Published in final edited form as:

J Mol Biol. 2010 June 25; 399(5): 741–758. doi:10.1016/j.jmb.2010.04.041.

Role of the protective antigen octamer in the molecular mechanism of anthrax lethal toxin stabilization in plasma

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Abstract

Anthrax is caused by strains of *Bacillus anthracis* that produce two key virulence factors, anthrax toxin (Atx) and a poly- γ -D-glutamic acid capsule. Atx is comprised of three-proteins: protective antigen (PA) and two enzymes, lethal factor (LF) and edema factor (EF). To disrupt cell function, these components must assemble into holotoxin complexes, which contain either a ring-shaped homooctameric or homoheptameric PA oligomer bound to multiple copies of either LF and/or EF, producing lethal toxin (LT), edema toxin, or mixtures thereof. Once a host cell endocytoses these complexes, PA converts into a membrane-inserted channel that translocates LF and EF into the cytosol. LT may assemble on host cell surfaces or extracellularly in plasma. We show that under physiological conditions in bovine plasma that LT complexes containing heptameric PA aggregate and inactivate more readily than LT complexes containing octameric PA. LT complexes containing octameric PA possess enhanced stability, channel forming activity, and macrophage cytotoxicity relative to those containing heptameric PA. Under physiological conditions, multiple biophysical probes reveal that heptameric PA can prematurely adopt the channel conformation, but octameric PA complexes remain in their soluble prechannel configuration allowing them to resist aggregation and inactivation. We conclude that PA may form an octameric oligomeric state as a means to produce a more stable and active LT complex that may circulate freely in the blood.

Keywords

channel formation; electron microscopy; ion channel electrophysiology; mass spectrometry; synchrotron radiation circular dichroism spectroscopy

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Introduction

Bacterial pathogens utilize highly evolved systems to translocate protein effectors into host cells. Anthrax toxin¹ (Atx) is an elegant example of such a translocation system, and it is a key virulence factor produced by pathogenic strains of *B. anthracis*. Atx consists of three nontoxic proteins²⁻⁴ genetically encoded on a virulence island of the plasmid, pXO1.⁵ These proteins are secreted by vegetative *B. anthracis* in response to mammalian-host cues, such as bicarbonate and physiological body temperature.⁶ Atx is responsible for imparting host cell damage,^{7,8} disabling immune system function,⁸ facilitating bacteremia,⁹ and eventually causing host death, even after an infection is cleared with antibiotics.^{8,10}

Protective antigen (PA₈₃) is an 83-kDa, cell-binding component of Atx that ultimately forms a translocase channel capable of delivering the other two enzyme components, lethal factor (LF) and edema factor (EF), into the cytosol of a host cell. LF is a 90-kDa, Zn²⁺-dependent protease,¹¹⁻¹³ which cleaves host mitogen-activated protein kinase kinases.^{11,12} While PA and LF are individually nontoxic, the combination of LF and PA creates lethal toxin (LT), which can cause death.⁸ EF is a 89-kDa, Ca²⁺/calmodulin-activated adenylyl cyclase,¹⁴⁻¹⁶ which increases the cellular pool of 3'-5'-cyclic adenosine monophosphate (cAMP) in the host up to ~200-fold.¹⁴ In an analogous manner, PA and EF combine to form edema toxin, which can cause tissue swelling^{7,8} and lead to death in a manner distinct from LT.¹⁰

To achieve cytotoxicity, PA, LF, and EF must first self-assemble into holotoxin complexes. In one possible assembly mechanism, PA forms complexes on the surface of host cells in a receptor-dependent manner. PA first binds to one of two known Atx receptors (ANTXR).^{17, 18} PA is then cleaved by a furin-type protease to make the proteolytically-activated form, called _nPA. After a 20-kDa portion of _nPA (PA₂₀) dissociates, the remaining 63-kDa (PA₆₃), receptor-bound portion assembles into a mixture of ring-shaped heptameric (PA₇)¹⁹⁻²³ and octameric (PA₈)²⁴ oligomers. These prechannel oligomers can bind up to three and four LF and/or EF molecules, respectively.^{22,24} The complexes are endocytosed²⁵ and brought to an acidic compartment.²⁶ The PA oligomer then transforms into a translocase channel,²⁷ allowing the transmembrane proton gradient to drive LF and EF translocation^{20,28} into the cytosol, where LF and EF can catalyze reactions^{13,16} that disrupt the host cell.

A second key virulence factor of *B. anthracis* is the poly- γ -D-glutamic acid^{29,30} (γ -DPGA) capsule surrounding the bacillus. Strains deficient in the pXO2 plasmid,³¹ which encodes the machinery³²⁻³⁶ that produces and exports γ -DPGA, have significantly reduced virulence.³⁷ Long, linear γ -DPGA chains (~50 kDa) can be isolated from vegetative *B. anthracis* grown in vivo.³⁸ For *B. anthracis*, the genes responsible for producing the γ -DPGA polymer,³² anchoring it to the peptidoglycan cell wall,³⁵ and cleaving it into smaller dissociable fragments,³³⁻³⁶ augment its virulence perhaps through a variety of mechanisms, namely allowing the bacillus to resist complement^{33,39} and phagocytosis.³⁶ Interestingly, during infection lower-molecular-weight capsular fragments (L-capsule) are released from the bacillus by the hydrolytic enzyme dep, or capD.³³⁻³⁶ These L-capsule γ -DPGA fragments can bind to PA, and they co-purify with PA isolated from anthrax-infected blood.⁴⁰ Nonetheless, it is unknown whether γ -DPGA interacts with assembled LT complexes and whether this interaction may affect LT assembly, stability, or activity.

In the blood of infected animals at the later stages of anthrax, there is sufficient PA (80-100 μ g/ml),⁴¹ LF (10-20 μ g/ml),⁴¹ and PA-activating proteolytic activity^{24,42,43} to allow for LT assembly. The concentrations of these toxin components vary, however, depending upon the host species,⁴¹ and the stage of the disease.⁴⁴ Also increased levels of bacteremia tend to correlate with increased levels of these Atx components and L-capsular γ -DPGA.⁴⁴ With these high levels of Atx components and the presence of proteolytic activity capable of activating

PA, we have proposed that there is a receptor-free Atx assembly pathway in the blood, which is parallel in certain respects to the cell-surface pathway.²⁴ Animal infection models show that PA is rapidly cleared in the bloodstream, implying that the toxin has a finite lifetime there and competes with various host clearance mechanisms.⁴³ Thus toxin stabilization mechanisms would allow the toxin to persist and remain efficacious in the plasma and other extracellular environments.

PA₇ complexes, nevertheless, are unstable at physiological temperatures and pH,²⁴ where they readily convert to an inactive, aggregated state.^{24,45} How do LT complexes endure the conditions encountered in blood at physiological pH and temperature? PA₈ complexes have more favorable characteristics than their PA₇ counterparts, as they can remain soluble at physiological pH and temperature,²⁴ albeit it is unknown whether PA₈ complexes can form in plasma. Plasma is a complex, crowded environment, and it is unclear what the relative half lives of PA₇- and PA₈-containing LT complexes will be there. Developing an understanding of the physiological stabilities and molecular configurations of anthrax lethal and edema toxins is, therefore, of paramount importance to understanding their function throughout the stages of anthrax infection.

Results

PA₇ and PA₈ complexes assemble in bovine plasma

Previous studies have shown that PA complexes can assemble in plasma,²⁴ because sufficient proteolytic activity is present in bovine plasma to form proteolytically-nicked PA (nPA). Using a His₆-tagged PA construct, we assembled PA in defibrinated bovine plasma at 37 °C, purified the resulting complexes, and characterized their oligomeric composition using electron microscopy (EM) (Fig. 1a). The resulting micrographs were analyzed by crystal-structure-referenced alignment and classification. Prior experiments revealed that reference-free image analysis and this crystal-structure-referenced image analysis yield similar percentages of the two oligomers.²⁴ Based upon this analysis (Table S1), we find that plasma-assembled PA contains both the PA₇ and PA₈ oligomers (Fig. 1b). We found that assembly reactions conducted at pH 8 resulted in a predominately heptameric oligomer population; however, at pH 7.3, a predominately octameric oligomer population was observed (Fig. 1b). Also we found that EM images taken of the pH-7.3 assembly reactions contained large aggregates and particles that were more consistent with the elongated shape and size of the PA channel²⁰ (Fig. S1). Thus we conclude that there is a pH-dependent shift in the oligomer population, which favors the octameric form at pH 7.3.

