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The Protective Antigen Component of Anthrax Toxin Forms Functional Octameric Complexes

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Abstract

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, PA 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.

Keywords

Anthrax toxin; cell-surface assembly; translocation; lethal toxin; oligomerization

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Introduction

Anthrax toxin¹ (Atx) is a binary, A_2B toxin, comprised of three nontoxic proteins that are secreted by *Bacillus anthracis* and combine on eukaryotic host cell surfaces to make noncovalent, toxic complexes (Fig. S1). Protective antigen (PA) is the 83-kDa, cell-binding, *B* component, which ultimately forms a translocase channel for the two ~90-kDa, enzymatically-active, *A* components—lethal factor (LF) and edema factor (EF). Following secretion, PA binds to the host cell via one of two known Atx receptors, ATR1 (ref. ²) and ATR2 (ref. ³), and is then cleaved by a furin-type protease to make the proteolytically-activated form, called $_n$ PA. The 63-kDa, receptor-bound portion of $_n$ PA then self-assembles into a ring-shaped homooligomer, or pre-channel, which has been shown to be heptameric.⁴⁻⁸ The pre-channel can bind LF or EF to make lethal or edema toxins, respectively. These toxin complexes are endocytosed and brought to an acidic compartment.⁹ Under acidic conditions, the PA pre-channel inserts into the membrane¹⁰ to form a translocase channel. LF and EF translocate through the channel¹¹ to enter the cytosol, where they catalyze reactions that disrupt the host cell (Fig. S1).

Analogous to the staphylococcal α -hemolysin pore,¹² PA assumes a similar mushroom-shaped architecture⁸ and β -barrel transmembrane motif.^{13,14} The PA channel's β -barrel is similarly narrow (~15-Å in diameter^{8,15}) but considerably longer (~100-Å-long) than the α -hemolysin. The narrow channel requires LF and EF to unfold prior to translocation.¹⁶⁻¹⁸ Acidic endosomal conditions serve two purposes: first, they destabilize LF and EF by acid denaturation;¹⁸ and second, they drive translocation via a proton gradient (Δ pH).¹¹ PA also contains a required ring of phenylalanine side chains, or ϕ clamp, which catalyzes translocation,¹⁹ exemplifying how the channel's structure is crucial to its translocase function.

Assembly is paramount to Atx function^{20,21} and its cellular internalization.²² ATR receptors are slowly internalized by the host cell, but PA-bound ATR can internalize rapidly once it dimerizes,²² making proper oligomerization a critical step in the internalization pathway. ATR is dimeric,^{23,24} further complicating the assembly mechanism, since PA oligomers are believed to be odd-numbered and heptameric. ATR2 and Atx were recently implicated as factors that help the *B. anthracis* bacterium escape the acidic phagolysosome following spore germination, suggesting Atx components may assemble in hostile environments as well.²⁵ Another potentially interesting extracellular assembly mechanism has become apparent in the investigation of animals infected with *B. anthracis*, where it has been shown that anthrax toxin accumulates in the blood of animals.²⁶ Specifically, LF is found alongside proteolytically-activated $_n$ PA,^{27,28} implying that the PA is potentiated for assembly. Relative to toxin produced in vitro, the toxin produced in vivo (i.e., isolated from the blood of animals suffering from anthrax) forms unique assemblies as evidenced by their unique resistance to antibody binding, and the in vivo derived toxin is more lethal.²⁹ We probe assembly in various cellular and extracellular contexts, using electrophysiology, electron microscopy, mass spectrometry, and crystallography, and we conclude that the activity of the toxin may be regulated through assembly, potentially affecting the degree of cytotoxicity throughout the stages of anthrax.

Results

PA can form two different channel sizes

PA channels were inserted into planar lipid bilayers following one of two different assembly methods outlined in Fig. 1A. A preoligomerized sample, called $_Q$ PA (trypsin-nicked PA assembled on Q-sepharose anion-exchange resin¹⁰) was applied to the bilayer, and discrete single-channel steps were observed (Fig. 1B). $_Q$ PA channels had a mean conductance of 95.5 pS ($n = 360$). Single-channel conductance values (γ) for wild type (WT) PA channels were reported to range from 85 to 110 pS.³⁰ We also observed that the $_Q$ PA sample contained two

discrete channel sizes: a prevalent, smaller one and a rarer, larger one. The overall γ -value distribution recorded from many individual membranes was broad (Fig. 1C).

To determine if the method of PA assembly affected the γ -value distribution, we studied trypsin-nicked PA ($_{\text{n}}\text{PA}$) samples assembled in the presence of LF's amino-terminal domain (LF_{N}), called $_{\text{n}}\text{PA}+\text{LF}_{\text{N}}$. Again, larger-conductance channels appeared among smaller-conductance channels in two discrete sizes, but the frequency of observing larger channels increased. The γ value for $_{\text{n}}\text{PA}+\text{LF}_{\text{N}}$ [98 pS ($n = 107$)] increased relative to that observed for $_{\text{Q}}\text{PA}$. Moreover, a lower-tailed Whitney-Mann test (WMT) shows that $_{\text{Q}}\text{PA}$ and $_{\text{n}}\text{PA}+\text{LF}_{\text{N}}$ distributions are unique from one another ($p > 0.95$). Finally, three separate $_{\text{Q}}\text{PA}$ samples contained a consistently lower mean conductance of ~ 95 pS relative to two other $_{\text{n}}\text{PA}+\text{LF}_{\text{N}}$ samples, which had a mean conductance of ~ 98 pS. Thus the method of assembly shifts the single-channel-conductance distribution (Fig. 1C).

Inherent variability in bilayer thickness, combined with the presence of another more-conducting conformation, likely contributes to the broad aggregate distributions (Fig. 1C). To mitigate this, we examined the discrete differences in channel conductance within each membrane by tabulating the set of all the pairwise differences (δ) in γ values per membrane, $\{\gamma_i - \gamma_j\}$, where $i \neq j$. A histogram of δ values recorded for the $_{\text{n}}\text{PA}+\text{LF}_{\text{N}}$ sample shows that two sizes of channel conductance are present, as the distribution fits best to a two-Gaussian distribution (Fig. 1D). Based upon our fit, these two populations of γ values differ from one another by $8 (\pm 2)$ pS, or about $\pm 10\%$, where the larger-sized conductance state represents $25 (\pm 6)\%$ of the population. This result led us to conclude that PA forms two discrete conductance states that may be differentially populated, depending upon the method of assembly.

We tested whether larger-conductance channels were a substate of less-conducting channels due to a conformational rearrangement. To test this possibility, we measured ~ 6 -minute-long recordings of single channels (Fig. 1E). While fluctuations to a more conducting substate are observed, these fluctuations are relatively small; and large-conductance channels (> 102 pS) did not interconvert to smaller ones (< 95 pS) during these and all other recordings. Therefore, either two unique channel sizes exist in PA samples or the timescale of the conformational rearrangements between the large- and small-conductance states is slow.

Electron microscopy reveals two PA oligomers

Under the assumption that the $\sim 10\%$ difference in conductance states may correspond to slow-timescale structural changes in the channel diameter,^{19,31} we examined the $_{\text{Q}}\text{PA}$ oligomers for structural heterogeneity by electron microscopy (EM). Reference-free analysis of $\sim 10^4$ negatively-stained particles identified two classes of ring-shaped oligomers—the heptamer and a novel octamer (Fig. 2A).

