Current Topics

β-Barrel Pore-Forming Toxins: Intriguing Dimorphic Proteins†

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Many bacteria secrete polypeptides with intrinsic properties that generate a remarkably wide range of stable structural states. Specifically, these polypeptides can exist either as a water-soluble monomer or as a multimeric integral membrane protein. Furthermore, the ability to convert from a stable folded state in water to a different stable folded state inside a membrane is also an intrinsic property of the polypeptide. This conversion is spontaneous, and though it is triggered by an interaction with a specific protein, lipid, and/or carbohydrate at the membrane surface, the insertion of the polypeptide into the membrane proceeds without the assistance of other proteins as chaperones or energy transducers. Protein biochemists, protein folders, structural biologists, and others are therefore intrigued by the structural dimorphism of these polypeptides, while clinicians, microbiologists, and others are focused on the functional ramifications of these interesting structural properties. For these proteins are bacterial pore-forming toxins, and their purpose is to damage or infiltrate mammalian cells and interfere with their function.

The insertion of proteins into membranes is, in nearly all cases, a highly regulated process that involves multiple proteins besides the polypeptide being inserted. For example, membrane protein integration into the membrane of the endoplasmic reticulum (ER) in eukaryotic cells usually requires complex molecular machinery (termed the translocon) and a series of coordinated ribosome and translocon interactions and movements to accomplish integration (*1*). Similar protein-mediated processes effect the insertion of membrane proteins into mitochondrial membranes (*2*), chloroplast membranes (*3*, *4*), and bacterial membranes (*3*, *5*). These processes also usually require a chaperone(s) or mechanism (e.g., translational arrest by eukaryotic signal recognition particle) to maintain the polypeptide in an insertion-competent state (*1*−*5*).

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However, there is a class of proteins that insert spontaneously into membranes without the assistance of other polypeptides. The most striking examples of such proteins are the pore-forming toxins (PFTs), the above-mentioned proteins that are secreted from bacterial cells, fold into a stable water-soluble intermediate state, and ultimately convert spontaneously into a membrane-inserted conformation that punctures the membrane of the target cell. In most cases, if not all, multiple copies of a protein are required to form a pore. Thus, normal PFT function involves protein-membrane interactions, protein−protein interactions, and an unusual protein folding pathway that involves a controlled transition from a water-soluble conformation to a membrane-inserted structure.

PFTs therefore offer a unique opportunity to explore a wide range of significant structural and mechanistic issues that are also likely to be important in other non-PFT processes. These include the following: how does a PFT recognize and bind to a specific membrane(s)? How does the presence of the appropriate target membrane trigger the conversion from a soluble, monomeric folded state to a membrane-inserted, oligomeric folded state? Does oligomerization occur prior to or following membrane insertion? How are residues of the PFT that interact with the hydrophobic core of the membrane bilayer prevented from premature exposure? What drives the substantial PFT structural changes that accompany pore formation?

PFT Classification. PFTs have been isolated from a wide variety of bacteria. A PFT is classified as an α-PFT if it forms pores by the insertion of amphipathic α-helices (e.g., channel forming colicins; for a recent review see refs *6*) or as a β-PFT if it forms pores by the insertion of amphipathic β-hairpins into the membrane to create a β-barrel [e.g., *Staphylococcus aureus* α-hemolysin (α-HL) (*7*)]. Two different types of β-PFTs have been described to date. One type forms pores and disrupts cellular membranes, thereby causing leakage and lysis of the target cell. Another type of β-PFT belongs to the family of A−B toxins. These proteins also form pores in membranes, but for the purpose of delivering a catalytic functional domain into the cytosol of the target cell. The A−B toxins have at least two functional domains, the A domain which carries the enzymatic activity [e.g., *B.*

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anthracis lethal factor (LF) or edema factor (EF)], and the B domain which binds to the membrane and effects translocation of the A domain across the membrane, in some cases by forming a transmembrane (TM) β-barrel [e.g., anthrax protective antigen (PA)]. Whereas the former type of β-PFT acts directly on the plasma membrane of cells [or in the case of listeriolysin O, on an internal vacuolar membrane (*8*)], the A−B toxin complexes are internalized by receptor-mediated endocytosis and exposed to the acidic pH inside the endosome that triggers membrane insertion of the B domain and translocation of the A domain into the cytosol [e.g., see (*9*−*11*)].

TM β-barrels are also often found in proteins located in the outer membranes of bacteria (*12*). These β-barrels may contain as few as eight (OmpA and OmpX) or as many as 22 (FhuA and FepA) β-strands. However, these β-barrels differ markedly from those formed by β−PFTs because OmpA et al. are formed by a single polypeptide chain, while PFT β-barrels are formed by β-strands contributed by several protein monomers. Membrane proteins involved in the translocation of polypeptides through the mitochondrial and plastid outer membranes are also presumed to form β-barrel pores, but direct experimental evidence has yet to be obtained (*13*). Interestingly, it has recently been reported that streptolysin O (SLO), a β-PFT, forms part of the secretion machinery in Gram-positive bacteria (*14*). It remains to be seen whether this is a common functional role for β-PFTs homologous to SLO.

Bacterial PFTs that utilize β-barrels during pore formation represent a growing family of proteins involved in bacterial pathogenesis, and several reviews on individual β-PFTs have been published (e.g., refs *15*−*17*). Most β-PFT pores are formed by heptameric oligomers and are 15−35 Å in diameter [e.g., α-HL and anthrax PA (*18*, *19*)]. In contrast, cholesterol-dependent cytolysins (CDCs) form pores as large as 300 Å in diameter by the oligomerization of 40−50 monomers (*20*, *21*). Here we wish to summarize the current status of various structural and mechanistic features of β-PFTs, as well as consider some experimental approaches for examining such proteins. We will focus primarily on data obtained with those toxins whose structures are best known (i.e., α-HL, perfringolysin O, and anthrax PA).

