The Identification and Structure of the Membrane-Spanning Domain of the

*Clostridium septicum* Alpha Toxin

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Abbreviations

AT: alpha toxin

TMD: transmembrane domain

GPI: glycophosphatidylinositol

CDCs: cholesterol-dependent cytolysins

IANBD: N, N’-dimethyl-N-(iodoacetyl-N’-(7-nitrobenz-2-oxa-1,3-diazoyl)ethylendiamine

IAF: 5-iodoacetamidofluorescein

FI: fluorescence intensity

D1-D4: domains 1-4

DSA: DOXYL-stearic acid
Summary

Alpha toxin (AT) is a pore-forming toxin produced by *Clostridium septicum* that belongs to the unique aerolysin-like family of pore-forming toxins. The location and structure of the transmembrane domains (TMD) of these toxins have remained elusive. Using deletion mutagenesis, cysteine-scanning mutagenesis and multiple spectrofluorimetric methods a membrane-spanning amphipathic $\beta$-hairpin of AT has been identified. Spectrofluorimetric analysis of cysteine-substituted residues modified with an environmentally sensitive fluorescent probe via the cysteine sulfydryl showed that the side chains of residues 203-232 alternated between the aqueous milieu and the membrane core when the AT oligomer was inserted into membranes, consistent with the formation of an amphipathic transmembrane $\beta$-hairpin. AT derivatives that contained deletions that removed up to 90% of the $\beta$-hairpin did not form a pore but were similar to native toxin in all other aspects of the mechanism. Furthermore, a mutant of AT that contained an engineered disulfide, predicted to restrict the movement of the $\beta$-hairpin, functioned similarly to native toxin except that it did not form a pore unless the disulfide bond was reduced. Together these studies revealed the location and structure of the membrane-spanning domain of AT.
Introduction

_Clostridium septicum_ is a Gram-positive anaerobe that typically causes fulminant, often fatal infections such as nontraumatic gas gangrene or necrotizing enterocolitis in compromised patients (1). More recently it has also been shown to be a superinfection of hemolytic uremic syndrome (2). Alpha toxin (AT) is the only known lethal factor secreted by _C. septicum_ (3). AT belongs to a distinctive class of related cytolytic toxins whose prototype is aerolysin, secreted by the Gram-negative bacterium _Aeromonas hydrophila_ (4). Interestingly, enterolobin, a cytolytic protein derived from the Brazilian _Enterolobium contortisiliquum_ tree also appears to be related to aerolysin (5,6).

The mechanisms of AT and aerolysin have been shown to be highly similar. Both toxins bind to cells via glycoposphatidylinositol (GPI)-anchored protein receptors (7,8) although aerolysin appears to bind to some receptors that are not recognized by AT and vice versa. Following cell binding both toxins are activated by the proteolytic cleavage of an amino terminal propeptide by furin or furin-like protease (9,10). Activation then allows the toxin monomers to oligomerize on the membrane and form a pore (11,12). The difference seen in receptor specificity of the two toxins appears to be linked to the presence of an amino terminal peptide of aerolysin that is not conserved in AT. The crystal structure of aerolysin, the only member of this toxin family whose crystal
structure has been solved (13), shows that it is a bi-lobal protein consisting of four distinct domains (D1-4), three of which are rich in β-sheet structure (Figure 1). The small lobe of aerolysin is missing from the amino terminus in AT and this implies that AT is a single-lobed structure consisting of three domains that are homologous to D2-D4 of aerolysin (Figure 1). The small lobe of aerolysin (D1) contains a lectin-binding domain that enables it to bind to various receptors that are not recognized by AT (14,15). However, fusion of D1 of aerolysin to the amino terminus of AT converts it to a molecule with aerolysin-like receptor specificity and activity (15). Comparatively little is known about the cytolytic mechanism of enterolobin, but its primary structure appears to be more related to aerolysin than AT. Enterolobin displays sequence similarity with both the small and large lobes of aerolysin and appears to form a dimer in solution (6,16).

Although many aspects of the cytolytic mechanism of AT and aerolysin have been elucidated, the domain(s) of these toxins directly involved in the formation of the pore have yet to be identified. Two structural motifs used by pore-forming toxins to span membranes are amphipathic β-strands and α-helices. Both motifs have been identified in toxins as the secondary structures used to cross the membrane during pore formation. For example, the pore-forming domain of the colicins and the T-domain of diphtheria toxin use a pair of amphipathic α-helices to span the membrane (17,18), whereas the protective antigen component of anthrax toxin (19), the α-hemolysin from
*Staphylococcus aureus* (20,21), and perfringolysin O from *Clostridium perfringens* (22,23) all use amphipathic \( \beta \)-strands that contribute to the formation of a transmembrane \( \beta \)-barrel (Figure 1). However, identification of the TMD has not been necessarily easy or without controversy. The TMD of the cholesterol-dependent cytolysins (CDCs) was first suggested to be comprised of a \( \beta \)-sandwich (24), or an amphipathic \( \alpha \)-helix (25) and Gilbert *et al.* (26) suggested that the CDCs did not penetrate the membrane at all. The pore-forming region of the CDCs was unambiguously shown to be comprised of 2 amphipathic \( \beta \)-hairpins located in D3 of the perfringolysin O crystal structure that are derived from 6 short \( \alpha \)-helices of the soluble monomer (22-24).

