



Review

Structure and mechanism of *Escherichia coli* type I signal peptidase[☆]

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ARTICLE INFO

Article history:

Received 25 August 2013

Received in revised form 26 November 2013

Accepted 4 December 2013

Available online 11 December 2013

Keywords:

Signal peptidase

Leader peptidase

Signal peptide

Leader peptide

Protein secretion

Preprotein processing

ABSTRACT

Type I signal peptidase is the enzyme responsible for cleaving off the amino-terminal signal peptide from proteins that are secreted across the bacterial cytoplasmic membrane. It is an essential membrane bound enzyme whose serine/lysine catalytic dyad resides on the exo-cytoplasmic surface of the bacterial membrane. This review discusses the progress that has been made in the structural and mechanistic characterization of *Escherichia coli* type I signal peptidase (SPase I) as well as efforts to develop a novel class of antibiotics based on SPase I inhibition. This article is part of a Special Issue entitled: Protein trafficking and secretion in bacteria. Guest Editors: Anastassios Economou and Ross Dalbey.

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1. Introduction

Protein secretion is an essential process in all cells. Important insights into protein secretion mechanisms have been gleaned from studies performed in bacteria, given that many of the fundamental mechanistic elements of the process appear to have been conserved throughout evolution. The hallmark of a secretory protein is the amino-terminal extension – the signal (or leader) peptide. The majority of bacterial proteins, whose final destination resides on the trans-side of the cytoplasmic membrane, are secreted post-translationally via the general Sec-system [1,2]. Protein secretion, catalyzed by the Sec-system, can be thought of as occurring in three separate steps: 1) targeting to the membrane, 2) translocation across the membrane, and 3) release from the membrane. Very briefly, the molecular chaperone SecB binds to the mature region of a secretory preprotein, keeping it in a translocation competent (molten globular) state and helping to guide it to the translocase (SecYEG/SecA) at the membrane surface. SecA, an ATPase, appears to push the preprotein through the SecYEG channel. Accessory proteins SecDFyajC may also play a role, but do not appear to be essential. The translocated preprotein is tethered to the cytoplasmic membrane via its signal peptide until the signal peptide is cleaved off by type I signal peptidase (SPase I). Besides the Sec-system, there are many more specialized bacterial secretion systems and even other signal peptidases, such as the type II [3] and type IV [4] signal peptidases that specialize in the processing of pre-lipoproteins and pre-pilin proteins respectively. These enzymes

(aspartic acid proteases) are neither structurally nor mechanistically related to SPase I (a serine protease).

This review will focus on what is currently known about the third step of the general protein secretion process (SPase I catalyzed cleavage of secretory preproteins) in the model organism *Escherichia coli*.

2. The substrate–membrane bound secretory preproteins

The substrates for SPase I are secretory preproteins tethered to the membrane via their signal peptide (Fig. 1). SPase I catalyzes the cleavage of the secretory preproteins to create two products, the released mature secretory protein and the membrane bound signal peptide. Early sequence analysis revealed the fundamental features of the signal peptide located at the amino-terminus of every secretory protein: a length of approximately 20–30 residues, an amino-terminal region with a net positive charge (N-region), followed by a hydrophobic region (H-region), and then a protease recognition sequence (C-region) with a preference for small residues at the –3(P3) and –1(P1) positions relative to the cleavage site (scissile bond). The specificity is sometimes referred to as the “(–3, –1) rule” or the “Ala-X-Ala rule” [5–8] because of its preference for alanine at the P1 and P3 positions. The average eukaryotic signal peptide is ~23 residues in length while the average Gram-negative eubacterial signal peptide is ~25 residues in length, and the average Gram-positive eubacterial signal peptide is ~32 residues in length [9,10]. It is believed that the variations in the signal peptide lengths may reflect the differences in the thickness of the lipid bilayers in which the signal peptides reside.

Modern genomic sequencing methods (UniProt [11,12]) along with computational signal sequence prediction programs (SignalP [13,14]) have provided lists of potential SPase I substrates for a large number of species whose genome has been sequenced. There is also a growing

Abbreviations: SPase I, bacterial type I signal peptidase

[☆] This article is part of a Special Issue entitled: Protein trafficking and secretion in bacteria. Guest Editors: Anastassios Economou and Ross Dalbey.

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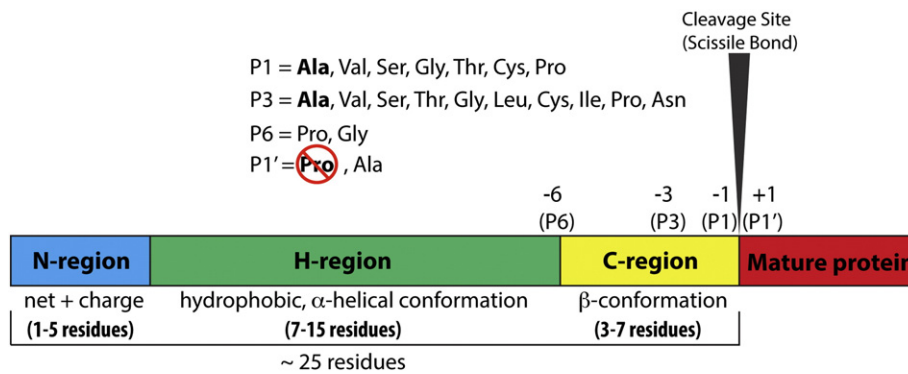


Fig. 1. A schematic diagram of the amino-terminal region of an *E. coli* secretory preprotein. The signal peptide (region before the SPase I cleavage site) is shown in blue/green/yellow. The N-region (blue) has a net positive charge. The H-region (green) is hydrophobic in nature with leucine being the most common residue found in this region. Biophysical studies are consistent with the membrane embedded H-region being α -helical. The C-region (yellow) contains the cleavage specificity residues and most likely undertakes an extended β -conformation when bound within the SPase I substrate-binding groove. The residue just preceding the cleavage site is referred to as the P1 or -1 residue. The mature region (region after the SPase I cleavage site) is shown in red. The residue just after the cleavage site is referred to as the P1' or the $+1$ residue. The allowed residues at the P1, P3, P6 and P1' are listed above the schematic. A secretory preprotein with a proline at the P1' position acts as a SPase I competitive inhibitor and is not cleaved by SPase I.

list of experimentally verified secretory preproteins (and therefore signal peptides) accelerated by the use of tandem mass spectrometry proteomic techniques [15]. Based on these methods, it is currently estimated that approximately 10% of the *E. coli* genes contain signal peptides, fewer than previously thought.

Lists of secretory preprotein and signal peptide sequences (proposed and experimentally verified) from a large number of genomes are available from the Signal Peptide database (SPdb) [16]. For a current list of predicted and experimentally verified secretory preprotein and signal peptide sequences from *E. coli* K-12, see the Ecogene database [17,18].

Both secretory preproteins as well as synthetic peptides have been used in experimental investigations of the *E. coli* SPase I amino acid preferences for each of the signal peptide residue positions, before and after the cleavage site. Synthetic peptides, based on the pre-maltose binding protein, were used to discover the minimal size peptide, $(-3)ALAKI(+2)$, that can be correctly processed by *E. coli* SPase I. The most efficiently cleaved synthetic peptide that was kinetically characterized in this study was: $(-7)FSASALAKI(+2)$ [19]. Site-directed mutagenesis was used to investigate *E. coli* SPase I processing of the M13 procoat [20]. It was found that *E. coli* SPase I was able to process substrates with almost any residue in the $+1$, -2 , -4 , and -5 positions, but only alanine, serine, glycine, and proline were allowed at the -1 position and only serine, glycine, threonine, valine, and leucine at the -3 position. Proline was also required at the -6 position for efficient cleavage. Site-directed mutagenesis experiments were also used to investigate the *E. coli* SPase I catalyzed processing of *E. coli* alkaline phosphatase preprotein. It was found that large residues at the -2 position and mid-sized residues at the -5 position allowed for the most efficient cleavage [21]. This same preprotein was used to further investigate residue preferences at the -6 to -4 positions [22]. A proline at the $+1$ position has been shown to inhibit preprotein processing [23–25].

