Bryan Krantz

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Education

2003-2006	**Harvard Medical School** NIH Postdoctoral Fellow, Department of Microbiology & Molecular Genetics Laboratory of John Collier	Boston, MA
1997-2002	**University of Chicago** Ph.D. Department of Biochemistry & Molecular Biology Laboratory of Tobin Sosnick	Chicago, IL
1992-1996	**Emory University** B.S. Chemistry with Highest Honors / B.A. English	Atlanta, GA

Prior Research Experience

Harvard Medical School (2003-2006) **Advisor: John Collier**

My postdoctoral research centered on how anthrax toxin proteins translocate across cellular membranes into the host cell. Using fluorescence, circular dichroism, and planar lipid bilayer electrophysiology studies, I showed that the substrate proteins unfold under acidic conditions found in endosomes, proton gradients drive transport, and that the channel-forming membrane component of the toxin requires a heptad ring of phenylalanine residues to chaperone substrate protein components through the channel.

University of Chicago (1997-2003) **Advisor: Tobin Sosnick**

Using biophysical approaches, such as stopped-flow, fluorescence, circular dichroism, and small angle x-ray scattering, I studied the folding kinetics of small globular proteins, developing two novel methods. I measured deuterium/hydrogen isotope effects to detect backbone hydrogen bond formation during folding; and I designed bi-histidine metal ion binding sites to delineate the structure of the rate-limiting folding nucleus.

Emory University (1994-1997) **Advisor: Keith Wilkinson**

I addressed the substrate specificity of ubiquitin specific proteases using protein chemistry and enzyme coupled reactions to synthesize novel substrates and inhibitors.

Honors

National Institute of Health—National Research Service Award Postdoctoral Fellowship, (2004-2006)
Departmental Thesis Award in Biochemistry & Molecular Biology, University of Chicago (2003)
National Institute of Health Training Grant, University of Chicago (1997-2001)
Highest Honors in Chemistry, Emory University (1996)
Chemistry Departmental Award, Emory University (1996)
Sigma Tau Delta English Honor Society, Emory University (1995)
Howard Hughes Undergraduate Research Fellowship, Emory University (1995)
Phi B Kappa, Emory University (1995)
Dean's Scholarship, Emory University (1992-96)

Press Highlights on Research

Press highlight: "Bryan Krantz: From folding to unfolding proteins. Interview by Liz Savage" by Liz Savage in *The Journal of Cell Biology* 184: 618 (2009).

Enhanced Perspectives article: "Translocation of Anthrax Toxin: Lord of the Rings" by Gunnar von Heijne in *Science* 309: 709 (2005).

Press highlight: "Toxin Channeling" by Celia Henry in *Chemical & Engineering News* 83: 13 (2005).

Grant Support

National Institute of Health. Title "Physical Principles of Bacterial Toxin Translocation across Membranes" Award period (Sep 2008 to Sep 2012) NIH Award # (R01 AI077703-01). SPO Award # (025620-003). Award Amount per year ($360,000).

Invited Seminars and Meetings

Invited Lectures

Gordon Research Conference. Microbial Toxins & Pathogenicity. Gave lecture titled, "Anthrax toxin assembly & translocation: an interplay of form & function" At Proctor Academy, Andover, NH. 2008-Jul-13-18.

Bay Area Microbial Pathogenesis Symposium (BAMPS). Gave lecture, titled, "Molecular mechanisms of anthrax toxin translocation" at University of California, San Francisco. 2008-Mar-29.

How the anthrax toxin pore unfolds and then translocates its enzymatic factors across bilayer membranes. Biopolymers Gordon Research Conference. Newport, RI. Jun 2006.

Anthrax toxin translocation. 50th Meeting of The Biophysical Society. Salt Lake City, UT. Feb 2006.

Anthrax toxin's protective antigen pore: a protein translocase with a chaperone-like active site. Harvard Medical School Dept. of Biological Chemistry & Molecular Pharmacology Seminar Series. Boston, MA. Oct 2005.

