Size and Conformation Limits to Secretion of Disulfide-bonded Loops in Autotransporter Proteins

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Background: There is a general paucity of cysteine residues within the passenger domains of autotransporter proteins.

Results: Distantly spaced cysteine pairs forming disulfide-bonded loops or those enclosing structural elements are secretion-incompetent.

Conclusion: Only closely spaced cysteine pairs are compatible with the autotransporter pathway.

Significance: Secretion of folded peptides by the autotransporter pathway is limited; hence autotransporters lack large disulfide-bonded loops to remain secretion-competent.

Autotransporters are a superfamily of virulence factors typified by a channel-forming C terminus that facilitates translocation of the functional N-terminal passenger domain across the outer membrane of Gram-negative bacteria. This final step in the secretion of autotransporters requires a translocation-competent conformation for the passenger domain that differs markedly from the structure of the fully folded secreted protein. The nature of the translocation-competent conformation remains controversial, in particular whether the passenger domain can adopt secondary structural motifs, such as disulfide-bonded segments, while maintaining a secretion-competent state. Here, we used the endogenous and closely spaced cysteine residues of the plasmid-encoded toxin (Pet) from enteropathogenic Escherichia coli to investigate the effect of disulfide bond-induced folding on translocation of an autotransporter passenger domain. We reveal that rigid structural elements within disulfide-bonded segments are resistant to autotransporter-mediated secretion. We define the size limit of disulfide-bonded segments tolerated by the autotransporter system demonstrating that, when present, cysteine pairs are intrinsically closely spaced to prevent congestion of the translocator pore by large disulfide-bonded regions. These latter data strongly support the hairpin mode of autotransporter biogenesis.

Gram-negative bacteria possess seven secretion pathways (numbered I–VI and the chaperone-usher pathway) that facilitate navigation of secreted proteins through the inner membrane, periplasm, and outer membrane (OM). These pathways generally use specialized machineries that span the width of the cell envelope and that differ in complexity, structural features, and mechanism of protein translocation. At first glance, the simplicity of the type Va secretion pathway appeared to be the exception; all the functional elements required for secretion appeared to be contained within a single protein with the N-terminal signal peptide mediating inner membrane translocation, the central passenger domain being the secreted functional moiety, and the C terminus forming a β-barrel structure in the OM, the latter element being essential for passenger domain translocation to the bacterial cell surface. Accordingly, the superfamily of proteins that exploit this pathway for their delivery to the surface of Gram-negative bacteria was termed autotransporters (ATs) (1).

However, recent studies demonstrating that passenger domain secretion requires the aid of accessory factors have challenged the theory that ATs are self-contained secretion systems; periplasmic chaperones SurA, Skp, and DegP have been implicated in AT biogenesis (2–5), as have BamA and BamD (5–8), the essential components of the OM β-barrel assembly machinery (BAM), which functions in concert to insert into the OM correctly folded integral outer membrane proteins (OMPs) that adopt a β-barrel conformation (9). Although it is clear that ATs associate closely and tightly with BamA and BamD during OM translocation (5, 10–12), the precise nature of these interactions is unknown. Certainly, it still remains to be elucidated how these components facilitate the integration of AT β-domains and other β-barrel OMPs into the lipid bilayer. Nevertheless, when inserted into the OM, the AT β-domain forms a β-barrel structure reminiscent of most other integral OMPs (13–18). Consistent with early bioinformatic studies, which suggested that the AT β-domain is evolutionarily and structurally conserved (19), almost perfectly superimposable crystal structures of the β-domain of five monomeric ATs have been reported (14–18). Each structure demonstrated a 12-stranded

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The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1–S3 and Figs. S1–S4.

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2The abbreviations used are: OM, outer membrane; OMP, outer membrane protein; AT, autotransporter; BAM, β-barrel assembly machinery; Pet, plasmid-encoded toxin; D2A, domain-2A; β-ME, β-mercaptoethanol; Bis-Tris, 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)propane-1,3-diol; TCEP, tris(2-carboxyethyl)phosphine; mPEG-Mal, mPEG-maleimide.
β-barrel adjoined by periplasmic turns and extracellular loops of varying length, with a narrow hydrophilic pore.

