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Autotransporter structure reveals intra-barrel cleavage followed by conformational changes

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Abstract

Autotransporters are virulence factors produced by Gram-negative bacteria that consist of two domains, an N-terminal “passenger domain” and a C-terminal “ β -domain”. β -domains form β -barrel structures in the outer membrane while passenger domains are translocated into the extracellular space. In some autotransporters, the two domains are separated by proteolytic cleavage. Using X-ray crystallography, we solved the 2.7 Å structure of the post-cleavage state of the β -domain of EspP, an autotransporter produced by *E. coli* O157:H7. The structure consists of a 12-stranded β -barrel with the passenger / β -domain cleavage junction located inside the barrel pore, approximately mid-way between the extracellular and periplasmic surfaces of the outer membrane. The structure reveals an unprecedented intra-barrel cleavage mechanism and suggests that two conformational changes occur in the β -domain post-cleavage, one conferring increased stability on the β -domain and another restricting access to the barrel pore.

Keywords

autotransporter; secretion; outer membrane; β -domain

Autotransporters represent a large family of virulence proteins secreted by Gram-negative bacteria¹⁻⁵. All autotransporters contain a cleavable signal peptide, an N-terminal passenger domain that carries the virulence function, and a C-terminal β -domain. Passenger domains vary in size but are usually very large (~600 - 3000 residues). The β -domains of “classical” autotransporters are typically ~250 - 300 residues whereas the β -domains of a distinct subfamily of trimeric autotransporters are only ~70 residues. Autotransporters are translocated across the inner membrane by the Sec machinery. After transiting the periplasm, β -domains are inserted into the outer membrane (OM) as β -barrels and passenger domains are translocated across the OM into the extracellular space. Once translocated, a passenger domain can remain associated with the OM or be cleaved and released from the β -domain⁶. The structures of four passenger domains have been solved as either full-length proteins or fragments⁷⁻¹⁰ and were

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AUTHOR CONTRIBUTIONS

T.J.B. purified, crystallized, and determined the crystal structure of EspP. N.D. and H.D.B. designed and performed mutagenesis and cell biological assays. P.L. and S.K.B. were involved in crystallization, data collection, structure analysis, and discussions.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

shown to contain β -solenoid motifs (see Supplementary Fig. 1 online)¹¹. The structures of the β -domains of NalP, a classical autotransporter from *Neisseria meningitidis*¹², and Hia, a trimeric autotransporter from *Haemophilus influenzae*¹³, have also been solved. The NalP β -domain crystallized as a monomeric 12-stranded β -barrel with a ~30 residue segment of the passenger domain traversing the pore in an α -helical conformation. The Hia β -domain is also a single 12-stranded β -barrel, but it is assembled from three subunits that each contribute four β -strands. A short passenger domain segment derived from each of the three subunits is embedded inside the β -domain pore.

Several models have been proposed to explain passenger domain translocation¹²⁻¹⁵ (recently reviewed by Dautin and Bernstein¹⁶). In one model, the C-terminus of the passenger domain is folded into the β -domain pore in the periplasm in a post-translocation conformation. The prefolded β -domain is then inserted into the OM and the passenger domain is transported across the OM by a concerted mechanism that possibly involves Omp85, an essential protein that promotes OM protein integration and assembly¹⁷. An advantage of this model is that it circumvents the need for one or more passenger domains to be translocated through a relatively small barrel pore in the absence of an external energy source. A second translocation model focuses on the β -solenoid motifs found in passenger domains, which could supply the energy needed for translocation by folding on the extracellular side of the OM once a small portion has reached the cell surface¹¹. In this model, a short hairpin comprising the C-terminus of the passenger domain is positioned inside the barrel pore with its tip protruding into the extracellular space. Folding at the tip of the hairpin would then pull the rest of the passenger domain through the pore. A third model is based on the observation that the β -domain of IgA protease forms multimeric ring-like structures when the protein is produced in *E. coli*¹⁸. The central cavity is about 20 Å in diameter, and was postulated to transport multiple passenger domains.