Small molecules target protective antigen's phenylalanine clamp—the site required to translocate lethal & edema factor into the host cell. 2nd Annual Retreat of the New England Regional Center of Excellence for Biodefense and Emerging Infectious Disease Research. Durham, NH. Sep 2005.

The protective antigen binding domains of the lethal and edema factors of anthrax toxin unfold under acidic pH conditions. 49th Meeting of The Biophysical Society. Long Beach, CA. Feb 2005.

Realizing protein hydrogen bond network formation with kinetic D/H amide isotope effects. University of Chicago Molecular Genetics & Cellular Biology Retreat. Chicago, IL. Dec 2001.

When Do Hydrogen Bonds Form? D/H Backbone Amide Isotope Effects in Protein Folding Kinetics. University of Chicago Annual Retreat. Lake Geneva, WI. Oct 1998.

Poster Presentations

Mechanisms of Membrane Transport Gordon Research Conference, 2005; Protein Society, 2003; Proteins Gordon Research Conference, 2001; Johns Hopkins Protein Folding Meeting, 2000-2001; U. of Chicago, Annual Retreat, 1997-2002.

Departmental, University and Public Service

Graduate Student Recruitment (2007-08) – Dept. of Molecular & Cell Biology
Structural & Quantitative Biology Seminar Coordinator (2007-08) – Dept. of Chemistry
Graduate Student Recruitment (2008-09) – Dept. of Molecular & Cell Biology
Structural & Quantitative Biology Seminar Coordinator (2008-09) – Dept. of Chemistry
New Faculty Search Committee (2009) – Dept. of Chemistry

Current Teaching

Classroom Training

Upper Division Courses

Semester and Year	Course Number	Course Title	# of Units	Registered Enrollment (use OIR info)	Number & length of formal lectures and/or labs taught per week	Team taught courses: # of lectures & labs taught during semester	# of Students from whom evaluations are available for this course
Fall 2007	CHEMC 130	Biophysical Chemistry: The Molecules of Life: Physical Principles and Cellular Functions	4	175	2 x 1.5 hr lects. per week	10 lectures	~100
Spring 2008	MCB102	Survey of the Principles of Biochemistry and Molecular Biology	4	375	3 x 1 hr lects. per week	15 lectures	~200
Fall 2008	CHEMC 130	Biophysical Chemistry: The Molecules of Life: Physical Principles and Cellular Functions	4	175	2 x 1.5 hr lects. per week	10 lectures	~100
Spring 2009	MCB110 L	General Biochemistry & Molecular Biology Laboratory	4	69	2 x 1 hr lects. per week + 3 hours of lab after each lecture	10 lectures	63

Graduate Courses

Semester and Year	Course Number	Course Title	# of Units	Registered Enrollment (use OIR info)	Number & length of formal lectures and/or labs taught per week	Team taught courses: # of lectures & labs taught during semester	# of Students from whom evaluations are available for this course
Fall 2007	MCB290	Principles of Cellular Protein Unfolding and Translocation Across Membranes	1	12	1x 1 hr session per week	13	12

Research Training

Graduate Students

(7) Adam Schawel (Chemistry Dept.) 2008-present.
(6) Jen Colby (Molecular Toxicology Graduate Group) 2008-present.
(5) Michael Brown (Molecular & Cell Biology Dept.) 2008-present.
(4) Alexander Kintzer (Chemistry Dept.) 2007-present.
(3) Geoffry Feld (Chemistry Dept.) 2007-present.
(2) Katie Thoren, Dept. of Chemistry, 2006-present.
(1) Samuel Stephenson, Dept. of Molecular & Cell Biology, 2007-2008.

Rotation Graduate Students

(8) Lara Collazo (Molecular & Cell Biology) 2008.

(7) Bryan Schmidt (Molecular & Cell Biology) 2008.
(6) Michael Brown (Molecular & Cell Biology Dept.) 2007.
(5) Jen Colby (Molecular Toxicology Graduate Group) 2007.
(4) Alex Kintzer (Chemistry) 2007.
(3) Geoffry Feld (Chemistry) 2007.
(2) Katie Hart (Chemistry) 2007.
(1) Albert Lang (Molecular & Cell Biology) 2007.