Signature of a β-PFT: An Oligomeric TM β-Barrel. A breakthrough in our understanding of pore formation by β-PFTs was provided by the determination of the crystal structure of the oligomeric complex of *S. aureus* α-HL (*7*). With a shape resembling that of a mushroom, the α-HL heptamer measures approximately 100 Å in height and up to 100 Å in diameter. A solvent-filled channel parallels the 7-fold axis and ranges from ∼15 to ∼46 Å in diameter. The stem domain, a 14-strand antiparallel β-barrel, constitutes the TM pore. The cap domain protrudes from the extracellular surface to form a large hydrophilic domain, while the seven rim domains define the underside of the cap where it interfaces with the outer leaflet of the cell membrane (*7*) (Figure 1).

The crystal structure of the α-HL oligomer was extremely important because it revealed the structural basis for the formation of an aqueous pore through the phospholipid bilayer: a β-barrel that spanned the membrane and that was created by β-hairpins contributed by seven α-HL polypeptides. As the first crystal structure of a β-PFT oligomer, this

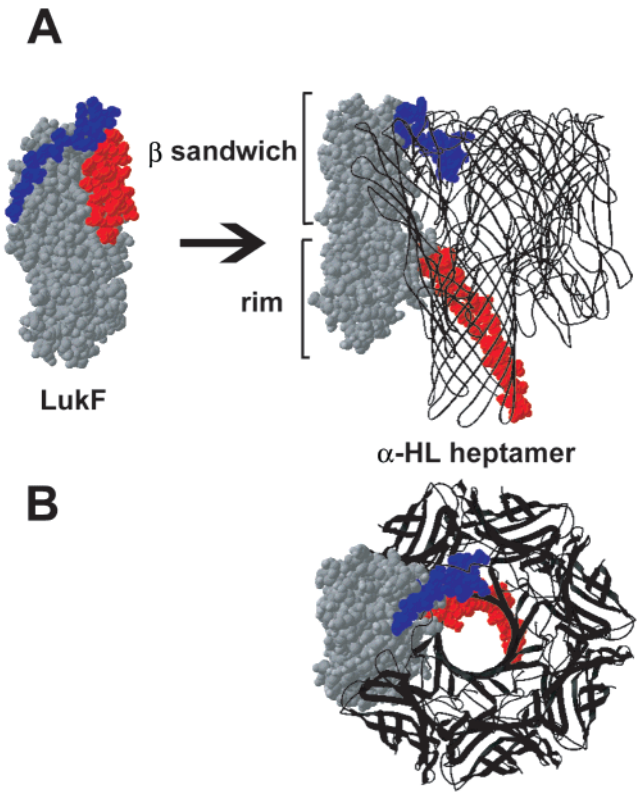

FIGURE 1: Conformational changes associated with the transition of α-HL from a water-soluble monomer (represented by the LukF structure) to the membrane-inserted heptamer. (A) Space-filling representation of the monomeric structure of LukF (left) and of one α-HL monomer in the oligomer (right). The rest of the protomers in the heptameric complex are shown using a ribbon representation. The β-sandwich domain and the rim domain of one monomer, as defined in ref *7*, are indicated. The amino-latch (blue) and the TMH (red) are shown in both the LukF monomer and the α-HL heptamer. (B) Top view of the heptameric oligomer represented in panel A.

structure established the paradigm that has guided subsequent thinking about β-PFT structure and function.

Identification of Proteins as β-PFTs. For many putative β-PFTs that appear to oligomerize as heptameric complexes (e.g., refs *18* and *19*), the adoption of the α-HL oligomeric structure as a paradigm for those β-PFTs seems reasonable. For example, the unrefined crystallographic structure of a water-soluble anthrax PA heptamer is available at 4.5 Å resolution (*19*) and it has a shape similar to the heptameric oligomer of α-HL. The structure showed a hollow ring, 160 Å in diameter and 85 Å high, with a central cavity about 35 Å in diameter.

Whereas the X-ray crystal structures for the water-soluble form of several β-PFTs have been solved (e.g., refs *19* and *22*−*24*), only one high-resolution structure for an oligomeric complex is available to date (*7*). This scarcity of high resolution structural information for membrane-inserted oligomers is not surprising because obtaining crystals of membrane proteins is rather difficult and also because the size of the oligomers exceeds what can currently be analyzed successfully using NMR. Given the difficulties associated with membrane protein structural analysis by crystallography and NMR, other experimental approaches have been required to obtain residue-specific structural information about β-PFTs inserted into membranes.

Benson et al. (*25*) employed conductivity measurements to determine whether the anthrax PA $2\beta_2$-$2\beta_3$ loop (residues 298−327) in domain 2 is located at the interface between the lipid core of the membrane and the aqueous pore. In this approach, individual residues in the $2\beta_2$-$2\beta_3$ loop were replaced with cysteine and the resulting protein was allowed to form a pore. The membrane-inserted oligomer was then tested for reactivity with a bilayer-impermeant, positively charged reagent that specifically reacts with water-accessible sulfhydryls. If the cysteine of interest was located along the ion-conducting pathway, derivatization with this reagent would introduce a positive charge within the cation-selective channel and thereby reduce channel conductance. The results of this study suggested that each $2\beta_2$-$2\beta_3$ loop of anthrax PA contributed two antiparallel β-strands (residues 302−325) to make a 14-stranded β-barrel. However, as noted by Benson et al. (*25*), this approach does not prove that the hydrophobic positions face the interior of the bilayer. To test this assumption, alternative techniques such as fluorescence spectroscopy (see below) or electron paramagnetic resonance (*26*) must be used.

