Stoichiometry of Anthrax Toxin Complexes†

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ABSTRACT: After being proteolytically activated, the protective antigen (PA) moiety of anthrax toxin self-associates to form symmetric, ring-shaped heptamers. Heptameric PA competitively binds the enzymatic moieties of the toxin, edema factor and lethal factor, and translocates them across the endosomal membrane by a pH-dependent process. We used two independent approaches to determine how many of the seven identical EF/LF binding sites of the PA heptamer can be occupied simultaneously. We measured isotope ratios in complexes assembled from differentially radiolabeled toxin subunits, and we determined the molecular masses of unlabeled complexes by multiangle laser light scattering. Both approaches yielded the same value: the PA heptamer in solution binds three molecules of protein ligand under saturating conditions. This suggests that each bound ligand sterically occludes the binding sites of two PA subunits. According to this model, a ligand-saturated heptamer is asymmetric, with the sites of six of the seven subunits occluded. These results contribute to the conceptual framework for understanding the mechanism of membrane translocation by anthrax toxin.

The AB toxins produced by certain bacteria and plants have the unusual property of being able to transfer their A moieties across a membrane to the cytosol of susceptible cells. The mechanism by which this occurs differs from toxin to toxin and remains incompletely understood for any toxin. Anthrax toxin belongs to the so-called "binary" AB toxins, a subset in which the enzymatic A and receptor-binding B moieties are released from the bacteria as discrete monomeric proteins, which later assemble into toxic complexes at the mammalian cell surface. This subset also includes *Clostridium perfringens* ι-toxin, *Clostridium botulinum* C2 toxin, and *Bacillus cereus* vegetative insecticidal proteins. Anthrax toxin differs from other binary toxins in having two A moieties, edema factor (EF)[1] and lethal factor (LF), and a single B moiety, protective antigen (PA). PA is capable of binding and mediating the action of EF and LF independently of each other.

According to the current model, assembly of the anthrax toxin components is initiated when PA binds to a proteinaceous receptor, which is present on most or all mammalian cells (*1*). The anthrax toxin receptor, ATR, was identified recently as a type I membrane protein with an extracellular domain that binds directly to PA and that is homologous to the von Willebrand factor A domain (*2*). Once bound to ATR, PA is cleaved into two fragments by a member of the furin family of cell-associated proteases. The resulting N-terminal fragment, PA_{20} (20 kDa), dissociates, diffuses away, and plays no further part in toxin action. The complementary fragment, PA_{63} (63 kDa), remains receptor-bound and displays two new properties: (i) it self-associates, yielding ring-shaped heptameric oligomers, $[PA_{63}]_7$ (*3*); and (ii) these oligomers bind the enzymatic moieties, EF and LF, competitively and with high affinity ($K_d \sim 1$ nM) (*4*). This interaction is via the homologous amino-terminal domains of EF and LF (domains termed EF_N and LF_N, respectively).

Once assembled on the cell surface, the noncovalent complexes of $[PA_{63}]_7$ with EF and/or LF are endocytosed and trafficked to a low-pH compartment. There, the acidic environment induces a conformational change in $[PA_{63}]_7$ that allows it to form a membrane-spanning β-barrel (*5*−*7*). Translocation of the enzymatic moieties is associated with formation of the β-barrel, though the mechanism and route of membrane traversal by these moieties remain unclear. Once within the cytosol, EF binds calmodulin and converts ATP to cyclic AMP (*8*). LF is a Zn^{2+}-dependent protease that cleaves certain mitogen-activated protein kinase kinases and perhaps other proteins (*9*, *10*), causing death of macrophages and ultimately death of the host. Both EF and LF are thought to help to protect the bacterium from the immune system during an infection.

An important parameter in describing the assembly and translocation processes of anthrax toxin is the ratio of enzymatic to PA_{63} subunits in the fully formed complexes. We employed two independent methods to examine this question and found that both gave the same result: $[PA_{63}]_7$ binds a maximum of three ligand molecules at saturation. This conclusion is consistent with a model in which each bound ligand molecule occludes the sites of two subunits of $[PA_{63}]_7$.

EXPERIMENTAL PROCEDURES

Preparation of Proteins. LF_N (34 kDa; Figure 1), EF (91 kDa with a hexahistidine tag), and $[PA_{63}]_7$ (444 kDa) were purified as described previously (*11*, *12*). LF_N (30 kDa) used

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[1] Abbreviations EF, edema factor; LF, lethal factor; LF_N, amino-terminal domain of LF; PA, protective antigen.

