Anthrax lethal toxin assembles at the surface of mammalian cells when the lethal factor (LF) binds via its amino-terminal domain, LF₆₃, to oligomeric forms of activated protective antigen (PA). LF-PA complexes are then trafficked to acidified endosomes, where PA forms heptameric pores in the bounding membrane and LF translocates through these pores to the cytosol. We used enhanced peptide amide hydrogen/deuterium exchange mass spectrometry and directed mutagenesis to define the surface on LF₆₃ that interacts with PA. A continuous surface encompassing one face of LF₆₃ became protected from deuterium exchange when LF₆₃ was bound to a PA dimer. Directed mutational analysis demonstrated that residues within this surface on LF₆₃ interact with Lys-197 on two PA subunits simultaneously, thereby showing that LF₆₃ spans the PA subunit:subunit interface and explaining why heptameric PA binds a maximum of three LF₆₃ molecules. Our results elucidate the structural basis for anthrax lethal toxin assembly and may be useful in developing drugs to block toxin action.

\[ \text{Bacillus anthracis}, \text{ the causative agent of anthrax, secretes three monomeric proteins that assemble at the host cell surface to form a series of toxic, non-covalent complexes. Toxin assembly begins when one of these proteins, termed protective antigen (PA₈₃) (83 kDa), binds to either of two cell-surface receptors (1, 2) and a 20-kDa piece is proteolytically removed from the N terminus (3). The remaining receptor-bound fragment, PA₆₃ (63 kDa), then spontaneously self-assembles into a ring-shaped homoheptamer (PA₆₃₇), termed the pre-pore (4). The latter can bind up to three copies of either, or both, of the two remaining toxin components, edema factor (EF) and lethal factor (LF) (5). The combination of LF and PA is sometimes called lethal toxin, and the combination of EF and LF, edema toxin. LF and EF bind competitively to oligomeric forms of PA₈₃ via their homologous N-terminal ~250-residue domains, termed LF₆₃ and EF₆₃ (6, 7). The resulting toxic complexes are then internalized into acidified compartments (8) where the prepore converts into a membrane-spanning pore (9), allowing EF and LF to translocate to the cytosol (10, 11). Once in the cytosol, LF, a Zn²⁺-dependent protease (12), and EF, a calmodulin-dependent adenylate cyclase (13), exert their toxic effects by modifying specific cytosolic targets.}

Describing how the three components of anthrax toxin recognize each other at the molecular level can elucidate the self-assembly process at the cell surface and how they dissociate prior to, or during, translocation in the endosome. Such information could also aid in designing inhibitors that block toxin action. Previously, conservation-guided mutagenesis was used to screen for residues on LF/EF and PA that mediate complex assembly (14–19). Lacy et al. (14) identified a small rectangular patch of seven residues on the surface of LF₆₃/F₆₃ that were critical for interacting with PA. However, later mutagenesis studies revealed a larger footprint on PA than could be accounted for by the surface patch (15). These findings, along with the nanomolar affinity (5, 7) observed for the complex, led us to consider the possibility that additional residues on LF₆₃/EF₆₃ may be involved in binding to PA.

We have employed enhanced methods of peptide amide hydrogen-deuterium (H/D) exchange mass spectrometry to identify regions within LF₆₃ that are involved in binding to PA (20–26). Protein surfaces that are buried at an interface are protected from solvent, causing a decrease in the rate of H/D exchange (27). From H/D exchange profile analysis of LF₆₃-derived peptide fragments, we identified four regions of primary structure that displayed protection from solvent in the presence of PA. These regions form a single continuous surface on LF₆₃ that contacts PA. Further, by mutagenesis we identified two acidic residues, Glu-135 and Asp-182, at diametrically opposite points on this surface that interact with the same residue, Lys-197, on adjacent subunits of oligomeric PA. These findings show how LF and EF bind to oligomeric forms of PA and explain the observation that the heptameric PA prepore can bind only three molecules of EF or LF simultaneously.

