# Chapter 7
# Bacterial Signal Peptidases


**Mark Paetzel**

**Abstract** Signal peptidases are the membrane bound enzymes that cleave off the amino-terminal signal peptide from secretory preproteins. There are two types of bacterial signal peptidases. Type I signal peptidase utilizes a serine/lysine catalytic dyad mechanism and is the major signal peptidase in most bacteria. Type II signal peptidase is an aspartic protease specific for prolipoproteins. This chapter will review what is known about the structure, function and mechanism of these unique enzymes.

**Keywords** Protein secretion · Signal peptide · Signal peptidase · Bacterial lipoprotein · Periplasmic protease

## Introduction

The basic elements of the general protein secretion system are conserved in all cells; archaeal (Pohlschroder et al. 2018), bacterial (Chatzi et al. 2013; Driessen and Nouwen 2008; Tsirigotaki et al. 2017) and eukaryotic (Nyathi et al. 2013; Voorhees and Hegde 2016). A key feature of this system is the signal peptide. The signal peptide or signal sequence is approximately 20–30 residues in length and located at the amino-terminus of secretory proteins (preproteins) . The signal peptide is essential for targeting to the cytoplasmic membrane or endoplasmic reticulum membrane and also translocation across the membrane. Upon translocation of secretory proteins, the signal peptide is cleaved off.

The protein machinery of the general secretion system helps catalyze the targeting and translocation events. Bacterial protein secretion occurs predominantly as a post-translational translocation event. In other words, preproteins are fully synthesized and released from the ribosome within the cytoplasm, and then targeted to and translocated across the cytoplasmic membrane. Typically, the preprotein first encounters the protein SecB that functions as a holdase, keeping the preprotein sol-

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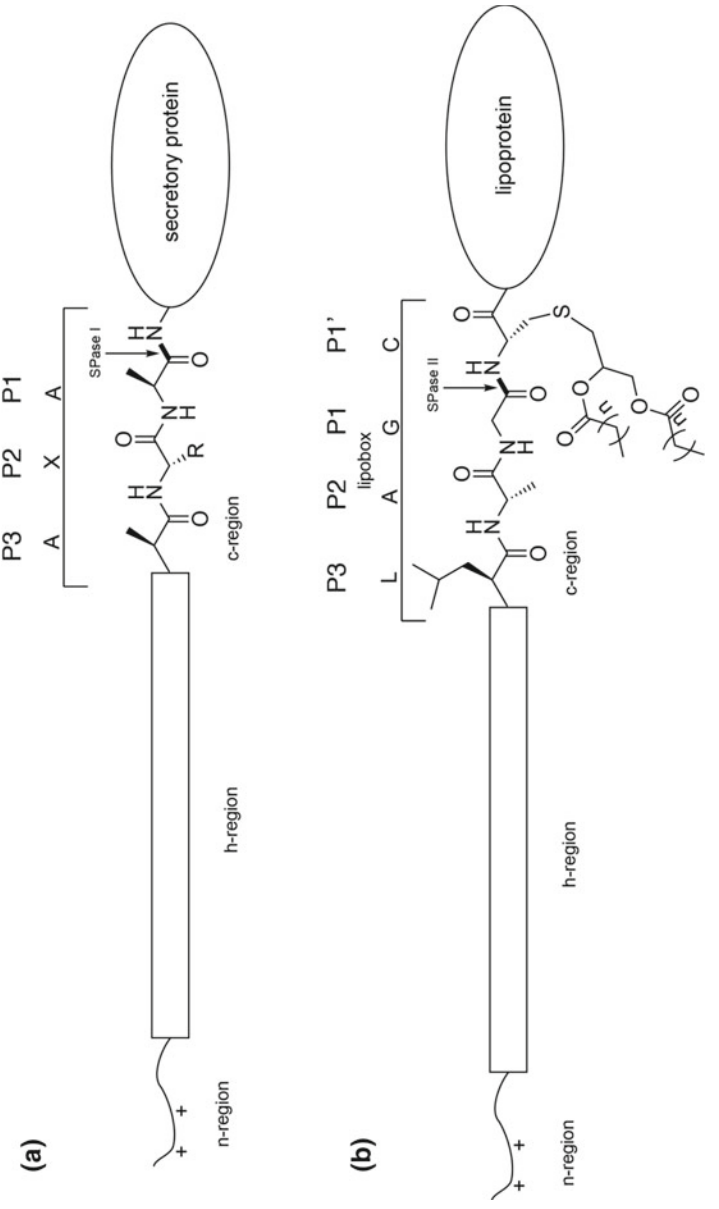
uble and in a translocationally competent form that can fit through the translocation channel SecYEG. The ATPase SecA feeds the preprotein through SecYEG stepwise. The energy for translocation across the membrane comes from ATP hydrolysis and the proton motive force. Once the carboxy-terminal region of the preprotein has been pushed through the translocase, the signal peptide is thought to partition into the lipid bilayer via a lateral gate in the SecYEG channel. The preprotein is then tethered to the membrane via the signal peptide. This membrane-tethered preprotein is the natural substrate of signal peptidase (SPase). It has been shown that removal of the signal peptide is essential in order for secretory proteins to be released from the cytoplasmic membrane (Dalbey and Wickner 1985). Limited proteolysis (Randall 1983) and deletion studies (Kaderbhai et al. 2008) are consistent with the carboxy-terminal region of preproteins being nearly or completely translocated across the cytoplasmic membrane before the signal peptide is cleaved off.

There are two types of bacterial SPase referred to as type I and type II. SPase I cleaves off the signal peptide from the majority of secreted proteins. It is an endopeptidase that utilizes a serine nucleophile and has the enzyme commission number EC 3.4.21.89. In the MEROPS protease database it is in clan SF and the family S26. Archaea also have an SPase I, but their sequences reveal that they maybe more related to a component of the eukaryotic signal peptidase complex. SPase II cleaves off the signal peptide from bacterial lipoproteins, proteins that associate with the membrane via an amino-terminal lipid modification. SPase II is an endopeptidase that utilizes aspartate catalytic residues and has the enzyme commission number EC 3.4.23.36. In the MEROPS protease database, it is in clan AC and the family A8. No homolog of SPase II has been found in an archaeal genome. Before discussing the research related to the structure, function and mechanism of the bacterial and archaeal SPase, the preprotein signal peptide will be briefly described.

The signal hypothesis states that secretory proteins contain an amino-terminal sequence (signal sequence) which helps the target secretory protein to the membrane and that after translocation across the membrane, the signal sequence is cleaved off by a specific peptidase (signal peptidase, SPase) (Blobel and Dobberstein 1975a, b; Milstein et al. 1972; Sabatini et al. 1971). Since this groundbreaking discovery, a great deal has been learned about the structure and function of signal peptides.

The general features of signal peptides are conserved across evolution. They have an amino-terminal region of sequence (1–5 residues in length) called the n-region that has a net-positive charge. The n-region is followed by a stretch of 7–15 hydrophobic residues (often leucines) labeled the h-region. Just beyond the h-region are 3–7 residues termed the c-region. This c-region contains the specificity residues for SPase recognition and cleavage. The consensus sequence for the cleavage site consists of small aliphatic residues at the −3 and −1 positions relative to the cleavage site (or P1 and P3 positions in Schechter and Berger nomenclature (Berger and Schechter 1970). This is often called the Ala-X-Ala rule because of the preference for alanine at the −3 and −1 position (Perlman and Halvorson 1983; von Heijne 1983, 1985), while there is a lack of preference for specific residues at the −2 position, thus the X designation (Fig. 7.1a). Signal peptide length is conserved as well. The average eukaryotic signal peptide is approximately 23 residues in length, while the average


(a)
P3 P2 P1
A X A
SPase I
secretory protein
c-region
h-region
n-region
(b)
P3 P2 P1 P1'
L A G C
lipobox
SPase II
lipoprotein
c-region
h-region
n-region

◀**Fig. 7.1** Bacterial secretory preproteins. **a** Secretory proteins have three regions within their signal peptide: the n-region has a net-positive charge, the h-region contains predominantly aliphatic residues such as leucine, and the c-region contains the specificity cleavage site for signal peptidase. Small aliphatic residues such as alanine are preferred at the P1 and P3 positions (also referred to as the −1 and −3 positions). The molecular structure of the last three residues of the c-region are drawn in an extended conformation in order to show the alternating up and down topology that leads to the "Ala-X-Ala" SPase preference. The h-region is shown as a rectangle to symbolize an α-helical conformation. **b** Bacterial prolipoproteins are synthesized with a signal peptide that has similarities to secretory proteins sequences with the exception that the c-region contains the so-called "lipobox" with the consensus sequence leucine(P3)-alanine(P2)-glycine(P1)-cysteine(P1ˆ′). The P1ˆ′ cysteine is covalently modified by diacylglycerol. This is the substrate of SPase II

Gram-negative bacterial signal peptide is approximately 25 residues in length and the average Gram-positive bacterial signal peptide is approximately 32 residues in length (Nielsen et al. 1997a, b). It has been hypothesized that the differences observed in signal peptide length may reflect the differences in the thickness of the lipid bilayer in the different membranes.