The focus of this study, EspP, is a classical autotransporter associated with diarrheagenic strains of *E. coli*¹⁹. It belongs to the SPATE (serine protease autotransporters of Enterobacteriaceae) family of autotransporters, whose passengers encode serine proteases that cleave various mammalian proteins^{6,20}. Biochemical studies have indicated that EspP is a monomer like NalP¹⁵. Once the EspP passenger domain is translocated across the OM, it is cleaved from the membrane embedded β -domain between two asparagine residues (Asn1023/Asn1024) and released from the cell surface. The Asn/Asn cleavage site defines the boundary of the EspP passenger domain (residues 56 -1023) and β -domain (residues 1024 - 1300)²¹. Although the passenger domain contains a serine protease motif located at residues 261 - 264, this motif is not used to cleave the two domains^{22,23}.

To learn what happens to the β -domain after cleavage and release of the passenger domain, we determined the crystal structure of the native EspP β -domain at 2.7 Å resolution. This is the first structure of an autotransporter β -domain post-cleavage, and it consists of a monomeric 12-stranded β -barrel with its N-terminal 15 residues inserted into the barrel lumen from the periplasmic side. In agreement with a recently proposed autocatalytic cleavage mechanism²⁴, residues implicated in cleavage are located deep inside the β -barrel, in a region of EspP that would be embedded in the OM. The structure suggests that two discrete conformational changes occur after cleavage and release of the passenger domain: one confers increased stability on the β -domain and another restricts access to the barrel pore. Our structure does not support an oligomeric translocation model, but rather a model in which a single β -barrel facilitates the translocation of a single passenger domain to the extracellular surface¹⁵.

RESULTS

The EspP β -domain consists of a β -barrel with a small domain in the pore

Crystals of the EspP β -domain were grown from protein that was targeted to *E. coli* outer membranes and naturally cleaved between the passenger- and β -domains. The best crystals grew in the presence of the detergents octyl- β -D-glucoside and Cymal®-1, and we solved the structure by multiwavelength anomalous dispersion using selenomethionine-substituted protein. Representative electron density can be found in Supplementary Figure 2 online.

The β -domain of EspP begins with a short α -helix (residues 1024 - 1028) that is connected through a linker loop (residues 1029 - 1038) to a 12-stranded β -barrel (residues 1039 - 1300). The strands of the β -barrel are anti-parallel and connected by short periplasmic turns and extracellular loops of variable length. Beta strands range in length from 11 to 21 residues; the shortest extracellular loops contain just 4 residues, while the longest loop contains 18 residues (Fig. 1a,1b). Extracellular loop 5 (residues 1236 - 1253) folds into the pore of the β -barrel, where it makes extensive contacts with the β -barrel and restricts solvent access from the extracellular side (Fig. 1c,1d). Loop 5 is not involved in any crystal contacts (data not shown). In contrast to the numerous interactions observed between loop 5 and the β -barrel, extracellular loops 3 and 4 exhibit high mobility and are therefore partially unresolved in the crystal structure.

The β -barrel has a shear number of 14²⁵, with an ellipsoid shape described by major and minor axes of approximately 27 Å and 19 Å, as calculated from C_{α} positions. The actual pore size (in the absence of the N-terminal domain) is smaller toward the periplasmic surface and larger toward the extracellular surface, with an effective diameter ranging from ~6.4 Å to ~11.0 Å²⁶. The height of the β -domain is approximately 70 Å while the OM thickness of an *E. coli* strain K-12 is estimated to be 69 Å²⁷. Therefore, the EspP β -domain is unlikely to protrude substantially beyond the cell surface. The interior of the β -barrel is populated primarily by hydrophilic residues; there are a total of 53 hydrophilic and 23 hydrophobic sidechains that line the pore. The exterior of the β -barrel is charged at the top and bottom with a hydrophobic belt in the middle. This belt marks the region of EspP that is embedded in the hydrophobic portion of the OM. As seen for many other OM proteins, numerous aromatic residues delineate the hydrophobic belt (Fig. 1b).

