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Determining the Essential Unfolding Step in Protein Translocation using Anthrax Toxin

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Protein translocation across membranes is crucial to many cellular processes, which includes bacterial pathogenesis. However, its exact molecular mechanism is not well understood. An established model to study this process is the anthrax toxin, a three-protein virulence factor that intoxicates cells via transmembrane protein translocation. Following endocytosis of the toxin, the protective antigen (PA) forms a transmembrane channel, triggered by the acidic endosomal pH, through which the two active enzyme components, the lethal factor (LF) and the edema factor (EF) are translocated to the cytosol of the host cell. Two key non-specific polypeptide binding sites – the α-clamp and Φ-clamp – catalyze protein translocation across the PA channel. The Φ-clamp is a very narrow opening, thus requiring proteins to unfold during translocation. We hypothesize that the PA channel can be used to translocate small heterologous proteins, such as Im7, and that translocation occurs by protein unfolding through a rate-limiting transition state. We expressed and purified small proteins composed of Im7 wild type attached to the first 30 amino acids of LF (LF1-30.Im7 WT), and its 10 single amino acid mutants. Planar lipid bilayer electrophysiology was used to perform single channel translocation assays of the WT and its mutants. Our results revealed that the mutants translocated faster and at lower voltages than the wild type, indicating protein destabilization due to mutation of the core residues. This destabilization facilitated the unfolding process, which reduced the driving force necessary for their translocation. Moreover, the critical step in translocation can be determined by mapping the transition states through denaturation studies using guanidine hydrochloride. Understanding the mechanism of protein unfolding can significantly contribute to the development of a new model for targeted drug delivery.

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Determining the High-Resolution Structures of the Anthrax Toxin Protective Antigen Pore Bound to its Lethal and Edema Factors

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Anthrax toxin, one of the virulence factors of *Bacillus anthracis*, consists of three individual protein factors: the cell binding/pore-forming protective antigen (PA), and the enzymatic factor lethal factor (LF), and edema factor (EF). Due to its formation of a protein-conducting pore, PA provides an excellent system to study protein translocation. To date, the only structural information available of the PA bound to its enzymatic substrate is of the amino terminal domain of LF, LFn, bound to the pre-pore octamer. This structure has shown that the binding of LF to the PA pre-pore serves to stabilize its unfolding before being translocated through the channel. Using cryo-EM, we determined high-resolution structures of the heptameric PA in its pore conformation bound to LF at 4.6-Å, and bound to EF at 3.6-Å. The PA_7LF_3 and PA_7EF_3 pre-pore complexes were inserted into lipid nanodiscs, causing them to take random orientation on cryo-EM grids and allowing us to perform single-particle analysis. The structures contain the pore form PA heptamer bound to a single LF or EF, respectively. The C-terminal domains of LF is disordered in the PA_7 LF structure, resulting in no visible electron density. When comparing the LFn domain when bound to PA in the pore state versus the pre-pore complex, there appears to be no significant changes in structure. The first α-helix in LF its unfolded and docked within the α-clamp site. In the structure of PA_7EF, the full length of EF protein appears to be ordered, showing domain reorganization within EF upon binding to PA. In addition, the α-clamp is also populated by the first α-helix in EF. These structures can help in our understanding of the mechanisms involved in protein translocation.

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Elucidating the Effect of Listeriolysin O Structural Intermediates on Lipid Diffusivity

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Listeriolysin O (LLO) falls in the sub-class of cholesterol dependent cytolysin (CDC) pore-forming protein. It is secreted by *Listeria monocytogenes* which causes listeriosis, a fatal disease to immune-compromised individuals as well as infants. Pore formation has been studied and found to be initiated by the binding of LLO to cholesterol, followed by oligomerization of the monomers and insertion of transmembrane segments inside the bilayer to form a pore. Studies suggest that LLO transitions through an inactive intermediate state, called a prepore, in the pore formation process. Although LLO has been widely studied, there is very little information in the literature that connects the manner in which membrane lipid dynamics are modulated during pore formation. To address some these unresolved issues pertaining to LLO interaction and assembly on phospholipid membrane, we used fluorescence correlation spectroscopy (FCS) and FRET on artificial membrane systems. Giant unilamellar vesicles (GUVs) were used as model systems to study LLO interactions with membranes. LLO induced dye leakage of GUVs, revealed two distinct population of vesicles: leaked and unleaked. Interestingly, LLO was found to preferentially bind to the Ld region of GUVs on both leaked and unleaked vesicles. FRET was monitored between Alexa488-tagged-LLO and DiI-labelled-lipid. We observed significant FRET efficiency on leaked vesicles whereas it was rarely observed in unleaked vesicles supporting the connection between the pore states and leakage. Interestingly, lipid diffusivities as measured from FCS, also showed corresponding difference between leaked and unleaked vesicles. Leaked vesicles demonstrated enhanced lipid diffusivity in comparison to the unleaked vesicles. These results are attributed to the different structural changes that happen during the pore formation. Based on our results, lipid dynamics can potentially be used as a marker to distinguish between oligomeric states.

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Recombinant Expression and Refolding of a Potassium Channel-Activating Three-Finger Toxin

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Tx7335 is a novel three-finger peptide toxin that was originally isolated from the Eastern Green Mamba venom. A unique characteristic of Tx7335 is the ability to increase duration and frequency of openings in the potassium channel KcsA. Potassium channels play an important role in electrical signaling of excitable cells. Dysfunction of the channels can lead to major health problems such as certain heart and autoimmune diseases. Potassium channels are natural targets for venomous animals seeking to paralyze their prey. As a result, toxins have been shown to be an effective method of modulating and studying potassium channels. To obtain sufficient amounts of Tx7335 for future studies into its mechanism of action, we are recombinantly expressing and purifying this toxin. A synthetic gene encoding for Tx7335 was cloned into an expression vector expressing the toxin as a thioredoxin fusion protein with a TEV protease cleavage site in between them. The fusion protein was overexpressed in E coli. BL21(DE3) C43 expression strain and purified by Nickel affinity chromatography. The toxin was released from the fusion protein by cleavage with TEV protease and refolded by addition of reduced and oxidized glutathione in 1 to 10 ratio. Tx7335 was further purified through Cation exchange chromatography. Refolding was assessed by mass spectrometry, and the recombinant toxin will be used for future functional and structural studies of toxin-KcsA interactions.

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Ebola Virus Delta-Peptide Acts as an Enterotoxic Viroporin *In Vivo*

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Ebola virus (EBOV), part of the filovirus family, has a genome that encodes for a partly conserved, 40-residue polypeptide, called the delta peptide, which is produced during Ebola virus disease pathogenesis. Sequence-structure-function analysis and *in vitro* data suggest that the delta peptide is a viroporin, a term used to describe a diverse family of virally encoded pore-forming peptides and proteins involved in the replication and pathogenesis of numerous viruses. Full-length and conserved C-terminal EBOV delta peptide fragments permeabilize synthetic lipid bilayers and multiple cell types *in vitro*. Activity requires that the two conserved cysteines are connected by a disulfide linkage. Here, we follow up those results by testing delta peptides using an established mouse model of diarrheal pathology focusing on the small intestine. We hypothesized that the cell permeabilization observed against numerous cell types *in vitro* would correspond to intestinal pathology, *in vivo*. We found that the delta peptide exerts potent enterotoxic activity against mouse intestine upon introduction to a closed intestinal ileal loop, resulting in a diarrheal syndrome