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Membrane translocation by anthrax toxin

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Abstract

Much attention has been focused on anthrax toxin recently, both because of its central role in the pathogenesis of *Bacillus anthracis* and because it has proven to be one of the most tractable toxins for studying how enzymic moieties of intracellularly acting toxins traverse membranes. The Protective Antigen (PA) moiety of the toxin, after being proteolytically activated at the cell surface, self-associates to form a heptameric pore precursor (prepore). The prepore binds up to three molecules of Edema Factor (EF), Lethal Factor (LF), or both, forming a series of complexes that are then endocytosed. Under the influence of acidic pH within the endosome, the prepore undergoes a conformational transition to a mushroom-shaped pore, with a globular cap and 100 Å-long stem that spans the membrane. Electrophysiological studies in planar bilayers indicate that EF and LF translocate through the pore in unfolded form and in the N- to C-terminal direction. The pore serves as an active transporter, which translocates its proteinaceous cargo across the endosomal membrane in response to ΔpH and perhaps, to a degree, $\Delta\psi$. A ring of seven Phe residues (Phe427) in the lumen of the pore forms a seal around the translocating polypeptide and blocks the passage of ions, presumably preserving the pH gradient. A charge state-dependent Brownian ratchet mechanism has been proposed to explain how the pore translocates EF and LF. This transport mechanism of the pore may function in concert with molecular chaperonins to effect delivery of effector proteins in catalytically active form to the cytosolic compartment of host cells.

Keywords

Brownian ratchet; planar bilayer; proton gradient

1. Introduction

For many disease-causing bacteria a key strategy for survival within mammalian hosts is to deliver selected enzymes (effector proteins) into the cytosol of host cells, primarily with the aim of killing or disabling key cellular elements of the immune system. Delivery of an effector protein requires specialized machinery to enable it to cross a membrane at some level within the host cell, and much attention has been devoted to defining such machinery and understanding how it functions. Some of the simplest and most extensively studied systems for protein translocation are found in intracellularly acting bacterial toxins. Our current understanding of translocation by anthrax toxin, which has proven to be one of the most tractable toxins for studying this process, is summarized here. Readers may also wish to consult

other recent reviews relevant to this topic (Collier, 2003; Finkelstein, 2009; Puhar, 2007; Young, 2007).

Anthrax toxin is an ensemble of three large, multidomain proteins, which are secreted from *Bacillus anthracis* as monomers and self-assemble on receptor-bearing cells into a series of toxic, hetero-oligomeric complexes (Pimental, 2004; Smith, 2000). Two of the proteins are enzymic intracellular effectors: Lethal Factor (LF, 90 kDa), a Zn^{++} -dependent protease (Duesbery, 1998; Vitale, 1998), and Edema Factor (EF, 89 kDa), a Ca^{++} - and calmodulin-dependent adenyl cyclase (Leppla, 1982). The third is a receptor-binding and pore-forming protein, called Protective Antigen (PA, 83 kDa), which transports EF and LF from the extracellular milieu to the cytosolic compartment of mammalian cells. EF and LF can be transported to the cytosol by PA and act independently of one other, a fact that has given rise to the terms Edema Toxin, EdTx, and Lethal Toxin, LeTx, for the binary combinations, EF + PA and LF + PA, respectively (Ezzell, 1984; Friedlander, 1986). However, all three components of the toxin are produced during *B. anthracis* infections, and can combine to form ternary complexes as well as binary complexes during self-assembly at the cell surface (Pimental, 2004). In addition, any given host cell that is affected by EF is almost certainly affected by LF, and vice versa; and there is evidence of synergy between these two effector proteins (Cui, 2007; Rossi Paccani, 2007; Tournier, 2005). Thus, while the terms Edema Toxin and Lethal Toxin are useful in analyzing and describing experimental findings, it is also appropriate to think of the ensemble of PA, EF, and LF as a single system.

