The protective antigen (PA) moiety of anthrax toxin delivers the toxin’s enzymatic moieties to the cytosol of mammalian cells by a mechanism associated with its ability to heptamerize and form a transmembrane pore. Here we report that mutations in Lys-397, Asp-425, or Phe-427 ablate killing of CHO-K1 cells by a cytotoxic PA ligand. These mutations blocked PA’s ability to mediate pore formation and translocation in cells but had no effect on its receptor binding, proteolytic activation, or ability to oligomerize and bind the toxin’s enzymatic moieties. The mutation-sensitive residues lie in the 2βα-2βα and 2β10-2β11 loops of domain 2 and are distant both in primary structure and topography from the 2β2-2β3 loop, which is believed to participate in formation of a transmembrane β-barrel. These results suggest that Lys-397, Asp-425, and Phe-427 participate in conformational rearrangements of a heptameric pore precursor that are necessary for pore formation and translocation. Identification of these residues will aid in elucidating the mechanism of translocation and may be useful in developing therapeutic and prophylactic agents against anthrax.

The mechanisms by which intracellularly acting toxins cross membranes to access their cytosolic substrates are poorly understood. Most members of this class of toxins are bipartite entities (so-called AB toxins), composed of an enzymatic A polypeptide covalently or noncovalently linked to a B polypeptide or complex (1). The B moiety binds to cell-surface receptors and in general serves as a vehicle to deliver the A moiety to the cytosol. There, the latter covalently modifies its target substrate(s), eliciting disease symptoms.

Anthrax toxin (ATx)1 belongs to a unique subset of AB toxins, termed binary toxins, in which the A and B moieties are released from the bacteria as discrete monomeric proteins. These proteins assemble at the surface of mammalian cells into receptor-bound toxemix complexes. The anthrax bacillus secretes two different A proteins, edema factor (EF) and lethal factor (LF), plus a single B protein, protective antigen (PA), which delivers them both to the cytosol. EF is an edenylate cyclase, whereas LF is a Zn2+-dependent protease, which cleaves certain mitogen-activated protein kinase kinases within mammalian cells (2–4).

In some AB toxins, including ATx, diphtheria toxin, and several clostridial toxins, the B moiety is capable of forming channels (pores) in lipid bilayers under conditions that promote translocation (5–9). Evidence from mutational studies with anthrax and diphtheria toxins implies a close relationship between channel formation and the process by which the A moieties cross cellular membranes (10, 11).

PA, a monomeric 83-kDa protein, binds to an as yet unidentified cell-surface receptor on mammalian cells and is activated by furin or a furin-like protease (12, 13). The resulting N-terminal fragment (PA20; 20 kDa) diffuses into the extracellular milieu, leaving the complementary C-terminal fragment (PA63; 63 kDa) bound to the receptor. PA63 manifests a set of properties, not found in the native protein. PA63 spontaneously oligomerizes to form a ring-shaped heptamer, (PA63)7 (14, 15), and binds EF and LF tightly and competitively (16, 17). According to our current model, (PA63)7 complexed with EF and/or LF is taken into the cell by receptor-mediated endocytosis and trafficked to an acidic compartment (18). There, acidification induces a conformational change in the (PA63)7 moiety, causing it to convert to a membrane-spanning pore (19). Pore formation triggers translocation of EF and/or LF across the membrane to the cytosol, but the mechanism of this process remains to be elucidated.

The crystallographic structure of PA reveals a long, flat molecule, consisting largely of β-structure, and is organized into four structural domains (Fig. 1A) (15). Domain 1 contains the furin site, and the surface of this domain exposed when PA63 is removed has been hypothesized to serve as the binding site for EF and LF (15). Domain 2 forms the central structure of the heptamer and contains a disordered loop (2β2-2β4, residues 302–325) that has been implicated in forming the transmembrane pore. Mutations have been found recently within domain 3 that affect oligomerization, suggesting this to be the function of this domain.2 Domain 4 functions in receptor binding, as demonstrated by mutational analysis and by studies with monoclonal antibodies (11, 20).

The crystallographic structure of a heptameric form of PA63 has also been solved (Fig. 1B) (15). The structure shows a hollow ring, 160 Å in diameter and 85 Å high, with a central negatively charged lumen of average diameter 35 Å. There are no major conformational differences between PA63 in (PA63)7.
and in native PA. The monomers pack such that domain 1′ (that portion remaining after removal of PA20) and domain 2 face the lumen, whereas domains 3 and 4 are on the periphery. No hydrophobic surface that could interface with the core of a bilayer is seen, leading to the suggestion that this form may represent an intermediate in pore formation assembled on the cell surface.

