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Perfringolysin O Structure and Mechanism of Pore Formation as a Paradigm for Cholesterol-Dependent Cytolysins

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Abstract

Cholesterol-dependent cytolysins (CDCs) constitute a family of pore forming toxins secreted by Gram positive bacteria. These toxins form transmembrane pores by inserting a large β -barrel into cholesterol-containing membrane bilayers. Binding of water-soluble CDCs to the membrane triggers the formation of oligomers containing 35–50 monomers. The coordinated insertion of more than seventy β -hairpins into the membrane requires multiple structural conformational changes. Perfringolysin O (PFO), secreted by *Clostridium perfringens*, has become the prototype for the CDCs. In this chapter, we will describe current knowledge on the mechanism of PFO cytolysis, with special focus on cholesterol recognition, oligomerization, and the conformational changes involved in pore formation.

Keywords

Cholesterol; Cholesterol-dependent cytolysins; Membrane; Lysteriolysin O; Perfringolysin O; Pneumolysin; Pore formation; Streptolysin O; β -barrel; Toxin

4.1 Introduction

Perfringolysin O (PFO) is the prototypical example of a growing family of bacterial pore-forming toxins known as the Cholesterol Dependent Cytolysins (CDCs, Tweten 2005; Heuck et al. 2010; Gilbert 2010). CDCs are secreted by Gram-positive bacteria including *Bacillus*, *Listeria*, *Lysinibacillus*, *Paenibacillus*, *Brevibacillus*, *Streptococcus*, *Clostridium*, *Gardnerella*, *Arcanobacterium*, and *Lactobacillus* (see Heuck et al. 2010; Rampersaud et al. 2011; Jost et al. 2011). There are 30 members of the CDC family reported for Gram-positive bacteria and, surprisingly, two CDC-coding DNA sequences have been found in the Gram-negative *Desulfobulbus propionicus* and *Enterobacter lignolyticus*. However, in contrast with the Gram-positive bacteria that produce CDCs, the Gram-negative ones have not been shown

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Index keywords: β -hairpin, β -sandwich domain, β -barrel, Cholesterol, Cholesterol accessibility, Cholesterol-dependent cytolysins, Cholesterol recognition, Cholesterol threshold, *Clostridium perfringens*, Gram positive bacteria, Intermedilysin, Lysteriolysin O, Membrane structure, Oligomerization, Perfringolysin O, Pneumolysin, Phosphatidylcholine, π -stacking interaction, Pore-forming toxins, Pre-pore complex, Streptolysin O, Transmembrane β -barrel, Transmembrane pore, Trp rich loop.

to inhabit humans or indeed animals of any kind (Hotze et al. 2013). Despite their extremely diverse lineage, the majority of CDCs show an amino acid sequence identity greater than 39% when compared to PFO (Heuck et al. 2010). The C-terminus (domain 4 or D4) of PFO is responsible for membrane binding and is the domain with the highest percentage of amino acid identity when sequences are compared with other CDC members.

Most CDCs possess a cleavable signal sequence which targets the toxins for secretion to the extracellular medium. The secreted water-soluble toxins diffuse until encountering their target, a cholesterol-containing mammalian cell membrane (Fig. 4.1, step I). An exception to the cholesterol requirement for targeting was found for intermedilysin which uses the human receptor CD59 for membrane targeting (Giddings et al. 2004). However this toxin still requires cholesterol to insert into the membrane and form a transmembrane pore (Giddings et al. 2003). After binding, CDC monomers diffuse across the surface of the membrane and interact reversibly with other monomers until formation of a stable dimer (Fig. 4.1, step II, Palmer et al. 1995; Hotze et al. 2012). These initial dimers grow by the incorporation of additional monomers into a large ring shaped complex (known as the pre-pore complex, (Fig. 4.1, step III, Shepard et al. 2000). Each of these complexes contains 30-50 monomers, and upon insertion into the membrane, they form large β -barrel pores, (up to 250-300 Å in diameter, Fig. 4.1, step IV, Dang et al. 2005; Shatursky et al. 1999; Shepard et al. 1998).

In this chapter, we will discuss CDCs through the lens of one of the most studied and well understood CDCs, PFO (Tweten 2005; Heuck et al. 2010; Gilbert 2010). We will focus on the targeting of PFO to cholesterol-containing membranes and on the multiple conformational changes the protein undergoes in order to spontaneously transition from a water-soluble monomer to a large multimeric transmembrane complex. We will also comment on the most recent findings about the PFO cytolytic mechanism.