2. Protective Antigen

2.1 Activation, acidic pH, and pore formation

Leppla and coworkers showed that PA must be proteolytically activated in order to interact with LF and EF (Singh, 1989). The activation involves cleavage of the native protein into N-terminal 20-kDa and C-terminal 63-kDa fragments (PA_{20} and PA_{63} , respectively) and may be effected in vivo by cell-associated furin-family proteases (Klimpel, 1992) or by proteases in the blood of animals (Ezzell, 1992; Moayeri, 2007). For research purposes trypsin is commonly used to activate PA in solution. The relative importance of activation by protease(s) in the blood vs. cell-associated proteases during *B. anthracis* infections remains unknown. PA_{20} and PA_{63} remain tightly associated by noncovalent interactions, but may be separated by anion-exchange chromatography (Leppla, SH et al., 1988). PA_{20} has been reported to affect gene transcription in human peripheral blood leukocytes and to induce apoptosis (Hammamieh, 2008). However, it is clear that PA_{63} mediates the biological effects of LF and EF, which can lead to death of the host.

Besides proteolytic activation, another factor affecting anthrax toxin action is acidic pH within an intracellular, membrane-bound compartment. This was first shown in studies in which the lethal action of LeTx on mouse peritoneal macrophages was found to be blocked by pretreatment of the cells with various amines or monensin, agents that raise the pH of acidic intracellular compartments and thereby dissipate transmembrane proton gradients (Friedlander, 1986). The dependence on acidic pH is reminiscent of that found in certain other bacterial toxins, such as diphtheria, botulinum, and tetanus toxins, which are endocytosed and undergo translocation across endosomal membranes in response to acidic intravesicular conditions (Sandvig, 1980). Thus passage through an acidic intracellular compartment was inferred as a required step in anthrax toxin action.

PA_{63} , but not native PA, is able to form ion-conductive pores (channels) in membranes, as first demonstrated in electrophysiological studies in planar phospholipid bilayers (Blaustein, 1989). In those studies trypsin-activated PA or purified PA_{63} , but not PA_{20} , formed cation-selective channels that were both pH- and voltage-dependent. PA_{63} has also been shown to

permeabilize liposomes and the plasma membrane of mammalian cells to monovalent cations under acidic conditions (Koehler, 1991; Milne, 1993; Sun, 2007; Zhao, 1995).

2.2 The prepore

Electron microscopic analysis revealed that purified PA₆₃ in solution contains heptameric, ring-shaped oligomers, a finding that served as a clue to the mechanism of channel formation (Milne, 1994). The crystallographic structures of both monomeric PA and heptameric PA₆₃ have been determined (Lacy, 2004; Petosa, 1997). Native PA contains four folding domains (Figure 1). Domain 1 is N-terminal and contains the furin recognition site, RKKR, within a protease-accessible loop (Klimpel, 1992), and two Ca⁺⁺ ions that help stabilize the structure (Gao-Sheridan, 2003). Domain 2 is directly responsible for pore formation and contains a disordered loop, the 2β2-2β3 loop, that ultimately forms the membrane-penetrating element of the pore. In addition, domain 2 forms a luminal structure that actively catalyzes polypeptide translocation (Krantz, 2005). Domain 3 functions in oligomerization of PA₆₃ (Mogridge, 2001), and domain 4, in receptor recognition (Singh, 1991).

The crystallographic structure of PA₆₃ revealed details of the ring-shaped heptamer originally seen in electron micrographs: a hollow ring, 85 Å high and 160 Å in diameter, with a central lumen averaging ~35 Å in diameter (Petosa, 1997). Removal of PA₂₀ eliminates a steric clash that prevents native PA from oligomerizing. Domain 1' (that portion of domain 1 remaining as a part of PA₆₃) and domain 2 are lumen-facing, and domains 3 and 4 are on the outside. The 2β2-2β3 loop was not seen in the original 4.5-Å structure, but was later observed in a 3.6-Å structure to project outward and to pack between domains 2 and 4 of the neighboring monomer (Lacy, 2004).

No belt of hydrophobic amino acids was apparent on the PA₆₃ heptamer, suggesting that this structure corresponded to a precursor of the pore, or “prepore”. How the transition of the prepore to the pore might occur was suggested by the structure of the Staphylococcal α-hemolysin pore, solved at 1.9 Å resolution by Gouaux and coworkers (Song, 1996). The hemolysin structure showed a mushroom-shaped homoheptamer with a stem comprising a transmembrane 14-strand antiparallel β barrel. Each of the seven protomers contributed two β strands. Although there was no evidence of sequence similarity between PA and the hemolysin, the primary structure of the 2β2-2β3 loop was consistent with the hypothesis that the loop could undergo a conformational rearrangement to form a similar 14-strand β barrel, with outward-facing hydrophobic side chains interacting with the hydrophobic core of the membrane, and inward-facing hydrophilic side chains contacting the aqueous lumen of the pore. This model received support from electrophysiological studies in which cysteine-scanning mutagenesis of the 2β2-2β3 loop was coupled with chemical derivatization by a thiol-reactive reagent that reported on the orientation of individual side chains (Benson, 1998; Nassi, 2002).

