**CHARGE REQUIREMENTS FOR PROTON GRADIENT-DRIVEN TRANSLOCATION OF ANTHRAX TOxin**

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Anthrax lethal toxin is used as a model system to study protein translocation. The toxin is composed of a translocase channel, called protective antigen (PA), and an enzyme, called lethal factor (LF). A proton gradient (ΔpH) can drive LF unfolding and translocation through PA channels; however, the mechanism of ΔpH-mediated force generation, substrate unfolding, and the establishment of directionality are poorly understood. One recent hypothesis suggests that the ΔpH may act through changes in the protonation state of residues in the substrate. Here we report the charge requirements of LF’s amino terminal binding domain (LFN) using planar lipid bilayer electrophysiology. We find that acidic residues are required in LFN to utilize a proton gradient for translocation. Constructs lacking negative charges in LFN’s unstructured presequence translocate independently of the ΔpH driving force. Acidic residues markedly increase the rate of ΔpH-driven translocation, and the presequence is optimized in its natural acidic-residue content for efficient ΔpH-driven unfolding and translocation. We discuss a ΔpH-driven charge-state Brownian-ratchet mechanism for translocation, where glutamic and aspartic acid residues in the substrate are the “molecular teeth” of the ratchet. Our Brownian-ratchet model includes a mechanism for unfolding and a novel role for positive charges, which we propose chaperone negative charges through the PA channel during ΔpH translocation.

Transmembrane protein translocation (1-4) and intracellular protein degradation (5) are essential processes that allow the cell to traffic protein, form new organelles, maintain protein quality control, and regulate the cell cycle. As folded proteins are thermodynamically stable under typical cellular conditions, protein translocation and degradation often require complex, energy-consuming molecular machines to catalyze the required series of unfolding and translocation steps in these pathways. Some of these protein transporters and degradation machines mechanically unfold their protein substrates via an ATP-dependent motor. However, a transmembrane proton gradient (ΔpH) may also be used to generate an appropriate driving force that can unfold (6) and translocate a substrate protein across a membrane (1).

A transmembrane proton gradient forms a proton motive force (PMF) comprised of two different types of available free energy: an electrical free energy (ΔG_ψ) dependent on the membrane potential (Δψ) and a chemical potential (Δµ_ΔpH) dependent on the proton concentration gradient (ΔpH). The former ΔG_ψ can be derived from the charge of the translocating protein, z, and Faraday’s constant, F, according to the relation, ΔG_ψ = ΔG_ψ° + zFΔψ (1,6,7). The latter Δµ_ΔpH is expressed as a chemical potential, Δµ_ΔpH = Δµ_ΔpH° + 2.3 nRT ΔpH (where n is the number of protons involved in the reaction, and R and T are the gas constant and temperature, respectively) (1,6). Either of these energies can develop enough force under physiological conditions, on the order of tens of pN (6,8), and is sufficient to unfold a protein during translocation (6,9). The molecular mechanism of ΔpH-driven translocation, in particular the enforcement of directionality and substrate unfolding, is not well understood.

Anthrax toxin (10) is a useful model system (4) to study ΔpH-driven protein unfolding (11) and translocation (1,2,6,12). The toxin is a key virulence factor secreted by *Bacillus anthracis* (the...
causative agent of anthrax). It is comprised of three proteins: the translocase component, protective antigen (PA, 83 kDa); and two enzyme components, lethal factor (LF, 91 kDa) and edema factor (EF, 89 kDa). These secreted proteins must assemble into holotoxin complexes either on the surface of cells or in the blood serum (13). In each assembly mechanism, PA is initially cleaved by a protease, allowing a small 20-kDa portion to dissociate. The remaining 63-kDa PA fragment can then self-assemble into either a ring-shaped homoheptamer, PA₇ (14-16) or homooctamer, PA₈ (12,13). PA oligomers then bind to LF and EF and form toxic complexes. Binding occurs at the top of the pre-channel and in a deep amphipathic cleft, termed the α clamp, which also aids in unfolding the substrate (17).

The complex is then endocytosed by the target host cell, and subsequent acidification of the endosome causes the PA oligomer to convert into a transmembrane channel. Moreover, the ΔpH generated by the acidification of the endosome facilitates the unfolding (11) and translocation of LF and EF through PA (1) into the cytosol. ΔpH-driven translocation is aided by two catalytic active sites, the α clamp (17) and the Φ clamp (2). The Φ clamp is located inside the PA channel (1,2). It is comprised of a narrowly-apposed ring of 7 or 8 Phe-427 residues, where one Phe is contributed by each PA monomer in the oligomer (2). The Φ clamp prefers to make interactions with hydrophobic and aromatic groups, and its activity facilitates translocation (2) by helping to unfold the substrate (6). How the Φ clamp stimulates (1) ΔpH-driven translocation is unclear.

While a ΔpH can drive translocation, the groups that are protonated and/or deprotonated in the substrate and/or channel are unknown. One clue is provided by the known cation selectivity of the PA channel (18). This selectivity is likely due to the ~100-150 glutamic and aspartic acid residues (depending upon PA’s oligomeric state) known to be located inside the PA channel (2,19). This cation selectivity is unusual given the acidic isoelectric points of both LF and EF (1). Thus we previously proposed a ΔpH-driven charge-state Brownian ratchet translocation mechanism, (1) which is based upon the theoretical work of Feynman (20), Oster (21), and Astumian (22). In this mechanism, the large available thermal energy of the translocating protein is biased in a useful and directed way by the ΔpH gradient, thereby supporting productive translocation.

Because the channel is cation-selective and, therefore, anion-repulsive, we expect that anionic residues within the portion of LF or EF passing through the channel must be protonated, thus making the translocating chain net cationic. The segment of LF within the channel is in fact net positive, as shown by Δψ-dependent measurements, which report positive z values (6). Once the Brownian thermal energy of the translocating protein causes the substrate to exit to the higher pH cytosolic side of the membrane, deprotonation to a more anionic state is favored. The resulting charge repulsion can then enforce directionality during translocation by preventing backward movement through the anionic channel. This process then repeats in multiple cycles toward completion. In this report, we investigate how ΔpH-driven translocation depends upon charged residues in the substrate.

**Experimental Procedures**

*Constructs and proteins.* Site-directed mutagenesis is performed using a Quikchange procedure. WT PA is expressed and purified as described (2). PA₇ oligomers are produced as described (12). His₆-LF₇ (residues 1-263 of LF) and mutants thereof are purified from overexpressing bacteria using standard Ni²⁺-nitrilotriacetic acid affinity chromatography and Q-sepharose, anion-exchange chromatography, when required (GE Healthcare, USA) (1). When indicated, His₆ tags, which are an amino-terminal, 17-residue leader containing a hexahistidine sequence, MGSSHHHHHHHSSGLVPR, are removed from His₆-LF₇ with bovine α-thrombin treatment (0.5 units/mg of protein) for 30 minutes at room temperature in 20 mM Tris, 150 mM NaCl, 2 mM CaCl₂ and 1 M glucose at pH 8.0. Note that after thrombin cleavage, an additional GSHM sequence is left at the amino terminus of all cleaved LF₇ constructs derived from the pET15b plasmid.