After insertion of the AT β-barrel into the OM, translocation through the β-barrel pore was thought to begin by the formation of a hairpin structure at the most C-terminal portion of the passenger domain, termed the autochaperone domain. There is now a substantial body of evidence demonstrating that the autochaperone domain emerges from the bacterial cell first and folds into a stable protease-resistant structure, which may drive folding of the remaining passenger domain and subsequent translocation across the OM in the absence of external energy sources (11, 20–23). OM translocation is dependent on a passenger domain conformation that differs from the final protein structure, which for most ATs is a characteristic right-handed β-helical stalk-like structure connected by various loops, some of which are presumed to confer functionality (21, 23–27).

The conformation of the passenger domain has important implications for secretion. Originally, it was hypothesized that the passenger domain was maintained in a linear state during secretion and that folding occurred only after translocation (1). However, other studies have challenged this model, suggesting that folded elements can readily be secreted to the bacterial surface (29, 30). Studies investigating this relationship between the folding and secretion of AT passenger domains have largely focused on disulfide bond formation during transit through the periplasm, between exogenous Cys residues introduced into passenger domains (20, 28), or between Cys residues within heterologous passenger domains (29–36). Although the translocation of sizable folded domains comprising disulfide-bonded segments of heterologous passenger domains was reported (29–32), a greater number of studies have demonstrated that periplasmic formation of either long disulfide-bonded loops or tightly folded/rigid structures is incompatible with OM translocation/secretion of AT passenger domains (20, 28, 29, 33–36). These dichotomous sets of studies have given rise to two mechanistic models of AT biogenesis, one proposing that passenger domain translocation occurs through a folded AT β-barrel pore, and the other proposing BamA as an accessory factor mediating passenger translocation before the β-domain adopts a final folded conformation.

Here, we used the plasmid-encoded toxin (Pet), a prototypical member of the serine protease ATs of the Enterobacteriaceae (SPATEs) (37), to ascertain the tolerance for folded elements during OM translocation. Pet possesses a pair of endogenous Cys residues separated by 4 amino acids that are located within a region of the passenger protein termed domain-2A (D2A), which is roughly 50 amino acids in length and is present only in the serine protease ATs of the Enterobacteriaceae that elicit toxic effects on eukaryotic cells (supplemental Table S1). The conformation of the passenger domain has important implications for secretion. Originally, it was hypothesized that the passenger domain was maintained in a linear state during secretion and that folding occurred only after translocation (1). However, other studies have challenged this model, suggesting that folded elements can readily be secreted to the bacterial surface (29, 30). Studies investigating this relationship between the folding and secretion of AT passenger domains have largely focused on disulfide bond formation during transit through the periplasm, between exogenous Cys residues introduced into passenger domains (20, 28), or between Cys residues within heterologous passenger domains (29–36). Although the translocation of sizable folded domains comprising disulfide-bonded segments of heterologous passenger domains was reported (30–32), a greater number of studies have demonstrated that periplasmic formation of either long disulfide-bonded loops or tightly folded/rigid structures is incompatible with OM translocation/secretion of AT passenger domains (20, 28, 29, 33–36). These dichotomous sets of studies have given rise to two mechanistic models of AT biogenesis, one proposing that passenger domain translocation occurs through a folded AT β-barrel pore, and the other proposing BamA as an accessory factor mediating passenger translocation before the β-domain adopts a final folded conformation.

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Construction of an E. coli TOP10 dsbA Mutant—The λ Red recombinase system (41) was used to construct an in-frame dsbA knock-out mutant of E. coli TOP10.