PFT Characterization Using Fluorescence Spectroscopy. Fluorescence spectroscopy is an extremely valuable technique for examining protein structure and conformational changes, especially when proteins are undergoing a transition from a water-soluble to a membrane-inserted state. The primary reason for using fluorescence spectroscopy is its sensitivity, both in terms of the wide range of information that one can extract from an analysis of the fluorescence signal and also in terms of the small amount of material required to obtain quantifiable data (less than a picomole at nanomolar concentrations). Most important, the fluorescence approach provides a means to characterize the surroundings of any PFT residue before, during, and after pore formation in either synthetic or natural membranes. This method therefore provides important information that is not accessible using crystallography, NMR, or other techniques.

Since pore formation involves a transition from a water-soluble PFT to a membrane-inserted protein, a water-sensitive fluorescent probe can be used to identify the membrane-embedded regions of a PFT. By site-directed mutagenesis of a cysteine-less toxin molecule, a specific PFT residue can be replaced with cysteine and then labeled with a cysteine-specific fluorescent reagent. If this residue is located in an aqueous environment in the water-soluble monomer and is inserted into the interior of the membrane during pore formation, a significant change in the fluorescence emission properties of the fluorophore will be observed. For most water-sensitive dyes, the emission intensity and fluorescence lifetime increase when the fluorophore moves from an aqueous to a nonaqueous milieu, and the wavelength of maximum emission intensity is shifted to the blue.

As noted above, two different secondary structures have been observed for the regions of a PFT polypeptide that span the membrane and form a boundary between the lipid bilayer and an aqueous pore: an amphipathic β-sheet conformation or an amphipathic α-helical conformation (*27*). For a β-sheet conformation, the environment of residues along the polypeptide at the interface is expected to alternate between aqueous (facing the pore) and nonpolar (facing the core of the bilayer). Alternatively, for an amphipathic α-helix, the residues facing the aqueous pore or the nonpolar bilayer would conform to a helical wheel analysis of residue projection from the α-helix. Thus, by determining the environments of a sequence of adjacent residues in the protein, the fluorescence approach can directly identify the secondary structure of the TM segments of bacterial toxins.

This approach was first employed to identify the membrane-inserted region of α-HL before the crystal structure of the heptameric complex was available (*28*). Various single-cysteine mutants of α-HL were labeled with the polarity-sensitive fluorescent probe acrylodan, and the emission maxima of the membrane-inserted oligomers were measured to distinguish between water-exposed and membrane-inserted acrylodan. In addition, the oligomer spectrum was examined after treatment with deoxycholate to determine whether the solubilization of the membrane would cause a red-shift in the emission of membrane-facing acrylodans (no change was expected if the acrylodan was buried in a nonpolar protein pocket). These initial experiments did not reveal the alternating pattern of probe environment expected for an amphipathic membrane-inserted β-hairpin (*28*), but a later study completed after the crystal structure was known did report the expected alternating spectral patterns for one strand of the α-HL β-hairpin (*29*). The discrepancy was attributed to the use of small unilamellar vesicles in the earlier study (*29*), but the highly hydrophobic acrylodan dye may have contributed by burying itself in the bilayer even when it should have been facing the pore. Thus, the choice of probe and/or membrane system can influence the observed results.

The ideal fluorescent probe for PFT studies will not alter the conformation or location of the polypeptide to which the dye is covalently attached. In addition, the probe will be soluble in both aqueous and nonaqueous milieus so as to serve as a stable reporter in both environments and to minimize the possibility that the probe's properties will drive an alteration in protein structure or folding. NBD (7-nitrobenz-2-oxa-1,3-diazole) is one fluorescent probe that fulfills these requirements because NBD has a relatively small size for a dye, it is uncharged, and its N and O atoms give the dye sufficient polar character to be soluble in an aqueous environment. Equally important, the emission intensity and fluorescence lifetime of NBD increase substantially upon moving from an aqueous solvent to the nonpolar core of the bilayer (*30*). NBD has been used as a reporter group in many studies, including investigations of nascent protein chain environment during co-translational protein translocation and integration at the ER membrane (e.g., refs *1* and *30*).

Multiple Independent Fluorescence Techniques (MIFT). A change in fluorescence intensity is frequently used to detect and monitor a change in the local environment of a fluorophore. Yet by itself, a fluorescence intensity measurement does not reveal the extent of fluorophore heterogeneity. For example, a 2-fold increase in observed emission intensity could result from 100% of the probes doubling their intensity or from 50% of the probes quadrupling their intensities while 50% experienced no change in intensity. Thus, one needs to complement intensity measurements with measurements that can assess sample homogeneity.

Fluorescence lifetime measurements are particularly useful because they reveal the extent of heterogeneity in a sample. A probe in a particular environment has a specific fluorescence lifetime, so the detection of multiple lifetimes in a

sample demonstrates directly that the probes are in multiple distinct environments. By determining the fraction of dyes in each environment, one can quantify the extent of sample heterogeneity. In the case of PFTs, one can ascertain the homogeneity of the sample both before and after membrane insertion and, hence, can determine whether each protein in a sample undergoes the same transition during membrane insertion. Such quantitative data cannot be directly elicited from measurements of changes in emission wavelength or intensity before and after membrane insertion.