PA₇ complexes aggregate more readily than PA₈ complexes in bovine plasma

The prior experiment suggests that heptamers may aggregate at pH 7.3, causing the measured shift in the composition of the oligomers. To further test this notion, we challenged pre-assembled PA complexes in plasma at pH 7.3 and 8.0. Our pre-assembled complexes (called His₆-nPA-LF_N) were 74% PA₇ and 26% PA₈ (Table S1). We then incubated this His₆-nPA +LF_N mixture for 5 minutes at 37 °C in defibrinated bovine plasma, adjusted to either pH 7.3 or pH 8.0. At pH 8.0, we found that PA₇ complexes were the predominant form (Fig. 1c), consistent with the previous assembly experiment (Fig. 1b). However, at pH 7.3, PA₈ was the predominant species among the soluble complexes (Fig. 1c). Large aggregates were also observed more frequently under the lower pH condition. We conclude that PA₈ is better able to resist aggregation than PA₇ in bovine plasma under physiological pH and temperature conditions (pH 7.3, 37 °C).

The stability of LT's cytotoxicity activity depends upon PA's oligomerization state

We next examined the stability of LT's macrophage cytotoxicity activity, using purified heptameric (PA₇LF₃) and octameric (PA₈LF₄) LT. Our enriched PA₇LF₃ sample contained 98% PA₇ and 2% PA₈,²⁴ and our enriched PA₈LF₄ sample contained 69% PA₈ and 31% PA₇ (Table S1). Immortalized bone-marrow macrophages were then incubated with increasing amounts of PA (0.01-100 µg/mL) and constant LF (1 µg/mL) and assayed for lytic activity. PA₇(LF)₃, PA₈(LF)₄, and mixtures of the unassembled monomers (LF plus PA₈) all possess similar macrophage cytotoxicity activity (Fig. 2a).

However, when these LT complexes were challenged at 37 °C in either pH-7.3 buffer or pH-7.3 defibrinated bovine plasma, their cytotoxicity changed markedly. For the PA₇(LF)₃ complex, the challenge incubation caused an ~10-fold loss of cytotoxicity with a half-life of ~2 minutes (Fig. 2b, c). However, the PA₈(LF)₄ complex exhibited a significantly longer half-life, losing ~10-fold of its activity with ~12- and ~45-minute half-lives for buffer alone and buffered bovine plasma, respectively (Fig. 2b, c). We also found that PA₇LF₃ complexes were as susceptible to inactivation when incubated in either 10% BSA, bovine plasma or buffer alone (Fig. S2). The activities of monomeric PA and LF were not affected under these incubation conditions. Interestingly, when PA₇LF₃ was incubated in bovine plasma at 42 °C, it lost 1000-fold of its activity with a 10-minute half-life (Fig. 2c); but under identical conditions, PA₈(LF)₄ only lost 10-fold of its activity with a 10-minute half-life (Fig. 2c). We conclude that PA₈(LF)₄ complexes have a more thermostable and robust macrophage cytolysis activity than PA₇(LF)₃ complexes.

The stability of PA's channel-forming activity depends on its oligomerization state

We then asked how well PA₇(LF_N)₃ and PA₈(LF_N)₄ complexes insert into planar lipid bilayers (PLB) following a challenge at 37 °C, pH 7.4. Steady-state current levels were recorded for PA₇(LF_N)₃ or PA₈(LF_N)₄ complexes before and after a timed incubation at 37 °C, pH 7.4. The normalized fraction of inserted channels, *f*, was determined for each type of oligomer by measuring the ratio of steady-state currents obtained from incubated and non-incubated samples (Fig. 3a). The ratio of octamer to heptamer insertion efficiency (*f*_{PA8}/*f*_{PA7}) is plotted in Fig. 3b. Within the first few minutes of incubation, the PA₇ complexes lost their ability to insert, but the PA₈ complexes remained active (Fig. 3b). Overall PA₈ complexes exhibited ~15-fold greater insertion efficiency than their PA₇ counterparts (Fig. 3b). We assume that this experiment underestimates the difference in the stability of the channel-forming activity, since the PA₇ and PA₈ samples are not 100% pure. We conclude that the PA₇ complexes are rapidly inactivated under physiological conditions, while PA₈ complexes maintain a consistent level of channel-insertion activity even following an extended incubation at 37 °C, pH 7.4.

BSA and LF_N augment PA channel-forming activity

We then asked whether the observed losses in PA-insertion activity could be preserved by the including 10% BSA in the incubation reaction (Fig. S3a). When 10% BSA was included in the incubation, the insertion rate actually slowed by a factor of two, relative to the standard (Fig. S3a). The specific insertion activity, however, increased by a factor of two. We also found that PA insertion activity was augmented ~2-fold for PA₇(LF_N)₃ co-complexes relative to unliganded PA₇ complexes (Fig. S3b). Therefore, PA channel insertion activity is stabilized when liganded with either specific or non-specific binding partners.

EM analysis of PA₇ and PA₈ complexes as a function of pH

Previously, we found that as soluble PA₇ complexes were depleted from mixtures of PA oligomers, large aggregates appeared. Here we examined, in finer detail, the pH dependence of the correlated disappearance of the two different PA oligomers and appearance of the

aggregates. Purified n PA-LF_N complexes (containing a roughly 2:1 mixture of PA₇ and PA₈ complexes) were incubated at 37 °C for 5 minutes across a range of pHs, and then the complexes were imaged by negative-stain EM (Fig. 4a). Aggregates first appeared at pH 7.4; and these aggregates increased in size until large precipitates were observed below pH 6.5. Free, resolvable PA prechannel complexes were selected at each pH (down to pH 6.5, which was the lowest pH individual complexes could be identified). We generated class-average images of the PA₇ and PA₈ complexes at each pH (Fig. 4a). A plot of the relative fraction of PA₇ and PA₈ complexes versus pH reveals a steep, sigmoidal relationship with a pH midpoint of 7.7 (Fig. 4b). By pH 7.4, only trace levels of PA₇ complexes were observed, and >90% of the remaining particles were PA₈ complexes (Table S1). We also measured the average number of soluble PA₈ and PA₇ complexes per micrograph (Fig. S4). The average number of PA₈ and PA₇ complexes was consistent from pH 9.0 to pH 8.0; however, a steep transition occurred below pH 8.0, corresponding to an abrupt decrease in the number of PA₇ complexes per micrograph (Fig. S4). The number of complexes was relatively constant from pH 7.4 to 6.5, as was their composition, which was >90% PA₈ (Fig. 4b). Starting at pH 6.5, we found, however, that the number of soluble PA₈ complexes began to decrease, reaching undetectable levels, when the pH was less than 6.0 (Fig. 4b, S4).