Analysis of Pet Biogenesis—Growth, expression, and precipitation of Pet from TOP10 and TOP10 dsbA::kan transformed with pBADPet derivatives were performed as described previously (39). Where necessary, 10 mM sulfoxide (DMSO) as a 40 mM stock solution and used immediately. Where necessary) and incubated overnight at 4 °C. TCEP-treated (reduced) and untreated (oxidized) samples were mixed with the same volume of non-reducing SDS-sample buffer (2×), resolved on 4–20% gradient Precise protein gels in Tris-HEPES-SDS buffer (Thermo Fisher Scientific), and localized by Western immunoblotting using anti-Pet passenger antibody.

Pet Purification and Circular Dichroism Spectroscopy—TOP10 cells transformed with pBADPet and pBADPetΔD2A were purified, and clarified supernatants were concentrated as described above. Pet and PetΔD2A were purified, and far-UV CD spectra were collected as described previously (21) except that Pet derivatives were purified using a HiLoad 16/60 Superdex 75 gel filtration column (GE Healthcare), and far-UV CD measurements were collected from 190 to 260 nm on a JASCO J-715 spectropolarimeter at room temperature with a 1-mm path length cell, 2-nm bandwidth, 1-nm increments, 2-s response, 100 nm/min scanning speed, and continuous scanning mode. Eight scans were averaged, and the spectrum was subtracted for buffer contribution.

Co-immunoprecipitation—Chemical cross-linking, spheroplast formation, and lysis of E. coli cultures for co-immunoprecipitation of BamA and BamD were performed as described previously (44). A total of 4 μg of anti-Pet passenger domain antibody was added into the cleared lysate, and the mixture was shaken for 1 h. A total of 60 μl of protein A-Sepharose beads (Sigma-Aldrich) was added to the lysate-antibody mixture, which was shaken for an additional 1 h. Beads within this mixture were pelleted (3,000 × g, 1 min) and washed three times with 1 ml of Pierce IP lysis buffer (Thermo Fisher Scientific) followed by one wash with 1 ml of PBS. Boiling in 50 μl of sample buffer containing DTT served to elute the proteins and to reverse the cross-linking reaction. The eluted fractions were centrifuged as above to completely remove the beads and separated on NuPAGE® 4–12% gradient Bis-Tris gels in MES buffer (Invitrogen). Western immunoblotting was carried out using rabbit polyclonal antibodies against the Pet passenger domain, BamA and BamD.

RESULTS

Native Cys Residues Are Not Required for Secretion or Folding of Pet—With the exception of several polymorphic ATs of Chlamydia (45), a feature of AT proteins is the general paucity of Cys residues within the secreted passenger proteins. Where multiple Cys residues are present, the distances between the Cys pairs generally range from a minimum of 3 residues to a maximum of only 17 residues (46) (supplemental Table S3). It has previously been suggested that paired Cys residues found within AT passenger proteins are required for the correct biogenesis of ATs (46, 47). The Pet AT possesses a pair of endogenous Cys residues separated by 4 amino acids that are located within D2A. To determine the location of D2A, a three-dimen- sional model of Pet was built using the crystal structure of the homologous Hbp AT protein (26) (Fig. 1A). The model reveals that like Hbp, Pet possesses a serine protease domain (D1), which is N-proximal to the β-helix. In the case of Hbp, discursive loops extend from the central β-helix at different points to form D2 and D3 (25, 26). Both domains are lacking from Pet. However, Pet possesses D2A in place of D3, and the Pet amino...
acid sequence directly adjacent to D2A (QPDEW) is almost completely conserved with Hbp (supplemental Fig. S1); it has been shown that these residues provide a platform for the projection of a stable external loop capable of independent movement (48). Thus, our model predicts that D2A contacts D1 and returns to the β-helical stem close to the point of departure such that the Cys residues do not form part of the central β-helical stem.

