the product, which is two carbon atoms longer than the initial, priming substrate. *The thiolase reaction*: In thiolase the substrate for the priming reaction is acetyl-CoA. In the second step a further acetyl-CoA is activated by a deprotonation step for which the base is also a cysteine, Cys378. The acetyl group on the enzyme is subsequently transferred to the activated acetyl-CoA in the third reaction, by which acetoacetyl-CoA is formed. In mammals there are two biosynthetic thiolases, T2 in the mitochondria and CT in the cytosol.

In the FAS II pathway of *E. coli*, three different KAS enzymes are found: KAS I (E.C. 2.3.1.41), KAS II (E.C. 2.3.1.179) and KAS III (E.C. 2.3.1.180) which are very well characterized [1]. KAS I and KAS II are closely sequence- and structure related, but KAS III is more different in fold and function. KAS III is only involved in the first FAS cycle, where the substrates of KAS III are acetyl-CoA (for the priming reaction) and malonyl-ACP, whereas KAS I and KAS II are involved in the subsequent cycles (Fig. 3). The substrate specificities of KAS I and KAS II are overlapping, but KAS II is uniquely responsible for the last extension step of unsaturated C16-enoyl-ACP to 3-keto-C18-enoyl-ACP, whereas KAS I is uniquely required for the synthesis of unsaturated fatty acids [42,45]. Crystal structures are available for each of the three *E. coli* KAS enzymes. These crystal structures concern the unliganded enzymes [46] as well as complexes in which the active site is acylated. Structures are also available of complexes with specific inhibitors, like cerulenin and thiolactomycin. There are no structures of *E. coli* KAS I or KAS II showing the mode of binding of free or acylated ACP or CoA. Only for *E. coli* KAS III the mode of binding of the CoA moiety is known [47,48], but also for KAS III there are no structures known of the complexes with acetyl-ACP or acetyl-CoA.

2.3.1. Crystal structures, active site geometries and reaction mechanisms of KAS enzymes

KAS proteins share many structural features with thiolases, which are abundant enzymes that can participate in several different pathways, either degradative like in β-oxidation, or in biosynthetic pathways, like in the synthesis of cholesterol. The Claisen condensation reaction catalysed by thiolase in the synthetic direction is essentially identical to the reaction catalysed by the KAS enzymes (Fig. 3). The best studied biosynthetic thiolase is the bacterial thiolase of *Zoogloea ramigera*, which uses as a priming substrate acetyl-CoA, like *E. coli* KAS III. There are two important general differences between KAS enzymes and thiolases: (i) thiolases use as priming substrates acyl-CoA molecules (not acyl-ACP) and (ii) the substrate to be extended is acetyl-CoA (and not malonyl-ACP). The precise reaction mechanism of the bacterial thiolase is better understood because crystal structures capturing the various reaction intermediates are available [49]. Therefore, in this review, the active site geometry of the KAS enzymes will be compared with the active site of this thiolase. The key difference between the KAS enzymes and thiolase with respect to the condensation reaction mechanism concerns the activation of the

substrate: in the KAS enzymes the activation of the substrate (malonyl-ACP) is achieved by decarboxylation, whereas in thiolase the activation of the substrate (acetyl-CoA) is achieved by deprotonation. In both cases an enolate intermediate is formed. The deprotonation step requires a base, which in thiolase is a cysteine. The thiolase studies have shown that two oxyanion holes play a key role in the condensation reaction, referred to as oxyanion hole 1 and oxyanion hole 2, as described by [50]. Oxyanion hole 1 facilitates the deprotonation of acetyl-CoA, generating a nucleophilic carbon ion, which attacks the carbonyl carbon atom of the acylated cysteine. This results in a tetrahedral intermediate in which the negatively charged oxyanion is stabilized by oxyanion hole 2, formed by two main chain NH-groups, N(Gly380) and N(Cys89). Oxyanion hole 2 is therefore very similar to the classical oxyanion hole as originally observed in the serine proteases [51]. Oxyanion hole 1 has unique properties, as it is formed by a conserved water molecule as well as by the side chain NE2-atom of His348; such hydrogen bonding partners have not been observed in oxyanion holes stabilizing tetrahedral oxyanion intermediates, but is more common in oxyanion holes stabilizing enolate intermediates, like in this reaction mechanism. The conserved water molecule, Wat82 is hydrogen bonded to the side chain of Asn316, by which the conserved Wat82-Asn316 diad is defined.

Residues Cys89, Asn316, His348 are three important residues for catalysis in the thiolase family of enzymes. These residues are conserved in the superfamily, but it has been noted that the precise nature of the conservation of the corresponding residues is different in different subfamilies, thereby generating three families of enzymes: the CNH-family (the thiolases), the CHH-family (the KAS I/ KAS II enzymes), and the CHN-family (KAS III, and PKS III enzymes) [52,53]. Each of the above described structural features of the thiolase active site can be seen also in the active site of the KAS enzymes, except for the catalytic base. This is also visualized in Fig. 4. However, not in all cases the precise functionality of these structural features of the KAS enzymes has been probed by the available structures. With respect to thiolase it can be noted that oxyanion holes 1 and 2 could be identified because in one of the available structures the complex of acetyl-CoA bound to the acetylated enzyme was captured: in this intermediate the carbonyl oxygen of acetyl-CoA is bound in oxyanion hole 1, whereas the carbonyl oxygen of the acetylated enzyme is bound in oxyanion hole 2 [54].

As there are no structures of KAS enzymes complexed with acetyl-CoA/ACP or malonyl-CoA/ACP, the decarboxylation mechanism of the KAS enzymes remains speculative. It is possible that the proposed binding of the thioester oxygen atom in oxyanion hole 1 sufficiently destabilises the malonyl moiety, such that carbon dioxide becomes an easy leaving group. Alternatively, a nucleophilic water is involved, generating a carbonate ion; in this respect it has been suggested that His298 of KAS I is also important for activating this water [1,55]. In any case, mutagenesis studies of KAS I show that the residues corresponding to Asn316 and His348, respectively His298 and His333, are absolutely essential for the decarboxylation reaction [56], and Rock and coworkers [57] proposed that the His–His pair generates an oxyanion hole to facilitate the decarboxylation reaction, as suggested by binding studies of *E. coli* KAS I and KAS II with cerulenin. It should be noted that in the superposition study of the structure of the intermediate thiolase complex and the KAS-active sites the carbonyl oxygen atom of the thiolase acetyl-CoA is precisely placed at the predicted oxyanion hole (Fig. 4). In the unliganded KAS enzymes a water is seen to be bound at this site. Further experimental binding studies are required to prove that this site corresponds to an oxyanion binding site. Structural studies and mutagenesis studies with KAS III of *E. coli* [47] and Mycobacterium [58] also have been interpreted such that the residues His244 and Asn274 shape an oxyanion hole.

A unique conserved feature in the active site of the KAS I and KAS II enzymes are Lys328 or Lys335, respectively (Fig. 4). Mutagenesis studies with KAS I show that the K328A variant is unable to catalyse the decarboxylation reaction [55,56]. Structural studies show that only small structural changes are introduced by this mutation. The disabling of the decarboxylase activity could be related to these structural changes, and/or due to the change of the electrostatic properties of the active site, or both. Another highly conserved residue is Phe392, Phe400, also shown in Fig. 4. This side chain rotates on acylation of the catalytic cysteine, and it has been suggested that the rotation of this side chain is essential for competent binding of the malonyl-moiety of malonyl-ACP/CoA [59,60]. In the KAS III enzymes the residues corresponding to Lys328 and Phe 392 are not conserved, implying that the details of the reaction mechanism of the KAS III enzymes are different. As shown in Fig. 4, Lys328 superimposes with Asn316 of thiolase (pointing with its NH group to the active site), whereas in the KAS III subfamily this corresponds to a peptide moiety, pointing with its NH-group to the active site (Fig. 4). Phe392/Phe400 is only conserved in the KAS I/KAS II sequences, but not in KAS III, and its side chain is placed near the entrance of the fatty acid binding pocket. On fatty acid binding, this residue changes conformation. The KAS I/KAS II enzymes have an extended fatty acid binding pocket, whereas the synthetic thiolase and the KAS III enzyme only have a small binding pocket, consistent with their substrate specificity.

