

Single Particle CryoEM of the Anthrax Toxin Initial Engagement Complex

Alexandra J Machen¹, Narahari Akkaladevi², Tommi A White^{2,3}, and Mark T Fisher¹

¹ Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City, KS, USA.

² Department of Biochemistry, University of Missouri, Columbia, MO, USA.

³ Electron Microscopy Core Facility, University of Missouri, Columbia, MO, USA.

The lethality of anthrax, a zoonotic disease and bioterrorism agent, is due to the anthrax toxin. This tripartite toxin consists of protective antigen (PA), lethal factor (LF), and edema factor (EF). The 440 kDa heptameric PA prepore binds up to three molecules of LF, a mitogen-activated protein kinase kinase protease, and/or EF, an adenylate cyclase. This entire complex, bound to receptor proteins, is endocytosed. As the endosome acidifies to pH 5.0 (late endosome), the receptor bound PA prepore unfolds and refolds into an extended beta-barrel pore structure that penetrates the endosomal membrane. This pore unfolds and translocates the 90 kDa LF enzyme across the pH gradient (endosomal pH 5.0 to cytosolic pH 7.0) of the endosomal membrane through the narrow pore lumen in a pH driven hypothesized Brownian ratchet mechanism [1]. The translocated LF then refolds on the cytosolic side of the membrane and disrupts cell signaling resulting in cell lethality. To initiate translocation, the N-terminal region of LF moves away from the main body of LF and binds to the alpha clamp region of the PA pore cap [2]. This initial engagement positions the lysine rich N-terminal portion of LF to interact with the 7-fold phe-clamp located further down the PA pore. The directional translocation of LF depends on protonation of acidic residues, the electrostatic character of the PA pore lumen, and remaining positive charges. Subsequent deprotonation of the translocating peptide after the polypeptide passes the phe-clamp region prevents back transfer. To define the nature of the initial engagement and phe-clamp channel blocking steps that occur at different pHs we are using single particle cryoEM.

With recent advancements of cryoEM, a significant bottleneck in obtaining atomic resolution structures is sample preparation, especially for aggregation prone and membrane proteins like PA [3]. In order to solve the atomic structure of the initial engagement complex, large quantities of soluble PA pore with single bound LF_N (the N-terminal domain of LF) are needed to avoid imposing sevenfold symmetry while obtaining diverse conformational coverage. To this end, 1LF_N-PA pore complexes were produced using an immobilized bead based system and solubilized using authentic lipid bilayer nanodiscs (Figure 1a). E126C LF_N and PA prepore were incubated prior to immobilization to reduce the number of complexes with multiple bound LF_N and ensure particle homogeneity. Homogeneous complexes were immobilized by coupling E126C LF_N to thiol sepharose beads. Beads were rinsed in acid to transition the PA prepores to pores. Pre-nanodisc micelles were then added and bound to the aggregation prone hydrophobic transmembrane beta barrel of PA. The micelles were collapsed into nanodiscs. Soluble complex were released by reducing E126C LF_N. The assembly of 1LF_N-PA-nanodisc complexes was first confirmed and optimized using negative stain TEM. 1LF_N-PA-nanodisc complexes were then vitrified to for single particle cryoEM using a FEI Mark IV Vitrobot. Imaging was performed using a FEI Tecnai F30 G2 Twin Transmission Electron Microscope at 200kV. Single particle analysis (EMAN2/SPARX) of cryoEM micrographs clearly indicated 2D class averages with singly bound LF_N. Figure 1b highlights side view class averages of 1LF_N-PA-Nanodisc complexes. While LF_N is not visible in all of the averages, our immobilization protocol ensures each complex has at least 1 LF_N. Several of the class averages in Figure 1b do not show a distinct nanodisc or PA beta barrel. To create

expected 2D class averages, a reference model of 1LF_N-PA-Nanodisc complex (Figure 1c) was created in Chimera by docking published structures of LF_N (PDB 3KWV), PA, excluding domain 4 (PDB 3J9Z), and nanodisc (PDB 2MSC). When filtered to 20 Å, back projection of this reference model also had several side views that did not show a distinct LF_N, nanodisc, or beta barrel density (Figure 1d) indicating our sample has the conformational diversity necessary for near atomic to atomic resolution structures.

Our successful assembly of the singly bound LF_N-PA pore inserted into a nanodisc will allow us to obtain atomic structures of the initial engagement of LF with the pore prior to translocation. An immobilized bead based system and nanodisc technology allowed us to achieve stable conformationally diverse particles. Now that we have made it through bottle neck of sample preparation, the next step is to collect on a higher powered scope with a direct electron detector. Comparing initial engagement complexes at pH 7.5 and 5 will help us understand how the endosomal pH facilitates unfolding and directional translocation of the bound LF through the PA translocon.

References:

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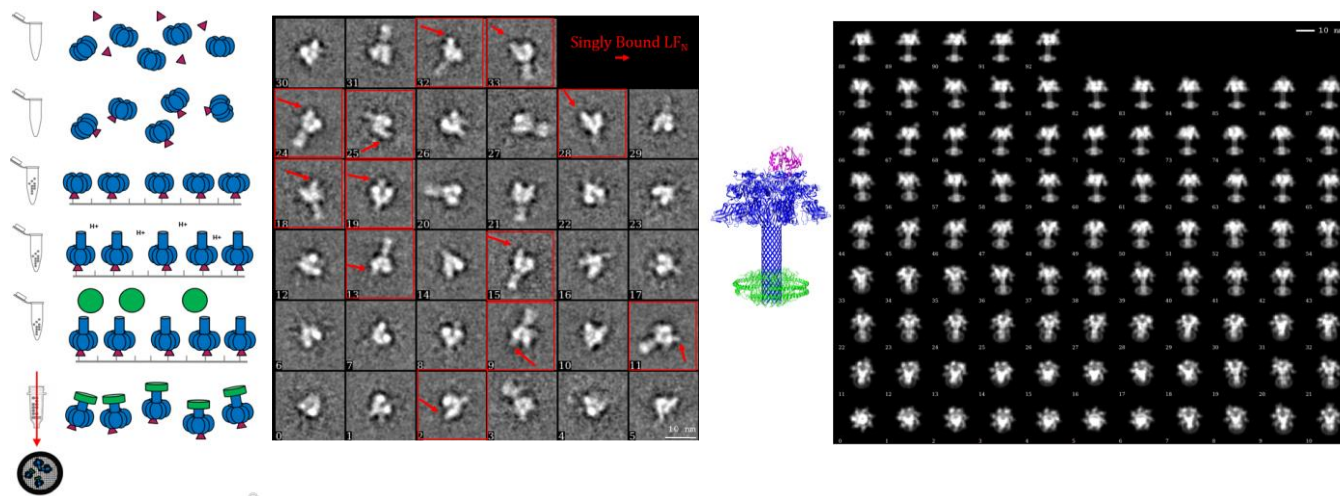


Figure 1. A) Schematic of complex formation. LF_N (pink triangles) and PA (blue ellipse) complexes were produced using immobilized bead based system and solubilized using lipid bilayer nanodiscs (Green). B) 2D class averages of ~1200 side view particles using SPARX. Red arrows highlight clear single LF_N density. Scale bar in bottom right box is 10 nm. C) Side view of reference model of LF_N(Pink)-PA(Blue)-Nd(Green) complex. D) Projection of 20Å filtered reference model. Scale bar in top right corner is 10 nm.