Streptococcus agalactiae CAMP factor: Functional Roles of N- and C-terminal Domains & Target Membrane Effect on Toxin Activity

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners. I understand that my thesis may be made electronically available to the public.

Abstract

CAMP factor is a well-known pore-forming toxin secreted by *Streptococcus agalactiae* that forms discrete pores on susceptible membranes whether natural or artificial.

This work reports haemolysis and liposome permeabilization experiments with intact CAMP factor and with fragments representing its N- and C-terminal domains, as well as fluorescence experiments using an NBD-labelled cysteine mutant. The collective findings indicate that the N-terminal domain of CAMP factor is responsible for membrane permeabilization while the C-terminal accounts for membrane binding and likely participates in oligomerization.

Based on previous work, which suggested that varying target membrane lipid composition affects CAMP factor activity and evidence pointing to a cholesterol requirement, further studies were conducted on the requirement of specific lipids for CAMP factor activity, as well as varying lipid chain length and degree of saturation on liposomes with and without cholesterol. The results show that cholesterol is not required for membrane permeabilization. Additionally, no clear difference is observed when chain length or degree of saturation is varied as it concerns dimyristoyl and palmitoyl/oleoyl lipids; however, dioleoyl lipids seems to limit CAMP factor pore formation in the presence of cholesterol. An explanation for this result is proposed.

Acknowledgements

First and foremost, in accordance with my faith and hope in Jesus Christ of Nazareth, I would like to give thanks to God Almighty for giving me the strength, the perseverance, the ability, and the opportunity to undertake this research project as well as bring it to a successful end (1 Thessalonians 5:18).

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Dedication

This work is dedicated to the Brefo-Mensah family:

Kwabena, Ann, Harriet, Isaac, and *Michael*.

Your unending support made this possible.

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List of Abbreviations

3D ..... Three-dimensional

BADAN 6-bromoacetyl-2-dimethylaminonaphthalene

BLAST ..... Basic Local Alignment Search Tool

CAMP ..... Christie, Atkins, and Munch-Petersen

CATH ..... Protein Structure Classification Database at University College London

CDC ..... Cholesterol-Dependent Cytolysin

CH ..... Cholesterol

CTD ..... C-Terminal Domain

DMPC 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine

DMPG 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol

DOPC 1,2-dioleoyl-*sn*-glycero-3-phosphocholine

DOPG 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol

DT ..... Diphtheria Toxin

EYPC ..... Egg Yolk Phosphatidylcholine

FRET ..... Fluorescence (Förster) Resonance Energy Transfer

GAS ..... Group A Streptococci

GBS . . . . . Group B Streptococci

GPI . . . . . Glycosylphosphatidylinositol

HBS . . . . . HEPES-Buffered Saline

IANBD . . . . . N,N'-dimethyl-N-(iodoacetyl)-N'-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine

MAC . . . . . Membrane Attack Complex

NTD . . . . . N-terminal Domain

NBD 7-nitrobenz-2-Oxa-1,3-diazole

PDB . . . . . Protein Data Bank

PFP . . . . . Pore-Forming Protein

PFT . . . . . Pore-Forming Toxin

PFO . . . . . Perfringolysin O

PLAP . . . . . Placental Alkline Phosphatase

POPC 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine

POPG 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol

SCOP . . . . . Structural Classification of Proteins

SDM . . . . . Site-Directed Mutagenesis

sRBC . . . . . Sheep Red Blood Cell

TCEP . . . . . Tris(2-carboxyethyl)phosphine

Chapter 1

Introduction

This thesis deals with *Streptococcus agalactiae* CAMP factor, a pore-forming protein, and in particular its structure and function. In this introductory chapter, I will first give some background on pore-forming proteins in general, with an emphasis on pore-forming bacterial toxins; this will be followed by an overview of previous work on CAMP factor.

1.1 Pore-Forming Proteins

Pore-forming proteins (PFPs) are generally secreted as water-soluble monomeric molecules that target and bind to a specific host cell surface element (i.e., sugars, lipids, or proteins) in order to use it as a receptor, then undergo a conformational change which favours oligomerization and thus allows them to insert into biological membranes to form pores; the ultimate effect of this membrane perforation is usually cell death [1, 2].

PFPs play several different roles in nature; they are often a part of attack or defense mechanisms deployed by a variety of organisms. These organisms range from humans to earthworms to sea anemones to fungi and bacteria [3, 4].

Humans, and vertebrates in general, employ PFPs both in the regulation of apoptosis (programmed cell death) and within their immune systems [1]. An essential step in the activation of apoptosis is the permeabilization of mitochondrial outer membrane, which is caused by Bax, a member of the Bcl2 family. An important part of the immune system is the complement cascade,

which, upon activation by antigens, triggers the formation of a pore (the membrane-attack complex or MAC) on microbial cell membranes [3, 1]. Perforin, which is structurally similar to the MAC, is unleashed by lymphocytes upon virus-infected cells [3].

The role of complement and perforin in host-pathogen interactions is mirrored by that of microbial pore-forming toxins (PFTs), which enable the pathogens to combat the cells of the host's immune system, and in some cases to persist inside these cells [5]. Other microbial pore-forming proteins are not directly toxic but instead permit enzymatic toxins to enter the host cells; examples are the AB toxins such as diphtheria toxin or anthrax toxin, as well as type III secretion systems that create direct pathways for the transport of toxic proteins from the microbial to the host cell cytoplasm [3].

While conceptually similar, pore-forming proteins exhibit a great deal of structural diversity. Their secondary structure may be predominantly α-helical or β-sheet; the pore size may vary from 10 to 300 Å [6, 2]; they may be monomeric or, more commonly, oligomeric. In the latter case, oligomerization typically precedes pore formation; thus, an oligomeric 'pre-pore' stage can often be detected which then turns into an actual pore by a cooperative process of membrane insertion [2].

1.1.1 Pore-Forming Toxins

In general, bacterial protein toxins are defined as secreted poisonous substances that directly interfere with the host organism by damaging or disrupting the membrane bilayer [7, 8]. However, though some PFPs are referred to as toxins, these typically act on animals or humans. There are exceptions, as toxins secreted by certain bacteria that attack other strains of the same species (i.e., protein toxins/bacteriocins secreted by organisms such as *Escherichia coli, Pseudomonas pyogenes, and Enterobacter cloacae*) [9]. In the following, the term 'toxin' may refer to both 'proper' toxins and to bacteriocins.

The PFT family, a subgroup of the PFPs, have very divergent amino acid sequences (or primary structures) but can be broadly classified into two large groups: α-PFTs and β-PFTs [3, 2]. This classification is based on the secondary structure elements used by the PFT to traverse the membrane bilayer. In the case of α-PFTs, α-helices are used to span the membrane while β-PFTs

form membrane-inserted β-barrels [2, 6]. Other domains within the toxin molecules usually mediate receptor binding and oligomer formation. In α-PFTs, these auxiliary domains are typically largely α-helical also, whereas in β-PFTs they may comprise both β-sheet and α-helical elements [3]. Selected examples of the PFTs can be found in Table 1.1 with their class, family, and the organism that secretes them.

Table 1.1: Prototypes of Pore-Forming Toxins (Modified from [2, 6])

PFT	CLASS	FAMILY	ORGANISM
Colicin E1	α-PFTs	Colicins	*Escherichia coli*
Equinatoxin II	α-PFTs	Actinoporins	*Actinia equina*
Diphtheria toxin	α-PFTs	Colicin-like fold[4]	*Corynebacterium diphtheriae*
α-haemolysin (Hla)	β-PFTs	Haemolysins	*Staphylococcus aureus*
Aerolysin	β-PFTs	Aerolysins	*Aeromonas hydrophila*
Perfringolysin O	β-PFTs	Cholesterol-Dependent Cytolysins	*Clostridium pefringens*

1.1.2 α-Pore-Forming Toxins

Typically, the pore-forming domain of α-PFTs contains a sandwich-like conformation within which a central hydrophobic helical hairpin is surrounded/protected by amphipathic anti-parallel hairpins of α-helices, usually 6-10 in number (Figure 1.1) [6, 1].

A change in conformation is required for the release of the hydrophobic helical hairpin from the sandwich-like conformation into the membrane bilayer [6]. This change is triggered by contact with the target membrane and allows the surrounding helices to spread out on the membrane surface, such that charged residues interact with the polar head groups, while the hydrophobic residues face the bilayer interior [6]. The most thoroughly studied group of the α-PFTs are the colicins secreted by *E. coli*. The colicins, like other bacteriocins, are secreted by the same species they attack and have a unique structure-function relationship responsible for causing cell death [10, 2]. They are secreted by *E. coli* in two forms: nuclease and pore-forming [10]. The unique three-dimensional (3D) structure of the pore-forming colicins contain three separate domains: the N-terminal, middle, and C-terminal domain [11, 10]. These domains serve three distinct functions: translocation, receptor-binding, and pore formation, respectively [10, 12].

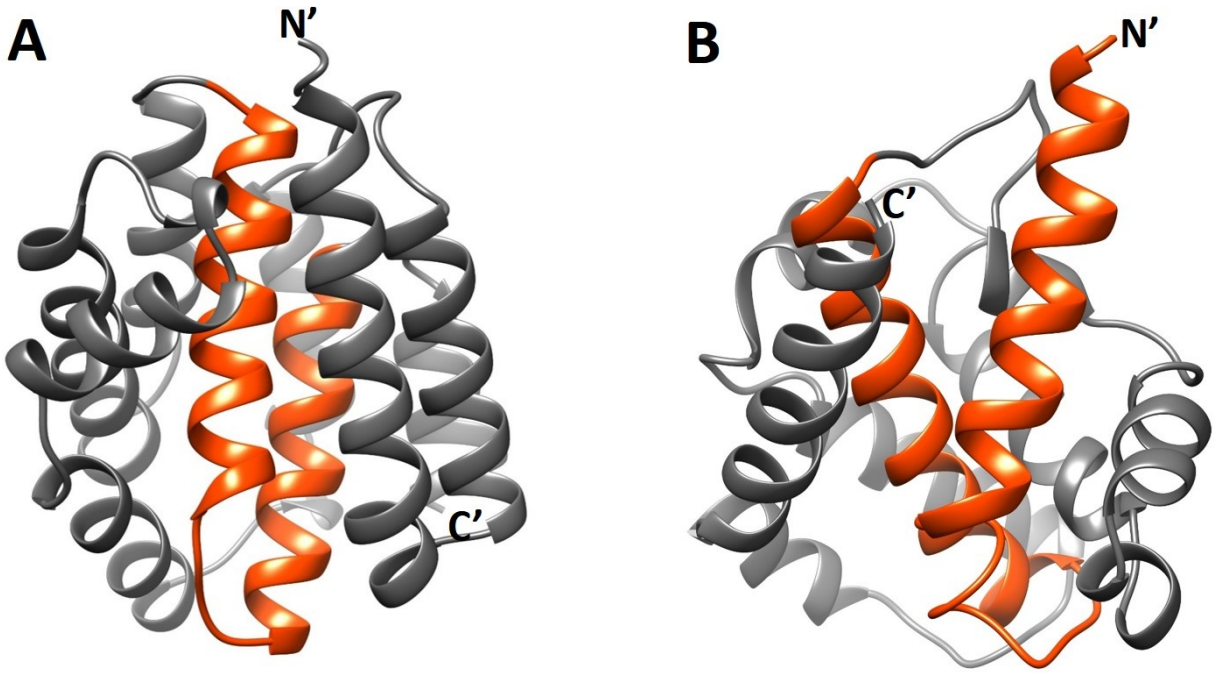

Figure 1.1: Hydrophobic helical hairpins. A: Pore-forming domain of colicin A with the hydrophobic helical hairpin coloured in orange and surrounded by amphipathic anti-parallel helices coloured in gray (PDB ID: 1COL). **B:** Structure of diphtheria toxin transmembrane/translocation domain displayed in like manner to **A** for comparison (PDB ID: 1DDT). A common theme among predominantly helical PFTs, hydrophobic helical hairpins are normally sheltered in a bundle of amphipathic helices [2].

Other α-PFTs also contain this theme of functional assignment to specific domains with the most notable being diphtheria toxin. Diphtheria toxin (DT) is secreted as a single polypeptide and cleaved by a protease into the A and the B chains, which remain connected by a disulfide bond [13]. The A chain, or the catalytic domain which contains enzymatic activity, is known to target the cytosol. The B chain, which contains the receptor-binding and translocation domains, is responsible for membrane binding and facilitation of the A chain transport into the cytosol [13, 14].

Once released from the B chain after internalization in the cytosol, the A chain ADP-ribosylates elongation factor 2, which inhibits protein synthesis and thus causes cell death [15, 14]. The B chain is responsible for membrane binding, insertion into the membrane bilayer, and facilitation of the translocation of the A chain into the cytosol by receptor-mediated endocytosis (Figure 1.1

B) [14]. The low pH inside the endosome causes DT to detach from its receptor and the B chain to insert into the endosome membrane. This facilitates the translocation of the A chain into the cytosol after disulfide reduction [14].

Eukaryotic organisms can also express proteins that are structurally related to the colicins. These proteins are from the Bcl-2 and Bcl-x protein family and show 3D structural similarities to the pore-forming/translocation domains of the colicins despite the absence of homology between amino acid sequences [6, 10].

1.1.3 β-Pore-Forming Toxins

As stated earlier, β-PFTs form pores in membranes via a bilayer-spanning β-barrel [6]. Once bound to their target membrane receptor, β-PFT monomers aggregate on the membrane and begin to form an intermediate structure known as the oligomeric pre-pore (Figure 1.2); this structure then undergoes the necessary conformational rearrangements which lead to concerted membrane insertion and subsequent pore formation [2, 6].

In certain cases the pores that are formed are incomplete, leading to partial membrane-spanning structures known as arc-pores. These arc-pores may be stable enough for release of internal cellular material due to the abundance of inter-strand hydrogen bonds [6, 3, 2].

Apart from the shared trait of the β-barrel, the members of this PFT family have little resemblance in terms of their primary, tertiary, and quaternary structures; there are, however, three common features that unite this family: their lack of long stretches of hydrophobic residues in their primary structure, their richness in β-sheet content, and their tendency to assemble into higher order oligomeric pre-pore structures prior to pore formation [2, 6]. These unifying features do not imply a common structure in the β-PFT monomer used to insert into membranes, as they can be used by members of the β-PFT family in different capacities such as domain rearrangements and proteolytic activation during the pore formation process [6].