Channel conductance experiments indicate that the 2β2-2β3 loops of the seven monomers of heptameric PA63 interact to generate a transmembrane β-barrel similar to that observed in Staphylococcus aureus α-hemolysin (21, 22). These loops project outwards laterally from the midsection of the crystallographic (PA63)7, and would have to move to the base of this structure to assemble into a β-barrel on the heptamer axis. This movement could be effected by an unfolding of the Greek-key motif comprising the first four β-strands of domain 2, in which the 2β2 and 2β3 strands flanking the loop peel away from the edge of domain 2 (15).

In the current study, we show that mutation of any of three solvent-exposed residues within domain 2, Lys-397, Asp-425, and Phe-427, blocks pore formation and translocation across native membranes. These residues are in the 2β10-2β11 loops, which lie on the luminal aspect of the crystallographic heptamer, and are far removed from the 2β2-2β3 loop implicated in formation of the transmembrane β-barrel. The characteristics of these mutant proteins suggest that Lys-397, Asp-425, and Phe-427 participate in conformational rearrangements of the PA63 heptamer that are required for translocation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Media, and Chemicals—**Chinese hamster ovary-K1 (CHO-K1) cells were obtained from American Type Culture Collection. The cells were grown in Ham’s F-12 medium supplemented with 10% calf serum, 2 mM l-glutamine, 500 units/ml penicillin G, and 500 units/ml streptomycin sulfate and maintained at 5% CO2 in a humidified atmosphere. Cells were seeded into 24- or 96-well microtiter plates (Costar, Cambridge, MA) 16–18 h prior to the experiment. All supplies for cell culture media were obtained from Life Technologies, Inc., unless noted otherwise. All chemicals were obtained from Sigma Chemical Co., unless specified.

**Preparation of PA Proteins—**Mutations were constructed using the QuikChange method of site-directed mutagenesis following the manufacturer’s protocol (Stratagene, La Jolla, CA). All proteins were cloned into the pET22b (+) (Novagen, Madison, WI) expression vector, transformed into Escherichia coli BL21(DE3) (Novagen), and expressed as described previously (23). Briefly, cultures were grown in LB (24) at 37 °C to A600 of 1.0. Expression of the recombinant protein was induced by the addition of isopropyl-β-D-thiogalactopyranoside to 1 mM. Following induction, the cells were grown for an additional 3 h at 30 °C and harvested by centrifugation for 10 min at 8000 × g.

Proteins were released from the periplasm by osmotic shock. The cells were resuspended in 20 mM Tris, pH 8.0, 30% glucose, and 1 mM EDTA and incubated at room temperature for 10 min with continuous stirring. The cells were then harvested by centrifugation, resuspended in ice-cold 5 mM MgSO4 containing 20 mM benzamidine (Research Organics, Cleveland, OH), and incubated at 4 °C for 10 min with constant stirring. After the cells were pelleted by centrifugation at 8000 × g, the periplasmic extract was decanted. Tris-HCl, pH 8.0, was added to the extract to a final concentration of 20 mM, and the entire sample was loaded onto a column packed with Q-Sepharose High Performance (Amersham Pharmacia Biotech, Piscataway, NJ). After the column was washed with buffer A (20 mM Tris, pH 8.0), bound proteins were eluted with a linear gradient of 0–25% buffer B (20 mM Tris, pH 8.0, 1 M NaCl). The PA-containing fractions were concentrated, and the sample was exchanged over a pd-10 column (Amersham Pharmacia Biotech) equilibrated in buffer A. The PA-containing eluate was loaded onto a Mono-Q (Amersham Pharmacia Biotech) column and eluted with a 0–25% B gradient. Fractions containing PA were determined by SDS-PAGE and stored at −80 °C.

Proteins were assayed using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) as per the manufacturer’s instructions. All liquid chromatography was done using an AKTA purifier (Amersham Pharmacia Biotech).

**Activation of PA—**PA was activated by treatment with trypsin, yielding nicked PA (nPA). Trypsin was added to PA (0.2–0.5 mg/ml) at a final trypsin:PA ratio of 1:1000 (w/w). This mixture was incubated at room temperature for 20 min followed by addition of a 10-molar excess of soybean trypsin inhibitor.

