

Functional Characterization of  
*Arcanobacterium pyogenes* Pyolysin in an  
Oligomeric Form, and the Binding of  
CAMP Factor to IgG.

by

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# Abstract

The work described in this thesis deals with two pore forming toxins, namely *Arcanobacterium pyogenes* pyolysin and *Streptococcus agalactiae* CAMP factor.

Pyolysin (PLO) belongs to the homologous group of cholesterol-dependent cytolysins. In chapter 2, it is shown that PLO can form small oligomers in solution, without the requirement for any membranes or membrane lipids. These small oligomers may aggregate into larger ones on membranes; however, in solution, they apparently do not grow by addition of further monomers, as their size is virtually unaffected by variations of incubation time or toxin concentration. The small, solution-derived oligomers retain hemolytic activity. The membrane lesions observed by electron microscopy are similar to those that are formed by monomeric PLO, except that they are mostly incomplete and arc-shaped, as opposed to the predominantly ring-shaped ones formed by monomeric PLO when directly incubated with membranes. This structural difference corresponds to a detectable difference in functional pore size, as determined by marker release experiments. Thus, arc-shaped PLO oligomers may form functional pores of reduced size.

In chapter 3, we show that liposomes that contain phosphatidylcholine and ceramide but no cholesterol or other sterol are susceptible to the cholesterol-dependent cytolysin pyolysin. Pyolysin, at a low rate, forms small oligomers in solution. The solution-derived oligomers are more effective on ceramide-containing liposomes than the monomeric

toxin. In contrast, they have much lower activity on liposome membranes that contain cholesterol but no ceramide. Our findings therefore show that at least one member of the ‘cholesterol-dependent cytolysins’ is in fact not strictly dependent on the sterol. In addition, in conjunction with previous data, they suggest that the requirement for cholesterol involves early or intermediate stages of oligomer formation, rather than the final event of membrane insertion.

Chapter 4 of this thesis concerns *Streptococcus agalactiae* CAMP factor. CAMP factor has previously been reported to bind the Fc fragments of immunoglobulin G (IgG) and has therefore also been called ‘protein B’, in analogy to protein A of *Staphylococcus aureus*. We attempted to characterize the interaction of protein B with IgG in more detail. In contrast to protein A, CAMP factor does not inhibit the activation of complement by hemolysin antibodies bound to sheep red cell surfaces. IgG also failed to inhibit the co-hemolytic activity of CAMP factor, which it displays on sphingomyelinase-treated sheep red cells; this is in disagreement with previous findings. After co-incubation, CAMP factor and IgG were cleanly separated by gel filtration. Therefore, CAMP factor does not detectably bind to IgG.

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# Dedications

To my beloved wife and my little two angels

To my mother and brothers

To the soul of my father

To everyone supported me

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# Abbreviations

C-12 Ceramide : N-lauroyl-D-erythro-shingosine;

CD : Circular Dichroism;

CDC : Cholesterol Dependent Cytolysin;

CYT : Cytotoxin;

DMPC : 1,2-dimyristoyl-*sn*-glycerol-3-phosphocholine;

EM : Electron microscopy;

EF : Edema Factor;

FRET : Fluorescence Resonance Energy Transfer;

GBS : Group B Streptococcus;

GPI : Glycosylphosphatidylinositol;

HBS : HEPES Buffer Saline;

IgG : Immunoglobulin G;

IgM : Immunoglobulin M;

ILY : intermedilysin;

IPTG : Isopropyl- $\beta$ -D-thiogalactopyranoside;

LB : Luria bertani broth;

LF : Lethal Factor;

LLO : Listeriolysin;

MDT : Membrane Damaging Toxins;

OD : Optical density;

PA : Protective Antigen;

PBS : Phosphate Buffered Saline;

PC : Phosphatidylcholine;

PDB : Protein Data Bank;

PFO : Perfringolysin;

PFT : Pore Forming Toxins;

PLO : Pyolysin;

PMN : Polymorphonuclear cell;

RPM : Revolution Per Minute;

SDO : Solution Derived Oligomers;

SDS : Sodium Dodecyl Sulfate;

SDS-PAGE : Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis;

SLO : Streptolysin O;

TBS : Tris Buffer Saline;

UV : Ultra Violet;

VCC : *Vibrio cholera* Cytolysin;

# **Chapter 1**

## **Introduction and Literature Review**

## 1.1 Introduction to Pore-forming Toxins

An evolutionary outcome of the host-pathogen relationship between higher eukaryotes and bacteria is the development and production of virulence factors that enable the pathogen to invade the host. These bacterial virulence factors are most often protein molecules (or toxins).

Of the more than 300 protein toxins characterized to date, at least one third act by disrupting membranes (and are therefore known as membrane-damaging toxins (MDT))[3]. MDT can be classified into three types, based on their mechanisms of action against target cell membranes:

1. Enzymatically active cytolysins, which act on lipids in the cell membrane, leading to its degradation.
2. Tensio-active cytolysins, which solubilize cell membranes by a detergent-like action.
3. Pore-forming toxins [13, 4].

In contrast to tensio-active cytolysins, pore-forming toxins form discrete pores or channels in cell membranes, disrupting the permeability barrier and leading eventually to cell death (Fig. 1.1). Pore-forming toxins typically behave as hydrophilic proteins prior to membrane binding. In order for these water-soluble proteins to insert into their target

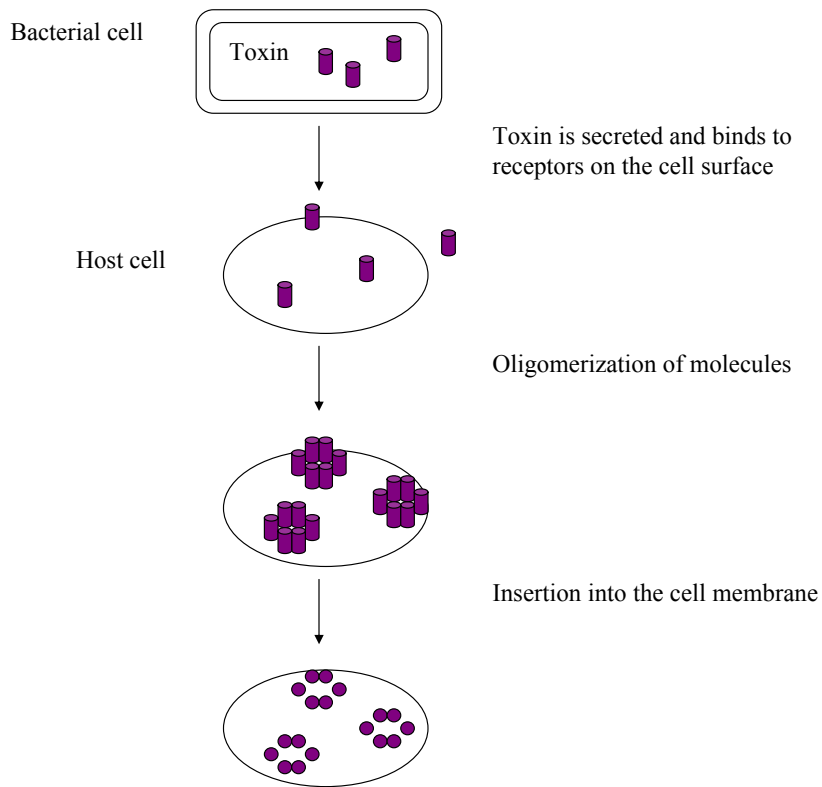


Figure 1.1: The general steps involved in membrane pore formation by PFTs.

membranes, they must undergo a series of conformational changes that will expose or generate new hydrophobic surfaces which can penetrate the apolar core of the lipid bilayer.

Organisms other than bacteria can also produce cytolytic toxins. These organisms include certain insects, poisonous reptiles and stinging marine invertebrates.

In addition to 'pure' pore-forming toxins, there also are bacterial toxins that contain

pore-forming subunits or domains in addition to enzymatic ones; well-known examples are cholera toxin, anthrax toxin, and diphtheria toxin. The toxic enzyme components of these toxins act intracellularly, and the pore-forming components enable the enzymes to cross the membrane barrier and reach the cytosol. Toxins that follow this functional paradigm are often referred to as 'A-B toxins'; the 'A' refers to the enzymatically active component, whereas the B represents the membrane-binding and pore-forming component.

## 1.2 Classification of Pore Forming Toxins

Pore-forming toxins (PFTs) can be classified in several ways. For instance, they may be classified according to the organism that produces them, or to some specific characteristic that is required for activity. However, the most useful classification is based on structural features as described by Gouaux in 1997 [55]. At the most general level of this scheme, pore-forming toxins are considered as either  $\alpha$ - or  $\beta$ -PFTs.

$\alpha$ -PFTs are predicted to form pores using  $\alpha$ -helices. These toxin molecules tend to be highly  $\alpha$ -helical, with the larger ones having pore-forming domains consisting of a three-layer structure of up to ten  $\alpha$ -helices sandwiching a centrally positioned hydrophobic helical hairpin (Fig. 1.2). This hairpin is thought to drive the initial steps of the insertion process.

Some examples of members in this class are the pore-forming colicins [124], *Pseudomonas aeruginosa* exotoxin A [1], the insecticidal *Bacillus thuringiensis*  $\delta$ -endotoxins (Cry)[86] and diphtheria toxin [27].

Proteins related to bacterial pore forming toxins also occur in eukaryotic organisms. An example are the members of the Bcl-2 and Bcl-x family of apoptotic proteins, among which Bcl-2 was the first one to be identified in B-cell leukemic cells. These proteins have been shown to be structurally similar to  $\alpha$ -PFTs and also form ion channels in a fashion similar to the toxins, although there is no homology at primary and structure level [95, 105].

The other major class of pore-forming toxins, the  $\beta$ -PFTs, have been shown or are predicted to insert into membranes to form  $\beta$ -barrels.  $\beta$ -PFTs have quite different tertiary and quaternary structures from  $\alpha$ -PFTs. The monomeric structures are more compact than those of  $\alpha$ -PFTs. As indicated by their name, all  $\beta$ -PFTs consist mainly of  $\beta$ -structures, (Fig. 1.3). The class includes *Aeromonas hydrophila* aerolysin, *Clostridium septicum*  $\alpha$ -toxin, *Staphylococcus aureus*  $\alpha$ -hemolysin, *Pseudomonas aeruginosa* cytotoxin, the protective antigen component of anthrax toxin, the insecticidal *Bacillus thuringiensis* Cyt  $\delta$ -endotoxins, and the family of cholesterol-dependent cytolysins.

The oligomers of  $\beta$ -PFTs are usually fairly stable and remain intact even after solubilization of membranes with detergents. For example,  $\alpha$ -hemolysin heptamers are stable



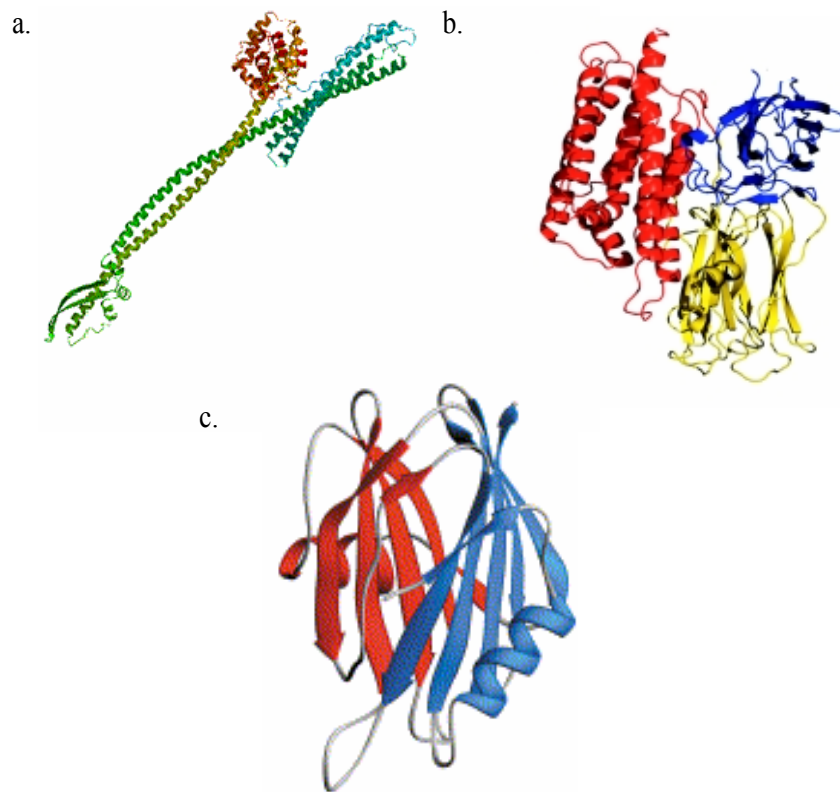


Figure 1.2: Ribbon representations of  $\alpha$ -PFT structures. (a) Colicin Ia (1CII)[178]. (b) Cry insecticidal  $\delta$ -endotoxin (1JI6) [86]. (c) *Actinia equina* equinatoxin II (1IAZ)[6]. These structures were obtained from the Protein Data Bank (PDB) and rendered using RASTOP software (RasTop 2.2 By Philippe Valadon, Version 2.2, January 2007).

even in the presence of the denaturing detergent sodium dodecylsulfate (SDS) at up to 65 °C [174]. Because of this, the pores formed by  $\beta$ -PFTs are in many cases more thoroughly characterized. For instance,  $\alpha$ -hemolysin, anthrax toxin protective antigen and aerolysin pores are heptamers with diameters of 1.5 to 4.6 nm [159, 127, 179], whereas the cholesterol-dependent cytolysins can form pores composed of up to 50 monomers with a diameter up to 30 nm [116, 104, 17].

In contrast, oligomers of  $\alpha$ -PFT are far less stable and fall apart when the membranes are exposed to detergent. The structures of these pores are therefore far less well characterized, and even their subunit stoichiometries are unknown.

In the following section, we will briefly review the current knowledge about the structure and molecular mechanisms of  $\beta$ -PFTs.

### **1.3 $\beta$ -Pore Forming Toxins**

$\beta$ -Pore Forming Toxins share a broadly similar mode of action. All  $\beta$ -PFTs studied so far are produced and secreted by bacterial cells as stable water-soluble monomers; upon binding to their target membranes, they start to oligomerize and insert into the cell membranes and form oligomeric trans-membrane pores. The following subsections will provide a brief review of representative  $\beta$ -PFTs.

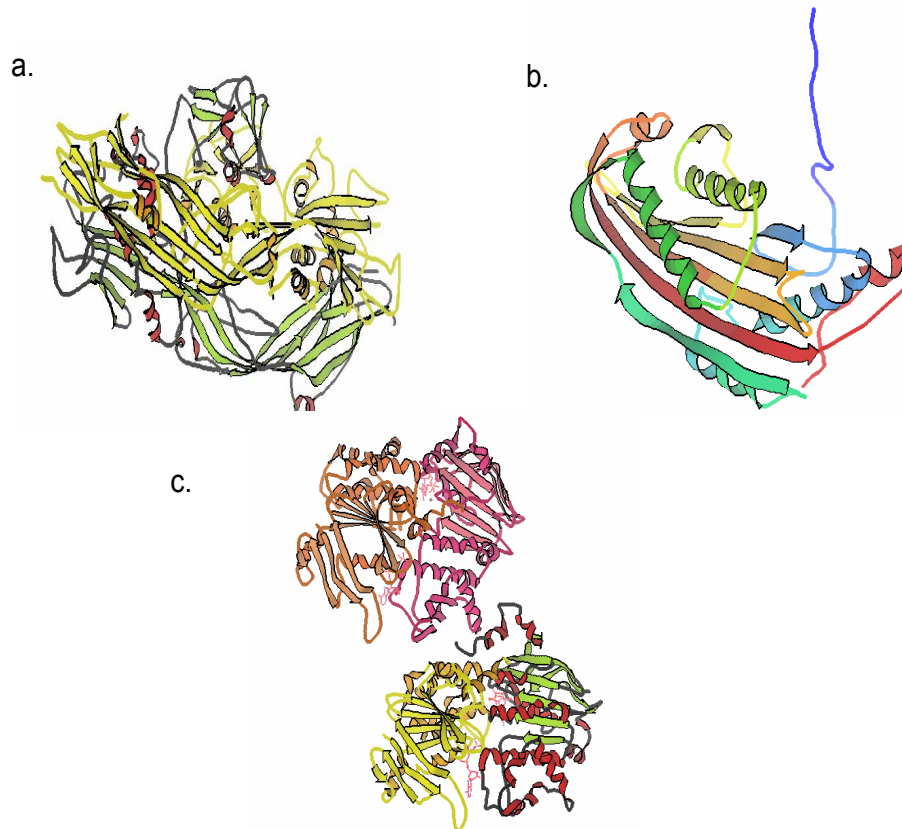


Figure 1.3: Ribbon representations of  $\beta$ -PFT structures. (a) *Aeromonas hydrophila* proaerolysin monomer (1PRE)[125]. (b) *Bacillus thuringiensis* Cytotoxin (1CBY) . (c) *S. aureus*  $\alpha$ -hemolysin monomer . These structures were rendered from PDB records using RASTOP software (RasTop 2.2 By Philippe Valadon, Version 2.2, January 2007).

### 1.3.1 *Staphylococcus aureus* $\alpha$ -Hemolysin

Staphylococcal  $\alpha$ -hemolysin is produced as a water-soluble, single-chain polypeptide of 293 amino acid residues, with no cysteine [56]. This toxin can self-assemble on membranes to form a heptameric pore. The mechanism of pore-formation by  $\alpha$ -hemolysin has been extensively studied. The structure of the fully assembled and inserted  $\alpha$ -hemolysin heptamer has been solved, and the monomer structure can be inferred from the crystal structures of homologous staphylococcal toxins in their monomeric forms [159].

The pore formed by  $\alpha$ -hemolysin has a mushroom-shaped structure consisting of a cap, a rim and a stem domain (Fig. 1.4) [159]. The cap has a diameter of about 10 nm and is composed of  $\beta$ -sandwich structure. Together with the rim it forms the core of the protein. The stem or transmembrane domain is constructed of a 14-stranded  $\beta$ -barrel formed from 7  $\beta$ -hairpins, with each hairpin contributed by a single monomer [159].

Differences in susceptibility to permeabilization by  $\alpha$ -hemolysin have been observed between different mammalian cells. For example, rabbit erythrocytes are lysed by nanomolar concentrations of the toxin, whereas lysis of human erythrocytes first occurs at 200-fold higher concentrations; this has been ascribed to the existence of saturable high-affinity binding sites on the rabbit cells [30, 60, 26]. Some studies have suggested the existence of a protein receptor for  $\alpha$ -hemolysin [171, 123]. Recently, however, Valeva *et al.* provided evidence that clustered phosphocholine head groups may serve as sites for

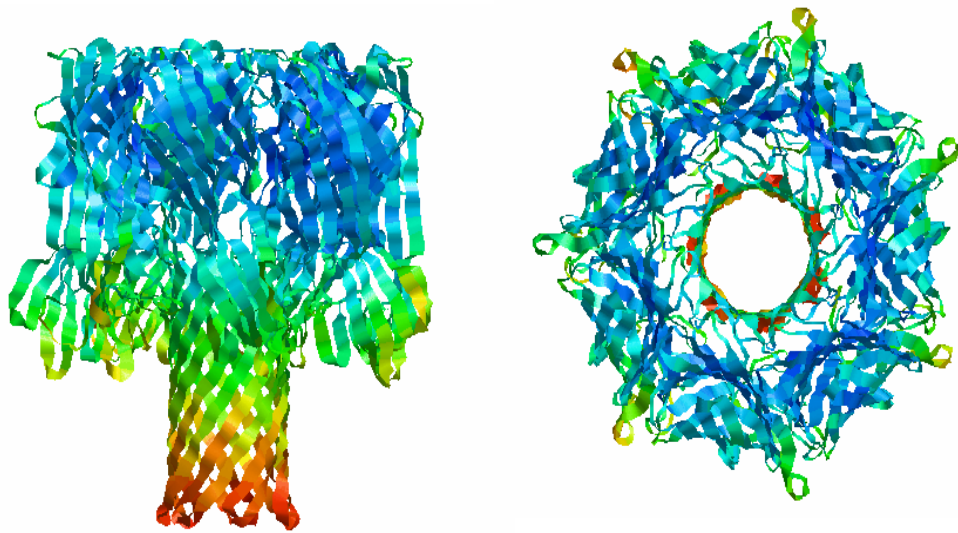


Figure 1.4: *Staphylococcus aureus*  $\alpha$ -hemolysin heptameric pore viewed down and perpendicular to the pore axis (7AHL) [159]. This structure was rendered from PDB records using RASTOP software (RasTop 2.2 By Philippe Valadon, Version 2.2, January 2007).

binding and assembly for  $\alpha$ -hemolysin. According to these authors, varying susceptibilities of different cell species toward  $\alpha$ -hemolysin is likely due to variations in the number of phosphocholine head group clusters that are exposed to the extracellular environment [163].

After initial membrane binding,  $\alpha$ -hemolysin first oligomerizes to form a heptameric non-lytic ‘pre-pore’ complex, which subsequently inserts into the membrane to form the pore. This pore formation is the result of multiple steps and cooperative process in which the environment of residues located in the N-terminus changes, as well for the residues of the rim and the central domain [168, 165, 164, 169].

Studies that were conducted before the X-ray structure of the  $\alpha$ -hemolysin pore was resolved, showed that the a deletion mutations in the N-terminus or within the glycine-rich stem region (residues 118-142) were arrested in the pre-pore stage [170, 175, 174, 173], indicating that these two regions are important in the pre-pore to pore transition. Afterwards, the crystal structure of  $\alpha$ -hemolysin heptamer showed that the central glycine-rich stem region from each monomer contributes a two-stranded antiparallel  $\beta$ -sheet to the  $\beta$ -barrel of the trans-membrane pore, which therefore comprises 14  $\beta$ -strands in all.

Based on sequence identity and other similarities, many toxins are considered as homologous to  $\alpha$ -hemolysin. Homologous toxins include the staphylococcal leukocidins,

*Clostridium perfringens*  $\beta$ -toxin and *Bacillus cereus* hemolysin II and cytotoxin K [3].