A short α -helix is inserted inside the barrel pore from the periplasmic side of the OM in an orientation perpendicular to the barrel axis. It is connected to the first strand of the β -barrel by the linker loop (Fig. 1b,1e). The α -helix and linker loop are positioned inside the β -barrel by 12 hydrogen bonds (data not shown). The α -helix is predominantly positively charged and forms hydrogen bonds with an “acidic cluster” of residues (Asp1120, Glu1154, Glu1172) on the inside wall of the β -barrel (Fig. 2). The α -helix and linker loop block access from the periplasm, while extracellular loop 5 blocks access from the extracellular space, such that there is no clear path through the barrel pore after cleavage. Since the β -domain remains in the OM after translocation of the passenger domain, the absence of an accessible pore would protect the bacterium from unwanted influx or efflux.

EspP appears to change conformation after releasing its passenger

The post-cleavage structure of the EspP β -domain does not reveal what EspP looks like prior to passenger domain cleavage. However, a comparison with NalP¹² suggests that conformational changes occur in the EspP β -domain once the passenger domain is cleaved and released. EspP and NalP are only 17% identical based on a structural alignment (see Supplementary Fig. 3 online)²⁸, but superimposition of the β -barrels using DaliLite²⁹ resulted in an rmsd of 1.7 Å for β -strand C_{α} positions and 2.4 Å for all C_{α} atoms, indicating marked

structural homology. Whereas EspP cleavage occurs in the barrel pore (discussed below), NalP cleavage occurs in the extracellular space by a mechanism involving the serine protease motif encoded in its passenger domain³⁰.

Secondary structure predictions suggest that like NalP, the region of EspP surrounding the cleavage site forms a long (~25 residues) α -helix prior to cleavage (see Supplementary Fig. 4 online). After cleavage, the EspP passenger domain is released (Fig. 3a,3b) and the β -domain appears to undergo two conformational changes. First, the residual α -helix remaining after cleavage likely adopts an orientation perpendicular to the barrel axis (Fig. 3c), allowing the newly created and positively charged N-terminus of the α -helix to interact optimally with the acidic cluster of residues on the interior wall of the β -barrel. Second, extracellular loop 5 probably extends outward prior to passenger domain cleavage. Once the passenger domain has been released, loop 5 folds into the barrel as seen in the crystal structure, closing the pore from the extracellular side of the OM (Fig. 3c).

Passenger domain cleavage confers added stability on the β -domain

β -barrel OM proteins often remain folded in SDS-PAGE sample buffer unless the sample is heated. Additionally, folded β -barrel proteins are more compact and migrate faster than unfolded β -barrel proteins on SDS-PAGE gels, allowing the two forms to be easily separated and visualized. We took advantage of these properties to examine the stability of the EspP β -domain.

As a control, we used EspP Δ 1, a truncation mutant of native EspP that contains 116 residues of the passenger domain followed by the wild-type β -domain. The passenger domain of EspP Δ 1 is translocated and cleaved similarly to full-length EspP²². After cleavage, the β -domain of EspP Δ 1 is extremely stable, exhibiting a 50% denaturation temperature of 84°C in SDS-PAGE sample buffer (Fig. 4, panel 1). To investigate the stability of the β -domain prior to cleavage we analyzed EspP Δ 1 containing either one point mutation, N1023D (EspP Δ 1(N1023D)), or two point mutations, N1023S and N1024S (referred to as EspP* Δ 1), that prevent cleavage but allow translocation of the passenger domain across the OM^{22,24}. The β -domains of these mutants were less stable than EspP Δ 1 and showed 50% denaturation temperatures of 66°C (Fig. 4, panels 7 and 8). These data suggest that after cleavage and release of the passenger domain, the β -domain adopts a more stable conformation.

To examine the contributions to β -domain stability of the passenger- and β -domain segments of the long α -helix that presumably spans the barrel prior to cleavage, two truncation mutants of EspP Δ 1 were made and the stabilities of their β -domains were examined. One mutant, EspP β (+N1023D), which expresses the β -domain alone with an additional aspartate residue at its N-terminus, showed similar 50% denaturation temperatures as EspP Δ 1(N1023D) (Fig. 4, panels 5 and 8), suggesting that the presence of the passenger domain segment of the α -helix does not confer increased stability on the β -domain. In contrast, a second truncation mutant, EspP β (Δ 1024-1031), which lacks the β -domain segment of the α -helix and 3 residues of the linker loop, fully unfolded at the lowest temperature tested (25°C) (Fig. 4, panel 6). These data indicate that residues 1024 - 1031 are critical for the β -domain to form a structure that is stable in the presence of SDS.