Despite a lack of conformity in the β-PFT monomeric structure used for membrane insertion, there seems to be a common secondary structure that appears during the pore-forming process. This structure is known as the β-hairpin, which is used to insert into the membrane bilayer and

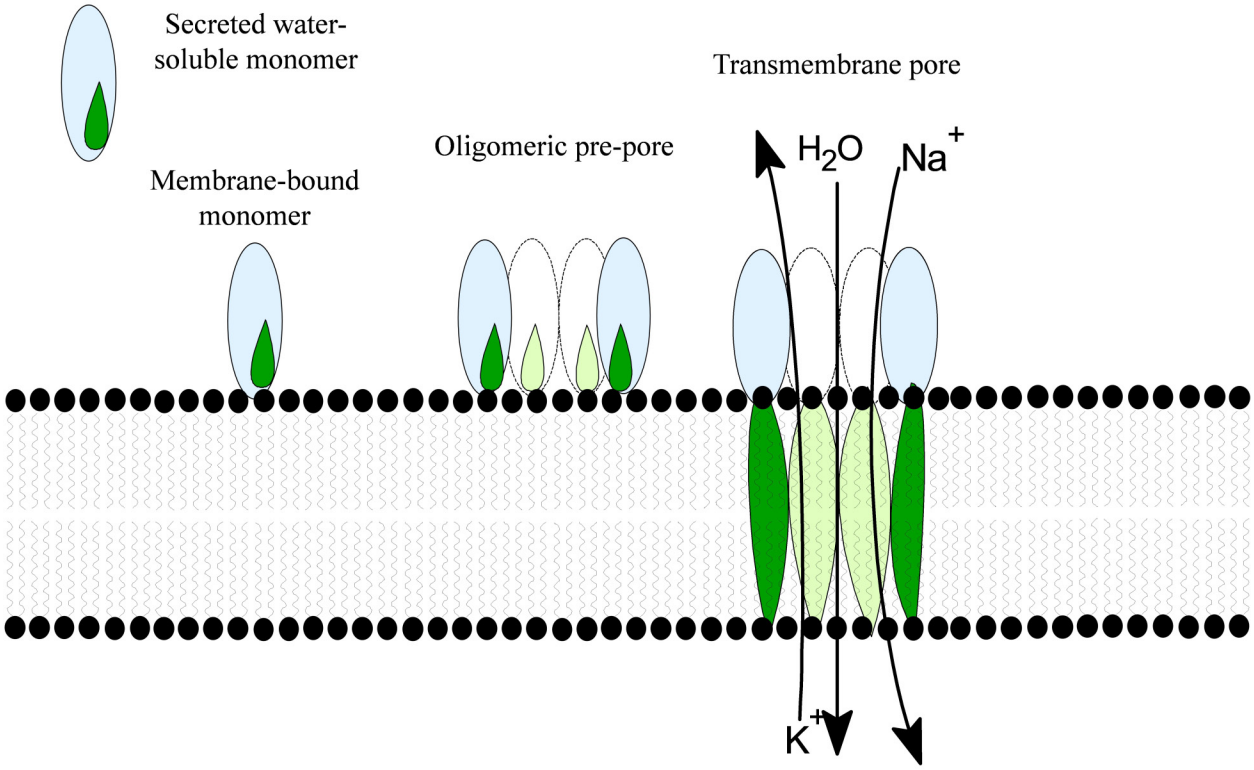

Figure 1.2: Process by which β-PFTs typically form pores. Once secreted as water-soluble monomers, they locate their receptor and concentrate on the membrane, form an oligomeric pre-pore and undergo the necessary rearrangements to form a transmembrane pore.

is consistent among the β-PFTs be it the aerolysin, CDC, or the haemolysin family; this insertion occurs through a conformational change in the monomer (Figures 1.2 and 1.3) [2, 6].

In the aerolysin family, the water-soluble monomer is first cleaved and undergoes a conformational change via a β-strand containing loop that transforms into a β-hairpin for the purpose of forming a pre-pore; this is followed by membrane insertion then pore formation [2]. In the case of the CDCs, two sequence motifs, which are α-helical in the monomer, later concomitantly transform into amphipathic β-hairpins during membrane insertion[6]. Finally, the haemolysins-the toxin family that includes *S. aureus* α-toxin-form 14- or 16-stranded β-barrel pores, conceal their membrane-inserting motif, prior to pore formation, in the form of a pre-stem loop [2]. The loop then becomes an antiparallel β-hairpin in the transmembrane domain of the oligomer once pore formation is complete (Figure 1.3) [2, 6].

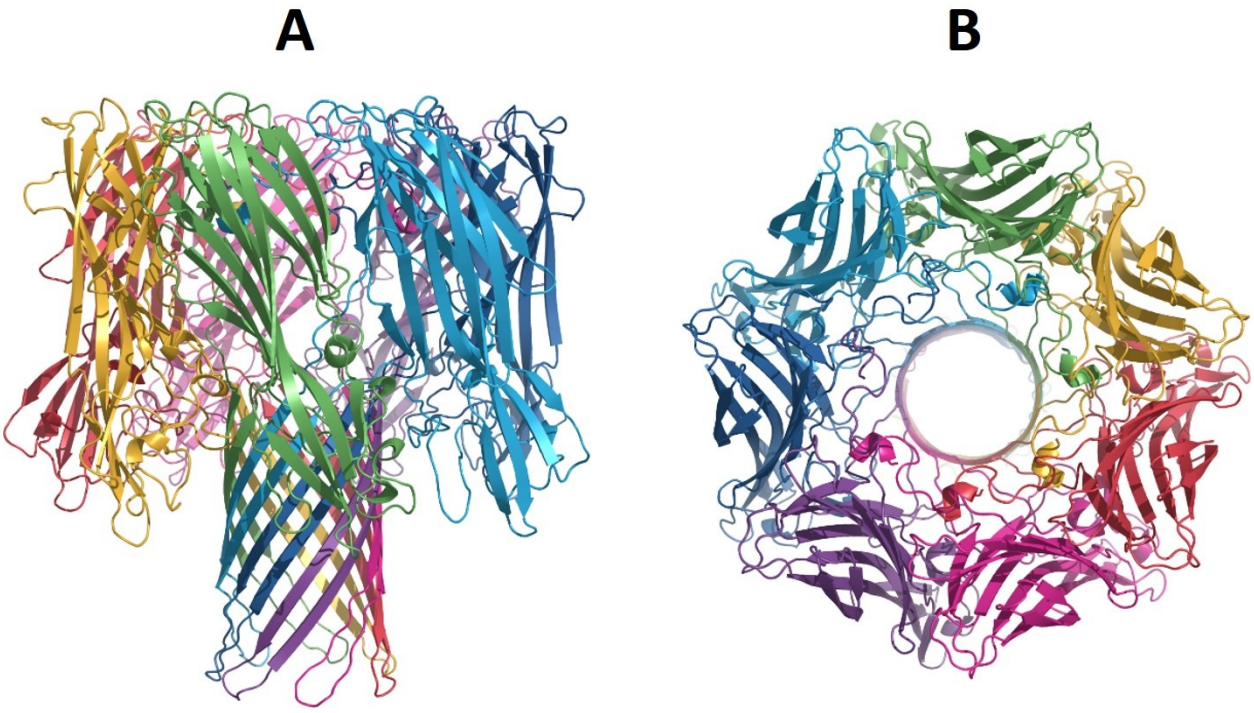

Figure 1.3: Ribbon representation of the α-haemolysin transmembrane pore. Structure of the *Staphylococcus aureus* α-haemolysin pore. The water-soluble monomeric form of α-haemolysin inserts into the lipid bilayer to form a mushroom-shaped homo-oligomeric heptamer with a transmembrane solvent-filled channel that is 100 Å in length. The transmembrane domain comprises the lower half of a 14-strand antiparallel β-barrel, to which each monomer contributes two β strands [16]. The lumen of the channel is hydrophilic while its exterior is hydrophobic. **A:** Perpendicular view of heptamer; **B:** Parallel view. PDB ID: 7AHL.

1.2 CAMP factor: Background

Streptococcus agalactiae CAMP factor was discovered by Christie, Atkins, and Munch-Petersen in 1944. The work of these scientists revealed that when *S. agalactiae*, which secretes CAMP factor, was grown in close proximity to *Staphylococcus aureus*, which secretes sphingomyelinase, synergistic haemolysis takes place [17]. The phenomenon was named the CAMP reaction after the surname initials of the authors and has been widely used as a test to presumptively identify clinical isolates of *S. agalactiae* [18, 19]. The CAMP reaction can be demonstrated by streaking *S. aureus* close to *S. agalactiae* on sheep blood agar plates (Figure 1.4). These streaks then result in an area

of enhanced haemolysis after incubation at 37 °C via the action of extracellular products from the aforementioned organisms (i.e., sphingomyelinase and CAMP factor, respectively) [20].

1.2.1 CAMP Reaction Mechanism

An important component of the CAMP reaction is sphingomyelinase—a membrane-damaging toxin [21]. Sphingomyelinase's membrane-damaging action on sheep red blood cells (sRBCs) sensitizes them by conversion of sphingomyelin to ceramide. The sensitization of sRBCs by sphingomyelinase then leads to a pronounced zone of haemolysis when these cells are exposed to CAMP factor. This zone of synergistic haemolysis is due to pore formation and is distinct from the action of any other extracellular product released by either organism [22].

The synergistic action of sphingomyelinase and CAMP factor is observed only on cells with at least 45 mol% sphingomyelin in the membrane [23]. This requirement explains why the initial observers of the CAMP reaction did not detect synergistic haemolysis in human, rabbit, horse, and guinea pig RBCs, which contain 27 mol%, 19 mol%, 14 mol%, and 11 mol% sphingomyelin, respectively [17, 24]. Bovine and sheep RBCs displayed distinct lysis because of their 46 mol% and 51 mol% sphingomyelin, respectively [24]. CAMP factor is able to act on RBCs that are high in phosphatidylcholine rather sphingomyelin when these are pre-treated with phospholipase C (PLC), which converts glycerophospholipids to diacylglycerol. Thus, CAMP factor does not require ceramide specifically for membrane permeabilization [22].

From liposome permeablization studies, Lang and Palmer inferred that cholesterol concentration plays a role in CAMP factor's activity. Results from calcein release experiments showed that liposomes containing 45 mol% cholesterol were three times more sensitive to CAMP factor than those with 25 mol% [22]. Thus, it seems that cholesterol is an important element in CAMP factor activity on cell membranes but more work is needed to further understand the effect of cholesterol and lipid composition on the CAMP reaction mechanism.

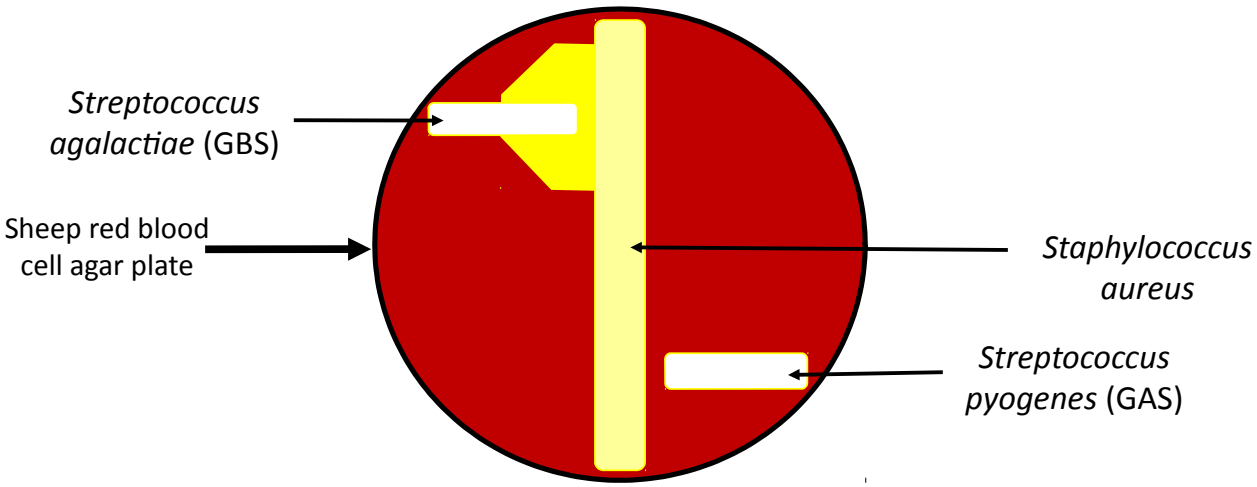

Figure 1.4: Schematic representation of CAMP reaction on sheep red blood cell agar plate. A positive result of *Streptococcus agalactiae* or GBS's CAMP factor action on the *Staphylococcus aureus* sphingomyelinase-sensitized RBCs is shown by the expansive bright yellow co-haemolysis zone. This zone represents enhanced haemolysis as opposed to the minor haemolytic activity seen around each streak. A negative result is observed via a streak of *Streptococcus pyogenes* or GAS in close proximity to *S. aureus*.

1.2.2 Characterization of CAMP factor and its Mode of Action

CAMP factor is an extracellular protein secreted by *S. agalactiae*. Several other streptococci, such as *S. pyogenes* and *S. uberis*, contain and express the highly conserved CAMP factor gene (*cfa*) whose protein product is homologous to that of *S. agalactiae* CAMP factor (Table 1.2).

Table 1.2: Comparison of the *cfa* product in streptococci (compiled with data from [25])

Organism	Number of residues in nascent CAMP factor	Number of residues in mature CAMP factor	Sequence similarity *S. agalactiae* CAMP factor	Molecular weight of gene product (kDa)
S. agalactiae	255	226	100%	28.4
S. pyogenes	257	229	59.9%	28.5
S. uberis	256	228	66.4%	28.3

After the cleavage of its N-terminal signal peptide, mature CAMP factor contains 226 amino acids with a molecular weight of approximately 26 kDa [22]. CAMP factor was first characterized as a PFT by Lang and Palmer in 2003 through electron microscopy and osmotic protection studies that demonstrated that CAMP factor forms pores that vary in size on sRBCs pre-sensitized with

sphingomyelinase. The electron microscopy studies showed both regular and arc-shaped pores. The largest of these pores were reported as being 12 nm or more in diameter [22]. Comparable results had previously been described for Streptolysin O (SLO) as well the Membrane Attack Complex (MAC) of complement proteins [26, 27].