More complex synthetic LF₇ constructs are made using a three-step, gene-synthesis procedure, according to the following scheme:

$$\text{HM}^{1}\text{AGGHGDVG}^{10}\text{HVKEKEKND}^{20}\text{ENKRK}$$
$$\text{DEERN}^{30}\text{KTQEEHLKEI}^{40}\text{MKHIVKIEVK}^{50}\text{GEE}$$


AVKKEAA\textsuperscript{60}EKLLEKPSD\textsuperscript{70}VLEMYKAIGG\textsuperscript{80}KIVYD

The underlined pairs of amino acids on either end are encoded by the restriction sites, Nde I and a silent Sal I site (V84 and D85), respectively, which are used for cloning. Superscripted numbers indicate the numbering convention of LF residues in 1J7N (23). Overlapping oligonucleotides encoding the desired sequences with the amino-terminal replacement are synthesized (Elim Biopharmaceuticals, Inc., Hayward, CA) and amplified by two rounds of polymerase chain reaction (PCR). In Round I, 22 nM of nested oligonucleotides with consistent annealing temperatures of \(\sim 55^\circ C\) are amplified in a standard PCR reaction. In Round II, 1 \(\mu\)L of the PCR product made in Round I is amplified with the two outermost PCR primers (1 \(\mu\)M each) to make the synthetic double-stranded DNA fragment. These LFN synthetic DNA fragments are ligated via a 5’ Nde I site and 3’ Sal I site into the pET15b-LFN(Sal I) construct, which contain an in-frame, silent Sal I restriction site in LF N at V84 and D85. The synthetic LFN constructs are overexpressed, purified, and their His\textsubscript{6} tags were subsequently removed as described above (when required).

**Electrophysiology.** Planar lipid bilayer currents are recorded using an Axopatch 200B amplifier (Molecular Devices Corp., Sunnyvale, CA) (6,12). Membrane bilayers are painted onto a 100-\(\mu\)m aperture of a 1-mL polyethersulfone cup with 3% 1,2-diphytanoyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids, Alabaster, AL) in neat n-decane. Cis (side to which the PA oligomer is added) and trans chambers are bathed in the indicated buffers as required. By convention, \(\Delta \psi \equiv \psi_{\text{cis}} - \psi_{\text{trans}}\) (\(\psi_{\text{trans}} \equiv 0\) V), and \(\Delta \text{pH} \equiv \text{pH}_{\text{trans}} - \text{pH}_{\text{cis}}\). Translocation experiments are carried out as described (6,12) generally using a universal pH bilayer buffer system (UBB: 10 mM oxalic acid, 10 mM phosphoric acid, 10 mM MES, 1 mM EDTA). However, for translocations requiring a pH > 7.5, we used an altered UBB (6 mM oxalic acid, 6 mM phosphoric acid, 6 mM MES, 6 mM boric acid, 6 mM TAPS, 1 mM EDTA), which is better at buffering in the 7.5-9 range. We found that these two types of buffers produced consistent translocation results. Generally, an additional 100 mM equivalent of KCl is added to the UBB and maintained symmetrically in most translocation experiments, excepting when translocation kinetics are monitored in the absence of a \(\Delta \psi\). In that case, additional KCl is only added to the cis-side buffer. The \(\text{pH}_{\text{cis}}\) is generally 5.6 unless indicated otherwise.

In a typical experiment, we first insert PA\textsubscript{7} channels into a planar bilayer by adding the prechannel oligomeric form to the cis side of the membrane under a small \(\Delta \psi\) of 20 mV. Insertion is observed as an increase in current. Then WT or a mutant LFN is added also to the cis side of the membrane, and a decrease in current is observed. Excess LFN in the cis compartment is removed by perfusion, and the translocation process is initiated by either raising the \(\Delta \psi\) or by creating a \(\Delta \text{pH}\) by raising the pH of the trans chamber. When translocation is initiated by raising the pH of the trans chamber, we set the time when 1% of maximum translocation occurs as \(t = 0\) s in order to control for different mixing times.

An alternative method of creating a proton gradient was also used and provided similar results: the \(\text{pH}_{\text{cis}}\) and \(\text{pH}_{\text{trans}}\) buffer were preadjusted to their final conditions; after substrate binding to the channel was complete, then the cis buffer was perfused at \(\sim 0\) mV at pH 5.6 to maintain the \(\Delta \text{pH}\); then the voltage was raised to the final voltage upon the completion of perfusion (after \(\sim 30\) s).

**Single-channel recordings.** Single-channel LFN-docking experiments were performed as described (6,12). Planar lipid bilayers were bathed in symmetric buffer (100 mM KCl, 1 mM EDTA, 10 mM succinic acid, pH 5.6). PA oligomer was applied directly to membranes at \(\sim 10^{-16}\) M. Single channel insertion is observed by a discrete step in current under an applied voltage. Once a single channel has inserted into the membrane, LFN was added to the cis side of the membrane at 200 pM. Blocking events were recorded at a constant \(\Delta \psi\) of 20 mV for various LFN mutants with and without their His\textsubscript{6} tag. Data were acquired at 400 Hz using a low-pass filter of 200 Hz. For LFN that stably blocked a PA channel, the voltage was reversed to -80 mV after several minutes of conductance block in order to clear the substrate from the channel; this procedure verifies that the channel is
still present. Histograms of the current versus time data were fit to a two or three Gaussian function to obtain the relative percentages of time spent in the open, blocked, and partly-blocked states.

RESULTS

Chemical potential component of PMF is sufficient to drive translocation. To study translocation, we use planar lipid bilayer electrophysiology (1,2,6) and a model substrate, LF N, the amino-terminal, 263-residue, PA-binding domain of LF (24). In this assay, a planar lipid bilayer separates two aqueous chambers (called cis and trans). We first insert PA channels into a planar bilayer by adding the prechannel heptameric form of PA to the cis side of the membrane under a Δψ of 20 mV (Δψ ≡ ψcis - ψtrans, where ψtrans = 0 V). Channel formation, observed as an increase in current, then stabilizes, and either wild type (WT) LF N or an LF N mutant (MUT) is added also to the cis side of the membrane. Subsequently, an exponential decrease in current is observed as LF N’s amino-terminal presequence inserts into the ion-conducting channel and blocks ion flow (25). Excess LF N in the cis compartment is removed by perfusion, and the translocation process is initiated by either changing the Δψ and/or ΔpH. The ensuing current increase generally observed results from LF N translocation to the trans side of the membrane, as inferred by control experiments (1,2,6). Thus two types of parameters can be obtained from translocation records: the time for half of the protein to translocate (1/2, measured in seconds) and the efficiency of translocation, which is equivalent to the fraction of LF N that successfully translocates.