Researchers continue to develop increasingly more accurate signal peptide prediction servers (Choo et al. 2009; Leversen et al. 2009; Petersen et al. 2011; Savojardo et al. 2018; Wang et al. 2018). The availability of genome sequences (UniProt 2013) and improved proteomic methods have vastly increased the number of predicted and experimentally verified signal peptide sequences. Biophysical and modeling studies are consistent with the h-region of the signal peptide being helical within the hydrophobic environments of detergent micelles or membrane (Bechinger et al. 1996; McKnight et al. 1991a, b; Rizo et al. 1993; Sankaram et al. 1994; Wang et al. 1993). Modeling and structural analysis is consistent with the c-region of the signal peptide being in the extended β-strand conformation (Paetzel et al. 1998, 2002; Ting et al. 2016). The substrate cleavage-site being in a β-conformation is the classical conformation within protease binding sites (Tyndall et al. 2005). The structural and biophysical information now available regarding the conformation of signal peptides is in agreement with the prediction that was made in 1983 (von Heijne 1983).

Occasionally, unusual signal peptide variants are recognized. For example, it has been observed that in *Porphyromonas gingivalis* and other *Bacteroidetes* species >50% of the preproteins have a glutamine at the P1′ position (Bochtler et al. 2018). This generates an amino-terminal glutamine after SPase I processing. The newly formed amino-terminal glutamines are cyclized to pyroglutamate by glutaminyl cyclase. These pyroglutamate containing proteins do not appear to be specific to inner membrane proteins, periplasmic proteins, outer membrane proteins or extracellular proteins. It is not yet clear what role the amino-terminal pyroglutamate plays in the *Bacteroidetes* physiology but it has been shown that glutaminyl cyclase is essential in these species.

There has been significant effort put into the optimization of signal peptides for the secretion of industrially important recombinant proteins (Freudl 2018; Ghahremanifard et al. 2018; Low et al. 2013; Molino et al. 2018; Selas Castineiras et al.

2018; Ujiie et al. 2016; Zhang et al. 2018). A recent comprehensive review on signal peptides has been published (Owji et al. 2018).

Bacterial secretory proteins that utilize bound cofactors often need to fold within the cytoplasm in order to assemble properly and bind their cofactor. These folded proteins use the twin arginine translocation (Tat) secretion system rather that the general secretion system. The name comes from the sequence within the signal peptide. These signal peptides have a so-called twin-arginine motif (SRRxFLK) located between the n- and h-regions. There are 27 known Tat substrates in *E. coli* (Tullman-Ercek et al. 2007) and it has been shown that SPase I cleaves off Tat signal peptides (Luke et al. 2009).

Archaea have an evolutionarily similar general secretion system to that seen in bacteria and eukaryotes which includes similar signal peptide characteristics (Bardy et al. 2003). The similarity allows for bacterial SPase I to recognize and cleave archaeal preproteins. *E. coli* SPase I is capable of cleaving pre-α-amylase from *Thermococcus kodakarensis* (Muhammad et al. 2017). Genomic analysis suggests that protein secretion in archaea may be more similar to that of eukaryotes than to prokaryotes. For example, archaea lack a SecA (ATPase) homolog, therefore archaeal translocation maybe more energetically similar to the eukaryotic system where co-translational translocation is the main mechanism of transport across the membrane. Consistent with this idea, archaea also appear to lack a post-translational holdase type of targeting chaperone like SecB (Pohlschroder et al. 2018). Subtle differences in archaeal signal peptides have led researchers to develop a signal peptide prediction server trained on a set of characterized archaeal signal peptides (Bagos et al. 2009).

Bacterial lipoproteins are soluble hydrophilic proteins located in the bacterial cell wall. These proteins are tethered or anchored to the inner or outer membrane by permanent covalent lipid modifications. They have a diacylglycerol linked to an amino-terminal cysteine via a thioether linkage. Many of the bacterial lipoproteins perform functions that are essential to cellular viability. They have roles in: membrane and cell shape maintenance, outer membrane protein assembly, outer membrane stabilization, transport of molecules, energy production, signal transduction, virulence mechanisms, adhesion, digestion, sensing, growth, cell motility and many more cellular functions.

Bacterial lipoproteins are initially synthesized as preprolipoproteins that have a signal sequence for targeting and translocation via the general secretion system pathway (Driessen and Nouwen 2008) (Fig. 7.1b). The signal peptides of lipoproteins are very similar to those for secretory proteins in that they are approximately 20 residues in length and have a net positively charged region (n-region) followed by a stretch of hydrophobic residues (h-region) and then a recognition sequence region (c-region). Within the c-region is the SPase II recognition sequence called the "lipobox". The consensus sequence for the lipobox is leucine most often at the −3 (P3) position relative to the cleavage site, alanine most often at the −2 (P2) position, glycine most often at the −1 (P1) position and cysteine always at the +1 (P1′) position. The sequence variations that are observed in the lipobox are [**L**VI] [**A**STVI][**G**AS]**C** (Chimalapati et al. 2013; Kovacs-Simon et al. 2011; LoVullo et al. 2015; Nakayama et al. 2012; Narita and Tokuda 2017; Zuckert 2014). The +1 (P1′) cysteine is modified

with diacylglycerol (DAG) via a thioether linkage. This reaction is catalyzed by lipoprotein diacylglyceryl transferase (Lgt) and occurs in the inner membrane using the phospholipid within the inner membrane as substrate (Mao et al. 2016; Sankaran et al. 1997). The DAG-modified protein is then referred to as a prolipoprotein and is the substrate for type II signal peptide (SPase II) which cleaves off the signal peptide (Chimalapati et al. 2013). The scissile bond is the peptide bond preceding the invariant cysteine within the lipobox. Most SPase II will not cleave preprolipoproteins (protein without DAG-modification) (Inouye et al. 1983). For many Gram-positive bacterial species this is the end of lipoprotein synthesis; these mature lipoproteins are tethered to the outer leaflet of the cytoplasmic membrane, preventing them from diffusing away from the surface of the bacteria (Nguyen and Gotz 2016). For Gram-negative species another modification can occur. A fatty acid is attached, via an amide linkage, to the α-amino group of amino-terminal cysteine. This reaction is catalyzed by the enzyme lipoprotein N-acyl transferase (Lnt) (Buddelmeijer and Young 2010; Hillmann et al. 2011; Noland et al. 2017). The mature lipoprotein with three acyl-chains can then be shuttled to the outer membrane via the lipoprotein outer membrane localization (LOL) machinery (Konovalova and Silhavy 2015; Tokuda and Matsuyama 2004), or stay within the inner membrane if there is a retention signal which often consists of an aspartic acid at the +2 (P2′) position (Seydel et al. 1999; Terada et al. 2001).

The database DOLOP lists and categorizes both predicted and experimentally verified bacterial lipoproteins (Babu et al. 2006; Madan Babu and Sankaran 2002), but this database has not been updated since 2005. A video procedure for the purification of lipoproteins via Triton X-114 detergent extraction and electrophoresis, followed by chemical characterization by proteolysis, solvent extraction and mass-spectrometry is available (Armbruster and Meredith 2018). There are many recent reviews on bacterial lipoproteins (Hutchings et al. 2009; Konovalova and Silhavy 2015; Kovacs-Simon et al. 2011; LoVullo et al. 2015; Narita and Tokuda 2017; Zuckert 2014). For a review on the chemical characterization of both Gram-negative and Gram-positive bacterial lipoproteins by mass-spectrometry see the review by Nakayama et al. (2012). A lipoprotein signal peptide prediction server called LipoP is available (Juncker et al. 2003; Rahman et al. 2008). Great progress has been made in understanding the enzymes in the bacterial lipoprotein synthetic pathway and crystal structures have been solved for each of the enzymes involved: Lgt (Mao et al. 2016), Lnt (Noland et al. 2017) and SPase II (Vogeley et al. 2016). Only SPase II will be discussed in this chapter.