Does the observed pH-dependent aggregation of PA-LF_N complexes result from PA prematurely adopting the channel state? PA channels are discernable by EM,²⁰ and we found that even at pH 9, similar elongated structures, corresponding to the PA channel state appeared in our EM micrographs, albeit they were observed infrequently (Fig. 4a). To better image PA channels, we found that the addition of 10% BSA minimized their aggregation (Fig. 4c) and improved our identification of mono-dispersed channel-shaped particles. These solution conditions revealed similar proportions of soluble octameric and heptameric prechannels when compared with samples prepared in the absence of BSA (Table S1; Fig. 4c). Interestingly, we found that our 2D-class-average images (Fig. 4c) were consistent with previous images of heptameric channels.²⁰

We then estimated the percentages of the oligomers that adopt the channel state under these conditions (pH 7.4, 37 °C, 10% BSA). First, we employed reference-free alignment, classification, and averaging to generate class-averages of the observed elongated channel particles and the axially-oriented rings. We identified four classes, consistent with PA₇ and PA₈ prechannels, elongated channel structures, and contracted PA₇ rings, comprising 11.3, 35.1, 6.1, and 47.4% of the population, respectively. While it is assumed the elongated structure represents a sagittal section of the PA channel, we could not determine the precise subunit stoichiometries of these images. Using SPIDER,⁴⁶ we instead analyzed the axial classes, which comprised 93.1% of the population, and we determined the centers of each subunit in the respective axial views of each oligomer class. From these values, we calculated the center of mass of the complex and the mean radius from each subunit to the center of the complex. For PA₇ and PA₈ prechannel complexes, we find that these mean radii are 33.7 (±0.8) Å and 39 (±1) Å, respectively. However, when the complexes were incubated at 37 °C, pH 7.4, 10% BSA, the mean radii of the three different axially-oriented oligomer classes, (i) contracted PA₇, (ii) uncontracted PA₇, and (iii) PA₈, were 27 (±2) Å, 33 (±2) Å, and 39 (±2) Å, respectively. The mean-radius metric for the heptameric class, but not the octameric class, contracts significantly by 7 (±2) Å. The contraction of the PA₇ rings to 27 Å is consistent with the contraction observed for the heptameric channel state.²⁰ Since the PA₈ rings do not contract, we do not assume that they form the channel state under these conditions. Of the total population of prechannel and channel states, PA₇ channels are observed 53.5% of the time; however, the channel classes we observe are predominantly heptameric. The 10% BSA additive modestly stabilizes the heptameric prechannel, since it represents approximately 10% of the sample, following the 5-minute, 37-°C incubation. Thus we conclude that PA₇ readily converts to the

channel state at pH 7.4, 37 °C; and, while BSA minimizes aggregation, it does not prevent PA heptamers from converting to the channel state.

Probing PA channel conversion with circular dichroism (CD) spectroscopy

We then used CD spectroscopy to probe PA's structural transition to channel state. Upon lowering the pH from 7.5 to 6.5, the CD signal at 222 nm (CD_{222}) for PA₇ and PA₈ prechannels increased ~10-20% (Fig. 5a, b, S5). The time dependence of this increase is rapid with respect to the drop in pH (occurring on a timescale of ~1 s). Control experiments on monomeric PA₈₃ (Fig. 5a) confirmed that the CD_{222} signal change is oligomer-specific, and we hypothesize that the CD_{222} signal change corresponds to the pH-dependent prechannel-to-channel transition.

To analyze the nature of the pH-dependent CD signal change, we obtained synchrotron radiation circular dichroism (SRCD) spectra at various pHs. SRCD, as opposed to conventional CD spectroscopy, enables for higher quality far UV CD measurements in the high salt conditions required, thereby facilitating the determination of more accurate secondary structure values. We found that NaF (the preferred salt in CD measurements) could be used during PA₈₃ monomer measurements, but the F⁻ ion caused the PA₇ oligomer to precipitate, and consequently, we used NaCl in all PA oligomer measurements. SRCD spectra of PA₈₃ and PA₇ were obtained at pH 7.5 and pH 6.5 (Fig. 5a). The helix and sheet content deduced from the spectrum of PA₈₃ was 9% and 37%, respectively, in close agreement with its crystal structure.²³ The spectrum also did not change as a function pH (7.5 or 6.5) or salt (NaF or NaCl). Analyses of the SRCD spectra of PA₇ samples at pH 7.5 and pH 6.5 indicated an increase in ordered secondary structure content, i.e., one with more helix and sheet. From the analysis, PA₇ at pH 7.5 is 17% helix, 25% sheet, and 33% loop or coil; and PA₇ at pH 6.5 is 22% helix, 27% sheet and 30% loop/coil. We conclude that coil/loop structures in the prechannel convert to sheet and helix structures in the channel.

To verify that the CD-detected increases in helix and sheet structure corresponded to the conversion of the prechannel to the channel state, we measured the pH dependence of the SRCD spectra of PA₇ in the presence of the extracellular, soluble domain from human anthrax receptor domain, sANTXR2 (Fig. S6). The SRCD spectral change is only evident when the pH is lowered to 5.5, consistent with previous studies.¹⁹ The pH-6.5 spectrum for sANTXR2-liganded heptamer is similar to that observed at pH 7.5, and thus the binding of sANTXR2 to PA shifted the pH dependence of the SRCD spectrum shift by approximately one unit (Fig. S6). We conclude that the pH-dependent CD-signal change corresponds to the prechannel-to-channel transition.

We then compared the pH-dependent CD_{222} signal change for PA₇(LF_N)₃ and PA₈(LF_N)₄ complexes at 25 °C and 37 °C. The change in the CD_{222} signal occurred at distinct pH thresholds for PA₇(LF_N)₃ and PA₈(LF_N)₄ complexes (Fig. 5c, S5). At 25 °C, both oligomers show a similar increase in CD_{222} signal at pH 7.0. However, at 37 °C, PA₇(LF_N)₃ complexes exhibit an increase in CD_{222} at pH 7.4, whereas PA₈(LF_N)₄ complexes show a similar increase in CD_{222} signal at pH 7.0 (Fig. 5c). We conclude that PA₈(LF_N)₄ complexes are more pH-resistant to conversion to the channel state at 37 °C.

Mass spectrometry studies of pH-dependent changes in PA-LF_N complexes

We then used nanoelectrospray ionization mass spectrometry (nanoESI-MS) to monitor how pH affected the conformation, stability and solubility of PA₇ and PA₈ complexes. A 2:1 mixture of PA₇(LF_N)₃ and PA₈(LF_N)₄ complexes was diluted into buffers, ranging from pH 8.0 to 6.2. The mass spectrum acquired at pH 8.0 exhibits charge state distributions for LF_N, nPA, and the complexes, PA₇(LF_N)₂, PA₇(LF_N)₃, and PA₈(LF_N)₄ (Fig. 6). We also observed a 63-kDa

species consistent with the mass expected for n PA monomer that has dissociated into free PA_{63} and free PA_{20} species. This 63-kDa species was determined to be produced by gas-phase dissociation of n PA, consistent with a weak noncovalent interaction between PA_{63} and PA_{20} . The relatively low average charge for this ion (10.2+), compared to that for the n PA ion (17.8+), is the result of asymmetric charge partitioning.^{47,48} The spectrum is, however, dominated by signal for $PA_7(LF_N)_3$, where the remaining ~20% relative abundance corresponds to the $PA_8(LF_N)_4$ complex, which is partially obscured by overlap with peaks from the heptameric distribution. A partial complex, $PA_4(LF_N)_2$, was observed in all of the spectra at low abundances.

The spectrum acquired at pH 7.6 (Fig. 6) exhibits the same monomer distributions with no significant change in the average charge states (Table S2). In contrast, there is a dramatic decrease in the relative abundance of $PA_7(LF_N)_3$ concomitant with a slight increase in the average charge (43.1+ to 43.4+); and the $PA_7(LF_N)_2$ complex is fully depleted. The relative abundance of $PA_8(LF_N)_4$ increases dramatically, but neither its absolute abundance nor its average charge changes significantly (46.6+ to 47.1+). A distribution of ions for $PA_8(LF_N)_3$ is observed, which may be due to loss of a single LF_N from $PA_8(LF_N)_4$ in solution or may be visible at this pH due to the loss of signal for the adjacent $PA_7(LF_N)_3$ distribution. The loss of signal for $PA_7(LF_N)_3$ at this pH and temperature (37 °C) is consistent with results obtained by EM (Fig. 4a,b, S4) and CD (Fig. 5), likely corresponding to the prechannel-to-channel transition.