Mass spectrometry reveals Atx heterogeneity

To further establish the observed heterogeneity, we used nanoelectrospray ionization mass spectrometry (nanoESI-MS). We assembled WT $_{\text{n}}\text{PA}$ with WT LF_{N} and then analyzed the mixtures by nanoESI-MS (Fig. 3A). We identified two large molecular mass species, 537,082 (± 186) and 631,167 (± 217) Da, corresponding to the oligomers, $\text{PA}_7(\text{LF}_{\text{N}})_3$ and $\text{PA}_8(\text{LF}_{\text{N}})_4$, respectively, as well as two minor (and likely intermediate) complexes of 158,193 (± 38) and 315,395 (± 27) Da, corresponding to $\text{PA}_2\text{LF}_{\text{N}}$ and $\text{PA}_4(\text{LF}_{\text{N}})_2$, respectively (Table S1). The $\text{PA}_7(\text{LF}_{\text{N}})_3$ species was observed previously.⁶ Moreover, similar results were obtained when full-length LF and EF were used as ligands (data not shown). The $\text{PA}_8(\text{LF}_{\text{N}})_4$ complex is novel and not only corroborates the presence of the octameric PA species, but also demonstrates that an octamer can carry a payload of four LFs or EFs—one more than its heptameric counterpart.

Finally, the previously undetected forms, PA_2LF_N and $PA_4(LF_N)_2$, suggest a pathway of assembly for the octamer via even-numbered intermediates.

We then probed the kinetics of PA assembly with nanoESI-MS (Fig. 3B). Here oligomerization was initiated by mixing $_nPA$ and LF_N in a 1:1 ratio, using the assembly method described in the single-channel studies. The ion abundances in the ESI mass spectra were recorded for ~1 hour at several time points. The abundances of both $PA_7(LF_N)_3$ and $PA_8(LF_N)_4$ increase concomitantly. Also the appearance of either oligomer correlates to a decrease in the relative ion abundances for free PA monomer and $PA_4(LF_N)_2$ species. We observed an initial burst in abundance of the $PA_4(LF_N)_2$ species followed by a subsequent decrease with time; the decrease was concomitant with the increase in the formation of $PA_7(LF_N)_3$ and $PA_8(LF_N)_4$. The trend in $PA_4(LF_N)_2$ abundance suggests that it is a stable intermediate in the formation of the higher-order complexes. $PA_8(LF_N)_4$, however, is not an intermediate species in the heptamerization pathway, since its relative abundance did not decrease during the experiment.

Stabilizing dimeric PA intermediates promotes octamer formation

To further investigate how even-numbered intermediates influence assembly, we conducted EM studies using PA oligomers prepared in the presence of a dimeric soluble Atr receptor domain (dsATR), LF_N , or EF_N (EF_N 's PA-binding, amino-terminal domain). A dimeric ATR construct was also chosen based upon evidence that the receptor may exist in a dimeric state on cell surfaces.²⁴ PA pre-complexed to dsATR (Fig. 2B), but not to monomeric ATR (msATR) (Fig. 2C), showed increased proportions of octamers upon assembly. Further studies demonstrated that when dsATR was loaded under less saturating conditions, the octamer levels decreased (Fig. S2B). Thus the more saturating conditions allow the dsATR sites to fully populate with PA prior to assembly, which increases the probability of forming the even-numbered, octameric form.

LF or EF can form a ternary complex with PA dimers.²¹ We have also observed this species in our mass spectrometry experiments (Fig. 3A; Table S1). When LF_N or EF_N is used to assemble $_nPA$ into oligomers (Fig. 2D,E), ~25% of the population became octameric. This increase is five- to ten-fold more than that observed for PA oligomerized in the absence of LF_N or EF_N . Also a PA mutant, S170C, which can form a disulfide-bonded homodimer (as modeled in Fig. S2C), increases the proportion of octamers relative to unlinked WT PA (Fig. 2F). Finally, our reference-free EM analysis was supported, when possible, with reference-based analysis, mass spectrometry, and electrophysiology (Table S2). Therefore, we conclude that by increasing the population of even-numbered PA_2 -precursor complexes, we observe an increase in the proportion of octamers.

PA forms octamers on cells

These in vitro results led us to probe the oligomerization pathway on cell surfaces. We used a Chinese hamster ovary (CHO) cell line, expressing ATR2, called C-CHO.³² PA with a carboxy-terminal six histidine tag (His6), called His6-PA, was added to the extracellular medium of cultured C-CHO cells and incubated to assemble. Endosomal acidification was blocked with ammonium chloride to prevent the conversion of the pre-channel oligomers to the channel state. The cells were harvested, lysed in detergent and purified on His6-affinity resin. SDS-PAGE gels of His6-pure extracts were western blotted, confirming that the purified fraction contained His6-PA in the 63-kDa form (Fig. S3).

EM studies of these extracts identified oligomeric rings consistent with the size and shape of PA oligomers; however, these complexes were less well oriented than the other in vitro samples, perhaps due to the presence of cellular components, like full-length ATR, which may form the observed extensions from the oligomeric structure. Several tilted class averages were

obtained to capture this heterogeneity. From these, we determined that ~20–30% of the oligomers were octameric (Fig. 2G). Control experiments show that His6-PA on its own forms ~1% octamer (Fig. S2A), confirming that the His6 tag is not responsible for the high levels of octamer observed on cells. We also examined extracts from T-CHO cells (expressing ATR1) and from C-CHO cells that were co-treated with both PA and LF_N; and we found that octamer levels were ~20–30% under all cell-surface conditions tested (data not shown). Thus PA forms a mixture of octamers and heptamers on cell surfaces.

Crystal structure of the octamer

Mutations were then introduced into PA to probe the molecular mechanism of assembly. The most interesting mutations identified disrupted the interface between two PA subunits at the interface of domain 4 (D4) and the neighboring membrane insertion loop (MIL) in the adjacent PA subunit. One mutant replaced the MIL (i.e., residues 305–324) with a type II turn, deleting all possible hydrophobic interactions between L668 of D4 and F313 and F314 in the MIL. This type of PA oligomer (PA^{ΔMIL}) was reported to form heptameric rings;³³ but our version made an enriched source of octameric rings as observed by EM (Fig. 5A; Table S2).

We used PA^{ΔMIL} oligomers as a concentrated source of octameric PA, and solved the octamer's crystal structure to 3.2-Å resolution (Fig. 4A; Table S3). Molecular replacement identified eight PA monomers arranged as a ring in the asymmetric unit. We find that the octamer is best described as having fourfold noncrystallographic symmetry (NCS), because there are two types of PA monomer conformations [called A and B (Fig. 4C)] that occupy alternating positions around the ring (Fig. 4D). From structural alignments, these two conformers differ in the orientation of D4 (Fig. 4C). This conformational heterogeneity is notable, since D4 interacts with the MIL and may be a structural feature in the assembly mechanism. D4's flexibility is also consistent with the higher than average B factors observed there and also in the MIL of the heptamer structure.⁷ Thus plasticity in these two regions may provide a mechanism for octamer formation, where modulation of the structure in these regions may occur either (i) via ATR binding, which reorients D4 relative to D2 (refs. ^{7,34}); or (ii) via exposure to a more acidic pH, which may alter the conformation of the MIL.

Consistent with our single-channel data, the mean pore diameter of the octamer pre-channel is ~10% larger than the heptamer [46 (±4) and 40 (±4) Å, respectively] (Fig. 4A,B). The residues lining the pre-channel of the octamer are similar to those in the heptamer; all types of chemistries are represented, though the charge composition is more anionic overall. The octamer buries ~3300 Å² of solvent accessible surface area (ΔASA) per monomer, which is ~800 Å² less than the heptamer; the MIL and its interactions with its neighboring docking groove in D4 largely account for this difference. The octamer forms additional contacts (not found in the heptamer) at sites more proximal to the central pore, accounting for ~350 Å² of additional buried surface per dimer interface (Fig. 4E). For WT octameric PA, the MIL should form analogous interactions with this D4-docking groove; and therefore, the octamer may bury at least 350 Å² more surface area per monomer than the heptamer when the MIL is present. Thus WT octamer will bury ~6100 Å² of additional surface relative to the heptamer, when including the eighth subunit and additional increases in burial per monomer.

