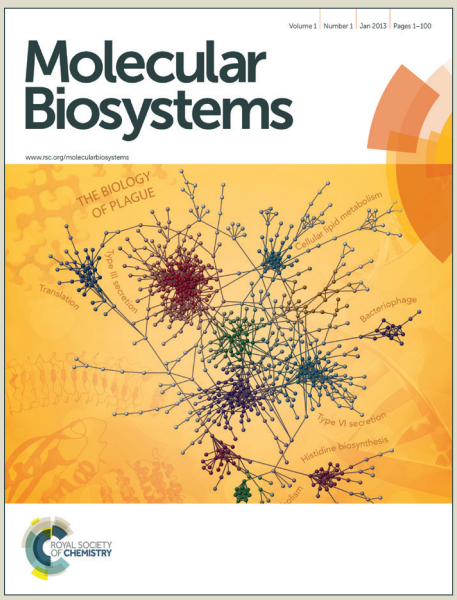
Molecular BioSystems

Accepted Manuscript



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Fatty Acid Biosynthesis Revisited: Structure Elucidation and Metabolic Engineering

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Abstract

Fatty acids are primary metabolites synthesized by complex, elegant, and essential biosynthetic machinery. Fatty acid synthases resemble an iterative assembly line, with an acyl carrier protein conveying the growing fatty acid to necessary enzymatic domains for modification. Each catalytic domain is a unique enzyme spanning a wide range of folds and structures. Although they harbor the same enzymatic activities, two different types of fatty acid synthase architectures are observed in nature. During recent years, strained petroleum supplies have driven interest in engineering organisms to either produce more fatty acids or specific high value products. Such efforts require a fundamental understanding of the enzymatic activities and regulation of fatty acid synthases. Despite more than one hundred years of research, we continue to learn new lessons about fatty acid synthases' many intricate structural and regulatory elements. In this review, we summarize each enzymatic domain and discuss efforts to engineer fatty acid synthases, providing some clues to important challenges and opportunities in the field.

Introduction

Sparked by renewed interest in biofuels, green chemicals, and antibiotic research, the past decade has seen new research on fatty acid synthases (FASs). Although innovative technologies in petroleum discovery have temporarily met some of our demand for oil,[1] these appear to provide short term solutions for increasing hydrocarbon demands. Our future requires alternative energy sources not only

to replace liquid fuels, but also to provide renewable feedstocks for chemicals and consumables.[2] With current capital investments focused on petrochemical processes, a direct petroleum replacement, or "drop-in" solution, would prove most attractive. Crude oil is a fatty-acid rich mixture of hydrocarbons formed over millions of years from ancient biomass. One critical challenge of our time is to harness the power of photosynthesis to produce large quantities of fatty acids on a much shorter timeframe. In order to improve yield or design tailored products, we must understand the underlying metabolic process of fatty acid biosynthesis and its peripheral partners.

Nature produces fatty acids for biological scaffolding, such as cell walls, membranes, and protein modification; as energy storage, such as triacylglycerides; and as building blocks for primary and secondary metabolites including biotin and lipoic acid. All organisms make fatty acids using highly conserved chemistries. Typically, fatty acid biosynthesis begins with acetyl-CoA, carboxylation produces the malonyl-CoA building blocks that are subsequently condensed and reduced in an iterative fashion until the fatty acid chain matures for use by the cell. In bacteria, plants, and algae the different enzymes that catalyze this cycle are expressed as discrete proteins (type II), whereas non-plant eukaryotes use a FAS in which all functionality is supplied by multi-domain megasynthases (type I) (Figure 1). In fungi, the synthase is encoded on two genes and assembles as a heterododecamer of 2.6 MDa, whereas the human FAS is encoded on one gene and forms a homodimer of 540 kDa. It is believed that type I synthases derive from an ancient gene fusion event of type II enzymes.[3] Most eukaryotes harbor an additional type II FAS in their mitochondria, presumably dedicated to the biosynthesis of lipoic acid.[4]

Both type I and type II FAS rely on a small protein, the acyl carrier protein (ACP, Table 1), to shuttle the fatty acid cargo from enzyme to enzyme. ACP must be activated via post-translational modification by a phosphopantetheinyl transferase (PPTase) to install a coenzyme A (CoA) derived 4'-phosphopantetheine arm (PPant) (see Figure 3). The terminal thiol forms the tether upon which the growing fatty acid chain is iteratively extended by ketoacyl synthases (KS, Table 1), reduced by ketoacyl ACP reductases (KR), dehydrated by dehydratases (DH), and further reduced by enoyl ACP reductases (ER), until being offloaded by dedicated thioesterases (TE) or acyltransferases (AT) (Figure 10).

Archaea are well known to lack traditional fatty acid-based lipids so it was long assumed that they did not have an FAS. However, a non-typical FAS was recently discovered in archaea that appears to be ACP independent (Figure 1).[5, 6] This discovery may offer an opportunity to study FAS in a unique regulatory and metabolic environment.

In this review we focus on what is known about the different enzymatic domains, their structure and function, and protein-level engineering efforts. Unless otherwise stated discussion will focus on the FAS pathway from *E. coli*, the most well studied system. Finally, we briefly discuss metabolic engineering of the fatty acid synthase for the production of fuel or other products.

Table 1 – Fatty acid synthase enzymes and their common abbreviations.

Abbrv1	Abbrv2	Name	E.c.	Ref
ACP	AcpP	Acyl carrier protein	-	[7-9]
AcpH	AcpH	ACP phosphodiesterase, ACP hydrolase	3.1.4.14	[10]
AcpS	AcpS	ACP synthase, phosphopantetheinyl transferase	2.7.8.7	[11]
DH	FabA	β-Hydroxydecanoyl-ACP dehydratase II	5.3.3.14	[12]
KS1	FabB	β-Ketoacyl-ACP synthase I	4.2.3.14	[13, 14]
-	FabC	Acyl carrier protein (old name, found to be AcpP)	-	-
AT	FabD	Malonyl-CoA:ACP transacylase	2.3.1.39	[15]
AccB	FabE	Biotin carboxyl carrier protein of ACCase	-	[16]
KS2	FabF	β-Ketoacyl-ACP synthase II	2.3.1.179	[14]
KR	FabG	β-Ketoacyl-ACP reductase	1.1.1.100	[17]
KS3	FabH	β-Ketoacyl-ACP synthase III	2.3.1.180	[18, 19]
ER	FabI	Enoyl-ACP reductase I	1.3.1.9	[20]
-	FabJ	Ketoacyl synthase (found to be FabF)	-	[21]
ER	FabK	Enoyl-ACP reductase II (e.g. *Streptococci*)	1.3.1.9	[22]
ER	FabL	Enoyl-ACP reductase III (e.g. *Bacilli*)	1.3.1.104	[23]
-	FabM	cis-3-decenoyl-ACP isomerase	5.3.3.14	[24]
-	FabQ	Dehydratase/isomerase	n/a	[25]
-	FabR	Transcriptional repressor	-	[26]
-	FabT	Transcriptional regulator	-	[27]
ER	FabV	Enoyl-ACP reductase IV (e.g. *Vibrii*)	1.3.1.9	[28]
DH	FabZ	β-Hydroxyacyl-ACP dehydratase I	4.2.1.59	[12]
TE	FatA/B	Acyl-ACP thioesterase	3.1.2.14	[29-32]

<Figure 1>

Figure 1 – Comparison of FAS domain organizations. a) Mammalian type I synthase (PDB: 2VZ8) structure with a diagram below of the domain organization. Domains that were not observed in the crystal structure are denoted by hashed borders. b) *E. coli* type II discrete structures with a comparative diagram below denoting multimeric states of the proteins. (PDB: KR, 1Q7B; DH, 1MKB; ER, 1DFI; ACP, 2FAD; KS, 2VB9; MAT, 2G2Z) c) Two fungal type I synthases demonstrating increased scaffolding elements and organization compared to the mammalian type I synthase in (a). At left, top-view cross-section of FAS from yeast *Saccharomyces cerevisiae* (PDB: 2UV8). At right, thermophilic fungus *Thermomyces lanuginosus* (PDB: 2UVB, 2UVC). d) Domain organization for the ACP-independent type II-like FAS discovered in Archaea, from which no structures are currently available.

FAS transport: ACP

Central to fatty acid biosynthesis, ACPs transport and present the growing acyl chain to appropriate reaction partners for elongation and fatty acid production. These relatively small proteins exhibit α-helical bundle topography and are dynamic in nature, making them challenging to study by crystallography.

ACPs require dynamic movement because they are acted upon by many enzymes, each of which is a binding partner. ACPs from type I synthases are tethered to the megasynthase by flexible linkers in the peptide chain, allowing the ACP to sample enzymatic domains for biosynthesis. ACPs from type II synthases, however, are discrete proteins that must deliver intermediates to independent catalytic partners. Due to its central metabolic role and vital interactions with many partner proteins, ACP is both a prime target and an important consideration for metabolic engineering efforts.

Because type I FASs are so large, much structural information is based on individual domains cloned out of the full synthase. A few such truncated ACP structures are available, such as the rat ACP (PDB: 2PNG)[33] and the human ACP in complex with its PPTase (PDB: 2CG5).[34] One 3.2 Å crystal structure of the mammalian type I FAS is available with five domains resolved (PDB: 2VZ8), but the ACP and TE remained unresolved.[35] This structure demonstrates a global "ginger-bread man" quaternary structure facilitating the interaction of the ACP with the partner domains (Figure 1).

An organizationally different approach is observed in the fungal type I FAS, acquired at 3.1 Å resolution forming a large "soccer-ball" heterododecamer ($\alpha_6\beta_6$) (PDB: 2UVB).[36] In this structure three ACPs are tethered in each of two chambers within the protein, with three sets of enzymatic domains lining the chambers. The distances between reactive domains require that ACPs travel up to 130 Å, which is

facilitated by flexible linkers.[37] Extensive computer simulation work found that each ACP bounces stochastically within this chamber, but with asymmetric probability observed for multiple ACPs to simultaneously interact with equivalent catalytic sites.[38] The authors conclude this to be an entropic phenomenon and hesitate to suggest cooperativity between subunits. The yeast type I FAS (PDB: 3HMJ)[39] offers a similar barrel shape with a different domain arrangement, further studied by electron microscopy (EM).[40] A larger reaction chamber than the fungal FAS was observed by crystallography, and observed variable domain occupancy by the ACPs further suggests a stochastic role for the ACP within type I complexes. An excellent current review by Grininger discusses type I structure and the evolution of these megasynthases.[41]

Available structures of ACPs from type II synthases demonstrate a different approach to the challenge of processivity. Higher copy numbers, up to 0.25% of soluble protein,[42, 43] are observed. In order to transport reactive intermediates through the cytosol, type II ACPs protect their cargo by sequestration within a central hydrophobic cleft. Sequestration satisfies multiple roles: protecting the thioester linkage from hydrolysis and premature product release; shielding reactive intermediates from side-reactions; providing a limiting “ruler” to control final metabolite size; and inducing conformational changes in the ACP that trigger downstream catalytic steps.[44]

The nature of sequestration has been studied from many different approaches. Looking into the hydrophobic pocket, crystallographic studies of acylated ACPs yielded several structures showing an expandable hydrophobic pocket within the helical bundle accommodating the growing chain (PDB: 1LOH, 2FAC, 2FAD, 2FAE).[45] This was also observed by mutagenesis, which found a chain length dependence on ACP fatty acid stabilization.[46] Solution-state NMR studies of a spinach ACP loaded with decanoate and stearate found that the hydrophobic pocket could comfortably sequester ten carbons, but no conformational changes allowed it to successfully sequester the full stearate.[47] The 18 carbon chain forms a flexible hairpin structure, which may represent a recognition motif for thioesterase activity. In contrast with these type II systems, the type I mammalian ACPs do not sequester the acyl chain as demonstrated by NMR.[33] Acyl states of the ACP showed no chemical shift perturbations when compared to the *holo* state, and Nuclear Overhauser Effects (NOEs, spin-spin coupling through space observed by NMR) between the ACP and the acyl chain were not observed.

Recent work from our group provides the first mechanism-based structural glimpse of how a sequestered intermediate in the hydrophobic pocket is presented to a partner protein (PDB: 4KEH).[48] The *Escherichia coli* ACP was covalently crosslinked to a DH, FabA (Table 1), by a mechanism-based

PPant probe that mimics the natural chemistry. In the structure, the long helix II of the ACP is anchored by electrostatic interactions with the DH, while an arginine patch pulls helix III away. This allows the translocation of the acyl chain from the hydrophobic pocket of the ACP into the active site of the DH. This translocation (see Figure 2) has been called the 'switchblade mechanism', a mnemonic introduced by Ban and co-workers in 2006.[49] Recently, Cronan argued against this nomenclature, indicating that a 'switchblade' suggests an active expulsion of cargo from the inner core of the ACP for which there is no evidence.[44] Thus, 'chain-flipping' has been adopted as a more accurate representation of this unique carrier protein behavior.

There are seven other structures of ACPs interacting with partner proteins available (PDB: 3EJB, 3EJD, 3EJE; 4IHF, 4IHG, 4IHH; 4ETW; 3NY7; 2XZ0; 2FHS; 1F80; 2CG5). Remarkably, all show strong interactions with helix II and significant structural perturbation of helix III. Indeed, in the crystal structure of *E. coli* ACP in complex with ER (PDB: 2FHS) much of the ACP is too dynamic to be resolved, with the exception of helix II.[50] The binding interaction of ACP with its partners appears to be very consistent, based on currently available structural data (see Figure 8).