At pH 7.2, the observed average-charge states for the protein monomer's mass spectra do not change significantly (Fig. 6, Table S2). The population of $PA_7(LF_N)_3$ is fully depleted while higher charge ions for $PA_8(LF_N)_3$ and $PA_8(LF_N)_4$ are observed, causing an increase in the average charge of the distributions to 47.9+ for both complexes (Table S2). The small decrease in the relative abundance of $PA_8(LF_N)_4$ may be due to the lower overall signal-to-noise ratio observed for all of the charge state distributions in this spectrum. Significantly, the charge state distributions for $PA_8(LF_N)_3$ and $PA_8(LF_N)_4$ increase from 47.9+ to 49.0+ and 50.1+, respectively, in the spectrum acquired from a pH-7.0 solution (Fig. 6). This change is not observed for the monomer charge state distributions in this spectrum, where the average charge for LF_N is unchanged (10.4+), n PA increases from 17.6+ to 17.9+ (note that at pH 8.0, n PA has an average charge of 17.8+), and PA_{63} increases from 10.3+ to 10.4+ (Table S2). In the spectra acquired with solutions at pH 6.8, 6.6, and 6.4, no significant changes were observed in the average charges of the monomer proteins or complexes (Table S2), while we observed a continual decrease in the signal-to-noise ratio of the complexes, but not the monomers. No signal was observed for the complexes in the pH-6.2 solution, while the monomers appeared unchanged (Table S2). Thus we conclude that the increase in average charge observed for octamer complexes represents either a soluble form of the PA_8 channel or a soluble intermediate formed as PA_8 prechannel converts to the channel state.

The heptamer, but not the octamer, produces detergent-resistant aggregates

We used the sodium-dodecylsulfate-(SDS)-resistance assay—an established method to detect PA channel formation²⁷—to corroborate the pH/temperature dependencies of prechannel-to-channel conversion for the two different oligomers. In the SDS-resistance assay, $PA_7(LF_N)_3$ and $PA_8(LF_N)_4$ prechannel complexes were incubated for 1 hour at either room temperature or 37 °C in solutions buffered from pH 8 to 5. The SDS-treated samples were then separated by SDS-polyacrylamide gel electrophoresis (PAGE). The resulting gels showed two major bands: (i) the lower, higher-mobility band corresponds to the dissociated monomer, PA_{63} ; and (ii) the upper, low-mobility band, is the SDS-resistant PA channel²⁷ (Fig. 7). We reproduced the previous results, finding that incubating heptameric prechannel preparations at 25 °C causes an SDS-resistant PA channel band to appear when the pH ≤ 7 .²⁷ However, when we repeated

the experiment on the heptameric form at 37 °C, we found that the SDS-resistant band began to appear at higher pHs ($\text{pH} \leq 7.4$; Fig. 7a). We then tested the PA octamer, and found that it did not fully convert into an SDS-resistant species at 25 or 37 °C (Fig. 7b). The residual, SDS-resistant band in $\text{PA}_8(\text{LF}_N)_4$ lanes likely corresponds to the 5-10% $\text{PA}_7(\text{LF}_N)_3$ contamination. We conclude that at 37 °C, PA_7 converts to the SDS-resistant state at pH 7.4, while PA_8 does not.

Discussion

Reports⁴⁹ on inhalational anthrax infections reveal that *B. anthracis* spores are first taken up by alveolar macrophage cells in the lungs; the spores are then transported by macrophage cells to lymph nodes in the mediastinum, where they germinate, allowing vegetative *B. anthracis* to ultimately disseminate into the blood. Blood is a complex mixture of plasma, immune cells, red blood cells, and platelets. The plasma fraction of blood is the liquid component, which constitutes more than half of the blood's total volume and consists mainly of water, electrolytes, and high concentrations of proteins, including albumins, immunoglobulins, proteases, and factors required for clotting. Here in this complex, crowded environment, *B. anthracis* secretes the three protein components that ultimately constitute anthrax lethal and edema toxins.

Analyses of blood taken from anthrax-afflicted animals in the latest stages of anthrax infection reveal a relatively high concentration of LT components and lower molecular weight γ -DPGA fragments.⁴¹ The PA and LF components are at 80-100 $\mu\text{g}/\text{ml}$ and 10-20 $\mu\text{g}/\text{ml}$ concentrations, respectively.⁴¹ Due to the presence of a serum protease, most of the PA is proteolytically-activated and potentiated for co-assembly with LF.^{42,43} These conditions suggest that LT complexes can assemble in lymph and plasma prior to reaching the cell surface, as we have demonstrated *in vitro* in bovine plasma.²⁴ Studies of PA proteolysis and clearance in blood show that PA is rapidly cleared,⁴³ implying that they have a finite lifetime and must endure the conditions encountered in the bloodstream long enough to reach cell surfaces. Of course, once PA complexes bind to their cell-surface receptors, they are stabilized and premature channel conversion is attenuated.¹⁹ However, it is unknown how soluble forms of assembled LT, which are found in the blood, are stabilized. Here we report the thermostabilities and half-lives of LT complexes, which contain either the PA_7 or PA_8 oligomer. We propose that a critical mechanism for LT stabilization involves the ability of PA to assemble into the more stable octameric complex.

PA_8 is the preferred oligomerization state in bovine plasma

We have shown that PA_7 and PA_8 complexes form on cell surfaces in roughly a 2:1 ratio,²⁴ however, the ratio of these two different PA oligomers in plasma is unknown. In bovine plasma at pH 7.3, we find that the PA_7 : PA_8 ratio favors PA_8 30:70; but at pH 8, PA_7 is favored 70:30 (Fig. 1b). The pH-dependent shift in favor of PA_8 may result from several possible mechanisms: (1) The assembly rate of octamers is faster at physiological temperature and pH; or (2) the two oligomers interconvert; or (3) the heptameric complexes are inactivated through a pH-dependent process. The first possibility is unlikely as mass spectrometry studies have shown that heptamers and octamers form at similar rates.²⁴ The second possibility is also implausible as it has been found that oligomers do not readily exchange monomers.⁵⁰ The third possibility involving a pH-dependent inactivation of PA_7 complexes is more likely based upon our prior assembly results in buffer²⁴ and in plasma (Fig. 1). This pH-dependent process manifests in the formation of large aggregates, containing inactivated PA complexes (Fig. S1). Thus in plasma under physiological conditions (pH 7.3, 37 °C), the octameric oligomerization state is the predominant form, because it is able to resist aggregation, unlike its heptameric counterpart (Fig. 1, S1).

The stability of LT activity in bovine plasma

Previous reports suggest that LT complexes can form in the blood of infected animals,^{7,40} and this *in vivo* form of the toxin is more potent than that isolated *in vitro* from *B. anthracis* cultures.⁷ Knowing that the toxin can form in two unique configurations, we tested their individual stabilities in macrophage cytotoxicity assays. In agreement with our EM analysis, we found that the macrophage cytotoxicity activity of PA₇LF₃ LT was rapidly inactivated following a brief, 5-minute incubation in plasma at 37 °C (Fig. 2c). Conversely, PA₈LF₄ complexes have a longer lifetime in either pH-7.3 buffer or bovine plasma (Fig 2b, c). In buffer, PA₈LF₄ loses ~10-fold of its activity with a half-life of ~12 minutes [albeit this half-life was extended ~6 times longer than PA₇LF₃ under these conditions (Fig. 2b, c)]. Interestingly, in bovine plasma, the lifetime of PA₈LF₄ complexes is significantly prolonged. Only ~10-fold of its activity is lost with a half-life of ~45 minutes (Fig. 2c). Additionally, PA₈LF₄ LT complexes are even more resilient than PA₇LF₃ LT complexes at elevated, fever-like temperatures (42 °C). Since our most enriched samples of PA₈LF₄ contain ~30% PA₇LF₃ by EM (Table S1) and both PA₇LF₃ and PA₈LF₄ complexes have comparable LT activity prior to the challenge incubation (Fig. 2a), the observed loss of PA₈LF₄ LT activity may be attributed to PA₇LF₃ contamination. Therefore, our results underestimate the relative thermostability of PA₈LF₄ complexes. Consistently, the incubation of PA₇(LF_N)₃ in pH-7.4 buffer causes a rapid decrease in its PLB-insertion activity (Fig. 3a), but PA₈(LF_N)₄ complexes retain ~15-fold more PLB-insertion activity under identical conditions (Fig. 3b). Therefore, PA₈ complexes maintain a more robust macrophage-lysis, channel-forming, and translocase activity than PA₇ complexes. We conclude that PA's octameric oligomerization state is a means to stabilize receptor-free LT complexes, allowing active LT complexes to circulate freely in the blood.