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in Figure 2 and Table 1 was treated with 1 unit of thrombin/mg of LF_N overnight at 4 °C to remove the amino-terminal hexahistidine tag. Thrombin was removed by incubation with benzamidine−Sepharose 6B (Pharmacia). Proteins were quantified by Bradford assay (Bio-Rad) and the Edelhoch method (*13*). In the Edelhoch method, absorbance at 276 nm of a protein solution in 6 M guanidine hydrochloride and 30 mM MOPS, pH 7.0, was measured. The extinction coefficient of a protein was calculated by adding the extinction coefficients (in 6 M guanidine hydrochloride) of each tryptophan (5405 M^{-1}) and tyrosine (1455 M^{-1}) in the protein. Concentration was determined by dividing the absorbance at 276 nm by the extinction coefficient.

Reductive Methylation. Proteins were dialyzed into 20 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 8.2, and 150 mM NaCl. Sodium cyanoborohydride was added to the protein (approximately 1 mg) to a final concentration of 5 mM. One microcurie of [^{3}H]-formaldehyde (98 Ci/mol) or [^{14}C]formaldehyde (52 Ci/mol) (NEN Dupont) was added to $[PA_{63}]_7$ or LF_N, respectively, and the solution was incubated overnight at 4 °C. Proteins were dialyzed against 50 mM potassium phosphate, pH 8.0, and 150 mM NaCl. The incorporation of either ^{3}H or ^{14}C was measured by scintillation counting. A solution of 2.4×10^{-5} M PA_{63} was determined to contain 910 dpm of ^{3}H/μL. A solution of 5.0×10^{-5} M LF_N was determined to contain 3540 dpm of ^{14}C/μL. The specific activities were calculated to be 3.8×10^{13} dpm of ^{3}H/mol of PA_{63} and 7.1×10^{13} dpm of ^{14}C/mol of LF_N. The molar ratio of radiolabeled methyl group per molecule of PA_{63} or LF_N was determined by the formula:

$$\text{methyl group/protein} = [\text{specific activity of protein (dpm/mol of protein)}]/[\text{specific activity of formaldehyde (Ci/mol)} \times 2.22 \times 10^{12}\ \text{dpm/Ci}]$$

The number of methyl groups per polypeptide chain was calculated to be 0.6 for LF_N and 0.2 for PA_{63}.

Isolation of LF_N·$[PA_{63}]_7$ Complexes. Mixtures (100 μL) of various concentrations of ^{14}C-LF_N (1.0×10^{-7}, 2.0×10^{-7}, 4.0×10^{-7}, 8.0×10^{-7}, 1.2×10^{-6}, 1.6×10^{-6}, 2.0×10^{-6}, and 4.0×10^{-6} M) and 4×10^{-7} M ^{3}H-$[PA_{63}]_7$ in 50 mM potassium phosphate, pH 8.0, 150 mM NaCl, and 0.2% Elugent (Calbiochem) were loaded onto a TSK QC-PAK300GL column (Toso Haas). The column buffer (50 mM potassium phosphate, pH 8.0, 150 mM NaCl, 0.2% Elugent) was pumped at a flow rate of 1 mL/min. Fractions of 125 μL were collected, and radioactive content was determined by scintillation counting (Wallac Winspectral 1414 liquid scintillation counter).

Multiangle Laser Light Scattering. LF_N (200, 200, or 460 μg injected in separate runs), $[PA_{63}]_7$ (40, 80, or 160 μg), or LF_N·$[PA_{63}]_7$ (230:80, 100:50, or 100:50 μg/μg) was loaded onto a Shodex KW-803 column at a flow rate of 0.5 mL/min. EF (685, 685, or 685 μg) or EF·$[PA_{63}]_7$ (260:93, 260:93, or 42:93 μg/μg) was loaded onto a Superdex 200 column (Pharmacia) at a flow rate of 0.5 mL/min. The column buffer was 20 mM Tris-HCl, pH 8.2, and 200 mM NaCl. The column was connected to a DAWN EOS 18-angle light scattering detector (Wyatt Technology) and an OPTILAB DSP interferometric refractometer (Wyatt Technology). Detectors 8−18 of the light scattering detector were used. A d*n*/d*c* (refractive index increment) value of 0.185 mL/g was used. Bovine serum albumin was used as an isotropic scatterer (i.e., scatters light equally in all directions) to normalize the light scattering detectors. Normalization compensates for slight differences in electronic gain among the detectors. Molecular mass calculations were performed by ASTRA software.