### **1.3.2 Anthrax Toxin Protective Antigen (PA)**

*Bacillus anthracis* produces three monomeric proteins that collectively constitute anthrax toxin. This A-B toxin is somewhat unusual in having two A components, namely the lethal factor (LF) and the edema factor (EF). The B component is represented by a 83 kDa pore-forming protein called the 'protective antigen'. This protein acts as the vehicle for the delivery of LF and EF to the host cells cytosol. Host cell invasion begins when protective antigen (PA) binds to cellular membrane receptor, is proteolytically activated, and oligomerizes to form a ring shaped pre-pore heptamer (Figure 1.5). Lethal factor and edema factor then compete for the binding sites on the PA heptamer, resulting in the formation of a complete anthrax toxin complex with 1 to 3 LF and/or EF molecules per heptamer [32, 101, 102]. The resulting complexes are transported to the endosome. There, under the influence of the acidic pH, the PA pre-pore is converted to a mature trans-membrane pore, and LF and EF unfold and traverse the endosomal membrane across the pore into the cytosol.

The crystallographic structure of monomeric PA shows a long, flat molecule containing four folding domains (Figure 1.6). Domain 1 contains a cleavage site for the protease furin as well as binding sites for two calcium ions. The calcium ions are important in the

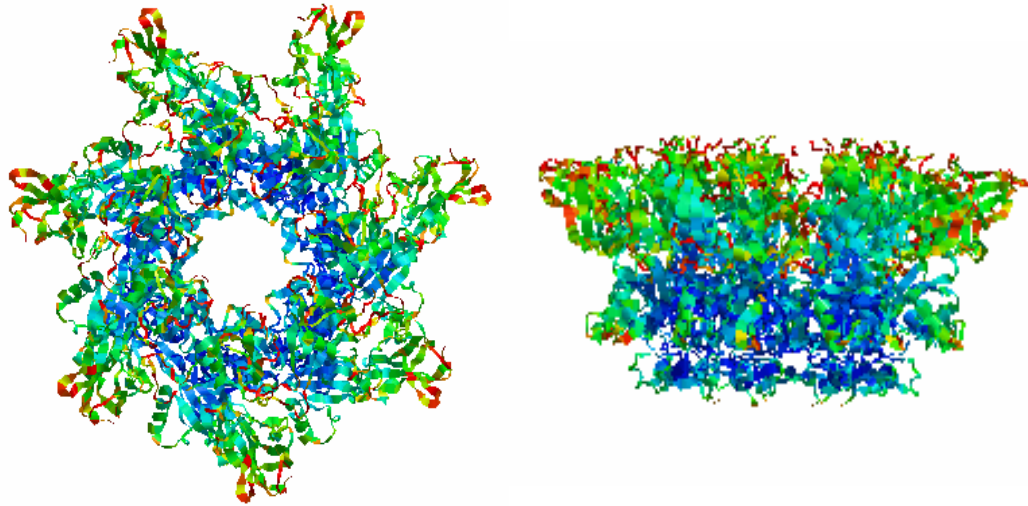


Figure 1.5: Protective antigen heptamer viewed down and perpendicular to the pore axis (1TZO) [180]. These structures were rendered from PDB records using RASTOP software (RasTop 2.2 By Philippe Valadon, Version 2.2, January 2007).

maintenance of the proper conformation for oligomerization and for ligand binding after proteolysis [127, 47].

Domain 2 consists of several long  $\beta$ -strands. The main function of this domain is to form a trans-membrane pore to serve as the portal of entry of EF and LF into the cytosol. Recently, domain 2 has been shown to participate in binding of PA to the cellular receptor [79, 145, 11]. Domain 3 is believed to mediate self-association of PA. Mutations in this



domain strongly inhibit oligomerization of PA [79, 103]. Domain 4 is primarily involved in binding to cellular receptors, and this domain has little contact with the rest of the protein [156, 137].

The transition of PA from the pre-pore to trans-membrane pore has been shown to be dependent on an acidic pH and the specific receptor to which it is bound. Similar to  $\alpha$ -hemolysin, the PA pore is heat stable and SDS-resistant. The pre-pore of PA is not SDS-resistant; it may be kept in aqueous solution at alkaline pH [84, 93]. When the environment becomes acidic, the water-soluble heptamer precipitates and becomes resistant to SDS. This precipitation is probably indicative of the hydrophobic domains becoming exposed. Moreover, the addition of a non-denaturing detergent such as deoxycholate will also induce transition of the pre-pore to an SDS-resistant heptamer, which might resemble the conformation of the membrane-inserted pore [93, 94].

### **1.3.3 *Vibrio cholerae* Cytolysin**

*Vibrio cholerae* is a Gram-negative bacterium that colonizes the small intestine [144], and it is the causative agent of the often lethal disease cholera. In addition to cholera toxin, highly pathogenic strains of *V. cholerae* produce a pore-forming toxin, known as *V. cholerae* cytolysin (VCC).

Despite of the differences between VCC and PFTs in functional properties, molecu-

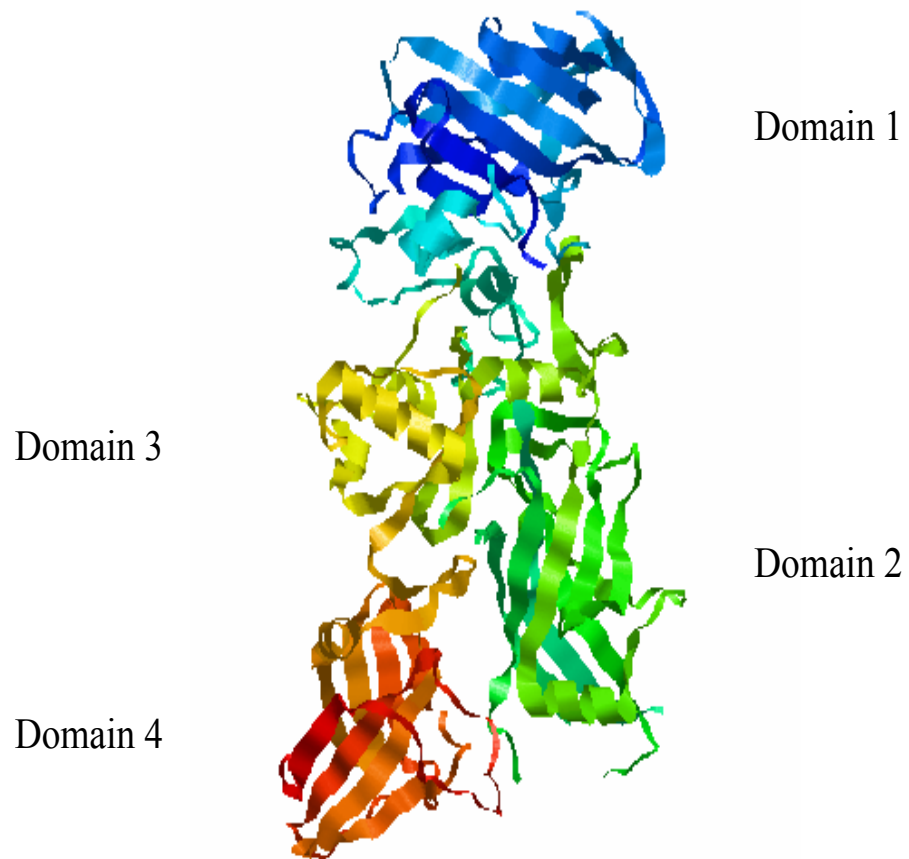


Figure 1.6: Ribbon representative of Monomeric PA (1ACC) [180]. This structure was rendered from PDB records using RASTOP software (RasTop 2.2 By Philippe Valadon, Version 2.2, January 2007).

lar mass and low sequence identity, the X-ray structure of the VCC pro-toxin, resolved in 2005, confirmed that VCC belongs to the  $\beta$ -barrel PFTs [117]( Figure 1.7). In addition, Valeva and colleagues in 2005 have provided further evidence in favor of a  $\beta$ -barrel organization of the transmembrane pore of VCC using site-specific labeling with polarity-sensitive fluorophores bound to cysteine mutants within the putative pore region [167].

VCC is secreted as a pro-toxin ( 80 kDa) that is proteolytically cleaved to yield the active 65 kDa toxin. The estimated size of the pore formed by VCC is 1.2-1.6 nm [64], and is composed of heptameric oligomers [58].

Cholesterol in the target cell and liposomal membranes was reported to play an important role in the assembly of the toxin oligomers needed for pore formation by triggering oligomerization [64, 65]. In addition, cholesterol was proposed to be the binding site of VCC on target membrane specially after the observation that VCC binds more efficiently to phosphatidylcholine (PC) liposomes containing cholesterol than to liposomes composed of PC only [64, 183]. However, it has been reported that VCC can bind to sphingolipids [184], glycophorin B [181] and glycoproteins [143].



Figure 1.7: Ribbon representation of VCC pro-toxin (1XEZ) [117]. This structure was rendered from PDB records using RASTOP software (RasTop 2.2 By Philippe Valadon, Version 2.2, January 2007).

## 1.4 Cholesterol-Dependent Cytolysins

The major part of the research described in this thesis was conducted on pyolysin, which belongs to the homologous family of the cholesterol dependent cytolysins. Therefore, this family of toxins will be reviewed separately, despite the fact that it forms a subset of the  $\beta$ -pore forming toxins.

The cholesterol-dependent cytolysins (CDCs) constitute a large family of pore-forming toxins that are produced by more than 20 species from the Gram-positive bacterial genera *Clostridium*, *Streptococcus*, *Listeria*, *Bacillus*, *Paenibacillus* and *Arcanobacterium* (see table 1.1)[3, 162, 51]. Peculiar features of CDCs include their absolute dependence on membrane cholesterol for their cytolytic activity and the formation of very large pores, which are composed of up to 50 monomers and are up to 30 nm in diameter [116, 104, 17].

### 1.4.1 CDC Monomer Structure

In 1997 and 2005, the crystal structures of the two cholesterol-dependent cytolysins perfringolysin (PFO) and intermedilysin (ILY) were resolved [138, 128]. As shown in figure 1.8, the monomer structures are rich in  $\beta$ -sheet, have an elongated shape, and are comprised of four domains. Domain 1, located at one end of the molecule, has both  $\alpha$ -helix and  $\beta$ -sheet content. Domain 2 is a long curved single layer of anti-parallel  $\beta$ -sheet

Table 1.1: List of known members of the cholesterol-dependent cytolysins [18].

Organism	Toxin	Abbreviation
<i>Arcanobacterium pyogenes</i>	Pyolysin	PLO
<i>Bacillus cereus</i>	Cereolysin	CLY
<i>B. thuringiensis</i>	Thuringiolysin O	
<i>Brevibacillus laterosporus</i>	Laterosporolysin	
<i>Clostridium biferementans</i>	Biferementolysin	
<i>C. botulinum</i>	Botulinolysin	
<i>C. chauvoei</i>	Chauvoelysin	
<i>C. histolyticum</i>	Histolyticolysin	
<i>C. novytype A</i>	Oedematolysin	
<i>C. perfringens</i>	Perfringolysin O	PFO
<i>C. septicum</i>	Septicolysin O	
<i>C. tetani</i>	Tetanolysin	
<i>Listeria ivanovi</i>	Ivanolysin O	ILO
<i>L. monocytogenes</i>	Listeriolysin O	LLO
<i>L. seeligeri</i>	Seeligerilysin O	LSO
<i>Paenibacillus alvei</i>	Alveolysin	ALY
<i>Streptococcus canis</i>	Streptolysin O	
<i>S. equisimilis</i>	Streptolysin O	
<i>S. intermedius</i>	Intermedilysin	ILY
<i>S. pneumoniae</i>	Pneumolysin	PLY
<i>S. pyogenes</i>	Streptolysin O	SLO
<i>S. suis</i>	Suilysin	SLY

connecting domains 1 and 4. Domain 3 extends from the core  $\beta$ -sheet of domain 1, and adopts  $\alpha$ - $\beta$  topology with a core consisting of 5 strands of anti parallel  $\beta$ -sheet. Domain 4 contains the highly conserved tryptophan-rich undecapeptide sequence (ECT-GLAWEWWR) which appears to mediate the binding of some CDCs to cholesterol-containing membranes and also has been shown to insert into membranes [59, 109].

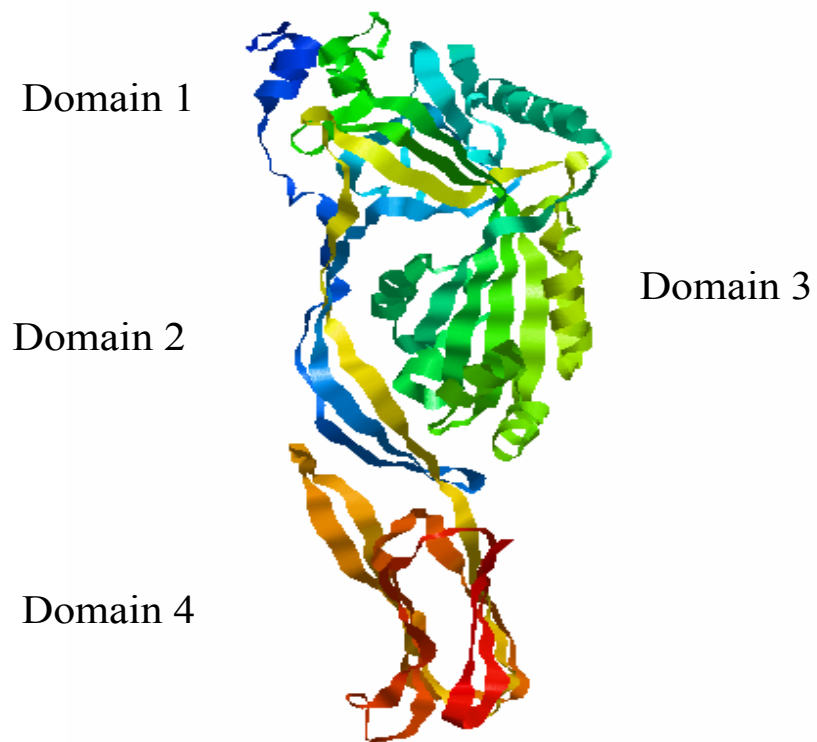


Figure 1.8: Crystal structure of PFO. A ribbon representation indicating the location of domains (1PFO). This structure was rendered from PDB records using RASTOP software (RasTop 2.2 By Philippe Valadon, Version 2.2, January 2007).

### **1.4.2 The Role of Cholesterol in the Activity of CDCs**

Traditionally, the role of cholesterol in pore formation was believed to be in the binding step. This assumption was supported by two main observations: Firstly, the inhibition of the hemolytic activity of the toxins by incubation with free cholesterol [5, 31, 35, 48, 62, 100, 98, 129, 151, 152, 176], and secondly, the observation of direct binding of CDCs to pure cholesterol and to liposomes containing cholesterol [67, 71, 115].

Studies with liposomes have shown that the total concentration of cholesterol in liposome membranes required for binding of CDCs is quite high, on the order of 40% by mole of the total membrane lipid. Interestingly, it has been shown that the transition from little or no binding to full binding to lipid-cholesterol liposomes occurs within a narrow range of cholesterol concentration [113].

The dogma that cholesterol is the receptor for CDCs has been re-evaluated in the current decade, prompted by the discovery that the CDC intermedilysin (ILY) specifically recognizes human cells; this specificity is based on the ability of ILY to bind to the human form of the glycosylphosphatidylinositol-anchored protein CD59, indicating that at least with this toxin cholesterol does not function as the sole receptor [50].

The same authors examined the role of cholesterol in the three CDCs streptolysin O (SLO), ILY and PFO. It was found that the depletion of cholesterol from erythrocyte membrane to an extent of 90% abolished the haemolytic activity of those toxins, while



the binding of SLO and ILY reportedly remained largely unchanged. The extent of PFO binding decreased tenfold, but binding was still detectable. Nevertheless, the cytolytic activity of all three toxins was lost, and it was observed that the toxins did oligomerize but remained arrested at the pre-pore stage. Based on these findings, cholesterol was proposed to be involved in and essential for membrane insertion of the oligomer rather than monomer binding [49]. In contrast to these assertions, Nagumune and colleagues reported that SLO has significant affinity to liposomes consisting of lecithin plus cholesterol but not to liposomes lacking cholesterol [107]. In addition, they found that ILY had no significant affinity to lecithin liposomes with or without cholesterol. Interestingly, ILY showed no specific binding to liposomes of human erythrocyte membrane lipids [107]. These findings confirm the exceptional binding behavior for ILY but, for SLO, reaffirm the role of cholesterol in binding to liposomes reported previously for PFO by Ohno-Iwashita in 1988 [114]. Therefore, consensus on which aspects of toxin activity are dependent on cholesterol has not yet been reached.

### **1.4.3 The Pore Forming Mechanism of CDCs**

As members of the  $\beta$ -pore forming toxins, CDCs share the same general mechanism of pore-formation outlined before. In this subsection, I will try to highlight some structural details associated with this process.

Upon binding to cell membranes, monomers diffuse laterally to initiate oligomeriza-

tion. Discernible structural rearrangements occur in domain 3 during this process[38], including the rotation of a loop, comprised of a short  $\beta$ -strand and  $\alpha$ -helix, away from the edge of the D3 core  $\beta$ -sheet to allow oligomerization of the monomers [132].

These oligomerized monomers form what has been termed the pre-pore complex [53, 52, 155, 61]. These pre-pores consist of toxin molecules in largely the same conformation as before membrane binding [33, 160].

Within the pre-pore oligomers, the main interaction occur through domain 1 and 3 [177, 131, 160]. The subsequent oligomerization of the monomers into pre-pore complexes aligns the twinned D3 trans-membrane  $\beta$ -hairpins (TMH) segments of adjacent monomers and triggers their insertion into the membrane to form a large trans-membrane  $\beta$ -barrel as shown in Figure 1.9 [154, 153].

Over the last 30 years , both circular oligomeric rings and incomplete ‘arc’ rings of CDCs have been observed on membranes using electron microscopy [99, 31, 16, 112, 150, 121]. This observation, which is an unusual feature not observed with most other toxins, sparked widespread debate among scientists about the mechanism of assembly of the CDC oligomeric pore complexes in the membrane bilayer. models have been proposed. Tweten and his colleagues, found that PFO can indeed assemble into a large oligomeric complex in the absence of significant insertion of its membrane-spanning *beta*-hairpins [155] so they proposed that the formation of a complete, circular, oligomeric ‘pre-pore’

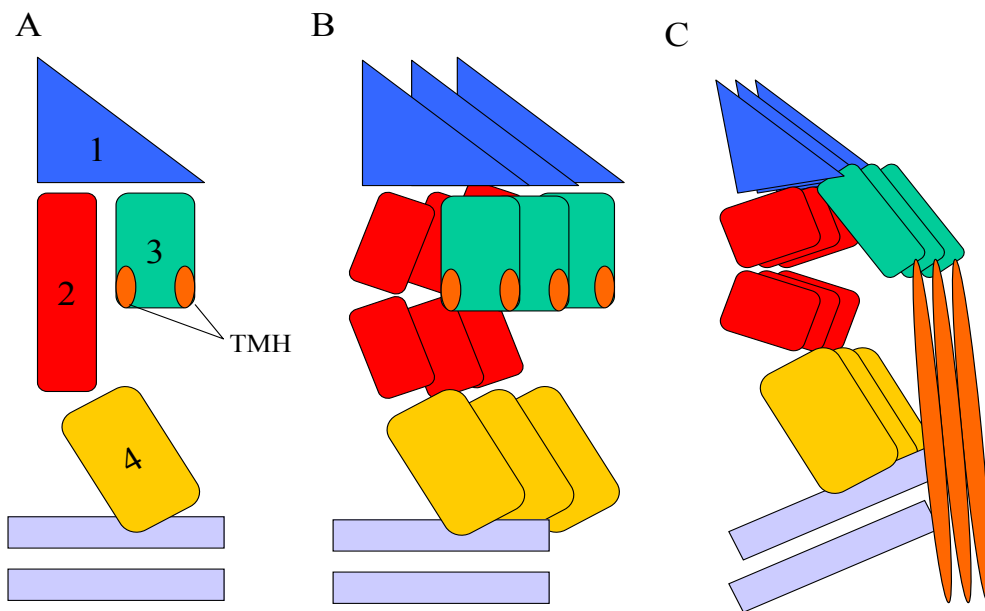


Figure 1.9: The first step in CDC pore formation mechanism is the binding of the monomers to the host membrane (A). The monomers oligomerize to form a membrane bound pre-pore (B). Finally a dramatic conformational change occurs to form a membrane-inserted pore (C) [160].

complex is obligatory and occurs prior to the insertion of the trans-membrane  $\beta$ -barrel [59]. The second model was proposed by Palmer and colleagues and suggested that oligomerization and membrane insertion of a CDC molecule occur concomitantly. According to their model, pore formation is initiated by the formation of a dimer that immediately inserts into the membrane and serves as a nucleus for further oligomerization. Further monomers are successively added to the dimer and concomitantly insert into the membrane, thereby giving rise to a 'growing' arc-shaped pore that eventually becomes the complete, ring-shaped pore [121]. This interpretation was based on the finding that a mutant that inhibited the oligomerization of native toxin induced the formation of arc-shaped oligomers in the absence of complete rings, and that these arc-shaped oligomers formed functional pores of reduced size, indicating that oligomerization does not need to be complete for insertion to occur. In the third model, proposed by Gilbert, there are two oligomeric states, pre-pore and pore, and the moment of transition depends on factors other than the completeness of the oligomeric ring [54]. Furthermore, once the transition (*i.e.*, membrane insertion) has occurred, oligomerization ceases. This model appears to be the only one compatible with all available experimental evidence.

#### **1.4.4 Role of CDCs in Pathogenesis**

One of the remarkable features of CDCs is the large size of the pores they form in mammalian cell membranes. This aspect has always been somewhat puzzling, since the ap-

parent cytotoxic effect - membrane permeabilization and cell lysis - can be achieved more economically with much smaller pores comprised of far fewer monomers, as exemplified by  $\alpha$ -hemolysin.

Despite the progress made in understanding the individual steps that define the cytolytic mechanism of CDCs, the role these toxins play in pathogenesis as pore formers is not yet completely understood.

However, the effect of pore-forming toxins in pathogenesis is not limited to simple cell destruction. Like other pore-forming toxins, CDCs are sometimes referred to as hemolysins due to their lytic activity on red blood cells, which are often used as convenient models in experimental studies. However, other cell types are susceptible to CDCs as well; this includes cells of the immune system. For instance, Streptolysin O kills human neutrophil granulocytes [41], while pneumolysin exerts its cytotoxicity on human immune cells (such as macrophages and neutrophils), respiratory endothelial and epithelial cells [126].

