Assembly and Topography of the Prepore Complex
in Cholesterol-Dependent Cytolysins*

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SUMMARY

Cholesterol-dependent cytolysins are a family of pore-forming proteins that have been shown to be virulence factors for a large number of pathogenic bacteria. The mechanism of pore formation for these toxins involves a complex series of events that are known to include binding, oligomerization, and insertion of a transmembrane β-barrel. Several features of this mechanism remain poorly understood and controversial. Whereas a prepore mechanism has been proposed for perfringolysin O, a very different mechanism has been proposed for the homologous member of the family, streptolysin O. To distinguish between the two models, a novel approach that directly measures the dimension of transmembrane pores was used. Pore formation itself was examined for both cytolysins by encapsulating fluorescein-labeled peptides and proteins of different sizes into liposomes. When these liposomes were re-suspended in a solution containing anti-fluorescein antibodies, toxin-mediated pore formation was monitored directly by the quenching of fluorescein emission as the encapsulated molecules were released and the dyes were bound by the antibodies. The analysis of pore formation determined using this approach reveals that only large pores are produced by perfringolysin O and streptolysin O during insertion (and not small pores that grow in size). These results are consistent only with the formation of a prepore complex intermediate prior to insertion of the transmembrane β-barrel into the bilayer. Fluorescence quenching experiments also revealed that PFO in the prepore complex contacts the membrane via domain 4, and that the individual transmembrane β-hairpins in domain 3 are not exposed to the nonpolar core of the bilayer at this intermediate stage.
INTRODUCTION

Cholesterol-dependent cytolytins (CDCs) are produced by a variety of pathogenic Gram positive bacteria (reviewed in 1,2). The monomeric forms of the CDCs are highly water-soluble, but the proteins bind to cholesterol-containing membranes and then spontaneously self-associate to form large aqueous pores in the bilayer. These oligomeric complexes vary in size and may contain up to 50 individual monomers (3-5).

The only crystal structure of a water-soluble, monomeric form of a CDC was solved by Rossjohn et al (6) for perfringolysin O (PFO) from Clostridium perfringens, and their data revealed that PFO is comprised of four domains. The crystal structure of a membrane-inserted oligomer of a CDC is not presently available. However, several fluorescence-based studies have identified the regions of PFO that form a transmembrane β-barrel, and have also provided other structural information about the membrane-inserted oligomer. Domain 3 of PFO contains two stretches of amino acids (190-217 and 288-311) that interact with the membrane during pore formation and create an amphipathic β-sheet that serves as an aqueous-lipid interface after insertion into the membrane (7,8). Domain 4 (residues 391-500) is involved in membrane recognition and binding, and remains close to the membrane surface in the membrane-inserted oligomer where it contacts, but is not deeply embedded in, the bilayer (9,10).

The mechanism of action of the CDCs involves a complex series of events that are known to include the binding and stable association with cholesterol-containing membranes by the toxin monomers (11,12), the lateral diffusion on the bilayer with concomitant formation of cytolysin oligomers (13-17), and ordered and coupled conformational changes that result in pore formation (9,17-19).
The relative timing of oligomer formation and of the insertion of individual transmembrane β-hairpins (TMHs) has been a subject of controversy (1,2,20,21). Two different models have been proposed to explain the insertion of the CDC β-sheet into the membrane, one based on the prepore mechanism that has been shown to mediate the formation of small pores by several toxins (Fig. 1A; 22-25), and the other on the gradual enlargement of a small oligomer and pore into a large oligomer and pore by the sequential addition of monomers to an initial inserted complex (Fig. 1B; 15).

Shepard et al (16) showed that a PFO oligomeric complex is formed on liposomes at both 4°C and 37°C, and also showed by SDS-agarose electrophoresis that this oligomer is large and relatively uniform in size. At low temperatures, these authors were able to identify the formation of a prepore complex formed in the absence of significant insertion of the TMHs. Furthermore, they showed that PFO was found to increase the ion conductivity through a planar bilayer by large and discrete stepwise changes in conductance that are consistent with the insertion of a preassembled pore complex into the bilayer. In contrast, Palmer et al (15) proposed that individual SLO monomers are inserted and added to the pre-existing oligomers on erythrocyte membranes to produce a pore that grows in size continuously until it reaches its final state.

To establish unambiguously whether PFO and SLO only form pores of a discrete, large size or, alternatively, pores whose diameter is continually increasing, we have analyzed the mechanism of pore formation using fluorophores of different sizes to monitor pore dimensions. Several fluorescein-labeled peptides and proteins with different hydrodynamic radii were encapsulated into cholesterol-containing liposomes, and the kinetics of pore formation was detected by the rate of fluorophore exposure to
quenchers (fluorescein-specific antibodies) located in the external medium. If a prepore complex is an obligatory intermediate for the insertion of the transmembrane β-barrel, all sizes of trapped molecules should be released at the same time from the liposomes. On the contrary, if the pore starts small and grows continuously, the smaller trapped molecules should be released faster than the larger ones.

As we have pointed out (10), major conformational changes in the elongated monomeric PFO structure (6) are required to bring the TMHs to the membrane surface while leaving only the tip of domain 4 exposed to the bilayer. Thus, an important question is to what extent the conformational changes have occurred by the time the prepore complex is formed. In other words, what structural changes have occurred at the stage where the PFO prepore complex is poised to insert its TMHs into the bilayer, but cannot because of a lack of energy (16), a mutation (19), or a disulfide bond (9,17). Are its TMHs lying on the surface of the membrane prior to the cooperative insertion of the TMHs to form the β-barrel (19)? If so, have the nonpolar sides of the hairpins already been exposed to the hydrophobic core of the bilayer? Does domain 4 sit on the membrane surface with the same orientation that it has in the membrane-inserted oligomer? Because so little is known about the prepore structure, we have also addressed the above important questions.

