On The Role of Sphingomyelinase in CAMP-factor Membrane insertion and Oligomerisation

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.

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ABSTRACT

CAMP factor is a 25kDa extracellular protein from *Streptococcus agalactiae* (Group B streptococci) that contains 226 amino acid residues. CAMP factor has been characterized as a pore-forming toxin (PFT). The typical mechanism of pore formation of PFTs involves three main stages, namely binding of toxin monomers to the membrane surface, oligomerization of the monomers on the cell membrane, and finally the insertion of oligomers into the membrane.

This study focused on second stage, and investigates the oligomerisation of CAMP factor on sheep red blood cell membranes. It is known that the hemolytic activity of CAMP factor is greatly enhanced by interaction with sphingomyelinase from *Staphylococcus aureus.* We here focused on understanding the role of sphingomyelinase in the oligomerisation step.

Experimental data were obtained using Förster resonance energy transfer (FRET) studies. The fluorescence dyes IAEDANS and Fluorescein-5-maleimide were used as donor/acceptor fluorophores and attached to mutant single cysteine residues in CAMP factor. Samples of donor- and acceptor-labelled protein were mixed and incubated with red cell membranes that had or had not been pre-treated with sphingomyelinase. Energy transfer was monitored with time-resolved and steady-state fluorescence measurements. In the time-resolved experiments, the fluorescence lifetime of the donor was measured in the

presence and the absence of the acceptor, on membrane samples that were or were not treated with sphingomyelinase.

We observed a decrease in the fluorescence lifetime of the donor with the presence of the acceptor. The decrease in lifetime due to acceptor interaction signifies the occurrence of energy transfer between the donor and acceptor fluorophores, which indicates proximity due to oligomerisation of the CAMP factor protein on the cell membrane. This was only observed when the membranes had been treated with sphingomyelinase.

When membranes were used that had not been treated with sphingomyelinase, the donor lifetimes are very low, suggesting the inability of the CAMP factor to undergo membrane insertion and oligomerisation.

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DEDICATION

To my father and mother

To my beloved wife Fouzia Idrees, who love and encourage me all the time without reservation

To my kids Muhammad Saad Idrees, Rameen Idrees, Maaz Khan

To my brothers and sisters

To everyone who supported me

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

GBS	Group B Streptococci
S. agalactiae	*Streptococcus agalactiae*
S. aureus	*Staphylococcus aureus*
SLO	Streptolysin O
E. coli	*Escherichia coli*
PFT	Pore Forming Toxin
GPI	Glycosyl Phosphatidylinositol
GST	Glutathione-S-transferase
SRBC	Sheep Red Blood Cells
IPTG	Isopropyl-β-$_D$-thiogalactofuranoside
LB	Luria Bertani broth
PBS	Phosphate Buffer Saline
DTT	DL-Dithiothreitol
Rpm	Rotation Per Minute
SDS PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
IAEDANS	N-(iodoacetylaminoethyl)-5-naphthylamine-1-sulfonic acid
DMSO	Dimethylsulfoxide
SDS	Sodium Dodecyl Sulfate
CDC	Cholesterol-dependent cytolysin
D1 to D4	Domain 1 to Domain 4

LukF	Leukocidin F
LukS	Leukocidin S
PFO	Perfringolysin O
TMH	Transmembrane β-hairpin
ILY	Intermedilysin
Cry	Cry toxin , crystal
Cyt	Cytotoxin
F109C	Phenylalanine substituted with cystein at position 109
L107C	Leucine substituted with cystein at position 107
EDTA	Ethylendiamine tetra acetic acid
FI	Fluorescence Intensity
F	Fluorescein
I	IAEDANS
RBC	Red blood cell
Bp	Base pair
SLO	Streptolysin O

CHAPTER 1

INTRODUCTION

1.1 Membrane Damaging Toxins

Gram-negative and Gram-positive bacteria both produce membrane-damaging toxins or cytolysins, which disrupt or damage the plasma membrane of mammalian target cells, leading to cell swelling and lysis [1].

Membrane-damaging toxins have historically been identified through their cytolytic action on human and animal erythrocytes, which has led to many of them being referred to as hemolysins; however, the effect of most such toxins is not restricted to red cells, and some even act preferentially on other cells, for example leukocytes. According to their mode of action, membrane-damaging toxins can be divided into three groups

(i) Enzymatically active cytolysins (phospholipases)

These toxins disrupt the membrane by enzymatically hydrolyzing the phospholipids of the membrane bilayer. The most important toxins of this family are phospholipases, for examples are *Clostridium-perfringens* a-toxin (phospholipases C), *Staphylococcus aureus* β-toxin (sphingomyelinase C), and *vibro damsela* hemolysin (phospholipases D) [1].

(ii) Tensio-active cytolysins, which solubilize the cell membranes by detergent-like action [2], and partial insertion into the hydrophobic regions of the target membranes, for example, the δ-toxins of 26 amino acids of *Staphylococcus aureus*, *Staphylococcus haemolyticus* and *Staphylococcus lugdunensis* [2].

Two models have been proposed for membrane penetration of these toxins, (1) the carpet model and (2) the barrel-stave model. Both models for membrane-lytic peptides are not fully understood.

(1) Membrane destruction and solubilization by the carpet model.

i. According to this model the toxin adsorbs onto the membrane surface, where it non-specifically aggregates into large carpet-like structures.

ii. The peptide monomers will bind to the phospholipids head groups and the membrane permeation will occur, when the local concentration of membrane-bound peptide will increase.

iii. This will destabilize the membrane through defects in lipid packing of membrane, due to which the cytoplasm can leak out from the cell. This finally breaks the membrane into micellar protein-lipid complexes, and the cell membrane will completely disintegrate.

(2) In the barrel-stave model, the bundles of amphipathic a-helices will insert into the hydrophobic core of the membrane, and will recruit additional monomers, to form trans-membrane channels or pores [3, 4].

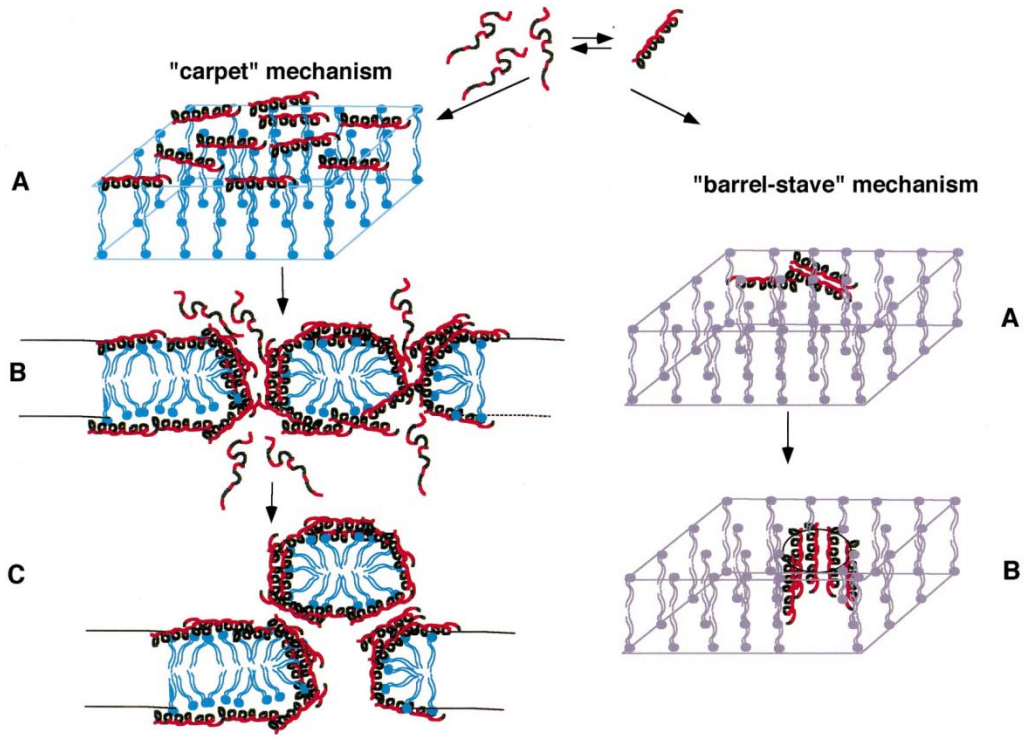


Figure 1: The carpet mechanism (left) and the barrel-stave mechanism (right) of tensio-active peptide action.

In the carpet model the peptides are attached to the hydrophobic faces of membrane surfaces, and their hydrophilic surfaces facing the aqueous solution (step A). When a certain concentration of peptides monomers is reached the membrane is rapture and finally disintegrated, and formed the transient pores and micelles (step B and C). In a barrel-stave model the amphipathic a-helices (peptides) first assembled on the surface of the membrane (A), then insert into the lipid core of the membrane to recruit additional monomers to form trans-membrane pores (B), adapted from [2].

(iii) Pore forming toxins

Researchers have been studying pore-forming toxins (PFTs) for a long time, and with some toxins, quite a bit is known about their mechanisms of action.

They are widespread among bacteria, but can also be secreted by some higher organisms such as plants, insects, fungi or even animals such as sea anemones or earthworms as a means of attack or defence [5].

Pore-forming proteins and peptides form discrete pores in cell membranes. These toxins are typically secreted as water-soluble, monomeric proteins and, upon membrane binding, become membrane-inserted state after large conformational changes. The PFTs will interact with cell or synthetic lipid membranes, change conformation and oligomerize in the membrane to create water filled pores, which are permeable to solutes. This creates an osmotic imbalance causing the highly concentrated ions to diffuse out of the cell into the low concentrated extra cellular medium and water to flow inside the cell, leading to swelling, lysis and death of the host cell [6].

The flow of ions goes both ways. The osmotic imbalance is caused by more ions rushing into the cell than out of it.

1.1-1 The general mechanism of pore formation

According to the available experimental evidence, most of the characterized pore-forming toxins (PFTs) have similar mechanism of action [7]. Figure 2 outlines the model of pore formation [8]. Most bacterial PFTs are released as water-soluble monomeric proteins, which later on diffuse and bind to target cell membrane. Membrane binding of PFTs may occur by the binding of specific receptors on the target cell surface, such as cholesterol or the glycan core of Glycosylphosphatidylinositol (GPI)-anchored proteins [9, 10]. Upon binding, the

toxin molecules oligomerize to form a pre-pore complex [11]. The pre-pore complex undergoes a major conformational change that leads to the exposure of hydrophobic surfaces. Thus the pre-pore complex inserts into the lipid matrix of the cell membrane, generating trans-membrane channels lined by hydrophilic residues. These channels differ in size, from 1-2 nm with staphylococcal a-hemolysin and Vibrio cholera cytolysin, [12], to 25-30 nm with SLO (Streptolysin O) and PFO (Perfringolysin O) [13-14].

According to the general mechanism of action described above, PFTs can assume two different stable states, namely the water-soluble, monomeric and the membrane inserted, oligomeric state [15]. In the water-soluble state, the hydrophilic surface is exposed in the aqueous environment, thus avoiding the aggregation and precipitation that is common in proteins, such as integral membrane proteins that expose their hydrophobic surfaces. In the oligomeric state, a conformational change has occurred that exposes hydrophobic surfaces, which now mediate the interaction with the non-polar fatty acyl chains in the lipid membrane.

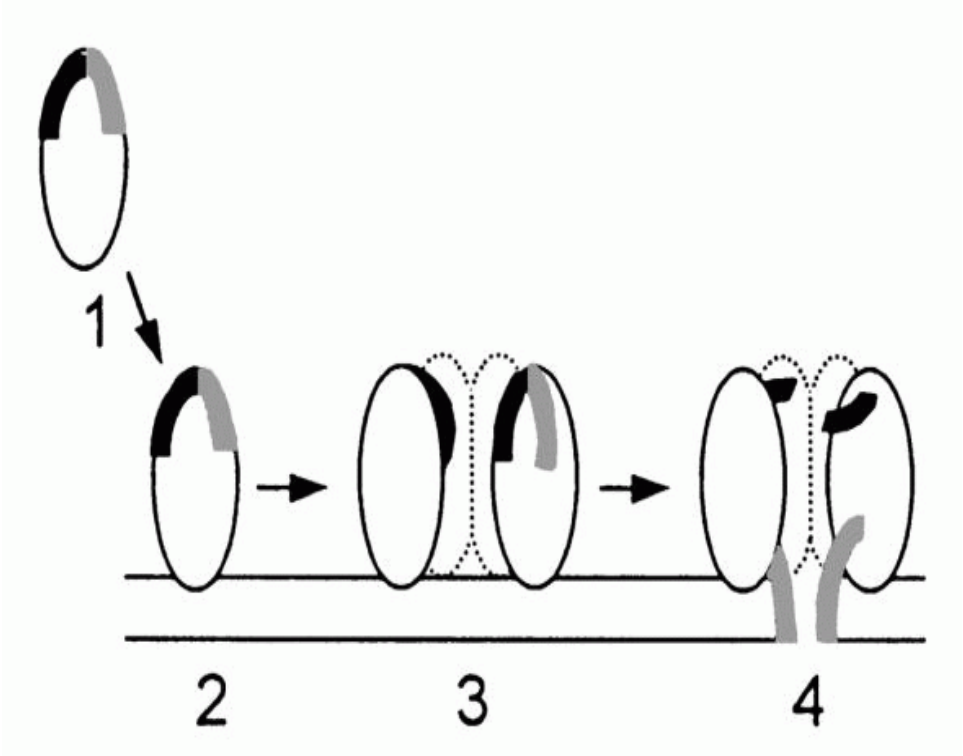


Figure 2: General mode of action of PFTs.

(1) Water-soluble local monomers (2) bind to and adjust themselves on Lipid bilayer, sometime via specific receptors (3) Membrane bound monomers have a collision by means of lateral diffusion in the membrane plane to form a pre-pore complex and undergo a conformational change, which leads to the exposure of hydrophobic surfaces and the formation of the membrane inserted pore, adapted from [8].

