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Diversity in Enoyl-Acyl Carrier Protein Reductases

R. Prisca Massengo-Tiassé¹ and John E. Cronan^{1,2}

¹Department of Microbiology, University of Illinois at Urbana-Champaign, Urbana, IL 61801 USA

²Department of Biochemistry, University of Illinois at Urbana-Champaign, Urbana, IL 61801 USA

Abstract

The enoyl-acyl carrier protein reductase (ENR) is the last enzyme in the fatty acid elongation cycle. Unlike most enzymes in this essential pathway, ENR displays an unusual diversity among organisms. The growing interest in ENRs is mainly due to the fact that a variety of both synthetic and natural antibacterial compounds are shown to specifically target their activity. The primary anti-tuberculosis drug, isoniazid, and the broadly used antibacterial compound, triclosan, both target this enzyme. In this review, we discuss the diversity of ENRs, and their inhibitors in the light of current research progress.

Keywords

Enoyl-acyl carrier protein reductase; fatty acid biosynthesis; FAS II; triclosan; SDR; MDR

Introduction

Enoyl-acyl carrier protein (ACP) reductases (ENRs) catalyze the last step of the elongation cycle in the synthesis of fatty acids. Fatty acid biosynthesis is essential for survival in mammals, plants, fungi and bacteria (the archaea make isoprenoid-based lipids). All these organisms use a similar set of reactions that utilize acetyl-CoA as initiating substrate and malonyl-CoA as building block to extend fatty acyl chains by two carbon atoms per cycle. The last step in this cycle is the reduction of the substrate enoyl-thioester to an acyl moiety (Fig. 1). Although this reaction sequence is conserved among diverse organisms, the organization and structure of the enzymes involved differ markedly. The cytosol of mammalian cells contains a large multifunctional homodimeric protein (called FAS I) which contains all of the enzymatic activities in the pathway whereas the fungi have a different FAS I in which the needed active sites are split between two multifunctional proteins that function as a hexameric structure [1, 2]. In contrast bacteria, plants, apicomplexan protozoa and mitochondria synthesize fatty acids using a series of discrete monofunctional proteins that each carry out a single reaction. This is called the type II fatty acid synthesis or FAS II pathway [3]. The FAS I and FAS II pathways are closely related in that the high resolution structures of an individual FAS II protein can often be superimposed on that of the cognate domain of the FAS I proteins [2]. Indeed, the dimeric structure of the type II 3-ketoacyl-ACP synthases was an important clue in deduction of the mammalian FAS I structure [4]. Most of the FAS proteins are of a single generic design. For example, the structures (and to some degree the sequences) of the type II FabF 3-ketoacyl-ACP synthases have been conserved in all fatty acid synthesis pathways and even in related pathways such as polyketide synthesis. However, such conservation is not seen within the

Address correspondence to: John E. Cronan, Department of Microbiology, University of Illinois, B103, Chemical and Life Sciences Laboratory, 601 S. Goodwin Ave, Urbana, IL 61801 USA, Tel: 217 333-7919, Fax: 217 244-6697, j-cronan@life.uiuc.edu.

enoyl-ACP reductases. An impressively wide diversity of proteins that catalyze reduction of this double bond are found in nature.

