

Published in final edited form as:

Biochemistry. 2006 February 21; 45(7): 2380–2386.

The Assembly and Disassembly Kinetics of Anthrax Toxin Complexes†

Kenneth A. Christensen^{1,2}, Bryan A. Krantz¹, and R. John Collier^{1,*}

¹Harvard Medical School, Department of Microbiology and Molecular Genetics, Boston, MA 02115

²Clemson University, Department of Chemistry, Clemson, SC 29634

Abstract

Proteolytic activation of the protective antigen (PA) component of anthrax toxin allows it to self-associate into a ring-shaped homoheptamer, [PA₆₃]₇, which can bind the enzymatic components, lethal factor (LF) and edema factor (EF). [PA₆₃]₇ is a pore-precursor (prepore), and under the low pH conditions of the endosome, it forms a transmembrane pore that allows LF and EF to enter the cytosol. We labeled PA with donor and acceptor fluorescent dyes and used Förster resonance energy transfer to measure the assembly and disassembly kinetics of the prepore complex in solution. The dissociation rate constant for [PA₆₃]₇ was $1 \times 10^{-6} \text{ s}^{-1}$ ($t_{1/2} \sim 7$ days). In contrast, a ternary complex containing the PA-binding domain of LF (LF_N) bound to a PA₆₃ dimer composed of two non-oligomerizing mutants, dissociated rapidly ($t_{1/2} \sim 1$ minute). Thus, the substantial decrease in the rate of disassembly of [PA₆₃]₇ relative to the ternary complex is due to the cooperative interactions among neighboring subunits in the heptameric ring. Low concentrations of LF_N promoted assembly of the prepore from proteolytically activated PA, whereas high concentrations inhibited assembly of both the prepore and the ternary complex. A self-assembly scheme of anthrax toxin complexes is proposed.

Anthrax toxin, a major virulence factor of *Bacillus anthracis*, consists of three nontoxic proteins that interact at the mammalian cell surface or in solution to form toxic noncovalent complexes (1,2). The three proteins are edema factor (EF), a calmodulin-dependent adenylate cyclase; lethal factor (LF), a Zn²⁺-metalloprotease; and protective antigen (PA₈₃, 83 kDa), a non-enzymatic protein that, after proteolytic activation, can bind EF and LF and deliver them to the cytosol of mammalian cells. Once within the cytosol, EF and LF catalyze reactions that cause deleterious effects on the host (3-5).

The following model of anthrax toxin assembly and action at the cellular level has emerged in recent years. PA₈₃ binds to either of two known cell surface receptor proteins (6,7). A cellular protease of the furin family then cleaves PA₈₃ into a “nicked” form (nPA₈₃) containing 2 fragments: PA₂₀ (20 kDa), corresponding to the N-terminus, and PA₆₃ (63 kD), corresponding to the C-terminus (8,9). PA₂₀ dissociates into the medium and plays no further known role in toxin action. Its removal eliminates a steric barrier to self-association of PA₆₃, allowing this fragment to oligomerize and form a heptameric ring-shaped structure, [PA₆₃]₇, termed the prepore (10). The PA₆₃ prepore is able to bind up to three molecules of LF and/or EF competitively and with nanomolar affinity (11,12). The resulting complexes are localized to detergent-resistant lipid microdomains, where they undergo receptor-mediated endocytosis (13). Acidification of the toxin-containing compartment causes a structural rearrangement of the prepore that enables it to insert into the membrane and form a pore (14,15). Pore formation

†This work was supported by NIH grant R37-A1022021 (R.J.C.).

*Corresponding author (E-mail: jcollier@hms.harvard.edu, Phone: (617) 432-1930; Fax: (617) 432-0115).

is linked to translocation of the bound enzymatic cargo (LF or EF) to the cytosol by an acid-induced unfolding mechanism (16-18). LF cleaves members of the mitogen-activated protein kinase kinase family (4,5), leading by a still poorly defined sequence of events to host cell death. Elevation of the cAMP concentration by EF protects the bacteria from phagocytic destruction (19).

The assembly of toxic complexes from PA, EF, and LF can be replicated in solution when trypsin or a serum protease is used to activate PA (14). In the absence of EF or LF, the PA₂₀ and PA₆₃ fragments tend to remain associated via noncovalent interactions (equilibrium dissociation constant \approx 190 nM; (20)). When PA₆₃ is separated from PA₂₀ by anion-exchange chromatography of nPA₈₃, it spontaneously oligomerizes to the prepore state (14).

Alternatively, oligomerization of PA₆₃ can be stimulated by adding LF, EF, or their PA₆₃-binding N-terminal domains (LF_N and EF_N, respectively) to nPA₈₃ in solution (21,22). As no quantitative study of PA₆₃ oligomerization or dissociation has been reported, we describe here measurements of the kinetics of PA₆₃ oligomer formation and dissociation in solution.

Materials and Methods

Protein expression and purification

PA₈₃ (K563C, D512K K563C, K199E R468A R470D K563C) and LF_N (residues 1-263 of LF) were produced recombinantly in *E. coli* BL-21 DE3 Star using the pET 22b expression system for PA₈₃ and pET 15b expression system for LF_N as described previously (22). Briefly, the proteins were expressed in cultures grown in a 5-liter fermentor using ECPM1 media supplemented with 50 mg/l carbenicillin until reaching an OD₆₀₀ of 3-10 (22,23). The cultures were induced with IPTG (0.8-1 mM) for 3-4 h at 27-30°C for PA₈₃ and 37°C for LF_N until reaching a final OD₆₀₀ of 6-20. Periplasmic lysates were buffered with 20 mM Tris-Cl (pH 8.5) and supplemented with 2 mM dithiothreitol to prevent oxidation of the Cys residues. PA₈₃ was purified by anion exchange chromatography (Q Sepharose) with a 0M-250 mM NaCl gradient using Buffers A (20 mM Tris-Cl, 1 mM CaCl₂, pH 8.5) and B (A + 1 M NaCl). Hexahistidine tagged LF_N was purified from cell pellets lysed by French press and sonication using Ni²⁺-affinity chromatography.(24) Pure fractions, as determined by SDS-PAGE, were concentrated and stored at -80°C. Protein concentrations were determined by absorbance spectrophotometry (ϵ_{280} (PA₈₃) 75,670 M⁻¹cm⁻¹ and (LF_N) 18,040 M⁻¹cm⁻¹).