1.2 Classification of pore forming toxins

Pore forming toxins (PFTs) can be classified in a number of ways; for example, on the basis of organism that produces them or according to some particular features that are required for their activities. A particularly useful classification is based on the membrane damaging mechanism and certain structural features.

1.2-1 The a-Pore-forming toxins

The a-PFTs are interacting with the membrane through a-helices to form pores. These toxins are generally rich in a-helices. Their pore forming domains consist of a three-layered structure, which has up to approximately ten a-helices sandwiching a central hydrophobic helical hairpin. It is assumed that this hairpin will take the initial step of the insertion process in the membrane [16]. For example, the pore forming colicins are typical member of the class that also includes *Pseudomonas aeruginosa* exotoxin A, and some insecticidal δ-endotoxins (Figure 3).

The δ-endotoxins from *Bacillus thuringiensis* are insecticidal proteins that kill some insects by forming pores in the midgut epithelial cell membrane [17]. When the δ-endotoxin crystals are ingested by an insect, these crystals are dissolved in the midgut (due to low basic pH) releasing the protoxin molecules of which they are made. The protoxin is activated by proteolysis, and then the activated toxin binds to the midgut epithelial cells. This activated toxin creates pores in the cell membranes leading to cell lysis [18]. As a result the gut becomes immobilised, the larvae stop feeding, allowing the bacteria to be retained and to break down the insect gut wall. The insect will die within a few hours of ingestion due to starvation and septicaemia [18].

The binding affinity of these activated toxins is often directly related to the toxicity, though binding does not assure toxicity.

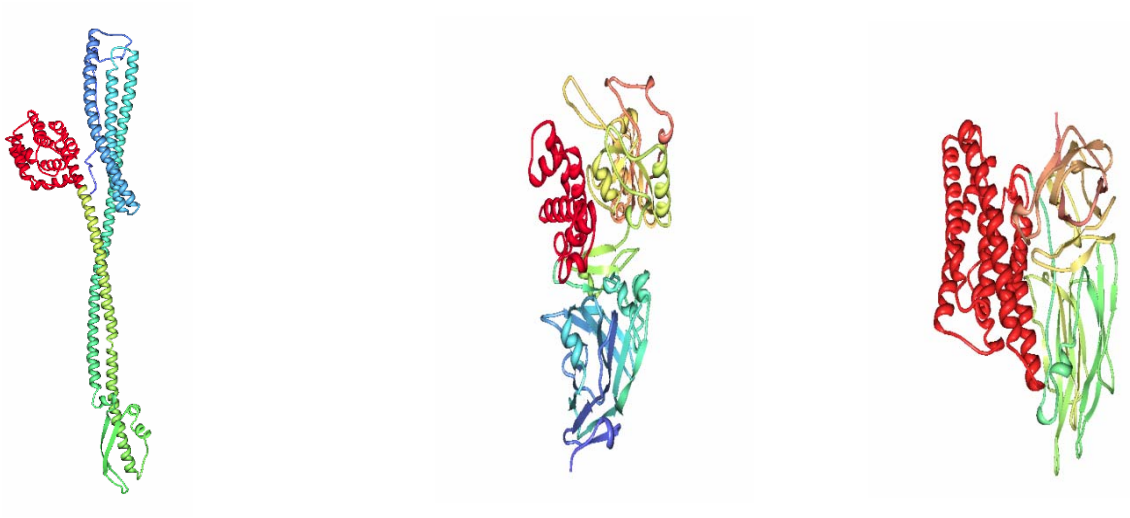
Insecticidal proteins of *Bacillus thuringiensis* are divided into two families; crystal forming (Cry) toxin family that is receptor specific and cytolytic (Cyt) toxin that probably bind to phospholipids [19, 20].

The Cry toxins are discussed here as a model for a-PFT. The Cry δ-endotoxins are the largest group with 89 known members. They are produced as protoxins having a molecular weight of about either 70kDa or 130kDa; after proteolytic activation, they form active toxins having molecular weight of 66-67kDa [21]. In the case of larger precursors, the active region resides in the N-terminal half. Cry proteins are made of three functional domains: The N-terminal domain (domain I) is a bundle of seven amphipathic and hydrophobic a-helices, in which a5 is in the middle of the bundle and is surrounded by other outer helices. Mutagenesis data suggest that this domain might play a major role in membrane insertion and pore formation [16].

Domain II has variable loops that are involve in the binding of receptor. Domain II adopts a triangular β-prism shape with anti-parallel β-sheets that are packed around a central hydrophobic core [16, 22].

The receptors for some Cry toxins that are well known include a cadherin-like protein (CADR), GPI-anchored aminopeptidase-N (APN); GPI-anchored alkaline phosphatase (ALP) and a 270kDa glycoconjugate. In insects and nematodes, glycolipids are proposed to be very important as Cry receptor molecules [16, 22].

The C-terminal domain (domain III) is a β-sandwich of two anti-parallel sheets. The mechanism of this domain has still not known although mutagenesis data suggests that it may also play a role in receptor binding [16] (Figure 4).



A B C

Figure 3: Ribbon representations of PFT structures. The domains thought responsible for the pore-forming activity of each toxin are highlighted in red. A : Colicin Ia [23] ; B : *Pseudomonas aeruginosa* exotoxin A [24]; C : Cry insecticidal δ-endotoxin adapted from [22].

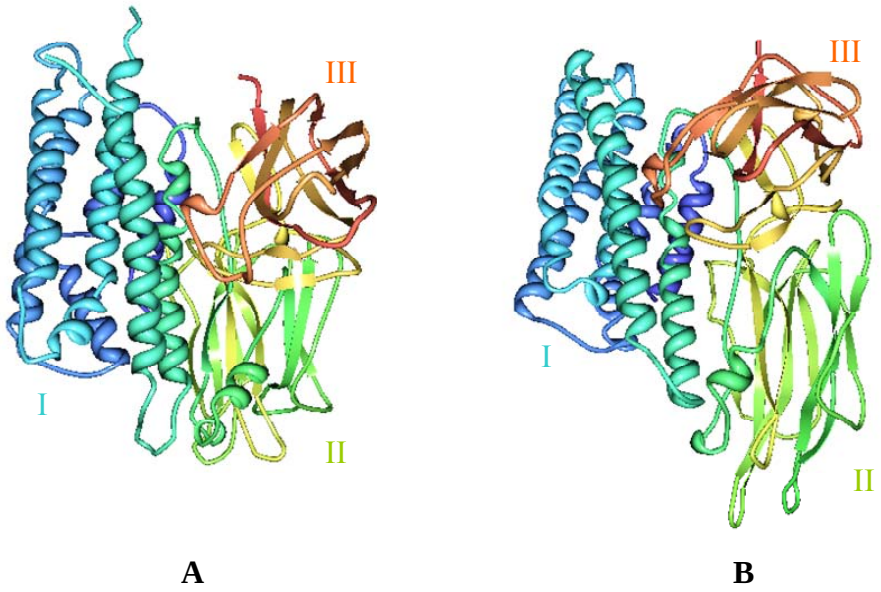


Figure 4: Structure of Cry endotoxins in the soluble state A: Cry3Bb, **B**: Cry4Aa adapted from [16].

1.2-2 β-Pore-forming toxins

Another major class of pore forming toxins are known as the β-PFTs; these toxins act by inserting into the membranes to form a β-barrel. At first sight, β-PFT bear little resemblance to one another; they are different in their primary, secondary, tertiary and quaternary structures. The three common characteristics of β-PFTs are (1) they are rich in β-sheet content, (2) in their primary structure they have a lack of stretch in hydrophobic residues that could be used to partition into a membrane, (3) during pore formation they must assemble into high order of oligomeric state.

The pore structure and the insertion pathway of β-PFTs are much better understood as compare to a-PFTs. But it is not easy to distinguish a common characteristic between β-PFTs that might elucidate exactly how they insert into membranes.

The examples of β-pore forming toxins are aerolysin, *Clostridium septicum* a-toxin, *Staphylococcus aureus* a-hemolysin, *Pseudomonas aeruginosa* cytotoxin, anthrax toxin protective antigen, some insecticidal δ-endotoxins (Cyt) [16] and the cholesterol dependent cytolysins. Recent studies suggest that a-PFTs and β-PFTs have a similar mechanism for the insertion in to membrane [16].

1.2-3 A-B toxins

These toxins are composed of two major domains. The A portion is an active enzymatic moiety, and the B portion is responsible for membrane binding and pore formation [25]. As is the case with "pure" pore-forming toxins, the B subunits can insert into the membrane either with a-helices or with β-strands. Once the pore is formed, the "B" portion translocates the "A" portion into the cytosol of the target cell, and the enzymatic activity of the "A" portion then causes cell death [25].

Examples of AB toxins include *Cholera*, *diphtheria*, *tetanus* and *anthrax* toxins [26].

1.3 Structure and action of two prototype pore-forming toxins

Two particularly well-studied examples of bacterial pore-forming toxins (β-PFTs) are a-hemolysin and the cholesterol dependent cytolysins.

1.3-1 a-Hemolysin

Staphylococcus aureus secretes many PFTs including the single component toxin a-hemolysin and the bi-component leukotoxins are related in amino acid sequence [16, 27]. The bi-component leukotoxins consists of two different subtype proteins, one each belonging to the classes S and F [27].

a-Hemolysin is released as a 33kDa water-soluble monomer from the *Staphylococcus aureus* cells [28]. Upon binding on the surface of the susceptible cells, the membrane bound monomers oligomerize to form a heptameric β-barrel trans-membrane pore, which leads to cell death and lysis [29]. For the molecular study of the pore-forming proteins, the crystal structure of a-hemolysin heptamer and monomer of the homologous toxin leukocidin are well explained structural models. Both the water soluble monomeric form and the membrane inserted heptameric form of a-hemolysin and leukocidin have been thoroughly investigated [30].

The conformational changes that occur during the pore formation by a-hemolysin can be shown by comparing the crystal structure of these two states. It may also be useful for structure functional relationships with the other β-barrel PFTs.

The crystal structure of the detergent solubilised heptamer has been solved at a 1.9Å resolution by X-ray crystallography [28].

The heptamer has a mushroom like shape and measure up to 100Å in diameter and 100Å in height. A solvent filled trans-membrane channels along the seven fold axis, ranges from 15 to 46Å in diameter. The heptamer consists of the cap,rim, and stem domains (Figure 5) [28].

The β-barrel is the part of the stem domain, a 14 strand antiprallel β-barrel to which each protomer contributes two β-strands; each is 70Å and form the lytic trans-membrane domain, the closed β-barrel is 52Å high and 26Å has of channel diameter [28].

The cap domain protrudes from the extracellular surface forming a large hydrophilic domain and the 7 rim domain underside of the cap. These domains are in close proximity and/or direct contact with the outer leaflet of the membrane bilayer [31].

Because of amino acid sequence identity and other similarities, many toxins are homologous to a-hemolysin. These toxins include the *Staphylococcal* leukocidins, *Clostridium perfringens* β-toxin and *Bacillus cereus* hemolysin II and cytotoxin K [32]. But there is a difference between the stem domain and the amino terminal latch of the a-hemolysin and Luk F. The amino latch of the Luk F monomer in the water soluble state is closely packed against the β-standwich core of the molecule, while in heptamer it is extend to interact with the

adjacent protomers of the a-hemolysin. In LukF the stem domain is folded in close proximity with β-standwich core to maintain the monomer water solubility, while in the heptamer of a-hemolysin the stem domain is unfolded to insert into the membrane with one β-hairpin [30].

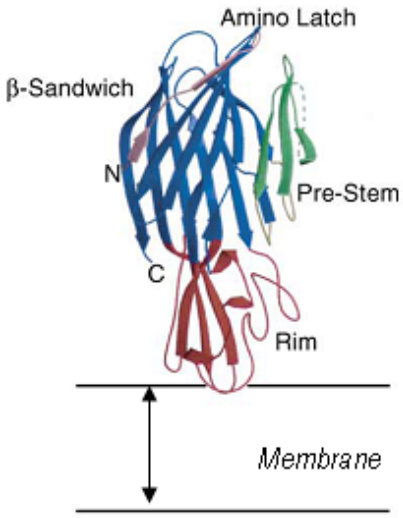
Pore formation of a-hemolysin is processed by four steps. Water soluble monomer →Membrane bound monomer → Heptameric prepore → Heptameric pore [28].

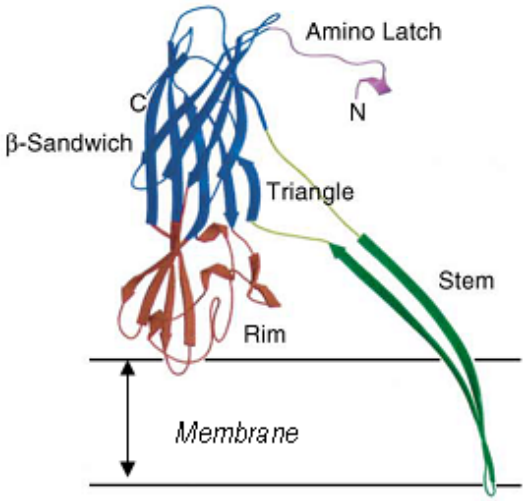
1-Water soluble monomer: the a-hemolysin secreted by *Staphylococcus aureus* is a primilarly hydrophilic molecule that binds to target membranes as a water soluble monomer [33].

2- Membrane bound monomer: The toxin monomer binds to the host cell membrane without major conformational changes.

