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Structural basis of lipoprotein signal peptidase II action and inhibition by the antibiotic globomycin

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Supplementary Materials Information: 8 Figures, 1 Table, 5 Movies, Materials and Methods, Author Information

Abstract

With functions that range from cell envelope structure and stability to signal transduction and transport, lipoproteins make up 2-3 % of bacterial genomes and play critical roles in bacterial physiology, pathogenicity and antibiotic resistance. Lipoproteins are synthesized with a signal peptide securing them to the cytoplasmic membrane with the lipoprotein domain in the periplasm or outside the cell. Post-translational processing requires a signal peptidase II (LspA) that removes the signal peptide. Here, we report the crystal structure of LspA from *Pseudomonas aeruginosa* in complex with the antimicrobial, globomycin at x Å resolution. Conserved residues in the active site and mutagenesis studies identify LspA as an aspartyl peptidase. In a striking example of molecular mimicry, globomycin appears to inhibit by acting as a non-cleavable peptide that includes a tetrahedral transition-state analogue. This complex structure should inform rational antibiotic drug discovery.

One Sentence Summary

A crystal structure shows how a bacterial enzyme functions in lipoprotein maturation and is inhibited by the antibiotic globomycin.

We are interested in proceeding as a report – manuscript must be cut to fit our report format. Need to cut about 500 words of text beyond the cutting I have done (the main text double space, no figs, should start on page 3 and end on page 9 – this is giving some space beyond our usual report constraint)

The threat of antibiotic resistance is recognized as a serious public health issue. Governments have introduced incentives to encourage work on anti-infectives by major drug makers in the face of a rise in antimicrobial-resistant infections globally.

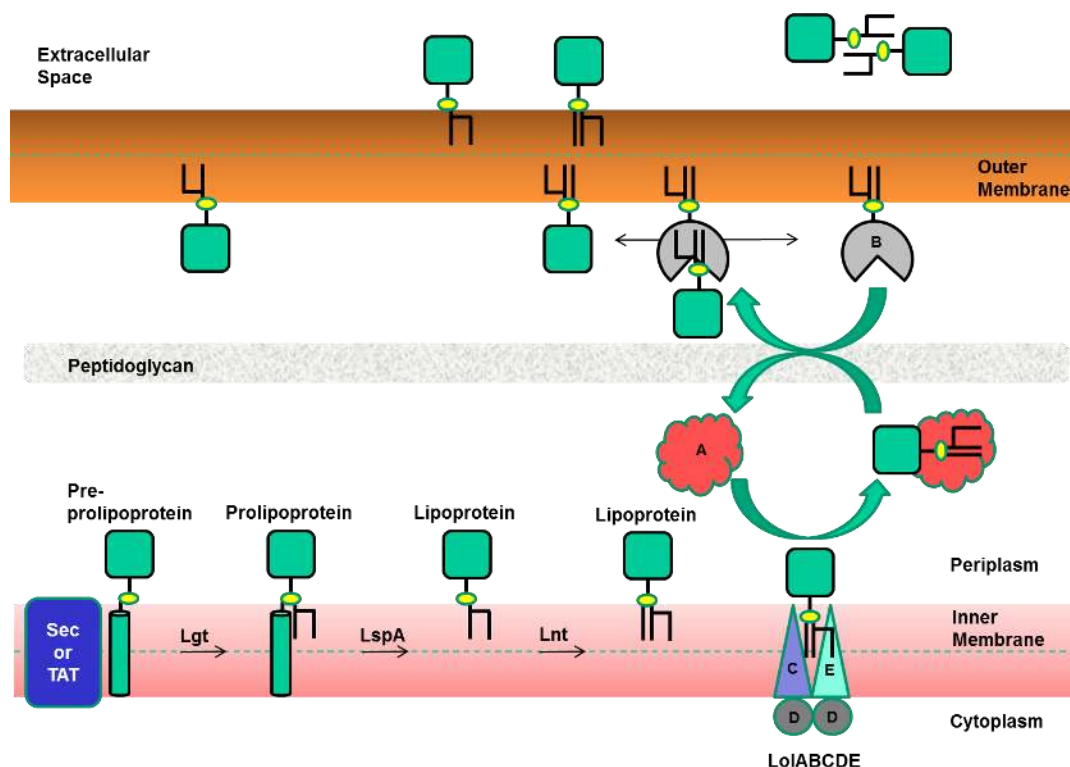


Fig. 1. Post-translational processing leading to lipoprotein maturation in Gram-negative bacteria.