Preparation of nPA₈₃ and [PA₆₃]₇

Nicked PA₈₃ (nPA₈₃) was prepared using TPCK-treated trypsin (Sigma) by incubating PA₈₃ (1-2 mg/ml) in Buffer C (A+ 150 mM NaCl) for 35 min at room temperature at a 1:1000 w/w ratio trypsin:PA₈₃ followed by addition of 1:100 w/w ratio of soybean trypsin inhibitor (Sigma) to prevent further proteolysis. This procedure yielded a >90% nicking efficiency. [PA₆₃]₇ was prepared at >95% purity using anion exchange chromatography. Briefly, nPA₈₃ was loaded onto the column (Q sepharose) followed by elution using a 0M-500 mM NaCl gradient with Buffers A and B. Separation of PA₂₀ from PA₆₃ was followed by spontaneous oligomerization of PA₆₃ on column. Pure fractions, as determined by SDS-PAGE were concentrated and stored at -80°C. [PA₆₃]₇ concentrations were determined by absorbance spectrophotometry (ϵ_{280} 347,480 M⁻¹cm⁻¹).

Fluorescent labeling of proteins

Proteins with single Cys mutations were labeled with Alexa fluor 488 C₅ maleimide, Alexa fluor 546 C₅ maleimide, or Alexa fluor 594 C₅ maleimide (Invitrogen) using manufacturer's procedures. Protein solutions were exchanged back into Buffer C by gel filtration. The protein conjugates' dye-to-protein ratios were 0.9-1.2 as determined by absorbance spectrophotometry. Dye-labeled proteins were stored at -80°C.

Measurements of k_d for $[\text{PA}_{63}]_7$

$[\text{PA}_{63}]_7$ was purified from a 1:1 mixture of nPA₈₃ K563C labeled with Alexa fluor 488 (nPA₈₃ K563C*488) and nPA₈₃ K563C labeled with Alexa fluor 546 or Alexa fluor 594 (nPA₈₃ K563C*564, nPA₈₃ K563C*594). The purified $[\text{PA}_{63}]_7$ contained both donor and acceptor labels ($[\text{PA}_{63}]_7$ *FRET). For rapid dilution experiments, $[\text{PA}_{63}]_7$ *FRET was diluted from 10 μM to 10 nM in Buffer D (50 mM Tris-Cl pH 8.5, 150 mM NaCl, 0.5 mg/ml BSA, and 0.02% NaN₃). In competition experiments, 10 nM labeled $[\text{PA}_{63}]_7$ *FRET and 250 nM unlabeled $[\text{PA}_{63}]_7$ were combined in Buffer D. In both experiments, fluorescence emission ratio (570 / 525 \pm 10 nm emission, 488 \pm 4 nm excitation from Xe arc lamp) was monitored using a spectrofluorometer over 16 days. Solution temperatures were $\sim 20^\circ\text{C}$ throughout the entire experiment. Data were fit to a single exponential decay model using non-linear least squares to recover the observed dissociation constant, k_d .

Additional experiments contained the protein denaturant Guanidine HCl (GdmHCl). Varying amounts of GdmHCl (0 to 0.6 M, as determined by refractive index) were added to 10 nM heptamer solutions in Buffer D. Fluorescence emission ratio (615 / 525 \pm 16 nm emission, 488 nm excitation from Ar⁺ laser) was monitored using a spectrofluorometer over 10 days. All solutions were maintained at 20°C throughout the entire experiment. Data were fit to a single exponential decay model using non-linear least squares to recover the observed dissociation constant, k_d .

Since measurements of k_d required long incubation periods at room temperature, all solutions were sterile filtered and supplemented with 0.02% NaN₃ to inhibit microbial growth that could lead to degradation of the non-covalent protein complexes. SDS-PAGE was used to monitor PA₆₃ proteolysis over a 16 day period. Briefly, a 20 nM solution of $[(\text{PA}_{63})_7]$ *488 in Buffer D was incubated at either $\sim 20^\circ\text{C}$ or at -20°C for 16 days. At the conclusion of this period, the room temperature incubated sample was again frozen at -20°C until electrophoresis. These solutions were loaded onto a 4-20% polyacrylamide gel. The gel was washed in ddH₂O and observed on a UV transilluminator to excite the Alexa dye without fixing or staining which quenches the fluorescence.

Measurements of k_d for $[(\text{PA}_{63})_2\cdot\text{LF}_N]$ ternary complex

$[(\text{PA}_{63})_2\cdot\text{LF}_N]$ was prepared by incubating a 1:1:1 mixture of nPA₈₃ D512K K563C labeled with Alexa fluor 488 (nPA₈₃ D512K K563C*488), nPA₈₃ K199E R468A R470D K563C labeled with Alexa 546 (nPA₈₃ K199E R468A R470D K563C*546), and LF_N at 5 μM (total nPA₈₃ was 10 μM). All $[(\text{PA}_{63})_2\cdot\text{LF}_N]$ contained both donor and acceptor labels ($[(\text{PA}_{63})_2\cdot\text{LF}_N]$ *FRET). $[(\text{PA}_{63})_2\cdot\text{LF}_N]$ *FRET was diluted from 5 μM to 10 nM in Buffer D and the fluorescence emission ratio measured over time (570 / 525 \pm 16 nm emission, 488 nm Ar⁺ laser line excitation). The kinetic transients were fit to a single exponential decay model (Eqn 1) using non-linear least squares analysis to recover the observed rate constant, k_d , where the amplitude at time 0, A , decays with respect to time, t . The offset, C , is the final value reached when the system achieves equilibrium.

$$A(t) = Ae^{-kt} + C$$

Eqn 1

Measurement of k_a for $[(\text{PA}_{63})_7\cdot(\text{LF}_N)_3]$ and $[(\text{PA}_{63})_2\cdot\text{LF}_N]$

Association rates of $[(\text{PA}_{63})_7\cdot(\text{LF}_N)_3]$ were determined by mixing 1:1 ratios of nPA₈₃ K563C*488 and nPA₈₃ K563C*594 at concentrations between 1.2 and 120 nM, followed by addition of variable concentrations of LF_N (1×10^{-9} to 1×10^{-5} M) to prompt oligomerization in solution. Oligomerization was monitored by measuring sensitized emission (FRET) via a fluorescence emission ratio of intensity (615 nm / 525 nm). Similarly, association rates were determined for $[(\text{PA}_{63})_2\cdot\text{LF}_N]$ by mixing 1:1 ratios of nPA₈₃ D512K K563C*488 and nPA₈₃