While a ΔpH can drive substrate translocation in the presence of a small, positive Δψ (1), it has not yet been shown if a ΔpH alone is sufficient. To test this possibility, we set up the bilayer with a potassium chloride gradient; this procedure allows current to flow when Δψ is 0 mV. It should be noted that by design, the voltage-clamp amplifier maintains the system at 0 mV even under a ΔpH, which we define as ΔpH ≡ pHtrans - pHcis. At a Δψ of 0 mV and positive ΔpH values, we found that both the rate and efficiency of LF N translocation increase with larger positive ΔpH gradients (Fig. 1 inset). We estimate the activation energy of translocation (∆G‡) using the following relationship, ∆G‡ = RT ln(t1/2/c), where c is an arbitrary constant of 1 s. The ΔpH dependency of the ∆G‡ for LF N translocation is biphasic with two different limiting slopes (Fig. 1). This dependency is similar to what has been observed under varying ΔpH conditions with a constant, nonzero Δψ (1,6). The relationship fits to a two-barrier model:

$$\Delta G^\ddagger(\Delta pH) = RT \ln[\exp((\Delta G^\ddagger_{o1} - 2.3 n_1 \Delta pH)/RT) + \exp((\Delta G^\ddagger_{o2} - 2.3 n_2 \Delta pH)/RT)]$$

(Eq. 1)

where the indexed ∆G^\ddagger_{o1} and n values are the activation energy and number of protons required to cross each barrier, respectively (6). The steeper ΔpH dependency of the ∆G‡ on the left-hand side of the graph (less than 1.2 ΔpH, n1 value of 4.0 (±0.5)) corresponds to a barrier limited by LF N unfolding, (6) whereas the shallower ΔpH dependency on the right-hand side (at higher ΔpH values, n2 value of 0.22 (±0.03)) may correspond to a barrier limited by a yet uncharacterized translocation process (6) (Fig. 1). We conclude that the chemical potential component of the PMF is sufficient to drive LF N translocation. 

ΔpH-driven translocation depends on charged residues in LF N. The charge-state Brownian ratchet model (1) predicts that negatively-charged acidic residues in the substrate are critical to ΔpH-driven translocation. To test this model, we replaced the unstructured amino-terminal presequence of LF N (residues 1 to 27) with a randomized neutral/uncharged background comprised of Gly, Ser and Thr residues (Fig. 2A). We call this neutral mutant background LF NSyn°. This sequence is still polar and flexible but lacks charged residues. We found that LF N Syn° translocates slower than LF N WT under conditions of ΔpH = -0.6 to 1.0, pHcis = 5.6, and Δψ = 60 mV (Fig. 2B). Additionally, the construct possessed a significantly reduced ΔpH dependence (LF N WT: n1 = 1.9 (±0.2); LF N Syn°: n = 0.59 (±0.06); Fig. 2B). (It should be noted that we included the amino-terminal His6 tag in these LF N constructs due to defects in channel blocking for certain constructs—an effect that we explore more fully below. To our knowledge, the His6 tag does not obfuscate our results or interpretations, since we are looking at effects relative to similarly His6-
tagged control constructs.) Initially, we predicted that acidic residues alone would be sufficient to restore LF_N Syn°'s defect in translocation rate and its weaker ΔpH dependence; however, we found this was not the case. LF_N Syn+ has acidic residues restored at their WT positions, further reduced the rate and efficiency of translocation relative to LF_N Syn° and had essentially no ΔpH dependence (n value of 0.0 (±0.1); Fig. 2B). Restoring only basic residues in the LF_N Syn+ construct increased translocation rate relative to LF_N Syn° but further reduced the ΔpH dependence (n value of 0.14 (±0.07); Fig. 2B). Only when both acidic and basic residues are restored in LF_N Syn± do we see translocation that is identical to the WT substrate (Fig. 2B). Moreover, since LF_N WT and LF_N Syn± translocate with identical ΔpH dependencies, we can safely assume that the noncharged residues in the WT leader sequence are unimportant to ΔpH-driven translocation, as these have been substituted en masse with Gly, Ser and Thr residues in LF_N Syn±. Thus acidic residues alone in LF_N’s presequence are not sufficient for efficient ΔpH-driven translocation; instead, a balance of acidic- and basic-charged residues is required.

To further explore the differences between the Syn constructs, we translocated them under more physiologically relevant conditions (ΔpH = 2, ΔΨ = 0 mV). Suspecting that difficulties in protonating all of the acidic residues in LF_N Syn-'s account for its poor translocation, we also varied the cis pH to see if lower pHs would increase its translocation speed relative to the chargeless LF_N Syn°. Here, we report the fraction of substrate translocated after five minutes. This is a better method for comparing substrates that display large differences in both translocation efficiencies and rate. Unsurprisingly, LF_N WT and LF_N Syn° show no cis pH dependence (Fig. 2C). LF_N Syn°, however, displays a moderate, linear cis pH dependence, likely due to the increased substrate destabilization at lower pHs. As predicted, LF_N Syn± has a much stronger cis pH dependence. It displays little translocation within five minutes at cis pH 5.6 (though it does eventually reach 15-20% translocation). By cis pH 5.0, LF_N Syn° and LF_N Syn+ translocate similarly to LF_N WT and LF_N Syn±. When the membrane potential is 0 mV, LF_N Syn+ translocates poorly at all cis pHs. We assume that LF_N Syn°’s comparatively better performance relative to LF_N Syn+ under higher voltage conditions (Fig. 2B) is an artifact of the supplemental Δψ-driving force. Thus we conclude that LF_N Syn+ is greatly stimulated by a PMF dominated by a ΔpH with lower cis pH conditions, i.e., below ~pH 5.2.

Cationic groups in presequence allow anionic residues to penetrate channel. The translocation defect observed for LF_N Syn+ at pH 5.6 was surprising and led us to hypothesize that a presequence dominated by acidic groups was unable to traverse the cation-selective PA channel, likely due to electrostatic repulsion, which impeded entry into the channel. To test this possibility, we examined the ability of the His6-tagged and untagged versions of the LF_N Syn constructs to bind inside the PA channel. In this assay, when LF_N’s presequence binds the PA channel it blocks ion conductance. In bulk, ensemble channel-binding assays, we found that all of the His6-Syn constructs could fully engage and dock into the PA channel, as evidenced by the complete block of channel’s conductance (Fig. 3A). Upon removing the His6-tag, we found that LF_N WT and LF_N Syn± maintained a stable complex with the PA channel. We conclude that uncharged and purely anionic leader presequences are unable to stably engage the PA channel; however, the defects in these constructs may be complemented by a 17-residue His6-tag.

