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The structure and mechanism of bacterial type I signal peptidases A novel antibiotic target

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Abstract

Type I signal peptidases are essential membrane-bound serine proteases that function to cleave the amino-terminal signal peptide extension from proteins that are translocated across biological membranes. The bacterial signal peptidases are unique serine proteases that utilize a Ser/Lys catalytic dyad mechanism in place of the classical Ser/His/Asp catalytic triad mechanism. They represent a potential novel antibiotic target at the bacterial membrane surface. This review will discuss the bacterial signal peptidases that have been characterized to date, as well as putative signal peptidase sequences that have been recognized via bacterial genome sequencing. We review the investigations into the mechanism of *Escherichia coli* and *Bacillus subtilis* signal peptidase, and discuss the results in light of the recent crystal structure of the *E. coli* signal peptidase in complex with a β-lactam-type inhibitor. The proposed conserved structural features of Type I signal peptidases give additional insight into the mechanism of this unique enzyme.

Keywords: Signal peptidase; Leader peptidase; Signal peptide; Protein translocation: Serine protease; Antibiotic target

Abbreviations: DFP, diisopropyl fluorophosphate; ER, endoplasmic reticulum; FRET, fluorescence resonance energy transfer; Imp, mitochondrial inner membrane peptidase (the mitochondrial signal peptidases, e.g., Imp1 and Imp2); MBP, maltose-binding protein; PMSF, phenylmethylsulphonyl fluoride; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SPC, signal peptidase complex.

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1. Introduction

Bacterial proteins that are targeted to and translocated across the cytoplasmic membrane contain an amino-terminal peptide extension called the signal (or leader) peptide. The majority of protein translocation in bacteria occurs post-translationally via the Sec system (for a recent review, see Economou, 1999). The Sec system is made up of the proteins SecA, SecB, SecD, SecE, SecF, SecG, and SecY. Typically, the homotetramer SecB interacts with the newly synthesized preprotein in the cytoplasm and targets the protein to the SecAYEG-translocase at the membrane surface. The secretory preprotein then interacts with the membrane-associated homodimer SecA, which uses the energy from ATP hydrolysis to translocate the preprotein through the polytopic integral membrane protein channel thought to be formed from the components SecYEG (Fig. 1). Type I signal peptidases are membrane-bound endopeptidases that presumably are localized in close proximity to SecYEG. They are typically anchored to the membrane by amino-terminal transmembrane segments, and have a carboxy-terminal catalytic domain that resides on the outer surface of the cytoplasmic membrane. Type I signal peptidases function to cleave away the signal peptide from the translocated preprotein, thereby releasing secreted proteins from the membrane and allowing them to locate to their final destination in the periplasm, outer membrane, or extracellular milieu. Inhibition of bacterial Type I signal peptidase activity leads to an accumulation of secretory proteins in the cell membrane and eventual cell death (Koshland et al., 1982; Dalbey & Wickner, 1985; Kuhn & Wickner, 1985; Fikes & Bassford, 1987).

Bacterial Type I signal peptidase is an attractive target for the design of novel antimicrobial compounds for several reasons: it is an essential enzyme for the viability of the bacterium (Date, 1983; Cregg et al., 1996; Zhang et al., 1997; Klug et al., 1997) and its active site is relatively accessible on the outer leaflet of the cytoplasmic membrane (Wolfe et al., 1983a, 1983b; Moore & Miura, 1987; San Millan et al., 1989). Type I signal peptidases also have a Ser/Lys dyad mechanism that is unique and conserved among this family of enzymes in both gram-positive and gram-negative species (Sung & Dalbey, 1992; Black et al., 1992; Tschantz et al., 1993; Black, 1993; van Dijl et al., 1995; Paetzel et al., 1997; Dalbey et al., 1997). The majority of eukaryotic signal peptidases contain a conserved histidine in place of the conserved lysine general base. It is possible that the eukaryotic signal peptidases utilize the more classical Ser/His type of proteolytic mechanism (Dalbey et al., 1997). There are also significant cell localization and substrate specificity differences that distinguish the bacterial and higher order

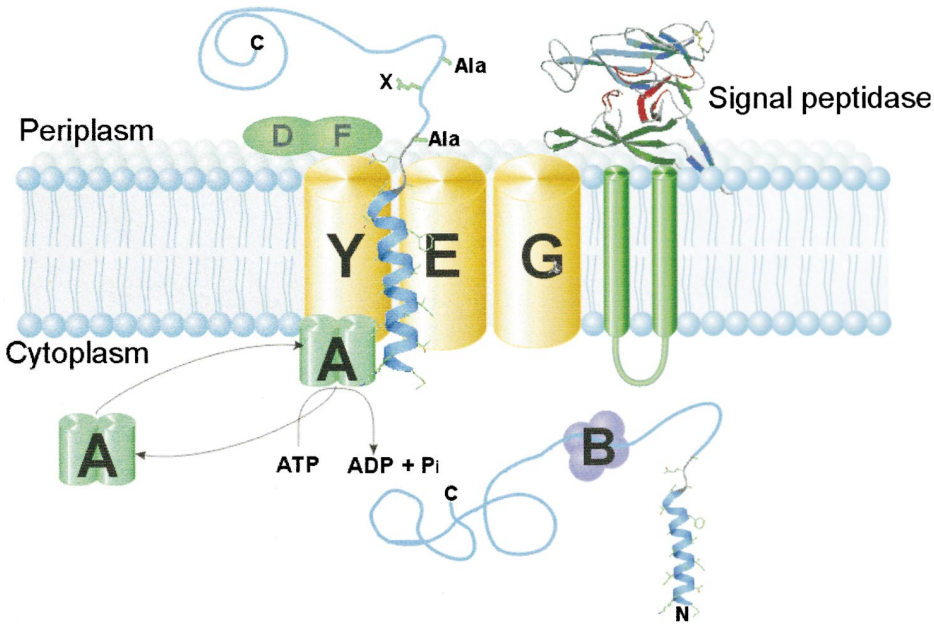

Fig. 1. The vast majority of bacterial secretory proteins are exported post-translationally across the cytoplasmic membrane via the Sec-dependent pathway. The preprotein is targeted to the cytoplasmic membrane surface with the assistance of the export chaperone SecB. SecA, an ATPase, drives the preprotein chain across the membrane through the SecYEG channel, using the energy of ATP hydrolysis. Once the preprotein is translocated across the membrane, the signal peptide is cleaved off by the Type I signal peptidase.

Type I signal peptidases (von Heijne, 1990). These differences support the choice of bacterial Type I signal peptidase as an attractive target for the development of novel antibiotic therapies.

It should be noted that there are three different types of bacterial signal peptidases. In addition to the Type I signal peptidase, there is a Type II signal peptidase that cleaves the signal peptides from lipid modified proteins and a Type III signal peptidase that specializes in the cleavage of the prepillin proteins. There is no sequence similarity among the three types of signal peptidases. Unlike the Type I signal peptidase, both Type II and Type III signal peptidases are not thought to be essential for bacterial viability. For an extensive review of the individual types of signal peptidases, see the comprehensive book by von Heijne (1994).

Type I signal peptidases (EC 3.4.21.89) are integral membrane serine endopeptidases. They belong to the serine protease family S26 (Rawlings & Barrett, 1994). Based on tertiary structure and conserved sequence motifs (Figs. 2 and 3), Type I signal peptidases have been classified into the evolutionary clan of serine proteases SF, which utilize a Ser/Lys catalytic dyad mechanism as opposed to the more common Ser/His/Asp catalytic triad mechanism (Table 1). To date, the majority of the work toward the understanding of the mechanism of bacterial Type I signal peptidases has been performed using the gram-negative *Escherichia coli* signal peptidase and the gram-positive *Bacillus subtilis* SipS. With the rapid increase in the number of putative signal peptidases being identified via the bacterial genome projects and the recent elucidation of the first crystal structure of a Type I signal peptidase (Paetzel et al., 1998), we are now more fully armed to investigate the potential of Type I signal peptidase as a universal and novel antibiotic target at the bacterial membrane surface.

2. Type I signal peptidases

2.1. Type I signal peptidases from gram-negative bacteria

Escherichia coli signal (leader) peptidase is by far the most thoroughly characterized Type I signal peptidase. *Escherichia coli* signal peptidase was the first to be cloned (Date & Wickner, 1981), sequenced (Wolfe et al., 1983a), overexpressed (Wolfe et al., 1982; Dalbey & Wickner, 1985), purified (Zwizinski & Wickner, 1980; Wolfe et al., 1982, 1983b; Tschantz & Dalbey, 1994), and biochemically (Sung & Dalbey, 1992; Tschantz et al., 1993; Chatterjee et al., 1995; Paetzel et al., 1997) and structurally characterized (Paetzel et al., 1998). Western blot analyses of *E. coli* cells have revealed that *E. coli* signal peptidase is constitutively expressed from the single-copy lepB gene, and each cell contains approximately 1000 Type I signal peptidase molecules (van Klompenburg et al., 1995).

A number of experiments have revealed that *E. coli* signal peptidase is essential for cell viability. When lepB gene expression is put under the control of the araB promoter (Dalbey & Wickner, 1985), or the natural promoter is partially deleted (Date, 1983), the limited expression of signal peptidase is associated with limited cell growth and division. The temperature-sensitive *E. coli* strain IT41, which has a mutation in the lepB gene, shows an accumulation of preproteins and a reduced growth rate when grown at the nonpermissive temperature of 42°C (Inada et al., 1989).

Typical of many gram-negative species, *E. coli* signal peptidase (323 amino acids, 35,988 Da) has two predicted amino-terminal transmembrane segments (residues 4–28 and 58–76), a small cytoplasmic domain (residues 29–57), and a large carboxy-terminal catalytic domain (residues 77–323) (Fig. 3). The *E. coli* signal peptidase membrane topology has been investigated both by proteolysis (Wolfe et al., 1983a; Moore & Miura, 1987) and by gene-fusion studies (San Millan et al., 1989). A model for the *E. coli* transmembrane domain has been proposed based on disulfide cross-linking studies (Whitley et al., 1993). This study suggests that aliphatic amino acids primarily form the interface between the two transmembrane segments and that the two helices pack against each other in a left-handed supercoil. Electrospray-mass spectrometry analysis has shown that the amino-terminus is blocked by a modification consistent with acetylation (Kuo et al., 1993).

The function of the small 28 amino acid cytoplasmic loop that links the two amino-terminal transmembrane segments currently is unknown. Early work with the *E. coli* Type I signal peptidase was made difficult by an autocatalytic degradation that occurred during the purification of the protein. It was shown by amino-terminal sequencing to occur between residues 40 and 41, which are predicted to lie in the small cytoplasmic domain of the enzyme (Talarico et al., 1991; Kuo et al., 1993) (Fig. 3). The protein is now most efficiently purified by replacing the residues in this region with a series of histidine residues and then utilizing nickel-affinity chromatography (Paetzel et al., 1997).

Deletion studies on the *E. coli* signal peptidase have shown that the first transmembrane segment and the cytoplasmic domain are not directly involved in catalysis (Bilgin et al., 1990). Kuo and co-workers (1993) were able to construct and purify a soluble, catalytically active fragment of *E. coli* signal peptidase that lacked both transmembrane segments and the small cytoplasmic segment. This construct, referred to as Δ2–75, was further characterized biochemically and kinetically (Tschantz et al., 1995) and then crystallized (Paetzel et al., 1995). It was found using both preprotein and peptide substrates that Δ2–75 required detergent or phospholipid for optimal activity, even though it lacks its transmembrane segments (Tschantz et al., 1995). It has been shown that Δ2–75 signal peptidase will bind to the inner and outer membranes of *E. coli*, as well as to vesicles composed of purified inner membrane lipids (van Klompenburg et al., 1998). Lipid surface tension experiments are consistent with the Δ2–75 penetrating into the lipid in a phosphatidylethanolamine-dependent fashion (van Klompen-