A key question of processivity thus arises: does the ACP in type II FAS communicate the status of its cargo to prevent unproductive interactions or enhance productive ones? NMR studies of ACPs loaded with short biosynthetic intermediates found that helix II was conformationally consistent regardless of the intermediate, but significant changes were observed at the C-terminus of helix III.[51] The authors conclude that while helix II must be used for docking, close interactions with helix III and the PPant arm may be used to communicate the cargo's status.

Foundational work on the biochemical properties of ACPs[46] identified differences between the *Vibrio harveyi* ACP and the *E. coli* ACP. Divalent cations were critical for only the *V. harveyi* ACP structure at neutral pH. Later, NMR studies comparing the *V. harveyi* and *E. coli* ACPs confirmed both the importance of divalent cation binding and implicating a histidine near the C-terminus.[52] Mutation studies found that acyl-ACP synthetase functionality is dependent on the ACP structure and the presence of a hydrophobic pocket.

The first successful chimeric ACP produced was used to functionally replace the root nodulation protein NodF in Rhizobium,[53] a carrier protein unrelated to the primary FAS. The chimera was composed of both the *E. coli* ACP and the NodF gene, with large portions of the proteins requiring changes to achieve functionality. This study clearly demonstrated that protein-protein recognition is necessary for these

systems to function, and that such protein-protein recognition will need to be considered in FAS engineering efforts.

Work generating peptidyl carrier protein (PCP) ACP chimeras produced a *Bacillus subtilis* PCP carrying the recognition helix from the *B. subtilis* ACP.[54] This work benefitted from co-crystal structures between the carrier protein and the PPTase, which clearly showed the arrangement of residues involved in recognition. Functionality was established through the ability of these PPTases to convert the *apo*-chimera to the *holo*-form. Although tempting to attempt to generate a "universal" carrier protein, structural research on the carrier protein[55] suggests that mutagenizing partner proteins will be more successful.

Another approach is to create a "minimal" carrier protein with which to study truly essential protein-protein interactions. Found in a *B. subtilis* open reading frame, the 11 amino acid sequence dubbed "ybbR" maintains an alpha-helical structure in solution and offers the shortest polypeptide tag identified that can be acted on by PPTases.[56, 57] YbbR and similar domains are widespread in other species and may be involved in cell cycle regulation.[58] Further studies are needed to explore ybbR as a true "minimal" ACP and not just a PPTase substrate.

In *E. coli,* ACP interacts with SpoT during carbon starvation, triggering (p)ppGpp accumulation and the stress response of the organism. [59, 60] SpoT is a bifunctional enzyme that can both synthesize and degrade the alarmone guanosine penta- or tetraphosphate (p)ppGpp, which is involved in the stringent response in bacteria causing the inhibition of RNA synthesis and conservation of amino acids. The interaction between SpoT and ACP only occurred with functional ACP suggesting that the ratio of acyl-ACP to *holo*-ACP may be sensed by the bacteria. Studying the ACP as a regulatory target complements earlier work demonstrating that the *apo*-ACP would inhibit cell growth by stopping acyltransferase activity in lipid metabolism.[61]

ACP is an essential protein central to metabolism and organism growth, which presents a challenge for studying modifications *in vivo*. A temperature-sensitive ACP mutant and an ACP knockout carrying an arabinose-controlled supplementary ACP were generated.[62] Interestingly, three of the four residues identified as critical when generating these strains were also identified by Burkart and Tsai in the ACP-DH structure as critical to ACP-DH interaction.[48] The ACP knockout strain allows for complementation studies *in vivo*. *V. harveyi* ACP can complement the absence of endogenous ACP in *E. coli*, both spinach[63] and algal ACP cannot.[32] The algal ACP interacts efficiently *in vitro* with an *E. coli* KS, suggesting that a

different protein-protein interaction in its fatty acid biosynthesis is non-productive. Whether this is determined by the ACP dynamics or the partner protein recognition, remains a question.

Early NMR studies of a spinach ACP observed a slow exchange between two structures.[64] This was confirmed by later studies that observed an exchange between a folded and an unfolded state that was altered by the acylation state of the ACP.[47] It might well be that these subtle differences in the ACP structure govern the processivity of the fatty acid synthase. The defining feature of an ACP is flexibility; in substrate, structure, and partner. The subtle communication in this flexibility cannot be overlooked, but offers opportunity and challenge, expanding both the potential of successful engineering and the strict requirements for any engineering to be successful.

Several detailed reviews of the ACP are available. A modern and exhaustive review was published by Crosby and Crump in 2012,[9] a broader review was published by Chan and Vogel in 2010,[7] and an excellent comparative review was published by Byers and Gong in 2007 looking at sequence and structural relationships.[8]

Challenges and Opportunities

It is clear that ACPs must be considered in any efforts to engineer FASs. A more complete understanding of ACP's many roles is necessary, which will require additional co-crystal and NMR studies. For true processive engineering, our understanding of ACP's structure when alone needs to be complemented by an understanding of the ACP's structure when with a partner.

<Figure 2>

Figure 2 – Cartoon of cargo sequestration and chain-flipping mechanism by acyl carrier proteins. a) the acyl carrier protein (ACP, grey protein cartoon structure) is post-translationally modified with a 4'-phosphopantetheine arm, bearing a terminal thiol. b) when a fatty acid intermediate (yellow rectangle) is loaded onto the 4'-phosphopantetheine moiety, it is protected from the environment by sequestration in the inner core of the ACP. c) a partner protein (orange) binds to the ACP. d) the protein-protein binding event induces a conformational change in the carrier protein, allowing the cargo to flip out of the inner core into the active site of the partner enzyme (the "chain-flipping mechanism"). e) chemical transformation of the fatty acid intermediate (yellow > red) occurs in the active site of the partner enzyme. f) after the reaction is complete the partner enzyme leaves, and another partner protein (blue) can bind to the ACP.

Modification of ACP: PPTases and AcpHs

ACPs transport the growing fatty acid chain by a covalent thioester tether. This thioester linkage is at the end of a PPant arm, which is post-translationally attached to the ACP (Figure 3).[11] A dedicated PPTase is responsible for this modification, transferring PPant from CoA to a conserved serine residue on *apo*-ACP, forming *holo*-ACP. The PPTase is essential for "activation" of carrier proteins and biosynthesis to occur.

Almost every organism maintains at least one PPTase, and often multiple PPTases are found. PPTases are classified in three different subfamilies. The AcpS-type PPTases, which show limited promiscuity to their carrier protein and CoA substrates, are always involved in activating carrier proteins of type II FAS. The Sfp-type PPTases, named for the archetypical surfactin synthase activator in *B. subtilis*, show highly promiscuous behavior and are therefore used in many heterologous synthase expression studies. Finally, in some type I FAS megasynthases (e.g. from fungi) the PPTase is part of the synthase. The use of the PPant arm is not limited to FAS, but is also observed in secondary metabolism including polyketide- and non-ribosomal peptide synthases. We have recently published a comprehensive review on the superfamily of PPTases, encompassing many challenges and opportunities.[11]

In the field of metabolic engineering, PPTases have not surfaced as a prime target. This might be because the endogenous PPTase activity is sufficient, and thus not rate limiting. For example, *E. coli* has three PPTases: "AcpS" for FAS, the Sfp-type "EntD" for the siderophore enterobactin synthase, and the Sfp-type "AcpT" with currently unknown function. Endogenous *apo*-ACP is toxic to *E. coli*,[61] so the ACP pool is almost completely in *holo*- or acyl-form. Heterologous overexpression of many ACPs in *E. coli* typically results in a mixture of *apo*- and *holo*-carrier protein, suggesting that AcpS, EntD or AcpT often manage to post-translationally modify these foreign carrier proteins.

In some organisms, primarily bacteria, an enzyme is found that facilitates the reverse transformation of a PPTase. The ACP hydrolase (AcpH), or phosphodiesterase, removes the PPant arm from *holo*-ACP, regenerating *apo*-ACP. Although this enzyme was discovered in the 1960s, difficulties with expression in *E. coli* have prohibited its study and use in the laboratory. The native *E. coli* enzyme is very active *in vitro* but unstable and difficult to work with.[10] The *Pseudomonas aeruginosa* AcpH is much more amenable *in vitro*,[65] facilitating its use in the laboratory to detach labeled cargo or recycling previously labeled carrier proteins.[66-68]

<Figure 3>

Figure 3 – Acyl carrier protein post-translational manipulations and modifications. Carrier proteins (CPs) come from the ribosome in its naked *apo*-form (blue circle). By the action of a PPTase, utilizing coenzyme A, a highly conserved serine residue of the CP is post-

translationally modified with a 4'-phosphopantetheine arm forming *holo*-ACP (yellow circle). This active form of the carrier protein is used to carry the growing fatty acid chain, by action of the FAS enzymes (acyl-ACP, red circle). A thioesterase (TE) or acyltransferase (AT) can cleave off the fatty acid, regenerating *holo*-CP. The adenylate-forming enzyme acyl-ACP synthetase (AasS) can directly load a fatty acid onto *holo*-CP, resulting in acyl-ACP (red circle). In the laboratory, it is possible to attach pantetheine-probes, in a CoaA, -D, -E and PPTase Sfp dependent fashion, to *apo*-CP, leading to crypto-CP (pink circle). Acyl carrier protein hydrolase or phosphodiesterase, AcpH, can regenerate the *apo*-form of the carrier protein from acyl-, crypto- and *holo*-ACP.

Challenges and Opportunities

It is becoming clear that the different states of ACP can be regulatory. For example, *apo*-ACP is toxic,[61] while C18:1-loaded ACP allosterically regulates the activity of the acetyl-CoA carboxylase (ACCase),[69] crucial in priming fatty acid biosynthesis. We expect that in the coming years many more of these interactions will be discovered, informing the organization of a regulatory network of acyl-ACPs in fatty acid biosynthesis (FAB).

AcpH is able to efficiently remove the PPant from *holo*-ACP, inactivating ACP. Cronan and co-workers observe that if AcpH is as active *in vivo* as *in vitro* there would be no *holo*-ACP present, since AcpH is much more active than the PPTase AcpS.[70] Thus, it seems likely that there is tight regulation of AcpH activity. In the context of metabolic engineering this enzyme has been overlooked and opens up some interesting possibilities for tricking species into up-regulating FAB.

Chain initiation: MCAT

Malonyl-CoA ACP transacylase (MCAT) catalyzes the initiation of fatty acid biosynthesis by conversion of malonyl-CoA to malonyl-ACP (Figure 4), the key building block in fatty acid synthesis for all three ketoacyl synthase (KS) enzymes.[71, 72] MCAT is transiently malonylated at an active site serine residue (Ser92 in *E. coli*). *E. coli* MCAT can then transfer the malonyl group to a variety of substrates *in vitro*, including CoA, ACP, pantetheine, N-(N-acetyl-β-alanyl)-cysteamine or N-acetylcysteamine.[71] Interestingly, the enzyme does not catalyze loading of acetyl-CoA.

MCAT was first isolated from spinach in 1982, quickly followed by avocado and the cyanobacterium *Anabaeana variabilis*.[73-75] Soybean harbors two isoforms of MCAT, presumably from gene duplication or alternative splicing.[76] These two similar proteins demonstrate different activity and inhibition profiles,

and both accept a variety of CoA analogs, with preference for malonyl-CoA. Only a single isoform of MCAT is found in the genome of *Cuphea lanceolota*[77] and *Brassica napus*, two vascular plants with well-studied FAS pathways.[78] The proteins show respectively 25% and 47% sequence homology to *E. coli* FabD, and overexpression of the *B. napus* enzyme in *E. coli* can complement an *E. coli* FabD mutant. Recently, the structure of MCAT from the cyanobacteria *Synechocystis* sp. PCC 6803 was solved, showing a 40% sequence identity with FabD from *E. coli*.[79] Also, MCAT from *S. aureus* and *Streptococcus pneumoniae* were recently described, suggesting subtle differences in substrate specificity.[80] Interestingly, the identification and characterization of a type II homologous MCAT in human mitochondria helped decipher the presence of a type II FAS in a type I FAS utilizing organism.[81]

To assay MCAT activity *in vitro*, a cumbersome radioactive assay was originally used.[71] A new assay was more recently developed, coupling MCAT activity with α-ketoglutarate dehydrogenase monitoring by NAD^+ reduction.[82] This assay was used for screening inhibitors against MCAT from the apicomplexan parasite *Eimeria tenella*[83] and *P. falciparum*[84,85].