PFO is secreted by *Clostridium perfringens* as a 52.6 kDa protein, and the crystal structure of the water-soluble monomer revealed four distinct domains (Fig. 4.2A, Rossjohn et al. 1997). The overall three dimensional structure observed for PFO is conserved for all other CDCs whose high resolution structures have been solved (Polekhina et al. 2006; Bourdeau et al. 2009; Xu et al. 2010). Domain 1 (D1) consists of the top portion of the elongated molecule. D1 is the only domain that does not undergo large structural rearrangements during pore formation. Domain 2 (D2) adopts mostly a β -strand secondary structure that collapses vertically during pore-formation to allow the insertion of the β -hairpins that form the transmembrane β -barrel (Ramachandran et al. 2005; Dang et al. 2005; Czajkowsky et al. 2004; Tilley et al. 2005). Domain 3 (D3) contains both the β -sheet involved in the oligomerization of the toxin and the six short α -helices that unfurl into two amphipathic β -hairpins to form the β -barrel (Shepard et al. 1998; Shatursky et al. 1999; Ramachandran et al. 2004). Domain 4 (D4) consists of a β -sandwich and contains a conserved Trp rich loop as well as three other conserved loops at the distal tip (Fig. 4.2B and C). D4 is responsible for cholesterol recognition and the initial binding of the toxin to the membrane (Heuck et al. 2000; Ramachandran et al. 2002).

4.2 Membrane Recognition and Binding

One of the unique features of the mammalian cell membrane is the presence of cholesterol. *C. perfringens* and other pathogens have exploited this property of mammalian membranes to target their CDCs without compromising the integrity of their own membranes. It has long been known that binding of PFO and other CDCs requires high levels of cholesterol in model membranes prepared with phosphatidylcholine (Alving et al. 1979; Rosenqvist et al. 1980; Ohno-Iwashita et al. 1992). Based on the requirement of high cholesterol levels, targeting of PFO to cholesterol rich domains or “lipid rafts” has been suggested (Ohno-Iwashita et al. 2004). However, it has become clear that exposure of cholesterol at the membrane surface is a key factor to trigger PFO binding, and “lipid rafts” may not be necessary for toxin binding (Heuck et al. 2007; Nelson et al. 2008; Flanagan et al. 2009; Moe and Heuck 2010; Sokolov and Radhakrishnan 2010; Olsen et al. 2013). Moreover, the localization of PFO oligomers on the membrane surface may change from the original binding site after insertion of the β -barrel (Nelson et al. 2010; Lin and London 2013).

It has also been shown that the binding of PFO to cholesterol containing membranes is modulated by amino acids located in the loops that connect the β -strands at the bottom of D4 (Fig. 4.2C, Soltani et al. 2007b, a; Moe and Heuck 2010; Farrand et al. 2010; Johnson et al. 2012; Dowd and Tweten 2012), however the precise molecular mechanism of CDC-cholesterol interaction remains poorly understood.

4.2.1 Cholesterol Recognition

The first step in the binding of a water-soluble CDC to the membrane involves the formation of a non-specific collisional complex between a monomer and the lipid bilayer. This step is diffusional and electrostatic interactions may play an important role (e.g., introduction or elimination of negative charges alters binding, Soltani et al. 2007b; Johnson et al. 2012). While on the membrane surface, insertion of non-polar and aromatic amino acids and/or specific interactions with membrane lipids may anchor the protein to the membrane (Cho and Stahelin 2005). However, non-polar amino acids are rarely exposed on the surface of water-soluble proteins, and therefore conformational changes are often required to expose these residues to the hydrophobic core of the membrane bilayer. As a result, multiple conformational changes are triggered during the transition of PFO from a water-soluble monomer to a membrane-inserted oligomer.

In model membranes prepared exclusively with phosphatidylcholine > 30 mol% cholesterol is required to trigger binding of PFO (Ohno-Iwashita et al. 1992; Heuck et al. 2000), streptolysin O (Rosenqvist et al. 1980), lysteriolysin O (Bavdek et al. 2007), or tetanolysin (Alving et al. 1979) but the amount of cholesterol needed does vary depending on membrane phospholipid composition. The “cholesterol threshold” can be reduced by the presence of double bonds in the acyl chains of the phospholipids or by the presence of phospholipids with smaller head groups (Flanagan et al. 2002; Nelson et al. 2008; Flanagan et al. 2009). Therefore, it has become clear that modifications to the phospholipids that form the membrane can alter the ability of PFO to detect cholesterol at the membrane surface (Moe and Heuck 2010). Despite their influence on membrane binding the presence of phospholipids is not required, since cholesterol alone (in the absence of any other lipid) is

sufficient to trigger PFO oligomerization and formation of ring-like complexes (Heuck et al. 2007 and references therein). Accessibility of cholesterol at the membrane surface seems to be the key to trigger the binding of PFO to membranes (Flanagan et al. 2009; Moe and Heuck 2010; Sokolov and Radhakrishnan 2010; Olsen et al. 2013).