RESULTS AND DISCUSSION

Our attempts to determine subunit ratios of anthrax toxin complexes formed at the surface of CHO-K1 cells were unsuccessful due to several problems, including difficulties in measuring the fraction of PA converted to heptamer, nonspecific binding by preformed $[PA_{63}]_7$, and high background due to nonspecific binding of ligands to the cell surface. We therefore focused on measuring ligand:PA_{63} ratios of complexes formed in solution.

As a prelude we purified PA_{63}, EF, and LF_N and quantified their concentrations both by the Bradford assay and by the Edelhoch method (*13*). While the Bradford assay relies on using protein (bovine serum albumin) standards and the Edelhoch method relies on using extinction coefficients of tryptophan and tyrosine in guanidine hydrochloride, the two methods yielded consistent results. Native PA, LF, and LF_N were synthesized in *Escherichia coli* and purified as described (*11, 12*). PA_{63} was obtained in pure form by anion-exchange chromatography of trypsin-activated PA. The purified protein obtained in this way is heptameric ($[PA_{63}]_7$) (*3*) and stable in solution if the pH is maintained moderately basic (pH ≥8.0). This form binds EF and LF avidly and mediates their actions on cells.

As an initial approach to measuring the subunit ratios in ligand-saturated PA_{63}, we differentially radiolabeled PA_{63} and LF_N, mixed them in various ratios, isolated the complexes by size-exclusion chromatography, and measured the isotope ratios. PA_{63} was reductively methylated with ^{3}H-HCHO and LF_N with ^{14}C-HCHO. Under the conditions employed, less than one radiolabeled methyl group was attached per polypeptide chain. The methylation does not change the electrostatic charge of the modified amino group and is therefore gentle and unlikely to perturb function. ^{14}C-LF_N was added in various ratios to a constant amount of ^{3}H-PA_{63}, and the mixtures were chromatographed on a TSK column equilibrated with 50 mM potassium phosphate, pH 8.0, in 150 mM NaCl. The nonionic detergent, Elugent, was added to minimize interactions of $[PA_{63}]_7$ with the column matrix. The concentration of PA_{63} (expressed in terms of the monomeric subunits) was approximately 2800-fold higher than the dissociation constant of the LF-PA_{63} interaction (*4*), ensuring virtually complete binding of the LF_N up to the point of saturation of PA_{63}. The exclusion limit of the column was such that both the LF_N·PA_{63} complexes and unliganded PA_{63} heptamer eluted together in the void volume, while unbound LF_N was delayed by the column and separated from these complexes. The high molecular mass complexes were collected, ^{3}H and ^{14}C were measured by scintillation counting, and the molar ratios of LF_N to PA_{63} were calculated. As shown in Figure 1, the ratio of LF_N to PA_{63} subunits in the complexes plateaued at a value of 0.43, equivalent to a ratio of three LF_N molecules per PA_{63} heptamer. Saturation occurred at approximately the same value of the ratio of

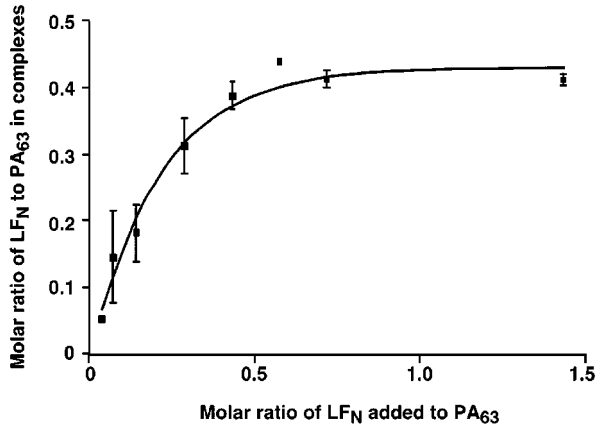

FIGURE 1: Subunit ratios in complexes of differentially radiolabeled LF_N and $[PA_{63}]_7$ isolated by size-exclusion chromatography. ^3H-$[PA_{63}]_7$ and various amounts of ^{14}C-LF_N (indicated in the *x*-axis as molar equivalents of PA_{63}) were chromatographed over a gel filtration column, as described under Experimental Procedures. The complexes in the void volume were isolated, and their radioactive content was measured by scintillation counting to determine the molar ratio of LF_N to PA_{63} (ordinate).