K199E R468A R470D K563C*546 at concentrations between 1.2 and 120 nM, followed by addition of variable concentrations of LF_N (1×10^{-9} to 1×10^{-5} M) to prompt complex formation in solution. [(PA₆₃)₂-LF_N] formation was monitored by measuring the sensitized emission (FRET) via a fluorescence emission ratio (570 nm / 525 nm ± 16 nm). All data were fit using non-linear least squares analysis to a second order exponential decay model (Eqn 2), where the amplitude at time 0, *A*, decays with respect to time, *t*. (22) The offset, *C*, is the final value reached when the system achieves equilibrium.

$$A(t) = A / (1 + k_d t) + C \quad \text{Eqn 2}$$

Results

Dissociation kinetics of [PA₆₃]₇

Equal parts of two differently labeled PA₈₃ mutants, PA₈₃ K563C*488 and PA₈₃ K563C*546 (or PA₈₃ K563C*594), were combined, proteolytically activated with trypsin, and loaded onto an anion exchange column. FRET-labeled [PA₆₃]₇ ([PA₆₃]₇*FRET) was isolated by gradient elution and found to show significant FRET (Figure 1, dashed line) compared to the control of an unnicked equimolar mixture of donor and acceptor labeled PA₈₃ (Figure 1, solid line). The dissociation rate constant (*k_d*) of [PA₆₃]₇*FRET was measured after dilution of the complex from 10 μM to 10 nM. The decline in FRET as a function of time was monitored over 16 days, with the fluorescence emission ratio (570 nm / 525 nm, 488 nm excitation) serving as the metric. We fit the observed kinetic transients (Figure 2A) to a single exponential decay function (Eqn. 1) and calculated the *k_d* to be $1 \pm 1 \times 10^{-6} \text{ s}^{-1}$, corresponding to a *t*_{1/2} of 7 days. When a 25-fold excess (250 nM) of unlabeled [PA₆₃]₇ was present, the results were indistinguishable from those obtained in the absence of the unlabeled protein (Figure 2B).

We also performed dilution experiments in the presence of low concentrations of GdmHCl to check the accuracy of the measured value of *k_d*. [PA₆₃]₇*FRET was diluted from 10 μM to 10 nM in solutions of 0.2 to 0.6 mM GdmHCl, and the loss of FRET due to complex dissociation was monitored, as before. The low concentrations of GdmHCl employed accelerated the rate of dissociation (the majority of signal change occurred during the first 2-4 days), while avoiding protein unfolding. The observed kinetic transients were fitted to a single exponential decay function to recover *k_d* and the rate constants were plotted vs. GdmHCl concentration (Figure 2C). Extrapolating kinetics data to 0 M GdmHCl yielded a value of *k_d* for [PA₆₃]₇ identical to that determined in the absence of GdmHCl. This agreement suggested that proteolysis was not likely to contribute significantly to the observed kinetics. Additional support for this came from SDS-PAGE (Figure 3) of [(PA₆₃)₇]*488 that had been incubated at room temperature for 16 days and a control which was stored at -20°C during the experiment. Figure 3 shows showed that there was little change of intact PA₆₃ following the long incubation. To minimize proteolysis, all solutions had been sterile filtered and supplemented with 0.02% NaN₃ to inhibit microbial growth.

To obtain a baseline for the observed changes in FRET, we mixed equal parts of unnicked PA₈₃ K563C*488 and PA₈₃ K563C*546 and monitored the fluorescence emission ratio over time. Consistent with the inability of PA₈₃ to self-associate, the fluorescence emission ratio was in the limit of no detectable [PA₆₃]₇ in solution and was stable over the time course of the experiment. Also, changes in fluorescence emission intensity were minimal (< 10%) over 400 hours, indicating that losses to adsorption to cuvette walls were negligible (Figure 2D inset). Small changes in total emission intensity over time did not affect kinetics values, as they were calculated from the ratio of fluorescence emission intensities.

Association kinetics of $[\text{PA}_{63}]_7\text{-LF}_N$

Because the equilibrium of PA_{63} oligomerization is so strongly in favor of $[\text{PA}_{63}]_7$, neither monomeric PA_{63} nor sub-heptameric oligomers of the wild-type fragment have ever been isolated. Hence it has not been possible to study the kinetics of PA_{63} association in a homogeneous system (20). Nonetheless, LF or EF, or their N-terminal PA_{63} -binding domains promote oligomerization of PA_{63} , starting with nPA_{83} , presumably by displacing PA_{20} and bridging associated PA_{63} subunits. Therefore we chose to examine the oligomerization process in the presence of LF_N . Various concentrations of LF_N (10 nM to 10 μM) were added to a mixture of equal parts of nPA_{83} K563C*488 and nPA_{83} K563C*594 (12-150 nM), and increases in FRET were monitored over time by the 615 nm / 525 nm fluorescence emission ratio. Recorded kinetic transients were fitted to Eqn 2 (second-order kinetics) to recover k_a (Figure 4A), and the observed rates were plotted vs. the concentration of LF_N (Figure 4C). Since LF_N has been shown to promote PA_{63} oligomerization in solution, we expected that raising the LF_N concentration would increase the observed association rate, and indeed this was observed at relatively low concentrations of LF_N . However, as the LF_N concentration was raised above 50 nM, the observed oligomerization rate declined. In contrast, significant differences in observed rates were not observed for a ten-fold difference in nPA_{83} concentration, suggesting that the rate determining step in association does not directly involve PA_{63} (Figure 4C).

Association kinetics of $[\text{PA}_{63}]_2\text{-LF}_N$

To investigate the basis of this phenomenon, we studied the assembly of a ternary complex containing an equimolar ratio of LF_N and each of two non-oligomerizing, mutant forms of PA_{63} . Mogridge et al. described D512K as a point mutation on one face of PA_{63} that blocked self-association of the protein; and a set of three mutations (K199E, R468A, and R470D) on the complementary oligomerization surface of the protein had the same effect (21). Neither the single mutant nor the triple mutant alone bound LF_N , but when the two were combined in the presence of LF_N , a ternary complex containing one equivalent of each of the three proteins was formed (21). In this complex, $[\text{PA}_{63}]_2\text{-LF}_N$, the single and triple mutant forms of PA_{63} presumably associate via their complementary, wild-type faces, with LF_N bound to them in the same way it binds to pairs of adjacent subunits within the prepore (21).