We then examined the channel-blocking activity of the LF_N Syn constructs at symmetrical pH 5.6 in single-channel experiments. The His6-tagged Syn constructs all formed stable, fully-blocked complexes with the PA channel, where few opening (or unblocking) events were observed (Fig. 3C). However, for the untagged LF_N Syn°
and LFN Syn⁻ constructs, we again found noticeable defects in channel blocking activity. The two untagged constructs differ notably in their behavior. Untagged LFN Syn⁰ is able to form transient blockades of the PA channel (which fully block the conductance) for durations of 5-10 s. Untagged LFN Syn⁻ is not able to form lasting blockades, and only blocked 29 (±2)% of the channel’s conductance. These partly-blocked flickering events lasted 10-30 s (Fig. 3C). From these single-channel studies, we conclude that the two defective mutant LFN presequences (Syn⁰ and Syn⁻) possess distinguishable characteristics: the purely anionic version (Syn⁻) cannot penetrate the PA channel and only produces a partial flickering 30% blocked state, whereas the neutral Syn⁰ presequence can completely penetrate and block the channel, albeit ~70% less often than WT, Syn⁺, and Syn⁵. These results, taken together, imply that anionic residues are effectively repulsed by the channel; however, cationic residues can effectively chaperone anionic residues through the channel.

**Acidic-residue dependence of ΔpH translocation extends beyond presequence.** Based upon our initial characterization of the sequence dependence of ΔpH-driven translocation, we determined that acidic residues are responsible for the ΔpH dependence, but counter-balancing positively-charged residues can assist passage through the cation-selective PA channel. It should be noted that the requirement of acidic residues on the ΔpH dependence of LFN translocation exists when all the negative charges are mutated to serine (Fig. S1A) and when the His₆ tag is cleaved and the negative residues mutated to their uncharged analogs (i.e. Glu→Gln and Asp→Asn; Fig. S1B). Here we produced a series of amino-terminal LFN mutants in which successively larger amino-terminal stretches of acidic residues are replaced with serine residues (Fig. 4A). We call these anionic-residue replacement constructs LFN des(1⁻)ₓ⁻ᵧ, where x and y designate the region of sequence in which the natural Asp and Glu residues were replaced with Ser. Such extensive mutations in LFN destabilized the substrates to varying degrees, as shown in equilibrium protein-folding stability measurements (Fig. S2A). In fact, our analysis is limited to the first 68 residues of LFN because removing acidic residues beyond this point resulted in too substantial a loss in protein solubility and stability.

To normalize for these differences in protein stability, we translocated each His₆-tagged LFN des(1⁻)ₙ mutant in the presence (i and ii) or absence (iii) of a ΔpH driving force:

(i) lower Δψ and higher ΔpH, ΔpH = 1, pH_cis = 5.6, Δψ = 20 mV (Fig. S2B);

(ii) higher Δψ and lower ΔpH, ΔpH = 0.6, pH_cis = 5.6, Δψ = 40 mV (Fig. S2C);

(iii) higher Δψ and no ΔpH, ΔpH = 0, pH_cis = 5.6, Δψ = 60 mV (Fig. S2D).

By comparing the translocation rates of a single mutant under two different conditions, we can calculate a ΔΔG‡ value by ΔΔG‡ = ∆G‡(ΔpH=0) - ∆G‡(ΔpH=0), which normalizes for stability. We then referenced the ΔΔG‡ values for each mutant to that of LFN WT by determining a ΔΔΔG‡ value, where ΔΔΔG‡ = ΔΔG‡(MUT) - ΔΔG‡(WT). Here, larger ΔΔΔG‡ values indicate a reduced capacity to translocate using a pH gradient compared to LFN WT. These comparisons revealed a clear trend showing that the more acidic residues that are removed from LFN, the less it was able to effectively use a pH gradient for translocation (Fig. 4B). Also, we generally found that condition (i), which contains a higher ΔpH, increases ΔΔΔG‡ more so than the lower ΔpH condition (ii), indicating that the effect was specifically due to an inability to use the pH gradient (Fig. 4B). Based on these results, we conclude that acidic residues are critical to the mechanism of ΔpH-dependent translocation both within the presequence region and well into the folded structure of the substrate protein.

**Optimal positions for acidic residues in the presequence.** We then asked which acidic residues in the leader presequence were most critical to ΔpH-driven translocation. Using the LFN des(1⁻)ₙ construct as our background, we introduced single glutamates at various positions and measured the translocation kinetics of these new constructs under the influence of a ΔpH. We found that little or no gain (and perhaps a small decrease in rate) is observed when introducing a single Glu at positions 1 to 13 (inclusive, Fig. 5B). A small but
reproducible ~2-fold increase in translocation rate (relative to the LFN des(3)1-46 background) is observed when introducing a Glu into positions 15 to 20 (inclusive, Fig. 5B). The optimal region for the introduction of a Glu residue appears to occur between residues 23 and 31 (inclusive), where the most optimal site is position 23 (increase of 5-fold; Fig. 5B). More modest increases in the translocation rates are observed from residues 34 to 43 (the extent of the analysis in this mutant background, Fig. 5B). Similar results were obtained in the LFN des(3)1-32 background, and multisite replacements with as few as four of the eight wild-type charges could fully restore the translocation rate to that of LFN WT (Fig. S3). We conclude that residues 23 to 31 in LFN’s presequence are the most optimal positions to place acidic residues for ∆pH-mediated translocation.

Anionic-charge density in presequence is optimized for ∆pH-driven translocation. We then determined whether the natural sequence of LFN is optimized in terms of the placement of the acidic residues in the presequence. First, we determined the density of various classifications of amino acids: acidic, basic, acidic plus basic, and hydrophobic. Our density metric is calculated as the number of residues in each classification that are ±4 positions, or one turn of an α helix, from the probed site. We then performed linear regression analysis to determine the degree to which the density of these various types of residue classifications correlated with the relative change in translocation rate resulting from the introduction of Glu residues into the LFN des(3)1-46 background (Fig. 5B). We find that acidic residue density provides the strongest correlation with a significant p value of 0.001 (Fig. 5C). While the density of total charges, acidic plus basic, is a fairly good predictor (p = 0.003), this correlation likely reflects the contribution of the negative-charge density and not the positive-charge density, since the latter correlation is not significant (p = 0.3). The degree of hydrophobicity also poorly correlated (p = 0.09) with the most optimal positions for acidic residues in ∆pH-mediated translocation. We conclude that the natural placement of acidic residues in LFN’s presequence is optimized for ∆pH-dependent translocation.

Charge-dense regions are optimally positioned proximal to folded structure. Since we find that when translocating under a ∆pH driving force that the region of highest charge density in LFN’s presequence (residues ~15 to 30) is also the optimal region for introducing Glu residues into the LFN des(3)1-46 mutant background (Fig. 5), we hypothesized that the location of this cluster of charged residues is only critical in relation to LFN’s folded domain. That is, if the charged cluster were moved further away from the folded structure of LFN, then the force generated by the applied ∆pH gradient would not be as fully realized on the folded structure of LFN. Thus acidic residues more proximal to the substrate’s folded region would better facilitate proper LFN unfolding. To test this hypothesis, we created the LFN 27Ins° construct, which inserts a series of 16 random Gly, Ser, and Thr residues between positions 27 and 28 of LFN WT (Fig. 6A). Despite possessing all of the acidic residues of LFN WT and only extending the substrate’s overall length by ~6%, translocation is slowed approximately 50-fold under a 1.06 unit ∆pH and no ∆ψ (Fig. 6B). An identical insertion placed earlier (LFN 0Ins° and LFN 11Ins°) in the LFN’s amino terminus does not appreciably effect ∆pH-dependent translocation. Restoring charges to the inserted region in a pattern matching that of LFN residues 12 to 27 (LFN 27Ins°) returns translocation speed to that of LFN WT. Interestingly, all constructs with inserts (regardless of position, charge, or background) displayed a reduction in efficiency of translocation: approximately 50-65% that of LFN WT.

