How Interaction of Perfringolysin O with Membranes Is Controlled by Sterol Structure, Lipid Structure, and Physiological Low pH

INSIGHTS INTO THE ORIGIN OF PERFRINGOLYSIN O-LIPID RAFT INTERACTION

Perfringolysin O (PFO) is a sterol-dependent, pore-forming cytolysin. To understand the molecular basis of PFO membrane interaction, we studied its dependence upon sterol and lipid structure and aequous environment. PFO interacted with diverse sterols, although binding was affected by double bond location in the sterol rings, sterol side chain structure, and sterol polar group structure. Importantly, a sterol structure promoting formation of ordered membrane domains (lipid rafts) was not critical for interaction. PFO membrane interaction was also affected by phospholipid acyl chain structure, being inversely related to tight acyl chain interaction was also affected by phospholipid acyl chain structure, being inversely related to tight acyl chain packing with cholesterol. Experiments using the pre-pore Y181A mutant demonstrated that sterol binding strength and specificity was not affected by whether PFO forms a transmembrane β-barrel. Combined, these observations are consistent with a model in which the strength and specificity of sterol interaction arises from both sterol interactions with domain 4 and sterol chemical activity within membranes. The lipid raft-binding portions of sterol bound to PFO may remain largely exposed to the lipid bilayer. These results place important constraints upon the origin of PFO raft affinity. Additional experiments demonstrated that the structure of membrane-inserted PFO at low and neutral pH was similar as judged by the effect of phospholipid and sterol structure upon PFO properties and membrane interaction. However, low pH enhanced PFO membrane binding, oligomerization, and pore formation. In lipid vesicles mimicking the exofacial (outer) membrane leaflet, PFO-membrane binding was maximal at pH 5.5–6. This is consistent with the hypothesis that PFO function involves acidic vacuoles.

The cholesterol-dependent cytolysins (CDCs) are a family of bacterially secreted pore-forming proteins that require cholesterol to function (1). Perfringolysin O (PFO) is a CDC that contributes to the pathogenesis of the anaerobic Gram-positive bacterium *Clostridium perfringens* (2). While PFO has been presumed to act extracellularly on immune cells (2), it has more recently been shown to be necessary for both phagocytic escape and survival of *C. perfringens* within host macrophages (3).

PFO contains four domains. Secreted as aqueous monomers, PFO recognizes membrane cholesterol through a tryptophan-rich motif within Domain 4 (4, 5). Once associated with the membrane, PFO oligomerizes into complexes of 20–50 subunits (forming a pre-pore structure) (6). In the pre-pore state, the insertion domain (Domain 3) is held ~60 Å above the surface of the membrane by Domain 2 (7). To induce pore formation, Domain 2 undergoes a vertical collapse, which brings Domain 3 within range to insert into the bilayer (8). Additionally, a major structural rearrangement takes place within Domain 3 whereby six α-helices rearrange into two amphipathic β-hairpins that insert into the membrane to form a transmembrane (TM) structure (8). The resulting pore ranges in size from 250–300 Å in diameter (6).

While cholesterol has been presumed to be the cellular receptor for PFO and some other CDCs (5), how PFO binds cholesterol has yet to be fully explained. Studies using model membrane systems typically require considerably high concentrations of cholesterol (up to 50 mol%) in order for efficient pore formation by CDCs (9). Recent studies also show that only the tip of Domain 4 is exposed to the nonpolar core of the bilayer (10, 11). A model in which PFO binds to a membrane surface involving several sterol molecules has recently been proposed (12). Additionally, cholesterol is required for the PFO pre-pore to pore conversion, and has also been shown to be necessary for pore for-
mation by intermedilysin, a related CDC, which does not use cholesterol as a receptor, but requires it for pore formation (6).

CDC proteins are also of interest because they are believed to bind to lipid rafts via their affinity for sterol. Lipid rafts are tightly packed sphingolipid and sterol-rich liquid-ordered (Lo) membrane domains which are believed to co-exist in eukaryotic cellular membranes with loosely packed disordered (Ld) domains composed mostly of unsaturated lipids (for recent reviews see Refs. 13, 14). Rafts are believed to serve many functions in cellular processes at the plasma membrane and have been proposed to serve as platforms that regulate protein-protein interactions (15). While these lipid domains have been highly studied in model membranes, where their existence is widely accepted, their formation and functional role in cells remains controversial. Both intact PFO and isolated Domain 4 have been used as markers of cholesterol-rich regions of cell membranes (4, 16, 17).