Enoyl-ACP Reductases

Enoyl-ACP reductases catalyze the reduction of a *trans*-2-acyl-ACP (an enoyl-ACP) to the fully saturated acyl-ACP species (note that *trans*-2-butyryl-ACP is often called crotonyl-ACP). The reductant is either NADH or NADPH, although in one case a reduced flavin (FMNH₂) is used as an intermediate in the reduction. The pyridine nucleotide reduction of the double bond is thought to proceed by conjugate addition of a hydride ion from NADH or NADPH to carbon 3 of the *trans*-2-acyl group with the intermediate formation of an enzyme-stabilized enolate anion on the C1 carbonyl oxygen [5,6] (Fig. 2). Collapse of the enolate via protonation at C2 would yield the saturated product with the C2 proton being derived from the hydroxyl group of an active site tyrosine side chain. The tyrosine proton is replenished from solvent via a proton wire involving Lys163 and the ribose hydroxyl groups plus a chain of water molecules [7]. The pyridine nucleotide cofactor hydride ion utilized by the *E. coli* [8] and *Mycobacterium tuberculosis* [5] enoyl-ACP reductases (FabI and InhA, respectively) is the 4*S* hydrogen whereas the mammalian type I synthase uses the 4*R* hydrogen [9]. The *trans*-2 unsaturated acyl chain is linked to ACP via a thioester linkage that is required for enolization. ACP is a key feature of the fatty acid synthetic pathway in that all of the intermediates are covalently bound to this small, very acidic and extremely soluble protein [7]. The carboxyl groups of the fatty acyl intermediates are in thioester linkage to the thiol of the 4'-phosphopantetheine (4'-PP) prosthetic group that in turn is linked to Ser-36 of ACP through a phosphodiester bond. ACP thioesters are the substrates of the enzymes of the pathway. The ACPs of type II systems are discrete proteins whereas those of the type I systems are a domain of these polyfunctional "megasynthase" proteins. These ACP domains have a structure very similar to the type II ACPs [10,11]. Although the physiological substrate of ENRs is the cognate *trans*-2-acyl-ACP, these enzymes will often show activity with model substrates such as CoA or N-acetylcysteamine *trans*-2-acyl-thioesters. However, the K_M values for the model substrates are generally much higher than those of the ACP substrates and the V_{MAX} values are usually lower. In fatty acid synthesis ENRs not only complete the reaction cycle but also serve to pull the reversible reactions that process carbon atom 3 of the acyl chain to completion. In the absence of ENR activity, *E. coli* [12] and the mammalian type I FAS [13] accumulate the products of the 3-ketoacyl-ACP synthase (the only irreversible reaction of the fatty acid synthesis cycle) and 3-ketoacyl-ACP reductase steps as 3-hydroxyacyl-ACPs. This is due to the equilibrium of the 3-hydroxyacyl-ACP dehydratase step which greatly favors formation of the 3-hydroxyacyl species.

A structure of 2-dodecenoyl-ACP cocrystallized with *E. coli* FabI has recently been reported [14]. Unfortunately, the resolution of structure was such that it yielded little direct information and much of the proposed structure was based on model building and molecular dynamics simulations. Indeed, only parts of the ACP backbone and none of the side chains were visible in the x-ray structure and these were modeled in using the available butyryl-ACP structure. Moreover, neither the prosthetic group nor the attached fatty acid were visible and hence these moieties were also modeled into the structure. These manipulations plus the dynamic nature of ACP structure result in only a speculative model. Given this caveat, the two proteins are reported to interact via a very small interface composed of electrostatic interactions between three acid residues of ACP helix 2 and three basic residues adjacent to the FabI substrate-binding loop. Although this interface is similar in composition to that formed between ACP and AcpS, it is much smaller and probably less stable. Although mutation of the implicated FabI basic residues did result in increased Michaelis constants for 2-dodecenoyl-ACP, some of these mutations had only modest effects and others did not conform to the purely electrostatic interactions proposed in that substitution of alanine for a putatively key lysine residue had a

greater effect than substitution with a glutamate residue. An interesting feature of the crystal structure is that only one of the two FabI monomers had an associated ACP suggesting that only one of the two subunits of the FabI homodimer is active at a time [14].

Within the field of fatty acid biosynthesis ENRs were rather neglected enzymes until the *Mycobacterium tuberculosis* ENR, InhA, was discovered to be the target of the primary anti-tuberculosis drug, isoniazid [15]. This was soon followed by the realization that the ENRs of *E. coli* and many other bacteria were targets of the antimicrobial, triclosan, a synthetic compound very widely used in such every day products as soap, toothpaste and plastics [16]. These findings argued that differences between mammalian FAS I ENRs and bacterial FAS II ENRs rendered the bacterial enzymes good antimicrobial target candidates. However, other bacteria were found to be resistant to triclosan suggesting the presence of triclosan-resistant ENRs and these were soon demonstrated [17]. These factors have led to a striking recent expansion of the ENR literature.