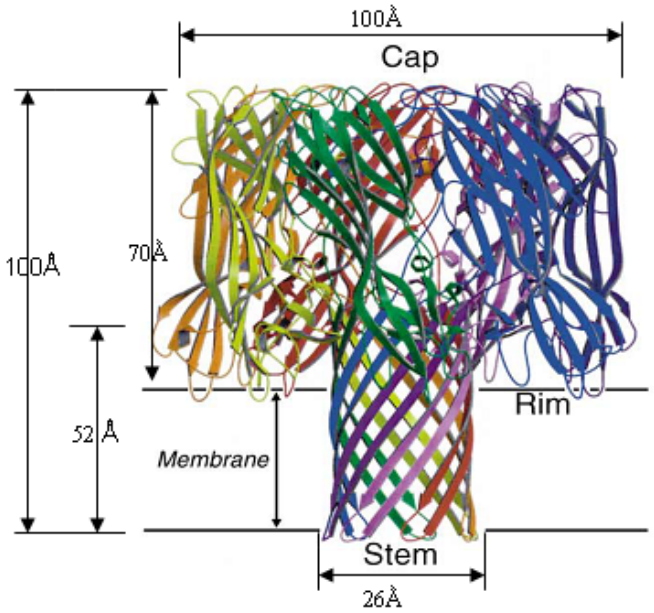
3- Heptameric pre-pore: Membrane bound monomers oligomerize with N-terminal latch, but the β-barrel is not yet inserted in this pre-pore state. Due to collision of membrane bound monomers a non lytic heptamer is formed on the membrane surface or non-lytic oligomeric intermediate is found before pore formation.

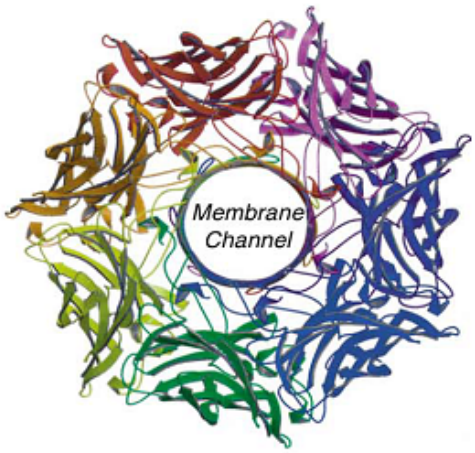
4- Heptamirc pores: The final pore is formed by membrane insertion of an amphipathic β-barrel, which comprises of the central loop and domains of all heptamer subunits [34].





A: Leukocidin F B: a-hemolysin protomer





C: a-Hemolysin heptamer side view D: Top view of Heptamer

Figure 5: Crystal structure of a-hemolysin and the homologous toxin LukF

(a) The LukF monomer represented in the water-soluble state. (b) Membrane inserted state represented by one protomer of the a-hemolysin heptamer. (C) Ribbon representation of the a-hemoysin heptamer (Side view). (D) Different colors highlight protomers (Top view of Hetamer), adapted from [28].

1.3-2 Cholesterol Dependent Cytolysins

The cholesterol-dependent cytolysin (CDC) are a large family of pore-forming toxins that form large pores of approximately 25 nm in diameter, comprising rings of 30 to 50 subunits. CDCs are single chain polypeptides of 50 to 60 kDa. CDCs are produced by more than 20 species of gram-positive bacteria, including *Clostridium*, *Streptococcus*, *Listeria*, *Bacillus*, and *Arcanobacterium*. Among the CDCs, perfringolysin O (PFO), sterptolysin O, and intermedilysin (ILY) have been extensively characterized with respect to their structure and function [35].

The structure of PFO in its monomeric form has been identified by X-ray crystallography. The crystal structure of monomeric PFO revealed that CDCs has elongated, rod-like shape molecules [36]. These molecules are rich in β sheets and are hydrophilic without having hydrophobic patches on the surface. There are four domains in the PFO molecule, D1 to D4 (Figure 6). No crystal structure is available for the membrane inserted oligomers of any CDC.

There cytolytic activities depend on the presence of cholesterol in the membrane.

According to spectroscopic and mutagenesis studies [37, 38] CDCs exhibit several unique features during their transition from water-soluble monomer to the membrane inserted oligomers.

To a large extent, in water-soluble monomer form, each pre-stem domain is folded into three short a-helices within domain 3. During pore formation, each group of three helices extends into a β-hairpin. Ultimately each monomer contributes two β-hairpins (TMHs) to the β-barrel trans-membrane channel instead of one β-hairpin (Figure 6) [39]

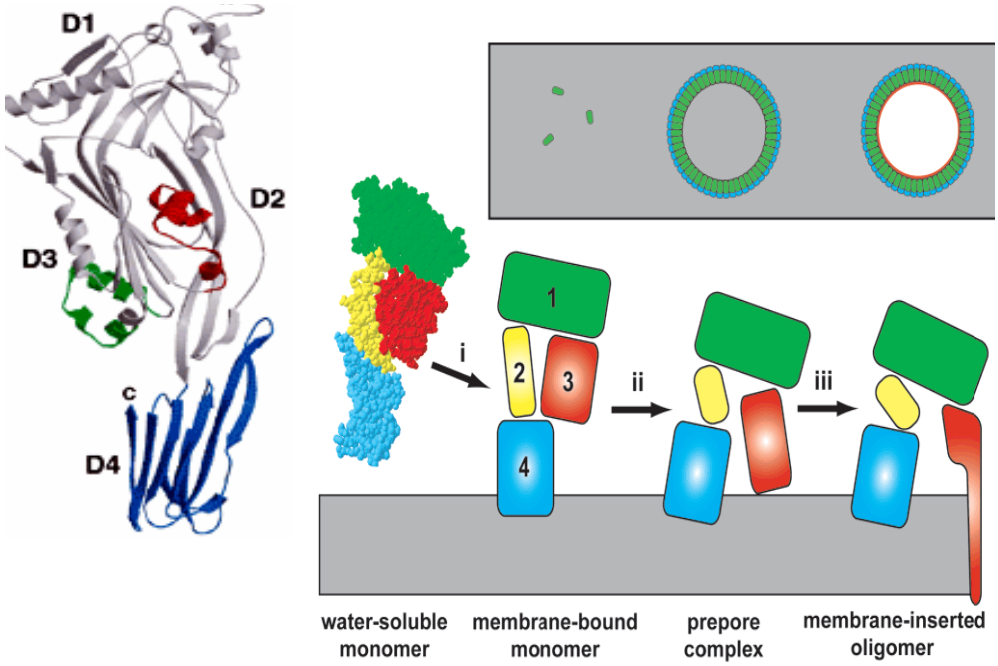


Figure 6: Pore formation by CDTs. Ribbon representation of the water-soluble of PFO monomer.

Four domains D1, D2, D3, and D4 are shown (Left panel), the red is β hairpins-TMH1 and TMH2 green that each monomer contribute to the β-barrel [39]. A schematic model of stages of PFO insertion into the membrane, (Right panel): TMH1 and TMH2 insert in domain 3, insert in to the membrane once the prepore complex is formed, adapted from [40].

1.4 CAMP factor

The CAMP factor is an extracellular, cysteine-free protein secreted by *Streptococcus agalactiae* or group B *streptococci* (GBS) [41]. Its molecular mass is approximately 25000 Da, and composed of 226 amino acids with a pI of 8.9 [42.43].

Streptococcus agalactiae is a non-motile, catalase-negative and Gram-positive bacterium. It can be found in the normal flora of urogential, respiratory, and gastrointestinal tracts of humans [44]. *Streptococcus agalactiae* is a major cause of cattle mastitis, resulting in reduction of milk production and a greater source of economic loss for dairy industry [44].

It also accounts for a number of invasive infections in newborn babies and young infants, pregnant women, and non-pregnant adults who have typical medical conditions like cancer, liver disease, and diabetes mellitus [45, 46, and 47]. It is very slightly hemolytic on non-sensitized sheep red blood cells (SRBC), but shows strong hemolytic activity on sphingomyelinase-sensitized red blood cells. This phenomenon is known as the CAMP reaction. The CAMP reaction is presently used as a diagnostic test for the presumptive identification of Group B streptococci in clinical isolates [48].

1.4-1 The CAMP reaction

The CAMP reaction was first observed in 1944 by Christie, Atkins, and Munch-Petersen [48], whose initials are combined in the name of the reaction and of the streptococcal protein involved in it (CAMP factor).

When a colony of *S.agalactiae* is grown on a sheep blood agar plate in the vicinity of a colony of *S.aureus* typically a hemolytic zone can be observed where two bacteria strain meet.

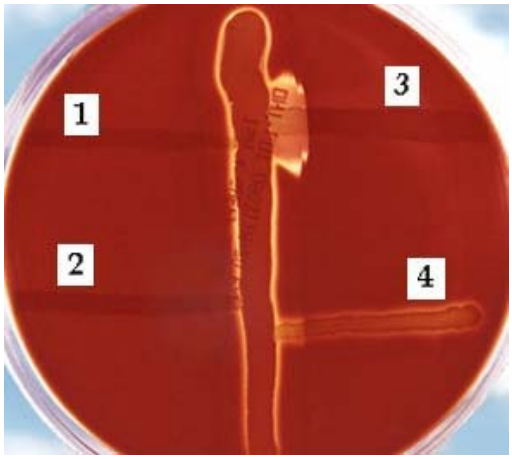


Figure 7: Illustration of CAMP reaction on a sheep blood agar plate

A vertically streaked strain of *S.aureus* meets streaks of (1) *Enterococcus faecalis*, (2) *Streptococcus salivarus*, (3) *Streptococcus agalactiae*, and (4) *Enterococcus durans*, adapted from [48].

The white region where *S.aureus* and *S.agalactiae* meet is characteristic of the lytic activity of the CAMP factor on sensitized cells.

S. aureus produces sphingomyelinase or β-hemolysin, and *S. agalactiae* produces CAMP factor. Sheep red blood cells contain sphingomyelin (Figure 8), and therefore are sensitive to the action of sphingomyelinase. The sphingomyelin of the sheep red blood cells membrane will be converted into ceramide by sphingomyelinase. CAMP factor then binds to these pre-treated cell membranes, and carries out its lytic activity.

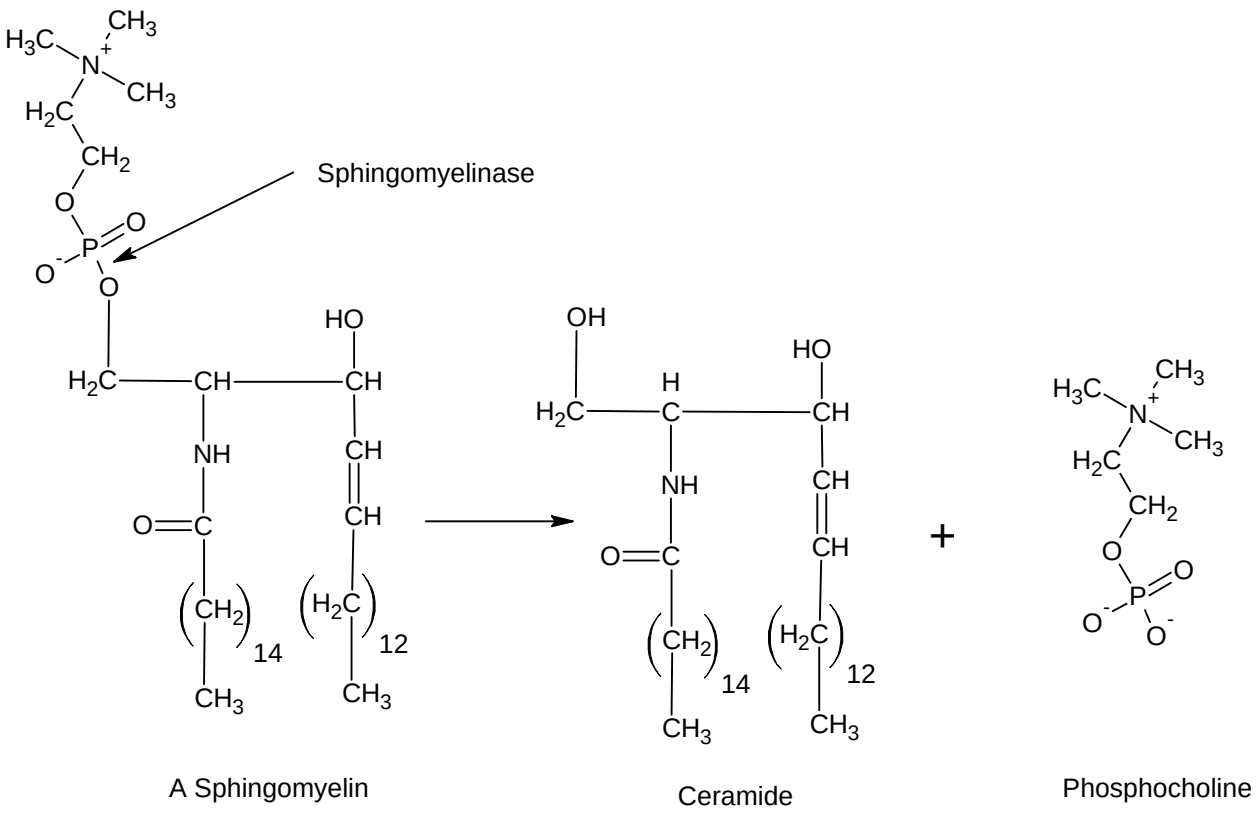


Figure 8: Conversion of membrane sphingomyelin to ceramide catalyzed by sphingomyelinase.

1.4-2 CAMP factor as a pore forming toxin

CAMP factor is a part of PFT family. It forms pores of various sizes in diameter in sphingomyelinase containing membrane [49].The amount of sphingomyelin is a determinant of lytic activity of CAMP factor. It is reported that at least 45% of the membrane lipids should be sphingomyelin for the CAMP factor to react [41]. This is the reason that CAMP factor is toxic for sphingomyelinase treated bovine and sheep (50% and 45% sphingomyelin respectively) but not toxic to horse, rabbit, and human erythrocytes (less than 25% sphingomyelin).

