Conformational Changes That Effect Oligomerization and Initiate Pore Formation Are Triggered throughout Perfringolysin O upon Binding to Cholesterol*

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Pore formation by the cholesterol-dependent cytolysins (CDCs) requires the presence of cholesterol in the target membrane. Cholesterol was long thought to be the cellular receptor for these toxins, but not all CDCs require cholesterol for binding. Intermidelysin, secreted by Streptococcus intermedius, only binds to membranes containing the human protein CD59 but forms pores only if the membrane contains sufficient cholesterol. In contrast, perfringolysin O (PFO), secreted by Clostridium perfringens, only binds to membranes containing substantial amounts of cholesterol. Given that different steps in the assembly of various CDC pores require cholesterol, here we have analyzed to what extent cholesterol molecules, by themselves, can modulate the conformational changes associated with PFO oligomerization and pore formation. PFO binds to cholesterol when dispersed in aqueous solution, and this binding triggers the distant rearrangement of a β-strand that exposes an oligomerization interface. Moreover, upon binding to cholesterol, PFO forms a prepore complex, unfolds two amphipathic transmembrane β-hairpins, and positions their nonpolar surfaces so they associate with the hydrophobic cholesterol surface. The interaction of PFO with cholesterol is therefore sufficient to initiate an irreversible sequence of coupled conformational changes that extend throughout the toxin molecule.

Cholesterol is a distinguishing feature of mammalian membranes. It is therefore not surprising that some bacterial pore-forming toxins have evolved to take advantage of this property of mammalian cells. For example, the cholesterol-dependent cytolysins (CDCs) secreted by a number of Gram-positive bacteria absolutely require cholesterol in the target membrane to create a pore (1–3). The abundance of CDCs reveals their importance in the pathogenic mechanisms of these organisms.

The absolute requirement for cholesterol led researchers to postulate that cholesterol functioned as a receptor for CDC molecules. Consistent with this view, preincubation of CDCs with cholesterol inhibited their binding to membranes when exposed to mammalian cells (1). However, this long-standing paradigm has recently been challenged by the finding that not all CDCs require cholesterol for membrane binding. The CDC from Streptococcus intermedius, intermedilysin (ILY), binds to a membrane protein receptor, the human CD59 (4). Yet ILY forms pores only if the membrane contains substantial cholesterol (5). Thus, the contribution of membrane cholesterol to the mechanism of CDC cytolysis appears to be more complicated and multifaceted than simply acting as a CDC-binding site.

Perfringolysin O (PFO), secreted by pathogenic Clostridium perfringens (2), is a prototypical CDC. Upon encountering a cholesterol-containing membrane, the toxin oligomerizes and spontaneously inserts into the bilayer to form a large transmembrane pore (diameter ~ 300 Å) (6–8). The C terminus of PFO (domain C4 or D4) encounters the membrane first (9–11), and D4 binding triggers the structural rearrangements required to initiate the oligomerization of PFO monomers (12, 13) and formation of a prepore complex on the membrane surface (14, 15). Pore formation occurs when two amphipathic β-hairpins from each PFO molecule are inserted to span the membrane (16–18). The concerted insertion of transmembrane β-hairpins (TMHs) from ~35 PFO monomers then creates a large transmembrane β-barrel (8).

Although significant progress has been made in characterizing the structural changes in PFO that occur at different stages of pore formation, the mechanism by which cholesterol promotes PFO binding to the membrane and ultimately pore formation remains unknown. PFO binds only to liposomal and cellular membranes that contain more than 30 mol % cholesterol (10, 19). It has therefore been suggested that PFO binds to cholesterol-enriched microdomains or lipid rafts (20). However, the different phases detected in cholesterol:phospholipid

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2 The abbreviations used are: CDC, cholesterol-dependent cytolysin; PFO, perfringolysin O; ILY, intermedilysin; CMC, critical micellar concentration; NBD, 7-nitrobenz-2-oxa-1,3-diazole; BDF, N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-yl)methyl iodacetamide; TMR, tetramethylrhodamine-5-iodoacetamide dihydroiodide; MβCD, methyl-β-cyclodextrin; FRET, Förster resonance energy transfer; TMH, transmembrane β-hairpin.
mixtures are already present in membranes at cholesterol concentrations lower than 30 mol% of total lipids (21–23). It is therefore difficult to envision why PFO binding to a membrane exhibits such a sharp dependence on bilayer cholesterol content (10, 24). An alternative explanation is that PFO binding to membranes is dictated by the cholesterol chemical potential rather than by the formation of a particular phospholipid(s)-cholesterol phase or raft. Consistent with this possibility, a sharp increase in the chemical potential of cholesterol has been observed when the cholesterol concentration is raised above 30 mol% of the total lipids (25–27). Moreover, whereas arc and ring structures similar to those observed in natural membranes are formed when PFO is incubated with cholesterol dispersions, the lytic, lethal, and cardiotoxic properties of PFO and related toxins are inhibited by exposure to pure cholesterol (1, 28, 29).