Lipoproteins are synthesized as pre-prolipoprotein precursors via the Sec or TAT pathways with an N-terminal signal peptide (green cylinder) securing them to the cytoplasmic membrane and the lipoprotein domain (green square) in the periplasm. Post-translational processing requires, at a minimum, the sequential action of lipoprotein diacylglycerol transferase, Lgt, to lipid modify with a diacylglycerol and signal peptidase II, LspA, to remove the signal peptide. For some lipoproteins, the lipoprotein N-acyl transferase, Lnt, is required to N-acylate the N-terminal cysteine of the lipoprotein. LspA cuts in the lipobox, a consensus sequence of form LAGC* where C* (yellow oval) has been dagylated by Lgt, to the amino side of the C*. Trafficking to the outer membrane uses the lipoprotein outer membrane localization (Lol) pathway that involves the ABC transporter, LolCDE, a carrier chaperone, LolA, and a receptor, LolB, itself a lipoprotein. Lipoproteins reside mostly on the outer leaflet of the inner membrane and on the inner leaflet of the outer membrane facing into the periplasm. Some are located facing and in the extracellular space.

The enzymes involved in bacterial lipoprotein post-translational processing (**Fig. 1**) are essential in many pathogenic organisms and have no equivalents in humans, making them potential drug targets (2-4). LspA, as a key enzyme involved in the post-translational processing of close to two hundred lipoproteins in *P. aeruginosa*, an opportunistic human pathogen, is a target for antibiotic development (2, 5). LspA does not have sequence homology to proteins of known structure. A DALI search failed to identify structural homologs, suggesting the peptidase exists in an entirely

new fold. Globomycin, an antibiotic produced by select strains of *Streptomyces*, inhibits LspA (2). This cyclic depsipeptide (**fig. S1**) is an antimicrobial effective against Gram-negative bacteria. Synthetic analogs also inhibit the growth of Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA) (2).

P. aeruginosa has 175 lipoproteins (5) and presumably all are processed by a promiscuous LspA. The prolipoprotein substrates of LspA are cleaved at a four-residue consensus sequence known as the lipobox that always ends with a cysteine. The prolipoproteins consist of an N-terminal signal peptide that includes the first three residues of the lipobox and a C-terminal lipoprotein starting with the lipobox cysteine. Signal peptides range in length from 13 to 34 residues and are predicted to be transmembrane helices. The N-terminal stretch of amino acids, the N-segment, is 2 to 21 amino acids long and includes at least one signature cationic residue. Between the N-segment and the lipobox is the 6 to 17 residue long hydrophobic, or H-segment, that is rich in apolar residues. The lipobox has a consensus sequence Leu-Ala-Gly-Cys (LAGC, $L^{-3}A^{-2}G^{-1}C^{+1}$) and, in the prolipoprotein form, the cysteine is in thioether linkage to diacylglycerol (DAG). We refer to the dagylated cysteine as Cys*.