It was previously hypothesized that the D4 \( \beta \)-sandwich of aerolysin penetrates the membrane and forms the TMD of aerolysin (13). More recently, Tsitrin *et al.* (27) also suggested that the D4 \( \beta \)-sandwich forms the TMD of aerolysin based on an electron density map of what was proposed to be a soluble oligomer of aerolysin. However, to date, no experimental data have been reported that directly demonstrate the interaction of a specific region of aerolysin, or the related AT or enterolobin, with the membrane.

The aerolysin-based structural model of AT and the aerolysin crystal structure exhibit a conspicuous amphipathic loop that is found in D2 of AT and the corresponding D3 of aerolysin (Figure 1). Using multiple fluorescent and biochemical approaches we
have confirmed that this amphipathic loop in D2 of AT, comprised of residues K203 to Q232, forms an amphipathic β-hairpin that spans the membrane and is necessary for pore formation. Similar analyses of residues in the D3 β-sandwich of AT, corresponding to the D4 region of aerolysin indicates that it is only peripherally associated with the membrane and is unlikely to participate directly in the formation of the transmembrane β-barrel.

Experimental Procedures

**Bacterial strains, plasmids, cell lines, and chemicals.** The gene for AT was cloned into the pET-22(b)+ expression vector (Novagen, Madison, WI) (designated pBRS10) and placed into *Escherichia coli* BLR-DE3 cells for high-level expression as previously described (28). The SupT1 cell line was a generous gift of Dr. William Hildebrand (University of Oklahoma Health Sciences Center, Oklahoma City, OK). All chemicals were obtained from Sigma Chemical Company (St. Louis, MO) and all enzymes from Gibco BRL (Rockville, MD) unless otherwise specified.

**Generation of deletion and point mutations.** All deletion mutations in AT were generated using a four-primer site-directed PCR mutagenesis procedure previously described
except that pBRS10 was used as the template and pfu turbo thermostable polymerase (Stratagene, La Jolla, CA) was substituted for taq. PCR overlap products were purified using the Quantum-Prep PCR Kleen Spin columns (Bio-Rad, Hercules, CA), digested with NcoI and XhoI, and ligated into pET-22(b)+ digested with NcoI and XhoI.

An active, cysteine-less derivative of AT, termed AT\textsuperscript{C86A}, was generated using the above mutagenesis procedure and the resulting plasmid was designated pBRS20. All cysteine substitutions were produced using pBRS20 as template, using either the four-primer PCR mutagenesis procedure above or QuikChange site-directed mutagenesis (Stratagene).

**Expression and purification of AT.** The growth and harvesting of *E. coli* BLR-DE3 expressing polyhistidine-tagged native AT and the various AT derivatives was performed according to Sellman *et al.* (28). The cell pellets were resuspended in 150 ml of buffer A (10 mM MES (2-(N-morpholino)ethanesulphonic acid) (Research Organics, Cleveland OH), 150 mM NaCl, pH 6.5). Lysis of cells was carried out in an EmulsiFlex-C5 high pressure homogenizer (Avestin, Ottawa, ON, Canada) at 15,000 psi. Cell debris was removed by centrifugation at 21,000 x g for 10 min. Purification of the His-tagged AT from the supernatant was accomplished using a cobalt-chelating column and
cation-exchange column was done as previously described (28). AT-containing
fractions eluted from the cation-exchange column were combined in a Micro-ProDiCon
System (Spectrum, Gardena, CA) with a 10,000 MWCO Micro-ProDiCon membrane for
simultaneous dialysis and concentration. Samples were dialyzed against 10 mM MES,
500 mM NaCl, 1 mM EDTA, pH 6.5 (buffer B) overnight at 4°C. For cysteine-substituted
proteins, 1 mM dithiothreitol was included in the dialysis buffer. 10% glycerol was added
to the concentrated toxin before storage at −80°C. Protein concentration was
determined by absorbance at 280 nm using a molar extinction coefficient of 63,000 M⁻¹
cm⁻¹ (Tweten, unpublished data).

**SupT1 Membrane Preparation.** SupT1 cells were cultured in RPMI 1640 growth medium
supplemented with 20% fetal calf serum and 100 units penicillin and streptomycin. Four
roller bottles of cells were grown to approximately 2 × 10⁶ cells/ml, the cells were
harvested, pelleted at 250 x g for 10 min, and resuspended to a final volume of 50 ml in
Hanks Balanced Salt Solution (HBSS) (BioWhittaker, Walkersville, MD). Membranes
were prepared by lysing the cell suspension in an EmulsiFlex-C5 high pressure
homogenizer set at 15,000 psi. Cell membranes were collected by centrifugation at
30,000 x g for 20 min. Supernatant was removed and the cell membranes were washed
five times by resuspending the membrane pellet in 30 ml of buffer C (50 mM HEPES,
0.5 M NaCl, pH 8.0) and centrifuging at 30,000 x g for 20 min. Following the final wash, the membranes were resuspended with buffer C to an A$_{600}$ of 10.