2.3 The pore

The heptameric PA₆₃ prepore (Figure 1B) is stable in solution at pH≥8, and readily dissociates into its constituent 63-kDa subunits when exposed to SDS (Milne, 1994). As the pH is lowered to neutrality and into the acidic range, the prepore undergoes a conformational rearrangement that enables it to insert into membranes, and concomitantly the heptamer becomes insoluble and forms aggregates. After treatment at low pH, PA₆₃ runs on SDS polyacrylamide gels as a series of SDS-resistant, high molecular-weight aggregates. The tendency to aggregate in aqueous buffers, which is typical of integral membrane proteins, has so far precluded crystallization of the pore and has made analysis of its structure by electron microscopy difficult.

The first direct images of the PA₆₃ pore were obtained by negative-stain electron microscopy (Figure 1D), following discovery that the *E. coli* chaperonin, GroEL, a quaternary chaperonin with radial seven-fold symmetry, binds to the prepore and the pore and inhibits aggregation of the latter (Katayama, 2008). There is no evidence that GroEL or related chaperonins are relevant to the physiological functioning of PA, but GroEL proved useful as a molecular scaffold for structural analysis, permitting free pore and pore:GroEL complexes to be imaged. Prepore-to-pore conversion was induced in these experiments by adding urea to 1 M, instead of lowering the pH, because GroEL is sensitive to acidic conditions. Single-particle analysis of the pore:GroEL and unbound-pore populations yielded virtually identical reconstructions of pore structure, at 25-Å and 28-Å resolution, respectively.

As predicted, the pore was seen to be a mushroom-shaped object with a 125-Å-diameter cap and 100-Å-long stem (Katayama, 2008). Contradicting the initial hypothesis that GroEL might bind the 2β2-2β3 loops during prepore-to-pore conversion, the chaperonin was seen to bind coaxially to the cap. GroEL may have interacted with N-termini of the PA subunits, suggesting mobility of these segments of polypeptide. The seven copies of domain 3 were observed as “knobs” radiating out from the cap, but domain 4 was not seen. Presumably this was because domain 4 was mobile or disordered under the conditions of the experiment, and the corresponding density was diminished during particle averaging.

The length of the stem is consistent with conversion, via conformational transformation, of the Greek key motif comprising the 2β2-2β3 loop and flanking β strands, β1-β4, into a 100-Å β barrel (Figure 1C) (Nassi, 2002; Petosa, 1997). The membrane-spanning region, corresponding to the 2β2-2β3 loop, comprises roughly the distal third of the stem. The turn region of the loop contains two Phe residues, F313 and F314, which form a hydrophobic tip of the stem and may aid in insertion and stabilization of the stem in membranes. In addition, the stem contains another Phe residue, F324, which is predicted to interact with the *cis* leaflet of the membrane. These Phe residues presumably form the aromatic girdles, which are common in integral membrane proteins and interact with the interface between the hydrophobic core of the membrane and the head-group regions (Schulz, 1993).

2.4 EF and LF binding

LF and EF bind to PA₆₃ competitively via homologous N-terminal domains (Bragg, 1989; Quinn, 1991), termed LF_N and EF_N, respectively. LF_N and EF_N correspond to discrete folding domains of the parent proteins and are loosely tethered to the C-terminal, multidomain catalytic regions of these proteins (Drum, 2002; Pannifer, 2001). PA is able to bind LF and EF only after it has been proteolytically activated, the PA₂₀ fragment has dissociated, and the PA₆₃ fragment has self-associated to form oligomers (Mogridge, 2002).

By two independent methods, it was shown that heptameric PA₆₃ binds a maximum of three molecules of either LF or EF, or a combination of both (Mogridge, 2002). This stoichiometry, combined with the finding that both EF and LF can bind to the same prepore, implies that as many as nine different complexes can be generated by interactions of the heptamer with 1, 2, or 3 molecules of EF or LF alone or of both proteins in 1:1, 2:1 or 1:2 ratios, respectively (Pimental, 2004). That more than one bound ligand molecule may be translocated by a single prepore is suggested by experiments showing that the fraction of bound LF_N molecules translocated across the plasma membrane of CHO-K1 cells in response to acidification of the external medium remained constant at ~0.4 as the degree of saturation of the prepore was varied (Zhang, 2004).