The angles, θ , between n adjacent monomers arranged symmetrically about a ring are ideally equal to $180 - 360/n$, and these angles widen ~6° for the octamer (Fig. 4A) with respect to the heptamer (Fig. 4B). We found this is accomplished by a subtle shift in the inter-monomer packing interfaces (Fig. 4E), where the octamer buries more surface area in regions proximal to the central channel. During assembly, the steric mass of the MIL may act as a non-specific wedge that effectively nudges the adjacent monomer toward a more acute θ in the heptamer. Thus PA^{ΔMIL}, in the absence of this constraint, is able to relax θ to achieve the octameric

configuration. The MIL has two functions: (i) to form the channel in the membrane; and (ii) to control the oligomerization number of pre-channel assemblies.

The relative stability of the PA heptamer and octamer

To test whether, octameric and heptameric complexes differed in their stabilities, we incubated mixtures of heptameric and octameric PA at different temperatures and pHs. Under mildly acidic conditions (pH 5.7), we found that the heptameric form precipitated almost quantitatively as judged by both EM and nanoESI-MS (Fig. 5A; Table. S2); however, the octameric form maintained its solubility and persisted. This difference provided us with the means to isolate the octameric form for crystallization. Since these experiments used the PA^{ΔMIL} construct, we tested the relative stability of WT PA oligomers formed in the presence of LF_N at physiological pH. After the assembly under physiological conditions, we found that the sample could be purified by S400 gel filtration, generating ~90%-pure octamer, as judged by EM and nanoESI-MS (Fig. 5B; Table. S2). Therefore, we conclude that the heptameric form assembled and was subsequently inactivated by aggregation under physiological conditions; however, the octameric form persisted as a soluble complex.

Translocase activity of octameric channels

The pre-channel PA oligomer forms the translocase channel state under acidic pH conditions.^{10,33} The β -barrel, which penetrates the membrane, is comprised of the MIL and adjacent β -strands of in D2. Our structure reveals that the contacts made in D1' (Δ ASA of ~1100 Å²) and those immediately adjacent to it in D2 (~1400 Å²) are largely identical and sufficient to form and maintain stable oligomeric complexes even when the MIL is not present. Therefore, the octamer may form channels, and the two Gaussian populations of conductance levels observed in planar bilayers (Fig. 1B,C) may reflect octamers and heptamers, which have stably inserted into membranes.

We then tested this further and asked whether octamers and heptamers possessed similar translocase activity. Here we compared QPA, which is ~98% heptameric, to a sample, nPA + LF_N, ~90% enriched in octamer. In an ensemble translocation experiment, channels are first inserted into a membrane at 20 mV; the channels are loaded with LF_N, which blocks the conductance; excess LF_N is removed by perfusion; and then the LF_N is translocated at a higher voltage, or $\Delta\psi$. We observe that LF, EF, LF_N, and EF_N translocate through the two different PA oligomers with similar efficiencies and rates (Fig. 5C). (Efficiency is the measured amplitude translocated divided by the maximum theoretical amplitude; the rate is estimated by the time it takes for half of the protein to translocate).

To further verify that larger-sized and smaller-sized channels are capable of translocating protein, we performed single-channel translocation experiments (Fig. 5D). Here we formed single PA channels at 20 mV and then added LF_N. Once the channel closed due to LF_N binding, the voltage was raised to 50 mV. Translocation events ($n \sim 10$) were recorded until the channel became inactive. This procedure was repeated on a second channel obtained in the same membrane, which had a ~10% larger conductance. Therefore, we conclude that large and small PA channels are functional translocases, and both the octameric and heptameric forms of the PA channel are functional.

Discussion

We suggest that the octameric form of PA has not been previously observed,⁴⁻⁸ because the standard method of PA assembly, which uses an anion-exchange column,¹⁰ yields oligomers virtually devoid of octamers (Fig. 2A). Assembly in the presence of ligand, LF_N, EF_N, LF, EF or dimeric ATR, produces a 25–30% population of octameric oligomers in vitro (Fig. 1, 2B-

D). This heterogeneous assembly mechanism is physiological, because a similar proportion of octameric oligomers is observed on the surface of cells (Fig. 2G). Our crystal structure of the octamer is not a regular octagon, but rather is composed of four PA dimer pairs arranged in a square planar symmetry. This symmetry suggests that dimeric PA intermediates populate the assembly pathway, and indeed mass spectrometry reveals that dimeric PA species are general assembly intermediates (Fig. 3B). While heptameric and octameric channels have similar translocase activity, octameric oligomers are more stable under physiological pH and temperature (Fig. 5). We propose that these two different oligomerization states are functionally relevant to anthrax pathogenesis, namely in the two different environments in which the toxin assembles (Fig. 6). (i) On cell surfaces, assembly bottlenecks may be mitigated allowing for proper endocytosis of functional complexes by having the two assembly routes. (ii) In blood plasma, PA may assemble prior to reaching the cell surface and require a more stable oligomeric configuration, since the heptamer is weakly stable under physiological conditions, especially in the absence of its cellular receptor.

Cell-surface assembly and endocytosis

The current model for anthrax toxin assembly proposes that PA assembles into a heptamer on cell surfaces expressing ATRs (Fig. S1). Initially, PA binds a cell-surface receptor, ATR1 or ATR2, is cleaved by a furin-type protease, and then begins to assemble into the ring-shaped oligomer. While the current model predicts that these oligomers will be heptameric,¹ the oligomeric states populated on cell surfaces have not been reported. We specifically addressed this question by extracting oligomers from cell surfaces and analyzing the distribution of oligomeric states by electron microscopy. We find that PA forms both a heptamer and the novel octamer in about a 2:1 ratio, using cell lines expressing either ATR1 or ATR2. What selection pressures on the toxin might maintain this dual-oligomerization-state mechanism? Currently, it is known that cell-surface assembly and endocytosis may be coupled processes. For example, the rate of cellular internalization through endocytosis for either free ATR or PA bound ATR is slow; and this basal rate is only accelerated if the PA is preassembled into PA heptamers or PA-bound ATR subunits are aggregated by antibody cross-linking.²² Therefore, PA assembly and the corresponding aggregation of ATRs triggers endocytosis.

Our experiments extend the current understanding of assembly and we now can improve upon the model. First, we assume that ATR1 and ATR2 are effectively dimeric on cell surfaces, because ATR-mediated assembly in solution only promotes octameric assembly when the receptor is dimeric (Fig. 2B,C). A dimeric receptor model agrees with previous studies, which examined the aggregation state of ATR1's transmembrane helix.²⁴ Therefore, we propose that the antibody cross-linking experiment reported by Abrami et al.²² more likely involves a higher order aggregation of PA subunits beyond the formation of PA dimers. We anticipate this, because it is known that LF and EF promote the dimerization of PA (Fig. 3; ref. ²¹); and an unintended consequence of this dimerization may be that PA₂LF or PA₂EF ternary complexes would be improperly endocytosed prior to assembly into functional ring-shaped oligomeric complexes. Thus an efficient coupling of cell-surface assembly and endocytosis would limit premature endocytosis of non-functional dimers and tetramers.

A key function of the dual assembly mechanism may ultimately be to allow for assembly under a range of PA monomer concentrations. If we consider the two extreme cases of either low or high PA monomer concentrations, assembly would be hindered if only one oligomeric state were possible. On one hand, supposing only the octameric assembly pathway were possible and the PA monomer concentration was low, then the dimeric ATR sites may not be saturated, and assembly would be inhibited at the critical oligomerization step, allowing unassembled complexes to be endocytosed. On the other hand, supposing only the heptameric form were possible and the PA monomer concentration was high, the dimeric ATR sites would be fully

saturated with PA, and assembly would be inhibited until one PA could dissociate from its ATR binding site, allowing for an odd number of subunits to assemble immediately before endocytosis. This latter scenario is especially prohibited knowing that the dissociation lifetime for the PA-ATR2 interaction is on the order of days.³⁵ In sum, the mixed-oligomerization mechanism mitigates these potential assembly bottlenecks, allowing the toxin components to fully assemble and remain efficacious under the wide dynamic range of extracellular PA concentrations.