4.2.2 What is Cholesterol Accessibility?

It has long been recognized that cholesterol modulates important membrane properties including permeability, fluidity, thickness, and domain formation, among others. The cholesterol-dependent association of certain proteins and peptides with membranes has been often associated with the effect of cholesterol on one or more of these membrane physical properties. More recently, studies with molecules that directly interact with cholesterol, like cyclic sugar polymers (e.g., cyclodextrins, Radhakrishnan and McConnell 2000), enzymes (e.g., cholesterol-oxidase, Lange et al. 2005), and bacterial toxins (e.g., PFO, Nelson et al. 2008; Flanagan et al. 2009; Moe and Heuck 2010; Sokolov and Radhakrishnan 2010) have shown that the accessibility of cholesterol at the membrane surface also plays a critical role in cell biology.

Cholesterol is insoluble in aqueous solutions, but it is readily soluble in phospholipid bilayers. The solubility limit of cholesterol in lipid bilayers is dictated by the nature of the phospholipids (acyl chain length and saturation, and head group size, Ohvo-Rekilä et al. 2002). If the concentration of cholesterol in a bilayer increases to levels above its solubility limit, cholesterol aggregates would form crystals and precipitate out into the aqueous solution (Mason et al. 2003; Bach and Wachtel 2003; Ziblat et al. 2010).

Given its hydrophobic nature, in a lipid bilayer cholesterol orients parallel to the acyl chains of the phospholipids with the only polar group (an OH) facing the surface of the membrane, in close proximity to the phospholipid head groups (Fig. 4.3). At low concentrations, the interaction of cholesterol with other membrane components (lipids, proteins, etc.) reduces the ability of cholesterol to interact with water-soluble molecules at the membrane surface. In other words, when present in low amounts, cholesterol is not accessible to interact with molecules like PFO or cyclodextrins. As the concentration of cholesterol increases, its accessibility remains low until a saturation point is reached. The concentration of cholesterol at the saturation point will depend on the phospholipid or phospholipid mixture present in the membrane (Fig. 4.3A). At this point, a small increase in the sterol concentration causes a sharp increase in the ability of water-soluble molecules to interact with cholesterol (Heuck et al. 2000; Radhakrishnan and McConnell 2000; Lange et al. 2005). Different models have been proposed to explain changes on cholesterol accessibility at the membrane surface: the cholesterol:phospholipid complex model and the umbrella model (Huang and Feigenson 1999; McConnell and Radhakrishnan 2003). Despite their thermodynamics or steric basis, the models are not mutually exclusive (Lange and Steck 2008; Mesmin and Maxfield 2009). Recent molecular dynamics simulations of simple membrane models (Olsen et al. 2013) suggested that cholesterol accessibility is related to the overall cholesterol depth within the membrane bilayer and not to the appearance of a new pool of cholesterol molecules (sometimes referred as free cholesterol or active cholesterol). In favor of clarity in this chapter we will refer to the effect that cause an increase in the interaction of cholesterol with

water-soluble molecules, as an increase in cholesterol accessibility at the membrane surface (Fig. 4.3).

4.2.3 Domain 4 and the Conserved Loops

PFO D4 consists of two four-stranded β -sheets located at the C-terminus of the protein (Fig. 4.2). There are four loops that interconnect the eight β -strands at the distal tip of the toxin, three short loops (L1, L2, and L3) and a longer Trp rich loop (also known as the conserved undecapeptide). These loops insert into the membrane upon binding and are presumably responsible for the interaction of the toxin with cholesterol (Ramachandran et al. 2002; Soltani et al. 2007b; Farrand et al. 2010). Two of these loops (L2 and L3, Fig. 4.2C) connect β -strands from opposite β -sheets, while L1 and the Trp rich loop connect β -strands from the same β -sheet. L1 and the Trp rich loop are parallel to each other and abutted perpendicularly by L2 forming a pocket in the bottom of the protein. The loops that form the pocket are the most conserved segments in D4, and modifications to any of these loops affects the cholesterol binding properties of PFO (Polekhina et al. 2005; Farrand et al. 2010; Moe and Heuck 2010; Johnson et al. 2012, see below). The remaining L3 is far less conserved and distant from the pocket formed by the other three loops.