Biophysical methods have been employed to investigate the structure of signal peptides within solution ($+/-$ detergent micelles), within phospholipid vesicles, or within membrane mimetic environments [26–31]. Most of the findings from these studies are consistent with the H-region of the signal peptide being helical in structure while within the membrane. Based on the crystal structure of *E. coli* SPase I inhibitor complexes and molecular modeling studies, the C-region is likely in an extended β -conformation when bound within the SPase I substrate-binding groove [32]. This is discussed in detail below. Fig. 1 summarized the general features of *E. coli* signal peptides.

It has recently been shown that preproteins which are translocated via the twin-arginine translocation (Tat) system are also processed by SPase I [33]. Tat substrates contain a signal peptide with the twin-arginine motif (SRRxFLK) located between the N- and H-regions. The

H- and C-regions are similar to the corresponding regions in the Sec-system signal peptides except that the Tat signal peptide C-region often contains a positively charged residue. The Tat system is responsible for catalyzing the translocation of fully folded proteins, many of which contain redox sensitive co-factors [34]. The Tat system is found in bacteria, archaea and the thylakoid of chloroplasts. There are 27 known Tat substrates in *E. coli* [35].

3. *E. coli* type I signal peptidase (SPase I)

3.1. Discovery and characterization

E. coli SPase I activity was first observed in 1978 [36], shortly after the discovery of signal peptides [37], and is currently the most characterized SPase I. It was the first SPase I to be cloned [38], sequenced [39], overexpressed [40,41], purified [39,40,42,43], and biochemically [44–47] and structurally [32] characterized. Each *E. coli* cell contains approximately 1000 SPase I molecules [48]. SPase I is constitutively expressed from the single-copy *lepB* gene which has been mapped to a location between *purl* and *nadB* at min 54 and 55 on the *E. coli* chromosome [49]. It is an essential gene for *E. coli* survival [41,50,51]. *E. coli* SPase I has the enzyme commission number EC 3.4.21.89 and is categorized within the evolutionary clan SF and the serine protease family S26 (subfamily S26A), according to the MEROPS peptidase database [52].

3.2. Primary sequence

E. coli SPase I is 324 residues in length with a calculated molecular mass of 35,960 Da and a theoretical isoelectric point of 6.85, which is consistent with the measured value [40]. The numbering system used for the *E. coli* SPase I amino acid sequence in this review corresponds to the sequence in the UniProt database (accession number, P00803). The discrepancy between the numbering system used in many *E. coli* SPase I publications and the numbering used in P00803 is based on an error in the original DNA sequencing in 1983 [39]. Arg42 in the 1983 report is actually Ala42–Gly43. This inserts a residue within the cytoplasmic loop region, between the amino-terminal transmembrane segments, and moves the sequence up one number after that point. The sequence 1–41 is correct in register and numbering. The sequence 44–324 is correct in register, but the numbering is one residue different from the old numbering. It has been observed that the N-terminus of *E. coli* SPase I is blocked [39] and it has been suggested that it is acetylated at its N-terminus, but this has so far not been confirmed [53]. A disulphide bond links Cys171 and Cys177 [54]. Fig. 2 shows the primary sequence for *E. coli* SPase I with annotation for secondary structural elements and catalytically important residues.

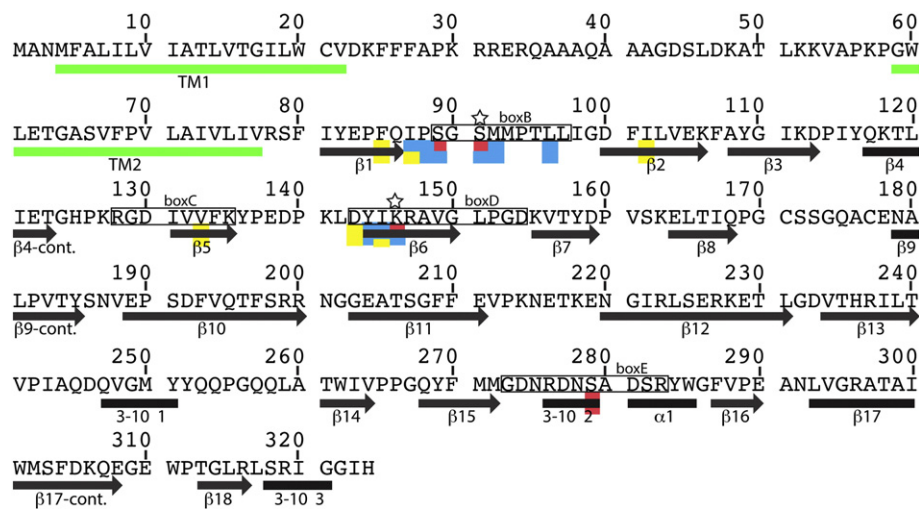


Fig. 2. The primary sequence for *E. coli* SPase I. The green bars below the sequence signify the residues that make up the transmembrane segments (TM1 and TM2). Black arrows signify β -sheets, black bars signify helices. Small red blocks signify the catalytic residues, stars are above the residues involved in the Ser/Lys catalytic dyad. Blue blocks signify the residues that contribute atoms to the S1 substrate specificity pocket. Yellow blocks signify the residues that make up the S3 substrate specificity pocket. This sequence corresponds to UniProt data base accession number: P00803.

3.3. Membrane topology

The membrane topology of *E. coli* SPase I has been characterized based on its sensitivity to protease digestion [39,55]. Its topology has also been investigated by carrying out gene-fusion studies [56]. Based on these studies, it has been suggested that *E. coli* SPase I has two N-terminal transmembrane segments (residues 4–28 and 58–76), a small cytoplasmic domain (residues 29–57), and a large C-terminal catalytic domain (residues 77–324). Hydrophathy analysis of the *E. coli* SPase I sequence suggests slightly different transmembrane segments, two 19 residue long sections: 4–22 and 59–77 (Fig. 2). Site-directed disulphide cross-linking studies were used to propose a structural model for the two transmembrane segments [57]. Deletion analysis has shown that the first transmembrane segment and the cytoplasmic loop region are not essential for activity in vivo [58]. The second transmembrane segment functions as a non-cleavable signal sequence. SPase I from many other eubacterial species only contain one transmembrane segment. A soluble catalytically active domain of *E. coli* SPase I (Δ 2-76, formally known as Δ 2-75) that lacks both transmembrane segments has been characterized in vitro [53,59].

3.4. Purification

Overexpression and purification of full-length wild-type *E. coli* SPase I is made difficult by autocatalyzed degradation. It has been shown by N-terminal sequencing that the main self-cleavage site is right after residue 40, which resides within the cytoplasmic loop region. This site corresponds to a typical SPase I cleavage recognition sequence (Ala38–Gln39–Ala40↓Ala41). The cleaved SPase I has 100-fold lower activity than the full-length enzyme [60]. To avoid this self-cleavage and expedite purification of the overexpressed protein from the chromosome-expressed wild-type SPase I, residues 35–40 were mutated to histidine [47]. *E. coli* SPase I is extracted from the inner membrane by non-ionic detergents such as Triton X-100. Evidence to date suggests that *E. coli* SPase I functions as a monomer and does not require co-factors. It has been observed that wild-type *E. coli* SPase I incorporated within phospholipid vesicles does not show autodigestion [61].

3.5. Site directed mutagenesis and chemical modification to identify catalytic residues

Site-directed mutagenesis studies have shown that *E. coli* SPase I has an essential Ser91 [44] and Lys146 [45,47,62] (Ser90 and Lys145 in the

old numbering system), but no essential cysteine or histidine. Mutating Lys146 to alanine, histidine, asparagine, methionine, aspartic acid, glycine or serine all produced inactive enzymes. Site directed chemical modification studies are consistent with Ser91 being the nucleophile. When Ser91 was mutated to a cysteine (S91C) the enzyme was still active but became susceptible to inhibition by the cysteine specific reagent N-ethylmaleimide [45]. A similar approach also supports Lys146 functioning as the general base. When Lys146 was mutated to cysteine (K146C) the resulting enzyme was inactive but partial activity was restored upon modification of the cysteine by 2-bromoethylamine to produce the lysine analog (γ -thia-lysine) at position 146 [47].

3.6. In vitro assays and kinetics analysis

Early analysis of secretory protein cleavage by *E. coli* SPase I was performed using cell-free assays with radioactive (35 S-methionine labeled) preproteins. The extent of the preprotein substrate cleavage by *E. coli* SPase I was accessed by SDS-PAGE, followed by autoradiography [36,43,63].