Yet fluorescence lifetime analysis cannot by itself distinguish between probes in similar environments that have very similar fluorescence lifetimes, such as a probe located in the hydrophobic core of a bilayer and a probe in the hydrophobic core of a protein. Thus, an additional independent experimental approach is necessary to determine probe location unambiguously. One such approach is collisional quenching, a technique that relies on the ability of some molecules and ions to dissipate the excited state energy of an excited fluorescent dye upon colliding with it. Such collisions therefore reduce or quench the fluorescence emission of the sample. Since sample emission is decreased only when a dye collides with a quencher molecule or ion, this technique constitutes a direct measure of the accessibility of the probe to the quencher species.

The extent of probe exposure to a quencher can be estimated by the magnitude of the quencher-dependent reduction in fluorescence emission, if any. The location of probes in a sample can therefore be determined by using collisional quenchers that are themselves restricted to certain locations within the sample (e.g., refs *1*, *31*, and *32*). For example, a probe attached to a residue that faces the aqueous medium in either a soluble or membrane-inserted PFT will be quenched by hydrophilic quenchers (e.g., iodide ions). Similarly, a quencher restricted to the nonpolar interior of the membrane (e.g., a nitroxide moiety covalently attached to an acyl chain of phosphatidylcholine) will quench a probe that faces or is embedded in the lipid bilayer, but will not quench a probe that is located in the nonpolar interior of a protein domain. In addition, one can discriminate between an amphipathic polypeptide that lies on the membrane surface and one that spans the bilayer by using a lipophilic quencher that is localized at some point within the bilayer (*32*). This approach is particularly advantageous because one can address directly the location of each probe in the membrane-inserted oligomer, thereby eliminating the need to solubilize the oligomeric PFT complexes in detergent.

Additional information can be obtained from fluorescence resonance energy transfer (e.g., ref *33*) or fluorescence polarization measurements. By combining the data provided by different fluorescence techniques, one can reduce ambiguities in interpretation and hence generate accurate information about monomeric and oligomeric PFT structure. The application of MIFT therefore provides a mechanism to obtain important information about PFT structure, especially the membrane-inserted structure, in the absence of a crystal structure.

A Different PFT β-Barrel Motif. The CDCs comprise a large PFT family [currently 23 members (*34*)] that form pores only in membranes that contain cholesterol. As noted above, the CDCs form very large pores (~300 Å in diameter) containing ~50 subunits (e.g., see refs *20* and *21*), so an oligomeric membrane-inserted CDC complex clearly differs substantially from that of α-HL. Yet the nature of some of the differences has only recently been established.

MIFT has been used to identify unambiguously which portion of one CDC, perfringolysin O (PFO), is directly involved in pore formation and what secondary structure is adopted by the polypeptide after insertion into the membrane (*31*, *32*). The analysis showed that PFO, in contrast to α-HL, uses two amphipathic β-hairpins per monomer to create the very large β-barrel that forms the CDC pore. The insertion of two TM hairpins (TMHs) per toxin monomer therefore constitutes a new paradigm for the formation of β-PFT pores (*32*). By providing two TMHs per monomer, CDCs can create larger pores than an equivalent number of β-PFT monomers that have only a single TMH. Interestingly, the use of two TMHs per monomer is not restricted to CDCs. The recent crystal structure of the bacterial protein TolC has revealed that the β-barrel portion of this protein that spans the outer bacterial membrane is assembled from three TolC monomers, each of which contributes two antiparallel β-hairpins (*35*).

Thus, β-barrel pore formation by β-PFTs can be accomplished in at least two ways that differ in the amount of polypeptide contributed to the β-barrel per protein subunit. However, it is not yet clear to what extent the mechanism for formation of the β-barrel differs for β-PFTs that supply either 1 or 2 TMHs per polypeptide to the β-barrel.

Mechanism of Pore Formation. The general mechanism employed by β-PFTs to form a TM pore can be summarized as follows:

Water-soluble monomer →
Membrane-bound monomer →
Membrane-bound pre-pore oligomer →
Membrane-inserted oligomer

However, this general mechanism is not absolute, and different toxins may exhibit variations of this general pathway, as noted below. On the basis of what is known about pore formation for different β-PFTs, we will briefly summarize our current understanding of each of the steps that conform to this general mechanism: targeting, oligomerization, and insertion.

Membrane Targeting. To direct bacterial toxins to specific mammalian cells, mechanisms for targeting the toxins to particular membranes have evolved. However, to date, no receptor has been unambiguously identified for any known β-PFT. Some evidence suggests that toxin targeting is mediated by protein−protein interactions. For example, the sensitivity of α-HL for different cells varies over many orders of magnitude: human erythrocytes require 1000 times more toxin for lysis than do rabbit erythrocytes (*36*). This suggests that rabbit erythrocytes possess a specific α-HL receptor, although it has not been conclusively identified. Anthrax PA also binds to a yet-unknown ubiquitous cell surface receptor (*37*).

The CDC toxins appear to recognize another structural feature found only in mammalian membranes: the presence of cholesterol. Whether cholesterol functions as a receptor or participates in a post-binding event remains a controversial subject. There are a plethora of studies that have examined cholesterol binding and cholesterol-dependent inhibition of

the CDCs (for a review, see ref *34*), yet none of these studies has proven conclusively that cholesterol itself is a receptor for the CDCs. In addition, there is at least one recently discovered member of the CDC family, intermedilysin, which appears to exhibit a significantly restricted range of cytolytic activity toward erythrocytes from various species (*38*). Intermedilysin primarily targets human erythrocytes and exhibits virtually no activity on at least nine different animal erythrocytes. Trypsin treatment of the human erythrocytes decreases binding of the toxin, suggesting that the intermedilysin receptor has a proteinaceous component (*38*). Further investigation is necessary to decipher the nature of the receptor for the CDCs.