We next asked whether the proposed conformational changes might account for the increase in post-cleavage stability. In the β -domain structure, the α -helix remaining after cleavage of the passenger domain is rotated by about 90° from its presumed pre-cleavage orientation and forms 3 hydrogen bonds with residues Asp1120, Glu1154, and Glu1172 (Fig. 2). To ascertain the importance of these interactions, we utilized two point mutants that decrease the amount of negative charge in the acidic cluster, EspP Δ 1(E1154Q) and EspP Δ 1(E1172Q). These mutants inhibit, but do not prevent cleavage, allowing the stability of their β -domains to be

tested after normal passenger domain cleavage and release (Fig. 5, lanes 4 and 6). Both mutants begin to unfold at a lower temperature (50% denaturation temperatures of 75°C and 77°C) than wild-type EspPΔ1 (Fig. 4, panels 2 and 3). Thus, the specific interactions between the α -helix and acidic cluster are important for maximal β -domain stability. To investigate contributions from loop 5, we compared the stability of a loop 5 deletion mutant, EspPΔ1(ΔL5), to EspPΔ1. We found that deleting loop 5 had no effect on passenger domain translocation or cleavage (see Supplementary Fig. 5 online). Like EspPΔ1, EspPΔ1(ΔL5) was extremely thermostable, with a 50% denaturation temperature of 85°C (Fig. 4, panel 4), indicating that loop 5 does not make substantial contributions to β -domain stability.

To further characterize EspP, we analyzed several mutants for their ability to form pores in the OM through antibiotic sensitivity assays (see Supplementary Table 1 online). While EspPΔ1 and EspP*Δ1 showed similar profiles, EspPΔ1(ΔL5) exhibited a substantial increase in sensitivity to nalidixic acid and rifampicin. In addition to showing even greater sensitivity to these two drugs, EspPβ(Δ1024-1031) displayed increased sensitivity to bacitracin and vancomycin. These data suggest that both loop 5 and the N-terminal α -helix restrict antibiotic access to the periplasm but that the N-terminal α -helix is more restrictive than loop 5.

EspP cleavage occurs in the barrel pore

From an extensive series of mutagenesis experiments, Dautin *et al.* showed that the cleavage of the EspP passenger domain is autocatalytic, and proposed that a residue of the β -domain (Asp1120) interacts with a residue of the passenger domain (Asn1023) to mediate cleavage inside the barrel pore through an unusual catalytic mechanism (see Supplementary Fig. 6 online) ²⁴.

In the crystal structure, catalytic residue Asp1120 protrudes into the barrel pore, forming a salt bridge with Arg1028 (data not shown). Because Asn1023 (the second catalytic residue) becomes the C-terminus of the passenger domain after cleavage, it is not present in the crystal structure. However, Asn1024 is located inside the barrel lumen, approximately 5 Å from Asp1120, placing Asn1023 near Asp1120 prior to cleavage in agreement with the model. All three residues of the acidic cluster (Glu1154, Glu1172, and Asp1120) are within 5 Å of Asn1024 and could potentially mediate cleavage (Fig. 2). To test the participation of each residue, we used Western blots and an antibody directed against the C-terminus of the β -domain to examine the amount of cleaved versus uncleaved EspP. Consistent with previous results ²⁴, we found that single substitutions of alanine or glutamine for Glu1154 or Glu1172 inhibited cleavage, but did not prevent it entirely, whereas mutation of Asp1120 to asparagine abolishes cleavage (Fig. 5, lanes 3-6 and 9). To test the possibility that Glu1154 and Glu1172 act redundantly to mediate cleavage and that Asp1120 plays an indirect role in the cleavage reaction, double alanine or glutamine substitutions were made in Glu1154 and Glu1172. Neither set of double mutations prevented cleavage entirely (Fig. 5, lanes 7 and 8). These data argue that Glu1154 and Glu1172 are not catalytic and support the original conclusion ²⁴ that Asp1120 directly interacts with Asn1023 to mediate cleavage (Fig. 5, lane 9). The salt bridge observed between Asp1120 and Arg1028 in the crystal structure provides further evidence that a conformational change occurs post-cleavage.