Construction of the AT homology model. The homology model of AT was based on the 2.3 Å resolution crystal structure of proaerolysin (Feil, S.C., Rossjohn, J., Buckley, J.T. and Parker, M.W., unpublished results). The sequence alignment, which included the related toxin, enterolobin, and subsequent homology model were generated using the aerolysin structure as a template within the HOMOLOGY module of Insight II (Accelrys Inc., San Diego, U.S.A.) on a Silicon Graphics Indigo 2 Maximum Impact Workstation. The model was built in two stages: (i) identification of significant regions of sequence identity between AT and aerolysin, and assigning coordinates to these regions, (ii) assigning coordinates to the homology model where the sequence identity to aerolysin is less convincing using a database of peptide fragments. The models were visually checked to remove any steric clashes within the software package ‘O’ (30), using the lego side chain and lego main chain options. This was followed by energy minimisation (500 cycles of Powell minimisation with tight harmonic restraints on the C$_\alpha$ atoms, followed by 500 cycles with no harmonic restraints) using X-PLOR (v. 3.851) (31). The quality of the model was assessed using PROCHECK (Laskowski et al., 1993) and the 3D-1D environment plot (32). The similar molecular mechanism, the pairwise sequence
identity, the good stereochemistry and 3D-profiles of the AT model, are factors indicative of a good quality homology model.

Modification of Cysteine-Substituted AT with NBD or IAF. All cysteine-substituted mutants of AT were stored in 1 mM dithiothreitol (DTT). Therefore, excess DTT was removed before labeling the unique cysteines of each AT mutant with the sulfhydryl-specific derivative of the fluorescent dye N,N'-dimethyl-N-(iodoacetyl-N'-(7-nitrobenz-2-oxa-1,3-diazoyl)ethylenediamine (NBD) or 5-idoacetamido fluorescein (IAF) (Molecular Probes, Eugene, OR). Approximately 1 mg of toxin was passed through a gel filtration column (1.5 cm inside diameter x 20 cm) containing Sephadex G-50 equilibrated in buffer C at room temperature. NBD or IAF was then added to the protein to a 20-fold molar excess. The reaction was incubated in the dark for 1 hour at room temperature and passed again over a Sephadex G-50 column equilibrated in buffer C to remove unbound dye. The extent of labeling was determined spectroscopically using an ε_{478\text{nm}} of 25,000 M^{-1} cm^{-1} for NBD or an ε_{492\text{nm}} of 75,000 M^{-1} cm^{-1} for IAF (33). Protein concentration was determined by absorbance at 280 nm using a molar extinction coefficient of 63,000 M^{-1} cm^{-1} (Tweten, unpublished data).
**SupT1 Cell Viability Assay.** SupT1 cells were cultured in RPMI 1640 growth medium supplemented with 20% fetal calf serum and 100 units penicillin and streptomycin. SupT1 cells at 500,000 cells/ml were placed into the wells of a flat-bottom microtiter plate in 100 µl aliquots for a total number of cells per well of approximately 50,000. Toxin was diluted in a separate round-bottom microtiter plate in which the wells were prefilled with 10 µl HBSS. Toxin (1 µg) was placed into the first well of a single row and brought up to 20 µl final volume with HBSS. The toxin was carried through two-fold serial dilutions in the remaining wells of the row. Next, 10 µl from each well was added to respective wells of the flat-bottom plate containing the SupT1 cells and incubated for 4 hours at 37°C. Following incubation, 10 µl Cell Counting Kit-8 reagent (Dojindo Molecular Technologies, Gaithersburg, MD) was added to each well and incubated for 4 hours at 37°C. The plates were read at A450nm and the concentration of toxin that resulted in 50% cell death (tissue culture lethal dose 50% (TCLD50)) determined. Results from cells treated with mutant toxins were expressed as a percentage of the value obtained from cells treated with wild type AT.

**Steady-State Fluorescence Spectroscopy.** Steady-state fluorescence was measured using an SLM 8100 photon-counting spectrofluorometer with a double monochromator in the excitation light path, a single emission monochromator, cooled PMT housings,
and a 450 W xenon lamp (Spectronic Instruments, Rochester, NY), with the band-pass set at 4 nm. The emission wavelength was 540 nm and the excitation wavelength was 480 nm. Emission scans were taken at 1 nm intervals between 500 and 600 nm for both monomeric and oligomeric forms of each mutant. For each NBD-labeled residue tested, the net NBD-intensity was determined by subtracting the fluorescence intensity of an equivalent NBD-free sample.

For each sample, toxin was preactivated with trypsin at a 1:3500 ratio (wt:wt/trypsin:toxin) for 30 min at 37°C. The reaction was stopped with the addition of a 30-fold molar excess of the protease inhibitor TLCK (tosyllysine chloromethyl ketone). 10 µg preactivated toxin was incubated alone (monomer) or in the presence of 50 µl SupT1 membranes (oligomeric) and brought up to 400 µl final volume with buffer C. All samples were incubated overnight at 37°C in the dark to allow binding, oligomerization and insertion to go to completion. For intensity measurements, reactions were placed into quartz cuvettes (1 cm x 1 cm) filled with 1.6 ml buffer C at 37°C and placed into the spectrofluorometer set at a constant temperature of 37°C.