The model of the Pet passenger domain does not suggest an obvious reason why the Cys residues may influence passenger domain translocation. To determine whether the paired Cys residues or D2A were required for Pet biogenesis, culture supernatant fractions from E. coli cells expressing wild-type Pet (pBADPet) (39) and a D2A deletion construct (PetΔD2A; pBADPetΔD2A) were analyzed for the presence of proteins (Fig. 1B). SDS-PAGE demonstrated that Pet lacking D2A accumulated in the extracellular fraction at levels indistinguishable from wild-type Pet, indicating that D2A is not required for the biogenesis of Pet.

Large Disulfide-bonded Loops Abolish Secretion of Pet—The nature of AT passenger domain translocation across the OM remains controversial. To address the conflicts arising from these datasets (20, 28–36) and to avoid artifacts that could result from heterologous constructs, we wished to address the issue of passenger domain translocation using an AT passenger domain that possesses endogenously paired Cys residues. The distances between endogenous Cys pairs in AT passenger domains generally range from a minimum of 3 residues to a maximum of only 17 residues (46) (supplemental Table S3). As such, we hypothesized that Cys pairs separated by more amino acids would form disulfide-bonded loops in the periplasm that would block secretion. To test this hypothesis, we created pBADPet20aa and pBADPet48aa encoding mutant Pet proteins (Pet20aa and Pet48aa) in which the positions of the endogenous Cys residues within D2A of the Pet passenger domain were altered such that they were positioned 20 and 48 amino acids apart (Fig. 2A and supplemental Fig. S2). SDS-PAGE analysis of concentrated supernatant fractions harvested after growth of TOP10 (top panel) and TOP10ΔdsbA (bottom panel) expressing wild-type Pet (Wt4aa) and Pet20aa to Pet48aa, M, molecular mass markers, C, mPEG-Mal labeling of wild-type Pet (Wt4aa), and PetΔD2A, TOP10 expressing empty vector (EV), Pet, and PetΔD2A. M, molecular mass markers. C, SDS-PAGE analysis of concentrated supernatants from TOP10 expressing Pet and PetΔD2A before (lane a) and after (lane b) gel filtration chromatography. D, far-UV CD spectra of Pet (open squares) and PetΔD2A (closed triangles) in millidegrees (mdeg) showing maxima and minima at 199 and 217 nm and 196 and 214 nm, respectively.
ysis of culture supernatant fractions derived from *E. coli* pBADPet20aa and pBADPet48aa revealed that Pet20aa and Pet48aa were not secreted into the culture medium (Fig. 2B, *top panel*). In contrast, when expressed in a strain lacking DsbA, stalling of passenger domain translocation across the OM was circumvented, and both proteins accumulated in the extracellular fraction (Fig. 2B, *bottom panel*). These results suggest that the 20 and 48 amino acids separating the Cys pairs in Pet20aa and Pet48aa, respectively, form disulfide-bonded loops that are too large to migrate through the diameter of the translocator pore, thereby stalling OM translocation and the subsequent secretion of the Pet passenger domain.

Having established that disulfide-bonded loops of 20 and 48 amino acids were incompatible with AT passenger domain translocation, we next set about determining the maximum number of endogenous residues that can form a disulfide-bonded loop that is tolerated by the translocator pore. We used site-directed mutagenesis to sequentially increase the distance between the Cys pair in D2A such that the Cys residues were separated by 6 (Pet6aa), 8 (Pet8aa), 10 (Pet10aa), 12 (Pet12aa), 14 (Pet14aa), 16 (Pet16aa), and 18 (Pet18aa) amino acids (Fig. 2A and supplemental Fig. S2). SDS-PAGE analyses of culture supernatant fractions from *E. coli* cells expressing Pet6aa to Pet18aa revealed that all mutant proteins could be secreted into the culture medium, albeit at reduced levels when compared with wild type for proteins containing larger disulfide-bonded loops (Fig. 2B, *top panel*). Furthermore, in the absence of DsbA, Pet14aa, Pet16aa, and Pet18aa accumulated in the extracellular compartment at levels resembling the wild-type protein. Perylation experiments revealed that Pet6aa, Pet8aa, Pet10aa, Pet12aa, Pet14aa, Pet16aa, and Pet18aa interacted with mPEG-Mal only under reduced conditions (+TCEP), evidenced by a shift in the molecular weight of each protein (Fig. 2C), confirming the presence of a disulfide bond in the mature secreted species. Overall, our data demonstrate that the longest disulfide-bonded loop that does not significantly interfere with OM translocation of the Pet passenger domain is formed between a Cys pair separated by 18 residues.