The Trp rich loop is the longest of the D4 loops, containing 11 residues (E C T G L A W E W W R). It is a signature feature of the CDCs and is highly conserved among species. The three-dimensional structure of this loop seems to be more variable (Rossjohn et al. 1997; Polekhina et al. 2006; Bourdeau et al. 2009; Xu et al. 2010), but this may simply reflect its flexibility (Polekhina et al. 2006). Initially, the Trp rich loop was thought to be responsible for cholesterol recognition and binding, and this idea was supported by several studies showing that modifications in it greatly decreased the pore-forming activity of the protein (Saunders et al. 1989; Pinkney et al. 1989; Michel et al. 1990; Sekino-Suzuki et al. 1996; Korchev et al. 1998; Billington et al. 2002; Polekhina et al. 2005). However, recent studies showed that the other loops in D4 are also responsible for cholesterol recognition (Farrand et al. 2010). The Trp rich loop has now been suggested to play a role in both the pre-pore to pore transition (Soltani et al. 2007b) and the coupling of monomer binding with initiation of the pre-pore assembly (Dowd and Tweten 2012). Dowd and colleagues recently showed that modification of a charged amino acid in the Trp rich loop (Arg 468, Fig. 2B) resulted in complete elimination of the pore-forming activity of PFO and had a significant effect on the membrane binding of the toxin (Polekhina et al. 2005; Dowd and Tweten 2012). The R468A PFO derivative was not able to oligomerize after membrane binding, suggesting that this modification disrupts the previously reported allosteric coupling between D4 and D3 (Heuck et al. 2000). Despite the novel functions assigned to the Trp rich loop, its role in binding cannot be neglected since many modifications to this segment have been shown to have a significant effect in toxin-membrane interaction (Polekhina et al. 2005).

Unlike the flexible Trp rich loop, the three-dimensional structure of the other three short loops is more conserved. The L3 is located on the far edge of D4, away from a pocket formed by the Trp rich loop, L1, and L2 (Fig. 4.2C). Modifications introduced into L3 have been shown to have either a negligible effect on cholesterol interaction, or to decrease the amount of cholesterol required for binding (Farrand et al. 2010; Johnson et al. 2012). For

example, the elimination of the charge of D434 in L3 reduced the amount of cholesterol required to trigger binding (Johnson et al. 2012). These results suggest that L3 plays a limited role in cholesterol recognition, and its effect on binding may be related to nonspecific interactions with the membrane that stabilize the bound monomer at lower cholesterol levels.

4.2.4 Proposed Cholesterol Recognition Motif

It has been proposed that PFO contains a cholesterol recognition motif composed of only two adjacent amino acids in L1, T490 and L491 (Farrand et al. 2010). These amino acids are completely conserved throughout all reported CDCs, and modifications to them greatly affect the binding of the protein to both cell and model membranes (Farrand et al. 2010). These data suggest a prominent role for these two amino acids in cholesterol recognition, however other well conserved amino acids in that region have not been analyzed yet (e.g., H398, Y402 and A404). Moreover, no direct interaction between cholesterol and these two residues has been shown so far. The fact that both amino acids must be mutated to eliminate binding in a motif containing only two amino acids, coupled with the fact that there are many additional conserved amino acids in the vicinity, suggest that other amino acids may also play a role in cholesterol recognition and form part of the cholesterol binding site. Further studies are required in this area.

4.2.5 The Effect of Cholesterol Accessibility on Perfringolysin O Binding

While cholesterol accessibility is necessary for PFO binding, the analysis of PFO derivatives with modifications on D4 revealed that sterol accessibility is not sufficient to trigger stable PFO-membrane association (Johnson et al. 2012). As mentioned above, native PFO readily binds to model membranes containing 40 mol% cholesterol (and an equimolar mixture of other phospholipids, see Johnson et al. 2012), revealing that cholesterol is accessible at the membrane surface. However, the PFO^{C459A-L491S} derivative was not able to bind to the same membranes, clearly indicating that the cholesterol molecules were not sufficiently accessible to trigger toxin binding. Binding of the PFO^{C459A-L491S} derivative was recovered when the cholesterol concentration was increased to 50 mol%, suggesting that the affinity of this derivative for cholesterol is lower than that of native PFO, and more cholesterol was required at the membrane surface to trigger stable binding (note that L491 is one of the two amino acids proposed to be essential for cholesterol recognition). It is not clear how cholesterol accessibility varies with increasing amount of cholesterol in the membranes. For simplicity we have represented this variation as a linear function of cholesterol concentration (Fig. 4.3) however cholesterol accessibility may have a non-linear dependence in these systems. Further investigations are required in this area to establish the precise mechanism of PFO-cholesterol interaction as a function of cholesterol accessibility.