	#a.a.	MW	pI	TM	Box B	Box C	Box D	Box E
					88 95	127 134	142 153	272 282
Gram Negative								
* *Escherichia coli*	323	35,973	7.65	4-28, 58-76	SGSMMPTL	RGDIVVFK	DYIKRAVGLPGD	GDNRDNSADSR
* *Salmonella typhimurium*	324	35,778	6.01	4-28, 59-77	SGSMMPTL	RGDIVVFK	DYIKRAVGLPGD	GDNRDNSADSR
* *Pseudomonas fluorescens*	284	31,903	6.85	7-28, 58-76	SGSMKPTL	RGDVMVFR	NYIKRVVGLPGD	GDNRDNSNDSR
* *Haemophilus influenzae*	349	39,734	5.88	4-23, 31-52, 84-105	SGSMESTL	RGDVIVFK	DYIKRIVGKGGD	GDHRDHSDDSR
Helicobacter pylori	290	33,710	8.36	10-34	SRSMVGTL	RGEVVVFI	YYVKRNFAIGGD	GDNRDNSSDSR
* *Bradyrhizobium japonicum* SipF	254	28,831	6.91	16-35	SGSMKATL	RGDIVVFR	DYIKRVIGLPGD	GDNRDNSTDSR
* *Bradyrhizobium japonicum* SipS	259	27,614	9.84	ND	SGSMEPTL	QGDVVVFR	AWVKRVVGLPGD	GDNRDNSADSR
Borrelia burgdorferi 1	211	25,043	9.89	19-43, 57-81, 112-132, 187-207	SNEMLPTI	MNDIVLYE	YKVSRIAAVQGD	NDNLSVLNDSR
Borrelia burgdorferi 2	326	38,481	8.89	5-23,39-58	SGSMENTL	ESDIIIFE	FLVKRGAFADGK	GDNRDNSHDGR
Borrelia burgdorferi 3	168	19,837	9.64	11-34	GESMTPAI	QKYLLLWK	IAIKKIFAIPGE	GENKQISLDSR
Treponema pallidum 1	235	26,880	9.56	16-37	ADSMQPTL	RGDLVLAT	GLFKRAMNAVLG	CDNRIVSSDSR
Treponema pallidum 2	512	57,619	9.22	2-23, 53-77	SESMVPSF	RGDIVVFS	PLVKRIVALPGE	GLDTGQEGPSL
Thermotoga maritima	306	36,082	6.48	13-34	TGSMIPTI	IGEIVVFW	KYVKRLVGKGGD	GDNTKESLDCR
* *Phormidium laminosum*	203	22,485	5.64	ND	SESMLPTL	RGDIIVFH	AFIKRVIGLPGE	GDNRNNSYDSH
* *Azotobacter vinelandii*	284	32,039	6.54	7-28, 59-77	SGSMKPTL	RGDVMVFR	NYIKRVVGLPGD	GDNRDNSNDSR
Rickettsia prowazekii	264	30,870	8.73	16-34	TGSMKATI	RGDIVVFR	RYIKRLIGLPGD	GDNRDRSNDSR
* *Rhodobacter capsulatus*	265	28,878	7.66	15-36	SGSMKDTL	RGDVVVFR	DFIKRLIGLPGD	GDNRDNSEDSR
Gram Positive								
* *Bacillus subtilis* SipS	184	21,047	9.35	15-39	GDSMYPTL	RGDIVVLN	HYVKRIIGLPGD	GDNRRNSMDSR
* *Bacillus subtilis* SipT	193	21,854	9.46	16-37	GSSMYPTL	RGDIVIIN	HYVKRLIGKPGE	GDNRLNSMDSR
* *Bacillus subtilis* SipP (pTA1015)	186	21,251	9.41	11-30	GKSMDPTL	RGDIIILN	HYVKRLIGLPGD	GDNRQESMDSR
* *Bacillus subtilis* SipP (pTA1040)	185	21,568	9.34	15-34	GESMKPTL	RGDIVVLN	HYVKRLIGLPGD	GDNRQNSMDSR
* *Bacillus subtilis* SipU	187	21,182	9.43	16-36	GSSMAPTL	RGDIIVIH	SFVKRLIGLPGD	GDNRLNSLDSR
* *Bacillus subtilis* SipV	168	18,956	9.71	5-26	GVSMNPTF	RFDIVLFK	VLIKRVIGLPGE	GDNRIYSFDSR
* *Bacillus subtilis* SipW	190	20,678	5.59	3-24, 144-165	SGSMEPEF	KGDVITFM	AVTHRIVDITKQ	GDPGVMVYAFV
Bacillus amyloliquefaciens SipS1	185	21,104	9.63	12-31	GESMEPTL	RGQIVVLN	HYVKRIIGLPGD	GDNRRNSMDSR
Bacillus amyloliquefaciens SipS2	193	21,860	9.51	16-37	GSSMYPTL	RGDIVIIN	HYVKRLIGKPGE	GDNRLNSMDSR
Bacillus licheniformis	186	21,145	8.63	16-35	GTSMDPTL	RGDIVIID	HYVKRLIGLPGD	GDNRQRSMDSR
Bacillus caldolyticus	182	21,263	9.84	12-34	GKSMMPTL	RFDIIVFH	DYVKRVIGLPGD	GDNRLSSWDSR
Staphylococcus aureus SpsA	174	20,146	9.83	7-28	NNDMSPTL	NGDIITYR	IYTSRIIAKPGQ	NDHDNNQHDSR
* *Staphylococcus aureus* SpsB	191	21,691	9.02	8-27	GESMDPTL	KGNVVVFH	DYVKRVIGVPGD	GDNREVSKDSR
Staphylococcus carnosus sipA	174	19,931	9.72	7-31	NNEMSPTL	NGDVVMYK	TYFGRVIGLPGQ	NDNRANQSDTR
Staphylococcus carnosus sipB	189	21,431	9.33	8-27	GDSMYPTL	KGNVIVFH	DYVKRVIGMPGD	GDNREVSKDSR
* *Streptococcus pneumoniae* Spi	204	23,497	5.84	11-29	GHSMDPTL	RFDIVVAH	DIVKRVIGMPGD	GDDRLVSSDSR
Streptomyces coelicolorgi	148	16,069	11.67	ND	GPSMVPTL	PGDVVVLR	LVVKRAAERRGA	GDNAFAGGDST
Streptococcus mitis				ND, (partial sequence)	GHSMDPTL	RFDIVVAH	DIVKRVIGMPGD	GDDRLVSSDSR
* *Streptomyces lividans* SipX	320	34,548	9.25	57-81	SGSMEQTI	RGDVVVFR	DLIKRVVGVGGD	GDHRSNSADSR
* *Streptomyces lividans* SipZ	258	26,536	9.73	31-52, 219-240	TSSMTPTI	RGDVVVFK	PMVKRVVAVGGD	GDERRNSVDST
* *Streptomyces lividans* SipW	259	27,662	4.72	49-68	SGSMERGL	RGDIVVFD	DYIKRVVGVGGD	GDHRSDSSDSR
* *Streptomyces lividans* SipY	291	31,470	5.52	41-62, 259-280	SSSMENTL	RGEVVVFH	DLIKRVIGVAGD	GDHRQNSRDSR
Streptomyces coelicolor 1	258	26,550	9.73	31-52, 219-240	TSSMTPTI	RGDVVVFK	PMVKRVVAVGGD	GDERRNSVDST
Streptomyces coelicolor 2	336	35,865	5.28	86-107, 304-325	SSSMENTL	RGEVVVFH	DLIKRVIGVAGD	GDHRQNSRDSR
Streptomyces coelicolor 3	446	49,018	11.11	57-81, 291-310	SGSMEQTI	RGDVVVFR	DLIKRVVGVGGD	GDHRSNSADSR
Streptomyces coelicolor 4	259	27,662	4.72	49-68	SGSMERGL	RGDIVVFD	DYIKRVVGVGGD	GDHRSDSSDSR
Aquifex aeolicus	256	29,676	6.02	8-28	SASMEPTL	RGDMIVFK	DFIKRIIARGGD	GDNRDNSQDSR
Chlamydia trachomatis	628	71,504	8.41	81-99, 602-620	TGSMRPTI	RGELVVFT	RYIKRCMGKPGD	GDNCPMSADSR
Chlamydia pneumoniae	636	72,507	9.17	80-99, 600-619	TGSMRPTI	RGGLVVFT	RYIKRCMGRPGD	GDNYPMSADSR
Mycobacterium leprae	289	31,086	5.67	56-75	SESMEPTL	PGDVIVFK	DLVKRVIAVGGQ	GDNRIHSADSR
Mycobacterium tuberculosis	294	31,880	5.63	68-87	SESMEPTL	PGDVIVFR	DLVKRVIAVGGQ	GDNRTHSADSR
Synechocystis sp PCC6803 1	196	22,230	5.67	16-35	SDSMLPTL	VGDIIVFH	AFIKRVIALPGQ	GDNRNNSNDSH
Synechocystis sp PCC6803 2	218	24,733	5.41	ND	SSSMEPTL	RGEIVVFN	AFIKRIIGLPGD	GDNRNNSYDSH
Mitochondrial								
* *Saccharomyces cerevisiae* IMP1	190	21,433	7.01	2-26	GESMLPTL	MGDCIVAL	RICKRVTGMPGD	GDNLSHSLDSR
* *Saccharomyces cerevisiae* IMP2	177	19,930	10.01	14-35	GTSMQPTL	RDDIILFK	VYCKRVKGLPFD	GDNYFHSIDSN
Schizosaccharomyces pombe IMP2	157	17,269	8.63	2-21	GPSMMPTL	VGDVVVSA	HVCKRIIGMPGD	GDNIAHSLDSR
Thylakoid								
* *Arabidopsis thalia 1*	340	37,853	6.03	ND	STSMYPTL	VSDIVIFK	VFIKRIVASEGD	GDNRNKSFDSH
Arabidopsis thalia 2	250	27,909	4.85	ND	STSMYPTL	VSDIVIFK	VFIKRIVASEGD	GDNRNKSFDSH
Arabidopsis thalia 3	313	34,515	7.1	16-40	GPSMIPTL	RGDIVVIR	TPIKRVVGVEGD	GDYTHNSRDSR
Archaea								
Pyroccus horikoshii	154	17,184	6.42	8-29	SGSMEPVF	VGDVIVYR	PIIHRVRGIKYI	GDHNPVPDIYY
Methanococcus jannaschii	203	23,724	6.51	2-23, 174-195	SDSMYPIM	VGDIVVYK	PVIHRVIDKVEF	GDNNPIHDPEL
Methanobacterium thermoautotrophicum	144	16,164	4.95	10-31	SGSMEPVF	KGDIIIYD	PVIHRVIGVETD	GDNNQDPDPAP
Pyroccus abyssi 1	327	36,503	9.3	6-25, 135-153, 169-189, 291-310	SDSMTPTI	VGDIIVFN	WTVHRVYAITES	GDNNVATDQQD
Pyroccus abyssi 2	155	17,110	9.23	8-29	SGSMRPVF	VGDVIVYK	PIIHRVRGIKQV	GDNNPVPDLYE
Archaeoglobus fulgidus 1	151	16,534	6.59	5-26, 120-141	GTSMLPEL	TGDLILIF	LITHRVVEKTSE	GDNLPREDEPV
Archaeoglobus fulgidus 2	189	20,618	5.58	14-38	SGSMEPHL	YGDVIVYK	PIIHRAIAYVHK	GDNVRTNQLPD
Archaeoglobus fulgidus 3	290	32,667	9.1	3-22, 129-148, 180-198, 248-272	SSSMEPLM	LGDVVAFK	LITHRVVEIGDG	GDAVEDVDPFD
ER								
Human SPC18	167	19,131	9.58	6-30, 139-163	SGSMEPAF	VGEIVVFR	PIVHRVLKIHEK	GDNNAVDDRGL
* *Dog* SPC18	179	20,625	9.48	18-42	SGSMEPAF	VGEIVVFR	PIVHRVLKIHEK	GDNNAVDDRGL
* *Dog* SPC21	191	21,600	9.22	4-22, 34-55, 164-188	SGSMEPAF	AGEIVVFK	PIVHRVIKVHEK	GDNNEVDDRGL
Rat SPC18	179	20,599	9.15	18-42, 151-175	SGSMEPAF	VGEIVVFR	PIVHRVLKIHEK	GDNNAVDDRGL
* *Saccharomyces cerevisiae* Sec11	167	18,762	9.19	10-34	SGSMEPAF	VGDVVVYE	PIVHRVLRQHNN	GDNNAGNDISL

Fig. 2. The regions (or boxes) of conserved residues in Type I signal peptidases. There is limited sequence identity between Type I signal peptidases, except in the regions of sequence referred to as boxes A–E (box A is not shown). The isoelectric point (p*I*) and molecular weight (MW) were calculated using the Compute pI/MW tool at http://www.expasy.ch/tools/pi_tool.html. The transmembrane segments (TM) were predicted using the program HMMTOP (Tusnady & Simon, 1998). ND; no transmembrane segment detected using the HMMTOP program. The sequences marked by an asterisk have been shown to encode a protein with signal peptidase activity. The putative Type I signal peptidase sequences (not marked by asterisks) are available via the tigr web-site at http://www.tigr.org/.