The first kinetic analysis of *E. coli* SPase I was performed with synthetic peptides, based on the SPase I cleavage site region within the maltose binding protein [19]. The progress of the cleavage reaction was analyzed by reverse phase high-performance liquid chromatography. The resulting kinetic constants were quite poor in comparison to other characterized proteases. Interestingly, kinetic assays using macromolecular preprotein substrates revealed more efficient processing rates than the smaller synthetic peptide cleavage assays [46,64]. The down side of the preprotein assays is that they usually required time consuming SDS-PAGE and autoradiography or densitometry steps. The most frequently used preprotein substrate is pro-OmpA-nuclease A [46]. This substrate has been used to measure the activation energy of *E. coli* SPase I catalyzed preprotein cleavage and the value of 10.4 kcal/mol shows that *E. coli* SPase I has a catalytic efficiency close to that of other serine proteases [65]. Continuous assays that utilize synthetic peptides with fluorescence resonance energy transfer (FRET) donor and acceptor pairs are the most convenient and most sensitive substrates for SPase I kinetic characterization [66,67]. Including a sequence, in the substrate, that mimicked a signal peptide N and H-region in front of the SPase I recognition sequence, was shown to drastically improve the cleavage efficiency [67]. A similar effect was observed when a fatty acid was included at the N-terminus [68]. This effect is likely due to a more optimal presentation of the substrate (local effective concentration) to the detergent micelle embedded SPase I.

Combinatorial libraries of synthetic peptides on beads have been used to optimize fluorogenic peptide substrates [69,70]. See Table 1 for a description of a selection of substrates used to characterize *E. coli* SPase I.

3.7. In vivo assays

A temperature sensitive strain of *E. coli* (IT41) has been used in complementation assays to confirm the role of putative SPase I gene products from a number of different bacterial species [51]. This mutant strain is normally only able to grow at permissive temperatures (28 to 32 °C), but when transformed with a plasmid containing a functional SPase I gene, IT41 is able to grow at a non-permissive temperature (42 °C). Briefly, IT41 cultures, transformed with a plasmid +/- the gene for a functional SPase I, are grown at non-permissive temperature and the optical density (540 nm) of the cultures is periodically measured (every 30 min) for 8 h.

The cause of the temperature sensitivity within the IT41 strain appears to be an amber mutation at nucleotide position 115 (C to T) within the *lepB* gene, which is within the codon for Gln39 and results in a TAG amber termination codon [71].

An *E. coli* strain was developed that has regulatable expression of the *lepB* gene [72] and can be used to test cellular inhibition of SPase I. The strain has a *lepB* gene within an L-arabinose inducible pBAD plasmid while the chromosomal copy of *lepB* has been removed.

3.8. Inhibitors

SPase I is a promising antibiotic target because, as mentioned above, it is an essential bacterial enzyme and its active site is located on the extra-cytoplasmic surface of the membrane and thus relatively accessible to drugs. The fact that it has a different catalytic mechanism and oligomeric nature from the functionally homologous enzyme found within human cells suggests that there would likely be few side effects from a SPase I specific inhibitor. Therefore many industrial, as well as academic, labs have been actively searching for compounds that inhibit SPase I. These compounds could potentially lead to a novel class of antibiotics.

The observation that *E. coli* SPase I was not inhibited by standard commercially available protease inhibitors was one of the first clues that this enzyme may be mechanistically unique [53,73–75]. Other characteristics discovered about the inhibition of *E. coli* SPase I include: *E. coli* SPase I activity appears to decrease in the presence of sodium chloride above 160 mM and magnesium chloride above 1 mM [73]; *E. coli* SPase I shows product inhibition – the M13 procoat signal peptide has been demonstrated by in vitro assays to be a competitive inhibitor [24]; and preproteins with a proline at the +1 (P1') position are effective competitive inhibitors of *E. coli* SPase I [23,25].

The first small molecule inhibitor of *E. coli* SPase I was reported in 1994. It was shown that β -lactam compounds could inhibit *E. coli* SPase I in a pH and time-dependent manner [76]. The β -lactam (or penem) class of compounds was subsequently investigated for their

SPase I inhibition potential [77–80]. The most effective penem compounds are the 5S stereoisomers. The compound allyl (5S,6S)-6-[(R)-acetoxyethyl]-penem-3-carboxylate has a IC_{50} of less than 1 μ M (Fig. 6A). The stereochemistry of this inhibitor led to the proposal that the nucleophile of *E. coli* SPase I (Ser91) attacks the scissile bond of its substrate from the *si*-face rather than the more commonly seen *re*-face nucleophilic attack [77]. The crystal structure of the *E. coli* SPase I–5S,6S penem complex would later confirm this hypothesis [32] (Fig. 6). New routes to the synthesis of the 5S-penem SPase I inhibitors have been reported and studies of structure–activity relationships have begun to explore the binding specificity for these compounds that represent a potential new class of antibiotic [81].

Arylomycins are lipohexapeptides that have been shown to have antibiotic properties [82,83] (Fig. 8A). These compounds were first isolated from extracts of *Streptomyces* sp. Tu 6075. The hexapeptide has the sequence: D-MeSer, D-Ala, Gly, L-MeHpG, L-Ala, and L-Tyr. The amino acid L-MeHpG is N-methyl-4-hydroxy-phenylglycine (a tyrosine missing the C β carbon). The L-MeHpG is cross-linked with the C-terminal tyrosine to form a three residue macrocycle via a 3,3-biaryl bridge. A fatty acid is attached to the N-terminus. Crystallographic and biophysical analysis of the mode of binding of arylomycin within the *E. coli* SPase I substrate-binding groove has revealed that the inhibitor binds in a non-covalent fashion. Specifics of the interactions are discussed below. Arylomycins have now been synthesized and work is underway to optimize their SPase I inhibitory effectiveness and antibiotic properties against a broad range of bacterial species [84–90]. Recently arylomycin compounds have been identified from *Streptomyces roseosporus* [91].

A substrate based peptide aldehyde SPase I inhibitor has recently been developed that has an IC_{50} value around 13 μ M against *E. coli* SPase I and is approximately 100-fold more effective against *Saccharomyces aureus* SPase I (SpsB) [92].

Table 2 lists a number of *E. coli* SPase I inhibitors and their reported IC_{50} values.

3.9. A soluble catalytically active domain

A catalytically active soluble form of *E. coli* SPase I (Δ 2-76), which lacks the two amino-terminal transmembrane segments, was first characterized in 1993 [53]. Electrospray ionization mass-spectrometry analysis revealed a mass of 27,952 a.m.u., 42 a.m.u. different from the theoretical value based on sequence. This construct, like the full-length construct, is blocked to amino-terminal sequencing suggesting that the N-terminus of *E. coli* SPase I (Δ 2-76) is acylated. *E. coli* SPase I (Δ 2-76) was further characterized in 1995 [59]. It was shown that the catalytic efficiency of this construct is only 15-fold lower than full-length enzyme and that, for optimal activity, it requires detergent or *E. coli* phospholipids. The isoelectric point of this construct was measured to be 5.6, in contrast to 6.9 for the full-length enzyme. An optimized large-scale refolding and purification procedure was developed that allowed for the crystallization of *E. coli* SPase I (Δ 2-76) [93].

Table 1
E. coli SPase I in vitro activity assays.

Substrate	Assay method	k_{cat} (s^{-1})	K_m (μ M)	k_{cat}/K_m ($s^{-1} M^{-1}$)	Ref.
Preprotein: (Pro-OmpA-nuclease A) Outer membrane protein A signal peptide from <i>E. coli</i> fused to nuclease A from <i>Staphylococcus aureus</i> .	SDS-PAGE gel-assay	8.7	16.5	5.3×10^5	[46]
Preprotein: (AS-b ₅) alkaline phosphatase signal peptide fused to full-length mammalian cytochrome b ₅	SDS-PAGE gel-assay	10.6	50	2.1×10^5	[64]
Fluorometric peptide: Y ^(NO₂) FSASALA ₁ KIK ^(Abz)	FRET spectroscopy	0.0098	144	85	[66,67]
Fluorometric peptide: K(5)-L(10)-Y ^(NO₂) FSASALA ₁ KIK ^(Abz)	FRET spectroscopy	1.5	0.6	2.5×10^6	[67]
Decanoyl-LTPATAKA ₁ ASKIDD-OH	HPLC-MS	418	988	4.2×10^5	[68]
Ac-WSASA ₁ LA ₁ KI-AMC	Fluorescence (coupled with leucine aminopeptidase digest of KI-AMC product) or HPLC analysis	4.6×10^{-3}	78	59	[76]
FSASALA ₁ KIEEG	HPLC	1.25×10^{-4}	1400	8.9×10^{-2}	[19]
FSASALA ₁ KI	HPLC	3.2×10^{-2}	800	40	[19]

FRET: fluorescence resonance energy transfer; Abz: 2-aminobenzoyl; AMC: aminomethylcoumarin; Y^(NO₂): 3-nitro-L-tyrosine; K^(Abz), ϵ -(2-aminobenzoyl)-L-lysine.