## Gram-Negative Type 1 Signal Peptidase

*Escherichia coli* SPase I was the first bacterial SPase I to be studied and is the most thoroughly characterized SPase to date. The first observation of bacteria SPase I activity was in *E. coli* membranes using a nascent bacteriophage f1 pre-coat protein as the substrate (Chang et al. 1978). *E. coli* SPase I was also the first bacterial SPase I

to be purified. Bacteriophage M13 procoat protein was used as the substrate to assess the 6,000 fold purification (Zwizinski and Wickner 1980). A proteoliposome assay showed that only purified *E. coli* SPase I and phospholipid were needed for preprotein processing (Watts et al. 1981). The pH optimum of *E. coli* SPase I is 8.5–9.0 and it is inhibited by NaCl concentrations above 160 mM and $MgCl_2$ concentrations above 1 mM. The enzyme has been shown to be essential for cell growth (Date 1983). *E. coli* SPase I is the product of the *lepB* gene, is 324 residues in length, and has a calculated molecular mass of 35,960 Da and a theoretical isoelectric point of 6.9. Quantitative western blot analysis is consistent with approximately 1000 SPase I molecules per cell (van Klompenburg et al. 1995). The current residue numbering system for *E. coli* SPase I is different by one residue from the numbering system used in earlier work. This is due to an error in the originally reported sequence of the *E. coli* enzyme (Wolfe et al. 1983); Arg42 in the originally reported sequence is actually Ala42. In addition there is glycine residue (Gly43) just after the Ala42 that was not in the originally reported sequence. The insertion moves the sequence up one number after that point. Therefore, in the current system, residues 1–41 are consistent with the old numbering and residues 44–324 are correct in sequence but the numbering is one residue different from the old numbering. The sequencing error corresponds to residues within the cytoplasmic region, between the two transmembrane segments. The numbering system used in this chapter matches that in the UniProt sequence database (accession number: P00803).

Protease accessibility assays show that *E. coli* SPase I is an integral membrane protein and the majority of the protein chain is on the outside of the cytoplasmic membrane (Moore and Miura 1987; Wolfe et al. 1983). Enzyme-fusion assays are consistent with both the amino- and carboxy-termini facing the periplasm (San Millan et al. 1989). The interface between the two transmembrane segments in *E. coli* SPase I has been modeled based on disulfide mapping (Whitley et al. 1993). It was found that residue pairs 3–76, 4–76, 4–77, –4–80, and 7–76 are likely in close proximity. Modeling is consistent with the two transmembrane helices packing against each other in a left-handed supercoil with an i, i + 3/i, i + 4 grooves-into-ridges arrangement. Site-directed mutagenesis and deletion studies show that the first transmembrane segment (residues 4–28) and cytoplasmic region (residues 29–57) are not essential for catalytic activity. The second transmembrane segment (residues 58–76) is essential for activity and for initiating the translocation of the periplasmic region (residues 77–324) where the protease active site resides (Bilgin et al. 1990) (Fig. 7.2). It has been shown that highly purified and concentrated *E. coli* SPase I will cleave itself in a intermolecular fashion within its cytoplasmic region (Talarico et al. 1991). Since this cleavage site region is on the opposite side of the cytoplasmic membrane from the SPase I active site, it is not likely this is a common occurrence in vivo, although disruption of the membrane or mistakes in SPase I assembly could allow for this type of cleavage.

Early studies tried to classify the mechanism of *E. coli* SPase I based on sensitivity to standard protease inhibitors (Zwizinski et al. 1981). These experiments were inconclusive and suggested that this enzyme may utilize a unique mechanism. Site-directed mutagenesis of conserved ionizable residues was consistent with Ser91

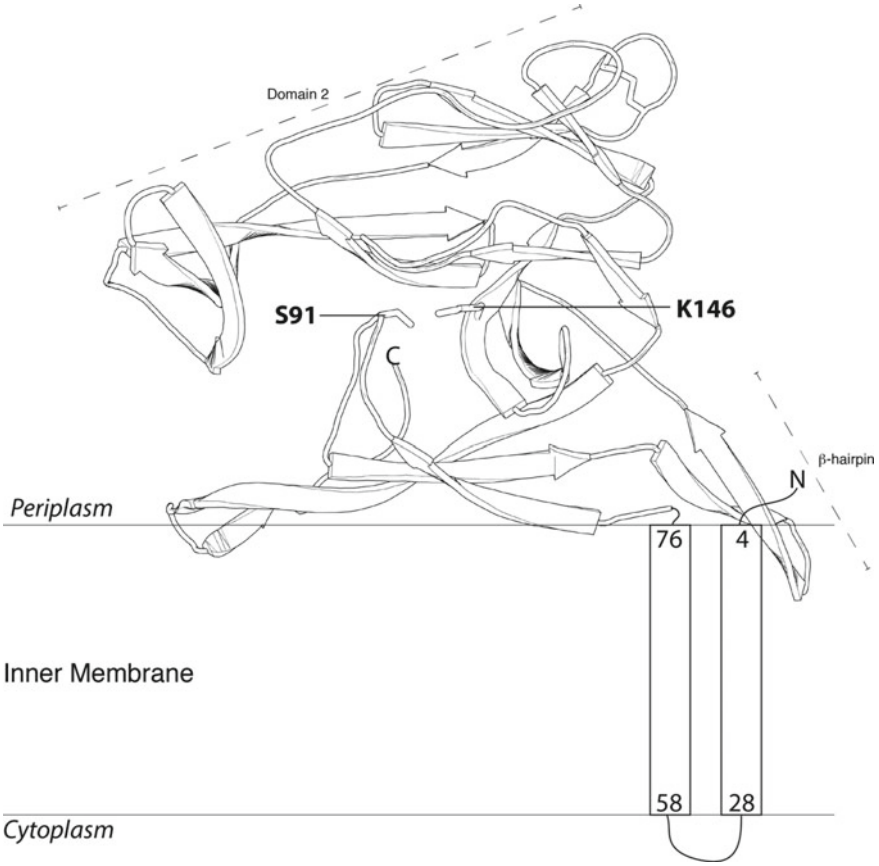



**Fig. 7.2** The membrane topology and protein fold of *E. coli* SPase I. The transmembrane segments, not part of the crystal structure, are represented by rectangles. The nucleophilic Ser91 and general base Lys146 are labeled. The β-ribbon and domain 2 region not normally observed in the Gram-positive SPase I enzymes are labeled (PDB: 1B12)

and Lys146 being essential for catalytic activity (Black 1993; Black et al. 1992; Paetzel et al. 1997; Sung and Dalbey 1992; Tschantz et al. 1993). Therefore, a Ser/Lys catalytic dyad mechanism was proposed.

The first three-dimensional structure of a SPase I was that of *E. coli* SPase I (Paetzel et al. 1998). A soluble catalytically active enzyme was produced, lacking the two amino-terminal transmembrane segments (Δ2-76) (Tschantz et al. 1995). This active periplasmic region of *E. coli* SPase I produced ordered diffraction quality crystals (Paetzel et al. 1995). Crystal structures of this enzyme are available in complex with a β-lactam type of inhibitor (Paetzel et al. 1998), in complex with a lipopeptide base inhibitor called arylomycin (Paetzel et al. 2004), in complex with arylomycin and β-sultam inhibitor (Luo et al. 2009), and in complex with a lipoglycopeptide version

of arylomycin (Liu et al. 2011a). In addition, a structure of *E. coli* SPase I with a free active site is available (Paetzel et al. 2002).

The crystal structures reveal that SPase I is primarily constructed of β-sheets. There are two antiparallel β-sheet domains (domain I and domain II) and an extended β-hairpin that extends from domain I (Fig. 7.2). Domain I contains the catalytic residues and has a similar protein fold to that of the proteinase domain in the UmuD protein of the SOS mutagenesis system (Paetzel and Strynadka 1999). Sequence alignments reveal that domain II is much smaller or missing in Gram-positive SPase I. A disulfide bond (Cys171–Cys177) resides within domain II of *E. coli* SPase I but mutagenesis has shown it to be nonessential (Sung and Dalbey 1992). Almost all of the conserved SPase I residues are located near the active site in domain I.

The catalytic center of SPase I resides at the end of a substrate binding groove that is provided by a missing β-stand of a would-be β-barrel within domain I. The strand and the loop region that makes up the edges of the binding groove run parallel to one another. Modeling studies based on the inhibitor complexes and the constraints related to the lipid bilayer of the inner membrane suggest that the c-region of the signal peptide within the preprotein substrate binds in a parallel β-strand conformation (Paetzel et al. 1998, 2002). This binding mode is in agreement with previous predictions (von Heijne 1983). The nucleophile Ser91 resides on a loop following the first β-stand. The general-base Lys146 resides on a β-strand with its Nζ atom within hydrogen bonding distance to the Ser91Oγ (Fig. 7.3). Lys146 Nζ is also within hydrogen bonding distance to Ser279Oγ. This is a conserved residue and site-directed mutagenesis experiments show that it is important for optimal activity (Klenotic et al. 2000). The oxyanion hole is constructed from the mainchain NH group of Ser91 and the side chain hydroxyl group of the Ser89. In vivo and in vitro analysis of site-directed mutants at this position are consistent with Ser89 playing a role in transition state stabilization (Carlos et al. 2000).