Premature channel formation is the molecular basis for heptameric LT inactivation

At physiological pH and temperature, PA complex aggregation (Fig. S1) correlates with a rapid, irreversible loss of macrophage-cytotoxicity (Fig. 2c) and PLB-insertion (Fig. 3) activities. PA₈ complexes, however, are less susceptible than PA₇ complexes to inactivation under these conditions. What molecular mechanism best explains these differences? We found that elongated, channel-shaped structures were observed alongside aggregates in plasma samples (Fig. S1), suggested that conversion to the channel state may facilitate aggregation and inactivation of PA₇ complexes (Fig. 2c, 3). To corroborate this molecular mechanism, we measured the pH dependence of the prechannel-to-channel transition by several methods: EM (Fig. 4b), CD and SRCD (Fig. 5), SDS-resistance PAGE (Fig. 7a), and mass spectrometry (Fig. 6). The pH-dependency results of these various studies for the two different purified PA oligomers suggest the following hypothesis: PA₇ converts to the channel state at pH 7.4, while PA₈ may only convert to the channel state at pHs ≤ 7 (Fig. 8). We conclude that PA₈'s decreased pH-sensitivity to channel formation may allow it to persist stably in plasma (Fig. 9).

Insights into the mechanism of channel formation

PA channel formation is a pH-dependent process, likely involving the protonation of groups that drive the partial unfolding of certain regions of domain 2 in PA.^{19,27,51} In this model, a large-scale rearrangement occurs, requiring the unfolding of the β1 and β4 strands and dissociation of the loops that interact with residues in domain 4 of the neighboring subunit;¹⁹ β2, β3, and the membrane insertion loop then form the final β-barrel channel structure. By EM and crystallographic studies, the PA prechannel forms relatively compact heptameric and octameric rings with dimensions of 170 × 170 × 80 Å and 180 × 180 × 80 Å, respectively.^{19,24} A recent EM structure of the heptameric channel suggested that the large conformational change associated with the prechannel-to-channel transition produces an elongated structure with approximate dimensions of 50 × 50 × 170 Å.²⁰

Here we further analyzed structural changes in PA-LF_N complexes using nanoESI-MS. Owing to the gentle nature with which biomolecules are transferred to the gas phase, nanoESI-MS permits structural analysis of intact proteins⁵² and protein complexes.⁵³⁻⁵⁵ By nanoESI-MS, the observed pH-dependent shift in average charge in the mass spectra of PA₈(LF_N)₄ complexes at pH 7 is consistent with the putative structural rearrangement proposed for the prechannel-to-channel transition (Fig. 6, Table S2), which we have monitored using EM (Fig. 4b) and CD (Fig. 5c). Finally, we found that PA₈(LF_N)₄ may form a more soluble channel state, as its abundance did not decrease, as we observed with the PA₇(LF_N)₃ complex (Fig. 6). Despite the change in average charge observed in MS experiments, the PA₈(LF_N)₄ complexes showed minimal loss of their LF_N ligands, demonstrating that these complexes remain intact when PA forms the channel state. The mechanism of channel formation has remained elusive, due to PA's propensity to form aggregates,^{20,45} however, PA₈ oligomers form more soluble channel complexes that may be amenable to future mechanistic and structural studies.

The large-scale rearrangement of secondary structure upon channel conversion likely corresponds to a net-increase in β -sheet content. SRCD measurements can calculate the change in secondary structure content of the prechannel and channel states by providing spectral information in the far UV wavelengths due to the increased brightness of the synchrotron light source. Detection of CD signal at these lower wavelengths is critical to assigning secondary structure content.⁵⁶⁻⁵⁸ PA₇ complexes exhibited a pH-dependent increase in CD signal within the range 200-230 nm, which corresponded to increases in β (and surprising α) structure (Fig. 5a). However, this signal change only occurs in the oligomer, as pH-dependent increases in CD signal were not observed for monomeric PA₈₃. The pH dependence of the CD signal change also shifted one pH unit in the presence of sANTXR2 (Fig. S6), consistent with the known stabilization imparted by ANT XR binding.¹⁹ Previous studies concluded that these CD signal changes indicated that the channel state has more α -helical content than the prechannel state.⁴⁵ We also observed a large change in CD signal at wavelengths below 200 nm, which also corresponded to a loss of disordered coil structure, upon acidification of the PA oligomer (Fig. 5a). We conclude that the transition observed in the CD spectrum likely corresponds to omega loop/coil structures and disordered regions in the prechannel state^{19,23,24} that refold into β barrel structures in the channel state.^{20,51}

Specific and non-specific LT stabilization mechanisms

Atx components are secreted into a complex milieu of plasma components, including high concentrations of protein (~100 mg/ml), the most common of which are serum albumins. LT complexes have been shown to assemble in plasma,²⁴ but the effect of plasma components on their stability has not been studied. It is known that crowded environments, containing high concentrations of a protein, such as BSA, can stabilize folded proteins and protein complexes, preventing the aggregation of their unfolded states by reducing the diffusional mobility of partially unfolded forms.⁵⁹ Aggregation of the PA₇ channel was inhibited in the presence of BSA, resulting in discreet channel-shaped particles by EM (Fig. 4c). Interestingly, BSA also prevented losses in the PLB-insertion activity of pre-incubated PA-LF_N complexes (Fig. S3a). The channel-insertion rate decreased by a factor of two, suggesting that reduced diffusional mobility may play a role in the stabilization mechanism. The formation of LT complexes may also prevent aggregation, as PA₇(LF_N)₃ complexes insert more efficiently into PLBs than non-liganded PA₇ (Fig. S3b). This result indicates that fully assembled LT is more stable than partially assembled forms with substoichiometric loadings of LF. Perhaps BSA may stabilize partially unfolded PA intermediates that populate as PA converts to the channel state. The unfolding of PA's β -strands may expose surfaces, which BSA may bind, thereby impeding channel formation; however, it may also minimize PA inactivation and aggregation. The bacterial chaperone, GroEL, has been reported to bind PA channels in the D1' domain responsible for binding LF.²⁰ GroEL binding limited PA channel aggregation,²⁰ which is

consistent with the observation that the inclusion of LF_N increases PA channel formation activity. Thus LF, BSA, and other protein components in blood may specifically or non-specifically block competing inactivation processes such as premature channel formation and aggregation, albeit these mechanisms of stabilization are modest relative to the gain in stability imparted by PA₈ formation.

LT stabilization during anthrax pathogenesis

Why would anthrax toxin preferentially assemble into the shorter-half-lived, heptameric form? We have proposed that PA₇ complexes may form on cell surfaces to expedite endocytosis of intact PA complexes.²⁴ Another rationale for the formation of the heptameric LT may be that it allows for spatial control of toxin levels. During the initial stages of anthrax infection, bacterial replication is localized to various organs,⁴¹ facilitating bacteremia⁹ and suppression of the immune system.^{8,9} LT components initially exist at low levels in the blood.⁴⁴ We postulate that Atx may act locally to compromise the host's immune system, while simultaneously limiting systemic damage to the host and premature death. Therefore, heptameric complexes may act nearest to its sites of secretion in regions of more intense bacterial infection. Active, heptameric complexes may only be able to travel limited distances from the sites of infection due to their attenuated half-lives in plasma (Fig. 2c). Octameric complexes, on the other hand, persist for longer periods in plasma, allowing them to potentially impart cytotoxic effects further away from the sites of infection. Thus Atx may assemble into more stable and less stable forms of Atx to limit the highest intensities of cytotoxicity to regions immediately surrounding the sites of infection. Through this mechanism, zones of infection can be established and immune system function can be dampened without causing system-wide shock. The supply of the longer-half-lived octameric form will depend on the free concentrations of monomeric PA and LF. Indeed, at the terminal stages of an anthrax infection, high concentrations of LT, L-capsular γ -DPGA, and *B. anthracis* levels have been reported in the hours preceding death of the host. Thus as the infection intensifies, the stabilized form of the octameric toxin may then accumulate, inflicting more system-wide shock. Moreover, our results indicate that PA₇LF₃ complexes cannot persist under fever-like temperatures, but PA₈LF₄ complexes remain quite viable at elevated temperatures (Fig. 2c). Thus PA₈LF₄ complexes may provide a means for Atx to endure the elevated temperatures associated with natural host-responses to *B. anthracis* infection.⁶⁰ Finally, while PA octamer formation may provide a molecular mechanism for Atx stabilization in plasma, our studies do not preclude other potential stabilization mechanisms, such as PA's interaction with albumins, other chaperones, and the capsular material, γ -DPGA, which is also secreted by *B. anthracis* during infection.⁴⁰