Undergraduate Students
(7) Iok I. Tang (Chemistry) 2009-present (current student)
(6) Evan Worden (MCB, Amgen visiting summer scholar, paid helper in 2009) 2008, 2009.
(5) Paulina Bukshpun (Chemistry) 2008.
(4) Amy Chang (MCB) 2007-2008.
(3) Sarah Watson (MCB) 2008.
(2) Allen Kwong (MCB) 2006-2009 (graduated, Honors Thesis).
(1) Shon Greenberg (MCB) 2006-2008 (graduated, Honors Thesis).

High School Students
(1) Keng Lam (Albany High) 2008.

Thesis Committees
(6) Jake Siegel (Biophysics Graduate Group).
(5) Randall McNally (Chemistry)
(4) Christina Baer (Biophysics Graduate Group).
(3) Veronica Zepeda (MCB).
(2) Bryan Schmidt (MCB).
(1) Sebastian Deindl "Structural basis for the inhibition of tyrosine kinase activity of ZAP-70" (Comparative Biochemistry) graduated 2009.

Past Teaching Experience
(5) Supervision of Research Technician, Harvard Medical School, 2003-2004.
(4) Supervision of Undergraduate's Research, Harvard Medical School, 2003-2004.
(3) Supervision & Training of Research Technician, University of Chicago, 2002-2003.
(2) Nucleic Acid Structure/Function, Teaching Assistant, University of Chicago, 2001.
(1) Introduction to Biophysical Methods, Teaching Assistant, University of Chicago, 2000.

Publications

***Peer-reviewed designated* [PR]**

[PR 22] Kintzer AF, Thoren KL, Sterling HJ, Dong KC, Feld GK, Tang II, Zhang TT, Williams ER, Berger JM, **Krantz BA**. (2009) "The protective antigen component of anthrax toxin forms functional octameric complexes." *J. Mol. Biol.* 292: 614 (doi:10.1016/j.jmb.2009.07.037).

[PR 21] Pandit AD, **Krantz BA**, Dothager RS, Sosnick TR. (2007) "Characterizing protein folding transition States using Ψ-analysis." *Methods Mol. Biol.* 350: 83.

[PR 20] Sosnick TR, **Krantz BA**, Dothager RS, Baxa M. (2006) "Characterizing the protein folding transition state using Ψ analysis." *Chem. Rev.* 106: 1862.

[PR 19] **Krantz BA**, Finkelstein A, Collier RJ. (2006) "Protein translocation through the anthrax toxin transmembrane pore is driven by a proton gradient." *J. Mol. Biol.* 355: 968.

[PR 18] Christensen KA, **Krantz BA**, Collier RJ (2006). "The assembly and disassembly kinetics of anthrax toxin complexes." *Biochemistry*. 45: 2380.

[PR 17] Wolfe JT, **Krantz BA**, Rainey GJA, Young JAT, Collier RJ. (2005) "Whole-cell voltage clamp measurements of anthrax protective antigen pores." *J. Biol. Chem*. 280: 39417.

[PR 16] **Krantz BA**, Melnyk RA, Zhang S, Juris SJ, Lacy DB, Wu Z, Finkelstein A, Collier RJ. (2005) "A phenylalanine clamp catalyzes protein translocation through the anthrax toxin pore." *Science*. 309: 777.

[PR 15] Christensen KA*, **Krantz BA***, Melnyk RA, Collier RJ. (2004) "Interaction of the 20 kDa and 63 kDa fragments of anthrax protective antigen: kinetics and thermodynamics." *Biochemistry.* 44: 1047. ***Contributed equally to this work.**

[PR 14] **Krantz BA**, Trivedi AD, Cunningham K, Christensen KA, Collier RJ. (2004) "Anthrax toxin's lethal and edema factors unfold under acidic pH conditions." *J. Mol. Biol.* 344: 739.