The details of PFO-raft affinity are of particular interest because PFO is a TM protein, and the origin of TM protein-raft affinity is not clear. Although biochemical studies detect TM proteins within detergent-resistant membranes that may be derived from ordered domains in cells, TM proteins should not be able to pack well with lipids in an ordered state (18). Because the TM insertion of PFO can be controlled, it is an ideal protein to study this issue. Furthermore, like other CDCs, PFO interaction with membranes is affected by sterol structure (19–24), and the relationship between the raft-forming abilities of sterols (25–27) and sterol interaction with PFO should yield useful information on PFO affinity for rafts.

In this study we found that the interaction of PFO with membranes does not require that the sterol to which it binds has the ability to promote raft formation. Furthermore, tightly packing phospholipids, which interact strongly with sterols, tended to weaken the PFO-membrane interaction. These results do not mean that PFO does not interact with rafts, but, together with the observation that a pre-pore mutant has a similar sterol specificity as wild-type protein, it does place important constraints on the origin of PFO affinity for rafts. In the course of these experiments we also found that a low pH strongly promoted the interaction of PFO with membranes. Combined with recent cellular studies (3), this supports the hypothesis that at least one physiological function of PFO involves low pH.

**EXPERIMENTAL PROCEDURES**

**Materials**—Unlabeled phospholipids, cholesterol, and 1,2-dibromostearoyl-sn-glycero-3-phosphatidylcholine (BrPC) were purchased from Avanti Polar Lipids (Alabaster, AL). 1,2-Dipalmitoyl-N-pyrenesulfonyl phosphatidylethanolamine (pyrene-PE) was purchased from the Molecular Probes Division of Invitrogen (Carlsbad, CA). Lanosterol (97% pure) and β-sitosterol were purchased from Sigma-Aldrich. All other sterols were purchased from Steraloids (Newport, RI). Lipids were stored dissolved in ethanol or chloroform at −20 °C. Concentrations were determined by dry weight (or for pyrene-PE by absorbance using an ε of 35,000 cm⁻¹ M⁻¹ at 350 nm). Streptavidin BODIPY-FL conjugate (BOD-SA) (discontinued except as a custom labeling product) was purchased from Invitrogen and reconstituted with water to give a 100 μM solution. Biocytin (ε-biotinyl-L-lysine) was purchased from AnaSpec (San Jose, CA). TALON bead resin was purchased from Clontech (Mountain View, CA). All other chemicals were reagent grade.

Sterol purity was analyzed on HP-TLC plates (Merck & Co, Whitehouse Station, NJ). Approximately 2 μg of sterol dissolved in ethanol was applied to the plate, dried, and then chromatographed using a sequential solvent system. The first solvent (50:38:3:2 (v/v) chloroform/methanol/acetic acid/water) was allowed to migrate halfway up the plate. The plate was then dried, introduced into a second chamber containing the solvent system 1:1 hexane/ethyl acetate (v/v), and chromatographed until the solvent migrated to near the top of the plate. For each step, solvent chambers were equilibrated with solvents for at least 2 h before chromatography. The plate was then dried and sprayed with 5% (w/v) cupric acetate, 8% (v/v) phosphoric acid in water. To detect sterol, plates were charred at 180 °C for 5 min. Sterols deemed impure (zymosterol and desmosterol) were purified by TLC (28), and purity was confirmed by HP-TLC.

A functional cysteine-less derivative of wild-type PFO (PFO C459A) and a pre-pore mutant (PFO C459A Y181A, gift of A. Heuck, University of Massachusetts, Amherst) were expressed in *Escherichia coli* as described previously (29). Both WT and pre-pore PFO were then purified by a modification of the previously reported protocol (29). Three hours after induction of expression with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG), two liters of cultured *E. coli* expressing PFO were pelleted at 4 °C. The bacteria were resuspended in NiA buffer (10 mM MES, 150 mM NaCl, pH 6.5) containing 150 μg/ml phenylmethylsulfonyl fluoride and 100 μg/ml chicken egg white lysozyme (Sigma-Aldrich), incubated for 30 min at room temperature, subjected to tip sonication with a cell disruptor (Heat Systems, Ultrasound, Inc, Plainview, NY) for 15 s while cooled on ice, and then cooled a further 15 s. The sonication and cooling steps were repeated two times. Next, the mixture was spun down at 15,000 rpm in a SS-34 rotor at 4 °C using a Dupont RC-5 centrifuge. The supernatant from this step was incubated for 20 min. at room temperature while mixing with TALON metal affinity resin (3 ml). Resin was pelleted with a tabletop centrifuge, added to a 0.8 × 4 cm poly-prep plastic column (BioRad, Hercules, CA), washed with about 5 ml of NiA buffer followed by a 1-ml aliquot of NiA buffer containing 50 mM, and then washed with a 1-ml aliquot of NiA buffer containing 100 mM imidazole. The PFO was then eluted with several 1-ml aliquots containing NiA buffer with 400 mM imidazole. Fractions containing PFO were pooled and dialyzed overnight against 4 liters of Buffer B (10 mM MES, 1 mM EDTA, pH 6.5) with one buffer change. The pooled PFO-containing fractions were then subjected to gravity anion-exchange chromatography using SP-Sephadex resin (GE Healthcare, Piscataway, NJ) in a 0.8 × 4 cm poly-prep plastic column and stepwise eluted using 1-ml aliquots of Buffer B containing increasing concentrations of NaCl in 100 mM steps, with duplicate aliquots at 300 mM and 400 mM NaCl. The majority of purified PFO eluted in Buffer B containing 300–400 mM NaCl. It was then dialyzed against PBS, pH 7.4 (10 mM sodium phosphate, 1 mM potassium phosphate, 137 mM...
sodium chloride, 13 mM potassium chloride), and stored at −20 °C.