**A Rigid Linker Interferes with OM Translocation**—Based on the data above, we hypothesized that the disulfide-bonded loops of stalled intermediates would be located in the periplasmic space. To address this hypothesis, we monitored disulfide bond-induced folding of the Pet passenger domain during transit through the periplasm when the distance between the endogenous Cys pair was increased via the insertion of one, two, or three HA epitope tags with the following amino acid sequence, YPYDVPDYA (Fig. 3A, *right panel*). Because the endogenous Cys pair within D2A of Pet is separated by 4 amino acids, the plasmids pBADPet1HA, pBADPet2HA, and pBADPet3HA encode Pet derivatives where the Cys pairs are separated by 13 (Pet1HA), 22 (Pet2HA), and 31 (Pet3HA) residues, respectively. We theorized that Pet1HA would be secreted but that Pet2HA and Pet3HA would form stalled intermediates. As expected, Pet2HA and Pet3HA did not accumulate in the culture supernatant fractions of *E. coli* pBADPet2HA or *E. coli* pBADPet3HA (Fig. 3A, *top and bottom panels*). However, surprisingly, Pet1HA was not secreted to the extracellular milieu from *E. coli* pBADPet1HA. In all three cases, stalling of passenger domain OM translocation could be circumvented under conditions preventing intramolecular disulfide bond formation through growth in the presence of reducing agent, β-ME (Fig. 3A, *top panel*), or in a dsha- background (Fig. 3A, *bottom panel*). A schematic description of the HA epitope tag insertions is shown on the right. M, molecular mass markers. B, SDS-PAGE analysis of TCA-precipitated culture supernatant fractions harvested after growth of TOP10 and TOP10 ΔdsbA expressing Pet, Pet1HA (1HA), Pet2HA (2HA), and Pet3HA (3HA). Intramolecular disulfide bond formation was prevented through growth in the presence of β-ME (top panel) or in a dsha- background (bottom panel). A schematic description of the HA epitope tag insertions is shown on the right. M, molecular mass markers. B, SDS-PAGE analysis of TCA-precipitated culture supernatant fractions harvested after growth of TOP10 expressing empty vector (EV), Pet, and Pet9G in the presence or absence of β-ME. C, mPEG-Mal labeling of Pet9G and PetC12G/C17G in the presence and absence of TCEP. Supernatants were harvested and concentrated after growth of TOP10 cells expressing Pet9G and PetC12G/C17G. Samples were resolved on a gradient 4–20% Tris-HEPES-SDS-PAGE gel, and Pet was localized by Western immunoblotting using anti-Pet passenger antibody. The arrow indicates labeled/pegylated Pet, and the asterisk shows unlaible/deppegylated Pet.

Our results were difficult to reconcile considering that the disulfide-bonded loop formed with one HA epitope tag was only 13 residues in length and that we had demonstrated the capacity of Pet to support the secretion of a passenger domain with a disulfide-bonded loop composed of 18 amino acids. Therefore, we decided to investigate whether it was the position, the nature, or the length of the HA epitope tag insertion that obstructed OM translocation of the Pet passenger domain by creating pBADPet9G, which codes for a Pet derivative (Pet9G) where the Cys pair is separated by 13 residues due to an insertion of 9 Gly residues. SDS-PAGE analysis of culture supernatant fractions from *E. coli* pBADPet9G revealed that Pet9G is expressed, processed, and secreted at wild-type levels in the presence or absence of β-ME (Fig. 3B). To determine whether secretion was unaffected because Pet9G failed to form a disulfide bond, Pet9G was isolated from the culture supernatant fractions harvested after growth of TOP10 and TOP10 ΔdsbA expressing Pet, Pet1HA (1HA), Pet2HA (2HA), and Pet3HA (3HA). Intramolecular disulfide bond formation was prevented through growth in the presence of β-ME (top panel) or in a dsha- background (bottom panel). These latter data indicate that HA epitopes are not innately resistant to secretion via the AT translocator.
indicate that when trapped within a disulfide-bonded loop, it is the rigid nature of the HA epitope that prevents secretion. Overall, our results indicate that both the nature and the length of the amino acid sequences contained between Cys residues are critical parameters for the translocation competence of passenger domains containing disulfide-bonded segments.