To create a FRET-based assay of PA_{63} self-association, we introduced the K563C mutation into both of the oligomerization-deficient mutants and labeled the single (now double) mutant with Alexa 488 and the triple (now quadruple) mutant with Alexa 546. Each preparation was nicked with trypsin, and equal parts of nPA_{83} D512K*488 and nPA_{83} K199E D468A R470D K563C*546 were mixed at concentrations between 12 and 120 nM. LF_N was then added at various concentrations to initiate dimerization of the PA_{63} mutants. The fluorescence emission ratio was recorded as a function of time, and the data were fit to Eqn 2 to recover k_a . As shown in Figure 4D, the association rate declined as the LF_N concentration was raised, as seen in higher LF_N concentration ranges with wild-type nPA_{83} .

Dissociation kinetics of double-labeled $[(\text{PA}_{63})_2\text{-LF}_N]$

$[(\text{PA}_{63})_2\text{-LF}_N]^*\text{FRET}$ was diluted from 5 μM to 10 nM, and the fluorescence emission ratio (570 nm/520 nm emission, 488 nm excitation) was monitored. The kinetics transients were fit using a single exponential decay model to recover k_d (Figure 5). The measured k_d for the $[(\text{PA}_{63})_2\text{-LF}_N]^*\text{FRET}$ was $1.3 \pm 0.01 \times 10^{-2} \text{ s}^{-1}$ ($t_{1/2} = 53 \text{ s}$), showing that dissociation was 10,000-fold faster than with the $[\text{PA}_{63}]_7^*\text{FRET}$.

Discussion

We have measured the kinetics of $[\text{PA}_{63}]_7$ dissociation and association in the presence of LF_N using FRET between donor and acceptor fluorophores introduced into PA_{83} . Since PA_{83} contains no Cys residues, we used mixtures of Cys mutants (K563C) labeled with either Alexa fluor 488 (donor), Alexa fluor 546 (acceptor), or Alexa fluor 594 (acceptor) in our experiments. The K563C mutation has been shown not to affect activity of PA_{83} (25). From the $[\text{PA}_{63}]_7$ crystallographic structure, the distance between adjacent K563 residues is approximately 59 Å. Since the Förster distance for these two dyes was estimated to be 55 Å, FRET is an effective reporter of PA_{63} oligomerization (22). We also made kinetic measurements of $\text{PA}_{63}\cdot\text{PA}_{63}$ dissociation and association using the $[(\text{PA}_{63})_2\cdot\text{LF}_N]$ construct (PA_{83} D512K and PA_{83} K199E R468A R470D) in the presence of LF_N . We expect that the distance between the adjacent K563 residues in the $[(\text{PA}_{63})_2\cdot\text{LF}_N]$ complex is similar to that in the $[\text{PA}_{63}]_7$ complex, such that FRET effectively monitors its association state. Since the FRET labels were on PA_{63} and not LF_N , we only monitored the PA_{63} dissociation.

Disassembly of $[\text{PA}_{63}]_7$ was very slow, with a k_d of $\sim 10^{-6} \text{ s}^{-1}$. A 10,000-fold faster dissociation rate, $\sim 10^{-2} \text{ s}^{-1}$, was observed for the PA_{63} dimer bridged by LF_N . The markedly slower dissociation rate of the heptamer presumably results from cooperative interactions of each subunit with neighboring subunits in the homoheptameric ring. We surmise that the slower dissociation rate of the PA_{63} heptamer translates into a substantially increased thermodynamic stability of the heptamer relative to individual PA_{63} dimer interactions.

The substantial stability of $[\text{PA}_{63}]_7$ is of more than theoretical interest, as it impinges on the ability of dominant-negative mutant forms of PA to serve as potent antitoxins, and hence as potential therapeutics for treating anthrax infections. Certain point mutations in the pore forming domain (domain 2) of PA block the ability of the $[\text{PA}_{63}]_7$ prepore to undergo the conformational transition to the pore (e.g., D425A), or else block the translocation activity of the pore, once it is formed (e.g., F427A) (26,27). A subset of the mutant forms of PA were found to be dominant-negatives (DN), such that co-oligomerization of low stoichiometric ratios of mutant to wild-type PA yielded inactive prepores or pores. This DN phenotype depends not only on the co-oligomerization of mutant PA_{63} with wild-type being stochastic, but also on the stability of the prepore, which precludes subunit-subunit exchange that might allow wild-type PA_{63} subunits to recombine to form uniformly wild-type heptamers.

Determination of association rates for PA_{63} is complicated by the intrinsic affinity of PA_{20} and its ability, when bound, to block self-association of PA_{63} . In cells, when membrane-bound $n\text{PA}_{83}$ molecules dissociate to PA_{20} and PA_{63} , the PA_{63} is constrained to the two-dimensional membrane surface, while the PA_{20} is free to dissociate into extracellular solution. As a result, the concentration of PA_{63} in the membrane is effectively elevated relative to PA_{20} , and this may facilitate PA_{63} oligomerization. The picture is somewhat different in solution, where, except at sub-micromolar concentrations, PA_{20} and PA_{63} remain associated via non-covalent interactions. In this case, oligomeric PA_{63} does not exist at any measurable concentrations without the addition of at least one of the ancillary proteins: LF, LF_N (N-terminal binding domain of LF), or EF (20). Our working hypothesis is that in solution, LF (or LF_N) prompts oligomerization by binding to PA_{63} and preventing it from associating with PA_{20} .

We made association rate measurements of $[(\text{PA}_{63})_7\cdot(\text{LF}_N)_3]$ and $[(\text{PA}_{63})_2\cdot\text{LF}_N]$ using donor and acceptor labeled mixtures of $n\text{PA}_{83}$ with variable LF_N concentrations. Unlike the measured k_d 's, only small differences (factor of 2) were observed between the k_a 's for $[(\text{PA}_{63})_7\cdot(\text{LF}_N)_3]$ and $[(\text{PA}_{63})_2\cdot\text{LF}_N]$. Notably, the observed association rate constants decreased with increasing concentrations of LF_N . This apparently indicates that LF_N inhibits PA_{63} oligomerization at higher concentrations. The measured heptamer k_a initially increased with

LF_N as expected, but then decreased at higher LF_N concentrations. The dimer k_a consistently decreased with increasing LF_N concentration. Initially we hypothesized that high LF_N concentrations selectively populated PA₆₃ dimers bridged by LF_N, thereby reducing free PA₆₃ concentration. Because an unliganded PA₆₃ subunit is necessary to complete the [PA₆₃]₇ ring, this could effectively inhibit assembly of toxic complexes. However, subsequent measurements of [(PA₆₃)₂·LF_N] k_a also demonstrated a negative dependence on LF_N concentration, indicating the existence of another or competing source of the observed rate decreases.