Table 2
E. coli SPase I inhibitors.

Inhibitor	IC ₅₀ (μM)	Ref.
Allyl (5S,6S)-6-[(R)-acetoxyethyl] penem-3-carboxylate	0.38	[77–80,122]
morpholino-β-sultam	610 (±18) ^a	[104]
Arylomycin A ₂	1 (±0.2) ^a	[104,123]
Decanoyl-PTANA-aldehyde	13.4 (±1.3)	[92]

IC₅₀: half maximal inhibitory concentration.

^a Based on kinetic analysis with the soluble domain of *E. coli* SPase I (Δ2-76).

^b Based on kinetic analysis with the full-length *E. coli* SPase I.

4. The three-dimensional structure of *E. coli* type I signal peptidase

Five crystal structures of *E. coli* SPase I (Δ2-76) have been determined to date (Table 3). The first three-dimensional structure of a signal peptidase was reported in 1998 [32]. The crystal structure of *E. coli* SPase I (Δ2-76) was solved by multiple isomorphous replacement methods and refined to 1.9 Å resolution, in complex with the β-lactam (5S,6S penem) inhibitor discussed above.

4.1. Protein fold

E. coli SPase I (Δ2-76) has a mainly β-sheet protein fold made up of two anti-parallel β-sheet domains (domain I and II), and a number of small helices (Figs. 2–5). There is also an extended β-ribbon that gives the protein an overall conical shape of approximate dimensions 60 × 40 × 70 Å. A disulphide bond (Cys171–Cys177) precedes a β-turn within domain II. The structure shows that the highly conserved regions of sequence revealed in earlier sequence alignment analysis [94] (boxes B–E, Figs. 2 and 5) are all contained within domain I, clustered around the active site. The extended β-ribbon (residues 108–124, made up of β-stands 3 and 4) and domain II (residues 151–269, β-stands 7–14) are both insertions within domain I, and are variably present in SPase I from different species (Fig. 5). Domain I shows structural similarity to *E. coli* UmuD, a protease involved in damage inducible SOS mutagenesis [95]. Despite only 17.4% sequence identity in this region, 69Cα atoms superimpose with a root mean square deviation of 1.6 Å [96].

4.2. Catalytic residues and surrounding conserved residues

The only titratable functional group within hydrogen bonding distance to Ser91 O_γ is the ε-amino group of Lys146 (2.9 Å), which is consistent with its proposed function as the general base (Fig. 6). Lys146 N_ζ is also coordinated to the O_γ of Ser279 and atom O10 of the penem. Ser279 resides within the box E region of conservation seen in SPase I sequence alignments (Fig. 2, 5). Subsequent site directed mutagenesis experiments have shown that this residue is essential for

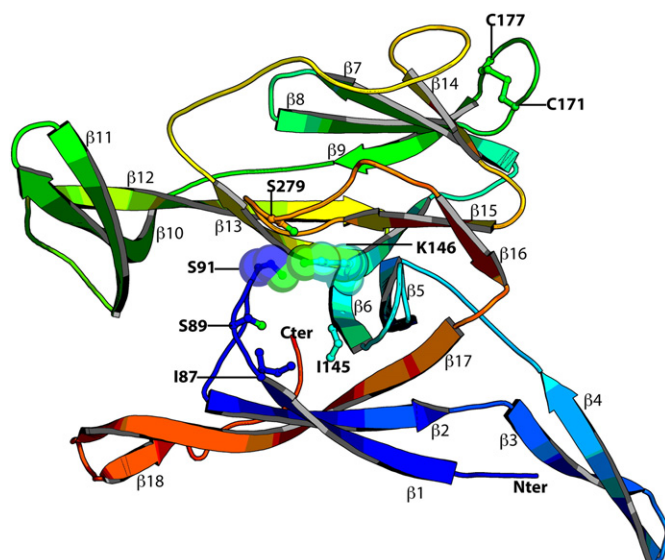


Fig. 3. A ribbon diagram of *E. coli* SPase I. The β-strands are numbered sequentially. The small helices are not shown for clarity. The structure is colored in a gradient from the amino-terminus (Nter, residue 78, blue) to the carboxy-terminus (Cter, residue 324, red). The side chains of the catalytic residues (Ser91, Lys146, Ser89, Ser279) as well as the side chains of the residues that divide the S1/S3 substrate specificity pockets (Ile87, Ile145) are shown in ball & stick. Semitransparent van der Waals spheres highlight the Ser/Lys catalytic dyad. The disulfide bond (Cys171/Cys177) is shown in ball & stick.

optimal activity [97]. These experiments also demonstrated that the salt bridge formed between Asp281 and Arg283 is critical for optimal activity. As can be seen in Fig. 6, Asp281 O_{δ2} is directly hydrogen bonded to Ser279 O_γ, which in turn supports the position of the general base Lys146 N_ζ. The C_α of Gly273 is located directly behind Lys146 N_ζ. Site-directed mutagenesis studies have shown that even a mutation to alanine at position 273 adversely affected the *E. coli* SPase I activity [97]. Lys146 N_ζ is completely buried in a hydrophobic environment within the penem-complex. This is likely how the lysine is capable of functioning as a general base. Earlier pH-rate profile experiments gave an apparent pK_a of ~8.7 [47], which is nearly two pH units lower than the pK_a of a solvent exposed lysine ε-amino group. NMR titrations will be needed to obtain the directly measured pK_a of the ε-amino group of Lys146, with and without inhibitors or substrates.

4.3. Ser91 covalently bonded to a β-lactam type inhibitor

The penem-SPase I complex structure directly exhibits the role of the Ser91 O_γ as the nucleophile in that the electron density within the active site is consistent with a covalent bond between the Ser91 O_γ and the C7 carbonyl carbon of the inhibitor (Fig. 6). This also confirms the *si*-face nucleophilic attack. The oxyanion hole was identified by the

Table 3
Crystal structures of *E. coli* SPase I.

Structure	PDB	Res. ^a (Å)	Space group	Unit cell a,b,c (Å) ^b	a.s.u. ^c	pH ^d	Ref. ^e
Penem complex	1B12	1.95	P2 ₁ 2 ₁ 2	111, 113, 99	4	4.6	[32] 1998
Free-active site	1KN9	2.40	P4 ₁ 2 ₁ 2	112, 112, 199	4	5.4	[102] 2002
Arylomycin complex	1I7D	2.47	P4 ₃ 2 ₁ 2	70, 70, 258	2	6.0	[103] 2004
Arylomycin/β-sultam complex	3IIQ	2.00	P4 ₃ 2 ₁ 2	70, 70, 260	2	6.5	[104] 2009
Glyco-Arylomycin complex	3S04	2.44	P4 ₃ 2 ₁ 2	72, 72, 263	2	7.4	[84] 2011

^a Resolution.

^b Unit cell dimensions are rounded to closest whole number. All unit cell angles are orthogonal.

^c Number of protein chains in the asymmetric unit.

^d pH of crystallization reservoir solution.

^e Reference and reference year.