However, the CAMP factor can be toxic for phospholipases C treated rabbit or human erythrocytes. The ceramide is therefore not specifically required for the CAMP reaction to occur.

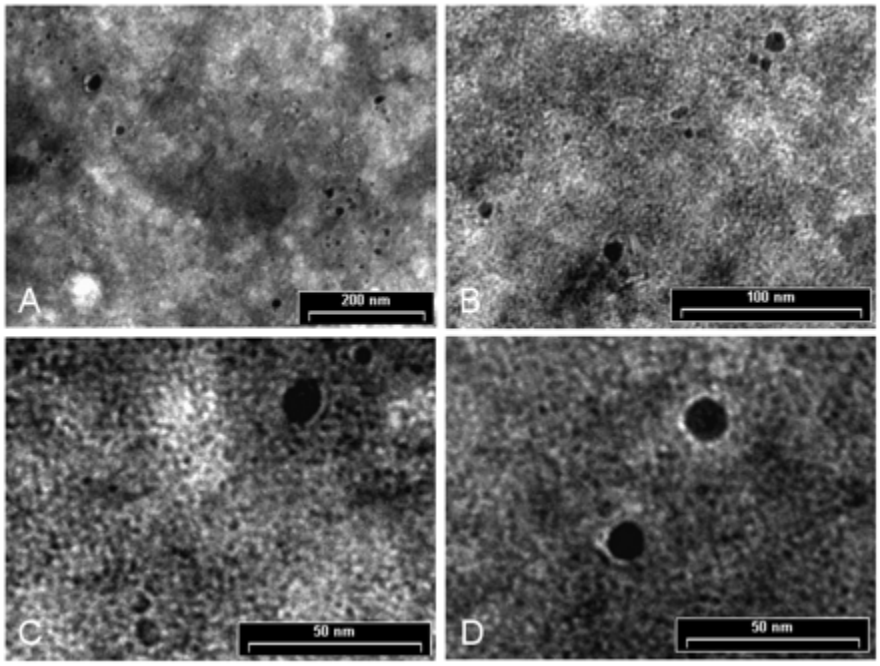


Figure 9: Electron microscopy of CAMP factor treated sheep red blood cells. The size of the pores varies according to protein concentration. Magnification varies among panels A-D by the scale bars, adapted from [49].

1.5 Fluorescence

Fluorescence is widely using in biochemistry research laboratories and biological sciences research.

Fluorescence is also used in clinical chemistry, environmental monitoring, DNA sequencing and genetic analysis; additionally fluorescence is also used for cell identification (51).

1.5-1 Fluorescence Resonance Energy Transfer (FRET)

Fluorescence resonance energy transfer is a useful technique to study the dynamics of protein molecules (52). FRET is a distance-dependent excited state interaction in which the emitted energy of donor (D) is transferred to an acceptor (A) in a non-radiative process.

The donor-acceptor pair must observe some particularities, such as the absorbance spectrum of the acceptor must overlap the emission spectrum of the donor (Figure 10), for the FRET to occur. The extent of energy transfer depends on distance (r) between the donor and acceptor by a factor of $1/r^{\square}$, and the dipole-dipole interaction between the donor and acceptor [51].

FRET can be observed with steady-state spectroscopy by comparing intensities and time-resolved measurements by comparing lifetimes.

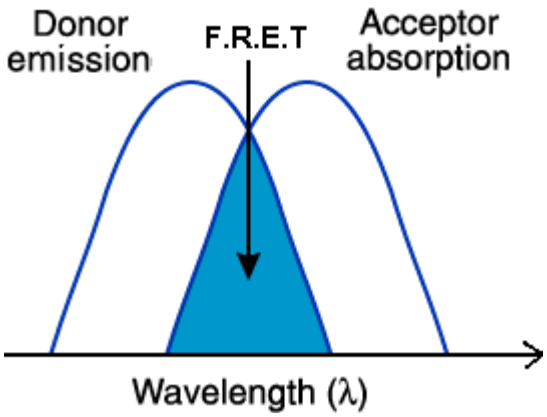


Figure 10: Schematic representation of FRET phenomenon.

FRET occurs if the emission spectrum of the donor overlaps the absorption spectrum of the acceptor, but the energy transfer is non radiative (does not occur via a photon), adapted from [51].

1.6 Research Objectives

The previous electron microscopy and osmotic protection studies proved that CAMP factor forms pores in red blood cell membranes. The results identified two stages of the proposed mechanism for CAMP factor activity, membrane binding and pore formation.

In contrast to many other pore forming toxins, such as *streptolysin O*, preliminary studies of the CAMP factor oligomers showed that they are unstable outside the membrane environment. Additionally these studies suggests that the oligomerization and pore formation is affected by the change in lipid composition [49], implying the involvement of sphingomyelinase in these steps.

As CAMP factor oligomers are not stable following membrane solubilisation [49], the aim of this research has to observe oligomerisation on the cell membranes. Additionally it is known that sphingomyelinase changes in the lipid composition of the cell membrane; therefore we will also observe its role in CAMP factor oligomerization and membrane insertion, by comparing FRET on membranes treated with sphingomyelinase with that on membranes not treated with the enzyme.

CHAPTER 2

Experimental procedures and materials

2.1 Reagents

Glutathion-agarose resin, glutathione, DL-dithiothreitol (DTT), thrombin from human plasma, sphingomyeline phosphodiesterase (sphingomyelinase) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Okaville, ON). Aminobenzylpencillin (Ampicillin) was from Bioshop (Burlington Ontario). The BL21 (DE3) strain of *Escherichia coli* was obtained from Novagen (Madison, WI). The XL1 blue strain of Escherichia coli was attained from Clontech (Palo Alto, CA). pGEX-KG vector was a gifit from Jingya Li (National Center for Drug Screening, Shanghai, China). Restriction endonucleases, EcoRI+HindIII (DNA marker).

Protein marker was obtained from MBI Fermentas (Burlington Ontario). Isopropyl β-thiogalactoside (IPTG), ethylene diaminotetraacetic acid (EDTA) and Triton X-100 received from Bioshop (Burlington Ontario). PD-10 desalting columns were received from Amersham Biosciences. 5(2-iodoacetyl) amino) ethyl) amino) (1, 5-IAEDANS) from Molecular probes (Eugene, Oregon, USA). Fluoresceine-5-maleimide from Biotium, Inc. Sheep red blood cells purchased from Cedarlane (Hornby, ON).

β-Mercaptoethanol was obtained from EM science (Merck KGaA, Dramstadt Germany), DMSO (dimethyl sulphoxide) from Caledon Laboratories LTD, Georgetown Ontario, Canada. Plasmid DNA was prepared using QIAprep Spin

Miniprep Kit from Qiagen (Mississauga, Ontario). P1U1trapure MiliQ water was used in all reactions.

2.2-Preparation of competent cells

2.2-1 Preparation of XL1 Blue cells and their transformation

1ml of overnight XL1 blue cells culture was inoculated to 100 ml of fresh LB broth and grown at 37□ with vigorous shaking at 200 rpm to reach an optical density (OD 600) of 0.4. The cells were then centrifuged at 3000 rpm at 4□ for 10 minutes after which the supernatant was removed and the fresh LB and 2X TSS buffer were add to the cell pellets and re-suspended gently on ice. The XL1 blue cell suspension was aliquot into a portion of 100 µl and stored in -80□. To transform the cells, 1 µl of DNA was added to 100 µl of thawed competent cells and incubated on ice for 45 minutes. The cells were heat shocked to keep the cell tubes in 42□ water bath for 45 seconds. Immediately the cells were plated on already prepared 50 µg/ml ampicillin agar plates [53].

One positive colony was selected from L.B agar plate containing 50 µg/ml (ampicillin) to inoculate 5 ml of sterilized L.B medium with ampicillin and grown overnight with shaking at 225 rpm at 37°C. 1 ml of overnight culture was taken and centrifuged at 10,000 g for 3 minutes. Centrifugation was repeated 2 to 3 times.

The pellets were re-suspended in RNase buffer P1, and the plasmid was extracted by using QIAprep Spin Miniprep Kit. The plasmid size was confirmed by 0.8% agarose gel electrophoresis. The plasmid was used to transform *E-coli* BL21 (*DE3*) cells for expression.

2.2-2 Preparation of BL21 (DE3) cells and their transformation

1 ml of fresh overnight BL21 *(DE3)* cell culture was diluted into 250 ml of sterilized L.B broth and grown at 37°C with vigorous shaking for 4 to 5 hours to reach an OD 600 of 0.5. The cells were spilt in to two centrifuge tubes and centrifuge at 3000 g for 15 minutes at 4°C. The Supernatant were discarded and the pellets were re-suspended in 2x125 ml of ice-cold 10% glycerol. The cells were harvested again followed by centrifugation and the supernatant discarded. The cells were re-suspended in 125 ml of 10% ice-cold glycerol.

Finally the cells were re-suspended to the final volume of 2x1 ml 10% ice-cold glycerol. The suspension was spilt into 100 µl aliquots and stored at -80°C [54].

1 µl of plasmid DNA was added to 50 µl BL21 competent cells, mixed well and incubated on ice for 1 minute. The electroporation instrument, a BioRad cell pulsar electroporation unit, was set on 1.80 KV. The mixture of cells and plasmid DNA was transformed into the inner slit of elcotroporation cuvette. The cuvette was placed into the instrument chamber and pulsed once for every sample. The cuvette was removed from the chamber immediately. The cells contents were removed from the cuvette and mixed with 1 ml SOC medium(0.5% (w/v yeast extract), 2% (w/v) Bio-trypton, 10 mM NaCl, 2.5 mM KCl,10 mM MgCl□, 20 mM MgSO□, 20 mM glucose). The sample was incubated at 37°C with vigorous shaking for 1 hour at 225 rpm. The solution was then centrifuged at 6000g for 1 minute, the 900µl supernatant was discarded and the pellets were re-suspended

in 100 µl SOC solution. The 100 µl transformed cells were placed on ampicillin L.B agar plates.

2.3 Protein induction and purification

Protein induction and purification were performed according to a protocol by K. Guan and J.E. Dixon [55].

A single colony from ampicillin plate was picked and inoculated in 100 ml 2xYT sterilized media containing 100 µg/ml ampicillin, and grown overnight at 37°C with vigorous shaking at 225 rpm.

100 ml overnight culture was inoculated into 1 litre sterilized 2xYT (liquid medium, 10 g yeast extract, 16 g tryptone, 5 g NaCl, in 1 litre) with 100 mg/ml ampicillin, and grown at 37 °C with shaking at 200 rpm untill the OD□□□ reached a value between 0.5 and 0.8. A 1 ml sample before inducing the protein synthesis with Isopropyl-β-D-thioglactopyranoside (IPTG) (Bioshop Canada Inc) was taken for SDS-PAGE analysis (sodium dodecyl sulphate polyacrylamide gel electrophoresis). Protein synthesis was induced with 1 mM IPTG. The culture was grown at 37°C on 200 rpm for four hours; the 1ml sample was taken for SDS-PAGE analysis after induction. 20 µl samples taken before induction and after induction were added to 1X SDS-PAGE sample buffer. The samples were heated at 95°C for 5 minutes, and loaded on 12% SDS-Gel at 200 V to analyse the protein synthesis.

The cells were harvested by centrifugation at 10,000 g, at 4°C for 10 minutes (Beck Man Coulter, Avanti J-E Centrifuge) and then stored at -20°C.

For CAMP factor, the cells were thawed on ice and resuspended in 15 ml phosphate buffer saline (PBS) (16 mM K□HPO□, 150 mM NaCl, pH 7.2). The cells were lysed by using an emulsifier at 17000 psi. The lysed cells were centrifuged on 20,000 g at 4°C for 30 minutes.

Growth of E-coli, protein induction, purification and thrombin cleavage of the recombinant protein was done as described Guan and Dixon [55], with some modification.

The glutathione column was equilibrated with PBS buffer before protein purification, by washing 4 to 5 times with PBS buffer. The fusion protein (lysate) was gently mixed with glutathione-agarose beads and shaken for 30 minutes at 4°C, 5 washes were performed using 10 ml of PBST buffer (16 mM K_2HPO_4, 150 mM NaCl, pH 7.2,1% tritonX-100, and 2 mM DTT).

The protein bound to the glutathione beads was shaking for 1 hour at 4°C with the last 10 ml wash of PBST buffer. The PBST buffer was removed and one wash of 10 ml was performed with thrombin cleavage buffer (50 mM Tris, 150 mM NaCl, 2.5 mM $CaCl_2$, pH 8.0). 12 µg/ml of thrombin was added to the protein solution and the GST beads resuspended and incubated for 1 hour at 4°C. 2 ml of cleaved protein was eluted [55, 56], and the native concentration was determined using the Bradford assay [57]. The eluted fractions brought to the final concentration of 10% glycerol and stored at -80°C .

2.4 Gel filtration

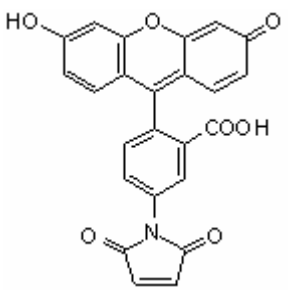
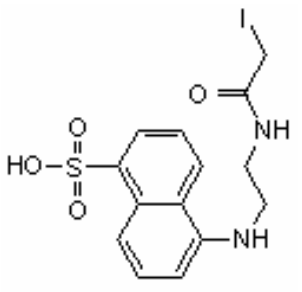
After the GST column purification, the CAMP factor protein was passed through a PD-10 column (Amersham biosciences Baie D' Urfe QC) (diameter 16 mm: length 25 cm) or a desalting gel Bio-Gel P-6DG column (Bio-Rad Laboratories). The column was equilibrated with the elution buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 7.5). The CAMP protein was eluted with 2.5 ml of elution buffer and collected in 0.5 ml fractions. Bradford method (Coomassie brilliant blue G-250) was used to check the protein presence in the eluate.