The structure of *E. coli* MCAT[86] reveals the roles of conserved residues: Gln11/Leu93 (stabilization oxyanion hole), Ser92 (active site), Arg117 (binding of substrate) and His201 (activating Ser92).[15] MCAT from *Heliobacter pylori* has been crystallized and its interaction with its ACP studied by computation.[87] Also, the structure of MCAT from *Streptomyces coelicolor* and its binding to its ACP was studied in detail by computation.[88, 89] Interestingly, detailed mutagenesis of the active site residues of *S. coelicolor* MCAT suggests that only Ser97 is required for activity and not two implicated nucleophiles (Ser and His) as previously published.[90,91]

M. tuberculosis has two MCATs at its disposal, FabD[92] and FabD2, which show little sequence similarity or clear phylogeny.[93, 94] The structure of *M. tuberculosis* FabD has been reported by two labs.[94, 95] In one (PDB: 2QC3),[94] the enzyme shows a slightly different active site, with the serine side chain rotated away 100°, which would suggest that the enzyme has a different active site topology than *E. coli* or *Saccharomyces cerevisiae* MCAT homologs. However, overlaying both MtMCAT structures (PDB: 2QC3 and 2QJ3) shows that only the orientation of the serine residue differs significantly between these structures.

It has been suggested that MCAT sits at the crossroads of the FAS and polyketide synthase (PKS), since both metabolic machines require conversion of their ACPs into malonyl-ACP by malonyl-CoA. However, the affinity of MCAT towards PKS ACPs is lower than FAS ACP, and mutagenesis studies suggest that the

binding mode of PKS ACP is different than FAS ACP.[89] For example, the modeled FAS ACP-MCAT interaction from *Xanthomonas* sp. differs substantially from that of PKS ACP-MCAT in *S. coelicolor*.[96]

Interestingly, when assembling a small PKS *in vitro* it was found MCAT was not essential for product formation, suggesting that *holo*-ACP can directly load malonyl-CoA.[97] It appeared that self-malonylation ability is an intrinsic property of polyketide carrier proteins.[98] This phenomenon has also been observed in *P. falciparum* FAS *holo*-ACP.[99] Subsequent work showed that PKS ACPs can catalyze the malonylation of FAS ACPs via trans-thioesterification of malonyl-CoA, malonyl-ACP (PKS) and malonyl-ACP (FAS).[100] However, the same group later showed that this catalytic effect was in fact an artifact presumably due to a small contamination of the (PKS and FAS) carrier proteins with FabD itself.[101] This topic is still controversial.

When *E. coli* MCAT was expressed in rapeseed and tobacco,[102] no significant changes in fatty acid content or profile were observed suggesting that this enzyme does not catalyze a rate limiting step. However, overexpression of the protist *Schizochytrium* sp. MCAT in the yeast *S. cerevisiae* led to substantial increases in fatty acid production and biomass.[103] Recently, the first microalgal MCAT was identified and expressed.[104] Although transcription of MCAT increased when *Nannochloropsis* sp. was nitrogen starved to trigger lipid production, no correlation could be found between MCAT expression and fatty acid profile or quantity, similar to that seen in other plants.[102] When the MCAT FabD and general purpose thioesterase TesA were overexpressed in *E. coli*, ~11% increase in fatty acid content was observed over the TesA-only strain.[105]

<Figure 4>

Figure 4 – Malonyl-CoA acyltransferases (MCAT/FabD). A-D) Similar to Arthur et al.[89] we used several protein-protein docking servers to visualize the tentative interaction between ACP and MCAT. Using Patchdock,[106] Grammx[107] and Cluspro,[108] *E. coli* ACP (PDB: 1T8K) was docked onto *E. coli* FabD (PDB: 2G1H) and compared with the modeled structure of ScACP and ScFabD, PDB: 1NNZ, shown in A.[88] B) Low energy state observed by all three methods,while C and D were observed by two. E) reaction catalyzed by MCAT/FabD. F) Published structures of malonyl-CoA acyltransferases (MCAT/FabD) show very close homology. Here, we docked using Cluspro[108] to various MCAT X-ray crystal structures (in dark brown) *E. coli* ACP, showing the top 5 hits (protein bundles in multicolor). From top left to bottom right: 1NM2 (from *Streptomyces coelicolor*), 2G1H (from *E. coli*), 2H1Y (from *Helicobacter pylori*), 3TQE (from *Coxiella burnetii*),

3PTW (from *Clostridium perfringens*), 2QC3 (from *Mycobacterium tuberculosis*), 3EZO (from *Burkholderia pseudomallei*) and 3IM8 (from *Streptococcus pneumoniae*).

Challenges and Opportunities

With the relative wealth of structural information for MCATs (Figure 4) in contrast to other FAS enzymes, it should be possible to engineer MCATs to accept other CoA starter units. It has become clear that protein-protein interactions between ACP and MCAT govern productive catalysis. With the various docking models and binding modes of ACP, there is a strong need for a co-crystal structure (Figure 4A-D), NMR titration experiments, or mechanistic crosslinking to further enhance this understanding. [48]

Chain extension: Ketoacyl synthases

KSs catalyze the chain extension step in FAS, via Claisen condensation to form the carbon-carbon bond (Figure 5). Typically two or three KSs are found in type II FAS, whereas type I only contains one. The three subfamilies of initiation and elongation enzymes are all derived from a thiolase precursor enzyme.[14] In this class of enzymes, the nucleophilic α-anion of the acyl-thioester is generated in either a non-decarboxylative or decarboxylative fashion, with KSs employing the latter.

Along with MCAT, the KSIII "FabH" is responsible for the initiation of FAS. Here acetyl-CoA is loaded onto a cysteine active site residue, and malonyl-ACP is extended by two carbon units via decarboxylative addition, releasing CO_2. Some organisms harbor a suite of FabHs, to facilitate the biosynthesis of various unusual fatty acids. FabH is most active on substrates with less than four carbons and is inactive with acyl-ACPs.

Overexpression of FabH leads to a general increase in shorter chain fatty acids,[19] but other interesting findings have also been observed. Overexpression of FabH from *B. subtilis* in *E. coli* led to branched C15 and C17, metabolites not found in wild-type *E. coli*.[109] Polyhydroxyalkanoate synthase, responsible for the production of polyesters of 3-hydroxyalkanoic acids as intracellular granules in many bacteria, has an important reliance on FabH. Overexpression of PhaC and FabH in *E. coli* led to the production of P(3HB) homopolymers in the presence of glucose.[110] In the same system, mutagenesis of FabH resulted in the production of various unusual polymers.[111] Most likely, FabH overexpression leads to a larger pool of acetoacetyl-ACP, which can be converted into 3-hydroxybutyryl-CoA, the monomer of PHB, or more frequent initiation of fatty acids, leading to higher C14 and C16, and lower C18, levels.[112] Deletion of FabH hampers growth significantly and leads to enhanced C18 levels.

The two other ketoacyl synthases in *E. coli* are KSI FabB and KSII FabF. Both FabB and FabF show activity with saturated C4 to C14 fatty acids. FabB catalyzes the condensation of cis-3-decenoyl-ACP, cis-5-dodecenoyl-ACP and cis-7-tetradodecenoyl-ACP, with malonyl-ACP. Interestingly, strains lacking FabB are unsaturated fatty acid auxotrophs and seem to be unable to elongate cis-3-decenoyl-ACP.[113] This implicates FabB in *de novo* production of unsaturated fatty acids in bacteria, as these organisms generally do not possess desaturases. However, overexpression of FabB does not result in an increase in unsaturated fatty acids.

In *E. coli* FabF has been found to be mainly responsible for the elongation of C14:1 (Cn:x, in which n = number of carbons and x = number of unsaturations in the chain). The last step to vaccenic acid (C18:1) has been demonstrated to be catalyzed by FabF and not FabB.[114] Deletion of FabF leads to a temperature-sensitive mutant, whereas overexpression shows lethality. When FabF was overexpressed, a 4-fold increase in malonyl-CoA was observed due to arrest of fatty acid biosynthesis.[115, 116] Coexpression of FabD alleviates this effect, and it has been hypothesized that FabD forms a complex with FabF, FabH, or FabB. FabF is involved in thermal regulation of fatty acid composition of the cell membrane in *E. coli*.[114] Interestingly, FabF and FabH are co-transcribed within the Fab cluster, whereas FabB is modulated by the FadR and FabR repressors.

Lactococcus lactis has only FabF and no FabB. Expression of this FabF can replace both FabB and FabF of *E. coli*, although the strain loses its thermal regulation of fatty acid unsaturation.[117] Also the FabF of *Clostridium acetobutylicium* (which shows the same fatty acid profile as *E. coli*) can replace both FabB and FabF in *E. coli*.[118] The FAS inhibitor cerulenin, which targets KSs, was found to increase the production of medium chain fatty acids *in vivo*. FabF was then mutagenized for octanoyl-ACP specificity and FabB put under inducible degradation, yielding a 12% theoretical yield of octanoate production.[119]

The Khosla laboratory recently reconstituted the complete *E. coli* FAS *in vitro*.[120] Increasing the concentration of FabF or FabH enhances FAS activity at low concentrations but inhibits at high concentrations. On the other hand, the amount of FabB does not seem to influence activity. It is hypothesized that at high concentrations *holo*-ACP is removed from the pool of free *holo*-ACP by binding with FabF and FabH, thus lowering overall FAS activity. Supporting this conclusion are observations of acyl-ACP inhibition by FabH *in vitro*.[121, 122]

Reconstituting the FAS of a cyanobacterium allows for a comparison of these bacterial synthases. Surprisingly, where FabI (ER) and FabZ (DH) are the rate limiting enzymes in *E. coli*, in *Synechococcus* sp.

PCC 7002, FabH was found to be solely rate limiting.[18] Additionally, FabB is not essential in *Synechococcus*, whereas FabF is required.

A few KSII enzymes from plants have been cloned and characterized. A mutant of *Arabidopsis thaliana* KSII shows an increase in palmitic acid (C16:0) and a decrease in C18 fatty acids. Similarly RNAi down-regulation of this enzyme results in a 7-fold increase in C16:0. Overexpression of CwKSII in *A. thaliana* reduces the amount of C16:0 and enhances the amount of C18 fatty acids. However, only a slight reduction in C16 was observed when a KSII was overexpressed in *B. napus*. When *Jatropha curcas* KSII was overexpressed in *A. thaliana*, the ratio of C18/C16 increased, albeit slightly.[123]

KSs that show promiscuity for fatty acid substrates must be able to initiate with different acyl-CoAs, as in the case of FabH; or to extend from C4 to C16, as in the case of FabB. Since these enzymes are essential to FAS, there seems to be some redundancy – e.g. the *E. coli* KSI can supplement the KSII. Protein-protein interactions between KS and ACP are required for catalysis, and unrelated carrier proteins from non-ribosomal peptide synthases are not substrates, as showcased by the absence of mechanistic crosslinking *in vitro*.[124] A chloroplastic algal FAS ACP successfully crosslinked with *E. coli* KSII, suggesting that the protein-protein interactions between ACP and KS are relatively permissive.[32] Engineering a plant KSII into green algae resulted in minimal effects on lipid and fatty acid accumulation. Similarly, when spinach KSIII was overexpressed in tobacco, only a 5% increase in fatty acid content was observed, despite the 50-fold increase in enzymatic activity.[125] Overexpression of *Cuphea* KSIII resulted in a 9% decrease in fatty acid content. Expression of *E. coli* KSIII in *B. napus* resulted in shorter fatty acids and growth arrest, suggesting complex regulation on fatty acid biosynthesis.[126]

Fungi and animals utilize a type I FAS megasynthase for fatty acid production, which contains only one KS. Recently, it has been found that this KS is responsible for the C16/C18 ratio.[127] *S. cerevisiae* has a 2.0-2.5 ratio of C16/C18 whereas *Hansenula polymorpha* has a ratio of 0.2-0.3, although their FAS I and FAS II subunits have 62 and 67% sequence identity. Swapping PPTase, ACP, KR and KS domains between these two fungi showed that only KS-swapping resulted in a change in C16/C18 ratio.[127]

All three ketoacyl synthases from *E. coli* have been crystallized (KSI,[128] KSII,[129] KSIII[130, 131]), and demonstrate a thiolase fold that is similar to all condensing enzymes (Figure 5).[14, 132] The important active site residues are Cys112, His244 and Asn274 in *E. coli* FabH. A Cys112Ser mutant cannot transacylate, but both His244Ala and Asn274Ala mutants show increased activity for the half-reaction. Similarly, decarboxylation is much increased in the Cys112Ser mutant but abolished in the His244Ala and

Asn274Ala mutants, showing the necessity of all three residues for catalysis. The docking site of ACP onto FabH has been elucidated.[133] Despite this work, there is no systematic study on how structure or active site composition controls specificity and product formation.

<Figure 5>

Figure 5 – Ketoacyl synthases. The ketoacyl synthase dimer is highly conserved throughout all branches of life. *Top*: FabH (KSIII, PDB: 1EBL) and FabD (MCAT) are responsible for initiating fatty acid biosynthesis. The acetyl group of acetyl-CoA is transferred to the active site cysteine residue of KSIII. Malonyl-ACP (the product of FabD/MCAT) binds to KSIII and a new carbon-carbon bond is formed, while CO_2 is released. *Bottom*: FabB (KSI, PDB: 1G5X) and FabF (KSII, PDB: 2GFW) extend the chain further by first transfer of a fatty acid from an alkyl-ACP species to a conserved cysteine residue on the ketoacyl synthase. A different carrier protein loaded with a malonyl moiety binds to the loaded KS and a new carbon-bond is formed, while CO_2 is released. The type I FAS KS from pig is shown as insert, showcasing the highly conserved fold and dimer structure of these enzymes.