[PR 13] Sosnick TR, Dothager RS, **Krantz BA**. (2004) "Differences in the folding transition state of ubiquitin indicated by ϕ- and ψ-analyses." *Proc. Natl Acad. Sci.* 101: 17377.

[PR 12] Wigelsworth DJ*, **Krantz BA***, Christensen KA, Lacy DB, Juris SJ, Collier RJ. (2004) "Binding stoichiometry and kinetics of the interaction of a human anthrax toxin receptor, CMG2, with protective antigen." *J. Biol. Chem.* 279: 23349. ***Contributed equally to this work.**

[PR 11] Jacob J, **Krantz B**, Dothager RS, Thiyagarajan P, Sosnick TR. (2004) "Early collapse is not an obligate step in protein folding." *J. Mol. Biol.* 338: 369.

[PR 10] **Krantz BA**, Dothager R, Sosnick TR. (2004) "Discerning the structure and energy of multiple transition states in protein folding using ψ-analysis." *J. Mol. Biol.* 337: 463.

[PR 9] **Krantz BA**, Mayne L, Rumbley J, Englander SW, and Tobin R. Sosnick. (2002) "Fast and slow intermediate accumulation and the initial barrier mechanism in protein folding." *J. Mol. Biol.* 324: 359.

[PR 8] **Krantz BA**, Srivastava AK, Nauli S, Baker D, Sauer RT, Sosnick TR. (2002) "Understanding protein hydrogen bond network formation with kinetic D/H amide isotope effects." *Nature Struct. Biol.* 9: 458.

[PR 7] Shi Z, **Krantz BA**, Kallenbach N, Sosnick TR. (2002) "Contribution of hydrogen bonding to protein stability estimated from isotope effects." *Biochemistry.* 41: 2120.

[PR 6] **Krantz BA**, Sosnick TR. (2001) "Engineered metal binding sites map the heterogeneous folding landscape of a coiled coil." *Nature Struct. Biol.* 8: 1042.

[PR 5] **Krantz BA**, and Sosnick TR. (2000) "Distinguishing between two-state and three-state models for ubiquitin folding." *Biochemistry.* 39: 11696.

[PR 4] Yin L, **Krantz B**, Russell NS, Deshpande S, Wilkinson KD. (2000) "Nonhydrolyzable diubiquitin analogues are inhibitors of ubiquitin conjugation and deconjugation." *Biochemistry.* 39:10001.

[PR 3] **Krantz BA**, Moran LB, Kentsis A, Sosnick TR. (2000) "D/H amide isotope effects reveal when hydrogen bonds form during protein folding." *Nature Struct. Biol.* 7: 62.

[PR 2] Larsen CN, **Krantz BA**, Wilkinson KD. (1998) "Substrate specificity of deubiquitinating enzymes: ubiquitin C-terminal hydrolases." *Biochemistry.* 37: 3358.

[PR 1] Amerik A, Swaminathan S, **Krantz BA**, Wilkinson KD, Hochstrasser M. (1997) “In vivo disassembly of free polyubiquitin chains by yeast Ubp14 modulates rates of protein degradation by the proteasome.” *EMBO J.* 16: 4826.

Non-peer-reviewed designated **[NPR]**

[NPR 2] Pimental RL, Christensen KA, **Krantz BA**, Collier RJ. (2004) “Anthrax toxin complexes: heptameric protective antigen can bind lethal factor and edema factor simultaneously.” *Biochem. Biophys. Res. Comm.* 322: 258.

[NPR 1] **Krantz BA.** (2002) *Protein Folding: New Methods Unveil Rate-limiting Structures*. Ph.D. diss., U. of Chicago.

Research Summary

OVERVIEW

To function, a protein must be correctly localized in the cell, especially in ones that are internally compartmentalized by membrane bilayers. Proteinaceous, membrane-embedded transporters, called translocase channels, can traffic proteins across membranes by a process known as transmembrane protein translocation. Translocase channels also play key functional roles in microbial pathogenesis, because a host cell's lipid bilayer membrane functions as a formidable, first line of defense, isolating the pathogen from its cytosol. For example, the bacterium, *Bacillus anthracis*, secretes a three-protein toxin, called anthrax toxin, which is composed of protective antigen (PA), lethal factor (LF), and edema factor (EF). PA assembles into an oligomeric translocase channel, forming a narrow passageway across the host cell's endosomal membrane bilayer. The channel, however, is so narrow that LF and EF traverse it as unfolded polypeptide chains. Once inside the target cell's cytosol, LF and EF refold and then catalyze reactions that disrupt the cell's normal physiology.