The peculiar stoichiometry of three ligands per heptameric prepore is consistent with a model in which a high-affinity ligand (LF/EF) binding site (Elliott, 2000) is formed at the intersection of two PA₆₃ subunits (Cunningham, 2002). Indeed, it has been possible to isolate a ternary

complex containing one ligand molecule and one molecule of each of two different oligomerization-deficient PA₆₃ mutants with lesions on a different PA₆₃-PA₆₃ contact faces (Mogridge, 2002). A robust model of how LF and EF dock onto the prepore was generated after identifying three LF-PA contact points, defined by a specific disulfide crosslink and two pairs of complementary charge-reversal mutations (Lacy, 2005). The contact points were consistent with the lowest energy LF-PA complex found by protein-protein docking with the Rosetta energy minimization program. Additional data consistent with this model were obtained using enhanced peptide amide hydrogen/deuterium exchange mass spectrometry and directed mutagenesis to define the surface on LF_N that interacts with PA₆₃ (Melnyk, 2006). By directed mutations it was shown that the Lys197 residues on two neighboring PA₆₃ subunits can interact simultaneously with separate sites on a single LF_N molecule, thereby providing further evidence that the LF/EF binding site encompasses two adjacent subunits of the prepore.

An important conclusion of the combined studies on the orientation of LF_N docked to the prepore is that helix 1 of this domain, which extends into solution from the main body of the domain, positions the highly charged and disordered N-terminal region (residues 1-26 of LF) directly over the lumen of the prepore (Lacy, 2005; Melnyk, 2006). As detailed below, this is ideal for entry of the N-terminus into the pore and initiating N- to C-terminal translocation.

2.5 Receptor binding

The action of anthrax toxin is initiated by binding of PA to cellular receptors (see Young and van der Goot, this volume). Two receptors have been identified, termed ANTXR1 (or TEM8, tumor endothelial marker 8) and ANTXR2 (or CMG2, capillary morphogenesis factor 2) (Bradley, 2001; Scobie, 2003). Both are type 1 membrane proteins, and share 60% amino acid identity and a metal ion-dependent adhesion site (MIDAS), which is important for the interaction with PA. PA binds directly to an extracellular domain related to von Willebrand factor type A or integrin inserted domains (VWA/I) present in both. The prepore binds 7 equivalents of ANTXR2 VWA/I, and the affinity of the interaction is high ($K_d \sim 0.2$ nM) (Elliott, 2000; Lacy, 2004; Wigelsworth, 2004). The affinity for ANTXR2 is significantly lower, however.

Crystallographic structures have been determined for the ANTXR2 VWA/I domain alone and complexed with native PA or the prepore (Lacy, 2004; Lacy, 2004; Santelli, 2004). Most of the contact surface of the VWA/I domain is with domain 4 of PA, but there is also significant contact with the 340-348 loop of domain 2. The fact that the VWA/I domain contacts domain 2 as well as domain 4 suggested that VWA/I might stabilize PA₆₃ and alter the pH at which the prepore converts to the pore. Indeed, by two independent methods, this interaction was shown to lower the threshold of prepore-to-pore conversion by a pH unit (Lacy, 2004). The lower affinity of ANTXR1 for PA has physiological consequences, as prepore-to-pore conversion occurs at a higher pH. The pH threshold for conversion of the prepore to the pore and for translocation differ by about a pH unit, depending on whether ANTXR2 or ANTXR1 is the receptor (Rainey, 2005). Because ANTXR2 requires a lower pH, intoxication via this receptor is more sensitive to ammonium chloride than via ANTXR1. These findings suggest that translocation may occur at different points in the endocytic pathway, depending on the specific receptor to which the PA is bound.

3. Translocation

3.1 Systems for study

A cell-based assay developed by Olsnes and coworkers for probing the translocation of diphtheria toxin across the plasma membrane was adapted to anthrax toxin (Falnes, 1994; Wesche, 1998). In that assay, radiolabeled translocation ligands were bound to proteolytically

activated PA at the surface of CHO or L6 cells, and translocation was induced by lowering the pH of the medium. The cells were then treated with Pronase E to degrade exposed label at the cell surface, and protease-protected ligands were quantified after fractionation on SDS polyacrylamide gels. LF_N was found to be translocated most efficiently (35-50%), whereas LF, EF, or fusion proteins containing LF_N fused to certain heterologous proteins, such as DTA or dihydrofolate reductase (DHFR) were translocated less efficiently (15-20%). LF_N fusions to certain other proteins did not translocate at all. The efficiency of translocation of EF and LF from the endosome is not known.