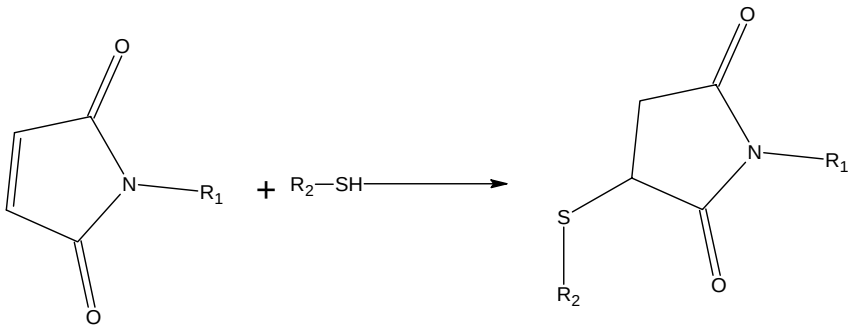
2.5 Cysteine-specific labelling of the CAMP factor F109C mutant

Cysteine is the only one among the 20 standard amino acids that has a thiol group, which can easily and selectively be modified by a variety of reagents, for example maleimides [58] such as fluorescein-5-maleimide and haloacetamides [59] (Figure 11).

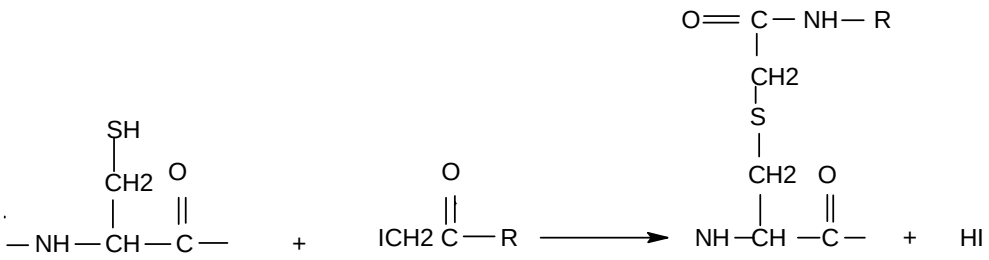
The desalted CAMP factor mutant F109C was labelled with 1 mM Fluorescein-5-maleimide (Biotium, Inc) and 5 mM IAEDANS (N-(iodoacetylaminoethyl)-5-naphthylamine-1-sulfonic acid (Molecular probes). Incubation occurred at room temperature for one hour with fluorescein maleimide and overnight with IAEDANS (Molecular Probes). The unreacted dye was removed by gel filtration over P-6DG with labelling buffer as eluant. The presence of labelled protein was determined by observing the fractions on a UV-transilluminator. The fluorophores used possess the following properties (Table 1).

Table 1: Some properties of the fluorophores used in the project, (1) Fluoresceine-5-maleimide and (2) IAEDANS

Name	Fluorescein-5-maleimide	IAEDANS
Chemical formula		
Maximum excitation wavelength (nm)	490	336
Maximum emission wavelength (nm)	520	496



Maleimide Thioether



Cysteine residue Haloacetamide Thioether

Figure 11: Reactions of Sulfhydryl groups

A cysteine residue with a maleimide (e.g. fluorescein-5-maleimide) and a haloacetamide (e.g. IAEDANS)

2.6 Hemolysis assay

10% sheep red blood cells were prepared by washing 400 µl sheep blood in 600 µl PBS buffer and spun down at 2000 g for 4 minutes. 900 µl of the supernatant was removed and the 100 µl pellets was resuspended in 900 µl fresh PBS buffer. The washing steps were repeated until the supernatant was clear (typically 3 washings). A 1% RBC suspension (100 µl, 890 µl PBS buffer, 10 µl 1 M $MgCl_2$) was then sensitized with 0.5 µl (2 $mg.ml^{-1}$) sphingomyelinase and incubated at 37°C for 15 minutes.

Serial dilution of 50 µl CAMP factor was performed in 8 wells of a 96-well plate containing 50 µl of PBS buffer.

50 µl of sensitized sheep RBC were added to each well and incubated for 30 minutes at 37°C. The absorbance was read at 650 nm (SPECTRAmax 190, Microplate Spectrophotometer; Molecular Devices, Sunnyale, (CA).

The positive control consisted of 50 µl PBS buffer, 50 µl Na-Deoxycholate (10%) and 50µl sensitized sheep RBC. The negative control consisted of 100µl PBS buffer and 50µl sensitized sheep RBC.

2.7 Preparation of erythrocyte ghost membranes

400 µl of sheep red blood cells were washed three times with PBS buffer by centrifugation at 2000 g for 4 minutes. 600 µl a solution containing 10% red blood cells and 1 M $MgCl_2$ was treated with sphingomyelinase (3 µl, 2 mg/mL), and a second sample not treated with sphingomyelinase, when incubated for 15 minutes at 37°C. The red blood cell samples were osmotically lysed in 5 mM phosphate buffer (pH 7.5). The membrane pellets were repeatedly washed by centrifugation at 13000 g for 10 minutes with the same buffer until they appeared white. Eventually the membrane pellets were resuspended in 500 µl of phosphate lysis buffer.

2.8 Membrane binding of CAMP factor

Labelled CAMP factor and unlabelled W.T CAMP factor protein were both added to 100 µL of sphingomyelinase-treated or untreated membranes. Both samples were incubated for 30 minutes at 37°C.

After incubation, the membranes were spun down at 13000g for 10 minutes to remove the unbound CAMP factor. The pellets were resuspended in Tris buffer (50mM Tris, 150mM NaCl, pH 7.5). The dissolved membrane pellets were analyzed by SDS-PAGE.

2.9 Electrophoresis

SDS-PAGE was performed according to Laemmli [60], using a polyacrylamide concentration of 4% in the stacking gels and 12% in resolving gels. Gels were stained with Coomassie Blue R250 and de-stained with de-staining buffer (water 88%, methanol 5%, and acetic acid 7%).

2.10 Protein Assay

The CAMP factor protein concentration was determined by absorbance at 280 nm or using the Bradford assay [61].

2.11 Spectrofluorimetry

A PTI QuantaMaster spectrofluorimeter was used to take the steady-state fluorescence measurements. The excitation and emission for IAEDANS was 336 and 496 nm, and for fluorescein was 490 and 520 nm. Each of the labelled mutants were examined free in solution and after being incubated with membrane, and fluorescence intensity of the membrane bound toxin was calculated by the following equation.

% of membrane bound toxin = (FI of membrane pellets or oligomer / FI protein solution, or monomer) X 100 [1]

To obtain the emission changes as a result of membrane binding, the emission from the monomer protein was compared with the emission of membrane bound toxin.

Time resolved measurements for IAEDANS and fluorescein-labelled CAMP factor were performed using a FT-100 compact fluorescence lifetime spectrometer (PicoQuant, Berlin, Germany). A LDH-P-C-370 LED laser light source was used for IAEDANS labelled protein, and the IAEDANS emission was isolated using a band pass filter 450FL07-25 AM-60192 S/N: 05 (Melles Griot Canada Inc, Montreal, QC).

A LDH-P-C-470 LED laser light source was used for fluorescein-labelled samples and the emission was isolated using a 520-5 nm band pass filter (Andover Corporation, NH, USA).

Decays counts were fitted using the FluoFit software (PicoQuant), allowing for three fluorescence lifetime components, from which the average lifetime $< \tau >$ was calculated according to the following equation.

$$< \tau >= \sum a_i \tau_i / \sum a_i \quad [2]$$

Here, a_i represents the amplitude at time zero and τ_i is the lifetime of i^{th} component [61].

CHAPTER 3

RESULTS

3.1 Construction and choice of the mutant

Mutants of CAMP-factor F109C and L107C were constructed by Tianhua Zhang. We chose the two mutants L107C and F109C, in which the 107th residue and the 109th residue (leucine and phenylalanine, respectively) had been replaced with cysteine by site directed mutagenesis. These mutants were chosen from a larger set of mutants, since they retain a hemolytic activity similar to the wild type CAMP factor both before and after labelling with the fluorescent reagents. The steady state fluorescence spectra were observed with fluorescein-labelled L107C and F109C mutant (Figure 12). Both the labelled samples were treated with membrane, F109C observed high intensity spectra as compare to L107C intensity spectra. F109C showed better labelling than to L107C, so we chose to use the F109C mutant for our experiments.

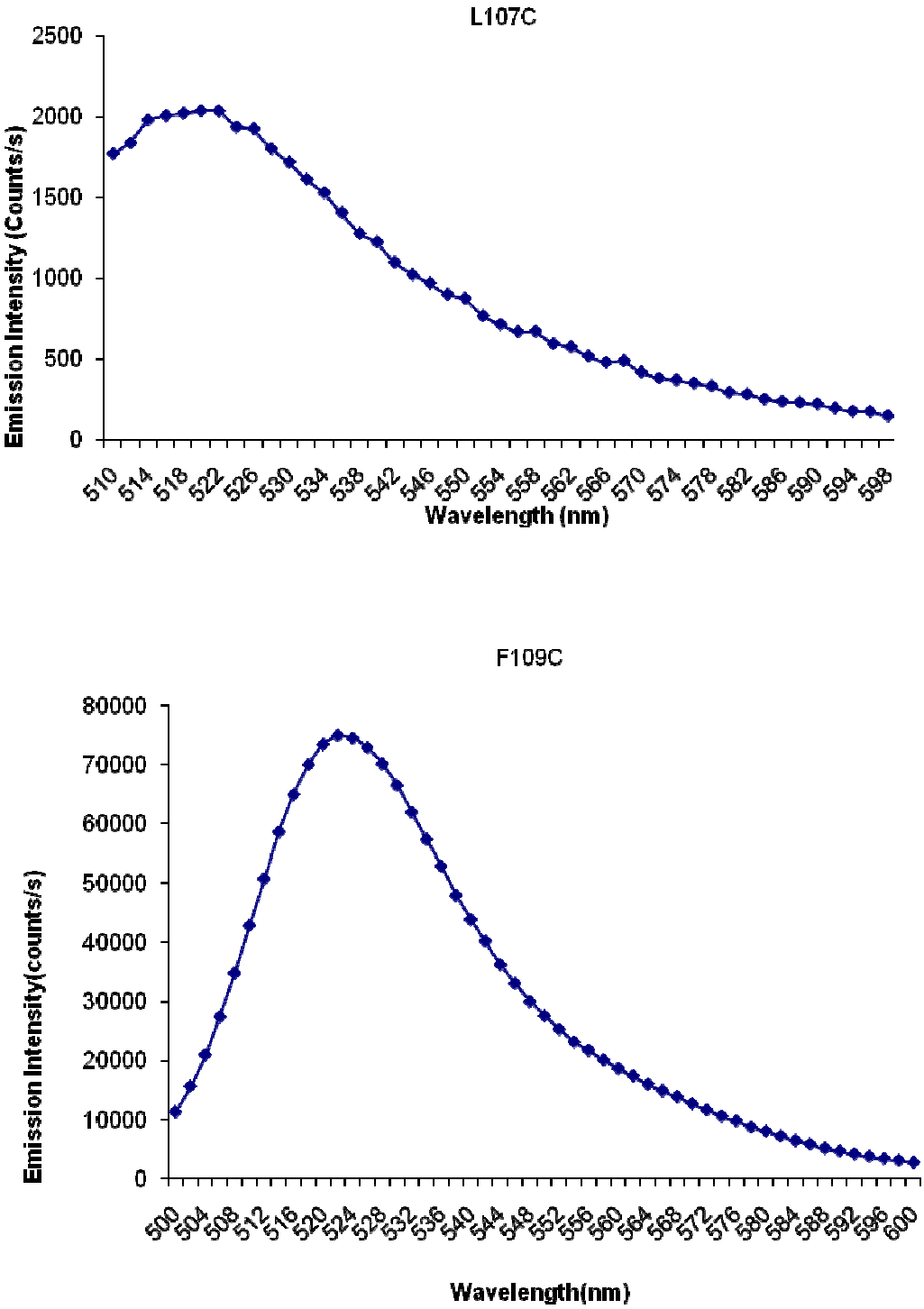


Figure 12: Choice of Mutant; Comparison of L107C and F109C. (Excitation 490 nm)

3.2 purification of CAMP factor DNA

The F109C mutant cells were transferred to *E-coli* BL-21 cells a number of colonies grown on the plate after 24 hours. One colony was picked and grown for plasmid extraction. DNA was purified by following QIAprep Spin Miniprep Kit protocol [Figure 13]. The 0.8% agarose gel was run to analyze the DNA fractions.