To clarify the role of cholesterol in PFO cytolysis, we decided to determine the extent to which different steps in the cytolytic pathway could be elicited solely by the presence of cholesterol. Site-directed fluorescence labeling, stage-specific PFO derivatives, and several different fluorescence spectroscopic techniques were used to examine cholesterol-dependent changes in PFO conformation. Our studies reveal that a selective interaction between the loops at the tip of PFO D4 with cholesterol initiates coupled conformational changes that extend throughout the toxin molecule. These conformational changes are indistinguishable from those shown to occur when PFO binds to, oligomerizes on, and forms pores in liposomal and cellular membranes containing sufficient cholesterol. Furthermore, once initiated, these allosteric conformational changes are not reversible.

**EXPERIMENTAL PROCEDURES**

*Preparation of PFO Derivatives*—A construct containing the wild-type PFO sequence was obtained by PCR-based mutagenesis using the QuickChange (Stratagene) procedure and the pRSETB plasmid coding for the rPFO (11). The codon GCT for Ala-459 was replaced by the codon TGC for Cys-459. The derivative containing the native undecapeptide sequence (residues 458–468) is referred as nPFO. PFOC459A is designated a derivative containing the native undecapeptide sequence (residues 458–468) is referred as nPFO. Briefly, rPFO derivatives described previously (11, 12, 16, 17, 30). rPFO, and its derivatives were expressed and purified as described previously (11, 12, 16, 17, 30).

*Fluorescent Labeling of rPFO Derivatives*—rPFO derivatives were labeled with NBD as before (17). Briefly, rPFO derivatives (5–10 μM) in Buffer A (50 mM Hepes (pH 8.0), 100 mM NaCl, and 1 mM EDTA) were reacted for 2 h at room temperature (~22 °C) with a 10-fold molar excess of N,N'-dimethyl-N-(iodoacetyl)-N'-(-7-nitrobenz-2-oxa-1,3-diazol-4-yl) ethylenediamine. In the case of rPFOV322C, the reaction was made 3 M in guanidine hydrochloride to facilitate access of the N,N'-dimethyl-N-(iodoacetyl)-N'-(-7-nitrobenz-2-oxa-1,3-diazol-4-yl) ethylenediamine to the partially buried Cys (12). NBD-labeled rPFO derivatives were separated from free dye by gel filtration through a Sephadex G-50 column (1.5 cm inner diameter × 25 cm) equilibrated with Buffer B (50 mM Hepes (pH 7.5), 100 mM NaCl). The efficiency of labeling was determined spectrophotometrically to be 80–100% by using molar absorb-

*tivity coefficients of 74,260 M\(^{-1}\) cm\(^{-1}\) at 280 nm and 25,000 M\(^{-1}\) cm\(^{-1}\) at 478 nm for rPFO and NBD, respectively (31, 32).

For Förster resonance energy transfer (FRET) experiments, N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-yl)methyl iodoacetamide (BDF) and tetramethylrhodamine-5-iodoacetamide dihydroiodide (TMR) labeling of rPFO derivatives was also done using a 10-fold molar excess of the thiol-specific fluorescent reagent. The efficiencies of labeling were determined spectrophotometrically to be greater than 70% by using a molar absorbivity coefficient of 76,000 M\(^{-1}\) cm\(^{-1}\) at 502 nm for BDF and 87,000 M\(^{-1}\) cm\(^{-1}\) at 543 nm for TMR (32). All fluorescent probes were obtained from Molecular Probes (Invitrogen). All fluorophore-labeled rPFO derivatives were frozen in small aliquots in Buffer B containing 10% (v/v) in glycerol and stored at −80 °C.