The KAS I, KAS II and KAS III enzymes of plant plastids have been characterized [61]. More recently, since the discovery of the mitochondrial FAS II, the structural properties of two mitochondrial KAS enzymes have also been reported [60,62] (Fig. 4). A single mitochondrial KAS enzyme catalyses the elongation step for all FAS cycles. This KAS is most closely related to the KAS I/KAS II family, having the CHH-motif of catalytic residues (Fig. 4). The closest homologue is the *E. coli* KAS II. Interestingly, the substrate specificity for the initiation reaction of the human mitochondrial and the plant mitochondrial enzyme is different. The human enzyme requires acetyl-ACP for this reaction [7], whereas the plant enzyme requires malonyl-ACP [45]. It has been found that the catalytic efficiency of the mitochondrial enzyme is optimal for the C6-acyl-ACP and the C12-acyl-ACP [7,45]. In conjunction with the available data on other mitochondrial FAS II enzymes, this highlights two important aspects related to the possible function of mitochondrial FAS II. (i) It agrees with the notion that C8-fatty acids are an important product of mitochondrial FAS II and (ii) mitochondrial FAS II is also capable of synthesis of longer fatty acids up to C14. Protein crystallographic binding studies with the mitochondrial KAS enzymes have shown that a hydrophobic pocket exists for binding these tails. This hydrophobic pocket, which extends towards the dimer interface, is divided into two compartments by Met154, Ile154 in respectively the human, plant mitochondrial KAS enzyme [60]. This residue is proposed to be able to adopt two different conformations, thereby extending the pocket in the case of binding a fatty acid with a longer tail. In the known structures (of the C6-acylated complexes) the pocket is large enough for binding a C6 tail, but a C8 tail cannot be accommodated. A conformational switch of this residue is predicted to extend the pocket and allow for binding of a C12 tail.

2.4. 3-Ketoacyl reductase (KAR)

3-Ketoacyl reductase (KAR) is responsible for the second step of FAS (Fig. 1) in which the C3–O3 carbonyl–oxygen double bond is reduced. The bacterial enzymes utilize NADPH as the cofactor. The best characterized free standing KAR is the *E. coli* KAR, also referred to as FabG [1]. *S. cerevisiae* Oar1 (*Sc*Oar1p) is the yeast mitochondrial homologue of FabG. A yeast *oar*1Δ strain showed a

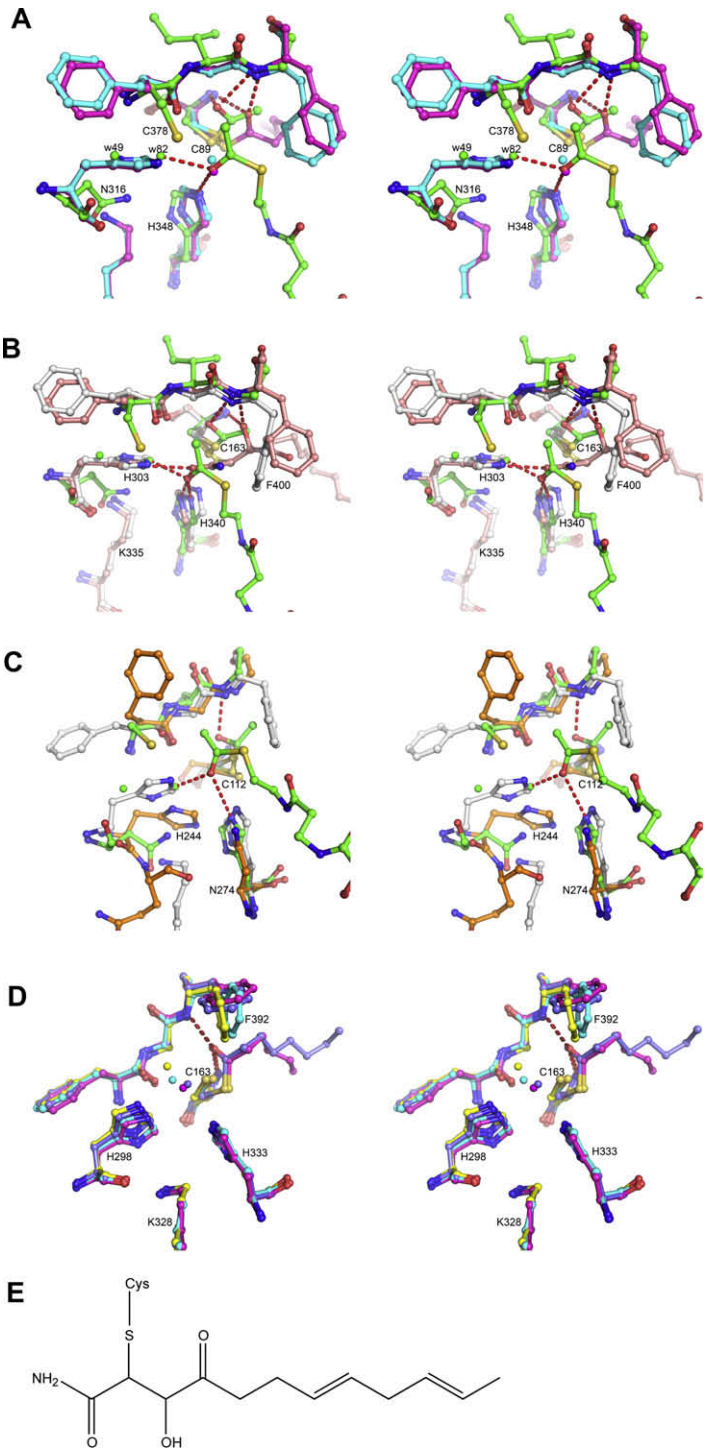

Fig. 4. The catalytic site of the KAS enzymes and comparison with the active site of the bacterial thiolase complexed with acetyl-CoA. The thiolase structure (pdb entry 1dm3) is highlighted in green in A–D. Dotted lines indicate the hydrogen bonding interactions in oxyanion hole 1 and oxyanion hole 2. Active site waters are colored as the corresponding enzyme. (A) Liganded (C6 acylated cysteine, 2iwz, magenta) and unliganded (2iwy, cyan) human mitochondrial KAS. The position of the active site water (cyan) of the unliganded human mitochondrial KAS structure coincides with the oxyanion hole of the thiolase structure. The catalytic residues of the bacterial thiolase are labeled, Cys378 is the catalytic base. (B) Liganded (1b3n, pink) and unliganded (1kas, grey) *E. coli* KAS II. The ligand is cerulenin, the covalent structure of which is also shown in Figure 4E. Note that oxygen atoms of cerulenin bind in oxyanion hole 1 and oxyanion hole 2. The catalytic residues of KAS II are labeled. (C) Unliganded KAS II (1kas, grey) and KAS III (1ebl, orange; this KAS III structure is of the complex with CoA, but CoA is not shown). Note that in this comparison each of the three motifs CHH (KAS II, grey), CHN (KAS III, orange) and CNH (thiolase, green) is included. The catalytic residues of KAS III are labeled. (D) Liganded (2iwz, magenta) and unliganded (2iwy, cyan) mitochondrial KAS compared with liganded (2bui, cyan) and unliganded (2buh, yellow) *E. coli* KAS I. Note the characteristic rotation of the active site phenylalanine and cysteine when comparing the liganded and unliganded structures. The catalytic residues of KAS I are labeled. (E) The covalent structure of cerulenin. Cerulenin is a potent inhibitor of KAS enzymes. It covalently modifies the catalytic cysteine. The picture images the structure of the product of cerulenin as modified by the reaction with this cysteine.

respiratory deficient phenotype and the cytochrome loss phenotype characteristic for mitochondrial FAS II mutants [28]. However, the biochemical properties of *Sc*Oar1p have not yet been determined. A human mitochondrial KAR (*Hs*KAR) was described very recently. Unexpectedly, *Hs*KAR was shown to be heterotetramer consisting of 17β-hydroxysteroid dehydrogenase type 8 and

carbonyl reductase type 4. The NADH-dependent *Hs*KAR share sequence similarity with Oar1p and FabG [10].