**Stalled Intermediates Possess a Hairpin Conformation**—Conceivably, OM translocation of Pet20aa, Pet48aa, Pet1HA, Pet2HA, and Pet3HA is initiated by the formation of a hairpin structure at the autochaperone domain and continues until the long disulfide-bonded loop or inflexible structure in D2A is reached, thereby jamming the translocator pore and preventing further transport of the passenger domain. Thus, it is conceivable that for Pet1HA, the disulfide-bonded loop containing the HA epitope is located in the periplasmic space when expressed in wild-type cells. Furthermore, we hypothesized that if a hairpin conformation was adopted, the C-terminal portion of the passenger domain would be exposed on the bacterial cell surface. To test these hypotheses, we generated pBADPet1HA-FLAG, a construct that encodes Pet1HA-FLAG, a Pet1HA derivative harboring a FLAG epitope tag (DYKDDDDK) in the C terminus of its passenger domain in addition to the HA derivative harboring a FLAG epitope tag (DYKDDDDK) in the C terminus of its passenger domain in addition to the HA epitope already present in D2A. The FLAG epitope tag was inserted between 2 residues (Gly-837 and Phe-838) that are present in a long loop projecting from the β-helix (supplemental Fig. S3A). Importantly, insertion of a FLAG epitope tag at this location did not abolish Pet secretion in a 

**FIGURE 4.** Stalled intermediates possess a hairpin conformation that can be detected using fluorescence microscopy. TOP10 cells expressing empty vector (EV), PetSB, PetSB-FLAG, PetL4HA, Pet1HA, and Pet1HA-FLAG were collected 1 h after induction with 0.02% arabinose and subjected to indirect immunofluorescence using anti-Pet passenger antibody (left panels), anti-HA antibody (middle panels), or anti-FLAG tag antibody (right panels; pseudo-colored red) and an Alexa Fluor 488-labeled conjugate (all panels). In the case of Pet1HA and Pet1HA-FLAG, half of the samples were fixed and permeabilized prior to labeling. Corresponding fields are also shown by phase contrast microscopy. Completely black boxes indicate that staining was not assessed.

Pet passenger antibody was suggestive of partial exposure of the passenger domain of these trapped intermediates at the cell surface and was comparable with that of the positive control, PetSB. Strong labeling of Pet1HA-FLAG with anti-FLAG tag antibody was also indicative of exposure of the C terminus of the passenger domain at the cell surface with labeling comparable with that of the positive control, PetSB-FLAG (Fig. 4, right panels, compare Pet1HA-FLAG with PetSB-FLAG). Collectively, these data demonstrate that Pet1HA and Pet1HA-FLAG are bona fide translocation intermediates that accumulate in the OM in a hairpin conformation with the N terminus exposed in the periplasm, whereas the C terminus of the passenger domain is exposed at the cell surface (supplemental Fig. S4).

