

The anthrax toxin channel: a barrel of LFs

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The use of anthrax as a model system dates back to the late 19th century and Koch's initial investigations into the transmission of infectious diseases (Koch, 1877). These studies, coupled with his subsequent investigations into tuberculosis, enabled Koch to formulate the first set of rules that were used to determine the etiology of a human infectious disease. Koch's postulates, as these rules came to be known, have been used successfully by generations of microbe hunters, and have secured a special place for anthrax in the history of medicine.

Anthrax continues to serve as a useful model system, with studies of anthrax toxin, the causative agent of anthrax, yielding insights into bacterial pathogenesis (Young and Collier, 2007), gating and permeation of ion channels (Blaustein et al., 1989, 1990; Blaustein and Finkelstein, 1990a,b; Anderson and Blaustein, 2008), and transport of proteins across membranes (Collier, 2009). It is this last topic—protein translocation—that is the focus of the paper by [Basilio et al.](#) in this issue. In particular, the authors address the following question: must the lethal factor (LF) component of anthrax toxin shed all of its secondary structure as it tunnels through the channel formed by the protective antigen (PA) component, or can it retain most of its α -helical nature? Because the usual *JGP* reader is likely less familiar with anthrax toxin channels than with the typical panoply of voltage-gated channels in excitable tissue, it will be helpful to first review some features of this system before delving into the experiments that address the translocation question.

Anthrax infection results from exposure to *Bacillus anthracis*, a gram-positive spore-forming bacterium that is found in soil and in the hair or hides of contaminated animals (Collier and Young, 2003). Although isolated skin contact typically causes only a localized infection in humans, inhalation of aerosolized spores can lead to a hemorrhagic invasive lung disease that is often fatal (Meselson et al., 1994). Cellular injury and death result from the action of anthrax toxin, a tripartite toxin composed of a pore-forming subunit called protective antigen (PA), so-named because of its use in generating vaccines, and two distinct enzymatically active subunits: LF and edema factor (EF) (Smith and Stoner, 1967; Smith, 2000).

Pathogenesis relies on receptor-mediated endocytosis, vesicular acidification, pore formation in the endosomal membrane by a PA-derived oligomer, and eventual translocation of EF and LF through this pore into the cytosol (Young and Collier, 2007). Once there, LF acts as a zinc-dependent protease that disrupts cellular signaling by cleaving proteins of the mitogen-activated protein kinase family (Duesbery et al., 1998), and EF is a calcium- and calmodulin-dependent adenylate cyclase that induces high levels of cAMP in mammalian cells (Leppla, 1982).

This route of intoxication is characteristic of the A/B family of toxins, a term coined by Gill to describe toxins that contain an active (A) domain—typically an enzyme that disrupts an important cellular process—and a binding (B) moiety that somehow mediates entry of the A fragment into the cytosol. Members of this family include the dichain toxins ricin, diphtheria, tetanus, and botulinum (Gill, 1978).

A fascinating property shared by the B fragments of many A/B toxins is that they form ion-conducting channels in lipid bilayers, with pore formation optimal under conditions that mirror those in an acidified endosome (Donovan et al., 1981; Kagan et al., 1981; Hoch and Finkelstein, 1985; Hoch et al., 1985; Blaustein et al., 1987; Gambale and Montal, 1988). With the exception of anthrax (discussed below), little is known about the molecular architecture of these channels. Nevertheless, given this channel-forming ability, along with the knowledge that the soluble A fragments must cross a lipid membrane to reach the cytosol, it is tempting to speculate that these B fragments act as tunnel proteins to accommodate the membrane translocation of their A fragment partners. Sorting out the connection between channel formation (which, after all, involves the movement of small inorganic ions) and translocation of proteins has proved to be very challenging, however, and anthrax is the only toxin for which translocation of A through B has been convincingly demonstrated.

The pore-forming capability of PA resides in a 63-kD fragment (PA₆₃) generated by proteolytic cleavage of whole PA (Blaustein et al., 1989). Purified PA₆₃ forms

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heptamers at neutral pH, but at acidic pH and in the presence of a lipid bilayer, this heptameric prepore undergoes a transition into a mushroom-shaped 14-stranded β barrel that forms a transmembrane channel (summarized in Young and Collier, 2007). (Although PA₆₃ is heptameric in several experimental systems, there is evidence for octamer formation *in vivo*; Kintzer et al., 2009, 2010.) The dimensions of this channel can be gleaned from modeling efforts (Krantz et al., 2004; Nguyen, 2004) as well as from negative-stain electron microscopy data (Katayama et al., 2008, 2010) (see Fig. 1 of Basilio et al., 2011). The channel is ~ 170 -Å long, with a stem region that is ~ 100 Å in length and a cap region that is ~ 70 -Å long and 125-Å wide. The inner diameter of the pore is ~ 15 Å, which is large enough to accommodate the passage of an α helix.