Extracellular assembly in blood plasma

Anthrax toxin complexes were first identified in the blood of *B. anthracis*-infected guinea pigs; and this blood (after sterilization with antibiotic treatment) could then be subsequently administered to a second uninfected animal to impart its lethal affect.²⁶ Throughout the later stages of infection, both the proteolytically-activated form of PA ($_n$ PA) and LF are found in the sera of infected animal models.²⁸ This form of PA, of course, may co-assemble with LF; however, the prior study did not test this possibility. We demonstrate here, using native gel electrophoresis, that PA can be proteolytically activated in bovine blood plasma, and toxin complexes can form stably (Fig. S4). Under aqueous conditions, we have shown that octamers form in the presence of LF_N or EF_N (Fig. 2D, E). Thus octamer formation is not limited to a receptor-dependent mechanism, and octamers may assemble extracellularly in blood plasma in an LF- or EF-dependent manner (Fig. 6).

Toxin stability

Recent work in vitro revealed that heptameric pre-channel complexes are unstable under physiological conditions, readily converting to the channel state.⁸ Here we demonstrate that physiological temperatures and pH lead to irreversible aggregation of the heptameric form, such that the octameric form is left behind in stable, isolable complexes (Fig. 5A,B). We think this effect may be linked to pH-dependent differences in the octameric pre-channel to channel transition or inherent differences in the stability of octameric toxin complexes. ATRs have been shown previously to stabilize the pre-channel conformation under physiological conditions.^{7,33,36,37} Toxin complexes assembled in the bloodstream, however, do not benefit from ATR stabilization. Thus octameric complexes may represent a more stable toxin configuration that may persist in the blood of infected animals, serving a primary role to maintain cytotoxicity under physiological conditions.

Octamer structure

Our crystal structure shows that the octamer is a ring of eight PA monomers, consisting of a square-planar arrangement of four PA dimers (Fig. 4C,D). This structure is in contrast to the near-perfect, regular heptagon model of the heptamer.⁷ Our EM and crystallographic studies of these toxin complexes suggest that the octamer is composed of four pairs of PA subunits that are conformational heterodimers; and the heptamer may actually consist of three PA heterodimers and one asymmetric monomer (Fig. 2). Our analysis of the precise symmetry of the heptamer by EM should be tempered by the resolution of the technique, however; and further crystallographic studies are required. Nonetheless, the octamer and its tetrameric arrangement of PA pairs suggests that dimeric PA intermediates produced by interactions with LF, EF or a dimeric ATR subsequently assemble in a pairwise fashion to form the octamer (Fig. 2,6).

From the crystal structure, we conclude that the WT octamers should bury 6100 Å² of additional surface per oligomer relative to the heptamer, indicating that octamers may possess greater inherent stability, preventing disassembly and/or premature conversion to the channel state. Each PA heterodimer, of course, is poised to bind an LF/EF molecule (or a pair of ATRs), affording the octamer four binding sites and the heptamer three (Fig. 3, 6). The improved

stability of octameric toxin complexes may result from full occupancy of four heterodimeric, hydrophobic, LF binding sites; whereas for the heptamer, only three of such sites can be occupied, leaving one PA subunit exposed. Moreover, additional interfaces between adjacent LF subunits in the octameric complex may create a novel ring of interfaces between adjacent LF subunits, adding stability that cannot be attained in the heptameric complex (Fig. 6). Future structural studies will elucidate how these two lethal toxin configurations may differ. We conclude that the added surface burial and structural symmetry of the even-numbered octameric configuration of toxin complexes may explain the improved stability over the heptameric configuration.

Toxin activity

We propose that the general paradigm for the aggregate physiological anthrax toxin activity is a product of the catalytic rate of translocation and the inherent stability of the two possible oligomeric configurations: the heptamer and the octamer (Fig. 6). A secondary effect relates to the fact that the octameric configuration provides an additional LF- and EF-binding site per complex (Figs. 3A,6), increasing the potential toxicity of saturated octameric toxin complexes. We find in our planar lipid bilayer translocation assays that the two oligomers translocate LF and EF at similar rates (Fig. 5C). Therefore, we propose differences in toxin activity will likely result from intrinsic differences in oligomer stability. Without ATR stabilization, heptameric toxin complexes may be inactivated under physiological conditions (Fig. 5A,B), whereas octameric toxin complexes may remain soluble and fully functional. We hypothesize that these physiological conditions are present when the toxin assembles in an ATR-independent manner (e.g., in the blood, lymph, or phagolysosomal compartment²⁵), and these conditions may favor the octameric form.

Staphylococcal α -hemolysin

The pathogenic factor produced by *Staphylococcus aureus*, called α -hemolysin, is comprised of multiple copies of a 293 residue polypeptide.³⁸ The assembled toxin ultimately forms a circular, β -barrel-type, ion-conducting pore with a mushroom-like architecture.¹² Interestingly, this pore-forming toxin is believed to have multiple oligomeric states. EM,³⁹⁻⁴¹ atomic force microscopy⁴² studies, and electrophysiology studies⁴³ determined that the α -hemolysin can be hexameric. However, chemical cross-linking studies;⁴⁴ crystallographic studies;^{12,44} single-molecule, photo-bleaching fluorescence methods;⁴⁵ and electrophysiology studies⁴³ suggest the toxin forms a heptamer. The relative populations of the two oligomeric states and the conditions affecting these relative distributions have not been reported, however.

Pathogenesis

Why may PA assemble into a mixture of oligomers? For *B. anthracis* to optimize its lifecycle and proliferate in its host, it secretes a toxin, which at the initial stages of pathogenesis, may first help germinated bacteria escape from macrophages {Banks, 2005 #4284} and second allow a small population of bacteria to selectively suppress the immune system. However, these toxin conditions are nonlethal. During infection, *B. anthracis* secretes low, undetectable concentrations of the toxin components; and at the latest stages of infection, 100 $\mu\text{g/mL}$ levels of PA are detectable in the blood of infected animals.²⁸ This large increase in PA concentration immediately precedes death in animal models.²⁷ Such a wide dynamic range in toxin levels suggests a relationship between toxin assembly and cytotoxicity. We propose that, on the cell surface, assembly of octameric oligomers alleviates a potential assembly bottleneck imposed by ATR dimerization, while in the bloodstream, lymph, or phagolysosome²⁵ the octameric toxin complexes may function as a more stable species. Thus heterogeneous assembly may function in two key contexts: the known cell surface assembly pathway (Fig. S1) and a putative toxin ATR-independent assembly pathway (Fig. 6,S4).

PA assembly pathways are further complicated by dimerization of ATRs (refs. ^{23,24}) and induced dimerization of PA subunits by LF_N and EF_N. The heptameric assembly route may be efficient at low concentrations, where PA likely assembles predominantly from monomers. On the other hand, PA assembly would be attenuated at high concentrations, where PA assembles via dimeric intermediates, in the absence of the octameric assembly route. Endocytosis coincides with PA assembly,²² and a loss in toxin activity could occur if partially-assembled complexes were endocytosed. Therefore, we reason that the observed heterogeneity (Fig. 2G) may serve to alleviate these potential assembly bottlenecks, incurred during oligomerization.

At later stages in anthrax infection, when PA and LF concentrations are high, a significant proportion of toxin complexes may assemble prior to encountering cell surface ATRs. In fact, the blood of infected animals contains PA that is almost exclusively proteolytically activated.²⁸ Here we demonstrated that PA, LF, and EF assemble into lethal and edema toxin complexes in bovine blood (Fig. S4). Assembly in the bloodstream should produce a similar proportion of octameric toxin complexes as we observe in vitro (Fig. 2). However, we expect that octameric complexes could persist in these conditions, while heptameric complexes may form inactive aggregates. Therefore, octamer formation could provide a mechanism of overcoming these harsh, attenuating conditions encountered extracellularly (in the absence of an ATR). The proposed two-oligomer assembly model suggests that the anthrax toxin can modulate its aggregate activity through assembly due to these differences in toxin stability. Further structure/function studies of heptameric and octameric oligomers will clarify this molecular mechanism.