Table 1: Molecular Mass Determinations Using Multiangle Laser Light Scattering

	measured molecular mass (kDa)	theoretical molecular mass (kDa)
EF	93 ± 0.3	91
LF_N	31 ± 2	30
$[PA_{63}]_7$	460 ± 3	444
EF·$[PA_{63}]_7$	720 ± 20	717[a]
LF_N·$[PA_{63}]_7$	530 ± 10	534[a]

[a] Theoretical values for saturated complexes are based on a stoichiometry of three ligand molecules per PA_{63} heptamer.

added LF_N to PA_{63}, consistent with the high affinity of the binding.

As an independent way to determine subunit ratios, we used multiangle laser light scattering to measure the absolute molecular masses of PA_{63} complexes formed in solution. The light scattered by a protein is directly proportional to the product of the weight-average molar mass and the concentration of the protein and also shows angular dependence on particle mass (*14*, *15*). Samples of PA_{63}, EF or LF_N, or mixtures thereof were chromatographed on Superdex 200 (EF·PA_{63}) or Shodex KW-803 (LF_N·PA_{63}) size-exclusion columns, and the effluent was continuously monitored with an 18-angle laser light scattering detector and an interferometric refractometer connected in series. The refractometer provided a continuous index of protein concentration. The molecular masses of the proteins were calculated by ASTRA software.

EF, LF_N, and the PA_{63} heptamer each gave a single peak, and the molecular masses determined were within 2−5% of their theoretical values (Table 1). Consistent with its heptameric state, PA_{63} gave a molecular mass of 460 kDa, close to the theoretical value of 444 kDa. When PA_{63} was mixed with excess EF (molar ratio of EF to PA_{63} monomeric subunits = 2), two peaks were observed in the column effluent corresponding to liganded PA_{63} (elution volume, 8.6 mL) and excess free EF (elution volume, 12.3 mL) (Figure 2). The peaks contained similar amounts of protein, as determined by the refractomer, but the complex scattered

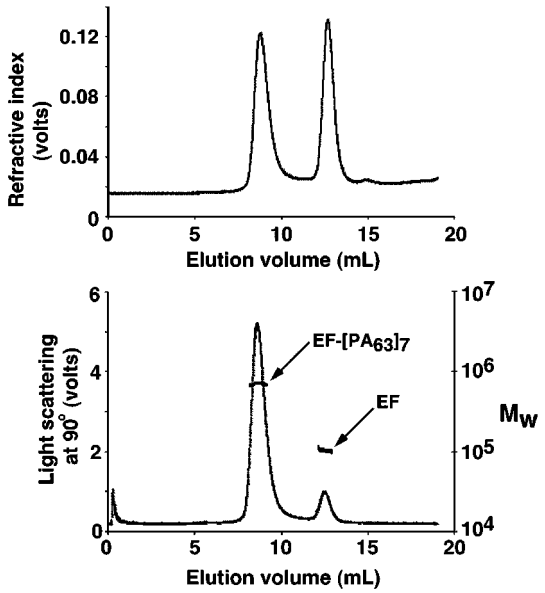

FIGURE 2: Multiangle laser light scattering of EF-$[PA_{63}]_7$. A mixture (120 μL) of EF (260 μg) and $[PA_{63}]_7$ (93 μg; approximately 2-fold molar excess of EF over PA_{63} monomer) was chromatagraphed over a Superdex 200 gel fitration column, which was connected to a light scattering detector (lower panel) and an interferometric refractometer (upper panel). The values of molecular mass determined in volume increments across each peak are shown (arrows).

light much more efficiently, consistent with its higher molecular mass. Molecular masses were almost constant at points across each peak, indicating homogeneity of the molecular species within the individual peaks. The measured molecular mass of the protein in the earlier peak was 720 kDa, corresponding almost exactly to the theoretical molecular mass of a complex of three EF molecules plus one PA_{63} heptamer (Figure 2). Increasing the molar ratio of EF to PA_{63} subunits to 2.5 did not affect the measured molecular mass (data not shown). Chromatography of a mixture of LF_N and PA_{63} heptamer also yielded two peaks (data not shown), the first yielding a measured molecular mass of 530 kDa, equal to that of a complex of three molecules of LF_N and one $[PA_{63}]_7$, and the second peak, a molecular mass of 31 kDa, corresponding to that of free LF_N (Table 1).