Studies of protein unfolding and transmembrane translocation probe exciting biophysical questions, which apply broadly to the studies of soluble molecular motors, which unfold, disassemble, and degrade proteins. How is a stable substrate protein unfolded in the cell? What structural features in the translocase channel (or translocase machinery) determine the complex energy landscape that guides a chemically-complex, unfolded chain through the narrow confines of the channel? The biophysical chemistry of transmembrane protein translocation, however, has been challenging to characterize, and the three-dimensional structures of many translocase channels are unknown. Bacterial toxins, like anthrax toxin, are particularly well-suited for these studies, because they carry their own translocase-channel machinery, which is able to spontaneously insert into lipid bilayer membranes.

We use many different biophysical methods to understand the complex chemistry of protein translocation. We will analyze in detail the thermodynamic and kinetic mechanisms, which describe how the translocase channel of anthrax toxin unfolds its substrate proteins, exploring the role of chaperone-like, active-site surfaces in the PA channel. We will dissect Brownian-ratchet translocation models through ensemble and single-channel electrophysiology studies of artificial, designed polypeptide substrates. The

structure and assembly of the PA channel will be pursued using spectroscopy, electrophysiology, electron microscopy, and crystallography.

Funding support

National Institute of Health. Title "Physical Principles of Bacterial Toxin Translocation across Membranes" Award period (Sep 2008 to Sep 2012) NIH Award # (R01 AI077703-01). SPO Award # (025620-003). Award Amount per year ($360,000).

Outline of Research

- **I. Translocation-coupled protein unfolding**
- **II. Molecular mechanisms of polypeptide translocation**
- **III. Structure and function of the protective antigen oligomerization pathway**
- **IV. Structure and function of the protein translocation initiation complex**
- **V. Structure of the protective antigen channel**

I. Translocation-coupled protein unfolding

This work is being reviewed for publication in *PNAS*. The paper is titled "Lethal factor unfolding is the most force-dependent step of anthrax toxin translocation." The paper examines how a protein unfolds during translocation. The paper develops a formalism to dissect individual translocation steps, it determines that the unfolding step is the most force-dependent, and it reveals the structures in the substrate that are rate-limiting to unfolding. These data are reconciled with the literature's current understanding of mechanical unfolding.

Cellular compartmentalization requires machinery capable of translocating polypeptides across membranes. In many cases, transported proteins must first be unfolded by means of the proton motive force and/or ATP hydrolysis. Anthrax toxin, which is composed of a channel-forming protein and two substrate proteins, is an attractive model system to study translocation-coupled unfolding since the applied driving force can be externally controlled and translocation can be monitored directly using electrophysiology. By controlling the driving force and introducing destabilizing point mutations, we

identified the barriers in the mechanism, determined which barrier corresponds to protein unfolding, and mapped how the substrate protein unfolds during translocation. In contrast to previous studies, we find that the protein's structure next to the signal tag is not rate-limiting to unfolding. Instead, a more extensive part of the structure, the amino-terminal β-sheet subdomain, must disassemble to cross the unfolding barrier. We also examined the role of the channel's phenylalanine-clamp (ϕ-clamp) active site; these types of sites, composed of a narrow ring of phenylalanine residues, which is a common feature to many translocase machines, is critical for translocase activity. We find that unfolding is catalyzed by the phenylalanine-clamp active site. Finally, we propose a broad molecular mechanism for translocation-coupled unfolding, which is applicable to both soluble and membrane-embedded unfolding machines.