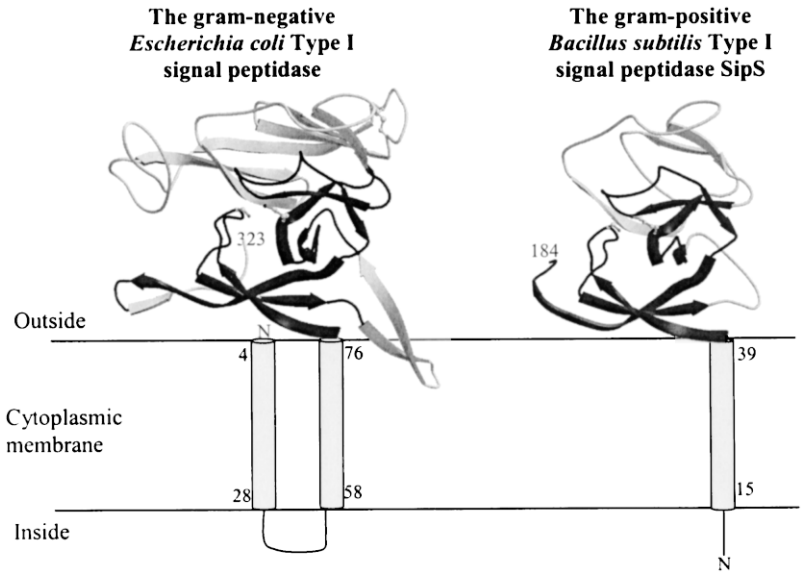

Fig. 3. The membrane topology of the gram-negative *E. coli* Type I signal peptidase and the gram-positive *Bacillus subtilis* Type I signal peptidase SipS. The majority of gram-negative signal peptidases have multiple transmembrane segments. Most gram-positive signal peptidases are smaller and have a single transmembrane segment. The periplasmic region of all gram-negative signal peptidases contains a β-sheet Domain I and II. Domain I contains all of the conserved boxes of sequence and the catalytic and binding-site residues. Domain II and the extended β-ribbon are smaller in the gram-positive signal peptidases. The orientation of the catalytic domain relative to the plan of the lipid bilayer is proposed based on the location of the catalytic residues, as well as the large exposed hydrophobic surface (Paetzel et al., 1998). The gram-positive *Bacillus subtilis* SipS structure was modeled based on the *E. coli* signal peptidase crystal structure (Paetzel et al., 1998).

burg et al., 1998). It should be noted that all in vitro studies to date are consistent with bacterial Type I signal peptidases acting as monomeric enzymes and requiring no additional proteins or cofactors for catalytic activity.

Many other Type I signal peptidases from gram-negative species have been cloned, expressed in *E. coli*, and shown to have signal peptidase activity by genetically complementing the temperature-sensitive *E. coli* strain IT41. This has become a standard method for confirming that putative Type I signal peptidase genes impart Type I signal peptidase activity when expressed. The *E. coli* strain IT41 contains a mutation in the gene for Type I signal peptidase (lepB), which gives a temperature-sensitive phenotype (Inada et al., 1989). The strain shows normal growth at 32°C, but slow growth at 42°C. Transformation of this strain with a plasmid containing a Type I signal peptidase complements the mutant lepB gene, allowing growth at the elevated temperature. The nature of the mutation in the lepB gene has been shown to be a nonsense mutation at codon 39 (Cregg et al., 1996). Apparently, at the permissive temperature, there is read-through that is not allowed at the elevated temperature. The studies that have utilized this method for demonstrating Type I signal peptidase activity in gram-negative bacteria include those for the signal peptidase from *Pseudomonas fluorescens* (Black et al., 1992), the thermophilic cyanobacterium *Phormidium laminosum* (Packer et al., 1995), *Azotobacter vinelandii* (Jock et al., 1997), and *Bradyrhizobium japonicum* SipS/SipF (Bairl & Muller, 1998). In a similar experiment, the signal peptidase from *Salmonella typhimurium* was cloned, expressed, and was demonstrated to display Type I signal peptidase activity by using a second temperature-sensitive *E. coli* mutant where the expression of signal peptidase is repressed at 28°C (N4156::pGD28) (van Dijl et al., 1990). The Type I signal peptidase from *Rhodobacter capsulatus* has also been cloned and expressed (Klug et al., 1997). The inability to culture an *R. capsulatus* strain with a disrupted lepB gene is consistent with Type I signal peptidase being essential for the viability of the *R. capsulatus* cell (Klug et al., 1997).

In gram-negative bacteria, such as *E. coli*, *Pseudomonas fluorescens*, *Salmonella typhimurium*, *R. capsulatus*, and *Haemophilus influenzae*, there is only one identifiable Type I signal peptidase. Recently, it has been shown that *Bradyrhizobium japonicum* (Muller et al., 1995; Bairl & Muller, 1998) has two functional and distinct Type I signal peptidases (SipS and SipF). Mutant phenotype analysis suggests that the SipS and SipF exhibit different substrate specificity (Bairl & Muller, 1998). This would be similar to the situation with the mitochondrial inner membrane protease (Imp1 and Imp2) from yeast mitochondria (Nunnari et al., 1993). From an analysis of newly emerging bacterial genome sequences, it appears that there are likely several examples of multiple Type I signal peptidase genes in a single gram-negative species. For example, *Borrelia burgdorferi* has three putative Type I signal peptidases and *Treponema pallidum* and a *Synechocystis* sp. (PCC 6803) have two each (Fig. 2).

Table 1
Evolutionary clans of serine proteases

Clan	Catalytic residues	Example
SA	Ser/His/Asp	Chymotrypsin (PDB: 2cga) 
SB	Ser/His/Asp	Subtilisin (PDB: 2st1) 
SC	Ser/His/Asp	Carboxypeptidase C (PDB: lysc) 
SE	Ser/Lys	D-Ala-D-Ala transpeptidase (PDB: 3pte) 
SF	Ser/Lys	Type I signal peptidase (PDB: 1b12) 
SH	Ser/His/His	Cytomegalovirus protease (PDB: 1 cmv) 

2.2. Type I signal peptidases from gram-positive bacteria

As in the gram-negative species, Type I signal peptidases from gram-positive bacteria appear to be monomeric enzymes containing an amino-terminal transmembrane anchor and a carboxy-terminal catalytic domain. However, typically, the gram-positives have only a single transmembrane segment and a short amino-terminal cytoplasmic tail. The catalytic domain is also significantly smaller in size compared with the gram-negative species (Fig. 2).

In gram-positive bacteria, the occurrence of multiple Type I signal peptidases within a single species is very common. By far, the most thoroughly characterized gram-positive Type I signal peptidases are those from *Bacillus subtilis. Bacillus subtilis* contains seven Type I signal peptidases (five chromosomal [SipS, SipT, SipU, SipV, and SipW] and two plasmid encoded [SipP pTA1015 and SipP pTA1040]) (Meijer et al., 1995; Tjalsma et al., 1997, 1998). The *Bacillus subtilis* signal peptidases are all approximately the same size, ranging from 18,956 to 21,854 Da. They all have one proposed transmembrane segment, except for SipW, which has a second predicted transmembrane segment at its carboxy-terminus (Tjalsma et al., 1998). SipW is more like the eukaryotic endoplasmic reticulum (ER) or archaea bacterial signal peptidase in that it has a histidine residue replacing the conserved lysine general-base (see Section 2.3). This makes *Bacillus subtilis* the only known organism to contain in the same membrane signal peptidases with lysine and histidine general bases (Tjalsma et al, 1998). Using temperature-sensitive SipS variants, van Dijl and co-workers (Tjalsma et al., 1997) have been able to show that only strains of *Bacillus subtilis* lacking both SipS and SipT are nonviable. They proposed that the functionally redundant signal peptidases may provide a growth advantage for this gram-positive eubacterium, which secretes large amounts of protein. Two different Type I signal peptidases have been cloned and sequenced from the related species *Bacillus amyloliquefaciens* (Hoang & Hofemeister, 1995).

Two of the most notoriously antibiotic-resistant pathogenic "superbugs" are the gram-positive species *Streptococcus pneumoniae* and *Staphylococcus aureus.* Considerable resources worldwide are being expended to develop new families of antibiotics against these clinical pathogens (Neu, 1992). The Type I signal peptidase from *Streptococcus pneumoniae* (spi) has been cloned and overexpressed in *E. coli.* It was shown to be essential for the viability of *Streptococcus pneumoniae.* Introduction of the spi gene into the previously described temperature-sensitive *E. coli* strain (IT41) allowed normal growth rates at elevated temperatures, indicating that spi has signal peptidase I activity (Zhang et al., 1997). The Type I signal peptidase from *Staphylococcus aureus* (spsB) has also been cloned, expressed in *E. coli*, and shown to cleave *E. coli* preproteins in vivo (Cregg et al., 1996). Use of a temperature-sensitive plasmid has revealed that spsB is essential and contains the only Type I signal peptidase activity in *Staphylococcus aureus* (Cregg et al., 1996). *Staphylococcus aureus* contains a second open reading frame (spsA), just 15 nucleotides upstream of spsB, that has 62% similarity and 31% identity to spsB. Interestingly, sequence alignments indicate that the spsA gene encodes a protein that does not contain the essential active-site Ser and Lys (Cregg et al., 1996). Newly sequenced bacterial genomes indicate that there are likely more examples of these proteins with high sequence similarity to the Type I signal peptidases, except for the catalytic residues. For example, *Borrelia burgdorferi*, the Lyme disease spirochete, and *Staphylococcus carnosus* (sipA) contain such genes (Fig. 2). It is not clear what function these proteins play in the bacterial cell.

Recently, four genes encoding distinct Type I signal peptidases have been cloned from *Streptomyces lividans* TK21 (Schacht et al., 1998; Parro & Mellado, 1998; Parro et al.,

1999). All four genes (sipW, sipX, sipY, and sipZ) are clustered such that three of them constitute an operon and the fourth is the first gene of another operon. Three of the four (sipX, sipY, and sipZ) have been reported to contain a putative carboxy-terminal transmembrane segment in addition to the amino-terminal transmembrane segment. Using the program HMMTOP (Tusnady & Simon, 1998), we detected a putative carboxy-terminal transmembrane segment for only SipY and SipZ (Fig. 2). Preliminary studies suggest that all four genes may be essential for cell viability (Parro et al., 1999). Only when the four genes are expressed together in *E. coli* IT41 are they able to compensate for the temperature-sensitive *E. coli* signal peptidase mutation (Parro et al., 1999). *Streptomyces lividans* Type I signal peptidases will be unique amongst bacterial Type I signal peptidases if further experimental analysis confirms that all four of the sip genes are required for Type I signal peptidase activity.

2.3. Type I signal peptidases from higher order species

The Type I signal peptidases from the eukaryotic ER belong to the serine protease family S27 and possess a conserved histidine in place of the proposed lysine general base found in the bacterial Type I signal peptidases of the family S26 (Rawlings & Barrett, 1994). Unlike the "stand-alone" bacterial Type I signal peptidases, the active sites of the eukaryotic Type I signal peptidases are sequestered inside the lumen of the ER, and exist as a hetero-oligomeric membrane protein complex known as the signal peptidase complex (SPC). For example, in the yeast *Saccharomyces cerevisiae*, the SPC is composed of four proteins (Sec11, SPC1, SPC2, and SPC3) (YaDeau & Blobel, 1989). Both Sec11 and SPC3 are required for optimal signal peptidase activity, but only Sec11 contains the conserved Ser/His catalytic residues (Fang et al., 1996, 1997; Meyer & Hartmann, 1997). It has been shown that SPC1 and SPC2 are nonessential for Type I signal peptidase activity (Mullins et al., 1996). The mammalian SPC consists of five polypeptide chains (SPC18, SPC21, SPC 12, SPC25, and SPC22/23) (Evans et al., 1986; Greenburg & Blobel, 1994; Kalies & Hartman, 1996; Lively et al., 1994). The proteins SPC18 and SPC21 are homologs to the yeast Sec11 protein and have active-site residues. The function of the proteins SPC 12, 25, and 22/23 as of yet are unknown. The proteins SPC18, 21, and 22/23 are single transmembrane proteins that are exposed to the luminal side of the ER (Shelness et al., 1993), whereas the SPC12 and SPC25 span the membrane twice, with the majority of the protein facing the cytosol rather than the ER lumen, suggesting that they may play some role other than catalytic (Kalies & Hartmann, 1996). In contrast, based on sequence similarity and substrate specificity, the mitochondrial inner membrane peptidases (Imp1 and Imp2; Behrens et al., 1991; Nunnari et al., 1993; Schneider et al., 1994) and the thylakoidal-processing peptidase (Halpin et al., 1989; Chaal et al., 1998) appear to be more related to the bacterial Type I signal peptidases. This is consistent with the endosymbiont hypothesis, which states that the mitochondria and chloroplast organelles evolved from bacteria (Cavalier-Smith, 1987).