Quenching of NBD Emission by Membrane-Restricted Spin-Labeled Phospholipids. To determine if a particular NBD dye was present within the nonpolar core of the bilayer, we incorporated nitroxides into the SupT1 membrane suspensions as follows.
DOXYL-stearic acid and 16-DOXYL-stearic acid (DSA) (Sigma, St.Louis, MO) were suspended together to a final concentration (each) of 50 mg/ml in methanol. SupT1 membrane suspensions were made 4% in the DSA solution by directly adding the DSA mixture to the membranes. The membranes were vortexed immediately for approximately 30 seconds and rocked at room temperature for one hour.

For each experiment, 10 µg preactivated toxin was incubated with 50 µl DSA-containing membranes in a total volume of 400 µl (brought up to volume with buffer C) overnight at 37°C in the dark. The samples were placed into quartz cuvettes (1 cm x 1 cm) prefilled with 1.6 ml buffer C at 37°C and placed into the spectrofluorometer sample holder set at a constant temperature of 37°C. Emission scans were taken at 1 nm intervals between 500 and 600 nm with an integration time of 1 sec. Due to the fact that the DSA is dissolved in methanol, samples containing membranes made 4% in methanol alone were used as controls for net NBD-intensity on membranes. Emission scans of NBD-free samples were taken and subtracted from the equivalent NBD-labeled samples to determine the net NBD intensity.

*Saturable Binding of AT\textsuperscript{T224C-IAF} and K\textsubscript{d} Determination.* The affinity of AT\textsuperscript{T224C-IAF} for SupT1 cells was determined using a liquid phase binding assay. Increasing amounts
of AT\textsuperscript{T224C}-IAF (10-500 nM) were incubated with 1x10\textsuperscript{6} SupT1 cells, brought up to 100 \(\mu\)l final volume in ice-cold HBSS and incubated for 30 min at 4\textdegree C. Following incubation, cells were pelleted and washed once with 200 \(\mu\)l ice-cold HBSS to remove unbound toxin and brought up to a final volume of 400 \(\mu\)l with ice-cold HBSS. The geometric mean fluorescence of the cells was determined by analysis on a FACS Calibur (Flow Cytometry and Confocal Microscopy Laboratory, WMRI, OUHSC, Oklahoma City, OK) equipped with a 488 nm laser.

Each experiment was carried out in duplicate and all data points represent the mean of four independent experiments. Non-specific binding was determined by adding 25 \(\mu\)M unlabeled toxin to each amount of labeled toxin tested and this value was subtracted from that obtained with labeled toxin alone to calculate specific binding. GraphPad PRISM software, version 3.0, was used for non-linear regression, curve-fitting analysis to determine an approximate \(K_d\).

**Competitive Binding Assay and \(K_i\) Determination.** The ability of wild type toxin or deletion mutants to compete for binding with AT\textsuperscript{T224C}-IAF to SupT1 cells was determined using a modified version of the above liquid phase binding assay. AT\textsuperscript{T224C}-IAF at 208 nM was mixed with increasing concentrations of either unlabeled wild
type or mutant toxin (0.5 nM to 10 µM) and brought up to a final volume of 100 µl in ice-cold HBSS before adding to 1x10^6 SupT1 cells. Cells incubated with AT^224C-IAF alone were considered maximum binding and the mean fluorescence value was set at 100%.

Each ligand concentration was analyzed in duplicate. To determine the inhibition constants (K_i) for each competing toxin, the geometric mean fluorescence of the cells was plotted versus the concentration of competitor toxin. GraphPad PRISM, version 3.0, was used for non-linear regression, curve-fitting analysis for determination of K_i values for each competitor with a K_d for AT^224C-IAF set at 92 nM.

**Activation and Oligomerization of TMD Deletion Mutants and AT^S220C-S269C on SupT1 Membranes.** Activation and oligomerization of toxin in the presence of membranes was carried out as follows. Wild type and mutant protoxin (AT^Pro) (5 µg) was activated using trypsin at a ratio of 1:1000 trypsin:toxin (wt/wt). The mixture was incubated at 37°C for 30 min and the trypsin was inhibited by the addition of a 30-fold molar excess of the protease inhibitor TLCK. SupT1 membranes (10 µl) were added to the activated toxin and the volume was adjusted to 30 µl with buffer C and incubated at 37°C for 2 hours. SDS sample buffer (8 µl 6X) and 7 µl of 10% SDS were added to the
samples, boiled at 90°C for 2 min and separated on a 4-15% gradient gel. The proteins were transferred to nitrocellulose paper and the blot was incubated with affinity purified anti-AT antibody. After 1 hour, unbound primary antibody was removed by washing the blot three times in buffer D (10 mM Tris-HCl, 150 mM NaCl, .05% Tween 20, pH 8.0) and then secondary antibody conjugated to horseradish peroxidase was added to the blot and incubated for an additional 45 min. The blot was again washed three times in buffer D to remove unbound antibody. Colorimetric development of the bands recognized by the antibody was accomplished by developing the blot with the color development solution 4-chloro-1-naphthol according to manufacturer’s instructions (Bio-Rad, Hercules, CA).