Diversity of Enoyl-ACP reductases

Most ENRs are members of the Short chain Dehydrogenase Reductase (SDR) superfamily and thus are closely related to the other SDR enzyme of the fatty acid synthesis cycle, 3-ketoacyl-ACP reductase, in both structure and mechanism [7]. The many members of the SDR family show a significantly conserved structure despite little sequence homology (15–30%) [18–20]. The largely conserved SDR folding pattern allows specific sequence motifs to be assigned, with those for the coenzyme-binding and active site regions being the most definitive. Other classes of ENRs are the TIM barrel flavin containing enzyme, FabK, the ENRs found in mitochondria and the ENR domains of the mammalian and fungal megasynthases. The mitochondrial ENR and the mammalian FAS ENR domain are members of the Medium chain Dehydrogenase Reductase (MDR) family. Both NADH and NADPH are used as the hydride source in the reactions of SDR-type ENRs. Some enzymes strongly favor one coenzyme over the other whereas others function with either coenzyme. For example, *E. coli* FabI is active with either NADH or NADPH [21], but the two activities are not equally efficient. The NADPH-dependent activity is weaker than the NADH-dependent activity and a single mutation at the C-terminal end of the protein (Ser₂₄₁→Phe) results in loss of the NADPH activity [21]. FabI is reported to respond differently to diazaborine inhibition (see below) depending on which cofactor is bound [21]. The NADPH enzyme is refractory to inhibition suggesting that FabI adopts different conformations depending on the coenzyme bound [21]. There seems to be no discernable physiological rationale for coenzyme choice among ENRs, although all of the known 3-hydroxyacyl-ACP reductases, the other pyridine nucleotide-requiring fatty acid cycle enzyme, use NADH. Note that the coenzyme(s) used by a given SDR enzyme can often be deduced from its sequence [20].

SDR superfamily ENRs

The crystal structures of four SDR superfamily ENRs are available. The structures of *E. coli* FabI [22], the ENR of rape seed plastids [6,23], and *M. tuberculosis* InhA [24] indicate that all are homotetramers of essentially the same structure. *E. coli* FabI and *M. tuberculosis* InhA are the most thoroughly studied of these enzymes. Both have the highly conserved SDR motif, Tyr-X₆-Lys in which the primary role of the lysine side chain is to stabilize the binding of the cofactor through hydrogen bond interactions with the hydroxyl groups of the nicotinamide ribose moiety whereas the tyrosine acts as the proton donor to C2 [7]. Structures of FabI, InhA and the plant plastid ENR complexed with various inhibitors have allowed more detailed descriptions of the active sites of these enzymes that will be discussed below. In comparison to the other enzymes, InhA has a deeper binding cleft that is postulated to allow accommodation of the long-chain substrates of mycolic acid biosynthesis. However, it has recently been shown

that InhA can support short chain fatty acid synthesis when expressed in yeast mitochondria [25].

Two other members of the SDR class of ENRs, FabL [26] and FabV [27] illustrate the sequence diversity within this enzyme family. *Bacillus subtilis* FabL is only 25% identical with *E. coli* FabI and uses NADPH whereas the *B. subtilis* FabI protein uses NADH [26]. Although FabL has the Tyr-X₆-Lys motif in common with FabI proteins, the coenzyme-binding site of FabI has the classical Rossmann fold motif whereas the FabL coenzyme-binding fold departs from that motif. *Vibrio cholerae* FabV is 60% larger than the typical SDR family member (which are generally about 250 residues in length) and the spacing between the FabV active site tyrosine and lysine residues is eight residues (Tyr-X₈-Lys) [27] which is two more than FabI and FabL and one more than the maximum reported in other SDR proteins [20]. However, like FabI, FabV has the classical Rossmann fold motif [27]. A recent bioinformatic analysis of the SDR superfamily placed the enzymes into five families [20]. One of these families (called divergent) is composed of the FabI-type enoyl-ACP reductases of bacteria and plants [20]. FabL and FabV do not fall cleanly into this or any of the four other SDR families.