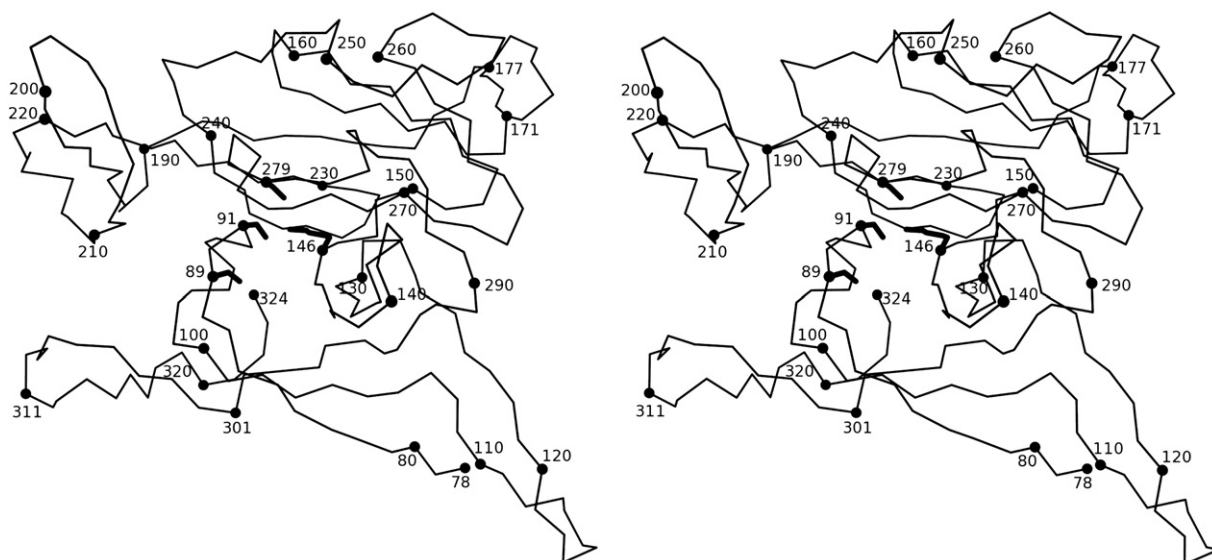


Fig. 4. A C α trace of *E. coli* SPase I. The figure is prepared in stereo. Every tenth residue is designated with a sphere and labeled. Side chains are shown for catalytic residues (Ser89, Ser91, Ser279, and Lys146) and the disulfide bond (Cys171/Cys177).

O8 carbonyl oxygen of the inhibitor pointing towards the NH of Ser91. The only other potential contributor to the oxyanion hole is the hydroxyl group of Ser89, but the inhibitor has forced the χ_1 -angle of the Ser89 side chain into a position such that it is not available to form the hydrogen bond to the would-be oxyanion. This may be one of the means by which this inhibitor stabilizes its covalent attachment to the nucleophile. Subsequent site-directed mutagenesis experiments showed that mutating Ser89 to threonine results in an enzyme with almost wild-type activity, whereas mutating Ser89 to an alanine or cysteine results in an enzyme with drastically lower activity [98]. The change in catalytic activity was found to be mostly due to a decrease in the k_{cat} while the K_{m} did not change significantly. The calculated differential free energy of transition stabilization provided by the Ser89 hydroxyl group was found to be 5.2 kcal/mol. Another interesting observation, within the active site of the structure, was that there were

no suitably placed waters that could potentially function in the deacylation step. Displacement of the deacylating water again may help explain the inhibitory properties of this compound.

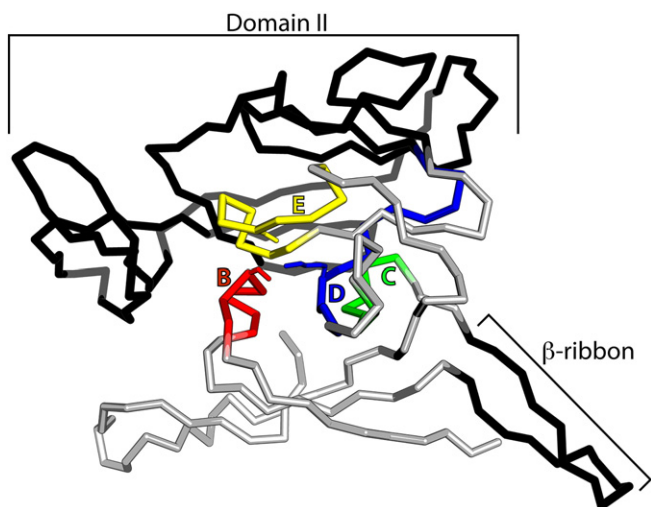


Fig. 5. The conserved boxes of sequence seen within SPase I sequence alignments are mapped onto a C α trace of the structure of *E. coli* SPase I. Boxes B (residues: 89–97), C (residues: 128–135), D (residues: 143–154) and E (residues: 273–283) are shown in red, green, blue and yellow respectively. Domain I is rendered in white but includes the conserved box regions shown in color. Domain II (residues: 155–263) and the β -ribbon (residues: 108–124) are shown in black.

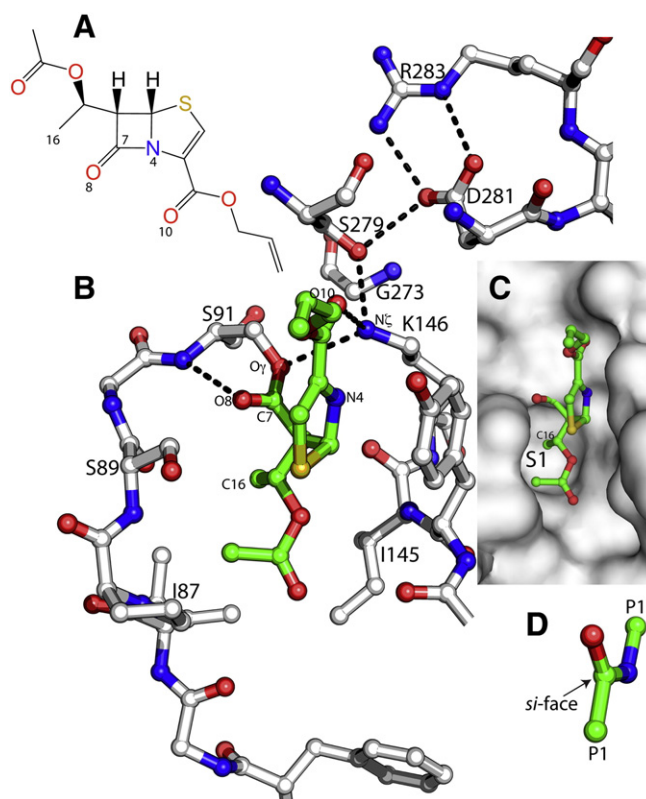


Fig. 6. Structure of a β -lactam (penem) type inhibitor co-crystallized with *E. coli* SPase I. A. The structure of the β -lactam-type inhibitor allyl (5S,6S)-6-[(R)-acetoxyethyl]-penem-3-carboxylate. B. Structure of the residues of the *E. coli* SPase I active site with the 5S,6S-penem covalently bound to the Ser91O γ . Note that the bond between the carbonyl carbon (C7) and nitrogen (N4) within the penem is broken upon forming the acyl-enzyme ester bond to the Ser91O γ . The carbon atoms of SPase I are in white. The carbon atoms of the penem are in green. Nitrogen atoms are blue, oxygen atoms are red, and sulfur atoms are gold. C. Structure of the penem-SPase I complex with the SPase I rendered as solvent accessible surface. The C16 methyl group of the inhibitor is pointing into the S1 substrate specificity pocket. D. The si-face of a scissile bond.

4.4. Substrate binding groove and the S1 and S3 specificity pockets

The structure explained a great deal about the SPase I substrate preferences. A methyl group on the penem inhibitor (C16), which was shown to be essential for the effectiveness of the inhibitor, is pointing into a shallow hydrophobic pocket on the *E. coli* SPase I surface – the S1 substrate specificity pocket [77] (Fig. 6C). The S1 pocket is made of atoms from the residues: Met92, Ile145, Leu96 and Ile87 (Fig. 7). The shallow hydrophobic S1 pocket is consistent with the strong preference for alanine at the P1 position of the SPase I preprotein substrates (Fig. 1).