4.2.6 Mutations in Domain 4 Affect the Cholesterol Threshold Required to Trigger Binding

The effect that a particular amino acid modification has on PFO activity is often characterized by alterations to the hemolytic properties of the toxin (i.e., pore formation). The EC₅₀ or effective toxin concentration required for 50% lysis is a good indicator of these effects. Another method frequently used to characterize the effect of modifications in PFO derivatives is the percentage of hemolysis as compared with the one obtained for the native

toxin under the same experimental conditions. However, it is worth noticing that the latter method is highly influenced by the toxin/red blood cell ratio used in the assay (see Fig. S2 in Johnson et al. 2012). Similarly, when the characterization of PFO derivatives is done using model membranes, the protein/lipid ratio should be carefully taken into account.

The effect of a particular amino acid modification is dependent on how much cholesterol is accessible at the membrane surface (Johnson et al. 2012, and see below). As mentioned above, the PFO^{C459A-L491S} derivative showed negligible binding to liposomes containing 40 mol% cholesterol, but the binding of PFO^{C459A-L491S} was indistinguishable from that of native PFO when membranes containing 50 mol% cholesterol were used. The deleterious effect of many mutations to the toxin can be overcome by an increase in the cholesterol content in the membrane (Moe and Heuck 2010; Johnson et al. 2012). Interestingly, while most modifications to the D4 loops do not affect the sharp sigmoidal shape of the binding isotherm, the amount of cholesterol required for 50% binding (or “cholesterol threshold”) may change significantly for different PFO derivatives. Therefore, when comparing PFO derivatives using model membranes it is more accurate to quantify the effect of a particular modification as the relative change in the “cholesterol threshold” compared to one obtained for native PFO using the same batch of membranes (Johnson et al. 2012).

We have shown recently that modifications to the binding domain of PFO were able to increase or decrease the “cholesterol threshold” of a PFO derivative (Johnson et al. 2012). These derivatives were successfully used to detect changes in the cholesterol content of cells and model membranes. While PFO has long been put forth as a probe for cholesterol-rich membranes, the advent of new PFO derivatives with varied “cholesterol thresholds” adds a layer of selectivity to the cholesterol sensing measurements.

4.3 Oligomerization on the Membranes Surface

Upon binding to a cholesterol containing membrane, PFO diffuses across the surface of the lipid bilayer and oligomerizes into a large ring shaped complex (Fig. 4.1). This complex contains 35-50 individual PFO monomers (~250-300 Å inner diameter) and it is referred to as the pre-pore complex (Olofsson et al. 1993; Shepard et al. 2000; Dang et al. 2005). Transition of the pre-pore complex to the final membrane-inserted complex occurs by the insertion of numerous β -hairpins (two per monomer) that perforate the membrane forming a large transmembrane β -barrel (Shatursky et al. 1999). The conformation of the individual PFO monomers in the pre-pore complex is not vastly changed from that of their water-soluble form. There are subtle structural changes triggered by membrane binding and oligomerization of the protein that allow for proper alignment of the monomers and the overall geometry of the pore (Ramachandran et al. 2004). Formation of complete rings at the membrane surface seems to be regulated by the relatively slow formation of an initial CDC dimer (Palmer et al. 1995; Hotze et al. 2012).

4.3.1 Nucleation of the Pre-pore Complex

Oligomerization of the CDCs is triggered by membrane binding and interaction with cholesterol (or exceptionally by interaction with a protein receptor for intermedilysin). Cholesterol binding is sufficient to trigger the conformational changes that unblock the

hidden oligomerization interface in the water-soluble monomer (Ramachandran et al. 2004; Heuck et al. 2007). Blockage of the oligomerization interface in the monomer prevents premature oligomerization of the toxin in solution. This regulatory mechanism can be overridden if the monomers are present at high concentration in solution (e.g., for pneumolysin, Gilbert et al. 1998; Solovyova et al. 2004), but oligomerization is rare at physiological concentrations (i.e, nM range or lower).

The most significant of the conformational changes that follows membrane binding involves the exposure of the core β -sheet that comprises a larger part of D3. A short β -strand ($\beta 5$) separates from the core β -sheet in D3 and exposes $\beta 4$ for its interaction with the always-exposed $\beta 1$ strand of another PFO molecule, promoting oligomerization (Ramachandran et al. 2004; Hotze et al. 2001). This conformational change is thought to be facilitated by a pair of Gly residues, G324 and G325, located in the loop between $\beta 4$ and $\beta 5$. These Gly residues are highly conserved, and act as a hinge between the two β -strands (Ramachandran et al. 2004). In addition to the separation of $\beta 5$ from $\beta 4$, it has been suggested that there is a disruption of the D2 and D3 interface. This disruption is thought to be caused by the rotation of D4 which breaks the weak interactions between D2 and D3. These conformational changes cause the rotation of D3 away from D1 and ultimately the unfurling of the transmembrane hairpins (Rossjohn et al. 2007; Hotze et al. 2012).

