Crystal Structure of a Bacterial Signal Peptide Peptidase

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Signal peptide peptidase (Spp) is the enzyme responsible for cleaving the remnant signal peptides left behind in the membrane following Sec-dependent protein secretion. Spp activity appears to be present in all cell types, eukaryotic, prokaryotic and archaeal. Here we report the first structure of a signal peptide peptidase, that of the Escherichia coli SppA (SppAEC). SppAEC forms a tetrameric assembly with a novel bowl-shaped architecture. The bowl has a dramatically hydrophobic interior and contains four separate active sites that utilize a Ser/Lys catalytic dyad mechanism. Our structural analysis of SppA reveals that while in many Gram-negative bacteria as well as characterized plant variants, a tandem duplication in the protein fold creates an intact active site at the interface between the repeated domains, other species, particularly Gram-positive and archaeal organisms, encode half-size, unduplicated SppA variants that could form similar oligomers to their duplicated counterparts, but using an octamer arrangement and with the catalytic residues provided by neighboring monomers. The structure reveals a similarity in the protein fold between the domains in the periplasmic Ser/Lys protease SppA and the monomers seen in the cytoplasmic Ser/His/Asp protease ClpP. We propose that SppA may, in addition to its role in signal peptide hydrolysis, have a role in the quality assurance of periplasmic and membrane-bound proteins, similar to the role that ClpP plays for cytoplasmic proteins.

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Introduction

The cellular strategy of using an amino-terminal extension (signal peptide) to help facilitate targeting and translocation of proteins across cellular membranes is a process conserved across all forms of life.1 In order for secreted proteins to be released from the membrane after translocation the signal peptide is cleaved off by signal peptidase. It is the role of signal peptide peptidase to further cleave the signal peptides into smaller peptides that are able to be released from the membrane. Novak and Dev confirmed, based on their *in vitro* biochemical analysis of *Escherichia coli* signal peptide degradation, that *E. coli* signal peptide peptidase (SppAEC) is the endopeptidase that cleaves within the hydrophobic region (H-region) of the signal peptide.2

SppAEC, also referred to as protease IV, is 618 residues in length (molecular mass 67,219 Da).3 Biochemical analysis has revealed that SppA is anchored to the cytoplasmic membrane.4 SppA homologues exist in eubacteria,5,6 archaea5 and the thylakoid membrane of chloroplasts7 and they collectively belong to the evolutionary clan of proteases SK and the protease family S49.8 Unlike the eukaryotic functional homologue that has been proposed to be an aspartic protease,9 site-directed mutagenesis along with sequence analysis is consistent with the bacterial, archaeal and thylakoid SppA being a serine protease.6

Results and Discussion

A catalytically active and soluble version of the membrane protein SppAEC (SppAECΔ2–46) that lacks residues 2 through 46 (containing the predicted N-terminal transmembrane segment, residues 29–45) was used for crystallization. SppAECΔ2–46 was crystallized in the space group *P*2₁ with four...
molecules in the asymmetric unit and its structure was solved by single-wavelength anomalous diffraction (SAD). The final refined structure, at 2.6 Å resolution, includes residues 56 to 549 for each of the four molecules in the tetrameric assembly. The previously predicted second and third transmembrane segments (residues 398–414 and 421–441, respectively; Swiss-Prot accession number P08395) are actually buried segments within the major globular domain of SppAEC. Thus, we believe these earlier predictions likely hampered creation of soluble constructs of the bacterial signal peptide peptidases by inferring deletion points that spanned crucial folding elements of the enzyme.

The protein fold of SppAEC

SppAEC has an α/β protein fold. The major globular domain is made up of three consecutive β-α-β units in a superhelix motif. An extension domain made up of a long α-helix, a long β-strand and two small α-helices sticks out of the major globular domain and creates a dome-like structure in the supermolecular tetrameric complex (Figs. 1 and 2).