Fluorescence quenching analyses of the mechanism of pore formation for a functional PFO molecule reveal that: (i) the insertion of the oligomeric transmembrane β-barrel for PFO requires the formation of a prepore complex; (ii) neither PFO nor SLO are able to form small pores on cholesterol-containing membranes; and (iii) the interaction of PFO with the bilayer during binding and prepore assembly does not result in small molecule leakage through the membrane. In addition, the topographical
examination of the prepore complex reveals that only the tip of domain 4 is embedded in the membrane bilayer, a topography similar to that observed in the final membrane-inserted oligomer. In contrast, the domain 3 TMHs are not exposed to the nonpolar interior of the membrane in the prepore complex.

**EXPERIMENTAL PROCEDURES**

*Preparation of Toxins Derivatives* - The gene for PFO\(^{C459A}\), the cysteine-less derivative of PFO in which C459 was replaced by alanine, was cloned in pTrcHisA (Invitrogen, Carlsbad, CA) as described previously (7). This plasmid (named pRT20) was used as the template for all cysteine-substitution mutagenesis. The generation of cysteine-substituted derivatives of PFO\(^{C459A}\), their expression, and their purification have been described previously (7). Single-cysteine mutants of PFO were 90-100% labeled with NBD and purified as before (7). The toxin concentration was calculated using a molar absorptivity (\(\varepsilon\)) at 280 nm of 84,000 cm\(^{-1}\)M\(^{-1}\) for PFO (7) and of 71,300 cm\(^{-1}\) M\(^{-1}\) for SLO (26).

*Peptide and Protein Labeling with FITC* - The labeling of carbonic anhydrase from bovine erythrocytes (CA), \(\beta\)-amylase from sweet potato (Amy), and thyroglobulin from bovine thyroid (Thy) (all from Sigma, St. Louis, MO) with fluorescein isothiocyanate isomer I (FITC, Molecular Probes, Eugene, OR) was performed as described by Heuck and Wolosiuk (27). Briefly, the proteins (10 mg/ml) were incubated with FITC (molar ratio 1:2, protein:FITC) in 50 mM NaHCO\(_3\) (pH 9.0) for 12 hr at room temperature with gentle mixing. Unreacted FITC was removed by gel filtration (Sephadex G-25 or G-50, 1.5 cm ID x 20 cm) equilibrated with 50 mM HEPES (pH 7.5), 100 mM NaCl (buffer A), and the homogeneity of the sample was determined by FPLC.
using a Superdex-200 column (Amersham Pharmacia, Piscataway, NJ) equilibrated in the same buffer. Fractions corresponding to a single symmetric peak of the expected molecular weight were pooled and stored at -20°C until use. Reduced L-glutathione (Sigma) was incubated with FITC (molar ratio 1:1) in buffer A for 18 hr and stored at –20°C until use.

**Liposome Preparation** - All phospholipids were obtained from Avanti Polar Lipids (Alabaster, AL). Cholesterol was obtained from Steraloids (Newport, RI). Large unilamellar liposomes were generated using an Avestin Inc. (Ottawa, Canada) Liposofast extruder. A mixture of cholesterol and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) (55:45 mole%, respectively) in chloroform was dried at 37°C under N₂ and kept under vacuum for at least 3 hr. To hydrate the lipid mixture, 0.55 ml of buffer A was added to the dried phospholipid/sterol mixture (final total lipid concentration 10 mM), and the sample was incubated for 30 min at 37°C. The lipids were then resuspended by vortexing. The suspended phospholipid/sterol mixture was frozen in liquid N₂ and thawed at 37°C a total of five times to reduce the number of multilamellar liposomes and to enhance the trapped volumes of the vesicles (28). Then the sample was passed 21 times through the extruder, equipped with both a 100 nm and a 200 nm pore size polycarbonate filter, at room temperature (20-25 °C). Whereas the 100 nm filter dictates the final size of the vesicles (mean diameter ~110 nm; 28), the 200 nm filter faces the initial incoming solution and reduces the chances of contamination with larger particles or foreign material. The resulting liposomes were stored at 4°C and used within 2 weeks of production.

Liposomes used in lipophilic quenching experiments were prepared using POPC and cholesterol in the same way, except that 10 mole% of the POPC (i.e., 4.5% of the
total lipids) was replaced by a nitroxide-labeled phospholipid, 1-palmitoyl-2-stearoyl-(x-doxyl)-sn-glycero-3-phosphocholine (x-doxyl-PC, where x is equal to 5, 7 or 12 and indicates the location of the quencher in the acyl chain of the phospholipid). The composition of these liposomes was therefore 55:40.5:4.5 mole % for cholesterol, POPC, and doxyl-PC, respectively.

Liposomes containing Tb(DPA)\textsubscript{3}\textsuperscript{3-} and the fluorescein-labeled peptides or proteins were prepared as above, except that buffer A included 3 mM TbCl\textsubscript{3} (Alfa Aesar, Ward Hill, MA), 9 mM 2,6-pyridinedicarboxylic acid (DPA, Sigma, neutralized to pH 7), and 20 µM fluorescein (attached to the indicated protein), and was added to the lipid film to yield a final total lipid concentration of 30 mM (final volume 0.35 ml). The resulting liposomes were separated from non-encapsulated Tb(DPA)\textsubscript{3}\textsuperscript{3-} and fluorescein-labeled proteins by gel filtration (Sepharose CL-6B-200, 0.7 cm ID x 50 cm) in buffer A. Traces of L-3-phosphatidylcholine-1,2-di[\textsuperscript{14}C]oleoyl (Amersham Pharmacia) were added to the lipid mixture in order to quantify the lipid concentration and recovery after gel filtration. The Tb(DPA)\textsubscript{3}\textsuperscript{3-} complex was included in each preparation to allow simultaneous analysis of the formation of small and large pores, as well as an internal control for differences in the leakage properties of each preparation. When these liposome preparations were exposed to PFO\textsuperscript{C459A}, the rate of quenching of the Tb(DPA)\textsubscript{3}\textsuperscript{3-} complex by 5 mM EDTA in the extra-vesicular medium was identical in each case (not shown).