We are extending and proposing to extend this with some shorter- and longer-term experiments. First, we would like to determine if the substrate unfolds in solution as it does on the surface of the PA channel. This study will address whether unfolding machines follow the unfolding pathway naturally taken. We are addressing this question using stopped-flow protein folding studies. Secondly, we are asking whether β-sheet structures in general are rate-limiting to translocation coupling unfolding mechanisms. We have mainly studied the translocation of the amino-terminal domain of LF, and its β-sheet subdomain is rate-limiting to the unfolding mechanism. A second β-sheet domain in LF (domain 3) may be responsible for the slow translocation kinetics of full-length LF. LF C-terminal truncation mutants are being tested to determine if this structure is limiting to full-length LF translocation. Mutagenesis will then be used to probe the unfolding mechanism in greater detail. Finally, we would like to examine the LF_N's mechanical unfolding mechanism using optical tweezers. We believe that the underlying translocation-coupled unfolding mechanism is mechanical; and thus we expect that the structures limiting LF_N unfolding in pulling experiments will reveal a similar rate-limiting core surrounding the β-sheet subdomain.

II. Molecular mechanisms of polypeptide translocation

A basic summary of preliminary findings is given below for this aim. Part (a) is on the role of negatively-charged residues in proton gradient-driven protein translocation. Part (b) is on the role of hydrophobic residues in protein translocation. In each

subaim, we are systematically addressing the effect of sequence composition on the translocation kinetics of anthrax toxin.

(Part a) Protein translocation, specifically the movement of proteins across biological membranes, is an important process; however, few of these systems are well-characterized. The kinetic mechanisms of translocation are not understood. Anthrax toxin is a useful model system due to its ability to function as a translocase channel in planar lipid bilayer assays. During these assays, it is possible to control the driving forces and study distinct processes in the mechanism independently. While it is established that a proton gradient (ΔpH) drives anthrax toxin translocation, the mechanism for how this occurs has not yet been demonstrated. To elucidate the mechanism, we re-engineered the pre-sequence of LF, eliminating the naturally occurring negative charges from the amino terminus. The removal of these negative charges causes LF's amino-terminal domain (LF_N) to translocate 2 orders of magnitude slower than wild type under a ΔpH. This result is exciting because it reveals that negative charges are the molecular teeth in the Brownian ratchet mechanism of translocation. The charge composition is now being varied in this presequence to determine the charge requirements and the number of protons involved upon crossing the translocation barrier.

(Part b) Several protein translocases, including anthrax toxin, have been shown to interact nonspecifically with translocating polypeptides via a hydrophobic ring or gasket known as the ϕ clamp. Anthrax toxin provides a particularly good model system for studying this interaction because the planar lipid bilayer assay can be used to study each step of the translocation mechanism individually. We are probing the molecular details of the hydrophobic interaction and how it affects the movement of substrate proteins across the membrane. We found that natural sequences in LF and EF can contain a high density of aromatic residues. These sequences were cloned onto the carboxy-terminus of LF_N. We find that the most hydrophobic sequences inhibit translocation; however, when the sequences are cloned between two folded domains, we find that they can accelerate translocation. We interpret the preliminary results to mean that hydrophobic sequences upstream of a folded domain can aid in the unfolding by interacting with the hydrophobic ϕ-clamp and α-clamp sites we have identified on the PA channel. Future work is aimed at varying the hydrophobicity and aromaticity of these natural sequences to determine

those dependencies. Also the stability of the downstream protein will be modified to determine if unfolding is limiting translocation of the second folded domain.

III. Structure and function of the protective antigen oligomerization pathway

This work is published in *Journal of Molecular Biology*. The paper is titled "The protective antigen component of anthrax toxin forms functional octameric complexes." The manuscript shows that PA may form both the traditional heptamer and a novel octamer. We then characterize the octamer side by side to the heptamer with detailed structure and function studies.