Thus two unrelated methods of estimating the subunit ratios yielded the same value, three, as the maximal number of enzymatic ligand molecules that can bind per PA_{63} heptamer at ligand saturation. Determination of subunit ratios of complexes from isotope ratios clearly depends on accurate specific radioactivities of the labeled proteins and, thus, of protein concentrations. The Edelhoch and Bradford methods of protein estimation gave consistent and accurate results, and we estimate that the specific radioactivities of the proteins were accurate to within ±5%. Thus the error in our estimate of three molecules of LF_N bound per PA_{63} heptamer is within ±0.2. Although protein concentration enters into the calculation of molecular masses determined by multiangle laser light scattering, in this case the concentration was determined by monitoring the refractive index of the column effluent and did not depend on our measurements by the Edelhoch and Bradford methods. In addition, the solvent employed in the light scattering measurements, unlike that in the isotope ratio experiments, contained no detergent. Thus

we believe there is no systematic error that might have affected both of the methods we used to measure subunit ratios.

The value of three ligand molecules per PA_{63} heptamer differs from that reported in another publication (*16*). In that study, Singh et al. added increasing amounts of LF to either trypsin-nicked PA or purified PA_{63} heptamer and subjected the mixtures to native gel electrophoresis. They found free LF only when the amount of LF added to the mixture exceeded 7 molar equiv of PA_{63} heptamer and concluded that the PA_{63} heptamer can bind seven molecules of LF. When we performed a similar experiment with PA_{63} plus various amounts of LF_N, we observed free LF_N when the amount added exceeded 3 molar equiv of $[PA_{63}]_7$ (data not shown). The basis of this discrepancy is unknown but may lie in the sensitivity of detection of free ligand. Despite this discrepancy, it is worth noting that Figure 2 of the report of Singh et al. shows what appear to be three major bands of putative LF·PA_{63} complexes, possibly corresponding to the heptamer with one, two, or three copies of bound LF. The Phast electrophoresis method used by the group is rapid, and electrophoretic separation may have occurred within a sufficiently short period relative to the half-life of LF dissociation (~25 min at 23 °C) to allow the individual complexes to remain discrete (*4*).

A quantitative study by surface plasmon resonance has revealed a single class of sites for EF and LF on PA_{63} with a K_d on the order of 1 nM (*4*). These sites most likely reside on the hydrophobic surface of domain 1′ that is exposed by removal of PA_{20} and is located on the "top" face of the heptamer. While binding of seven whole EF/LF ligands (90 and 89 kDa, respectively) to this surface is conceivable, it would not be surprising, given the masses of EF and LF relative to the PA_{63} monomers, if steric overlaps between bound ligands constrained this number to a lower value. The value of three ligand molecules per PA_{63} heptamer implies that each EF or LF molecule occludes the sites on two subunits. This "footprint" of binding is apparently dictated by the LF_N and EF_N domains alone, as we found the same stoichiometry with LF_N as with full-length EF.

Because there is no evidence of sequence duplications within LF_N or EF_N, it is unlikely that either of these domains contains two quasi-identical sites capable of binding to two complementary sites on two PA_{63} subunits. Rather, we suggest that each ligand binds to an asymmetric site composed of residues on a single PA_{63} subunit (or possibly two adjacent subunits) and sterically occludes the same site on an adjacent subunit. According to this model a PA_{63} heptamer saturated with the LF_N and/or EF_N contains three of these ligands bound to three sequential pairs of PA_{63} subunits around the ring, leaving the seventh subunit unoccupied. Thus the saturated complex is asymmetric.

Because there is no evidence to suggest that ligand molecules bound to adjacent pairs of PA_{63} subunits interact in either a positive or negative manner, it seems likely that each pair acts as an independent site, and consequently a PA_{63} heptamer can bind one, two, or three molecules of ligand (either EF or LF). Wesche et al. have performed studies in which translocation of radiolabeled PA_{63} ligands bound to the cell surface was induced by lowering the pH and found that the efficiency of translocation varied from ligand to ligand, ranging up to 50% with LF_N (*17*). Translocation efficiency as a function of the degree of saturation of PA_{63} has not been carefully examined, however, and the fraction of the ligand molecules translocated in complexes saturated to various degrees remains speculative. This question, together with the problems of determining the mechanism and pathway of translocation across the endosomal membrane, remains an interesting challenge in understanding the overall mechanism by which anthrax toxin acts.

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