**Steady-State Fluorescence Spectroscopy** - Intensity measurements were performed using the same instrumentation described earlier (7). The excitation wavelength and bandpass, and the emission wavelength and bandpass, were,
respectively: 470, 4, 530, and 4 nm for NBD; 295, 2, 348, and 4 nm for Trp; 495, 4, 520, and 4 nm for fluorescein; and 278, 2, 544, 4 nm for Tb(DPA)$_3^{3-}$. For Tb(DPA)$_3^{3-}$ measurements, an Oriel 5215 cutoff filter (0% transmittance below 350 nm) was placed in the emission light path to block any second-order excitation light. When measurements were performed at low temperature, the cuvette chamber was continuously flushed with N$_2$ to prevent condensation of water on the cuvettes. Kinetics measurements were done using 1 cm x 1 cm quartz cells and the samples were continuously stirred using a magnetic stirring bar (1.5 mm x 8 mm). End-point measurements were done in 4 mm x 4 mm quartz microcells that were coated with POPC vesicles to minimize protein adsorption (29). When additions were made to microcells, the contents were mixed thoroughly with a 2 mm x 2 mm magnetic stirring bar as described previously (30).

**Time-Dependent Detection of PFO NBD or Trp Emission** - A sample containing the water-soluble PFO monomer (final concentration 25-100 nM) in buffer A was placed in the temperature-controlled cuvette chamber and measurements were taken until a stable signal was obtained ($F_0$). The kinetic analysis was started by the addition of liposomes (final concentration 50-100 µM in buffer A, final volume 1.6 ml), and data acquisition was initiated 15 sec later, after complete mixing of the sample. Emission intensities were recorded at the appropriate intervals and at least sixty measurements were taken for each experiment. Blank measurements were made using an otherwise identical sample that lacked the fluorophore. The blank data were subtracted from the corresponding sample data, and the net $F_0$ value was then dilution-corrected.

For experiments using doxyl quenchers, the emission intensities of 50 nM aliquots of the indicated monomeric NBD-labeled PFO sample in buffer A were measured at the
indicated temperature as explained above. Time-dependent data acquisition was started after the addition of liposomes (final concentration 50 µM) that either contained or lacked 5-, 7-, or 12-doxyl-PC.

Detection of Pore Formation - (A) End point measurements: liposomes (100 µM total lipids) were suspended in 280 µl of buffer A containing 5 mM EDTA and 4 µl of a 1:10-diluted (in buffer A) solution of anti-fluorescein antibody (0.5 µl of this rabbit polyclonal IgG fraction quenches ~95% of the emission intensity of 0.8 fmoles of GSH-Fl in buffer A; lot 84B1, Molecular Probes). The net initial emission intensity (F₀) was determined after equilibration of the sample at 25°C for 5 min. Aliquots of 20 µl containing the amount of toxin that gives the indicated final concentration were added and the samples were incubated 30 min at 37°C. After re-equilibration at 25°C, the final net emission intensity (Fₖ) of the sample was determined (i.e., after blank subtraction and dilution correction) and the fraction of marker quenched was estimated using (F₀-Fₖ)/(F₀-Fₜ), where Fₜ is the net emission intensity obtained when the same liposomes are treated with an excess of toxin (i.e., under conditions of maximal release of the fluorophore). Typical values of Fₜ/F₀ for Tb(DPA)₃³⁻, GSH-Fl, and Amy-Fl are 0.07, 0.12, and 0.23 respectively.

(B) Kinetics measurements: liposomes (50 µM total lipids) loaded with Tb(DPA)₃³⁻ and the fluorescein-labeled protein were suspended in buffer A supplemented with 5 mM EDTA and 20 µl of a 1:10-diluted (in buffer A) solution of anti-fluorescein antibody. After equilibration of the sample at 25°C, the net initial emission intensity was determined and PFOC₄₅₉A was added to a final concentration of 25 nM (final volume of 1.6 ml). Unless indicated, data collection and analysis were performed as described above.
RESULTS

Determining the Size of Pores in Liposomal Membranes - A detailed analysis of the mechanism of PFO pore formation requires an assay that can directly measure and efficiently discriminate between transmembrane pores having different diameters. To achieve this, we have used a spectroscopic approach that detects the release of liposome-encapsulated fluorophores following toxin addition. By surrounding the liposomes with quenchers of the fluorophores, any release of fluorophore into the medium is detected by a reduction in emission intensity as a quencher contacts a fluorophore. Such an approach monitors the co-localization of fluorophore and quencher because no quenching will be observed if the quencher cannot access and interact with the fluorophore (e.g., 31,32,33). However, as soon as a pore is formed in the liposomal membrane, a fluorophore can diffuse out of the liposome and its release will be detected by the quenching of the emission intensity. The size of the pore can then be estimated by using fluorophores of different sizes within the liposomes because a fluorophore will only move through pores that are larger than the fluorophore (32).

*Staphylococcus aureus* α-hemolysin (αHL) is a β-barrel pore-forming toxin that forms a transmembrane pore that is ~16 Å in diameter (24). When liposomes encapsulating fluorescent molecules of different sizes were incubated with αHL, only the small fluorophores with a diameter of about 10 Å were able to pass through the αHL pore and be quenched (Fig. 2A, Table I). A large molecule like Amy-Fl (~100 Å in diameter) was unable to pass through the αHL transmembrane pore.