Results

Residues between K203 and Q232 alternate between nonpolar and polar environments. Membrane-interactive amphipathic α-helices and β-strands are defined by the periodicity of the interaction of their side chains with the membrane and aqueous environments; approximately every 3.5 residues of an amphipathic α-helix interacts with the membrane (18), whereas alternating residues of an amphipathic β-strand interact with the membrane (22). Residues K203 to Q232 of AT exhibit characteristics of an amphipathic transmembrane β-hairpin. The fluorescence-based method of Shepard...
et al. (22) was used to determine if this loop penetrates the membrane and then to examine the periodicity, and therefore its secondary structure, if it is inserted into the membrane. This technique takes advantage of the environmentally sensitive properties of the sulfhydryl-specific fluorescent dye iodoacetamide-NBD (NBD). The fluorescence intensity (FI) of NBD is quenched when it is in an aqueous environment, such as the channel of the pore, but increases when it is in a nonpolar environment, such as the core of the bilayer.

Residues K203 to Q232 were individually mutated to cysteines, and the proteins were purified and labeled with NBD. The FI of each NBD-labeled mutant was examined in the absence and presence of SupT1 cell-derived membranes that contain a receptor(s) for AT (7). Figure 2 shows example emission scans from adjacent residues, E227 and F228, in the monomeric and membrane-bound forms. In an amphipathic β-hairpin the side chain of F228 is predicted to interact with the bilayer during pore formation, while the side chain of E227 is predicted to reside in the hydrophilic channel of the pore. Consistent with this prediction, no significant increase in FI was seen for AT E227C-NBD following its incubation with membranes (Figure 2A), whereas the FI of ATF228C-NBD increased approximately three-fold under the same conditions (Figure 2B).

The combined fluorescence data for NBD when attached to the individual cysteines that were substituted for all residues within this region are summarized in
Figure 2C. The change in the emission intensity of NBD was determined from the ratio of the FI in the membrane-bound ($F_{\text{memb}}$) and soluble ($F_{\text{soluble}}$) states. These data reveal an alternating pattern of FI in which the side chains of these residues appear to weave in and out of a nonpolar environment. The pattern is most dramatic between residues K203 to K211 and E223 to E231.

**Residues of AT domain 2 span the membrane.** While an increase in the FI of NBD indicates movement of the NBD into a nonpolar environment such as the bilayer, it does not necessarily confirm the location of the labeled residue in the bilayer. The membrane location of those residues shown to enter a nonpolar environment was confirmed by collisional quenching analysis. Nitroxides are efficient quenching agents of NBD as previously shown by Shepard et al. (22), and when attached to the fatty acyl chain of stearic acid, nitroxides can be introduced into natural membranes and will only quench fluorophores exposed to the bilayer core (22,23).

As seen in Figure 3, the pattern of quenching by DOXYL-stearic acid (DSA) was consistent with the alternating periodicity previously seen for the FI in Figure 2. The greatest level of quenching was seen between residues G205 and E231. V212, which did not show an increase in FI on membranes, was quenched significantly by the membrane-restricted nitrooxide. This observation suggests that the V212 side chain initially resides in a nonpolar environment in the soluble monomer of AT, so the FI of the
NBD-modified cysteine at this position does not change significantly as the side chain moves from its nonpolar location in the soluble monomer to its position in the membrane.

Based on the pattern of quenching, E215 and I217 would be expected to face the channel where they would not come into contact with the quencher, however, both exhibit a small degree of quenching when incubated with these membranes. Since the alternating pattern is lost at this point and then resumes at residue A218, it is possible that the turn region connecting the 2 transmembrane β-strands may exist within the span of residues from 215 to 218, and as a result, these residues may be only partially exposed to the membrane.

The cytolitic activity of each of the NBD-labeled mutants was tested on SupT1 cells. Of the 30 residues mutated and labeled in D2, fifteen retained 25% or greater activity, twelve retained 5-25% activity, and three retained ≤1% activity (data not shown). Those mutants that lost 99% or greater activity are indicated in Figures 2 and 3 and each are predicted to face the membrane when inserted (I204, F210, and F226). Due to the severe loss of activity by these mutants, they were not characterized further.

**Deletion of residues between K203 and Q232 abolishes pore formation.** Based on the molecular model of AT (Figure 1) it appears that deletion of the amphipathic loop would not significantly affect folding of AT, since it does not appear to be a core domain
with extensive contacts. We therefore hypothesized that deletion of these residues would only disrupt pore formation if the residues are in the membrane-spanning region. Three mutants within this region were constructed, deleting increasing lengths of the loop region: \( \text{AT}^{212-222} \), \( \text{AT}^{208-226} \) and \( \text{AT}^{204-230} \). The largest deletion (\( \text{AT}^{204-230} \)) removed approximately 90% of the predicted TMD. All deletion mutants were found to lack cytolytic activity on SupT1 cells (data not shown).