Consistent with these data, we propose the following mechanism (Scheme 1), to account for the observed rate behavior. In this case, oligomerization would proceed through formation of PA₆₃ dimers bridged by LF_N (similar to the [(PA₆₃)₂·LF_N] complex), and PA₆₃·LF_N complex formation would be a competing pathway that inhibits heptamer formation. At high LF_N concentrations, closing the heptamer ring structure could not proceed without dissociation of LF_N from PA₆₃ and is not favored. In addition, since the observed k_a is independent of nPA₈₃ concentration, binding of PA₆₃ to LF_N must occur only after a rate determining unimolecular rearrangement of LF_N.

Since our proposed mechanism predicts the existence of a [PA₆₃·LF_N] species, we attempted to detect this species using FRET. We titrated nPA₈₃ D512K K563C*488 with LF_N E126C labeled with Alexa fluor 594 (LF_N E126C*594) and also measured dissociation of the putative [LF_N E126C*594·PA₆₃ D512K K563C*488] by rapid dilution of the complex while measuring the fluorescence emission ratio (data not shown). Although significant populations of FRET complex were not detected, these data do not rule out the existence of a [PA₆₃·LF_N] complex. Supposing the [PA₆₃·LF_N] species had a K_d of 10 μM-1 mM, accurate measurement of K_d would require such high concentrations of protein to make FRET observation unlikely. In addition, a low affinity complex may have a very short individual lifetime and relatively fast off-rates, and would not be detectable using a slow kinetic approach. The complex could also be oriented such that the donor-acceptor distance approaches or exceeds twice the Förster distance, making complex association or dissociation invisible to FRET measurement. Hence, while we are currently unable to observe this interaction, we assert that the most likely mechanism for LF_N-mediated oligomerization of PA₆₃ is one in which both the [PA₆₃·LF_N] and [(PA₆₃)₂·LF_N] intermediates may precede or compete with [PA₆₃]₇. A LF_N·LF_N interaction could contribute to the observed negative rate dependence, but no precedent exists for such an interaction. Thus we propose that the overpopulation of putative [(PA₆₃)₂·LF_N] and [PA₆₃·LF_N] intermediates may be the source of the observed LF_N-dependent slowing of PA₆₃ oligomerization.

Acknowledgments

K.A.C. thanks P.C. Ackroyd for help preparing and evaluating this manuscript. We also thank R.-A.L. Pimental for her help with protein production and purification. One of us (RJC) holds equity in PharmAthene, Inc.

Abbreviations

PA, Protective Antigen
 [PA₆₃]₇, Protective Antigen homoheptamer
 LF, Lethal Factor
 EF, Edema Factor
 LF_N, Lethal Factor N-terminal domain
 PA₈₃, 83 kD full length Protective Antigen
 nPA₈₃, nicked Protective Antigen (83 kD)
 PA₂₀, 20 kD N-terminal fragment of Protective Antigen
 PA₆₃, 63 kD C-terminal fragment of Protective Antigen

EF_N, Edema Factor N-terminal domain
 FRET, Förster resonance energy transfer
 cAMP, cyclic adenosine monophosphate
 TPCK, *t*-1-Tosylamide-2-phenylethylchloromethyl ketone
 IPTG, Isopropyl β-D-thiogalactoside
 DTT, Dithiothreitol
 nPA₈₃ K563C*488, nicked Protective Antigen, residue 563 labeled with Alexa fluor 488 C₅ maleimide
 nPA₈₃ K563C*546, nicked Protective Antigen, residue 563 labeled with Alexa fluor 546 C₅ maleimide
 nPA₈₃ K563C*594, nicked Protective Antigen, residue 563 labeled with Alexa fluor 594 C₅ maleimide
 [PA₆₃]₇*FRET, Protective Antigen homoheptamer containing a 1:1 ratio of Protective Antigen where residue 563 is labeled with Alexa fluor 488 plus either Alexa fluor 546 or 594
 GdmHCl, Guanidine HCl
 [PA₆₃]₂·LF_N, Ternary complex of 1:1 ratio of Protective Antigen non-oligomerizing mutants (D512K and K199E R468A R470D) plus the N-terminal domain of Lethal Factor
 nPA₈₃ D512K K563C*488, non-oligomerizing Protective Antigen mutant, residue 563 labeled with Alexa fluor 488
 nPA₈₃ K199E R468A R470D K563C*546, non-oligomerizing Protective Antigen mutant, residue 563 labeled with Alexa fluor 546
 [PA₆₃]₂·LF_N*FRET, Protective Antigen ternary complex with the Lethal Factor N-terminal domain labeled at Protective Antigen residue 563 with Alexa fluor 488 and 546
 [PA₆₃]₇·(LF_N)₃, Protective Antigen homoheptamer saturated with N-terminal domain of Lethal Factor ligands
 LF_N E126C*594, N-terminal domain of Lethal Factor, residue 126 labeled with Alexa fluor 594 C₅ maleimide
 PA₆₃·LF_N, putative 1:1 complex with Protective Antigen and LF_N
 k_d , dissociation rate constant
 k_a , association rate constant
 DN, Protective Antigen mutant that displays dominant negative phenotype
 K_d , equilibrium dissociation constant
 Cys, cysteine

