Multiple Driving Forces Required for Efficient Secretion of Autotransporter Virulence Proteins*

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**Background:** Many virulence proteins are secreted using the autotransporter system.
**Results:** Autotransporter proteins do not fold until secreted and are secreted poorly in the absence of folding.
**Conclusion:** Folding helps drive autotransporter secretion, but another energy source is required for its initiation.
**Significance:** Our conclusions reconcile apparent contradictions in the literature and importantly contribute to understanding and manipulating autotransporter secretion.

Autotransporter (AT) proteins are a broad class of virulence proteins from Gram-negative bacterial pathogens that require their own C-terminal transmembrane domain to translocate their N-terminal passenger across the bacterial outer membrane (OM). But given the unavailability of ATP or a proton gradient across the OM, it is unknown what energy source(s) drives this process. Here we used a combination of computational and experimental approaches to quantitatively compare proposed AT OM translocation mechanisms. We show directly for the first time that when translocation was blocked an AT passenger remained unfolded in the periplasm. We demonstrate that AT secretion is a kinetically controlled, non-equilibrium process coupled to folding of the passenger and propose a model connecting passenger conformation to secretion kinetics. These results reconcile seemingly contradictory reports regarding the importance of passenger folding as a driving force for OM translocation but also reveal that another energy source is required to initiate translocation.

Autotransporters (ATs)4 are the largest family of virulence-related proteins secreted from Gram-negative bacterial pathogens (1). They perform a diverse spectrum of pathogenesis functions, including cell adhesion, biofilm formation, invasion of host cells, and disruption of the host immune system (2). To perform these functions, AT proteins must first be secreted to the cell surface across the inner and outer bacterial membranes. Blocking AT secretion could therefore disrupt a wide range of virulence functions from a diverse group of pathogens. Moreover, the same secretion mechanism can be engineered to secrete other proteins of interest into the medium or display them on the cell surface (3, 4). Although much work has been devoted to elucidating the AT (or Type Va) secretion mechanism, the dynamic nature of the process has hampered our understanding of what energy source(s) drives secretion of the passenger across the outer membrane (OM) given that there is no ATP available in the periplasm and no proton gradient across the OM (5).

Autotransporters have developed a number of common features that facilitate their secretion (Fig. 1) (6). Each autotransporter includes an N-terminal signal sequence that directs transport across the inner membrane. Following the signal sequence are the two major functional units: (i) the passenger, which is secreted into the extracellular space and performs the virulence function, and (ii) the C-terminal translocator, which is essential for transport of the passenger across the OM and includes a transmembrane β-barrel. One model proposes that the AT passenger crosses the OM in an unfolded conformation through the central pore of its own β-barrel domain. Stepwise folding of the passenger C terminus on the cell surface could then be used to prevent the passenger from sliding back into the periplasm, effectively creating a Brownian ratchet to drive OM secretion (7–11). Consistent with this model, it has been shown that the passenger C terminus crosses the OM first (12). However, several periplasmic chaperones, including the BAM complex (13–15), have been shown to interact with the passenger before and during OM translocation (16–19). Additionally, although there are limits to the size and bulkiness of prefolded passenger structural elements that can cross the OM without blocking translocation (20, 21), some of these structures are surprisingly large when compared with the 1–2-nm diameter of the translocator pore (22, 23). Moreover, recent reports have shown that the range of passenger sequences and folding properties compatible with OM translocation is broader than previously thought (24, 25). These results have introduced a considerable level of complexity and confusion about what was once thought to be a fairly straightforward and self-sufficient protein secretion mechanism.

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1 This work was supported, in whole or in part, by National Institutes of Health Grants R01 GM097573 (to P. L. C.) and T32 GM075762, a Chemistry-Biology Interface Graduate Program fellowship (to E. B.).
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The abbreviations used are: AT, autotransporter; OM, outer membrane; BAM, β-barrel assembly machinery; TAM, translocation and assembly module; Bla, β-lactamase; LB, Luria-Bertani; IPTG, isopropyl β-D-thiogalactopyranoside; Amp, ampicillin; ProK, proteinase K; NQ, N631Q; 4K, L157K, L228K, H35K, and L498K.
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In AT secretion, as in any other process, the cell must obey the basic laws of thermodynamics. Namely, the final state of a protein after its secretion must be energetically more favorable (have a lower free energy) than its initial state; otherwise secretion will not proceed. Often, a cell will compensate for an energetically unfavorable process by coupling it to another, highly favorable process, such as ATP hydrolysis or the flow of ions down a concentration gradient. The challenge of AT translocation across the OM is that beyond the inner membrane no such obvious external energy source is available to couple to and therefore drive AT secretion. This means that during OM translocation the AT protein must progressively move from a less stable (high energy) to a more stable (low energy) state throughout this process; if it falls into an off-pathway low energy trap, there is no ATP-driven mechanism available to restore efficient secretion. Because small molecules and ions can pass freely through OM pores that connect the periplasm and extracellular space (26), these two environments are very similar for many factors that could affect protein stability (temperature, pH, activities of water, and cosolvents). There is therefore little reason to assume that the AT passenger will be more stable on one side of the OM than on the other. In addition, from an entropic perspective, concentrating all AT molecules on the outer cell surface is inherently unfavorable compared with having them evenly distributed on both sides of the OM. How then does the cell ensure efficient OM translocation of AT proteins?