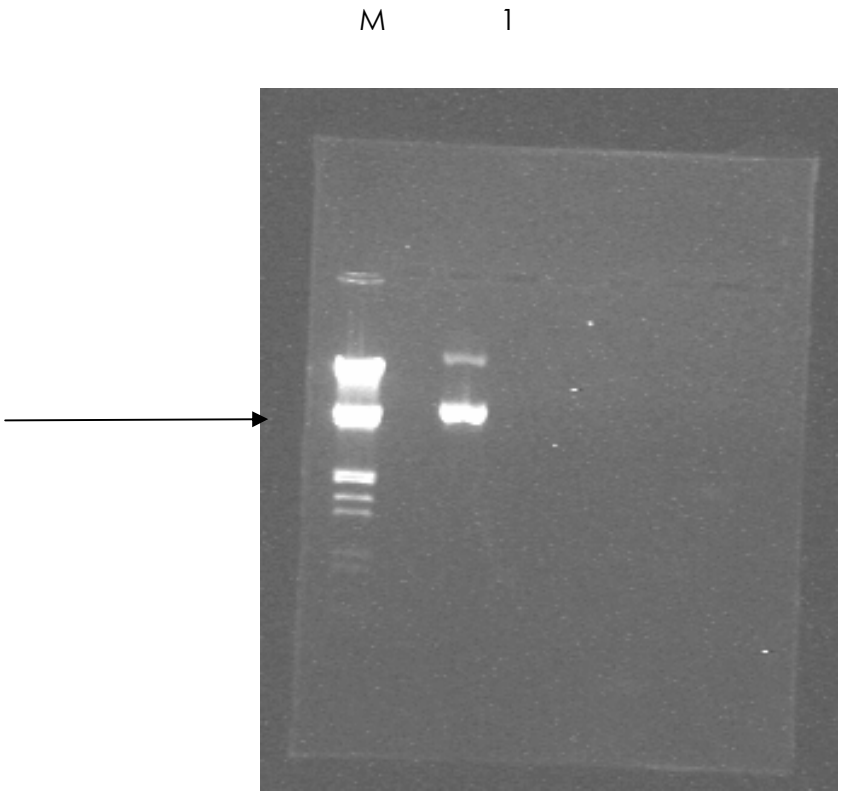


Figure 13: 0.8% agarose gel for CAMP factor DNA, M; Lambda DNA/EcoR1+HindIII Marker, 1; DNA Fragment 3530bp

3.3 CAMP factor protein expression and purification

A number of colonies were grown on ampicillin plates after 24 hours. One colony was cultured for 24 hours in 100ml 2XYT media. During the cell culture samples were collected before and after IPTG induction. After cell lysis and centrifugation, the protein was expressed as glutathione-S-transferase/CAMP factor fusion protein.

A 53 kDa fusion protein was purified with a well established protocol [55, 61]. The fusion protein absorbed to the glutathione agarose beads was cleaved by thrombin, after which the 25 kDa CAMP factor fragment was released from the agarose beads with a concentration of 2mg/ml of CAMP factor protein [Figure 14].

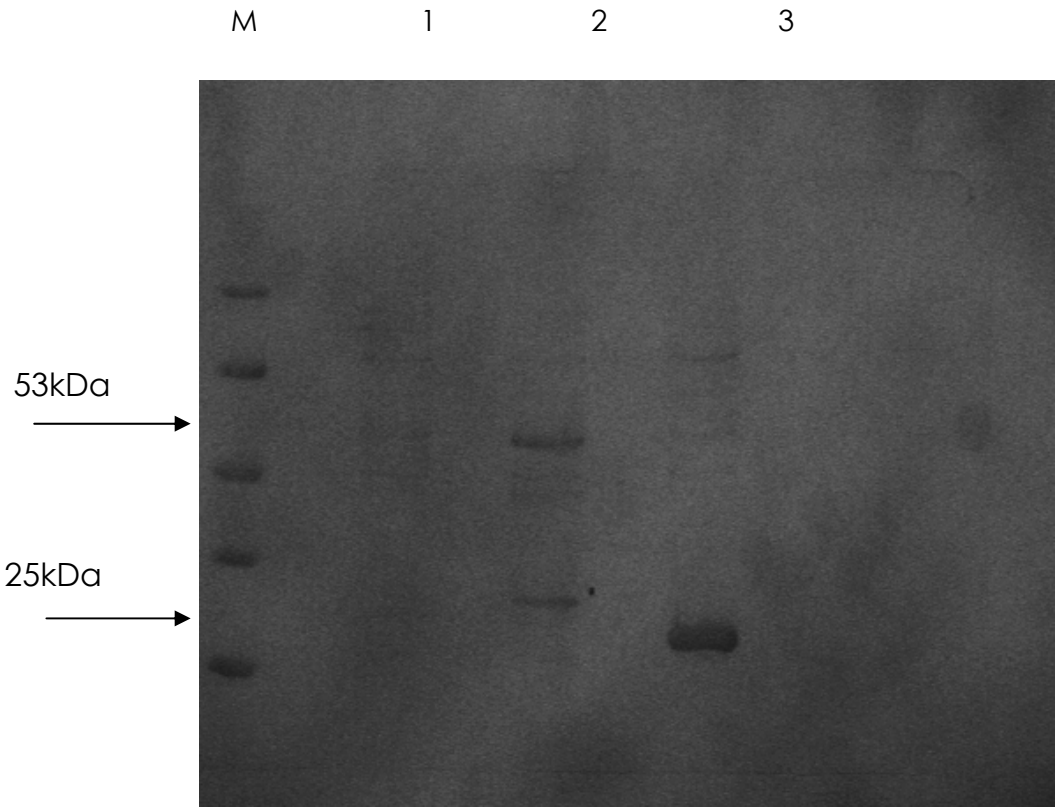


Figure 14: Expression and purification of CAMP factor in E.Coli by SDS-PAGE analysis.

M, molecular weight marker; Lane 1, *E.Coli* protein extract before induction with IPTG and Lane 2, after induction with IPTG, Lane 3, CAMP factor protein released from GST-CAMP after thrombin cleavage.

3.4 Cysteine-specific fluorescent labelling

F109C was labelled with 5 mM IAEDANS or 1 mM fluorescein-5-maleimide fluorophores, respectively (Figure 15). After gel filtration (P-6DG) used for desalting and labelling, the Bradford reagent was used to identify the protein fractions.

The labelled protein concentrations were determined by absorbance at 280, and with the Bradford assay. The concentrations were 0.1mg/ml for the IAEDANS-labelled protein and 0.6 mg/ml for the fluorescein-labelled protein.

We used 2 µg IAEDANS-labelled protein as donor, 8 µg fluorescein-labelled protein as acceptor (1:4), and 20 µg wild type CAMP factor (1:4:10) as unlabelled protein. To observe the labelling the SDS gel was analyzed by ultraviolet transilluminator without distaining by coomassie blue.

1 2

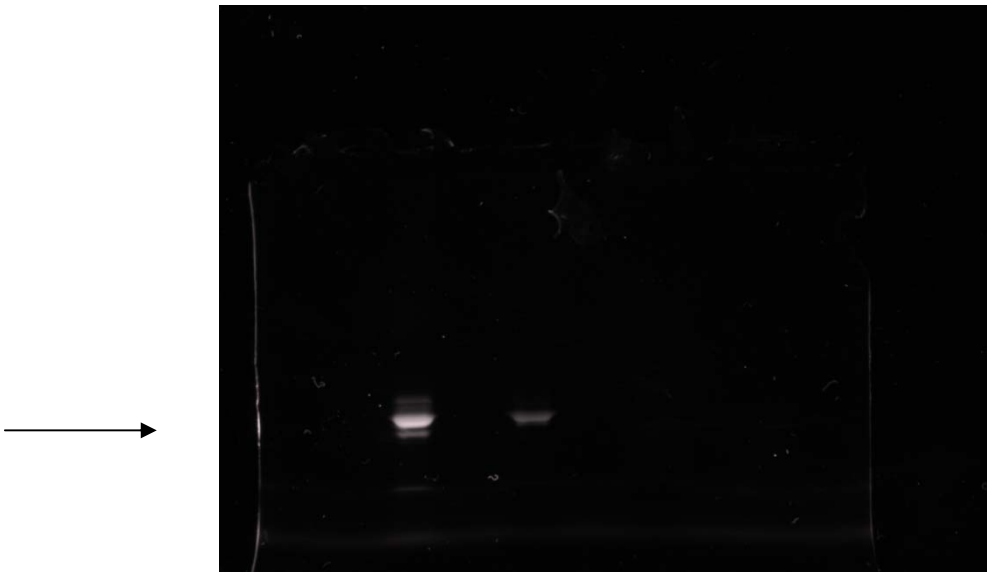


Figure 15: F109C labelled with Fluorescein, 2- F109C labelled with IAEDANS

3.5 Hemolytic activity of F109C on sheep erythrocytes

Hemolysis assays were performed on the labelled F109C mutant and the unlabelled wild type CAMP factor protein. F109C mutant have hemolytic activity similar to that of wild type CAMP factor. Therefore the mutation and labelling didn't affect the activity of the CAMP factor.

Hemolysis was observed with F109C with cells treated with sphingomyelinase. However, on cells not treated with sphingomyelinase, F109C didn't show any hemolytic activity, as there was no change in turbidity. F109C at the concentration of 0.35 mg/ml has lysed 50% of the sheep red blood cells in 1 min 40s (Figure 16).

Hemolysis was monitored decrease in turbidity (OD_{650}).

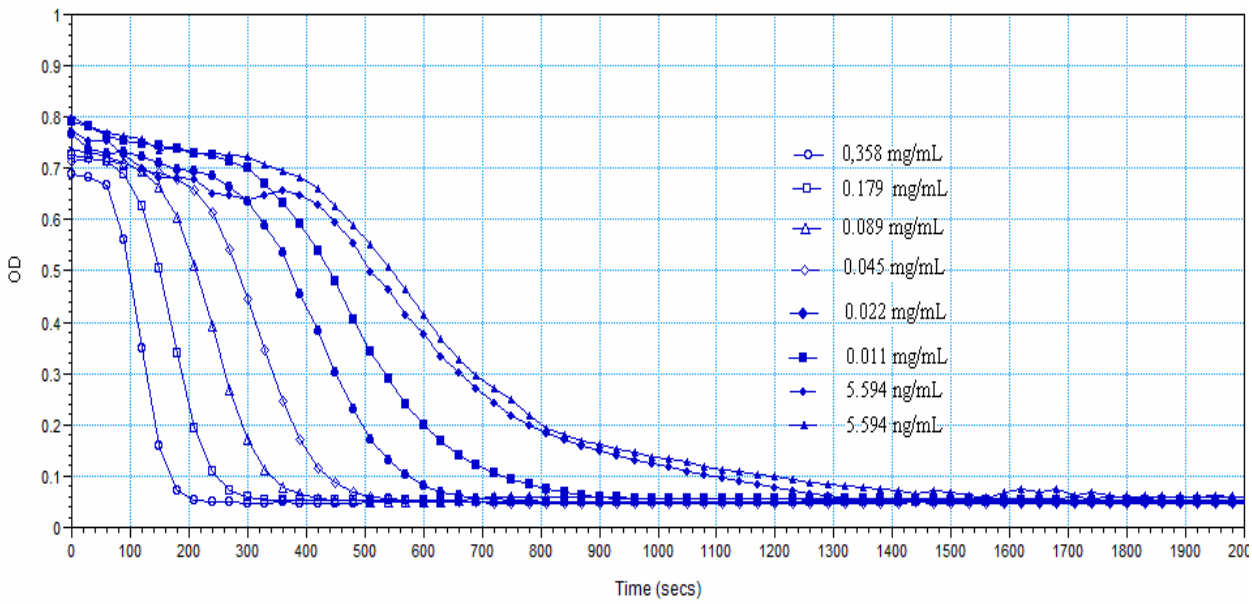


Figure 16: Hemolytic activity of F109C. F109C has lysed 50% of the sheep red blood cell in 1 minute 40 seconds at a concentration of 0.35mg/ml in 1 minute 40 seconds

3.6 Binding of F109C CAMP factor to erythrocyte membranes

The CAMP factor F109C protein was incubated with red blood cell membrane with and without treatment with sphingomyelinase. After incubation the sample was centrifuged and washed once to remove the unbound protein.

The membranes samples were then analyzed SDS-PAGE [Figure 17]. F109C CAMP factor was found to bind to membranes treated with sphingomyelinase. Membrane binding was also observed with wild type CAMP factor with sphingomyelinase-treated membranes.

Membranes without sphingomyelinase treatment did not show membrane binding with either F109C or wild type protein. Due to the interference of erythrocyte membrane protein, its binding couldn't be reliably analyzed by SDS-PAGE. Therefore the toxin binding was analyzed by spectrofluorimetry.

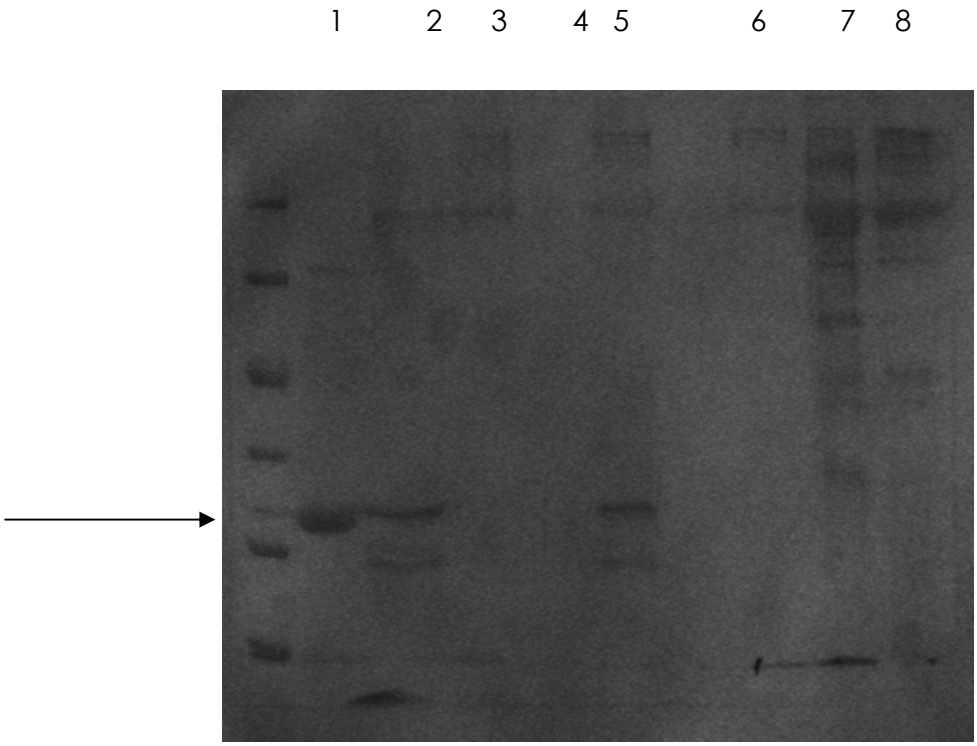


Figure 17: Binding of CAMP factor F109C protein with sphingomyelinase treated and untreated membrane.1-Molecular weight marker, 2- F109C protein, 3- F109C with membrane treated with sphingomyelinase, 4- F109C with membrane treated without sphingomyelinase, 5- W.T CAMP factor with membrane treated with sphingomyelinase, 6- W.T CAMP factor with membrane treated without sphingomyelinase, 7- Membrane treated with sphingomyelinase without protein, 8- Membrane without sphingomyelinase without protein.