Studies of the crystal structures of the apo enzyme and liganded FabG revealed a conformational change of the active site induced upon NADPH binding to the enzyme prior to substrate recognition [63,64]. This suggests an ordered reaction mechanism in which NADPH must bind first to prime the enzyme for acceptance and processing of the substrate. FabG contains a conserved triad of catalytic residues Ser138–Tyr151–Lys155. Although the structure of a ternary complex is missing, the available data suggest that during the catalytic cycle, Tyr151 donates a proton to the substrate O3 atom, whereas NADPH provides a hydride to the C3 carbon of the substrate. The crystal structures of this reductase from *Mycobacterium tuberculosis* and from chloroplasts of *Brasscia napus*, the rape seed plant, have been solved [65]. Each of these reductases belongs to the SDR superfamily and display a tetrameric architecture. In the SDR superfamily each subunit is compactly folded into one domain of about 250 residues. Each domain has a central, parallel β sheet consisting of 7 β-strands covered by α helices, comprising a classical Rossmann dinucleotide binding fold. The Ser–Tyr–Lys triad is conserved in all the above-mentioned enzymes. Detailed structural studies of *E. coli* FabG suggest that a chain of several water molecules acts as a proton wire to regenerate the tyrosine side chain once it has donated its proton to the substrate during the catalytic cycle [63].

2.5. 3-Hydroxyacyl thioester dehydratase (HTD)

The characterized dehydratases of mitochondrial FAS belong to the MaoC dehydratase-like subfamily of thioesterase/thiol ester dehydratase/isomerase (TED1) superfamily. The latter is comprised of bacterial, archeal and eukaryotic proteins with diverse functions from thioester hydrolysis to phenylacetic acid breakdown and transcriptional regulation of fatty acid biosynthesis. The TED1 superfamily consists of 17 subfamilies, two of which are presented by the *E. coli* FabA and FabZ dehydratases. Park and co-authors have shown that the MaoC gene encodes an enoyl-CoA dehydratase involved in polyhydroxyalkanoate synthesis in *E. coli* [66]. The eukaryotic mitochondrial HTD was resistant to computational and biochemical identification approaches. However, in a cloning by function approach, yeast mutants defective in mitochondrial HTD were identified by screening for mutants required to maintain a mitochondrially targeted form of *E. coli* FabA to survive on media containing only a non-fermentable carbon source [67]. The mutations underlying this phenotype were all found to be in the yeast *YHR067w*/*HTD2*gene, encoding a protein which was targeted to mitochondria, as shown by immunofluorescence microscopy studies with an Htd2-protein A fusion chimera. Protein sequence analysis revealed the presence of a MaoC-type amino acid motif, and over-expression of Htd2p resulted in increased hydratase-2 activity in yeast mitochondrial extracts. The *htd2* null mutant yeast was respiratory deficient, unable to maintain mitochondrially encoded cytochromes and experienced mitochondrial morphological alterations similar to other yeast mutants deficient in the mitochondrial FAS pathway, leading to the conclusion that this protein was indeed the yeast HTD.

The identification of human HTD2 did not proceed in the expected fashion, as no apparent homolog to yeast Htd2p or fabA/fabZ could be found by computational means. Instead, cDNAs encoding the human dehydratase were identified yet again by a functional cloning approach, in which the respiratory deficient *htd2*-1 yeast mutant strain was transformed with human cerebellum and kidney cDNA libraries, and transformants were selected for their ability to grow on medium containing a non-fermentable carbon source [3,8]. Surprising, however, was the finding that the plasmids rescuing the *htd2*-1 yeast mutation contained human *RPP14* cDNAs, which encode one subunit of the mammalian RNase P complex. Closer analysis of the cDNAs revealed an additional 3'ORF encoding a human mitochondrial HTD2 (*Hs*HTD2). The *HsHTD2* ORF was shown to be responsible for rescuing the yeast mutation. The encoded protein was overexpressed as a fusion to maltose binding protein, and the recombinant protein exhibited hydratase-2 activity. In addition, a GFP-tagged variant of the protein was shown to localize to mitochondria in HeLa cells. The *RPP14-HTD2* bicistronic mRNA encodes two proteins with seemingly widely disparate roles and is expressed most abundantly in heart and liver, human tissues with robust mitochondrial function. The isolation of the human HTD2 allowed the identification of a highly similar mitochondrial homolog in *T. brucei*, which had evaded characterization before the identity of the human enzyme was established [68]. In addition, the recently characterized *Mycobacterium tuberculosis* 3-hydroxyacyl-ACP dehydratase is also homologous to *Hs*HTD2 [69]. Interestingly, all three proteins resemble the PhaJ type dehydratases involved in polyhydroxyalkanoate synthesis, rather than the *E. coli* FabA or FabZ dehydratases.

The emergence of this unusual bicistronic arrangement in bony fish implies that the mRNA structure has been preserved for 400 million years. Almost all eukaryotic mRNAs are monocistronic, because translation is initiated by the small ribosomal subunit, which scans from the cap along the 5′ UTR in search of the first AUG start codon [70]. How the translation of the *HsHTD2* dehydratase coding sequence is initiated 121 nucleotides 3′ of the RPP14 stop codon is a topic for future investigation. A stunning discovery that may have some bearing on the meaning of the vertebrate arrangement of an RNAse P component linked to a mitochondrial FAS II gene was revealed recently in a publication that describes the necessity of an intact mitochondrial FAS II in yeast for the function of yeast mitochondrial RNAse P [3](see below).

2.5.1. Structures of HTDs

The common feature in all family members is so-called hot dog fold structure [71,72]. 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 the human and the recently identified *T. brucei* mitochondrial dehydratases correspond to the mass of the FabA and FabZ proteins (18.8 kDa and 17.0 kDa, respectively). However, the predicted molecular mass of the yeast mitochondrial dehydratase is 33.1 kDa [67] which is close to the mass of the hydratase-2 part (31.0 kDa) of the peroxisomal multifunctional enzyme type 2 [73]. Yeast Htd2p is therefore predicted to form a "double hotdog fold" similar to the structure observed in the hydratase-2 part of the yeast Mfe2p [74].

2.6. Enoyl thioester reductase (ETR)

The last step of the FAS cycle is catalysed by enoyl thioester reductases. Enzymes originating from three protein families are found to catalyse this reaction in fatty acid synthesis of various organisms and cellular compartments. Thus ETR1s are an example of functional convergence during the evolution. Each of the ETRs, short chain dehydrogenase/reductase (SDR), medium-chain dehydrogenases/reductases (MDR) and TIM barrel families will be discussed shortly below.

SDRs and MDRs are very large protein families. Members of both SDR and MDR catalyse NAD(P)(H)-dependent oxidation/reductions. Around 3000 members of the SDR superfamily, including 63 human proteins, have been defined to date [75]. The "short" refers to the shorter length of the polypeptide chain of SDRs compared to members of the MDR superfamily. SDRs can be further divided into two families, "classical" (~250 residues) and "extended" (~350 residues) [76]. Most SDRs are homodimeric and homotetrameric by quaternary structure but there exist some monomeric

members. SDRs as well as MDRs possess a conserved Rossmann fold that is formed by α/β folding with a central parallel β-sheet sandwiched between two arrays of parallel α-helices, albeit they are divergent in terms of substrate specificities and sequence identities [77,78]. In SDRs, a proton relay system is formed by Asn, Ser, Tyr and Lys, the catalytic tetrad [78]. The tyrosine and lysine residues of the catalytic tetrad are strictly conserved and they are usually arranged as the Tyr–X–X–X–Lys consensus sequence. The separation between the tyrosine and lysine varies from three to seven residues [79]. In the crystal structures of SDR family members, it has been noticed that the location of the lysine residue is almost identical, but the position of the tyrosine residue in the polypeptide sequence is more flexible [80].

Compared to SDRs, the physiological roles of the MDRs are more versatile including metabolic enzymes, detoxification, and regulation [75]. Thus far, approximately 1000 members of the MDR superfamily, among them 23 human proteins have been found [75]. Some MDRs contain one or two zinc ions required either for the catalytic activity or the stabilization of parts of the tertiary structure [81]. MDRs can be divided into eight subfamilies. The eukaryotic mitochondrial ETR belongs to the mitochondrial respiratory function (MRF) subfamily of MDR superfamily. For most of the subfamilies a conserved sequence pattern has been detected. The MRF sequence pattern has a highly conserved Thr–Tyr–Gly–Gly–Met motif [81] involved in cofactor binding [34].