Hotze et. al. have recently suggested that the initial interaction between two membrane-bound PFO monomers is weak and transient (Hotze et al. 2012) and rarely of sufficient length to allow for the transition to a stable dimer with $\beta 1$ and $\beta 4$ strands properly aligned. However, if the transition occurs, addition of further PFO monomers to the complex becomes favorable and oligomerization ensues. Therefore, formation of a stable initial dimer constitutes the rate limiting step in oligomerization that diminishes the formation of uncompleted rings on the membrane surface (Fig. 4.1, step II, Hotze et al. 2012). While it has been originally proposed that the separation of $\beta 5$ from $\beta 4$ happens upon membrane binding (Ramachandran et al. 2004), it is still unclear whether these structural changes are caused by toxin binding or as a consequence of monomer-monomer oligomerization.

4.3.2 Alignment of Core β -sheets

Addition of monomers to the growing oligomer requires the proper alignment of the core β -strands of the newly added PFO monomer with a β strand at the edge of the oligomer. Formation of hydrogen bonds between adjacent β -strands is energetically favorable but non-specific in nature. If the alignment is not correct, proper growing of the oligomer would not be possible. Thus, it is critical to regulate the alignment of neighbor β -strands to prevent the formation of truncated pre-pore complexes. It has been suggested that the correct alignment of adjacent β -strands among individual PFO monomers is dictated by π -stacking interactions between aromatic residues located in $\beta 1$ (Y181) and $\beta 4$ (F318) (Ramachandran et al. 2004). Modifications on either of these residues have proven to be extremely deleterious to the ability of PFO to form pores (Ramachandran et al. 2004; Johnson et al. 2012). Interestingly, despite being a critical interaction, it appears that only Y181 is completely conserved among the CDCs. A few CDC family members do not contain an aromatic residue in the corresponding location of F318 in PFO, suggesting that proper alignment of adjacent β -

strands may follow another regulatory mechanism for these members (i.e., lectinolysin, intermedilysin, vaginolysin, pneumolysin, mitilysin, pseudoneumolysin, and the two newly identified members, see Heuck et al. 2010; Rampersaud et al. 2011; Jost et al. 2011).

4.4 Mechanism of Pore Formation

The last step in the cytolytic mechanism of PFO is the formation of the transmembrane pore. The pre-pore complex transitions into a membrane-inserted complex forming a large transmembrane β -barrel (Fig. 4.1, step IV). This transition involves the unfurling of six short α -helices located in D3 down to two amphipathic β -hairpins, and the collapse of D2 to bring down the β -hairpins so they can span the hydrophobic core of the membrane. Large secondary and tertiary structural changes are required to coordinate the insertion of more than 140 individual β -strands and the removal of thousands of lipid molecules to form a β -barrel pore. The use of two β -hairpins per monomer to create a transmembrane β -barrel was first described for PFO (Shatursky et al. 1999; Heuck et al. 2001), and it is likely that this mechanism is also employed by other important pore-forming proteins like the Membrane Attack Complex/Perforin (MACPF) proteins (Hadders et al. 2007; Rosado et al. 2007; Dunstone and Tweten 2012).

A key step in the pore formation mechanism of the CDCs is the unfurling of six short α -helices in D3 to form two extended amphipathic β -hairpins (Shatursky et al. 1999; Heuck et al. 2007; Sato et al. 2013). These conformational changes are necessary to minimize the exposure of hydrophobic residues in the water-soluble form of the PFO monomer (Shatursky et al. 1999; Heuck and Johnson 2005). After insertion, the hydrophobic side of the amphipathic hairpin faces the non-polar lipid core, and the hydrophilic side faces the aqueous pore (Fig. 4.4, Shatursky et al. 1999; Shepard et al. 1998). The exact molecular mechanism for the pre-pore to pore conversion remains unknown, but thermal energy plays a key factor since at low temperatures (e.g., 4 °C) the PFO oligomer remains locked at the pre-pore complex state (Shepard et al. 2000; Heuck et al. 2003).