**Stalled Intermediates Interact with BamA and BamD during OM Translocation**—We recently demonstrated that BamA and BamD are the only components of the BAM complex essential for AT biogenesis (8). This is further supported by in vivo cross-linking experiments documenting direct BamA/BamD-AT interactions (5, 10–12). Because AT secretion is extremely rapid (31), we performed co-immunoprecipitation assays via mild chemical cross-linking of proteins using formaldehyde to determine whether our stalled intermediates specifically interact with BamA and BamD in vivo during active translocation. Chemical cross-linking facilitates the identification of acces-
Discussion

The dimensions of the translocator pore and the mechanism of OM translocation have direct implications for the degree of passenger domain folding prior to secretion. It is universally accepted that the fully folded AT β-domains, observed by x-ray crystallography, have pore diameters too narrow to accommodate significant structural elements within the passenger domains (14–18). However, translocation of sizable folded domains comprising disulfide-bonded segments of heterologous passenger domains has been reported (30–32). In contrast, most available evidence directly or indirectly indicates that OM translocation is dependent on a passenger domain conformation, which does not possess significant structural elements, is largely unfolded, and differs from the final protein structure (4, 11, 20, 21, 23, 49). The conflict between these data may arise through the use of non-native passenger domains and thus non-native structural elements. To address the dichotomy of these data sets, the secretion of an AT passenger domain that possesses a disulfide-bonded structural element formed by native Cys residues was examined.

We and others had previously noted the general paucity of Cys residues within the passenger domains of ATs (46). Here, updated bioinformatic analyses of the ATs confirmed that when present, Cys residues are juxtaposed with an average distance of ~7 residues and maximum distance of 17 residues (supplemental Table S3). The exceptions to this observation are the polymorphic ATs of Chlamydia (45). Interestingly, although Chlamydia do contain DsbA orthologues, which are expected to function to oxidize Cys-rich periplasmic proteins to form a substrate for peptidoglycan in the elementary body, the novel chlamydial periplasmic oxoreductase, DsbH, may be responsible for maintaining a reducing periplasm (50). Although there is no direct evidence, it is tempting to speculate that DsbH keeps the polymorphic ATs of Chlamydia in a reduced state in the periplasm to maintain their translocation competence. These data strongly suggest that AT proteins have evolved to lack large disulfide-bonded loops within their passenger domains, but that they maintain the ability to secrete passenger domains with small disulfide-bonded structural elements. Certainly, the formation of small disulfide-bonded loops between closely spaced endogenous Cys residues does not interfere with passenger domain translocation (24, 25, 31, 51).

To systematically determine the maximum length of a disulfide-bonded loop that does not interfere with passenger domain translocation, we analyzed the secretion of Pet. The Pet passenger domain possesses a pair of native Cys residues that form a disulfide bond, obviating the need for the introduction of endogenous Cys residues that might obscure the data. Analysis of Pet and its single-site mutant derivatives demonstrates an apparent inverse relationship between the length of a disulfide-bonded loop and secretion efficiency and shows that the longest disulfide-bonded loop that does not significantly interfere with OM translocation of the Pet passenger domain is formed between a Cys pair separated by 18 residues. This is a distance that is in agreement with the preference for shorter connections in globular proteins where 49% of all disulfide bonds are formed between Cys pairs separated by less than 24 residues (52) and is not far removed from the maximum distance separating endogenous Cys pairs within some wild-type passenger domains. Thus, our results suggest that when present, Cys pairs are intrinsically closely spaced to prevent congestion of the translocator pore by large disulfide-bonded regions.

Having demonstrated that the AT β-domain is capable of secreting passenger domains with disulfide-bonded loops containing 18 amino acids, it was perplexing to find that a smaller loop containing an HA epitope tag insertion obstructed OM translocation of the Pet passenger. However, we demonstrated that it is the rigid and/or bulky nature rather than the position or length of the HA epitope tag insertion that is the critical parameter for translocation competence, indicating that only flexible regions with minimal tertiary structure are compatible with the AT pathway. This observation could explain the discrepancy in OM translocation efficiencies observed by others for disulfide-bonded heterologous passenger domains (29–36).
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Indeed, our observations are consistent with the findings of Jong et al. (28), who showed that replacement of D2 with a 145-amino acid calmodulin moiety completely blocked the secretion of Hbp into the extracellular milieu when calcium was added to the growth medium to induce a conformational change in calmodulin in the form of a rigid dumbbell structure. The data are also consistent with observations by Rutherford et al. (53), who demonstrated that a folding-defective derivative of MalE could be translocated across the OM but that native MalE could not. These combined data strongly suggest the passenger must be maintained in a linear conformation with minimal structure to remain secretion-competent.