The N-terminal (residues 56–316) and C-terminal (residues 326–549) halves of SppAEC are tandem repeats of the above-described protein fold with the residues 317–325 forming a linker between the halves (Fig. 1). Despite only 18% sequence identity between the halves they superimpose with an rmsd of 2.5 Å (Fig. 1b). Each half has nine α-helices and 11 β-strands. The protein/protein interface between the N-terminal and C-terminal halves buries 1800 Å² of molecular surface on each half and is linked by 21 hydrogen bonds and one salt bridge. The interface is 60% nonpolar and the residues that contribute to the interface include the catalytic dyad residues Ser409 and Lys209, the nucleophile Ser409 coming from the C-terminal half and the general base Lys209 arriving from the N-terminal half (Fig. 1). The Ser409 O and the Lys209 N² are within hydrogen-bonding distance to one another (2.8 Å, average for the four molecules in the asymmetric unit). Besides the Lys209 N², there are no other titratable functional groups within the vicinity of the Ser409 O that could play the role of a general base.

The shape, dimensions and surface features of the SppAEC tetramer

Analysis by analytical size-exclusion chromatography in line with multilangle light scattering is consistent with SppAECΔ2–46 being a tetramer in solution (data not shown). Previous chemical cross-linking data on the full-length membrane-bound form of SppAEC was consistent with SppAEC being a tetramer. Size exclusion chromatographic analysis of the homologous enzyme from Arabidopsis thaliana (SppAAT) was also consistent with a tetrameric assembly. The long extended interface between the SppA monomers (∼66 Å × ∼27 Å) buries approximately 1450 Å² of surface (∼60% nonpolar) on each monomer. The interface is predominately α-helical in nature but with one long antiparallel two-stranded β-sheet that is formed from the long β-strand in the extension domain that protrudes from the major globular domain in each neighboring monomer. There are a total of 13 hydrogen bonds and one salt bridge between the monomers (Fig. 2).

The SppAEC tetramer is bowl shaped with an opening at its base of approximately 96 Å in diameter (predicted membrane association surface) (Fig. 2). A ridge on the inside of the bowl restricts the opening to approximately 40 Å in diameter. A concave surface is formed between the arched roof of the inverted bowl structure and the ridge (Fig. 2g). This concave surface creates the substrate-binding pockets for four active sites within the SppAEC tetramer. The top of the inverted bowl-shaped structure contains an axial opening approximately 22 Å in diameter that is rimmed with positive charges (Fig. 2a and g). The depth of the bowl-shaped protein is approximately 50 Å from base to axial opening. The wide opening of the bowl has an outer rim of positive charge followed by a broader inner rim of negative charge (Fig. 2e). The molecular surface of the deep interior of the bowl is predominantly nonpolar (Fig. 2e).

The assembly of Ser/Lys dyad active sites from adjacent domains and monomers

It was previously observed that SppA from eubacteria, archaea and thylakoids appears to form two main families of SppA based on size. The smaller SppA enzymes, which include the characterized Gram-positive Bacillus subtilis SppAbs and the archaeal Thermococcus kodakaraensis SppATK, are approximately half the size of the larger SppA enzymes and are most homologous to the C-terminal half of SppAEC. Our structure of SppAEC and our sequence analysis (Fig. 3) bring new insights into the relationship between the smaller and larger forms of SppA. As mentioned above, the N-terminal and C-terminal halves of SppAEC appear to be tandem repeats and the nucleophile Ser409 is located on the C-terminal half, whereas the general...
Fig. 1 (legend on previous page)
Fig. 2 (legend on next page)
base Lys209 is located on the N-terminal half. As can be seen from Fig. 1a, the side-chain functional groups of these residues come together, within hydrogen-bonding distance, at the interface between the N-terminal and C-terminal halves. When the halves are superimposed, the Ser409

**Fig. 2.** The oligomeric state, electrostatic surface properties and dimensions of SppAEC. (a), (c) and (e) show the electrostatic properties of SppAEC mapped on its molecular surface (white, neutral; blue, positive; red, negative). (b), (d) and (f) show different views of ribbon diagrams of SppAEC. Each monomer of the tetramer is a different color. (g) A section through the middle of SppAEC revealing the dimensions of the opening to the bowl-shaped structure. The catalytic residues S409 (green) and K209 (magenta) are shown as van der Waals spheres.