Results obtained with this cell-surface translocation assay strongly suggested that an effector protein must unfold in order to be translocated, a concept commensurate with the estimated size of the PA₆₃ channel (~12-15 Å) (Blaustein, 1990; Song, 1996; Wesche, 1998). Thus, introduction of an artificial disulfide into the DTA moiety of LF_N-DTA blocked translocation of this fusion protein, and translocation of LF_N-DHFR or LF_N-DTA was blocked by their ligands, methotrexate and adenine, respectively. The failure of other LF_N fusions to translocate may have resulted from inability of the passenger proteins to unfold properly under conditions of the assay.

An electrophysiological system for studying translocation across planar phospholipid bilayers involving only toxin proteins has yielded invaluable data on the process (Krantz, 2005; Zhang, 2004; Zhang, 2004). In this system one first forms PA₆₃ pores in a bilayer by adding purified prepore or trypsin-activated PA to one chamber (defined as the *cis* chamber). Typically both compartments contain 100 mM KCl, buffered to pH 5.5, and a low transmembrane potential (e.g., +20 mV) is applied. Once PA₆₃ has been depleted from solution and the current has plateaued, the *cis* compartment is perfused to remove residual protein from the aqueous phase. Adding LF_N to the *cis* compartment leads to a rapid and almost complete blockage of current, as LF_N binds to the pore. Raising the transmembrane potential to +50 mV, or raising the pH of the *trans* compartment, then restores the current to approximately the original level. A variety of controls support the conclusion that this restoration of current corresponds to translocation of the channel-blocking ligand through the pore.

3.2 The N terminus of effector proteins initiates N to C translocation

Deleting more than about 20 residues from the N-terminus of LF_N ablated the protein's ability to block conductance and strongly inhibited its translocation across the plasma membrane of CHO-K1 cells in response to low pH (Zhang, 2004; Zhang, 2004). These results strongly suggested that translocation is initiated by entry of the N-terminus into the pore and that translocation proceeds in an N- to C-terminal direction. Supporting this concept were the findings (i) that binding of streptavidin to a biotinylated Cys residue at the N-terminus of LF_N prevented the protein from blocking ion conductance, and (ii) when the disulfide bridge linking the protein to biotin was reduced, conductance blocking activity was restored.

There is evidence that entry of the N-terminus of LF_N into the pore depends primarily on its having a net positive charge. The disordered N-terminal regions of LF and EF are not identical in sequence, but are densely populated by charged residues - approximately equal numbers of positively and negatively charged residues. At acidic pH values, neutralization of the acidic residues would be expected to give the region a net positive charge and cause the N-terminus to be drawn by electrostatic attraction into the negatively charged pore, perhaps aided by a positive transmembrane potential. There is general agreement that the potential across the endosomal membrane is positive, but estimates of the magnitude vary widely (Krantz, 2006; Rybak, 1997; Sonawane, 2002; Van Dyke, 1994). The notion that positive charge is key to initiating translocation through the PA₆₃ pore is supported by the finding that fusing a His₆ tag to the N-terminus of N-terminally truncated forms of LF_N restored both the ability to block ion conductance and the ability to undergo translocation across planar bilayers (Zhang,

2004). In addition, fusing short polycationic tracts (e.g., Lys₆) to the N terminus of DTA, the catalytic domain of diphtheria toxin, enabled it to undergo PA-dependent entry into CHO-K1 cells, albeit with lower efficiency than LF_N-DTA (Blanke, 1996). Free LF_N competitively inhibited the entry of LF, EF, or LF_N-DTA, but not Lys₆-DTA, suggesting that the latter bound to a different site on the PA₆₃ pore. It now seems likely that the polycationic tracts electrostatically “guided” the N-terminus of the DTA fusion proteins into the negatively charged pore lumen, initiating translocation through the pore, thereby avoiding the intermediate, LF_N-dependent step of binding to the mouth of the pore.