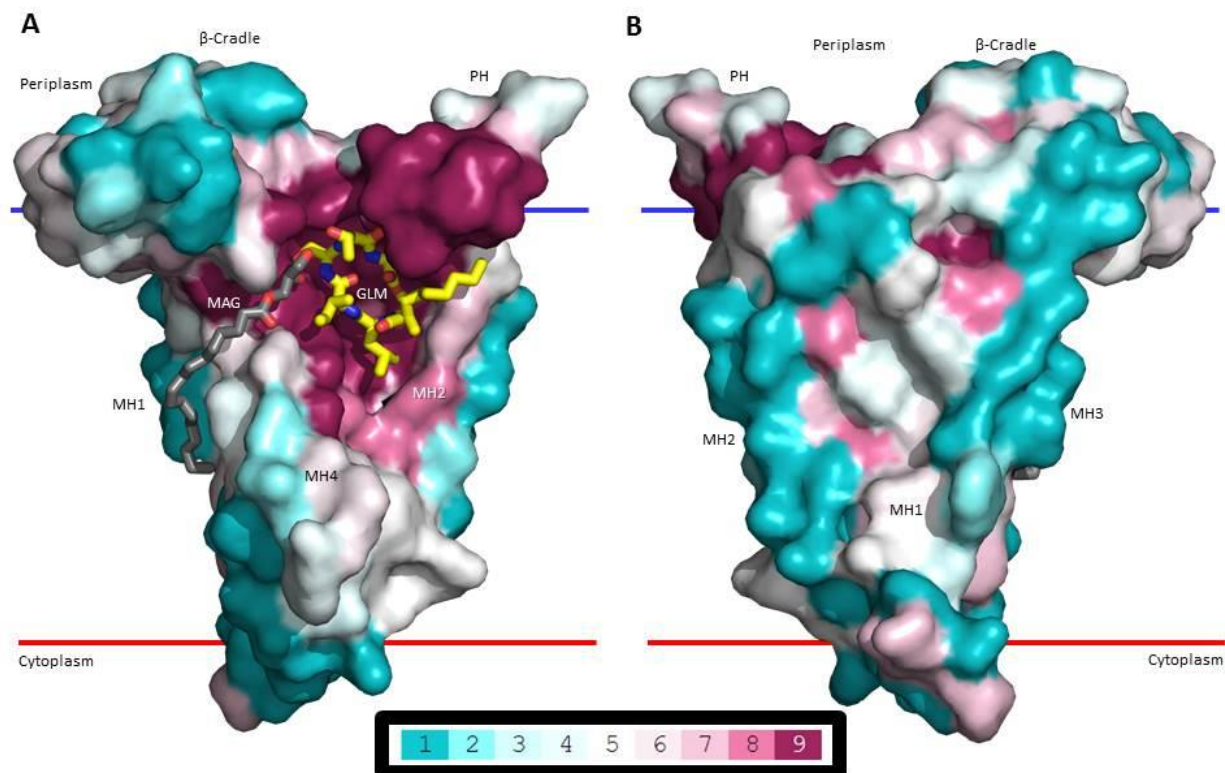
While predicted to be part of the signal peptide helix, it seems likely that the lipobox adopts an extended conformation to provide access for cleavage to the scissile Gly-Cys* bond and to enable the C-terminal lipoprotein to reside in the periplasm. In the case of shorter signal peptides, the distance between the cationic N-segment which is anchored at the cytoplasmic interface and the lipoprotein that is in the periplasm, may induce the helix to unwind locally. The high frequency of Gly in the lipobox could facilitate uncoiling. Residues at the N-terminus of the cleaved lipoprotein provide signals for downstream trafficking machinery (2-4) and are known (6) or have been speculated to be uncoiled (3). The structures of only a few lipoproteins are available. Examples include CytC (PDB ID, 3CP5), AlgK (3E4B), Lpp (1EQ7), CsgG (4Q79) and the four lipoproteins of the BAM complex, BamB (3PRW), C (2YH5), D (3QKY) and E (2YH9) (7). Together, these observations suggest that the lipobox and the first few residues that follow it into the lipoprotein are in an extended conformation.

We sought to obtain a high-resolution crystal structure of LspA to gain insights into mechanism of action. Crystallization trials were conducted using recombinant LspA from *P. aeruginosa* (strain PAO1) (5). Fos-choline 12 proved an effective solubilizing and refolding detergent, and affinity and size-exclusion chromatography provided material of high purity. *In meso* (lipid cubic phase) crystallization trials (8) with the apo-enzyme failed to produce crystals. In the presence of globomycin however, crystals grew at 20 °C with monoolein (9.9 MAG) as host lipid. A structure of the complex was obtained with a resolution of 2.8 Å using seleno-methionine single-wavelength anomalous diffraction phasing (**Fig. 2**).