In contrast to erythrocytes, nucleated cells can recover from attack by pore-forming toxins by a membrane repair mechanism [135]. With immune cells, sub-lethal doses of CDCs can trigger a cascade of cellular responses such as expression and release of cytokines by pneumolysin, activation of the components of arachidonic acid cascade in the host defence cells by pneumolysin [96] and streptolysin O [140], cytokine receptor

shedding [172] and nitric oxide generation [23]. Such responses very likely contribute to inflammation and indirect tissue damage in bacterial infections.

In addition to this general mode of action, some CDCs have specific features related to pathogenicity. Pneumolysin, apart from its direct membrane-damaging action, also activates complement, which results from its binding to the Fc arm of IgG [97].

Recent studies have revealed that streptolysin O (SLO) translocates the *Streptococcus pyogenes* NAD-glycohydrolase into the cytoplasm of host cells, leading to an increase in cytotoxicity [89, 24, 92]. SLO can therefore be likened to the protective antigen component of anthrax toxin; this underscores the close relationship of pore-forming and A-B toxins. NAD-glycohydrolase translocation requires a special N-terminal domain of SLO not found in any of the other CDCs, suggesting those toxins do not function in a similar way.

The most numerous and substantial studies on the role of a CDC in pathogenicity have been performed on listeriolysin (LLO). This toxin is produced by the intracellular bacterium *Listeria monocytogenes*. It deviates from other CDCs by its acidic pH optimum[48]. An acidic environment is encountered by the bacteria inside the phagosome [10], from which they release themselves into the cytosol by the secretion of LLO. At later stages of the infection, LLO is used again to break down the cell membranes between adjacent host cells, facilitating the spreading of bacteria among host cells without

exposure to extracellular immune mechanisms [46].

## 1.5 Pyolysin

Pyolysin is a CDC produced by *Arcanobacterium pyogenes*. This bacterium is Gram-positive, non-motile, non-spore-forming and rod-shaped. This organism was reclassified from the genus *Actinomyces* on the basis of 16S rRNA sequences [134], and it is the most prevalent pathogen within the *Arcanobacterium* genus. *A. pyogenes* grows under aerobic and strictly anaerobic conditions, but optimal growth is obtained in a CO<sub>2</sub>-enriched atmosphere. All *A. pyogenes* isolates exhibit beta-haemolysis on agar media containing bovine or ovine blood [133], even though isolates of porcine origin are generally more haemolytic [161].

*A. pyogenes* is an opportunistic pathogen of economically important livestock such as cattle and swine. This organism is also a common inhabitant of the mucous membranes of these animals, and is routinely isolated from udders, urogenital, and upper respiratory tracts of healthy animals [25, 111, 161, 130]. *A. pyogenes* is also commonly found associated with the bovine rumen [110] and can be isolated from the porcine stomach [73].

Since *A. pyogenes* is a commensal, the source of infection is often autogenous. Although *A. pyogenes* is capable of acting as a primary pathogen, infection often follows

a physical or microbial trauma to the mucous membrane, allowing dissemination of the organism. *A. pyogenes* is one of the most common opportunistic pathogens of domestic ruminants and pigs, causing a variety of suppurative infections involving the skin, joints and organs. These infections include abortion, abscesses, arthritis, endocarditis, mastitis, pneumonia, osteomyelitis, uterine infections leading to infertility, and vesiculitis [161, 85]. Infection is not confined to domestic animals: *A. pyogenes* is a particularly versatile pathogen, being able to cause disease in a multitude of different animal species including antelopes, bison, camels, cats, chickens, deer, dogs, elephants, gazelles, horses, macaws, reindeer, turkeys and wildebeest. *A. pyogenes* is an infrequent cause of infections in humans [45], and these infections are often a result of occupational exposure, as *A. pyogenes* is not a part of the human normal flora.

Pyolysin (PLO), is a primary virulence factor of *A. pyogenes* [19, 34, 9]. PLO is a haemolysin capable of lysing red blood cells of a variety of animal species, and is responsible for the characteristic  $\beta$ -haemolysis exhibited by *A. pyogenes* grown on blood-containing media [134]. Despite its initial identification as a haemolysin, PLO resembles most other CDCs in not being confined to any particular cell type.

PLO is produced by all *A. pyogenes* strains examined to date [19, 34, 9]. It is expressed primarily in stationary phase cultures [34, 54], where it is detected in culture supernatant as a 55 kD protein [19, 9].



PLO has been demonstrated to be a major determinant of virulence in *A. pyogenes* infections. An *A. pyogenes plo* deletion mutant constructed by allelic exchange, was nonhaemolytic compared to its isogenic wildtype strain, indicating that PLO is the major haemolysin produced by *A. pyogenes* [9]. The *plo* mutant could be restored to full virulence by supplying the *plo* gene in trans, confirming that the reduction in virulence observed with the *plo* mutant was due to the defect in PLO production. Significantly higher doses of the *plo* mutant were required to establish infection in mice [9]. This decrease in virulence is similar to the effects of CDC knockouts in other bacteria such as *Streptococcus pneumoniae* [14] and *Clostridium perfringens* [7].

While clearly a member of the CDC family, PLO is unique in several ways. It is the most divergent member, exhibiting only 31-41% amino acid similarity to other CDCs (figure 1.10). In addition, PLO, along with the human specific cytolysin intermedilysin (ILY), have undecapeptide sequences which diverge significantly from the consensus for this family (table 1.2).

In both PLO and ILY, the otherwise conserved single cysteine residue is substituted with alanine. This cysteine residue is responsible for the 'thiol-activated' nature of most CDCs, and its lack in PLO or ILY explains why these two toxins are insensitive to thiol modification [19, 18, 20, 21]. In fact, the replacement of the alanine with cysteine by site-directed mutagenesis of PLO, results in an oxygen-labile PLO molecule [20, 21].

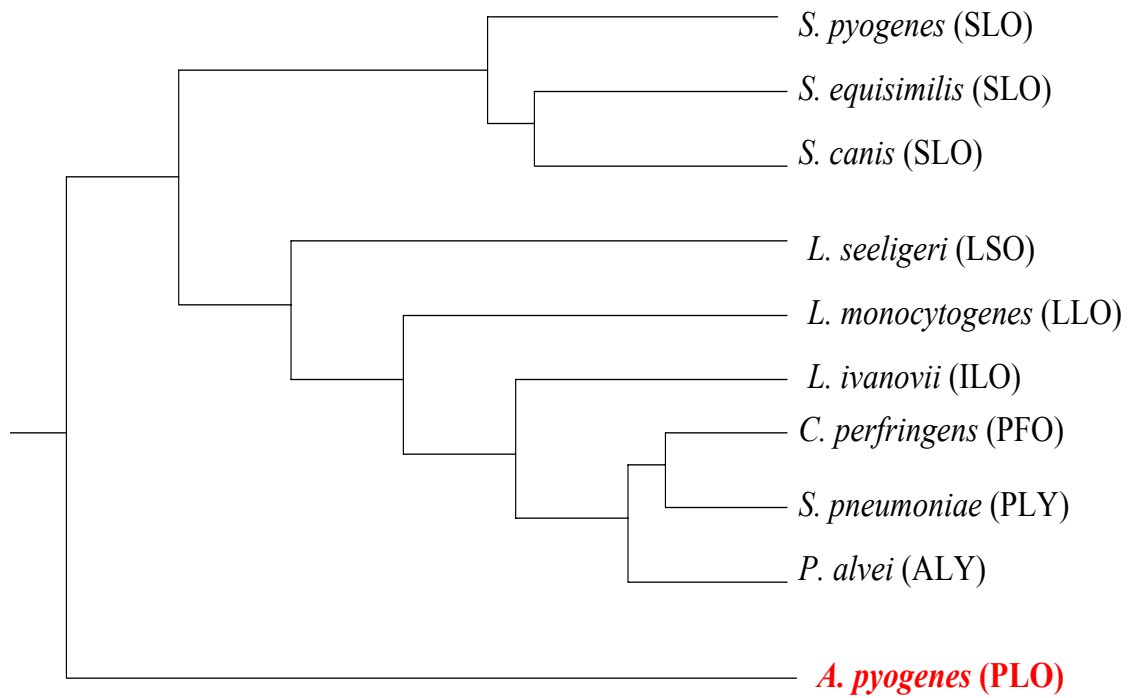


Figure 1.10: Phylogenetic analysis of protein sequences showing the relationship between the cholesterol-dependent cytolysins. Cytolysins are indicated by the name of the bacterium producing it [2].

Table 1.2: The conserved undecapeptide sequence of CDCs. Amino acids which do not match the consensus undecapeptide sequence are in red. Amino acid numbers are given for the first amino acid in the sequence for each protein [18].

SLO	529	ECTGLAWE-WWR
PLY	427	ECTGLAWE-WWR
SLY	455	ECTGLAWE-WWR
LLO	483	ECTGLAWE-WWR
LSO	484	ECTGLFWE-WWR
ILO	458	ECTGLAWE-WWR
PFO	458	ECTGLAWE-WWR
ALY	460	ECTGLAWE-WWR
CLY	465	ECTGLAWE-WWR
PLO	491	EATGLAWDPWW-
ILY	485	GATGLAWEPW-R

As with other CDCs, amino acids within the conserved undecapeptide sequence of PLO, especially the three conserved tryptophan residues, are important for haemolytic activity and cholesterol binding [20, 21]. However, the divergent undecapeptide sequence of PLO appears to be required for its full cytolytic activity, as mutations that bring the undecapeptide sequence closer to the family's consensus sequence reduce the haemolytic activity of PLO [20, 21]. Recent experiments with ILY suggest that this toxin lacks a specific affinity for cholesterol primarily as a result of its divergent undecapeptide sequence [107]. While the PLO undecapeptide is more similar to that of ILY than other CDCs, PLO still retains significant affinity for cholesterol [20, 21]. In addition to the undecapeptide, two regions at the N-terminus of the protein, amino acids 55-73 and 123-166, appear to be important for PLO activity, as monoclonal antibodies directed to these

regions block haemolytic activity [66]. Site-directed mutations within these regions also result in significant reductions in the haemolytic activity of PLO [66]. These regions comprise a portion of domain 1 of the PLO molecule, for which no direct function has been assigned.

While PLO is an important virulence factor for *A. pyogenes*, the precise role it plays in pathogenesis is unclear. In addition, while the intraperitoneal mouse model is representative of disseminated *A. pyogenes* infections such as liver abscessation, it may less accurately reflect other *A. pyogenes* infections such as mastitis or pneumonia. PLO is toxic for mouse peritoneal macrophages, the J774A.1 murine macrophage cell line, and bovine and ovine PMNs [34, 9], and this activity may provide protection for the invading organism against the host immune response. Consistent with this hypothesis, the *plo* mutant is cleared rapidly from infected mice, within 48 h post-challenge, suggesting that it is overwhelmed by the immune response. In addition, an *A. pyogenes* strain expressing a mutant PLO molecule, with a tryptophan to phenylalanine substitution in the undecapeptide sequence and <0.3 % haemolytic activity, was reduced for virulence to a similar extent as the PLO knockout mutant [75]. This result suggests that the cytolytic activity of PLO is important for this toxin to exert its pathogenic effects. In addition to direct toxicity for immune cells.

## 1.6 *Streptococcus agalactiae* CAMP Factor

### 1.6.1 *Streptococcus agalactiae*

*Streptococcus agalactiae* (*S. agalactiae*) is a gram positive, non-motile, catalase-negative bacterium, which can be found as part of the normal flora in respiratory, urogenital and gastrointestinal tracts of humans. *S. agalactiae* is also known as Group B streptococcus (GBS), where the 'B' refers to the Lancefield classification. This classification is based on the so-called C carbohydrate, which is associated with the cell wall and is soluble in dilute acid; there are 13 types of this carbohydrate, designated A through O, which can be serologically differentiated. *S. agalactiae* can be identified by means of cultural characteristics (such as the tolerance to high salt concentration), pigment production and biochemical reactions such as the hydrolysis of sodium hippurate, the CAMP test (see below) and susceptibility to bacitracin [39].

*S. agalactiae* is an important pathogen in cattle mastitis, resulting in loss of milk production [22], and it accounts for a substantial number of cases of invasive infections in newborn and young infants, pregnant women, and nonpregnant adults who typically have underlying medical conditions (diabetes mellitus, cancer, liver disease, etc)[147, 8, 148]. Many virulence factors have been found to be associated with *S. agalactiae* infections. The best characterized one is the capsular polysaccharide, which prevents deposition of human opsonic complement component C3b, thus protecting the bacteria from comple-

ment lysis [36, 37, 91]. In addition to capsular polysaccharides, *S.agalctiae* produces a wide range of surface proteins, including the so called  $\alpha$ - and  $\beta$ -proteins. The  $\alpha$ -proteins seem to play an important role in the early stages of infection by *S.agalactiae* [87], while  $\beta$ -protein (also called Bac protein) was shown to bind to the Fc arm of immunoglobulin [15, 70, 88, 141, 142].

The lytic phenomenon exhibited by group B streptococci on sheep red blood cells was first reported by Christie, Atkins and Munch-Petersen in 1944 [28]. These authors found that GBS induces a distinct zone of complete hemolysis when grown near colonies of *Staphylococcus aureus* strain secreting sphingomyelinase ( $\beta$ -Toxin). This co-hemolytic effect was called the CAMP reaction after the initials of the last names of its discoverers. Since then, the CAMP reaction has been used as a diagnostic test for the presumptive identification of Group B streptococci in clinical isolates.

A 26 kDa extracellular protein produced by *Streptococcus agalactiae* was found to be responsible for the CAMP reaction, and therefore was named CAMP factor. The CAMP factor gene of *S. agalactiae* encodes a 28 kDa protein with an N-terminal signal peptide that is cleaved upon secretion. The mature peptide of CAMP factor is a monomeric 26 kDa protein composed of 226 amino acids with a pI of 8.9 [12, 76]. The amino acid sequence of CAMP factor was determined by Ruhlmann *et al.* [139]; no cysteine is present in this protein.

Like *S. agalactiae*, some *Streptococcus* species and *Propionibacterium acnes* produce a CAMP reaction on sheep red blood agar plates due to proteins that are closely homologous to CAMP factor. On the other hand, several other bacteria, such as *Rhodococcus equi* [44], *Listeria monocytogenes*, *Listeria seeligeri* [136], *Aeromonas* sp.[42], *Actinobacillus pleuropneumoniae* [68], also produce CAMP reactions; however, they do not possess proteins closely homologous to CAMP factor.

Even though CAMP factor has long been presumed to be a virulence factor, a highly purified CAMP protein was not lethal to rabbits, unlike a partially purified CAMP factor preparation, which could exert lethal effects when administered to rabbits and mice [77, 157].

Lang and Palmer have shown that CAMP factor forms pores of finite size on the sheep red blood cells membranes (figure 1.11). In addition, further studies with liposomes confirm that the process of CAMP factor pore formation is highly cooperative, supporting an oligomeric nature of the membrane lesion [82].

GPI-anchored proteins act as cell surface receptors for CAMP factor, and the binding site appears to be located in the glycan core of the GPI anchor moiety [83].

In addition to its pore-forming activity, CAMP factor was reported to bind to the Fc fragment of IgG and IgM; therefore, it was called Protein B [77], in analogy to *Staphylococcus aureus* protein A. The reported Fc binding property of CAMP factor suggested a

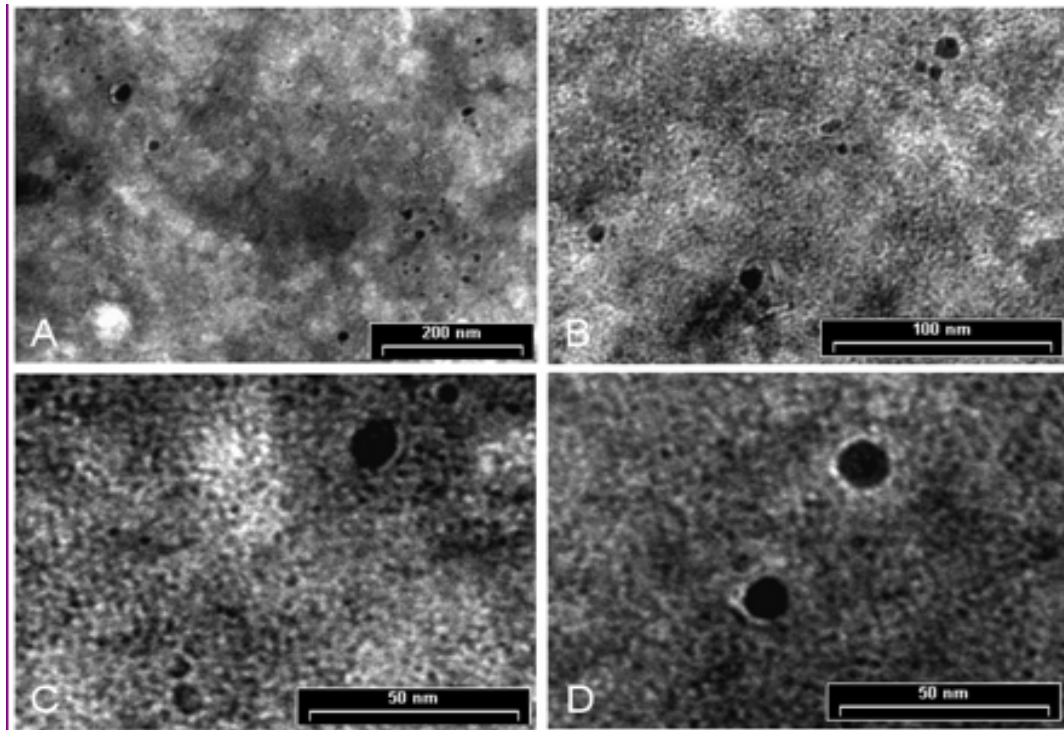


Figure 1.11: Electron microscopy of the pore formed by CAMP factor on sheep red cell membranes. Magnification varies among panels A - D as indicated by the scale bars. this image obtained from Lang and Palmer 2003 [82]



possible role of CAMP factor in resistance to phagocytosis.

## 1.7 Research Objectives

The work described in this thesis consists of two parts. The first part was conducted on pyolysin, while the second part concerns CAMP factor.

In the first part, the main objective was to characterize the role of lipids other than cholesterol in the activity of PLO; such possible roles had not been thoroughly studied before. This interest came from our preliminary observation that treating red blood cells with sphingomyelinase, which converts sphingomyelin to ceramide, will greatly enhance the susceptibility of erythrocytes to PLO. This work is discussed in detail in chapter 3.

Our preliminary experiments on the lipid specificity of PLO with model membranes led us to consider the possibility that pyolysin might also oligomerize in solution, which we found indeed to be the case. The functional characterization of PLO oligomers formed in solution became the other main focus of this study and is described in details in chapter 2.

In the second part, our original objective was to examine the possibility that the reported binding of *Streptococcus agalactiae* CAMP factor to the Fc fragment of IgG [77] might inhibit the activation of complement (as is the case with staphylococcal protein A), and if applicable, to characterize the region of the CAMP factor molecule responsi-

ble for such an effect. As it turned out, no such inhibitory effect occurred, which led us to re-examine the binding of CAMP factor to IgG. This part will be discussed in detail in chapter 4.

## **Chapter 2**

**The Cholesterol-Dependent Cytolysin  
Pyolysin forms small,  
hemolytically active oligomers in  
solution**

## 2.1 Introduction

Pyolysin (PLO) is an important virulence factor of *A. pyogenes* [19, 34, 74]). PLO belongs to the family of cholesterol-dependent cytolysins (CDCs), which comprises toxins produced by more than twenty species of gram-positive bacteria. Within this family, PLO is unique in several ways. It is the most divergent member, exhibiting only 31-41% amino acid identity to other CDCs. Also, unlike most other CDCs, it is insensitive to either reducing agents or thiol-blocking agents [19, 18, 20] . This is due to the lack of a cysteine residue that is conserved in all other CDCs but one, the sole other exception being *Streptococcus intermedius* intermedilysin (ILY; [108]). For additional detail see section 1.5.

The mechanism by which CDCs form pores was reviewed in Chapter 1. Briefly, All CDCs are secreted as soluble monomeric molecules. Upon binding to target membranes, they oligomerize to form oligomeric pre-pores, which then insert into the lipid bilayer to form the final trans-membrane pores. Depending on whether or not oligomerization was completed at the time of insertion, pores may be ring-shaped or arc-shaped; in the latter case, the opposite side of the pore is lined by a free edge of the lipid membrane [121].

While efficient oligomerization of CDCs requires the presence of membranes, it was previously reported that pneumolysin, which is produced by *Streptococcus pneumoniae*, self-associates in solution to form oligomeric structures similar in size and shape to those

that appear on the membrane coincident with pore formation [51, 53]. Preliminary experiments with PLO and model membranes led us to consider the possibility that pyolysin might also oligomerize in solution. In this report, we present evidence that this is indeed the case. The oligomers formed appear to be of a fairly homogenous size, which is much smaller than the typical size of membrane-derived oligomers. The solution-derived oligomers retain the ability to assemble into larger oligomers on membranes and thereby permeabilize them, but the pores thus formed do show some structural and functional differences from those formed by monomeric PLO. The findings of this study have significant implications for the interaction of CDCs with lipid membranes and the mechanism of pore formation. <sup>1</sup>

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<sup>1</sup>This work has been submitted for publication.

Guenter Scholz, Stephen Billington, Naghmeh Sarraf and Michael Palmer are co-authors for this work.

## **2.2 Materials and Methods**

### **2.2.1 Production and Purification of Histidine-tagged PLO (His-PLO)**

Cultures (1000 ml) of *E. coli* BL 21 carrying the pTrcHis B plasmid with wild type [20] PLO or N90C mutant PLO were grown in Luria-Bertani (LB) broth (Bioshop, Burlington, Ontario) with ampicillin (100  $\mu\text{g/ml}$ ) at 37°C. Once the OD<sub>600</sub> had reached a value of 0.6, protein expression was induced with 1.0 mM IPTG, and incubation continued for another 3 h. The cells were then shaken overnight at 25°C and 150 rpm. Cells were harvested by centrifugation at 5000 x g for 10 mins and the cell pellet was resuspended in column binding buffer (20 mM Tris/HCl, 150 mM NaCl, 10 mM imidazole, pH 8.0). The cells were lysed using an emulsifier (Avestin Emulsiflex-C5) at 17,500 psi and the insoluble material was removed by centrifugation at 12000 xg for 30 mins. His-PLO was purified from the soluble fraction by metal-chelating chromatography on Ni-NTA agarose (Novagen, Oakville, Ontario), using a linear gradient of imidazole (10 mM - 450 mM) in binding buffer for elution.