3.3 Luminal mutations in the pore elucidate its function

Certain mutations in PA have given important clues to the mechanism by which the pore functions in translocation. Of particular interest are point mutations in two luminal loops of PA, which are distant both in primary structure and topography from the loop (2β2-2β3) that forms the transmembrane 14-strand β barrel (Sellman, 2001). Initially mutations within the 2β7-2β8 loop and the 2β10-2β11 loop were found to inhibit the ability of PA to mediate pore formation and translocation in cultured cells, without altering other functions of the protein, such as receptor binding and the ability to be proteolytically activated and oligomerize. Mutations in Asp425 and Phe427, which lie in the 2β10-2β11 loop, were of particular interest. The D425A mutation completely blocked conversion of the SDS-dissociable prepore to the SDS-resistant pore and thus prevented ion-conductive channels from forming in planar bilayers. The F427A mutation partially inhibited prepore-to-pore conversion, but strikingly, the pores that formed lacked protein translocation activity. The K397A mutation, in the 2β7-2β8 loop, also affected prepore-to-pore conversion.

The potency of these mutations in blocking the biological activity of PA, together with the knowledge that PA₆₃ forms homo-oligomers as an essential step in anthrax toxin action, suggested that PA carrying mutations in Lys397, Asp425, and/or Phe427, might co-assemble with wild-type PA and act as dominant-negative inhibitors of toxin action in vivo (Sellman, 2001). When translocation-deficient mutants were tested by mixing various amounts of each one with a constant amount of wild-type PA and testing the mixtures for ability to mediate the action of LF_N-DTA on CHO-K1 cells, four of the mutants showed strong dominant-negative activity. Two carried point mutations, F427A and D425K; one carried two point mutations, K397D/D425K; and the fourth carried a deletion of residues 302-325, corresponding to the 2β2-2β3 loop. The results suggested that with the most potent of the inhibitors the incorporation of a single mutated PA₆₃ protomer was sufficient to inactivate a prepore. In support of this notion, mixing the F427A mutant, the double mutant, or the 2β2-2β3 loop deletion mutant, in a 1:1 ratio or even a substoichiometric, 1:4 ratio, with wild-type PA allowed Fischer 344 rats to survive Lethal Toxin action, while control animals reached a moribund state within 90-100 minutes. Co-oligomerization of wild-type PA₆₃ with a mutant in which residues of the 2β2-2β3 loop were replaced with ones from its homologue, iota-b toxin, also proved strongly inhibitory (Singh, 2001).

Recently the strongly dominant effects of the D425A and F427A mutants were demonstrated directly (Janowiak, 2009). Each mutant protein was mixed with a 20-fold excess of wild-type PA, the mixture was activated with trypsin, and the prepore fraction, containing co-oligomerized mutant and wild-type PA₆₃ protomers, was isolated. Heteroheptamers with a single mutated subunit were then purified from the heterogeneous mixture of prepores and characterized. The presence of the single D425A protomer within an otherwise wild-type prepore was found to inhibit pore formation by $>10^4$, to block prepore-to-pore conversion, and to abrogate PA activity in a standard cytotoxicity assay. The single F427A protomer inhibited cytotoxicity ~100-fold, resulting from strong inhibition of translocation, together with smaller effects on pore formation and ligand affinity.

3.4 Phenylalanine-427 and the Phe clamp

The effects of F427 mutations on the properties of PA have yielded insights into the translocation mechanism. Most importantly, the translocation of LF_N across planar bilayers was found to be strongly impaired by mutations at position 427 (Krantz, 2005), and the effects of various amino acid replacements for F427 on translocation in this system correlated with effects on cytotoxicity in cellular assays. Trp, Leu, and Tyr proved to have good activity in promoting translocation through the PA pore in planar bilayers and were effective in promoting cell killing by LF_N-DTA, whereas residues with small aliphatic or hydrophilic side chains were inefficient, or inactive, in both assays. Remarkably, Ile was far less active than Leu, indicating a sensitivity to the location of the branch point in the aliphatic side chain.