LspA is a small protein consisting of 169 amino acids (5). It is a monomer in the crystal. Structurally, the protein can be broken down into two domains each assembled from secondary structure elements from discontinuous stretches of the protein chain (**Fig. 2**). The first consists of

four transmembrane helices (MH1-4) with the N- and C-termini in the cytoplasm. The second is the periplasmic domain which has two sub-domains. The bigger of the two is a 4-stranded, amphiphilic β -sheet which rests on the membrane. The sheet has the appearance of a cupped hand or cradle (β -cradle) that extends away from the helical core of the protein. Its hydrophobic surface is in contact with the membrane, while its polar surface faces the periplasm. The second sub-domain consists of a long loop with a single helical turn, the periplasmic helix (PH). The PH also sits on the membrane with the long axis of the periplasmic PH loop orthogonal to that of the β -cradle.

Fig. 2. LspA-globomycin complex structure. (A) Cartoon representation of LspA viewed from within the membrane with the membrane interface shown as horizontal lines and globomycin as a stick figure (yellow carbons). Transmembrane helices are labelled MH1-4. The β -stranded cradle (labelled) at the membrane interface is poised to accommodate the soluble domain of the prolipoprotein substrate and lipoprotein product. The C-terminal “strand” of the β -cradle leading into the proposed active site is interrupted by conserved Pro137 and does not conform to standard β -sheet hydrogen bonding patterns. (B) As in (A) rotated by $\sim 60^\circ$. (C) As in (A) viewed from the periplasm. (D) 2Fo-Fc simulated-annealing composite OMIT map contoured at 1σ and carved at 2.3 \AA around globomycin. Globomycin appears to inhibit by mimicking a non-cleavable, tetrahedral transition-state in the reaction. Proposed catalytic aspartates, Asp124 and Asp143, are shown on either side of the β -hydroxyl of globomycin serine (g-Ser). Globomycin and catalytic aspartates are shown in stick representation with yellow and slate (A-C) or cyan (D) carbons, respectively.



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Fig. 3. Conserved residue surface representation of LspA based on an analysis of 485 orthologs. The orthologs had between 35% and 95% sequence identity with the signal peptidase II of *P. aeruginosa* (PAO1). (A) View into the globomycin binding site from within the membrane. LspA in surface representation. Globomycin and MAG as sticks with yellow and grey carbons, respectively. (B) As in (A) rotated by 180°. The color coding is as follows: cyan, white and purple correspond to low, average and high conservation, respectively. The analysis was done using the ConSurf server (9).

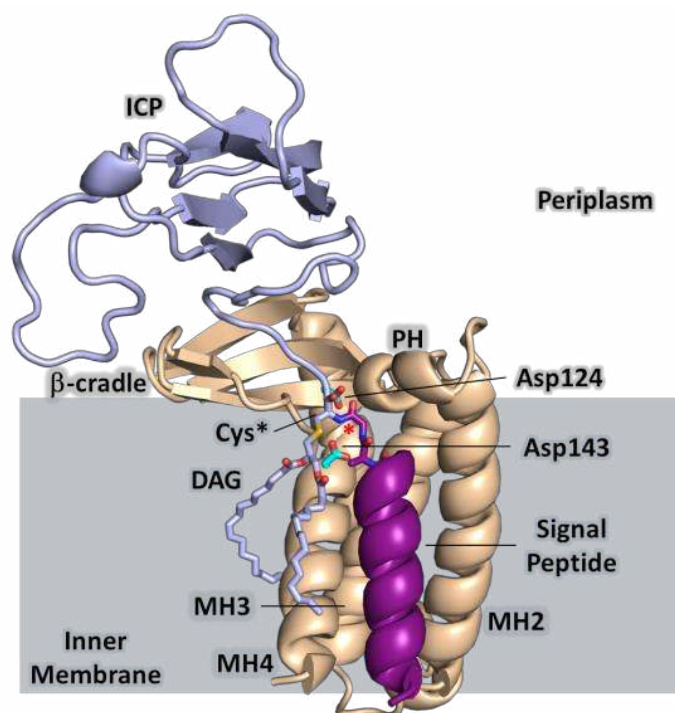


Fig. 4. The prolipoprotein, proICP, from *P. aeruginosa* docked into LspA. The signal peptide helix (magenta) docks into the pocket created by MH2, MH3 and MH4. The lipobox cysteine, Cys*, is dagylated (light blue sticks) and the lipoprotein (light blue cartoon) extends across the β -cradle into the periplasm. The complex, as shown, is stable in MDS, especially in the region of the signal peptide helix and the lipobox. Full details of docking and simulations are available under Methods. Catalytic dyad aspartates shown as stick with cyan carbons.