References

- (1). Smith H. Discovery of the anthrax toxin: the beginning of in vivo studies on pathogenic bacteria. *Trends Microbiol* 2000;8:199–200. [PubMed: 10785632]
- (2). Smith H, Stoner HB. Anthrax toxic complex. *Fed. Proc* 1967;26:1554–1557. [PubMed: 6051334]
- (3). Duesbery NS, Resau J, Webb CP, Koochekpour S, Koo HM, Leppla SH, Vande Woude GF. Suppression of ras-mediated transformation and inhibition of tumor growth and angiogenesis by anthrax lethal factor, a proteolytic inhibitor of multiple MEK pathways. *Proc. Natl. Acad. Sci. U. S. A* 2001;98:4089–94. [PubMed: 11259649]
- (4). Pellizzari R, Guidi-Rontani C, Vitale G, Mock M, Montecucco C. Lethal factor of *Bacillus anthracis* cleaves the N-terminus of MAPKKs: analysis of the intracellular consequences in macrophages. *Int. J. Med. Microbiol* 2000;290:421–7. [PubMed: 11111921]
- (5). Vitale G, Pellizzari R, Recchi C, Napolitani G, Mock M, Montecucco C. Anthrax lethal factor cleaves the N-terminus of MAPKKs and induces tyrosine/threonine phosphorylation of MAPKs in cultured macrophages. *Biochem. Biophys. Res. Commun* 1998;248:706–11. [PubMed: 9703991]
- (6). Bradley KA, Mogridge J, Mourez M, Collier RJ, Young JA. Identification of the cellular receptor for anthrax toxin. *Nature* 2001;414:225–9. [PubMed: 11700562]
- (7). Scobie HM, Rainey GJ, Bradley KA, Young JA. Human capillary morphogenesis protein 2 functions as an anthrax toxin receptor. *Proc. Natl. Acad. Sci. U. S. A* 2003;100:5170–4. [PubMed: 12700348]

- (8). Klimpel KR, Molloy SS, Thomas G, Leppla SH. Anthrax toxin protective antigen is activated by a cell surface protease with the sequence specificity and catalytic properties of furin. *Proc. Natl. Acad. Sci. U. S. A* 1992;89:10277–81. [PubMed: 1438214]
- (9). Molloy SS, Bresnahan PA, Leppla SH, Klimpel KR, Thomas G. Human furin is a calcium-dependent serine endoprotease that recognizes the sequence Arg-X-X-Arg and efficiently cleaves anthrax toxin protective antigen. *J. Biol. Chem* 1992;267:16396–402. [PubMed: 1644824]
- (10). Petosa C, Collier RJ, Klimpel KR, Leppla SH, Liddington RC. Crystal structure of the anthrax toxin protective antigen. *Nature* 1997;385:833–8. [PubMed: 9039918]
- (11). Elliott JL, Mogridge J, Collier RJ. A quantitative study of the interactions of *Bacillus anthracis* edema factor and lethal factor with activated protective antigen. *Biochemistry* 2000;39:6706–13. [PubMed: 10828989]
- (12). Mogridge J, Cunningham K, Collier RJ. Stoichiometry of anthrax toxin complexes. *Biochemistry* 2002;41:1079–82. [PubMed: 11790132]
- (13). Abrami L, Liu S, Cosson P, Leppla SH, van der Goot FG. Anthrax toxin triggers endocytosis of its receptor via a lipid raft-mediated clathrin-dependent process. *J. Cell Biol* 2003;160:321–8. [PubMed: 12551953]
- (14). Blaustein RO, Koehler TM, Collier RJ, Finkelstein A. Anthrax toxin: channel-forming activity of protective antigen in planar phospholipid bilayers. *Proc. Natl. Acad. Sci. U. S. A* 1989;86:2209–13. [PubMed: 2467303]
- (15). Milne JC, Collier RJ. pH-dependent permeabilization of the plasma membrane of mammalian cells by anthrax protective antigen. *Mol. Microbiol* 1993;10:647–53. [PubMed: 7968541]
- (16). Krantz BA, Trivedi AD, Cunningham K, Christensen KA, Collier RJ. Acid-induced Unfolding of the Amino-terminal Domains of the Lethal and Edema Factors of Anthrax Toxin 2004;344:739.
- (17). Zhang S, Finkelstein A, Collier RJ. Evidence that translocation of anthrax toxin's lethal factor is initiated by entry of its N terminus into the protective antigen channel. *PNAS* 2004;101:16756–16761. [PubMed: 15548616]
- (18). Zhang S, Udho E, Wu Z, Collier RJ, Finkelstein A. Protein Translocation through Anthrax Toxin Channels Formed in Planar Lipid Bilayers. *Biophys. J* 2004;87:3842–3849. [PubMed: 15377524]
- (19). O'Brien J, Friedlander A, Dreier T, Ezzell J, Leppla S. Effects of anthrax toxin components on human neutrophils. *Infect. Immun* 1985;47:306–10. [PubMed: 3917427]
- (20). Christensen KA, Krantz BA, Melnyk RA, Collier RJ. Interaction of the 20 kD and 63 kD fragments of the anthrax toxin protective antigen: kinetics and thermodynamics. *Biochemistry* 2005;44:1047–1053. [PubMed: 15654761]
- (21). Mogridge J, Cunningham K, Lacy DB, Mourez M, Collier RJ. The lethal and edema factors of anthrax toxin bind only to oligomeric forms of the protective antigen. *PNAS* 2002;99:7045–7048. [PubMed: 11997437]
- (22). Wigelsworth DJ, Krantz BA, Christensen KA, Lacy DB, Juris SJ, Collier RJ. Binding Stoichiometry and Kinetics of the Interaction of a Human Anthrax Toxin Receptor, CMG2, with Protective Antigen. *J. Biol. Chem* 2004;279:23349–23356. [PubMed: 15044490]
- (23). Bernard, A.; Payton, M. Production of recombinant proteins. John Wiley & Sons, Inc.; New York: 2004.
- (24). Wesche J, Elliott JL, Falnes PO, Olsnes S, Collier RJ. Characterization of membrane translocation by anthrax protective antigen. *Biochemistry* 1998;37:15737–15746. [PubMed: 9843379]
- (25). Mourez M, Yan M, Lacy DB, Dillon L, Bentsen L, Marpoe A, Maurin C, Hotze E, Wigelsworth D, Pimental RA, Ballard JD, Collier RJ, Tweten RK. Mapping dominant-negative mutations of anthrax protective antigen by scanning mutagenesis. *Proc. Natl. Acad. Sci. U. S. A* 2003;100:13803–8. [PubMed: 14623961]
- (26). Krantz BA, Melnyk RA, Zhang S, Juris SJ, Lacy DB, Wu Z, Finkelstein A, Collier RJ. A Phenylalanine Clamp Catalyzes Protein Translocation Through the Anthrax Toxin Pore. *Science* 2005;309:777–781. [PubMed: 16051798]
- (27). Sellman BR, Mourez M, Collier RJ. Dominant-negative mutants of a toxin subunit: An approach to therapy of anthrax. *Science* 2001;292:695–697. [PubMed: 11326092]

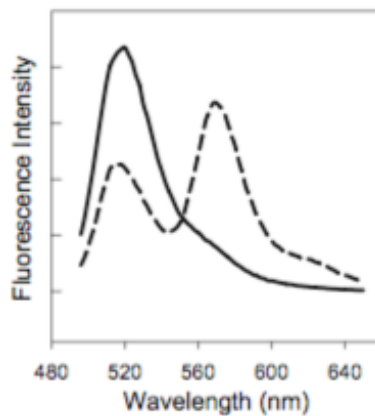


Figure 1.