Judging from the crystallographic structure of the prepore, the F427 residues in neighboring residues are 15-20 Å apart and are solvent-exposed within the lumen. Evidence from electron paramagnetic resonance spectroscopy (EPR) measurements suggested that these residues moved into close proximity (<10 Å) during prepore-to-pore conversion (Figure 2) (Krantz, 2005). There is evidence suggesting that this movement involves formation of a salt bridge between Asp426, a residue immediately adjacent to Phe427, and Lys397 within a neighboring subunit (Melnyk, 2006). Consistent with the notion that the Phe427 residues are in close proximity to each other in the pore, single-channel ion conductance values were roughly inversely proportional to the size of the substitution at position 427. For example, channels formed from F427W or F427L mutants showed smaller ion-conductance values than channels with Phe or Ala at this position. The F427A mutation almost doubled the single-channel flux of monovalent ions (mainly K⁺) through the pore. These findings imply that the seven Phe427 residues of the prepore form a narrow constriction in the pore lumen - a “ring of rings,” which has been dubbed the Phe clamp (or “ ϕ clamp”). The Phe clamp may be viewed as an active site, crucial for protein translocation in this system.

The hypothesis that the Phe clamp interacts directly with the translocating polypeptide is strongly suggested by single-channel recordings in planar bilayers, in which addition of LF_N to the *cis* compartment was found to block conductance (Krantz, 2005). With wild-type PA channel, blockage was almost complete (>95% at +20 mV and ~10 nM LF_N) and was stable, as manifested by a continuously closed state. In contrast, F427A PA formed channels in which a saturating level of LF_N caused incomplete blockage (on average ~50%) and dynamic flickering among open, closed, and partially closed states. These findings suggest that the positively charged N terminus of LF_N enters the pore and interacts either directly with the Phe residues at position 427 or with nearby residues in such a manner that the pathway of ion flow through the pore is blocked.

How the Phe clamp functions is crucial to understanding how the pore transports its proteinaceous cargo. The superiority of the Phe side chain to others suggests that its π clouds also contribute through aromatic-aromatic, cation- π , or π - π interactions. Consistent with this notion, the integrity of the Phe clamp was found to be important for the binding of hydrophobic cations, such as tetrabutylammonium, tetraphenylphosphonium, and others that serve as channel blockers. In addition the Phe clamp was found to be the site of binding of the polyaromatic, 4-aminoquinolone drug, quinacrine (Orlik, 2005). The fact that the Phe clamp requires an aromatic surface or centrally oriented aliphatic surface to function properly suggests that it may serve as a site for transient interactions with hydrophobic side chains of translocating polypeptides (Krantz, 2005). Studies on LF_N and EF_N as a function of pH showed that these domains undergo a transition to a molten globule state in the pH range of the endosome (Krantz, 2004). Further unfolding of this molten globule state to a fully unfolded state commensurate with translocation can be conceived to occur if the Phe clamp creates an environment mimicking the hydrophobic core of the unfolding protein, thereby reducing the energy penalty of exposing hydrophobic sequences to solvent.

That this hypothesis may provide an incomplete explanation of the role of the Phe clamp is suggested by results showing that protein translocation through the PA pore is driven by a proton gradient (Krantz, 2006). Conductance measurements in planar bilayers indicated that LF_N, and less efficiently LF and EF, were translocated from the *cis* compartment to the *trans* compartment, under the influence of a proton gradient even under a negligible applied membrane potential. Translocation was found to depend on the magnitude and sign of the pH gradient. ΔpH promoted translocation more effectively at lower voltages than at higher voltages and occurred even under negligible membrane potential.

How could translocation of a polypeptide through the PA pore in a phospholipid bilayer be driven solely by a proton gradient? The bilayer system employed consisted of nothing more than diphytanoyl-phosphatidyl choline membrane, the pore, and the translocation substrate (e.g., LF_N). No cellular proteins and no alternative energy source, such as ATP, were present. A potentially relevant observation was that ΔpH was less able to promote translocation through F427A channels, and certain other F427 mutant channels, than through wild-type channels (Krantz, 2006). Generally, with large aromatic or aliphatic residues (except for Ile) were present at position 427. LF_N blocked ion conductance effectively, and ΔpH was effective in promoting translocation. In contrast, LF_N only partially blocked ion conductance through F427A channels and was not translocated when the transmembrane potential was raised. The F427A channels showed an imperfect seal and a flickering pattern of conductance, suggesting that the positively charged N-terminal region of LF_N was not firmly held at the Phe-clamp site (Krantz, 2005). These findings suggested a possible alternative function for the Phe clamp, namely that it blocks the passage of protons, and thus maintains the pH gradient that drives translocation.