**Fig. 3.** A sequence alignment of signal peptide peptidases SppA. The secondary structure as calculated by DSSP12 is shown above the alignment. The sequences were acquired from the Swiss-Prot database with the accession numbers for each sequence in parenthesis: E. coli SppAEC (P08395); Vibrio cholerae SppAVC (Q9KQK4); Haemophilus influenzae SppAHI (P45243); A. thaliana SppAAT (Q9SEL8); B. subtilis SppABS (O34525); T. kodakaraensis SppATK (Q5JE91).

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Fig. 4 (legend on next page)
nucleophile and Lys209 general base are actually quite far apart (Fig. 1b). Interestingly, this superposition shows that Lys209 from the N-terminal half overlaps well with Ala461 from the C-terminal half, the region most similar to the “half-sized” archaeal SppA. The sequence alignment in Fig. 3 reveals that Ala461 aligns with Lys214 of T. kodakaraensis SppATk. Previously, Matsumi et al. proposed, based on their mutagenesis work on SppATk, that Lys214 was the general base in SppATk. Therefore, together these results are consistent with the position of the nucleophile (Ser409) and general base (Lys209) as seen in the SppAEC structure and provide a structural context for the previous SppATk mutagenesis results. In the case of SppATk, both nucleophile (Ser162) and general base (Lys214) reside on the same domain (or “half”), which would have the effect of putting these residues quite distant from each other, unless they assembled into an oligomeric assembly similar to that seen in SppAEC. Only when the oligomeric assembly comes together would the serine nucleophile come into close proximity to the lysine general base. This would suggest that the smaller SppA enzymes form an octameric assembly that would be similar in architecture to the tetrameric assembly seen in the larger ("double sized") SppA relatives, such as SppAEC. This also has the effect of doubling the number of active sites in the assembly of the smaller SppA enzymes (Fig. 4). Previous size-exclusion chromatography analysis of SppATk is consistent with it being an octamer.11

Comparison of a Ser/His/Asp protease and a Ser/Lys protease that utilize a similar protein scaffold

Despite limited sequence identity, the N- and C-terminal regions of the Ser/Lys protease SppAEC monomers are similar in protein fold to the monomers seen in the cytoplasmic "classical" Ser/His/Asp ClpP protease. The SppAEC N-terminal or C-terminal halves can be individually superimposed on a ClpP monomer with an rmsd of 3.0 Å (15.6% identity for 147 aligned residues) and 1.8 Å (25% identity for 155 aligned residues), respectively (Fig. 5a and b). ClpP is an ATP-dependent, general protease that works with the chaperones ClpA and ClpX to recognize, unfold and cleave misfolded proteins and transient proteins in the bacterial cytoplasm. Like SppA, ClpP protease also forms a large circular assembly with an axial port hole but the packing of the monomers differs from that of SppA such that a completely unique oligomeric assembly in terms of orientation, size, symmetry and exposed molecular surfaces is created (Figs. 6 and 7). In the case of ClpP, the axial hole is much smaller (~13.5 Å, Fig. 7a and b) and is formed by the globular portion of the structure (Fig. 6a), with the internal sequence extension (or "handle") creating the intercalating components that allow for two seven-fold, bowl-shaped structures to form a 14-mer spherical assembly (Fig. 6c). Additionally, each monomer of ClpP protease encodes a complete active site involving a Ser/His/Asp catalytic triad, again differing from SppA, which utilizes residues at the interface of its tandemly repeated domains to create the Ser/Lys active-site dyad (Fig. 5). The internal surface of the ClpP protease bowl-shaped complex that contains the 14 active sites is much more polar than that seen near the 4 active sites in the SppAEC bowl-shaped complex (Fig. 7).

Remarkably, when a ClpP monomer is superimposed onto the C-terminal half of a complete SppAEC monomer (Fig. 5c), the chemically important atoms within the Ser/His/Asp catalytic triad residues of ClpP superimpose with the catalytic atoms within the Ser/Lys catalytic dyad of SppAEC (Fig. 5d). Specifically, the Oγ of the serine nucleophiles superimpose as do the Nε2 of the histidine general base in ClpP and the Nε of the lysine general base of SppAEC, even though the positions of these general base residues come from sequentially and structurally disparate regions of the molecules (Fig. 5d). As is seen in other Ser/Lys proteases,15 in replacement of the aspartic acid observed in ClpP there is a second serine (Ser431) in SppA that helps coordinate the lysine general base (Fig. 5d). This residue is also conserved across the SppA enzymes (Fig. 3).