Non-SDR FAS ENRs

Although, due to their presence in bacteria and plants, ENRs of the SDR superfamily are probably the dominant form in biology, other proteins catalyze this reaction. The most extreme example is *Streptococcus pneumoniae* FabK which is an FMN-containing protein, the crystal structure of which was recently reported [28]. In this enzyme NADH is the reductant, but acts indirectly by reducing the tightly bound flavin cofactor. The reduced flavin then reduces the double bond. Unlike the SDR enzymes, FabK has a TIM barrel ($\alpha 8$ - $\beta 8$) structure and is an FMN-dependent oxidoreductase of the NAD(P)H-dependent flavin oxidoreductase family. The closest relative of FabK is the ENR domain of the Type I FAS of the fungi, *Saccharomyces cerevisiae* [29] and *Thermomyces lanuginosus* [30]. Another close relative is 2-nitropropane dioxygenase which was the original annotation of FabK. FabK was discovered due to its genomic location within a cluster of fatty acid synthetic genes and the lack of an SDR-type ENR encoded in the *S. pneumoniae* genome [17,31].

In mammalian and fungal mitochondria the ENRs are members of the MDR superfamily which contains a GHE motif roughly 60 residues from the N-terminus plus a mid-chain GX1–3GX1–3G pattern. Structures of both the yeast and human mitochondrial ENRs are available [32, 33]. The main function of mitochondrial fatty acid synthesis is thought to be production of the octanoyl moiety of lipoic acid [34–37].

In the mammalian FAS megasynthase, in contrast to all of the other functional domains of the fatty acid elongation cycle [2], the ENR domain has a different fold from its functional analogues of the bacterial type II FAS. Rather than the SDR fold (FabI, FabL, FabV), the typical MDR fold (in mitochondrial ENRs) or even the TIM barrel protein fold (FabK), the mammalian ER establishes a new MDR subfamily that is structurally related to bacterial quinone oxidoreductases [38]. The mammalian FAS I ENR contains two subdomains, a nucleotide binding Rossmann-fold and a substrate-binding portion. The NADP⁺ cofactor is bound between the two subdomains [38].

Physiological manifestations of ENR levels

The effects of ENR deficiency have been examined in several genetically accessible organisms. In *E. coli* a *fabI* mutation that encodes an enzyme that loses activity at high temperature blocks growth at high temperatures [12]. As expected the growth-inhibited cells accumulated the four carbon 3-hydroxyacyl-ACP and *trans*-2-acyl-ACP species [12]. Recently, it has been reported that overproduction of FabI is toxic to growth of *E. coli* and this toxicity is offset by low doses

of the FabI inhibitor, triclosan, thereby indicating that overproduction of enzyme activity rather than of FabI protein causes inhibition [39]. Although the reason for this toxicity has not been established, two hypotheses involving disruption of the normal cellular fatty acid composition seem worth testing. The first hypothesis is that the extra FabI activity pulls the elongation cycle such that 3-hydroxytetradecanoyl-ACP is converted to tetradecanoyl-ACP and thus the supply of 3-hydroxytetradecanoyl-ACP required for synthesis of lipid A, an essential outer membrane component, becomes limiting. The second hypothesis depends on the fact that FabI potentially competes with the isomerase activity of FabA, the dehydratase/isomerase responsible for introduction of the double bond in *E. coli* unsaturated fatty acid synthesis. FabA dehydrates 3-hydroxydecanoyl-ACP to *trans*-2 decenoyl-ACP and then isomerizes the double bond to give *cis*-3-decenoyl-ACP, the double bond of which is perpetuated as the double bond of the long chain fatty acids [40]. However, the *trans*-2 decenoyl-ACP intermediate is not tightly bound to FabA [41–43] and upon dissociation could be reduced by the excess FabI before it could be recaptured by FabA. This would result in an unsaturated fatty acid deficiency which, if severe, would result in cell lysis [44]. Although this scenario has not yet been tested, it is consistent with our finding that FabI deficiency results in increased unsaturated fatty acid synthesis at the expense of saturated fatty acid synthesis (unpublished data). This presumably results from accumulation of *trans* 2-decenoyl-ACP [12] thereby making more substrate available for the FabA-catalyzed isomerization reaction.

A more direct connection between ENR activity and unsaturated fatty acid synthesis arises in *Streptococcus pneumoniae*. This bacterium uses a monofunctional isomerase introduction of the double bond in its unsaturated fatty acids [45]. This enzyme isomerizes *trans*-2 decenoyl-ACP to *cis*-3-decenoyl-ACP and thus directly competes with FabK for substrate. Hence, the relative levels of the two proteins must be closely controlled to allow the synthesis of unsaturated fatty acids, which are essential for cell growth [46].