Oligomerization. After successful recognition of the target membrane, some β-PFTs oligomerize on the membrane surface and form a membrane-bound pre-pore complex prior to pore formation. It has been shown for α-HL (*39*−*41*) and PFO (*33*, *42*−*44*) that oligomerization precedes pore formation, and that the pre-pore complex is a legitimate intermediate in assembly and not a dead-end product generated by the experimental conditions (*40*, *43*).

However, some β-PFTs require additional processing before oligomerization can take place. Anthrax PA is activated by proteolysis after binding to specific receptors (*45*). This activation seems to be related to the proteolytic exposure of the binding sites for EF and LF (*19*, and references therein), as well as to the proteolysis-dependent oligomerization of the receptor-bound toxin (*46*).

During assembly of the oligomer, the TM domains are protected in a preinsertion state. Only after the oligomer is completed do the TM domains insert and form the membrane-spanning β-barrel. It was shown for α-HL that a nonlytic heptameric pre-pore complex yields the final TM pore after a conformational transition (*39*, *40*). For PFO, a pre-pore complex intermediate has recently been observed directly at low temperature (*42*). In addition, a disulfide bond between one of the TMHs and domain 2 of PFO prevents pore formation (*43*) and arrests the toxin in its pre-pore oligomeric state (*44*). Reduction of this disulfide bond then results in rapid pore formation because the TMH is no longer tethered to the core of the PFO protein (*43*, *44*). Ion conductivity measurements also indicate that a discrete PFO pre-pore complex is formed prior to the insertion of the TM β-barrel because the conductance through a planar bilayer was found to increase by large and discrete stepwise changes after addition of PFO (*42*). The common requirement of a pre-pore intermediate in β-PFT pore formation presumably allows the alignment of TM β-strands prior to insertion and hence the coordination of their insertion during β-barrel formation.

Controlling Insertion. Formation of a TM β-barrel involves the exposure of its hydrophobic surface to the hydrophobic core of the membrane bilayer. The premature exposure of this surface in the absence of a membrane could inactivate the toxin by the formation of nonspecific protein aggregates. Thus, one expects that the hydrophobic residues of the TMHs will be appropriately inaccessible in the water-soluble monomer and that the process of insertion will be carefully regulated. Either membrane binding and/or monomer-monomer interactions during oligomerization may elicit the conformational changes necessary to trigger the exposure of the nonpolar residues and the insertion of the TMHs into the bilayer. The importance of TMH structure on regulating pore formation is also revealed by the observation that reversing the sequence of the TMH of α-HL causes premature oligomerization in the absence of membranes (*47*).

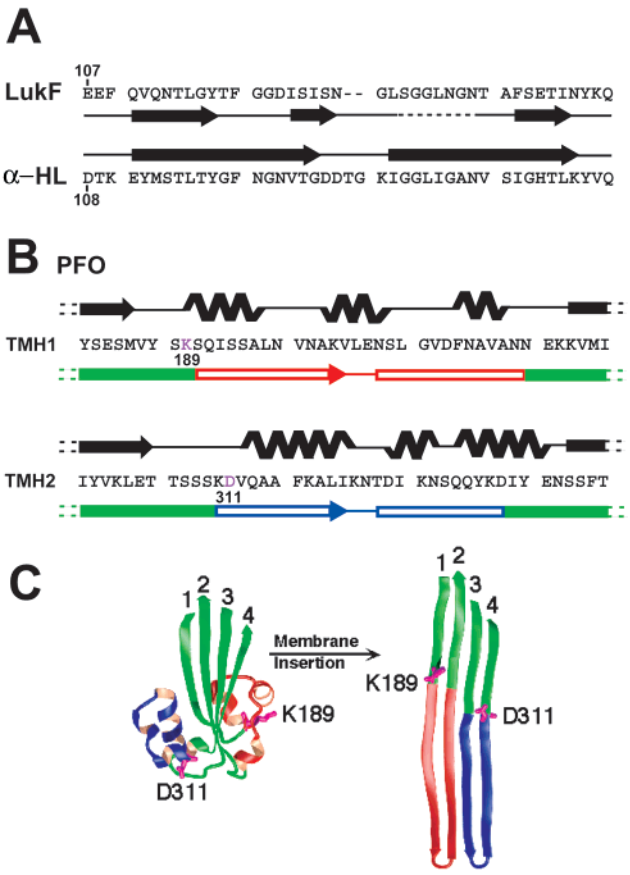

FIGURE 2: (A) Secondary structure of the TMH polypeptide in the LukF monomer and in the α-HL heptamer. The arrows indicate residues in a β-strand conformation (*23*). For LukF, the dotted line indicates residues not visible in the crystal structure. (B) The secondary structures adopted by the TMH1 and TMH2 residues in the PFO monomer (black upper lines) and membrane-inserted oligomer (colored lower lines) (*22*, *31*, *32*). The arrows show residues in β-sheets and the zigzag ribbons indicate residues in α-helices. The elements corresponding to the proposed TM structure of PFO are color-coded as in panel C and shown as open arrows. (C) Conformational changes in domain 3 of PFO that accompany pore formation. The structures of TMH1 (red), TMH2 (blue), and the domain 3 core β-sheet (green) in the water-soluble PFO monomer are shown on the left (*22*). As shown on the right, the preexisting core of β-sheet of domain 3 is proposed to be extended by the unfolding of the domain 3 α-helices and the formation of the membrane-spanning dual hairpin structure of each membrane-inserted PFO protein (*32*). The locations of residues K189 and D311 of TMH1 and TMH2, respectively, are shown in both structures in purple.