EspP and NalP β -domains have distinct electrostatic surfaces

With the availability of two monomeric autotransporter β -domain structures, we asked whether common features exist that might suggest a possible translocation mechanism. We analyzed the electrostatic surfaces of the EspP and NalP β -domains (Fig. 6). The interior surface of the EspP β -barrel is predominantly negatively charged, while the interior of the NalP β -barrel is positively charged toward the periplasmic surface, and negatively charged or neutral toward the extracellular surface. The dissimilar electrostatic properties of the two barrel pores suggest

that, while EspP and NalP may share a common general translocation mechanism, the specific interactions between their passenger- and β -domains are different. We note that if the β -domain acts as a translocation pore, the narrow pore dimensions could only accommodate polypeptides lacking tertiary structure.

The diverse electrostatic properties of the two β -domains may also reflect the different passenger domain cleavage mechanisms used by EspP and NalP. Since passenger domain cleavage occurs in the extracellular space for NalP, the pore α -helix is retained inside the barrel. Charged residues cluster on one side of the helix and contribute 7 salt bridges with the barrel wall¹². For EspP, we modeled 5 turns of the predicted α -helix directly upstream of the passenger domain cleavage site, which would allow it to span the barrel pore prior to cleavage. The predicted helix is also amphipathic (see Supplementary Fig. 4 online) with the charged face containing two acidic residues (Asp1014 and Glu1021) and just one basic residue (Lys1016). Since the EspP pore is acidic, the lack of appreciable positive charge in the pore helix upstream of the cleavage site would be expected to reduce the affinity of this portion of the passenger domain for the β -domain pore and facilitate its release.

DISCUSSION

In this report, we describe the crystal structure of the EspP β -domain as well as experiments assessing the cleavage mechanism and post-cleavage events. This is the first structure of an autotransporter β -domain exhibiting post-cleavage conformational changes. EspP crystals were grown from protein that was targeted to *E. coli* outer membranes and naturally cleaved. NalP crystals, used to determine the only other monomeric β -domain structure to date, were produced from refolded protein. Because the structure of EspP is very similar to that of NalP, our results imply that autotransporters can be refolded to a native (or native-like) conformation.

From a comparison with NalP, we were able to infer conformational changes taking place in the β -domain of EspP after cleavage and release of the passenger domain. These changes involve (1) closure on the extracellular side of the β -barrel by loop 5, which folds into the barrel pore, and (2) rotation of the N-terminal α -helix that remains in the pore after cleavage, such that it interacts with three acidic residues on the interior barrel wall. Our SDS-denaturation experiments suggest that the β -domain adopts a more stable conformation after cleavage and release of the passenger domain. We found that extracellular loop closure does not contribute to stability of the β -domain, while α -helix reorientation / H-bonding to acidic residues on the barrel wall stabilizes the β -domain after cleavage. The interactions between the passenger domain and β -domain prior to cleavage appear not to contribute to β -domain stability, consistent with the idea that the passenger domain is weakly held inside the barrel pore. The charge distribution of the predicted α -helix upstream of the EspP cleavage site may reduce its affinity for the acidic barrel lumen and facilitate passenger domain release. In contrast, deleting the first 8 residues of the α -helix and linker loop of the β -domain prevented the formation of a SDS-stable β -barrel, consistent with the idea that, prior to cleavage, these residues are tightly bound inside the base of the barrel, aligning Asn1023 with Asp1120 for initiation of the cleavage reaction.