Preparation of Liposomes—Multilamellar vesicles (MLV) were prepared at a concentration of 500 μM lipid in PBS, pH 7.4 or 5.1 (PBS at pH < 7.4 being prepared by titrating PBS pH 7.4 with acetic acid) similarly as described previously (30). Dried lipid mixtures (redissolved in CHCl₃ and redried under N₂ and with acetic acid) similarly as described previously (30). Dried in an Eppendorf centrifuge model 5415C (Westbury, NY) for 20 min at room temperature. Samples were spun down at 14,000 rpm and dried for 3 h at 70 °C in a Savant slab gel dryer (Holbrook, NY), stained with 0.2% (w/v) Coomassie Blue dissolved in 30% (v/v) methanol, 10% (v/v) acetic acid for 2 h, and then destained for 30 min to 1 h in the fixing solution.

Assay for Pore Formation—PFO-induced pore formation was measured by assaying the efflux of vesicle-entrapped biocytin via the increase in the BODIPY fluorescence emission intensity upon binding of biocytin to BODIPY-labeled streptavidin (BOD-SA) in the external solution. LUVs with trapped biocytin were prepared by freezing and thawing MLVs (10 mM lipid), as described above, in the presence of 537 μM biocytin. The mixture was dialyzed against 4 liters of PBS overnight with one change of dialysis buffer to remove external biocytin. 10 μl of LUVs with entrapped biocytin were diluted to a lipid concentration of 100 μM and a volume of 990 μl with PBS and then BOD-SA (10 μl from the stock solution) was added externally to vesicles to give a BOD-SA concentration of 10 nM. BODIPY emission intensity was then measured. PFO was added to a concentration of 5 μg/ml, samples were briefly mixed, and then BODIPY intensity was monitored as a function of time for up to 45 min.

RESULTS

PFO Interacts with Membranes at Both Low and Neutral pH—Because low pH-induced unfolding often aids protein toxin insertion into membranes, we compared the behavior of PFO at low and neutral pH. First, the interaction between (Cys-less) PFO and model membrane vesicles was measured. The removal of the Cys eliminates the sensitivity of PFO to spontaneous inactivation by oxidation (32).) Previous studies have shown that the interaction of PFO with membranes can be detected by the large increase in Domain 4 Trp emission intensity that accompanies association with membranes (31). A similar (4-fold) increase in Trp emission intensity relative to that in aqueous solution is observed when PFO is incubated with vesicles at pH 7.4 and pH 5.1, suggesting PFO interacts with membranes in a similar fashion at low and neutral pH (Fig. 1). Notice that there is a small red shift in the emission spectrum at low pH in aqueous solution relative to that at neutral pH. This is consistent with an increased Trp exposure to a polar environment, e.g. aqueous solution, at low pH, and suggests that there is a small unfolding event at low pH. In the presence of lipid vesicles, this red shift is not observed, and spectra at low and neutral pH are nearly identical.

The kinetics of PFO interaction with vesicles at neutral and low pH were also compared. Measurements of the time dependence of the increase in emission intensity upon incubation of PFO with vesicles demonstrates that PFO-membrane binding occurs faster at pH 5.1 (t½ = 1.5 min) than at pH 7.4 (t½ = 6.5 min) (data not shown). A similar difference in the rate of interaction at pH 5.1 and 7.4, is observed at 37 °C, although the half-times for membrane interaction decrease by a factor of about two relative to those at room temperature and the increase in fluorescence is about 3-fold (data not shown).
PFO-Membrane Interaction

**PFO-Vesicle Interactions at Low pH Occur at Lower Cholesterol Concentrations Than at Neutral pH**—The above results suggest that low pH might enhance the interaction PFO with membranes. To examine this, the interaction of PFO with vesicles containing various amounts of cholesterol was compared at low and neutral pH. Fig. 2A shows that the cholesterol concentration that induces PFO interaction with DOPC/cholesterol vesicles is less at low pH than at neutral pH, with the increase in fluorescence emission intensity being half-maximal at 15–20 mol% cholesterol at pH 5.1 (○) and 25–30 mol% cholesterol at pH 7.4 (●).