In *B. subtilis*, which has two ENRs, FabI and FabL, the gene encoding either enzyme can be inactivated without blocking growth, but a strain lacking both enzymes could not be constructed [26]. In the yeast, *Saccharomyces cerevisiae*, a single mutation suffices to block growth on non-fermentable carbon sources [47]. Another yeast, *Candida tropicalis*, has two mitochondrial ENRs that seem to be a result of gene duplication. Either gene can replace the *S. cerevisiae* enzyme *in vivo*. When given a mitochondrial targeting sequence the *S. cerevisiae* enzyme can also be functionally replaced by *E. coli* FabI [48] and *M. tuberculosis* InhA [25]. The mammalian homologue also restores mitochondrial fatty acid synthesis and respiratory function to the *S. cerevisiae* mutant strain [49]. In the plant, *Arabidopsis*, a mutation resulting in 90% loss of ENR activity gives only a 10% reduction in fatty acid content indicating that *Arabidopsis* ENR is in large functional excess [50].

Regulation of ENR levels

Although, there is some evidence for transcriptional regulation of ENR expression during plant development [51–53], the regulatory mechanisms remain unclear. Differential transcription of the two mitochondrial ENRs of *C. tropicalis* has also been reported specifically during growth on nonfermentable carbon source [48], suggesting different specific roles for the isoforms, but again the regulatory mechanisms have not been defined. *In vitro* results indicate that ENR activity is regulated at the enzyme level. *E. coli* FabI is inhibited by long chain acyl-ACPs [54]. This may be a case in which the product inhibition seen with all enzymes has been utilized for regulatory purposes. *E. coli* FabI has also been reported to be inhibited by palmitoyl-CoA *in vitro* [21]. Although this inhibition has been ascribed a physiological role, it remains to be demonstrated that the observed inhibition is not due to the well-known detergent properties of long chain acyl-CoAs. Several bacterial FabIs were reported to be inhibited by long chain fatty acids *in vitro* [55]. However, since activity was assayed using a hydrophobic model compound,

trans-2-octenoyl N-acetylcysteamine, the observed inhibition might be due to partition of the substrate into fatty acid micelles rather than a direct effect on the enzymes. These studies should be repeated with an ACP substrate.

Polyketide synthase ENRs

There is no doubt that polyketide synthesis is an evolutionary descendent of fatty acid synthesis. The fatty acid synthase (FAS I) responsible for *de novo* fatty acid synthesis in the cytosol of animal cells is homologous in sequence and analogous in architecture with the very large family of type I modular polyketide synthases (PKSs) [56]. Both “megasyntase” assembly lines use homologous domains (ACP or PCP) to carry the growing fatty acid or polyketide via a pantetheine-linked thioester [56]. The common thioester chemistry, similar structures and the adaptable architecture have resulted in the proliferation of hybrid PKS-FAS pathways found in phylogenetically diverse bacteria [56]. Indeed, the extremely complex lipids of mycobacteria are made via a partnership between a FAS and a modular PKS [57] and a class of fatty acids are produced by modular PKS systems [58,59]. Therefore, a comparison of the FAS and polyketide synthase ENRs is of interest. The ENRs of the type I polyketide synthases are of an MDR sub-family and are closely related to that found in the mammalian FAS I enzyme. These ENR domains have the same location within the progression of domains as the ENR of the mammalian FAS megasyntase. Inactivation of the ENR of either a type I polyketide synthase [60] or the mammalian FAS I [13] results in accumulation of the expected *trans*-2 unsaturated species thereby establishing the function of these domains.

Recently several ENRs involved in polyketide synthesis have been described and two of these have recently been shown to have ENR activity [61]. These enzymes are either monomeric proteins or bifunctional proteins containing ENR and acyl transferase domains. These enzymes violate the straightforward scenario of type I polyketide synthesis in which the domains of the multifunctional megasyntase contain all of the activities required to assemble the complete carbon skeleton. These ENRs act on intermediates tethered to an ACP domain of the megasyntase and therefore act in *trans* [61]. Threading on known structures suggests that these enzymes are related to FabK, although the presence of a flavin cofactor has not yet been reported.