The triose phosphate isomerase (TIM) barrel fold was first discovered in triosephosphate isomerase [82]. The core of the fold resembles a barrel and it consists of eight parallel β-strands; the barrel structure is surrounded by eight helices. The TIM β/α barrel is the second widely shared fold [83].

2.6.1. Prokaryotic ETRs

At least four different ETRs exist in bacteria: FabI, FabK, FabL and the very recently characterized FabV (Table 2). *E. coli* and *B. subtilis* contain FabI an NADH-dependent homotetrameric ETR of SDR superfamily [84,85], whereas *Streptococcus pneumoniae* does not have a homolog of FabI but instead contains a FabK. FabK and FabI do not share any sequence similarities and they belong to different protein families. Moreover, FabK, which is active as a homodimer, requires flavin mononucleotide (FMN) as a second cofactor in addition to NADH [86]. Interestingly, *B. subtilis* was found to have a second form of ETR in addition to FabI, called FabL [85]. FabL is an SDR superfamily enzyme with low similarity to FabI and contains the key catalytic residues arranged in Tyr-X_6-Lys dyad. In contrast to FabI which is NADH-dependent, FabL reduced the double bond only in the presence of NADPH. The nature of quaternary structure of FabL has not been reported yet. The enoyl reductase FabV was characterized from *Vibrio cholerae*. *V. cholerae* does not have homologs of previously identified ETRs. FabV, like FabI and FabL, is a member of SDR superfamily, but about 60% larger than a typical SDR protein, shows a strong cofactor preference for NADH over NADPH [87] and is active as a monomer. Additionally, *M. tuberculosis*, *Pseudomonas aeruginosa* and *Enterococcus faecalis* have been reported to have another set of two ETRs, FabI (InhA) and FabK [88].

Interestingly, triclosan, a chlorinated bisphenol, inhibits FabI-type enzymes by forming a FabI–NAD^+–triclosan ternary complex [89], whereas FabK, FabL and FabV are triclosan-resistant [87]. Moreover, the InhA homologue of FabI in *M. tuberculosis* is inhibited by isoniazid [90]. A phenylimidazole derivative inhibitor (AG205) binds to the catalytic site of FabK and probably competes with NADH [91].

The crystal structures of FabI and FabK have been determined. FabI is a homotetramer with single subunit monomers. The subunit topology shows striking similarity to other enzymes of the SDR superfamily [92]. The structure of FabK is, however, similar to its functional counterpart of the FAS I pathway in fungi [91]. Both monomers of dimeric FabK have a TIM-barrel fold. FabL has been crystallized and a dataset to 2.5 Å resolution has been collected but the crystal structure of FabL has not been published yet [93].

2.6.2. Eukaryotic ETRs

In the yeast mitochondrial FAS system, the reduction of the double bond is catalysed by an MDR enzyme, Etr1p [94], for which the crystals structures of the apo and holo enzymes have been determined [34]. The monomer of homodimeric Etr1p of *Candida tropicalis* consists of two domains, the N-terminal catalytic and C-terminal NADPH cofactor binding domains. The helices and sheets of the cofactor binding domain are assembled into the classical Rossmann fold. *C. tropicalis* contains two isoforms of Etr1p encoded by different genes that differ by only three amino acids [95]. These two isoforms of *Ct*Etr1p form both homodimers and heterodimers. Interestingly, the respiratory deficient phenotype of *S. cerevisiae* strains lacking the Etr1p homolog can be rescued by mitochondrially targeted FabI, a tetrameric SDR, but in contrast to the wild type, these cells become triclosan sensitive when grown on a non-fermentable carbon source [94]. This observation indicated that the enzyme activity per se is more important than the structural properties of the protein complexes *in vivo*. The mammalian ortholog of Etr1p, MECR/ETR1 (also known as nuclear receptor binding factor 1, Nrbf-1) was identified on the basis of similarities to the fungal protein. MECR/ETR1 (also an MDR enzyme) forms a dimer with a molecular mass of 37 kDa for each monomer [9]. The overall structure of MECR/ETR1 is very similar to its yeast functional counterpart, Etr1p. MECR/ETR1 shows a bimodal distribution of catalytic specificity with maxima at C8 and C12 [96]. In the crystal structure of human MECR/ETR1 a deep curved pocket was identified extending from the catalytic site to the interior of the catalytic domain [96]. The pocket is long enough to accommodate fatty acyl moieties of up to 16 carbons chain length. *C. tropicalis* Etr1p also has such a pocket but the shape of

Table 2
ETRs from various FAS systems. Each protein family has different color. FabV shows preference for NADH over NADPH.

Protein	Origin	Protein family	Cofactor(s)	Subcellular location
FabI	Bacteria	SDR	NADH	
FabL	Bacteria	SDR	NADPH	
FabV	Bacteria	SDR	NADH/NADPH	
FabK	Bacteria	TIM	NADH + FMN	
ER of FAS I	Yeast	TIM	NADPH + FMN	Cytosol
Etr1p	Yeast	MDR	NADPH	Mitochondrion
ER of FAS I	Mammalian	MDR	NADPH	Cytosol
MECR/ETR1	Mammalian	MDR	NADPH	Mitochondrion
2E-enoyl-CoA reductase	Mammalian	SDR	NADPH	Peroxisome

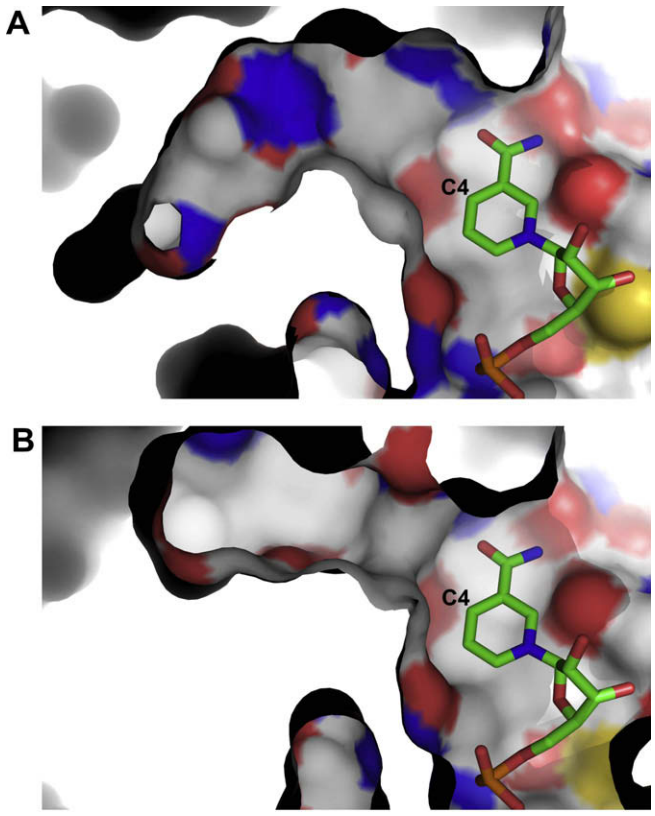

Fig. 5. The catalytic pocket in ETR1. Molecular surface presentations of the active sites of human MECR/ETR1 (A) and *C. tropicalis* Etr1p (B) are presented. The structure of Etr1p was solved in the presence of NADPH. The position of NADPH in the active site of MECR/ETR1 was obtained by superpositioning holo Etr1p on apo MECR/ETR1. The C4 of the nicotinamide group of NADPH is the site of hydride transfer. During the reaction cycle the hydride ion of C4 is transferred to the β-carbon of the substrate. A deep pocket extends from the active site of MECR/ETR1 and Etr1p. The pocket of MECR/ETR1 is approximately 18 Å deep and therefore can accommodate fatty acyl groups of up to 16 carbons.

the pocket differs from the human counterpart in some details (Fig. 5).