Materials and Methods

Proteins

PA—Recombinant WT PA, carboxy-terminally His6-tagged PA,⁴⁶ and all other PA mutants described herein were overexpressed in the periplasm of *E. coli* BL21(DE3), and they were purified as 83-kDa monomers as described.³⁵ A modified QuikChange procedure⁴⁷ using Pfu Turbo polymerase (Agilent Technologies, Santa Clara, CA) was implemented to make a deletion construct (PA^{ΔMIL}) from the PA expression vector, PA₈₃ pET22b+ (EMD Chemicals, Gibbstown, NJ).¹³ In PA^{ΔMIL}, residues 305–324 were deleted and two point mutations (V303P and H304G) were introduced simultaneously, leaving a Type II turn in place of the MIL [residue numbering as in 1ACC (ref. ⁵)].

Dimeric PA—The QuikChange procedure was also used to engineer in a S170C point mutation into WT PA. PA S170C, purified under oxidizing conditions, was judged to be dimeric by SDS-PAGE.

Soluble dimeric ATR2—Recombinant soluble anthrax toxin receptor domain (sATR2), residues 40–217 (ref. ³⁵), was expressed from a pGEX vector (GE Healthcare) as a glutathione-S-transferase (GST) fusion protein, affinity purified on glutathione sepharose as described.³⁵ The GST-sATR was shown to be fully dimeric by nanoESI-MS and is called dsATR2.

Soluble monomeric ATR2—A monomeric version of sATR2 (msATR) was made by subcloning residues 40–217 of ATR2 into pET15b (EMD Chemicals) via the Nde I and BamH I restriction sites, using a pGEX expression clone.³⁵ This construct has an amino-terminal His6 tag as described previously.³⁴ The protein was similarly purified on a His6 column and judged to be pure by SDS-PAGE, and it was confirmed to be monomeric by nanoESI-MS.

LF_N and EF_N—Recombinant LF_N (LF residues 1–263) and EF_N (residues 1–254 of EF) were overexpressed from pET15b constructs⁴⁸ and then purified from the cytosol using His6 affinity

chromatography. LF_N was further processed by incubating with bovine α -thrombin to remove the amino-terminal His6 tag and purified over Q-sepharose anion exchange as described previously.¹⁹ EF_N was used with its His6 tag and not processed any further. Each protein sample was verified by MALDI mass spectrometry.

PA oligomerization on a Q-sepharose—PA monomers were first treated with trypsin at a 1:1000 mass ratio for 15 minutes at room temperature, making _nPA. The trypsin was blocked by the addition of a 1:100 mass ratio of soybean trypsin inhibitor and 1 mM phenylmethylsulphonyl fluoride (PMSF). Then the small, 20-kDa fragment, released by trypsinization, was separated by anion exchange chromatography, using Q-Sepharose High Performance resin (GE Healthcare), such that the remaining ~60 kDa portion oligomerized as previously described.^{10,35} This crude, oligomeric PA mixture (_QPA) was used throughout and contains mixtures of both heptameric and octameric complexes as judged by EM and nanoESI-MS.

_nPA oligomerization in the presence of LF_N/EF_N or ms/dsATR2—_nPA was prepared as describe above and then diluted to into an appropriate buffer containing stoichiometric amounts of LF_N/EF_N or ms/dsATR2. LF_N/EF_N assembly experiments were carried out by diluting _nPA to ~1 mg/mL in 20 mM phosphate, 150 mM sodium chloride, pH 7.5, containing excess LF_N/EF_N. The assembly reaction was allowed to equilibrate for two hours at room temperature to afford complete oligomerization, as assessed by native gel. The dsATR2- and msATR2-assembly experiments were carried out by diluting _nPA to ~2 mg/mL in 20 mM cacodylate buffer, 150 mM sodium chloride, 1 mM calcium chloride, pH 7.5, containing the determined stoichiometric amounts of msATR2 or dsATR2. The assembly reaction was allowed to equilibrate for ten minutes at room temperature to afford complete oligomerization (as assessed by native gel electrophoresis). Assembly reactions were purified on an S200 gel filtration column equilibrated in assembly buffer to remove unassembled components. msATR2 does not promote oligomerization of _nPA, so it was assembled in the manner that _QPA is assembled as described above.

Isolation of wild type octameric PA—_nPA+LF_N complexes were prepared as described above, dialyzed against 10 mM Tris, pH 8, and diluted to 2 mg/mL in 0.1 M cacodylate buffer, pH 7. The sample was incubated for 5 minutes at 37 °C, concentrated, and purified on an S400 gel filtration column. The purity and oligomeric homogeneity of the resulting complexes were assessed by electron microscopy and mass spectrometry.

Electrophysiology—An Axopatch 200B amplifier (Molecular Devices Corp., Sunnyvale, CA) was used in the voltage-clamp, capacitor-feedback mode. The amplifier was interfaced to a CyberAmp 320 signal conditioner (Molecular Devices), which typically filtered the data at 200 Hz via a low-pass, 4-pole, Bessel section. The filtered, analog signal was typically recorded by computer at 400 Hz using a Digidata 1440A analog-to-digital converter (Molecular Devices) and AXOCLAMP software (Molecular Devices). Most data analysis, post-acquisition filtering, and curve fitting used a combination of CLAMPFIT (Molecular Devices), ORIGIN6.1 (OriginLab Corp., Northampton, MA), and custom Perl scripts.

Relative macroscopic membrane insertion activities for WT PA and mutant forms were assayed as follows. Planar lipid bilayers (PLB) were painted⁴⁹ using a 3% solution of the lipid, 1,2-diphytanoyl-*sn*-glycerol-3-phosphocholine (DPhPC; Avanti Polar Lipids, Alabaster, AL), in *n*-decane solvent. The bilayer was painted inside either a 100 or 200 μ m aperture of a 1-mL, white delrin cup while bathed in aqueous Buffer S: 100 mM KCl, 1 mM EDTA and 10 mM succinic acid, pH 6.6. Cis (side to which the PA oligomer is added) and trans compartments were bathed in symmetric Buffer S. For macroscopic current measurements, PA oligomer (25 pM) was added to the cis compartment, which was held at a $\Delta\psi$ of +20 mV. ($\Delta\psi$, the membrane

potential, is defined as $\Delta\psi = \Delta\psi_{\text{cis}} - \psi_{\text{trans}}$, where $\psi_{\text{trans}} \equiv 0$ mV.) Channel insertion increased over a period of minutes and stabilized after 20 to 30 minutes. Macroscopic currents were then measured at this point or the channel-inserted membrane was used in translocation experiments as described below.

Single-channel measurements were obtained using DPhPC/decane films formed on a 100 μM aperture in a white delrin cup. Single-channel conductance measurements were carried out at a $\Delta\psi$ of +20 mV in symmetric Buffer S. Single-channel current recordings were determined by adding a dilute solution ($\sim 10^{-14}$ M) of PA oligomer to the cis chamber, stirring briefly, and then waiting 5 to 30 minutes until discrete steps in current were observed. The clamping voltage and current responses were acquired at 400 Hz under low-pass filtering at 200 Hz. To observe subtle, slow-timescale fluctuations in the single-channel current records, we implemented a Gaussian-filter algorithm; this filter does not cause ‘overshoot’ during channel opening and closing transitions. The filter's Gaussian kernel was defined with a σ having a width of 100 data points. A Perl script was written to apply the Gaussian filter to our datasets.

To calculate the mean unitary conductance levels, time courses not treated with the Gaussian filter were analyzed by fitting Gaussians to 0.003 to 0.03 pA binned histograms of the current responses, using either ORIGIN6.1 or CLAMPFIT. Some records contained multiple, but readily separable, current steps up to as many as ten single channels. Means, μ , from Gaussian curve fits were then subtracted from similar fits to the noise observed at ‘zero’ current to obtain each single-channel current, i . The single-channel conductance, γ , is calculated from the single-channel current, i , and voltage, V , by $\gamma = i / V$. Errors from μ 's were propagated to establish errors in γ 's for each measurement, which were ~ 0.5 pS.