### **2.2.2 Cysteine-specific Labeling of Mutant N90C**

The single cysteine mutant N90C was constructed by site-directed mutagenesis as described in [63] and confirmed by DNA sequencing (Mobix lab, Hamilton, ON). The mutant protein was purified by metal-chelating chromatography as describe above, and

2 mg/ml of PLO with the unique cysteine residues were labeled with a mixture of fluorescein-5- maleimide (Invitrogen) and tetramethylrhodamine-5-maleimide (Sigma) (1:3) in Tris-buffered saline (TBS; pH 7.5). The absolute concentrations for fluorescein-5- maleimide and tetramethylrhodamine-5-maleimide were  $125\mu\text{M}$  and  $375\mu\text{M}$ , respectively. Incubations were for 30 min at room temperature. Excess label was then removed by gel filtration on a PD10 column equilibrated with TBS, and the monomers and solution derived oligomers separated by size exclusion chromatography as described below.

### **2.2.3 Size Exclusion Chromatography**

Size exclusion chromatography was performed using a Superdex 200 column (40cm x 0.8cm), equilibrated with PBS, on an AKTApurifier chromatography system (GE Healthcare, Baie d'Urfe, QC, Canada). The flow rate was maintained at  $0.3\text{ ml}\cdot\text{min}^{-1}$ , and the elution profile was monitored by online measurement of the  $A_{280}$ . The eluate was collected in fractions of 0.3 ml and analyzed by SDS-PAGE.

### **2.2.4 Lysis of Red Blood Cells**

Venous blood was obtained from volunteers, and the red blood cells were washed five times in hemolysis buffer (10 mM Tris-HCl, 150 mM NaCl, pH 7.4) by centrifugation. The erythrocytes were then resuspended in hemolysis buffer to 0.5% (v/v).  $20\mu\text{l}$  of cell suspension was added to the wells of a microtiter plate containing serial dilutions of

PLO in 180  $\mu$ l of the former buffer. Hemolysis was measured by the decrease in turbidity ( $A_{650}$ ) using a 96-well plate reader (Spectramax 190, Molecular Devices, Sunnyvale, CA). Alternatively, un-lysed cells and cell membranes were pelleted by centrifugation, and the absorbance of hemoglobin released into the supernatant ( $A_{540}$ ) was measured. A sample of red blood cells incubated in the absence of PLO was used as a blank, and readings from cells osmotically lysed in 10 mM phosphate buffer, pH.7.4 were considered as 100% lysis.

### **2.2.5 Marker Release Experiments**

Washed, packed human red blood cells were osmotically lysed on ice with 10 vol. of 10 mM sodium phosphate buffer (pH 7.4), and the membranes repeatedly washed by centrifugation (4°C at 15 000 x g for 3 min) in the same buffer. After a final wash with 16 mM Na-phosphate/150 mM NaCl (PBS), the membrane ghosts were resuspended in PBS containing 0.25 mg/ml fluorescein isothiocyanate-dextran (Sigma; average mol. weights, 4, 75, 250 or 2000 kDa, respectively) and incubated at 37°C for 45 min. The resealed ghosts were washed with PBS by centrifugation four times, incubated for 30 min on ice, and washed again. The pellet was then resuspended in 20 volumes of the same buffer. Serial twofold dilutions were prepared from 200  $\mu$ g/ml of monomeric PLO or of solution-derived oligomers in PBS. To each dilution (100  $\mu$ l), 50  $\mu$ l of the marker-laden ghost suspension was added, followed by incubation at 37°C for 30 min. The samples



were then centrifuged at 12,000 x g for 2 min, and the supernatants were diluted into 3 ml of 10 mM Tris/50 mM NaCl/0.2% SDS (pH 8.0) and analyzed fluorimetrically. The percentage of marker released was obtained by comparison with a Triton-lysed reference; spontaneous leakage was assayed on a parallel sample incubated without toxin and subtracted.

### **2.2.6 Electron Microscopy**

Red blood cells were incubated and lysed with 50  $\mu\text{g}/\text{ml}$  PLO, and the membranes were collected by centrifugation. The membranes were washed three times with 5 mM HEPES buffer (pH 7.4) and mounted onto carbon-coated grids (Formvar/copper, 200 mesh; Marivac Ltd., St.Laurent, QU) followed by negative staining with sodium phosphotungstate (2% w/v, pH 7.4) for 5 s. Membranes were then examined in a LEO 912 AB Omega electron microscope operated at 100 kV.

For solution-derived oligomers without membranes, samples (7  $\mu\text{l}$ ) were placed on 400-mesh carbon coated formvar copper grids (Marivac Ltd., St.Laurent, QU). After several minutes the grids were dried, washed three times with 10 l of water and stained for 15 second using saturated uranyl acetate (pH. 4.5). Specimens were viewed with a Philips CM20 electron microscope (Philips Electronics Ltd., Guildford, UK) at an accelerating voltage of 200 kV. Images were digitized using a Gatan 679 slow-scan CCD camera and analyzed using DIGITALMICROGRAPH software (version 2.1, Gatan inc., Pleasanton,

CA).

### **2.2.7 Preparation of Liposomes**

All lipids were purchased from Avanti Lipids (Alabaster, AL). The lipids, dissolved in chloroform, were mixed in various molar ratios (see "Results"), dried down under nitrogen in a round-bottom flask, and then dried under vacuum for an additional 3 h to remove the residual solvent. The dried lipids were hydrated at room temperature for 1 h in 1 ml of phosphate buffer saline (PBS), pH.7.3. The lipid suspension was sized down to unilamellar liposomes using a liposome extruder (Northern Lipids, Vancouver, British Columbia, Canada) by extrusion 10 times through a polycarbonate membrane with a 100-nm pore size.

### **2.2.8 Preparation of Erythrocyte Membranes**

Human red blood cells were osmotically lysed in 5 mM phosphate buffer (pH 7.3) and the membrane were pelleted and repeatedly washed until they appeared white. The last wash was conducted using PBS (pH 7.3).

### **2.2.9 Circular Dichroism Spectroscopy**

Circular dichroism (CD) spectra were obtained in a Jasco J-715 CD spectropolarimeter using J-715 analysis software. Samples were measured in a 1 mm quartz cuvette (Hellma,

Concord, Ontario, Canada) kept at 25°C. Samples consisted of 100  $\mu\text{g}$  monomeric or SDO PLO alone or PLO with DMPC liposomes or 1:1 DMPC:cholesterol liposomes and incubated for 30 minutes at 37°C. Spectra were recorded over a 190-250 nm range with a 1.0 nm bandwidth, 0.2 nm resolution, 100 mdeg sensitivity at a 0.125 s response, and a rate of 100 nm/min with a total of 25 accumulations. Spectra for liposomes alone were obtained as references and subtracted from the spectra obtained of the toxin in the presence of liposomes.

### **2.2.10 Electrophoresis**

SDS-PAGE was performed according to Laemmli [80], using polyacrylamide concentrations of 4% in the stacking gels and of 12% in the resolving gels. Gels were stained with Coomassie blue.

### **2.2.11 Protein Assay**

PLO concentrations were determined using the absorbance at 280 nm and a calculated extinction coefficient of  $68,480 \text{ M}^{-1}\cdot\text{cm}^{-1}$ . The extinction coefficient was calculated from the amino acid composition according to Pace *et.al* [118].

### 2.2.12 Spectrofluorimetry

Steady-state fluorescence measurements were taken using a PTI QuantaMaster spectrofluorimeter. The excitation and emission for fluorescein wavelengths were 492 and 520 nm, respectively. Time-resolved measurements were performed in a FT-100 compact fluorescence lifetime spectrometer (PicoQuant, Berlin, Germany), using a LDH-P-C-470 LED laser light source. Fluorescein emission was isolated using a 520.5 nm bandpass filter (Andover Corporation, NH, USA). Decays were fitted using FluoFit software (PicoQuant), allowing for two fluorescence lifetime components, from which the average lifetime  $\langle \tau \rangle$  was calculated according to the formula

$$\langle \tau \rangle = \frac{\sum \alpha_i \tau_i}{\sum \alpha_i}$$

where  $\alpha$  represents the intensity contribution of a given lifetime component at  $t=0$ .

Where necessary, a third component with a lifetime of 50 ps was allowed for to accommodate light scattering; this component was excluded from the lifetime calculation. The fit residuals were evenly distributed, and the  $\chi^2$  values below 1.3 in all cases.

## 2.3 Results

### 2.3.1 Spontaneous Oligomerization of PLO in Solution

. Oligomers were present in cell lysates of *E. coli* cells recombinantly expressing his-tagged PLO. Figure 2.1 shows the elution profile of a sample of PLO as obtained from *E. coli* lysate by metal-chelating chromatography, resolved on a Superdex 200 column. Ahead of the main peak, which corresponds to the monomer with a molecular weight of 57 kDa, there is a well-defined peak containing oligomeric PLO. The elution volume of this peak corresponds to an apparent molecular weight of approximately 350 kDa, which indicates that the oligomeric species contains approximately 6 monomer subunits. Both peaks, when re-chromatographed immediately after isolation, were stable, *i.e.*, they eluted as single, symmetrical peaks at their previous positions (Figure 2.2).

Incubation of isolated monomers (2 mg/ml) for 12 hours at 20 °C resulted in the formation of some more oligomer, as shown in Figure 2.3. The extent of oligomerization observed *in vitro* increased with the monomer concentration but was not detectably affected by variation of pH (pH 5-10) or salt concentration (0-0.5M NaCl; data not shown). Since at pH 5 the histidine residues in the purification tag will be protonated, it seems unlikely that the his-tag is responsible for oligomerization. This conclusion is also supported by the previous observations that oligomers of similar size form with pneumolysin expressed without a his-tag, and that a his-tag does not induce oligomerization of an-

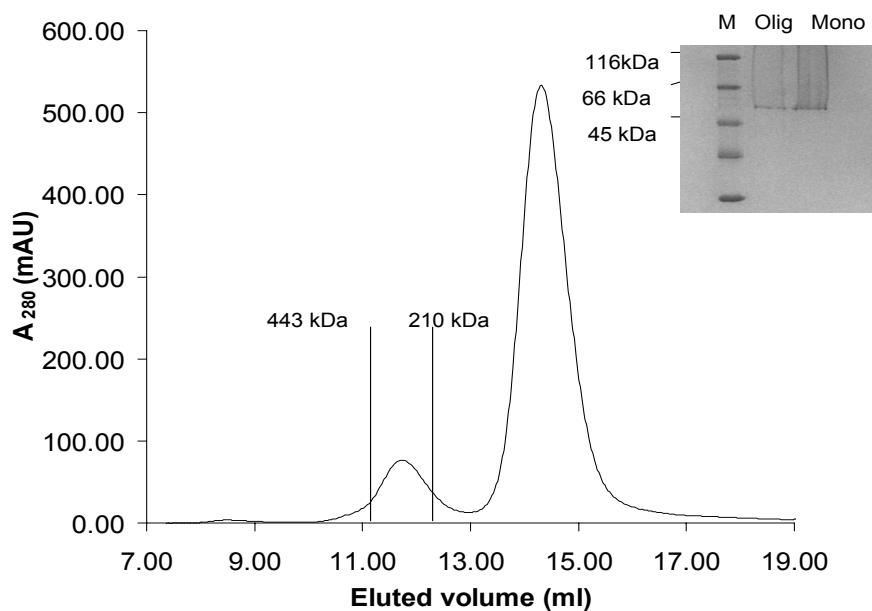


Figure 2.1: Size exclusion chromatography elution profiles of 2 mg/ml PLO, resolved on a Superdex 200 column, as purified from *E. coli* cultures by metal-chelating chromatography. Ahead of the main peak, which corresponds to the monomer, there is a peak representing the solution-derived oligomer. The peak positions of apoferritin (443kDa) and amylase (210 kDa) are indicated by vertical lines. Inset: SDS-PAGE of monomers and oligomers.

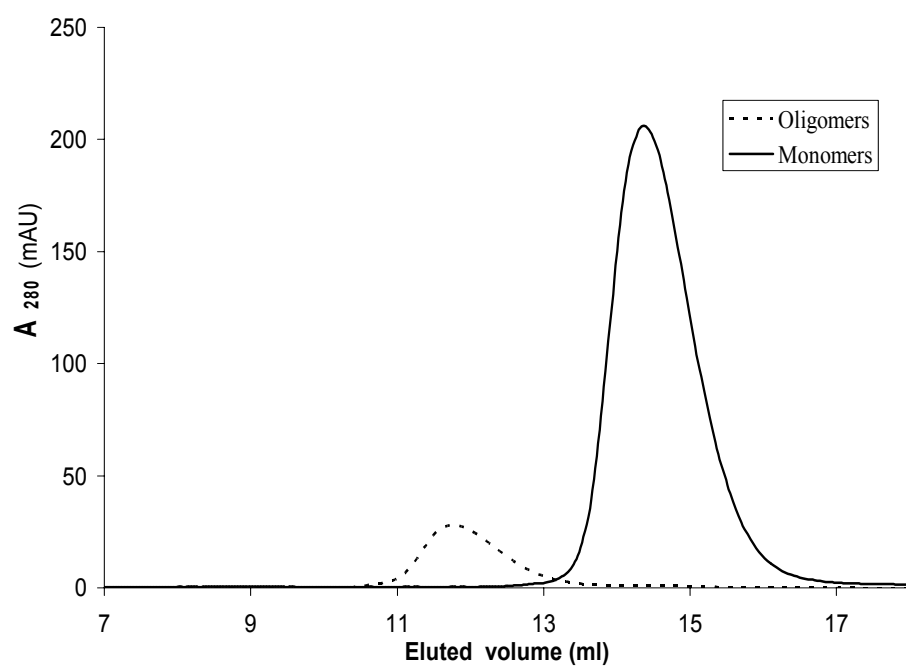


Figure 2.2: Size exclusion chromatography elution profiles of rechromatographed monomers and oligomers immediately after isolation, resolved on a Superdex 200 column equilibrated and eluted with PBS.

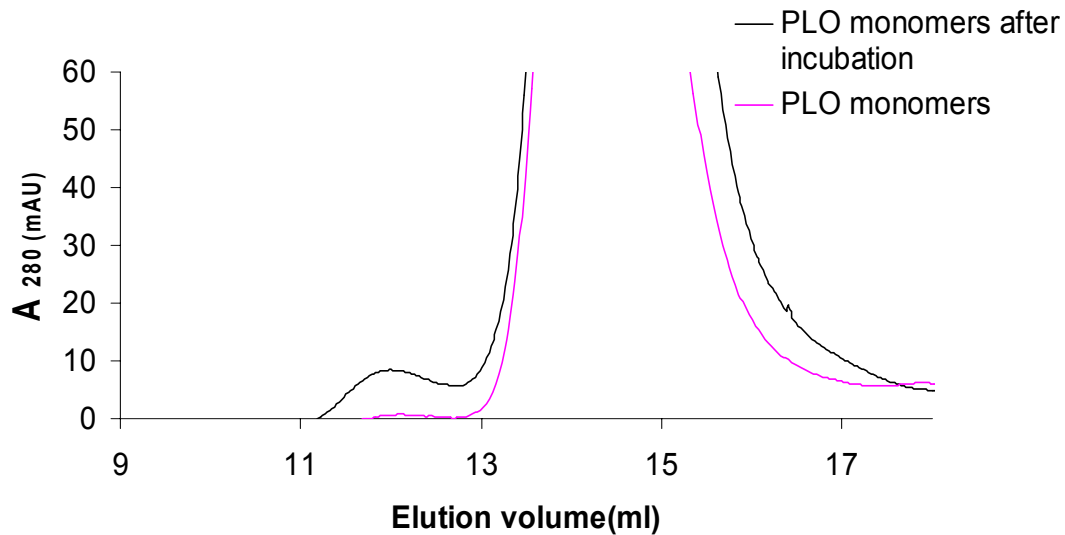


Figure 2.3: Size exclusion chromatography elution profiles of 2 mg/ml monomeric PLO (isolated by gel filtration) after incubation at 20°C for 12 hours.

other CDC (perfringolysin O) in solution [158]. The extent of oligomerization obtained with purified monomer in vitro was significantly smaller than that observed with the unfractionated protein extracted from *E.coli*. This may be related to the higher monomer concentration present in the bacterial cytoplasm (5mg/ml).

The size of the oligomer did not change significantly between different preparations or experiments. When a PLO sample that had not been subjected to gel filtration was concentrated to 10 mg/ml and then resolved by gel filtration, some additional species



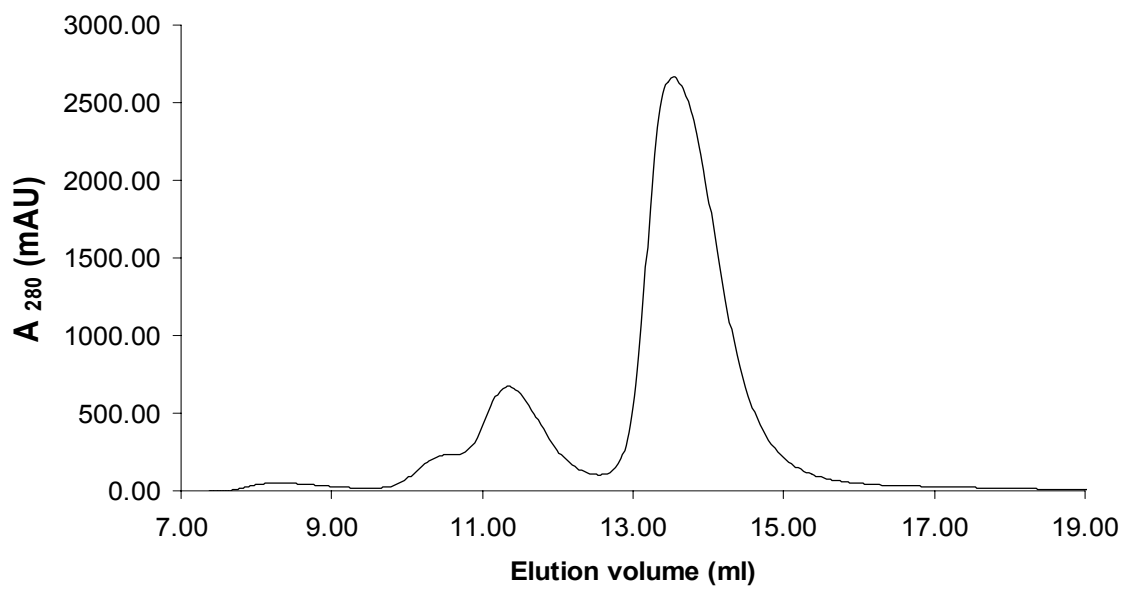


Figure 2.4: Size exclusion chromatography elution profiles of unfractionated PLO was concentrated to 10 mg/ml PLO and incubated for 4 hours at 37°C before gel filtration.

of higher molecular weight were apparent in low abundance, but the position of the major oligomer peak was unchanged (Figure 2.4). This suggests that the solution-derived oligomers do not grow by successive apposition of further monomers; rather, it appears that larger oligomeric species may form from the aggregation of small oligomers. In all further experiments, the oligomers used were obtained as described in the legend to Figure 2.1; this preparation will be referred to as ‘solution-derived oligomers’, or SDO for short.

### **2.3.2 Conformation of the Subunits in Solution-Derived Oligomers**

While the toxin monomers undergo local conformational changes as they bind to membranes and assemble into pre-pores [160], the major conformational change is associated with the final event of co-operative membrane insertion of the oligomer [160]. As evident in Figure 2.5, this latter change is reflected in a difference between CD spectra of monomeric PLO and membrane-associated oligomers obtained by incubation with cholesterol-containing liposomes [109]. On the other hand, the CD-spectrum of solution-derived oligomers closely resembles that of monomeric PLO in buffer or after incubation with cholesterol-free liposomes. This indicates that, in the SDO, the conformational change that corresponds to the event of membrane insertion of the oligomer has not yet occurred.