ENR Inhibitors

A striking feature of the well-studied inhibitors of SDR family ENRs (InhA and FabI) is that inhibition requires the NAD⁺ product to be bound within the active site. That is, the inhibitor forms a stable ternary complex with the protein and the oxidized cofactor. Some inhibitors form a covalent bond with NAD⁺ whereas others do not. The fact that each of these inhibitors must complete a catalytic cycle and bind the inhibitor before the weakly bound NAD⁺ product is released accounts for the observed slow inhibition of ENR activity. The extensive interactions made by the inhibitors with both the NAD⁺ moiety and the protein in the ternary complex readily explains the tight binding properties of these inhibitors.

INHIBITORS THAT FORM A COVALENT BOND

The anti-mycobacterial properties of isoniazid (INH) have been known for almost fifty years. The drug is active only against mycobacteria and the slow growing mycobacteria are especially sensitive [62]. Isoniazid-resistant strains were isolated almost immediately after clinical use of the drug was initiated and today as much as 30% of all clinical isolates of *M. tuberculosis* are isoniazid-resistant. The rapid development of isoniazid resistance is due to the fact that the compound is a pro-drug requiring activation by the KatG catalase/peroxidase. Most isoniazid resistance is due to inactivating mutations within the catalase/peroxidase gene, which have no effect on *M. tuberculosis* virulence. Activation of isoniazid by whole cells of *M.*

tuberculosis containing KatG produces a variety of isonicotinoyl-NAD adducts [63]. One of these adducts, the acyclic 4S isomer, is the InhA inhibitor [62]. Resistance to isoniazid can occur by mutation of any one of at least three genes other than *katG* and *inhA* [62]. X-ray diffraction and mass spectrometric analyses of specific InhA NAD-acylpyridine adducts show that the compound covalently binds C4 of the cofactor nicotinamide ring. Subsequent π -stacking with the ring of an active site Phe residue strengthens interactions with the protein. The position blocked by covalent adduct formation is that involved in the hydride transfer from NADH to enoyl-ACP in the course of the InhA reaction cycle. Attempts to make isoniazid-NAD⁺ analogues have met with some success, but this work is chemically challenging and in its early stages [64,65].

Diazaborines are a class of heterocyclic boron-containing compounds that inhibit FabI by the formation of a covalent bond between the boron atom and the 2'-hydroxyl of the NAD⁺ ribose moiety [22,66]. The drug π -stacks with the nicotinamide ring of the coenzyme and also has van der Waals interactions with the hydrophobic substrate-binding pocket. The boron atom and its associated hydroxyl group occupy the space of the enolate in the putative substrate complex. While both isoniazid and diazaborines form covalent adducts with the NAD-bound form of enoyl-ACP reductase, the point of attachment is different and the interaction of the two drugs with the target enzyme differs. Diazaborines seem to have been abandoned as a medically useful set of compounds, perhaps due to their undesirable inhibition of RNA processing in eukaryotic cells [67].

Non-covalent ENR inhibitors

Triclosan (TCL)

The four types of bacterial ENRs show different interactions with TCL, a trichlorinated biphenyl ether. In 1998 McMurray and coworkers reported that TCL resistance in *E. coli* mapped to *fabI*, the gene encoding enoyl-ACP reductase [68]. *In vivo* and *in vitro* confirmation of this finding quickly followed [17,69–71]. Heath and co-workers unequivocally demonstrated that TCL inhibited the enoyl-ACP reductase step of fatty acid biosynthesis and were the first to recognize that the specific mutations resulting in TCL resistance within *fabI* were analogous to the *inhA* mutations imparting resistance to INH in *M. tuberculosis* [69]. The InhA enzymes of both *M. smegmatis* and *M. tuberculosis* were subsequently demonstrated to also be inhibited by TCL [72,73]. Likewise, the *Arabidopsis* plant ENR was highly sensitive to inhibition by triclosan [74]. Kinetic and structural studies of the interaction of TCL with four different FabI proteins have shown that TCL forms a tightly associated ternary complex with the protein and the charged nicotinamide cofactor (47, 62, 94, 122). Triclosan is composed of two linked chlorine-substituted aromatic rings. As seen in the diazaborine structures (although without covalent bond formation) the phenol ring makes π -stacking interactions with the nicotinamide ring of NAD⁺ and the hydroxyl group of the drug forms hydrogen bonds with both the phenolic oxygen of Y156 and the 2'-hydroxyl of the nicotinamide ribose. Extensive van der Waals interactions with the protein also occur. It has been proposed that triclosan binding represents a model for substrate binding [16,66,75].