Fungal cytosolic FAS I is organized in a way more suitable for the coordinated biosynthesis of fatty acids. Fungal FAS I is a barrel-shaped α6β6 multifunctional complex with a molecular mass of 2.6-megadalton, contains two separated reaction chambers (three active sites per chamber) [97]. The ETR domain of fungal FAS I contains cofactor binding sites for FMN and NADPH [98]. Interestingly, the FMN-binding site of the fungal FAS I consists of a TIM-barrel fold, whereas the NADPH-binding site is formed predominantly by an α-helical domain that is different from a Rossmann fold [97]. Apparently, the TIM-barrel fold is related to FMN binding as exemplified by the prokaryotic FabK ETR. The mammalian cytosolic FAS I is formed by X-shaped intertwined multifunctional α2 homodimer with a molecular mass of 0.54 MDa. Unlike the ETR of yeast FAS I, the ETR domain of the mammalian FAS I resembles MDR enzymes and contains a NADPH-binding Rossmann fold as well as a substrate binding domain. [99].

The peroxisomal ETR in mammals, a monomeric NADPH specific SDR enzyme with a molecular mass of 32.5 kDa, displays a maximum activity with the medium chain length substrate (C10) [100].

3. Acyl carrier protein (ACP) as protein and interaction with FAS enzymes

ACP is one of the abundant small proteins present inside the cell. The protein was first discovered in *E. coli* in the early sixties and

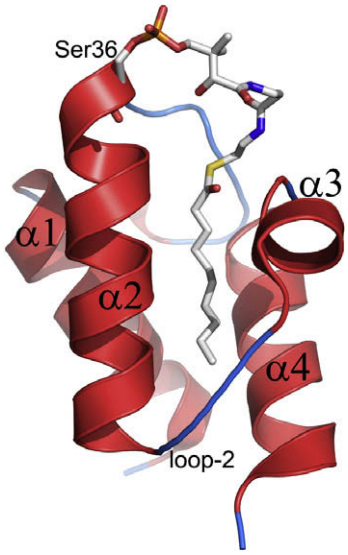

Fig. 6. Crystal structure of ACP. The crystal structure of E. coli acyl-ACP (PDB entry code 2fae). The *E. coli* ACP has four helices (α1–α4) arranged in a bundle; the interior of the bundle is hydrophobic. Upon transforming apo-ACP into acyl-ACP, 4′-phosphopantetheine is first transferred to a conserved serine (Ser36) of the amino acid backbone and then the acyl group is attached to terminal SH-group of phosphopantetheine. In this structure, the hydrophobic core of the protein is occupied by the fatty acyl group of ten carbon atoms and the ω-end of the decanoyl group is pointing toward the loop-2 that is between α2 and α3.

soon after was named ACP [101]. ACP is synthesized as an inactive apo protein and subsequently activate by the transfer of 4′-phosphopantetheine from CoA to a specific and conserved serine of the amino acid backbone (Fig. 6) by PPTase (holo-ACP synthase) [17,102]. The size of the *E. coli* ACP is approximately 9 kDa and the size varies between the species. The ACP of bacteria and plants are very similar both in their primary and tertiary structures [103]. Much less sequence identity is detected between the bacterial and mammalian ACPs [103]. The highest conservation between species on the amino acid sequence level can be observed in vicinity of the serine residue to which 4′-phosphopantetheine is attached.

The first mitochondrial ACP was discovered in *N. crassa* [104]. The 9.6-kDa protein was found to be localized in the peripheral arm of NADH:ubiquinone reductase (complex I) [105,106] of the respiratory chain. Studies with bovine heart mitochondria showed that two forms of mitochondrial ACP, one associated with respiratory complex I and the other as a soluble matrix protein, coexist in mammalian mitochondria [107–109]. The human ACP was also shown to be localized to mitochondria by immunofluorescence detection [6].

ACPs have versatile roles in different organisms. Most of all, ACP acts as a central carrier of fatty acids during their synthesis and utilization; it shuttles the intermediates of the FAS II pathway between the active sites of the other FAS II components. The growing fatty acid chain is attached to the terminal sulfhydryl group of phosphopantetheine, and during the entire reaction cycle the substrate stays attached in ACP. The over-expression of ACP in *E. coli* inhibited the cell growth [110] due to the insufficient attachment of 4′-phosphopantetheine to ACP, resulting in the inhibition of lipid metabolism by excess amounts of apo ACP. In eukaryotes, deletion of the *S. cerevisiae* gene (*ACP*1) coding for mitochondrial ACP led to a 10–20-fold decrease of lipoic acid content compared to the wild-type and concomitant loss of all mitochondrially encoded cytochromes [111] while the general cellular fatty acid composition was unaffected. In *N. crassa*, the loss of ACP caused several defects: complex I was not properly assembled and the lysophospholipid content of the membrane was increased fourfold [105].

The effects of knockdown of ACP expression in human cells are discussed further below.

3.1. The structures of apo-ACP, holo-ACP and acyl-ACP

Tens of NMR and crystal structures of ACP from different species and with different ligands are available at the PDB. Typically, ACPs exhibit a conserved fold with four alpha helices arranged in a roughly 4-fold symmetrical bundle, as exemplified by the *E. coli* protein (Fig. 6). The four-helix bundle consists of three major helices and one shorter helix, helix-3. The helix-2 is termed as a recognition helix [112] because of its important role in contacting the partner enzyme of a complex. The helices enclose a hydrophobic core forming a tunnel that provides a sheath for fatty acid binding. This cavity runs almost parallel to helix-2 and tapers down towards the exit of the tunnel.

The high-resolution crystal structures of butyryl(C4)-ACP, hexanoyl(C6)-ACP, heptanoyl(C7)-ACP and decanoyl(C10)-ACP shows the mode of binding of the fatty acyl tail as they occupy the hydrophobic core of the four-helix bundle [113,114]. In all cases the acyl chain adopts an extended conformation and the omega end of the fatty acyl tail points toward the loop-2 after helix-2 (Fig. 6). A slightly different binding mode of the acylated 4′-phosphopantetheine group was found in hexanoyl-ACP and heptanoyl-ACP crystals compared to butyryl-ACP and decanoyl-ACP structures. In these cases the thioester-linked acyl chain located approximately 5 Å deeper in the tunnel. When the sizes of the hydrophobic tunnel-like cavities were compared between the unliganded and liganded ACPs, the volume increased from close to zero to 164 $Å^3$ and largest expansion was observed with decanoyl-ACP [113]. This volume change was referred to as a hydrophobic cavity expansion and gradual swelling of the global protein fold. The swelling did not affect changes in the secondary structures or the overall protein fold.

3.2. Interaction of ACP with FAS enzymes

The docking studies with the NMR structure of *E. coli* ACP with the crystal structure of *E. coli* FabH (KAS III) [115] indicated that ionic interactions are important in the recognition procedure upon complex formation. To identify the residues of ACP that undergo conformational changes upon formation of the complex with FabG (KAR), a chemical shift perturbation method was employed [112]. The largest changes were observed for the residues of the recognition helix, including serine that locates at the N-terminus of this helix and is the attachment point for the covalent 4'phosphopantetheine modification of holo-ACP. The changes extended also to loop-2 next to helix-2. Especially the position of isoleucine of loop-2 was significantly perturbed.

The structure of the *E. coli* ACP bound to FabI (ETR) was the first crystal structure of a FAS II complex [116]. In this complex, however, the density for most of the side-chains in ACP were missing and therefore the authors used computational method to fill-in the missing details. The results showed that the binding of ACP did not alter the structure of the partner enzyme and also that the complex structure was predominantly stabilized through hydrogen bonding interactions between the acidic residues of the recognition helix of ACP and the basic ones on the partner.

There exists, however, a crystal structure of a FAS II complex in which ACP is involved and completely visible; it is a structure of *B. subtilis* holo-ACP bound to the AcpS holo-ACP synthase/PPTase [13]. The nature of the contacts between holo-ACP and AcpS are very similar to the interactions of complexes of ACP with FAS II components. Specifically, almost all of the residues of ACP involved in the hydrophilic contacts are located within the recognition helix. There are, however, few additional significant hydrophobic contacts.

The structures of the complexes show that the protein–protein interactions are not very extensive. The weak affinity is expected because ACP has to reversibly interact with various kinds of enzymes [103]. Most likely, the interactions will become more specific when the substrate molecule starts to contribute to interactions [113]. Moreover, ACPs have been found to be functionally interchangeable in the reactions of fatty acid metabolism between different species [117].