In addition, the osmotic protection studies using polyethylene glycols (PEGs) of varying size showed that CAMP factor activity on RBCs results in delayed haemolysis in the presence of PEG 6000 and 8000 [22]. Previous experiments using PEGs 4000 and 6000, with hydrodynamic radii of 1.6 nm and 2.7 nm, respectively, and the observation that PEG 4000 and 6000 had no protective effect on CAMP factor suggested that the effective pore radius is within this range [22]. The results of the aforementioned experiments therefore suggested that CAMP factor is a PFT which causes lysis on susceptible cell membranes.

In addition to the aforementioned studies, subsequent work on CAMP factor in the Palmer group focused on the identification of the membrane-spanning domain of CAMP factor [28]. These studies initially examined residues 119 to 144 of CAMP factor by using hydropathy plots of its primary sequence which suggested that these residues span the membrane bilayer [29]. This cursory examination was followed by cysteine-scanning mutagenesis, fluorescent labelling, and haemolytic assays of said mutants to characterize the region of interest as membrane-inserting [28, 30]. The final conclusion of this work was that CAMP factor used the aforementioned residues to traverse the membrane bilayer as an amphipathic α-helix [28, 30]. The results from the above studies therefore indicated that more work was required to increase our understanding of the membrane-binding as well as the membrane-inserting domain of *S. agalactiae* CAMP factor through a study that reveals the protein structure.

Previous work on CAMP factor also focused on the search for a cell surface receptor as experiments using calcein-loaded, ceramide-containing liposomes, suggested that ceramide was not specifically required for CAMP factor activity; additionally, the amount of cholesterol in the membrane was also shown to influence CAMP factor activity, but the toxin did not have significant homology with PFTs that use cholesterol as a cell surface receptor [22, 31]. Taken together, these findings suggested that perhaps a membrane component other than a bulk lipid was involved in CAMP factor's binding to erythrocyte membranes [31].

Several homologous PFTs (e.g., *A. hydrophila* aerolysin and *Clostridium septicum* α-toxin) were long known for their receptor-mediated binding to membranes through their high affinity interaction with the glycan core of various glycosylphophatidylinositol(GPI)-anchored proteins [32, 33]. GPI anchors have a shared core that is composed of an ethanolamine phosphate, three mannose residues, and a non-acetylated glucosamine linked to phosphatidylinositol [34]. The basic structure of a membrane-embedded GPI-anchored protein is shown in Figure 1.5.

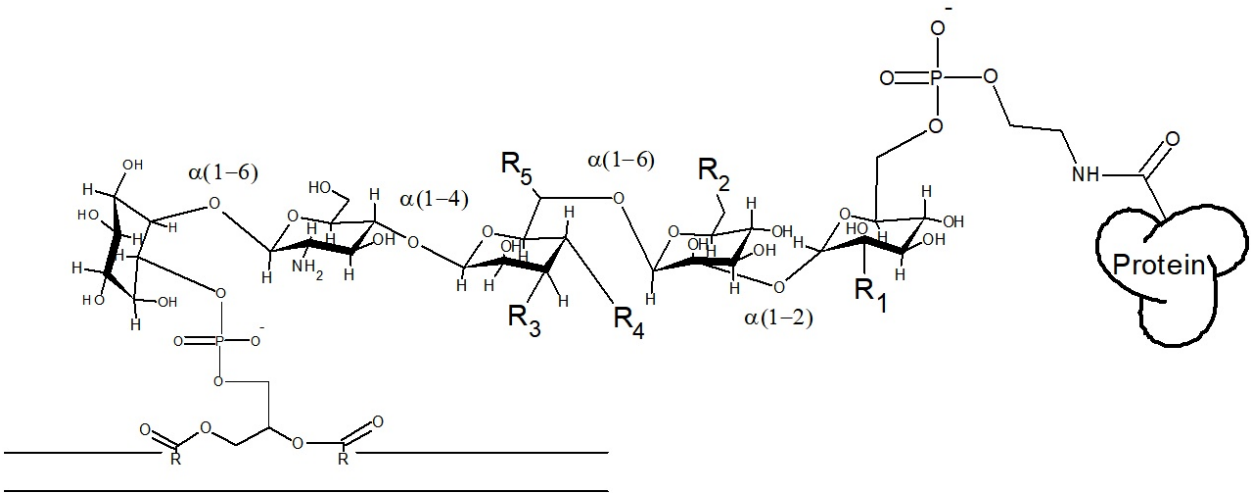

Figure 1.5: General structure of a GPI-anchored protein in a membrane bilayer. R-R_6: Possible modifications, R: acyl chain R_1: mannose, R_2: phosphoethanolamine, R_3: phosphoethanolamine, R_4: tetragalactose, R_5: N-acetylgalactosamine, and R_6: fatty acyl residue

Lang and coworkers therefore pursued the possibility of a glycan receptor by cleaving the GPI anchor on the surface of RBCs as well as the incorporation of placental alkaline phosphatase, a GPI-anchored protein, into model membranes loaded with calcein and treating them with CAMP factor, separately [31]. The results from this work showed that CAMP factor uses the carbohydrate moiety of GPI-anchored proteins as a cell surface receptor in binding to membranes [31].

1.3 The CAMP factor 3D Structure

Despite the aforementioned insights concerning *S. agalactiae* CAMP factor's mode of action, the molecular and structural details of its ability to form pores remained unknown. This is because CAMP factor does not share sequence homology with any proteins whose 3D structure have been

solved. Accordingly, homology-based assignment of structure and function was not possible. Furthermore, the absence of a 3D structure limited the range of strategic experiments that could be used to gain further insights about CAMP factor's mechanism.

1.3.1 Insights from the 3D Structure of CAMP factor

Through the work of Dr. Tengchuan Jin, the three-dimensional structure of CAMP factor has been solved (manuscript submitted). This was achieved through X-ray crystallography and showed that the overall structure of *S. agalactiae* CAMP factor is composed of two domains separated by a linker region and constitutes a novel structural fold not found in the Protein Data Bank (PDB). The N-terminal domain (NTD) consists of a bundle of five helices while the C-terminal domain (CTD) contains a bundle of three (Figure 1.6).

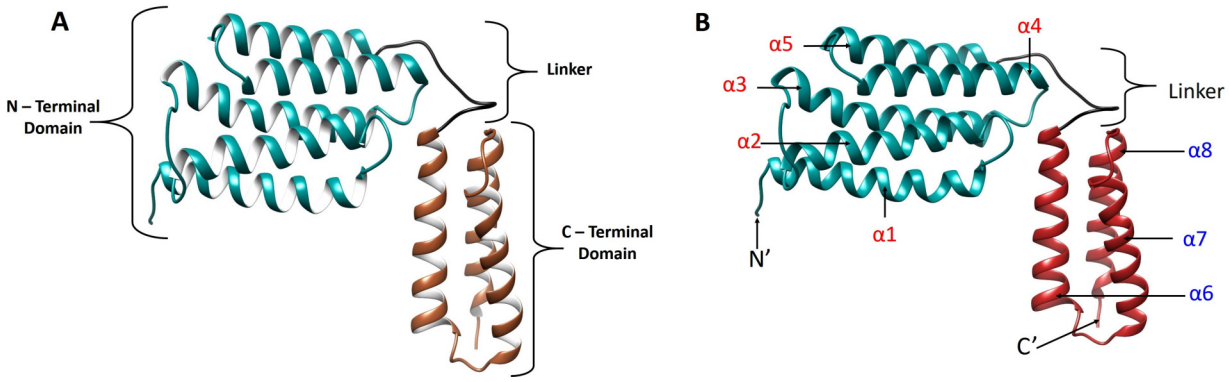

Figure 1.6: Three-dimensional structure and helix nomenclature of *S. agalactiae* CAMP factor. **A:** The two distinct domains highlighted by the curly brace (i.e., the NTD (blue) and CTD (brown)) as well as the linker region (black) which joins them. **B:** Helices are numbered consecutively from the N- to the C-terminus. Helices α1-α5 form the NTD in teal; the CTD corresponds to α6-α8 coloured in brick red). The linker region is highlighted in black. PDB ID: 56HI.

Accordingly, a portion of this thesis work is dedicated to the experimental characterization of the functional roles of the domains of CAMP factor. This work was done via haemolytic experiments, liposome permeabilization studies, as well as mutagenesis and fluorescence studies. The findings, which are presented in Chapter 2, suggest that the NTD is chiefly responsible for membrane permeabilization while the CTD accounts for membrane binding and likely participates in oligomerization on membranes.

1.3.1.1 The CAMP factor Structure Represents a New Structural Fold

A BLAST search did not yield a homologous structural model by sequence similarity. Thus, the submission of the coordinates of the 3D structure of CAMP factor to several online servers (i.e., DALI, pdbefold, and VAST) was necessary to determine whether homologous structures exist in the PDB [35, 36, 37].

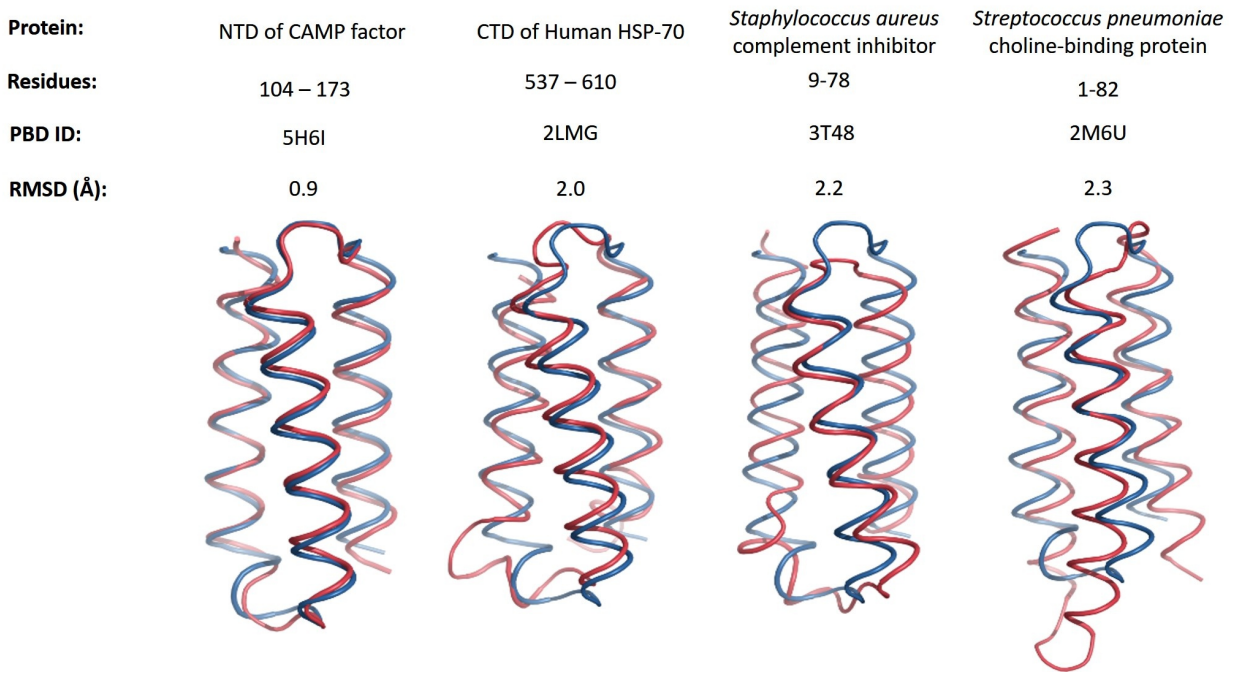

Figure 1.7: Structural similarity of the C-terminal domain. Structural alignment of the CAMP factor C-terminal domain with helices α3-α5 of the NTD and with domains of three other proteins that belong to group 46996 of the SCOP database [40]. The CTD is coloured in blue. The alignments were carried out using PyMOL®.

In general, these servers compare the 3D structure of a protein to structural fold databases (i.e., SCOP and CATH) to identify similar structural folds [38, 39]. Surprisingly, no protein was found with architecture or topology identical to CAMP factor suggesting that CAMP factor's 3D structure defines a novel structural fold.

Furthermore, when the two domains are considered independently, no known protein structural fold was found for the NTD in the aforementioned databases. However, the 3-helix bundle of the CTD shares high structural similarity with the immunoglobulin/albumin-binding domain-like

fold (i.e., group 46996 of in the SCOP database) [40]. In addition, a careful examination of the two domains of CAMP factor shows that the 3-helix bundle, α6-α8, of the CTD, is practically superimposable on α3-α5 of the NTD (Figure 1.7).

1.3.1.2 CAMP factor Lacks Extended Hydrophobic Sequence Motifs

CAMP factor is a PFT that has been shown to form membrane-inserted pores and has high helical content (Figure 1.6) [22]. Consequently, its monomeric structure would be expected to contain a hydrophobic helical hairpin as observed among α-PFT family (Figure 1.1). However, this theme is not exemplified in CAMP factor; rather, a seemingly random arrangement of hydrophobic and hydrophilic residues is found on its surface (Figure 1.8).