*Preparation of Liposomes*—Synthetic liposomal membranes were composed of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids) and the indicated sterol and cholesterol Is Sufficient to Trigger PFO Cytolysis

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*Preparation of Liposomes*—Synthetic liposomal membranes were composed of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids) and the indicated sterol and cholesterol were generated as described previously (15). Cholesterol (5-cholesten-3β-ol) and 3-epicholesterol (5-cholesten-3α-ol) were obtained from Steraloids.

**Incubation with Sterol Dispersions in Aqueous Solutions**—Water-soluble PFO monomer samples (0.3 ml final volume, 0.1 μM final concentration) in buffer C (50 mM Hepes (pH 7.5), 100 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol) were equilibrated at 37 °C for 5 min before the net initial emission intensity (F0) was determined (i.e., after blank subtraction). Sterols were then added to the indicated final concentration, and the sample was then incubated at 37 °C for 15 min. The net emission intensity (F) of the sample was determined after blank subtraction and dilution correction. Sterols were dissolved in absolute ethanol to 10 mM and diluted with additional ethanol as necessary. When added to solutions of nPFO or rPFO derivatives, the final concentration of ethanol was always lower than 5% (v/v). Control samples were incubated with an identical volume of ethanol. When indicated, sterol aggregates were dissolved by the addition of methyl-β-cyclodextrin (MβCD, Sigma) to a final concentration of 3 mM.

*Assay for Pore Formation*—The pore formation activity of PFO and their derivatives was assayed as described before (15).

**Steady-state Fluorescence Spectroscopy**—Intensity measurements were performed using the same instrumentation described earlier (17). 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; 470, 2, 510, and 2 nm for BDF/TMR FRET determinations; 500, 1, 500, 1 nm for right angle light scattering measurements and 278, 2, 544, 4 nm for Tb(DPA)3\(^2+\). For Tb(DPA)3\(^2+\) 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. Kinetics measurements and end point measurements were done as described earlier (15). Coating of the cuvettes with 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine vesicles was omitted for all experiments containing only sterols.

**Energy Transfer Measurements**—Four biochemically equivalent samples were prepared in parallel for each energy transfer measurement: sample D (donor only) contained 50 nM
rPFOE167C-BDF and 50 nm of unlabeled rPFOE167C, sample DA (donor + acceptor) contained 50 nm rPFOE167C-BDF and 50 nm rPFOE167C-TMR; sample A (acceptor only) contained 50 nm rPFOE167C and 50 nm rPFOE167C-TMR; and sample B (blank) contained 100 nm rPFOE167C. In all four samples, the final cholesterol concentration was 3 μM. All samples were incubated at 37 °C for 15 min in Buffer C to permit complete binding of rPFO derivatives to cholesterol before spectral measurements were initiated.

Electron Microscopy—nPFO (10 nm) was incubated with 3 μM cholesterol in standard Buffer C for 30 min at room temperature (20–23 °C) after which 5 μl of sample were applied onto a Formvar carbon-coated copper grid that had been rendered hydrophilic by glow-discharging for 25 s. After 60 s, excess solution was blotted, and samples were stained with 1% (w/v) ammonium molybdate (pH 7.0). Specimens were observed in a JEOL 1200-EX transmission electron microscope operating at an acceleration voltage of 100 kV. Micrographs were recorded at a calibrated magnification.

RESULTS

PFO Binds Selectively to Cholesterol Dispersed in Aqueous Solution—CDCs bind to membranes via D4, and this interaction typically involves the exposure of Trp residues located at the tip of D4 to the nonpolar core of cholesterol-containing membranes (9–11, 15, 30). Because this decrease in polarity increases the intrinsic Trp emission intensity of the PFO molecule, this spectral change directly detects PFO binding to the membrane surface (9, 10). We therefore determined whether Trp emission intensity changes could be used to detect direct interactions between cholesterol and nPFO.

Upon addition to an aqueous solution, cholesterol undergoes a reversible self-association at the critical micellar concentration (CMC) of ~35 nm at 25 °C to form colloidal aggregates (33). Such cholesterol aggregates are rod-shaped and heterogeneous in size (34). The maximum solubility for cholesterol in aqueous solutions is ~5 μM. Above this concentration, crystal formation leads to cholesterol precipitation (33). The appearance of such cholesterol precipitate is accompanied by a dramatic increase in the amount of 500 nm light scattered by the solution (Fig. 1C).