The assembly of bacterial toxins and virulence factors is critical to their function, but the regulation of assembly during infection has not been studied. We begin to address this question using anthrax toxin as a model. The protective antigen (PA) component of the toxin assembles into ring-shaped homooligomers that bind the two other enzyme components of the toxin, lethal factor (LF) and edema factor (EF), to form toxic complexes. To disrupt the host, these toxic complexes are endocytosed, such that the PA oligomer forms a membrane-spanning channel that LF and EF translocate through to enter the cytosol. We show using single-channel electrophysiology that PA channels contain two populations of conductance states, which correspond with two different PA pre-channel oligomers observed by electron microscopy—the well-described heptamer and a novel octamer. Mass spectrometry demonstrates that the PA octamer binds four LFs, and assembly routes leading to the octamer are populated with even-numbered, dimeric and tetrameric, intermediates. Both heptameric and octameric PA complexes can translocate LF and EF with similar rates and efficiencies. Here we also report a 3.2-Å crystal structure of the PA octamer. The octamer comprises ~20-30% of the oligomers on cells, but outside of the cell, the octamer is more stable than the heptamer under physiological pH. Thus the PA octamer is a physiological, stable, and active assembly state capable of forming lethal toxins that may withstand the hostile conditions encountered in the bloodstream. This assembly mechanism may provide a novel means to control cytotoxicity.

Presently we are determining the functional robustness of the two different isoforms of lethal toxin (LT)—i.e., one form, of course, contains LF bound to the PA heptamer, and the other form contains LF bound to the PA octamer. In these experiments, the two purified isoforms of LT are treated at 37 °C for 5

minutes, and then various functional assays are performed. The octameric form appears to have robust tolerance to these physiological conditions; the octamer retains significantly more marcrophage cytotoxicity activity and channel formation activity relative to the heptamer. We then asked how the PA heptamer inactivates itself upon heating to 37 °C. The loss of channel forming activity for the heptamer, of course, suggested that the heat treated form had converted to the channel state prematurely. To test this idea, we examined electron micrscopy images of LT containing PA heptamer in the presence of bovine serum albumin (BSA). BSA prevents the formation of large indiscernible aggregates, and allowed the conformation of the heat treated heptamer to be elucidated. The heat inactivated form is the channel state, which thus explains the low solubility upon heat treatment. Other biophysical techniques, including circular dichroism (CD) spectroscopy are been used. In collaboration with a group using synchatron-based CD, we show that the pH dependence for the increase in β sheet secondary structure formation in PA oligomers is altered depending on the oligomeric state. The octamer requires lower pH and higher temperatures to convert to the channel state. Thus we have also expanded upon our current knowledge of the molecular mechanism of heptameric LT inactivation and further developed our understanding of PA channel formation.

IV. Structural studies of protein translocation initiation

This work is being reviewed for publication at *Nature*. The paper is titled "Structural basis for the pre-translocation unfolding of proteins by anthrax lethal toxin." We describe the first lethal toxin structure. The structure reveals a novel feature of the PA oligomer, we call the α clamp, which has important implications in assembly and protein translocation.

Anthrax toxin is a three-protein virulence factor secreted by *Bacillus anthracis*, the causative agent of anthrax. In order to achieve cytotoxicity and lethality, the toxin's three components must assemble into holotoxin complexes. Specifically, the protective antigen (PA) component forms either a ring-shaped homoheptameric or homooctameric oligomer, which can bind multiple copies of the two enzymatic components, lethal factor (LF) and edema factor (EF), producing various lethal toxins (LT) and edema toxins, respectively. After binding a cell-surface receptor, toxic complexes are endocytosed; the soluble PA oligomer converts into a membrane-inserted channel; and LF and EF unfold and translocate

through the channel, reaching the cytosol. Once in the cytosol, LF and EF disrupt cell function through their enzymatic activities. We report a 3.1-Å X-ray crystal structure of the octameric LT complex, which we show here is the more thermostable isoform. The structure contains the PA octamer bound to four PA-binding domains from LF. Each LF binding site encompasses ~1900 $Å^2$ in two discontinuous regions. The twin calcium-ion binding sites on adjacent PA subunits frame deep hydrophobic clefts (which we call α clamps) on the surface of the oligomer. The first α helix and β strand of each LF unfold and dock into an α-clamp site. We show that the α clamp is functionally relevant to efficient holotoxin assembly, PA octamer formation, and LF translocation. The α clamp is thus an active site that may catalyze translocation by binding amphipathic helices or hydrophobic surfaces on the substrate, allowing mechanical denaturation to proceed efficiently. This structure captures an unfolding intermediate, provides new insights on protein unfolding during translocation, and inspires therapeutic strategies that target toxin assembly.