Membrane binding was also analyzed by spectrofluorimetry (Figure 18). F109C was labelled with fluorescein maleimide. The binding efficiency was determined by emission intensity. It showed that CAMP factor could bind to the untreated cell membrane, although the binding efficiency was very low, while the treated membrane observed 60% binding efficiency. The binding efficiency was calculated by equation 1 section 3.11.

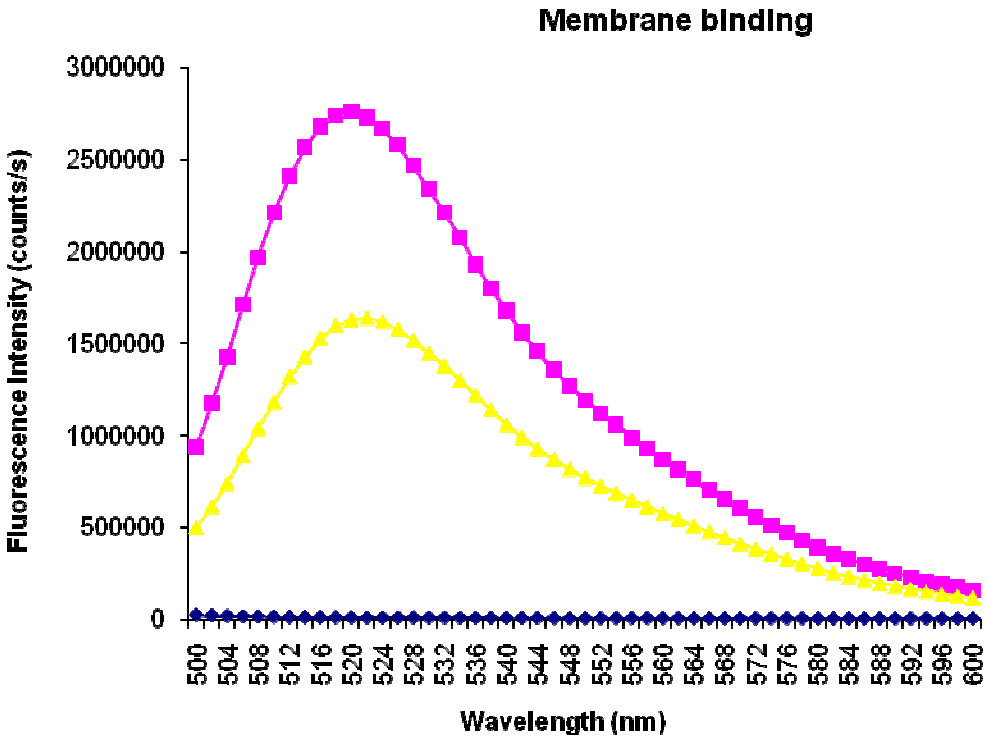


Figure 18 Emission spectra of CAMP F109C labelled with Fluorescein maleimide incubated with and without membrane (excitation wavelength 490 nm): The reduction in intensity corresponds to the fraction of the toxin bound to the membranes. The dark blue spectra representing membrane, (lower) yellow spectra representing F109C incubated with membrane have lower intensity counts (middle), and the purple spectra representing F109C without membrane (upper) have high intensity counts (upper)

3.7 Steady State study for F109C mutant

Emission spectra were obtained for F109C mutant oligomers labelled with IAEDANS and Fluorescein, which were bound to sheep erythrocyte ghost membrane treated with sphingomyelinase and without sphingomyelinase. Data were obtained for the donor and acceptor labelled proteins individually as well as for donor and acceptor mixture..

To account for self-quenching, the samples F109C were also made of the individual fluorophores and with the addition of unlabelled wild type CAMP factor.

The comparison between the labelled protein alone and the label protein with the wild type unlabelled protein observed in the following figure 19 and 20. F109C labelled with fluorescein (Figure 19) and IAEDANS showed very little change in emission intensity when incubated with wild type unlabelled protein (Figure 20). The wild type showed lower intensity than with fluorescein labelled protein.

This decrease in intensity is caused by competition of labelled and unlabelled proteins for binding sites on the membrane.

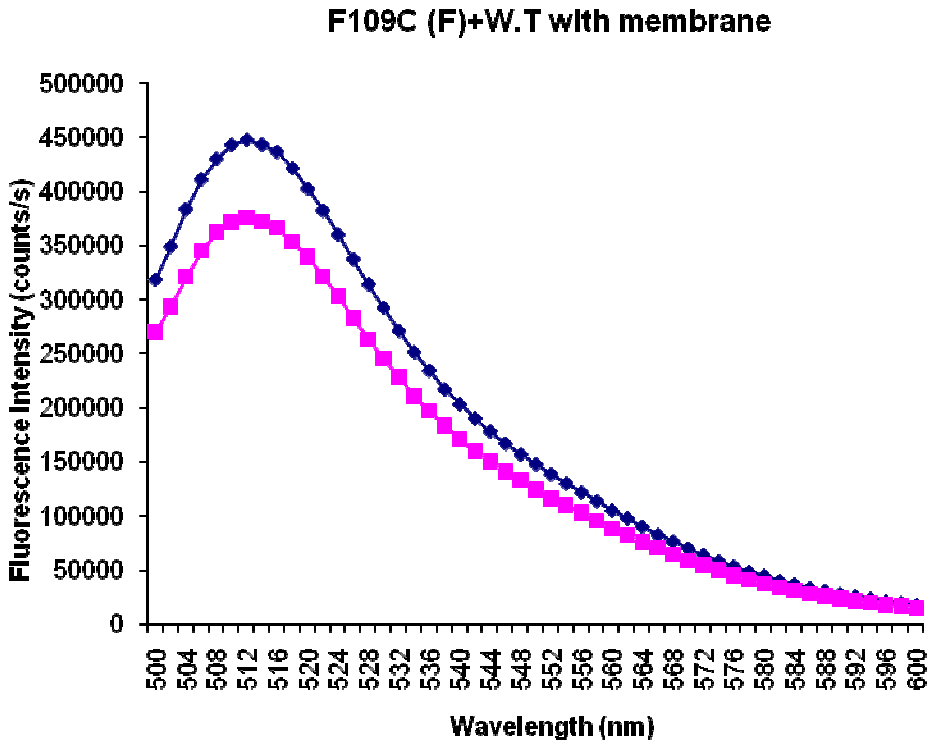


Figure 19: Fluorescein-labelled F109C with and without wild type CAMP factor incubated with membranes treated with sphingomyelinase (excitation wavelength 490 nm). Purple spectra (lower) representing F109C and wild type protein with membrane, and dark blue spectra (upper) representing F109C alone with membrane

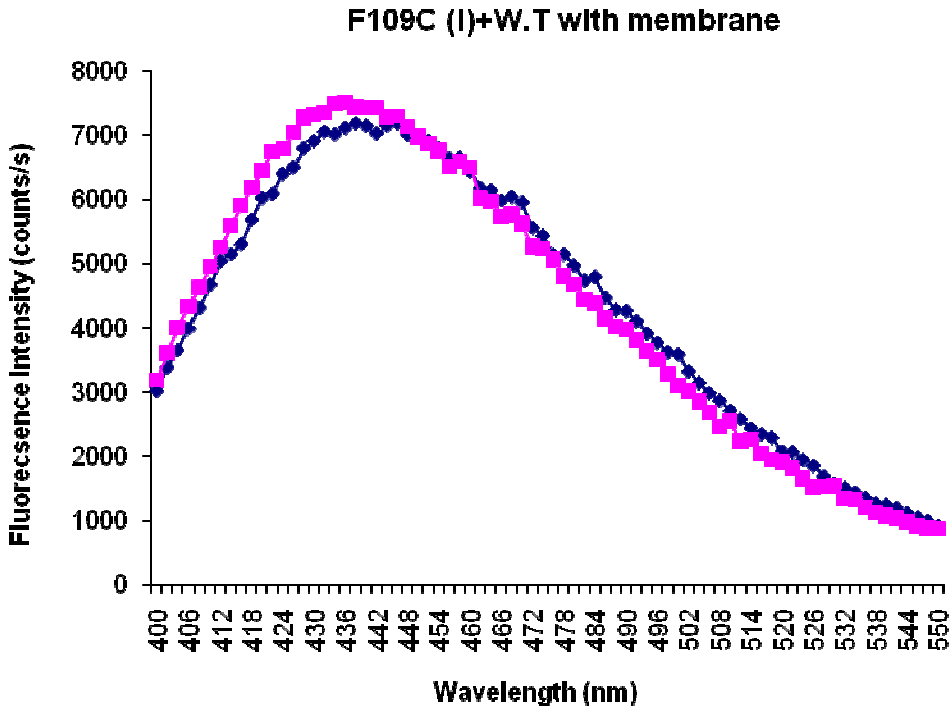


Figure 20: IAEDANS-labelled F109C and wild type with membrane treated with sphingomyelinase (excitation wavelength 336 nm). Purple spectra representing F109C and the wild type protein with membrane (upper) and the dark blue spectra representing F109C alone with membrane (lower)

Comparing the fluorescence intensities of F109C labelled samples (fluorescein and IAEDANS) on membranes treated with sphingomyelinase and without sphingomyelinase yielded the following results:

Fluorescein labelled F109C in membrane treated with sphingomyelinase showed 60% toxin binding efficiency. While IAEDANS labelled F109C with treated membrane observed 35% toxin binding efficiency. The untreated membrane observed 6% and 5% binding efficiency respectively.

Sphingomyelinase treated membrane with fluorescein labelled F109C showed 12 time higher intensity (Figure 21), while with IAEDANS labelled F109C showed 6

times higher intensity than untreated membranes (Figure 22). The changes in emission intensity of fluorescein-labelled samples and IAEDANS-labelled samples were determined from the ratio of the fluorescence in the membrane-bound (FI membrane-bound protein) and without membrane (FI protein solution) [37]. The percentage of binding was calculated by equation 1 (section 3.11).

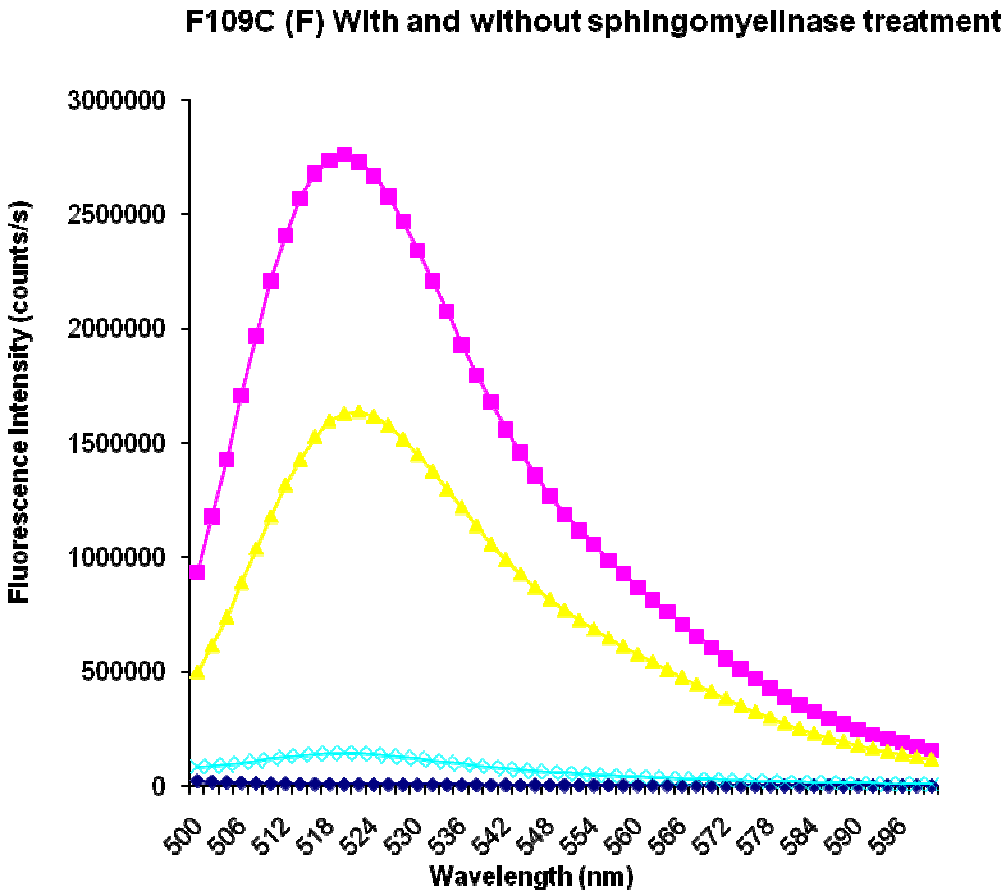


Figure 21: Fluorescein labelled F109C without membrane and F109C with membrane treated with sphingomyelinase and untreated with sphingomyelinase (excitation wavelength 490 nm). Dark blue spectra representing membrane, light blue spectra representing F109C with untreated membrane have low intensity counts (lower), the yellow spectra representing F109C with treated membrane have high intensity counts (middle), and the purple spectra representing F109C without membrane have highest intensity counts (upper)