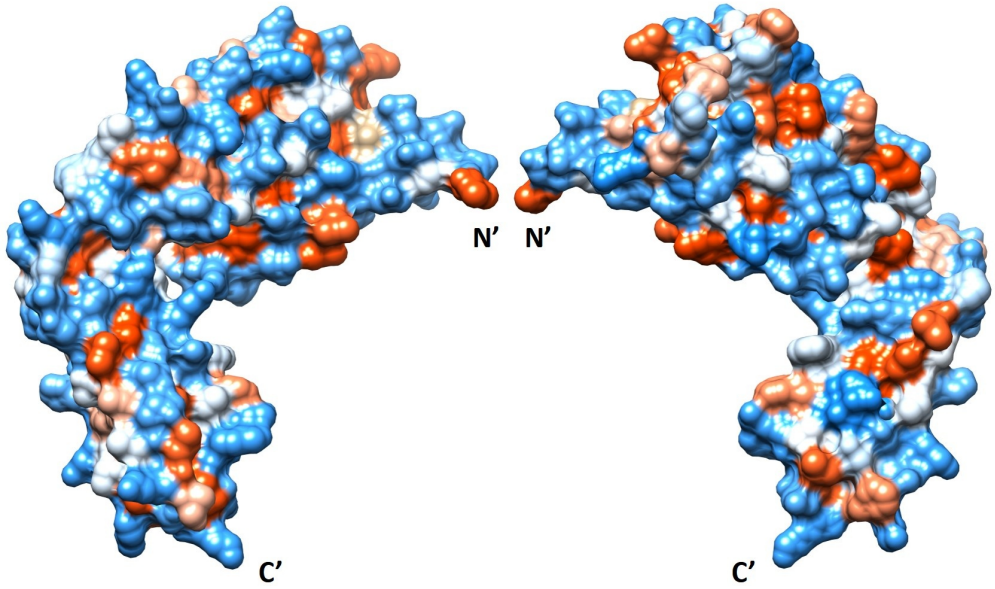

Figure 1.8: Hydrophobic and hydrophilic residue distribution on the CAMP factor protein surface. Two perspectives of the CAMP factor surface structure are displayed. The residues are coloured using the scale defined by [41] with the most hydrophilic residues are shown in blue, neutral ones in white, and hydrophobic ones in dark orange. Decreasing hydrophobicity or hydrophilicity is shown by a decrease in the shade of either blue or dark orange. PDB ID: 56HI.

1.3.1.3 CAMP factor Lacks a Clear Hydrophobic Helical Hairpin

A common theme among the predominantly helical PFTs (e.g., colicin A and diphtheria toxin) is the use of hydrophobic helical hairpins to insert into the lipid bilayer and form membrane-inserted pores (Figure 1.6) [42, 43, 44]. However, no clear hydrophobic helical hairpin was found in CAMP factor by using either sequence- or structure-based prediction tools [35, 36, 37, 45]. Additionally, none of the bioinformatics tools used could locate any carbohydrate-binding motifs in CAMP factor to account for its experimentally observed binding to GPI anchors [31, 46, 47, 48].

1.4 The Research Project

1.4.1 Research objectives

The focus of this research project is to gain further insights into the function of the domains of *Streptococcus agalactiae* CAMP factor as well as the effect of target membrane lipid composition on toxin activity.

The revelation of a novel structural fold composed of two separate domains that make up a 5+3 helix bundle connected by a linker region by X-ray crystallography provoked further inquiry into the functional role of the N- and C-terminal domains of CAMP factor. The functional role of each domain was probed by the use of haemolytic assays, calcein release studies, as well as liposome permeabilization experiments via a fluorescently labelled mutant and domain constructs. Additionally, since previous studies have suggested that CAMP factor activity varies with lipid composition and requires cholesterol for pore formation, this assertion was further examined using cholesterol-deficient and cholesterol-containing model membranes with some interesting new findings. Accordingly, this thesis focuses on the following areas:

1. The functional roles of the N- and C-terminal domains of CAMP factor. This topic will be addressed in Chapter 2.

2. The effect of membrane lipid composition on the activity of *S. agalactiae* CAMP factor. This topic is the subject of Chapter 3.

Chapter 2

Functional Roles of N- and C-Terminal Domains of CAMP factor

2.1 Introduction

Streptococcus agalactiae is known for its ability to cause disease in newborn infants as well as pregnant or postpartum women, immune-compromised individuals, and the elderly [49, 50]. Also known as group B streptococcus (GBS), it is the cause of contagious cattle mastitis as well as a fish pathogen [51, 52]. *S. agalactiae* strains express CAMP factor[1], a 26 kDa pore-forming toxin (PFT) which plays a major role in the CAMP reaction [22, 53].

The CAMP reaction is the synergistic lysis of sheep red blood cells (sRBCs) by sphingomyelinase from *Staphylcoccus aureus* and CAMP factor. The initial description of this co-haemolytic phenomenon was first described by Christie, Atkins, and Munch-Petersen, whose surname initials now form its moniker [17].

[1] The work presented in this chapter reflects a combined effort between Tengchuan Jin, PhD and Eric K. Brefo-Mensah. The manuscript has been submitted for review to *The Journal of Biological Chemistry*. Dr. Tengchuan Jin generated and purified the CAMP factor domain constructs (i.e., N- and C-terminal domains) as well as the cysteine mutants and MBP-CAMP. He also crystallized the forms of CAMP factor necessary for solving the 3D structure. Eric K. Brefo-Mensah, was responsible for purification and verification of WT, SeMetCAMP, F138C activity as well as the other haemolytic assays, liposome permeabilization, fluorescent labelling, and spectrofluorimetry.

On membranes of sRBCs treated with sphingomyelinase, the addition of CAMP factor results in circular pores of variable diameter that are visible when observed by electron microscopy [22]. Formation of these pores is facilitated by the attachment of CAMP factor to the cell membrane via the glycosyl moieties of glycosylphosphatidylinositol-anchored proteins (GPI-APs) [31].

The structure of CAMP factor, which has recently been solved, contains two well-delineated domains that are joined by what appears to be a flexible linker. The structure also shows no close similarity to any other pore-forming toxin that would allow the functional roles of each domain to be assigned. The studies described in this chapter aim to characterize these functional roles through experiment.

2.1.1 Site-Specific Labelling of Proteins via Fluorescent Probes

Spectroscopic methods are often employed when studying protein structure and function. Fluorescence spectroscopy is one such method and requires chemical modification of biomolecules, specifically proteins and their constituent amino acids [54, 55]. Conventional protein chemical modification focuses on covalent attachment of fluorescent probes to the amino, carboxyl, imidazole, and thiol groups of target proteins [56, 55].

In order to properly study membrane insertion, methods such as steady-state fluorescence and Fluorescence (Förster) Resonance Energy Transfer (FRET) are used, which require that specific residues of proteins be labelled [57]. Of the 20 standard amino acids, lysine, which contains a primary amine side chain, and cysteine, which contains a thiol side chain, are the two amino acids for which there are well known and efficient labelling strategies as well as commercially available dyes [58].

Though lysine can be selectively labelled, its high abundance in most proteins means that site-specific labelling is unrealistic. However, cysteine's rare occurrence in proteins, as well as its nucleophilicity, makes it a viable candidate for site-specific fluorescent labelling with various fluorescent probes [56, 59]. Additionally, cysteines can be introduced into proteins by site-directed mutagenesis for the sake of fluorescent labelling (Figure 2.1) [60].

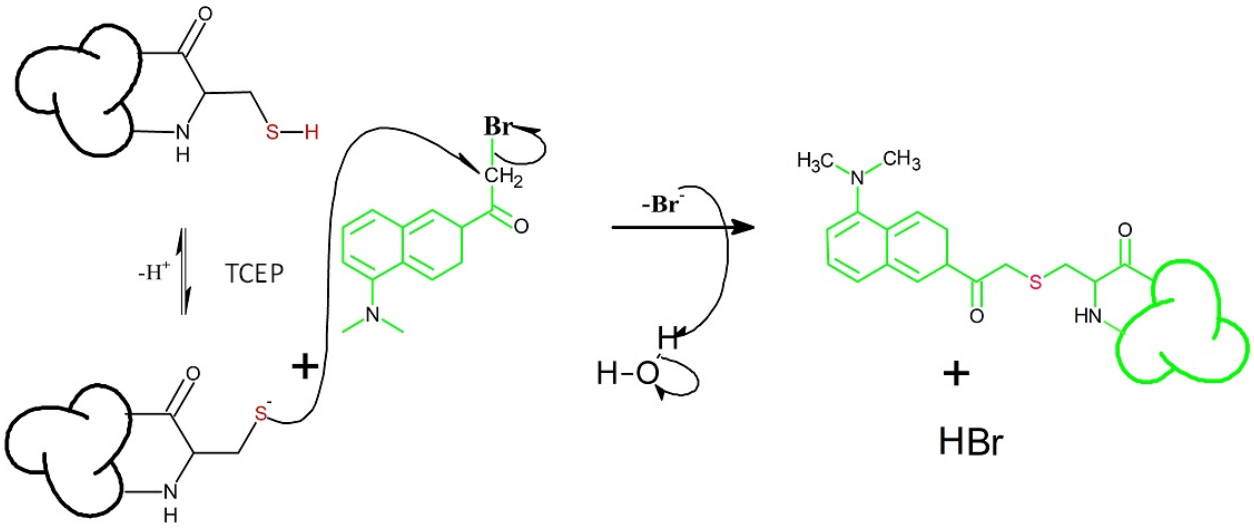

Figure 2.1: Site-specific labelling of single-cysteine containing protein via BADAN (6-Bromoacetyl-2-dimethylaminonaphthalene). In this reaction the nucleophile is the thiol group of the cysteine. The reaction an be performed in 1 mM TCEP at approximately pH 7.3.

In this chapter, site-specific labelling is employed in attaching a fluorescent probe to an active cysteine mutant of CAMP factor for the purpose of studying membrane insertion, without the loss of protein function.

2.2 Materials and Methods

2.2.1 Protein Expression and Purification

The plasmid, pGEX-KG, a gift from Dr. Jingya Li (Head of Drug Screening at the National Centre for Drug Screening, Shanghai, China) contains the coding sequence of wild type (WT) CAMP factor CAMP$^{(M50L,M136L)}$, a double mutant, which was expressed for the current study[22]. CAMP $^{(M50L,M136L)}$ is active and equivalent to the CAMP factor from *Streptococcus agalactiae*. The coding sequence of PGEX-KG is derived from mature wild type CAMP factor, amplified from *Streptococcus agalactiae* genomic DNA, and matches the published mature peptide sequence for CAMP factor [22]. An additional mutant, F138C, was also expressed with phenylalanine residue 138 replaced with cysteine having no effect on activity [30]. Both WT and F138C were expressed

using BL21 (DE3) *Escherichia coli* and purified using glutathione agarose (GSH-Agarose) (Sigma-Aldrich®, St. Louis, MO) Table 2.1.

Constructs of the two domains of CAMP factor were fused, expressed, and purified with maltose-binding protein (MBP; PDB ID: 1MPD) by Dr. Tengchuan Jin from a pET30-derived vector as previously described, then cleaved by Tobacco Etch Virus protease [61]. MBP-CAMP was purified using immobilized metal affinity chromatography Table 2.1.

Protein purity for all forms of CAMP factor mentioned were assessed by mass spectrometry and SDS-PAGE analysis to be greater than 95%. Furthermore, protein concentration was measured by the Bradford Assay [62].

Table 2.1: CAMP factor variants used in this study. Pseudo-wild type (usually referred to as 'WT' in the text) contains two mutant leucine residues where wild-type CAMP factor contains methionines; these substitutions do not affect activity [63]. In the mutant F138C, residue phenylalanine 138 has been replaced with cysteine. All mutant residues are highlighted.

CAMP factor Variant	Protein Sequence
Pseudo-WT	DQVTTPQVVNHVNSNNQAQQ**L**AQKLDQDSIQLRNIKDNVQ GTDYEKPVNEAITSVEKLKTSLRANPETVYDLNSIGSRVE ALTDVIEAITFSTQHLANKVSQANID**L**G**F**GITKLVIRILD PFASVDSIKAQVNDVKALEQKVLTYPDLKPTDRATIYTKS KLDKEIWNTRFTRGKKVLNVKEFKVYNTLNKAITHAVGVQ LNPNVTVQQVDQEIVTLQAALQTALK
N-terminal domain (NTD)	GSVDSNNQAQQMAQKRDQDSIQLRNIKDNVQGTDYEKPVN EAITSVEKLKTSLRANSETVYDLNSIGSRVEALTDVIEAI TFSTQHLANKVSQANIDMGFGITKLVIRILDPFASVDSIK AQVNDVKALEQKVLTYPDLKPTDAAAS
C-terminal domain (CTD)	GSVDRATIYTKSKLDKEIWNTRFTRDKKVLNVKEFKVYNT LNKAITHAVGVQLNPNVTVQQVDQEIVTLQAALQTALKAA AS

2.2.2 Haemolysis Assay

Sheep RBCs (Cedarlane®, Burlington, ON) were washed five times in HEPES-buffered saline (HBS, 20 mM HEPES, 150 mM NaCl, pH 7.4) by centrifugation at approximately 600 $\times$ g for four minutes. The erythrocytes were then re-suspended in HBS, with 10 mM $MgCl_2$ at pH 7.4,

to 1% (v/v). The re-suspended cells were then treated with 50 mU/ml sphingomyelinase (EC 3.1.4.12, Sigma-Aldrich®, St. Louis, MO) from *Staphylococcus aureus* and incubated at 37°C for 30 minutes. A 125 µL of cell suspension was added to CAMP factor - WT, NTD, or CTD, alone or in combination (compositions and final concentrations are indicated in the Results section) - in the wells of a 96-well microtiter plate to a 250 µL final volume.

The progress of haemolysis was measured by the decrease in optical density of 650 nm, a wavelength that lies outside the absorption band of haemoglobin and thus reflects cell turbidity alone. These measurements were performed using a SpectraMax® Plus 384 Microplate Reader (Molecular Devices®, Sunnyvale, CA). In all cases, the experiments were performed in quadruplicates, in parallel, using the same samples and repeated at a later date for confirmation. The results in parallel and from different days were identical. Control samples without CAMP factor remained stable during the assay period.

2.2.3 Liposome Permeabilization

In order to prepare liposomes, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), cholesterol, and 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG) were purchased from Avanti Polar Lipids (Alabaster, AL). The lipids were dissolved in chloroform and mixed at a molar ratio of 6.5:2.5:1. The mixture was dried under a stream of nitrogen in a round-bottom flask for five minutes and then dried under vacuum for an additional three hours to remove residual chloroform.

The dried lipids were re-suspended by vortexing for 30 minutes with 3.0 mL of HEPES-buffered saline (HBS, 20 mM HEPES, 150 mM NaCl, pH 7.4) containing 50 mM calcein (Sigma-Aldrich®, St. Louis, MO) at room temperature to a final total lipid concentration of 3 mg/mL. The resulting suspension of multilamellar liposomes was converted to unilamellar liposomes by extrusion through a 100 nm polycarbonate membrane filter (Whatman), 15 times. Subsequently, non-encapsulated calcein was removed by chromatography using a Bio-Gel A-1.5m (BIO-RAD®, Mississauga, ON) column pre-equilibrated with HBS.