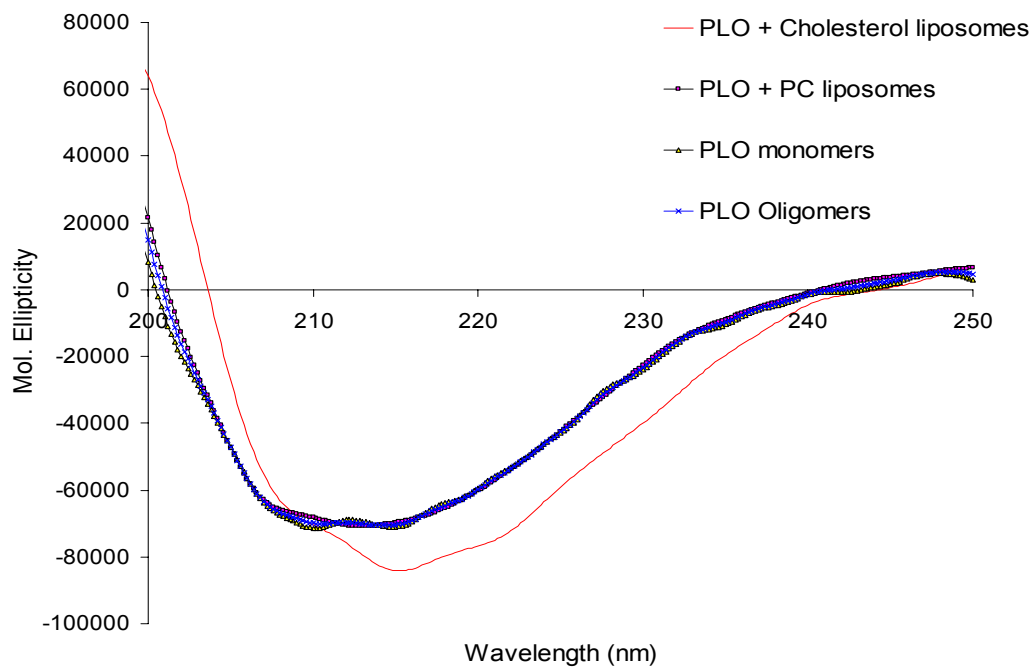


Figure 2.5: Far-UV CD spectra of SDO, monomers, monomers in the presence of cholesterol liposomes (1:1, cholesterol and PC) and PC liposomes. In all samples, the toxin concentration used was 100  $\mu\text{g/ml}$

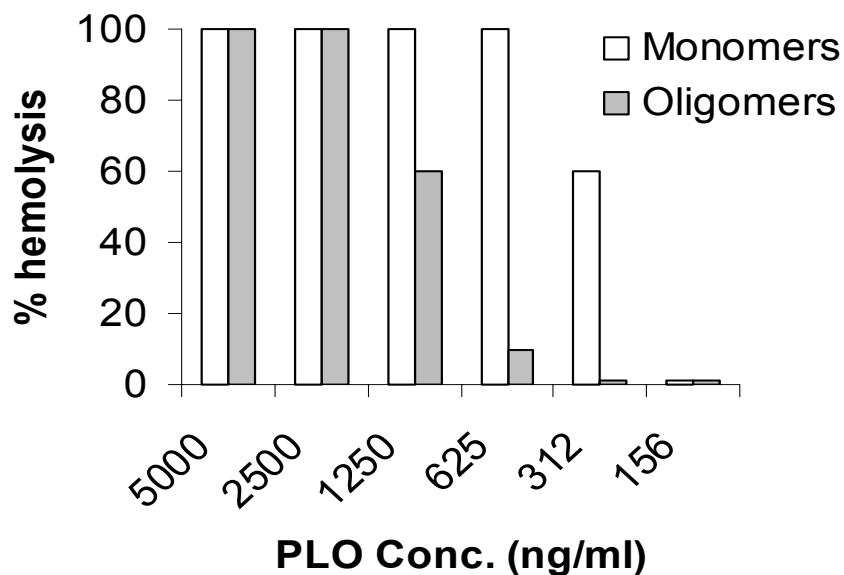


Figure 2.6: Hemolysis of human erythrocytes by monomeric and oligomeric PLO. Red blood cells (0.5% by volume) were exposed to various concentrations of monomeric and oligomeric PLO. Hemolysis was assayed by measuring the OD<sub>540</sub> of hemoglobin released into the supernatant.

### 2.3.3 Hemolytic Activity of Solution-Derived PLO Oligomers

To test the activity of SDO, a hemolysis assay was performed (Figure 2.6). Surprisingly, it was found that SDO exhibit hemolytic activity, even though their hemolytic titer was approximately four times lower than that of the monomers, and hemolysis was slower than that causes by an equal amount of monomers (Figure 2.7).

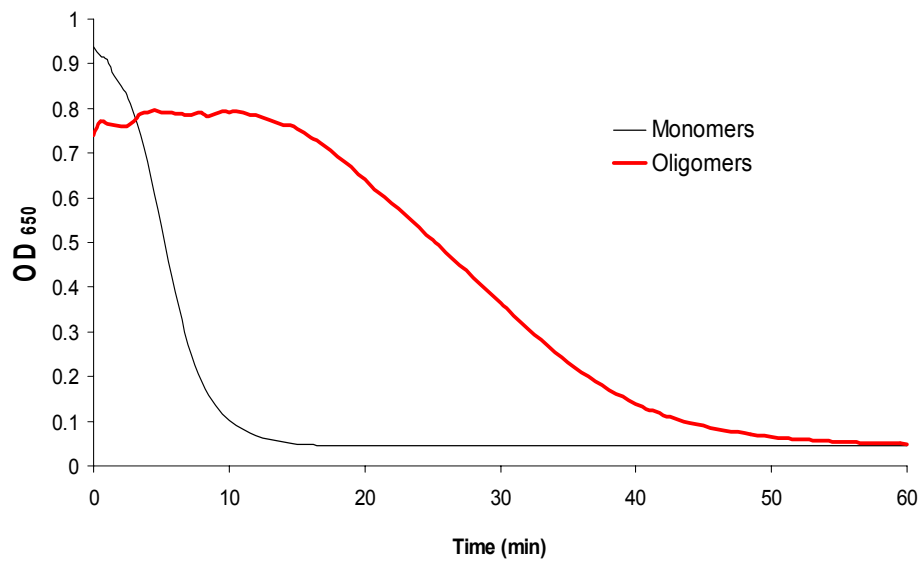


Figure 2.7: Hemolysis kinetics of monomeric and oligomeric PLO. Human erythrocytes (0.5% by volume) were exposed to 25  $\mu\text{g}/\text{ml}$  of monomeric and oligomeric PLO, respectively, and the time course of hemolysis was measured by the decrease in turbidity ( $\text{OD}_{650}$ ).

### **2.3.4 Electron Microscopy of Pores Formed by Solution-Derived Oligomers**

With approximately 6 subunits, the SDO are far smaller than the typical, mostly ring-shaped CDC oligomers that form on susceptible cell membranes and which contain approximately 50 subunits [119]. With pyolysin, the membrane-associated oligomers had not been characterized before. Therefore, the observation of hemolytic activity associated with the small, solution-derived oligomers raised the question whether or not the pores formed by PLO are of the same size as those of other CDC. Figure 2.8a shows oligomeric pores on red cell membranes that were incubated with monomeric PLO. Most oligomers are complete circles, with diameters of approximately 30 nm; a few incomplete, arc-shaped oligomers also present. This pattern very much resembles that observed previously with other CDC [112, 121, 99, 31, 16, 150]; therefore, the oligomers that form from monomers on membranes are far larger than the solution-derived oligomers.

The question then arose whether the SDO form pores individually, or whether they give rise to larger structures in order to form pores. Figure 2.8b shows pores formed by incubation of red cell membranes with solution-derived oligomers. In this sample, most oligomers are incomplete and arc-shaped; a few apparently complete rings are seen as well. At the same time, most of the arcs approach or slightly exceed half-circle size. This size corresponds to approximately 20-25 subunits; therefore, the observed oligomers

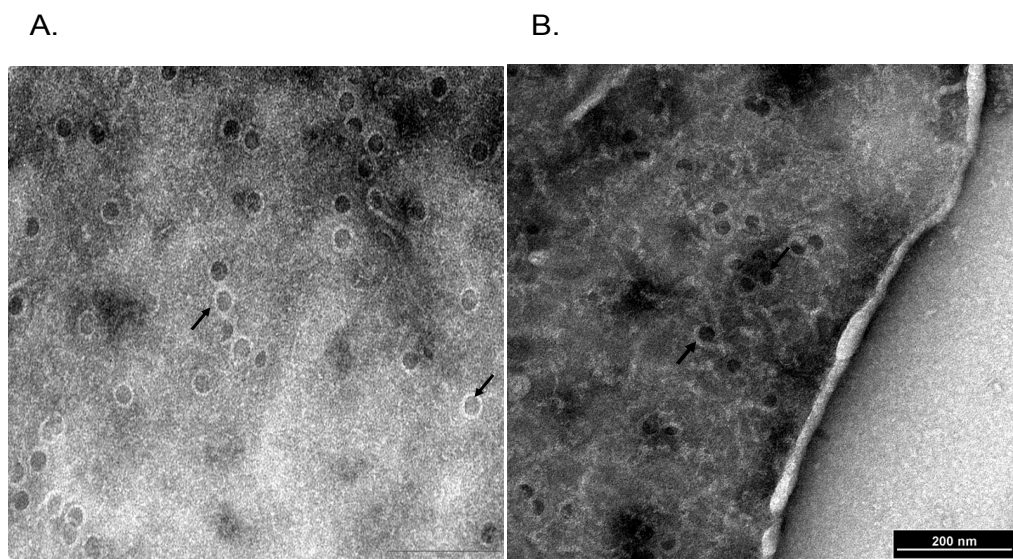


Figure 2.8: Electron microscopy of oligomeric forms of PLO. (a) Sheep red blood cell membranes, incubated with  $80 \mu\text{g/ml}$  of monomeric PLO. (b) Sheep red blood cell membranes, incubated with  $80 \mu\text{g/ml}$  of solution-derived oligomers.

cannot correspond directly to single SDO but rather must have arisen by association of several of the latter.

Figure 2.9 shows SDO that have not been incubated with membranes. They appear as oblong structures with a length mostly between 7 and 15 nm (see arrows.). Assuming a distance between two neighboring subunits in an oligomer of 2.5 nm [33], this corresponds to 3-6 subunits per oligomer. This size is definitely much smaller than that of oligomers observed on membranes, which further supports the idea that several solution-derived oligomers jointly give rise to one larger pore upon binding to target membranes.

### **2.3.5 Mechanism of Pore-Formation by Solution-Derived Oligomers**

The observed formation of one large pore from several SDO might occur by way of directly merging several SDO into one membrane-associated oligomer. Alternatively, it is also conceivable that SDO, though stable in buffer, would dissociate upon membrane binding into monomers, which would then assemble into oligomers in the usual way. To distinguish between these possibilities, we employed a hemolytically active single cysteine mutant of pyolysin (N90C) that was labeled with a mixture of fluorescein and rhodamine. These two labels constitute an efficient pair for fluorescence energy transfer; they were attached to the unique cysteine residue of the mutant protein, so that each monomer in an SDO carried either fluorescein or rhodamine, but not both. Table 2.1



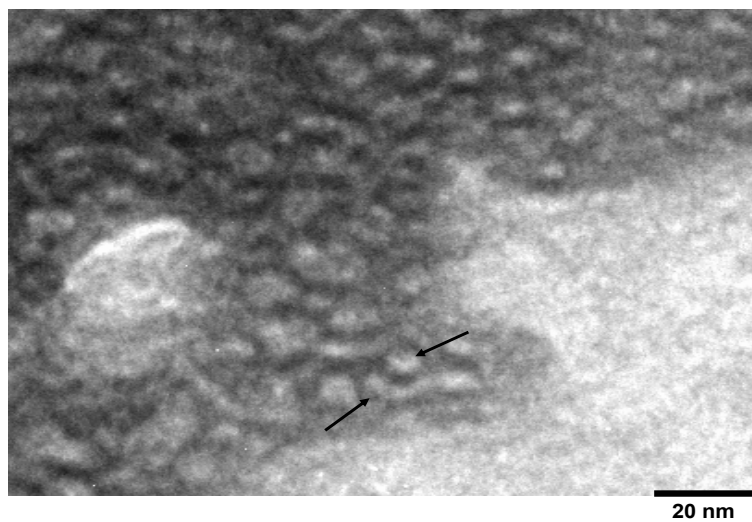


Figure 2.9: Electron microscopy of solution-derived oligomers in the absence of red blood cells.

lists the fluorescence lifetimes of labeled SDO in various experiments, as well as those of monomeric control samples. The average fluorescence lifetime of fluorescein in a monomer sample is 3.4 ns; it drops to 2.5 ns upon interaction with membranes, which is due to FRET between fluorescein- and rhodamine-labeled subunits drawing near within mixed oligomers. Inclusion of unlabelled monomers in excess over the labeled ones will prevent the two labels from getting close within hybrid oligomers, and accordingly oligomerization does not result in a shorter lifetime in this case.

Table 2.1: Fluorescence lifetimes of pyolysin mutant T90C, labeled with fluorescein and rhodamine, under various experimental conditions. The labeled protein concentration used was 10  $\mu\text{g/ml}$ , the molar ratio between the labeled and the unlabeled protein used was 1:4, and all the samples with membranes were incubated for 30 minutes at 37°C. The lifetimes given represent the weighted averages of two lifetime components, determined as detailed in the Methods section.

Sample	$\tau_1, I_{o1}$	$\tau_2, I_{o2}$	$\chi^2$	Average lifetime (ns)
Labelled monomers	1.201, 5591	4.217, 15166	1.16	3.40
Labelled monomers, membranes	1.038, 8466	3.915, 8762	1.109	2.50
Labelled and unlabelled monomers, membranes	1.616, 4338	4.211, 10382	1.12	3.45
Labelled SDO	1.151, 7935	3.847, 9039	1.26	2.59
Labelled SDO, membranes	1.131, 5848	3.964, 8226	1.052	2.79
Labelled SDO, unlabelled monomers, membranes	1.111, 5994	3.964, 7824	1.116	2.73

The fluorescence lifetime of SDO is similar to that of the membrane-derived oligomers; it changes very little upon interaction with membranes in the presence of unlabeled monomers in excess. If the SDO had dispersed into monomers, these should have then formed hybrid oligomers with unlabeled monomers, and the fluorescence lifetime should have increased to a value similar to that observed before with labeled and unlabeled monomers on membranes. The fact that this did not happen indicates that the SDO do not dissociate. Therefore, SDO may interact directly with one another to form larger oligomers, which then give rise to trans-membrane pores.

### **2.3.6 Functional Size of Pores Formed by Solution-Derived Oligomers**

Previous experiments with an oligomerization-impaired mutant of streptolysin O (another CDC with homology to PLO) have demonstrated that incomplete, arc-shaped oligomers may form pores of reduced functional diameter[121]; however, this point has been contested [155]. We therefore asked whether or not the higher abundance of incomplete, arc-shaped pores observed with SDO resulted in a reduced functional pore diameter. To answer this question, dextran marker release assays were performed, using fluorescein-labeled dextrans of different molecular weights. Figure 2.10a shows that dextrans 250 and 2000 (with average molecular weights of 250 and 2000 kDa, respectively) are released to the same extent if the membranes are exposed to monomeric PLO;

the same also applies for smaller species such as dextran 4 and 75 kDa (data not shown). This suggests that essentially all toxin pores are permeable for the largest species. Dextran 2000 has an estimated molecular diameter of 58 nm [149], which exceeds the diameter of the PLO pores. The fact that dextran 2000 is released nevertheless is in line with previous findings that dextran and other linear polymers can permeate across pores of smaller diameter by partial uncoiling and ‘reptation’, *i.e.*, a snake-like wriggling action that results from Brownian motion [106]. With pores formed by solution-derived oligomers, release of dextrans also proceeded in parallel up to a molecular weight of 250 kDa (data not shown). However, dextran 2000 was released to a lesser extent than dextran 250 at all toxin concentrations tested (Figure 2.10b). Therefore, the incomplete, arc-shaped oligomers observed by EM do indeed represent pores of reduced functional diameter.

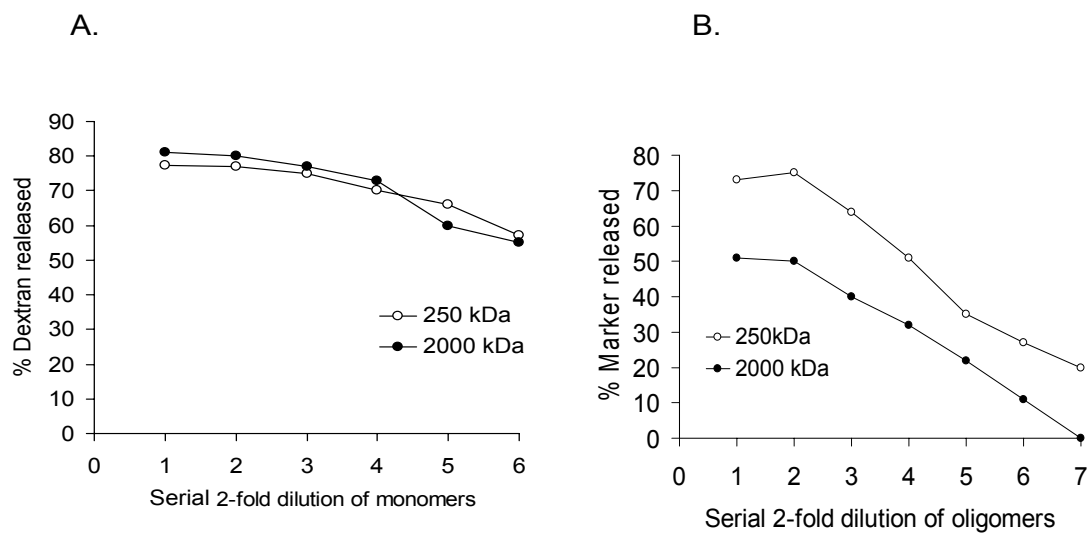


Figure 2.10: Release of FITC-dextran 250 and 2000 from human erythrocyte ghosts by monomeric PLO and by solution-derived oligomers. Ghosts were incubated for 30 min with twofold serial dilutions of (a) monomeric PLO and (b) solution-derived oligomers; the concentration in the first well was 133 g/ml in either case. The percentage of marker released was obtained by fluorescence as described in Materials and Methods.

## 2.4 Discussion

While CDCs require cholesterol (normally as part of lipid membranes) for efficient oligomerization, the slow formation of oligomers in solution has been reported before for one such toxin (pneumolysin) [51, 53, 158]. Analytical ultracentrifugation showed that the size of these oligomers ranges from dimers to hexamers [158]. In the present study, we show that PLO, too, may form oligomers in solution. While the exact subunit stoichiometry - or distribution thereof - of this species was not determined, size exclusion chromatography indicates a number of approximately 6 subunits, whereas EM suggests some degree of variation, which is in line with the previous findings on pneumolysin. A novel finding of this study is the observation of hemolytic activity in the solution-derived oligomers. Frequently, oligomerization of pore-forming proteins in solution is associated with loss of activity. This has been observed with prematurely oligomerizing mutants of *Staphylococcus aureus*  $\alpha$ -hemolysin [169]; similarly, the lack of hemolytic activity in complement membrane attack complexes that form prematurely in solution is the underlying principle of the complement-fixation assay [78].

With CDCs and other pore-forming toxins, it is commonly assumed that the unit of toxin polymerization on membranes is the toxin monomer itself. The observation of hemolytic activity in the SDO shows that this need not necessarily be the case - larger oligomers can assemble from smaller ones. One might therefore consider the possibility

that, not only in solution but also on membranes, small oligomers may form first and then function as the unit of polymerization in the formation of larger oligomers. However, the time course of oligomerization of streptolysin O on membranes [122] supports a mechanism of oligomerization that is directly driven by monomers. In addition, a difference in the mechanism of pore-formation by monomers and SDO is suggested by the predominance of incomplete, arc-shaped oligomers on membranes exposed to SDO. How can this latter observation be understood? With streptolysin O, oligomerization follows a two-step nucleation-propagation pattern. The rate-limiting step is nucleation; the relative rapidity of propagation ensures that most oligomers will complete a short time after initiation. Therefore, at any time, the number of incomplete oligomers competing for the limited pool of monomers is low [122]. In contrast, with SDO, there is no distinction between monomeric and oligomeric reactants, and thus there may not be much of a kinetic difference between nucleation and propagation. Furthermore, the assembly of complete rings may be sterically hindered, as two arc-shaped intermediates each spanning more than a half-circle will no longer be able to combine into a full one.

The observation that the pores formed by SDO have a lower effective diameter confirms the previous assertion that incomplete, arc-shaped oligomers can indeed form pores [121]. It does not, however, imply anything about the temporal coordination of oligomerization and membrane insertion; while the study cited proposed a simultaneous progression of both, it has since been shown that oligomerization does indeed precede mem-



brane insertion, resulting in the formation of a transient oligomeric ‘pre-pore’ complex [155, 61].

Incubation of PLO at various concentrations and for different lengths of time resulted in different yields of SDO. In contrast, the size was not detectably affected, indicating that the SDO is at least meta-stable and does not readily grow by the addition of further monomers. Why is this? One hypothesis would be that the SDO assume some closed shape, *e.g.*, ring shape (Figure 2.11a) that precludes any addition of further subunits. However, this does not fit with the extended appearance of the SDO observed by EM, or with the fact that SDO can readily react with each other on membranes.

The only other possibility appears to be that SDO undergo some conformational change that prevents them from reacting with monomers, without preventing them from reacting with each other (Figure 2.11b and c; in solution, the reaction between SDO may be slow because of their low concentration). While ‘global’ spectroscopic methods such as circular dichroism do not provide evidence of any conformational change accompanying the formation of SDO, differences observed in the interaction of monomeric PLO and SDO with membrane lipids suggests that some conformational differences do indeed exist (see chapter 3). The assumption of a conformational transition raises the question how it may be kinetically related to the progress of oligomerization.

Solution-derived oligomers (SDO) of pyolysin are limited in size; oligomerization

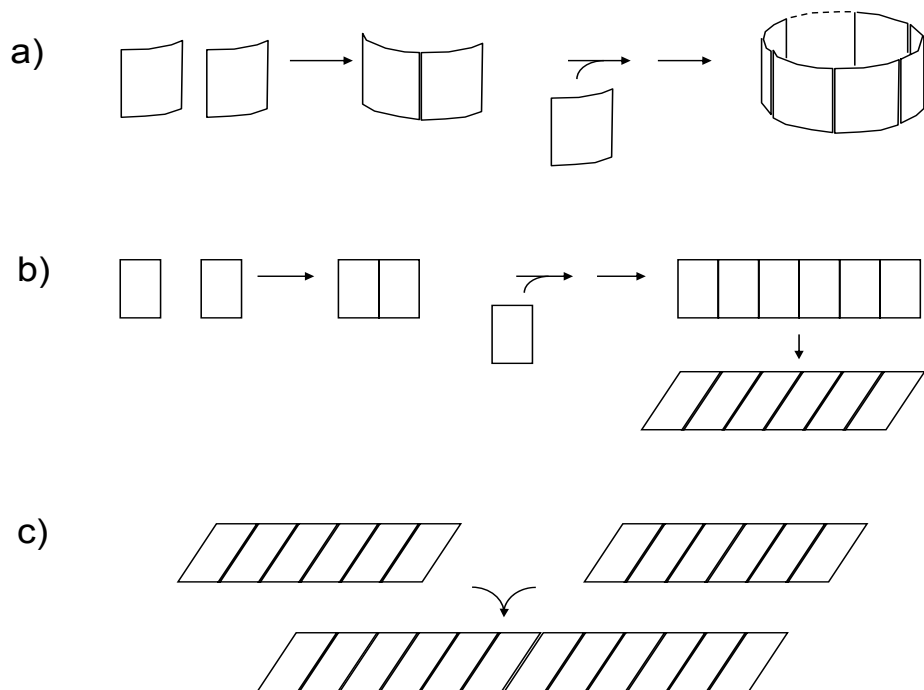


Figure 2.11: Hypothetical explanations for the limited size of oligomers forming in solution: The addition of further monomers may be prevented either by closed geometry of the oligomer (a) or by a conformational change that occurs at a certain stage of oligomerization (b). The latter would still permit the association of oligomers with each other (c), as observed at higher concentration in solution and on membranes.

ceases at a size of approximately 6 subunits. It was proposed that the premature termination of oligomerization is due to a conformational transition of the SDO. The probability (or kinetic rate of this change) may be related to the size of the SDO in various ways: It may be cooperative in nature and therefore require a minimum size of the oligomer. Once this minimum size is reached, the rate of the conformational change may or may not change with the number of oligomer subunits. If we assume that the conformational change may originate in a single subunit or a small number of cooperating subunits and then spread in a domino-like fashion among the residual subunits - as has been observed with oligomers of *Staphylococcus aureus*  $\alpha$ -hemolysin, [166] - the rate of the conformational change will be approximately proportional to the size of the oligomer.