It is possible binding to vesicles might occur without an increase in Trp fluorescence emission intensity. To confirm that the increase in Trp fluorescence emission intensity accurately reports when binding to vesicles occurs, more direct methods were used. First, a pyrene-labeled lipid was used as a fluorescence resonance energy transfer (FRET) acceptor for Trp, and binding as a function of cholesterol concentration assayed via the amount of FRET, as detected by quenching of Trp fluorescence emission intensity. Fig. 2B shows that the cholesterol concentration dependence of Trp fluorescence quenching is very similar to the cholesterol dependence of the Trp intensity increase in the absence of acceptor, with lipid interaction occurring at a lower cholesterol concentration at low pH than at neutral pH. As commonly observed (33), FRET-induced quenching is incomplete because not all of the donors are close enough to the pyrene-labeled lipid to take part in energy transfer. Thus, the maximal level of FRET-induced quenching, 80%, presumably represents complete binding of PFO to the vesicles. It should also be noted that the small amount of apparent FRET at low cholesterol concentration is largely an inner filter artifact arising from a small amount of pyrene absorbance.

These results were further confirmed by measuring the association of PFO with vesicles via centrifugation of PFO mixed with MLV. The amount of bound PFO in the MLV-containing pellet was detected by agarose gel electrophoresis in SDS (SDS-AGE). The cholesterol concentration dependence of PFO binding detected by sedimentation (Fig. 2C) is similar to that obtained from fluorescence intensity measurements in terms of the threshold sterol concentration for binding PFO and its pH dependence. The position of the main PFO band on the gels indicates that when membrane-bound, PFO efficiently forms characteristic SDS-resistant oligomers (29) at both pH values (although a variable amount of monomers can be often observed).

**PFO Forms Pores Efficiently at Low pH**—While the experiments above show that PFO exhibits similar binding and oligomerization behavior at low and neutral pH, under some circumstances PFO can form pre-pore oligomers that do not deeply membrane-insert (29). We therefore investigated whether PFO pore-forming behavior is retained at low pH. To assay pore formation, we measured the efflux of biocytin encapsulated inside LUVs. In this method, efflux is detected by the increase in the BODIPY emission intensity that occurs when biocytin binds to BODIPY-tagged streptavidin added externally to the vesicles (34–36).

Fig. 3 shows that PFO forms pores efficiently in DOPC vesicles containing 50 mol% cholesterol at both pH 5.1 and 7.4, with the rate of biocytin efflux being slightly faster at low pH. The difference between neutral and low pH is even larger at lower cholesterol concentrations (data not shown), presumably because PFO binds to a greater extent at low pH than at neutral pH. Fig. 3 also shows no pore formation occurred in the absence of cholesterol.

A control experiment using a previously identified mutant (PFO C459A/Y181A) that remains in the pre-pore state (37), shows a lack of pore formation at both low and neutral pH (Fig. 3, A and B). This confirms the validity of the pore-formation assay, and shows that the difference in pre-pore mutant and wild-type PFO behavior is retained at low pH. We conclude that the structure and membrane interactions of PFO at low and neutral pH must be very similar, although low pH enhances PFO membrane interaction and function.

**Effect of Sterol Structure upon PFO-Membrane Interaction: Fluorescence Studies**—It has been proposed that PFO binds to cholesterol-enriched ordered domains (lipid rafts) (16). Prior studies of sterol specificity have shown that sterol structure is important for interaction with PFO, but have not established whether or not PFO interacts most strongly with sterols promoting lipid raft formation (21). To investigate this, the interactions of PFO with sterols and sterol derivatives that either strongly stabilize ordered domain formation (cholesterol, dihydrocholesterol, epicholesterol, lathosterol, sitosterol (25–28)), weakly stabilize or have little effect of the stability of ordered domain formation (zymostenol, lanosterol, cholesteryl acetate, cholesterol methyl ether, allocholesterol (25–28)) or destabilize ordered domain formation (coprostanol (27)) were compared.
The binding of PFO to vesicles as a function of the concentration of sterol or sterol derivative within the vesicles was detected by sterol-induced increases in Trp fluorescence (Fig. 4). At low pH (Fig. 4A), PFO interacts well or moderately well with sterols that strongly promote lipid-ordered domain formation (cholesterol, dihydrocholesterol, sitosterol, lathosterol), weakly stabilize ordered domain formation (desmosterol, zymostenol, allocholesterol), or do not promote raft formation.

