Dynamic Phenylalanine Clamp Interactions Define Single-Channel Polypeptide Translocation through the Anthrax Toxin Protective Antigen Channel

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Abstract

Anthrax toxin is an intracellularly acting toxin where sufficient detail is known about the structure of its channel, allowing for molecular investigations of translocation. The toxin is composed of three proteins, protective antigen (PA), lethal factor (LF), and edema factor (EF). The toxin's translocon, PA, translocates the large enzymes, LF and EF, across the endosomal membrane into the host cell's cytosol. Polypeptide clamps located throughout the PA channel catalyze the translocation of LF and EF. Here, we show that the central peptide clamp, the ϕ clamp, is a dynamic site that governs the overall peptide translocation pathway. Single-channel translocations of a 10-residue, guest–host peptide revealed that there were four states when peptide interacted with the channel. Two of the states had intermediate conductances of 10% and 50% of full conductance. With aromatic guest–host peptides, the 50% conducting intermediate oscillated with the fully blocked state. A Trp guest–host peptide was studied by manipulating its stereochemistry and prenucleating helix formation with a covalent linkage in the place of a hydrogen bond or hydrogen-bond surrogate (HBS). The Trp peptide synthesized with L-amino acids translocated more efficiently than peptides synthesized with D- or alternating D,L-amino acids. HBS stapled Trp peptide exhibited signs of steric hindrance and difficulty translocating. However, when mutant ϕ clamp (F427A) channels were tested, the HBS peptide translocated normally. Overall, peptide translocation is defined by dynamic interactions between the peptide and ϕ clamp. These dynamics require conformational flexibility, such that the peptide productively forms both extended-chain and helical states during translocation.

Introduction

Membranes form hydrophobic barriers that separate aqueous compartments in cells, requiring membrane-embedded transporters and channels to allow for movement of biomolecules across these barriers. Some of the largest cargos to be delivered across membranes include multidomain folded proteins. Many bacterial toxins possess their own translocase channel, which can deliver a cytotoxic enzyme across a membrane into the cytosol of the host cell. For example, anthrax toxin [1,2] is made up of three proteins, protective antigen (PA; 83 kDa), lethal factor (LF; 90 kDa), and edema factor (EF; 89 kDa). The PA component self-assembles into a ring-shaped oligomer that inserts into the lipid bilayer to make a translocase channel; LF and EF translocate through the channel into the cytosol.

In order for anthrax toxin to properly function, it must first self-assemble. PA initially binds to a host cell receptor and is cleaved by a furin-like protease. A small 20-kDa portion of PA dissociates, leaving the remaining 63 kDa to self-assemble into a ring-shaped heptamer [3] or octamer [4]. This oligomer is called the prechannel. As the oligomer assembles, it presents binding sites for LF and EF [5,6]. The assembled complexes are endocytosed. Once the endosome matures and its pH becomes more acidic,

PA transforms from the prechannel state to the channel state [7]. The proton gradient established across the endosomal membrane drives LF and EF to unfold [8,9] and translocate [10] through the PA channel. Once in the cytosol, LF and EF refold and carry out their respective catalytic functions to disrupt cell signaling pathways. LF is a zinc metalloprotease that cleaves mitogen-activated protein kinase kinases [11], and EF is an adenylate cyclase [12].

LF and EF unfolding are required because the PA channel is so narrow that only structure as wide as an α helix can be sterically accommodated in most parts of the channel [9]. Substrate unfolding and translocation are catalyzed by a series of polypeptide clamp active sites that line the channel [2]. On the topmost surface of the channel is the α helix binding clamp (α clamp), which is a deep cleft between PA subunits that can bind to α helical structure. Within the channel is a ring of phenylalanine residues, called the phenylalanine clamp (ϕ clamp) [13], and this structure narrows considerably further to an opening of ~6 Å [14]—a diameter too narrow for α helical structure. The measured diameter of the channel was at pH 5.0, and no LF or EF was bound to that structure. The ϕ clamp, essential for translocation, preferentially binds to hydrophobic and aromatic functional groups [13], and it helps catalyze the unfolding step of translocation [8]. Beneath the ϕ clamp is a highly charged β barrel, called the charge clamp, which is required to harness the proton gradient driving force [15]. The charge clamp is highly anionic at the juncture nearest to the ϕ clamp.

Different models have been put forth on how proteins translocate through the PA channel. The extended-chain Brownian-ratchet model, on one hand, suggests that the translocating chain is in the fully extended conformation during translocation [16]. Carboxylates in the translocating chain are protonated on the low pH side of the membrane. Under Brownian motion, these protonated sites then diffuse past the charge clamp, which normally repels anionic groups, and then, the protonated residues deprotonate down the gradient, leaving them electrostatically committed to translocate toward the high pH side of the membrane. The cycle repeats until the entire protein is translocated. In this proposed mechanism, the clamp sites in the channel are largely static structures.

The helix-compression model, on the other hand, suggests that the polypeptide clamp sites are dynamic and under allosteric control [17]. During translocation, the translocating chain binds protons with its acidic groups and forms an α helix inside the channel. The dynamic ϕ clamp site adjusts to accommodate the wider helix. The 2 Å per residue compression of the translocating chain from the extended-chain state to a helical one is proposed to allow for a power stroke to be created. Once helix propagates to the α clamp, an allosteric change in the structure of the ϕ clamp drives it to the clamped state. The clamped state of the ϕ clamp causes the peptide to convert from the helical state to the extended-chain state. This conversion of the peptide from helical to extended-chain conformation allows it to extend past the charge clamp and deprotonate down the gradient. The deprotonated peptide is then electrostatically committed to translocate by the charge clamp via the development of a repulsive event that prevents retrotranslocation. The cycle then repeats on the next segment of polypeptide until the rest of the protein is translocated. Here, to further distinguish between these two models, we investigate the dynamics of the ϕ-clamp interactions using a 10-residue guest–host peptide probe.

Results

Single-channel translocation reveals subconductance intermediates

A previously described guest–host peptide system of the generalized sequence, KKKKKXXSXX, was used [18]. The guest-residue sites, designated "X", were substituted with Ala, Leu, Phe, Thr, Trp, or Tyr. Previously, we did not characterize the single-channel translocations of the guest–host peptide. Here, we use the peptide to probe the interactions at the ϕ-clamp site. A single PA channel was introduced into a planar bilayer, and 20 nM of one of the peptides was added to the *cis* side of the bilayer. In the initial characterization, the voltage membrane potential (Δψ) was adjusted to 70 mV so that individual blockade events would correspond to translocations. (By definition, $\Delta\psi \equiv \psi_{cis} - \psi_{trans}$, where $\psi_{trans} \equiv 0$.) We observed four conductance states in these experiments for each of the guest–host peptides. The four states included a fully open-channel state (O), a fully blocked-channel state (B), and two intermediate conductance states (Fig. 1). One intermediate was a ~10% subconductance state (I_1), and the other intermediate was a ~50% subconductance state (I_2). During the translocation of the guest–host peptides with aromatic insertions (Phe, Trp, and Tyr), these peptides exhibited an oscillation between the I_2 and B, where the oscillation occurred for the longest durations with the Trp peptide (Fig. 1).

Stereochemical variants of the guest–host Trp peptide

To further probe the oscillation in the single-channel translocations of the guest–host peptide, we chose the Trp peptide to vary its stereochemistry. The Trp peptide was selected because it exhibited the longest oscillation events of the peptides tested. Also, in a prior study, it was shown that Trp containing sequences