The substrate specificity binding pockets that lead to the active site explain the preference for alanine at the −1 (P1) and −3 (P3) position in preproteins (Fig. 7.3). The shallow and hydrophobic S1 substrate specificity binding pocket is made of atoms from the residues: Met92, Ile145, Leu96 and Ile87. The shallow and hydrophobic S3 substrate specificity binding pocket is constructed with atoms from residues: Phe85, Ile87, Ile102, Val133, Ile145 and Asp143. Site-directed mutagenesis, mass-spectrometry and molecular modeling were used to probe the importance of residues in the SPase I binding pockets. It was observed that Ile87 and Ile145, residues that divide the S1 and S3 binding pockets (Fig. 7.3), are important for specificity as well as cleavage site fidelity (Ekici et al. 2007; Karla et al. 2005). NMR analysis has recently been employed to look at the details of the interactions between SPase I and the signal peptide (De Bona et al. 2012; Musial-Siwek et al. 2008a, b).

*E. coli* SPase I has an extensive hydrophobic surface that runs along domain I and leads to the active site. This hydrophobic surface and the orientation of active site residues provide clues as to how the catalytic domain lays on the membrane surface. Chemical modification and site-directed mutagenesis experiments are consistent with Trp301 and Trp311 being important for activity and these residues reside along the hydrophobic surface (Kim et al. 1995a, b). Biophysical analysis has shown

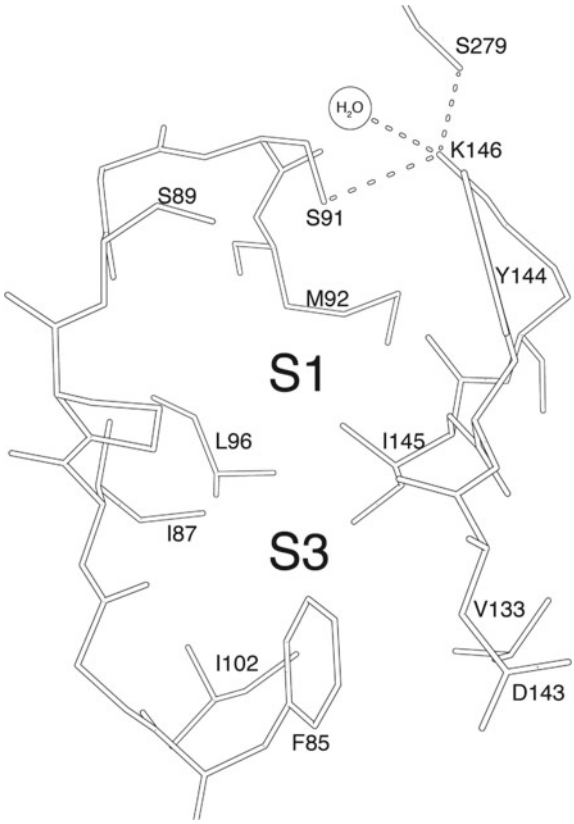



**Fig. 7.3** The active site region of *E. coli* SPase I. The regions corresponding to the S1 and S3 substrate specificity pockets are labeled. Dashed lines correspond to important hydrogen bonds within the active site. The coordinates used are from a structure with a free active site. An ordered, and potentially catalytic, water at the correct distance and angle to the general base and modeled scissile carbonyl is labeled (PDB: 1KN9)

that the catalytic domain of *E. coli* SPase I penetrates into the membrane and that phosphatidylethanolamine, the most abundant phospholipid in the *E. coli* inner membrane, mediates the association (van Klompenburg et al. 1997, 1998). Kinetic analysis shows that detergent or lipid is required for optimal activity of the soluble catalytic domain of *E. coli* SPase I even though it lacks the transmembrane domains (Tschantz et al. 1995).

Significant effort has been invested into SPase I inhibitor development. The first reported inhibitor of SPase I was a β-lactam compound (Kuo et al. 1994), and a number of penem type inhibitors have been developed (Allsop et al. 1995, 1996; Barbrook et al. 1996; Black and Bruton 1998; Perry et al. 1995). The first crystal structure of *E. coli* SPase I was solved with the compound allyl (5S,6S)-6-[(R)-acetoxyethyl]-penem-3-carboxylate (Fig. 7.4a) covalently bound to the Oγ of Ser91, thus proving directly for the first time its role as the nucleophile (Paetzel et al. 1998). The acyl-enzyme inhibitor complex also revealed that the nucleophile of *E. coli* SPase I attacks from the *si*-face of the scissile bond rather than the more commonly observed *re*-face attack. Work has continued on the development of the penem SPase I inhibitors (Harris et al. 2009; Yeh et al. 2018). Lipohexapeptides called arylomycins,

which have antibiotic properties, have been discovered to inhibit SPase I (Fig. 7.4b). These compounds were first isolated from extracts of *Streptomyces sp.* Tu 6075 and have 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 (Holtzel et al. 2002; Schimana et al. 2002). The aromatic ring of L-MeHpG is covalently bonded to the aromatic ring of L-Tyr to form a three-residue macrocycle via a 3,3-biaryl bridge. A fatty acid is attached to the amino-terminus. Crystallographic and biophysical analysis reveals that arylomycin binds to *E. coli* SPase I in a non-covalent fashion (Fig. 7.4b) (Paetzel et al. 2004). Analogs of arylomycin inhibitors have also been developed (Dufour et al. 2010; Liu et al. 2011b; Roberts et al. 2007, 2011a, b; Smith et al. 2010, 2011, 2018; Smith and Romesberg 2012). Other reported inhibitors of SPase I include boronic ester-linked macrocyclic lipopeptides (Szalaj et al. 2018) and aldehyde containing lipopeptides (Buzder-Lantos et al. 2009; De Rosa et al. 2017).

The crystal structures, site-directed mutagenesis and kinetic analysis are all consistent with the following proposed catalytic mechanism for *E. coli* SPase I. The c-region of the signal peptide binds within the SPase I substrate binding groove (Michaelis complex) which buries the catalytic residues and likely lowers the $pK_a$ of the lysine general base, Lys146. Ser91 serves as the nucleophile and is activated by the abstraction of its hydroxyl hydrogen by the deprotonated Nζ of the general base Lys146. The Ser279Oγ is within hydrogen bonding distance and helps orient the Nζ of Lys146 towards Ser91 (Fig. 7.3). The nucleophilic attack from the *si*-face of the scissile carbonyl by the activated Ser91 Oγ results in the tetrahedral oxyanion transition state 1. The oxyanion is stabilized via hydrogen bonds to the oxyanion hole (Ser91NH and Ser89OH). The protonated Lys146 Nζ donates a proton to the main chain nitrogen of the leaving group (P′ side of the scissile bond, the mature region of the secretory protein, product 1). The main chain carbonyl carbon of the P1 residue of the signal peptide is then covalently attached via an ester bond to the Ser91 Oγ (acyl-enzyme intermediate). A nucleophilic water (a.k.a. deacylating or catalytic water) is activated via the general base Lys146. The structure of *E. coli* SPase I with a free active site revealed a likely candidate for this water (Paetzel et al. 2002) (Fig. 7.3). The hydroxyl is in position to attack the carbonyl carbon of the ester bond. This forms the tetrahedral oxyanion transition state 2, stabilized via hydrogen bonds to the oxyanion hole. This state then leads to the regeneration of the enzyme and release of product 2.