Single-channel recordings of the $n\text{PA}+\text{LF}_N$ sample—We also analyzed the $n\text{PA}+\text{LF}_N$ sample, which was formed by taking 0.1 mg/ml of nPA and adding a stoichiometric equivalent of LF_N . The mixture was incubated at room temperature for 1 hour. The assembled complex (as judged by native gel electrophoresis, gel filtration, and EM) was applied to PLB membranes at $\sim 10^{-14}$ M. We initially observe channel insertion at a $\Delta\psi$ of -20 mV to preclude the LF_N moiety from binding within the channel and blocking the conductance. Note that because the PA sample was diluted 10^9 -fold, newly inserted channels could be shifted back to a $\Delta\psi$ of +20 mV to record their currents once the LF_N dissociated from the channel.

Electrophysiology-based translocation assay—For translocation experiments, a universal bilayer buffer (UBB) was used (10 mM oxalic acid, 10 mM MES, 10 mM phosphoric acid, 1 mM EDTA, 100 mM KCl). The pH of the UBB is either 5.6 or 6.6 depending on whether a ΔpH will be formed during the translocation assay. Once a membrane was formed, $Q\text{PA}+\text{LF}_N$ or $n\text{PA}+\text{LF}_N$ was added to the cis compartment (at pH 5.6); the cis compartment was held at a $\Delta\psi$ of ± 20 mV with respect to the trans compartment. (I.e., in the case of $n\text{PA}+\text{LF}_N$, the $\Delta\psi$ was held at -20 mV to allow for channel insertion to be observed, because at +20 mV, LF_N would block the channel). When $n\text{PA}+\text{LF}_N$ was used, the excess LF_N was perfused away, and residual bound LF_N was then translocated at 80 mV to clear the channels prior to initiating further translocation experiments. After the ensemble channel population was established, LF, LF_N , EF, or EF_N were added to the cis compartment. The progress of substrate binding to PA_{63} channels was monitored by the continuous fall in conductance. After the conductance block of PA channels was complete, excess substrate was removed from the cis compartment with perfusion with UBB, using push-pull, hand-crank-driven, 10-mL syringe pump. The cis compartment was perfused with 10 ml of UBB at a flow rate of 2 ml/min and $\Delta\psi$ held at +20 mV. Translocation of LF, LF_N , EF, and EF_N were initiated by jumping the $\Delta\psi$ to a higher positive voltage and/or jumping the ΔpH to a higher positive value (where $\Delta\text{pH} \equiv \text{pH}_{\text{trans}} - \text{pH}_{\text{cis}}$.) The ΔpH is induced by adding predetermined amounts of 0.4 M phosphoric acid to the

cis chamber. The final pH is determined using a pH meter following the completion of the translocation recording.

Electron microscopy—All the samples were prepared for EM in a similar manner. PA oligomer at 20–30 nM (in monomer concentration) was incubated in Buffer E (20 mM Tris, 100–250 mM NaCl, pH 8) for 5 minutes. 400 mesh copper grids were successively covered by a holey carbon film and a continuous carbon film. 4 μ l of sample was applied to a freshly glow-discharged support grid for 30 s and then stained in 5 successive drops (75 μ l) of either 1% uranyl formate (Structure Probe, Inc., West Chester, PA) or 2% uranyl acetate (Sigma-Aldrich, St. Louis, MO).

Negative-stain EM images were recorded with a Tecnai 12 (FEI Company, Hillsboro, OR) operated at 100 or 120 kV at a magnification of either 49,000 $\times$ or 50,000 $\times$. In some cases, data were collected on Kodak SO163 films (Eastman Kodak, Rochester, NY) at 600 to 800 nm underfocus. Film images were digitized with a Nikon Super Coolscan 8000 (Nikon U.S.A., Melville, NY) at a 12.7 μ m pixel size, resulting in a 2.54 Å/pixel at the specimen scale. In other cases, data was collected directly on a CCD camera, resulting in a 2.13 Å/pixel at the specimen scale. Particle images were selected for each data set using automatic or manual particle picking using boxer in EMAN.⁵⁰

Reference-free processing was done using the software package, IMAGIC (Image Science Software, Berlin, Germany) or SPIDER.⁵¹ Images were subjected to three successive cycles of multi-reference alignment, multivariate statistical analysis, and classification.^{52,53} The last classification was done using only the lowest order eigenvectors as described elsewhere⁵⁴ to separate the data by size and the heptameric and octameric oligomerization states.

A second method of image processing was used whereby reference images were made from 2D-projections of low resolution density maps generated from the crystal structures of the PA heptameric⁷ and octameric pre-channels, using SPIDER.⁵¹ Boxed images were then subjected to reference-based alignment and classification using the lowest order eigenvectors as stated above. Final class-average images were manually inspected for their oligomer number, designated as either heptamer or octamer, and tabulated to produce the final percentages of heptamers and octamers. Each method of classification, reference-free or crystal-structure-referenced, produced similar results (Table S2).

Extraction of PA oligomers from CHO cells—C-CHO and T-CHO cells were a kind gift from Arthur Frankel. The cell line was created from a spontaneous ATR-deficient CHO cell mutant line (PR230-CHO). The C-CHO and T-CHO lines were derived from stable transfections with the human ATR2 and ATR1 expression clones, respectively.⁵⁵ The cell lines were grown to confluence in Ham's F12 medium (Invitrogen), 10% fetal bovine serum (Invitrogen), 100 units/mL penicillin, 100 μ g/mL streptomycin (Sigma-Aldrich) in humid 5% CO₂ atmosphere at 37 °C, as described.³² Confluent cells were treated for one hour with 50 mM ammonium chloride, 50 mM 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.3 in Ham's Media to inhibit endosomal acidification, preventing conversion of PA to the channel state.⁵⁶ His6-PA monomers (WT PA with a carboxy-terminal His6 tag) were applied to cells in the same medium at 100 μ g/mL. Cells were incubated with His6-PA for one hour at 4 °C, washed with five volumes ice-cold phosphate buffered saline (PBS) to remove unbound PA, and lysed in Buffer L: 20 mM Tris, 0.35 M NaCl, 10 mM imidazole, 1% Nonidet P-40 (or IPEGAL), 0.25% deoxycholic acid, 1 mM PMSF, pH 8, as described previously.⁵⁵ Cells debris was removed by centrifugation and the supernatant incubated with 0.5 mL His6-affinity resin (Ni-NTA Superflow, Qiagen, Valencia, CA) overnight at 4 °C with stirring. The resin was washed with five volumes of Buffer L and eluted in Buffer L

supplemented with 300 mM imidazole. The elution was applied to electron microscopy grids for analysis.

Mass spectrometry—Mass spectra of the protein complexes were acquired using a quadrupole time-of-flight (Q-ToF) mass spectrometer equipped with a Z-spray ion source (Q-ToF Premier, Waters, Milford, MA). Ions were formed using a nanoelectrospray (nanoESI) emitters prepared by pulling borosilicate capillaries (1.0 mm O.D./0.78 mm I.D., Sutter Instruments, Novato CA) to a tip I.D. of $\sim 1 \mu\text{m}$ with a Flaming/Brown micropipette puller (Model P-87, Sutter). The instrument was calibrated with CsI clusters formed by nanoESI using a 24 mg/mL solution of CsI in 70:30 Milli-Q water:2-propanol prior to mass measurement. The protein solution for the stoichiometry determinations was prepared as described above and then concentrated to 10 μM followed by dialysis into 10 mM ammonium bicarbonate, pH 7.8. Immediately prior to mass analysis, the solution was diluted 1:1 with 200 mM ammonium acetate, pH 7.8. A platinum wire (0.127 mm diameter, Sigma, St. Louis, MO) was inserted through the capillary into the solution and electrospray was initiated and maintained by applying 1–1.3 kV to the wire (relative to instrument ground). Raw data was smoothed three times using the Waters MassLynx software mean smoothing algorithm with a window of 50 m/z (mass-charge ratio).