2.4. Regions of sequence similarity and identity (boxes B–E)

Using standard multiple-sequence alignment algorithms, it is possible to identify five regions of high-sequence similarity and identity in Type I signal peptidases from bacteria to human. These regions of sequence are referred to as boxes A–E (Dalbey et al., 1997). Box A corresponds to the transmembrane segment, and boxes B–E all reside in the carboxy-terminal catalytic domain. Fig. 2 shows the alignment of the conserved regions of sequence (boxes B–E) for the Type I signal peptidase sequences and putative signal peptidase sequences presently available. Box B (residues 88–95, *E. coli* numbering) contains the nucleophilic Ser90 and a conserved Met91. Box C contains residues 127–134, and box D (residues 142–153) contains the proposed general-base Lys145 and a conserved Arg146. Box E (residues 272–282) contains the highly conserved Gly272, Asp273, and Asn274, as well as Asp280 and Arg282. The precise structural details of the conserved boxes will be discussed in Section 4.4 in light of the recently solved crystal structure of the *E. coli* Type I signal peptidase. It has revealed that boxes B–E all lie near the signal peptidase active site and that they contribute to what is predicted to be a conserved catalytic Type I signal peptidase protein fold.

3. Mechanistic studies of Type I signal peptidases

3.1. The signal peptide

Although signal peptides do not show a great deal of sequence homology, they do contain three recognizable domains. They have a positively charged amino-terminus (n-region, 1–5 residues in length), a central hydrophobic domain (h-region, 7–15 residues in length), and a neutral, but polar, C-terminal domain (c-region, 3–7 residues in length) (Fig. 4). The n-region and h-region are required for efficient translocation, whereas the c-region specifies the signal peptide cleavage site (von Heijne, 1990). The signal peptides from gram-positive bacteria are significantly longer then those from other organisms, and they have a much longer h-region (von Heijne & Abrahmsen, 1989). The average eukaryotic signal peptide is 22.6 amino acids in length, the average gram-negative signal peptide is 25.1 amino acids in length, and the average gram-positive signal peptide contains 32.0 amino acids (Nielsen et al., 1997a, 1997b). The gram-positive signal peptides also in general have an amino-terminus containing more positively charged lysine and arginine residues (Edman et al., 1999). The eukaryotic signal peptides have fewer lysine residues in their n-region, and have a shorter h-region dominated by the presence of leucine residues (Nielsen et al., 1997a, 1997b). Statistical analysis of the amino acid sequences surrounding signal peptide cleav-

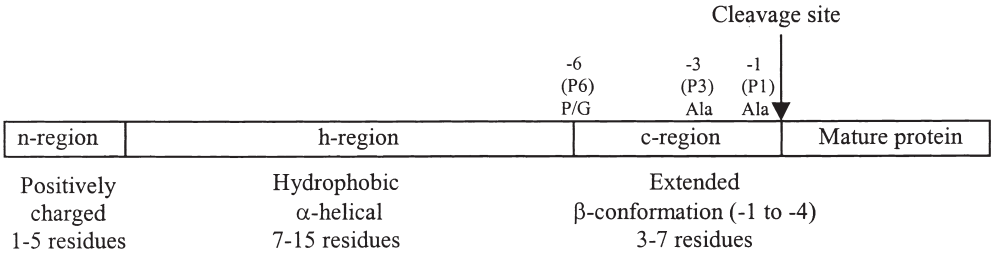

Fig. 4. The features of a typical bacterial signal peptide. Signal peptides have a positively charged amino-terminus (n-region), a hydrophobic central region (h-region), and a neutral, but polar, carboxy-terminus (c-region). The boundary between the h-region and the c-region is usually marked by a helix-breaking residue (Pro or Gly) at the −6 (P6) position relative to the cleavage site. The cleavage recognition sequence consists of small residues at the −1 (P1) and −3 (P3) positions relative to the cleavage site. By far, the most common residue at these positions is Ala.

age sites has led to the so-called (−3, −1) rule that states that the residues at the −3 and −1 positions relative to the cleavage site must be small and neutral for cleavage to occur (von Heijne, 1983, 1985; Perlman & Halvorson, 1983). Note that the −3 and −1 residues are synonymous with the P3 and P1 residues in the Schechter and Berger (1967) nomenclature. The residues at the −3 and −1 positions are most commonly Ala; therefore, the signal peptide cleavage-site specificity is sometimes called the (Ala-X-Ala) rule. The eukaryotic signal peptides show a less stringent requirement for Ala at the −3 and −1 positions, accepting Gly and Ser almost as frequently as Ala at the −1 position and Val, Ser, and Thr almost as frequently as Ala at the −3 position (Nielsen et al., 1997a, 1997b).

In addition to these statistical analyses, the substrate specificity or preference of Type I signal peptidase has been studied extensively in vitro and in vivo (Watts et al., 1983; Koshland et al., 1982; Dierstein & Wickner, 1986; Folz et al., 1988; Nothwehr & Gordon, 1989, 1990; Fikes et al., 1990; Kuhn & Wickner, 1985; Shen et al., 1991; Ryan & Edwards, 1995; Pratap & Dikshit, 1998; Karamyshev et al., 1998; Wrede et al., 1998). Using synthetic peptides based on the signal peptide cleavage site of pre-maltose-binding protein (MBP), Dev and co-workers (1990) found that the minimal segment of preprotein that can be cleaved by *E. coli* Type I signal peptidase is the −3 to +2 region (−3-Ala-Leu-Ala-/-Lys-Ile-+2). Using site-directed mutagenesis and both in vitro and in vivo Type I signal peptidase assays, Shen et al. (1991) investigated the limits of sequence variation tolerated for the processing of the M13 procoat protein by the *E. coli* Type I signal peptidase. They found that almost any residue is allowed at the +1, −2, −4, and −5 positions, whereas only the residues Ala, Ser, Gly, or Pro at the −1 position and Ser, Gly, Thr, Val, or Leu at the −3 position allowed processing of the M13 procoat protein. A recent mutagenesis study using the *E. coli* alkaline phosphatase preprotein revealed that large amino acids at the −2 position and middle-sized residues at the −5 position allowed for the most efficient processing by the *E. coli* signal peptidase (Karamyshev et al., 1998). These results are consistent with the statistical analysis of signal peptide cleavage-site sequences (Nielsen et al., 1997a, 1997b). The −6 position of the signal peptide is most often occupied by a Pro, Gly, or Ser residue, and it has been suggested that this position defines the transition from the h-region to the c-region (von Heijne, 1990). Shen et al. (1991) found that the M13 procoat will only be processed efficiently if there is a proline at the −6 position. Conversely, a proline residue at the +1 position relative to the cleavage site in pre-MBP prevents cleavage of the signal peptide; this construct was also shown to be an effective inhibitor of *E. coli* Type I signal peptidase (Barkocy-Gallagher & Bassford, 1992). It has been shown that a +1 proline has a similar effect in both Sec-dependent and Sec-independent preproteins (Nilsson & von Heijne, 1992). Prolines at the +2 or +3 position relative to the cleavage site have also been shown to negatively affect the efficiency of signal peptide cleavage (Hegner et al., 1992).

A number of studies have focused on the structure, orientation, and interactions of signal peptides in lipid bilayers or membrane mimetic environments (Cornell et al., 1989; Bruch et al., 1989; Jones et al., 1990; McKnight et al., 1991a, 1991b; Wang et al., 1993; Rizo et al., 1993; Jones & Gierasch, 1994; Bechinger et al., 1996; Keller et al., 1996; Voglino et al., 1998, 1999). Most studies to date are consistent with the central h-region adopting an α-helical conformation when in a lipid or hydrophobic environment (McKnight et al., 1989; Chupin et al., 1995; Voglino et al., 1998; Batenburg et al., 1988; Rizo et al., 1993; Wang et al., 1993). The boundary between the h-region and the c-region (−6 to −4), which often contains proline or glycine residues, has been suggested to have a β-turn structure (Rosenblatt et al., 1980; Perlman & Halvorson, 1983). The recent conformational, statistical, and mutational analysis by Karamyshev and co-workers (1998) is consistent with the signal peptide having an extended β-conformation in the −5 to −1 region, while bound to the signal peptidase-binding pocket. In addition, NMR (Killian et al., 1990) and ESR (Sankaram et al., 1994) experiments provide evidence for signal peptides inducing nonbilayer lipid structure (de Vrije et al., 1990). This may be a possible explanation for the processing of signal peptides with rather short h-regions. To date, there is no direct experimental evidence to confirm whether the bacterial signal peptide is associated with lipid

and/or the proteins of the secretion machinery (translocase) during the cleavage event. It is also presently unclear whether bacterial signal peptidases cleave the signal peptide during or after translocation.

There are a number of programs available for the identification of signal peptides and the prediction of their cleavage sites. The weighted matrix method of identifying signal peptides and predicting the location of the signal peptidase cleavage sites is commonly used (von Heijne, 1986). Recently, a neural network approach to identifying signal peptides and predicting their cleavage sites has also been developed (Nielsen et al., 1997a, 1997b). The network ensembles were trained on separate data sets from gram-positive, gram-negative, and eukaryotic organisms. This program (SignalP) shows a significant improvement in performance over the weighted matrix method and is publicly available (http://www.cbs.dtu.dk/services/SignalP/). The SignalP program has been further improved by including a hidden Markov model, making it possible to discriminate between cleaved signal peptides and uncleaved signal anchors that mediate the targeting to and translocation of membrane proteins across the cytoplasmic membrane (Nielsen et al., 1999).

3.2. Type I signal peptidase assays

Development of Type I signal peptidase assays was instrumental in the initial purification and characterization of signal peptidase activity (Chang et al., 1978; Zwizinski & Wickner, 1980; Jackson, 1983). Some of these initial assays, as well as the more recently developed signal peptidase assays, are listed in Table 2. It has been observed that the in vivo assay can be a more sensitive monitor of Type I signal peptidase activity than the available in vitro assays (Sung & Dalbey, 1992), and caution should be used when interpreting the in vitro results.

Although a number of synthetic peptide assays have been developed that were helpful in determining the Type I signal peptidase substrate specificity (Caulfield et al., 1988, 1989; Dev et al., 1990), they tend to be rather poor substrates in comparison with full-length preprotein substrates. The Type I signal peptidase substrate that gives the best catalytic constants to date is the pro-OmpA nuclease A (see Tables 2 and 3 in Chatterjee et al., 1995). It is a hybrid protein of the *Staphylococcus aureus* nuclease A attached to the signal peptide of the *E. coli* outer membrane protein A (OmpA). Using this substrate, Suciu et al. (1997) have estimated the activation energy of *E. coli* signal peptidase to be 10.4 ± 0.6 kcal/mol, which indicates that it is catalytically as efficient as the traditional Ser/His/Asp serine proteases. Also using the pro-OmpA nuclease A substrate, Paetzel and co-workers (1997) measured the pH-rate profile of *E. coli* signal peptidase, revealing a maximum efficiency at pH 9.0 and apparent pK_a values for titratable groups at ~8.7 and 9.3. Using the pro-OmpA nuclease A substrate at pH 9.0 and in the presence of the detergent Triton X-100, the purified wild-type *E. coli* signal peptidase gave the catalytic constants $k_{cat} = 120.0 \pm 10.7$, $K_m = 10.9 \pm 2.8$ mM, $k_{cat}/K_m = 1.1 (\pm 0.2) \times 10^7$ $sec^{-1}M^{-1}$. Although an excellent substrate, this assay requires sodium dodecyl sulphate-poly-

Table 2
Type I signal peptidase assays

Assay	Analysis method	Reference
In vivo		
Pro-OmpA expressed in the temperature-sensitive *E. coli* strain IT41	SDS-PAGE/immunoprecipitation/ fluorography	Bilgin et al., 1990; Sung and Dalbey, 1992; Tschantz et al., 1993
Complementation of temperature-sensitive *E. coli* strain IT41	Cell growth	Inada et al., 1989; Black et al., 1992; Black, 1993; Kim et al., 1995b
Pre(A13i)-β-lactamase plate assay	β-Lactamase activity	Van Dijl et al., 1992; Tjalsma et al., 1998; Meijer et al., 1995
Radiolabeled hybrid precursor pre(A13I)-β-lactamase specified by plasmid pGDL48. [Note: *Bacillus subtilis* signal peptidases will cleave pre(A13i)-β-lactamase, but *E. coli* signal peptidases will not]	SDS-PAGE/ immunoprecipitation/ fluorography	Tjalsma et al., 1997
In vitro		
Radiolabeled preproteins synthesized by in vitro transcription translation	SDS-PAGE/immunoprecipitation/ fluorography	Zwizinski and Wickner, 1980; Zwizinski et al., 1981; Sung and Dalbey, 1992; Tschantz et al., 1993; Vehmaanpera et al., 1993
Synthetic peptide	^{125}I label	Caulfield et al., 1988, 1989
	HPLC-UV detector	Dev et al., 1990; Kim et al., 1995a,b; Kuo et al., 1993, 1994
	Continuous spectrophotometric or spectrofluorometric	Kuo et al., 1994
	FRET-continuous spectrofluorometric	Zhong and Benkovic, 1998
Pro-OmpA nuclease A fusion protein	SDS-PAGE/densitometry	Chatterjee et al,. 1995; Suciu et al., 1997; Paetzel et al., 1997

acrylamide gel electrophoresis (SDS-PAGE) densitometry for its kinetic analysis (Table 2), and thus is not practical for the high-throughput screening of inhibitor libraries that is now commonly used for the elucidation of new lead compounds.