We are presently generating novel co-crystals with EF's amino-terminal domain and other LF analogs. In either case, we are interested in probing the extent of the α-clamp site's capability to recognize amphipathic helices. EF and LF have similar but non-identical α1 helices, the helix docked in the α-clamp site. We know the interaction is critical to form the translocation intiation complex that we crystallized. A substrate that interacts properly with the α-clamp site translocates more rapidly and efficiently. We suspect the interaction causes structural strain in the substrate, which effectively destabilizes the substrate. How do side chain chemistry, sterics, and intrinsic helicity affect translocation initiation? Are the structures obtained similar or does the PA surface adapt to the shape and chemistry of the substrate and vice versa? We have obtained three new crystal forms with different types of substrate proteins. One of our latest structures, solved by molecular replacement to ~4 Å, shows α helices docked into the α-clamp site. We are working to refine the structure and also to obtain better diffracting crystals. We are also exploring a variety of substrates in translocation assays, in which the critical α1 helix has been altered. The early results show a significant amount of tolerance to substitution there, suggesting that helical shape complementarity (rather than chemistry) is key to the α-clamp site's molecular recognition mechanism.

V. Structure of the protective antigen channel

We have identified two different forms of the PA oligomer. The octameric form is more symmetric and stable than the heptameric form. We have also determined that domain 4 in PA is superfluous to the structure of the PA channel and may interfere with crystallization due to its intrinsic flexibility. Constructs lacking domain 4 also are well-behaved and assemble into nearly uniform octameric PA preparations. Using this construct, we will crystallize the channel state of PA.

An important goal of the lab is to obtain a high resolution structure of the PA channel. The structure will be critical to understanding the molecular mechanism of translocation. As we have learned in many prior studies, the channel itself is not a passive player in translocation. Rather the channel uses specific “active sites” to bind to and engage the translocating chain. The structure of these sites, particularly the ϕ clamp (i.e., the narrow ring of phenylalanine residues inside the channel required for translocase function) will be critical to understanding polypeptide translocation. We have learned that PA can form octameric and heptameric oligomers, where the former is more robust, stable and symmetric than the later. We are presently screening the best protein construct for crystallization—ideally one that is soluble, rigid, and readily forms octamers. Electron microscopy studies on various PA channel preparations reveal several important properties. First, aggregates are more likely to form when the substrate is not bound to the channel. Either a specific substrate or a non-specific binding partner like BSA can prevent aggregates from forming. We prefer to use LF’s amino-terminal domain as a substrate although peptides designed for the alpha-clamp groove may prove equally effective. Second, the deletion of domain 4 creates a more soluble form of the PA channel that is highly octameric.

[As an aside, this may be an important result, since the truncated product appears in the blood animal models with anthrax. Blood serum proteases are able to remove domain 4, since there is a lysine- and arginine-rich loop there. We know that the octameric form of PA is better able to withstand the conditions of the blood. So this may be a novel pathway to form the octamer. This molecular mechanism is yet another means of forming the PA octamer.]

In any case, detergents are being screened with this new domain 4-less form of the octamer. We are also obtaining EM images of the preparation. Crystallization trials are underway.

Research Mentoring

I actively train six full-time graduate students, transient rotation students, and one dedicated undergraduate student. I try to emphasize experimental design including detailed positive and negative control experiments, good lab notebook practices and documentation of data, quality data collection as well as an understanding of the biophysical techniques and instrumentation. Our lab conducts weekly lab meetings, where individuals in the lab present their work to the group. I try to stimulate collaborations between members of the group. I take members of the lab to lunch in small groups or individually to encourage their efforts and spark new ideas.