The reactions for assembly kinetics were initiated by mixing 10 mM ammonium bicarbonate solutions of purified $_{\text{n}}$ PA and LF_{N} monomer in a 1:1 ratio to initiate oligomerization, and mass spectra were acquired continuously for 50 minutes. Variation in the voltage applied to the nanospray capillary and new capillaries at ~ 19 minutes and ~ 30 minutes were required to maintain ion current. Mass spectra were averaged for five-minute intervals and smoothed three times using the Waters MassLynx software mean smoothing algorithm with a window of 50 m/z . Each peak in a given charge-state distribution was integrated and the peak areas summed to give an absolute abundance for the corresponding analyte. Relative abundances were calculated as a fraction of the total abundances of the four analytes of interest monitored during the experiment.

Protein crystallization—Purified $\text{QPA}^{\Delta\text{MIL}}$ oligomer (judged to be rich in octameric complexes by EM) was prepared in Buffer X, which contained 74 mM sodium acetate, 7 mM Tris, 0.62 M NaCl, 37 mM tetrabutylammonium bromide, 7% ethanol, 0.07% *n*-dodecyl- β -D-maltopyranoside, pH 5.7, centrifuged to remove precipitated protein, and concentrated to 13 mg/mL. Initial crystallization conditions were established by sparse-matrix crystallization screens,⁵⁷ except our screens contained ~ 1000 unique conditions. A Mosquito nanoliter, liquid-handling robot (TTP Labtech, Cambridge, MA) was used to form 200 nL drops in 96-well format at 18 °C, using the hanging-drop, vapor-diffusion method.⁵⁸ Diffraction-quality crystals were formed with 1 μL hanging drops using the Mosquito (at 18 °C), containing a one-to-one mixture of 13 mg/mL $\text{QPA}^{\Delta\text{MIL}}$ oligomer in Buffer X with the reservoir solutions (ranging from 18 to 30 % *t*-butanol, 0.1 M Tris, pH 7.5 to 8.5). Often irregular, rectangular-prism-shaped crystals formed overnight; these grew as large as $\sim 200 \times 200 \times 75 \mu\text{m}$. Crystals were harvested in a 30% v/v polyethylene glycol (PEG) 400 cryoprotectant, where the PEG only replaced the water in the mother liquor, and immediately flash-frozen in liquid nitrogen.

X-ray diffraction data collection, solution and refinement—X-ray diffraction data were collected at the Advanced Light Source in Lawrence Berkeley National Lab, Beamline 8.3.1,⁵⁹ using a Quantum 315r CCD area detector (ADSC, Poway, California). The crystals diffracted to 3.2 Å in the triclinic space group, $P1$, with unit cell dimensions of 125.60, 125.67, and 125.82 Å for a , b , and c , respectively, and 106.64, 110.82, and 110.98° for α , β , and γ (Table S3). The diffraction data were indexed and scaled in HKL2000.⁶⁰ The scaled dataset was 98.7% complete to 3.2 Å. The self-rotation function in the CCP4 suite⁶¹ revealed strong peaks at a χ angles of 45°, 90° and 180°. Molecular replacement (MR) was performed using

PHASER⁶² in CCP4, where the search model was a loop-stripped chain A from 1TZO.⁷ This MR solution placed eight PA monomer chains in the asymmetric unit. The MR solution was refined with rigid-body constraints defined by the known domain boundaries in PA, using PHENIX.⁶³ NCS was established by first making structural alignments of individual monomers in CHIMERA⁶⁴ and then calculating the center of mass of each chain to compute the geometric arrangement of the chains about the oligomeric ring. We found the oligomer was an irregular octagon, and pairs of monomers of two different conformations, 'A' and 'B', formed the sides of a regular, square, planar tetramer. Subsequent rounds of model building in COOT⁶⁵ were followed by coordinate and B-factor refinement, with fourfold NCS restraints, using PHENIX. $2F_o - F_c$ and $F_o - F_c$ omit electron density maps were recalculated after iterations of model building and refinement, where F_o and F_c are the observed and calculated structure factors, respectively. MOLPROBITY⁶⁶ and PROCHECK⁶⁷ were used to validate the structure's geometry and stereochemistry during model building. Surface burial calculations were made using GETAREA1.1.⁶⁸ Molecular graphics renderings were computed using CHIMERA.⁶⁴

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

K.L.T did the single-channel studies. A.F.K., I.I.T., and T.T.Z. did the EM studies. G.K.F. prepared nanoESI-MS complexes. H.J.S. did the nanoESI-MS. A.F.K. and K.C.D. crystallized the octamer; A.F.K. and B.A.K. refined and analyzed the octamer with guidance from J.M.B. A.F.K. did the ensemble translocations. B.A.K., A.F.K., J.M.B., E.R.W., and H.J.S. wrote the manuscript. We thank J. Mogridge, K. Bradley, and R.J. Collier for discussions; E. Nogales and P. Grob for EM studies and advice; J. Liphardt for advice on single-molecule data; A. Frankel for the gift of the CHO cell lines; J. Fraser and S. Stephensen for X-ray diffraction screening, data collection and structure refinement; S. Greenberg and J. Yassif for purifying PA^{ΔMIL}; R. Zalpuri at the Robert D. Ogg Electron Microscope Laboratory; and A.T. Iavarone at the QB3/Chemistry Mass Spectrometry Facility for helpful discussions. This work was supported by University of California start-up funds and NIH research grants R01-AI077703 (B.A.K.) and R01-GM064712 (E.R.W.)

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Fig. 1. Heterogeneous PA channel conductance distributions

(A) Two PA samples were analyzed: (i) PA is nicked by trypsin to make nPA ; a 20-kDa piece (PA_{20}) dissociates, allowing PA to oligomerize into the pre-channel on a Q-sepharose column, making QPA ; and (ii) nPA is mixed with LFN to drive oligomerization, making $nPA+LFN$. Either pre-channel oligomer forms a channel upon inserting into the membrane. (B) Example of 200-Hz-filtered, single-channel data collected at a $\Delta\psi$ of 20 mV, 100 mM KCl, pH 6.60; γ values computed by $\gamma = i/\Delta\psi$ are listed next to each channel insertion. (C) Normalized histograms of the estimated single-channel γ values for the QPA and $nPA+LFN$ samples. Data bins are one-pS wide, and the number of channels, n , in each sample are normalized for comparison. The samples, QPA ($n = 360$; black bars) and $nPA+LFN$ ($n = 107$; red bars), are statistically distinct by a non-parametric, lower-tailed, Whitney-Mann test ($p > 0.95$). (D) Histogram of all the pairwise differences, δ , between measured γ values identified within the same membrane for the $nPA+LFN$ sample. The δ histogram was fit to one- (dotted line) and two-Gaussian (solid line) functions, using $A(\delta) = A_1/\sigma_1\sqrt{\pi/2} \exp(-2\delta^2/\sigma_1^2) + A_2/\sigma_2\sqrt{\pi/2}$

$[\exp(-2((\delta - \mu_2)/\sigma_1)^2) + \exp(-2((\delta + \mu_2)/\sigma_1)^2)]$, with R values of 0.89 and 0.96, respectively, yielding best-fit parameters: peak area $A_1 = 470 (\pm 80)$, $A_2 = 140 (\pm 40)$; mean, $\mu_2 = 8 (\pm 2)$ pS; and standard deviation, $\sigma_1 = 5.5 (\pm 0.5)$ pS, $\sigma_2 = 9 (\pm 2)$ pS. (See also Table S2.) (E) Single-channel current records for smaller- (black) and larger-sized (red) PA channels in 100 mM KCl, pH 6.6. Arrows indicate the two respective channel sizes. Data were acquired at 400 Hz and filtered further with a 100-point-per- σ Gaussian filter to better reveal conductance sub-states.