![FIGURE 2. PFO interaction with model membranes occurs at lower cholesterol concentrations at low pH. A, effect of pH upon Trp emission intensity versus cholesterol concentration. (●) DOPC/cholesterol vesicles at pH 7.4; (○) DOPC/cholesterol vesicles at pH 5.1. Trp emission intensity was normalized to a value of 1 in the absence of sterol in this and the following figures. B, effect of pH upon FRET-detected binding of PFO to vesicles versus cholesterol concentration. F/Fo is the ratio of Trp fluorescence intensity in the presence of vesicles containing pyrene-PE (F) to that in the presence of vesicles without pyrene-PE (Fo). Samples contained a total of 100 μM unlabeled lipid (DOPC and cholesterol) with or without 5 μM pyrene-PE. The x-axis gives the mol% of cholesterol in the samples without pyrene-PE. (●) DOPC/cholesterol vesicles pH 6.8; (○) DOPC/cholesterol vesicles at pH 5.1. C, effect of pH upon centrifugation/SDS-AGE-detected binding of PFO to vesicles versus cholesterol concentration. Samples of 50 μg/ml Cys-less PFO (C459A) were incubated with multilamellar vesicles (500 μM lipid) containing mixtures of cholesterol with DOPC and 10 mol% BrPC to aid pelleting. The pellet obtained after centrifugation was analyzed on SDS-AGE. Numbers above lanes show mol% cholesterol. Labels at left indicate migration position of: o, oligomers; m, monomers.]

![FIGURE 3. Formation of pores by PFO in LUV at neutral and low pH. A, pH 7.4. B, pH 5.1. Y-axis shows the increase in external BODIPY-SA emission intensity upon the release of trapped biocytin. Key: (open symbols) DOPC; (filled symbols) 1:1 mol/mol DOPC/cholesterol; (■) Cys-less PFO (C459A); (▲) Cys-less pre-pore mutant (Y181A/C459A). Samples contained 100 μM lipid and 10 nM BODIPY-tagged streptavidin added externally to LUV containing entrapped biocytin. BODIPY fluorescence was measured as a function of time after the addition of 5 μg/ml of PFO. Zero time is the time of addition of PFO. Pore formation experiments were carried out multiple times. Representative results in which experiments on Cys-less and Cys-less pre-pore mutants were carried out on the same day, and using the same preparation of lipid vesicles are shown.]

The binding of PFO to vesicles as a function of the concentration of sterol or sterol derivative within the vesicles was detected by sterol-induced increases in Trp fluorescence (Fig. 4). At low pH (Fig. 4A), PFO interacts well or moderately well with sterols that strongly promote lipid-ordered domain formation (cholesterol, dihydrocholesterol, sitosterol, lathosterol), weakly stabilize ordered domain formation (desmosterol, zymostenol, allocholesterol), or do not promote raft formation.
formation (coprostanol). The interaction with coprostanol and zymostenol requires somewhat higher sterol concentrations than is required for the other sterols. PFO does not interact or interacts very poorly with epicholesterol, which stabilizes ordered domains to a significant degree (27), or with lanosterol and sterol derivatives with a blocked 3\(-\)OH (cholesteryl methyl ether, cholesteryl acetate) that have little effect on ordered domain stability. This shows that PFO binding is not tightly correlated with the relative ability of sterols or sterol derivatives to form ordered domains.

The relative sterol specificity of PFO is similar at neutral and low pH. However, the dependence upon sterol concentration is shifted, such that much higher sterol concentrations are required to induce an increase in Trp emission intensity at neutral pH than at low pH (Fig. 4B).

Effect of Sterol Structure upon PFO-Membrane Interactions: Centrifugation Experiments—It is possible that the apparent dependence of PFO binding to membranes upon sterol structure is not due to a lack of PFO interaction with membranes, but rather to an inability of a particular sterol to induce a conformational change that alters Trp fluorescence emission intensity. To examine this possibility, the binding of PFO to membranes and the oligomeric state of the membrane-bound PFO was determined using centrifugation and SDS-AGE. Fig. 5 shows that at low pH there is near maximal PFO binding to vesicles containing cholesterol, dihydrocholesterol, sitosterol, or lathosterol at 20 mol% sterol, and some binding to vesicles with allocholesterol at 20 mol%. However, binding to vesicles containing coprostanol or zymostenol requires 30 mol% sterol, and no binding to vesicles occurs even with 40 mol% lanosterol, cholesteryl acetate or cholesterol methyl ether. This order of sterol recognition by PFO mirrors that derived from Trp fluorescence emission intensity (Fig. 4).