In an attempt to design a signal peptidase substrate suitable for high-throughput inhibitor screening, a direct continuous signal peptidase assay based on fluorescence resonance energy transfer (FRET) has been developed by Zhong and Benkovic (1998). The peptide substrate (Y*NO*$_2$-F-S-A-S-A-L-A-K-I-K-*Abz*) incorporates the signal peptidase cleavage site of the MBP signal peptide. The fluorescence of the anthraniloyl group (*Abz*) is quenched by the 3-nitrotyrosine until the peptide is cleaved by signal peptidase between the residues A and K. The k_{cat}/K_m for *E. coli* signal peptidase measured with this substrate was 71.1 $M^{-1}sec^{-1}$, four orders of magnitude lower than that achieved with the pro-OmpA nuclease A substrate (Table 3). Introduction of the hydrophobic h-region of the signal peptide into the Benkovic substrate may provide a more sensitive continuous assay that is required for high-throughput inhibitor screening. The recently acquired knowledge of the three-dimensional structure of Type I signal peptidases will also likely aid in the structure-based design of chromophoric or fluorogenic substrates.

3.3. Site-directed mutagenesis and site-directed chemical modification studies

The catalytic mechanism of bacterial signal peptidases has been studied by site-directed mutagenesis in the *E. coli* signal peptidase and the *Bacillus subtilis* SipS. Deletion studies showed that the catalytic activity of signal peptidase resides in the large carboxy-terminal periplasmic domain (Bilgin et al., 1990; Kuo et al., 1993; Tschantz et al., 1995). Sung and Dalbey (1992) have used site-directed mutagenesis to classify *E. coli* signal peptidase into a protease class, and found neither cysteine nor histidine residues to be essential for cleaving the bacteriophage M13 procoat protein. They found Ser90 to be critical for activity. Later work revealed that Lys145 is the only basic residue that is essential for *E. coli* signal peptidase activity, and suggested that it is involved in a Ser/Lys dyad mechanism (Tschantz et al., 1993; Black, 1993). Site-directed mutagenesis and chemical modification studies are consistent with Trp300 being important for efficient processing of the signal peptide (Kim et al., 1995a, 1995b). In parallel, extensive site-directed mutagenesis studies of the gram-positive signal peptidase *Bacillus subtilis* SipS has been performed by van Dijl and co-workers (1995). They found that the residues Ser43, Lys83, and Asp153 (the equivalent residues to *E. coli* Ser90, Lys145, and Asp280) were all essential for SipS activity.

To further explore the role of the proposed active-site residue Ser90 of *E. coli* Type I signal peptidase, Tschantz and co-workers (1993) constructed an active thiol signal peptidase by mutating the Ser90 to Cys. This variant was inhibited by the cysteine-specific reagent N-ethylmaleimide, whereas wild-type signal peptidase was not affected. Paetzel and co-workers (1997) combined site-directed mutagenesis and chemical modification to introduce unnatural amino acids at the 145 position. They changed Lys145 to Cys, which produced an inactive enzyme, and then showed that the active enzyme could be regenerated by reacting the Cys with 2-bromoethylamine-HBr to produce the lysine analog (γ-thia-lysine) at the 145 position. Modification of the Cys145 with (2-bromoethyl)trimethylammonium-HBr to form a positively charged nontitratable side chain at the 145 position failed to restore activity to the inactive Lys145Cys mutant. These observations are consistent with the essential Lys145 playing a role as the active-site general base.

3.4. Inhibitors of bacterial Type I signal peptidases

It has been observed in many laboratories that bacterial Type I signal peptidases are not inhibited by the standard protease inhibitors (Zwizinski et al., 1981; Talarico et al., 1991; Black et al., 1992; Kuo et al., 1993; Kim et al., 1995a). For example, Kim et al. (1995a) have found that *E. coli* signal peptidase is not inhibited by diisopropyl fluorophosphate (DFP), phenylmethylsulphonyl fluoride (PMSF), soybean trypsin inhibitor, APMSF, elastinal, benzamindine, leupeptin, tosyl-lysine chloromethyl ketone, *n*-tosylphenyalanine chloromethyl ketone, chymostatin, *p*-chloromercuribenzoate, EDTA, *O*-phenanthroline, phosphoramidon, and bestatin. They also found that *E. coli* signal peptidase was not labeled by [^{3}H]DFP, even at very high concentrations (20 mM), and that the activity of *E. coli* signal peptidase is adversely affected by high concentration of sodium chloride

Table 3
Kinetic constants for *Escherichia coli* signal peptidase measured with various substrates

Substrate	Method of analysis	Reaction conditions	k_{cat} (sec^{-1})	K_m (M^{-1})	k_{cat}/K_m (M^{-1} sec^{-1}) 3	Reference
Peptide (12-mer, MBP)	HPLC	a	1.25×10^{-4}	1.4×10^{-3}	8.9×10^{-2}	Dev et al., 1990
Peptide (9-mer, MBP)	HPLC	a	3.2×10^{-2}	8×10^{-4}	40	Dev et al., 1990
ProOmpA-nuclease A[c]	SDS-PAGE	b	8.73	1.65×10^{-5}	5.29×10^{5}	Chatterjee et al., 1995
Peptide (11-mer, MBP)	FRET-continuous	d	—	—	71	Zhong and Benkovic, 1998

[a]Sodium phosphate (pH 7.7, 25 mM), 1% Triton X-100, 37°C.

[b]Tris-HC1 (50 mM), 1% Triton X-100, pH 8.0, 37°C.

[c]ProOmpA-nuclease A is a fusion protein made up of the signal peptide of outer membrane protein A from *E. coli* and the nuclease A from *Staphylococcus aureus*.

[d]Triethanolamine-HCl (50 mM), 1 mM EDTA, 1% Triton X-100, pH 8.0, 37°C.

(>160 mM), magnesium chloride (>1 mM), and dinitrophenol (Zwizinski et al., 1981).

Escherichia coli signal peptidase can be inhibited in a competitive fashion by signal peptides. The 23 amino acid residue signal peptide from M13 procoat protein (MKKSLVLKASVAVATLVPMLSFA) was found to inhibit the in vitro processing of the procoat protein and pre-MBP by *E. coli* signal peptidase (Wickner et al., 1987). As mentioned in Section 3.1, a synthetic pre-MBP signal peptide with a proline at the +1 (P1′) position has been shown to inhibit *E. coli* signal peptidase activity in vivo (Barkocy-Gallagher & Bassford, 1992). The first report of an effective, nonpeptide, inhibitor of Type I signal peptidase was by Kuo and colleagues in 1994. They reported that β-lactam compounds could inhibit *E. coli* signal peptidase in a pH- and time-dependent manner. β-Lactam-type compounds have also been shown to be effective irreversible inhibitors of a number of serine proteases and hydrolases, such as elastase (Wilmouth et al., 1999; Taylor et al., 1999), phospholipase A_2 (Tew et al., 1998), and β-lactamase (Strynadka et al., 1992). In all these cases, the β-lactam bond mimics the peptide bond of the natural substrate. SmithKline Beecham Pharmaceuticals (Harlow, Essex, UK) has studied extensively the potential of β-lactam (or penem)-type inhibitors against bacterial Type I signal peptidase (Allsop et al., 1995, 1996, 1997; Perry et al., 1995; Black & Bruton, 1998). The most effective penem compounds are the 5*S* stereoisomers (Fig. 5a), which are capable of inhibiting both

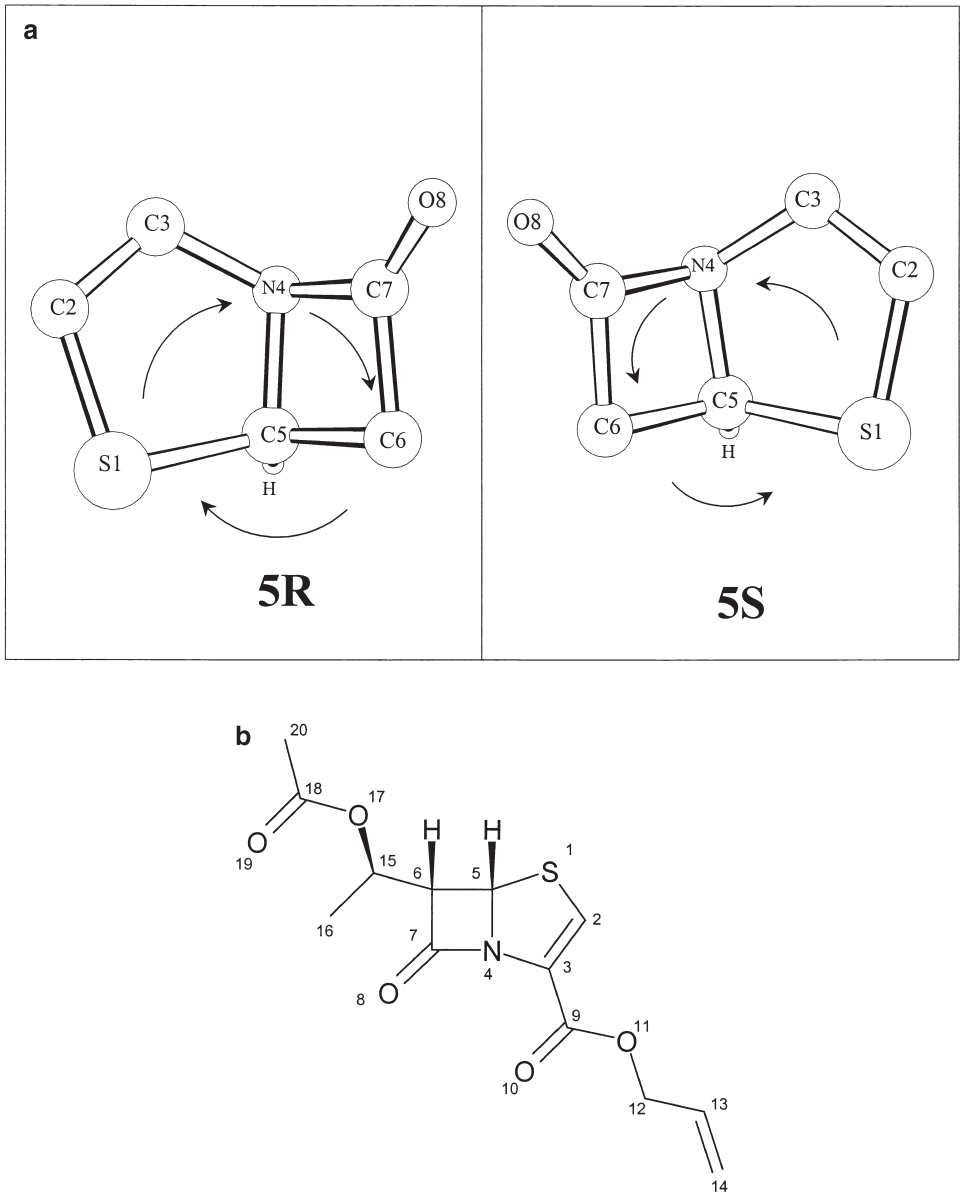

Fig. 5. (a) The 5*R* and 5*S* stereoisomers of the β-lactam type (penem) ring system. The arrows show the direction of the order of priority at the C5 chiral center. The bacterial signal peptidases bind the 5*S* isomers, whereas the β-lactamases and penicillin-binding proteins bind the 5*R* isomers. (b) Structure of the β-lactam (penem) inhibitor allyl(5*S*,6*S*)-6-[(*R*)-acetoxyethyl]-penem-3-carboxylate (Allsop et al., 1997; Black & Bruton, 1998).

the gram-negative *E. coli* and the gram-positive *Staphylococcus aureus* signal peptidases. The 5*S* stereoisomer is opposite stereochemically to that of the 5*R* β-lactams that are required for β-lactamases and penicillin-binding proteins (Fig. 5a). The compound allyl (5*S*,6*S*)-6-[(*R*)-acetoxyethyl]-penem-3-carboxylate (Fig. 5b) has an IC_{50} of less than 1 μM. A rationale for the face of nucleophilic attack by the *E. coli* signal peptidase Ser90 Oγ previously was proposed based on the stereochemistry of the penem inhibitors (Allsop et al., 1997). The X-ray structure of the *E. coli* signal peptidase–5*S*,6*S* penem complex confirmed the *si*-face attack of the nucleophile (Paetzel et al., 1998) (see Section 4.5). The penem inhibitors have also been reported to inhibit signal peptidases from cyanobacterium and the chloroplast thylakoids in vitro (Barbrook et al., 1996).