In the FAS I system, ACP is an integral part of long polypeptide chain. Very recently, the crystal structures of FAS enzymes have been determined from pig and *S. cerevisiae*, but only in the structure of the *S. cerevisiae* FAS I complex the ACP domain was visible [44,118]. This domain is located close to catalytic cleft of the KAS domain, with its phosphopantetheine part injected into the catalytic cleft of KAS. Therefore, this liganded structure of ACP differs from the *E. coli* acyl-ACP structures where the fatty acyl moiety is localized inside the ACP. The *E. coli* acyl-ACP structures may represent the delivery form of acyl-ACP during the transport of fatty acyl intermediates between the active sites of FAS II components. The structure of *S. cerevisiae* holo-ACP may then be the conformation in the "active" complex. The ACP of FAS I is surrounded by the long flexible linkers which allow the domain to shuttle a substrate molecule between active sites. Because of the high mobility of the linkers, they are not seen in the electron density maps [118].

4. The source of mitochondrial malonyl-CoA

The generation of malonyl-CoA is the first committed step of fatty acid synthesis, and the issue of the source of this compound used in yeast and higher eukaryote mitochondrial FAS is still surrounded by a number of intriguing questions and observations. The canonical mode of synthesizing malonyl CoA proceeds via the ATP-dependent condensation of acetyl-CoA and bicarbonate and is performed as a two-half step reaction by acetyl-CoA carboxylase (ACC). This enzyme is composed of two catalytic domains, the biotin carboxylase (BC) and carboxytransferase (CT), and one structural domain, the biotin-carboxy carrier protein (bcc). In the first, ATP requiring halfstep of the reaction, biotin is carboxylated by BC. Biotin, covalently attached to bcc, acts as a "swinging arm" cofactor to deliver the carboxy group to the CT domain, where the CO_2 – is transferred to acetyl-CoA. In bacteria, the three domains are formed by individual polypeptides. *E. coli* ACC is a heteroheptamer composed of one BC polypeptide, a dimeric BC and a heterotetrameric ($\alpha 2\beta 2$) CT [119]. The higher plant plastid FAS system appears to rely on an enzyme of similar structure. Plastid ACC however, has been reported to be membrane associated, and gel filtration studies indicate that these plant enzyme complexes may form higher order structures than the corresponding *E. coli* enzyme [120,121]. Overproduction of the ACC in *E. coli* results in a sixfold increase of FAS activity, indicating the potential for an important role of this metabolic step in the regulation of FAS.

The first mitochondrial ACC was identified in grasses as a protein similar to eukaryotic cytosolic ACCs [122], which are large enzymes carrying all ACC functions on a single polypeptide. Only one year later, yeast mitochondrial ACC was found to be encoded by the *HFA*1 gene [123]. Like the plant enzyme, Hfa1p resembles eukaryotic cytosolic ACCs (Acc1p in yeast). Deletion of *HFA*1 results in growth deficiency on lactate and ethanol, but not on glycerol. A remarkable feature of *HFA*1 is that the ORF encoding the ACC apparently starts far 5′ to the first ATG of the sequence. Translation of this sequence in frame with this codon reveals that homology of Hfa1p to Acc1p extends for at least 153 base pairs in the 5′ direction. Furthermore, the DNA sequence preceding the ACC-homology

region encodes a polypeptide predicted to form a mitochondrial import signal. It was shown that an *HFA*1 construct missing the sequence encoding the putative mitochondrial localization signal but including the sequence homologous to *ACC*1 was capable of rescuing the *acc*1Δ strain growth defect, indicating that *HFA*1 indeed encodes a functional ACC, but was not sufficient to complement the *pet* phenotype of the *hfa*1Δ strain on lactate. Conversely, a construct containing the predicted mitochondrial import sequence complemented the *hfa*1Δ phenotype but could not rescue the lethal phenotype of the *ACC*1 deletion. Therefore, it is clear that translation of *HFA*1 in yeast must be initiated more than 60 codons upstream of the annotated initiation ATG. Hoja and coworkers proposed that *HFA*1 was derived from an ancestral ACC, one copy of which diverged after the genome duplication in the *S. cerevisiae* ancestor, obtaining a mitochondrial import sequence [123].

A second mystery is the only partial respiratory deficiency of the *hfa*1Δ yeast strain. Deletion mutations in any one of the other mitochondrial FAS II genes leads to complete respiratory deficiency. The ability of the *hfa*1Δ strain to grow on glycerol indicates that there is still some residual function of the Krebs cycle and the respiratory chain, suggesting a secondary source of malonyl-CoA in yeast mitochondria. How this metabolite is generated is not known. The Schweizer group in Erlangen reported about a decade ago that a loss-of-function mutation in the *ACC*1 gene rendered yeast cells respiratory deficient [124]. Although the data is difficult to interpret, as the mutation has not been thoroughly characterized and confers a general fatty acid deficiency to the mutant, it is tempting to speculate that Acc1p can contribute some malonyl-CoA to the mitochondrial FAS II pathway. The route by which this substrate would the mitochondria, however, is not clear. Alternative sources of malonyl-CoA are discussed below.

A mammalian mitochondrial matrix ACC has not been characterized to date. The human genome harbors two genes encoding two distinct forms of ACC (ACC1, encoded by the *ACACA* gene, and ACC2, encoded by the *ACACB* gene). The N-terminus of ACC2 contains a mitochondrial targeting sequence that is immediately followed by a 100-aa hydrophilic stretch. ACC2 has been proposing to be anchored to the mitochondria outer membrane or the contact sites of the outer and inner mitochondrial membranes, with the bulk of the polypeptide facing the cytosol [125]. It is enriched in heart, skeletal muscle and brown adipose tissue [126]. This association with the mitochondrial membrane can be readily reversed, so any isolation procedure for ACC2 should not include a harsh treatment. In previous studies, ACC2 was purified using avidin-Sepharose chromatography from a heart non-mitochondria-homogenate after mitochondria are removed from the homogenate [127]. ACC2 resides primarily in the in cytosol rather than in the mitochondrial matrix or membrane. This cytosol – facing localization of the catalytic site of ACC2 argues against its role as a generator of intramitochondrial malonyl-CoA. The malonyl-CoA produced by ACC2 has been shown to be important as a negative regulator of carnitine/palmitoyl-transferase I and, by extension, mitochondrial fatty acid β-oxidation. In line with this notion, *ACACB* knockout mice exhibited a high fatty acid oxidation rate and a reduced fat content [128]. It was also recently reported that a cytosolically localized shorter isoform of ACC2 lacking the mitochondrial import sequence and the transmembrane domain is expressed in several tissues in mouse, and it was suggested that this form of ACC2 may play a role in lipogenesis [129]. Unless there is a malonyl-CoA import pathway, however, ACC2 cannot be responsible for the synthesis of this metabolite required in the matrix (see below). A recently published compendium of genes with a high likelihood of mitochondrial function, however, lists ACC1 as a candidate [130]. The human *ACACA* gene, encoding the soluble cytosolic ACC, is transcribed from three different promoters, and transcript heterogeneity is additionally enhanced by alternatively spliced variants, yielding in a plethora of different transcript isoforms in humans and mouse [131,132]. The physiological significance of the individual transcript forms is not clear, but it has been shown that mammalian ACC isoforms are regulated by diet and hormones (reviewed in [133]), and it is tempting to speculate about the existence of a mitochondrially targeted isoform. A model case of production two proteins of the same function for localization in different cellular compartments in mammals has been reported already more than 15 years ago for human uracil-DNA glycosylase [134,135].

In *organello* assays carried out with *T. brucei* showed that extramitochondrial malonyl-CoA can serve as a source for intramitochondrial malonyl-CoA [136]. Although no transporter for malonyl-CoA has been reported to date, it is possible that this type carrier exists, for instance, among the members of solute carrier family 25 (SLC25). Leu5p from yeast serves as a coenzyme A transporter across inner mitochondrial membrane [137], and it was recently shown that SLC25A42 from human mitochondria is a transporter of CoA and ADP [138]. As far we know, it has not been published whether malonyl-CoA is accepted as substrate for these or related carriers residing in inner mitochondrial membrane.