Materials and Methods

Proteins

Recombinant WT PA₈₃, carboxy-terminally His₆-tagged PA₈₃ (His₆-PA₈₃),⁶¹ and all other PA mutants were over-expressed in the periplasm of the *E. coli* strain, BL21(DE3). The 83-kDa monomers were purified from the periplasm as previously described.⁶² Recombinant LF and LF_N (LF residues 1-263) were overexpressed from pET15b constructs⁶³ and then purified from the cytosol as previously described.⁶² Soluble human anthrax receptor domain, sANTXR2, from the capillary morphogenesis protein 2, CMG2, was expressed, purified, and liberated of its affinity tag as previously described.⁶²

Isolation of PA₇ and PA₈ complexes

Heptameric PA-LF and PA-LF_N complexes were produced using Q-sepharose-purified PA oligomers²⁴ (which are >90% heptameric by EM) and then forming complexes with a two-fold stoichiometric excess of LF or LF_N (LF/LF_N:PA). The resulting complexes were purified

by S400 gel filtration chromatography and judged >90% PA₇ by EM. PA₈LF₄ and PA₈(LF_N)₄ were prepared by assembling _nPA in the presence of either LF or LF_N, respectively; the resulting complexes were incubated for 5 minutes at 37 °C in 0.1 M sodium cacodylate pH 7 at ~2 mg/mL PA concentration; and then the incubated mixture was purified on an S400 gel filtration column.²⁴ The homogeneity of all samples were verified by EM and nanoESI-MS measurements as previously described.²⁴ The PA-LF_N complexes were consistently ~90% PA₈.²⁴ The purified PA-LF complexes were ~70% PA₈ (Table S1). The overall yield of enriched PA₈-containing complexes was ~10% for PA-LF complexes and 20-30% for PA-LF_N complexes.

Purification of bovine-plasma-assembled PA complexes

Defibrinated bovine plasma (Rockland Immunochemicals, Inc.) was adjusted to pH 8.0 or pH 7.3 with 0.1 M Tris or 0.1 M Hepes, respectively. His₆-PA₈₃ monomer and LF_N were mixed in a one-to-one molar ratio, at a final PA concentration of 100 µg/mL in defibrinated bovine plasma and incubated for 1 hour at 37 °C or 42 °C. After one hour, the assembled His₆-PA-LF_N complexes were mixed with 50 µL Ni²⁺-NTA-sepharose affinity resin (GE Healthcare, Inc.) for one hour at room temperature. The resin was washed with five volumes of Buffer M: 20 mM Tris, 0.15 M NaCl, 20 mM imidazole, pH 8, and eluted with one volume of Buffer M supplemented with 50 mM increments of imidazole to separate plasma proteins from PA-LF_N complexes, which eluted at ~150-200 mM imidazole. The purity of the eluted fractions was assessed by SDS-PAGE. The oligomeric composition was assessed by EM.

Electron microscopy

Aliquots of PA complexes were adjusted to 20-30 nM (with respect to the monomer concentration) by diluting in either Buffer E (20 mM Tris, 150 mM NaCl, pH 8) for PA-LF_N complexes or Buffer E + 0.01% dodecyl-β-D-maltopyranoside (Sigma-Aldrich, St. Louis, MO) for PA-LF complexes. The presence of the detergent allowed for imaging of predominantly axially-oriented LT complexes. 4 µL of sample was applied to a freshly glow-discharged 400 mesh formvar-carbon coated grid for 1 minute. The grid was then washed in 5 successive drops of water and stained for 1 minute with 2% uranyl acetate (Sigma-Aldrich, St. Louis, MO). Negative-stain EM images were recorded on a Tecnai 12 electron microscope (FEI Company, Hillsboro, OR) operated at 120 kV at a magnification of 49,000× using a CCD camera. The micrograph resolution was 2.13 Å/pixel. Particle images were selected using automatic or manual particle picking using boxer in EMAN.⁶⁴

Reference images of 2D-projections of low resolution density maps generated from the crystal structures of the PA₇ (PDB 1TZO)¹⁹ and PA₈ (PDB 3HVD)²⁴ prechannel oligomers were produced using SPIDER.⁴⁶ Boxed images of the PA oligomer particles were subjected to successive cycles of alignment, multivariate statistical analysis, and classification.^{65,66} The last classification step was done using only the lowest order eigenvectors⁶⁷ to separate the data by size and the heptameric and octameric oligomerization states. Final class-average images, consisting of 20-30 classes, were manually inspected and tabulated to determine the oligomeric composition of each sample (Table S1). Ambiguous classes (3-5%) were omitted from the analysis.

For the PA oligomer samples treated at 37 °C, pH 7.4 in 10% BSA, we could classify channel conformations as well. However, since a crystal structure reference is not available for the PA channel, we used reference-free alignment to initially generate 20-30 reference classes, of which 5-10 were used for subsequent rounds of alignment, classification, and averaging. This procedure was sufficient to separate prechannels from axial and sagittal views of the PA channel and allow the generation of class-average images. Using SPIDER, we estimated the radii of each subunit in the axial class average images to their center of mass. From the mean

of these radii per class, we determined that the heptameric oligomer class formed a more contracted conformation than is typically observed for prechannel complexes, where the subunit to center of mass decreased by 7 Å. We deduced that these contracted PA₇ axial views represented the channel state. The PA₈ particles in the axial orientation did not appear to contract.

LT macrophage cytotoxicity

LT cytotoxicity was monitored by an enzyme-coupled lactate dehydrogenase (LDH) release assay.⁶⁸ Immortalized bone marrow macrophages from 129 mice (a gift from the Vance Lab at UC Berkeley) were grown to confluence in RPMI 1640 medium (Invitrogen, Inc.) supplemented with 10% fetal bovine serum (Invitrogen, Inc.), 100 units/mL penicillin (Sigma-Aldrich, St. Louis, MO), and 100 µg/mL streptomycin (Sigma-Aldrich, St. Louis, MO) in a humid, 5%-CO₂ atmosphere at 37 °C. One day prior to conducting assays, cells were trypsinized and re-plated at a density of 10⁵ cells/well. Cells were treated with a constant amount of LF (1 µg/mL) and varying concentrations of either PA₈₃ or oligomeric LT complexes (0.01-100 µg/mL) in ice-cold PBS. The toxin-treated cells were then incubated for 4 hours at 37 °C. Triplicate assays were performed for each condition. The cells were then centrifuged at 1400 RPM. The supernatant was removed and incubated with 20 µL of lactate solution (36 mg/mL in phosphate-buffered saline) and 20 µL of *p*-iodonitrotetrazolium chloride (2 mg/mL in phosphate-buffered saline with 10% dimethyl sulfoxide). The enzymatic reaction was started by the addition of 20 µL of nicotinamide adenine dinucleotide (NAD⁺)/diaphorase solution (13.5 units/ml diaphorase and 3 mg/ml NAD⁺). After a 15-minute incubation, the products were observed using an spectrophotometric microplate reader (Bio-Rad Laboratories, Richmond, CA) at a wavelength of 490 nm. The change in the absorbance signal is proportional to the number of lysed cells, where the amount of LDH released was normalized to the value obtained in wells treated with 1% Triton X-100 detergent. Effective-concentration-for-50%-lysis values (EC₅₀) were determined by fitting the normalized cell-lysis-versus-PA-concentration data in ORIGIN6.1 (OriginLab Corp., Northampton, MA). The stability of the LT complexes was assessed by measuring the EC₅₀ values of the LT complexes after they were incubated at 37 °C or 42 °C for specified times in various buffer conditions.