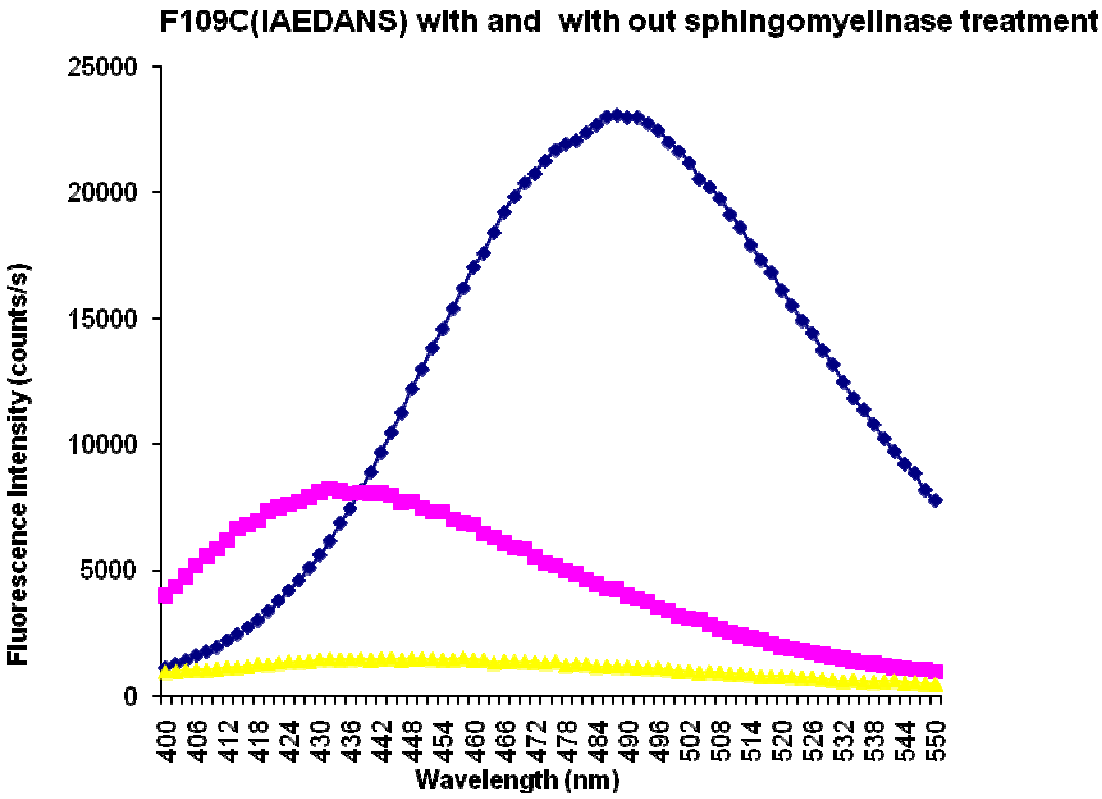


Figure 22: IAEDANS labelled F109C without membrane and F109C with membrane treated with sphingomyelinase and untreated membrane (excitation wavelength 336 nm) . The yellow spectra representing F109C with untreated membrane have low intensity counts (lower), the purple spectra representing F109C with treated membrane have high intensity counts (middle), and the dark blue spectra representing F109C without membrane have highest intensity counts (upper)

We obtained with the steady state observations that F109C CAMP factor in membranes treated with sphingomyelinase, showed high intensity due to the insertion of F109C into the hydrophobic environment of the membrane, which displayed membrane binding and oligomerization as compared to membranes not treated with sphingomyelinase. In untreated membranes we observed low intensity spectrum, which suggested that the toxin is still present in the polar

environment or hydrophilic environment of the membrane, and didn't observe membrane insertion and oligomerization.

These results clarify the role of sphingomyelinase on CAMP factor binding to RBC erythrocyte ghost membrane.

3.8 Time-resolved fluorescence studies for F109C

To further characterize the F109C CAMP factor mutant, we obtained the fluorescence lifetime. Time-resolved measurements are widely used in fluorescence spectroscopy, especially in studies of biological macromolecules, because they provide more detailed information than steady-state data.

The time-resolved study was used to obtain information on the donor (IAEDANS) lifetime with in the presence and absence of the acceptor (fluorescein).

The change in the fluorescence lifetime was used to observe energy transfer between donor and acceptor (FRET), for both samples membrane treated with sphingomyelinase and untreated membrane. The fluorescence decays were fitted with a three-exponential model. From the parameters of these three exponential components, the average fluorescence lifetime was calculated by equation 2 (section 3.11). The lifetime components are shown in the following table.

Table 2: Time-resolves data, Fluorescein (F), IAEDANS (I)

Sample	Lifetime Components						
	Decay1 (ns)	Amplitude1 I_0	Decay (ns)	Amplitude2 I_0	Decay3 (ns)	Amplitude3 I_0	Average Lifetime
F109C(F)-Sph	0.322	2414	3.827	12954	8.861	362	3.405
F109C (F)+WT-Sph	1.016	2263	3.966	12240	12.166	133	3.5844
F109C (F)-without-Sph	0.886	2833	3.8	11336	8.122	403	3.353
F109C (F)+WT-without-Sph	0.81	2373	4.002	11711	9.929	231	3.5685
F109C(I)+F109C(F)-Sph	0.272	16954	4.687	6867	14.75	2894	2.9752
F109C(I)-Sph	0.299	12883	4.012	3201	19.577	7296	6.8233
F109C(I)+WT-Sph	0.505	4281	4.343	9669	18.365	3154	5.9681
F109C(I)+ F109C(F)-without-Sph	0.179	34720	3.713	3410	8.986	3434	1.1966
F109C(I)+WT-without-Sph	0.19	33802	2.102	3112	7.65	3484	0.979
F109C (I)without-Sph	0.168	46983	2.809	2371	15.254	1348	0.6926

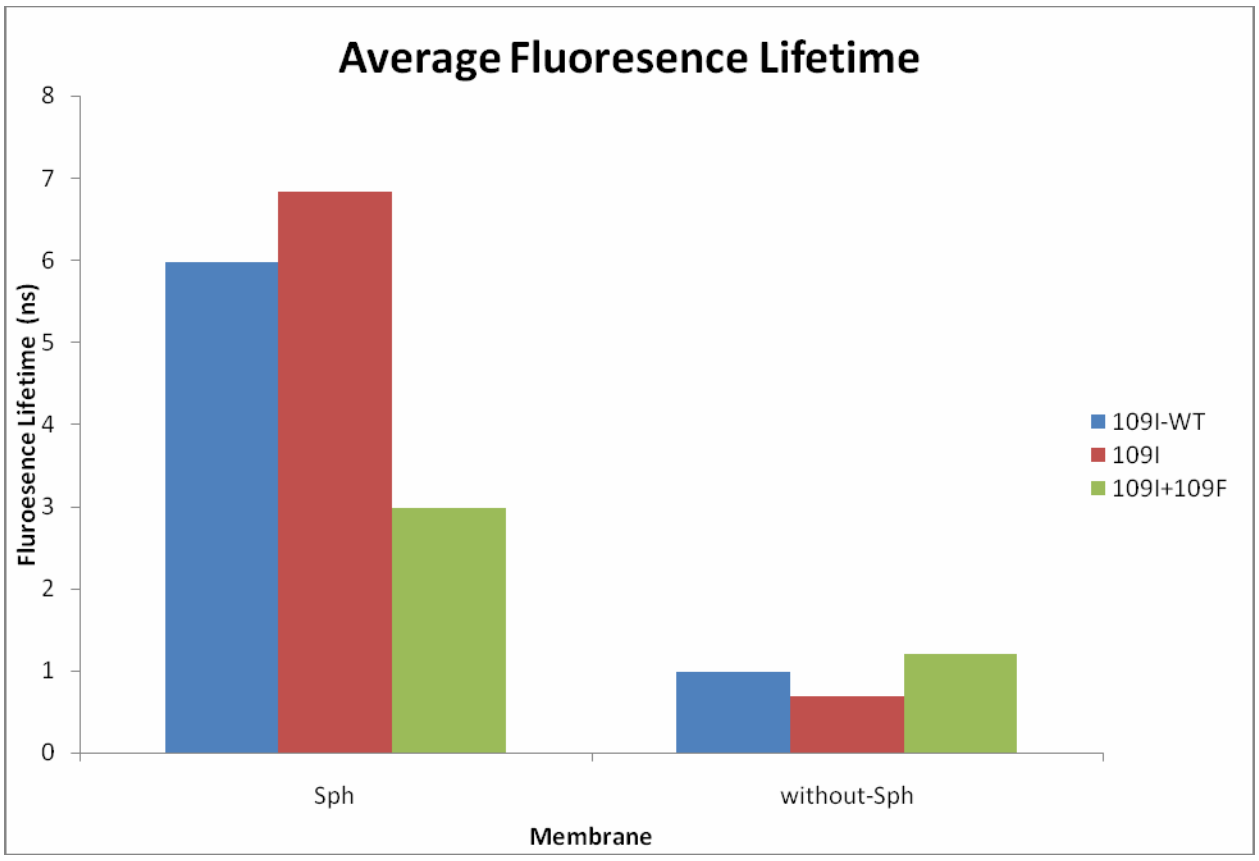


Figure 23: Average lifetime of donor (IAEDANS) labelled F109C plot shows fluorescence lifetime for the donor alone, donor-WT, and donor-acceptor

The collected data are displayed in figure 23; it contains the fluorescence lifetime results of different IAEDANS samples treated with sphingomyelinase versus results without sphingomyelinase. There is a significant difference in fluorescence lifetimes of samples treated with sphingomyelinase and untreated samples. The fluorescence lifetime for the donor alone, [F109C (IAEDANS), F109C (IAEDANS)-WT] is twice that of the donor interacting with acceptor, [F109C (IAEDANS) +F109C (Fluorescein)]. A decrease in donor lifetime in the presence of acceptor indicated energy transfer from donor to acceptor (FRET).

The observation from the above (figure 23) and tabulated data (Table 2) indicate that the FRET has occurred with samples treated with sphingomyelinase, and therefore that oligomerization occurs when CAMP factor associated with sphingomyelinase treated membranes. While these results are not observed in the untreated samples (without sphingomyelinase), which is suggesting that oligomerization cannot occur without sphingomyelinase. It is believed that without sphingomyelinase the protein (CAMP factor) cannot insert into the cell membrane and the protein is present in the surrounding polar environment, which upon excitation gave very short fluorescence lifetime. In contrary the sphingomyelinase treated samples shows longer fluorescence lifetime, which suggests that the protein (CAMP factor oligomers) are inserted into the cell membranes, and protected by the hydrophobic environment.

3.9 Discussion and Future Considerations

These results have shown that the FRET is a successful method for observing oligomerization of CAMP factor monomers on RBC membranes. The fluorescence assays have also confirmed that oligomerization is affected by the membrane lipid composition, such that sphingomyelinase activity is necessary for CAMP factor oligomer formation.

In contrast to many other pore-forming proteins, the oligomers of the CAMP factor are not stable following membrane solubilization, and therefore previous attempts to observe CAMP factor oligomers have been unsuccessful [49]

Therefore, the oligomeric state of CAMP factor on membranes must be experimentally determined. In the present study, the steady-state fluorescence was observed on membranes treated with sphingomyelinase and on untreated membranes. The treated membrane showed high toxin binding efficiency, while the untreated membrane showed very low toxin binding efficiency. This is in keeping with previous studies, which showed that sphingomyelinase increases membrane binding. At the same time, it confirms that the difference in membrane susceptibility is not fully accounted for by the decrease in binding alone, because different mammalian cell membranes have different susceptibilities and the membrane binding will also affect by change in lipid composition [49].

This suggested to us that sphingomyelinase also facilitates toxin binding and oligomerization. Addition of sphingomyelinase to the membrane leads to sphingomyelin cleavage (Figure 8) and produce a large amount of ceramide, [63, 64], which greatly increases the sensitivity of erythrocyte membrane [65] but it is not confirmed that ceramide has direct interaction with protein. It is still unclear which property of the red blood cell membrane mediates the sensitizing effect of ceramide [49].

In the present experiments we used fluorescence resonance energy transfer (FRET) on the cell membrane by using measurements of fluorescence lifetime. We observed that oligomerization only occurred for samples treated with sphingomyelinase (Figure 23).

The results of fluorescence lifetime showed that the samples containing both the donor and acceptor fluorophores had a lifetime half the time of the samples of just donor.

We didn't observe the FRET with the CAMP factor in membrane without sphingomyelinase treatment.

The lifetimes of the untreated membrane samples were considerably lower than the lifetimes observed for the membrane treated with sphingomyelinase. These experiments confirmed that without sphingomyelinase, membrane insertion and oligomerisation cannot occur. The labelled proteins are interacting with the polar environment and giving with rapid excitation decay.

The experiments with steady state and lifetime fluorescence also confirmed that the F109C residue sits right in a membrane-inserting region of the molecule.

In future studies we can use the same type of experiments, to investigate other CAMP factor mutants to observe and compare the results to the one stated here. The fluorophores used for this experiment were IAEDANS, as a donor, and fluorescein-5-maleimide as acceptor. Both fluorophores are thiol-reactive and therefore reacted well with cystein mutant CAMP factor protein.

IAEDANS was chosen as the donor because of its high water solubility, large Stokes shift (difference is the absorption and emission band maxima) and its emission overlaps with the absorption of many common fluorescent fluorophores

such as fluorescein, Alexa Fluor 546, and Oregon Green dyes, making it an excellent reagent for FRET measurements (Invitrogen, 2009).

However IAEDANS is known to be strongly dependent on the environment, and its conjugates undergo spectral shifts and changes in fluorescence intensity.

The results presented here demonstrate oligomerisation of CAMP factor on the cell membrane, and conclude that sphingomyelinase is essential for oligomerisation and pore formation.

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