Dautin *et al.*²⁴ recently showed that EspP is cleaved by an autoproteolytic mechanism. With the knowledge that β -domain / passenger domain cleavage occurs between Asn1023 and Asn1024, they identified residues in the active site that are responsible for cleavage and predicted them to be located within the barrel lumen. Our post-cleavage structure of EspP confirms the location of the cleavage site, and places it in the barrel pore approximately halfway between the periplasmic and extracellular sides of the OM. This location of catalytic residues is unique among OM proteins. The only other OM protease whose structure has been determined, OmpT³¹, uses four catalytic residues located on the extracellular surface of the

β -barrel to cleave its substrates. Similarly, the structure of outer membrane phospholipase A places active site residues on the extracellular surface of the β -barrel³².

Like the β -domain of NalP, the β -domain of EspP crystallized as a monomer with an α -helical segment inserted inside the barrel lumen. These structures suggest that classical autotransporters have a common architecture. However, we cannot determine from the crystal structures whether this architecture is established prior to passenger domain translocation or only after the translocation reaction is complete. In one scenario the α -helical segment is incorporated into the β -barrel during its assembly in the periplasm. The prefolded β -domain is then inserted into the OM by Omp85 and the passenger domain is transported across the OM by an exogenous factor. Alternatively, the C-terminus of the passenger domain might insert into the β -barrel as an extended hairpin to initiate passenger domain translocation and then adopt an α -helical conformation once the entire passenger domain is extruded through the pore. In this scenario, insertion of the β -domain into the OM probably still requires Omp85³³. In any case, our data effectively exclude an oligomeric translocation model.

In summary, this structure represents the first autotransporter β -domain solved after cleavage of its passenger domain and reveals details of an intra-barrel cleavage mechanism that appear to be conserved throughout the SPATE autotransporter family²⁴. Prior to cleavage, EspP is predicted to contain an amphipathic α -helix spanning the length of the barrel pore. Cleavage occurs between residues Asn1023 and Asn1024 of this α -helix, deep within the β -barrel. Weak interactions between the predicted α -helix upstream of the cleavage site and the barrel pore may facilitate release of the passenger domain from the cell surface. Once the passenger domain has been released, two conformational changes occur in the β -domain: loop 5 folds into the barrel pore, closing it from the extracellular side, while the residual α -helix docks with the barrel wall, increasing the stability of the β -domain.

METHODS

Plasmid construction

Detailed descriptions of plasmids used can be found in the Supplementary Methods and Supplementary Table 2 online.

Purification and crystallization of the EspP β domain

EspP Δ 1-6XHis was expressed from plasmid pC6H1 in BL21 cells. This construct uses the OmpA signal sequence to target EspP to the OM and expresses the C-terminal 116 residues of the passenger domain followed by the wild-type β -domain. A hexahistidine tag is attached to the C-terminus via a gly-ser linker. The purification procedure for the native protein and its selenomethionine derivative is described in the Supplementary Methods online. Crystals were grown at 21°C using the hanging drop method. The best crystals were grown by mixing 0.9 μ l of protein solution (~ 3 mg ml⁻¹), 0.2 μ l 3.4 M Cymal®-1 (Hampton Research), and 0.9 μ l well solution, containing 33 % (w/v) PEG 1000, 200 mM NaCl, 0.1 M Na cacodylate pH 6.4, and 5.0 % (v/v) glycerol. Crystals were frozen in propane cooled to 77 K and stored in liquid nitrogen.

Data collection and structure determination

Data were collected at 100 K at the Southeast Regional Collaborative Access Team (SER-CAT) 22-ID and 22-BM beamlines at the Advanced Photon Source, Argonne National Laboratory. The post-cleavage EspP protein (EspP Δ 1-6XHis) consists of 285 residues, 7 of which are naturally occurring methionines. A three-wavelength MAD dataset was collected from a single selenomethionyl crystal. The crystal had the symmetry of the monoclinic spacegroup P2₁, with unit cell dimensions $a = 85.2$ Å, $b = 53.2$ Å, $c = 209.2$ Å, $\beta = 101.2^\circ$,