Fluorescence emission spectra of $[PA_{63}]_7^*$ FRET and PA_{83} control. Fluorescence emission spectrum of $[PA_{63}]_7^*$ FRET (100 nM; dashed line) prepared by mixing equal parts nPA_{83} K563C*488 + nPA_{83} K563C*546 and allowing it to oligomerize on an anion exchange column followed by purification from PA_{83} and PA_{20} . Fluorescence emission spectrum of PA_{83} prepared by mixing equal parts PA_{83} K563C*488 and PA_{83} K563C*546 (100 nM total PA_{83} ; solid line). Excitation using 488 nm line of Ar^+ laser. Emission bandpass was 8 nm for both spectra.

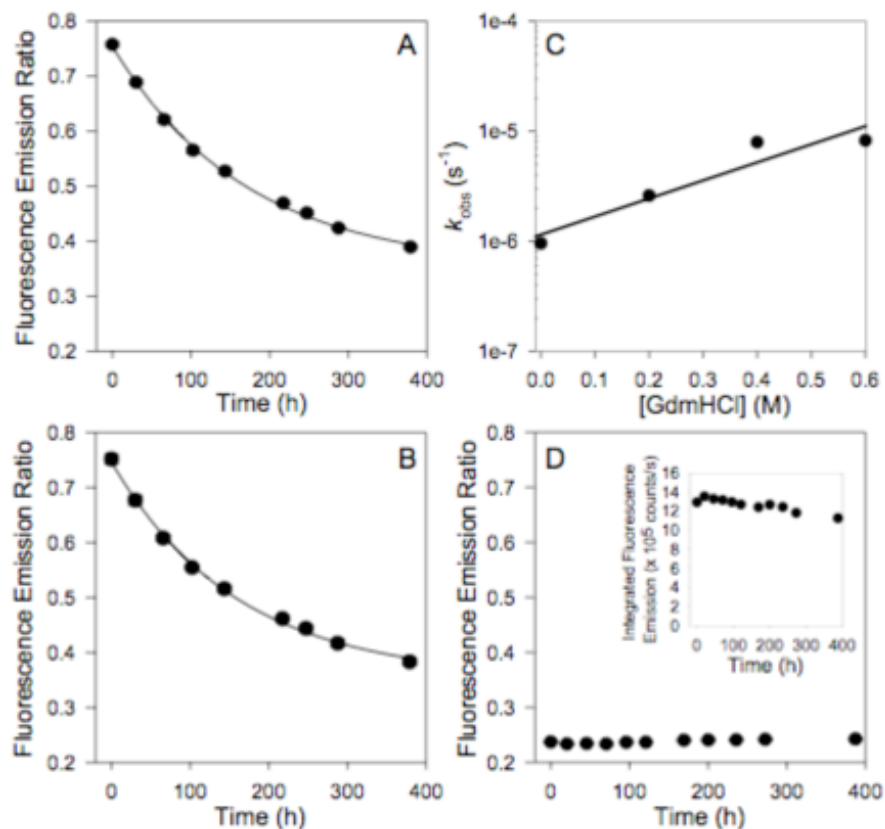


Figure 2. Dissociation of $[PA_{63}]_7^*$ FRET. A) Rapid dilution of $[PA_{63}]_7^*$ FRET from 10 μ M to a final concentration of 10 nM. Dissociation (decrease in FRET) was monitored over time by measuring the fluorescence emission ratio (570 nm/520 nm; 488 nm excitation). Error bars represent 95% c.l. B) Competition of 10 nM $[PA_{63}]_7^*$ FRET with 200 nM unlabeled $[PA_{63}]_7$. Dissociation (decrease in FRET) was monitored over time by measuring the fluorescence emission ratio (570 nm / 520 nm; 488 nm excitation). Error bars represent 95% c.l. C) Plot of recovered k_d vs. $[GdmHCl]$ for the rapid dilution of $[PA_{63}]_7^*$ FRET with low concentrations of GdmHCl (0 - 0.6 M) added to accelerate the observed dissociation rate. D) Controls: The fluorescence emission ratio of an equimolar mixture of PA_{83} K563C*488 and PA_{83} K563C*546 (unnicked) was monitored over time. The inset shows the integrated intensity over the course of an experiment. Error bars for both controls represent the 95% c.l. All experimental kinetic transients were fitted to a single-exponential decay model to recover k_d . All experiments were conducted at 20°C.

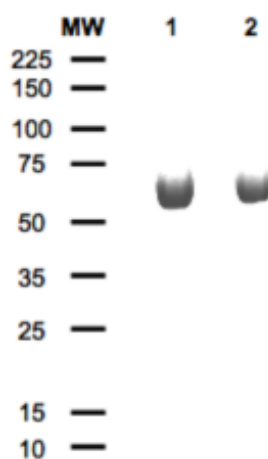


Figure 3. SDS-PAGE of [(PA₆₃)₇]*488 incubated in Buffer D for 16 days with MW markers indicated in kDa at left: room temperature incubation (lane 1); -20°C storage (lane 2).