Not all SDR family ENRs are sensitive to TCL. FabL is very poorly inhibited by TCL. The inhibition is reversible and does not require NAD⁺ whereas FabV is essentially refractory to inhibition by TCL (Figure 3). The FabK flavoprotein ENR is not inhibited by TCL and expression of *S. pneumoniae* FabK in *E. coli* raises the TCL concentration required to inhibit growth by over 4 orders of magnitude [31]. Moreover, *B. subtilis* FabI is less sensitive to TCL than is the *E. coli* enzyme [26]. Due to the widespread resistance of pathogenic bacteria to many of our present antibiotics, ENRs are the focus of large scale attempts to find compounds that specifically inhibit these enzymes. The ongoing attempts to find such molecules have generally begun with small molecule libraries based on structures that resemble TCL [65,76–

79]. Some of the resulting lead compounds inhibit FabKs as well as FabIs, although in only a few cases have the mechanisms of inhibition been defined. However, as yet, none of these compounds has surfaced as clinically useful antimicrobials. It should be noted that the presence of two entire classes of enoyl-ACP reductases that are insensitive to TCL inhibition, coupled with the promiscuous use of TCL in many everyday products, suggests it is only a matter of time until many bacteria acquire drug-resistant ENRs. Until very recently TCL was considered a nonspecific, broad spectrum, antibacterial and antifungal biocide. This compound is present in all manner of consumer items, including hand soaps, mouthwash, fabrics and plastics. The ecological damage is compounded by the fact that resistance to TCL is often concomitant with resistance to other FabI inhibitors including isoniazid, a drug generally reserved for use as a front line anti-tuberculosis drug. Moreover, resistance to TCL can also arise by overexpression of MarA, SoxS, and the AcrAB-encoded multidrug efflux pump, as well as by mutation of single residues within the FabI active site. Recently, TCL has been used to select for plasmid maintenance using *E. coli fabI* as the selective marker for plasmid retention [39]. This system may be of some advantage, but the possibility of resistance due to chromosomal mutations in *E. coli* and the existence of naturally TCL-resistant ENRs in other bacteria would seem significant limitations to its use.

Another possible objection to TCL, that it might inhibit the mitochondrial ENR of the host, does not seem a problem since the bacterial and mitochondrial ENRs belong to distinct protein families (SDR and MDR, respectively) and thus would seem unlikely to be affected by the same inhibitors. Moreover, *S. cerevisiae* is insensitive to TCL unless the yeast mitochondrial ENR is replaced with *E. coli* FabI. In this case the yeast cells become sensitive to TCL indicating that the compound crosses the yeast cell and mitochondrial membranes [48]. It would be of interest to repeat the ENR replacement experiment with the mammalian mitochondrial ENR [49] and test the TCL sensitivity of this construct. On the other hand, TCL has been reported to inhibit the ENR activity of the type I mammalian FAS megasynthase [80]. However, TCL is approved by regulatory authorities in the EU and the USA for many applications and an association between bacterial triclosan resistance and antibiotic susceptibility, though suggested, has not been found in practice [81].

Other inhibitors

Specific inhibitors have been described for FabK [79,82]. Kinetic analysis and a crystal structure indicate that one such compound acts by competitive inhibition of NADH binding [28]. The diversity of the bacterial enoyl-ACP reductases relative to the lack of structural and mechanistic diversity seen in the other enzymes of the FAS II elongation cycle argues that naturally occurring compounds exist that selectively inhibit one or another of these enzymes. This hypothesis has recently been confirmed by the discoveries of natural enoyl-ACP reductase inhibitors of fungal origin that specifically target FabK (Atromentin and Leucomelone) [83] and FabI proteins (Cephalochromin) [84] and cyperin [74]. Interestingly, another natural compound of fungal origin, cyperin, is also reported to inhibit the ENR of the plant *Arabidopsis* [74]. It would be interesting to test this compound on the bacterial ENRs. The mechanisms of ENR inhibition by these antibiotics remain to be described in detail. It is interesting that Cephalochromin was identified over 20 years ago as a natural compound of fungal origin with anti-tumor properties [85].