To test whether the reduction of the translocation rate in the LFN 27Ins° construct is due to an effective stabilization of the folded substrate, we introduced the destabilizing mutation, L145A (6), into the LFN WT and 27Ins° backgrounds (Fig. 6C). Under the same conditions described above, the translocation rate of LFN WT was increased by a factor of 2.3 (±0.7) by the L145A mutation while 27Ins° L145A’s rate increases by 6.4 (±1.9). We posit that 27Ins° greatly increases the height of the unfolding barrier (Fig. 6D). When the L145A mutation is introduced in LFN WT, a second barrier becomes limiting, but in 27Ins° the unfolding barrier
remains limiting. Thus despite reducing the height of the unfolding barrier to the same extent, the L145A mutation has a greater effect in 27Ins°. We conclude that the highly charged region must be present immediately prior to the folded domain for efficient protein unfolding to take place under a ∆pH.

Intermixed cationic and anionic groups in LFN’s presequence are optimal for ∆pH translocation. So far we have reported that basic residues are important for the initial insertion into the channel and acidic residues are most important just before LFN’s folded domain. From these observations, we might predict that translocation would not be inhibited when LFN’s presequence is rearranged so that all the basic residues are at the beginning and all the acidic residues are at the end. We made such a construct (LFN PosNeg) along with its inverse (LFN NegPos) and assayed their abilities to translocate with a proton gradient, ∆pH = 1.06 and ∆ψ = 0 (Fig. 7A). Both constructs were essentially unable to translocate (Fig. 7B). However, a construct with a randomized amino-terminal presequence (LFN Mix) is able to translocate much more similarly to LFN WT (Fig. 7B). Interestingly, none of the positions in this construct share the charge of their WT counterparts. The effectiveness of the LFN Mix construct suggests the specific WT arrangement of positive and negative charges is less important than simply maintaining an intermixed arrangement of positively- and negatively-charged residues. We propose that positively-charged residues act locally as ionic chaperones for negatively-charged acidic residues.

**DISCUSSION**

To perform work, molecular machines and protein translocases in the cell require a source of energy, generally in the form of chemical energy (ATP) or potential energy stored as either a PMF or other ionic gradient. These sources of energy are interchangeable as evidenced by the highly homologous (27) bacterial flagellum, (28,29) F₁Fₒ-ATP synthase, (30,31) and the vacuolar VₒV₁-ATPase membrane proton pump (32-34). In the former two cases, a PMF is used to drive the rotational motion of the flagellum (29) or the cycles of enzyme reactions (35) that produce ATP in the transmembrane F₁Fₒ-ATP synthase (36). In the latter system, ATP hydrolysis powers a rotary proton pump to generate a PMF, which is essentially the ATP synthase in reverse. The PMF is also an important driving force for transmembrane protein translocation in mitochondria (37), chloroplasts (38), and the endosomal compartment (1). Proton gradients also exist across the endoplasmic reticulum and other compartments in the cell (39) and in theory may comprise an important driving force for other types of translocases in the cell.

**Active pushing/pulling translocation models.** Protein translocation is basically a series of reactions that convert a source of free energy to mechanical work used to drive protein unfolding and unidirectional transport. This process is involved in transmembrane transport, protein degradation, and chaperone activities. Here, either ATP hydrolysis cycles (5,40-42) or utilization of the PMF (1) can propel polypeptides across membranes or into hydrolytic compartments for degradation. Often the molecular machine that carries out translocation processes contains loop regions with critical aromatic groups at their tips. Also, additional accessory proteins outside the channel may be utilized to engage the polypeptide during translocation. It is thought that cycles of ATP binding, ATP hydrolysis, and ADP and inorganic phosphate release cause these loops/proteins to move like simple linear actuators, which effectively pushes the translocating polypeptide in a particular direction (40,42-44). This mechanism is supported in part because these Φ-clamp-type structures are found in various conformations for different nucleotide bound states. For anthrax toxin, the utilization of the proton gradient occurs most optimally with an intact aromatic Φ-clamp structure, (1,2) suggesting that these aromatic internal pore loops may push the translocating polypeptide in a similar manner. At present, this active pushing mechanism of translocation is favored in many systems, but it cannot by itself explain all mechano-chemical coupled mechanisms.

**Brownian-ratchet models for translocation.** An alternative, but potentially complementary view, of mechano-chemical coupling should be considered in addition to the active pushing model. The Brownian ratchet model (20-22,45,46)
suggests that non-equilibrium fluctuations, namely in the form of chemical reactions or thermal gradients, can bias random thermal motion in a productive and directional way. In Feynman’s initial conception (20), stochastic fluctuations may be harnessed for useful work using a temperature gradient. While a thermal gradient is difficult to achieve across a biological membrane, chemical asymmetries and membrane gradients, such as a ΔpH or those created by ATP hydrolysis, are readily available to drive biological Brownian motors.

Ratchet model of ΔpH-driven anthrax toxin translocation. Anthrax toxin activity is most optimal when the endosomal compartment is allowed to naturally acidify (47). Acidification produces a transmembrane PMF that stimulates translocation (1,2,6). The chemical potential component of the gradient, ΔµH+, is sufficient by itself to drive translocation (Fig. 1). How might ΔpH-driven translocation follow a Brownian-ratchet mechanism? Brownian fluctuations in the substrate polypeptide chain may be coupled to protonation state changes in the substrate chain or channel (Fig. 8). We infer that acidic residues in the substrates (which have net acidic isoelectric points) are protonated upon entering the PA channel, because the charge-dependence, or z value, we observe for translocation is positive. This fact implies that the substrate chain is positively charged at the point of the potential drop in the channel (1,6). The positive-charge dependence also reflects the known fact that the PA channel is cation-selective (18), and a highly anionic polypeptide would be repulsed by the channel as we observe with our LFN Syn- construct (Fig. 2B). A similar effect has been observed when LFN is modified with a sulfate group (48). Therefore, a segment of substrate chain with protonated acidic residues will more readily achieve a positive charge enabling its passage through the PA channel under Brownian motion. It can then subsequently release protons into the trans side of the membrane (as favored in the higher pH condition), thus leading to a build-up of anionic charge in the trans-side substrate. The anionic charge in the trans substrate should effectively impede retrograde efflux (under Brownian motion) back into the anion-repulsive channel and enforce proper directionality. Cycles of this charge-state Brownian-ratchet mechanism would then translocate the remainder of the polypeptide across the membrane.