The three deletion mutants did not appear to be misfolded, since receptor binding, activation and oligomerization were not affected. Analysis of the binding data showed that \( \text{AT}^{T224C-IAF} \) bound to a single class of receptors these cells with a \( K_d \) of approximately 92 nM (Figure 4A). Competition experiments showed that the deletion mutants exhibited similar \( K_i \) values to that for wild type AT (Figure 4B). Thus, the deletions did not significantly affect receptor binding. Furthermore, the deletion mutants were activated \textit{in vitro} by trypsin similar to wild type and each oligomerized on membranes as well as or better than wild type AT, suggesting their overall structure had not been compromised by the loop deletions (Figure 5A). \textit{In vitro} activation by trypsin also did not yield peptides that were not found after activation of the native toxin in solution (data not shown). Hence, the general structure of these mutants was apparently not altered to an extent that resulted in the exposure of new trypsin sites.
Formation of a disulfide bond between the TMD and the β-sheet backbone of D2 prevents pore formation. Previous studies on the analogous loop region in D3 of aerolysin showed that it could be tethered to the backbone of the molecule by means of a disulfide bond between the loop and the backbone of the molecule (34). Once formed, the disulfide prevented oligomerization and pore formation until it was reduced with dithiothreitol (DTT). From these studies, it was concluded that the loop region must move away from the D3 β-sheet in order for aerolysin to form a stable heptamer and generate pores in membranes.

A similar experiment was performed with AT, in which residues analogous to those mutated to cysteine in aerolysin (based on the AT structural model in Figure 1) were replaced with cysteines. Purified ATS220C-S269C was found to be inactive in its oxidized form, but exhibited >80% of the activity of native toxin when reduced (data not shown). When activated by trypsin and incubated with SupT1 membranes, ATS220C-S269C bound and oligomerized on SupT1 cells under both reducing and non-reducing conditions in a manner similar to native AT (Figure 5B). Thus movement of the loop region in D2 of AT is only necessary for pore formation and not binding or oligomerization.

Domain 3 residues of AT do not appear to span the bilayer. The TMD of aerolysin was originally proposed to be comprised of the D4 β-sandwich which formed a
hydrophobic dagger that penetrated the membrane (13). This idea has been reiterated in a recent study of an aerolysin mutant that forms a water-soluble heptamer, in which an aromatic belt was said to distinguish the upper boundary of a transmembrane region that was proposed to be present behind the propeptide (27). Since D4 of aerolysin is homologous to D3 of AT, we examined residues within this region of AT to determine if they interact with the membrane during pore formation.

Two hydrophobic residues in D4 of aerolysin, L277 and L196, were suggested to interact with the membrane when pores are formed. These residues border three aromatic residues on different β-strands within D4 (Figure 6A), which were proposed (27) to make up an aromatic belt as is found in porins (35). L277 and L196 reside on two different β-strands in aerolysin (Figure 6A) and are not conserved in AT. For L277 of aerolysin, the equivalent residue in AT is a glutamic acid at position 246 (Figure 6B), whereas for L196, the homologous residue is a proline at position 162 (Figure 6B). As P162 represented a more conservative change, we mutated it to a cysteine and labeled the mutant with NBD. When labeled with NBD and incubated in the presence of membranes, AT^P162C-NBD displays a slight increase in FI that is not quenched upon addition of DSA to the membranes (Figure 6C), suggesting that it does not interact with the membrane during pore formation.

Five β-strands extend between D2 and D3 of AT (Figure 6B). P162 lies on one
of these β-strands (β-strand 1, Figure 6B) near the predicted D2-D3 junction. Seven residues downstream of P162 were examined on the same β-strand, which spans the length of the D3 β-sandwich of AT behind the propeptide, to determine if any of the residues within this region interact with the membrane. If D3 entered the membrane then these residues, which are on the same face as the predicted location of the propeptide, should interact with the membrane after toxin activation. These residues were analyzed in the same fashion as for those in Figures 2 and 3. As seen in Figure 6C, all of the residues tested on this β-strand, except AT L163C-NBD, display at least a 2-fold increase in FI on membranes compared to the soluble monomer. When incubated with membranes containing the membrane restricted collisional quenching agent DSA, most of the residues exhibited little or no quenching indicating that these residues do not interact with the membrane to a significant extent.

As described above, E246 of AT, corresponding to L277 of aerolysin, was also predicted to interact with the membrane (27). This residue is on a different β-strand than P162 (β-strand 2, Figure 6B). We examined two consecutive residues downstream of E246 to determine if this strand interacts with the membrane during pore formation. Using the same techniques as above, both residues showed an increase in FI upon incubation with SupT1 membranes (Figure 6C), but displayed little or no quenching when DSA was present in the bilayer. L264, which resides on β-strand 3 in the middle
of D3 of AT (Figure 6B), lies directly behind the propeptide. Residues on this strand are predicted to be masked by the propeptide until its dissociation and are immediately below one of the residues of the putative aromatic belt. AT<sub>L264C-NBD</sub> showed a very slight increase in FI that was not quenched on membranes containing DSA (Figure 6C). Therefore, the residues of the D4 β-strands do not appear to have significant interactions with the membrane.