Figure 2.12 shows numerical simulations based on the assumptions that:

1. Oligomerization starts with a nucleation event, which is of second order for the monomers (as with the membrane-dependent oligomerization of streptolysin O [122],
2. The nucleus grows by 'propagation', *i.e.*, successive addition of further monomers, which is of first order for both the monomers and the nuclei,
3. The rate of the conformational change of the SDO that terminates its growth is either constant, or proportional to the size of the SDO.

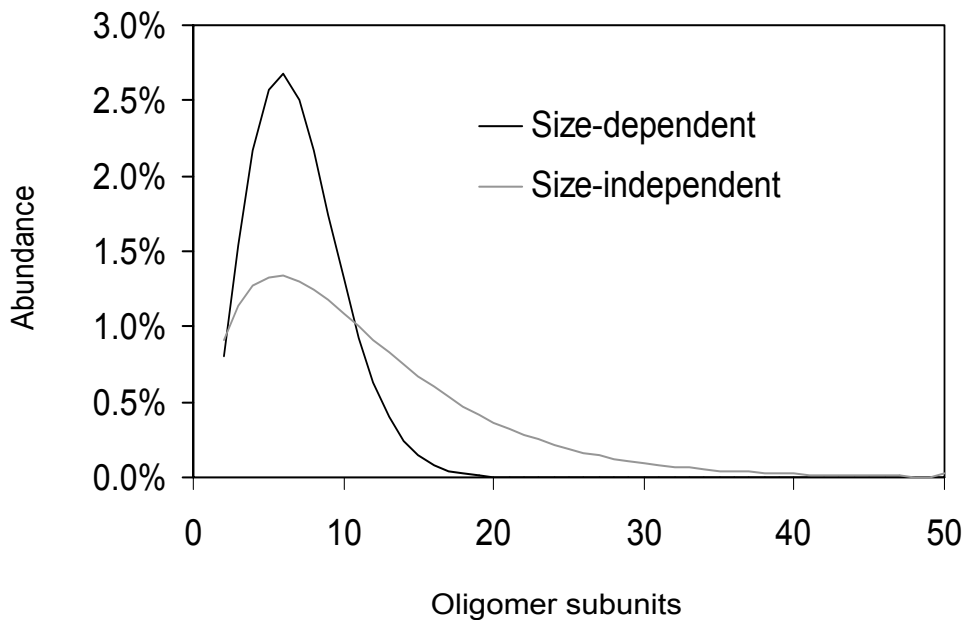


Figure 2.12: Hypothetical oligomer size distributions for the cases of a size-independent and a size-proportional rate constant, respectively, of the conformational change that results in the termination of oligomer propagation.

The constraints of the model do not suffice to determine the rate constants of all reactions: As long as the rate of nucleation is low, the resulting oligomer size distribution is determined by the ratio of the rate constants for propagation and conformational change. A program was written by Dr. Palmer that systematically varied this ratio, using a gradient descent method so as to make the hexamer the most abundant oligomer species. The simulation was stopped once the monomer level had dropped to below 80% of the total. The results of these calculations are shown in Figure 2.12.

With the assumption of a size-dependent rate constant, the oligomer size distribution peak is fairly sharp, which seems to be in agreement with the elution profile (it would be sharper yet if two or more adjacent subunits were required to trigger the conformational change; not shown). In contrast, with a size-independent rate constant of the conformational change, the resulting distribution is much broader. Considering that oligomers with more than 12 subunits should be eluted in the exclusion volume of the Superdex column, this simulation does not match the experimental chromatographic elution profile.

The model predicts that the maximum of the oligomer size distribution should change with the square root of the monomer concentration; whether or not this holds true is difficult to determine experimentally, since at low toxin concentrations oligomerization proceeds very slowly.

In sum, our study shows that PLO forms small oligomers in solution, the size of which may be limited by an internal conformational change that terminates oligomerization. These small oligomers retain hemolytic activity and are capable of assembling into larger ones on membranes. The oligomers thus formed remain incomplete and arc-shaped, and they give rise to pores of smaller functional size than the ring-shaped, complete ones that result from the incubation of monomeric PLO with membranes. This reaffirms the notion that incomplete, arc-shaped oligomers can indeed form functional trans-membrane pores.

## **Chapter 3**

# **A Cholesterol-Dependent Cytolysin (Pyolysin) Permeabilizes Cholesterol-Free Liposomes**

### 3.1 Introduction

*Arcanobacterium pyogenes* secretes a pore-forming toxin, pyolysin (PLO), which is a member of the family of cholesterol-dependent cytolysins (CDC) [19, 74, 34, 73, 72]. These toxins exhibit two characteristic features: the formation of very large pores, and their dependence on the presence of cholesterol in their target membranes [18, 119, 162].

Membrane binding of the CDC toxins involves a conserved region close to the C-terminus that contains three closely spaced tryptophan residues. With the exception of only two toxins (PLO and intermedilysin [19, 108]), this undecapeptide sequence also contains a unique cysteine residue, the modification of which renders the toxin molecule inactive. The fact that some such modifications may be reverted by thiol reagents, and that the toxins may thereby be reactivated, has earned the toxin family the alternative name of ‘thiol-activated toxins’.

Within the CDC family, PLO is the most divergent member, exhibiting only 31-41% amino acid similarity to the other ones. It is also one of the two only toxins lacking the conserved cysteine, and the three tryptophan residues within its undecapeptide region deviate from the otherwise perfectly conserved spacing [19, 18, 20]. The divergence within the putative membrane-binding region prompted us to examine the lipid specificity of the pyolysin-membrane interaction. We report here that pyolysin is able to permeabilize liposome membranes that contain no cholesterol or any other sterol.

In chapter 2. we discussed our findings that pyolysin is subject to a slow oligomerization in solution. The process of oligomerization and the behavior of the oligomers thus formed on cell membranes are the subject of chapter 2. Here, we report that the lipid specificity of monomeric toxin differs from that of the solution-derived oligomers, and we discuss the implication of this finding for the role of cholesterol in the different stages of toxin action.



## **3.2 Materials and Methods**

### **3.2.1 Production and Purification of Histidine-tagged PLO**

Pyolysin (PLO) was purified as described before in chapter 2 (subsection 2.2.1), and the solution derived oligomers were obtained using superdex 200 column as described in subsection 2.2.3.

### **3.2.2 Lysis of Red Blood Cells**

Hemolysis assay was conducted as described before in subsection 2.2.4.

For Sphingomyelinase treatment, 0.5 % washed red blood cells were incubated for 30 minutes at room temperature with 50 milliunits/ml sphingomyelinase from *S. aureus* (EC 3.1.4.12 , Sigma) in the hemolysis buffer in the presence of 10 mM MgCl<sub>2</sub>.

### **3.2.3 Preparation of Liposomes**

All lipids were purchased from Avanti Lipids (Alabaster, AL). The lipids, dissolved in chloroform, were mixed in various molar ratios (see "Results"), dried down under nitrogen in a round-bottom flask, and then dried under vacuum for an additional 3 h to remove the residual solvent. The dried lipids were hydrated at room temperature for 1 h in 1 ml of phosphate buffer saline (PBS), pH.7.3 containing 50 mM calcein. The lipid suspension was sized down to unilamellar liposomes using a liposome extruder (Northern Lipids,

Vancouver, British Columbia, Canada) by extrusion 10 times through a polycarbonate membrane with a 100-nm pore size.

For ceramide-containing liposomes, the suspension was warmed to 45°C during hydration. The suspension was frozen at -20°C and thawed at room temperature for several times. The lipid suspension was sized down to unilamellar liposomes using a liposome extruder (Northern Lipids, Vancouver, British Columbia, Canada) by extrusion 10 times through a polycarbonate membrane with a 100-nm pore size. The extruder was thermostatted to 45°C to facilitate processing of the ceramide-containing liposomes. The non-entrapped calcein was removed by gel filtration on Agarose 6-B pre-equilibrated with HBS.

### **3.2.4 Calcein Release Assay**

The liposomes with entrapped calcein were mixed with various amounts of PLO in 10 mM Tris-HCl, 150 mM NaCl, pH 7.4 buffer. After incubation at 37°C for 10 min, the samples were diluted with the same buffer, and calcein fluorescence intensity was measured immediately (excitation, 480 nm; emission, 520 nm). The fraction of calcein released was calculated by deducting the fluorescence intensity of a control sample incubated without PLO. 100% release was obtained from a liposome sample lysed by 6 mM sodium deoxycholate (pH. 8.0).

### **3.2.5 Electron Microscopy**

Red blood cells, with or without pre-treatment with sphingomyelinase, were incubated and lysed with 50  $\mu\text{g/ml}$  PLO, and the membranes were collected by centrifugation. The membranes were washed three times with 5 mM HEPES buffer (pH 7.4) and mounted onto carbon-coated grids (Formvar/copper, 200 mesh; Marivac Ltd., St.Laurent, QU) followed by negative staining with sodium phosphotungstate (2% w/v, pH 7.4) for 5s. Membranes were then examined in a LEO 912 AB Omega electron microscope operated at 100 kV.

### **3.2.6 Protein Assay**

Pyolysin concentrations were determined using Bradford assay or by the absorbance at 280 nm and a calculated extinction coefficient of  $68480 \text{ M}^{-1}.\text{cm}^{-1}$ . The extinction coefficient was calculated from the amino acid composition according to Pace *et.al* [118].

### **3.2.7 Spectrofluorimetry**

Fluorescence measurements were taken using a PTI QuantaMaster spectrofluorimeter (Photon Technologies Inc., London, Ontario, Canada). The excitation wavelengths were 289, 492 nm, and the emission wavelengths were 336, 520 nm for tryptophan and calcein, respectively. The band pass was 2 nm for both emission and excitation.

### **3.2.8 Binding of PLO to Liposomes**

Multilamellar PC liposomes, with or without 50% by mole cholesterol, were prepared as above, except that the membrane extrusion step was omitted. 100  $\mu\text{g/ml}$  of solution-derived oligomeric or of monomeric PLO were incubated with 500  $\mu\text{g/ml}$  of these liposomes. After 30 min incubation at 37°C, the liposomes were sedimented by centrifugation at 13,000 RPM in a table-top centrifuge for 15 mins, and the fraction of protein remaining in the supernatant determined by protein assay.

## **3.3 Results**

### **3.3.1 Activity of PLO on Sphingomyelinase-Pretreated Erythrocytes and Ceramide-Containing Liposomes**

Compared to other CDC, the activity of pyolysin on sheep red blood cells is rather low; in one of our initial experiments, we examined whether the susceptibility could be increased by treatment of the cells with sphingomyelinase, which proved to be the case (Fig 3.1). Figure 3.2 shows electron micrographs of red cell membranes with or without sphingomyelinase pretreatment after exposure to the same amount PLO (80  $\mu\text{g/ml}$ ). The difference in the number of pores on the membranes mirrors the difference in membrane susceptibility.

Sphingomyelinase converts sphingomyelin to ceramide. The enhancement of membrane susceptibility to pyolysin by ceramide was confirmed in a liposome model (Figure 3.3). Liposomes composed of 35% cholesterol and 20% ceramide by mole, with the balance being dimyristoyl-phosphatidylcholine (DMPC), leaked a fluorescent marker (calcein) to a much higher extent than liposomes containing cholesterol and DMPC only (35:65 by mole).

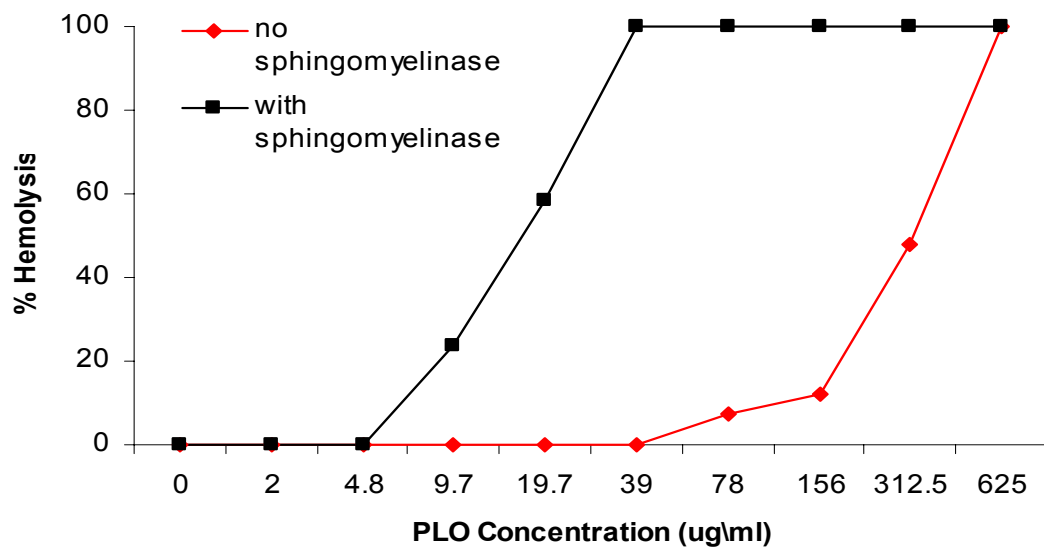
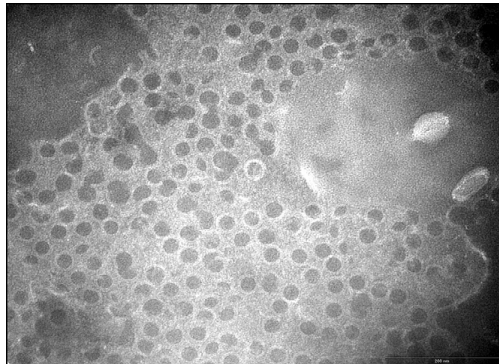


Figure 3.1: Hemolysis of human erythrocytes by PLO with and without pre-treatment with sphingomyelinase (see materials and methods). Red blood cells (0.5% by volume) were exposed to various concentrations of PLO. Hemolysis was assayed by measuring the  $OD_{540}$  of hemoglobin released into the supernatant.

With Sphingomyelinase



Without sphingomyelinase

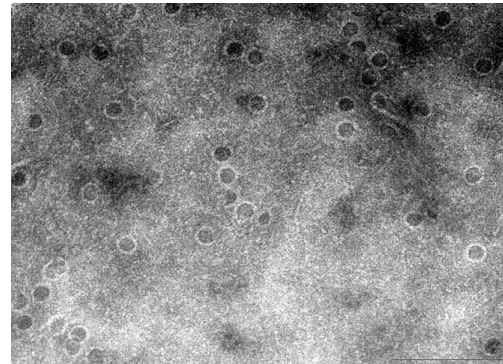


Figure 3.2: Electron microscopy of pores formed by PLO on red blood cells with or without pre-treatment with sphingomyelinase. Sheep red blood cell membranes, incubated with 80  $\mu\text{g/ml}$  of solution-derived oligomers.

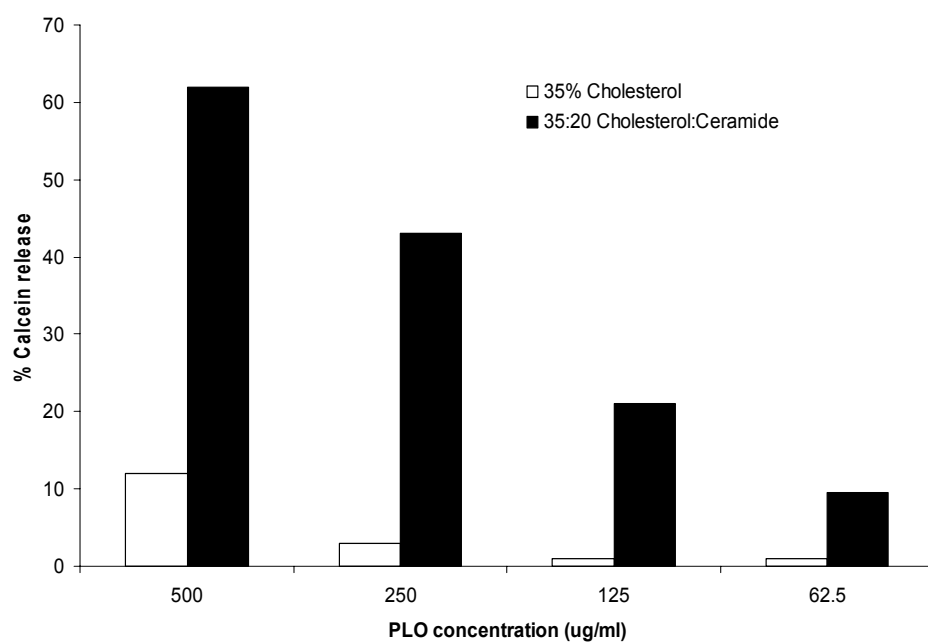


Figure 3.3: Calcein release assay for liposomes composed of 35% cholesterol and 35:20 cholesterol and ceramide by mole with PLO.



### **3.3.2 PLO Permeabilizes Liposomes Containing Ceramide but no Cholesterol**

The observation that ceramide increases the susceptibility of liposome membranes also containing cholesterol to PLO resembles previous findings with another CDC (streptolysin O) and with *Vibrio cholerae* cytolysin, a structurally unrelated toxin that also requires cholesterol for activity [184, 182]. In that study, the effect of ceramide was proposed to be entirely indirect and to occur by enhancement of the interaction of the toxins with cholesterol. Expecting a similar role of ceramide with pyolysin, we were surprised to observe that liposomes containing 35% ceramide but no cholesterol were more susceptible to PLO than those containing the same molar fraction of cholesterol but no ceramide (Figure 3.4). Both liposome species were substantially less susceptible than those containing the two lipids in combination, suggesting that a synergistic effect similar to the previously reported one was operative, too.

### **3.3.3 The Effect of Ceramide and of Cholesterol Concentrations on Membrane Susceptibility to PLO**

The effectiveness of cholesterol in sensitizing lipid membranes to CDC is strongly dependent on its concentration in the membrane [120, 119]; this was confirmed here with pyolysin, which became far more active when the molar fraction of the sterol in liposome membranes was raised from 35% to 50%. In contrast, the same increase in the concen-

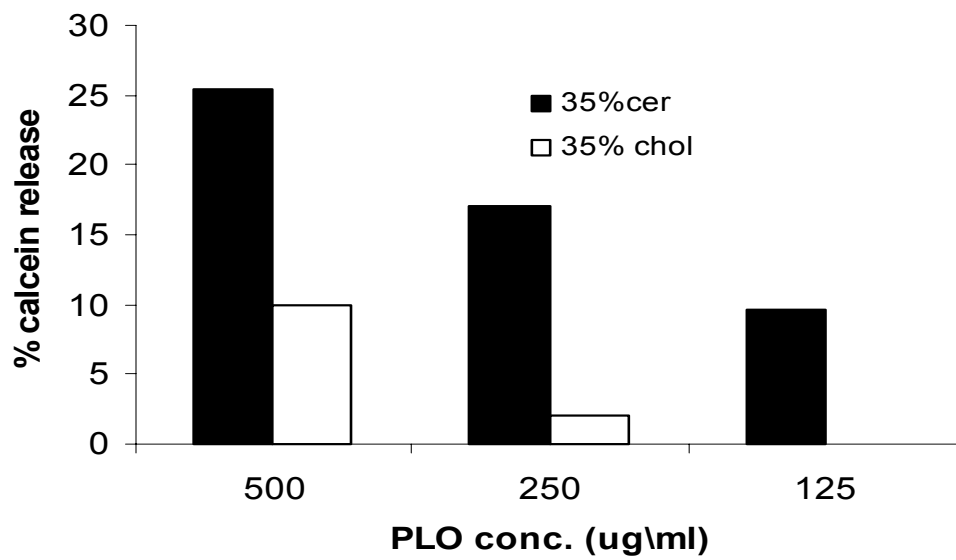


Figure 3.4: Calcein release assay for liposomes composed of 35% cholesterol and 35% ceramide by mole with PLO.

tration if ceramide showed only a minor enhancement on the susceptibility of liposomes to PLO (Figure 3.5).

To test the possible effect of PLO on membranes containing lipids other than ceramide, liposomes containing 50% dioleoylglycerol or phosphatidylethanolamine (which are not closely related in structure to ceramide) or sphingomyelin were prepared and tested by calcein release assay. None of these liposomes exhibited any calcein release upon incubation with PLO; therefore, ceramide remains the only non-sterol lipid identified so far that does confer a low degree of susceptibility to PLO.