Electrophysiology

Voltage-clamp electrophysiology experiments were performed using an Axoclamp 200B amplifier and AXOCLAMP acquisition software (Molecular Devices) as described.²⁴ Data analyses were performed using CLAMPFIT (Molecular Devices) and ORIGIN6.1. Relative membrane insertion activities for PA were assayed as follows. A planar lipid bilayer (PLB) was painted using a 3% solution of the lipid [1,2-diphytanoyl-*sn*-glycerol-3-phosphocholine (DPhPC; Avanti Polar Lipids, Alabaster, AL) dissolved in *n*-decane] inside either a 100-µm aperture of a 1-mL, white delrin cup bathed in universal bilayer buffer (UBB) (10 mM oxalic acid, 10 mM MES, 10 mM phosphoric acid, 1 mM EDTA, 100 mM KCl) at pH 5.5 or 6.6. PA oligomer complexes (0.1 nM) were added to the cis compartment, which was held at a membrane potential ($\Delta\psi$) of +20 mV and a proton gradient (ΔpH) of 1 unit. ($\Delta\psi \equiv \Delta\psi_{\text{cis}} - \psi_{\text{trans}}$, where $\psi_{\text{trans}} \equiv 0$ mV; $\Delta\text{pH} \equiv \text{pH}_{\text{cis}} - \text{pH}_{\text{trans}}$, where $\text{pH}_{\text{cis}} = 5.6$ and $\text{pH}_{\text{trans}} = 6.6$.) Channel insertion increased over a period of minutes and stabilized after 20 to 30 minutes, allowing the final current to be recorded.

The PLB-insertion activity of PA or PA-LF_N complexes was assessed before and after incubation at 37 °C in 0.1 M sodium cacodylate (Sigma-Aldrich, St. Louis, MO), pH 7.4. PA or PA-LF_N complexes were added to the cis compartment to a final concentration of 0.1 nM. Channel insertion activity was then reported by the current obtained per mole of PA complex. The activity for each sample was measured, and the fraction of inserted channels (*f*) was calculated. We define *f* as the ratio of the current obtained from a sample after 37-°C incubation

to the current obtained from the sample before the incubation. In a separate case, where the insertion efficiencies of PA₇ and PA₇(LF_N)₃ complexes were compared, the insertion efficiency was normalized relative to the insertion activity of unliganded PA₇ complexes.

Circular dichroism (CD) spectroscopy

CD measurements of the PA channel transition were obtained on a JASCO Model 810 spectropolarimeter (JASCO, Inc., Easton, MD). Triplicate-averaged scans were collected over the wavelength range from 260 to 190 nm at 1-nm increments. In time course experiments, the CD signal at 222 nm was recorded at a 1-Hz sampling rate. To determine the pH dependence of the prechannel-to-channel transition, samples were diluted to 50 nM in 2 mL of buffer (10 mM potassium phosphate, 10 mM potassium acetate, 0.1 M potassium chloride, pH 8), in a 1 cm quartz cuvette containing a Teflon stir bar, at 25 °C or 37 °C. The prechannel-to-channel transition of PA-LF_N complexes was observed as a nearly instantaneous change in CD at 222 nm (CD₂₂₂) upon changing the pH of the buffer by addition of aliquots of 0.4 M phosphoric acid.

Synchrotron radiation circular dichroism (SRCD) spectroscopy

Synchrotron radiation CD (SRCD) experiments were performed on beamline UV1 at the Institute for Storage (ISA) Ring Facilities, University of Aarhus, Denmark. SRCD spectra of WT PA₈₃ monomer were obtained at a protein concentration of 1.4 mg/ml using a 0.005 cm pathlength, cylindrical Suprasil (Hellma) cuvette at 20 °C. Spectra were recorded using the following buffers: 20 mM sodium phosphate, 150 mM sodium chloride, pH 7.5; 20 mM sodium phosphate, 150 mM sodium chloride, pH 6.5; or 20 mM sodium phosphate, 150 mM sodium fluoride, pH 7.5. Data were collected over a wavelength range of 260 to 175 nm at intervals of 1 nm; 10 scans of the sample and the baseline (consisting of the buffer alone) were averaged, subtracted and smooth with a Savitsky-Golay filter using CDTOOLS.⁶⁹ Secondary structure analyses used the CONTINLL^{57,58} and CDDSTR⁵⁶ algorithms available on DICHROWEB server⁷⁰ with reference dataset SP175.⁷¹

SRCD spectra of WT PA₇, ANT XR2, and WT PA₇-sANT XR2 complexes were obtained as above in buffer (20 mM sodium phosphate pH 7.5, 100 mM sodium fluoride, 1 mM magnesium sulfate). All reductions in pH were made just prior to measurement using small volumes of 1 M phosphoric acid (< 1% of the total volume). The protein concentrations were 0.19 mg/ml (monomer concentration) for the PA₇ oligomer, 0.095 mg/ml PA₇ oligomer plus 0.045 mg/ml sANT XR2 (an 11-fold molar excess), and 0.08 mg/ml for sANT XR2 alone. All protein concentrations were determined using duplicate quantitative amino acid analyses. Spectra for PA₇ oligomer alone, PA₇ oligomer plus sANT XR2, and sANT XR2 alone were obtained using a 0.05-cm-, 0.1-cm-, and 0.1-cm-pathlength cells as above, respectively.

Mass spectrometry

Mass spectra 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 nanoelectrospray (nano-ESI) 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 ~1 μm with a Flaming/Brown micropipette puller (Model P-87, Sutter). The instrument was calibrated with CsI clusters formed by nano-ESI using a 20 mg/mL solution of CsI in 70:30 Milli-Q water:isopropanol prior to mass measurement. The protein solutions were prepared by mixing 10 mM ammonium bicarbonate solutions (pH 7.8) of purified _nPA and LF_N, allowing the oligomers to assemble at room temperature for one hour. Immediately prior to mass analysis, an aliquot of the solution containing the complexes was diluted 1:4 into 200 mM ammonium acetate solutions at pH 6.2-8.0. 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. The nano-ESI capillaries were resistively heated to 37 °C by a NiCr-wire-wrapped aluminum collar.⁷² Raw data were smoothed three times using the Waters MassLynx software mean smoothing algorithm with a 40-unit window. Average charge states for ion populations were calculated as an intensity-weighted average of each charge state in a given distribution.

SDS-resistance PAGE analysis

SDS-resistance assays²⁷ were performed on purified PA₇(LF_N)₃ and PA₈(LF_N)₄ complexes. Complexes were diluted to 1 mg/ml final concentration (with respect to PA) in the following buffers, which vary depending upon the pH: 0.1 M Tris-Cl (pH 8.0), sodium cacodylate (pH 6.5 to 7.5), 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (pH 6.0), and sodium acetate (pH 5.0 to 5.5). The complexes were incubated for 1 hour. 1.25% SDS then was added, and the samples were run on a 12% polyacrylamide gel, which was stained in coomassie brilliant blue G-250.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

B.A.K., A.F.K., E.R.W., H.J.S., and B.A.W wrote the manuscript. A.F.K. and I.I.T. performed the EM experiments. A.A.G., A.J.M., and B.A.W. performed the SRCD experiments. A.F.K. performed the CD, electrophysiology, and LT cytotoxicity experiments. H.J.S. performed the nanoESI-MS experiments. We thank R. Zalpuri at the Robert D. Ogg Electron Microscope Laboratory for assistance with EM, and J. von Moltke in the Vance Laboratory, at University of California, Berkeley, for the macrophage cell line. We also thank J. Collier, K. Thoren, and G. Feld for their critical reading of the manuscript. This work was supported by University of California start-up funds, NIH research grants R01-AI077703 (B.A.K.) and R01-GM064712 (E.R.W.), and a project grant from the U.K. BBSRC to B.A.W and a beamtime grant to B.A.W. A.A.G. was supported by a BBSRC earmarked studentship. Access to the UV1 beamline at ISA is acknowledged under the EU Integrated Infrastructure Initiative (I3), Integrated Activity on Synchrotron and Free Electron Laser Science (IA-SFS), contract number RII3-CT-2004-506008.