Challenges and Opportunities

Although KSs are responsible for the crucial step of carbon-carbon bond formation in fatty acid biosynthesis, metabolic engineering has shown mixed results. Redundancy, (thermal) regulation, repression, and complex formation are all factors that significantly influence the activity of the various KSs. In this context it also interesting that the enoyl-ACP reductase (ER, FabI) is considered to be the rate limiting step of the fatty acid synthase in *E. coli*, while Kuo and Khosla showed recently that FabH is the rate limiting step in a cyanobacterial FAS.[18] The time is right for a comprehensive study of the influence of KS on FAS kinetics, overall processivity, and fatty acid profile.

3-ketoacyl ACP reductase (KR)

In contrast to enoyl-ACP reductases (see below), surprisingly little has been published on FAS KRs, the enzymes that are responsible for reducing 3-ketoacyl-ACP to 3-hydroxyacyl-ACP, using NAD(P)H. The *E. coli* KR FabG was isolated, purified, and characterized in 1966.[134] The enzyme appeared to be active on a range of 3-ketoacyl-ACPs with chain lengths varying between 4 and 10 carbons. FabG can function on acyl-CoAs, albeit with a large activity penalty.[134] The promiscuity of KRs is also demonstrated by the development of an activity assay based on ethylacetoacetate.[135]

Later, Cronan and co-workers showed that although there are many NAD(P)H Rossman-fold KRs annotated in the genome of *E. coli*, FabG is essential.[136] Since the family of short-chain alcohol

dehydrogenase/reductases is very large, annotation of FabGs is difficult. For example, in the genome of *L. lactis* two FabGs were found, but only one can complement an *E. coli* FabG sensitive strain.[137] The difference in FabG substrate specificity has been utilized in the engineering of polyhydroxyalkanoate (PHA) biosynthesis. PHAs are biological polymers that derive from the polymerization of R-3-hydroxy fatty acid analogs of coenzyme A. When *E. coli* is engineered with a PHA polymerase, no medium chain length PHA was formed. However, when *E. coli* was co-overexpressed with *P. aeruginosa* FabG accumulation did occur, suggesting that this FabG shows enhanced activity on 3-ketoacyl-CoAs.[110, 138]

The first structure of a KR was the KR of the plant *B. napus*.[139] Each monomer of the homotetrameric enzyme harbors a Ser-Tyr-Lys triad. A comparison of the structure of *E. coli* FabG [140] with the KR of *B. napus*, which was co-crystallized with $NADP^+$, showed large conformational changes corresponding to cofactor binding.[141] The tetramer from *B. napus* KR shows negative cooperativity in binding NADPH, which is further enhanced by the presence of ACP.[140]

Generation of temperature-sensitive FabG mutants in *E. coli* and *Salmonella* sp. revealed point-mutations on the twofold-axes of symmetry at the dimer interfaces, suggesting that these mutations destabilize the tetramer.[17] Recently, it was shown that FabG is also essential in *P. aeruginosa*, and a virtual inhibitor screen of the crystallized homo-tetramer gave low nanomolar binders, which bind on the dimer-dimer interface.[142]

Little direct research on type I synthase KRs has been accomplished. In one study, chicken liver fatty acid synthase was digested with proteases and the 3-ketoacyl reductase isolated.[143] This isolated enzyme was active on N-acetyl-S-acetoacetyl cysteamine but inactive on acetoacetyl-CoA, whereas the full synthase is active on both substrate mimics. The mammalian KR has received some attention as a potential anti-cancer target, and compounds have been identified as selective inhibitors.[144]

Challenges and opportunities

Although essential to FAS, KRs have received relatively little attention. There is only one mention of FabG overexpression in *E. coli* in relation to metabolic engineering.[145] We expect to see more work on this enzyme in the coming years, especially with its close resemblance to the well-studied ERs.

The structures of KR currently available do not explain the binding of ACP and how this influences cooperativity. To better understand this phenomenon, and its differences and similarities with ER, it is necessary to obtain structural data on the transient KR-ACP and ER-ACP complexes.

FAS dehydration: dehydratases

After the initial reduction of ketone to alcohol by KR, the β-hydroxyacyl alcohol is dehydrated to an enoyl moiety by DH enzymes via the elimination of water (Figure 6a). A conserved active site histidine catalyzes this reaction.

The resolved DH in the mammalian type I FAS is positioned above the 'waist' region of the FAS, with one pseudodimer observed per chain and two pseudodimers per complex.[49] These pseudodimers are two non-identical but similar subdomains that form a single active site together.[146] Several type II FAS DH structures are available, such as the homodimeric *E. coli* FabA (PDB: 1MKB)[147] and the hexameric (trimer of homodimers) *P. aeruginosa* FabZ (PDB: 1U1Z)[148]. Both type I and type II FAS DH complexes are made up of monomers demonstrating a double-hotdog topology in which α-helixes are wrapped by β-sheets.

Membrane plasticity is controlled in part by the ratio of saturated and unsaturated fatty acids. To this end, careful control of the synthesis of unsaturated fatty acids is critical for life. Organisms employing type I FAS systems synthesize fully saturated fatty acids, then achieve unsaturation using oxygen dependent desaturases (Figure 6b). Some bacteria, cyanobacteria, plants, and algae have desaturases as well. In contrast, many anaerobes and bacteria with type II FAS systems must use different approaches to achieve unsaturation.

In *E. coli*, the DH FabA offers a route to unsaturation. Both FabA and FabZ functionally eliminate the alcohol to the trans double bond, but FabA can further isomerize this to a cis 3-alkene which is not acted on by the downstream ER (Figure 6a).[149] In *E. coli*, FabA and the KSI FabB are co-expressed under the same operon and both are necessary for unsaturated fatty acid production. Gram positive bacteria, such as *S. pneumoniae* and *P. falciparum* lack the gene and must use a discrete isomerase to isomerize the double bond and produce unsaturated fatty acids.[149] *B. subtilis* was also found to have an independent desaturase.[150] The *C. acetobutylicium* FabZ can replace *E. coli* FabZ but cannot replace FabA, and it remains unknown how *C. acetobutylicium* produces unsaturated fatty acids since this organism lacks a FabA.

Comparing specificity has revealed that FabZ is more promiscuous than FabA, the latter being more active on acyl-ACPs of intermediate length.[151] This was demonstrated during the reconstitution of a FAS *in vitro*, which revealed activity relationships with the FabZ concentration consistent with promiscuity.[120] Additionally, the ratio of FabB to FabA correlated to the degree of unsaturation observed.[152] Taken together, these studies suggest that the positions of the unsaturations produced are controlled by

substrate specificity in the FabA, while FabZ simply dehydrates what is available. However, while manipulating the FabB to FabA ratio may prove useful in an engineering scenario, in *E. coli* protein level feedback inhibition is necessary since FabA and FabB are co-expressed under the same operon.

Engineering attempts to manipulate unsaturation have succeeded in identifying functional replacements for the FabA gene in *E. coli*.[13] Substitutions of the β-strands have suggested that the isomerase activity is achieved by the shape of the active site tunnel, and disruptions to the tunnel eliminated this activity.[153] Later studies have found that FabZ can be inhibited by either occupying this active site tunnel's entrance or blocking the tunnel itself, both with the net effect of preventing access to the active site.[154] Work on the *P. falciparum* FabZ has suggested that regulation of this DH is achieved by manipulating the oligomeric state of the FabZ, which may offer another mechanism to target with antibiotics.[155]

Similar to ERs, DHs offer an interesting drug target due to differences between type II and type I systems. To this end, activity assays using crotonoyl-CoA have been developed and *P. falciparum* FabZ[156] and *H. pylori* FabZ[157] have been screened against multiple inhibitors. Significant effort towards engineering mechanism-based inhibitors has led to the development of the crosslinking probe used to study the ACP FabA interaction discussed previously in the ACP section (Figure 6c).[48, 158, 159] A sulfonyl 3-alkynyl pantetheinamide generates a reactive allene that forms a trapped covalent bond with the active site histidine of the dehydratase (Figure 6c).

The crosslinking probe allowed us to see that structurally, the DH offers charge-complementary electrostatic interactions to anchor helix II of the ACP and a positive patch to pry helix III away and allow substrate translocation via the chain-flipping mechanism (PDB: 4KEH, Figure 2 and Figure 6d).[48] Interestingly, the interacting face is part of one DH monomer while the active site histidine is provided by the other, with the chain translocating to the interface between the monomers. This may offer insight into the requirement of pseudodimers in type I systems, since a simple dimer is prohibited by special requirements and two pseudodimers may offer reactive and interactive elements albeit with only one active site per chain.

Previous work on DHs included studying regulation, substrate specificity, and mechanism. With tools engineered from this data, including crotonyl-CoA assays and mechanism-based crosslinkers, we may be on the verge of engineering DHs in earnest. Studies New structural data is becoming available offering us our first real looks at how these machines function with their partners, and the promise of manipulating unsaturation highlights the potential of producing specific chemical feedstock targets.

Challenges and Opportunities

With the available structural and functional data, DHs are primed for engineering of unsaturation in model systems. Mutational studies of the FabA directed towards altering substrate specificity offer a direct path to different unsaturations in fatty acid profiles, at least in *E. coli* and organisms using two dehydratases. Furthermore, available structural data of both free DHs and a DH during interaction with an ACP provides an opportunity for directed development of novel antibiotics.

<Figure 6>

Figure 6 – Dehydratases. a) The bacterium *E. coli* has only one way to produce unsaturated fatty acids: FabA can not only reduce 3-hydroxydecanoyl-ACP, but also isomerize the double bond from trans-2-decenoyl-ACP to cis-3-decenoyl-ACP. Subsequent chain elongation by action of the other fatty acid synthase enzymes leads to the fatty acid C16:1. b) Other organisms (including cyanobacteria, plants, algae) have elongases and desaturases (green panel) that can extend and desaturate the saturated C16:0 or C18:0 fatty acids, as either acyl-ACP, acyl-CoA, acyl-lipid or free fatty acids. c) Mechanistic crosslinking between ACP and DH using a sulfonyl alkynyl pantetheinamide probe. d) The mechanistically crosslinked X-ray crystal structure of *E. coli* AcpP with FabA (PDB: 4KEH).

Enoyl ACP reductase (ER)

After DH activity, the resulting alkene is further reduced by an NAD(P)H dependent enoyl-ACP reductase (ER). Norris and Bloch [160-162] first discovered the ER during their initial studies of the FAS. Further work and characterization[163,164] demonstrated functionality only on crotonyl-ACP and not crotonyl-CoA or crotonyl-pantetheine. However, it was later shown that this enzyme also works on crotonyl-CoA.[165] Two different ERs were identified in *E. coli*, one binding NADPH and one NADH, with different specificity and substrate promiscuity characteristics.[164] Thirty years later it was found that only one ER is responsible for both saturated and unsaturated fatty acid biosynthesis in *E. coli*.[20]

Around the same time, the ER was also isolated from the chloroplastic type II FAS from the oilseed plant *B. napus* [166, 167], further characterized, and its homo-tetrameric structure (Figure 7) with Lys and Tyr active-site residues determined.[168] The ER resembles a hydroxysteroid dehydrogenase and is part of the large short-chain dehydrogenase reductase (SDR) family, although it uses a different Tyr and Lys-containing motif than the classical SDR members.[169] Similar to the *E. coli* enzyme, ER from *B. napus* exhibits some substrate promiscuity, with a Km of 1 µM for crotonyl-ACP and 178 µM for crotonyl-CoA. The ER gene was found by identifying the target of antibacterial diazaborines, and renamed from EnvM

to FabI. In addition to diazaborines, the enzyme is inhibited to a lesser extent by palmitoyl-CoA and -ACP, and one single point-mutation (Ser241Phe) results in the loss of NADPH-while retaining NADH-dependent activity.[165]

Although many structures are available, only a few structural studies focus on the substrates of ER and not the NAD(P)H co-factor or inhibitors. InhA, the ER from the type II FAS of *M. tuberculosis* has been co-crystallized with a C16 fatty acid moiety[170] and the *E. coli* FabI tetramer has been co-crystallized with two ACPs.[50] In the latter work, only two ACPs are observed, whereas the tetramer has four active sites. Comparing the orientation of ACP in relation to ER with other ACP-partner protein structures (see Figure 8) draws question to the significance of the structure.

ERs have received much attention in the field of antimicrobial research,[171, 172] especially with the discovery that the *M. tuberculosis* ER, InhA, is a target of isoniazid.[173] FabI of the malarial causative apicomplexan *P. falciparum* has also been targeted.[174] However, after the discovery that *P. falciparum* requires only *de novo* fatty acid biosynthesis in late growth stages, it is heavily under debate whether FAS is still a viable antimicrobial target.[175]

Triclosan is the most widely-used ER inhibitor, and was added to products such as hand-sanitizer and toothpaste even prior to understanding its target. Four papers in 1998 and 1999 signaled the start of triclosan-ER related research.[176-179] Interestingly, triclosan also inhibits the human fatty acid synthase *in vitro*, albeit with IC50 values many orders of magnitude larger than those observed in *E. coli* or *P. falciparum*.[180] While some bacteria are severely inhibited by triclosan, several other species have been identified that tolerate it. Either triclosan resistance originates from a mutation in FabI[181] or, more commonly, several species harbor multiple ERs or different types of ERs.[182] *B. subtilis* has two ERs: one highly homologous to *E. coli* FabI and an alternate named FabL.[23] Other bacteria, like *S. pneumoniae*, use the flavin dependent TIM-barrel reductase FabK. [22] Interestingly, the yeast type I FAS also contains an ER with homology to flavin-binding FabK.[37, 183] Other bacteria, such as *Vibrio cholerae*, use FabV, another short-chain dehydrogenase/reductase (SDR)-fold ER but much larger (Figure 7).[28] The mammalian type I FAS harbors a different MDR subfamily ER, dissimilar to the SDR-fold (FabI, FabL, and FabV), the TIM-barrel fold (FabK), or the mitochondrial MDR subfamily found in eukaryotes (Figure 7).[49] Thus, nature has found many different solutions to do the same reaction.