To shed light on which energy sources could drive AT OM translocation, we developed a kinetic model of this process and used simulations to identify scenarios that can lead to efficient secretion. We then tested these scenarios in vivo using pertactin, an archetypal AT from *Bordetella pertussis* (8, 12, 27–30). We found that pertactin OM translocation is tightly coordinated with folding: no folding occurred in a wild type passenger that is reversibly stalled in the periplasm, and a mutant deficient in passenger folding exhibited a marked decrease in OM translocation efficiency. Conversely, we demonstrated that two other potential energy sources, irreversible passenger cleavage and macromolecular crowding, have negligible effects on pertactin secretion. Taken together, these results provide strong evidence that passenger folding is a key driving force for autotransporter secretion but also suggest that a different energy source, such as the free energy released upon AT insertion into the OM, is required to initiate OM secretion.

EXPERIMENTAL PROCEDURES

Kinetic Simulations—Kinetic simulations were performed using Gillespie’s (31) stochastic simulation algorithm and the reaction model shown in Fig. 2a. The simulation program was written in C++ and is based on the open source StochKit package (32). One hundred replicates of each simulation were run to ensure sufficient statistical sampling. Fig. 2 presents the averages of all replicates. Results were independent of the total number of AT molecules in the system.

Values for reaction rate constants were estimated based on the following considerations. 1) Equilibrium constants for folding were set to $10^6$. This is an order of magnitude typical for the stability of many proteins and corresponds to a $\Delta G$ of $\sim -8 \text{ kcal mol}^{-1}$. Note that a folding equilibrium constant of $10^6$ means that unfolding will be $10^6$ times slower than folding, making unfolding practically impossible relative to the time scale of secretion. An analysis of equilibrium unfolding titrations for pertactin (8) suggests that its actual equilibrium folding constant is even higher, $\sim 10^{18}$ ($\Delta G < -24 \text{ kcal mol}^{-1}$), but this does not significantly affect the simulation results because $10^6$ is already large enough to ensure that practically no protein molecules would be unfolded at equilibrium. 2) The rate of folding is expected to be relatively slow. The rate of folding of an AT passenger in vitro is extremely slow ($k \sim 10^{-4} \text{ s}^{-1}$ for pertactin (30)) and can take days to reach equilibrium (8, 33). Folding in vivo obviously needs to be faster than that, but given the large size and high contact order of most AT passenger domains, we estimated the rate constant at $\sim 10^{-3} \text{ s}^{-1}$. 3) The equilibrium constants for folding in the periplasm and at the cell surface are the same, and the equilibrium constants for translocation across the OM are set to 1 for both the folded and unfolded proteins. There is no confirmed reason for a difference in protein stability between the extracellular and periplasmic spaces considering that small molecules can pass freely between them (26), providing a very similar environment in terms of water and cosolvent activities, pH, etc. 4) Within these
constraints, rates of folding and translocation were adjusted to match the experimentally observed time scale for pertactin secretion, measured as the rate of the appearance of the cleaved passenger (Fig. 3, a and b, Mature).

Molecular Biology—Most in vivo experiments were performed using Escherichia coli strain BL21(DE3)pLysS transformed with a pET21b-based plasmid that expresses a pertactin construct under an inducible T7 promoter. The constructs themselves are derived from either the full-length (pP.93WT) or the truncated passenger (pPERPLC01) constructs described previously (8, 12). The pertactin-H9252-lactamase (Bla) chimera used in the folding complementation assay was constructed by cloning the pertactin passenger sequence (residues 35–630) into the pDMB plasmid as described (34).

The plasmid expressing wild type pertactin, p.93EB, was created by cloning the entire pertactin coding sequence from pP.93WT (12) into the pET21b plasmid (Novagen/EMD Millipore, Billerica, MA) via the NheI and EcoRI restriction sites. This resulted in a pertactin construct under tight control of the inducible T7 promoter. Three point mutations present in pP.93WT that do not alter the secretion and folding properties of pertactin were fixed by PCR-amplifying the surrounding region from the P.69T construct and ligating it into pP.93WT through RsrlII and KpnI restriction sites. This ensures that the passenger sequence encoded in the p.93EB sequence matches that of P.69T and the published pertactin passenger sequence (Swiss-Prot accession number P14283). The construct P.69T (pPERPLC01 from (8)) was used to produce the truncated pertactin passenger (P.69T; encompassing residues Ala35–Pro573) for purification and use in vitro. p.93EB and P.69T were used as templates to produce other pertactin variants by PCR-based site-directed mutagenesis using the QuikChange II kit (Stratagene). These mutations as well as the primers used to introduce them are listed in Table 1. The success of each mutagenesis step was confirmed by sequencing the entire coding region.

We designed a mutant variant of the pertactin passenger, 4K, that cannot fold to the native H9252-helical conformation in vitro. This was achieved by replacing buried hydrophobic residues with positively charged Lys residues to disrupt the hydrophobic core of the folded structure, using the native passenger structure (29) as a guide. The positions of the four mutations (L157K, L228K, I435K, and L498K) were selected based on the following criteria: each mutated residue is hydrophobic (Val, Leu, Ile, or Phe), located within rungs 3–14 of the native H9252-helix, and pointed to the hydrophobic core, and neighboring mutations are at least two rungs apart (Fig. 4a).