Although *E. coli* SPase I is the most characterized signal peptidase there has been a significant amount of research on other Gram-negative type 1 signal peptidases. Most Gram-negative species have a single SPase I gene that codes for a membrane bound enzyme with two transmembrane segments but there are some exceptions. Photosynthetic bacteria have two types of membranes; the cytoplasmic membrane and the thylakoid membrane. Genomic analysis of the photosynthetic bacteria *Synechocystis sp*. strain PCC 6803 has revealed two SPase I like genes (sll0716 or lepB1 and slr1377 or lepB2) (Zhbanko et al. 2005). Gene knockout analysis was used to show that lepB2 is essential for cell viability and LepB1 is essential for photoautotrophic growth. It will be interesting to look at the relative distribution of these enzymes in the two different membrane systems of this cyanobacterium given that

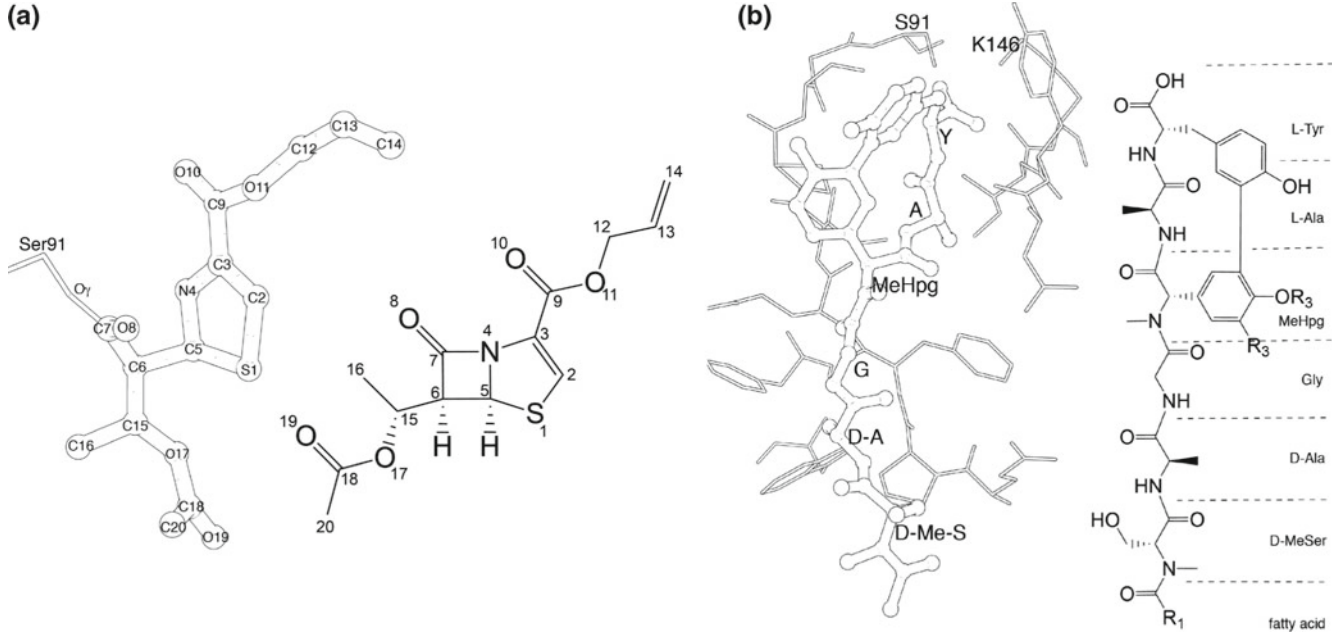



**Fig. 7.4** Inhibitors of SPase I. **a** The β-lactam type inhibitor, allyl (5S,6S)-6-[(R)-acetoxyethyl]-penem-3-carboxylate (PDB: 1B12). **b** The lipopeptide based inhibitor arylomycin (PDB: 1T7D)

proteomic analysis observes some potential crossover in activity and leaves open the possibility that there maybe other SPase I enzymes. SPase I activity has also been observed from solubilized membranes of the phototrophic α-purple bacterium *Rhodobacter capsulatus* (Wieseler et al. 1992).

The *Pseudomonas aeruginosa* genome contains the SPase I genes PA0768 and PA1303 (Waite et al. 2012). In vitro assays using FRET-peptide substrates showed that both enzymes are active. PA1303 is smaller than a typical Gram-negative SPase I and is non-essential. PA0768 has more of a typical length for a Gram-negative SPase I and is essential for cell growth. Site-directed mutagenesis suggests that PA1303 plays a role in the quorum-sensing cascade and that PA0768 is the main SPase I in *P. aeruginosa*. *Bradyrhizobium japonicum* is a soil bacterium that fixes nitrogen and is yet another Gram-negative bacterium with two SPase I genes (SipS and SipF) (Bairl and Muller 1998). These enzymes are also different in that they are predicted to have a single amino-terminal transmembrane segment, similar to the Gram-positive SPase I enzymes. The thermophilic cyanobacterium *Phormidium laminosum* also has a SPase I with a predicted single amino-terminal transmembrane segment (Packer et al. 1995). *Legionella pneumophila*, the facultative intracellular Gram-negative bacterium that causes Legionnaires' disease, has a SPase I with a unique sequence feature (Lammertyn et al. 2004). The conserved methionine that immediately follows the nucleophilic serine is a leucine. The structure of *E. coli* SPase showed that this residue is located right behind the Ser/Lys catalytic dyad (Paetzel et al. 1998) (Fig. 7.3).

Other Gram-negative species whose type 1 signal peptidase activity has been confirmed by in vivo complementation assays includes: *Azotobacter vinelandii* (Jock et al. 1997), *Bordetella pertussis* (Smith et al. 2000), *Pseudomonas fluorescens* (Black et al. 1992), *Salmonella typhimurium* (van Dijl et al. 1990), *Rickettsia rickettsii* and *Rickettsia typhi* (Rahman et al. 2003).

## Gram-Positive Type 1 Signal Peptidase

Unlike most Gram-negative species that possess a single gene for SPase I, Gram-positive bacteria tend to have multiple genes for SPase I. Sequence alignments reveal that Gram-positive SPase I are shorter in length than the Gram-negative SPase I. They are typically missing part of domain II in their extracytoplasmic region and they tend to have a single transmembrane segment at their amino-terminus rather than the two transmembrane segments normally observed in the Gram-negative SPase I. The best characterized Gram-positive SPase I are those from *Bacillus subtilis* and *Staphylococcus aureus*.

*Bacillus subtilis* SipS was the first Gram-positive SPase I to be characterized in detail (van Dijl et al. 1992). All together, *Bacillus subtilis* has five chromosomally expressed SPase I enzymes (SipS, SipT, SipU, SipV, and SipW) (Bolhuis et al. 1996; Tjalsma et al. 1997) and two plasmid expressed SPase I enzymes (SipP) (Meijer et al. 1995; Tjalsma et al. 1999b). Site-directed mutagenesis experiments are con-

sistent with the SipS utilizing a Ser/Lys catalytic dyad mechanism where Ser43 is the nucleophile and Lys83 serves as the general base. Interestingly it was also found that the mutant M44A showed increased activity (van Dijl et al. 1995). It has been shown that all of the *sip* gene products function as type 1 signal peptidases but only cells lacking both SipS and SipT are not viable (Tjalsma et al. 1998). SipW is very unique in this group of SPase I enzymes in that it has a histidine in place of the general base lysine (Tjalsma et al. 2000). This suggests it is more like the Sec11-like component of the eukaryotic ER signal peptidase complex or the archaeal SPase I (discussed below). SipW is required for processing of the spore-associated protein TasA (Tjalsma et al. 2000). SPase I genes from other *Bacillus* species have been characterized including: *Bacillus amyloliquefaciens* (Chu et al. 2002; Hoang and Hofemeister 1995; van Roosmalen et al. 2001), *Bacillus licheniformis* (Cai et al. 2016) and *Bacillus megaterium* (Malten et al. 2005; Nahrstedt et al. 2004).

*Staphylococcus aureus* has two SPase I-like genes (Cregg et al. 1996). The main SPase I is coded for by the *spsB* gene and is essential. Immediately preceding the *spsB* gene is another SPase I-like gene *spsA* but it lacks the catalytic residues. The nucleophilic serine is replaced by an aspartic acid and the lysine general-base is replaced by a serine. SpsB is involved in quorum sensing. It is responsible for the amino-terminal processing of the autoinducing peptide (AIP) molecule precursor AgrD (Kavanaugh et al. 2007). Kinetic analysis using both preprotein and peptide cleavage assays shows SpsB has a pH optimum around 8 and apparent $pK_a$ values of 6.6 and 8.7. Curiously, it has been shown that SpsB is not absolutely essential. *S. aureus* is able to survive without SpsB by over-expressing a native gene cassette that encodes a putative ABC transporter. Apparently it compensates for the lack of a SPase I by cleaving a subset of secretory proteins at a site distinct from the SpsB-cleavage site (Craney and Romesberg 2017; Hazenbos et al. 2017; Morisaki et al. 2016).