To confirm that the observed fluorescence quenching was dictated by the size of the pore, and not by the nature of the fluorophore-quencher pair used in the assay,
liposomes containing both a small fluorophore \([\text{Tb(DPA)}_3^{3-}\)] and a large fluorescent molecule (Amy-Fl) were incubated with \(\alpha\text{HL}\) (Fig. 2B). The fast quenching of the emission intensity of \(\text{Tb(DPA)}_3^{3-}\) after addition of \(\alpha\text{HL}\) clearly shows that the integrity of the membrane has been disrupted by the formation of pores that are at least 10 \(\Delta\) in diameter (the diameter of the pore required for the mixing of encapsulated \(\text{Tb(DPA)}_3^{3-}\) and extraliposomal EDTA). In contrast, Amy-Fl was not quenched because the fluorescent molecule and its quencher (the anti-fluorescein antibody) are too big to pass through the \(\alpha\text{HL}\) pore. Thus, the emission intensity of fluorescein was unaffected by \(\alpha\text{HL}\) addition. However, when PFO (pore diameter \(\sim 250 \Delta\)) was added to the same sample, efficient quenching of Amy-Fl intensity was observed.

This fluorescence approach therefore not only allows direct detection of the formation of a transmembrane pore, but it also permits a direct estimation of the diameter of the newly-formed pore. Hence, this spectroscopic assay is both efficient and informative.

**PFO Pore Formation: a Growing Pore or a Prepore Complex Assembly?** - Using the fluorescence approach described above, we can analyze pore-formation directly using a functional toxin and discriminate between the two models of pore formation proposed for the CDCs. If, as suggested by Palmer et al (15), the toxin initially forms a small pore that is gradually enlarged during oligomerization, a small encapsulated fluorophore will be released from the liposome before a large fluorophore. Therefore, immediately following addition of the toxin, the rate of fluorescence quenching should be faster for small fluorophores than for larger fluorophores. In contrast, if a prepore complex must be completely formed (i.e., a complete ring) before the insertion of the TMHs, any pores that are formed will have a diameter of \(\sim 250 \Delta\), and hence both large
and small fluorophores would be released from the liposome and quenched at the same rate (ignoring small differences due to different rates of diffusion).

To distinguish between these two possibilities, we encapsulated fluorescein-labeled peptides and proteins of different molecular sizes into liposomes (Table I). As indicated in Experimental Procedures, different batches of cholesterol-containing liposomes were prepared, each one containing a fluorophore of a different size (ranging from \( \sim 10 \Delta \) to \( \sim 140 \Delta \) in diameter). Using these liposome preparations, we examined the time-dependent release of the different fluorescein-labeled markers. As shown in Fig. 3, after the addition of PFO, the kinetics of quenching of the emission intensities for the different fluorescein-labeled proteins were almost identical. In these experiments, the ratio of toxin:total lipids was 1:2000, and under these conditions the amount of added PFO is sufficient to form, on average, at least one complete ring (\( \sim 45 \) monomers) per liposome. These results show that rate of fluorophore release from the liposome is the same for all sizes of fluorophores. We therefore conclude that PFO insertion into the membrane does not occur via sequential addition or a growing-pore mechanism. Instead, a prepore complex is assembled on the membrane surface prior to the insertion of the amphipathic \( \beta \)-barrel and pore formation.

Comparing PFO and SLO - As mentioned above, it has been suggested that SLO forms a pore that gradually grows in size, concomitantly with the oligomerization of the toxin (15; Fig. 1B). Based on the above results obtained for PFO, this would imply that CDCs do not use a common mechanism of pore formation. The primary sequences of PFO and SLO are 65% identical, and both structural and mechanistic similarities between the proteins have been reported (7-9,18,34). Although it seems unlikely that the PFO and SLO homologues would insert into the bilayer via vastly different mechanisms, the two toxins have to be examined in parallel using the same
experimental approach to assess the extent of similarity in their mechanism of pore formation.

In doing a comparative analysis of the release of different-sized markers from liposomes, one must be sensitive to the relative concentration of toxins and liposomes. For example, if both a small and a large marker are incorporated into the same liposomal preparation, and the liposomes are incubated with PFO, the emission intensity of both markers will be strongly quenched at high toxin/lipid molar ratio (i.e., at conditions where the formation of complete rings is favored). But at a low toxin/lipid ratio, the quenching of the markers will diminish, and will be limited by the rare event of binding of sufficient monomers on an individual liposome to form a pore. Nevertheless, independent of the total number of pores formed, the extents of quenching for the different markers should be identical if only large pores are formed.

In contrast, if only a few toxin molecules are able to form a pore (as suggested for SLO; 15), the pattern of fluorescence intensity quenching will be very different. The extent of quenching of the large marker will parallel the extent of quenching of the small marker only at high toxin/lipid ratio (note: the rates would still differ). At low toxin/lipid ratio, where only a few monomers will distribute, on average, on each liposome, the extent of quenching of the small marker is expected to be higher than that of the large one.

Using liposomes containing both a large marker (Amy-Fl) and a small marker [Tb(DPA)$_3^{3-}$], we quantified the total release of each fluorophore after incubating a set amount of liposomes with increasing amounts of PFO and SLO. Serial 1:2 dilutions of the toxins were prepared and an identical volume of each toxin solution was added to samples containing identical total lipid concentrations. As shown in Fig. 4A, the
percentage of release for both large and small markers was very similar at every toxin/lipid ratio, thereby indicating that PFO is not able to form small pores on cholesterol-containing membranes. The quenching pattern obtained for SLO (Fig. 4B) was very similar to that obtained for PFO, indicating that SLO also cannot form small pores in the liposomal membrane. Therefore, we conclude that pore formation by SLO also requires the formation of a prepore complex.