4. The three-dimensional structure of Type I signal peptidase

4.1. X-ray crystal structure of a soluble, catalytically active fragment of Escherichia coli *Type I signal peptidase*

Crystallization of membrane proteins is a challenging, but increasingly feasible, endeavor (Song & Gouaux, 1997; Ostermeier & Michel, 1997). The recent plethora of integral membrane protein structures (Xia et al., 1997; Iwata et al., 1995; Ferguson et al., 1998; Buchanan et al., 1999; Doyle et al., 1998; Chang et al., 1998; Olson et al., 1999; Dutzler et al., 1999) has occurred in parallel with advances in molecular biology, an increase in the number of commercially available surfactants needed to mimic the membrane milieu, and the development of novel crystallographic phasing strategies, including multiple anomalous diffraction, where structures can be solved from the data collected using a single crystal (Hendrickson & Orgata, 1997). However, structure determination of membrane proteins still remains a relatively daunting pursuit. In particular, there have been no reported successes in crystallizing proteins such as native signal peptidase, which contains a single transmembrane anchor domain.

In an effort to obtain Type I signal peptidase structural information, a soluble form of *E. coli* signal peptidase was constructed, expressed, purified, characterized (Kuo et al., 1993; Tschantz et al., 1995), and crystallized (Paetzel et al., 1995). The construct lacks the residues 2–75, which correspond to the 2 transmembrane segments, and is referred to as Δ2–75. The protein has a molecular weight of 27,952 Da, as measured by electrospray ionization mass spectrometry (Kuo et al., 1993), and a measured isoelectric point of 5.6 (Tschantz et al., 1995). The presence of detergent was essential for optimal activity and crystallization, specifically the addition of Triton X-100 at approximately the critical micelle concentration (Paetzel et al., 1995). Native data to 2.3-Å resolution have been collected. However, addition of an inhibitor greatly enhanced the order of the signal peptidase crystals. The structure of Δ2–75 in complex with a β-lactam type inhibitor (allyl (5*S*,6*S*)-6-[(*R*)-acetoxyethyl]-penem-3-carboxylate) (Fig. 5) was solved to high resolution (1.95 Å) by multiple isomorphous replacement (Paetzel et al., 1998).

4.2. The protein fold

The overall dimension of *E. coli* signal peptidase Δ2–75 is ∼60 × 40 × 70 Å, with the smallest dimension running perpendicular to the view seen in Fig. 6a. *Escherichia coli* Type I signal peptidase has a primarily β-sheet protein fold consisting of two anti-parallel β-sheet domains. Domain I, which we have termed the "catalytic core," contains the conserved regions of sequence (Boxes B–E), all of which reside near or are part of the active site (see Figs. 6 and 7). The structure has four extended loops or hairpins that are disordered (residues 108–124, 170–176, 198–202, and 304–313).

Despite very limited sequence identity (van Dijl et al., 1995), Domain I shares significant structural similarities with the UmuD′ protease, the proteolytic domain of a self-cleaving repressor protein involved in the SOS DNA-repair response in *E. coli* (Paetzel & Strynadka, 1999) (Fig. 6c). It has also been proposed to be a Ser/Lys protease (Takehiko et al., 1988; Peat et al., 1996). The large β-sheet Domain II (residues 154–264) and the long extended β-ribbon (residues 107–122) observed in the *E. coli* signal peptidase structure are insertions within Domain I (see Figs. 6 and 7). Domain II contains a disulfide bond between Cys170 and Cys176 that previously was detected by biochemical studies (Whitley & von Heijne, 1993).

4.3. The membrane association surface

The conserved Domain I also contains a large exposed hydrophobic surface that runs along the β-sheet made up from β-strands 1, 2, and 17. Exposure of significant areas of hydrophobic surface is rarely observed in soluble proteins, and it has been proposed that in signal peptidase, this unusual surface is likely to be involved in membrane association (Paetzel et al., 1998). β-Strand 17 contains the previously mentioned Trp300, which was shown to be essential for optimal activity in *E. coli* signal peptidase (Kim et al., 1995a, 1995b), even though the structure shows that it is localized more than 20Å from the enzyme active site (Paetzel et al., 1998). Aromatic amino acids are thought to play an important role in protein/membrane interfaces (Landolt-Marticorena et al., 1993), and presumably Trp300 facilitates the insertion or association of *E. coli* signal peptidase into the membrane. A tryptophan residue was found to be essential for the interfacial catalysis of phospholipase A_2 at the membrane surface (Gelb et al., 1999). Sequence alignments indicate that several conserved aromatic or hydrophobic residues exist in the proposed membrane-association domain in both gram-positive and gram-negative bacterial Type I signal peptidases (Fig. 7). Interestingly, *Bradyrhizobium japonicum* SipF contains a very high content of tryptophan residues near its carboxy-terminus (Bairl & Muller,

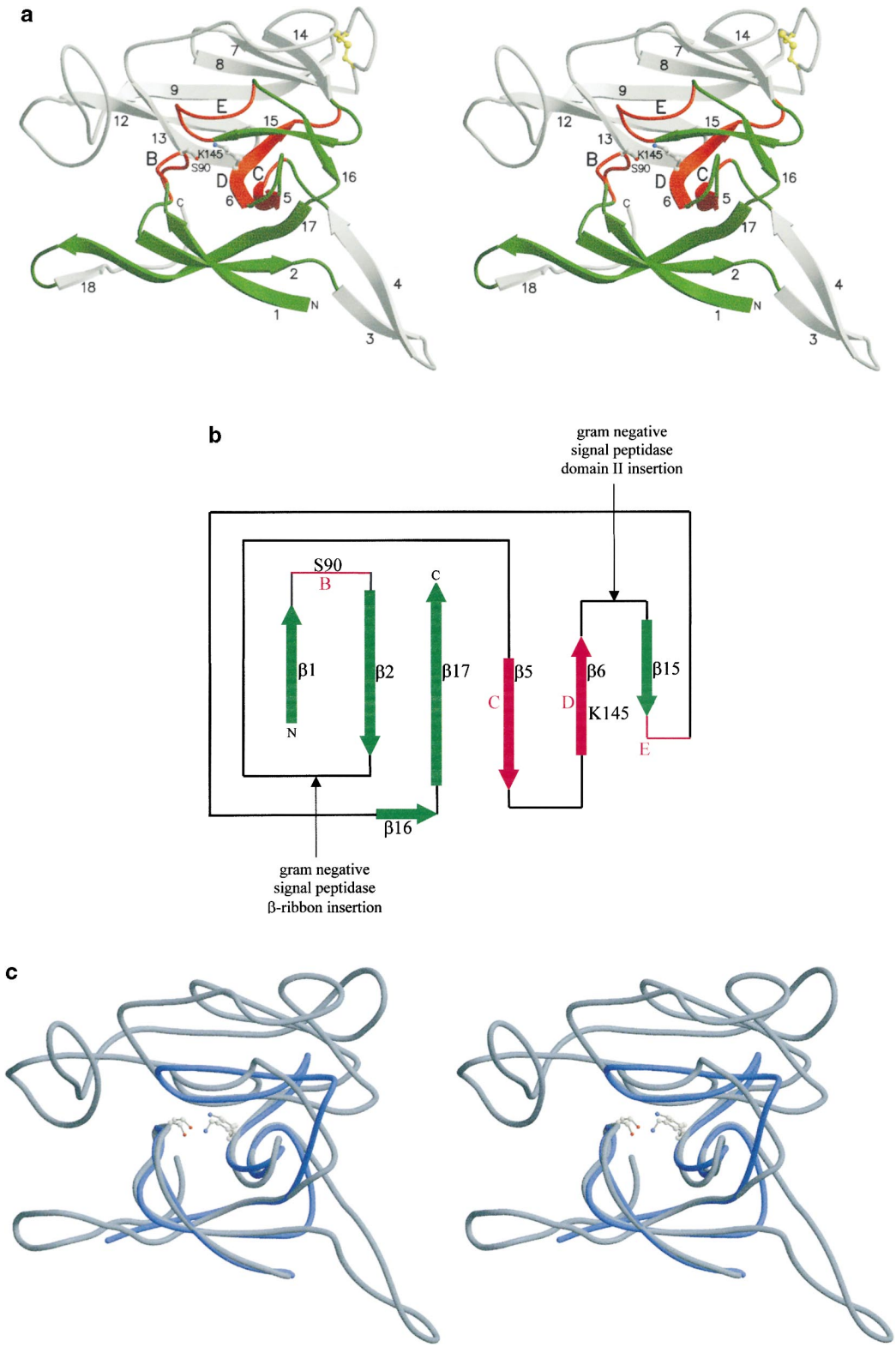

Fig. 6. (a) A stereo view of the overall protein fold of the *E. coli* type I signal peptidase catalytic domain (Δ2–75). Domain I is in green and red. The red regions correspond to the conserved boxes (B–E) of sequence in the type I signal peptidases (see Fig. 7). Domain II and the extended β-ribbon are in gray. The β-strands are numbered in the order of appearance in the sequence (see Fig. 7). Note that two β-strands (β10 and β11), a small α-helix (α1), and small 3_{10} helices (3_{10} 1–3) are not shown for clarity of the figure. The figure was prepared with the program MOLSCRIPT (Kraulis, 1991). (b) A schematic diagram of the protein fold in the conserved Domain I of Type I signal peptidases. Domain II and the extended β-ribbon, as seen in the gram-negative Type I signal peptidases, are insertions within Domain I. (c) A stereo view of the C_α backbone of the *E. coli* UmuD′ protease (dark blue) superimposed onto the C_α backbone tracing of the catalytic domain of *E. coli* Type I signal peptidase (light blue). The side chains of the Ser/Lys dyad in each protease are shown in ball-and-stick. This figure was prepared with the program MOLSCRIPT (Kraulis, 1991).

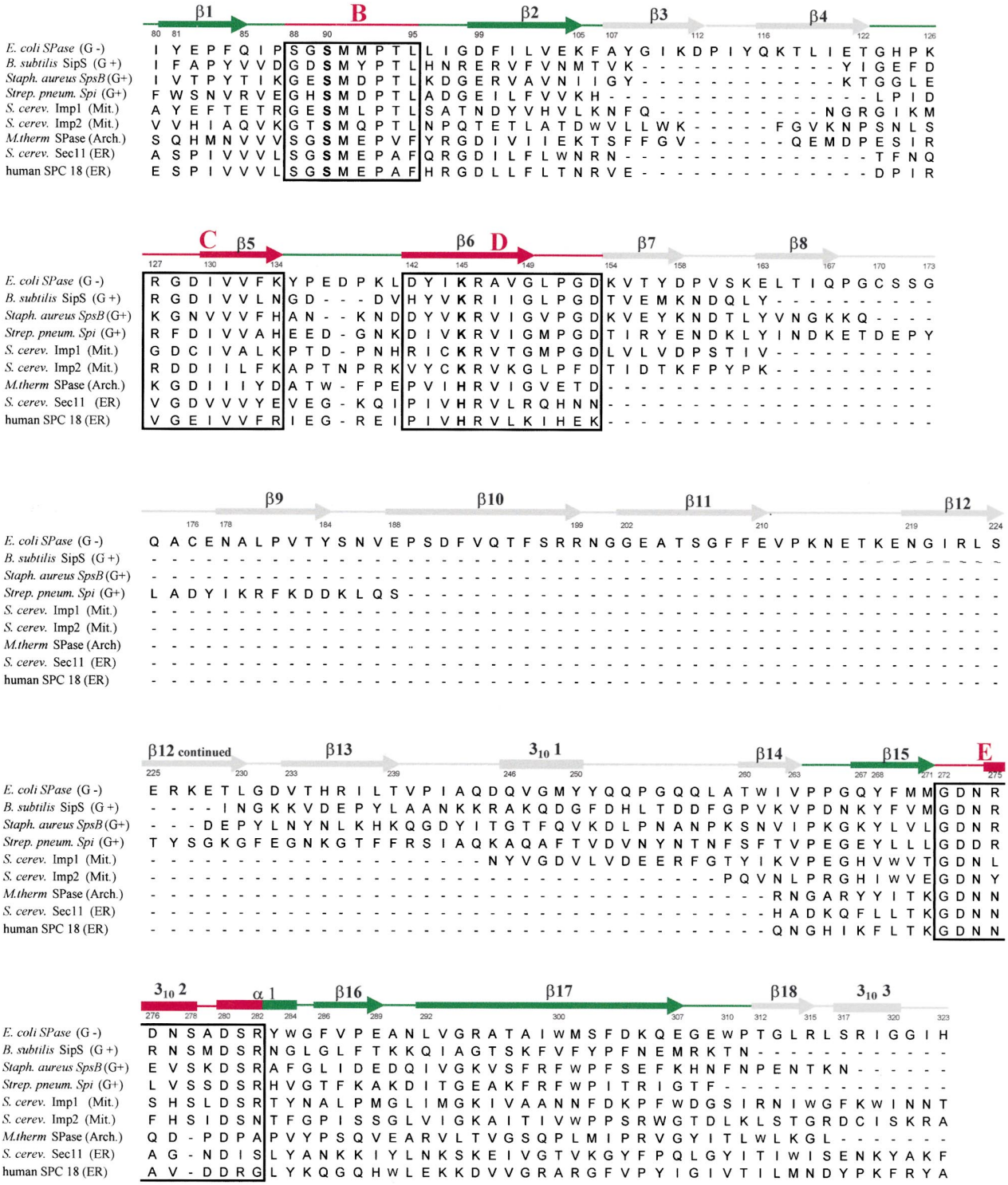

Fig. 7. A structure-based multiple sequence alignment of representative Type I signal peptidases from gram negative (G−), gram positive (G+), mitochondria (Mit.), Archaea (Arch.), and the endoplasmic reticulum (ER). The secondary structure of *E. coli* Type I signal peptidase, as determined by the program PROMOTIF (Hutchinson & Thorton, 1996), is shown above the alignment. The secondary structural elements within Domain I are shown in green and red. The red regions correspond to the conserved boxes (B–E) of sequence in the Type I signal peptidases. The secondary structural elements corresponding to Domain II and the extended β-ribbon are in gray.