**Discussion**

The formation of transmembrane pores by various bacterial toxins has been a subject of considerable interest in the years since the solution of the crystal structure of the pore complex of the *Staphylococcus aureus* α-hemolysin by Song *et al.* (20). The structure of the α-hemolysin membrane pore showed how the amphipathic β-hairpins of individual monomers of the toxin formed an amphipathic β-barrel structure in the membrane. Since that time, only anthrax toxin (19) and perfringolysin O (22,23) have been shown to utilize transmembrane β-hairpins to form a β-barrel. In the present study we have shown that residues 203-232 of AT also participate in the formation of a membrane-spanning β-hairpin (Figure 7). As with *S. aureus* α-toxin, the hairpins of the individual monomers of the AT oligomer likely combine and insert into the membrane to generate the pore-forming β-barrel. Consistent with this scenario is the alternating
pattern in the FI of the NBD attached to cysteine-substituted residues; those residues that were predicted to face the membrane generally exhibited a greater increase in FI than the residues predicted to face the channel of the bilayer. Furthermore, collisional quenching analysis with a membrane-restricted quencher confirmed that the same residues identified by their increased FI were indeed interacting with the nonpolar environment of the membrane. The size of the AT hairpin, approximately 30 residues, is also similar in length to that for the transmembrane hairpins of *S. aureus* α-hemolysin (20), the anthrax protective antigen (19) and the two transmembrane hairpins of perfringolysin O (22,23).

The fluorescence data were supported by deletion analysis of the putative TMD. Based on the crystal structure of the related aerolysin and the aerolysin-based molecular model of AT (Figure 1), we predicted that deletion of the transmembrane loop from AT would not significantly alter the folding pattern of AT and should have left intact its ability to bind receptor, undergo proteolytic activation and to form membrane oligomers. As expected, these mutants retained all the functions of native toxin, except that they were unable to form a pore and lyse cells. Similar results were observed for anthrax protective antigen when its transmembrane loop was deleted (36). Therefore, this region is necessary to the formation of the AT pore, but not for the other toxin functions that lead to pore formation.
In order to insert into the membrane this loop apparently moves away from the D2 backbone β-sheet. Rossjohn et al. (34) had previously shown that the introduction of a disulfide into aerolysin by the substitution of residues T253 and A300 with cysteines prevented pore formation. This disulfide locked the D3 loop region in aerolysin to the D3 β-sheet. They found that this disulfide prevented oligomerization of aerolysin and proposed that this loop must move in order for aerolysin to become insertion competent. Upon reduction of the disulfide, the disulfide-trapped aerolysin regained its cytolytic activity. Based on the molecular model of AT, the analogous residues of AT were mutated to cysteines. As expected, the structurally analogous disulfide in AT prevented pore formation when oxidized. However, like the deletion mutants, it retained the ability to bind membranes, undergo proteolytic activation and to form SDS-resistant oligomers. Also, like the aerolysin disulfide-trapped mutant, ATS220C-S269C regained nearly full cytolytic activity when the disulfide was reduced. Therefore, it also appears that this D2 transmembrane loop must move away from the backbone of AT to allow the β-hairpin to insert into the membrane.

One important difference between the aerolysin and AT disulfide mutants should be noted. We found that ATS220C-S269C can oligomerize when the engineered disulfide is oxidized, while disulfide mutant of aerolysin does not appear to oligomerize (34). This difference may reflect the fact that aerolysin exists as a head-to-tail dimer in
solution (13), whereas AT exists as a monomer (Melton and Tweten, unpublished data), and so the aerolysin dimer must dissociate prior to oligomerization. Aerolysin dimer formation is, in part, mediated via its small lobe (13,37) which is missing in AT. When the small lobe is removed from aerolysin-like AT, it exists as a monomer in solution (37). Therefore, it appears that the movement of the D3 loop of aerolysin may also be necessary for the disruption of the aerolysin dimer after receptor-binding (38).

Is the amphipathic structure of the AT TMD conserved in aerolysin and enterolobin? Both aerolysin and enterolobin maintain an amphipathic structure that is located in their primary structures and is positionally conserved with that of AT (Figure 7). Although enterolobin contains an arginine in the middle of the predicted hairpin, this residue may reside near the predicted turn and face the aqueous milieu. However, it could also penetrate the membrane with the aliphatic part of the side chain and snorkel the charged amine to the surface, similar to that found in the perfringolysin O TMDs (22). Hence, it appears reasonable that like AT, aerolysin and enterolobin may also use an amphipathic $\beta$-hairpin structure to form their transmembrane pore.

It has been previously suggested (13,27) that the D4 $\beta$-sandwich of aerolysin forms a hydrophobic dagger that penetrates and forms the membrane pore. D3 and D4 of AT and aerolysin, respectively, (Figure 1) contain the propeptides of these two toxins and the proteolytic cleavage of the propeptide has been shown to be essential for toxin
oligomerization, presumably by exposing sites protected by the propeptide (12,39,40).

Although we cannot rule out a superficial interaction of some residues within D3 of AT (D4 of aerolysin) with the membrane, the data herein do not support a role for D3 as the TMD of AT. Also, the fact that we can delete the D2 loop of AT and maintain its ability to bind and oligomerize without pore formation is also consistent with the previously established role of D3 in the oligomerization of AT (39) and D4 in the oligomerization of aerolysin (12,39,40),(13).