All constructs were additionally tagged with the HA immunofinity tag by substituting residues Ala265–Ala273 with YPYDVPDYA. The replaced residues are located in the RGD loop (Fig. 4a) previously shown not to be important for secretion or folding of the pertactin passenger (12). The substitution

FIGURE 2. Kinetic simulations of AT secretion. a, model used in simulations. The five states correspond to folded (F) or unfolded (U) passenger located in the periplasm (subscript in) or the extracellular space (subscript out). M is the final, proteolytically cleaved form. b, dependence of simulated secretion efficiency (after 60 min) on different rates in the kinetic model (double arrow, fast; double line, slow). Labels on bars correspond to simulation traces shown in c–f for various secretion scenarios. Traces in c–f use the same color-coding scheme as the labels in a. Red boxes highlight key differences in the rate constants used for alternative scenarios. c, passenger cleavage drives secretion. As long as the passenger is able to reach the cell surface, it is irreversibly cleaved, making secretion a unidirectional process. d, passenger folding can drive secretion even in the absence of cleavage. Slow periplasmic folding gives the unfolded passenger time to translocate before folding on the cell surface. If the folded passenger is unable to cross the OM, then it will stay surface-exposed. e, a combination of fast periplasmic folding and slow translocation of a folded passenger results in inefficient secretion. Most of the passenger folds while still in the periplasm and can neither cross the OM nor unfold. f, in the absence of cleavage and major kinetic barriers, the system will establish an equilibrium with only half of the passenger on the cell surface unless the stability of the secreted and periplasmic folded states differs.

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was initially made in the P.69T construct using three rounds of site-directed mutagenesis. The HA tag-containing region was then transferred into all other constructs using the MEGA-WHOP procedure (35). This consisted of first amplifying a short (507-bp) segment centered around the HA tag site using primers CTCGACGGCGGGCACATCACC and GACGATGGTCGCCCTGCGCATCG. The product of this PCR was then used as a megaprimer to amplify the target plasmid, introducing the entire HA tag region the same way as a mismatched oligonucleotide primer would introduce a point mutation in site-directed mutagenesis.

Purification of Pertactin Passenger—P.69T and its variants were purified as described previously (8). For P.69T-4K and P.69T-HA-4K, the purification protocol was slightly modified. Refolding of the solubilized inclusion bodies was performed by dropwise dilution from 25 ml of sonication buffer containing 6 M guanidinium hydrochloride into 4 liters of 50 mM Tris (pH 8.8). Other steps in the purification protocol were unchanged, but note that the 4K mutations and introduction of the HA tag modify the net charge of the protein, resulting in different elution profiles from an ion exchange column.

Pertactin Expression—To express pertactin, cells were grown in LB medium supplemented with 0.1 mg ml⁻¹ ampicillin (Amp). The medium was inoculated from an overnight culture using a 1:50 dilution and then incubated at 37 °C for 1.5 h until reaching midlog phase (A600 = 0.3–0.4). Expression was then induced by adding IPTG to the medium to a final concentration of 50 μM and incubating the culture at 37 °C for 40 min.

Pertactin Maturation Kinetics—To monitor pertactin maturation kinetics, E. coli cultures were first grown in LB (with 0.1 mg ml⁻¹ Amp) to midlog phase, and then pertactin expression was induced using 50 μM IPTG for 40 min. Cells were pelleted by centrifugation (4000 g for 10 min) and resuspended in 2 ml of LB after ProK shoving results in efficient secretion, whereas using PBS or PBS supplemented with 4 g liter⁻¹ glucose (Glc) stalls secretion. Stalling is maintained for more than 2 h, but the amount of stalled precursor decreases due to degradation. d, kinetics of pertactin OM translocation in PBS and LB monitored as in a and b. OM translocation stalls in PBS buffer but resumes once cells are resuspended in LB. Precursor levels decrease throughout the experiment as it is either translocated or degraded. Shaded areas represent time spent spinning and resuspending cells in fresh medium. e, ProK susceptibility of cell lysates taken at different times during the process shown in d. ProK-resistant pertactin fragments appear only when the passenger is folded (8). Only LB samples containing the mature secreted passenger show any significant amount of folded, ProK-resistant passenger fragments (arrows); no passenger folding is detected for the pertactin precursor even after >90 min of incubation in PBS.
incubated at 37 °C for ~90 min. To test the reversibility of secretion stalling in PBS, cells were once again pelleted, resuspended in LB − Amp, and incubated at 37 °C for 150 min. Throughout this process, culture aliquots were periodically analyzed by SDS-PAGE and Western blotting to measure the level of full-length precursor and mature passenger in the whole cell lysate. The primary antibody used for Western blotting was a rabbit polyclonal antibody raised against purified P.69T by the immunology facility at the University of Notre Dame. The secondary antibody was goat anti-rabbit

### TABLE 1

| Construct     | Parent | Mutation(s) | Forward primer  |
|---------------|--------|-------------|-----------------|
| P.69T         | pPERPLC01 (8) | n/a         | Used as is     |
| P.69T-4K      | P.69T  | L157K       | CATCCGCCAGCCCAACAGGGCTGTCG |
|               |        | L238K       | TGGGGGCGCAGTGAACACGTCGAGGC |
|               |        | I435K       | GTGAGGCTGCTGGATCAAAAGCAAGGCGGACCATGG |
|               |        | L498K       | GGGCTGAGCGACAGAAGTCGTCGAGGAGC |
| P.69T-HA      | P.69T  | A265Y,G266P | GCGGGAGGCCCTTTTATGCTATGTTCCGAGCTATG |
|               |        | G267Y,A268D | GAGGGGCTGCTGCTGCTAATGTTCTCCGAGCTATG |
|               |        | G271D,G272Y | CGCCGAGCTGCTGCTGCTAATGTTCTCCGAGCTATG |
| p.93EB        | p.93WT (12) | D262E,P264L | GAATTCATGCTCTTCTGGAGGCAGTGGC |
|               |        | S323P       | CCATGAGTCATTCCATCTGAGGAGC |
|               |        | n/a         | Entire gene ligated into pET21b |
| p.93EB-NQ     | p.93EB | N631Q       | CGCCGAAAGAGCAGCTCCAGTCAGG |
| p.93EB-4K     | p.93EB | L157K       | CATCCGCCAGCCCAACAGGGCTGTCG |
|               |        | L238K       | TGGGGGCGCAGTGAACACGTCGAGGC |
|               |        | I435K       | GTGAGGCTGCTGGATCAAAAGCAAGGCGGACCATGG |
|               |        | L498K       | GGGCTGAGCGACAGAAGTCGTCGAGGAGC |
| p.93EB-4K-NQ  | p.93EB-4K | N631Q       | CGCCGAAAGAGCAGCTCCAGTCAGG |

*a* Three point mutations in the original pP.93WT construct were fixed by PCR-amplifying the surrounding region from the P.69T construct using the two listed primers and then ligating the PCR product into pP.93WT through RsI and KpnI restriction sites.