In every case, the bound PFO is predominantly oligomeric (Fig. 5). It therefore appears that sterol structure does not greatly affect the ability of membrane-associated PFO to oligomerize. However, it should be noted that in several cases, there is some smearing of the oligomers on the gel at the highest sterol concentrations. The origin of this behavior is not understood.

We have also tested two additional sterols, ergosterol and 7-dehydrocholesterol, and found that they promote PFO binding to liposomes. However, this interaction was difficult to quantify because we found these sterols quench Trp fluorescence emission intensity (28), thereby masking the emission intensity increase usually observed when PFO binds to membranes. SDS-AGE showed that PFO binding and oligomer formation with liposomes containing 20 mol% of these sterols was as complete as for liposomes containing 20 mol% cholesterol (data not shown).

The sterol specificity of pre-pore PFO Y181A mutant, which cannot form a TM \(\beta\)-barrel (37) was also examined. It shows a sterol specificity profile at low pH (Fig. 6) that is almost identi-
cal to that of the Cys-less wild type PFO (Fig. 4A) as judged by the dependence of Trp emission intensity upon sterol or sterol derivative concentration within vesicles. Therefore, the step that is sensitive to sterol structure appears to be the initial recognition and binding of the membrane surface by PFO. Once bound, PFO can spontaneously form pre-pore complexes.

**Effect of Sterol Structure upon Pore Formation by PFO**—To determine if pore formation by PFO is also sensitive to sterol identity, DOPC vesicles encapsulating biocytin and prepared with different sterols, were exposed to PFO. Pore formation is observed at low pH with cholesterol, dihydrocholesterol (which is strongly raft promoting), desmosterol (which is weakly raft stabilizing) and coprostanol (which destabilizes rafts) (Fig. 7). Therefore, the raft-stabilizing abilities of a sterol are not tightly correlated with its ability to support PFO-induced pore formation. Vesicles containing DOPC mixed with 40 mol% of allocholesterol or lathosterol also show a significant degree of pore formation, but no pore formation was seen in vesicles containing DOPC and 40 mol% lanosterol (data not shown). The rate of pore formation is greater at 40 mol% for each sterol (Fig. 7B). In agreement with the binding experiments, samples with 25 mol% coprostanol, which is an insufficient concentration to promote maximal PFO binding, show a significantly reduced rate and extent of pore formation as judged by the rate of biocytin release when compared with samples containing other sterols, which promote near-maximal PFO-membrane interactions at 25 mol% for each sterol (Fig. 7B). Overall, the sterol dependence of pore formation by Cys-less PFO correlates with the level of its association with vesicles.

**Effect of Phospholipid Structure on PFO-Membrane Interaction**—To assess whether PFO-membrane interactions would be affected by the relative ability of phospholipids to form ordered domains, four phosphatidylcholines with differing abilities to form ordered domains by themselves and with cholesterol were examined. These four, listed in decreasing order of ability to form ordered domains and pack tightly with cholesterol, were (38): DPPC, which has two saturated palmitoyl acyl chains; POPC, which has a 1-position palmitoyl acyl chain and a 2-position unsaturated oleoyl acyl chain; DOPC, which has two oleoyl acyl chains; and diphytanoyl PC (DPhPC), which has two multibranch acyl chains. As judged by the cholesterol-induced increase in Trp emission intensity at low pH (Fig. 8), the cholesterol concentration needed to induce PFO binding to vesicles increases with PC type in the order: DPhPC < DOPC <
POPC/DPPC. This pattern indicates that PFO binds better to membranes that are loosely packed and have the least tendency to form ordered domains. This does not imply that PFO does not associate with lipid rafts, but does indicate that loose packing, which should increase cholesterol reactivity, promotes sterol binding to PFO (see “Discussion”).

PFO interactions with vesicles in which 50 mol% dioleoyl phosphatidylethanolamine (DOPE), or 10–30 mol% diphytanoyl PE, or 5–20 mol% palmitoyl (C16:0) ceramide were substituted for an equal mol% of DOPC were also examined. In all of these cases, there is a decrease in the % cholesterol need to induce PFO association with membranes (data not shown). These results are also consistent with a model in which cholesterol reactivity in membranes is an important parameter controlling association with PFO (see “Discussion”).