\[ \text{EXPERIMENTAL PROCEDURES} \]

\[ \text{Preparation of PA, LF₆₃, and Mutants—Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). Mutations in PA and LF₆₃ were made in the pET22b-PA and pET15b-LFN constructs using the QuikChange method (Stratagene) of site-directed mutagenesis. PA₆₃ and LF₆₃ were purified from Escherichia coli as described (28–30). LF₆₃ used in H/D experiments was further purified over an S-200 Sephacryl column that was pre-equilibrated with Tris buffer (10 mM Tris-HCl, 150 mM NaCl, pH 7).} \]

\[ \text{Activation of PA and Oligomer Formation—PA was activated by incubation with trypsin at a final trypsin:PA ratio of 1:1000 (wt:wt) for 30 min at room temperature. Adding a 10-fold molar excess of soybean trypsin inhibitor stopped the reaction. The ternary complex was formed by mixing the two pre-activated complementary oligomerization-deficient mutants (D512K and K199E/R468A/R470D) in equal quantities with a 3-fold molar excess of LF₆₃ (31). The complex was incubated for 3 h and then purified by chromatography on an S-200 Sephacryl column} \]
that was pre-equilibrated with Tris buffer (10 mM Tris-HCl, 150 mM NaCl, pH 7). Fractions containing the ternary complex were pooled and concentrated to 10–15 mg/ml.

**H/D Exchange Mass Spectrometry Experiments**—Fragmentation optimization and deuterium analysis were carried out as previously described (32–41). Undeuterated protein (20 μl, 70 μg) supplemented with 30 μl of quencher buffer containing 3.2 M guHCl, 0.8% formic acid, 16.6% glycerol, pH 2.3, followed by fragmentation on a 66-μl Peptide digestion column (flow rate 100 μl/min) produced optimal peptide probe coverage. For H/D exchange, a 70-μl aliquot of LFN was pipetted into 210 μl of D2O exchange buffer (200 mM NaCl, 0.1% formic acid, pD 4.0, 5 °C, final D2O 70%) to initiate H-to-D exchange. At various time points, a 20-μl aliquot of LFN was pipetted into 30 μl of quencher buffer (final concentration 2 M guHCl, H2O, 0.4% formic acid, pH 2.3, with 10% glycerol) to promote subsequent thawing at ~0 °C. Final protein concentration was ~5 mg/ml in 2 M guHCl. Samples were rapidly frozen on dry ice and stored at ~80 °C.

The number of deuterons incorporated was determined by electro-spray mass spectrometry (MS). Quenched protein samples were rapidly thawed, fragmented by passage through an immobilized protease column (0.05% trifluoroacetic acid, 100 μl/min, porcine pepsin 66 μl), and directly applied to a C18 column (C-18 300A; Grace Vydac, Hesperia, CA), followed by elution with a linear acetonitrile gradient (50 μl/min, 5–45% AcCN, 0.05% trifluoroacetic acid over 30 min) at pH 2.3, 0 °C. Eluate was electrosprayed directly into the mass spectrometer, either a Thermo Finnigan LCQ Classic ion-trap mass spectrometer (San Jose, CA), for data-dependent MS/MS or a Waters Micromass Q-TOF mass spectrometer (Manchester, UK) for MS1-only acquisition, as described (32–41).

MS/MS data were analyzed using the Sequest software program (Thermo Finnigan, Inc.) to identify the likely sequence of the parent peptide. Tentative identifications were tested with specialized H/D exchange mass spectrometry data-reduction software (32–41). The measured D-label for each fragment was corrected for the 70% D2O present during the exchange-in and for the loss of D-label suffered during the analysis employing the methods of Zhang and Smith (42) as shown in Equations 1 and 2.

\[
\text{Deuterium} (\%) = \left( \frac{m(P) - m(N)}{m(E) - m(N)} \right) \times 100 \quad \text{(Eq. 1)}
\]

\[
\text{Deuterium} (\%) = \frac{m(P) - m(N)}{m(E) - m(N)} \times \text{MaxD} \quad \text{(Eq. 2)}
\]

where \(m(P)\), \(m(N)\), and \(m(E)\) are the centroid values of partially deuterated peptide, non-deuterated peptide, and equilibrium–deuterated peptide, respectively. MaxD is the maximum deuterium incorporation calculated by subtracting the first two residues of the peptide, which have been shown to be fully exchanged due to end effects (43) and by subtracting the number of prolines from the total number of amide hydrogens in the peptide. The experimentally determined deuterium content of the equilibrium–deuterated sample was on average 80% (i.e., \(m(E) - m(N)/\text{MaxD} \times 0.70\); 0.70 is the deuterium content in the exchange buffer). Comparative on-exchange studies were performed three times, with representative results shown in Figs. 2 and 3.