**Cell Surface Translocation Assay—**A cell surface translocation assay to measure the PA-mediated translocation of [35S] LF was performed as described (25). CHO-K1 cells (2 × 105 cells/well) in a 24-well plate were chilled on ice for 30 min. Medium was removed by aspiration, and the cells were washed with ice-cold sterile Dulbecco’s PBS. nPA (2 × 106–8 M) in 250 μl of Ham’s F-12 medium buffered with 10 mM HEPES, pH 7.4, was layered on the cells and incubated for 2 h on ice. The medium was removed by aspiration, and the cells were washed with sterile ice-cold Dulbecco’s PBS. The cells were then incubated on ice for 1 h with Ham’s F-12 medium buffered with 10 mM HEPES, pH 7.4, containing [35S] LF, produced by in vitro transcription/translation using the TnT Coupled Reticulocyte Lysate System (Promega, Madison, WI). After the removal of the [35S] LF, the cells were washed with ice-cold PBS followed by a pH 5.0 or 7.0 pulse (10 mM Tris, 5 mM sodium glutcycine, 140 mM NaCl, pH 5.0 or 7.0) at 37 °C for 1 min. The cells were then treated with 2 mg/ml Pronase in 10 mM HEPES, pH 7.4, for

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**FIG. 1. Ribbon diagrams of the 63-kDa form of PA.** Domain 1′ is shown in yellow, domain 2 in red, domain 3 in blue, and domain 4 in green. The locations of Lys-397, Asp-425, and Phe-427 are shown in a ribbon diagram of a single monomer (A) from the PA63 heptamer. The dashed line represents the disordered 2β2-2β3 loop that is believed to form a transmembrane β-barrel upon pore formation. The crystallographic PA63 heptamer (B) is also illustrated to show the approximate locations of these residues in that structure (only Lys-397 is indicated, for simplicity). This figure was prepared with Molscript (34).
incubating nPA (0.5 mg/ml at pH 8.0) with an equimolar amount of LFN in calf serum, 2 mM L-glutamine, 500 units/ml penicillin G, 500 units/ml streptomycin sulfate, and [3H]Leu at 1 μCi/ml. After incubation for 1 h at 37 °C, the cells were washed with ice-cold PBS followed by ice-cold 10% trichloroacetic acid. Protein synthesis was measured as the quantity of [3H]Leu incorporated into the trichloroacetic acid-precipitable material.

**Heptamer Formation**—Oligomerization of PA, was measured by incubating nPA (0.5 mg/ml at pH 8.0) with an equimolar amount of LFN for 30 min at room temperature. The samples were then subjected to electrophoresis in a 4–12% native gradient gel (FMC Bioproducts, Rockland, ME) using 50 mM CHES, pH 9.0, 2 mg/ml CHAPS as the running buffer. SDS-resistant heptamer was formed by adding 100 mM sodium acetate, pH 4.0, until the pH of the solution reached 5.0, after which the samples were incubated at room temperature for 30 min. The samples were then dissolved in SDS-PAGE sample buffer (24) and run on a 4–12% SDS-PAGE gradient gel (FMC Bioproducts). Proteins were visualized with Coomassie Brilliant Blue.

**Rubidium Release**—CHO-K1 cells were plated at a density of 2 × 10^5 cells/well in 24-well plates and incubated at 37 °C for 4 h. The medium was aspirated and replaced with medium containing 1 μCi/ml ^86Rb and incubated for 16 h. Cells were then chilled on ice for 20 min followed by the removal of the medium, washing of the cells, and addition of nPA (2 × 10^-5 M) in HEPES-buffered medium. The cells were incubated with nPA for 2 h on ice followed by the addition of ice-cold pH 5.0 buffer. After a 30-min incubation, samples of the supernatant were collected and the radioactivity was measured.

**RESULTS**

**Mutations at Lys-397 and Asp-425 Block PA-dependent Cell Killing**—In exploring various regions of PA, we mutated residues within the 2β_2-2β_6 and 2β_10-2β_11 loops, which lie on the luminal aspect of the heptamer structure. Early in these studies, we found a residue in each loop that was crucial to PA’s ability to deliver a toxic ligand to the cytosol (23, 26). The ligand employed, LFNDTA, is a fusion protein containing LFN, the PA-binding domain of LF, fused to DTA, the enzymic domain of diphtheria toxin. Delivery of the fusion protein to the cytosol allows the DTA moiety to catalyze the ADP-riboseylation of elongation factor-2, thereby inhibiting protein synthesis. As shown in Fig. 2, substitution of Ala for either Lys-397 (within the 2β_7-2β_8 loop) or Asp-425 (within 2β_10-2β_11) completely blocked PA’s ability to mediate LFNDTA-dependent inhibition of [3H]Leu incorporation in CHO-K1 cells.