Very little metabolic engineering has been completed with ERs, but some insight in its effect on fatty acid levels has been gleaned from overproduction or knockout studies. While toxic, the *E. coli* FabI could

be overexpressed by supplementation with low levels of triclosan.[184] Two theories about FabI toxicity involve a reduction in lipid A building blocks or enhanced competition with FabA for substrate, leading to a deficiency in unsaturated fatty acids.[182] Recently, overexpression of FabI in *E. coli* did not result in growth retardation or any effect on fatty acid accumulation.[145] In plants, increased ER expression was observed during TAG deposition.[185] The ratio of plant FAS enzymes was observed to change significantly during growth, suggesting a life-cycle dependence or regulation.[186]

<Figure 7>

Figure 7 – Structures of enoyl-ACP reductases. Top: FabI from *E. coli* (PDB: 2FHS), FabL from *B. subtilis* (PDB: 3OIC) and FabV from *X. oryzae* (PDB: 3S8M) are all members of the SDR superfamily, utilizing NAD(P)H. Whereas FabI and FabL are tetramers, FabV is a monomer. ClustalOmega was used to generate a sequence identity matrix, including FAS1y (the yeast FAS ER), FAS1h (the human FAS ER) and MER (the human mitochondrial ER). Bottom: The ER dimer in type I FAS from pig (2VZ8) is shown in pink/purple, and FabK from *S. pneumoniae* (2Z6I).

<Figure 8>

Figure 8 – Comparison of all eight ACP-partner protein X-ray crystal structures deposted in the PDB, from the perspective of the ACP, showing the similar binding orientation of the ACP, but the different binding motifs of the partner proteins. From top to bottom and left to right: PDB: 1F80, *E. coli* PPTase AcpS with *E. coli holo*-AcpP, PDB: 2FHS, *E. coli* FabI with *E. coli* AcpP, PDB: 2XZ0, *Ricinus communis* stearoyl desaturase with *R. communis* ACP bearing a phosphoserine, PDB: 3EJB, *B. subtilis* P450BioI with *E. coli* AcpP bearing tetradecanoic acid, PDB: 3NY7, STAS domain of *E. coli* YchM with *E. coli* AcpP bearing a terminal acid propionic acid thioester, PDB: 4ETW, *E. coli* BioH with *E. coli* pimeloyl-AcpP, PDB: 4KEH, *E. coli* FabA mechanistically crosslinked with *E. coli* AcpP bearing a non-hydrolyzable pantetheinamide crosslinker (in light pink surface the second ACP) and PDB: 2CG5, *Homo sapiens* AASDHPPT with human FAS *apo*-ACP excised from the human type I FAS.

Challenges and Opportunities

Despite the large body of work on inhibition of ER, very little has been published on the engineering of this important step in fatty acid biosynthesis. Regarded by many as a rate limiting step,[20] it is necessary to understand whether this is really the case *in vivo,* and whether we can change this by metabolic engineering or systems biology.

FAS termination: acyltransferases and thioesterases

After chain elongation, fatty acid biosynthesis is terminated either by offloading fatty acids from ACP by acyl-ACP TEs, releasing free fatty acids, or by direct transesterification onto a lipid by acyltransferases (AT) (Figure 9a). In general, prokaryotes utilize ATs whereas eukaryotes make use of dedicated TEs.

E. coli however does harbor two α/β hydrolase TEs, TesA (Figure 9) and TesB,[187] but these have much higher activity with acyl-CoAs over acyl-ACPs and are not believed to be directly involved in fatty acid or lipid biosynthesis. TesA is a general purpose thioesterase/lipase/hydrolase that is localized in the periplasm. Overexpression of TesA in the cytosol increases the pool of free fatty acids in *E. coli*.[188-190] *E. coli* uses two different methods to transesterify acyl chains from acyl-ACP to lipids, involving PlsB, PlsY and PlsX, further discussed in the "Other Enzymes" section of this review.

Dedicated acyl-ACP TEs were discovered in 1991 in oil seed plants that show an unusual shorter fatty acid pattern in storage lipids.[191, 192] Dedicated short/medium chain TEs FatB [185, 188, 190] are responsible for the accumulation of these fatty acids. Later, it was found that plants harbor FatA in addition to FatB,[193] a more general TE showing some specificity for oleic acid. These enzymes have now also been annotated in some anaerobic bacteria, mosses and algae.[31] Although other eukaryotic species (e.g. fungi and mammals) also use dedicated TEs embedded in their type I fatty acid megasynthases, these TEs have a different fold (α/β hydrolase)[194] than their plant/algal counterparts, which have a typical hotdog-fold.[30, 195] In plants these hotdog-fold TEs solely can determine the chain length of fatty acids, and there has been considerable interest in metabolic engineering and mutagenizing these enzymes.

When plant TEs are overexpressed in *E. coli*, fatty acid biosynthesis is altered according to the specificity of the TE.[189, 196-199] For example, overexpressing the C12:0-ACP selective *Umbellularia californica* TE "UcFatB1" in *E. coli* lead to a 500-fold increase in C12:0 fatty acid production.[196] Mutagenesis on TEs has met mixed success and seems unpredictable, possibly because of limited structural information on these enzymes.[200, 201] In one case of success, it was shown that mutagenesis of UcFatB1 can change its specificity to C14:0-ACP.[202] Metabolic engineering of fatty acid biosynthesis in plants[203] has been demonstrated by the expression of FatB[192] and FatA[204] in *B. napus* seeds. Introduction of UcFatB1 in *B. napus* seeds resulted in lauric acid levels of ~60% of the total fatty acids. Such a remarkable change is the exception; for example introduction of C8:0-ACP selective *Cuphea hookeriana* TE "ChTE" into rapeseed gives only 12% octanoic acid whereas the native species contains 50%.[205] In general, production of short-chain fatty acids in plants has been marginally successful. When a FatB TE was

overexpressed in an acyl-ACP synthetase (AasS, see "Other Enzymes") knock-out strain of *A. thaliana*, a 2-fold increase in octanoic acid production was observed, suggesting that AasS not only works on exogenous acids but also plastidically produced acids.[206]

In contrast to plants, green algae only have one general purpose hotdog-fold TE (Fat1), and brown/red algae and diatoms seem to lack any.[32, 66] The green microalgae *C. reinhardtii* has recently been engineered with FatBs from different seed oil plants, with very limited success.[32, 66] Only the strain overexpressing its native Fat1 TE showed a modest increase in total fatty acids, due to the mismatch in protein-protein interactions between foreign TEs and ACP. This was observed by mechanistic crosslinking (Figure 9b), and highlights the importance of specific interaction. Engineering of cyanobacteria (blue green algae), with plant TEs resulted in the predicted increases in short/medium chain fatty acids,[207] although engineering of algal TE into cyanobacteria did not result in this effect.[208] Cyanobacteria are an attractive species since they are photosynthetic, can fix nitrogen, are genetically amenable and secrete large portions of produced fatty acids.[209]

<Figure 9>

Figure 9 – Fatty acid biosynthesis termination by thioesterases or acyltransferases. Top: a) In plants, green algae and some bacteria, dedicated hotdog-fold acyl-ACP thioesterases (e.g. CrTE, *Chlamydomonas reinhardtii* Fat1) are responsible for hydrolyzing fatty acids, with a certain chain-length off the ACP (e.g. CrACP, *C. reinhardtii* ACP). TesA is a multifunctional enzyme that can hydrolyze fatty acids off the ACP, but this is presumably not its primary function in *E. coli*. In bacteria, dedicated acyl-transferases, like PlsB, trans-esterify lipid headgroups with a fatty acid, directly from the ACP. Alternatively, *E. coli* uses PlsX to synthesize phospho-fatty acids, which are loaded onto lipids using PlsY. b) Mechanistic crosslinking of ACP to TE using α-bromo acid pantetheinamide probe. Bottom: structures of TesA (PDB: 1IVN), PlsB (PDB: 1K30) and a docked structure of chloroplastic *C. reinhardtii* ACP (model) with *C. reinhardtii* TE FAT1 (model).

Challenges and Opportunities

Although there are TE structures from anaerobic bacteria available (e.g. PDB:2OWN or 2ESS) there are currently no structures of FatA/FatB/Fat1s, prohibiting detailed structural or directed mutagenesis studies. To date, the results of TE mutagenesis are complex. There is a question whether or not steering product formation with TEs is viable, especially since these enzymes seem to be promiscuous for their substrates, accepting SNAcs, CoAs and acyl-ACPs. Fatty acids are not lipids, but nature efficiently stores fatty acids on various lipids using acyltransferases. These acyltransferases are poorly characterized and

we are at the beginning of understanding their roles.[210] Additionally, our understanding of the regulation of AT or TE activity by exogenous or endogenous signals (molecules or proteins) is in its infancy.

Metabolic engineering of fatty acid synthases

In the past decade, we have seen large strides in the metabolic engineering of fatty acid synthases in bacteria. However, the dream of designer strains producing specific fats and feedstock remains distant. Here, we will discuss select hallmarks of metabolic engineering of fatty acid synthases in various organisms, focusing on *E. coli*.

Important requisites for fatty acid biosynthesis are *holo*-ACP, malonyl-ACP and malonyl-CoA. It is assumed that the ACP itself is not rate limiting because it is present in high concentration, overexpression does not change the fatty acid profile, and because *apo*-ACP is toxic.[61] Overexpression of AcpS (the FAS PPTase responsible for transforming *apo*-ACP into *holo*-ACP) stops growth due to strong inhibition of glycerol-3-phosphate acyltransferase,[211] but can be partially alleviated by AcpH coexpression.

MCAT and KSIII are responsible for chain-initiation and thus formation of malonyl-ACP. Malonyl-CoA is produced by the ACCase complex. Overexpression of these proteins resulted in modest (1.2-1.6-fold) increases in total lipids, with a 5-fold increase in palmitic acid production when all three enzymes were overexpressed.[212] Similarly, engineering ACCase subunits accA, accB, accC, MCAT and a TE from *Streptococcus pyogenes* into *E. coli* resulted in a 2.4 fold increase in fatty acid production.[105] Interestingly, overexpressing the ACCase by itself did not change fatty acid production,[213] presumably due to inhibition by acyl-ACPs. A combination of deletion of β-oxidation (fatty acid degradation) and overexpression of an ACCase and a plant TE (at a low copy number) did however result in a 5-fold increase in fatty acid production.[213] Overexpression of MCATs from various species only results in slight increases in fatty acid production,[214] whereas deletion of MCATs is lethal.[136, 215]

Studies of *E. coli* strains overexpressing its own FAS enzymes found that overexpression of KR (FabG) and DH (FabZ) produced the largest amount of fatty acids. Additional overexpression of the ER (FabI) did not improve yield, and overexpression of the KSIII (FabH) actually reduced the amount of fatty acids, confirming the inhibitory effect of high concentrations of FabI and FabH on FAS in *E. coli*.[216] All strains containing FabZ show a significant increase in cis-C16:1 and cis-C18:1. Overexpression of KSII (FabF) gave a 1.3-fold increase in fatty acid production at 37 °C and a 2.7-fold increase at 20 °C, in which in the latter case the amount of unsaturated fatty acids increased by 7-fold.[216] Combination of FabF with other FAS

genes resulted in enhanced fatty acid production at lower temperatures, except in the case of FabI, which appeared to be inhibitory.

Reconstituting the FAS of *E. coli*[120] and a cyanobacterium[18] *in vitro* allowed for a comparison of these bacterial synthases. Surprisingly, where FabI and FabZ are the rate limiting enzymes in *E. coli*, in *Synechococcus* sp. PCC 7002, FabH is solely rate limiting.[18] Cyanobacteria have desaturases at their disposal, whereas *E. coli* needs to regulate fatty acid unsaturation via the ratio of FabA/FabZ and FabB.