**Characterization of the PFO Prepore Complex** - PFO pore formation can be halted at the prepore complex stage prior to TMH insertion into the bilayer by engineering a disulfide bond that prevents TMH extension (9,17), by mutation that prevents TMH insertion (19), or by incubation with membranes at low temperatures (16). One advantage of the last method of creating a prepore intermediate is that we can use a functional toxin instead of an inactive mutant.

The temperature-dependent interactions of domain 4 and of the domain 3 TMHs with membranes were analyzed using liposomes containing spin-labeled phospholipids. Intrinsic tryptophan fluorescence was monitored to follow the interaction of domain 4 with the membrane (9,35,36), while extrinsic NBD fluorescence was monitored to detect the interaction of a TMH with the bilayer (7-9). Since excited NBD and Trp moieties lose their excited state energy if they contact a nitroxide moiety, the emission intensity of the sample will be reduced if a Trp or NBD fluorophore is embedded in the bilayer where it can collide with the nitroxide moiety on a spin-labeled phospholipid (33,37). Since no fluorescence change will be observed if the collisional quencher and the Trp or NBD do not contact each other, this approach directly measures the accessibility of fluorescent probe to quencher, and hence provides topographical information.
The tryptophan emission intensity increased when PFO was incubated with liposomes prepared without or with spin-labeled phospholipids as shown in Fig. 5A. In each case, tryptophan emission intensity increased to a maximum by 60 min at 2°C. However, the magnitude of the final tryptophan emission intensity depended upon the location of the nitroxide quenchers, and hence the extent of collisional quenching in the liposomes. Thus, in the prepore complex the tryptophans in domain 4 are exposed to the bilayer interior, but are positioned close to the membrane surface, just as they are after pore formation has been completed (10).

In contrast, the emission intensity of an NBD probe located close to the tip of the TMH2 hairpin (PFO<sup>1303C-NBD</sup>; 8) was unaltered by prepore formation. Although the NBD emission intensity of this PFO mutant increases substantially when TMH2 is embedded in the interior of the bilayer (8), no increase in intensity was observed at low temperature even after 80 min (Fig. 5B). Consistent with the absence of any indication that this NBD was exposed to the bilayer core in the prepore complex, no nitroxide dependent quenching of NBD intensity was observed (circles) unless the sample is incubated at 37°C (squares). Similar results were obtained when the probe was located at different locations on TMH1 and TMH2 (Table II). [Even though the F/F<sub>0</sub> value for the PFO<sup>T301C-NBD</sup> derivative at 2°C was 2.8, the NBD moiety is not exposed to the lipid bilayer because the dye was not quenched when 5-doxyl-PC was incorporated into the liposomal membrane (data not shown)]. Thus, we conclude that the TMHs located in domain 3 are not exposed to the membrane interior in the prepore complex.

**Does the Interaction of Domain 4 with the Bilayer Affect the Permeability of the Liposomal Membranes?** - The mechanism by which lipids are ultimately eliminated from an aqueous CDC pore remains one of the most obscure aspects of pore formation. As
mentioned above, two different domains of PFO interact with the membrane bilayer, domain 3 and domain 4. Whereas domain 3 is directly involved in the formation of the transmembrane β-barrel (7,8), domain 4 binds first to the membrane and remains located at the surface of the bilayer (9,10). Images of a membrane-bound oligomer of a different CDC, pneumolysin, were obtained by cryo-electron microscopy, and the pore reconstruction did not show a large opening in the membrane (38). The authors suggested that these features could correspond to nonbilayer configurations such as inverted hexagonal lipid phases, and that this might lead to the breakdown of the permeability barrier.

Since PFO forms a prepore complex at low temperature, we wondered whether the binding of domain 4 or the formation of the prepore complex affects membrane permeability. Membrane permeability can be analyzed using fluorescence quenching as described above. To detect the formation of small pores or lesions, as well as any leakage of the membrane bilayer, we used the smallest fluorophore-quencher pair [Tb(DPA)$_3$$^3$/EDTA; Table I].

As shown in Fig. 6, almost no leakage of the membrane was detected when PFO bound to the cholesterol-containing liposomes at $2^\circ$C. Under these conditions, a prepore complex is formed (16) and domain 4 binds to the cholesterol-containing membrane with a topography resembling that of the membrane-inserted oligomer (Fig. 5A; 9). Only after incubation at $37^\circ$C (to trigger the β-barrel insertion), did the membrane became permeable to small as well as large molecules. Thus, the interaction of domain 4 with the membrane and the formation of the prepore complex do not alter the permeability of the bilayer. Moreover, the absence of pore formation at low temperature confirms our previous results that showed that the domain 4-
membrane interaction can be uncoupled from the insertion of the TMHs (9). Thus, under conditions where the insertion of the TMHs is prevented (9,16,19), PFO is able to bind to the membrane, oligomerize, and form a prepore complex without disrupting the membrane sufficiently to cause a significant leakage of the liposomal contents.

**DISCUSSION**

Our spectroscopic examination has provided five important insights into the mechanism of CDC pore formation. First, PFO does not form small transmembrane pores on cholesterol-containing membranes because the rate of release of liposome-encapsulated fluorophores is the same for all sizes of fluorophores. The mechanism of PFO pore formation therefore involves the formation of prepore complexes prior to insertion, not the growing-pore mechanism. Second, PFO and SLO follow a common mechanism of pore formation. The concentration-dependent pattern of fluorophore release is independent of the fluorophore size for both cytolysins, thereby indicating that the formation of a prepore complex is required to form a transmembrane pore in both cases. Third, domain 4 sits on the membrane surface in the prepore complex. The quenching of different fluorescence probes showed that only the tip of domain 4 is exposed to the hydrophobic core of the membrane when PFO is arrested at this stage. Fourth, the TMHs are not exposed to the membrane interior in the prepore complex because fluorescent probes located on the hydrophobic side of the TMHs are quenched by membrane-restricted doxyl quenchers only after the formation of the transmembrane β-barrel. Fifth, the binding of PFO to cholesterol-containing liposomes and the formation of the prepore complex do not alter the permeability of the liposomal membranes.
because no release of small fluorophores was detected under conditions where the

toxin binds and forms a prepore complex on the liposomal surface.