Acidic residues in the substrate are the teeth in the ΔpH-driven Brownian ratchet. One obvious and testable feature of this charge-state Brownian-ratchet mechanism is the role of acidic residues in the substrate. A previous study using a synthetic peptide attached to the folded domain of LFN via native chemical ligation showed that chargeless or positive-only presequences were defective in translocation (49). Here we more extensively explore charged-residue involvement in ΔpH translocation. By replacing the residues in the presequence en masse with neutral residues and measuring translocation under a variety of ΔpH conditions, we determined the ΔpH dependency of translocation, which is the best indicator of the ability to use a proton gradient as a driving force (Fig. 2). We found that sequences lacking acidic residues have comparatively weak ΔpH dependencies (Fig. 2), and the ability to translocate using a ΔpH decreases as more acidic residues are removed (Fig. 4). A more detailed scan, using an anion-less variant, LFN des (1-46), showed that the region spanning residues 23-31 received the largest gain in translocation kinetics when a single acidic residue was introduced (Fig. 5B). Furthermore, the natural sequence appears to maximize its inherent acidic-residue density in this very region to best take advantage of the ΔpH driving force (Fig. 5C). Thus based on this model, we conclude that acidic residues in the substrate form the molecular teeth in ΔpH-driven Brownian-ratchet mechanism.

Novel role for positive charges in the charge-state ratchet. We identified one other complicating factor in our analysis when we tested for the role of positive charges in the presequence. Using the synthetic presequence Syn series, we find that a Syn- presequence dominated by acidic residues and lacking basic residues is defective in translocation (Fig. 2B). However, this result does not mean that ΔpH-driven translocation requires only positive charges, as presequences engineered to only contain cationic residues tend to have flat ΔpH dependencies (Fig. 2B). Various lines of evidence indicate that acidic residues require
neutralization either by direct protonation (Fig. 2C) or by proximal positively-charged residues (Figs. 3 and 7). This requirement demonstrates that the channel has robust charge selectivity. We propose this selectivity enforces directionality through electrostatic repulsion, thereby making it an integral feature of the charge-state Brownian ratchet translocation mechanism.

Can ΔpH-driven Brownian ratchets generate robust unfoldase activity? Some formulations, (21,44) but not all, (50) of the Brownian-ratchet mechanism imply that unfolding must occur prior to the engagement of the ratcheting process. In the unfolding-limited conceptualization of the Brownian-ratchet model, it is suggested that the channel must wait for the protein to unfold and the channel itself is not a participant in the denaturation. In this view, it is thought that the Brownian-ratchet mechanism cannot fully explain the large translocation rate accelerations observed for stable substrates, such as dihydrofolate reductase (51), barnase (52), or cytochrome b₂ (53).

This formulation of the Brownian-ratchet model requires some updating with more current reports (1,2,6,17). The PA channel, for example, utilizes two principle unfoldase or denaturation sites within the channel, namely the Φ clamp (2,6) and α clamp (17). The former site is comprised of a ring of 7 or 8 Phe residues (depending on the oligomerization state of the PA complex), which can clamp onto the amino terminus of LF (2) and drive unfolding (6), presumably by binding to hydrophobic moieties in the substrate in a nonspecific manner (2). The latter α-clamp site is a deep cleft, situated between the twin Ca²⁺-ion binding sites on the surface of the PA oligomer; this clamp is capable of binding nonspecifically to a ~10 residue α helix and short ~5 residue β strand (17). The initial characterization of the α clamp revealed an interesting capability of the site; that is, upon binding to the surface of the PA oligomer, LF is partially unfolded. In this unfolding, LF’s first α helix and β strand unfurl and dock into the α-clamp cleft. The α clamp also has broad binding specificity, allowing the site to recognize many types of sequences and making it a general denaturation site on the translocase. The second factor to consider is that ΔpH-driven translocation facilitates unfolding (6). Two different types of barriers have been identified in LF translocation. The more force-dependent barrier (as determined by the n value, Eq. (1)) is also limited by protein unfolding (6). Thus substrate unfolding may not be out of the realm of possibility for a Brownian-ratchet powered translocase, since the closely-spaced denaturation sites in the channel allow for small displacements of sequence (small fluctuations) to be captured and stabilized via interactions with the channel.

How might denatured-protein binding sites on the channel operate in conjunction with a ΔpH-driven ratchet? Brownian thermal fluctuations are a significant source of energy that, if partially harnessed, could serve to denature a protein. For a polypeptide presequence (30 residues in length), Brownian thermal fluctuations provide ~12 kcal mol⁻¹ of thermal energy (assuming 2 degrees of freedom per backbone Φ and Ψ angle per residue). Thus while the α-clamp structure maintains contact with the sequence carboxy-terminal to the presequence, the amino-terminal end may move through the channel purely motivated by Brownian thermal fluctuations. Successful excursions require that the presequence region is electrostatically compatible with the cation-selective lumen of the channel. We believe that this is ensured either through the lowered pH of the cis side of the membrane or by adjacent positive charges, which may act to help chaperone the acidic groups across the membrane. Successful excursions are then anchored or captured on the trans side of the membrane once the acidic groups further deprotonate in the higher pH medium. The high degree of correlation between the best sites to introduce a Glu residue in LFₙ des⁴₁₋₄₆ and the naturally high density of negative charges in LF’s presequence (Fig. 5C) suggests that this anchoring step is more favorable with higher acidic-residue densities.

Interestingly, we find that the acidic-residue-rich sequence is located immediately adjacent to the folded structure. Thus after successful anchoring of the presequence takes place, an entropic tension may be effectively applied to the remaining folded structure. This entropic tension is derived from the fact that a more extended polypeptide chain would have fewer possible conformations; and to relieve this entropic tension, the protein would be driven to unfold. We tested
the positional dependence of this acidic residue cluster by creating an artificial gap between this naturally dense region and the folded structure of LF_N. When the gap contains neutral residues the translocation rate decreases 50 fold, but when the gap contains charged groups, the translocation rate is similar to WT (Fig. 6B). Furthermore, the decrease in the translocation rate coincides with an observed stabilization of the substrate (Fig. 6C). Previous studies show that a larger nucleus of structure unfolds during translocation in a large cooperative unfolding step (6); however, this unfolding occurs after a smaller portion of the amino-terminal structure is unfolded and docked into the α-clamp site (17). Therefore, we expect that the α- and Φ-clamp protein denaturation sites in the channel can reduce the overall unfolding barrier by allowing for small incremental unfolding steps to be stabilized. Effective trapping of Brownian fluctuations may force partially unfolded intermediates to then disengage from the channel, leading ultimately to the larger-scale cooperative unfolding event (6) we observe under a ΔpH.

Finally, the charge requirements we identify for the substrate strongly favor a Brownian-ratchet model. However, we cannot rule out the possibility that ΔpH-driven transport also involves coordinated, proton-dependent movement of loops within the PA channel, which help to push the substrate chain during translocation. A combination of the active-pushing and Brownian-ratchet models may apply to this system since these models are not mutually exclusive. In fact, other translocases already known to push proteins using ATP-driven loop movements may also use a Brownian ratchet to further drive transport.