Elimination of fatty acid β-oxidation as a means to improve the accumulation of fatty acids is a popular approach. For example, knocking out FadD (fatty acid CoA-ligase), while overexpressing a plant TE, the endogenous TE TesA and ACCase, resulted in high fatty acid titers in *E. coli*.[189] A combination of *fadE* (acyl-CoA dehydrogenase) deletion and overexpression of TesA resulted in a strain producing 1.2 g/L fatty acids,[188] whereas deletion of FadD , combined with the overexpression of dedicated seed plant TEs resulted in strains producing up to 4.5 g/L fatty acids.[213, 217]

Free fatty acid secretion is sought after, since it would facilitate the production of biofuel. Looking specifically at extracellular fatty acid production, a FadD knockout did not result in an increase, whereas a FadL (outer membrane fatty acid transporter) knockout, in combination with TesA overexpression resulted in a large increase in extracellular fatty acid production (from 5.5 mg/L to 4500 mg/L).[218] Interestingly, identification and overexpression of several *E. coli* fatty acid transporters did not result in a superior strain.[219]

Besides initiation, transport, and degradation, some core enzymes of the *E. coli* FAS have been overexpressed with the goal of enhancing yield. FabA, FabZ, and FabG were overexpressed in a hallmark study in which the fatty acid synthase from *E. coli* was reconstituted *in vitro*,[217] allowing for detailed studies of rate-limiting steps in the synthase. Overexpression of FabZ alone also resulted in a higher fatty acid production, and a combination of *fadD* knockout, *sucC* (glycolysis) knockout, and FabZ overexpression gave a strain producing 5.7 g/L C14-C16 fatty acids.[220] Overexpression of FabG leads to a 2-3 fold increase in fatty acid production,[145] whereas overexpression of FabI showed no effect, FabH was semi-lethal, FabF was lethal and FabB in combination with FabA showed modest increases in fatty acid production. Lastly, TEs which are responsible for off-loading the fatty acid from the ACP, have been engineered and overexpressed in many studies, utilizing TEs from various plant, algal or endogenous sources, resulting in shifting the fatty acid profile towards the specificity of the TE (except in algal engineering with foreign TEs and algal TE expression in cyanobacteria).[220]

Besides fatty acids themselves, there is an increasing interest in coupling fatty acid biosynthesis to other metabolic products. For example, fatty alcohols can be made by overexpressing acyl-CoA synthase and acyl-CoA reductase.[188, 221] The discovery of an acyl-ACP reductase facilitates this process further.[222] Methylketones, fatty acid methylesters, and alkanes can also be derived from fatty acids. Methylketones are made from fatty acids via β-oxidation. Fatty acids are initially converted into β-ketoacyl-CoAs, prior to hydrolysis by a TE (producing β-keto-fatty acids) and decarboxylation yielding the final methylketones.[223] Fatty acid methylesters can be made directly in a living organism by expressing a wax-ester synthase[224] or an S-adenosylmethionine-dependent methyltransferase.[225] Alkanes or alkenes can be derived from fatty acids using multiple pathways. These include, the combination of acyl-ACP reductase and aldehyde-decarbonylase,[222] or a P450-enzyme,[226] or head-to-head condensation of two fatty acids.[227] Recently, the identification of a wax-ester/diacylglyceride acyltransferase (WS/DGAT, AtfA) in *Acinetobacter baylii*[228, 229] and subsequent expression in *E. coli*, resulted in the production of triacylglycerides (TAGs) in bacteria.[230] Overexpression of AtfA, FadD, and deletion of diacylglycerol kinase, resulted in a strain with high TAG titers.[112]

Regulation of fatty acid biosynthesis is currently a crucial bottleneck in metabolic engineering (Figure 10).[112] For example, long chain acyl-ACPs seems to regulate the flux through fatty acid synthases. Cultures starved of glycerol (limiting phospholipid biosynthesis) show a decreased rate of acyl-ACP biosynthesis. Fatty acid biosynthesis is also downregulated when PlsB (sn-glycerol-3-phosphate acyltransferase) is inhibited by the alarmone ppGpp.[122] When TesA is overexpressed, acyl-CoAs and acyl-ACPs are hydrolyzed, depleting long-chain ACPs, increasing the rate of fatty acid biosynthesis. Acyl-ACPs directly inhibit the ACCase, KSIII and ER. *In vitro*, ER or ER/DH are the rate limiting enzymes in the fatty acid synthase, but how transcriptional or translational regulation influence fatty acid synthases is still an open question. FabR and FadR are regulators of fatty acid biosynthesis and fatty acid degradation but do not control all enzymes involved. Interestingly, coexpression of FadR and TesA resulted in a large increase of fatty acid production.[231] A FadR-derived regulator, FadE deletion and TesA overexpression also resulted in robust fatty acid production (5 g/l) in minimal media.[232] FadM is another regulator only recently discovered,[233] and more details of the complex regulation of fatty acid biosynthesis in *E. coli* continue to be elucidated.[234-237]

In cyanobacteria, bacterial and plant TEs have been overexpressed with success,[207, 238] but in general there has been relatively little development in metabolic engineering of cyanobacterial fatty acid synthases.[239] In one promising work, acyl-ACP synthetase was knocked out and a plant acyl-ACP TE

overexpressed, leading to accumulation of free fatty acids.[238] The same strategy was later applied to *Synechocystis* sp. PCC6803[240] and *Synechococcus elongatus* PCC 7942.[241] Based on this work, six generations of *Synechocystis* sp. PCC6803 were constructed with a variety of genes knocked out or overexpressed (either native or from foreign hosts).[209] The *in vitro* reconstitution of *Synechococcus* sp. PCC 7002 FAS opens up avenues to study the cyanobacterial FAS in more detail.[18] The increased interest in biofuels during the last five years has created an explosion of papers on improving lipid content of photosynthetic organisms,[242] primarily by engineering acyl-ACP thioesterases. In addition, the beneficial effect of omega fatty acids has led to many studies of overexpression of fatty acid modifying enzymes, like elongases and desaturases (see "Other Enzymes" section). We recently engineered various plant acyl-ACP thioesterases in the green microalgae *C. reinhardtii*, with limited success.[66] Only overexpression of the native acyl-ACP thioesterase, Fat1, increased fatty acid production. When Fat1 was expressed in cyanobacteria, very little effect was observed.[208] In diatoms work has focused on TEs,[243] desaturases, and elongases.[244] In plants, several efforts have led to strains with improved and manipulated oil content,[203, 241] again focused on acyl-ACP TEs,[203] desaturases,[245] and elongases.[246]

Engineering of type I synthases (e.g. fungal and mammalian) is in its infancy. Yeast FAS contains subunits FAS1 (β-subunit, AT-ER-DH and malonyl/palmitoyl-transferase) and FAS2 (α-subunit, ACP-KR-KS-PPT), forming a heterododecameric enzyme complex of 2.6 MDa. Creating chimeric FAS by mixing FAS1 and FAS2 from *S. cerevisiae* and *H. polymorpha*, produced interesting alterations to the fatty acid profiles, but rational design is still elusive. Swapping PPTase domains did not show any difference, whereas swapping KS domains gave marked changes in fatty acid chain length.[127] Recently, human type I FAS was inserted into a yeast FAS knockout strain, bearing different TE domains, and co-expressing different PPTases.[247] Although the yields were modest, (~50-100 mg/l) short-chain fatty acids showed a 64-fold increase compared to the wildtype.

Challenges and opportunities

We now know that many steps in fatty acid biosynthesis are regulated, either by dedicated regulators, acyl-ACPs, or other factors. The simple picture of an iterative cycle needs to be revised into a complex scheme (Figure 10). Whereas many have discussed these as bottlenecks, it may well be that we can take advantage of these regulatory mechanisms. One emerging and powerful method combines systems biology and metabolic engineering.[220] The *in silico* modeling of a whole organism is currently flawed due to approximation, but these models are becoming more powerful and accessible.[248] Since we have seen that overexpression (or downregulation) of genes often leads to unpredictable results and/or

competition with growth/survival, an approach that considers the organism as a whole, seems necessary and attractive.

<Figure 10>

Figure 10 – Fatty acid metabolism in bacteria. In the fatty acid synthase (*yellow circle*): ACCase is the acetyl-CoA carboxylase; MCAT, malonyl-CoA acyltransferase; KSIII (FabH), ketoacyl synthase; KR (FabG), ketoreductase; DH (FabA/FabZ), dehydratase; ER (FabI/K/L/V), enoyl-ACP reductase; KSI (FabB), KSII (FabF), ketoacyl synthases and PlsB, acyltransferase. In green the thioesterase (TE) pathway present in plants, algae and some bacteria. In orange the acyl-ACP synthetase (AasS) found in some organisms. The dotted arrow in the bottom right corner represent diffusion of fatty acids into the cell, as well as active FadL-mediated transport. Fatty acid catabolism (*blue circle*): FadD, CoA-ligase; FadE, acyl-CoA dehydrogenase; FadB, dual-function enoyl-CoA hydratase and hydroxyacyl-CoA dehydrogenase; FadA, acetyl-CoA acetyltransferase. FadR and FabR are the master regulators of fatty acid degradation and fatty acid biosynthesis, whereas ppGpp is an alarmone.

Other enzymes and other functions

The fatty acid synthase is surrounded by dozens of other enzymes that either input metabolites or remove fatty acids for further modification. ACCases are responsible for the production of malonyl-CoA (Figure 10), one of the essential starting materials of fatty acid biosynthesis. In *E. coli*, four proteins form the ACCase complex: biotin carboxylase, biotin carboxylic carrier protein, CoA-carboxylase and carboxyl transferase. In this reaction, biotin is carboxylated and the carboxyl group transferred to acetyl-CoA, forming malonyl-CoA. Acetyl-CoA stems from pyruvate, which is a product of glycolysis. The ACCase is inhibited by acyl-ACPs, presumably preventing the accumulation of fatty acids.[249] Overexpression of the ACCase in combination with TesA resulted in significant increases in fatty acid production.[250]

Some organisms are able to convert *holo*-ACP to acyl-ACP by shunting exogenous fatty acids directly into fatty acid or lipid metabolism (and not catabolism). This process is carried out by a dedicated acyl-ACP synthetase (AasS, Figures 3 and 10), which catalyzes the activation of a free fatty acid to its AMP-ester and subsequent thioester formation with the free thiol of the PPant arm of *holo*-ACP. *E. coli* harbors a unique bifunctional membrane-bound protein[251] which shows this activity *in vitro*, but is unable to utilize supplemented fatty acids *in vivo*. In contrast, *V. harveyi* has a soluble AasS, which can use exogenously supplied fatty acids with a preference for medium chain fatty acids.[252, 253] AasS has recently been found in cyanobacteria[254] and plants.[206]

In many bacteria, fatty acids get transferred directly from acyl-ACP to acyl-glycerol-3-phosphate. On the other hand, fatty acids from algae and plants get hydrolyzed off acyl-ACP by a dedicated TE (Figure 10). There are, at least, two ways for bacteria to transfer acyl chains from ACP to glycerophosphate. PlsX catalyzes the formation of acyl-phosphate, which is the substrate for glycerophosphate-acyltransferase PlsY. Alternatively, PlsB can directly transfer the fatty acid from ACP to glycerol-3-phosphate. The product acyl-glycerol-3-phosphate (also called mono-acylglyceride) is the substrate for enzymes that catalyze the formation of di-acylglycerides, and subsequently membrane lipids.

Lipoic acid is produced by mitochondrial type II fatty acid synthases in eukaryotes and relies on type II FAS in bacteria, starting with LipB catalyzed acyl-transfer from octanoyl-ACP to the *apo*-octanoyl-domain, and followed by the LipA catalyzed insertion of two sulfur atoms.[255] FASs are also directly involved in the biosynthesis of biotin, elucidated in 2010. BioC is responsible for producing malonyl-CoA methyl ester, which is condensed with malonyl-ACP by KSIII. This product gets taken through a complete cycle of fatty acid biosynthesis, forming pimeloyl-ACP methyl ester, the substrate for BioH. This then forms pimeloyl-ACP, the substrate for the subsequent biotin-biosynthetic enzymes.[256]

Fatty acids are often found in natural products, from which calcium dependent antibiotic (CDA) and daptomycin are possibly the most famous examples.[257] There is also a suite of natural products that are made from mature fatty acids, including falcarinol alkynes, cicutoxin, panaxytriol, wyerone, amides of fatty acids like oleamide, ricinoleic acid, vernolic acid, lipstatin, prostaglandins, thromboxanes, leukotrienes, urushiol, jasmonic acid, ginkgolic acids, coniine, and various modified fatty acids with epoxides, methyl side chains, cyclopropanes, and even unsaturated cyclopropanes embedded in the chain. Also, many polyketide synthases require hexanoyl-CoA starter units, which are most likely derived from FAS. Fatty acids are also often found attached to proteins. This post-translational modification plays important roles in regulation of protein trafficking, signaling and behavior.[258] Most common is myristoylation, the irreversible attachment of myrystic acid to an N-terminal glycine residue via an amide bond. Palmitoylation is reversible and encompasses the thioesterification of cysteine thiols.

Fatty acids are degraded by a dedicated pathway that closely resembles the reverse biosynthesis of fatty acids, but with the critical difference that CoA, and not ACP, carries the acyl cargo (Figure 10). FadL is a transporter that can transport exogenous acids into a cell while FadD is an acyl-CoA synthetase that loads these (and other cellular) free acids onto CoA. FadE catalyes the oxidation of acyl-CoA to enoyl-CoA. FadB hydrates enoyl-CoA to 3-hydroxyacyl-CoA and oxidizes it to 3-ketoacyl-CoA, which is the substrate for the ketothiolase FadA, forming acetyl-CoA and acyl-CoA. FadH, a 2,4-dienoyl-CoA

reductase, is necessary for the processing of unsaturated fatty acids and FadM is a recently identified TE. Fatty acid degradation is stringently regulated by the acyl-CoA controlled regulator FadR, the cyclic adenosine monophosphate receptor protein-cAMP complex, (p)ppGpp, and sigma-factor RpoS.

