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Lethal factor of anthrax toxin binds monomeric form of protective antigen

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Abstract

Anthrax toxin consists of three components: the enzymatic moieties edema factor (EF) and the lethal factor (LF) and the receptor-binding moiety protective antigen (PA). These toxin components are released from *Bacillus anthracis* as unassociated proteins and form complexes on the surface of host cells after proteolytic processing of PA into PA20 and PA63. The sequential order of PA heptamerization and ligand binding, as well as the exact mechanism of anthrax toxin entry into cells, are still unclear. In the present study, we provide direct evidence that PA63 monomers are sufficient for binding to the full length LF or its LF-N domain, though with lower affinity with the latter. Therefore, PA oligomerization is not a necessary condition for LF/PA complex formation. In addition, we demonstrated that the PA20 directly interacts with the LF-N domain. Our data points to an alternative process of self-assembly of anthrax toxin on the surface of host cells.

Keywords: Anthrax toxin; Monomers; Lethal factor; Protective antigen

Anthrax toxin from *Bacillus anthracis* belongs to a family of binary toxins in which enzymatic and receptor-binding moieties are released from bacteria as unassociated proteins. A self-assembly process takes place on the surface of receptor-carrying cells. The binding moiety determines the type of host cells and recruits the toxin moiety to the location of receptor molecules on the host cell surface. The current model of anthrax toxin self-assembly and entry into host cells includes initial binding of the full length 83-kDa protective antigen (PA) to cell surface receptors followed by its cleavage by cellular furin-like proteases. A 20 kDa amino (N)-terminal fragment of PA (PA20) dissociates into the extracellular medium and subsequently plays no further known role in the toxin action. The remaining carboxyl (C)-terminal, 63-kDa fragment (PA63) then assembles into a homo-heptameric ring structure accompanied by the clustering of the receptors into lipid raft domains of the plasma membrane. Proteolytic release of the N-terminal fragment of PA exposes a high affinity binding site for the lethal factor (LF) and edema factor (EF) enzymatic moieties on the surface of PA63. Once the toxin complex is assembled, it is internalized by receptor-mediated endocytosis and is trafficked into the endosome. The acidic environment of the endosome induces conformational changes in the complexes and translocation of EF and LF into the cytosol, where they catalyze reactions leading to toxic responses [1]. A stable complex between the PA heptamer and EF and/or LF contributes to the overall toxicity of the complex. Tight association between PA and EF/LF results in an enhanced effective concentration of the toxin on the cell surface and improved efficiency of their internalization. The sequential order of PA heptamerization and ligand binding, as well as the exact mechanism of anthrax toxin entry into the host cells, remain elusive.

LF is a 90 kDa protein [2]. The C-terminal region carries out the metalloprotease activity, while the N-terminal domain (residues 1–288, LF-N) is responsible for PA binding. It has been reported that LF-N showed the same affinity for the PA heptamer as full length LF [3]; thus LF-N has been used to study PA/LF interactions [4–6]. Furthermore, several heptamer-breaking mutations of PA, including

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single point mutation D541K and triple point mutation K228E/R497A/R499D on the PA heptamer-interface, have been identified [5]. It has been proposed [5] that each of the above two mutants could not form homo-oligomers but, once mixed, can form hetero-dimers with an interface similar to that observed in the heptamer because of the complementarity of mutations. These putative dimer-forming mutants were used to replace the PA63 heptamer in an attempt to simplify *in vitro* experiments on PA/LF interaction.

In the current study, we used oligomerization deficient PA mutants to examine the interaction of LF with PA in monomeric form. The binding of PA63 monomers with both the full length LF and LF-N was assessed by gel electrophoresis under non-denaturing conditions and confirmed by pull-down assays. Furthermore, we introduced point mutations into LF and LF-N to disrupt their PA binding sites. Interaction between these mutants was assessed by gel electrophoresis under non-denaturing conditions. Our results demonstrate that the PA63 monomer is sufficient to bind the full length LF molecule as well as its LF-N domain. We also showed that the PA20 directly interacts with the LF-N domain, but its functional role remains to be studied.

Materials and methods

Plasmid construction. The plasmids pET15b-LF and pET15b-PA, containing the entire genes of LF and PA (GenBank accession nos. AAY15237 and AAY15236), except for the portions that encode the signal sequences, were obtained from Dr. J.D. Ballard (Oklahoma University Health Science Center, OK). The vector pHMV6g, which was used for subcloning of PA mutants, is a derivative of the *Escherichia coli* expression vector pET28a encoding a fusion maltose binding protein (MBP) with an N-terminal His_6-tag and an Tev protease cleavage site followed by the multiple cloning sites under the control of an inducible T7 promoter. Mutants of PA, LF and LF-N were introduced by QuickChange site-directed mutagenesis performed according to the manufacturer's recommended protocol (Stratagene). The oligonucleotides GCGGCGGTTA ATCCTAGTAAGCCATTAGAAACG, CGGTTGATGTCAAAAATG AAAGAACTTTTCTTTCACC, GAAAATGGAGCAGTGGACCTCC ATACAGGC, and their reverse complements were used to introduce site directed mutations of D541K PA and K228E/R497A/R499D PA, respectively. LF-N (LF truncated at residue Ser288) was subcloned in pET15b vector (Novagen) which added a hexahistidine tag to the N terminus of the proteins. The oligonucleotides GTAATCCAATCTTC GGCAGATTATGTAG, CCATTAAAAATGCATCTGCTTCAGATG GAC and their reverse complements were used to introduce site directed mutations of E168A and D215A, respectively.