When nPFO was titrated with cholesterol dispersed in aqueous solution, no change in Trp intensity was detected when the concentration of cholesterol was below the CMC (Fig. 1C). Because the maximum increase in Trp intensity was the same with cholesterol aggregates as with cholesterol-containing membranes, these results suggest that the Trp environments are nearly equivalent when nPFO is bound to cholesterol-containing membranes and to colloidal aggregates containing only cholesterol.

Importantly, no spectral change was observed when nPFO was titrated with 3-epicholesterol, an isomer that differs from cholesterol only in that the hydroxyl group is directed axially instead of equatorially (Fig. 1). We therefore concluded that the cholesterol-dependent Trp emission intensity change was not the product of a nonspecific interaction between D4 and a nonpolar surface. Instead, the fluorescence change resulted from a selective interaction between the hydrophobic amino acids located in the D4 loops and the cholesterol molecules.

From these data, it is clear that PFO D4 can interact with cholesterol aggregates in solution. However, because D4 contains six Trp residues, it is difficult to identify unambiguously which D4 residues are exposed to cholesterol using Trp fluorescence.

Which Parts of D4 Interact with Cholesterol?—To identify D4 sites that contact purified cholesterol, the emission intensities of four different NBD-labeled rPFO mutants were examined before and after incubation with cholesterol. In this approach, a single amino acid in a Cys-free protein is replaced with a Cys residue, and its sulphydryl group is covalently modified with the water-sensitive fluorophore NBD (35). Here we examined four derivatives whose interactions with cholesterol-containing membranes had been characterized earlier by us (Fig. 2A) (11). Probes attached at residues 491 and 437 to yield rPFOA491C-NBD and rPFOA437C-NBD were used to ascertain whether the tip of D4 interacted with cholesterol, whereas two other derivatives, rPFOY442C-NBD and rPFOK455C-NBD, were used to assess the

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**FIGURE 1.** PFO binds selectively to cholesterol dispersed in aqueous solution. A, chair conformation of the cholesterol chemical structure. B, chair conformation of the 3-epicholesterol chemical structure. The only difference between 3-epicholesterol and cholesterol is the position of the 3-hydroxy group, which is in a 3α and in a 3β configuration, respectively. Trp emission intensity for 0.1 μM nPFO was measured before (Fo) and after (F) addition of the indicated amount of cholesterol (○) or 3-epicholesterol (■). The open symbols show the amount of light scattered at 500 nm in the samples with the corresponding closed symbols. The CMC of cholesterol (20–40 nm) and the solubility limit (4.7 μM) are indicated by the asterisk and the caret, respectively. Each data point shows the average of at least two independent measurements and their range.
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exposure of the external surfaces of the D4 β-sandwich to cholesterol in solution. The function of these proteins was not compromised by mutagenesis and modification (11).

Because NBD movement from an aqueous milieu to a more hydrophobic environment, such as the nonpolar core of a membrane bilayer, is accompanied by a pronounced increase in NBD emission intensity and lifetime (35, 36), the spectral properties of these derivatives were monitored before and after incubation with cholesterol dispersed in aqueous buffer. We observed that the emission intensities of probes located at position 437 and 491, each located in a loop at the tip of D4, increased 12- and 30-fold, respectively, after incubation with 3 μM cholesterol. No increase in either NBD or Trp fluorescence was observed when only ethanol lacking cholesterol was added to the buffer solution containing these rPFO derivatives. Thus, exposure to cholesterol caused these probes to move from an aqueous milieu into a hydrophobic environment. In contrast, the emission intensities of probes located at positions 442 and 455 did not change significantly (Fig. 2B). Hence, the latter two probes were in an aqueous milieu before and after cholesterol addition.

MβCD has been used extensively to manipulate the cholesterol content in cellular membranes (5, 37). MβCDs are able to bind cholesterol molecules and dissolve cholesterol aggregates in aqueous solution, as detected by the reduction in light scattered by the dispersed cholesterol molecules (data not shown). Interestingly, when MβCD was added to a sample containing both rPFO and cholesterol, the cholesterol-dependent emission intensity increases were reversed for the NBD dyes at the tip of D4 (Fig. 2, 437 and 491).