1998). Not surprisingly, given its large size, no ordered Triton X-100 detergent molecules were observed in the structure. However, there are pronounced solvent channels directly adjacent to the proposed hydrophobic membrane-association domain in the crystal. It is likely that the required detergent accumulates in these solvent channels, stabilizing the significantly exposed hydrophobic surface area on the *E. coli* signal peptidase molecule.

4.4. The tertiary structure of boxes B–E

The structural motifs within which the four conserved boxes reside can be summarized as follows. Box B (residues 88–95) contains the proposed nucleophilic Ser90 that resides on a well-ordered loop between the first 2 β-strands of the periplasmic catalytic portion of *E. coli* Type I signal peptidase. Box B also contains a conserved serine at position 88 in the gram-negative species. There is a conserved Met at position 91, which allows for the placement of its side-chain sulfur adjacent to the nucleophilic serine. This is a common feature observed in the active site of several Ser proteases and β-lactamases. Boxes C (residues 127–134) and D (residues 142–153) contain two antiparallel hydrogen bonded β-strands (β5 and β6; see Figs. 6 and 7). β-Strand 6 contains the proposed general base Lys145 and has main-chain atoms aligned perfectly to make β-strand-type hydrogen bonds with the signal peptide substrate, as seen in other serine proteases (Perona & Craik, 1995). Box E (residues 272–282) contains a small 3_{10} helix (residues 275–278) and part of a small α-helix (residues 280–282). The strict conservation of Gly272 is explained by the fact that it sits directly adjacent to the Nζ of Lys145 (Fig. 8). Any other residue at this site would necessarily position a side chain into this region that would clash with the essential Lys145 side chain. The conserved Ser278, which hydrogen bonds to the Lys145, most likely helps position the Lys145 Nζ for its general base role with Ser90. The highly conserved Asp280 and Arg282, which are positioned more than 5 Å from the catalytic residues (Ser90 or Lys145), form a strong salt-bridge (Fig. 8), and likely contribute to the structural stabilization of the active site rather than playing a direct role in catalysis.

4.5. The catalytic residues

As mentioned in Section 4.1, the ability to obtain high-resolution structural data for the *E. coli* signal peptidase (Paetzel et al., 1998) was facilitated by the addition of a covalently bound inhibitor. The electron density at the active site of the *E. coli* signal peptidase inhibitor complex revealed a covalent bond between the Ser90 Oγ and the carbonyl (C7) of the inhibitor (Fig. 8). This provided the first direct evidence that the Ser90 Oγ acts as the acylating nucleophile in the signal peptidase hydrolysis reaction. The signal peptidase inhibitor complex shows that Type I signal peptidases indeed are unique serine proteases, in that they attack the scissile bond of the substrate from the *si*-face of the amide bond. All known Ser/His/Asp serine proteases attack the scissile bond from the *re*-face (James, 1994) (see Fig. 9). The observation likely explains the preference for the 5*S* versus the 5*R* stereochemistry of the penem inhibitor, and supports the earlier proposals of Allsop and co-workers (1997).

The Nζ of Lys145 forms a strong hydrogen bond (2.9 Å) to the Ser90 Oγ, and is the only titratable group in the vicinity of the active-site nucleophile (Fig. 8). The Lys145 Nζ position is fixed relative to Ser90 Oγ by hydrogen bonds to the conserved Ser278 Oγ (2.9 Å) and the carbonyl oxygen (O10) of the inhibitor side chain (2.9 Å) (Fig. 8). Therefore, Lys145 Nζ is correctly positioned to act as the general base in both the acylation step and the deacylation step of catalysis. The side chain of Lys145 is completely buried in the inhibitor complex (Fig. 8), where it makes van der Waals contacts with the side-chain atoms of Tyr143, Phe133, Met270 and the main-chain atoms of Met270, Met271, Gly272, and Ala279, all of which are contained within the catalytic core (Domain I). The hydrophobic environment surrounding the Lys145 ϵ-amino group is likely essential for lowering its pK_a, such that it can reside in the deprotonated state required for its role as the general base (Paetzel et al., 1997; Paetzel & Dalbey, 1997).

The main-chain amide of Ser90 forms a strong hydrogen bond to the carbonyl of the acylated inhibitor. This suggests that the Ser90 amide may contribute to the formation of the

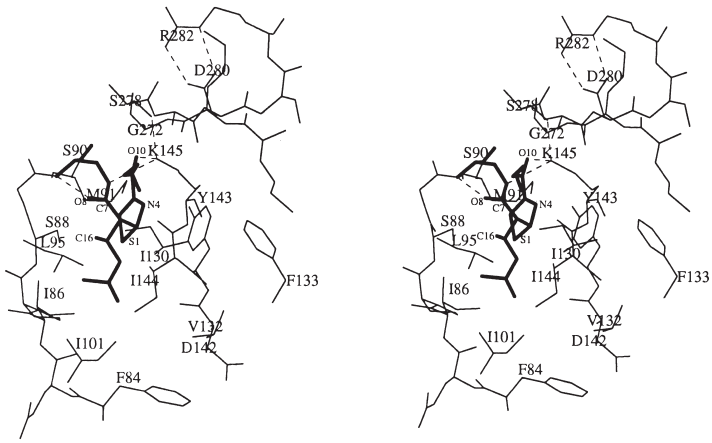

Fig. 8. A stereo view of the active-site of the *E. coli* Type I signal peptidase. Shown in thick black lines, the β-lactam-type inhibitor allyl (5*S*,6*S*)-6-[(*R*)-acetoxyethyl]-penem-3-carboxylate is covalently bound to the Oγ of Ser90. The carbonyl oxygen (O8) of the cleaved β-lactam (the bond between C7 and N4 has been broken) is sitting in the oxyanion hole formed by the main-chain NH of Ser90 (S90). The methyl group (C16) of the inhibitor sits in the S1 substrate-binding site. The Lys145 Nζ is hydrogen bonded to the Ser90 Oγ, Ser278 Oγ, and atom O10 of the penem inhibitor.

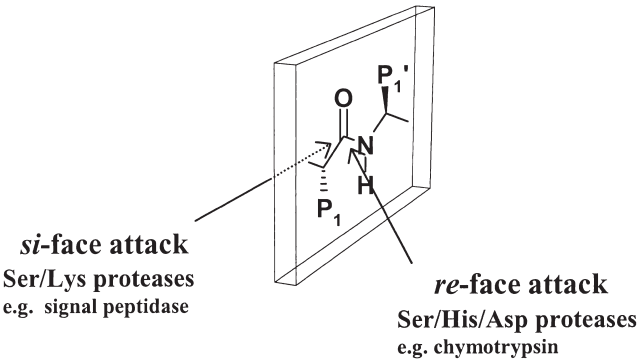

Fig. 9. The Ser Oγ nucleophile of bacterial Type I signal peptidases attacks the scissile bond of its substrate from the *si*-face rather than the *re*-face, as is seen with all known Ser/His/Asp catalytic triad serine proteases such as chymotrypsin.

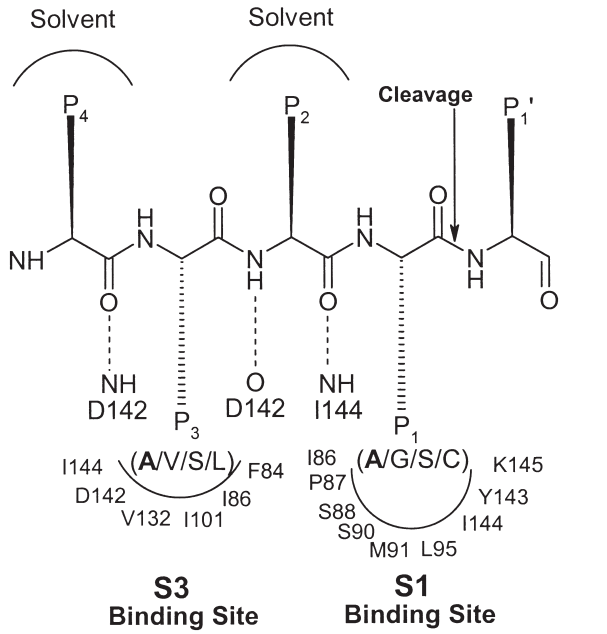

Fig. 10. A schematic drawing of the proposed interactions between the *E. coli* signal peptidase and a signal peptide substrate. The signal peptide has an extended β-conformation and makes β-sheet-type interactions with the β-strand that contains the general base Lys145. The P1 (−1) and the P3 (−3) residues of the signal peptide sit in the S1 and S3 specificity-binding sites, respectively. The P2 (−2) and P4 (−4) residue side chains point into solvent.

oxyanion hole (Fig. 8). A second contributor to the oxyanion hole is not obvious, but it is proposed that the Ser88 Oγ potentially could contribute to the oxyanion hole in the case of the natural preprotein substrate by a simple rotation of its χ_1 angle. The rotation is prevented in the β-lactam inhibitor complex structure by unfavorable van der Waals contacts between the Ser88 Oγ and the S1 and C15 atoms of the inhibitor (Fig. 8).

4.6. The substrate-binding sites and substrate specificity

The 5*S*,6*S* penem inhibitor that was co-crystallized with *E. coli* Type I signal peptidase has a methyl group (C16) that was found to be essential for effective inhibition (Allsop et al., 1995, 1996, 1997; Black & Bruton, 1998) (see Fig. 5). It was proposed that the C16 methyl group likely mimics the −1 (P1) alanine side chain of the signal peptide. The crystal structure of the complex reveals that the C16 methyl group sits in a small hydrophobic cleft, which can be recognized as the S1-binding pocket of the *E. coli* signal peptidase. The residues contributing atoms to the S1 pocket are primarily hydrophobic and include Ile86, Pro87, Ser88, Ser90, Met91, Leu95, Ile144, Tyr143, and Lys145 (see Figs. 8 and 10). The mapping of surface clefts on the signal peptidase structure has been performed using the algorithm CAST (Liang et al., 1998). This analysis indicates that the largest surface cleft corresponds to the proposed S1-binding site. The second most prominent cleft (the S3-binding site) was also easily recognized, both visually and by using the automated algorithm. It is also primarily hydrophobic in nature and sits on the other side of the S1 cleft relative to the active-site nucleophilic Ser90.

Modeling of a tetra-poly-alanine peptide into the signal peptidase active site also supported the identification of the S3 substrate-binding pocket. The position of the inhibitor methyl group (C16) in the S1 substrate-binding site, as well as the inhibitor carbonyl group (C7, O8), in the oxyanion hole was used as a guide. An extended β-strand conformation of the modeled peptide was needed in order to provide a favorable fit and β-sheet-type hydrogen bonding with the conserved β-strand containing Lys145 (β-strand 6; see Figs. 6a and 10) (Paetzel et al., 1998). These modeling studies supported earlier work that suggested that the carboxy-terminal 5-6 residues of the signal peptide would adopt a β-strand type conformation (Izard & Kendall, 1994) (Fig. 10). The β-sheet type of interaction at the binding site is common amongst Ser proteases (Perona & Craik, 1995). In the model, the side chain of the P3 (−3) Ala points into a shallow hydrophobic depression formed by Phe84, Ile86, Ile101, Val132, Ile144, and the Cβ of Asp142 (the proposed S3 substrate specificity site; Fig. 10). Although Ala is the most common residue at the P3 (−3) site of signal peptides, larger aliphatic residues, such as Val, Leu, and Ile, can also occur at this position (Nielsen et al., 1997a, 1997b). The hydrophobic depression for the S3 site is more shallow and broad as compared with the S1 site. This may help to accommodate the larger residues at the P3 site of the signal peptide substrate.