To study translocation through PA₆₃ channels, Zhang et al. (2004a,b) leveraged an interesting observation: PA₆₃ channels can be blocked by LF in a voltage-dependent fashion (for technical reasons, the authors chose to work with LF_N, the 263-residue N-terminal portion of LF that contains a binding site for PA₆₃ channels). At small positive voltages (eg +20 mV), the positively charged N-terminal portion of LF_N enters the channel and blocks conductance; at negative voltages, LF_N unblocks by coming back out of the channel to the cis solution. (By convention, voltages are those of the cis relative to the trans sides of the bilayer, with the cis side defined as the side to which PA₆₃ is added.) At larger positive voltages (e.g., $\geq +40$ mV), however, the degree of block actually decreases. Because the usual interpretation of relief of cationic block with larger positive voltages is that the blocker is permeant, the authors concluded that LF_N is driven through the channel and out the trans side in a manner reminiscent of the permeant, voltage-dependent block of PA₆₃ channels by tetraalkylammonium ions (Blaustein and Finkelstein, 1990b; Blaustein et al., 1990). To strengthen their translocation argument, the authors examined LF_N block using biotin tagging and streptavidin capture. When an LF_N that has been biotinylated at its N terminus is used to block PA₆₃ channels, streptavidin added to the trans side grabs the N terminus as it emerges from the pore and anchors it there so that it cannot unblock to the cis side upon application of negative voltages (Zhang et al., 2004b). Further evidence that LF_N is going through the PA₆₃ pore, rather than via some other route, is that chemical modification of pore-lining residues slows translocation.

This elegant wedding of the bilayer recording system with a biotin-streptavidin assay is an approach pioneered by the Finkelstein laboratory in the 1990's to study transmembrane protein movements associated with voltage gating of toxin channels (Qiu et al., 1994; Slatin et al., 1994). It takes advantage of the ease with which one can place a biotin residue at a site along a protein, the extremely tight binding of streptavidin with biotin, and

the large size and membrane impenetrability of streptavidin. This general method has since been extended to the study of voltage sensor movements of voltage-gated K⁺ channels in lipid bilayers (Jiang et al., 2003; Ruta et al., 2005; Banerjee and MacKinnon, 2008) and oocytes (Darman et al., 2006), and it forms the basis of the approach taken by Basilio et al. (2011).

To determine the secondary structure of LF_N during translocation, Basilio et al. (2011) introduced an interesting twist to the biotin-streptavidin capture strategy: molecular stoppers that allow LF_N to be trapped in the PA₆₃ pore. The authors used LF_N constructs in which a stopper—either biotin or yellow fluorescent protein (YFP)—was incorporated some distance away from the N-terminal biotin group. When added to the cis side of a PA₆₃-containing bilayer held at +20 mV, the biotinylated N terminus of this LF_N construct will enter the channel and work its way toward the trans side of the pore until the stopper impedes further translocation. If the distance between the N terminus and the stopper is long enough, streptavidin added to the trans side will grab the N-terminal biotin group and prevent LF_N from unblocking to the cis side. If that distance is too short, trans streptavidin will have no effect. By examining a series of LF_Ns in this manner, each with a different number of residues between its stopper and its N terminus, the authors were able to determine a cutoff for the number of amino acids required to span the channel.

Crucial to this approach is knowing where in the channel the stopper gets held up, which in turn relies on understanding both the nature of the stopper and the structure of the channel. There is reasonable evidence that both biotin and YFP can get inside the channel's wide vestibule on the cis side of the membrane, but that they are unable to pass through the ring of phenylalanines that comprise the Φ -clamp (indicated by the red arrow in Fig. 1 of Basilio et al., 2011). This likely accounts for the observation that, despite their rather different structures, the biotin and YFP stoppers yield similar cutoffs (~ 33 residues) when blocking from the cis side of the bilayer. (Fortunately, the authors do discuss the reason for the discrepancy between the N-terminal biotin, which can get through the Φ -clamp, and the downstream stopper biotins that cannot.) As the authors point out, an α helix consisting of 33 residues would, at ~ 50 Å, fall far short of spanning the ~ 120 -Å length of the channel's stem region. But as an extended chain, 33 amino acids would span ~ 119 Å, which jibes nicely with the length of the stem. The conclusion, therefore, is that LF_N translocates as an extended chain.