**PFO Does Not Form Small Pores during Oligomerization** - One of the still-

controversial aspects of the cytolytic mechanism of CDCs is how the large
transmembrane pores (~250 Å in diameter) are formed in a cholesterol-containing
membrane (1,2,20,21). Do CDCs oligomerize on the membrane and subsequently
insert the amphipathic β-barrel, or is the β-barrel created by the sequential insertion of
individual monomers to the growing oligomer?

It has been shown recently that PFO can form a prepore complex on the
membrane prior to the insertion of the transmembrane β-barrel. However, the presence
of a stable prepore complex was only observed under conditions where the insertion of
the β-hairpins was impeded (i.e., using non-pore-forming mutants, disulfide-trapped
derivatives, or working at low temperatures; 9,16,17,19). Therefore, we could not rule
out that under conditions where pore formation occurs (e.g., with the wild-type toxin at
temperatures higher than 20°C), the individual β-hairpins of PFO could insert into the
membrane concomitantly with the oligomerization process as suggested in the growing-
pore model (15, Fig. 1B).

To experimentally address this model and the alternative prepore model (Fig.
1A), we have analyzed the kinetics of pore formation of PFO<sup>C459A</sup> at 25°C by measuring
the toxin-dependent release of different fluorescein-labeled markers (with molecular
diameters ranging from approximately 10 to 140 Å) encapsulated into cholesterol-
containing liposomes. After the addition of PFO, we found that the kinetics of quenching
was identical for all of these fluorescent markers (Fig. 3). Since the rate-limiting step of
pore formation is oligomerization (9,17), these results clearly show that PFO\textsuperscript{C459A} also forms a prepore complex, as has been previously shown for several non-pore-forming PFO mutants (16,17,19). This prepore complex formation is then followed by the insertion of the β-barrel, which simultaneously allows all of the different-sized fluorophores to pass through the membrane.

\textit{Homologous CDCs Use a Similar Mechanism for Pore Formation} - We have here shown that the pore formation by PFO conforms to the prepore model (Fig. 1A) rather than the growing-pore model (Fig. 1B). Palmer et al (15) used a similar approach to conclude that SLO pore formation proceeds via the growing pore model. In their study, rhodamine-labeled dextrans of different sizes were encapsulated into erythrocyte ghosts and the extents of fluorophore release (the kinetics were not measured) differed for the large and the small dextrans. Do PFO and SLO use different mechanisms of pore formation, or are the observed differences caused by the different experimental procedures used? To resolve the discrepancy, we have examined PFO and SLO in parallel using the same experimental conditions.

Using liposomes that contained both small and large fluorophores, we analyzed the pattern of their release at different toxin/lipid molar ratios for both PFO and SLO. The quenching results that we obtained clearly show that neither PFO nor SLO is able to form small pores on cholesterol-containing membranes (Fig. 4). After the addition of PFO or SLO, the extents of release of large and small markers were very similar, thereby indicating that neither PFO nor SLO is able to form pores when only a few monomers are bound to each liposome. These similarities in the results indicate strongly that SLO also forms a prepore complex before inserting its TMHs to form the
transmembrane β-barrel. Hence, we conclude that the homologous CDCs use a common insertion mechanism to form a transmembrane pore.

The reason for the discrepancy between our results and those of Palmer et al (15) is not known. However, since dextrans are known to bind to erythrocytes (e.g., 39), it is possible that the different extents of dextran release into the supernatant may reflect their differing affinities for the erythrocyte ghosts. The higher affinity (40) and larger number of adsorption sites of the larger dextran would then explain its delayed and reduced release from the ghosts. An alternative possibility is that the presence of proteins in the erythrocyte membrane may cause cytolysins to form an incomplete prepore complex and the subsequent formation of a pore significantly smaller in size than the one observed in liposomal membranes.

In the Prepore Complex, Domain 4 Sits on the Membrane Surface and the TMHs Are Not Yet Inserted into the Membrane Core - We have used fluorescence quenching to examine the location of both membrane-interacting domains of PFO in the prepore complex. By monitoring intrinsic tryptophan fluorescence in the presence of different spin-labeled phospholipids with the nitrooxide quencher moiety located at different depths within the bilayer, we estimated the extent of domain 4 penetration into the membrane in the isolated prepore complex. The increase in intrinsic emission intensity observed when PFO\textsuperscript{1303C-NBD} binds to cholesterol-containing liposomes at 2°C revealed that domain 4 interacts with the bilayer and that some of the Trp residues are exposed to the hydrophobic membrane core. The kinetic profiles obtained in the presence and absence of different membrane-restricted quenchers also showed that domain 4 remains on the membrane surface throughout the entire process of oligomerization, with only its tip embedded in the bilayer (Fig. 5A). Thus, in the prepore complex, the
relative orientation of domain 4 to the membrane surface is very similar to the one observed in the membrane-inserted oligomer (9).

In contrast, a similar analysis performed using several NBD–labeled mutants on TMH1 and TMH2 (7,8) revealed that the NBD dyes located in either of these TMHs are not exposed to the membrane core either after binding to the membrane or after formation of the prepore complex (Fig. 5B, Table II).

*Formation of the Prepore Complex Does Not Disrupt the Membrane Permeability Barrier* - CDCs form large pores in cellular membranes that are big enough to allow the passage of large molecules (41). Moreover, we have shown that the insertion of PFO TMHs into the bilayer correlates with the formation of those large pores (9,19; Fig. 3 and 4). It has also been suggested that the superficial interaction of a CDC with the bilayer may alter the structure of the membrane and allow small molecules and ions to permeate through the bilayer (38).