Using the inhibitor as a guide, an extended poly-alanine β -strand was modeled into the *E. coli* substrate-binding groove, which is constructed on one side by the loop region following β -strand 1 that leads to the nucleophile Ser91, and on the other side by the residues from β -strand 6. Most proteases bind their substrates in an extended β -conformation [99]. This model allowed for the identification of the S3 substrate specificity binding pocket, which is made up of atoms from residues: Phe85, Ile87, Ile102, Val133, Ile145, and Asp143 (Fig. 7). The residues Ile87 and Ile145 form a ridge between the S1 and S3 pockets. The alternating up and down orientation of residue side chains within the extended β -strand model of the signal peptide C-region explains the Ala-X-Ala substrate preference of SPase I, in that the P1 and P3 alanine residues are pointing into the shallow hydrophobic pockets and the P2 residue is pointing out into the solvent allowing for any side chain at this position. The structural information on the S1 and S3 binding pockets was used to design site-directed mutants to elucidate the residues that control secretory preprotein cleavage fidelity [100]. It was found that mutating Ile145 to cysteine resulted in cleavage at multiple sites within the substrate and, if Ile145 and Ile87 were both mutated to alanine, SPase I was capable of cleaving after phenylalanine. It was also discovered that the double mutant I145C/I87C or I145C/I87T, which mimics the residues at these positions within the mitochondrial homolog (Imp1), was able to cleave substrates with an asparagine at the P1 position, as preferred by Imp1. Later, the mutants I145C/I87T, I145C, and I145A were shown by combinatorial peptide library analysis to have a relaxed substrate preference at the P3 position. The double mutant was able to process substrates with arginine, glutamine, or tyrosine at the P3 position [101].

4.5. Structure of *E. coli* SPase I with a free substrate binding site

The crystal structure of *E. coli* SPase I in the absence of bound inhibitor allowed for a structural comparison between the bound and unbound states of the active site [102]. This analysis revealed significant main chain and side chain differences within the substrate binding groove and the active site that result in a smaller S1 pocket in the inhibitor free enzyme. In addition, the position of the Ser89 side chain (O γ H), in the absence of the penem inhibitor, is consistent with its contribution to transition state oxyanion stabilization. A potential deacylating water was also identified (Fig. 7).

4.6. Structures of lipohexapeptide based inhibitors (arylomycins) bound to *E. coli* SPase I

The first crystal structure of arylomycin bound to *E. coli* SPase I revealed that the peptide based inhibitor binds non-covalently and is positioned such that one of its C-terminal carboxylate oxygens is within hydrogen bonding distance to all of the functional groups within the catalytic center of the enzyme (Ser91 O γ , Lys146N ζ , and Ser89O γ) (Fig. 8A, C) [103]. The inhibitor is therefore positioned so that the macrocycle is closest to the active site, with the main chain of the peptide having parallel β -sheet type hydrogen bonding interactions with both sides of the substrate-binding groove. All of the potential hydrogen bonding donor and acceptors within the three residue macrocycle are positioned to make hydrogen bonds with atoms in the binding groove, whereas only two of the six potential hydrogen bonding donors and acceptors in the N-terminal three residues of the inhibitor form hydrogen bonds. The side chain methyl group of the penultimate alanine of the inhibitor sits within the S3 pocket. Weak electron density was observed for the N-terminal fatty acid, suggesting it is dynamic in nature. The position of the N-terminal D-MeSer is located near the proposed membrane association surface. Both the crystal structure and spectroscopic data are consistent with arylomycin binding specifically to a single site. Fluorescence data are consistent with a two-step binding mechanism – a rapid binding step followed by a slower adjustment to a final bound state. Binding parameters for this inhibitor were also investigated using calorimetric methods.

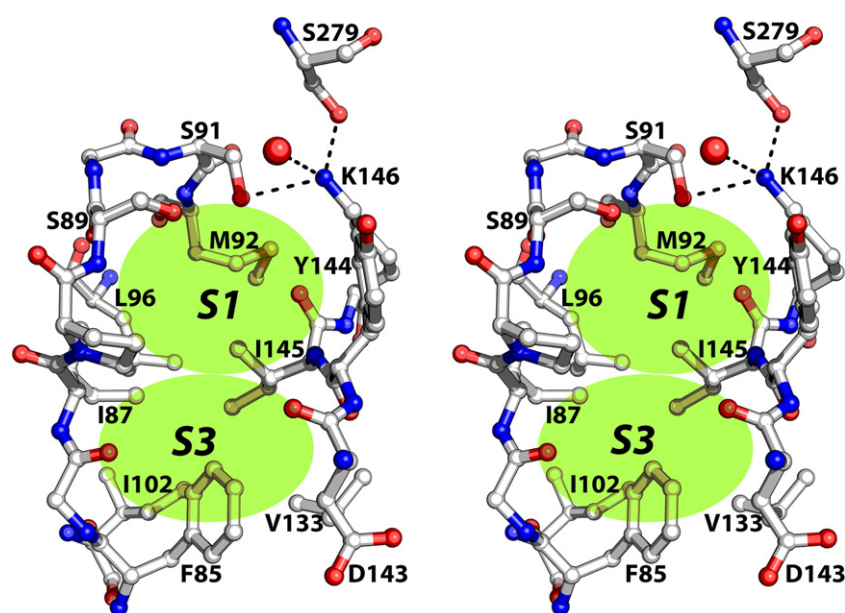


Fig. 7. A stereo ball & stick rendering of the empty substrate binding groove and active site of *E. coli* SPase I. Carbon atoms are shown in white, nitrogen atoms are in blue and oxygen atoms are in red. The proposed deacylating water is shown as a red sphere. The S1 and S3 substrate specificity pockets are highlighted with light green semi-transparent ovals. The hydrogen bonds that involve the general base Lys146 N ζ are shown as dashed lines. Ile87 and Ile145 contribute atoms to both pockets and make up the dividing point between the pockets.