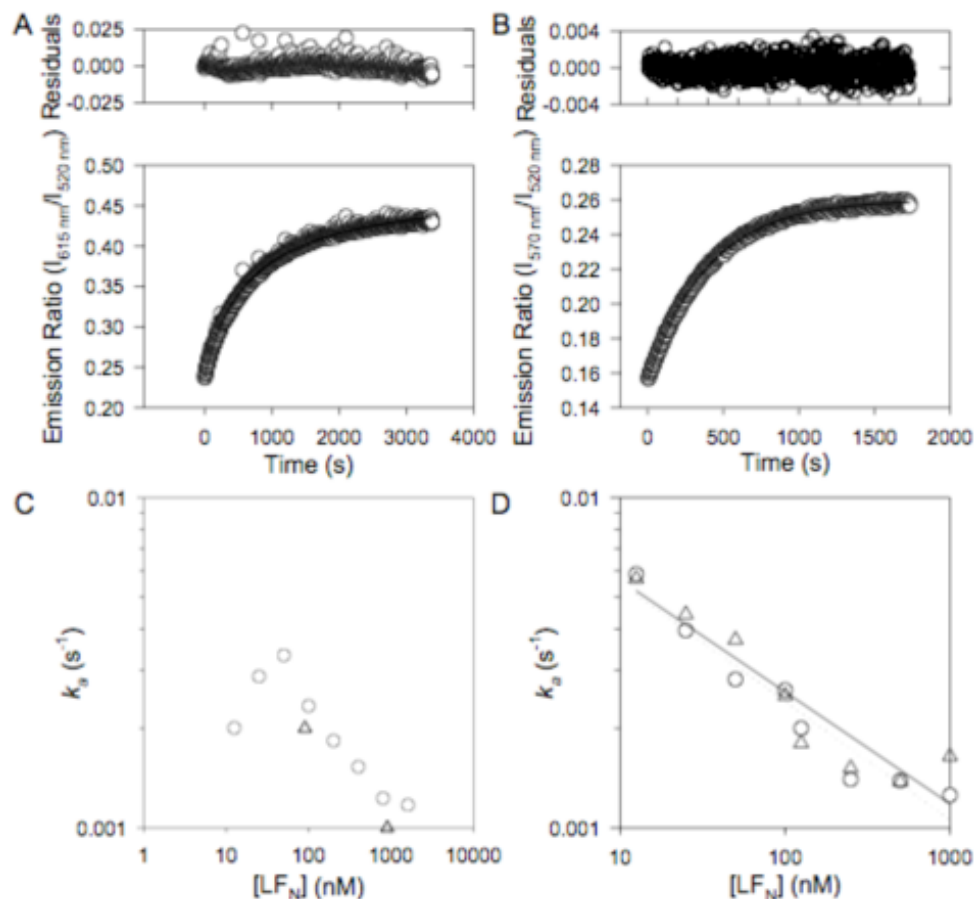


Figure 4. Determination of the association rates for $[PA_{63}]_7$ *FRET and $[(PA_{63})_2 \cdot LF_N]$ *FRET. A) Representative data for oligomerization of PA_{63} in the presence of LF_N . An equimolar mixture of nPA_{83} K563C*488 and nPA_{83} K563C*594 (12 nM total nPA_{83}) where LF_N (100 nM) was added at $t = 0$. The increase in FRET due to PA_{63} association was monitored by fluorescence emission ratio. Data fit to a second-order model (Eqn 2). Residuals are shown in the upper plot. B) Representative data for the dimerization of non-oligomerizing PA_{83} mutants in the presence of LF_N . An equimolar mixture of nPA_{83} D512K K563C*488 and nPA_{83} K199E R468A R470D K563C*546 (12 nM nPA_{83} total) where LF_N (100 nM) was added at $t = 0$. The increase in FRET due to the association of PA_{63} was monitored by fluorescence emission ratio. Data fit to a second-order model. Residuals are shown in the upper plot. C) Graph of observed rate constant (k_a) for PA_{63} association vs. concentration of LF_N . Circles represent data for 12 nM nPA_{83} ; triangles represent data for 150 nM nPA_{83} . D) Graph of observed rate constant (k_a) for PA_{63} dimerization of non-oligomerizing PA_{83} mutants. Circles represent data for 12 nM nPA_{83} ; triangles represent data for 120 nM nPA_{83} . Data are fit by linear regression (solid line, 12 nM nPA_{83} ; dotted line, 120 nM nPA_{83}).

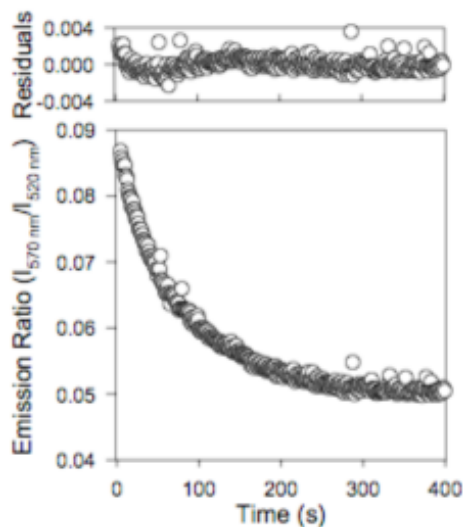
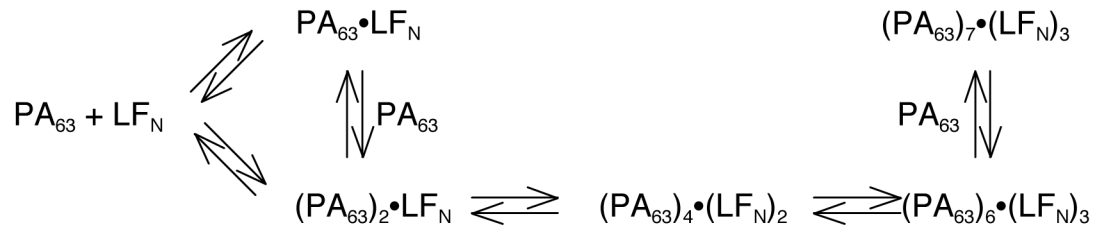
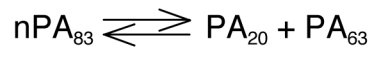


Figure 5. Dissociation of $[(PA_{63})_2-LFN]$. $[(PA_{63})_2-LFN]^*FRET$ was rapidly diluted from $5 \mu M$ to a final concentration of $10 nM$. A decrease in FRET was monitored over time by measuring the fluorescence emission ratio (lower plot; $570 nm / 520 nm$; $488 nm$ excitation). All data were fitted to a single-exponential decay model to recover k_d . Residuals were plotted (upper plot). Kinetic transients were acquired at $20^\circ C$.



Scheme 1.
Oligomerization of PA_{63} in the presence of LF_N .