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Figure 1. PA₈ is the predominate oligomer that forms in bovine plasma

Negative-stain EM analysis of His₆-PA-LF_N complexes either assembled in or incubated in defibrinated bovine plasma at 37 °C. Class-averaged images containing 10-100 particles of the resulting PA₇ and PA₈ oligomers; the total particle count, *n*; and percentages of each oligomer are indicated (black bars next to the representative images). (a) An electron micrograph at a magnification of 49,000× shows affinity-purified His₆-PA-LF_N oligomers, resulting from assembly in bovine plasma at pH 8.0. Representative soluble complexes are outlined by black boxes. (b) Class-average images of affinity-purified complexes assembled in bovine plasma at pH 8.0 (top, *n* = 1132; 28% PA₈; 72% PA₇) and at pH 7.3 (bottom, *n* = 434; 72% PA₈; 28% PA₇). (c) Class-average images of the soluble complexes remaining after incubating pre-assembled PA complexes in bovine plasma for 5 minutes at 37 °C. The starting mixture of His₆-PA-LF_N oligomers contained 74% PA₇ and 26% PA₈ by EM (*n* = 2572). Final class-averaged images are shown for the pH-8 incubation (top, *n* = 861; 38% octamer; 62% heptamer) and the pH-7.3 incubation (bottom, *n* = 300; 69% octamer; 31% heptamer).

Figure 2. The stability of LT's macrophage cytotoxicity activity

(a) Macrophage cytotoxicity assays were performed on PA₈₃, PA₇LF₃, and PA₈LF₄ in the presence of constant LF (1 µg/ml), using immortalized bone-marrow macrophages derived from 129 mice. PA concentrations reported on the x-axes are standardized in concentration to µg/ml of total PA. (b) Purified PA₇LF₃ and PA₈LF₄ complexes were challenged by incubation in pH-7.3 buffer or pH-7.3 buffered bovine plasma at 37 °C for the indicated durations. Macrophage cytotoxicity profiles of (left) PA₇LF₃ and (right) PA₈LF₄ complexes following their incubation in buffer (37 °C, pH 7.3) for the indicated durations. (c) Plots of the EC₅₀ values for PA₇LF₃ and PA₈LF₄ complexes as a function of the incubation duration: (left) buffer at 37 °C, pH 7.3; (middle) bovine plasma at 37 °C, pH 7.3; and (right) bovine plasma at 42 °C, pH 7.3. Dashed lines in the last panel estimate the EC₅₀ values, which are beyond the assayable range (i.e., > 300 µg/mL).

Figure 3. The stability of PA's channel-forming activity

Studies of PA channel formation in planar lipid bilayers compare the physiological stabilities of the PA₇(LF_N)₃ and PA₈(LF_N)₄ complexes at 37 °C, pH 7.4. **(a)** Time courses of the fraction of inserted channels for purified PA₇(LF_N)₃ complexes. The normalized fraction of inserted channels is calculated as the instantaneous current observed per mole of 37-°C pre-incubated PA oligomer sample divided by the final current per mole of PA oligomer for the 0-minute sample (which is the sample has not been subjected to a 37-°C incubation.) The 37-°C incubation durations (either 0, 5 or 30 minutes) are indicated on each time course. **(b)** A plot of the relative insertion efficiencies of PA complexes versus the duration of incubation at 37 °C, pH 7.4. The relative insertion efficiencies for each incubation duration is determined as the normalized fraction of inserted PA₈ channels divided by the normalized fraction of inserted PA₇ channels. Error bars represent a propagation of errors from standard deviations obtained for the averages of at least three PA₇ and three PA₈ samples.

Figure 4. pH-dependent PA oligomer aggregation coincides with a decrease in the proportion of soluble PA₇ complexes

(a) Electron micrographs of negatively-stained heptameric and octameric n PA-LF_N complexes following a five-minute, 37-°C incubation at the specified pHs. Class-average images containing 50-150 particles of PA₇ and PA₈ complexes are shown in the top left corner of each micrograph. The white scale bar in the inset is 5 nm. The final percentages and specific particle counts are reported in Table S1. Class-average images were not obtained at pH 6, 5.5 and 5 due to extreme aggregation. **(b)** A plot of the fraction of soluble PA₇ (black) and PA₈ (red) complexes versus pH resulting from the analysis shown in panel (a). **(c)** PA-LF_N complexes were incubated for 5 minutes at pH 7.4, as above, except 10% BSA was also included in the incubation. (left) Distinct, isolated particles with elongated structures were obtained alongside particles consistent with axial views of the PA₇ and PA₈ prechannel. The scale in this micrograph is the same as in panel (a). (right) A class-average image representing 49 elongated particles (dimensions of 15 × 5 nm) in the micrograph. This image is consistent with a sagittal section of the PA channel conformation.²⁰

Figure 5. pH-dependent changes in the circular dichroism spectra of PA oligomers

(a) SRCD spectra of (left) the PA₈₃ monomer and (right) the PA heptamer at pH 7.5 (black) and pH 6.5 (red). (b) Time-course records of CD signal at 222 nm (CD₂₂₂) after an acid pulse to pH 7 (red trace) and control (no pH pulse, pH 8.0 final, black trace) are shown. (c) The pH-dependence of the CD₂₂₂-signal change for PA₇(LF_N)₃ and PA₈(LF_N)₄ complexes at (left) 37 °C and (right) 25 °C. Traces were normalized to the initial and final CD₂₂₂ signals obtained.

Figure 6. Mass spectrometry analysis of pH-dependent changes in the populations and conformations of PA oligomers

n PA-LFN complexes were diluted into ammonium acetate buffered solutions (pH 8–6). Mass spectra of the PA-LFN complexes were taken as the sample was sprayed from an electrospray microcapillary that was heated to 37 °C. The PA₆₃ monomer charge state distribution in the mass spectra is the product of gas-phase dissociation of n PA into its two constituent noncovalently bound subunits in regions of the instrument preceding the time-of-flight analyzer. Fully and partially saturated PA₇(LFN)₃, PA₇(LFN)₂, and PA₈(LFN)₄ complexes are observed at pH 8. Heptamer signal rapidly degrades below pH 8.0, leaving predominately signal for PA₈(LFN)₄ and PA₈(LFN)₃ complexes from pH 7.6–6.4. PA₈(LFN)₄ and

$\text{PA}_8(\text{LF}_\text{N})_3$ complexes transition to a distinct, higher charge-state distribution at pH 7.0, likely indicative of a structural transition to the channel state. See Table S2 for the specific charge-state values obtained for each species at each pH.

Figure 7. The pH dependence of the SDS-resistance of PA depends on its oligomeric state and the temperature

SDS-resistance assays²⁷ were performed on (a) $PA_7(LFN)_3$ and (b) $PA_8(LFN)_4$ complexes incubated in the indicated pH at either 25 °C or 37 °C. The two species of interest on the SDS-PAGE gels are indicated as either the high-molecular-weight, SDS-resistant PA oligomer band (*) or low-molecular-weight SDS-soluble, PA_{63} monomer band (PA_{63}).

Figure 8. The pH dependence of PA channel formation depends on its oligomeric state
 Multiple biophysical probes report on conformational changes observed in PA oligomers: PA₇(LF_N)₃ (black symbols) and PA₈(LF_N)₄ (red symbols). The circular dichroism (CD) probe (■) is the observed pH-dependent increase in the CD₂₂₂ signal. The electron microscopy (EM) probe (○) is the pH-dependent change in average number of respective PA oligomers per micrograph (which is due to the loss of PA complexes upon channel formation and aggregation). The mass spectrometry (MS) probe (△) is the observed pH-dependent average charge-state increase for PA₈ complexes. Each dataset was normalized to the minimum and maximum values obtained for each signal. The solid-line sigmoid curves are meant to guide the eye; the estimated pH midpoints for the PA₇ and PA₈ curves are 7.5 and 7.0, respectively.

Figure 9. Molecular mechanism of LT stabilization in plasma

PA_{83} monomers are proteolytically activated in the bloodstream by a serum protease. The resulting nPA can assemble in the presence of LF to form heptameric and octameric LT complexes. Heptameric LT complexes are unstable, however, readily converting to the channel state under physiological temperature and pH. Inactivated heptameric channels subsequently form aggregates. Octameric LT complexes have a lower pH threshold for forming the channel state, and thus they are the predominant LT species in plasma. Models were made in CHIMERA⁷³ using the PDB coordinates of LF (1J7N¹³), PA_{83} (1ACC²³), PA_7 (1TZO¹⁹), and PA_8 (3HVD²⁴), and unpublished coordinates of the $PA_8(LF_N)_4$ complex.