Challenges and Opportunities

Besides storage and membrane lipids it is becoming clear that fatty acids are involved in many primary and secondary metabolic processes. The discovery that the biosynthesis of biotin exploits FAS, is a prime example of the potential undiscovered functions of fatty acids and the FAS machinery.

Conclusion and outlook

Fatty acid synthases are fascinating biosynthetic machines that offer many opportunities to study biology, biochemistry, and chemistry of biological processes. In contrast to the early discovery of fatty acid degradation (β-oxidation) in 1904 by Knoop, the elucidation of fatty acid biosynthesis[259] started much later with the publication of five papers by Barker and Stadtman in 1949.[260-264] They exploited the then newly available ^{14}C isotope, labeling acetate and feeding this to cell-free extracts of *Clostridium kluyverii*. This was followed by a large body of work by Vagelos and co-workers in the 1960s, using enzymology.[265] In the 1970s, Wakil[266] and co-workers showed that the eukaryotic synthase is a multidomain megasynthase, while Bloch and co-workers described fatty acid metabolism and regulation.[267] Building on these milestones, the labs of Cronan, Rock and Ohlrogge have dug deeper into various aspects of fatty acid biosynthesis in bacteria and plants.

It is fascinating to note that, although studied for 75 years and present in all biochemistry textbooks, we are only now uncovering the detailed features of the complex machinery of fatty acid biosynthesis. Regulation by many factors is being discovered, and we expect that many more regulatory elements will be elucidated in the coming years. Protein-protein interactions between ACP and partner proteins seem to govern processivity, and with access to new chemical biology tools we hope to see many more structures and predictions of these protein-protein interactions. Phylogeny of the individual FAS enzymes might also be a useful tool to anticipate differences and similarities between species' FAS. With enhanced insight into the biochemistry of this important biosynthetic process, metabolic engineering for designer oils or other FAS-derived products should become possible. Modern spectroscopic and ACP modifying techniques appear to be the ^{14}C of our time, offering us the exciting opportunity to explore

and engineer fatty acid biosynthesis in a new manner. Indeed, considering global energy demands and the increasing thirst for renewable liquid fuels, this opportunity may be crucial to our future.

Acknowledgements

We thank K. Finzel and N. Schoepp for critical advice. This work was supported by California Energy Commission CILMSF 500-10-039, DOE DE-EE0003373 and NIH R01GM095970.

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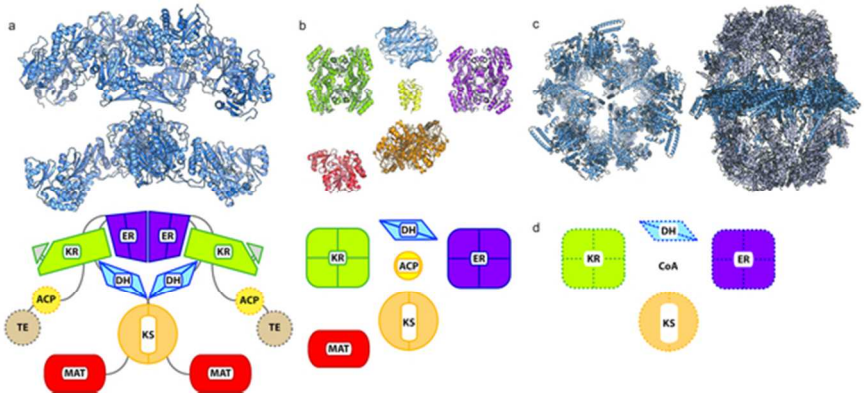

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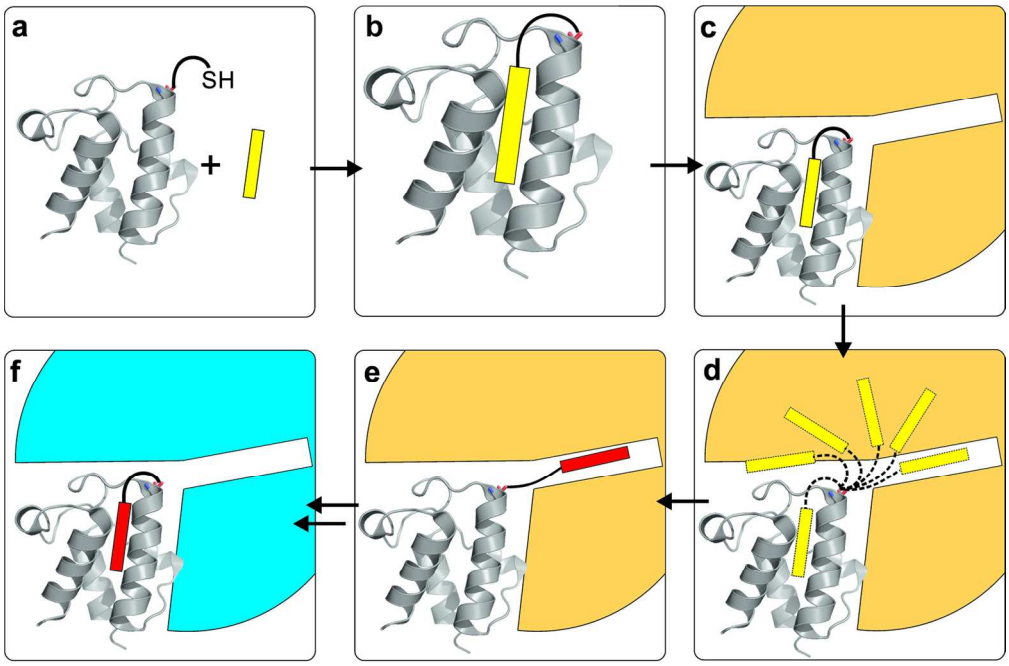

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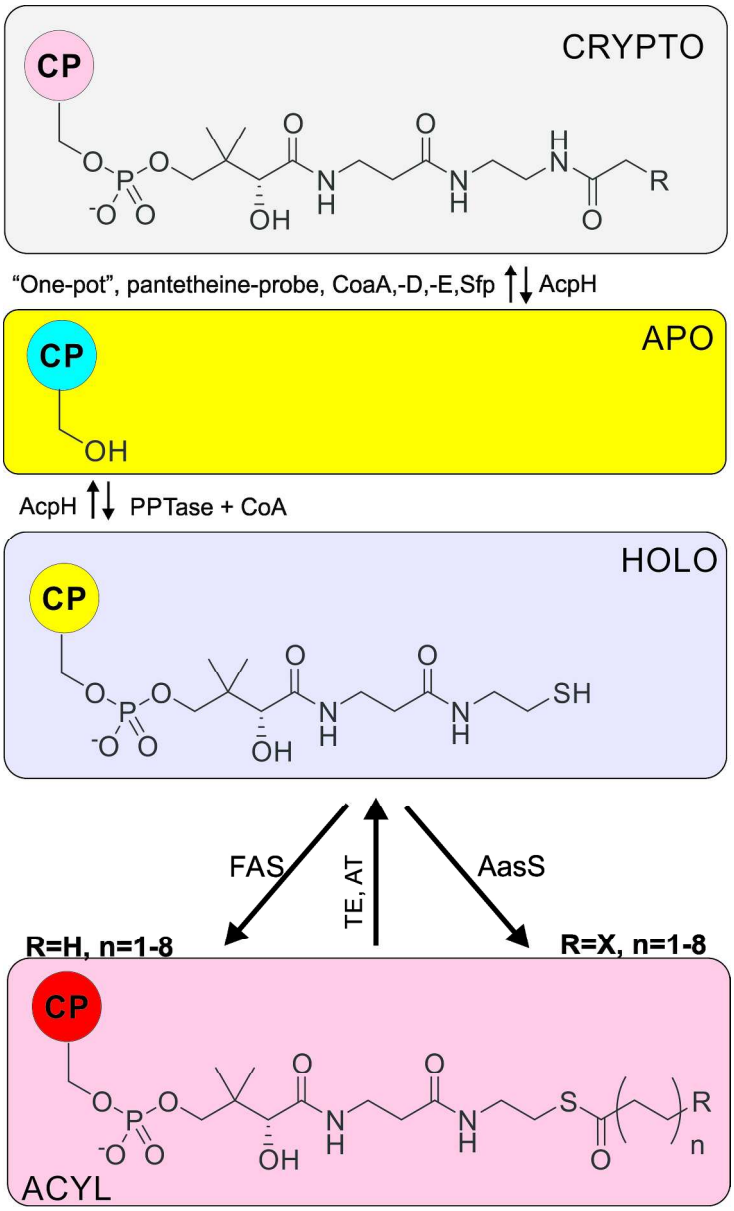

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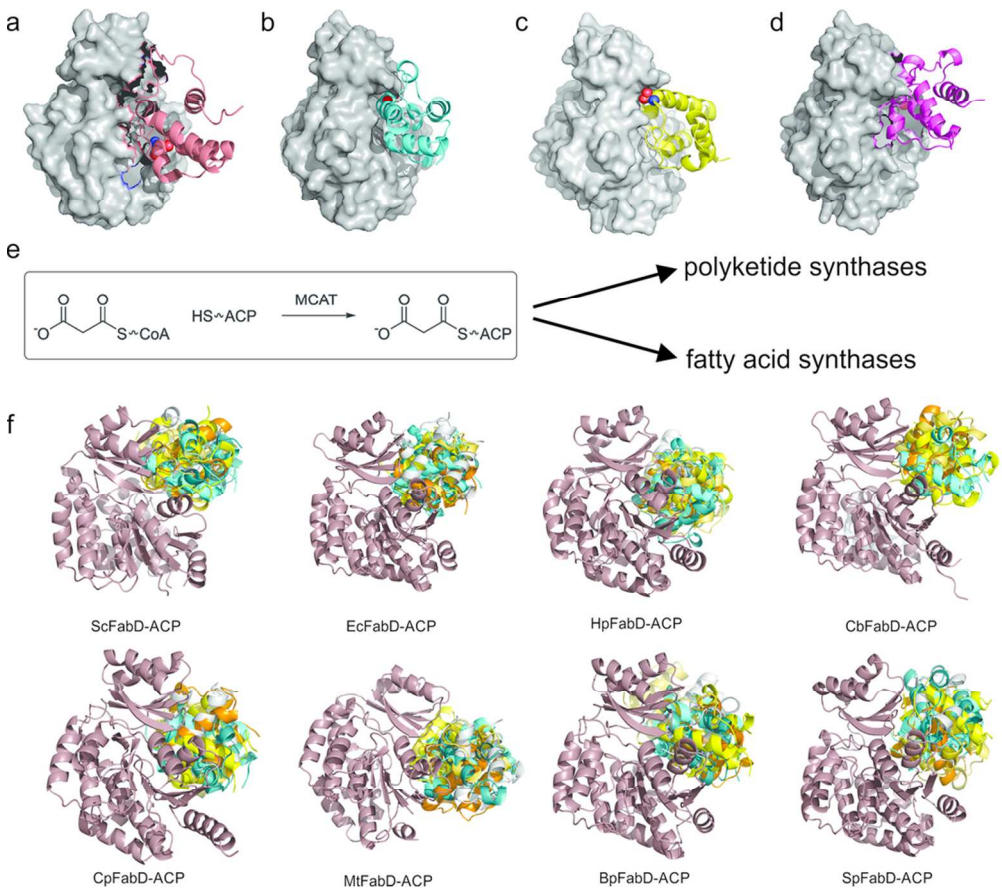

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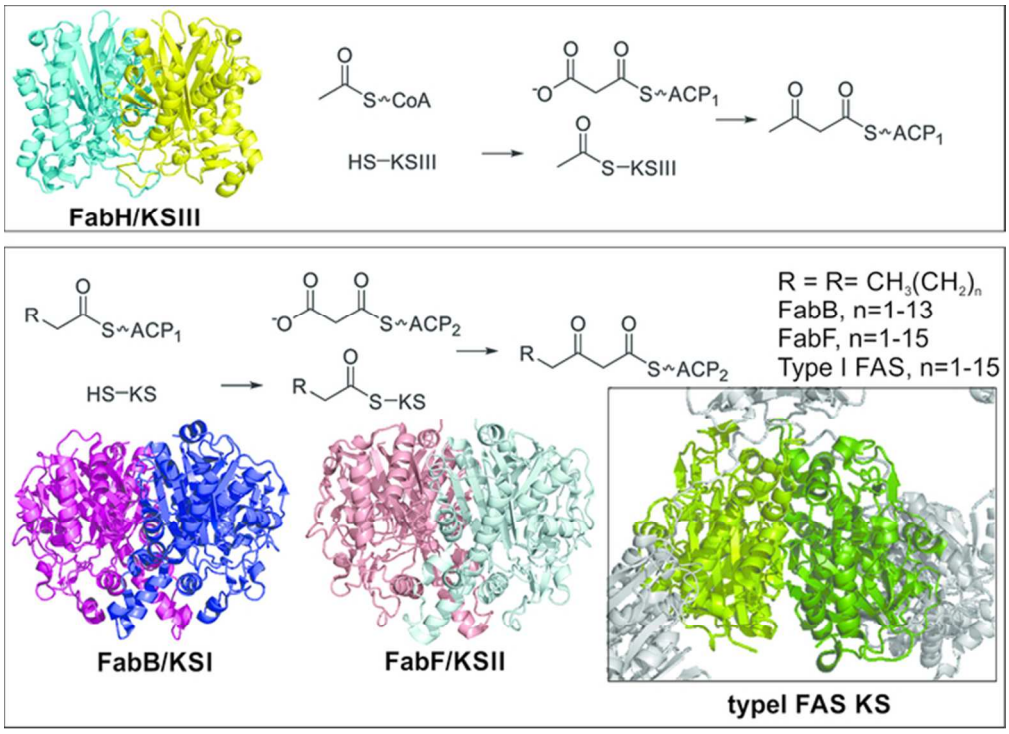