Sato et al have recently shown that in the pre-pore complex the β -strands that form the transmembrane pore are flexible and mobile (Sato et al. 2013). These transmembrane β -hairpins are located high above the membrane in the pre-pore complex (Tilley et al. 2005; Ramachandran et al. 2005) and are able to extend and test hydrogen bonding arrangements, but they do not fully form a β -barrel structure (Heuck et al. 2003; Sato et al. 2013). This partially unfolded state of the β -hairpins is thought to represent an intermediate step in pre-pore to membrane-inserted complex transition for PFO (Sato et al. 2013). The partial alignment of the β -hairpins in the pre-pore complex may constitute a kinetic barrier that deters the insertion of incomplete rings favoring the formation of complete pre-pore complexes.

The unfurling of the two α -helical bundles into two β -hairpins is favored by the formation of multiple hydrogen bonds, both between hairpins within a PFO monomer and between hairpins on adjacent monomers (Fig. 4.4). Crosslinking experiments revealed that the β -hairpins in the inserted β -barrel adopt a ~20 degree angle to the plane of the membrane, and the adjacent inter-monomer strands align themselves with a shift of two amino acids (Fig.

4.4, Sato et al. 2013). As mentioned above, PFO oligomerization is aided by the proper alignment of β -strands from adjacent monomers via π -stacking interaction between the completely conserved Y181 and the highly conserved F318. Inspection of the extended hairpins in the β -barrel conformation (Fig. 4.4) revealed another potential π -stacking interaction that may act to stabilize the hairpins in their extended conformation. These are the completely conserved F211 in the transmembrane hairpin 1 (TMH1) and highly conserved F294 (present in all but vaginolysin, lectinolysin, and intermedilysin of the 30 members) in the transmembrane hairpin 2 (TMH2). Interestingly, the F211C modification decreased the hemolytic activity of PFO (Shepard et al. 1998) and the PFO derivative containing the F294C modification could not be stably produced (Shatursky et al. 1999).

The vertical collapse of D2 to bring D3 closer to the membrane surface is another important step in pore formation (Czajkowsky et al. 2004; Ramachandran et al. 2005). In the pre-pore complex, PFO is positioned perpendicular to the membrane leaving D3 about 40 Å above the membrane surface (Ramachandran et al. 2005; Tilley et al. 2005). In this position, the β -strands that form the pore would barely reach the membrane surface and could not penetrate the membrane. The required vertical collapse of D2 would drop D3 to the membrane surface and allow the β -hairpins to punch through the membrane and form a β -barrel. Unfortunately, little is known about the mechanism of the transmembrane β -barrel insertion.

Formation of a pre-pore complex and formation of hydrogen-bonds between adjacent β -strands helps the toxin to overcome the energetic barrier of inserting non-hydrogen bonded β -hairpins (Heuck et al. 2010). The insertion of incomplete rings may also occur, especially when free monomers are no longer available to complete the circular complex. Trapped metastable arc-like structures may form a pore by themselves, but the formation of a lipid edge at one side of the pore is not energetically favored, and the arcs would have a tendency to associate with other arcs or any proximal complete rings (Palmer et al. 1998; Gilbert 2005; Praper et al. 2010).

One of the most intriguing aspects of the CDCs cytolytic mechanism is what happens to the lipids that are displaced to form the pore. The insertion of the β -barrel requires the displacement of more than 1000 lipid molecules from the membrane (Heuck et al. 2001). It is not clear how such a large amount of molecules are removed from the center of the pre-pore complex, but the hydrophilic nature of the inner portion of PFO the β -barrel could aid in this process.

4.5 Conclusions and Future Perspectives

Despite the lack of high resolution structures for intermediates or for the final membrane-inserted complex, the pore formation mechanism of the CDCs is becoming one of the better understood mechanisms among pore-forming toxins. The elucidation of the three-dimensional structure of the water-soluble PFO monomer (Rossjohn et al. 1997) in combination with the use of site-directed mutagenesis and fluorescence spectroscopy techniques played a critical role in these advances (Heuck and Johnson 2002; Johnson 2005).

More recently a lot of attention has been focused on the study of PFO-cholesterol interaction. These studies revealed that not only changes in cholesterol levels may increase or decrease the ability of the toxin to bind to membranes (Ohno-Iwashita et al. 1992; Heuck et al. 2000), but also changes in the phospholipid composition of the lipid bilayer (Ohno-Iwashita et al. 1992; Flanagan et al. 2002; Nelson et al. 2008; Flanagan et al. 2009; Moe and Heuck 2010; Sokolov and Radhakrishnan 2010).

Accessibility of cholesterol at the membrane surface seems to be a key factor to trigger PFO binding. However, simply having accessible sterol molecules is not enough to stabilize PFO monomers at the membrane surface. Different “grades” of cholesterol accessibility are required to trigger binding of PFO derivatives with modifications at the conserved loops of D4 (Johnson et al. 2012; Moe and Heuck 2010). More studies are required to elucidate the molecular details of how cholesterol accessibility modulates PFO binding.