For the toxins whose TMHs have been identified, the structures of the water-soluble monomers reveal that this segment adopts a variety of conformations to protect the hydrophobic residues of the β-hairpins. In the LukF monomer, the TMH segment folds into three short β-strands with the hydrophobic residues positioned against the protein core (*23*, *24*) (Figure 1A and Figure 2A). PFO uses a completely different strategy to hide the hydrophobic surface of the β-hairpins: each TMH is folded into three short α-helices in the water-soluble monomer (Figure 2, panels B and C). The segment proposed to form the TMH of anthrax PA could

not be observed in the crystal structure, suggesting that it adopts a disordered conformation (*19*).

The reason for the variation in the conformation of TMH segments in the different water-soluble monomers is not known. Yet these conformations are likely dictated primarily by two requirements for β-PFT function: the extension of a TMH into a β-hairpin at the proper time and the avoidance of entanglements that interfere with this extension. Thus, a rotation of domain 3 in PFO is necessary to allow the extension of the β-hairpins. This rotation has the effect of straightening out the very pronounced twisted core β-sheet that extends from domain 1 to domain 3 (*22*). As suggested by Rossjohn et al. (*22*), the feasibility of this movement is supported by the poor packing between domain 2 and domain 3, and the polarity of the interface between these domains. Domain interfaces usually have very complementary surfaces and multiple regions of hydrophobic contacts, and we presume that the absence of such interactions between domains 3 and 2 ultimately facilitates the release of domain 3 from domain 2 and allows domain 3 to flex away from domain 2 as a TM β-hairpin.

Yet the domain 3−domain 2 interactions in PFO must be sufficiently strong to prevent premature unfolding of domain 3. If the nonpolar residues of a TMH are exposed prior to their alignment with those of other subunits in a pre-pore complex, the chances for nonproductive hydrophobic association or aggregation with other proteins is greatly increased. This would be especially true for PFO and any other β-PFTs that have two TMHs per subunit. Thus, it is likely that the folding of each PFO TMH into a very compact individual domain consisting of three short α-helices in the monomer is designed to eliminate any premature and nonfunctional association between the two adjacent 26-residue TMHs.

Insertion. The formation of a pre-pore complex on the membrane surface appears to be a common intermediate in β-PFT pore formation. The insertion of a single amphipathic β-hairpin into a membrane is not energetically favored because in a hydrophobic environment that lacks hydrogen bond donors or acceptors, isolated β-hairpins cannot achieve the hydrogen-bond formation necessary to lower the cost of transferring the polar atoms of the polypeptide backbone into the hydrocarbon interior (*27*). However, this energetic barrier is circumvented if the β-strands are inserted as β-sheets and form closed structures such as the β-barrel. For monomeric β-barrel membrane proteins such as OmpA, a concerted folding mechanism has been observed in vitro in which the hydrogen bonds formed between adjacent β-chains presumably favor the insertion of the β-barrel into the membrane (*48*). Similarly, the formation of a β-PFT pre-pore complex may be required to allow the concerted insertion of the β-hairpins from individual monomers and thereby lower the energetic barrier for the insertion of non-hydrogen-bonded β-strands into the membrane. It is therefore of interest that coexpression of two complementary fragments of OmpA still results in the assembly of a functional protein (*49*). Also, the complementary combination of several truncation mutants on the TM β-hairpins of α-HL were analyzed, and only complementary fragments with nicks at the β-hairpin's loop were able to form pores with a hemolytic activity approaching that of the wild-type toxin (*50*). Thus, these results are consistent with the idea that insertion of a β-strand into the membrane is energetically unfavorable unless the polypeptide is completely hydrogen-bonded to adjacent polypeptides in a β-conformation.

An additional energetic constraint for the insertion of a β-hairpin into the membrane is the exposure of the hydrophilic surface of a single amphipathic β-hairpin to the nonpolar interior of the bilayer after insertion. The insertion of a single such hydrophilic surface into the membrane would be thermodynamically unfavorable. However, if the hairpins are inserted in a concerted process, the hydrophilic sides of the hairpins could remain in contact with the aqueous medium. Such a concerted insertion would require the displacement of lipids as the aqueous pore is formed in the bilayer. Since the cross-sectional area occupied by an inserted α-HL heptamer is π (outer radius of stalk)$^2 \approx 1385$ Å^2, and since a phospholipid occupies about 70 Å^2 of surface area (*51*), about 20 phospholipid molecules in each leaflet need to be moved to allow space for the α-HL β-barrel.

Whereas such a lateral displacement of a relatively low number of lipid molecules does not seem to create a major energetic barrier, lipid displacement during CDC pore formation appears to be a completely different matter. The creation of a CDC hole with an outer diameter near 300 Å requires the displacement of about 1000 phospholipid molecules in each leaflet [or about 800 phospholipids plus 800 cholesterol molecules because the average surface area occupied by one phospholipid molecule plus one cholesterol molecule is ~90 Å^2 in a 1:1 phospholipid/cholesterol mixture (*52*)]. Lipid removal from a CDC pore is therefore a major problem, and the mechanism by which lipids are eliminated from a CDC pore remains one of the most obscure aspects of pore formation.