### **3.3.4 Oligomerization of PLO on Liposome Membranes**

It has been reported that depleting cell membranes of cholesterol prevents permeabilization by CDC at the stage of membrane insertion, while oligomerization remains possible, so that the toxin molecules accumulate as ‘pre-pore’ oligomers on the membrane [49]. Since even at a ceramide fraction of 50% the susceptibility of liposome membranes to PLO was relatively low, we asked whether the inefficiency of permeabilization was caused at the stage of oligomerization or of membrane insertion. Oligomerization of pore-forming toxins can be detected by proximity-based fluorescence assays such as fluorescence quenching [69] or energy transfer (FRET) [69]). Figure 3.6 shows the fluorescence spectra of an equimolar mixture of fluorescein- and rhodamine-labeled PLO in solution and on different types of liposomes. While this mixture was intended for a

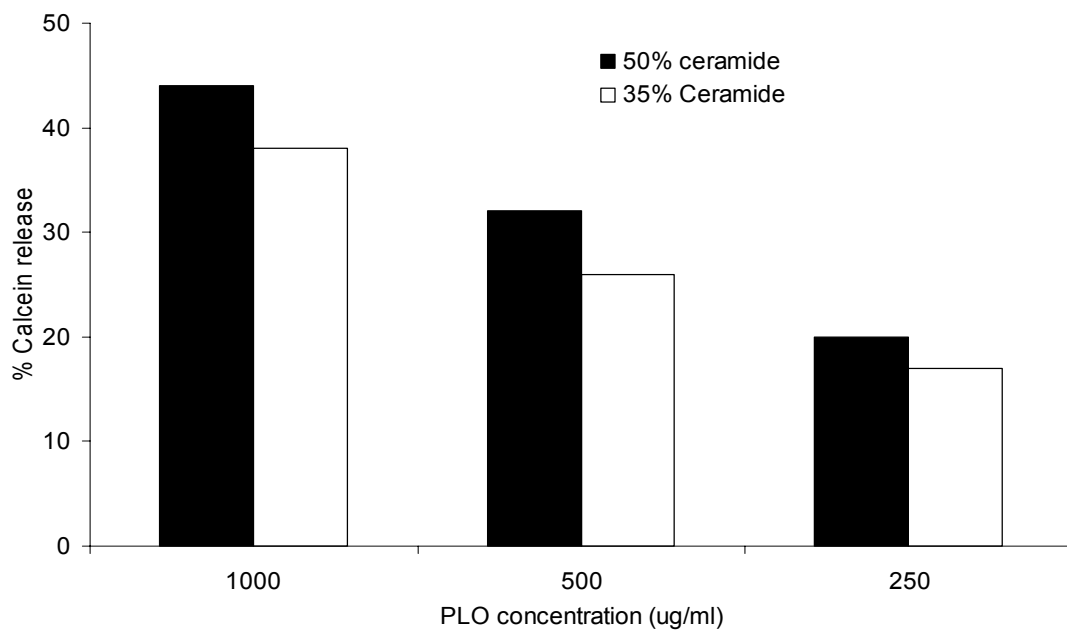


Figure 3.5: Calcein release assay for liposomes composed of 35% Ceramide and 50% ceramide by mole with PLO.

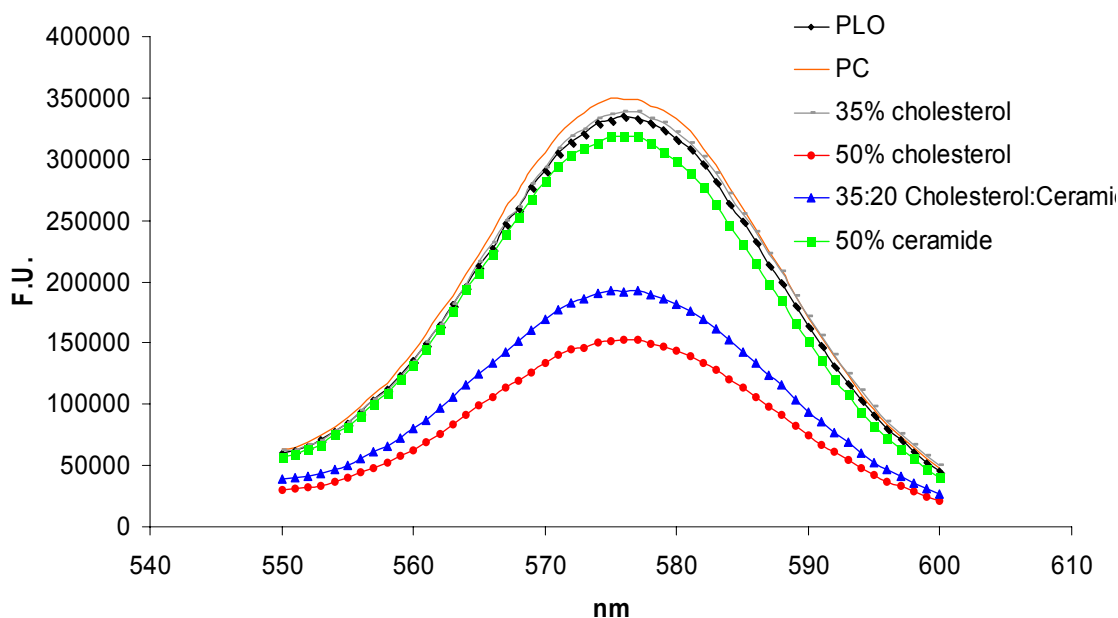


Figure 3.6: Quenching of Rhodamine-labeled N90C PLO on liposomes of different composition.

FRET assay, it turned out to provide a rather more sensitive readout when assaying the quenching of rhodamine fluorescence.

It is evident that the extent of fluorescence quenching is closely correlated with the extent of membrane permeabilization. Quenching (indicative of oligomerization) is very pronounced on the two most sensitive membranes studied, whereas it is minimal or absent with those membranes that have a low degree of susceptibility, including those containing ceramide but no cholesterol. This suggests that the low degree of susceptibility

to PLO conferred by ceramide is caused at or preceding the stage of oligomerization but not only at membrane insertion. This suggestions led us to test the efficiency of binding of PLO to ceramide containing liposome, the preliminary findings indicate low affinity between PLO and ceramide containing liposomes. Further experiments are required to test this process in more details.

### **3.3.5 The Role of Solution-Derived Oligomers in the Permeabilization of Ceramide-Containing Liposomes**

As shown in chapter 2, PLO was found to form solution-derived oligomers (SDO), which are stable in solution; on erythrocyte membranes, they join into larger oligomers to form pores and lyse the cells. The specific hemolytic activity of SDO is approximately four-fold lower than that of monomers. Here, we tested the effect of SDOs and monomeric PLO on liposomes with different lipid composition. Interestingly, we found that the difference in activity is greater with liposomes containing cholesterol but no ceramide (Figure 3.7).

In contrast, on liposomes containing ceramide but no cholesterol, the oligomer is approximately two times more active than the monomer (Figure 3.8).

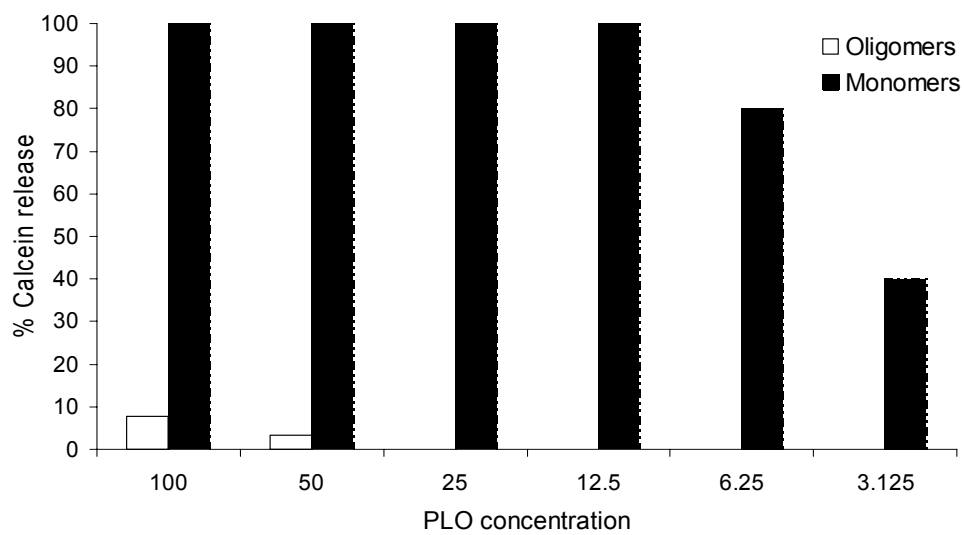


Figure 3.7: Calcein release assay for liposomes composed of 50% Cholesterol by mole with monomeric and oligomeric PLO.

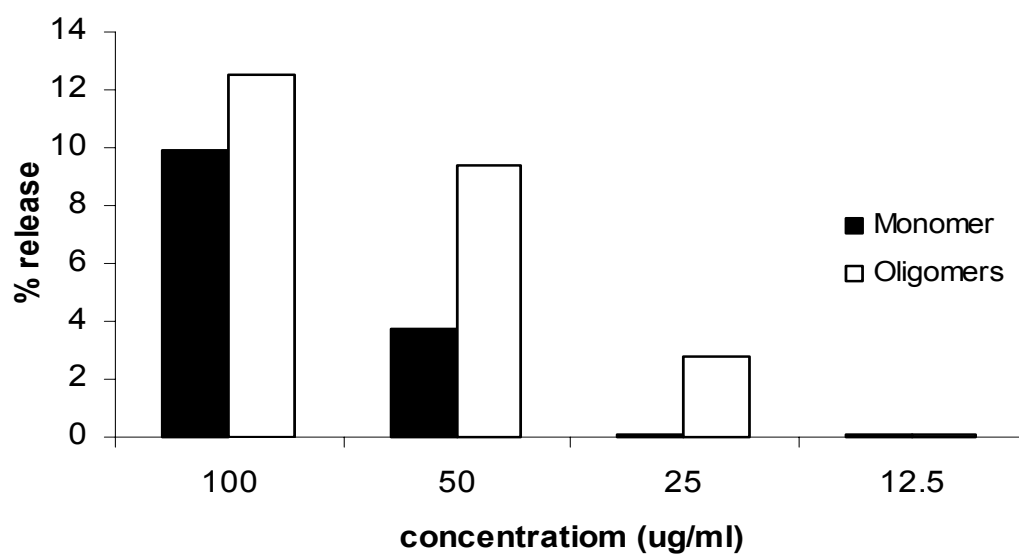


Figure 3.8: Calcein release assay for liposomes composed of 50% Ceramide by mole with monomeric and oligomeric PLO.



### **3.3.6 Binding of Solution-Derived Oligomers to Liposomes Containing Cholesterol**

The low activity of SDO on cholesterol-containing liposomes might conceivably be caused at any stage of pore-formation, *i.e.*, during binding, oligomerization, or membrane insertion. After incubation with multilamellar liposomes (which are convenient for binding assays because they can easily be sedimented by centrifugation), 30% of the monomeric PLO remained in the supernatant, indicating that 70% had bound to the liposomes. In contrast, the absorbance of SDO remained virtually unchanged before and after incubation with liposomes, indicating that the low activity of SDO towards cholesterol-containing liposomes is due to low binding affinity.

### 3.4 Discussion

Cholesterol-dependent cytolysins (CDC) are a group of bacterial toxins that have been so called because of their apparently strict requirement for cholesterol in the target membrane. We here provide evidence that, at least for one of these toxins, cholesterol is not absolutely required. While it has been reported before that several structurally related sterols can functionally replace cholesterol in model membranes [120], the current study is the first one to show that membranes entirely devoid of any sterol can be susceptible, too.

The lack of PLO activity towards liposomes containing dioleoylglycerol, phosphatidylethanolamine or sphingomyelin, indicates that ceramide is, to some degree, specifically required for this interaction. This supports the notion that the effect of ceramide reported here is truly distinct from that described in a previous study [182], in which the activity of streptolysin O and *Vibrio cholerae* cytolysin on membranes containing cholesterol was synergistically enhanced by ceramide but also by dioleoylglycerol or phosphatidylethanolamine. In that previous study, the synergistic effect of ceramide and several other lipids was proposed to be due to their cone shape and to be mediated by an increase in the chemical potential of cholesterol. According to this interpretation, synergism of ceramide and cholesterol should affect any protein that depends on membrane cholesterol; it should therefore be and was indeed here observed with pyolysin as well.

It was pointed out above (page 21) that some argument exists about the stage of pore-formation that involves cholesterol; while several studies support the notion that it is involved in membrane binding of the toxin, it has also been proposed that its main contribution consist in facilitating the insertion of the already assembled pre-pore [49]. The observations of the current study provides additional insight into the interaction of CDCs with lipid membranes and the role of cholesterol.

The first observation is that the activity of pyolysin on ceramide-containing membranes is much lower than that observed with cholesterol-containing membranes. This low activity might conceivably be caused at any of the three major stages of pore-formation, *i.e.*, membrane binding, oligomerization, or membrane insertion. Oligomerization was barely detectable in the fluorescence quenching assay. Preliminary experiments with multilamellar liposomes indicate that, at least with monomeric PLO, binding is inefficient, too, supporting the traditional notion that cholesterol is involved in binding. It still remains to be determined how effectively the minor fraction of PLO that does remain bound proceeds with oligomerization and membrane insertion. In addition, the binding affinity of solution-derived oligomers (SDO), which had a slightly higher specific membrane-permeabilizing activity, must be determined as well.

A role of cholesterol in toxin binding is also consistent with the observation that SDO bind much less efficiently than the monomeric toxin to liposomes containing cholesterol

but no ceramide. If binding and even formation of the oligomeric pre-pore could proceed in the absence of the sterol, it would be difficult to understand why pre-formed oligomers (SDO) should not bind equally well as the monomers. Even assuming that cholesterol does have a role in binding, the current observation remains intriguing: Pre-oligomerization might be expected to aggregate multiple sterol binding sites and therefore *increase* rather than decrease binding affinity. Therefore, the lowered affinity of SDO for cholesterol-containing membranes suggests that the binding site for the sterol that is available in the monomer may be hidden in the SDO. To the extent that the SDO structurally resembles the oligomeric pre-pore that forms on membranes, the low affinity of the SDO suggests that the pre-pore may no longer interact with the sterol. However, this would contradict the previous assertion of an involvement of cholesterol in the membrane insertion of the pre-pore [49]; therefore, this question needs to be investigated further.

Keeping in mind that SDO are still hemolytically active (see 57), the fact that they almost fail to bind to cholesterol-containing liposomes would suggest a possible role of membrane components other than cholesterol in the binding of PLO to erythrocyte membranes. The previous reports on the binding of intermedilysin [50] and of other CDCs to membrane proteins [90] suggests that some such receptor may also exist for pyolysin.

## Chapter 4

### ***Streptococcus agalactiae* CAMP Factor / Protein B Does Not Bind to Human IgG**

This work has been published

El-Huneidi W, Mui R, Zhang TH, Palmer M. (2007) *Streptococcus agalactiae* CAMP factor/protein B does not bind to human IgG. *Med. Microbiol. Immunol.* 196(2):73-7.

## 4.1 Introduction

CAMP factor is a 26 kDa extracellular cytolysin secreted by *Streptococcus agalactiae*. In addition to this cytolytic activity, CAMP factor was reported to bind to immunoglobulins G and M in a manner similar to protein A of *Staphylococcus aureus*, and it was therefore given the alternative name ‘protein B’ [77]. Somewhat unfortunately, that same name (Protein B) has also been applied to another protein produced by Group B streptococci, which has a molecular weight of 130 kDa and binds to IgA [29, 40, 57]. The interaction between CAMP factor and immunoglobulins reportedly inhibits the co-hemolytic activity of CAMP factor [77]. CAMP factor does not share significant homology with protein A, protein G, or any other known antibody-binding protein.

Binding of protein A to the Fc domains of immunoglobulins inhibits the hemolysis mediated by complement [146], suggesting that the finding that the binding sites for protein A and the complement component C1q overlap [81]. In the present study, we intended to examine the possibility of a similar effect with CAMP factor in order to gain insight into the relationship of the binding sites for C1q and CAMP factor. No inhibition of complement-mediated hemolysis was observed. We therefore re-examined the binding of CAMP factor to IgG in more detail. The results indicate that CAMP factor is not endowed with non-immune binding to IgG.

## **4.2 Materials and Methods**

### **4.2.1 Expression and Purification of Wild Type and Recombinant CAMP Factor**

Wild type CAMP factor was purified from a clinical strain of *S. agalactiae* (obtained from the Grand River Hospital, Kitchener, ON, Canada). A liquid culture (500 ml Todd-Hewitt broth with 2 % glucose) was grown at 37°C and adjusted to pH 6.2 every 15 minutes using a sterile-filtered equimolar solution of glucose and sodium bicarbonate. When the culture had reached the stationary phase, the cells were centrifuged at 15,000 g for 15 minutes and the supernatant precipitated using ammonium sulphate (65 % saturation). After centrifugation at 53,000 g for 15 minutes, the pellet was resuspended in 5 mL PBS (16 mM K<sub>2</sub>HPO<sub>4</sub>, 150 mM NaCl, pH. 7.5). The sample was applied to a chromatography column packed with ceramic fluoroapatite (BioRad) and equilibrated with 10 mM potassium phosphate buffer (pH 7.2). The column was eluted using a linear gradient of potassium phosphate buffer (10-400 mM, pH 7.2). The recombinant wild type CAMP factor and the single cysteine mutant F109C were prepared as described by Lang and Palmer [82].

### **4.2.2 Fluorescein Labeling of Mutant Recombinant CAMP Factor**

The CAMP factor mutant F109C was transferred into 50 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 7.5 by gel filtration on a PD10 column (Pharmacia). A 5-fold molar excess of fluorescein maleimide (Molecular Probes) was then added and the mixture was incubated at room temperature for 30 minutes. Unreacted fluorescein maleimide was removed by another round of gel filtration.

### **4.2.3 Hemolysis Assay of Inhibition of Complement Activation**

400  $\mu$ l of 10 % sheep red blood cells were washed four times with HEPES-buffered saline (HBS; 25 mM HEPES, 150 mM NaCl, 2.5 mM MgCl<sub>2</sub>, 0.75 mM CaCl<sub>2</sub>, pH. 7.2) and suspended to a final erythrocyte concentration of 4 % in HBS. 200  $\mu$ l of cell suspension was added to 200  $\mu$ l of 'hemolysin' (rabbit anti-sheep) antibodies (Cedarlane, Hornaby, ON) and incubated for 30 minutes at room temperature. The cells were pelleted by centrifugation at 2500 g for 4 minutes and washed for 4 times with HBS by resuspension and centrifugation. After resuspending in 200  $\mu$ l of HBS, 50  $\mu$ l were transferred to a series of microcentrifuge tubes either containing 50  $\mu$ l of HBS, wild type CAMP factor, recombinant CAMP factor, or protein A (final protein concentration 50 g/mL each). The tubes were incubated for 30 minutes at room temperature. Each tube was supplemented with 200  $\mu$ l of diluted rabbit serum and incubated for 1 hour at 37°C. After spinning



down membranes and unlysed cells at 2500 x g for 4 minutes, hemolysis was measured by the absorbance of the released hemoglobin ( $A_{445}$ ).

#### **4.2.4 Co-hemolytic Activity Assay**

Sheep red blood cells (Cedarlane, Hornby, Ontario, Canada) were washed five times in hemolysis buffer (10 mM Tris-HCl, 150 mM NaCl, pH 7.4) by centrifugation. The erythrocytes were then resuspended in hemolysis buffer to 0.5 % (v/v). The cells were incubated at room temperature for 5 min in the presence of 10 mM  $MgCl_2$  and with 50 milliunits/ml of sphingomyelinase from *S. aureus* (EC 3.1.4.12, Sigma). 180  $\mu$ l of cell suspension were added to the wells of a microplate containing serial dilutions of CAMP factor in 20  $\mu$ l of the former buffer and incubated for 30 minutes at 37°C. After spinning down membranes and unlysed cells at 2500 g for 4 minutes, hemolysis was measured by the absorbance of the released hemoglobin ( $A_{445}$ ). Control samples incubated without CAMP factor remained stable during the assay period.

#### **4.2.5 Size Exclusion Chromatography**

Size exclusion chromatography was performed using a Superdex 200 column (Amersham) equilibrated with PBS. The flow rate was maintained at 0.3 mLmin<sup>-1</sup> with an KTApurifier chromatography system (Amersham Biosciences, Baie d'Urfe, QC, Canada). The elution profile was monitored by online measurement of the  $A_{280}$  (for unlabeled pro-

teins) or  $A_{492}$  (for fluorescein-labeled CAMP factor). The eluate was collected in fractions of 0.3 ml, which were either examined by fluorimetry (see below) or precipitated with trichloroacetic acid (5 % final concentration) for analysis by SDS-PAGE.

#### **4.2.6 Electrophoresis**

SDS-PAGE was performed according to Laemmli [80], using polyacrylamide concentrations of 4% in the stacking gels and of 12% in the resolving gels. Gels were stained with Coomassie blue.

#### **4.2.7 Spectrofluorimetry**

Fluorescence measurements were taken using a PTI QuantaMaster spectrofluorimeter. The excitation wavelengths were 492 and 289 nm, and the emission wavelengths were 520 and 336 nm for fluorescein and tryptophan, respectively. The band pass was 2 nm for both emission and excitation.

#### **4.2.8 Protein Assay**

Recombinant and wild type CAMP factor concentrations were determined using the Bradford assay.

#### **4.2.9 Mass Spectrometry**

Positive ion electrospray mass spectrometry was performed on a Micromass Q-ToF Ultima GLOBAL mass spectrometer. The proteins were transferred to 20 mM ammonium acetate (pH 7.0) buffer using PD-10 columns, lyophilized overnight and dissolved to approximately 10 M in 1:1 acetonitrile/water solution containing 0.2 % formic acid. The samples were infused at 0.8 l/min. Raw data (m/z) were processed using MaxEnt1 software to yield spectra on a true molecular mass scale.

## 4.3 Results

Initial experiments were performed with recombinant CAMP factor expressed in *E. coli*. To rule out the possibility that the observed lack of binding to IgG is due to the slight changes of the primary structure that result from the recombinant expression system [82], wild type CAMP factor purified from *S. agalactiae* was included in the experiments as well. The wild type CAMP factor purified from *S. agalactiae* showed the expected molar mass of 23856 Da (Figure 4.1). Its co-hemolytic activity was similar to that of recombinant CAMP factor (Figure 4.2).