The calcein-loaded liposomes were diluted to approximately 30 µg/mL total lipid with HBS, mixed with CAMP factor - WT, NTD, or CTD, alone or in combination (compositions and final concentrations are indicated in the Results section), and then incubated for 30 minutes at room

temperature. The calcein fluorescence intensity was then measured (excitation, 478 nm; emission, 516 nm) on the QuantaMaster spectrofluorometer (Photon Technology International (PTI), London, ON). In all cases, the experiments were performed in triplicates, in parallel, using the same samples and then repeated at a later date for confirmation. The results in parallel and from different days were identical.

The extent of membrane permeabilization for each sample P was then calculated using the following formula:

$$P = \frac{(F_{\text{sample}} - F_0)}{(F_{\text{triton}} - F_0)} \tag{2.2.1}$$

where F_{sample} is the fluorescence of a liposome sample treated with toxin, while F_0 is the fluorescence of a liposome control without toxin, and F_{triton} is that of a liposome sample solubilized with Triton X-100 at a final concentration of 0.01%.

2.2.4 Fluorescent Labelling and Spectrofluorimetry

The cysteine mutant F138C (Table 2.1) was chosen for its activity comparable to that of WT CAMP factor and its membrane insertion capability by demonstrated by 5-doxyl stearic acid quenching of the labelled mutant in the membrane bilayer [30]. Mutant F138C was transferred to labelling buffer consisting of 100 mM sodium phosphate buffer with 1 mM Tris (2-carboxyethyl) phosphine (TCEP), pH 7.3, using gel filtration. Collected fractions were supplemented with 0.5 mM of N,N'-dimethyl-N-(iodoacetyl)-N'-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine (IANBD) (Invitrogen, Burlington ON). The samples were incubated at room temperature for 120 minutes with gentle stirring. The excess label was removed by gel filtration. In order to determine the labelling efficiency, the molar ratio of fluorophore to protein was calculated from UV-VIS spectra using and extinction coefficient of 23,500 l/mol $\times$ cm for IANBD at 478 nm. The extinction coefficient of CAMP factor was determined to be 25,040 l/mol $\times$ cm at 280 nm [64]. The measured absorbance values at 280 nm of the labelled proteins were corrected for absorbance of the dye at 280 nm. Labelling efficiencies ranged from 85 to 95%. The labelled protein was run

on SDS-PAGE, after gel filtration, to verify purity by absence of the residual free label normally expected at the dye front (Figure 2.2).

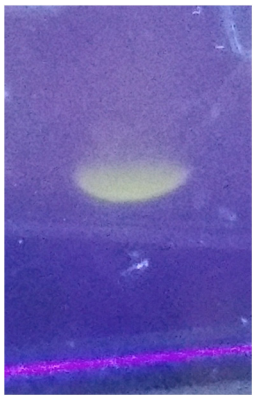

Figure 2.2: NBD-labelled CAMP factor Mutant on SDS-PAGE. Ultraviolet trans-illuminated SDS-PAGE gel of F138CNBD. Cysteine mutant F138 was labelled with IANBD with high labelling efficiency in TCEP-containing sodium phosphate buffer (100 mM sodium phosphate buffer with 1 mM Tris (2-carboxyethyl) phosphine (TCEP), pH 7.3) and purified via gel filtration.

Fluorescence measurements were performed using sRBC ghost membranes. The erythrocytes were lysed osmotically with low-salt phosphate buffer (5 mM Na_2HPO_4, 5 mM NaCl, pH 7.0) and washed repeatedly by centrifugation (i.e., 16,250 $\times$ g for 10 minutes) until a clear supernatant resulted. The sRBC membranes were then treated with sphingomyelinase as for haemolysis assays. The ghost membranes were suspended in HBS and incubated with the NBD-labelled mutant for 60 minutes at room temperature. The mixture was collected by centrifugation, the supernatant was retained, and the membrane pellet re-suspended in HBS buffer. Fluorescence emission spectra were acquired in a PTI QuantaMaster spectrofluorometer, using an excitation wavelength of 478 nm. The emission intensity was corrected for incomplete membrane binding; the extent of binding was determined by comparing the fluorescence of the saved supernatant with that of an equivalent amount of labelled mutant that had not been incubated with membranes. The bandpasses for excitation and emission were between 2 and 5 nm with the spectra acquired at room temperature.

2.3 Results

2.3.1 Functional Roles of the Two Domains

The novelty of CAMP factor's structure implies that the functions of its distinct domains cannot be inferred from any previously known model. In order to study the individual functions of CAMP factor's N- and C-terminal domains, they were expressed as separate fragments and their activities compared to that of WT toxin on sphingomyelinase-treated RBCs as well as liposomes composed of PC, PG, and Cholesterol (Figures 2.3 and 2.4).

On its own, CTD permeabilizes neither RBCs nor liposomes. NTD alone, however, permeabilizes both RBCs and liposomes; this indicates that membrane insertion and pore-forming activity predominantly reside within this domain. Furthermore, a fascinating find is made when the specific activity of NTD is compared to that of WT CAMP factor on the two membrane types: the haemolytic activity of NTD is about 10^4 times lower than that of WT CAMP factor, whereas on liposomes the activity is only about 15 times lower. Thus, the deletion of CTD has a much greater impact on RBCs, which contain GPI anchors, than on liposomes, which do not (Figure 2.3 and Figure 2.4).

Further characterization of NTD was pursued by monitoring membrane insertion of residue F138 in helix $\alpha 4$ (Figure 1.6). The cysteine mutant of F138 was then labelled with the polarity-sensitive dye NBD (henceforth F138CNBD) [66]. Once incubated with RBCs or liposomes, membrane-inserted F138CNBD produces a fluorescence emission that increased significantly compared to its monomeric counterpart in solution. Furthermore, a blue shift of approximately 10 nm was observed at the maximum emission wavelength. NBD typically responds this way when it inserts into the hydrophobic interior of the lipid bilayer [67]. On the other hand, since tryptophan fluorescence also tends to blue-shift upon membrane insertion, the CTD was incubated with liposomes to determine whether there was a change in fluorescence. The incubation did not change the fluorescence spectrum of the sole tryptophan residue (Figure 2.6B).

The question of the role of each domain in the interaction with neighbouring oligomer subunits provoked further inquiry into the effect of CTD in the presence of both WT CAMP factor and NTD.

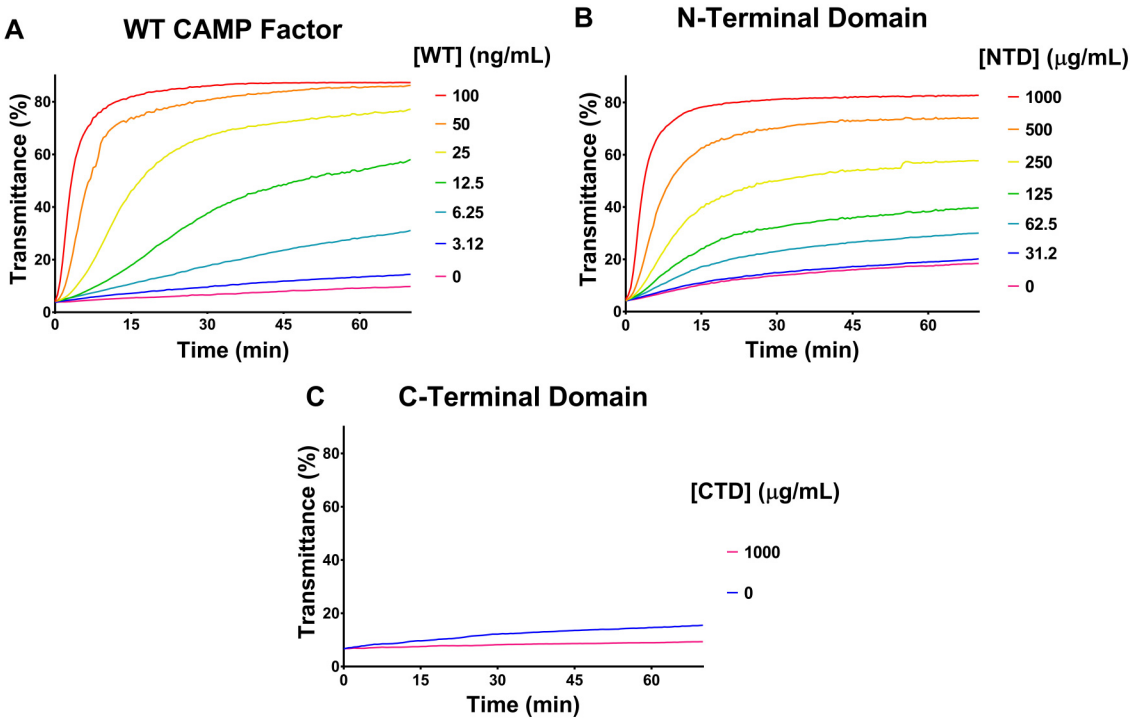

Figure 2.3: Haemolytic Assay of CAMP factor and its corresponding domains. WT CAMP factor was applied to sphingomyelinase-treated sheep red blood cells at the various concentrations **(A)**; the same was done for the N-terminal domain fragment **(B)** as well as the C-terminal domain **(C)**. No difference in effect was observed in the CTD fragment at various concentrations and thus only the maximum concentration used is shown. Note the significant difference in concentration between WT CAMP factor and the domain fragments. The transmittance of light through the cell suspension was monitored over time at a wavelength of 650 nm far outside the absorbance of haemoglobin (i.e., 540 nm) [65]. An increase in transmittance indicates reduced turbidity due to haemolysis. All transmittance values represent the means of quadruplicates.

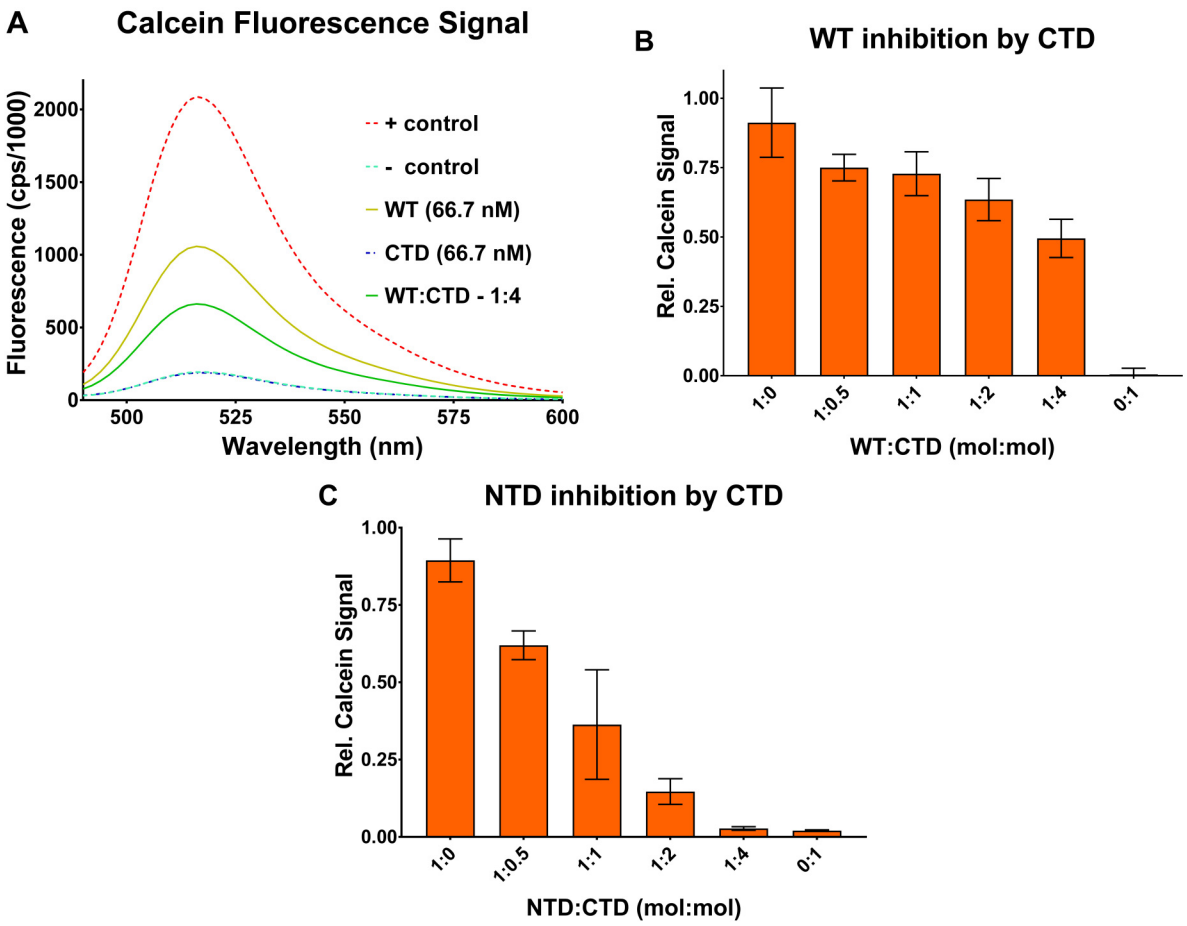

Figure 2.4: Inhibition of mature CAMP factor (WT) and N-terminal domain (NTD) by C-terminal domain (CTD) on calcein-loaded liposomes. Liposomes containing PC, Cholesterol, and PG were loaded with self-quenching concentrations of calcein. Once the membrane is permeabilized, calcein is diluted and fluorescence is increased in solution. Detergent solubilization (i.e., Triton X-100) is used as the positive control. **A:** WT causes half-maximal permeabilization at 66.7 nM. The CTD alone has no effect; its addition to WT at four fold molar excess partially inhibits WT. **B:** The same experiment plotted using a relative scale for calcein release, with additional molar ratios for WT:CTD included. **C:** NTD at 1 μM causes a similar extent of membrane permeabilization as a 15-fold lower amount of WT. NTD is also subject to inhibition by CTD. The error bars represent the standard deviation of the mean of calcein release between triplicates of each mixture of toxin and liposomes.