To examine this possibility, we tested the ability of PFO$^{C459A}$ to disrupt cholesterol-containing membranes under conditions where the insertion of the TMHs does not occur. As shown in Fig. 6, neither the binding of PFO$^{C459A}$ nor the formation of the prepore complex caused any leakage of the liposomal contents. Thus, these interactions of PFO with the bilayer do not alter membrane permeability prior to TMH insertion. Instead, the insertion of the TMHs and the formation of the transmembrane β-barrel are absolutely required to form a pore in the membrane.

Thus, the analysis of the mechanism of pore-formation of the CDCs reveals that only large pores are formed in cholesterol-containing membranes (and not small pores that grow in size). This observation is consistent only with the formation of a prepore complex prior to the insertion of the transmembrane β-barrel into the bilayer. The
topographical analysis of the PFO prepore complex showed that at this intermediate stage, PFO contacts the membrane surface via domain 4, and the individual transmembrane β-hairpins in domain 3 are not exposed to or embedded in the nonpolar core of the bilayer.

Acknowledgements. We thank Drs. S. Cheley and H. Bayley for their generous gift of S. aureus α-hemolysin.
REFERENCES


FOOTNOTES

1) The abbreviations used are: PFO, perfringolysin O; SLO, streptolysin O; αHL, S. aureus α-hemolysin; TMH, transmembrane β-hairpin; CDC, cholesterol-dependent cytolysin; NBD, 7-nitrobenz-2-oxa-1,3-diazole; FITC, fluorescein isothiocyanate; CA, carbonic anhydrase; Amy, β-amylase; Thy, thyroglobulin; GSH, reduced glutathione; Fl, fluorescein; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; x-doxyl-PC, 1-palmitoyl-2-stearoyl-(x-doxyl)-sn-glycero-3-phosphocholine; DPA, 2,6-pyridinedicarboxylic acid or dipicolinic acid.
FIGURE LEGENDS

Fig. 1. **Schematic models of the mechanism of pore formation by CDCs.** (A) The prepore model suggested for PFO (6,16). The toxin monomers bind to the membrane surface, oligomerize, and form a prepore complex prior to the insertion of the TMHs that form the transmembrane pore. (B) The growing-pore model suggested for SLO (15). In this model, the toxin molecules insert into the membrane as a dimer or small oligomer to initially form a small pore. The pore then grows continuously by the addition of monomeric cytolysins that insert individually into the bilayer and then diffuse laterally to associate with the growing end of the oligomer. The membrane surface is shown in light grey and is seen from above, and the aqueous transmembrane pore is shown in white.

Fig. 2. **Determination of the size of pores formed in liposomal membranes.** (A) The fraction of different encapsulated fluorophores quenched, and hence released, from liposomes (final concentration 50 µM total lipids) after incubation with αHL (final concentration 2 µM) as described in Experimental Procedures. The liposomes contained both the fluorescein-labeled marker and Tb(DPA)$_3^{3-}$. The reported values correspond to the average of at least two independent measurements. (B) Time-dependent profiles for the quenching of different fluorophores of different sizes released from liposomes upon the formation of transmembrane pores of different sizes. Liposomes loaded with both Tb(DPA)$_3^{3-}$ and Amy-Fl were treated with αHL (final concentration 2 µM), and the release of Tb(DPA)$_3^{3-}$ from the liposomes was detected by the quenching of Tb(DPA)$_3^{3-}$ emission intensity by EDTA located in the external buffer solution (filled circles). Amy-Fl was not released from the liposomes until PFO (final...
concentration 50 nM) was added 21 min after αHL addition. PFO-dependent release of Amy-Fl was then detected by the quenching of the fluorescein emission intensity by anti-fluorescein antibody (open circles). The extent of spontaneous Amy-Fl release from liposomes (i.e., leakage) is shown by the solid line (no toxin added).

Fig. 3. **Release kinetics of markers with different hydrodynamic radii from liposomes treated with PFO.** Liposomes loaded with different fluorescein-labeled proteins were treated with PFO as described in Experimental Procedures. The release of fluorescein-tagged markers from the liposomes was detected by the quenching of the fluorescein emission intensity by the anti-fluorescein antibodies present in the extraliposomal milieu of the sample. To directly compare the rate of release for different fluorescein-labeled markers, the time-trace profiles were normalized using \((F_0 - F)/(F_0 - F_f)\), where \(F_0\) is the net initial emission intensity, \(F_f\) the net final emission intensity, and \(F\) corresponds to the net observed emission intensity at a specific time. The plotted data correspond to the average of two independent experiments and are color-coded as indicated in the graph inset.

Fig. 4. **PFO and SLO do not form small pores in cholesterol-containing membranes.** (A) Released of different encapsulated fluorophores from liposomes at different PFO/lipid molar ratios. Both Amy-Fl (empty bars) and Tb(DPA)\(_3^3\) (filled bars) were encapsulated into the same cholesterol-containing liposomes, and the fraction quenched was estimated as described in Experimental Procedures. (B) Released of fluorophores at different SLO/lipid molar ratios for markers of different sizes as
described in A. The averages of at least three measurements are shown, and the error bars indicate the standard deviations of the measurements.