The structure of ClpP has been solved to 1.9 Å resolution in complex with a peptide-based inhibitor.13 Because of the similarity in the substrate-binding architecture in ClpP and SppAEC, it is possible to model a peptide substrate into the binding site of SppAEC based on the structure of the ClpP peptide–inhibitor complex (Fig. 8).

The model clearly shows the P1 side chain (modeled as an alanine for clarity) points into the predominantly hydrophobic S1 substrate specificity pocket. The P2 side chain is pointing out towards solvent, away from the binding site, and the P3 side chain is pointing towards the large S3 substrate specificity pocket, which has a negatively charged
surface at the bottom of the pocket. The two aliphatic side chains of residues Val379 and Ile434 form a ridge that corresponds to the dividing point between the S1 and S3 binding pockets. This is similar to what is seen in *E. coli* type I signal peptidase where the analogous residues have been shown to contribute to substrate specificity.16 The hydrophobic S1 pocket is consistent with previous analysis that showed a preference for substrates with aliphatic residues at the P1 position.2,4,17 It is possible that the relatively large and negatively charged S3 pocket may interact with the positively charged N-region of the signal peptide. Given these structural insights, it would be interesting to reinvestigate the substrate specificity of SppA.

The model is also consistent with the NH of Gly377 and Gly410 forming the SppAEC oxyanion hole by contributing hydrogen bond donors to the developing oxyanion during the transition state of the hydrolytic reaction. These two glycine residues are completely conserved (Fig. 3). The oxyanion holes in SppA and ClpP are unique among serine proteases in that they use a main-chain amide NH from the residue following the nucleophile rather than the main-chain NH of the nucleophile itself along with the penultimate residue to the nucleophile.18

**Fig. 5.** Comparison of the Ser/Lys protease SppA to the Ser/His/Asp protease ClpP. Superposition of a ClpP monomer (red) on the N-terminal region of SppA (green, residues 56–316) (a) and on the C-terminal region of SppA (blue, residues 326–549) (b). The position of the catalytic triad in ClpP is labeled. The position for the serine nucleophile in the C-terminal domain of SppA overlaps with the position of the serine nucleophile in ClpP (b), but the position of the general base in the N-terminal domain in SppA (K209) is distant from the position of the base (H122) seen in ClpP protease (a). (c) The superposition of ClpP (red) on the full SppA protein (green, blue). (d) A ball-and-stick rendering of the Ser/Lys catalytic dyad active site of sppAEC superposed on the Ser/His/Asp catalytic triad active site of ClpP protease.13

**SppA orientation relative to the membrane surface and substrate presentation**

SppAEC contains a proposed N-terminal transmembrane segment (residues 29–45) that likely tethers it to the cytoplasmic membrane (Fig. 9a). The N-terminus of our SppAEC structure (residue
56) is part of the globular domains that forms the rim of the bowl-shaped structure. Therefore, we propose SppAEC is oriented relative to the membrane surface such that its axial hole is pointing away from the inner membrane and the large opening of the bowl is associating with the membrane surface (Fig. 9a). Bioinformatic analysis and the positive inside rule19 are consistent with the C-terminal catalytic region facing the periplasmic or trans side of the inner membrane. Interestingly, surface analysis of SppAEC reveals that the largest depression (yellow surface in Fig. 9b) on the SppAEC molecular surface corresponds to the association interface between individual monomers on the inside of the bowl. Figure 9b shows the interface between two SppA monomers, one monomer in blue and the other in tan and rendered as a ribbon. These depressions lead up to the substrate-binding sites (labeled S1 and S3 in Fig. 9b). It is possible that these depressions on the surface help guide the signal peptide substrate towards the active site (red surface in Fig. 9b, the blue surface corresponds to the P1' side of the binding site) with the ring of negative charge near the base of SppA attracting the positively charged N-region of the signal peptide. Additional experiments will be needed to address the question of how signal peptides are pulled from the membrane and presented to the SppA-binding site. It is possible that