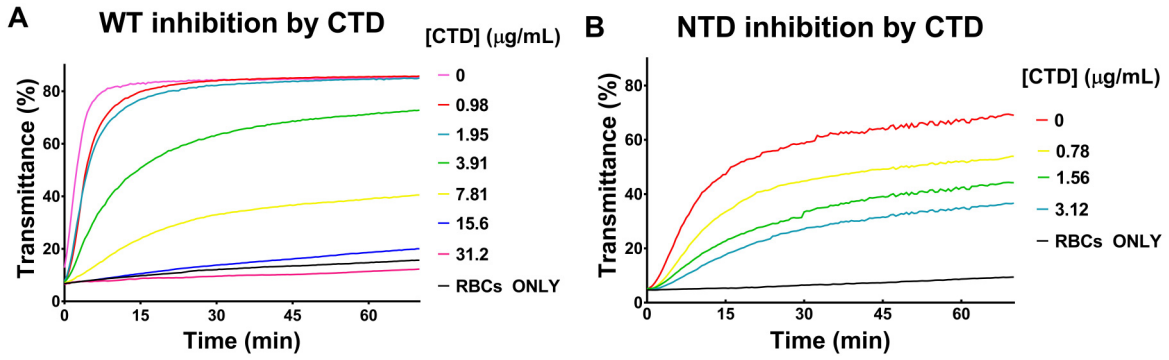

Figure 2.5: Inhibition of mature CAMP factor (WT) and N-terminal domain (NTD) haemolytic activity by C-terminal domain (CTD) on RBCs. WT (100 ng/mL) **(A)** and the NTD fragment (500 μg/mL) **(B)** were each admixed in the presence or absence of CTD at various doses and then combined with sphingomyelinase-treated sRBCs as in figure 2.3. At sufficiently high doses, CTD virtually eliminates the activity of both WT and NTD. All transmittance values represent the means of quadruplicates.

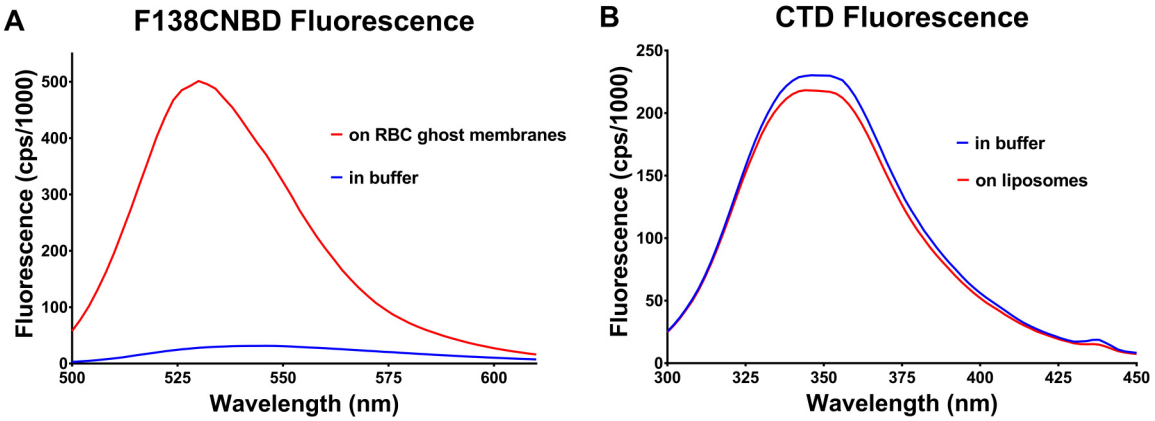

Figure 2.6: Fluorescence of NBD-labelled CAMP factor on RBC ghost membranes and the autofluorescence of the C-terminal domain (CTD) fragment on liposomes. CAMP factor residue F138, of the N-terminal domain, was replaced by cysteine and iodoacetamide derivative of the polarity-sensitive probe, NBD and termed F138CNBD. **A:** Incubation of F138CNBD with sphingomyelinase-treated sheep erythrocyte ghost membranes causes a large increase in fluorescence intensity as well as a significant blue shift, a behaviour consistent with the insertion of NBD in the membrane interior. **B:** Incubation of the CTD fragment with liposomes did not show any notable change in fluorescence or blue shift. All fluorescence signal values represent the means of triplicates.

Accordingly, WT toxin was combined with CTD prior to its addition to sphingomyelinase-treated RBCs. Interestingly, WT toxin was inhibited in a dose-dependent manner by CTD (Figure 2.5A). A similar result was observed when NTD was mixed with CTD prior to RBC addition (Figure 2.5B).

Although it is plausible that CTD's inhibition of WT toxin and NTD are a result of competition for binding to the GPI anchor on RBCs, inhibition was also observed on liposomes in both cases (Figure 2.4). Accordingly, since the liposomes used did not contain any GPI anchors, it is likely that the inhibition is due to a direct interaction between: 1) CTD and WT toxin, and 2) CTD and NTD.

2.3.2 Functional Significance of Conformational Flexibility within the Linker and NTD Regions

Pore-forming proteins are known for undergoing conformational changes when inserting into membranes [2]. A well-known technique for investigating these conformational changes is the restriction of the mobility of specific domains of the protein. This can be done by strategic introduction of disulfide bonds in certain closely interacting secondary structure elements and determining the effect on function [68]. As displayed in Figure 2.7A, disulfide bonds can be engineered into two distinct pairs of helices of the NTD in CAMP factor (i.e., DS1 and DS2) by mutagenesis. These modifications result in quantifiable decreases in haemolytic activity.

Interestingly, the introduction of a disulfide bond between the NTD and the CTD, as in residues 130 of α3 and 184 of helix α6, (i.e., DS3, Figure 2.7A) did not result in a notable change in haemolytic activity. This result is surprising since the linker region seems like a good site for local conformational flexibility (Figure 2.7).

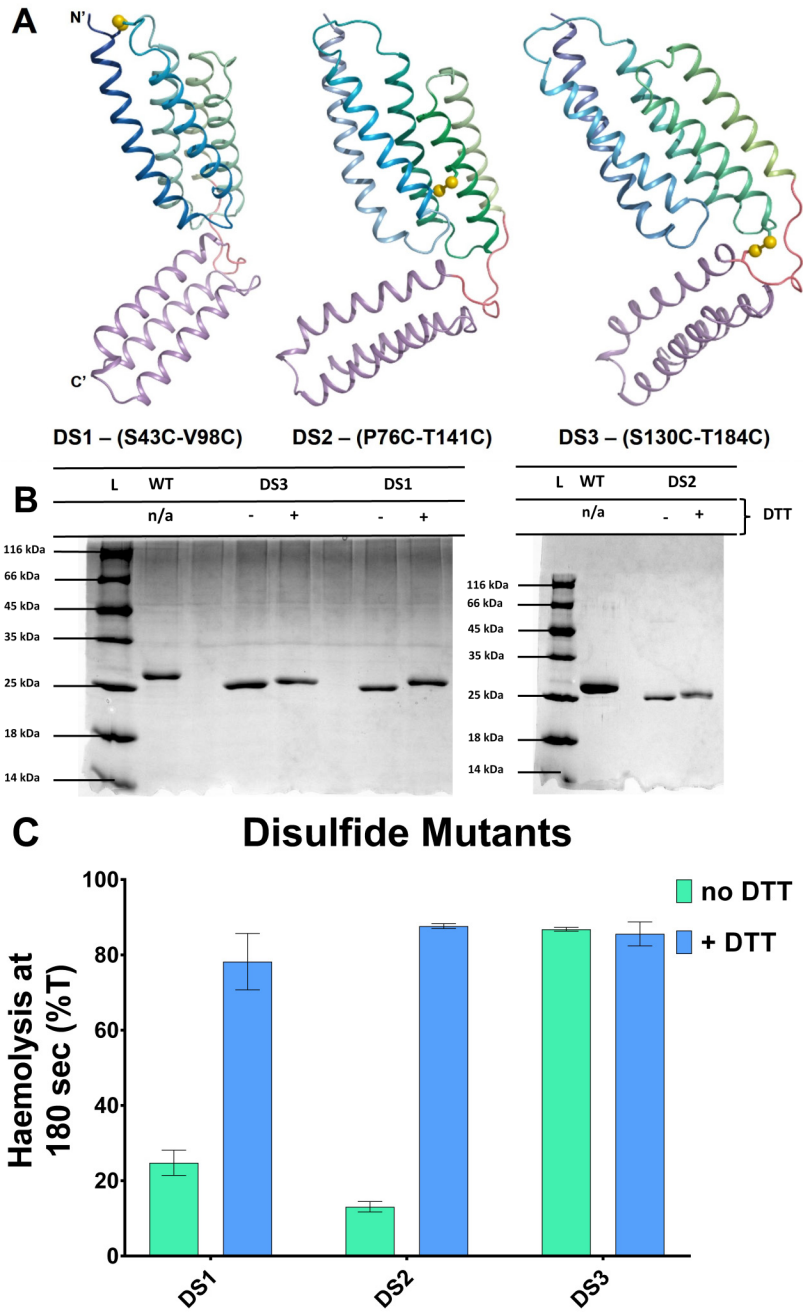

Figure 2.7: Disulfide mutants: verification and haemolytic activity. A: cysteine mutant pairs and their locations in the CAMP factor structure. **B:** SDS-PAGE of disulfide mutants in the absence and presence of the disulfide-reducing agent DTT. Accelerated migration indicates presence of disulfide bonds. **C:** Haemolytic activity of each mutant with and without DTT; **L** represents the protein ladder. The error bars represent the standard deviation of the mean of haemolytic activity at 180s within quadruplicates of each unique mixture of toxin and RBCs.

2.4 Discussion

2.4.1 Insights on Function: the N- and C-Terminal Domain

2.4.1.1 Domain Function is not Immediately Revealed Through Structure

CAMP factor's 3D structure reveals that the NTD and the CTD contain amphiphilic helices that may interact with the lipid membrane (Figure 1.6 and 1.8). These hydrophobic and hydrophilic regions are not clearly delineated and suggest that the CAMP factor molecule must undergo significant conformational rearrangement during pore formation [67]. These changes should orient hydrophobic regions so as to face the lipid bilayer, whereas the hydrophilic ones should face the pore lumen. This is not unprecedented as pore-forming proteins undergo conformational changes from their monomeric to oligomeric forms [2]. Therefore, further insights concerning CAMP factor show that the structure does not immediately lead to the assignment of function and thus further insights were needed from each of the domain fragments (i.e., NTD and CTD) through additional experimentation.

2.4.1.2 NTD is Responsible for Membrane Permeabilization; CTD is Responsible for more than Membrane Binding

The experiments with the separately expressed domain fragments showed that NTD permeabilizes both RBCs and liposomes, while the CTD permeabilizes neither, suggesting that the membrane-damaging activity resides mostly in the NTD. Furthermore, the haemolytic activity of NTD is about four orders of magnitude lower than that of WT toxin, whereas on liposomes the activity is only about 15 times lower. A plausible explanation for this discrepancy would be that the CTD mediates binding to GPI anchors; the loss of this function would be noticed on red blood cells, but not on liposomes. At the same time, the remaining difference, between WT and NTD, on liposomes suggests that the role of the CTD is not limited to GPI anchor binding. In keeping with this conclusion, CTD was found to inhibit the activity of both WT toxin and NTD, and this inhibition was manifest on both red blood cells and liposomes (Figures 2.3, 2.4, and 2.5).

These results also suggest that if the role of CTD were limited to binding, or in the case of competition with NTD, blocking GPI anchors, then it should not affect NTD on red blood cells. The other possibility is that CTD should only affect NTD on red blood cells if NTD also binds to the GPI anchor. Moreover, since liposomes have no GPI anchors, or other specific receptors, CTD should not inhibit NTD or WT toxin on liposomes. Therefore, these observations suggest that the CTD interferes with steps in pore formation other than binding and likely participates in oligomerization by interacting with the NTD. Participation of the CTD in oligomerization by interaction with the NTD is not unprecedented as it has been shown that the trypsin-cleaved domain 4-containing fragment of PFO and the domain 4 fragment of streptolysin O, both PFTs of the CDC subgroup, are capable of inhibiting the activity of their respective full length wild type toxins [69, 70]. These observations will require further validation by work on the two domains via binding to RBCs as well as the use of helix-specific disulfide cross-linking in studying activity on membranes and liposomes.

The fluorescently labelled CAMP factor residue F138 (i.e., in helix α4) was also shown to insert into the both RBCs and liposomes and confirms the idea that the N-terminal domain is the membrane-inserting domain (Figure 2.6A). In the case of CTD, its lone tryptophan residue did not reveal any change in fluorescence when incubated with liposomes which further supports the idea that the CTD is involved in membrane binding (Figure 2.6B). This finding does not negate the possibility that other residues could be participating in membrane insertion. The evidence for this possibility is bolstered by the seemingly random distribution of hydrophobic and hydrophilic residues on the surface of both domains of CAMP factor which suggests that a conformational change may be taking place in both domains during pore formation [67].

2.4.2 NTD Requires Internal Conformational Flexibility for Membrane Permeabilization

Once it was reasonably clear that the NTD was largely responsible for membrane permeabilization, the question arose whether the NTD requires conformational flexibility in order to permeabilize membranes. To answer this question, mobility restriction of specific helices via disulfide bridge engineering was applied to the NTD of CAMP factor [68]. The disulfide mutant, which connects the tops of two helices to that of the first (i.e., α2 and α3 to α1 or DS1, Figure 2.7A),

showed a moderate decrease in haemolytic activity, while its reduced form remained fully active (Figure 2.7C). Additionally, restriction of the midpoints of two helices in the NTD (i.e., α3 and α4 or DS2, Figure 2.7A) was also examined. This disulfide-restricted mutant showed significantly reduced activity, while its reduced form was fully active (Figure 2.7C). These results indicate that the NTD does in fact require internal conformational flexibility to permeabilize membranes. Such conformational flexibility is not surprising, considering that a similar behaviour is demonstrated in domain I, the membrane-permeabilizing region, of the *Bacillus thuringiensis* Cry1Aa toxin [68].