Dependence of PFO Interactions with Vesicles on pH: Physiological Implications—To ascertain how membrane composition affects the pH dependence of PFO-membrane interactions, vesicles were prepared with various phospholipids and cholesterol concentrations. The pH dependence of Trp fluorescence was then measured to identify the pH at which membrane interaction was maximal. Fig. 9A shows that PFO binding to vesicles containing POPC/cholesterol (7:3, mol/mol), DOPC/cholesterol (4:1), or DPhPC/cholesterol (17:3) is maximal over a broad low pH plateau. To better define the likely pH maximum under physiological conditions, the pH dependence of PFO-membrane interaction was then measured in vesicles containing a 1:1:1 molar ratio of sphingomyelin (SM): POPC/cholesterol, a mixture which mimics the outer (exofacial) leaflet of mammalian plasma membranes. Fig. 9B shows the binding of PFO to these vesicles has a somewhat sharper pH maximum near pH 5.5–6. Fig. 9B also shows that in SM/POPC/cholesterol vesicles in which cholesterol concentration is decreased to 25 mol%, there is an even sharper pH maximum of membrane interaction at just below pH 6. These results are consistent with the hypothesis that PFO functions in macrophage phagosomes, as phagosomes have a luminal pH between 5 and 6 (39) (see “Discussion”).

Negatively Charged Lipid Enhances Binding of PFO to Vesicles—Rossjohn et al. (40) very recently observed conformational changes in the PFO crystal structure at low pH, and suggested that these changes might aid PFO insertion into membranes at neutral pH when PFO encounters a membrane rich in anionic lipids, because the surface of anionic lipid vesicles have a lower local pH than that of the bulk aqueous solution. To determine if anionic lipid promotes PFO-membrane interactions, the binding of PFO to vesicles containing POPC and cholesterol with and without 20 mol% of the anionic lipid 1-palmitoyl-2-oleoyl phosphatidyl-l-serine (POPS) was compared (Fig. 10). At neutral pH, PFO interacts with vesicles at a slightly lower cholesterol concentration in the presence of POPS (▲) than in its absence (△). However, at low pH (5.1) the presence of POPS (■) results in an even larger decrease in the mol% of cholesterol required for PFO binding to vesicles. Very similar
Trp are more exposed to the aqueous environment at low pH, emission intensity in the absence of lipid indicate that the PFO addition, the low pH-triggered changes in Trp fluorescence conformational changes in Domains 2 and 3 prime PFO for crystallographic studies have proposed that low pH-induced for insertion (43, 44). This may also be the case for PFO. Recent protein and thereby primes the membrane-penetrating sequences for association with umbrella-forming lipids, as has been observed in lipid rafts (30, 47).

A result consistent with some degree of unfolding (45). It should be noted that the unfolding event that occurs at low pH is likely to be local. We were unable to induce PFO insertion into model membrane vesicles using conditions that induce more global unfolding, i.e. high temperature or urea (data not shown).

Effect of Phospholipid Structure upon PFO-Membrane Interactions: Implications for Sterol Binding—Another striking result was that PFO interactions with sterol are inversely related to the packing properties of the phospholipids. Specifically, the looser the packing of the phospholipids (38), the lower the concentration of cholesterol needed to induce insertion of PFO into the lipid bilayer. This behavior can be explained in terms of the effect of loose packing upon cholesterol chemical reactivity. The reactivity of membrane-associated cholesterol (as judged by its activity coefficient) should be increased by exposure to aqueous solution. The umbrella model postulates that the headgroups of phospholipids and sphingolipids act like umbrellas, limiting the exposure of the hydrophobic portions of cholesterol to water (cholesterol having too small a polar headgroup to fully shield itself from aqueous solution) and thus reducing its reactivity (46). Acyl chain and headgroup structures that limit the ability of cholesterol to pack closely with phospholipids should limit this shielding of cholesterol from water, thereby increasing cholesterol reactivity and thus its tendency to bind to other molecules. This effect can be very marked, and has been successfully invoked to explain how lipid structure can modulate cholesterol interaction with lipid rafts and with other toxins (30, 47).

It is also significant that we have identified conditions in which only relatively low concentrations of sterols (as low as 10–15 mol%) are required for PFO binding membranes. Studies involving PFO and model membrane systems have typically used liposomal formulations requiring very high cholesterol concentrations (about 50 mol%) to achieve efficient binding, oligomerization, and pore formation (9). Our study shows that there is no absolute requirement for a very high concentration of cholesterol. This result has practical importance because it will allow study of PFO-membrane interactions over a much wider range of in vitro lipid compositions.