Fig. 6. A comparison of the ClpP protease and the SppA protease oligomeric assembly in terms of monomer orientation, protein complex size and symmetry. (a) and (b) show the comparison between the sevenfold ClpP and the fourfold SppA from the top end view. In ClpP, the major globular domain of the protein forms the axial hole, whereas in SppA, the extension or handle domain forms the axial hole. (c) and (d) show a comparison from the side of the protein complexes. One can see that in ClpP the handle extension domain makes the intercalating interactions between the 7-mer halves of the ClpP ball-shaped 14-mer complex, whereas in SppA the handle extension forms the axial hole.
SppA may work in concert with other protein(s) that may help facilitate the extraction of the signal peptide from the membrane for subsequent SppA-mediated hydrolysis. This would be reminiscent of the ClpAP and ClpXP chaperone/protease complexes, where ClpX and ClpA are the ATP-dependent chaperones that are required to unfold proteins in the cytosol for hydrolysis by the ClpP protease.

**Fig. 7.** A comparison of the ClpP protease and the SppA protease oligomeric assembly in terms of the electrostatic nature of the exposed molecular surfaces. (a) and (b) show the difference in the size and charge at the axial hole. (c) and (d) show the difference in the dimensions between the ball-shaped ClpP protease 14-mer (two 7-mer oligomers combined) and the tetrameric SppA that forms a bowl-shaped structure. (e) and (f) show that SppA is much more hydrophobic in the interior of the proteolytic chamber.
The protein architectural similarity in the protein fold of SppA protease and ClpP protease begs the question: Does SppA have a possible role in protein quality control at the membrane surface similar to what ClpP protease performs in the cytoplasm?

**Experimental Procedures**

### Cloning and mutagenesis

The gene *sppA* was cloned out of *E. coli* genomic DNA (K12) using the oligonucleotides: forward primer, 5′-CCA TAT GGG TGG TGA TTC GAA AGA AA-3′; reverse primer, CTC GAG TTA ACG CAT GTT GGC GCA G-3′, which contain the restriction sites NdeI and XhoI for cloning. The PCR product was cloned into the expression vector pET-28a (Novagen). The final SppA construct (SppAΔ2–46) has the first 20 residues containing the 6xHis affinity tag and the thrombin cleavage site: MGSSHHHHH-SSGLVPR-GSH-sppA followed by a Met and then the *sppA* gene starting at the codon for residue Gly47. DNA sequencing confirmed the *sppA* sequence reported in the Swiss-Prot database (accession number P08395). The plasmid pET28-sppAΔ2–46 was transformed into BL21 (DE3) *E. coli* cells. The expressed protein (SppAECΔ2–46) is 593 residues in length (including the 6xHis affinity tag and the thrombin cleavage site) and has a molecular mass of 63,849 Da and a theoretical isoelectric point of 5.9.

### Expression and purification of sppA proteins

Overnight small-scale cultures were diluted (2:100 ratio) into LB media containing kanamycin (25 μg/mL), grown at 37 °C for 3.5 h and then induced with IPTG (0.75 mM final concentration) at 27.5 °C for 3.5 h. Cells were pelleted by centrifugation and then lysed using the Avestin Emulsiflex-3C cell homogenizer. The lysate was clarified by centrifugation and then lysed using the Avestin Emulsiflex-3C cell homogenizer. The lysate was clarified by centrifugation and then finally eluted with a step gradient (100 to 500 mM imidazole with 100 mM increments). The protein eluted from the column between 200 and 400 mM imidazole. The protein was then concentrated via an Amicon ultracentrifuge filter (Millipore) and applied to a Sephacryl S-100 HiPrep 26/60 size-exclusion chromatography column on an AKTA Prime system (Pharmacia Biotech) run at 1 mL/min, with a buffer containing 50 mM Tris–HCl, pH 7.5, and 100 mM NaCl. The lysate was applied three times to the column and then the column was washed with 50 mM imidazole, 50 mM Tris–HCl, pH 7.5, 100 mM NaCl (10 column volumes) and then finally eluted with a step gradient (100 to 500 mM imidazole with 100 mM increments). Fractions containing pure sppA were analyzed by SDS-PAGE and concentrated to 8 mg/mL. Analytical size-exclusion chromatography in line with multiangle light-scattering analysis is consistent with SppAECΔ2–46 being a monodispersed tetramer in solution in the absence of detergent (data not shown). Cleavage assays show that SppAECΔ2–46 is catalytically active (data not shown). The
selenomethionine (Se-Met)-incorporated SppA ECΔ2–46 was prepared by making an overnight culture of BL21 (DE3) cells containing pET28-sppA Δ2–46 in M9 minimal media (10 mL of overnight culture was used to inoculate 1 L of M9 minimal media). All growth media were supplemented with 25 μg/mL kanamycin for selection. Cultures were grown for 8 h at 37 °C with shaking and a mixture of the following L-amino acids were added directly: 100 mg lysine, phenylalanine, threonine; 50 mg isoleucine, leucine, valine; 60 mg Se-Met. After 15 min, protein expression was induced by the addition of 0.75 mM IPTG (final concentration). The purification procedure was the same as that used for the native protein.