REFERENCES


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FOOTNOTES

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The abbreviations used are: PA, protective antigen; LF, lethal factor; EF, edema factor; LF_N, lethal factor amino-terminal PA-binding domain; ΔpH, proton gradient; Δψ, membrane potential; t_{1/2}, time for half of substrate to translocate; His_6, six-histidine tag; Syn, synthetic sequence region; Ins, synthetic insertion sequence; des(-), sequence region lacking acidic charges.

FIGURE LEGENDS

Fig. 1. The chemical potential of the PMF is sufficient to drive LF_N translocation. Analysis of LF_N translocations driven purely by a ΔpH. Activation free energies (ΔG‡; expressed as RTln(t_{1/2}/c)) for individual translocations of His_6-LF_N are plotted against their respective ΔpH values and fit to Eq. (1). The fit parameters are: ΔG_{‡o1} = 12.2 (±1.1) kcal mol$^{-1}$, ΔG_{‡o2} = 2.5 (±0.2) kcal mol$^{-1}$, n_1 = 4.0 (±0.5), and n_2 = 0.22 (±0.03). (inset) Representative LF_N translocation records normalized as a fraction of the theoretical maximum of translocation under the following ΔpH values at a Δψ of 0 mV: 0.84 (black), 1.01 (red), 1.19 (green), 1.67 (purple), and 2.30 (blue). The universal bilayer buffer was consistently at a pH cis of 5.6, and pH_trans was adjusted to form the indicated ΔpH values. To control for buffer-mixing lag times, t = 0 was set as the time when 1% of maximum translocation occurred. Error bars are the mean ±s.d. (n = 2-5).

Fig. 2. Efficient ΔpH-driven translocation requires both acidic and basic charged residues. (A) Design of synthetic LF_N constructs (LF_N Syn) in which the first 27 amino acids are replaced using a sequence containing both acidic and basic residues (Syn±), neither acidic nor basic residues (Syn°), only acidic residues (Syn-), and only basic residues (Syn+). The exact sequence compositions of these constructs are shown alongside the LF_N WT sequence. (B) Translocation activation energy versus ΔpH results for the His_6-LF_N Syn constructs shown in (A): His_6-WT (black ■), His_6-Syn° (purple ●), His_6-Syn- (red ▲), His_6-Syn+ (blue ▼), and His_6-Syn± (green ♦). These ΔpH-driven translocation experiments were conducted at a ΔpH of -0.6 to 1.0, pH cis of 5.6, and a Δψ of 60 mV. Error bars are the mean ±s.d. (n = 2-5). The fit parameters for His_6-WT and His_6-Syn± using Eq. (1): His_6-WT, ΔG_{‡o1} = 1.5 (±0.2) kcal mol$^{-1}$, ΔG_{‡o2} = 0.8 (±0.3) kcal mol$^{-1}$, n_1 = 1.9 (±0.2), n_2 = 0.1 (±0.2); His_6-Syn±, ΔG_{‡o1} = 1.5 (±0.2) kcal mol$^{-1}$, ΔG_{‡o2} = 0.8 (±0.3) kcal mol$^{-1}$, n_1 = 1.7 (±0.2), n_2 = 0.1 (±0.2). His_6-Syn°, His_6-Syn-, and His_6-Syn+ were fit to a single-barrier model (ΔG_{‡} = ΔG_{‡o} - 2.3n_0ΔpH), with the parameters: His_6-Syn°, ΔG_{‡o} = 3.40 (±0.08) kcal mol$^{-1}$, n_0 = 0.59 (±0.06); His_6-Syn-, ΔG_{‡o} = 4.3 (±0.2) kcal mol$^{-1}$, n_0 = 0.0 (±0.1); His_6-Syn+, ΔG_{‡o} = 2.28 (±0.09) kcal mol$^{-1}$, n_0 = 0.14 (±0.07). (C) Maximum translocation efficiency achieved within five minutes at varying pH_cis values for the His_6-LF_N Syn constructs. These ΔpH-driven translocation experiments were conducted at a ΔpH of 2.0, pH_cis ranging from 5.0 to 5.6, and a Δψ of 0 mV. To control for buffer-mixing lag times, t = 0 was set as the time when 1% of maximum translocation occurred. Legend colors are identical to (B). Error bars are the mean ±s.d. (n = 2-8).

Fig. 3. Charge requirements for LF_N docking. (A) Ensemble bilayer recordings of PA channel conductance block by LF_N WT and LF_N Syn mutants with and without their His_6 tags were obtained at symmetrical pH 5.6 and saturating concentrations of LF_N (100 nM). The relative fraction of conductance...
Acidic-residue positions in LF N’s presequence are most critical to mutants, Syn+ and Syn± did not dissociate during the recording. (C) Single-channel blocking events recorded for LF N WT and LF N Syn mutants with and without their His6 tags at symmetrical pH 5.6. Once a single channel inserts into the membrane, LF N is added under a Δψ of 20 mV. Approximately 2 minutes of a typical blocking event is shown for each LF N. Data are acquired at 400 Hz under a low-pass filter of 200 Hz. For clarity, the displayed single-channel traces were downsampled by a factor of ten. To the right of each trace is a histogram of the current level for each recording. Gaussian functions fit to these histograms assess the percentage of the time the LF N-channel complexes in the open (o), blocked (b), and partly blocked states (*). The percentage time in the blocked and partly blocked states are given as follows: all His6-tagged LF N constructs as well as untagged LF N WT, LF N Syn+ and LF N Syn± were 100% blocked; untagged LF N Syn° was 28% blocked; and LF N Syn− was 1.6% blocked and 3% partly blocked (*). The errors for percentage of the time spent in the block states are all better than ±1%. The partly blocked (*) state is 29 (±2)% less conducting than the fully open state.

Fig. 4. Acidic residues within LF N’s folded domain are also critical to ΔpH-driven translocation. (A) The ribbons depiction of the structure of LF N is from 1J7N (23), where regions of sequence are colored by sequence position. The unstructured leader presequence (residues 1-32) is indicated as a thick colored line. Regions in which acidic residues were replaced with serine residues are colored as follows: 1-18 (red), 19-24 (green), 25-32 (purple), 33-46 (blue), 47-56 (brown), and 57-64 (gold). (B) The difference in activation energy ΔΔG‡ for each His6-LF N des(-) series mutant are obtained using two ΔpH-driven conditions (ΔpH = 1.0 and Δψ = 20 mV; ΔpH = 0.6 and Δψ = 40 mV) and one condition in the absence of a ΔpH (ΔpH = 0 and Δψ = 60 mV), where: ΔΔG‡ = ΔG‡(ΔpH>0) - ΔG‡(ΔpH=0). ΔΔG‡ values for each mutant (MUT) are then referenced to that of LF N WT to give the reported ΔΔG‡ values plotted above. ΔΔΔG‡ = ΔΔG‡(MUT) - ΔΔG‡(WT). In all cases, pH cis = 5.6. Error bars are the mean ±s.d. (n = 2).