The crystal structure of globomycin (10) was used to guide modelling into a distinctive, donut-shaped region of electron density in the between MH2, MH3 and MH4 on the periplasmic side of the membrane (**Fig. 2D**). The ligand density allowed unambiguous placement and orientation of the entire globomycin molecule, a 19-member cyclic depsipeptide that includes an α -methyl- β -hydroxy fatty acid, *N*-methyl-L-Leu (g·Leu), L-*allo*-Ile (g·Ile), L-Ser (g·Ser), L-*allo*-Thr (g·Thr) and Gly (g·Gly) (11) (**fig. S1**). Globomycin is amphiphilic with polar and apolar halves. g·Ser and g·Thr comprise the polar half while the hydroxy fatty acid, g·Leu and g·Ile make up the apolar half. As expected, the apolar half of globomycin extends into the membrane, and its polar end faces the interface. In the unbound state, MDS show globomycin partitions into the membrane oriented essentially as it is in the complex (**fig. S2**). The PH loop sits over and blocks direct access from the periplasm to the active site suggesting that globomycin first partitions into the lipid bilayer and then diffuses laterally into the active site to inhibit the enzyme.

Conserved residues decorate the globomycin binding pocket (fig 3), a region that has been implicated in peptidase activity (12).

Globomycin is firmly anchored to LspA by hydrogen bonds and hydrophobic interactions (**Fig. 2D and fig. S3**). The most notable hydrogen bond is that between the β -hydroxy of g·Ser and the carboxyl of Asp143, tentatively identified as one of the catalytic dyad aspartates (12). In MDS, this interaction persists throughout the 500 ns simulation. The backbone carbonyls of g·Leu, g·Ile and g·Ser, to one side of the globomycin ring, face the protein and hydrogen bond with the side chains of strictly conserved Asn112, Arg116. The side chain of g·Leu, as well as the acyl chain of the hydroxy fatty acid, have extensive hydrophobic interactions with apolar residues in the protein (**Fig. 2D**). These are particularly well developed in the case of the fatty acid which is in close contact with the membrane exposed surface of the PH and with residues on MH2. MDS confirms these interactions (**fig. S3**).

LspA was investigated by MDS in the complex and apo forms. In both, the enzyme was stable in a POPE:POPG membrane (**movies S1 and S2**). Through the 500 ns simulation, the transmembrane helix bundle held together as a unit while the β -cradle and PH loop remained anchored at the bilayer interface. In the apo form, simulations showed a minor rearrangement of the active site with a network of hydrogen bonds emerging that involve Asn54, Asp124, Asn140, Asp143 and Asn112, all highly conserved residues. In addition, there was enhanced flexibility of the PH loop, which moved laterally at the interface whilst remaining anchored to the membrane by highly conserved Phe59 and Phe61.

If globomycin in the complex indicates the active site in LspA, an examination of this part of the protein immediately suggests how the prolipoprotein substrate, orients itself for binding and Michaelis complex formation. The signal peptide helix of the prolipoprotein is the right size and shape to slot into the space between MH2 and MH4 of LspA (**Fig. 4**). Upon signal peptide helix binding, the lipobox of the substrate would sit in the active site. Presumably, it would reside with its backbone, in an extended conformation, similar to and perhaps mapping directly onto that of the g·Leu-g·Ile-g·Ser tripeptide in globomycin (**Fig. 5**), as observed in the complex structure. The Cys* of the lipobox, with its bound DAG, would now reside further up in the active site positioning the lipoprotein in the periplasm (**Fig. 4**). With the