Fig. 2. EM studies of heptameric and octameric PA

EM images of negative-stained samples of WT PA oligomers assembled either in vitro (upper panels A-F) or in vivo on cell surfaces (lower panel). Representative class averages of octamers (left) and heptamers (right) are shown. The total number of particles assessed, n , and relative percentages of heptamers and octamers are given. The proportions of heptamers and octamers are indicated by bars colored black and red, respectively. In vitro samples include: **(A)** n PA assembled on an anion-exchange column (QPA; $n = 12589$; 98% heptamer; 2% octamer); **(B)** n PA assembled in the presence of soluble dimeric ATR2 at a 4:1 stoichiometry (+dsATR; $n = 837$; 74% heptamer; 26% octamer); **(C)** n PA assembled in the presence of soluble monomeric ATR2 (+msATR; $n = 9401$; 99% heptamer; 1% octamer); **(D)** n PA assembled in the presence of LF_N; (+LF_N; $n = 8409$; 72% heptamer; 28% octamer); **(E)** n PA assembled in the presence of EF_N; (+EF_N; $n = 5363$; 81% heptamer; 19% octamer); and **(F)** disulfide-bonded PA S170C assembled on an anion-exchange column (QPA S170C; $n = 2933$; 78% heptamer; 22% octamer). Oligomers extracted from cells: **(G)** His6-PA assembled on cells expressing ATR2 (C-CHO; $n = 4729$; 74% heptamer; 26% octamer), where three classes of octamers and heptamers are shown, resulting from reference-based analysis. The 5-nm scale bar shown in panel A is consistent for all images. Percentages of oligomers are means of reference-free and crystal-structure-referenced alignments unless noted otherwise. (Specific percentages are listed in Table S2.)

Fig. 3. Mass spectrometry studies of Atx assembly

(A) NanoESI mass spectrum of _nPA co-assembled with LF_N. Multiple charge-state distributions are observed that correspond to five different PA-LF_N complexes. Charge states and molecular weights were calculated according to a prior method.⁶⁹ The PA₇(LF_N)₃ distribution clearly has the highest relative abundance and is shown above labeled with charge states. Insets show distributions of less intense oligomers PA₇(LF_N)₂ and PA₈(LF_N)₄, and low abundances of PA₂LF_N and PA₄(LF_N)₂ that may be stable intermediates in the formation of the higher order complexes. The molecular weight was assigned from the charge-state distribution that resulted in the smallest standard deviation in calculated molecular weight. (Table S1 summarizes the observed masses and describes the solvent correction.) (B) Assembly

kinetics for the 63-kDa PA monomer, $\text{PA}_7(\text{LF}_\text{N})_3$, $\text{PA}_8(\text{LF}_\text{N})_4$, and $\text{PA}_4(\text{LF}_\text{N})_2$ from a solution of PA_n mixed with excess LF_N . Data for all but $\text{PA}_4(\text{LF}_\text{N})_2$ were fit with exponential functions to guide the eye. The appearance of the oligomers, $\text{PA}_7(\text{LF}_\text{N})_3$ and $\text{PA}_8(\text{LF}_\text{N})_4$, coincides with the disappearance of PA monomer. Data at early times indicate a rapid increase in the abundance of $\text{PA}_4(\text{LF}_\text{N})_2$ followed by slow decay for $t \geq 5$ min., suggesting it is an intermediate in the formation of the higher order complexes; an interpolated line is given for $\text{PA}_4(\text{LF}_\text{N})_2$ also to guide the eye. All four analytes reach steady-state levels in ~ 30 min.

Fig. 4. X-ray crystal structure of PA in the octameric oligomerization state

Axial views of (A) the $PA^{\Delta MIL}$ octamer (PDB 3HVD) side-by-side with (B) the WT PA heptamer (PDB 1TZO; ref. ⁷). Monomer subunit chains are colored uniquely. The MIL is depicted with spheres in the latter structure of WT heptamer. (C) A backbone alignment of two adjacent PA monomers, chains A and B, called conformation A (red) and B (blue), showing the displacement of D4. (D) Superimposed on a surface rendering of half an octamer is the square planar arrangement of symmetrically related A and B conformers calculated from the positions of each chain's center of mass. Chains A, C, E, G are conformation A; and chains B, D, F and H are conformation B. Adjacent A-B pair center of masses are $64.3 (\pm 0.1) \text{ \AA}$ apart at angles of $90 (\pm 0.1)^\circ$. Domains are colored as: D1' (magenta), D2 (green), D3 (gold), and D4 (blue). (E) An A-B oligomerization interface split apart to compare relative differences in surface area burial at the oligomerization interface of the heptamer and octamer structure among the four domains. The domains (upper panel) are colored as in panel D. The relative degrees of surface area buried (lower panel) are colored as follows: surface buried equally (i.e., to within 10%) in either structure (green); surface buried 10% more buried in the heptamer (red); surface buried 10% more in the octamer (blue); and surface buried <75% in both structures (white). All molecular graphics were rendered using CHIMERA.⁶⁴

Fig. 5. Octameric and heptameric PA stability and activity

Negative-stained EM class-average images of PA heptamers and octamers before and after a reduction of pH and/or change in the temperature and solvent. The initial condition in panel A and B is pH 8, 0 °C. **(A)** Heptameric and octameric PA Δ MIL before (left) and after (right) exposure to pH 5.7, 7% ethanol, 4 °C. Before exposure, 25% octamer, 75% heptamer, $n = 10409$. After exposure, 89% octamer, 11% heptamer, $n = 14516$. **(B)** Heptameric and octameric WT PA oligomer complexes with LF_N before (left) and after (right) exposure to pH 7, 37 °C. Before exposure, 28% octamer, 73% heptamer, $n = 8409$. After exposure, 91% octamer, 9% heptamer, $n = 1084$. Relative percentages are given by the bars on the right for heptamers (black) and octamers (red). **(C)** Ensemble protein translocation records measured using planar lipid bilayer electrophysiology. Panels compare the relative translocase activities two types of samples: QPA+LF_N, which is >95% heptameric (black); and α PA+ LF_N, which had been purified and shown to contain ~90% octamer (red). Four substrates (LF, LF_N, EF, or EF_N) were used, and each was translocated at the indicated $\Delta\psi$ and Δ pH conditions. Records shown are the average of a set of three repetitions. The y-axes are normalized to the fraction of substrate-blocked channels that become unblocked due to translocation. Rates are given as $t_{1/2}$ (time for half of the substrate to translocate). LF translocated at $\Delta\psi = 30$ mV, Δ pH = 1 using heptameric ($t_{1/2} = 57$ s) and octameric PA ($t_{1/2} = 96$ s). LF_N translocated at $\Delta\psi = 50$ mV using heptameric ($t_{1/2} = 16$ s) and octameric PA ($t_{1/2} = 12$ s). EF translocated at $\Delta\psi = 50$ mV, Δ pH = 1 using heptameric ($t_{1/2} = 50$ s) and octameric PA ($t_{1/2} = 66$ s). EF_N translocated at $\Delta\psi = 60$ mV using heptameric ($t_{1/2} = 137$ s) and octameric PA ($t_{1/2} = 135$ s). **(D)** (Right) Single-channel translocations of LF_N at 50 mV through either a large- (red) or small-sized (black) channel. Black arrowheads on either end of the translocation indicate the beginning and end of each translocation. (Left) Histogram profiles of portions of the conductance levels of the large- and small-sized channels for comparison of the open-channel conductance levels.

Fig. 6. Heterogeneous assembly mechanism may modulate toxin activity

(A) On cells, PA may encounter dimeric ATR sites and assemble into PA₂ and PA₄ intermediates. Intermediates can combine to form either PA₈ or PA₇, which can load with EF and/or LF. (In principle, LF and EF may be involved in the mechanism as well to produce similar outcomes.) During extracellular or ATR-independent assembly, PA may encounter LF or EF, making the intermediates, PA₂LF_N and PA₄(LF_N)₂, which then form either PA₈(LF_N)₄ or PA₇(LF_N)₃. Models of LF-PA complexes are derived from a theoretical model.⁷⁰ Toxin activity is a combination of the oligomer's stability and translocase activity; instability may lead to the formation of inactive oligomeric complexes (*).