A detailed understanding of the cytolytic mechanism combined with the ability to modify the toxin and produce novel PFO derivatives, have now opened the door for the development of tools to study cell biology. Non-lytic PFO derivatives have been developed to study cholesterol accessibility at the surface of cell membranes (Ohno-Iwashita et al. 2010; Johnson et al. 2012). Other lytic PFO derivatives have been used to specifically permeabilize the plasma membrane of cells to study the biochemistry of intact organelles in their native environment (e.g., mitochondria Divakaruni et al. 2013). Moreover, the striking on-and-off binding properties of PFO have been exploited to study the role of cholesterol in cell physiology and the intracellular traffic of cholesterol (Pocognoni et al. 2013; Das et al. 2013). It is clear that the understanding of the molecular mechanism of these fascinating proteins secreted by pathogens has had, and will have a great impact on the studies of biochemistry and physiology in whole cells (Divakaruni et al. 2013), as well as on the studies of cholesterol-dependent mechanism in cell biology (Reid et al. 2004; Pocognoni et al. 2013; Das et al. 2013).

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Abbreviations

CDCs	cholesterol-dependent cytolysins
PFO	perfringolysin O
D1, D2, D3, and D4	domain 1, domain 2, domain 3, and domain 4
L1, L2, and L3	loop 1, loop 2, and loop 3
TMH1 and TMH2	Trans-membrane hairpin 1 and Trans-membrane hairpin 2

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Fig. 4.1.

Cartoon representation of the different steps/intermediates identified for the PFO mechanism of pore formation. A water-soluble monomer is secreted by the bacterium and binds to the target membrane via D4 (step I). Membrane-bound monomers diffuse across the membrane surface interacting transiently until they form a stable dimer (step II). The initial dimer starts growing with the addition of other monomers until completion of a circular ring or pre-pore complex (step III). In the last step, each monomer inserts two amphipathic transmembrane hairpins into the bilayer aided by the vertical collapse of D2 forming a large β -barrel pore (step IV). Domains are numbered and color coded as follows: D1 (green), D2 (yellow), D3 (red), and D4 (blue). Only a few PFO monomers are shown in the side view at the bottom to simplify the figure. On the top is a schematic top view for each step of the pore formation mechanism shown below. The membrane bilayer is depicted by a gray rectangle

Fig. 4.2.

Three dimensional structure of PFO showing the location of important elements that modulate cholesterol interaction (A) ribbon representation of the water-soluble PFO monomer with domains colored as indicated in Fig. 4.1. Also in color are three key residues that influence cholesterol interaction T490, L491, R468 (Red), and the Trp rich loop (TRP, orange). (B) A view of the tip of D4 from the bottom showing the exposed surface of the Trp rich loop residues (orange), the three small loops (green), and the residues indicated in A (red). (C) The ribbon rendering of the same bottom view of D4 shown in B. PFO (1PFO) structure representation was rendered using PyMol (DeLano Scientific LLC)

Fig. 4.3.

Cholesterol accessibility changes at the membrane surface as a function of the lipid composition. (A) when interactions with other membranes components saturate the accessibility of cholesterol increases at the membrane surface. (B) At constant cholesterol concentration, an increase in the number of double bonds on the acyl chains of the phospholipids increases cholesterol accessibility. (C) At constant cholesterol concentration, an increase in the concentration of phospholipids with smaller head groups increases cholesterol accessibility. The red lines depict a hypothetical increase on cholesterol

accessibility. The actual change on cholesterol accessibility for each schematic graph may differ from a simple linear response. Some cholesterol molecules are colored red to visualize the increase on accessibility but they are indistinguishable from other cholesterol molecules in the membrane

Fig. 4.4.

A schematic view that depicts the position and orientation of the transmembrane hairpins (TMH1 and TMH2) of PFO in the membrane-inserted complex as determined by Sato et al (2013). The tilted membrane and the rectangle representing the rest of the PFO molecule are depicted in gray and blue, respectively. The amino acids that compose the D3 β -sheet and the transmembrane hairpins are depicted by their single letter code and color-coded according to conservation in the CDC family. Amino acids conserved in more than 90% of the 28 CDC members are shown in red, in orange if conservation was higher than 70% but lower than 90%, and in black if not highly conserved. Highlighted in green are the aromatic amino acids that are thought to be involved in π -stacking interaction that stabilize PFO pre-

pore confirmation and help to align individual PFO monomers for pore formation (see text for details)

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