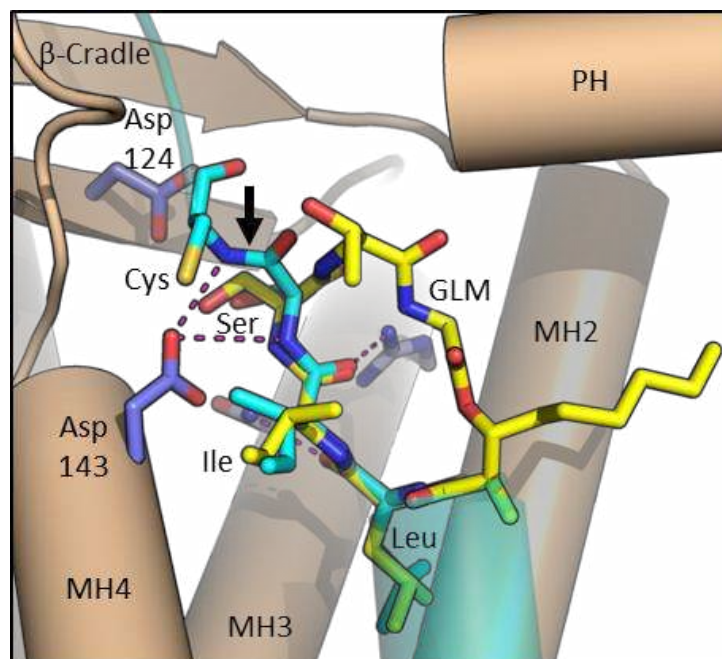


Fig. 5. Globomycin bound to LspA with a lipobox Leu-Ile-Ser-Cys tetrapeptide docked manually onto the g-Leu-g-Ile-g-Ser peptide backbone of globomycin. The docking exercise places the scissile Ser-Cys bond of the lipobox (black arrow) next to the catalytic dyad aspartyls, Asp124 and Asp143. LspA is shown in cartoon representation with cylindrical helices. Globomycin, the lipobox peptide and the catalytic aspartyls are shown as sticks with yellow, cyan and blue carbons, respectively. The proposed location of the prolipoprotein chain extending N- and C-terminally from the lipobox is indicated in transparent cyan cartoon representation. Need to cut to 4 figures this can be fig 4b

this alignment, the scissile Gly-Cys* bond is extended and positioned between the carboxyls of the proposed catalytic residues, Asp124 and Asp143. The lipid modification on Cys*, with its long hydrophobic chains, must remain anchored in the membrane. An indication as to where its glycerol head group and part of one of the chains might reside comes from a lobe of electron density modelled in the complex structure as a monoacylglycerol (MAG, the host lipid used for crystallization) which sits to one side of the active site (**fig. S4**). While host lipid density, at 2.8 Å resolution, is often poorly and incompletely defined, density for the active site lipid is clearly present in all four molecules of the asymmetric unit extending to C7 or C8 of the MAG acyl chain in omit maps. We suggest therefore that one of the DAG acyl chains of the prolipoprotein substrate occupies a similar position on LspA. Reminiscent of how MAG is bound in the complex, the head group of the DAG moiety is likely clamped in a groove where MH4 and the β -cradle come together thereby helping position and orient the scissile bond in the active site. The above model for how the prolipoprotein substrate interacts with LspA is supported by MDS (**Fig. 4 and movie S3**).

Residues 56 to 62, that include the PH, are highly conserved. This stretch of protein sits on the surface of the membrane directly over the active site (**Fig. 2**) and . Constrains extension of the substrate from the active site to the periplasm a confining space between the β -cradle and PH

loop sub-domains.. In this model the Cys* of the substrate is clamped between the β -cradle and the PH loop with the dipole of PH aligned with the carbonyl oxygen of Cys*. (fig 4)