Conformational Changes Associated with the Transition from Water-Soluble Monomer to Membrane-Inserted Oligomer. Biochemical analyses of α-HL pore formation have revealed two intermediate states: the membrane-bound monomer and the heptameric pre-pore complex (e.g., refs *39*, *40*, and *53*). Oligomerization precedes membrane insertion during pore formation, and TMH insertion involves interaction among neighboring protomers (*28*, *40*, *54*, *55*). A comparison of the monomeric structure of LukF and the oligomeric structure of the homologous α-HL revealed that the β-sandwich and rim domains behave as rigid bodies that adopt different relative conformations in the monomer and the heptamer due to small changes spread over a number of residues at the junction between the β-sandwich and rim domains (Figure 1). The only regions that show a dramatically different conformation when the monomer and oligomer are compared are the amino latch (Ala1-Val20 in α-HL) and the stem region (Lys110-Tyr148 in α-HL) (*7*, *23*, *24*). In the monomer, the two segments are packed against the core of the molecule with their tips in contact. In the oligomer, both segments have undergone a major conformational change and they are now in close contact with their respective counterparts of the neighboring subunits (Figure 1B). Mutations in either the N-terminal amino latch or the TMH region can markedly alter the efficiency of pore formation (e.g., see refs *39*, *56*, and *57*), and structural coupling of these two different regions may also be linked to the regions of α-HL that interact directly with the membrane (*39*, *55*).

Long-range coupling of conformational changes have been observed in a CDC. A schematic model of stages in PFO

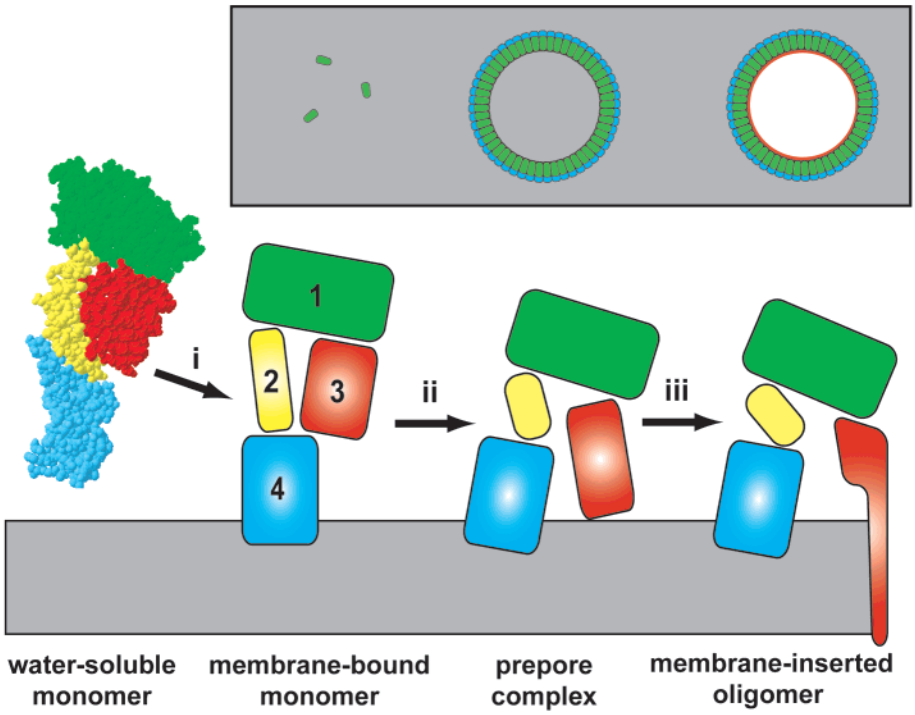

FIGURE 3: A schematic model of stages of PFO insertion into the membrane is shown. The domains of PFO are color-coded as indicated. The membrane bilayer is depicted in gray. To simplify the figure, only a single PFO polypeptide is shown in the side view (lower panel). The upper panel shows a schematic top view for each stage of pore formation.

insertion into the membrane is depicted in Figure 3. PFO is secreted as a water-soluble monomer and binds to cholesterol-containing membranes via domain 4. Successful recognition of the target membrane elicits a conformational change in a CDC (*43*, *58*, *59*) and the exposure of PFO domain 4 to the interior of the membrane (*43*, *58*), all of which accompany pre-pore formation (*42*, *44*). The cholesterol-dependent interaction of domain 4 with the bilayer is then communicated to the distant and noncontiguous domain 3, presumably via domain 2 (*43*). These structural changes, as well as those associated with oligomerization, may trigger the insertion of the TMHs into the membrane and the formation of the pore.

On the basis of these two examples, it appears that the structural changes associated with converting a β-PFT from a water-soluble monomer to a membrane-inserted oligomer extend through much of the molecule. Such an extensive network of structural linkages within a β-PFT protein can be advantageous because it reduces the chances of prematurely undergoing a structural transition that exposes a TMH. By allosterically linking different domains or regions of the protein, the system can couple separate interactions (e.g., binding to the membrane and binding to another subunit) and thereby ensure that pore formation proceeds only when the necessary criteria are met.

Unresolved Issues. Our knowledge of the structure, function, assembly, and regulation of the large number of β-PFT proteins is spotty and focused primarily on only a few of the β-PFTs. Although it is sometimes difficult, and perhaps even dangerous, to extrapolate from fragmentary information to the general principles of β-PFT structure and function, the available data at least provide us with working models that can be further tested experimentally. Among the many interesting unknowns that need to be addressed further are the following:

What Are the Roles of Lipids in Membrane Targeting? The involvement of lipids in the targeting of β-PFTs to membranes is not understood in molecular terms, nor is the role, if any, of lipids in the oligomerization and insertion phases of pore formation. For example, although cholesterol is required for CDC pore formation, direct binding of a cholesterol molecule to a CDC protein molecule has yet to be demonstrated unequivocally. Early studies revealed that CDC cytolytic activity is blocked by exposure to cholesterol (*34*), but they did not distinguish between CDC adsorption to a cholesterol micelle or cholesterol binding to a specific ligand binding site on the CDC. Thus, the mechanism of CDC interaction with cholesterol remains to be determined.