Fig. 5. **Location of domain 4 and the TMHs of PFO in the prepore complex.** (A) Time-dependent emission intensity profiles for Trp fluorescence (domain 4) at 2°C for PFO\(^{1303C-NBD}\). Toxin (50 nM) was incubated at 2°C in buffer A, and the initial emission intensity was determined (\(F_0\)). Liposomes were added to a final concentration of 50 µM total lipids, and intensity data collection (\(F\)) was initiated after 15 sec of mixing. The values obtained after incubation at 37°C (9), and reequilibration at 2°C are shown on the right. (B) Time-dependent emission intensity profiles for NBD fluorescence (TMH2) at 2°C for PFO\(^{1303C-NBD}\) obtained as in A. After completion of the incubation at 2°C, the sample was further incubated at 37°C for 20 min to allow complete insertion of the TMH. The final emission intensity was measured after re-equilibration of the sample at 2°C (squares). The plotted data correspond to the average values of two independent experiments. The uncertainties of the measured values are less than 0.04 for the time-dependent profile and less than 0.1 for the values measured after incubation at 37°C.

Liposomes containing the indicated quencher were prepared as in Experimental Procedures. The control refers to liposomes prepared without quencher.

Fig. 6. **The interaction of domain 4 with the membrane does not alter the bilayer permeability.** Time-dependent emission intensity changes observed for liposome-encapsulated Tb(DPA)\(^3\) and CA-FI after the addition of PFO to the liposomes at 2°C. Liposomes (50 µM) were incubated in the presence of EDTA (open circles) or anti-
fluorescein antibody (filled circles), and the initial emission intensity was determined 
\(F_0\). PFO was added to a final concentration of 25 nM and fluorescence intensity data 
\(F\) were measured after 15 sec of mixing. After completion of the incubation at 2°C, the 
samples were further incubated at 37°C for 20 min to allow complete pore formation. 
The final emission intensity was measured after re-equilibration of the sample at 2°C 
(arrow).
Table I

*Molecular dimensions of the fluorophores and quenchers employed to determine the size of transmembrane pores.*

<table>
<thead>
<tr>
<th>Molecule</th>
<th>MW (Da)</th>
<th>diameter (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tb(DPA)$_3^{3-}$</td>
<td>650</td>
<td>~ 10</td>
</tr>
<tr>
<td>Glutathione-Fl</td>
<td>600</td>
<td>~ 10</td>
</tr>
<tr>
<td>Carbonic Anhydrase-Fl</td>
<td>29,000</td>
<td>~45</td>
</tr>
<tr>
<td>β-Amylase-Fl</td>
<td>200,000</td>
<td>~100</td>
</tr>
<tr>
<td>Thyroglobulin-Fl</td>
<td>669,000</td>
<td>~140</td>
</tr>
<tr>
<td>EDTA$^4+$</td>
<td>290</td>
<td>~10</td>
</tr>
<tr>
<td>anti-Fl antibody</td>
<td>150,000</td>
<td>60 x 120 x 150</td>
</tr>
</tbody>
</table>
Table II

*Fluorescence-detected association of TMHs with the lipid bilayer.*

Relative emission intensity changes for different NBD-labeled PFO derivatives when incubated with cholesterol-containing liposomes at 2°C (prepore complex) and after incubation at 37°C (membrane-inserted oligomer). $F_0$ is the net emission intensity of each PFO derivative in aqueous solution (the monomeric form), and $F$ is the net emission intensity of the sample either after 60 min of incubation at 2°C with membranes (prepore complex) or after incubation at 37°C for 30 min and re-equilibration at 2°C (membrane-inserted oligomer). The probes that face the lipid bilayer in the membrane-inserted oligomer are indicated in boldface.

<table>
<thead>
<tr>
<th>PFO mutant</th>
<th>$F/F_0$ prepore complex</th>
<th>$F/F_0$ membrane-inserted</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TMH1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S194C-NBD</td>
<td>0.9</td>
<td>5.4</td>
</tr>
<tr>
<td>N197C-NBD</td>
<td>0.9</td>
<td>0.3</td>
</tr>
<tr>
<td>V198C-NBD</td>
<td>1.0</td>
<td>2.5</td>
</tr>
<tr>
<td>N199C-NBD</td>
<td>1.0</td>
<td>0.6</td>
</tr>
<tr>
<td>K201C-NBD</td>
<td>1.1</td>
<td>0.7</td>
</tr>
<tr>
<td>N205C-NBD</td>
<td>1.5</td>
<td>4.9</td>
</tr>
<tr>
<td><strong>TMH2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A296C-NBD</td>
<td>1.5</td>
<td>4.9</td>
</tr>
<tr>
<td>N300C-NBD</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>T301C-NBD</td>
<td>2.8</td>
<td>5.7</td>
</tr>
<tr>
<td>D302C-NBD</td>
<td>0.9</td>
<td>0.6</td>
</tr>
<tr>
<td>N305C-NBD</td>
<td>0.8</td>
<td>6.3</td>
</tr>
</tbody>
</table>
Fig. 2A

Fraction quenched

GSH-Fl  
Amy-Fl  
Tb(DPA)$_3^{-}$

Fig. 2B

Fraction quenched

Time (min)

control

no toxin

plus $\alpha$HL  
plus PFO
Fig. 3

![Graph showing the fraction of fluorophore released over time for different samples: Thy-Fi, Amy-Fi, CA-Fi, and GSH-Fi. The graph plots Time (min) on the x-axis and Fraction of fluorophore released on the y-axis. The data points are represented with error bars.]

- Thy-Fi
- Amy-Fi
- CA-Fi
- GSH-Fi
Fig. 4A

Fraction quenched vs. PFO/lipid molar ratio x 1000

Fig. 4B

Fraction quenched vs. SLO/lipid molar ratio x 1000
Fig. 5A

![Graph showing F/F₀ over time for different doxyls and controls.](image)

Fig. 5B

![Graph showing F/F₀ over time for control and 5-doxyl.](image)
Fig. 6
Assembly and topography of the prepore complex in cholesterol-dependent cytolysins
Alejandro P. Heuck, Rodney K. Tweten and Arthur E. Johnson

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