Fig. 9. The orientation of SppA EC with respect to the membrane surface. (a) The tetrameric SppA EC is shown in a ribbon diagram with each monomer a different color. The proposed position for the N-terminal transmembrane segment (residues 39–45) for each monomer (which is missing from the construct that was used for crystallization) is schematically shown. The position of the axial hole, the large opening of the bowl that leads to the active sites and the position of the catalytic residues relative to the membrane surface are labeled. The diagram is not to scale. (b) The interface between each SppA monomer creates the largest pocket on the SppA surface and a continuous path between the proposed membrane association surface and the active site. The interface between two SppA monomers is shown with each monomer (blue and tan) rendered in ribbon. The molecular surface of the pocket formed between the monomers is shown (yellow surface) and the position of the active site (Ser/Lys dyad in red surface) and the proposed membrane association surface is labeled. The S1 and S3 substrate-binding pockets are labeled and the S1’ side of the binding site is shown as blue surface.
Crystallization

The crystals used for SAD data collection were grown by the hanging-drop vapor-diffusion method. The crystallization drops were prepared by mixing 1 μL of protein (8 mg/mL) with 1 μL of reservoir solution and then equilibrating the drop against 500 μL of reservoir solution. The SppAEC Δ2–46 protein produced crystals in the space group P2₁ with unit cell dimensions of 93.0 Å × 153.0 Å × 102.2 Å, β = 104.2° (Se-Met), and 94.2 Å × 153.5 Å × 100.7 Å, β = 104.3° (native), with four molecules in the asymmetric unit and a Matthews coefficient of 2.7 Å³ Da⁻¹ (54.6% solvent). The optimized crystallization reservoir condition was 100 mM Tris, 3350, 200 mM K₂HPO₄. The crystallization was conducted within PHENIX version 1.324 automatically constructed density modification.24 The rest of the model was built and fit using the program COOT.25 The structure was refined using the program REFMAC5.26 The final models were approximately 80% of the polypeptide chain and per-residue contacts.32 The program CASTp33 was used to analyze the molecular surface and measure the substrate-binding site.

Data collection

Diffraction data were collected on Se-Met-incorporated crystals at beamline X4A of the Brookhaven National Laboratory, National Synchrotron Light Source, using a Q4 CCD detector and a Huber Eulerian Goniometer. The crystal-to-detector distance was 175 mm. Data were collected with 1° oscillations, and each image was exposed for 5 s. The native crystals were collected at the Advanced Light Source, Lawrence Berkeley National Laboratory, University of California at Berkeley, beamline 8.2.1 using an ADSC Q315R detector. The crystal-to-detector distance was 340 mm. Data were collected with 1° oscillation for each image was exposed for 5 s. The diffraction data were processed with the program HKL2000.22 See Table 1 for data collection statistics.

Structure determination and refinement

The SppAEC structure was solved by SAD using a data set collected at the peak wavelength (0.97925 Å), the programs HKL2MAP23 version 0.2 and AUTOSOL within PHENIX24 version 1.3.24 The program AUTOBUILD within PHENIX version 1.3.24 automatically constructed approximately 80% of the polypeptide chain and performed density modification.24 The rest of the model was built and fit using the program COOT.25 The structure was refined using the program REFMAC5.26 The final models were obtained by running restrained refinement in REFMAC5 with TLS restraints obtained from the TLS motion-determination server.27 The native structure was solved by molecular replacement using chain A from the SAD structure as a search model and then refined as described above. The data collection, phasing and refinement statistics are summarized in Table 1.
References