Fig. 5. Acidic-residue positions in LF N’s presequence are most critical to ΔpH-driven translocation. Acidic residues were added back into the His6-LF N des(-)1-46 construct background. (A) The sequences of the first 46 residues of LF N WT and LF N des(-)1-46 are shown, where acidic residues in the WT sequence are shaded red. (B) The relative translocation t1/2 times for acidic-residue introductions into the His6-LF N des(-)1-46 mutant backgrounds are given as the ratio t1/2(des(-)1-46) / t1/2(MUT) for ΔpH-driven translocation. The numbers on the x-axis indicate the position in which acidic residues are reintroduced into the His6-LF N des(-)1-46 mutant background. ΔpH-driven translocation conditions were ΔpH = 0.8, pH cis = 5.6, and Δψ = 50 mV. Error bars are the mean ±s.d. (n = 2-4). (C) A correlation of relative translocation rate (given as the ratio t1/2(des(-)1-46) / t1/2(MUT)) for the mutants in (B) versus the density of acidic residues normally found in the WT sequence. Acidic-residue density in this instance is the total number of acidic residues found in the four residues amino- and carboxy-terminal to the probed site. The linear regression fit to all the individual measurements (filled diamonds) is significant with a p value of 0.001 for the fit function, y = a + bx, where a = 0.5 (±0.5) and b = 0.6 (±0.2). Since multiple observations of particular acidic residue densities were obtained in certain instances, a heavy horizontal bar (mean) and error bars (±s.d.) are also given.

Fig. 6. Charged residues must be located immediately before the substrate’s folded domain for efficient translocation. (A) Sixteen-residue inserts with either no charge (Ins°) or a mix of positive and negative charges (Ins±) were inserted into LF N WT or LF N L145A. The resulting constructs are called yInsx, where y denotes the last WT residue prior to the insert and the superscript x represents the charge of the insert. Arrows indicate two other positions (0 and 27) where the Ins° sequence was inserted. (B) The
relative translocation $t_{1/2}$ times of insertion and LF$_N$ L145 mutants (MUT) are given as the ratio $t_{1/2}(\text{MUT}) / t_{1/2}(\text{WT})$. ΔpH-driven translocation conditions were: $\Delta p$H = 1.06, pH$_{\text{cis}}$ = 5.6, and $\Delta \psi$ = 0 mV. Error bars are the mean ± s.d. ($n$ = 2). (C) The relative translocation $t_{1/2}$ times of the LF$_N$ L145A and LF$_N$ 27Ins° L145A mutants compared to their respective (L145) counterpart given as the ratio $t_{1/2}(\text{L145}) / t_{1/2}(\text{LF}_N \text{L145A})$. Translocation conditions are as in (B). (D) Model energy diagrams depicting the changes in energy barriers caused by the L145A and 27Ins° mutations, wherein we interpret 27Ins° as greatly increasing the unfolding barrier. The L145A mutation reduces the unfolding barrier by the same extent in the WT and 27Ins° backgrounds. However, in the WT background, the rate becomes limited by the translocation barrier, so the relative increase in speed is not as large as that observed for the 27Ins° background.

**Fig. 7.** Proton gradient-driven translocation requires that acidic and basic residues be intermixed in the substrate. (A) Design of constructs that separate the acidic and basic amino acids in the first 27 residues of LF$_N$ to opposite ends of the presequence. PosNeg has a contiguous stretch of basic residues at the amino-terminal end and a contiguous stretch of acidic residues at the carboxy-terminal end; NegPos is the inverse sequence of PosNeg. A randomized construct was also prepared (called Mix), in which the acidic and basic residues are intermixed but no position has the same charge as its WT counterpart. (B) Translocation records for His$_6$-LF$_N$ WT (black), His$_6$-LF$_N$ PosNeg (blue), His$_6$-LF$_N$ NegPos (red), and His$_6$-LF$_N$ Mix (purple). ΔpH-driven translocation conditions were: $\Delta p$H = 1.06, pH$_{\text{cis}}$ = 5.6, and $\Delta \psi$ = 0 mV.

**Fig. 8.** The charge-state Brownian ratchet model for $\Delta p$H-driven translocation of anthrax toxin. At the time of endosome acidification, the substrate is bound to the top of the channel, with its $\alpha$1 helix in the $\alpha$ clamp. Upon channel conversion, the unstructured amino terminus docks in the $\Phi$ clamp. The low pH of the endosomal compartment will protonate most of the acidic residues while positive charges chaperone any remaining deprotonated aspartic or glutamic acids. This ensures that the translocating polypeptide will have a net positive charge, allowing it to move freely through the cation-selective channel. Due to Brownian motion, a portion of the substrate will eventually emerge in the cytosol. There, the higher pH of this region will result in frequently deprotonated acidic residues, thereby giving the emerged portion of the polypeptide a net negative charge and capturing it on the cytosolic side of the membrane. Repeated cycles of emergence from the channel though Brownian motion and capture via deprotonation allow the remaining portion of the substrate to translocate across the membrane.
Figure 1
Figure 2

A

WT  AGGHGDVGMHVKEEKKNKDEENKRKDEE
Syn- TGSGTTGSSTGGTSTGSSSTSGGSTG
Syn+ TGSGTDGSSTTSEGSSDTSGMTGDEE
Syn± TGSHTTGSSHKTSTKSTKSTKSTG
Syn* TGSHTDGSSHKEEKSKDETSRRKDEE

B

RT ln t1/2 /c (kcal mol-1)

C

Fraction Translocated

pHcis

5.0 5.2 5.4 5.6

0.0 0.2 0.4 0.6 0.8 1.0

-0.6 -0.4 -0.2 0.0 0.2 0.4 0.6 0.8 1.0

ΔpH
Figure 4

A  

B  

\( \Delta \Delta G^\ddagger \) (kcal mol\(^{-1}\))

\( \Delta \text{pH} = 1.0, \Delta \Psi = 20 \text{ mV} \)

\( \Delta \text{pH} = 0.6, \Delta \Psi = 40 \text{ mV} \)
Figure 5

A

\[
\begin{align*}
L_{F_{N\text{WT}}}^{\text{LF}_{1-46}} & \quad \text{AGGHGDVGHMHVKEKEKNKDENKRKDEERNKTQEEHLKEIMKHIVKI…} \\
\text{des}^{(-)}_{1-46} & \quad \text{AGGHGSVGMHVKEKEKNKDENKRKDEERNKTQSSHLKEIMKHIVKI…}
\end{align*}
\]

B

\[
\text{Acidic residue position in des}^{(-)}_{1-46} \text{ background}
\]

C

\[
\text{Relative translocation rate}
\]

\[
\text{Acidic-residue density}
\]
Figure 6

A  TTSTGSSSSTSGSTG
   0 0 0 0
   LFN  WT
   27Ins°
   27Ins°
   L145A

B

C

t_{1/2}(MUT) / t_{1/2}(WT)

D

Unfolding  Translocation  Unfolding  Translocation

LFN WT
LFN L145A
27Ins°
27Ins° L145A
Figure 7
Docking Brownian motion
Repeated cycles of 2 & 3

Types of Residues
- basic
- protonated acidic
- deprotonated acidic

Unfolding and Translocation

Figure 8
Charge requirements for proton gradient-driven translocation of anthrax toxin
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