4.1. Alternative modes of malonyl-CoA generation

Before the discovery of a mitochondrial acetyl-CoA carboxylase in grasses, it had been suggested that plant mitochondria may not contain an ACC [139,140], which may still be true for dicotyledonous plants, as the protein has not been detected in *Arabidopsis thaliana* [122]. It was therefore proposed that malonyl-CoA may be made available to the plant organelles by synthesis of malonyl-CoA in the cytosol, conversion to malonate by transfer of the CoA to succinate or acetoacetate, import into mitochondria via a dicarboxylic acid transporter [141] and re-activation to malonyl-CoA in the organelles. A malonyl-CoA synthetase activity responsible for this latter reaction has been found in plant mitochondria [142]. In principle, this mode of malonyl-CoA production may also be possible in mammals. The human genome harbors a homologue of the *Rhizobium leguminosarum* malonyl-CoA synthetase, with a high probability of mitochondrial localization. As these enzymes all belong to the acyl-CoA synthetase family of proteins, malonyl-CoA synthetase activity for the human enzyme needs to be established if this pathway should be considered as the source for malonyl-CoA.

A second possible alternative pathway for the generation of malonyl-CoA in humans is via the activity of propionyl-CoA carboxylase (PCC). It has been shown for the PCCs of several organisms that these enzymes can also accept acetyl-CoA as a substrate [143–145]. In mammals, PCC is a mitochondrially localized protein abundant in the heart and has also been demonstrated to metabolize acetyl-CoA [146].

4.2. Comments on biotinylation of apo-ACC, biotin:apoprotein ligase

To date, no investigation addressing the biotinylation of yeast Hfa1p ACC has been published. The only biotin:apoprotein ligase in *S. cerevisiae*, encoded by the *BPL*1 gene [147], has not been shown to enter the mitochondria and is not predicted to harbor an N-terminal mitochondrial import sequence. It has been reported that the lipoic acid content of a *bpl*1 point mutant is similar to wild-type [124]. Like in yeast, the human genome encodes only one biotin:apoprotein ligase (holocarboxylase synthase, HLCS) [148] which is predicted to be localized to the cytosol [149]. However, at least three biotin dependent human enzymes reside in the mitochondria, indicating that there must be either import of the holoenzymes or that a biotin:apoprotein ligase must be present in human mitochondria.

5. Octanoyl-ACP, lipoic acid and other products

All of the mitochondrial FAS II enzymes involved in the acyl chain elongation cycle characterized so far are clearly able to utilize substrates longer than C6 or C8. The physiological relevance of products longer than octanoic acid, however, is not clear. There is some *in vivo* and *in organello* data that appears to confirm the existence of longer chain fatty acid products of mitochondrial FAS II pathway in different organisms. For *N. crassa*, hexanoic and caprylic acid and hydroxymyristic acid, as well as a myristic acid end products attached to ACP were reported [150]. Analysis of [^{14}C] -malonyl-CoA incorporation into fatty acids produced in isolated pea leaf mitochondria indicated that octanoic, hydroxymyristic, lauric, and palmitic fatty acids [140] as well as stearic acid [142] were generated by mitochondrial FAS II. About 30% of the labeled product in pea plant was attached to the H protein of glycine decarboxylase by a DTT-insensitive linkage, leading to the conclusion that a large fraction of mitochondrial FAS II – generated fatty acid was used in the production of the covalently attached lipoic acid cofactor of the H protein [140]. It was subsequently shown in yeast that disruption of *ACP*1 [111] or other yeast mitochondrial FAS genes [8,21,68,123,151,152] all resulted in lipoic acid deficiency in *S. cerevisiae*. Investigation of the mitochondrial FAS products of *T. brucei* suggested the production of fatty acids of up to C16 in isolated mitochondria incubated in the presence of [^{14}C]-malonyl-CoA, and the RNAi knockdown of *T. brucei* mitochondrial ACP resulted in a marked reduction of lipoic acid recovered from these cells [136]. It was also demonstrated that highly purified bovine heart mitochondrial matrix preparations were sufficient to generate fatty acids of up to C14 length from 2-carbon precursors [153]. Octanoic acid was revealed to be one of the main products in these assays, and it was shown that this product could be recovered in the H-protein of the glycine cleavage system after addition of apo-H protein to the mitochondrial matrix extract. Noteworthily, the presence of H-protein itself in yeast is required for lipoylation of Lat1 and Kgd2, the respective E2 subunits of pyruvate dehydrogenase and α-ketoglutarate dehydrogenase [154]. Recently, it was demonstrated in RNAi knockdown experiments in human embryonic kidney cells that downregulation of ACP results in severely impaired protein lipoylation [155]. All this data indicates that the octanoic acid precursor for lipoic acid synthesis is one of the main products of mitochondrial FAS pathway in all the models investigated so far.

The relevance of the longer products generated in this pathway is not clear to date. Disrupting mitochondrial FAS appears to affect the mitochondrial phospholipids composition [105], but it is not known whether this is a direct effect due to decreased channeling of mitochondrially synthesized fatty acids into phospholipids or a pleiotropic effect due to a disturbed mitochondrial metabolism. It has been reported that a mutation in the yeast *CEM*1 gene, encoding the yeast KAS, resulted in a 50% reduction of cardiolipin and a 30% reduction of mitochondrial phosphatidylethanolamine, with a concomitant increase of mitochondrial phosphatidic acid, phosphatidylserine and phosphatidylinositol [156]. A similar observation was made in *T. brucei*, where RNAi knockdown of mitochondrial ACP results initially in an increase of total phosphatidylserine, followed by a return to normal levels and a drop of phosphatidylethanolamine levels to 35% of the level before RNAi [157]. The authors hypothesized that mitochondrial FAS pathway may be required for the repair of lipids damaged by reactive oxygen species. To date, however, incorporation of mitochondrially synthesized fatty acids into phospholipids has not been demonstrated.

As an alternative role for fatty acids longer than C8, it has been suggested that these compounds may be used for protein acylation and serve as a membrane anchor. This possibility has not been investigated thoroughly. A special case of an example for long chain acylation is 3-hydroxymyristyl-ACP itself in its incarnation as a component of complex I of mammalian mitochondria [158]. Future labeling experiments designed to follow the nature and fate of mitochondrially synthesized fatty acids may help to answer these important questions.

5.1. Lipoic acid as a vitamin

A host of pharmacological applications of lipoic acid, ranging from the treatment of the effects of diabetes [159], Alzheimer's disease [160] and mood disorders [161] through relieving oxidative stress in surgery [162] have been described. There is a large body of literature confirming that lipoic acid is a powerful antioxidant which has features of a vitamin for humans [163]. Mammals rely on lipoic-acid dependent enzyme complexes for mitochondrial and respiratory functions. Yet, we are not aware of any evidence that indicates that dietary lipoic acid is used in the activation of these mitochondrial enzyme complexes, and all the data available to date indicate that this may indeed not be the case. It has been shown that mammals have an intrinsic mitochondrial lipoyl activating and transferring machinery [164,165] was well as mitochondrial lipoic acid synthesis machinery [166] which is essential for embryo development in mouse [167]. The deleterious effects of a knockout mutation of mouse lipoic acid synthase on embryonic growth cannot be mitigated by supplementary feeding of lipoic acid to the mother. In addition, the lipoylation defect caused by the RNAi knockdown of human ACP RNAi in human tissue culture cells could not be rescued by supplementation with external lipoic acid [155]. The possibility should therefore be considered that mitochondrially synthesized lipoic acid and lipoic acid taken up as a nutrient may fulfill two different roles in mammalian metabolism, with the former being required for an ancient role in the activation lipoic-acid dependent enzyme complexes, and the latter in an acquired role as a protectant against oxidative damage in the cell. Future studies will shed more light on this issue.