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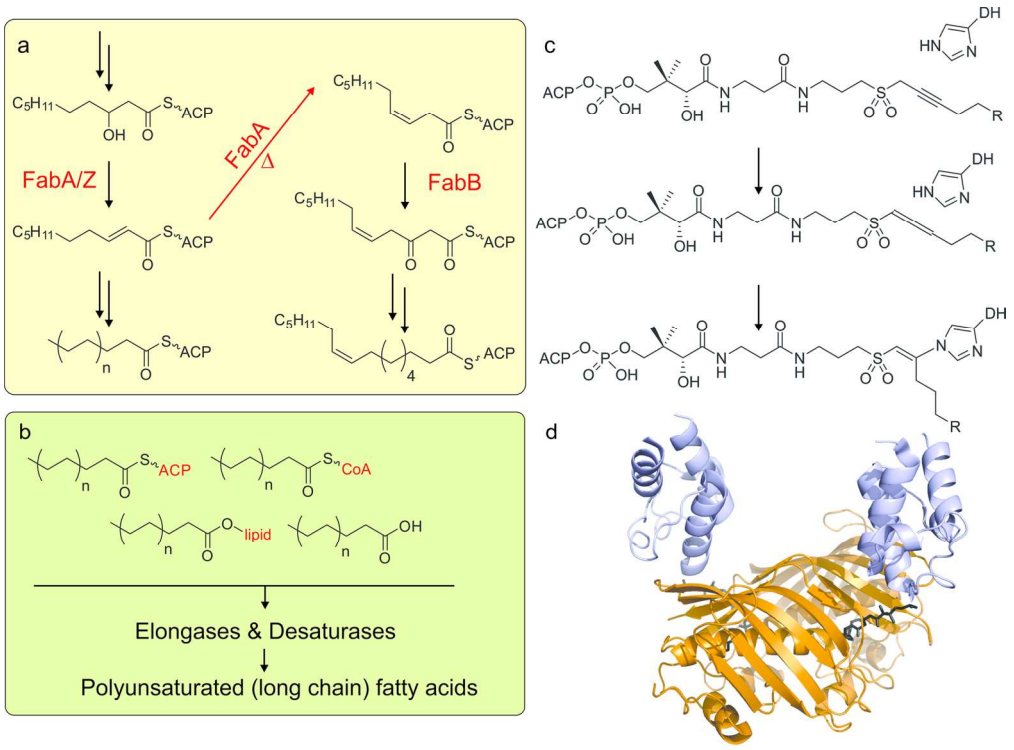

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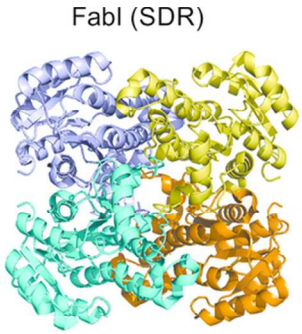

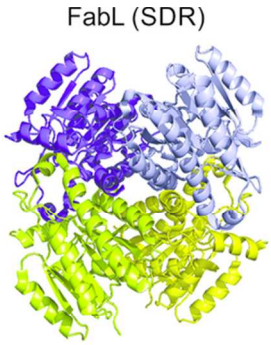

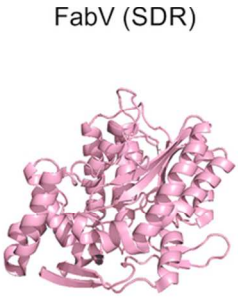

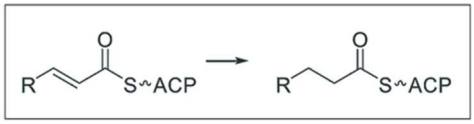

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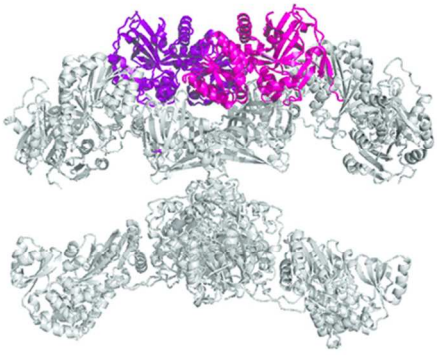
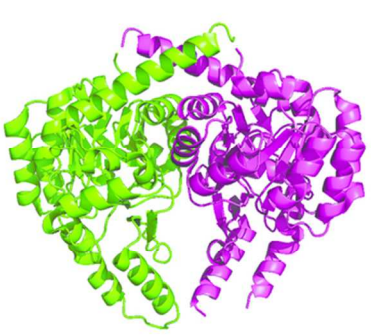
FabK	100	21	19	15	16	13	15
FAS1y	21	100	17	15	18	13	16
FAS1h	19	17	100	23	19	13	21
MER	15	15	23	100	20	16	17
FabV	16	18	19	20	100	19	20
FabI	13	13	13	16	19	100	30
FabL	15	16	21	17	20	30	100





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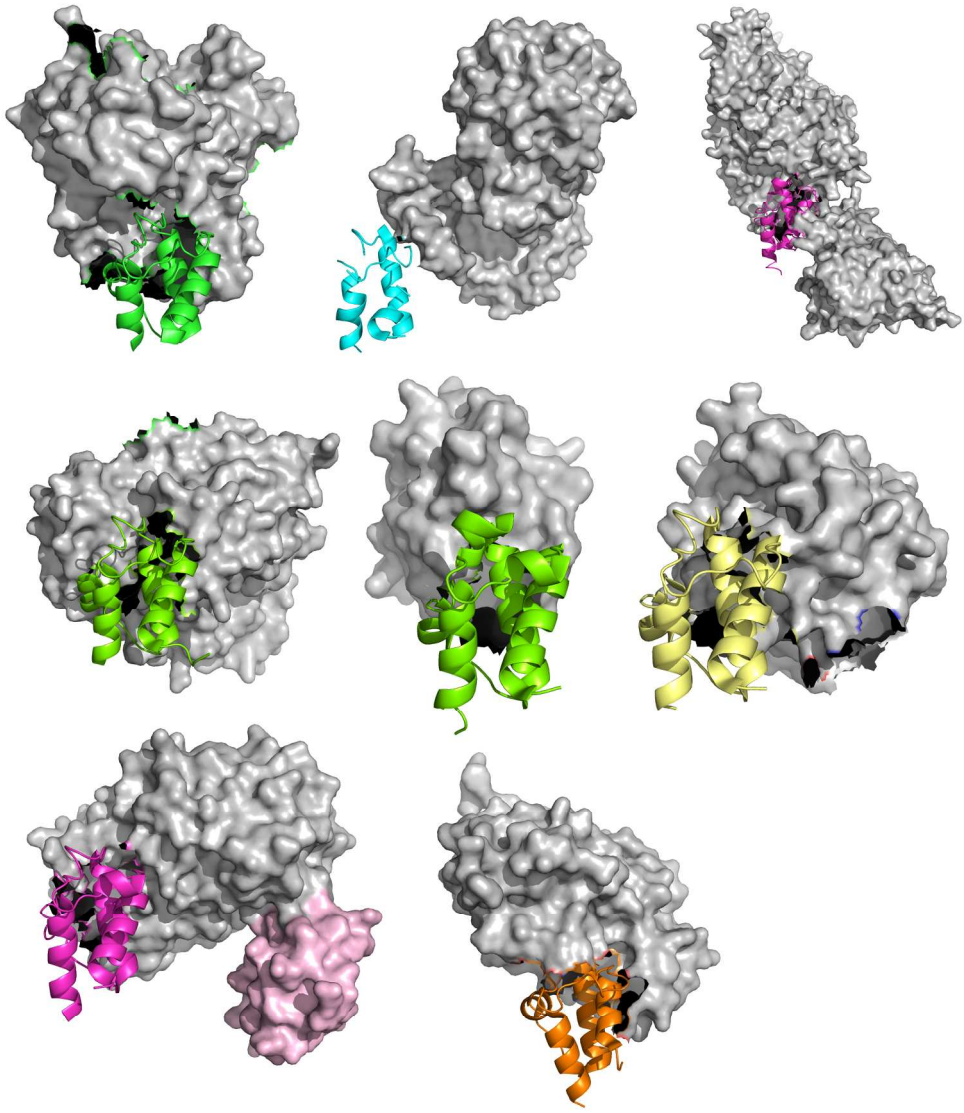

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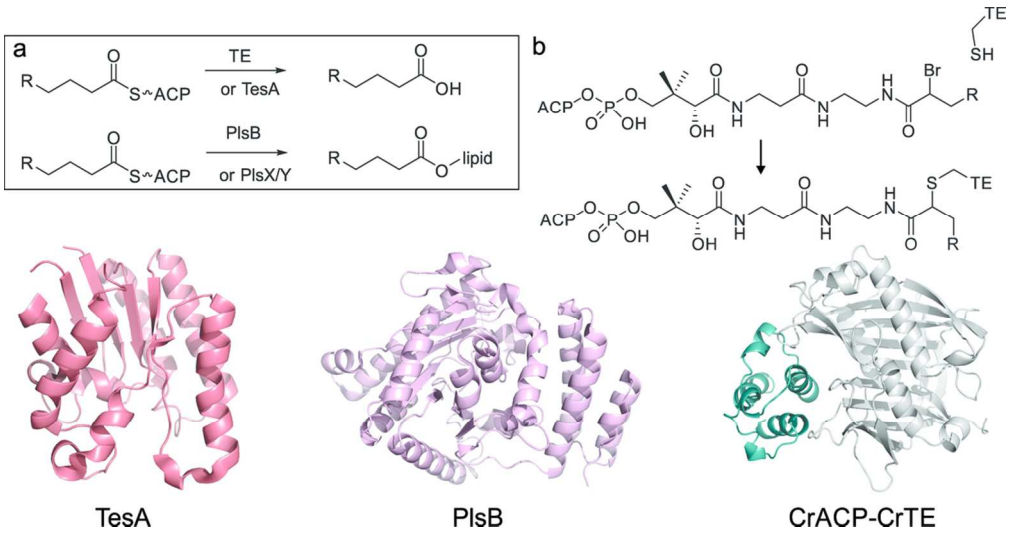

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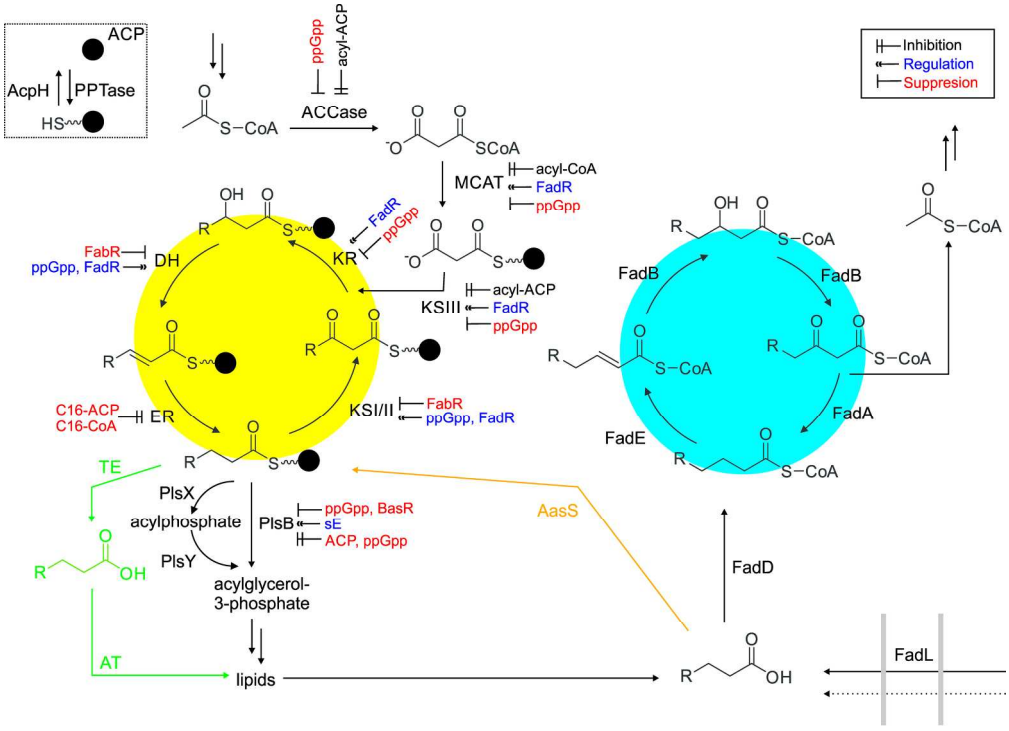

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