2.4.3 No Evidence of Functionally Crucial Mobility in the Linker Region

The insights revealed concerning the two domains made the role of the linker region in pore formation an area of great interest. Thus, the role of the linker region in pore formation was also studied by mobility restriction via disulfide bonds. Mobility restriction between the loop of α3 and α4 in connection to α6 (i.e., DS3, Figure 2.7A), causes no notable decrease in haemolytic activity. This suggests that the unrestricted mobility of the linker region may not be as significant to the modulation of domain activity as the 3D structure suggests. This effect on domain mobility may be limited as conformational changes could occur through helices α4 and α5 and thus minimize the effect of mobility restriction on haemolytic activity. Whatever the case may be, these results must be confirmed by more extensive work on specific residues in the linker region.

Chapter 3

The Effects of Target Membrane Lipid Composition on CAMP factor Activity

3.1 Introduction

CAMP factor is a PFT which forms large oligomeric pores on susceptible membranes [22], (i.e., cells or membrane vesicles that can be penetrated by the oligomeric form of the toxin). Pore formation has been demonstrated in both natural and artificial membranes. In both cases, the specific activity of the toxin was affected by membrane lipid composition [22]. Interestingly, CAMP factor activity on cells is increased by the conversion of sphingomyelin to ceramide [71, 22].

In a simple liposome model, the addition of C_{12}-ceramide did not increase susceptibility to CAMP factor [22]. These experiments did, however, suggest a requirement for cholesterol, whose omission rendered the liposomes (now composed of only C_{12}-ceramide and phosphatidylcholine) resistant to permeabilization by CAMP factor [22].

Accordingly, the goal of this work was to further examine the role of cholesterol in CAMP factor activity. This was done by the calcein release assay employed in Chapter 2. The results indicate, surprisingly, that cholesterol is not required for CAMP factor activity on membrane vesicles that otherwise consist of glycerophospholipids only [22]. The discrepancy with previous findings is likely due to the difference in the composition of the non-sterol lipids used in the two sets of

experiments. Overall, the previously postulated specific requirement for cholesterol is rejected by the findings presented here.

3.2 Materials and Methods

3.2.1 Materials

In order to prepare the necessary liposomes, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), and 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG), and cholesterol (cholesterol) were purchased from Avanti Polar Lipids (Alabaster, AL). Calcein (Figure 3.2B) was obtained from Sigma-Aldrich®, St. Louis, MO.

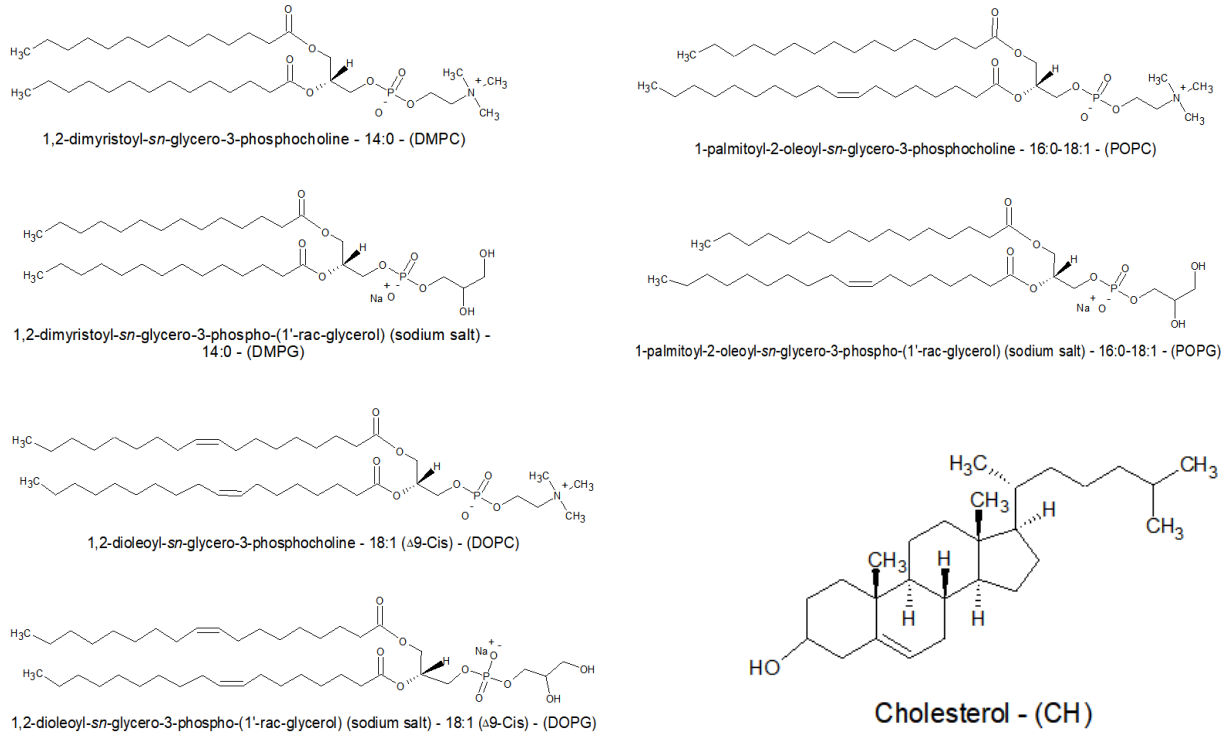

Figure 3.1: Chemical structures of lipid species used in this study.

3.2.2 Methods

All chemical structures were rendered using ACD/Chemsketch 2015 ®. All data were plotted using GraphPad Prism ® 7.

Protein expression and purification was performed as previously described [22, 61]. CAMP factor was subjected to the same haemolytic assay described in Chapter 2, section 2.2.2, for the sake of testing toxin activity on a standard model prior to use on liposomes.

The procedures used for preparing the large unilamellar vesicles (LUVs) are described in Chapter 2, section 2.2.3. All incubations involving these liposomes were carried out at 37°C, followed by measurement in a thermostatted cuvette holder at the same temperature.

The extent of membrane permeabilization for each sample P was then calculated using the formula given in Chapter 2, section 2.2.3.

The lipids used for preparing the cholesterol-containing liposomes were prepared at the following molar ratios: 6.5/2.5/1.0 (i.e., DMPC/cholesterol/DMPG), as well as 7.5/2.5 (i.e., DMPC:cholesterol). The mixtures without cholesterol were each prepared at a molar ratio of 7.5/2.5 of the following lipids: DMPC:DMPG, POPC:POPG, and DOPC:DOPG. Other preparations for liposomes included pure POPC and DOPC. Multiple attempts to prepare DMPC-only liposomes were unsuccessful, as these preparations were unstable during the assay period. The unstable nature of calcein-loaded DMPC liposomes is in line with work done by Clary *et al.*, whose work on carboxyfluorescein-loaded DMPC liposomes at the same temperature (i.e., 37°C) and a pH of 7.5 produced liposomes with a half-life of 24 minutes (Figure 3.2A) [72]. Liposomes were treated with CAMP factor dosages ranging from 0.2 µg/mL to 30 µg/mL (Figure 3.3A).

3.3 Results

3.3.1 Permeabilization of liposomes containing dimyristoyl lipids

As stated earlier, attempts to prepare DMPC only liposomes resulted in model membranes that were unstable during the assay period (Figure 3.1). However, stable liposomes were obtained

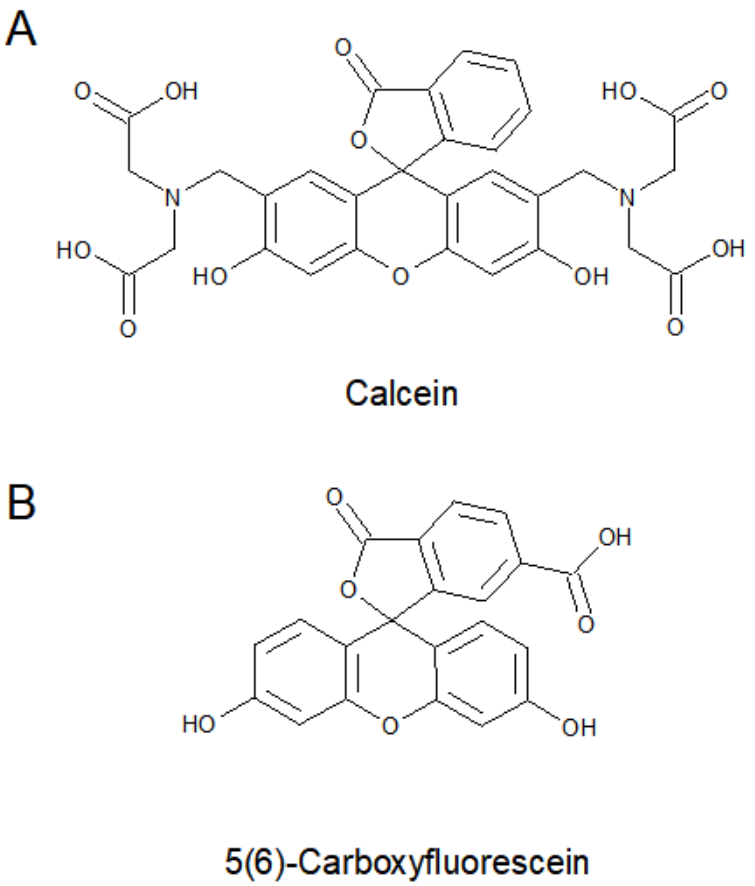

Figure 3.2: Structure of fluorescent dyes commonly used in permeability assays. Chemical structure of Calcein (A). Chemical structure of 5(6)Carboxyfluorescein (B).

when either or both of cholesterol (25 mol%) and DMPG (10 mol%) were included in the lipid mixture. Surprisingly, all three liposome species showed very similar susceptibility to CAMP factor; cholesterol had at most a minor effect on the dose-effect curve of permeabilization.

The data presented here therefore indicate that cholesterol is not required for CAMP factor to permeabilize liposomes (Figure 3.3B). These results are in stark contrast to previous findings which suggested that cholesterol was important for CAMP factor activity [22].

3.3.2 Permeabilization of liposomes containing palmitoyl/oleoyl lipids

While the experiments described in the previous section used DMPC and DMPG, earlier experiments by Lang and Palmer used egg yolk phosphatidylcholine (EYPC), which consists mostly

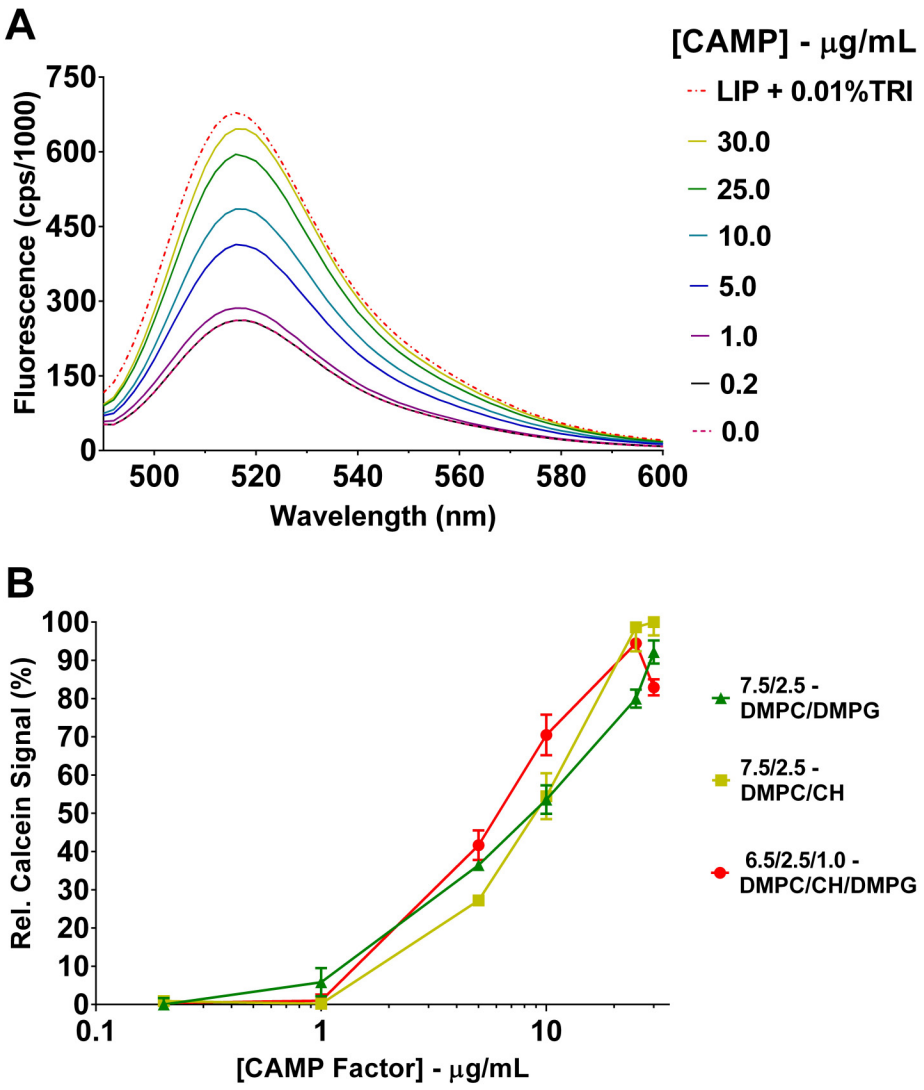

Figure 3.3: Effect of CAMP factor activity on dimyristoyl lipids in the presence or absence of cholesterol. Liposomes containing DMPC/DMPG, DMPC/Cholesterol, and DMPC/Cholesterol/DMPG were loaded with self-quenching concentrations of calcein. **A:** Fluorescence spectra obtained after incubation of CAMP factor with 7.5/2.5 DMPC/DMPG liposomes at various concentrations. LIP + 0.01%TRI is a mixture of calcein-loaded liposomes combined with 0.01% Triton X-100, which acts as the positive control. **B:** The same experiment plotted using a relative scale for calcein release for each liposome mixture. The error bars represent the standard deviation of calcein release within triplicates of each mixture of toxin and liposomes.

of POPC [22]. The question therefore arose whether the different observations concerning the role of cholesterol were due to differences in phospholipid acyl chain length and/or unsaturation.