Cholesterol-dependent Changes in PFO Domain 3—The interaction of PFO with cholesterol-containing membranes triggers conformational changes in the molecule that regulates the oligomerization of the toxin on the membrane surface. Upon membrane binding, a short β-strand (β5) located in domain 3 (D3) moves to expose the edge of a previously hidden β-strand (β4) that forms the monomer–monomer interface and is required for oligomer assembly (Fig. 3A) (12). This conformational change can be followed by the exposure of an NBD dye covalently attached to a single Cys substituted at position 322 in rPFO, rPFOV322C-NBD. In the water-soluble monomer, the NBD dye at position 322 exhibited a high fluorescence lifetime (τ ~ 8 ns), but this lifetime drops considerably (τ ~ 1 ns) upon pore formation. This conformational change can also be followed by the decrease in the fluorescence intensity of the NBD dye, which occurs at the same rate as the D4 Trp emission intensity increase upon membrane binding (12).

To determine whether the interaction of D4 with cholesterol aggregates is sufficient to trigger the conformational change in D3, rPFOV322C-NBD was incubated with different amounts of cholesterol, and the fluorescence intensities of the 322-NBD and Trp residues followed in parallel in the same sample. The extent of the D4 Trp intensity increases paralleled the extent of the 322-NBD intensity decreases in D3 (Fig. 3B). Thus, a D4 interaction with pure cholesterol is sufficient to trigger a conformational change in D3 that exposes the NBD dye to the aqueous solvent. This interaction is cholesterol-selective, because addition of 3-epicholesterol to rPFOV322C-NBD did not significantly affect either Trp emission or NBD emission (Fig. 3C).

Having noted that the cholesterol-dependent increase in NBD-labeled D4 emission was reversed by the addition of MβCD, we determined whether the cholesterol-induced increase in D4 Trp emission and the conformational change in D3 were also reversible by adding an excess of MβCD to cholesterol-bound rPFOV322C-NBD. Although the Trp exposure to nonpolar cholesterol was greatly reduced by dissolution of the cholesterol aggregates (Fig. 3D), the NBD emission intensity did not return to its original value (Fig. 3E). The nonreversibility of the NBD spectral change shows that β5 does not return to a location covering β4 after cholesterol is removed.

PFO Molecules Oligomerize Upon Binding to Cholesterol Aggregates—To assess whether PFO binding to cholesterol is sufficient to trigger toxin oligomerization, we used FRET to detect the close approach of PFO monomers to each other. We have previously used FRET to determine the topography of membrane-bound rPFO and to quantify the magnitude of the conformational changes that occur during pore formation (30). FRET is also an excellent method for monitoring molecular associations and has been used before with rPFO (5, 38).

The typical FRET experiment requires two fluorescent dyes, a “donor” (D) and an “acceptor” (A), that are located at specific sites (36). After excitation by the absorption of a photon, a D
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TABLE 1

Cholesterol binding triggers PFO association

| Liposomes | Plus sterol | Plus MJBCD |
|-----------|-------------|-------------|
| Liposomes | 0.85 ± 0.05 |              |
| Cholesterol | 0.81 ± 0.03 | 0.76 ± 0.03 |
| 3-Epicholesterol | 0 ± 0.06 | 0 ± 0.07 |

can nonradiatively transfer its excited-state energy to A if A has the appropriate spectral properties. The efficiency of this energy transfer depends primarily upon the extent of overlap between emission of the F and the absorption spectra of A, the relative orientation of D and A transition dipoles, and the distance between D and A. Donor emission intensity is reduced by FRET, and the magnitude of this decrease is used to measure the extent of energy transfer.

Two different derivatives of rPFO were prepared, rPFO$^{E167C-BDF}$ containing the donor dye and rPFO$^{E167C-TMR}$ containing the acceptor dye. The Glu-167 residue is located in domain 1, and neither the Cys mutation nor the labeling with the fluorophores altered the cytolytic activity of the toxin (30). No FRET was detected when the two rPFO derivatives were dissolved in aqueous solution at 100 nm because the average separation between D- and A-labeled rPFO molecules was too large (>100 Å) for FRET to be detected (38). However, after addition of liposomal membranes containing 50 mol % cholesterol, the FRET efficiency was 85% (Table 1). A similar FRET efficiency was obtained when the D- and A-labeled rPFOs were incubated with only 3 μM cholesterol (Table 1). Thus, cholesterol by itself is sufficient to trigger the same degree of PFO oligomerization as seen with liposomes containing a high cholesterol content. In contrast, no FRET-detected monomer-monomer association was observed when these rPFO derivatives were incubated with 3 μM 3-epicholesterol (Table 1). These results indicate that the selective association of PFO D4 with cholesterol aggregates is sufficient to trigger the allosteric conformational change in D3, about 70 Å above the membrane surface (30), that is required for monomer-monomer association (12).