6. Mitochondrial FAS and RNA processing

RNaseP is an essential endonuclease found in bacteria, archae, eukaryotes and their organelles and is responsible for the endonucleolytic cleavage at the 5′-ends of precursor tRNAs to generate the mature form. tRNA processing is crucial for the expression of mitochondrial mRNAs because tRNAs are co-transcribed and distributed between mRNAs. RNase P enzymes typically are ribonucleoproteins and consist of an essential RNA subunit and one or more protein subunits. In humans, the nuclear RNase P complex contains ten protein subunits: RPP14, RPP20, RPP21, RPP25, RPP29, RPP30, RPP38, RPP40, hPOP5, and hPOP1, as well as the H1 RNA component [168]. Yeast nuclear RNase P has a similar composition, but several homologs of human subunits are missing [169]. The RNase P complex found in yeast mitochondria bears more resemblance to prokaryotic enzymes, as it is composed of a single protein subunit, Rpm2p encoded in the nucleus, and the RPM1 RNA component, encoded by the mitochondrial genome. An interesting feature of yeast mitochondrial RNAse P is the fact that its activity itself is required for the 5′ trimming of the RPM1 RNA subunit, a modification step essential for further RPM1 maturation steps and ultimately for yeast mitochondrial RNAse P function [170]. Until very recently, the nature of human mitochondrial RNAse P had been subject to a heated debate. Mammals and many other eukaryotes lack a mitochondrially encoded RNAse P RNA [169], and it was reported that human mitochondrial RNAse P activity was resistant to rigorous RNAse treatment, indicating that the human enzyme did not contain an RNA subunit [171]. In a con-

trasting publication, however, a different group found a mitochondrial RNAase P containing the same RNA subunit as the human nuclear RNAse P [172]. The recent identification and *in vitro* reconstitution of an RNA-free human mitochondrial RNAse P [173] as a "patchwork enzyme" [174] composed of three protein subunits, MRPP1, MRPP2 and MRPP3, the former two presiously annotated with different functions, has put this debate to rest. It is noteworthy within the context of this review that the MRPP2 subunit of the human mitochondrial RNAse P is a highly conserved SDR protein which had previously been suggested to be a homotetrameric enzyme involved in lipid metabolism [175].

Mitochondrial FAS II is linked to RNAse P in both yeast and vertebrates, but the link is distinctly different between these groups of organisms. In *S. cerevisiae*, it has been shown recently that deletion of any one of the mitochondrial FAS II genes will result in a severe impairment of maturation of the RPM1 RNA subunit of mitochondrial RNAse P [3]. It was also reported earlier that a mutation affecting the yeast Lip5p lipoic acid synthase resulted in a defect of processing of mitochondrial tRNA, which may be conveniently explained by a lack of RNAse P function. This matter is complicated, however, by the existence of a positive feedback loop between lipoic acid synthase function and availability of acetyl-CoA, the basic substrate for fatty acid chain elongation, via the activity of pyruvate dehydrogenase, one of the lipoic-acid dependent enzymes [176]. A defect in lipoylation could therefore trigger a shortage of acetyl-CoA in the mitochondria and reduce fatty acid synthesis, which would decrease lipoylation even further, yielding in a downward spiral for acetyl-CoA production and mitochondrial FAS. Hence, it is not clear to date if lipoic acid or mitochondrially synthesized fatty acids themselves cause the tRNA processing defect in *lip5* mutants. It is even less clear how lipoic acid or fatty acids can affect the activity of mitochondrial RNAse P. Two models have been put forward to explain the requirement of mitochondrial FAS II for RPM1 processing in yeast [3]. The first model proposes a direct modification of RNAse P by a product of mitochondrial FAS II, while the second model invokes an indirectly acting fatty acid or downstream product affecting RNAse P assembly or activity. It has been demonstrated that at least the Rpm2p subunit of yeast mitochondrial RNAse P is not lipoylated [3].

The conserved genetic linkage of the vertebrate gene encoding human mitochondrial FAS II *Hs*HTD2 with a subunit of vertebrate RNAse P on the same bicistronic transcript appears even more mysterious. We usually associate the expression of polycistronic transcripts with prokaryotes, where many of the genes are clustered in operons often containing from two to more than ten genes. These operons allow for coordinated expression of proteins with related functions. The RPP14 – *Hs*HTD2 bicistronic transcript is not the first eukaryotic mRNA of this kind described [177]. In the reported cases, the transcripts can be sorted into two different groups depending on the fate of the RNA. In the first type, occurring in *Caenorhabditis elegans* [178] flatworms [179] and some primitive chordates [180], an initially polycistronic transcript is generated which is subsequently converted into a monocistronic mRNA by 3′ end cleavage and trans-splicing [177]. The second type represents a truly bicistronic mRNA encoding two different proteins which both are translated from the same transcript after transport into the cytosol. In mammals, the first bicistronic gene cluster was described by Lee in 1991 [181]. In several cases, alternative shorter variants of the mRNA are produced, containing only the 3′ open reading frame (ORF) where now an internal AUG codon a functions as the translational start site. Such transcripts should be considered functionally monocistronic [182]. Nevertheless, in addition to the *RPP14–HsHTD2* case, at least three more genuinely bicistronic transcripts have been described in humans [181,183–185].

Since it has become clear that RPP14 is not part of the mammalian mitochondrial RNAse P [173], the connection between human mitochondrial FAS II and RNA processing has become even more puzzling. The physical connection of these ORFs on the same transcript has apparently been established early in the vertebrate lineage and maintained for close to half a billion years [8], indicating a possible selective advantage for this arrangement. Linking the expression of a component of the nuclear RNAse P, a key enzyme in the control of general translation and cell division [168], to a component of mitochondrial FAS II, which controls respiration and lipoylation of mitochondrial proteins in mammals [155] may serve to coordinate nuclear gene expression and mitochondrial activity. The change of relationship between mitochondrial FAS II and RNAse P may be a reflection of the fundamental differences between the respiratory requirements of a yeast cell and human cells. Given the propensity of the human mind to want to see connections even where there is none, we should also not close our eyes to the possibility that, as striking this linkage of pathways in yeast and humans may seem it may just be a result of coincidence.

7. Comments and future directions

Although two decades have passed since the identification of ACP from *N. crassa*, the awareness of the mitochondrial FAS II pathway as a cellular process essential for mitochondrial functions also has only risen during the past few years. The KAS enzymes characterized in this pathway accept only acyl-ACPs as substrates, which metabolically separates β-oxidation and fatty acid synthesis in mammalian mitochondria. In principle, the operation of acyl-CoA dehydrogenases and ETR1 could constitute a futile cycle between acyl-CoAs and 2-enoyl-CoAs, but whether this occurs *in vivo* or how exactly it is prevented is not known. The issue whether the mitochondrial FAS II generates other physiologically relevant products than octanoyl-ACP and 3-hydroxymyristoyl-ACP *in vivo* remains an open question. At least the kinetic properties and structural features of the FAS enzymes studied *in vitro* as well as *in organelle* allow the generation of longer products. If these longer fatty acids are made in mitochondria, the destination of the generated acyl groups is an enigma. Do acyl-ACP accepting transacylases also exist in mammalian mitochondria?

Prokaryotic FAS enzymes are frequently used as drug targets as demonstrated by treatment of tuberculosis by isoniazid or use of triclosan. These drugs specifically target prokaryotes, but not because of the absence of a FAS type II in mammals as stipulated earlier, but because their target molecules FabI or InhA in bacteria, which belong to the SDR protein family, were replaced by the structurally different ETR1 MDR protein in eukaryotes. The prokaryotic FAS systems still represent promising drug targets, but the recognition of a similar system also in mitochondria calls for consideration of mammalian proteins. The ability to complement yeast FAS II mutants with prokaryotic of human counterparts may hold some potential to be developed into a tool that allows for a quick assessment of specificity of a bacterial anti- FAS II drug candidate, its ability to penetrate a cell wall and membrane, and its effect on eukaryotic metabolism in general.

As discussed above, the low level of cellular lipoic acid in the yeast strains deficient in functional mitochondrial FAS is probably not the only factors leading to the respiratory deficient phenotype. These cells are lacking mitochondrially encoded cytochromes and they also display impaired mitochondrial RNA processing. The elucidation of molecular link(s) between these processes will provide a challenge in future studies.

Thus far, there has been little evidence linking the mitochondrial FAS pathway to disease in mammals. A recent report on the development of cardiomyopathy in transgenic mice overpressing

Etr1/Mecr [186] now establishes a possible connection between mitochondrial FAS and heart disease. Furthermore, compromising protein lipoylation and respiratory complex I results in cell death in cultured human embryonic kidney 293T cells upon shutdown of ACP using RNAi technology [155]. We predict that the future will reveal novel associations with mitochondrial FAS and diseases in human.

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