SpsB is the only SPase I besides *E. coli* SPase I to be crystallographically characterized (Ting et al. 2016). Protein engineering was used to make a crystallizable construct with a peptide substrate tethered for presentation to its binding site (Fig. 7.5a). The catalytic domain of SpsB was fused with *E. coli* maltose binding protein (MBP) to promote solubility, stability, purification and crystallization. The construct included a poly-histidine-affinity tag followed by MBP (residues 33–393), followed by a three-residue linker (Ala-Gly-Ala) and then residues 26–191 of SpsB. The carboxy-terminal residues of SpsB (176–191) that are disordered in the free active site structure were replaced with a Strep-tag II (WSHPQFEK). Four different structures of SpsB were determined; a free active site structure (PDB: 4wvg) with the nucleophilic serine mutated to an alanine (S36A) and three structures with peptides bound and with the wild type residue at position 36. The peptides were tethered covalently to the MBP at an engineered cysteine residue (Q78C) via a thioether linkage. The covalent tether increased the local effective concentration of the peptide and led to high occupancy of the peptide within the binding site and clear electron density for the bound peptide. The peptides (peptide 1: GGGGADHDAHA↓SET, peptide 2: GGGGAVPTAKA↓ASK, and peptide 3: GGGGGAPTAKAPSK) were synthesized with an amino-terminal N-bromo-acetyl moiety that reacted with the Sγ

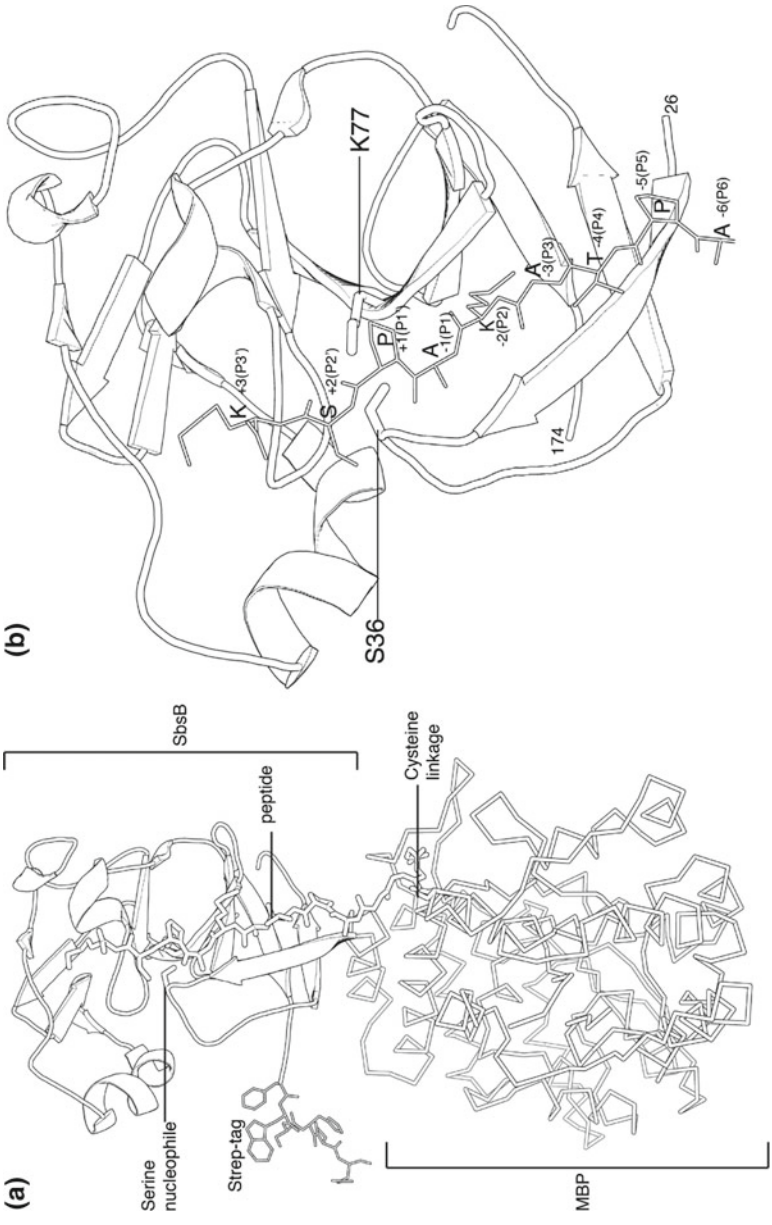
of the engineered cysteine. The structures of peptide 1 and peptide 2 bound to SpsB (PDB: 4wvh and 4wvi respectively) reveal that the substrate-like peptides have been cleaved at the scissile bond between the P1 and P1′ residues resulting in product complexes. The third peptide bound structure (PDB: 4wvj) has a noncleavable P1′ proline peptide (peptide 3) showing possible contacts that could be made by the P1′–P3′ residues of the substrate in a Michaelis complex. All of these complex structures reveal the methyl group of the P1 alanine pointing into the S1 binding pocket and the methyl group of the P3 alanine pointing into the S3 binding pocket. These structures are consistent with the binding mode previously hypothesized based on *E. coli* SPase I inhibitor complex structures (Liu et al. 2011a; Luo et al. 2009; Paetzel et al. 2002; Paetzel et al. 1998, 2004). SpsB is very similar to domain I of *E. coli* SPase I and the residues that makeup the active site and specificity binding pockets superimpose well. If the engineering of this construct does not interfere with the natural substrate cleavage site presentation, the SPase I-peptide complexes reveal the path that preprotein residues P6 to P3′ take along the enzyme surface (Fig. 7.5b).

There have been a number of other characterized Gram-positive type 1 signal peptidase including some from species that are associated with biofilm formation. *Staphylococcus epidermidis* has three SPase I genes (Sip1, Sip2, Sip3). The catalytic activity of Sip2 and Sip3 has been measured using both preprotein and synthetic peptide substrates. Sequence analysis shows that Sip1 does not have a lysine general base (Bockstael et al. 2009). The presence of SPase I paralogs without the full catalytic dyad has been observed in a number of species, such as *S. aureus* SpsA that was mentioned above, but it is not yet clear what role(s) these genes perform. *Streptococcus sanguinis*, contains two SPase I genes; SSA_0351 and SSA_0849. SSA_0351 has been shown to be essential for biofilm formation (Aynapudi et al. 2017). *Actinomyces oris* expresses two SPase I enzymes (LepB1 and LepB2). Mutational analysis is consistent with LepB2 being responsible for processing fimbrial proteins (Siegel et al. 2016).

*Streptococcus pneumoniae* SPase I (the *spi* gene product) has been expressed in *E. coli,* purified, and its activity characterized (Zhang et al. 1997). Site-directed mutagenesis and preprotein processing assays reveal the Ser38 and Lys76 of *S. pneumoniae* SPase I are essential for catalytic activity (Peng et al. 2001). Interestingly, biochemical studies provide evidence that this enzyme undergoes intermolecular self-cleavage that results in loss of activity and that this may play a role in regulation (Zheng et al. 2002).

*Listeria monocytogenes* contains three contiguous SPase I genes in its genome (SipX, SipY and SipZ). Gene knockout analysis has shown that SipZ is the major SPase I involved in the processing of most secretory proteins and that SipX and SipZ are specific for processing proteins related to pathogenicity (Bonnemain et al. 2004; Raynaud and Charbit 2005).

*Streptomyces lividans* contains four different chromosomally encoded SPase I genes (*sipW*, *sipX*, *sipY* and *sipZ*) (Parro et al. 1999). Sequence alignments and functional analysis are consistent with all four of the enzymes having a full set of catalytic residues and all four being able to process preproteins (Geukens et al. 2001b). The first of these genes to be cloned was that of sipZ (Parro and Mellado


(a)
SbsB
peptide
Cysteine linkage
Serine nucleophile
Strep-tag
MBP
(b)
K77
S36
K +3(P3')
S +2(P2')
P +1(P1')
A -1(P1)
K -2(P2)
A -3(P3)
T -4(P4)
P -5(P5)
A -6(P6)
26
174

◀**Fig. 7.5** The crystal structure of the catalytic domain of *S. aureus* SPase I (SpsB). **a** Protein engineering was used to make a crystallizable construct of SpsB. Maltose Binding Protein (MBP) was engineered at the amino terminus of SpsB. A strep-affinity-tag replaced the C-terminal residues of SpsB. A series of peptides corresponding to substrate and inhibitor sequences were covalently linked to the MBP via an engineered cysteine, such that they presented the specificity residues to the SpsB substrate-binding groove. **b** A close up view of the SpsB binding groove with the P1′ proline peptide (PDB: 4wvj)

1998). Membrane topology analysis shows that SipY has one amino-terminal transmembrane segment and one carboxy-terminal transmembrane segment. Sequence analysis suggests that SipX and SipZ have a similar topology (Geukens et al. 2001a). Kinetic analysis shows that SipW and SipY have a pH optimum of 8–9 whereas SipX and SipZ have a pH optimum of 10–11. All four enzymes show improved activity in the presence of phospholipids (Geukens et al. 2002). Mutagenesis and proteomic analysis is consistent with SipY being the main SPase I for general protein secretion in *S. lividans* (Escutia et al. 2006; Palacin et al. 2002).