Despite lacking the consensus sequence, Asp-Thr-Gly, of aspartyl proteases (13), LspA has been tentatively identified as an aspartyl endopeptidase (12). In the absence of evidence to the contrary, we assume here that the catalytic mechanism is as described for other aspartyl proteases (13). Accordingly, we envision Michaelis complex formation involving a conjunction in the active site of two aspartates, a catalytic water and the scissile bond. Using globomycin to demark the active site, two aspartate residues, Asp124 and Asp143, with suitably poised side chain carboxyls are apparent in the structure that, in all likelihood, represent the catalytic dyad (**Fig. 2D**). We propose therefore that these aspartates play a general acid-base role with the one closest to the proposed water (not seen in the complex but apparent in MDS, **movie S4**) being charged and abstracting a proton from it creating a potent nucleophile (**fig. S1B**). The nucleophile attacks the carbonyl carbon of the scissile bond forming a tetrahedral intermediate. Protonation of the amide nitrogen in the scissile bond and rearrangement causes the tetrahedral intermediate to collapse and hydrolysis products to form. Separation of the substrate's binding moieties by proteolysis would logically result in a sharply lower binding affinity of the two reaction products compared to substrate. The freed lipoprotein product is anchored in the membrane only by the DAG chains of its C-terminal Cys* and should readily diffuse out of the active site thereby resetting LspA for another round of catalysis. Further processing of the signal peptide and lipoprotein products of the LspA reaction, when it occurs, involves signal peptide peptidases and N-acylation by lipoprotein N-acyl transferase, Lnt, respectively (**Fig. 1**).

Many aspartyl proteases are important drug targets. The hypertension regulator, renin (14, 15), and HIV protease (13, 16) are examples. Of the thousands of inhibitors designed to target aspartyl proteases most contain a non-cleavable core structure of the hydroxyethylene or hydroxyethylamine type designed to mimic the tetrahedral intermediate in the proteolytic reaction (7) (**fig. S1C-E**). For those that have been crystallized bound to target proteases the hydroxyl group sits between and is within hydrogen bonding distance of the two catalytic aspartates. Strikingly, the hydroxyethylamide of g-Ser in globomycin incorporates elements of a non-cleavable transition-state isostere. In the complex, the hydroxyl group of g-Ser nestles between the two catalytic aspartates, Asp124 and Asp143, and although only hydrogen bonded to Asp143 in the complex, it is in position to coordinate both (**Fig. 2D**). This suggests that part of the globomycin molecule behaves as a non-cleavable tetrahedral transition state analogue (**fig. S1**) and that the antibiotic is a competitive inhibitor of LspA. Further mimicking other features of the prolipoprotein substrate, globomycin contains the peptide backbone and side chains corresponding to the P⁻³, P⁻² and P⁻¹ sites relative to the scissile bond in the lipobox. As noted, this feature of globomycin has been used to model a convincing lipobox and prolipoprotein into the substrate binding and active sites of LspA (**Figs. 4 and 5**). The prolipoprotein model has been exploited successfully in molecular dynamics simulations (MDS) (**Fig. 4**).

An analysis of deduced amino acid sequences of LspAs from 485 organisms having between 35% and 95% sequence identity with the signal peptidase II of PAO1 identified fourteen strictly conserved residues (D23, K27, N54, G56, G108, A109, N112, R116, V122, D124, 139F, N140, A142, D143) (12). The bulk of these map onto the proposed binding and active sites in the LspA complex (**Fig. 3**). This lends credence to the location of both sites in the protein as identified in the complex structure. All strictly conserved polar residues are involved in a hydrogen bonded network extending into the membrane between the two periplasmic sub-domains (**fig. S5**) that serves multiple purposes. It provides structural integrity and definition to the active and binding sites. At the same time, it positions and orients active site residues optimally for pK_a modulation and catalysis. Such a hydrogen bonded network of polar residues facilitates access to the active site of catalytic water molecules from the periplasm, confirmed in MDS (**movie S4**).