*b* Recloning into the pET21b plasmid removed low level constitutive expression by replacing the tac promoter with the tighter T7 promoter.

**FIGURE 4.** Comparison between in vitro folding properties of purified wild type (8, 30) and 4K pertactin passengers. a, the pertactin passenger native structure (Protein Data Bank code 1DAB (29)) showing the locations of four residues (Leu157, Leu228, Ile435, and Leu498; red) replaced with Lys in the unstable, non-native 4K construct. Also shown are loop residues (265–273; blue) replaced with an HA epitope tag. The figure was drawn using UCSF Chimera (54). b, difference in ProK susceptibility between wild type and 4K purified pertactin passengers. Folded wild type passenger produces characteristic ProK-resistant fragments (8), whereas 4K is rapidly degraded. c, far-UV circular dichroism (CD) spectra of wild type and 4K passengers. The wild type passenger displays a characteristic β-sheet signal (a minimum at 218 nm), whereas the 4K mutant does not. d, thermal denaturation monitored by the change in the CD signal at 218 nm. Two cooperative transitions above 60 °C are characteristic of the wild type passenger (8), whereas 4K displays only a very weak, broad transition at much lower temperatures. e, similar behavior is observed upon chemical denaturation using guanidinium hydrochloride (GdnHCl) monitored by the ratio of fluorescence intensities at 335 and 350 nm.
IgG conjugated to alkaline phosphatase (Novus Biologicals, Littleton, CO).

**In Vivo Folding Assay**—To test pertactin folding in the periplasm, samples of *E. coli* culture were periodically taken and lysed by freeze-thawing (three times) and sonication. The lysates were cleared by spinning at 16,100 × g for 5 min, and the supernatant was subjected to proteolytic digestion using 8 μg ml⁻¹ ProK at room temperature. Aliquots were taken after different ProK digestion times, and the digestion reaction was quenched by first adding 5 mM PMSF (an inhibitor of ProK), then shortly afterward adding SDS loading buffer, and boiling for 10 min. The samples were analyzed by Western blotting using an anti-pertactin antibody. The in vivo conformation of pertactin was also tested using the folding complementation assay as described below.

**Folding Complementation Assay**—The folding complementation assay was adapted from Mansell et al. (34). The pertactin passenger (residues Asp^{35}–Ser^{30}) was PCR-amplified from p.93EB (or p.93EB-4K) and ligated in pDBM via 5’ XbaI and 3’ BamHI restriction site overhangs, resulting in plasmid pDMB-P.69 (or pDMB-P.69–4K).

Overnight cultures of *E. coli* DH5α were grown in LB supplemented with 50 μg ml⁻¹ chloramphenicol for 16–22 h while expressing the pertactin–β-lactamase chimera constitutively from a trc promoter. Cell density was normalized so that A₆₀₀ = 1 and serially diluted in LB medium. From each dilution, 5 μl were spotted on plates with various concentrations of antibiotics (20 μg ml⁻¹ chloramphenicol or 25, 27.5, 30, 35, 40, 45, 50, 55, 60, 70, 100, or 125 μg ml⁻¹ Amp). The plates were then incubated at 37 °C for 16 h. The 20 μg ml⁻¹ chloramphenicol plate was used to determine how many colony-forming units (cfu) were present at each dilution and which dilution corresponded to 200–2000 cfu. The minimum inhibitory concentration was defined as the Amp concentration at which 200–2000 colonies no longer grew. The minimum inhibitory concentration (MIC) for pDMB-P.69 and pDMB-P.69–4K was determined for 10 min. The samples were analyzed by Western blotting then shortly afterward adding SDS loading buffer, and boiling for 10 min. The samples were analyzed by Western blotting using an anti-pertactin antibody. The in vivo conformation of pertactin was also tested using the folding complementation assay as described below.

**Western Blot**—Samples of cells expressing different pertactin variants were taken, resuspended in PBS to an A₆₀₀ of 0.3. The cells were then pelleted by centrifugation and resuspended in 25 ml of fresh growth medium supplemented with different concentrations of macromolecular crowding agent (0–400 g liter⁻¹ Ficoll 70 or 0–330 g liter⁻¹ dextran). After about 10 min of recovery at 37 °C, IPTG was added to a final concentration of 10 μg ml⁻¹; 30 min. Stained cells were counted using a Beckman-Coulter FC500 flow cytometer, and pertactin secretion was assessed by the emission of green light upon excitation with a 488 nm laser.

**Flow Cytometry**—Pertactin OM secretion was assessed by taking samples of pertactin-expressing cell cultures and resuspending them in PBS to an A₆₀₀ of ~0.3. The cells were stained by incubating with an anti-HA primary antibody (8.4 μg ml⁻¹; 30 min) and then with a FITC-conjugated secondary antibody (10 μg ml⁻¹; 30 min). Stained cells were counted using a Beckman-Coulter FC500 flow cytometer, and pertactin secretion was assessed by the emission of green light upon excitation with a 488 nm laser.