Lipid polar headgroup structure also affected PFO-membrane interactions. Our results showed that anionic lipids can promote PFO binding to membranes. The anionic charges near the surface may redistribute the lipid components in the bilayer to alter cholesterol exposure, alter the local pH at the membrane surface, and/or interact with PFO directly or indirectly to stabilize its binding to the membrane surface. We also found that PE and ceramide decreased the % cholesterol needed to induce PFO binding to vesicles. This agrees with previous studies of PFO (48) and that of another cytolysin (47) and can also be rationalized in terms of the umbrella model. The headgroup of PE is smaller than that of PC so should be less able to shield cholesterol from water, thereby increasing cholesterol reactivity. Similarly, as pointed out by Zitter et al. (47), ceramide has such a small headgroup it can even compete with cholesterol for association with umbrella-forming lipids, as has been observed in lipid rafts (30, 47).
**Effect of Sterol Structure upon PFO-Membrane Interactions: Implications for the Nature of the Sterol Binding Site**—Another conclusion from this study is that PFO-sterol interactions show a distinct specificity in terms of sterol structure. The structure of the polar headgroup, sterol rings and aliphatic side chains all affect how much sterol is needed to induce PFO membrane binding, oligomerization, and pore formation. The most critical feature is the OH group. In agreement with previous studies (21), our study confirms that PFO requires a free OH group in the β-OH configuration to recognize and interact with the sterol. The sterol ring structure is also important. A 5–6 double bond (cholesterol) favors PFO binding more than a double bond in the 4–5 (allocholesterol), 7–8 (lathosterol), or 8–9 (zymostenol) positions. Ring system planarity also has a significant effect, with the relatively flat dihydrocholesterol interacting with PFO better than coprostanol, an isomer of dihydrocholesterol that is highly bent between the steroid A and B rings. The methyl groups on the sterol rings, found in lanosterol, strongly interfere with PFO interactions, although this may also be partially due to the 8–9 double bond that it has in common with zymostenol. (It should be noted that the weak interaction of PFO with lanosterol is consistent with previous studies on ostreolysin (19).) Even aliphatic side chain structure had some effect, as shown by the slightly weaker interactions of PFO with sitosterol (which has a C24 ethyl group) than with cholesterol.

It would appear from these results that PFO recognizes groups all along the sterol molecule. This would be consistent with the presence of a sterol-binding pocket almost totally surrounded by residues in Domain 4 (49). On the other hand, only the tip of Domain 4 at one end of the elongated PFO molecule is embedded in the bilayer, and this is sufficient for cholesterol recognition and binding (7, 10). Because different sterols will occupy different steric spaces and hence will pack differently within the bilayer, bilayer surfaces will be created that differ in the exposure of the sterols, including the portions most likely to directly interact with PFO, to the aqueous solution. A bilayer-inserted sterol that is more exposed to aqueous solution will be more exposed to PFO in solution and thus interact more readily with PFO than one that is less exposed to solution. In this fashion the steric configurations of the hydrophobic portions of the various sterols would be expected to indirectly dictate the extent to which PFO recognizes the sterol molecule, even when the sterol is not totally buried within the protein. Defining the exact molecular origin of the sterol specificity of interactions with PFO will require further studies.

**Effect of Lipid Structure upon PFO-Membrane Interaction: Implications for PFO Interaction with Lipid Rafts**—It may seem puzzling that the sterol specificity of PFO binding to membranes does not support a model in which there is a close correlation between the raft (ordered domain) stabilizing abilities of a sterol (25–28, 50) and PFO binding. Several sterols that stabilize ordered domain formation (cholesterol, dihydrocholesterol, sitosterol, lathosterol (25, 26, 28)) interact well with PFO, but epicholesterol, which also stabilizes ordered domains (27), does not, and coprostanol, which destabilizes ordered domains (27), does.

However, if sterol binding enhances PFO interaction with rafts, an obvious mechanism would be that the raft-associating surfaces of the sterol remain exposed to the lipid bilayer upon binding PFO, and thus not interact with PFO. This would be analogous to the familiar mechanism by which binding to the headgroup of ganglioside GM1 anchors cholera toxin in rafts (51). A sterol bound in a deep cleft within the protein could not directly aid raft association in this way. Thus, one might not expect PFO to have any strong preference for sterols that form lipid rafts.

Furthermore, we have shown that PFO insertion is triggered more readily in a loosely packed lipid environment. This behavior also does not imply that PFO would have a higher affinity for disordered lipid domains than for lipid raft domains. It is possible that PFO could insert into disordered domains and then move into ordered domains subsequent to insertion. Furthermore, in membranes with co-existing disordered and ordered domains it is likely that the cholesterol concentration would be higher in the ordered domains (52, 53), and this would tend to cancel out the preference of PFO for cholesterol in a loosely packed environment. Also, it should be kept in mind that the ordered domains in cells would be more complex than in our binary lipid mixtures, and contain some unsaturated lipids that might increase PFO affinity for ordered domains. Indeed, our preliminary studies indicate that in membranes with co-existing ordered and disordered domains, PFO does have a tendency to partition into ordered domains to a significant degree.4

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**PFO-Membrane Interaction**

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