Based largely upon the contrasting observations for the secretion of disulfide-bonded segments, two prevailing models of passenger domain translocation have developed. The first encompasses translocation of a passenger domain through a folded AT β-domain pore via formation of a hairpin structure where a static strand is sequestered in the β-barrel lumen, whereas a sliding strand traverses the pore. Given the size constraints of the β-barrel pore, the passenger domain would have to be maintained in an extended conformation during the translocation event to prevent congestion. In this study, we determined the topology of bona fide translocation intermediates showing that they accumulate in the OM in a hairpin conformation with the N terminus of their passenger domains exposed in the periplasm, whereas their C terminus is exposed at the bacterial cell surface (supplemental Fig. S4). This result, in conjunction with the data demonstrating that secretion requires an unfolded passenger domain in a linear conformation, strongly supports the hairpin model of biogenesis.

The second model of passenger domain translocation suggests a role for BamA in maintaining an “open state” AT β-domain with complete folding occurring only after passenger domain translocation is complete; this model allows for the secretion of larger structural elements and glycosylated passenger domains. In support of this, several studies have demonstrated that BamA is essential for AT biogenesis (5–8). The interaction of our stalled intermediates with BamA in vivo during active translocation confirmed these observations. Recently, we demonstrated that BamD was also essential for passenger domain secretion, but that loss of the BamB, BamC, and BamE components had no effect on AT passenger secretion (8). The interaction of our stalled intermediates with BamD in vivo during active translocation confirmed these observations. Interestingly, interaction of BamD with the AT stalled intermediates was more prolonged than binding of BamA. These findings are consistent with a recent study demonstrating that the interaction of BamD with the AT β-domain persists after passenger domain translocation, cleavage, and secretion of the passenger (12). These authors also noted prolonged interaction of the AT β-barrel with BamB. However, BamB is not required for the insertion of Hbp or Pet β-barrels into the OM as folded species nor for translocation or folding of these passenger domains (5, 8). Furthermore, ATs are readily assembled in Neisseria sp., which lack BamB. Thus, we propose that the EspP-BamB and Hpb-BamB interactions observed in vivo (5, 10) are nonspecific and a consequence of BamB independently interacting with BamA.

The above data support a model in which the AT β-barrel docks with BamA and is maintained in an open and loosely folded conformation during passenger domain translocation with BamD required at a later step of AT biogenesis than BamA. Once the passenger domain is translocated, cleaved, and secreted, BamD would dissociate, triggering sealing of the AT β-domain and its folding into a stable barrel. This model conflicts with the simplistic hairpin model. The models may, however, be reconciled using recent observations for fimbrial biogenesis via the chaperone-usher pathway. Remarkably, Phan et al. (54) demonstrated that to allow translocation of the pilin subunit, the usher β-barrel rearranges from a kidney-shaped pore to a wider, near circular pore; such conformational distortions in β-barrel proteins were unprecedented as β-barrels were considered rigid structures. Notably, the AT barrel possesses a similar oval conformation to the usher; thus, we propose that the AT barrel adopts a circular conformation during passenger domain translocation, which allows migration of passenger domains with small disulfide-bonded segments and glycosyl moieties through the pore, and only after translocation and cleavage of the passenger domain are complete does the β-barrel “collapse” to the oval-shaped structure observed by crystallography. In this model, a stalled, trapped intermediate cannot shift to its final conformation and cannot be released from the BAM complex, a hypothesis consistent with the prolonged interactions observed between translocation-stalled intermediates and BamA and BamD.

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