**Macromolecular Crowding**—To test the effects of macromolecular crowding on pertactin secretion, cells were grown to log phase (A₆₀₀ = 0.4–0.6) in LB + 0.1 mg ml⁻¹ Amp at 37 °C. Cells were then pelleted by centrifugation and resuspended in 25 ml of fresh growth medium supplemented with different concentrations of macromolecular crowding agent (0–400 g liter⁻¹ Ficoll 70 or 0–330 g liter⁻¹ dextran). After about 10 min of recovery at 37 °C, IPTG was added to a final concentration of 50 μM to induce pertactin expression. After ~1 h of induction, samples of the cultures were prepared for immunofluorescence microscopy as described below.

**Immunofluorescence Microscopy**—Cells were resuspended in PBS to a final A₆₀₀ of ~0.5 and immobilized by incubating for 1 h on coverslips precoated with 1 mg ml⁻¹ poly-L-Lys. Coverslips were then washed with PBS and incubated for 15 min with an anti-pertactin rabbit polyclonal antibody raised against the purified pertactin passenger. After washing away the excess primary antibody, coverslips were incubated with a Cy3-conjugated secondary antibody (goat anti-rabbit; Jackson Immuno-Research Laboratories, West Grove, PA) for another 15 min. After washing away the excess secondary antibody, each loaded coverslip was placed face down onto a sample slide and sealed into place using nail polish. Bright field and fluorescence images were taken using an Applied Precision Delta Vision fluorescence microscope with 100× magnification, excitation with a 488 nm laser.
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wavelength of 555 nm, emission wavelength of 617 nm, and exposure time of 242 (for crowding experiments) or 933 ms (for PBS stalling experiments). Fluorescence images were taken in batches, focusing on different planes, and then deconvoluted to give a representative image of a narrow slice of the sample. All images are presented with the same exposure time, brightness, and contrast settings to enable direct comparisons of fluorescence intensities between samples.

RESULTS

Kinetic Simulations of Autotransporter Secretion

We developed a simple kinetic model of AT OM translocation that encompasses five possible states for passenger location and conformation (Fig. 2a). The passenger is treated as either folded (F) or unfolded (U), located either in the periplasm (subscript in) or outside the cell (subscript out). The fifth, mature form (M) corresponds to the final state after irreversible cleavage from the C-terminal β-barrel. Each transition between two states is assigned a kinetic rate constant, and these are used in conjunction with starting concentrations to simulate how the system composition changes over time. The starting concentrations were set to mimic our experimental setup (see below): a pool of non-native AT precursor in the periplasm with no surface-exposed passenger and no new protein being synthesized. Initial kinetic rate constants (Fig. 2c) were taken from published data on pertactin folding in vitro (8, 30) and from the overall secretion kinetics we measured in vivo (Fig. 3, a and b). The rationale behind the choice of rate constants is detailed under “Experimental Procedures.”

The initial parameter values yielded results that are in line with experimental observations (Fig. 2c), demonstrating their suitability as a starting point for the simulations. Nevertheless, other combinations of rate constants might also lead to efficient OM translocation especially for ATs other than pertactin. We therefore systematically varied these rate constants within reasonable limits and observed the resulting effects on secretion. From the results of these simulations, we identified three distinct scenarios that can lead to efficient OM translocation (Fig. 2, b–f).

Passenger Folding—The passenger crosses the OM in an unfolded conformation and then folds. Because the folded state is significantly more stable than the unfolded state, the protein remains folded; it is unable to spontaneously unfold to re-enter the periplasm (Fig. 2d). Translocation is effectively driven by the free energy of passenger folding on the extracellular side of the OM. Two requirements must be met: the passenger must not be able to recross the OM once folded, and the rate of folding in the periplasm must be at least an order of magnitude slower than extracellular folding; otherwise the passenger would fold in the periplasm and remain trapped there (Fig. 2e).

Proteolytic Cleavage—Cleavage of the passenger away from the β-barrel domain is a reaction so energetically favorable as to be irreversible. As long as the passenger is able to make its way to the cell surface where cleavage occurs, it will stay in the much more stable cleaved state, unable to reattach itself and return to the periplasm. The irreversible cleavage reaction can therefore push the system out of equilibrium and drive secretion to completion (Fig. 2c). This scenario requires that any kinetic traps that would prevent the passenger from reaching the cell surface, such as premature folding in the periplasm (Fig. 2e), must be avoided. Importantly, because the presence of an irreversible cleavage step could compensate for a lack of other driving forces, a background devoid of cleavage should be used when experimentally testing the effects of other driving forces on OM translocation.

Thermodynamic Control—In the absence of irreversible cleavage or any kinetic restrictions, only 50% of the passenger would reach the extracellular space (Fig. 2f). This is because an even distribution of molecules between the two compartments is the most energetically favorable state unless an unknown external factor can stabilize the extracellular form of the protein compared with the periplasmic form. We next tested each of these scenarios experimentally using pertactin as our model autotransporter.

Kinetics of Pertactin Maturation

Probing the kinetics of pertactin secretion first required developing an in vivo experimental system with which we could isolate and measure the rate of passenger translocation across the OM. We achieved this by first inducing pertactin expression for a limited time, which produced a stockpile of precursor molecules in the periplasm (with passenger and translocator still covalently connected). Whole cells were then exposed to ProK, which digested any passenger already secreted to the cell surface (“ProK shaving”), leaving only the periplasmic precursor (Fig. 3a). After removal of the inducer and protease, cells were resuspended into fresh growth medium, and samples were collected at different time points. These were analyzed by quantitative Western blotting to determine how much of the precursor had been secreted and processed into the final, mature form. The results (Fig. 3, a and b) show that secretion is slow with levels of mature protein peaking ~90 min after protease treatment. Precursor levels decrease throughout the experiment due to both its conversion to the mature form and degradation.