The FRET observed in samples containing an equimolar mixture of D- and A-labeled rPFOs as well as 3 μM cholesterol was not reversed upon adding MJBCD to solubilize the cholesterol (Table 1). Thus, once formed, the oligomeric complex is stable, and the monomers do not dissociate.

Visualization of Oligomers by Electron Microscopy—Because even a simple dimerization between D- and A-labeled rPFOs will show a high FRET efficiency, it is difficult to ascertain from FRET data the size of oligomers formed in the presence of only cholesterol. To determine the extent of toxin oligomerization, nPFO was incubated with cholesterol (3 μM final concentration), and the resulting samples were analyzed using transmission electron microscopy (EM). Negatively stained specimens showed that when incubated with cholesterol, nPFO formed rings and arc structures that were very similar in size to those formed in membrane bilayers (Fig. 4).

The combined FRET and EM data indicate that PFO binds similarly to cholesterol aggregates and to cholesterol-containing membranes. (i) D4 contacts the cholesterol aggregate so that only the loops at the tip of the D4 are exposed to a hydrophobic environment. (ii) The short β5-strand in D3 moves to expose the monomer-monomer interface. (iii) The monomers associate to form ring-like structures that are the prelude to pore formation.

Both TMHs Extend and Associate Amphipathically with the Nonpolar Surface of Cholesterol Aggregates—The last and final step in the cytolytic mechanism is the unfolding and insertion of both amphipathic TMHs. To assess the location of the TMH in the PFO oligomers formed on cholesterol aggregates, we
used rPFO_{T319C/V334C/A215C-NBD} because it cannot insert TMH1 when oxidized (12). When this oxidized rPFO derivative was incubated with cholesterol in the absence of a reducing agent, the Trp emission intensity increased 3.6-fold, whereas the NBD emission intensity was unaltered (Table 2). Reduction of the disulfide bond by the addition of dithiothreitol caused a 5.7-fold increase in the NBD intensity, thereby showing that the 215 residue of TMH1 is exposed to a hydrophobic environment when PFO is bound to cholesterol aggregates.

To ascertain the location and conformation of TMH1 and TMH2 in the cholesterol-bound PFO rings, we analyzed several rPFO derivatives with an NBD dye attached at different positions on the TMHs. NBD emission intensity increased dramatically for residues 194, 196, 198, 205, 215, 296, 301, 303, 305, and 307 upon addition of cholesterol aggregates (Fig. 5). The alternating exposure of TMH residues to a nonpolar environment reveals that both TMH1 and TMH2 adopt an amphipathic \( \beta \)-hairpin conformation similar to those observed in membrane bilayers (16, 17). Upon addition of M\( \beta \)CD to solubilize the cholesterol, the individual NBD intensities reverted to values similar to those observed for cholesterol-free monomeric rPFO (data not shown). This spectral change is not surprising because the disappearance of the hydrophobic cholesterol surface would leave the otherwise embedded residues on the nonpolar sides of TMH1 and TMH2 in an aqueous environment where their NBD intensity would return to its original monomeric value.

As noted above, the Trp emission intensity changes were very similar in magnitude when PFO was incubated with cholesterol in solution (Fig. 1C) or with cholesterol-containing membranes (24). The NBD emission intensity changes for each rPFO derivative were also very similar when the toxin was exposed to either cholesterol aggregates or liposomal membranes. The hydrophobic environment provided by the cholesterol molecules in solution therefore does not differ much from that “seen” by PFO when bound to membrane bilayers.

**DISCUSSION**

Our spectroscopic examination has provided four primary insights into the mechanism of PFO interaction with cholesterol. First, PFO D4 binds selectively to cholesterol when the sterol molecule forms colloidal aggregates in aqueous solution, with each loop at its tip exposed to the nonpolar surface of the cholesterol aggregate. Second, the D4-cholesterol aggregate interaction is necessary and sufficient to trigger the allosteric conformational change in D3 required for monomer-monomer association and toxin oligomerization. Third, PFO rings and arcs, similar in size to those observed with liposomal membranes, are formed when PFO is incubated with colloidal cholesterol aggregates in aqueous buffer. Fourth, both TMHs unfold and associate amphipathi-
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More than a century has passed since cholesterol inhibition of the CDCs was first observed (39), and several reports on the toxin-sterol interaction have been published subsequently (40). However, the molecular basis for the selective binding to cholesterol has remained puzzling. The stoichiometry of the toxin-sterol complex under equilibrium conditions has been difficult to determine because, among other reasons, the limited solubilities of cholesterol and other sterols in the aqueous phase, the nonuniform nature of their dispersions in water, and their tendency to stick to solid surfaces (40).