having four molecules in the asymmetric unit. All data were integrated and scaled with HKL2000³⁴. PHENIX³⁵ was used to locate 22 out of 28 selenium sites and for density modification and initial model building. The initial model was manually improved using Coot³⁶. This model was input into Phaser³⁷ for molecular replacement using a higher resolution native data set in spacegroup P2₁, with unit cell dimensions $a = 85.0 \text{ \AA}$, $b = 53.3 \text{ \AA}$, $c = 102.4 \text{ \AA}$, $\beta = 103.6^\circ$, having two molecules in the asymmetric unit. Iterative manual model building and refinement were performed using Coot and Refmac 38. Statistics for data processing and refinement are listed in Table 1. The following residues were disordered and therefore not included in the final model: residues 1074 - 1075 in periplasmic turn 1, residues 1135 - 1137 and 1184 - 1191 in extracellular loops 3 and 4, respectively. In the final model, 95.9 % of residues were in favored regions of the Ramachandran plot and there were no outliers³⁹.

Structure analysis

The optimal hydrogen bonding network was determined using the WHATIF web server⁴⁰. Structural alignments were determined using DaliLite²⁹. Electrostatic surfaces were calculated using GRASP⁴¹. Figures 1-3 and 6 were generated using Pymol⁴².

Analysis of EspP passenger domain cleavage and β -domain stability

AD202 cells⁴³ transformed with plasmids encoding the appropriate EspP derivatives were grown in 50 ml LB containing $100 \mu\text{g ml}^{-1}$ ampicillin to $\text{OD}_{550\text{nm}} = 0.2$. EspP synthesis was then induced for 30 min by the addition of $10 \mu\text{M}$ IPTG. To assay EspP passenger domain cleavage, aliquots of each culture were mixed with cold 10% (v/v) TCA and precipitated proteins were collected by centrifugation. To assay β -domain stability, cultures were centrifuged at $4000 \times g$ for 15 min at 4°C . Cells pellets were resuspended in PBS at a concentration of $10 \text{ OD}_{550\text{nm}}$ per ml and sonicated, and unbroken cells were removed by centrifugation at $4000 \times g$ for 10 min at 4°C . Portions of each supernatant ($10 \mu\text{l}$) were then added to an equal volume of SDS-PAGE sample buffer that contained 2% (w/v) SDS and heated 15 min at the indicated temperature. All samples received identical incubation times in sample buffer. In all experiments, proteins were resolved using 8-16% Tris-Glycine minigels (Invitrogen) and EspP-derived polypeptides were detected by Western blotting using an antipeptide antiserum directed against the C-terminus of EspP²². In passenger domain cleavage assays, antibody-antigen complexes were detected using HRP-linked protein A (Amersham) together with the Supersignal Pico Chemiluminescence kit (Pierce). In β -domain stability experiments, Alexa Fluor[®] 680-conjugated goat anti-rabbit IgG (Invitrogen) was used as a secondary antibody and antibody-antigen complexes were detected with an Odyssey scanner (Li-Cor).

Accession codes

Coordinates and structure factors for EspP have been deposited in the Protein Data Bank with accession code 2QOM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Structure of the EspP β -domain. β -strands are colored yellow, loops are green, and the α -helix is red. (a) Topology diagram of EspP. Regions missing in the structure are shown in blue. Filled (yellow) squares indicate residues whose side chains point toward the lipid bilayer. Unfilled (white) squares indicate residues whose side chains point toward the barrel pore. (b) Ribbon diagram of the β -domain. Sidechains for the aromatic girdle are shown in purple. Extracellular loops (L1 - L6) and the linker loop are labeled. (c) The EspP β -domain viewed down the barrel axis from the extracellular surface. Extracellular loop 5 makes 10 hydrogen bonds with 7 strands of the β -barrel; 5 of these interactions are shown. (d) Space filling representation of the β -domain viewed from the extracellular space. (e) Space filling representation of the β -domain viewed from the periplasm.

Figure 2.

Interactions between the luminal α -helix and acidic cluster. (a) Sidechains of the residues forming the acidic cluster are shown in stick representation. The α -helix and linker loop are shown as an electrostatic surface. Blue and red regions denote areas of positive and negative charge, respectively. White surfaces are neutral.

Figure 3.