This assay established key experimental boundaries for setting the initial rate constants of the kinetic model (Fig. 2). It also demonstrated that both the cleavage-driven and folding-driven scenarios described above are compatible with in vivo experimental data as we obtained very similar fits for both cases (Fig. 3b). We therefore performed additional experiments to distinguish between each of the potential driving forces described above.

Conformation of Pertactin in the Periplasm

It follows from our kinetic model that folding-driven OM translocation would require slow folding kinetics in the periplasm so that the passenger would maintain an unstable, non-native conformation prior to translocation. To test this, we exploited a fortuitous discovery: transferring E. coli into PBS reversibly stalls pertactin OM translocation with translocation resuming once cells are transferred back into LB (Fig. 3, c and d). The stalling is efficient enough that no mature pertactin passenger was detected on Western blots after 2-h incubation in PBS (Fig. 3c). We ruled out decreased cell viability as a potential cause of this effect because the number of cfu did not differ.
between cultures resuspended in PBS versus LB (data not shown). Furthermore, supplementing PBS with 4 g liter\(^{-1}\) glucose did not alleviate stalling, indicating that the loss of a carbon-based energy source is not the direct cause of secretion stalling (Fig. 3c). Regardless of the specific mechanism underlying secretion stalling, it allowed us to trap pertactin in the periplasm and probe its conformation in this secretion-competent precursor state by lysing the cells and exposing released proteins to a low concentration of ProK for brief periods of time. Such treatment has been shown previously to cleave the folded pertactin passenger into two characteristic fragments (8). In contrast, the unfolded pertactin passenger is degraded much faster, leaving no detectable fragments (Fig. 4b). We therefore used resistance to limited ProK digestion as an indicator of passenger folding. To obtain samples for this analysis, intact \textit{E. coli} expressing wild type pertactin were first subjected to ProK shaving as described above to remove mature secreted pertactin and then resuspended in PBS. Periodically, aliquots were taken, lysed, and subjected to limited ProK digestion to test for passenger folding in the periplasm. In parallel, whole cell lysate samples were taken to monitor for reappearance of the mature passenger, which would signify successful secretion.

While cells were kept in PBS, the cleaved pertactin passenger did not reappear, confirming that OM translocation was stalled (Fig. 3d), even as the precursor level decreased due to degradation. Once these cells were transferred back into LB, translocation and passenger processing resumed. Because no IPTG was present in the medium and there is no detectable \textit{de novo} pertactin synthesis in the absence of IPTG, this reversibility indicates that the stalled pertactin precursor remains secretion-competent. Limited ProK treatment of lysed cells in PBS showed that although stalled in the periplasm this AT passenger remains in an unstable, non-native conformation for 90 min, whereas secreted pertactin is stably folded as evidenced by the presence of characteristic native state fragments (Fig. 3e). These data demonstrate that the pertactin passenger remains in a non-native state in the periplasm and folds to its native state only upon reaching the cell surface. In terms of our kinetic model (Fig. 2), the fact that passenger folding is much slower in the periplasm than in the extracellular space satisfies a major requirement for secretion driven by passenger folding.

As an orthogonal approach to evaluate passenger conformation in the periplasm, we constructed a pertactin mutant, 4K, that substitutes four residues in the hydrophobic core of the passenger with positively charged lysine residues, rendering 4K incapable of folding under physiological conditions (Fig. 4). We compared this permanently unfolded mutant with wild type pertactin passenger in a Bla folding complementation assay.
where Bla activity is used to report on the conformational state of a periplasmic protein of interest (Fig. 5a; see also Ref. 34). The constructs lack a β-barrel domain so they are unable to cross the OM and remain trapped within the periplasm. Both constructs displayed nearly identical Bla activities, indicating that the conformational states of wild type and 4K were similar (Fig. 5b). Consistent with this result, lysing the cells and exposing these constructs to ProK digestion also produced no ProK-resistant pertactin fragments (Fig. 5c), confirming that both the wild type and 4K constructs remained unfolded in the periplasm.

**Effects of Passenger Folding and Cleavage on Secretion**

Our simulation results indicate that both passenger folding and cleavage represent potential driving forces for efficient secretion and that each could compensate for a lack of the other. To test all possible combinations of these driving forces, we constructed a series of pertactin mutants deficient in passenger folding, cleavage, or both. p.93EB-4K is a full-length pertactin construct bearing the passenger 4K mutations that render it incapable of folding, whereas p.93EB-NQ contains the point mutation N631Q shown previously to prevent the autocatalytic cleavage reaction (23). p.93EB-4K-NQ is deficient in both passenger folding and cleavage. Because the pertactin passenger is known to remain strongly non-covalently bound to the cell surface even after cleavage (12), the extent of secretion can be probed by exposing intact cells to protease activity or immunoaffinity labeling. Additionally, an HA tag was introduced into all constructs to ensure uniform antibody binding.

Control experiments confirmed that the introduced mutations did not significantly affect the ability of pertactin to be expressed or to remain attached to the cell surface for the duration of our experiments (Fig. 6).