**The Mutations Specifically Block Translocation and Pore Formation**—To identify the step in toxin action affected by these mutations, we first examined the effects of K397A and D425A on translocation, using the cell-surface assay of Wesche et al. (25). Radiolabeled LFNDTA was bound to trypsin-nicked PA (nPA) at the cell surface, and the cells were briefly treated with low pH buffer to induce translocation across the plasma membrane. The cells were then treated with Pronase to digest any LFNDTA remaining exposed, and the translocated LFNDTA was quantified by scintillation counting. Both K397A and D425A inhibited translocation at least 10-fold, as shown in Fig. 3B.

The mutations did not diminish the amount of LFNDTA initially bound by PA, at the cell surface (Fig. 3A) and thus did not alter PA’s receptor-binding and ligand-binding functions. Furthermore, the specificities of cleavage at the furin site of PA by trypsin or in the 2β_2-2β_6 loop by chymotrypsin are retained in LFNDTA. After a 30-min incubation, samples of the supernatant were collected and the radioactivity was measured.

**FIG. 2.** PA-mediated LFNDTA toxicity. WT-PA (○), PA-K397A (■), and PA-D425A (▲) were incubated over a range of concentrations from 1 × 10^-7 to 1 × 10^-12 M with CHO-K1 cells in the presence of 1 × 10^-8 M LFNDTA. After a 4-h incubation at 37 °C, protein synthesis was measured as the incorporation of [3H]leucine into newly synthesized protein. Cells incubated with LFNDTA in the absence of PA were used as standard for 100% protein synthesis.

**FIG. 3.** PA-dependent cell binding and translocation of [35S]LFN. nPA (2 × 10^-5 M) was incubated on CHO-K1 cells for 2 h on ice. The cells were then washed and incubated with [35S]LFN for another 2 h. A, samples of cells were then lysed, and the [35S]LFN in the lysate was measured by scintillation counting. % binding = (dpm [35S]LFN bound by mutant/[35S]LFN bound by PA) × 100. B, other samples were incubated for 1 min at 37 °C with buffer at pH 5.0 to trigger translocation. Unprotected [35S]LFN remaining on the surface of the cell was digested with Pronase. The cells were then washed and lysed, and the [35S]LFN remaining in the cytosol was measured by scintillation counting. % translocation = ([35S]LFN dpm protected from Pronase/total [35S]LFN dpm bound to cells) × 100.
the mutant PAs (data not shown) (23). These findings are strong indications that the K397A and D425A mutations did not cause general misfolding of the protein.

The ability of PA to translocate ligands across membranes has been correlated with its activity in forming transmembrane pores (11, 23). We measured the pH-dependent PA-mediated release of 86Rb from 86Rb-preloaded CHO-K1 cells as an assay of permeabilization of the plasma membrane (27). K397A and D425A entirely abrogated pore formation as assessed by this parameter (Fig. 4). Pore formation in a planar lipid bilayer is a highly sensitive method to assay for channel formation by PA (6), and the mutations also blocked pore formation by trypsin-activated PA in this system (data not shown). Thus the inhibition of translocation by K397A and D425A is correlated with a lesion in PA’s ability to form pores.

Oligomerization Is Not Affected by the Mutations but Transition to an SDS-resistant Form Is—To identify the step in pore formation affected by the mutations, we first probed the ability of the mutant forms of PA63 to oligomerize (15, 23). Addition of an equimolar amount of LFN to nPA in solution promotes oligomerization by PA63, and the resulting LFN(PA63)7 complex migrates as a low mobility band in nondenaturing polyacrylamide gels. As shown in Fig. 5, wild-type PA, PA-K397A, and PA-D425A formed similar amounts of the LFN complex, implying that oligomerization of PA63 is not affected by the mutations.

This conclusion is supported by results of anion-exchange (MonoQ) chromatography of the nicked mutant and wild-type PAs. PA63, elutes from the MonoQ resin in a single peak corresponding to an oligomeric form (or forms). Recently, PA mutants have been identified that are deficient in oligomerization, and such mutants yield little or no material in this peak. In contrast, K397A- and D425A-PA63 gave elution profiles closely resembling that of wild-type PA. The presence of (PA63)7 in the eluate was confirmed by electron microscopy (data not shown).