In the penem-signal peptidase structure, the thiozoladine ring of the penem inhibitor (Fig. 8) makes a stacking interaction with the aromatic side chain of Tyr143. Modeling studies show that the side chains of the residues at P2 and P4 point out of the active site towards the solvent (Fig. 10), which is consistent with the observed signal peptide sequence variability at these positions (von Heijne, 1985) (Fig. 10). In the model, the side chain of Tyr143 would be

adjacent to the P2 position in the signal peptide (Paetzel et al., 1998). Van der Waals interactions between the substrate and the enzyme at this position may be beneficial to the binding, given that the recent mutagenesis studies of the *E. coli* alkaline phosphatase signal peptide have shown that variants with large residues at the P2 position are processed more efficiently (Karamyshev et al., 1998). Interestingly, most bacterial signal peptidases have an aromatic residue at the 143 position (*E. coli* numbering), yet the eukaryotic signal peptidases have an Ile at this position (Figs. 2 and 7). The analysis of temperature-sensitive mutants identified in *Bacillus subtilis* SipS lends experimental support for the importance of the Tyr143 side chain. The mutants Leu74Ala and Tyr81Ala (the equivalent to Phe133 and Tyr143 in *E. coli* signal peptidase) are structurally stable, but catalytically impaired, at 48°C (Bolhuis et al., 1999). Collectively, this model explains the Ala-X-Ala (or −3, −1 rule) cleavage-site specificity of signal peptidases.

4.7. Predicted structural similarities and differences in Type I signal peptidases

A structure-based multiple sequence alignment of representative Type I signal peptidases reveal that Domain I is conserved throughout evolution (Fig. 7). A stereo view of Domain I can be seen in Fig. 6a (Domain I is in green and red), and a diagram of the protein fold of Domain I is seen in Fig. 6b. In general, the smaller size of the gram-positive Type I signal peptidases relative to the gram-negative Type I signal peptidases is a result of a smaller Domain II (154-264) and lack of an extended β-ribbon (residues 107–122). The eukaryotic ER and archaea bacterial Type I signal peptidases are completely missing Domain II. The signal peptidase from yeast, Sec11, has been shown to require a second component, SPC3, for catalytic activity. The SPC3 has some sequence similarity to the Domain II of *E. coli* signal peptidase (VanValkenburgh et al., 1999) and, therefore,

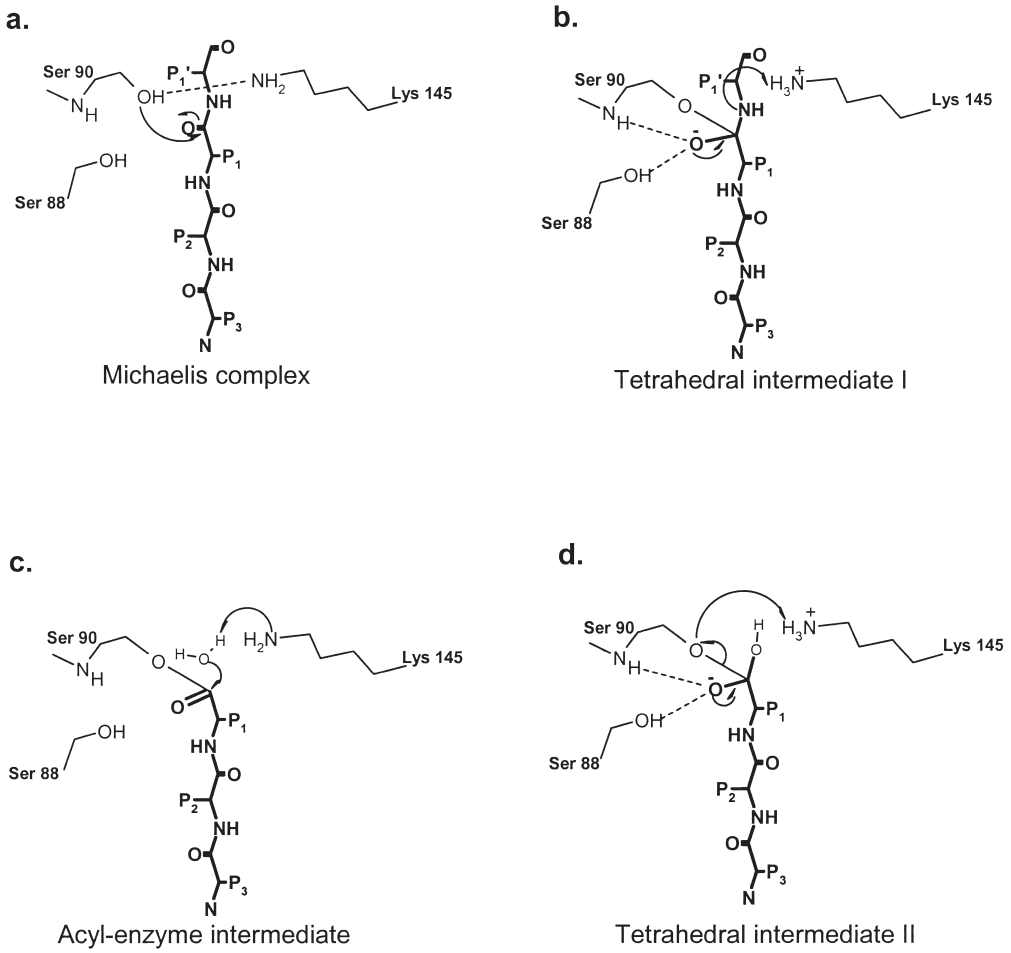

Fig. 11. The proposed mechanism of *E. coli* Type I signal peptidase. To date, all data are consistent with bacterial signal peptidases utilizing a Ser/Lys dyad mechanism. (a) The Nζ of the lysine general base (Lys145) abstracts the proton from the Oγ of the nucleophilic serine (Ser90). The activated Ser90 Oγ attacks the *si*-face of the scissile bond of the preprotein forming the oxyanion tetrahedral intermediate I. (b) The oxyanion tetrahedral intermediate I is stabilized by the oxyanion hole formed from the main-chain amide of Ser90 and the side-chain hydroxyl of Ser88. (c) The acyl-enzyme intermediate is attacked by a deacylating water made nucleophilic by its association with the Lys145 Nζ general base. (d) The tetrahedral intermediate II then collapses, releasing the cleaved signal peptide.

SPC3 may provide the same function as Domain II. The functional roles played by Domain II and the extended β-ribbon of gram-negative Type I signal peptidases are unknown. Given the position of the β-ribbon in the *E. coli* signal peptidase, it may be speculated that it could play a role in membrane association.

It has been proposed that the *E. coli* signal peptidase Ser88 Oγ contributes to the oxyanion hole (Paetzel et al., 1998). From the multiple sequence alignment, it appears that the 88 position is most often occupied by a Ser, Thr, or Gly residue (Figs. 2 and 7). The gram-negative bacteria, archaea, and ER signal peptidases usually contain a Ser or Thr, whereas the gram-positives tend to have a Gly at this position. The gram-positive Type I signal peptidases may utilize the main chain NH of Gly as a contributor to the oxyanion transition state stabilization.

4.8. A proposed mechanism for bacterial Type I signal peptidase

Based on the available biochemical data, the structure of the *E. coli* Type I signal peptidase inhibitor complex, and modeling of peptide substrates, we can propose a catalytic mechanism for the cleavage of signal peptides by *E. coli* Type I signal peptidase (Fig. 11). The signal peptide binds to the active site of the signal peptidase with the P1 (−1) to P4 (−4) residues of the signal peptide in an extended β-conformation, such that their main-chain hydrogen bonds with the conserved box D β-strand, which contains the Lys145 general base (β6; Fig. 10). The P1 (−1) and P3 (−3) side chains sit in the hydrophobic S1 and S3 substrate-binding clefts (Fig. 10). The Lys145 Nζ would act as the general base to abstract the proton from the Ser90 Oγ that performs a nucleophilic attack on the *si*-face of the scissile bond (Figs. 9 and 11a). The oxyanion tetrahedral intermediate I is then formed with electrostatic stabilization by hydrogen bonds to the oxyanion hole (the Ser90 main-chain amide and the Ser88 side-chain hydroxyl) (Fig. 11b). The ammonium group from the side chain of Lys145 would then donate a proton to the leaving group amide, the new amino-terminus of the now mature exported protein (Fig. 11b), forming the acyl-enzyme intermediate (Fig. 11c). Lys145 would then again act as the general base, this time activating the deacylating water that forms the tetrahedral intermediate II (Fig. 11d). The Lys145 side-chain ammonium group then donates a proton to the Ser90 Oγ, and the cleaved signal peptide dissociates, regenerating the signal peptidase active site. Multiple sequence alignments (Fig. 7) suggest that the residues involved in such a mechanism are conserved. It appears from the structure of the *E. coli* signal peptidase inhibitor complex that the reason the trapped acyl-enzyme was so stable (>60 days in the crystal) was that the deacylating water was completely excluded from the active site by the bound inhibitor.

Other enzymes with a potential Ser nucleophile and a Lys general base include the *E. coli* LexA repressor (Little et al., 1994), the *E. coli* UmuD protease (Peat et al., 1996), the *E. coli* Tsp protease (Keiler & Sauer, 1995), the class A and C β-lactamases (Strynadka et al., 1992; Oefner et al., 1990), the *Streptomyces* D-Ala-D-Ala peptidases (Kelly et al., 1989), the *Pseudomonas aeruginosa* TraF (Haase & Lanka, 1997), and the rat fatty acid hydrolase (Patricelli & Cravatt, 1999) (Table 4).

Enzymes other than proteases and hydrolases that appear to have a lysine general base are listed in Table 5. For a review of the evidence for the Ser/Lys dyad mechanism, see Paetzel and Dalbey (1997).

5. Conclusion

Bacterial Type I signal peptidase is a unique serine protease that represents a novel antibiotic target accessible at the bacterial membrane surface. As a result of the many new primary sequences available from the bacterial genome sequencing projects (http://www.tigr.org/), the recent three-dimensional structural information from *E. coli* signal peptidase, and the many site-directed mutagenesis experiments, we are now finally beginning to understand this unique class of serine protease.

With the first structure of a Type I signal peptidase now available, modeling of other bacterial Type I signal peptidases, as well as docking of potential inhibitors and the development of more effective peptide substrates for high-throughput screening, is now possible.

Table 4
Enzymes with a potential serine nucleophile and lysine general base

Enzyme	Residues	Mutational evidence	Structural evidence
E. coli Type I signal peptidase	S90, K145	Sung and Dalbey, 1992; Tschantz et al., 1993; Black, 1993	Paetzel et al., 1998
E. coli repressor LexA	S119, K156	Slilaty and Little, 1987	
E. coli Umu D	S60, K97	Takehiko et al., 1988	Peat et al., 1996
E. coli Tsp protease	S430, K455	Keiler and Sauer, 1995	
E. coli β-lactamase	S70, K73	Gibson et al., 1990	Strynadka et al., 1992
Streptomyces D-Ala-D-Ala peptidase	S62, K65		Kelly et al., 1989
Pseudomonas asparaginase	T100, K173	Swain et al., 1993; Miller et al., 1993	Lubkowski et al., 1994
Pseudomonas aeruginosa (RP4) TraF	S37, K89	Haase and Lanka, 1997	
Rat fatty acid amide hydrolase	S241, K142	Patricelli and Cravatt, 1999	

Table 5
Enzymes with a potential lysine general base

Enzyme	Residue	Mutational evidence	Structural evidence
Pseudomonas putida mandelate racemase	K166	Landro et al., 1994	Landro et al., 1994
Yeast enolase	K345	Poyner et al., 1996	Wedekind et al., 1994
E. coli aspartate aminotransferase	K258	Planas and Kirsch, 1991	Kirsch et al., 1984
Bacillus stearothermophilus leucine dehydrogenase	K80	Sekimoto et al., 1993	Baker et al., 1992 (related enzyme)
6-Phosphogluconate dehydrogenase	K183	Zhang et al., 1999	

Many fundamental questions remain to be answered regarding the function of bacterial Type I signal peptidase. Does signal peptidase cleave off the signal peptide from preproteins during or after translocation? And in what milieu does the cleavage occur? In the bulk solvent of the periplasm, in the head groups of the lipid bilayer, in the acyl chains of the lipid bilayer, or in the proteinaceous pore of the translocase? Is interfacial catalysis involved? What residues within the signal peptidase catalytic domain associate with the lipid bilayer? What roles, if any, do Domain II and the extended β-ribbon of the gram-negative signal peptidases play in catalysis? Why do some bacterial species have multiple Type I signal peptidases?

Acknowledgments

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