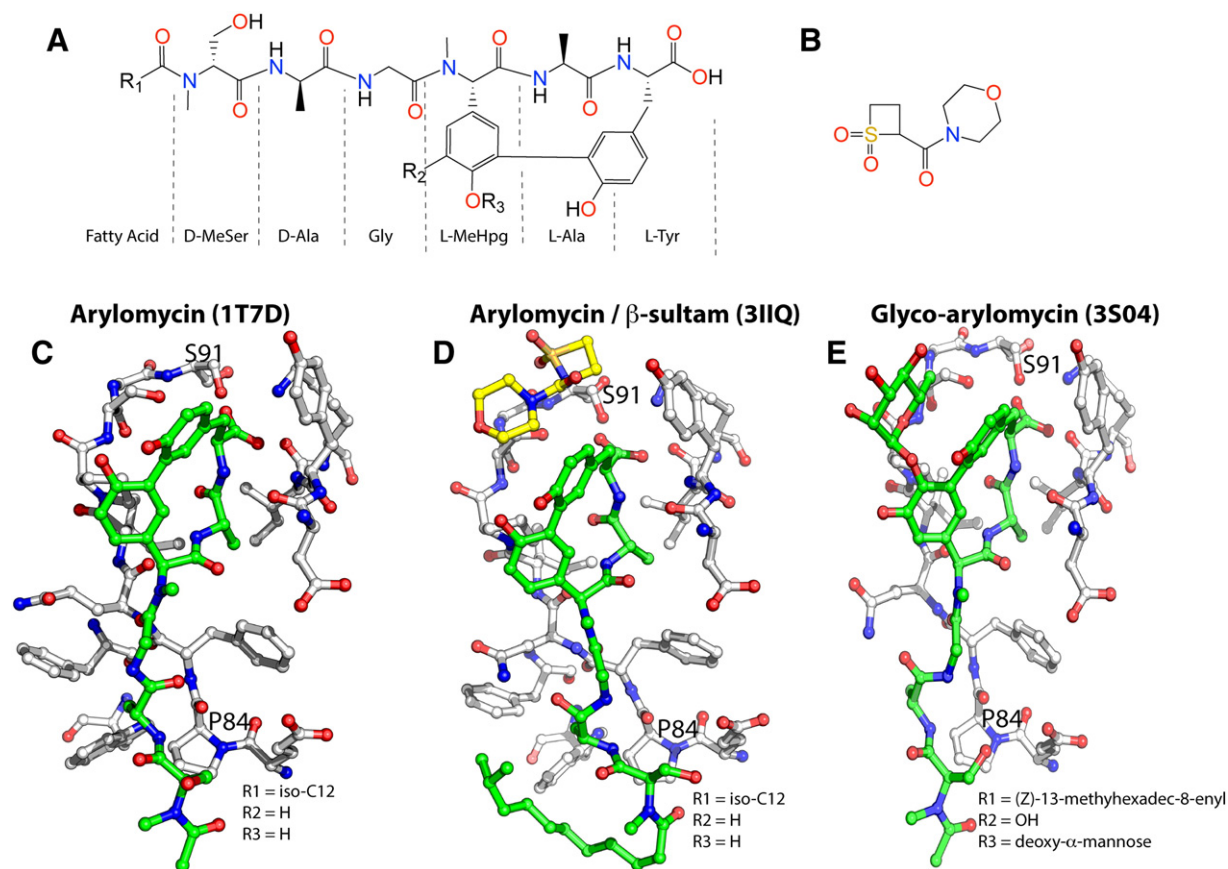


Fig. 8. Crystal structures of arylomycin variants bound to *E. coli* SPase I. A. The structure of arylomycin. D-MeSer represents D-methyl serine, MeHpg stands for N-methyl-4-hydroxyphenylglycine. R₁, R₂, and R₃ are defined below each structure. B. The morpholino- β -sultam derivative (BAL0019193). C. Arylomycin A₂ (green carbon atoms) bound within the active site of *E. coli* SPase I (white carbon atoms). D. The ternary complex of Arylomycin A₂ (green carbon atoms) and the morpholino- β -sultam derivative (yellow carbon atoms) bound within the active site of *E. coli* SPase I (white carbon atoms). E. The lipo-glycopeptide (glyco-arylomycin, green carbon atoms) bound within the active site of *E. coli* SPase I (white carbon atoms). The PDB accession code is listed above each structure. All nitrogen atoms are blue and oxygen atoms are red. The residues Ser91 and Pro84 are labeled.

The second structure of arylomycin bound to SPase I was in a ternary complex with another inhibitor, a morpholino- β -sultam derivative (Fig. 8A, B, D) [104]. The structure and binding and inhibition assays reveal that the compounds inhibit *E. coli* SPase I by binding to non-overlapping subsites near the catalytic center. The β -sultam compound binds in a noncovalent manner in close proximity to SPase I residues Ser89, Ser91, Lys146, Asn278, Ala280, and Glu308, as well as to a C-terminal carboxylate oxygen atom in arylomycin. There was clear electron density for the N-terminal fatty acid that runs along the proposed membrane association surface, near Trp301.

The most recently reported crystal structure of an arylomycin-SPase I complex was with a glycosylated form of arylomycin [84]. The structure reveals that the deoxy- α -mannose attached to the MeHpg residue of the lipoglycopeptide is directed away from the active site into the solvent, suggesting that the modification may function to increase the solubility of this natural product inhibitor (Fig. 8A, E).

Interestingly, it has been discovered that the presence of a SPase I binding site proline residue (at position 84, within β -strand 1 of *E. coli* SPase I) lends natural resistance to arylomycin's antibiotic activity in many species of bacteria [89]. Binding assays revealed that the proline mutation confers resistance by reducing the affinity of arylomycin to the SPase I binding site. A proline at this position eliminates one potential hydrogen bond donor on β -strand 1 of SPase I (Fig. 8).

4.7. Substrate recognition and the membrane association surface

Domain I of *E. coli* SPase I can be thought of as a β -barrel with one strand missing. This missing β -strand makes up the substrate-binding groove. Based on the crystal structures of the inhibitor complexes, a

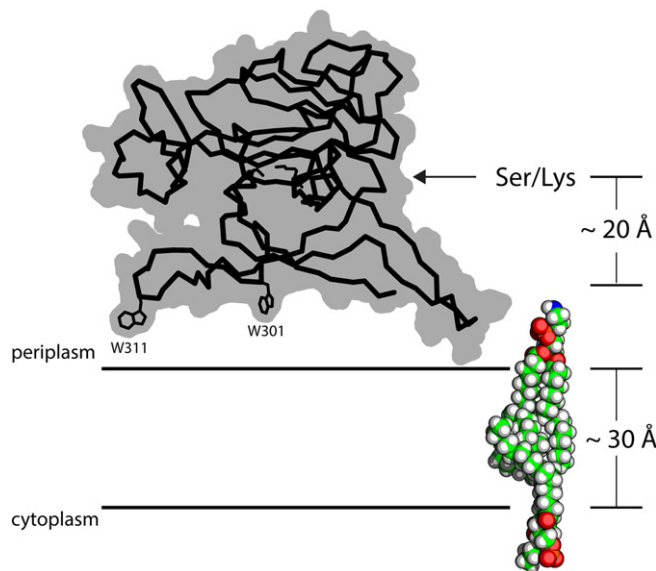


Fig. 9. The proposed membrane association surface of *E. coli* SPase I. To provide a perspective of the bilayer depth and the active site position relative to the proposed membrane association surface, a pair of phosphatidylethanolamine phospholipid molecules (van der Waals spheres – carbon, green; hydrogen, white; oxygen, red; nitrogen, blue) from a phospholipid bilayer structure simulation was rendered within the same file as the catalytic domain of *E. coli* SPase I. SPase I is shown as a black ribbon with the molecular surface shown as a semi-transparent gray outline, side chains for residues Trp301, Trp311, Ser91 and Lys146 are shown. Within the lipid bilayer, the distance from glycerol backbone to glycerol backbone is shown. The distance from the Ser91O γ to the Trp301C β (a residue on the proposed membrane association surface) is shown.

model for signal peptide C-region recognition and binding can be proposed. During substrate recognition and binding, the C-region of the signal peptide likely forms parallel β -sheet type hydrogen bonding interactions along β -strand 1 and the following loop that leads to the nucleophile Ser91. The other side of the binding groove is made up of residues from β -strand 6, which is also aligned in a parallel fashion to the signal peptide C-region. This side of the substrate-binding groove is fairly short and provides fewer potential hydrogen-bond donors and acceptors. A structural model for this type of interaction between the signal peptide of *E. coli* outer membrane protein A (OmpA) and *E. coli* SPase I was presented previously [102]. The model was guided by the *E. coli* SPase–penem complex crystal structure,

along with the crystal structure of LexA that was solved with its natural cleavage site bound within its substrate-binding groove [105]. LexA, like UmuD, is a structural homolog of SPase I domain I [96]. Based on this model, the potential hydrogen bonding donors and acceptors from β -strand 1 and loop 1 would be: Pro84O, Gln86NH, Gln86O, Ser89O γ H, and Ser91NH, while the potential hydrogen bonding donors and acceptors from β -strand 6 would be Ile145NH and Asp143O. This model suggests that the side chains of substrate residues P1, P3, P6 and P7 are positioned to make contact with the SPase I surface and that the side chains of substrate residues P2, P4 and P5 point towards the solvent. NMR analysis was used to investigate which residues of SPase I Δ 2-76 are perturbed upon signal peptide binding and it