We used competition ELISA and flow cytometry to quantify the OM secretion efficiencies of these constructs (Fig. 7). A competition ELISA (36) is performed by equilibrating the antigen-containing sample (in this case intact cells expressing pertactin variants) with an antibody; a higher antigen concentration will bind more antibodies, leaving fewer free in solution. The equilibrated mixture is then briefly incubated in microtiter wells that have been precoated with purified antigen (purified, native pertactin passenger). During this brief incubation time, only excess unbound antibody will bind to the immobilized antigen and contribute to the final measured signal. Applying this procedure to whole cells expressing different pertactin variants confirmed that both WT and NQ pertactin were efficiently secreted (Fig. 7a). In contrast, secretion of 4K and 4K-NQ was reduced more than 5-fold. Flow cytometry confirmed these results: among cells immunofluorescently stained for surface-exposed pertactin, cells expressing 4K or 4K-NQ exhibit significantly more fluorescence than empty vector (shown as dashed lines) but still secrete about 6–7-fold less pertactin compared with WT or NQ. The labels correspond to the mean fluorescence intensity of the indicated sample. c, summary of the results. Secretion depends on the ability of the passenger to fold regardless of cleavage. AU, arbitrary units.

![Diagram](image)

**FIGURE 6. Introduced mutations do not affect pertactin expression or its stability on the cell surface.** a, expression test of WT and 4K pertactin and their HA-tagged variants. All four constructs are expressed at about the same level after incubation with 50 μM IPTG for similar amounts of time. IPTG incubation beyond 50 min results primarily in the buildup of additional precursor and truncated fragments rather than substantially increased levels of mature, secreted passenger. b, stability of WT and 4K pertactin under experimental conditions. After IPTG-induced expression of each pertactin construct, the cell cultures were repeatedly spun down and resuspended in fresh PBS over a period of ~2 h. Despite a moderate decrease of the precursor levels, both the WT and 4K secreted passengers remain stable and securely attached to the cell surface even after cleavage (12), the extent of secretion can be probed by exposing intact cells to protease activity or immunoaffinity labeling. Additionally, an HA tag was introduced into all constructs to ensure uniform antibody binding. Control experiments confirmed that the introduced mutations did not significantly affect the ability of pertactin to be expressed or to remain attached to the cell surface for the duration of our experiments (Fig. 6).

**FIGURE 7. Effects of folding- and/or cleavage-deficient mutants on secretion.** a, quantification of pertactin secretion by competition ELISA (36) using whole cells. Secretion of 4K and 4K-NQ mutants is more than 5-fold lower compared with wild type or NQ pertactin. Raw signal was normalized to 0 for empty vector (EV) and 1 for WT, but note that the y axis scale is not linear as indicated by the standard curve for purified pertactin passenger shown on the right-hand y axis. Error bars are S.D. from four separate measurements of the same biological sample. b, flow cytometry of pertactin-expressing cells stained for surface-exposed pertactin. Cells expressing 4K or 4K-NQ exhibit significantly more fluorescence than empty vector (shown as dashed lines) but still secrete about 6–7-fold less pertactin compared with WT or NQ. The labels correspond to the mean fluorescence intensity of the indicated sample. c, summary of the results. Secretion depends on the ability of the passenger to fold regardless of cleavage. AU, arbitrary units.
Effects of Macromolecular Crowding on Secretion

We also tested whether secretion could be driven thermodynamically by differential macromolecular interactions that stabilize or destabilize the passenger on one side of the membrane versus the other. Such effects would necessarily be limited to macromolecules because molecules <600 Da can pass freely through the outer membrane (37) and would presumably interact with the passenger on both sides. Relative to the extracellular space, the periplasm is quite crowded with peptidoglycan and periplasmic proteins. This crowded environment could place entropically unfavorable constraints on the movement of the large passenger polypeptide in the crowded periplasm, which could raise its free energy and effectively push it out into the less crowded extracellular space. To test whether a change in macromolecular crowding serves as a driving force for pertactin OM translocation, we increased extracellular crowding by adding the common macromolecular crowding agents Ficoll 70 or dextran to the growth medium. We then monitored their effects on secretion of uncleavable full-length pertactin (P.93EB-NQ) using immunofluorescence microscopy. As shown in Fig. 8, addition of these crowding agents to the growth medium had no detectable effect on secretion, indicating that a difference in macromolecular crowding is not a major driving force.

DISCUSSION

The AT family of virulence proteins is large and diverse (38), but the degree to which this diversity extends to the secretion mechanism remains unknown. We developed a general computational framework within which to explore the impacts of and requirements for possible energy sources to drive translocation of an AT passenger across the OM. Our kinetic simulations (Fig. 2) identified three distinct scenarios that can lead to efficient AT secretion across the OM. Of these, secretion driven by passenger folding at the cell surface (7–11) is most compatible with available experimental data. The first requirement of secretion driven by passenger folding is the inability of the folded passenger to re-enter the periplasm, which is satisfied because the dimensions of the folded passenger exceed the pore diameter of the fully folded β-barrel (22, 29) and the OM is generally impermeable to proteins. The second requirement, that no folding of the passenger occur in the periplasm, has been confirmed here by showing that the stalled but secretion-competent pertactin passenger could remain in the periplasm in a non-native, protease-susceptible state for 90 min (Figs. 3 and 5). Hence for pertactin, both crucial requirements for OM translocation driven by extracellular passenger folding are met. Moreover, pertactin secretion was severely disrupted in folding-deficient mutants (Fig. 7). Taken together, these results strongly support a mechanism in which passenger folding provides a crucial free energy boost needed for efficient OM translocation.