Some insight into the interactions of β-PFTs with membranes has been gained by studies that vary the composition of synthetic liposomes. Zitzer et al. (*60*) have recently proposed that the critical membrane variable in both *Vibrio cholerae* cytolysin pore formation and SLO (a CDC) targeting and pore formation is the accessibility of the cholesterol at the membrane surface. Also, in natural membranes rich in cholesterol and glycosphingolipids, lipid material can segregate into microdomains that are termed lipid rafts (*61*). Bacterial toxins could, in principle, recognize and bind to these cholesterol-rich microdomains and thereby promote pore formation by concentrating the toxin monomers within a limited area on the membrane surface (*62*). Indeed, it has just been reported that PFO binds to a membrane via a lipid raft (*63*). Thus, the topography and properties of the membrane surface may play a critical, if undefined, role in β-PFT targeting and pore formation. But a detailed understanding of the role of lipids in the permeabilization process will require further experimentation.

Do CDCs Have a Common Mechanism for Pore Formation? There are currently two very different models for the mechanism of pore formation by CDCs. One model proposes

that CDC insertion occurs only after the formation of a circular pre-pore complex has been completed (Figure 3). The other model proposes that the CDC pore grows continuously by the consecutive addition of individual monomers until the circular complex is completed (*64*).

Evidence that supports the pre-pore model for PFO has been obtained using several different experimental techniques. First, fluorescence studies reveal that the rate of oligomerization of PFO on the membrane surface is faster than the rate of insertion of the β-hairpins (*33*, *43*). Second, a complete circular pre-pore oligomeric complex can be detected by electron microscopy, fluorescence, and SDS-agarose gel electrophoesis under conditions that prevent the insertion of the TM β-hairpins (*42*−*44*). Third, when PFO is added to one side of a planar lipid bilayer, ion conductivity through the membrane increases in large discrete steps rather than continuously (*42*). All of these results indicate that when water-soluble monomers are readily available, the formation of the oligomeric pre-pore complex proceeds to completion before the TMHs are inserted to create the aqueous pore.

When monomer availability is limited or is complicated by the presence of CDC mutants whose oligomerization has been impaired (e.g., ref *64*), the process of oligomerization may not go to completion. Could these incomplete oligomers insert their β-hairpins into the membrane and cause a membrane lesion? As discussed above, the insertion of individual β-hairpins into the membrane is unlikely. However, it is conceivable that partial β-barrels could be inserted into the bilayer if the number of monomers exceed a certain threshold number. Such a possibility would explain the presence of some arc-shaped incomplete oligomers in electron micrographs of liposome and erythrocyte membranes treated with PFO or another CDC (e.g., see refs *20* and *21*). On the other hand, samples prepared for electron microscopy typically have a much higher toxin/lipid molar ratio than is generally used in biochemical analyses. Since toxin availability is therefore not a limiting factor in electron microscopy experiments, it is conceivable that incomplete arcs are generated during sample preparation for the electron microscope, even when availability of toxin monomers is not limited. Thus, the origin of the CDC arcs is still an open question that has, as seen below, mechanistic ramifications.

On the basis of the existence of arc-shaped oligomers in electron micrographs, a different mechanism of oligomerization and insertion has been proposed for another member of the CDC family, SLO (*64*). According to this model, two membrane-bound monomers dimerize and then insert into the membrane. Subsequently, the dimer is rapidly elongated by association with and insertion of other monomers that have bound to the membrane. Continued elongation results in a growing arc-shaped oligomeric pore. When an SLO mutant that interferes with oligomerization is introduced into a sample of wild-type SLO, the maximum size of the molecular species that can pass through the resulting pores is reduced (*64*). This observation is consistent with the interruption of a continuously growing pore by the mutant proteins. It seems unlikely that the homologous PFO and SLO toxins would insert into the bilayer via vastly different mechanisms, but PFO and SLO have to be examined in parallel using the same experimental approaches to resolve this uncertainty.

What Are the Structures of Other Membrane-Inserted β-PFT Complexes? To assess the limits of the spectrum of structural motifs utilized by membrane-inserted β-PFT proteins, more structural studies are needed. Such studies may also identify intermediate stages in pore formation. For example, in the unrefined crystallographic structure of the water-soluble anthrax PA heptamer, no hydrophobic surfaces were found that might interface with the hydrophobic core of the lipid bilayer. This suggests that this heptamer represents an intermediate in the pathway of pore formation, probably the pre-pore state, rather than the pore itself (*19*).

Although more high-resolution X-ray crystal structures are desirable, the reality is that such membrane-bound β-PFT structure determinations are difficult to achieve. This is especially true for toxins such as the CDCs that form oligomers that are very large both in size and in number of subunits. Thus, fluorescence spectroscopy will be used with increasing frequency in studies of β-PFTs because the application of MIFT can provide unambiguous identification and conformational analysis of TMH segments. Moreover, fluorescence resonance energy transfer can be used to quantify the magnitude of conformational and topographical changes during pore formation. In addition, the fluorescence signal can be monitored in real time, so the kinetics of binding, insertion, and pore formation can all be determined directly using this approach. We look forward to the valuable information that will become available soon using this and other techniques to unveil many aspects of β-PFT structure and function. In particular, the dimorphic nature of the β-PFTs constitutes a rich area for exploring our understanding of controlled structural transitions in proteins.

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