**Cell Surface Binding Assay**—PA-mediated cell surface binding of 35S-labeled LFN was measured as described (15, 29). Briefly, Chinese hamster ovary-K1 cells were placed on ice for 30 min to inhibit endocytosis. The cells were washed once with cold Dulbecco’s phosphate-buffered saline and incubated with 2.4 × 10–8 M of trypsin-nicked PA on ice for 2 h. The cells were washed again with cold Dulbecco’s phosphate-buffered saline and incubated on ice with 35S-labeled LFN for 2 h. The 35S-LFN was produced by in vitro transcription/translation using the TNT T7-coupled reticulocyte lysate expression system (Promega). The cells were washed three times with cold Dulbecco’s phosphate-buffered saline and incubated on ice with lysis buffer for 20 min. Radioactive content was determined by scintillation counting.

**RESULTS**

Before measuring the H/D exchange characteristics of LFN, we varied the conditions of protease digestion, including guHCl concentration and duration, in order to maximize the number of peptides generated and the sequence coverage (see “Experimental Procedures”). Under optimal conditions we obtained 155 peptides, which completely covered the sequence between residues 63 and 263 (Fig. 1A). We were unable to obtain peptides within the first 60 residues of LFN due probably to the preponderance of basic residues in this region, which produce highly positively charged species that are difficult to identify in the mass spectrometer.

**H/D Exchange of LFN in the Absence of PA**—To characterize the kinetics of deuterium incorporation by LFN alone, we measured the deuterium content of LFN-derived peptides under native conditions at
H/D Exchange of LFN in the Presence of Oligomeric PA—We first examined the H/D exchange characteristics of LFN bound to the heptameric pre-pore, but because of the inherent tendency of these complexes to aggregate at high concentrations, we turned to a dimeric form of PA as an alternative binding partner for LFN. When two mutated, oligomerization-deficient forms of PA are combined in the presence of LFN (31), a ternary complex is formed containing one molecule of each species. The binding of the single LFN molecule stabilizes the PA dimer and is believed to occur via the same interactions that mediate binding to the heptameric pre-pore. The ternary complex was used earlier to identify some residues on each subunit of PA that were involved in binding to LFN (15). Digestion of the complex yielded 140 peptides from the LFN moiety. For several peptides, there was little change in deuterium uptake when LFN was bound to the PA dimer, but for certain others there was a marked decrease (Fig. 2A). Each peptide fragment contains multiple amide sites, which may exchange with different rates, but the data empirically fitted well with a two-component (fast and slow) exponential model. For each peptide the percentage of deuterons incorporated in the absence or presence of PA was determined over time, and values were plotted against the protein sequence to illustrate regions that became protected from H/D exchange in the presence of PA.

Four discrete sites in LFN (i.e. residues 95–120, DX site 1; 137–147, DX site 2; 177–189, DX site 3; and 223–235, DX site 4) became significantly more protected from solvent exchange in the presence of PA (Fig. 2B). When mapped onto the surface of LFN (Fig. 2C), these four sites converged to form a continuous surface that was larger than that implied by previous mutagenesis studies (14). All of the residues previously implicated in binding (i.e. Asp-182, Asp-187, Leu-188, Tyr-223, His-229, Leu-235, and Tyr-236) fell exclusively within DX sites 3 and 4, which comprise approximately half of the binding surface identified here. To address the role of the two additional sites identified here (i.e. DX sites 1 and 2), we constructed a series of mutants within and near these regions and tested their effects on PA binding.

Exploring the Roles of DX Sites 1 and 2—Twenty-seven mutants were generated at surface-exposed residues within and near DX sites 1 and 2. Mutated proteins were 35S-labeled using in vitro incorporation of [35S]Met at the five methionine sites in LFN, and the proteins were then tested for ability to bind cells in the presence and absence of PA. The extent of binding for each mutant is shown in Fig. 3A as the fraction of wild-type binding. Within DX site 1, only two residues, D106A and
Structural Basis for Anthrax Toxin Assembly