In contrast to the agreement in cutoff obtained with the two types of stoppers applied cis, the biotin and YFP stopper constructs behave quite differently when added from the trans side. The N terminus of an LF_N with a biotin stopper is able to enter the trans side of the pore and work its way all the way up the stem until the biotin

stopper reaches the Φ -clamp, whereas the YFP stopper is unable to enter the stem at all (compare the cartoon in Fig. 11 of Basilio et al., 2011, with that in Fig. 12). Thus, each stopper yields a different distance measurement; the biotin stopper, with its shorter cutoff, provides an estimate of the length of the mushroom cap, and the YFP stopper, with its longer cutoff range, provides an approximate readout of the length of the entire channel. Although the authors did not attempt to determine cutoffs as finely as with the cis-applied blockers, their results agree nicely with the dimensions derived from the EM structure.

Is it possible that LF_N remains mostly α helical during its passage through the PA_{63} channel but that trans streptavidin, by virtue of its ability to bind biotin nearly irreversibly, captures a rare extended configuration of LF_N ? This is a problem that can plague attempts to draw structural insights using approaches that rely on irreversibly trapping a particular conformation of a protein (disulfide cross-linking in proteins is a well-known example; Falke and Koshland, 1987; Careaga and Falke, 1992). In this case, a comparison of the kinetics of streptavidin capture for different blockers provides important insight and suggests that this is very unlikely. Using a *reductio ad absurdum* argument, the authors set up the straw man assumption that LF_N remains mostly α helical and only rarely adopts an extended conformation. A YFP blocker with 83 residues between its stopper and the N terminus would just span the stem region, and its N-terminal biotin would just be accessible to trans streptavidin. A blocker with 40 fewer residues between its stopper and N-terminal biotin would, if α helical, span a distance that is ~ 60 -Å shorter, and it would very rarely adopt a configuration for which its N terminus reached the trans solution. Therefore, the N-terminal biotin of the shorter blocker should be captured by streptavidin only very rarely and therefore at a much slower rate than that of the longer blocker (by a factor roughly proportional to the time it spends extended vs. helical). But the observed rates of streptavidin capture for these two blockers actually differ by only a factor of 2, thus generating a contradiction. The authors conclude, therefore, that LF_N is extended during translocation and that streptavidin is not capturing a rare configuration.

Why is anthrax the only A/B toxin for which translocation has convincingly been demonstrated? The answer likely lies in several unique features of this toxin. In other A/B toxins, the A and B domains are part of a single protein and therefore when purified toxin is added to a lipid bilayer, channel formation and translocation occur nearly simultaneously. But because PA, LF, and EF of anthrax toxin are synthesized de novo as individual proteins, channel formation and translocation can be readily dissociated. Furthermore, channels formed by PA_{63} contain binding sites for LF and EF, both in the pore and on the surface of the mushroom

cap domain, and these sites effectively increase the potency of LF and EF so that block can be observed at concentrations of these A fragments that are achievable in solution.

How does translocation through PA_{63} channels compare to protein translocation in other systems? Although voltage can drive translocation in anthrax, ΔpH turns out to be a more powerful driving force by creating a bias to the otherwise random thermal motion of the peptide in the PA_{63} channel. This Brownian ratchet mechanism relies on the deprotonation of anionic residues as they exit the channel to the more alkaline trans side, making them less likely to reenter the channel's anionic milieu (Krantz et al., 2006). An important component of this mechanism is the channel's Φ -clamp. This ring of phenylalanines, which is highly conserved among homologous toxins, forms a tight seal around the translocating peptide to prevent the pH gradient across the endosomal membrane from dissipating. It also catalyzes translocation by forming a binding site that mimics the hydrophobic core of a protein. As others have pointed out (Krantz et al., 2005; von Heijne, 2005), this latter function may be similar to the hydrophobic gasket present in the Sec61/SecY complex, the "greasy slide" seen in the structure of the sugar translocation channel maltoporin, and the hydrophobic residues that project into the central cavity of the GroEL/ES chaperonin. The extended nature of the polypeptide chain that LF_N adopts during translocation has also been observed in other systems, including transport across the mitochondrial membrane (Ungermann et al., 1994) and across the ribosome-translocon channel (Whitley et al., 1996; Mingarro et al., 2000). In the latter system, amino acid sequence plays an important role; peptides can traverse either as extended chains or, if their sequence contains residues with higher helical propensity, they can adopt a more compact helical form during translocation. Thus, the mechanism of protein transport in complex mammalian systems, which require auxiliary proteins such as ATPases to provide the energy that drives translocation, exhibits striking parallels to the mechanism of protein transport through the anthrax channel, which is a self-sufficient protein-translocating machine. It's a barrel of LFs, I tell ya.

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