Mutagenesis studies performed on LspA from *Bacillus subtilis* identified six amino acids, corresponding to Asp23, Asn112, Asp115, Asn140, Ala142, and Asp143 in *P. aeruginosa*, as being important for activity (12). Only Asp23 was required for stability, suggesting the other five residues were needed for catalysis, active site geometry, or the recognition of prolipoproteins (12). Four of the five residues map onto the proposed active site in the LspA-globomycin complex in ways that make sense. However, Asp115 is a highly conserved residue on the periplasmic end of MH3 (**fig. S5**). It coordinates with conserved Lys27 which interacts indirectly with catalytic Asp143 via conserved Asn112. Mutating it to Ala inhibited peptidase activity (12). In the Tjalsma *et al.* study (12), Asp115 and Asp143 were proposed to form the catalytic dyad. In light of the current structure, this is unlikely. We conclude that the catalytic dyad is Asp124 and Asp143. Both are strictly conserved and suitably disposed for proteolysis.

respectively, suggesting that this residue is functionally important but not catalytic. MDS revealed transient interactions involving Asp115 not seen in the crystal structure and provide a plausible explanation for how these two mutations compromise catalysis to different degrees (**movie S5**). The Arg116Ala mutant was inactive, as expected given Arg116's interaction with globomycin and its proposed role in orienting the lipobox backbone of the peptide substrate in the active site. Globomycin inhibited the enzyme (**Fig. 6C**).

Synthetic analogues of globomycin have been tested for efficacy against a number of bacterial species as part of a structure-activity relationship study (11). The most effective congeners, which were ten-times more potent than globomycin, were modifications where the fatty acyl chain was lengthened. This finding makes sense in light of the complex structure where a longer chain would provide for more extensive interactions with the apolar surface of MH2 (**Fig. 2D**). It would also increase solubility in the membrane which could contribute to potency. The hydroxyl group in g.Ser was shown to be essential for globomycin inhibitory activity which agrees with this residue's proposed role as a transition state mimic.

P. aeruginosa has 175 different lipoproteins (5). All must be processed by LspA. The signal peptide and extramembrane parts of lipoproteins differ from one another in size, sequence and structure to sizable degrees. How LspA is able to recognize and to process close to 200 distinct prolipoproteins as substrates and yet perform catalysis with each with fidelity is an intriguing question. A feature common to all is the dagylated lipobox cysteine. This cysteine is distinctive from LspA's perspective likely because it resides accessible at the membrane interface where it is oriented with its C-terminus extending out of the membrane attached to the extramembrane lipoprotein and with its side chain and N-terminus toward the membrane anchored by the DAG moiety and the signal peptide, respectively (**fig. S7**). Presumably, this unique disposition and set of linkages are what identify it, in all 175 prolipoproteins, for binding and cleavage by LspA. The LspA structure reported here has grooves and clefts radiating about the active site with remarkable shape complementarity to this trigonal feature of the prolipoprotein substrate. Upon docking, the scissile bond of the substrate sits in the active site of the enzyme poised for cleavage. Thus, while LspA is highly specific in terms of the proteolytic reaction it catalyzes, its impressive substrate promiscuity can be explained by a shape complementarity around the scissile bond shared by all prolipoproteins.

The complex structure reported here can be used to inform the design of globomycin analogues with improved binding, efficacy, selectivity and pharmacokinetic properties. As an alternative, since LspA is an aspartyl endopeptidase, it is a suitable target with which to explore the thousands of inhibitors developed for other medically relevant aspartyl proteases (13, 16). While the sequence identity of LspA from *P. aeruginosa* and MRSA is only 32%, the majority of active site residues are conserved. MDS of both the apo and globomycin-bound homology models of MRSA LspA show remarkable stability and fidelity to the observed dynamics of the crystal structure of *P. aeruginosa* LspA, suggesting the model is a faithful representation of the MRSA

homologue (**fig. S8**). In the absence of a crystal structure of MRSA LspA, these homology models should prove useful for drug design and discovery.

Based on the complex structure, globomycin is an antibiotic against which resistance, through point mutations in LspA that do not compromise catalysis, is hard to conceive. The binding sites of the prolipoprotein substrate and globomycin overlap while the active and binding site residues are networked to such degrees that any mutation perturbing globomycin's interactions with the enzyme would likely also impact on how LspA binds and cleaves its substrate. Since antibiotics that do not elicit resistance are invaluable medically (18), lessons in effective drug design might be learned from the Actinomycetes, microbes that may well have achieved this in globomycin.

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SUPPLEMENTARY MATERIALS

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References (19-39)

Materials and Methods

Figs. S1 to S8

Movies S1 to S5

Author information