In order to assess whether this was the case, POPC and POPG were used instead of DMPC and DMPG. The findings from this work indicate that the four liposome variants (i.e., POPC, POPC/POPG, POPC/cholesterol, and POPC/cholesterol/POPG), were all stable. Their susceptibility to CAMP factor showed little difference to each other and DMPC/DMPG liposomes (Figures 3.4 and 3.3B).

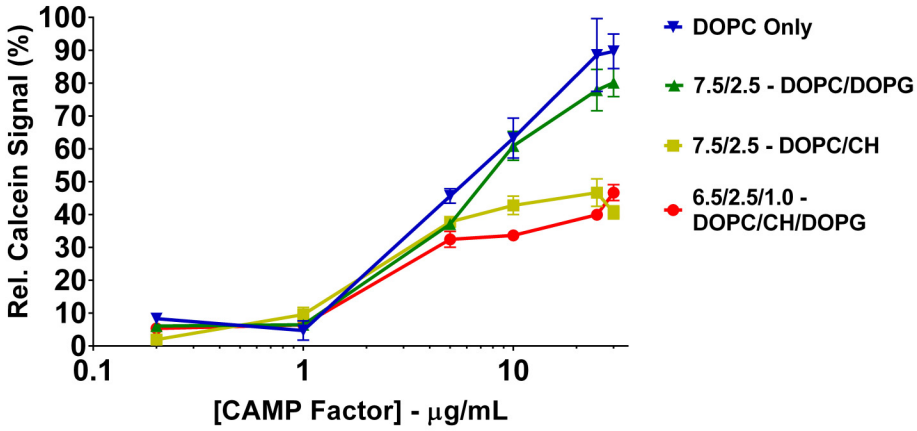

Figure 3.4: Effect of CAMP factor activity on palmitoyl/oleoyl lipids in the presence or absence of cholesterol. The experiment, in like manner to Figure 3.3**A**, was plotted using a relative scale for calcein release. The error bars represent the standard deviation of calcein release within triplicates of each mixture of toxin and liposomes.

3.3.3 Permeabilization of liposomes containing dioleoyl lipids

The use of dioleoyl lipids in membrane models is widespread for both the study of lipid systems as well as toxin activity [73, 74]. These phospholipids contain acyl chains that are longer and more unsaturated than those previously used to study CAMP factor activity on artificial membranes (Figure 3.1) [22]. Thus, model membranes composed of dioleoyl lipids were used to examine how CAMP factor activity is affected by the presence of cholesterol.

The data from these model membranes show that cholesterol-free dioleoyl liposomes respond to CAMP factor action in a similar manner to both dimyristoyl and palmitoyl/oleoyl liposomes.

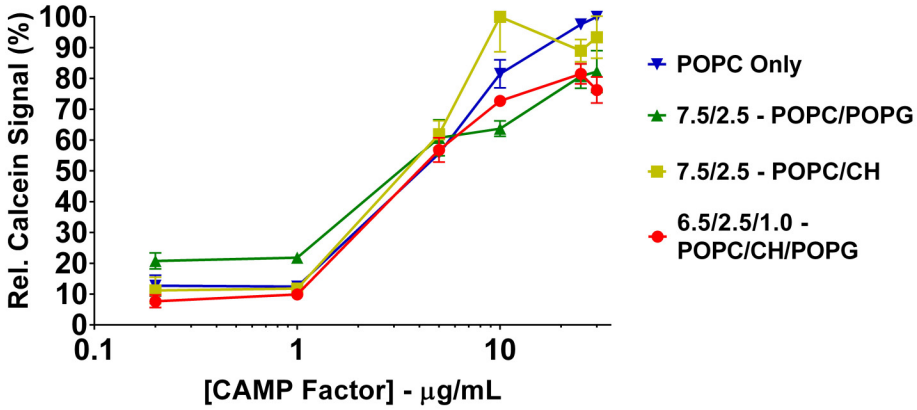

Figure 3.5: Effect of CAMP factor activity on dioleoyl lipids in the presence or absence of cholesterol. The experiment, in like manner to Figure 3.3**A**, was plotted using a relative scale for calcein release. The error bars represent the standard deviation of calcein release within triplicates of each mixture of toxin and liposomes.

However, cholesterol-containing dioleoyl lipids, although having a similar onset of permeabilization, have a lower maximal release (Figure 3.5). This is an unusual and unexpected finding; however, these liposomes also fail to substantiate any need of CAMP factor for cholesterol.

3.4 Discussion

The focus of the present study was the effect of membrane lipid composition on CAMP factor activity. All experiments were conducted at the physiological temperature of 37°C where all pure phospholipid membranes are in the fluid (i.e., liquid-disordered) phase [75]. Cholesterol-containing liposomes using the same phospholipids on the other hand, have reduced membrane fluidity (i.e., they are in the liquid-ordered phase) above their transition temperature because of the ordering effect of cholesterol [75].

Overall, the data from the current study show that cholesterol is not required for CAMP activity. Additionally, cholesterol has little effect on CAMP activity on PC and PC/PG membranes. Furthermore, PG, at least a 25% molar ratio, has little or no effect on CAMP activity. The effect of lipid chain length and unsaturation on CAMP factor activity was also tested and the results showed that both these factors have little or no effect when membranes are in the fluid state. Finally, when

the effect of membrane fluidity on CAMP factor activity was examined in liquid-disordered and cholesterol-induced liquid-ordered states, the data showed little effect on CAMP factor activity.

In Lang and Palmer's work, model membranes composed of C_{12}-ceramide, EYPC, and cholesterol were used to study CAMP factor activity [22]. In all cases, either C_{12}-ceramide and/or cholesterol were present in the liposome mixture. The results of this work suggested that cholesterol was important for CAMP factor activity on membranes [22]. One potential cause for the difference between these two studies is the use of unsaturated phospholipids (i.e., EYPC) in the former study, but this was ruled out by the use of very similar lipids (i.e., POPC and POPG) in assessing CAMP factor activity.

Thus, the only remaining difference was the use of C_{12}-ceramide in Lang and Palmer's study. Since ceramide promotes pore formation activity in cell membranes [22], a possible inhibitory role was never considered. Surprising as it may seem, the only remaining explanation is that C_{12}-ceramide actually inhibits CAMP factor activity in simple phospholipid membranes. This may explain why Lang and Palmer observed a slightly higher CAMP activity on PC/Cholesterol liposomes than those with a ternary mixture of PC/Cholesterol/Ceramide [22].

Taken together, the results from the work of Lang and Palmer and the current study produce the following observations compiled in Table 3.1. Each row in the table shows the presence or absence of a specific liposome component and its resulting effect on CAMP factor's ability to permeabilize the model membrane.

Table 3.1: CAMP Activity on Artificial Membranes

EYPC or POPC	C_{12}-Ceramide	Cholesterol	Permeabilization
+	-	-	+
+	-	+	+
+	+	-	-
+	+	+	+

Overall, the compiled data suggest that C_{12}-ceramide inhibits CAMP factor, and that cholesterol relieves this inhibition.

The neutralizing effect of cholesterol on ceramide-containing liposomes may be a result of the known tight interaction of cholesterol with sphingolipids [76, 77]. It is well-established that

cholesterol has an ordering and condensing property that allows for close packing of saturated acyl chains of sphingolipids (as well as phospholipids) by its smooth faces subsequently forming lipid rafts [76, 78]. Interestingly, it is also well-known that cholesterol associates weakly with highly unsaturated lipid species [77]. In cellular membranes, these lipid rafts are specialized membrane domains containing high concentrations of cholesterol, sphingomyelin, gangliosides, and proteins. Additionally, lipid rafts are also enriched in phospholipids that contain saturated fatty acyl chains [76, 75]. As a result of these liquid-ordered membrane domains, there is also lateral phase separation in the lipid bilayer which generates portions of the lipid bilayer that are not as ordered (i.e., liquid-disordered) [76, 78, 75]. This lateral phase separation may occur in cholesterol/ceramide-containing model membranes by cholesterol sequestering ceramide and thereby causing the remainder of membrane to be liquid-disordered and thus allowing pore formation to take place in these regions in the membrane [77].

It is noteworthy that there is little difference in CAMP factor activity on the various phospholipid compositions with the sole exception of cholesterol-containing dioleoyl liposomes incubated with higher CAMP factor concentrations (Figure 3.5). Though these dioleoyl liposomes have similar onset of permeabilization to dimyristoyl and palmitoyl/oleoyl ones, their maximal release is about half of what is expected. Thus far, such a phenomenon has no precedent as it concerns CAMP factor (or any other PFT for that matter).

One possible explanation for the reduced release of calcein from cholesterol-dioleoyl liposomes by CAMP factor pore formation may be that during the initial vortexing stage, lipid segregation occurs in the multilamellar vesicles such that some liposomes end up with plenty of cholesterol in the bilayer and others with very little. This subsequently gives rise to two different large unilamellar vesicle populations after extrusion and gel filtration resulting in one group with low cholesterol and the other high. Upon treatment of this mixture with CAMP factor perhaps the low-cholesterol vesicles are susceptible to pore formation due to their lower propensity for lipid raft formation [76, 77]. Currently, there is not enough evidence to prove such a theory. Thus, more work using dioleoyl lipids in combination with varying concentrations of cholesterol is needed in order to elucidate the cause of this reduced permeabilization of cholesterol-dioleoyl membranes.

Chapter 4

Summary and Future Work

4.1 Summary

4.1.1 Functional Roles of N- and C-terminal Domains of CAMP factor

CAMP factor was recently shown to have a novel structural fold composed of two distinct domains composed of a 5+3 helix bundle connected by a linker region (submitted manuscript). The work in Chapter 2 was thus performed based on the structural insights gained from the 3D crystal structure of the CAMP factor monomer solved by Dr. Tengchuan Jin (Figure 1.6). These insights were gained from sequence/structure-based analyses which did not immediately reveal a clear structure-function relationship.

The absence of a clear structure-function relationship necessitated the use of haemolytic assays and membrane permeabilization experiments using WT, the two distinct domain fragments (i.e., NTD and CTD), disulfide mutants (linker and NTD region), and a fluorescently labelled cysteine mutant on RBCs and liposomes as a method for studying the mode of action of CAMP factor. The data from this study showed that NTD permeabilizes both membrane systems while CTD permeabilizes neither, suggesting that membrane-damaging activity resides predominantly in the NTD. This view is supported by the membrane insertion of the NBD-labelled cysteine mutant F138C (Figures 2.2 and 2.6A). Additionally, NTD activity was shown to be approximately 10^4 times lower than that of WT on RBCs while on liposomes activity is only about 15 times lower;

thus, the removal of CTD has a greater impact on RBCs than liposomes suggesting that CTD mediates specific binding to GPI anchors (Figures 2.3 and 2.5).

However, CTD was also shown to inhibit both NTD and WT permeabilization on RBCs and liposomes (Figure 2.5). These data suggest that CTD does more than simply bind to GPI anchors. This is because if the role of CTD were limited to binding GPI anchors, then it should not affect NTD on red blood cells (or only so if NTD still binds to GPI anchors). Furthermore, since liposomes have no GPI anchors or other specific receptors, CTD should not inhibit NTD or WT toxin on liposomes. Thus, the observations indicate that CTD interferes with steps other than binding and likely participates in oligomerization.

The verified full length disulfide mutants (i.e., DS1 and DS2 which connect the tops of two and mid points of two other helices in the NTD respectively) were prepared by Dr. Jin for studying internal conformational flexibility in the NTD. The two disulfide mutants exhibit moderate and severe haemolytic activity, respectively, in comparison to the WT (Figure 2.7A). These results indicate that the NTD does in fact require intra-domain conformational flexibility in order to permeabilize membranes. In the case of the verified full length disulfide mutant DS3, prepared by Dr. Jin for studying the linker region (Figures 1.6 and 2.7A), little to no effect on haemolytic activity is observed. This suggests a limited effect on inter-domain mobility; this limited effect is not sufficient evidence that the linker region is not important in inter-domain mobility and pore formation. Thus, a more detailed study into this matter is necessary.

4.1.2 Further Studies on the Functional Roles of CAMP factor Domains

Further research into the functional roles of the N- and C-terminal domains of CAMP factor will require strategic placement of more disulfide mutations using cysteine-scanning mutagenesis. These mutations will be made for the purpose of studying conformational restraint between the helices of the two domains as well as the extent of membrane permeabilization on both natural and artificial membranes. This aforementioned principle can be applied to a study of the linker region to study inter-domain conformational flexibility of CAMP factor.

4.1.3 Effect of Target Membrane Composition on CAMP factor Activity

Based on previous work by Lang and Palmer [22], CAMP factor's activity was shown to be affected by target membrane composition; specifically, the data indicated that cholesterol was required as cholesterol-free liposomes were insensitive to CAMP activity. However, these cholesterol-free liposomes were only tested when ceramide was present. Thus, the purpose of Chapter 3 was to further examine the role of cholesterol on CAMP factor activity. This was done not only with the omission of ceramide, but with the additional intent of examining the effect of lipid chain length and degree of unsaturation.

The calcein release assay was used as the technique of choice and experiments were thus performed using LUVs composed of dimyristoyl, palmitoyl/oleoyl, and dioleoyl phospholipids in the presence or absence of cholesterol (Figures 3.3, 3.4, and 3.5). The data showed that in the presence or absence of cholesterol there is little difference in CAMP factor activity on membranes. Furthermore, lipid acyl chain length and unsaturation have little or no effect on CAMP factor activity when the lipid bilayer is in the fluid phase. It was also found that membrane fluidity, or at least the difference between the liquid-disordered and the cholesterol-induced liquid-ordered state, has little effect on CAMP factor activity. These findings are in contrast to Lang and Palmer's studies, which suggested that cholesterol was a requirement for pore formation by CAMP factor. However, as noted earlier, C_{12}-ceramide was always present in the liposome mixtures used to assess CAMP factor activity.

Interestingly, when the aforementioned studies are collectively considered, the overall evidence suggest that C_{12}-ceramide inhibits CAMP factor activity in the membrane bilayer while cholesterol relieves this inhibition (Table 3.1).

4.1.4 Further Studies on the Effect of Target Membrane on CAMP factor Activity

Future work expanding on Chapter 3 should include further studies on how ceramide inhibits CAMP factor on model membranes but activates it on natural cell membranes. One way to do this is to strip the GPI anchors and other carbohydrates from the membranes, and then see whether the effect of ceramide persists.

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