Conclusions

The diversity of ENRs in nature is surprising and suggests that evolutionary pressures such as the natural inhibitors mentioned above have exerted a selection for diversity. A sign of the evolutionary pressures is that some bacteria have only a single ENR whereas others have two, either of which suffices for growth in the laboratory. If these ENRs are functionally redundant,

why are both enzymes retained? This must be understood before ENR inhibitors with broad spectrum antibacterial activity can be developed. Moreover, it is likely that additional protein families that carry out the ENR reaction will be discovered. This seems certain to occur if ENR inhibitors turn out to be clinically useful antimicrobials. Finally it should be noted that annotation of bacterial ENRs is often problematical. Genomes generally encode many SDR family members and in the absence of a genomic context consistent with a role in fatty acid synthesis, a true ENR can be very difficult to distinguish from other family members that carry out the unrelated reactions such as reduction of a keto group. A parallel problem arises with the other SDR family fatty acid synthetic enzyme, 3-ketoacyl-ACP reductase [86]. It is also difficult to identify FabK homologues. Several proteins that align well with *S. pneumoniae* FabK have been reported to lack ENR activity [26,31].

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Fig. 1.

The enoyl-ACP reductase (ENR) reaction. The bond reduced is shown in red. The lengths of the red bonds are accentuated for purposes of illustration. Depending on the enzyme the reductant can be NADPH or FMNH₂ rather than NADH.

Fig. 2.

The reaction mechanism of *E. coli* FabI. The double bond reduction occurs by conjugate addition of a hydride ion from NADH (or NADPH) to C3 of *trans*-2-butenoyl-ACP. The added to C3 is the *pro-S* hydrogen of the coenzyme. The Tyr15 proton is replenished by a proton relay system through Lys163 and the hydroxyls of the coenzyme ribose moiety plus a chain of water molecules that provide access to solvent.

Figure 3.

Overview of the effects of triclosan (Tcl) on the SDR family ENRs, FabI, FabL and FabV. NAD binds to FabI only weakly [88], while FabV is strongly inhibited upon preincubation of the enzyme with NAD in the absence of substrate [27]. Triclosan does not directly bind to any of the three enzymes with appreciable affinity. The FabI-NAD-triclosan ternary complex is stable and highly inhibitory whereas FabL is unable to form a stable ternary complex [26]. Whether triclosan is actually able to bind the unstable FabV-NAD complex is unclear, but of little relevance since FabV is essentially refractory to inhibition by this compound [27].

Table I

Comparison of the catalytic signatures of enoyl-ACP reductases members of the large Short chain Dehydrogenase Reductase (SDR) superfamily. The bacterial ENRs are in bold whereas the others are plant ENRs. The underlined residues are the strongly conserved residues that have roles in ENR catalysis.

Name	Catalytic signature	Reference
Most SDR members	<u>Asn</u> -X _n - <u>Ser</u> -X ₁₀ - <u>Tyr</u> -X ₃ - <u>Lys</u>	[18,87]
<i>M. tuberculosis</i> InhA	<u>Phe</u> -X ₈ - <u>Tyr</u> -X ₆ - <u>Lys</u>	[24]
<i>S. typhimurium</i> FabI <i>H. pylori</i> FabI <i>E. coli</i> FabI	Ser-X ₁₀ - <u>Tyr</u> -X ₆ - <u>Lys</u>	[24]
<i>B. subtilis</i> FabI	Thr-X ₁₀ - <u>Tyr</u> -X ₆ - <u>Lys</u>	[24]
<i>B. subtilis</i> FabL	Ser-X ₁₀ - <u>Tyr</u> -X ₆ - <u>Lys</u>	[26]
<i>V. cholerae</i> FabV	Ser-X ₁₀ - <u>Tyr</u> -X ₈ - <u>Lys</u>	[27]
<i>B. napus</i> ENR <i>A. thaliana</i> ENR <i>Nicotinia tabacum</i> ENR	Thr-X ₁₀ - <u>Tyr</u> -X ₇ - <u>Lys</u>	[24]