3.5 A charge state-dependent Brownian ratchet mechanism

Krantz et al. have proposed a charge state-dependent Brownian ratchet mechanism to explain the multiple findings described above (Krantz, 2006). The PA channel is known to be strongly cation-selective and is thus biased against passage of negatively charged ions. With this property in mind, it is useful to envision an unfolded protein having partially translocated through the PA pore, such that the N terminus has emerged into the *trans* compartment, while the C terminus remains in the *cis* compartment. The protein is subject to Brownian (thermal) fluctuations, including a component orthogonal to the plane of the membrane. In the absence of a pH gradient or an applied potential, the polypeptide fluctuates back and forth within the pore, the amplitude being limited by various factors, including the locations and charge states of acidic side chains.

In the negatively charged state an Asp or Glu residue is unable to pass the electrostatic barrier within the channel that imposes cation selectivity, regardless of the direction of the approach. Under symmetric pH conditions, any given acidic side chain will spend the same percentage of time in the ionized state, regardless of its location, and thus, no net translocation occurs. However, if one now imposes a pH gradient across the membrane, simulating the gradient across the endosomal membrane, such that the *cis* compartment is acidic and the *trans* compartment neutral, acidic side chains approaching the barrier from the *cis* direction will have a higher probability of being protonated and thus diffusing past the barrier. Thus thermal fluctuations of the polypeptide chain will be biased in the *cis*-to-*trans* direction, causing the polypeptide to undergo a net translocation in that direction. Also, protons that become associated with acidic side chains in the *cis* compartment are released into the *trans* compartment, making the PA pore formally a protein-proton symporter (Krantz, 2006).

If this model is correct, then one would expect introduction of a non-titratable negatively charged side chain into LF_N to block the translocation process. This prediction has recently been fulfilled in experiments in which Cys residues introduced into various locations within LF_N were reacted with 2-sulfonato-ethyl-methanethiosulfonate, yielding a non-titratable

SO₃⁻ group (Basilio, 2009). Voltage-driven translocation was drastically inhibited by a single SO₃⁻ group introduced at a variety of locations within the protein.

At present the charge state-dependent Brownian ratchet mechanism is a plausible and well supported model for the role of the pore in translocation of LF and EF to the cytosolic compartment in cells. In the planar lipid bilayer system, translocation of LF and EF is far less efficient than translocation of LF_N or LF_N-DTA. Additional work is needed to determine whether this difference in efficiency exists in translocation from the endosomal compartment. Also, further research is needed to integrate results in model membranes that led to the Brownian ratchet model with findings that certain cellular proteins, besides the receptors, play a role in anthrax toxin translocation within mammalian cells. A requirement for cellular proteins for PA-dependent release of LF_N-DTA from isolated endosomal vesicles into the extravesicular milieu has been reported (Tamayo, 2008). Such a requirement is not at variance with the Brownian ratchet model of translocation. The release of an anthrax effector protein into the *trans* compartment of a planar bilayer system has not yet been demonstrated, and it is quite conceivable that this step requires ancillary cellular proteins in order, for example, to facilitate refolding or to block nonspecific interaction of the unfolded polypeptide emerging from the PA channel with the adjacent phospholipid bilayer.

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Figure 1.

Various oligomerization states and conformations of PA. A. Native PA, showing the approximate locations of F427 and the 2 β 2-2 β 3 loop (dashed line). In panels A and B, domain 1' is dark blue, domain 2 is red, domain 3 is green, and domain 4 is light blue. In panel A, PA₂₀ is in orange. B. Aerial view of the PA₆₃ prepore, showing the location of F427. C. Conformational transition of the prepore to the pore. The 2 β 2-2 β 3 loops are shown in red. (Modified from (Petosa, 1997)). D. Single-particle reconstruction of the PA₆₃ pore from negative-stain electron microscopy (from (Katayama, 2008)). Domain 4 was not seen, apparently because it is unordered with respect to the main body of the pore.

Figure 2.

Charge state-dependent Brownian ratchet model for translocation through the PA₆₃ pore. A. The pore with LFN bound and its N-terminal region threaded part way through the Phe clamp (yellow). One can envision the entire polypeptide undergoing Brownian motion from the impact of water molecules, the relevant component here being diffusion back and forth along the symmetry axis of the pore. A deprotonated acidic residue (red) cannot pass the electrostatic energy barrier (presumed here to be focused at the Phe clamp (Basilio, 2009)), until it is protonated. Protonation neutralizes the negative charge of the acidic residue (green in panels B and C), allowing it to freely pass the barrier, and deprotonation (panel C) converts it once again to a negatively charged form, which is unable to diffuse back across the barrier.