At first glance, these results may seem to contradict the recent finding that AT OM translocation is not significantly disrupted in EspP mutants with a largely disordered passenger (24). However, constructs used in that study preserved a significant portion (>100 residues) from the C terminus of the wild type passenger, including a segment shown previously to be important for passenger folding and OM translocation (9, 10, 39–41). In addition, many studies have found the AT secretion process to be severely disrupted under conditions that induced passenger folding in the periplasm (10, 24, 42, 43), consistent with our simulation results. On balance, it appears that AT OM translocation can be severely disrupted either by premature folding in the periplasm or by a disruption of folding at the cell surface, particularly in the C-terminal region of the passenger.

Our simulations indicated that the irreversible passenger cleavage reaction could also provide a powerful driving force for efficient secretion. Experimentally, however, cleavage-deficient pertactin mutants were secreted with the same efficiency as their cleavable counterparts (Fig. 7). Although this has been demonstrated previously for other ATs (23, 42, 44), the secretion efficiency of a cleavage-deficient mutant has to our knowledge never been tested in the absence of folding. The fact that cleavage could not rescue OM translocation of the folding-impaired 4K pertactin means that cleavage represents a separate process uncoupled from secretion. In other words, for 4K, the pool of secreted precursor molecules is depleted by cleavage but is not replenished presumably because for 4K there is a rate-limiting step earlier in the secretion process related to deficient passenger folding.

FIGURE 8. Secretion of the uncleavable full-length pertactin (P.93EB-NQ) monitored by anti-pertactin immunofluorescence microscopy. Extracellular crowding does not reduce secretion efficiency.
We must therefore consider AT secretion as an energy barrier problem. The rate constant of any biochemical process is directly related to the free energy needed to attain the transition state on the pathway between the more stable initial state and most stable final state: the higher this energy barrier, the lower the rate constant. Crossing a hydrophobic membrane represents a particularly high energy barrier, recently estimated to require 650 ATP equivalents for the transport of a single folded protein across two membranes (45). However, it appears that the AT secretion mechanism reduces this barrier by keeping the passenger in an unstable, non-native conformation until it reaches the cell surface. Because the AT protein starts out in an unstable high energy state, less additional energy is needed to reach the transition state. How the cell preserves the passenger in an unstable conformation while it resides within the periplasm is a matter of ongoing research, but it is reasonable to expect that it involves periplasmic chaperones known to interact with autotransporters before and during OM translocation (16–18).

Starting from an unfolded conformation in the periplasm, the passenger must first find and enter the OM translocation channel. Regardless of whether this channel is formed by the AT β-barrel domain alone or is held open and/or complemented by chaperones like BamA or TamA (14, 46, 47), entering this constrained space represents an energetically unfavorable confinement of the passenger (Fig. 9). To overcome this energy barrier, passenger insertion must therefore be coupled to an energetically favorable process. It has been shown that no external energy sources are required for secretion in vitro (19), and passenger folding is also not available as a driving force at this early stage (Fig. 3e). That leaves the energetically highly favorable folding and OM insertion of the β-barrel (48) as the most likely candidate to provide the energy required to confine a portion of the unfolded passenger within its OM translocation channel. This process is catalyzed in vivo by two related chaperone complexes, BAM and TAM (15), at least one of which appears to be required for OM translocation of AT passengers (18, 19, 47, 49). It is possible that these chaperone complexes serve to couple the energetically unfavorable insertion of the passenger into the translocation pore to the favorable process of folding and OM insertion of the β-barrel. Specific structural motifs recently discovered in the lumen of the β-barrel (50) could also be involved.

Once the passenger has been inserted into the translocator channel, small movements of additional residues in either direction across the OM would not significantly change the entropy of the system and hence would not represent a major energetic obstacle. However, OM translocation of the entire passenger requires moving a large number of residues to the cell surface, which is statistically less likely than moving a smaller number of residues back into the periplasm. Here, folding of the passenger C terminus becomes crucial as it lowers the free energy of subsequent OM translocation intermediates, ensuring that secretion proceeds smoothly downhill (Fig. 9). This implies that longer passengers will be more difficult to secrete and may rely more on folding as a driving force. Consistent with this hypothesis, large endogenous AT passengers appear to have stricter requirements for efficient secretion than small heterologous passengers (6, 51). For example, secretion of IcsA is compromised by mutations in the C-terminal end of the endogenous passenger (52), whereas substituting the entire passenger for the smaller heterologous MalE protein results in efficient secretion (43). The energetic model presented in Fig. 9 also explains why stability of the passenger C terminus is important for secretion efficiency, whereas folding of the N terminus has much less of an effect and can even be detrimental to efficient secretion (10).

In conclusion, we show here that the pertactin passenger retains a non-native, protease-susceptible conformation in the periplasm. This confirms a long-standing hypothesis about the
AT secretion mechanism and is a prerequisite for harnessing passenger folding as a driving force for OM translocation. Consistent with this mechanism, disrupting passenger folding greatly reduced the secretion efficiency of pertactin. Although other ATs may utilize additional energy sources to facilitate secretion, including charge-charge interactions with lipopolysaccharides (24), crowding effects, or association with other extracellular macromolecules, we can conclude that for pertactin coordinated protein folding constitutes a major driving force for secretion. Finally, we present a working model that clarifies the connection between the conformational energetics of AT passengers and the mechanism of OM secretion. This model is consistent with known features of the secretion process and moreover provides a framework within which to understand the multiple ways protein stability and other forces can affect the efficiency of OM translocation for members of this large and diverse family of virulence proteins.

Acknowledgments—We thank Ali Karim Ahmed for assistance with this large and diverse family of virulence proteins.

Driving Forces of Autotransporter Secretion

Note Added in Proof—Fig. 5C was not formatted correctly in the version of this article that was published on February 10, 2015 as a Paper in Press. The figure has been revised to conform with JBC policies.

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