Conformational changes occur upon cleavage of the passenger domain. **(a)** Cartoon representation of the pre-cleavage state of EspP. Like NalP, EspP is predicted to contain a long α -helix prior to passenger domain cleavage. **(b)** EspP during cleavage and release of the passenger domain. Autoproteolysis separates the β -domain from the passenger domain. Arrow 1 indicates passenger domain release from the barrel pore. Arrow 2 indicates the subsequent repositioning of the N-terminal α -helix. Arrow 3 indicates the folding of loop 5 into the barrel pore. **(c)** EspP β -domain after cleavage.

Figure 4.

Effect of point mutations and deletions on the stability of the EspP β -domain. AD202 was transformed with a plasmid encoding the indicated EspP derivative and autotransporter synthesis was induced by the addition of IPTG. Cell extracts were heated in SDS-PAGE sample buffer at various temperatures and the EspP fragment denoted by the shaded bar on the right was detected by Western blot using a C-terminal antipeptide antiserum. The faster migrating (lower) band corresponds to a compactly folded form of the fragment. Asterisks show the approximate positions of point mutations. The temperature at which 50% of each EspPΔ1 derivative was denatured was determined by first normalizing the signal in the upper and lower bands and then calculating the temperature at which the intensity of the two bands would be equal using Odyssey quantitation software.

Figure 5.

Effect of mutations in the 'acidic cluster' on EspP passenger domain cleavage. AD202 was transformed with a plasmid encoding EspPΔ1, EspP*Δ1 or the indicated EspPΔ1 mutant, and autotransporter synthesis was induced by the addition of IPTG. Passenger domain cleavage was then analyzed by Western blot using a C-terminal antipeptide antiserum.

Figure 6. Electrostatic properties of the EspP and NalP β -domains. **(a)** The electrostatic properties of the EspP barrel pore and luminal α -helix with its linker loop. The panel on the right is rotated by 180°. The barrel pore is strikingly acidic. **(b)** The electrostatic properties of the NalP barrel pore and luminal α -helix with its linker loop. The panel on the right is rotated by 180°. Unlike the interior of the EspP β -barrel, the interior of the NalP β -barrel shows an asymmetric charge distribution, basic toward the periplasm, and acidic / neutral toward the extracellular surface.

Table 1
Data collection, phasing and refinement statistics

	Native	Selenium		
Data collection^a				
Space group	P2 ₁	P2 ₁		
Cell dimensions				
<i>a</i> , <i>b</i> , <i>c</i> (Å)	85.0, 53.3, 102.4	85.2, 53.2, 209.2		
α, β, γ (°)	90, 103.6, 90	90, 101.2, 90		
		<i>Peak</i>	<i>Inflection</i>	<i>Remote</i>
Resolution (Å)	50.0-2.66 (2.8-2.7)	50.0-3.00 (3.11-3.00)	50.0-3.00 (3.11-3.00)	50.0-3.00 (3.11-3.00)
<i>R</i> _{merge}	0.105 (0.553)	0.140 (0.812)	0.100 (0.633)	0.110 (0.820)
<i>I</i> / <i>σI</i>	13.0 (1.92)	7.62 (1.08)	6.97 (0.824)	6.38 (0.659)
Completeness (%)	96.7 (79.4)	97.3 (85.8)	94.4 (71.9)	93.0 (67.6)
Redundancy	6.4 (5.2)	3.6 (3.0)	1.9 (1.7)	1.9 (1.7)
Refinement^b				
Resolution (Å)	15.0-2.66			
No. reflections	23299			
<i>R</i> _{work} / <i>R</i> _{free}	0.258 / 0.284			
No. atoms				
Protein	4035			
Water	6			
<i>B</i> -factors				
Protein	62.8			
Water	51.8			
R.m.s deviations				
Bond lengths (Å)	0.009			
Bond angles (°)	1.286			

^a One native and one selenomethionyl crystal were used to solve the structure. Values in parentheses are for the highest-resolution shell. Note that the native crystal contained two molecules in the asymmetric unit, while the Se-Met crystal contained four molecules in the asymmetric unit.

^b *R*_{free} was calculated for a random set of reflections that included 5 % of the data.