These studies provide the first experimental evidence for the location and structure of the transmembrane domain of a member of the aerolysin family of cytolytic toxins, *C. septicum* alpha toxin. Similar amphipathic regions also exist within the structures of the related aerolysin and enterolobin toxins suggesting that they may also utilize an amphipathic β-hairpin to span the bilayer. Therefore, we propose that this family of toxins belongs to the β-pore-forming class of toxins (41) that utilize an amphipathic β-hairpin to form a membrane-spanning β-barrel.

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References


Figures

Figure 1. Crystal structure of aerolysin and molecular model of AT. (A, B) The crystal structures of the membrane monomer of *Staphylococcus aureus* α-hemolysin (20) and the soluble monomer of *C. perfringens* perfringolysin O (24) are shown as a ribbon representation of the α-carbon backbone. The TMDs of each are shown in red. (C) The molecular model of AT was constructed based on the solved crystal structure of aerolysin and the homology between residues of domains 2-4 of aerolysin and the primary structure of AT. Color-coding of the various regions of the AT molecular model is as follows: purple, D1; cyan, D2; yellow, D3; orange, transmembrane domain (TMD) (determined herein); red, propeptide. (D) The solved crystal structure of aerolysin (13). Regions are color coded as follows: green, D1; purple, D2; cyan, D3; yellow, D4; orange, putative TMD; red, propeptide.

Figure 2. Fluorescence intensity of NBD-labeled cysteine-substituted residues in the predicted loop region of D2. Emission scans of soluble monomeric (solid line) and membrane-bound (dashed line) forms of AT E227C-NBD (A) and AT F228C-NBD (B). (C) NBD intensity changes for residues F200-S235 upon the transition of soluble AT to its membrane-inserted pore (F_{memb}/F_{soll})-1. Striped bars represent predicted
membrane-facing residues. Solid bars represent predicted channel-facing residues. n.d.- not determined; denotes NBD-labeled cysteine-substituted mutants with \( \leq 1\% \) cytolytic activity.

**Figure 3. Quenching of NBD-labeled mutants of AT by nitroxide-labeled SupT1 membranes.** The relative change in the FI of the NBD-modified residues 203-232 when AT is incubated in the presence of SupT1 membranes with (F) and without (F₀) DSA is shown by the equation \( \left( \frac{F₀}{F} \right) - 1 \). If the probe is quenched upon its entry into the membrane then \( \left( \frac{F₀}{F} \right) - 1 > 0 \), whereas if little or no quenching is observed then \( \left( \frac{F₀}{F} \right) - 1 \) will be close to 0. Striped bars represent predicted membrane-facing residues. Solid bars represent predicted channel-facing residues. n.d.- not determined; denotes NBD-labeled cysteine-substituted mutants with \( \leq 1\% \) cytolytic activity.

**Figure 4. Binding affinities of the TMD deletions.** (A) \( K_d \) determination of functional ATT224C-IAF on SupT1 cells. Error bars represent the standard error of the mean of the combined data set. (B) \( K_i \) of native and TMD deletion mutants when competed with ATT224C-IAF. The \( K_i \) for each competitor is indicated in the inset table. Each data point
represents the mean of two independent experiments carried out in duplicate. Error bars indicate the standard error of the mean for each combined data set.

**Figure 5. Activation and Oligomerization of TMD deletion and disulfide mutants.** Purified the AT deletion mutants AT^{212-222}, AT^{208-226} and AT^{204-230} (A) or the disulfide locked AT^{S220C-S269C} mutant (B) were monitored for proteolytic activation with trypsin and the formation of SDS-resistant oligomers on SupT1 membranes.

**Figure 6. Fluorescence intensity and quenching of NBD-labeled mutants in domain 3 upon membrane binding.** (A) Crystal structure of D4 of aerolysin. Residues in blue have been suggested to comprise an aromatic belt. L277 (green) and L196 (yellow) are hydrophobic residues that neighbor the aromatic belt (27). Yellow residues correspond to those tested in D3 of AT for membrane entry. (B) The molecular model of D3 of AT. Blue residues correspond to those in the putative aromatic belt of aerolysin. Residues in yellow were tested in this study for membrane penetration. Residue E246 (shown in green) corresponds to aerolysin residue L277. (C) The relative FI change of NBD-labeled residues of membrane-bound (F_{memb}) and soluble (F_{sol}) AT (solid bars) and of the same residues in the presence of membranes with (F) and without (F_0) the
collisional quenching agent DSA (striped bars) for residues on β-strands 1, 2 and 3 in D3 of AT (β-1, β-2, β-3).

Figure 7. Putative TMDs of the aerolysin-like cytolysin family. Asterisks denote the position of the AT residues shown to enter the membrane and those predicted to enter the membrane of the positionally conserved residues within the aerolysin and enterolobin primary structures.
A

K_d = 92.43 nM
B_max = 39.97

\[ \text{[T24C-IAF], nM} \]

FL1

B

\[ \log [\text{competitor}, \text{nM}] \]

FL1

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The identification and structure of the membrane-spanning domain of the Clostridium
genicolaum alpha toxin
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Tweten

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