If maintained in buffer of pH ≥ 8.0, oligomeric PA63 dissociates in the presence of SDS into monomeric subunits. When exposed to pH ≤ 7, or when treated with β-octylglucoside (even at pH ≥ 8.0), the material converts to an SDS-resistant state and runs as a high molecular mass band on SDS-PAGE (14, 22, 23). Unlike wild-type preparations, LFN(6)-liganded K397A or D425A prepore failed to convert to SDS resistance when treated at pH 5 (Fig. 6) or when incubated with β-octylglucoside (data not shown). Also, no SDS-resistant oligomer was formed when cells containing bound mutant protein were treated at low pH. These findings suggest that the K397A and D425A mutations affect a conformational transition in pore formation.

Effects of Other Mutations—We examined the effects of substituting other amino acids besides Ala at positions 397 and 425 (Table I). Although K397D caused such loss of activity, indicating that a positive charge is important for function at this site. The effects of K397Q coincided with those of K397D and K397A, except that K397Q did not block conversion of the prepore to an SDS-resistant state. At position 425, all other substitutions tested, including the most conservative ones (Glu and Asn), had the same effect as the Ala substitution. Thus the requirement for Asp at this position is highly specific.

We also mutated residues flanking Lys-397 in the 2β-β8 loop and Asp-425 in 2β8-2β9. Among the mutations examined (see Table I) only F427A caused a major functional defect. Like K397Q, F427A blocked cell killing by LFN(DTA), translocation.
PA-dependent toxicity of LF_δDTA was assayed by incubating CHO-K1 cells with increasing concentrations of PA for 4 h and measuring protein synthesis as described under “Experimental Procedures” (26). LF_δ binding was measured as the amount of [35S] LF_δ bound to CHO-K1 cells preincubated with nPA. Translocation was measured in a cell surface pronase protection assay (25). PA-dependent release of 86Rb from CHO-K1 cells was used as a measure of pore formation by PA at pH 5.0 (27). Heptamer formation was assessed by the incubation of nPA with an equimolar amount of LF_δ and visualized by native gel electrophoresis. Formation of the SDS-resistant heptamer was triggered by dropping the pH of the heptamer containing solution to 5.0 using 100 mM sodium acetate (23). The samples were then solubilized in SDS-PAGE sample buffer and subjected to SDS-PAGE.

### Table I

| LF_δDTA toxicity | LF_δ binding | Translocation | 86Rb release | Heptamer formation | SDS-resistant heptamer |
|------------------|--------------|---------------|--------------|-------------------|-----------------------|
| WT-PA            | ++++         | ++++          | ++++         | ++++              | ++++                  |
| K395C            | ++++         | ++++          | ++++         | ++++              | ++++                  |
| K397A            | +           | +             | +            | +                 | +                     |
| K397D            | +           | +             | +            | +                 | +                     |
| K397Q            | +           | +             | +            | +                 | +                     |
| K397R            | +           | +             | +            | +                 | +                     |
| E398C            | +           | +             | +            | +                 | +                     |
| D425A            | +           | +             | +            | +                 | +                     |
| D425N            | +           | +             | +            | +                 | +                     |
| D425K            | +           | +             | +            | +                 | +                     |
| D425E            | +           | +             | +            | +                 | +                     |
| D425K            | +           | +             | +            | +                 | +                     |
| D426A            | +           | +             | +            | +                 | +                     |
| F427A            | +           | +             | +            | +                 | +                     |
| S428C            | +           | +             | +            | +                 | +                     |
| T473C            | +           | +             | +            | +                 | +                     |
| Double           | +           | +             | +            | +                 | +                     |
| Quadruple        | +           | +             | +            | +                 | +                     |
| QuadrupleND      | +           | +             | +            | +                 | +                     |
| Quadruple        | +           | +             | +            | +                 | +                     |

**a** 75–100% activity of wild-type PA; **+**+, 50–74% activity of wild-type PA; **+**, 25–49% activity of wild-type PA; **−**, <5% activity of wild-type PA.

**b** Double, K397D/D425K.

**c** Quadruple, K395D/D425K/D425K/D425K.

**d** ND, not determined.

Mutations Affecting PA Translocation

### DISCUSSION

In the course of mutating residues observed on the luminal aspect of the PA_δ heptamer crystal structure, we found three sites at which substitutions caused a major disruption in LF_δDTA-mediated cell killing: Lys-397, Asp-425, and Phe-427. The mutations tested at these sites are unlikely to have caused general misfolding, given that the residues are solvent-exposed and did not affect the tryptic and chymotryptic cleavage patterns or expression of the protein. Furthermore, the mutations are highly specific in the functions they affect.