## Archaeal Type 1 Signal Peptidase

Genome analysis has shown that archaea possess a gene with sequence similarity to bacterial SPase I, but the lysine general-base that is observed in bacterial SPase I is replaced by a histidine in archaeal SPase I (Eichler 2002). In addition, most archaeal SPase I lack a large region of domain II (Eichler 2002). This suggests that archaeal SPase I maybe similar to the Sec11 component of the signal peptidase complex (SPC) that is present in eukaryotic species (Ng et al. 2007). It is interesting that the bacterial genus *Bacillus* contain SPase I genes that are similar to the eukaryotic or archaeal SPase I homologs. As mentioned above, the *B. subtilis* SipW is a characterized SPase I whose sequence suggests that it utilizes a histidine rather that lysine as its general base (Tjalsma et al. 1998, 2000).

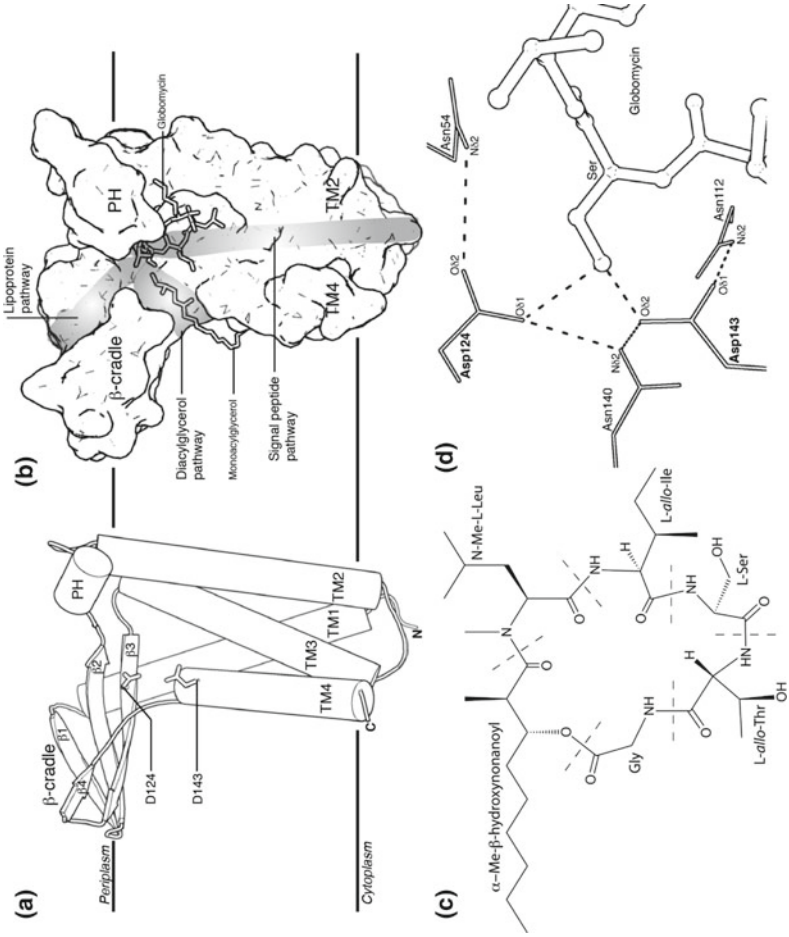
The first characterization of an archaeal type I signal peptidase was that of the methanogenic archaeon *Methanococcus voltae* (Ng and Jarrell 2003). The gene was cloned and expressed in *E. coli* and its activity analyzed using an in vitro assay with a truncated form of *M. voltae* S-layer protein as the substrate. This assay demonstrates that the archaeal SPase I gene product can function without the help of other components. Membrane topology prediction analysis suggests that this enzyme contains an amino-terminal and a carboxy-terminal transmembrane segment. Site directed mutagenesis studies on *M. voltae* SPase I show that Ser52 and His122 are essential residues and that Asp148 may also be essential (Bardy et al. 2005).

The halophilic archaeon *Haloferax volcanii* contains two SPase I genes in its genome (*sec11a* and *sec11b*). Gene deletion studies show that both of these genes are expressed but that only Sec11b is essential. Both enzymes were able to cleave preproteins but with different levels of efficiency (Fine et al. 2006). It is not yet clear why some arachaea have multiple SPase I paralogs. Of note, genomic analysis sug-

gests that haloarchaea use the Tat pathway rather than the general secretion system for nearly half of their secreted proteins. Since the Tat pathway specializes in the translocation of fully folded proteins, this maybe an adaption to the extreme salt concentrations outside the cell that could prevent proper protein folding. Interestingly, both SPase I enzymes (Sec11a and Sec11b) in *H. volcanii* have an acidic isoelectric point (4.5) consistent with the negative charge seen on most of the *H. volcanii* cell surface proteins. The calculated isoelectric point for most archaeal SPase I are significantly more basic. For example, the isoelectric point of *M. voltae* SPase I is 9.0. Site-directed mutagenesis and in vitro preprotein cleavage assays reveal that the residues Ser72, His137 and Asp187 in *H. volcanii* Sec11b are essential (Fink-Lavi and Eichler 2008).

## Type II Signal Peptidases

SPase II (also called type 2 signal peptidase, prolipoprotein signal peptidase, and LspA) catalyzes the removal of the amino-terminal signal peptide from bacterial proteins that are tethered to the inner membrane by the signal peptide and a diacylglycerol moiety. Many of the insights into the discovery and characterization of SPase II were facilitated by the cyclic pentapeptide (19-membered cyclic depsipeptide) globomycin (Fig. 7.6c). This natural product has four natural amino acid (glycine, L-serine, L-*allo*-isoleucine (2S,3R), L-*allo*-threonine (2S,3S)), N-methyl-L-leucine, and β-hydroxy-α-methyl carboxylic acid (α-methyl-β-hydroxynonanoyl). It is a product of non-ribosomal peptide synthesis in the Gram-positive mycelial bacteria *Actinomycetes*, first isolated in 1978, and shown to have antibacterial activity (Inukai et al. 1978a, b; Nakajima et al. 1978). It was revealed that globomycin's antimicrobial activity is the result of the inhibition of a prolipoprotein processing enzyme which converts prolipoprotein to lipoprotein (Inukai et al. 1978c). This was the first observation of SPase II activity. Globomycin was instrumental in the discovery that the cleavage of the signal peptide by SPase II is essential for the transport of lipoprotein to the outer membrane of Gram-negative bacteria (Hussain et al. 1980). Inhibition of SPase II results in the accumulation of prolipoprotein in the inner membrane which then leads to bacterial cell death (Hussain et al. 1980). This also helped confirm that modification with diacylglycerol occurs before cleavage of the signal peptide. Globomycin is a reversible noncompetitive inhibitor with a $K_i$ of 36 nM (Dev et al. 1985). Total synthesis and crystallographic analysis led to the relative and absolute configurations of globomycin (Kogen et al. 2000). NMR analysis suggests that globomycin exists as a mixture of two rotational isomers in solution. Synthetic globomycin has the same antimicrobial activity as the naturally purified globomycin and changes to the serine position decreases antimicrobial activity. Solid-phase methods of globomycin synthesis has allowed for further optimization of this inhibitor (Sarabia et al. 2011). Intriguingly, another cyclic secondary metabolite from the Myxobacteria called TA (myxovirescin) also inhibits SPase II (Xiao et al. 2012).


(a)
β-cradle
PH
Periplasm
Cytoplasm
D124
D143
TM1
TM2
TM3
TM4
(b)
Lipoprotein pathway
Diacylglycerol pathway
Monoacylglycerol
Signal peptide pathway
Globomycin
(c)
α-Me-β-hydroxynonanoyl
N-Me-L-Leu
Gly
L-allo-Ile
L-Ser
L-allo-Thr
(d)
Asn54
Asp124
Asn140
Asp143
Asn112
Ser
Globomycin

◀**Fig. 7.6** The crystal structure of *Pseudomonas aeruginosa* (strain PAO1) SPase II (PDB: 5DIR). **a** The protein fold of SPase II showing the transmembrane segments 1–4 (TM1-4). The β-cradle domain and the PH (periplasmic helix) region are labeled. The proposed catalytic residues Asp124 and Asp143 are labeled. **b** The surface of SPase II reveals three pathways that meet at the proposed position of the P1′ cysteine of the prolipoprotein. The proposed pathway for the lipoprotein (mature region of the prolipoprotein) is shaded and labeled, as are the proposed pathways for the signal peptide and the diacylglycerol. The bound lipid used in the crystallization is labeled. The position of the c-region of the signal peptide is proposed based on the position of bound inhibitor globomycin. **c** The molecular structure of SPase II inhibitor globomycin. **d** The active site of SPase II. The proposed catalytic residues Asp124 and Asp143 are within hydrogen bonding distance (dashed lines) to coordinating asparagine residues. The atoms involved in the hydrogen bonding interactions are labeled. The serine residue of globomycin, which mimics the P1 residue of the substrate, is shown in ball-and-stick

SPase II activity has been measured using both in vivo and in vitro assays (Dev and Ray 1984; Kitamura and Wolan 2018; Yamagata 1983). Recently a high-throughput in vitro SPase II activity assay has been designed, allowing for screening of chemical libraries. This has led to the development of inhibitors with nanomolar half-maximal inhibitory concentration values (Kitamura et al. 2018).