**LFN Binds to the Same Residue on Adjacent PA Subunits**—We next used the ternary complex to probe for pairs of basic residues on the PA dimer that were spaced such that they might interact with Glu-135 and Asp-182 on LFN. For simplicity, we only considered those pairs that were (i) positively charged, (ii) known to be involved in binding to LFN, and (iii) separated by ~40 Å. Only two pairs met these criteria, and both pairs consisted of the same residue from adjacent subunits, Lys-197 on subunit A with Lys-197 on subunit B and Arg-200 on subunit A with Arg-200 on subunit B. We excluded the latter pair based on their proximity to the dimer interface and instead focused on Lys-197. Mutating Lys-197 to Ala on either PA subunit caused a dramatic loss of binding to LFN, but with the K197A mutation on subunit A the loss was greater (Fig. 4A).

**DISCUSSION**

Anthrax toxin entry into mammalian cells depends on the ability of the individual toxin components to undergo self-assembly at the cell surface. After PA is proteolytically activated, the PA-Receptor complexes are assumed to diffuse in the plane of the membrane, allowing subunit-subunit collisions that result ultimately in the formation of the pre-pore. LF and EF can bind via their homologous N-terminal domains to oligomeric intermediates of PA, or to the fully assembled heptamer. Complexes of either LF or EF with oligomeric PA have thus far been refractory to crystallization, necessitating other approaches to characterize the interaction. On the basis of H/D exchange experiments, we have identified four distinct regions in LFN that become more protected from solvent H/D amide exchange in the presence of PA. These four DX sites converged to form a large (~1200 Å²) surface on LFN (Fig. 2C), and by site-directed mutagenesis we were able to identify additional putative contact residues, beyond those found earlier (Fig. 3C). The majority of the binding defective mutants identified here and elsewhere (15) localized to DX sites 2–4.

Our mutagenic analysis revealed evidence that two residues on the PA binding surface of LFN (Glu-135 and Asp-182), which are ~40 Å apart, interact with the same basic residue (Lys-197) on adjacent subunits of the pre-pore. This result supports earlier findings that oligomerization of PA is required for LF or EF binding, that the footprint of LFN and EF on PA is large enough to block the binding sites on two PA subunits, and that the heptameric pre-pore binds only three molecules of EF and/or LF at saturation. The Glu-135-Lys-197 interaction and Asp-182-Lys-197 interaction limit to two the possible orientations of LFN docked onto the pre-pore. The fact that mutations of Asp-182 and Lys-197 from subunit A have a greater effect on binding than mutations of Glu-135 and Lys-197 from subunit B favors the orientation shown in Fig. 4C. Other evidence favoring this orientation has come recently from Rosetta protein-protein docking simulations and identification of other contact pairs by directed mutagenesis (44). In this model, DX sites 1–4 make extensive contacts with subunit A from PA, with minimal contact observed between DX site 2 and subunit B from PA. This model places the N terminus over the pore lumen, whereas the other orientation would position the N terminus away from the lumen (Fig. 4D). Previous work has established that the amino terminus of LFN is the first to enter the pore lumen during translocation (45).

These results demonstrate how the four sites within LFN identified by DX participate in the binding interaction surface and also provide an explanation for how a single LFN bridges two PA subunits. In keeping with previous mutagenesis results, most of the residues implicated in binding are expected to be charged at neutral pH values. The affinity of such an interface is expected to decrease with decreasing pH where the acidic groups are more likely to exist in the protonated state. Thus, the
FIGURE 4. H/D exchange and mutagenesis define a model for the LF<sub>N</sub>-PA interaction. A, data represent the fraction of mutant 35S-LFN bound to PA and mutants of PA on cells relative to that seen for the wild-type ternary complex. Error bars represent S.E. of the mean. B, surface representation of dimeric PA with Lys-197 shown in yellow on subunits A and B. C, LF<sub>N</sub>-PA<sub>35</sub> model adapted from Lacy et al. (44) with the two subunits (A and B) of PA shown in pink and cyan. DX sites 1, 2, 3, and 4 identified using H/D exchange are colored blue, green, yellow, and red, respectively. Asp-182 and Glu-135 side chains are shown for LFN, and Lys-197 side chains on both PA subunits are shown. D, top view of LF<sub>N</sub> binding to domain 1 of heptameric PA. Black trace represents outline of LF<sub>N</sub>. Yellow circles indicate Lys-197 positions. Figures were generated using Chimera (46).

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