In aqueous solution, monomeric cholesterol concentration is limited to \( \sim 30 \text{ nM} \) (33, 41, 42). At increasing concentrations, cholesterol forms micelle-like aggregates with heterogeneous rod-like shapes and lengths between 100 and 600 nm (33, 34). Because of the rigidity of the sterol rings, these aggregates may not be a typical micelle but rather an aggregate of cholesterol molecules stacked side by side (41). These aggregates have a very limited range of stability, and when the total cholesterol concentration reaches \( \sim 5 \text{ \mu M} \), they coalesce and separate from the solution as a separate phase, presumably as cholesterol monohydrate precipitate (42).

Incubations of streptolysin O (43, 44), PFO (6), cereolysin (45), alveolysin (44), pneumolysin (44), and listeriolysin O (46) with cholesterol dispersed in aqueous solution produced aggregated sterol-toxin complexes. For PFO and streptolysin O, typical ring- and arc-like structures have been observed after incubation with cholesterol at concentrations above its solubility limit (i.e. higher than 5 \( \mu \text{M} \)) (6, 29, 43). Others have claimed that certain CDCs bind to cholesterol and form a 1:1 complex in aqueous solution. However, in the case of listeriolysin O, the experiments were done with a concentration of cholesterol much higher than the solubility limit (26 \( \mu \text{M} \)) (33, 47), and the final concentration of cholesterol was not provided for the experiments with pneumolysin (48).

The complexity of working with cholesterol in aqueous solution is evident from the diverse stoichiometry values obtained for various CDC-cholesterol complexes that ranged from 0.7 to 10,000 (40, 47, 48). Despite the uncertainty in CDC-cholesterol stoichiometry, it has become clear that cholesterol is required for all CDCs to create a pore in membrane bilayers (5). Yet different CDCs appear to require cholesterol at different points in the cytolytic mechanism. For example, cholesterol is not required for ILY binding to cellular membranes but is required for ILY TMH insertion and pore formation (4, 49). In contrast, PFO binding to membrane bilayers is totally dependent on the presence of sufficient cholesterol in the membrane (10, 19, 50, 51). Given that different steps in the assembly of various CDC pores require cholesterol, it is reasonable to ask to what extent cholesterol molecules, by themselves, can trigger the conformational changes in PFO associated with toxin oligomerization and pore formation.

To investigate how PFO association with cholesterol affects PFO structure, we trapped PFO at each step of the cytolytic mechanism by using different PFO derivatives. PFO binding to cholesterol dispersed in aqueous buffer was detected by monitoring Trp emission intensity. An increase in Trp intensity was observed only when cholesterol was present as colloidal aggregates in solution (between 0.1 and 3 \( \mu \text{M} \) cholesterol) and not when cholesterol was monomeric (i.e. below 40 \( \text{nm} \) cholesterol, Fig. 1C) (33). The Trp fluorescence increase reached a maximum at a cholesterol:PFO ratio of \( \sim 30:1 \) thereby indicating that more than one cholesterol molecule is required to provide the hydrophobic environment into which the tip of D4 embeds (Fig. 2). This interaction was reversible because solubilizing the cholesterol aggregates with M\( \beta \)CD returned the Trp emission intensity back to its original value.

The monomer-monomer interface in the PFO oligomer is formed when the \( \beta_{1}\)-strand in one subunit associates, presumably via hydrogen bonding, with the \( \beta_{4}\)-strand in a second subunit (Fig. 3A) (12). Premature association of PFO molecules (i.e. before they bind to an appropriate target membrane) is blocked by the presence of a short \( \beta \)-strand (\( \beta_{5} \)) that hydrogen bonds to \( \beta_{4} \) in each monomer and thereby prevents its interaction with the \( \beta_{1}\)-strand of a second monomer. But the binding of D4 to aggregates containing only cholesterol causes \( \beta_{5} \) to move away from \( \beta_{4} \) via a long distance (>70 Å) (12) membrane-to-D3 conformational change that promotes oligomerization (Fig. 3B). Both FRET and EM imaging showed that PFO oligomerized under our experimental conditions to form rings and arcs with dimensions similar to those observed in liposomal and natural membranes (Table 1 and Fig. 4). Interestingly, in contrast to the cholesterol-dependent Trp emission intensity increase (Fig. 3D), the exposure of V322C-NBD (\( \beta_{4} \)) to an aqueous environment was not reversed by solubilizing the cholesterol aggregates with M\( \beta \)CD (Fig. 3E).