The first SPase II to be investigated was the SPase II from *E. coli*. Evidence clearly showed that there existed in *E. coli* a distinct signal peptidase for the processing of lipoproteins (Tokunaga et al. 1982, 1984). Its gene was mapped and sequenced (Regue et al. 1984; Tokunaga et al. 1984) and the enzyme-fusion technique was used to determine its membrane topology. This analysis suggested that *E. coli* SPase II has four transmembrane segments with both the amino-terminus and the carboxy-terminus residing in the cytoplasm (Munoa et al. 1991). SPase II genes from other Gram-negative bacterial have been characterized including: *Enterobacter aerogenes* (Isaki et al. 1990), *Legionella pneumophila* (Geukens et al. 2006), *Myxococcus xanthus* (Paitan et al. 1999), and *Rickettsia typhi* (Rahman et al. 2007), and *Pseudomonas aeruginosa* (Vogeley et al. 2016).

Gram-positive SPase II enzymes that have been characterized include: *Bacillus subtilis* (Pragai et al. 1997; Tjalsma et al. 1999a, c), *Staphylococcus carnosus* (Witke and Gotz 1995), *Streptococcus pneumonia* (Khandavilli et al. 2008), *Streptococcus suis* (De Greeff et al. 2003), *Streptomyces coelicolor* (Munnoch et al. 2016), and *Streptomyces lividans* (Gullon et al. 2013). Sequence alignments and site-directed mutagenesis in *B. subtilis* SPase II suggest that SPase II is a unique aspartic protease (Tjalsma et al. 1999c).

One structure of a SPase II enzyme is available (Vogeley et al. 2016). LspA from *Pseudomonas aeruginosa* (strain PAO1) was crystallized using the *in meso* (lipid cubic phase) method (Caffrey 2015). The structure is refined to 2.8 Å resolution. The crystallization conditions were at pH5.6–6.0 and included MES buffer, PEG400, ammonium phosphate, and monoolein (monoacylglycerol, 9.9 MAG, 1-(9Z-octadecenoyl)-rac-glycerol). The enzyme is 169 residues in length (UniProt: Q9HVM5, molecular mass 18,997 Da, isoelectric point 8.0). The crystal structure has clear electron density for residues 2–158 (for chain A, the most ordered of the

four molecules in the asymmetry unit). Structural analysis suggests SPase II is a monomer.

The crystal structure of SPase II from *Pseudomonas aeruginosa* showed there are four transmembrane segments with a topology that places both the amino- and carboxy-termini in the cytoplasm (TM1: residues 9–34, TM2: residues 68–90, TM3: residues 95–119, and TM4: residues 140–158) (Fig. 7.6a). This topology is consistent with the gene-fusion topology analysis performed on the *E. coli* SPase II homolog (Munoa et al. 1991). The first three residues of TM1 are in the $3_{10}$-helical conformation. There is also a small periplasmic domain that is made up of a four-stranded antiparallel β-sheet (β1: residues 40–43, β2: residues 46–54, β3: residues 122–130, β4: residues 134–136). Because of its shape, this domain is referred to as the β-cradle. There is also a loop region with a single $3_{10}$-helical turn (residues 60–62), which is referred to as the periplasmic helix (PH). The β-cradle and PH are amphipathic in nature and reside on the periplasmic region of the protein, along the surface of the periplasmic side of the inner membrane lipid bilayer, approximately perpendicular to the orientation of the TM helices. The first two strands of the β-sheet and the PH are an insertion between TM1 and 2. The last 2 β-stands within the β-sheet are an insertion between TM3 and 4. The secondary structure assignments discussed here were assigned using the program PROMOTIF(Hutchinson and Thornton 1996).

The SPase II structure was cocrystallized with globomycin. This allowed for the clear interpretation of the substrate binding groove and the catalytic active site. The substrate-binding groove is located at the periplasmic end of TM2, 3 and 4. The residues that have direct interactions with the inhibitor, and therefore likely with the prolipoprotein substrate as well, include residues that reside on TM3 such as Arg116 and Asn112. Interactions also come from Asn140 and Asp143, residing on TM4. Contacts are made from Asp124 found on the third β-strand within the β-sheet. The residues Leu-Ile-Ser of globomycin likely fit into the substrate-binding groove of SPase II in a similar fashion to the lipobox residues in the prolipoprotein. The residues surrounding the bound inhibitor correspond to the residues that are most conserved in SPase II.

Modeling and molecular dynamics simulations suggests that the Cα atom of the P1′ cysteine within the prolipoprotein substrate sits at a three-way crossroad of three substrate binding surfaces on the surface of LspA (Fig. 7.6b). The periplasmic β-cradle and the PH-loop region form a groove where the mature region of the lipoprotein would be directed. A second groove is formed by the periplasmic β-cradle and TM4. The side chain of the P1′ cysteine could fit in this groove and the thioether linkage to diacylglycerol would direct the fatty acids to lay across the surface of TM4. A binding surface is formed from TM helices 2, 3 and 4. This creates a long surface for the signal peptide to bind. The surface complementarity between this enzyme and its unique substrate allows for LspA to recognize and cleave a broad range of prolipoproteins.

The clues provided by the inhibitor complex allowed for a hypothetical Michaelis complex to be modeled. This model in turn suggested the identity of the residues involved in the catalytic mechanism. The most likely residues are Asp124 and Asp143 (Fig. 7.6a, d). Asp124 resides on the amino-terminal end of β-strand 3 (β3) and Asp143 resides on the amino-terminal end of TM4. In the globomycin-SPase II complex the globomycin serine hydroxyl group hydrogen bonds to both of the proposed catalytic residues. Sequence alignments show 14 strictly conserved residues: Asp23, Lys27, Asn54, Gly56, Gly108, Ala109, Asn112, Arg116, Val122, Asp124, Phe139, Asn140, Ala142, and Asp143. Most of these residues are located near the active site. Site-directed mutagenesis was consistent with Asp124 and Asp143 being essential residues. This suggests that these residues likely play a direct role in catalysis similar to the classic aspartic protease mechanism (Rawlings and Barrett 2013; Wlodawer et al. 2013). A constellation of asparagine residues helps to coordinate and orient the proposed catalytic residues (Fig. 7.6d). Asp143 Oδ1 is hydrogen bonded to Asn112 Nδ2 (3.0 Å). Asp124 Oδ2 is hydrogen bonded to Asn54 Nδ2 (3.4 Å) and Asp124 Oδ1 is hydrogen bonded to Asn140 Nδ2 (3.2 Å). In the future, it will be helpful to see a structure of this enzyme with a free active site; this may reveal the coordination geometry for the catalytic water. It is likely that the bound globomycin has displaced the catalytic water. If the c-region of the prolipoprotein substrate lays within the binding site of SPase II in a similar orientation to that of the cyclic peptide globomycin, then the structure suggests that the nucleophilic water attacks from the *si*-face of the substrate's scissile carbonyl. The hydroxyl oxygen of the serine within the bound globomycin sits adjacent to the two proposed catalytic carboxylate groups of Asp124 and Asp143. The hydroxyl oxygen is closer to Asp143 Oδ2 (2.4 Å) than it is to Asp 124 Oδ1 (2.9 Å). If the globomycin serine hydroxyl is mimicking one of the hydroxyls of the *gem*-diol transition state, then it is possible that the Asp143 is stabilizing the transition state via a low barrier hydrogen bond as has been proposed in some aspartic proteases (Wlodawer et al. 2013). A full stereo-electronic understanding of the SPase II mechanism awaits structural analysis at higher resolution.

## Other Signal Peptide Cleaving Enzymes

Although they have not been covered in this chapter, there are a number of other bacterial peptidases associated with protein secretion such as those involved in prepilin processing (Dupuy et al. 2013) and hydrolysis of signal peptides (Dalbey and Wang 2013).

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