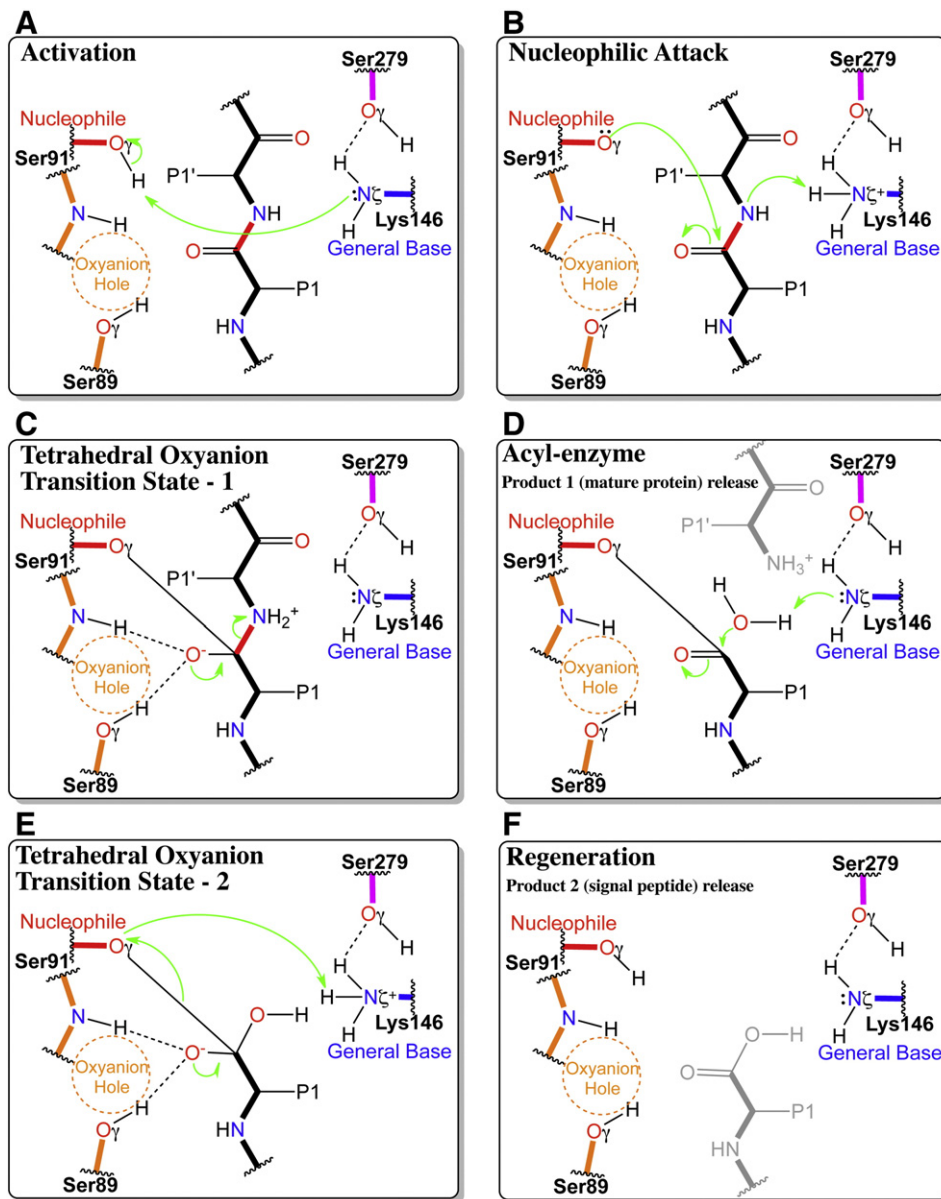


Fig. 10. The proposed catalytic mechanism of *E. coli* SPase I. A. The activation of the nucleophilic Ser91 (red) via the abstraction of the hydroxyl proton by the deprotonated N ζ of the general base Lys146 (blue). The Ser279O γ is within hydrogen bonding distance (dotted line) to the N ζ of Lys146. The oxyanion hole (orange) is constructed from the NH of Ser91 and the Ser89O γ H. The bound substrate (P1' to P1) is drawn in black, with the scissile bond in red. All electron pushing arrows are shown in light green. B. Nucleophilic attack on the scissile carbonyl by the activated Ser91 O γ . The protonated Lys146 N ζ donates a proton to the main chain amide nitrogen at the scissile bond (N-terminus of product 1). C. Tetrahedral oxyanion transition state 1. The oxyanion is stabilized via hydrogen bonds to the oxyanion hole. D. Acyl-enzyme complex and release of product 1 (mature protein, shown in light gray). The main chain carbonyl carbon of the P1 residue of the signal peptide is covalently attached via an ester bond to the Ser91 O γ . A nucleophilic (deacylating or catalytic) water is activated via the Lys146 N ζ and is in position to attack the carbonyl carbon of the ester bond. E. Tetrahedral oxyanion transition state 2. The oxyanion is stabilized via hydrogen bonds to the oxyanion hole. F. Regeneration of the enzyme and release of product 2 (signal peptide, shown in light gray).

was discovered that most of the perturbed residues map to the S1 and S3 substrate binding pockets [106,107]. In addition, NMR has been used to investigate the structure of the signal peptide upon binding to SPase I $\Delta 2-76$ [108].

If the arylomycin lipopeptides are mimicking the signal peptide association contacts, then it is clear that sites far from the active site, such as Pro84, are important for substrate affinity. Since the full length *E. coli* SPase I is more active than the SPase I soluble domain, and full length substrates are cleaved more efficiently than synthetic peptide substrates that only contain the C-region residues, one could ask: are there important contacts made between the SPase I transmembrane segment and the signal peptide H-region that dictate cleavage fidelity? Analysis of full length and soluble domains of both *E. coli* and *Bacillus subtilis* SPase I enzymes suggest there are not [109].

Studies have demonstrated that the accuracy of signal peptide cleavage by *E. coli* SPase I does not depend on the N-region, most of the H-region and also most of the mature region of preprotein substrates [110]. As mentioned earlier, site directed mutagenesis experiments show that the actual cleavage site fidelity is guided significantly by the S1 and S3 substrate specificity pockets [100].

The relative position of the cleavage site and the SPase I substrate binding groove within or on the membrane surface likely provides an important contribution to the efficiency of the reaction given that peptide substrates with a hydrophobic H-region mimic sequence, or a fatty acid attached to the N-terminus, are significantly more effective substrates [67]. Therefore, it is important to know what effect the membrane has on the secretory preprotein cleavage reaction and where the catalytic residues of SPase I are located, with respect to the preprotein cleavage site residues.

Purified *E. coli* SPase I incorporated within liposomes has been used in a number of early investigations into SPase I activity [111,112] but most assays are performed with SPase I solubilized within detergent micelles. Phospholipid vesicles with purified *E. coli* SPase I show that maximum catalytic activity is observed with approximately 55% phosphatidylethanolamine, the most abundant phospholipid in the *E. coli* inner membrane. Phospholipids with negatively charged head groups, found in abundance within the *E. coli* membrane, also enhance the catalytic activity [61]. Previous to this study, it was shown via membrane surface tension experiments and vesicle binding assays that phosphatidylethanolamine helps facilitate insertion of the catalytic domain of *E. coli* SPase I into the membrane [113].

The crystal structure of *E. coli* SPase I revealed that a hydrophobic surface runs along the full length of the enzyme, including the hydrophobic substrate binding site [32]. This surface is likely involved in membrane association. Located on this predicted membrane association surface are a number of aromatic residues. Trp301 has been shown to be essential for optimal activity in *E. coli* SPase I [75,114] even though the crystal structure maps it to a position more than 20 Å from the enzyme catalytic center (Fig. 9). It appears reasonable that Trp301 and possibly Trp311 may help facilitate the insertion of the catalytic region of *E. coli* SPase I into the membrane. Sequence alignments indicate that several conserved aromatic or hydrophobic residues exist on the proposed membrane-association surface in both Gram-positive and Gram-negative bacterial type I SPases [115–117]. Given the short length of most signal peptide H-regions, and the hydrophobic nature of the proposed SPase I membrane association surface, it is very possible that the cleavage event occurs well within the lipid bilayer.

There is precedence for the hydrolysis of a peptide bond within the hydrophobic confines of the phospholipid bilayer. Rhomboid proteases utilize a Ser/His catalytic dyad to perform their catalysis in the lipid bilayer [118], and there are other intramembrane proteases that utilize aspartyl- and metallo-protease mechanisms [119]. Interestingly, the remnant signal peptide left behind as a product of SPase I catalysis is cleaved within its H-region by an enzyme called

signal peptide peptidase (SppA). SppA is likely an intramembrane protease and also utilizes a Ser/Lys dyad mechanism [120,121].

5. Catalytic mechanism of *E. coli* SPase I

Based on kinetic analysis of site-directed mutants, chemical modification assays, as well as crystal structures (with and without inhibitors) a catalytic mechanism has been proposed for *E. coli* SPase I that is summarized in Fig. 10. Although the general components of the catalytic center (nucleophile: Ser91O γ , general-base: Lys146N ζ , oxyanion hole: Ser91NH/Ser89O γ H, and general-base positioning residue: Ser279O γ) appear to be defined, many mechanistic details remain to be investigated. For example, NMR titrations will help determine directly the pK_a of the general base Lys146 ϵ -amino group. It will be interesting to see how the pK_a of this functional group changes in the presence and absence of bound substrates and inhibitors, and also with the coordinating residue Ser279 mutated to other residues, in order to see if the mechanism utilized by SPase I is more accurately classified as a Ser/Lys/Ser catalytic triad rather than a Ser/Lys dyad.

Acknowledgement

This work was supported in part by the Canadian Institute of Health Research and the National Science and Engineering Research Council of Canada.

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