### 4.3.1 CAMP Factor Does Not Inhibit Antibody/Complement-Mediated Hemolysis

At the outset of this study, we intended to characterize the effect of the purported binding of CAMP factor to Fc domains of IgG on the latter's ability to inhibit hemolysis mediated by complement. We used the hemolysis assay that underlies the complement fixation test. In this assay, sheep red blood cells are coated with rabbit anti-sheep ('hemolysin') antibodies, which will activate complement and cause it to lyse the cells. Figure 4.3 shows that, as has been described previously [146], protein A indeed strongly inhibits complement-mediated hemolysis, indicating that its binding to the Fc domains of the hemolysin antibodies prevents these from activating serum complement. In contrast, neither recombinant nor wild type CAMP factor show any degree of complement inhi-

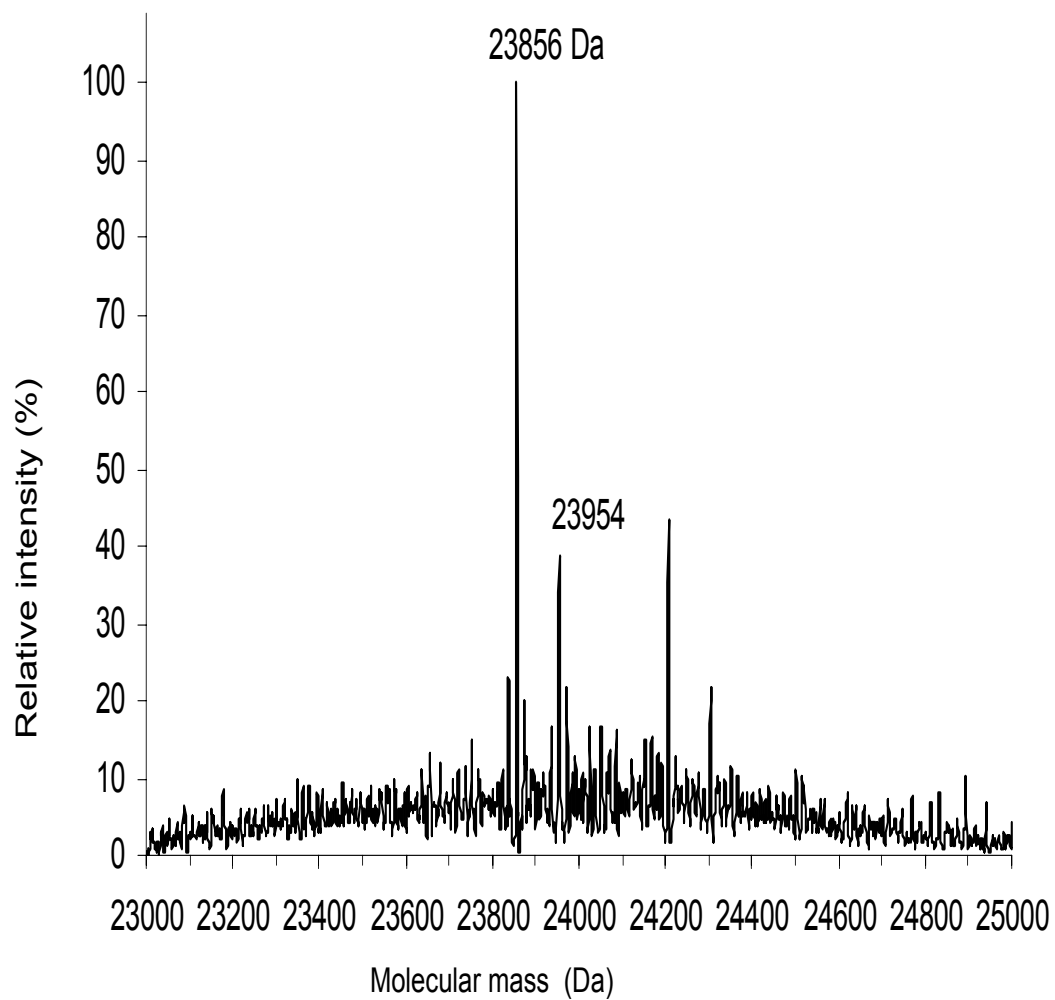


Figure 4.1: Mass spectrometry of wild type CAMP factor purified from *S. agalactiae*. The peak at 23,856 Da corresponds to wild type CAMP factor; the secondary peak at 23,954 Da is likely due to an associated phosphate ion.

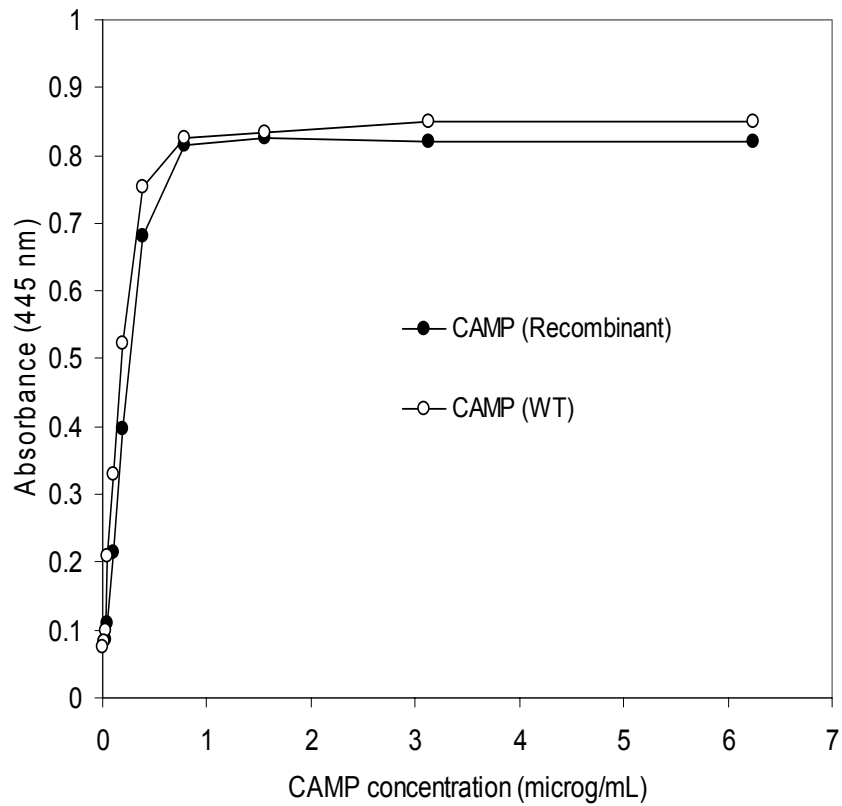


Figure 4.2: Co-hemolytic activity of wild type and recombinant CAMP factor. Sphingomyelinase-treated erythrocytes were incubated for 30 min with the amounts of wild type and recombinant CAMP factor indicated. The samples were centrifuged and the extent of hemolysis determined from the absorbance ( $A_{445}$ ) of hemoglobin released into the supernatant.

bition. Figure 4.3 also shows that CAMP factor alone did not cause hemolysis, as the cells were not sensitized with sphingomyelinase; therefore, the lysis observed with the samples containing both complement and CAMP factor was indeed due to complement activation.

### **4.3.2 Immunoglobulin G Does Not Inhibit the Co-Hemolytic Activity of CAMP Factor**

It has been reported that the non-immune binding to IgG inhibits the co-hemolytic activity of CAMP factor [77]. In this study, we tried to reproduce this inhibitory effect, using various molar ratios of IgG to CAMP factor. However, as shown in Figure 4.4, neither wild type nor recombinant CAMP factors were inhibited to any extent even with IgG present in large molar excess.

### **4.3.3 CAMP Factor Does Not Bind Human Immunoglobulin G**

The previous observations led us to re-examine the binding of CAMP factor to IgG. We chose to employ the same assay that was used in the preceding study, *i.e.*, co-incubation of IgG with CAMP factor, followed by analysis of the sample by size exclusion chromatography. First, fluorescein maleimide-labeled recombinant CAMP factor was incubated with a twofold molar excess of human IgG for 30 minutes at 37°C before application to the column. The fractions were examined for tryptophan and fluorescein fluorescence.

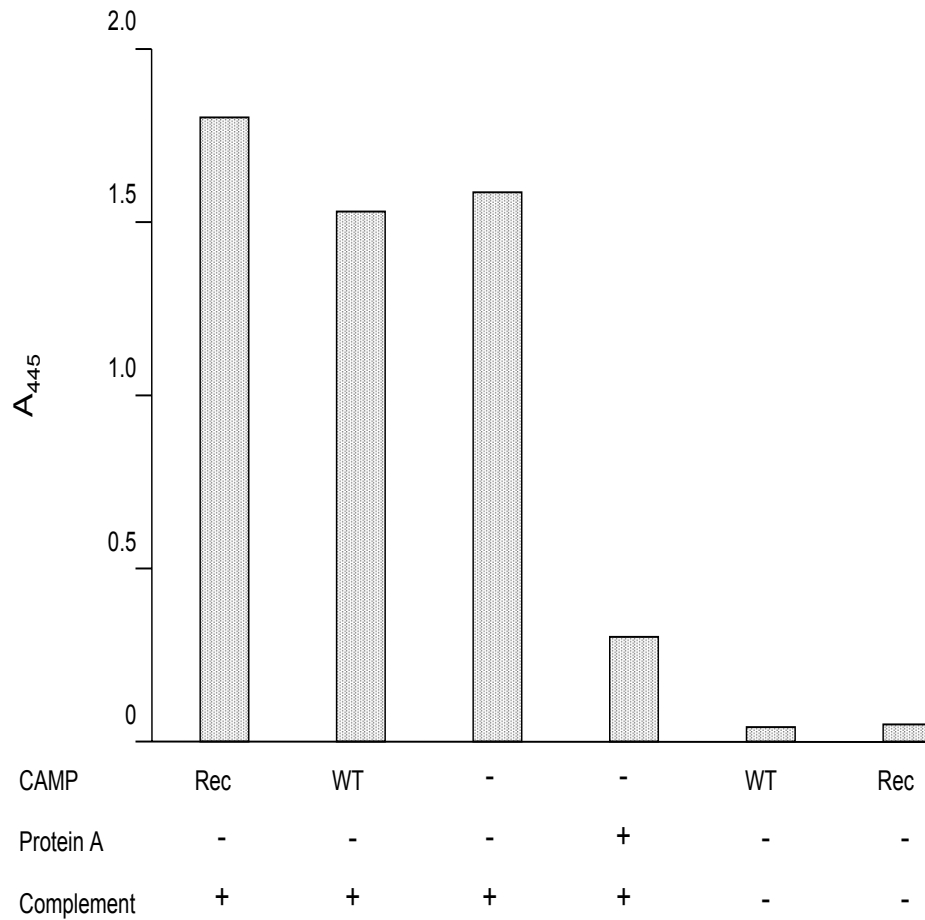


Figure 4.3: Hemolysis assay for inhibition of complement activation. Sheep erythrocytes were sensitized with rabbit anti-sheep IgG. They were then treated with protein A, recombinant (rec) or wild type (wt) CAMP factor as indicated before incubation with a source of complement (rabbit serum). Hemoglobin release from lysed cells was measured by the absorbance at 445 nm. Control samples with recombinant or wild type CAMP factor show that these alone do not cause hemolysis under the given conditions. complement-mediated hemolysis, indicating that its binding to the Fc domains of the hemolysin antibodies prevents these from activating serum complement. In contrast, neither recombinant nor wild type CAMP factor show any degree of complement inhibition.



Since IgG was used in excess and contains multiple tryptophan residues, whereas only one tryptophan residue occurs in CAMP factor, tryptophan fluorescence essentially coincides with the distribution of IgG; fluorescein is associated only with CAMP factor. The two proteins are clearly separated, with IgG being eluted at 12.7 ml and CAMP factor the 15.8 ml (Figure 4.5A), indicating a lack of binding between CAMP factor and the IgG.

To exclude any possibility that the recombinant expression or covalent labeling of CAMP factor had interfered with the binding of CAMP factor to IgG, the size exclusion chromatography was repeated using unlabeled wild type CAMP factor. In this experiment, the collected fractions were precipitated with TCA and subjected to SDS PAGE (Figure 4.5B). Again, no CAMP factor was found in the fractions containing IgG, thus confirming the preceding result.

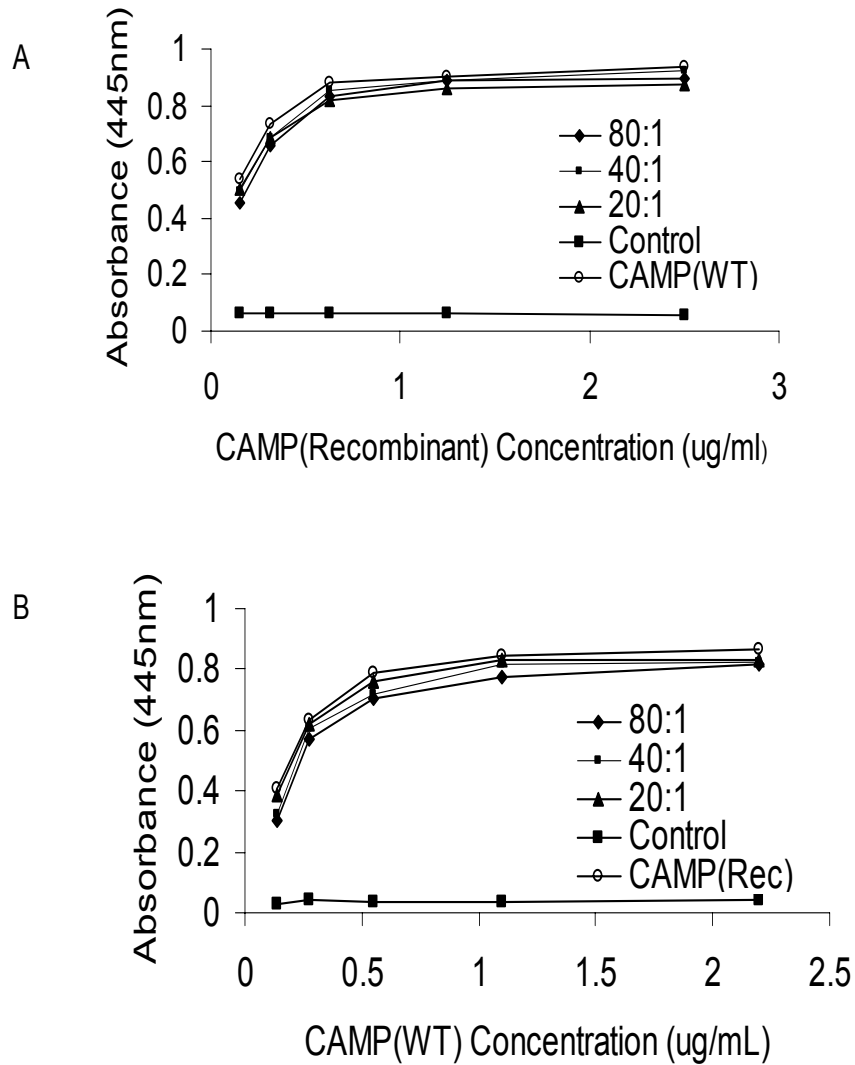


Figure 4.4: Co-hemolytic activities of wild type and recombinant CAMP factor in the presence of human IgG. Sheep red blood cells were pretreated with 25 mU/ml sphingomyelinase at room temperature for 30 minutes. They were then added to mixtures of IgG with wild type (A) and recombinant (B) CAMP factor (molar ratios as indicated). After 30 minutes incubation, the samples were centrifuged and A445 of the supernatant was measured.

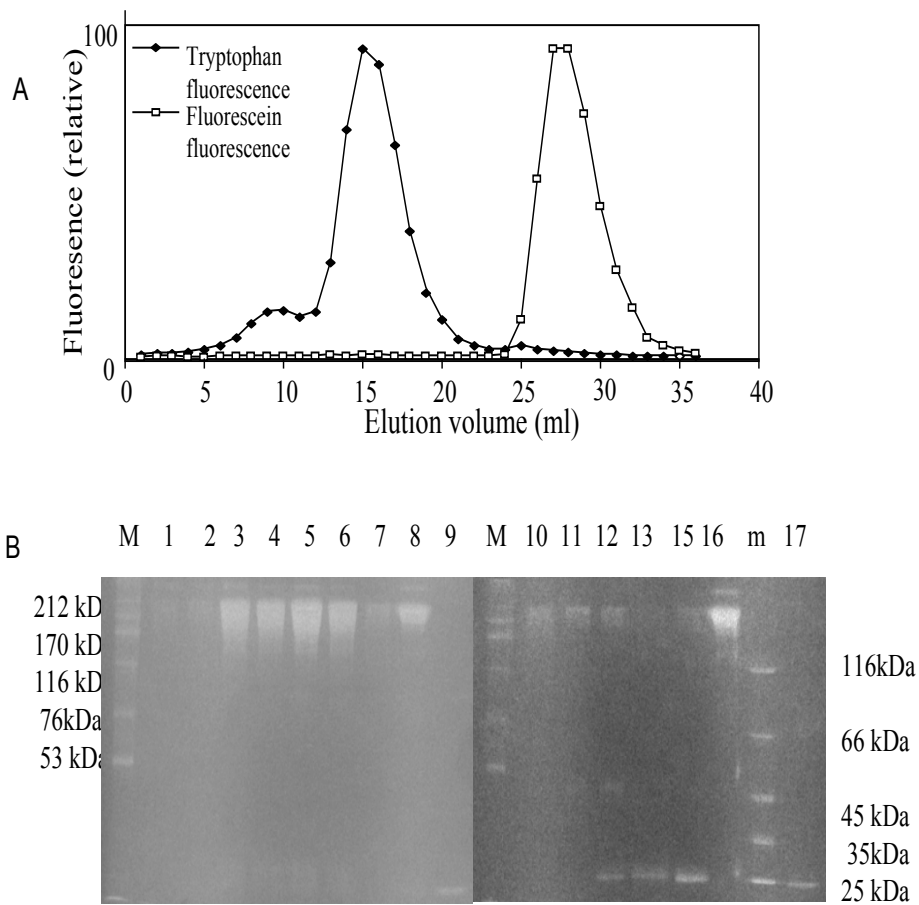


Figure 4.5: A. Analysis of CAMP factor binding to IgG by size exclusion chromatography. A: Tryptophan and fluorescein fluorescence profiles of a mixture of fluorescein-labeled recombinant CAMP factor with unlabeled IgG. Due to the use of IgG in 5 fold molar excess and the low abundance of tryptophan in CAMP factor, the tryptophan fluorescence trace mostly reflects the distribution of IgG. B. SDS-PAGE of fractions obtained from a mixture of wild type CAMP factor with a twofold molar excess of IgG. m, molecular weight marker. M, High molecular weight marker, lanes 1-7 represent the fractions collected between volume 11.3 and 14.9 ml, whereas lanes 11-15 represent the fractions collected between volume 15.4 and 16.6 ml. Lanes 9 and 17 are CAMP factor, lane 8 and 16 is human IgG.

## 4.4 Discussion

The results of the present study all support the conclusion that CAMP factor does not bind to immunoglobulin IgG. These results do not match with the reported findings regarding the binding of CAMP factor to the Fc fragment of immunoglobulin [77]. Indirect support for our conclusion comes from another previous study, which examined the reaction of CAMP factor with sera from adults and newborns infected with *S. agalactiae* as well as healthy control persons [43]. While sera of infected newborns and adults did react with CAMP factor, only 2 out of 11 sera from healthy teenagers gave a positive reaction, indicating that CAMP factor only reacts with specific antibodies, *i.e.*, via the Fab domain. If CAMP factor could indeed bind to the Fc domain, a (false) positive reaction binding should have been observed with all serum samples, regardless of the patients' previous exposure and acquisition of immunity to *S. agalactiae*. In all of the previous experiments that indicated binding of CAMP factor to Fc fragments of IgG and IgM [77], the immunoglobulins were used in 25-fold or higher molar excess over CAMP factor. CAMP factor is a small molecule (25 kDa), and the number of its binding sites for antibody molecules should be limited. The need for far higher than stoichiometric amounts of antibody might suggest a very low affinity of binding; however, as we were unable to reproduce the reported inhibitory effect of IgG on CAMP factor activity even at the same very high ratios, this explanation does not fit our results. Alternatively, the

previously reported effects may have been due to some contaminating protein in the antibody preparation. We conclude that CAMP factor does not bind to human IgG and, most likely, other immunoglobulins; its well-characterized co-hemolytic effect remains the only known activity of this protein. The name protein B in substitution for CAMP factor is therefore not appropriate.

## **Chapter 5**

### **Summary and Future Considerations**

The studies on pyolysin presented in this thesis have shown:

1. PLO forms small oligomers in solution. This observation agrees with previous reports on the formation of solution-derived oligomers by other CDCs [51].
2. The conformation of PLO within solution-derived oligomers (SDO) resembles the monomer or pre-pore and distinctively deviates from the inserted pore.
3. SDO will not dissociate on erythrocyte membranes but will coalesce into larger oligomers that then insert and form pores.
4. Pores derived from SDO tend to be arc-shaped and show reduced functional diameters, corroborating the notion [121] that arcs are indeed functional pores.
5. SDO have reduced activity on PC/cholesterol liposomes, which is due to reduced binding; this suggests that the cholesterol binding site is masked in the SDO, and that the oligomer at some point of its formation or maturation ceases to interact with cholesterol. It is still possible that cholesterol may become involved with the oligomer again at the final stage of membrane insertion.

Future experiments should elucidate the interaction of PLO with ceramide and with natural cell membranes:

1. In this study, N-Lauroyl-D-erythro-sphingosine (C-12 Ceramide) was the only ceramide tested. To determine the structural features of the ceramide molecule re-

sponsible for this sensitizing action, it would be worthwhile to compare the effects of other ceramides with different carbon chain lengths, as well as other ceramide derivatives (for example, using ceramides in which the C4-C5 double bond is replaced by a triple bond, a cyclopropyl group, or a benzene ring [182]).

2. Although the fact that SDO are more active than monomeric PLO suggests that oligomerization is required for permeabilization of ceramide-containing membranes, it has not been directly demonstrated that oligomerization does indeed occur on such membranes. This should be addressed with more sensitive assays of oligomerization, possibly involving separation of oligomers from monomers by size.
3. From the fairly high concentrations of pyolysin required to permeabilize membranes containing ceramide but not cholesterol, we can conclude that the specific activity of toxin on such membranes is low. While preliminary results suggest that this is due to low binding affinity, it would be valuable to use more sensitive and quantitative assays to measure binding affinity (this may include fluorescence techniques as well as surface plasmon resonance).
4. Our findings that SDO does not bind to cholesterol liposomes, while it is hemolytically active, would suggest a possible role of membrane receptors on red blood cells. It would be worthwhile investigating this possibility.



Streptococcal CAMP factor was previously reported to bind to the Fc fragment of IgG and therefore was called Protein B [77]. We have shown here that such binding does not occur. Likewise, we were unable to reproduce the reported inhibitory effect of IgG on the co-hemolytic activity of CAMP factor.

The negative findings of our study do not encourage any further research on the interaction of CAMP factor with immunoglobulins. Future studies should focus on more fully characterizing the structure, receptor interaction and membrane-permeabilizing activity of CAMP factor.

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