Expression and purification of proteins. Recombinant proteins were produced in *E. coli* BL21 DE3 Star cells using pHMV6g (PA mutants) and pET15b (LF and LF-N) vectors. Histidine-tagged PA mutants were purified from bacterial lysates by metal chelating affinity chromatography on His-Trap HP Nickel Column (Amersham Pharmacia) according to the manufacturer's instructions and cleaved between MBP and PA with Tev protease. The resulting PA83 protein was further separated from MBP and acTev (both histidine-tagged) by chromatography on His-Trap Nickel Column. The same metal chelating chromatography column was used to purify histidine-tagged LF (wild-type and mutants) from bacterial lysates. The proteins were further purified by ammonium sulfate precipitation followed by phenyl-sepharose chromatography. Similarly, histidine-tagged LF-N and its mutants were purified from bacterial lysates on His-Trap Nickel Column. Following chromatographic purification, the proteins were concentrated in Centriprep centrifugical concentrators (Amicon). Homogeneities of purified proteins were analyzed by SDS–PAGE with Coomassie blue staining, and all final samples were more than 90% pure. Protein concentrations were determined by absorbance spectrophotometry at 280 nm wavelength based on calculated molar extinction coefficients. Proteins were aliquoted and stored at −80 °C.

Preparation of PA63. Nicked PA was prepared by incubating PA with trypsin at a molar ratio of 1:1000 at room temperature (RT) for 30 min. The reaction was stopped by the addition of a 10-fold molar excess of soybean trypsin inhibitor relative to trypsin. PA63 was separated from the N-terminal 20 kDa fragment by Q-sepharose anion exchange chromatography (Amersham Pharmacia) in 20 mM Tris–HCl (pH 8.0) with a 0−0.2 M NaCl gradient followed by S-75 Superdex gel filtration (Amersham Pharmacia). Following chromatographic purification, the proteins were concentrated in Centriprep centrifugical concentrators.

Binding of LF to PA in solution analyzed by native gel electrophoresis. Purified PA63 mutants and LF or LF-N variants (wild type and mutants) were mixed at 1:1 molar ratio (PA monomer:LF monomer) in buffer A [20 mM Tris–HCl (pH 8.0) and 100 mM NaCl] and incubated for 15 min at RT. Mixtures, as well as PA63 mutants alone (negative control) in a volume of 5 μl, were mixed with 5 μl of loading dye (bromophenol blue in 50% glycerol) and loaded onto 20% acrylamide Tris–Glycine Phast gels (GE Healthcare). Gels were stained with either silver stain or Coomassie Blue. All experiments were repeated several times with similar results.

Pull-down assays. Pull-down assays were performed in buffer A. Purified hexahistidine-tagged LF or LF-N proteins were coupled with Nickel agarose beads (Sigma) according to manufacturer's instructions. The beads were washed twice with buffer A to remove unbound proteins. Purified PA63 mutants were added to the beads and incubated for 30 min at RT. The beads were washed with buffer A two more times, and bound proteins were eluted with 20 mM Tris–HCl (pH 8.0), 100 mM NaCl, and 500 mM imidazole. All fractions were analyzed by SDS–PAGE using 20% acrylamide Tris–Glycine gels. Gels were stained with Coomassie Blue.

All experiments were repeated several times with similar results.

Results

PA63 mutants, D541K and K228E/R497A/R499D, do not form stable dimers and remain in monomeric form

To study the LF/PA interaction in solution, we initially made two oligomerization deficient mutants of PA, D541K and K228E/R497A/R499D (PA TM), based on previously reported studies [5]. The recombinant proteins were expressed in *E. coli*. After trypsin cleavage, the 20 kDa N-terminal fragment and the 63 kDa C-terminal domain of PA were separated by anion exchange chromatography. Samples of PA63 mutants alone or in equimolar mixture of the two were analyzed either directly by gel electrophoresis under non-denaturing conditions (Fig. 1A) or by size-exclusion chromatography (Fig. 1B), followed with the analysis of the isolated peak fractions by native gel electrophoresis (Fig. 1C). The elution profiles of gel-filtration chromatography for individual mutants and their mixture were similar to each other (Fig. 1B); in all cases, we observed one major peak with an estimated molecular mass of 63 kDa corresponding to the PA63 monomer. The electrophoretic mobility of the oligomerization-deficient PA mutants varied only slightly from each other, probably because of the difference in surface charge (lanes 1 and 2, Fig. 1A), which concurs with results from a previous report [5]. However, we did not detect an additional band corresponding to a PA63 heterodimer (lane 3, Fig. 1A) that