The binding of PFO to cholesterol also results in the unfolding of D3 and the formation of two \( \beta \)-stranded TMHs. In addition, a major rearrangement of PFO domains occurs to allow the hydrophobic side of each TMH to associate with the nonpolar surface of the colloidal cholesterol aggregate (Fig. 5). Although the TMHs in an oligomer could not form an experimentally detectable pore in a colloidal cholesterol aggregate, it is important to note that the conformations adopted by the TMHs were the same on pure cholesterol and on membranes because the same TMH residues were exposed to nonpolar or to aqueous environments in the two cases. The PFO on the pure cholesterol surface therefore appears to be trapped at an intermediate state of pore formation in liposomal or natural membranes.

The existence of D4 residue movement into a hydrophobic milieu (Fig. 2) also confirms the presence of more than 1 cholesterol molecule per bound PFO (see above) because many cholesterol molecules would be required to provide the hydrophobic environments occupied simultaneously by both the D4 Trp residues and NBD dyes spread throughout each TMH (Figs. 2B and 5). As expected, the emission intensities of the NBD probes in the unfolded TMHs returned to their cholesterol-free values when the cholesterol aggregates were solubilized by M\( \beta \)CD and the TMHs were left in an aqueous milieu.

The combined data presented here reveal that a selective interaction between the loops at the tip of PFO D4 with cholesterol (and not one of its isomers) sets in motion an obligatory sequence of coupled conformational changes that extend throughout the toxin molecule. These structural changes...
include a long range allosteric linkage that causes β5 to move, thereby exposing D4 and allowing oligomerization to proceed. In addition, the six α-helices in D3 unfold and extend into amphipathic β-hairpins. Domain rearrangement within PFO then moves D3 close enough (30) to the colloidal cholesterol surface for the hydrophobic residues on the nonpolar side of the TMHs to insert into the nonpolar cholesterol aggregate. Thus, the cholesterol interaction with the D4 loops, by itself, is sufficient to trigger the primary structural changes in PFO that are indistinguishable up to the point of membrane puncture from those shown to occur when PFO binds to, oligomerizes on, and forms pores in liposomal and cellular membranes containing sufficient cholesterol. At this point we cannot rule out the possibility that a cellular factor may catalyze or improve the efficiency of PFO conformational changes upon membrane binding. However, no such factor has yet been identified by us or any other group.

The primary sequence of the D4 loops are conserved among different CDCs, suggesting that the three-dimensional arrangement of amino acids at the tip of D4 is critical for the interaction of the toxin with cholesterol molecules. In addition, the insertion of all D4 loops appears to be required for toxin oligomerization and pore formation (13). However, the molecular basis for the selective interaction of PFO D4 with cholesterol (and not with 3-epicholesterol) is not yet known, and PFO may recognize a single exposed cholesterol molecule at the membrane surface (52, 53) and/or the ordered arrangement of cholesterol molecules in a cholesterol-rich membrane. Interestingly, a monoclonal antibody induced by the injection of cholesterol monohydrate crystals also recognizes sterols stereospecifically because it binds to monolayers of cholesterol but not to monolayers of 3-epicholesterol (54).

It is also important to note that whereas D4 binding to cholesterol is reversible, the cholesterol-dependent movement of β5 and the oligomerization are not (Fig. 3, D and E). Thus, once initiated, the series of coupled conformational changes associated with PFO function go to completion. Monomeric PFO therefore folds into a conformation that is poised to spontaneously effect a series of structural changes that will ultimately lead to pore formation. The sole trigger for releasing this entire cascade of conformational changes is a selective binding interaction between cholesterol and the loops at the tip of D4. The PFO toxin has therefore evolved into an exquisitely sophisticated molecule that contains both the potential energy needed to puncture the membrane without an external energy source and also a cholesterol-based targeting mechanism that regulates the release of this potential energy.

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