The mutant forms of PA could be activated normally by trypsin, and the resulting PA_δ fragments were unimpaired in ability to bind ligands. We demonstrated ligand binding on wild-type PA; 2_δ interferes specifically with the ability of receptor-bound (PA_δb)2 to undergo conformational changes required for translocation.

Whereas mutations in Lys-397 and Asp-425 blocked pore formation in both cells and planar bilayers, F427A affected this process only in cells. A difference in the pH dependence of pore formation between cells and planar bilayers was observed earlier (6, 27, 28), suggesting a possible effect of the PA receptor on this process.

Mutations at positions 397 and 425 (except for K397Q and K397R) also differed from F427A and quadruple in their effects on the ability of PA_δ to form an SDS-resistant heptamer. Heretofore we used formation of an SDS-resistant heptamer as an apparent indicator of conversion of PA_δ to the pore (23). Milne et al. (14) had shown that an SDS-resistant high molecular weight PA band forms after PA activation, endocytosis, and trafficking to the endosome. Lysosomotropic agents or inhibitors of endocytosis blocked formation of the band, indicating that it represents a form generated under acidic conditions. Furthermore, a shift to this band was observed when the cells were treated at a pH known to induce pore formation.

Recently, however, we discovered that the crystallographic PA_δ heptamer is also SDS-resistant. Although purified oligomeric PA_δ is dissociable by SDS if maintained at pH > 8.0, the crystallization buffer (50 mM Tris-HCl, pH 8.0–8.5; 20–40 mM CaCl2, 2% polyethylene glycol 8000) induces SDS resistance, even in the absence of crystals. This suggests that two SDS-resistant forms of (PA_δ)7 may exist: the pore and the prepeptide form whose structure was determined. In light of these findings, it is not possible to give an unequivocal interpretation of the changes caused by the mutations at positions 397 and 425. Nonetheless, the inability of mutants to convert to an SDS-resistant form suggests that this property could be requisite for pore formation. It is clearly not sufficient, however,

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4 D. B. Lacy and R. J. Collier, unpublished results.
because PA-K397Q, PA-F427A, and PA in which the 2β2-2β3 has been deleted can all form SDS-resistant heptamer, despite their inability to form pores in the plasma membrane.

Precisely how mutations in Lys-397, Asp-425, and Phe-427 block conformational changes leading to pore formation and translocation is not evident. According to the model of Petosa et al. (15), for the 2β2-2β3 loops to form a transmembrane β-barrel domain 2 must undergo a major conformational rearrangement involving peeling out of the two flanking β-strands from the domain. The 2β2-2β3 and 2β10-2β11 loops, where Lys-397, Asp-425, and Phe-427 reside, are on the opposite side of domain 2 from the 2β2-2β3 loop, and it is unclear how they would participate in this rearrangement. The elegant analysis of pore formation by staphyloccocal α-hemolysin and the related protein LukF, showing intersubunit contacts via the N-terminal latch, provides an example of how such interactions can occur in a pore-forming toxin (21, 29). Dissimilarities in the fold prevent direct application of this model to PA, however.

Our data show that the requirement for Asp at position 425 is highly specific and the positive charge of Lys-397 is important, implying that these residues form specific contacts with sites on the same or neighboring subunits of the heptamer. Phe-427 presumably forms a hydrophobic contact near that of Asp-425. A double mutation (K397D/D425K) and a quadruple mutation (K395D/K397D/D425K/D426K) were constructed to probe the possibility that Lys-397 might interact with Asp-425, or alternatively Lys-395 and Lys-397 might interact with Asp-425 and D426. The results were negative but do not disprove that such interactions may occur, because the relevant residues in the mutants were out of their normal contexts.

Examination of PA-homologues (component-II of Clostridium botulinum C2 toxin (30), Sb component of Bacillus anthracis) showed that the single mutation, F427A; the double mutation, K397D/D425K; or deletion of the 2β2-2β3 loop endows PA with the property of being dominant negative. That is, PA63 derived from either of these mutants co-oligomerizes with wild-type PA63, but the resulting hetero-oligomers are unable to mediate translocation. These mutant forms of PA may therefore be useful as therapeutics for use in neutralizing anthrax toxin in individuals infected with Bacillus anthracis. Also, because the mutations alter the structure of PA only in minor ways, its immunoprotective properties are likely to be preserved, implying that the mutants could also be used for vaccination.

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