Investigations of the Membrane Binding of *Vibrio cholerae* cytolysin

by

Adele Veronica Bubnys
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Abstract

*Vibrio cholerae* cytolysin (VCC) is a pore-forming toxin originating from *Vibrio cholerae*, the causative agent of cholera. The toxin follows an intriguing dimorphic mechanism; it is secreted as a soluble monomer, binds to eukaryotic cell membranes, oligomerizes into a heptamer, and inserts seven β-hairpins into the membrane to form a β-barrel pore that lyases the cell by disrupting its osmotic potential. A number of studies have suggested that protruding rim loops of VCC’s central cytolysin domain may have an affinity for membrane cholesterol or other components. However, no specific sites of ligand interaction have been identified in these loops to date, and their precise role in the toxin pore formation mechanism remains unknown. We characterized the hemolytic function of the rim region loops using systematic alanine-scanning mutagenesis and identified a cluster of functionally essential residues, including two vital tyrosines, Y420 and Y421 and one serine, S357. These residues are located in the cleft between the rim and stem domains and may be involved in coordinating a membrane associated ligand. Further analysis of the essential residues suggests that they are probably not involved in directly coordinating cholesterol and instead may recognize a protein or combination of targets, although a cholesterol interaction may not be fully ruled out. The results thus confirm that the VCC rim region plays an essential role in membrane recognition prior to pore formation and provide a structural basis for future investigations to identify the specific ligand target of the rim region.
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Chapter 1
Introduction
*Vibrio cholerae* cytolysin (VCC) is an accessory toxin of the bacterium *V. cholerae* that is secreted as a soluble monomer, binds to target cells, oligomerizes into a heptamer, and extends β-strands into the target membrane to form a large, nonspecific pore (Heuck et al., 2001; Iacovache et al., 2008). VCC is known to bind to cell surface glycans (De and Olson, 2011; Nayanendu Saha, 1997; Olson and Gouaux, 2005). Although this glycan binding activity plays a major role in the toxin’s ability to bind to and recognize specific cells, the fact that VCC is still active against phosphatidylcholine (PC) and cholesterol liposomes suggests that this protein may be interacting with the target membrane by some other mechanism (Zitzer et al., 1999).

The VCC monomer and heptameric pore contain several long loops with a high number of aromatic and hydrophobic residues that protrude from the rim region of the cytolysin domain and may insert into the target cell membrane during pore formation (Olson and Gouaux, 2005). Similar protruding loops in the structurally related pore-forming toxin, *Staphylococcus aureus* α-hemolysin, contain a lipid binding pocket (Galdiero and Gouaux, 2004) so the presence of a potential cholesterol, lipid, or peptide binding site in VCC’s loops is not without precedent. This study seeks to determine what role the rim region plays in VCC binding and pore formation using systematic alanine scanning mutagenesis and functional characterization of selected residues in this domain.
1.1 Vibrio Cholerae Cytolysin

1.1.1 Pathology and Identification of VCC

"Vibrio cholerae" cytolytic (VCC) arises from the O1 El Tor "Vibrio cholerae" bacterium, the causative agent of the current 7th global Cholera epidemic (Kaper et al., 1995). This toxin is partially responsible for the hemolytic activity of El Tor V. cholerae against rabbit, sheep, dog, human, and other species’ erythrocytes, which distinguishes it from classical V. cholerae (Kaper et al., 1995; Zitzer et al., 1997). While cholera toxin is the primary virulence factor of V. cholerae, strains of V. cholerae deficient in cholera toxin may still trigger mild diarrhea. This mild toxicity is entirely eradicated through removal of the VCC gene, suggesting that VCC holds a minor, yet notable role in the pathogenesis of V. cholerae (Saka et al., 2008).

Crude preparations of VCC induce hemolysis in rabbit, guinea pig, pig, sheep, goat, dog, horse, calf, mouse, goose, and chicken erythrocytes, increase the vascular permeability of rabbit epithelium (Yamamoto et al., 1984), and cause fluid accumulation in rabbit ileal loops in a mode consistent with cell lysis (Saka et al., 2008). VCC also causes rapid and irreversible depletion of ATP in target cells and opens up an anion current that disrupts cellular osmotic potential, resulting in cell death (Zitzer et al., 1997b). The similarities in VCC’s channel forming abilities to those of "Staphylococcus aureus" α-hemolysin identify VCC as a member of the β-barrel family of pore forming toxins (Bhakdi et al., 1993). Many subsequent studies of VCC structure and mechanism have utilized the archetypical α-hemolysin as a model system.
1.1.2 Structure and Mechanism

Sequence analysis reveals that VCC shares approximately 15% sequence identity with the *S. aureus* pore forming toxins α-hemolysin and leukocidin F (LukF) (Olson and Gouaux, 2003). Both α-hemolysin and LukF contain similar cytolysin domain scaffolds, a rim domain with protruding loops that come into contact with the target membrane, and a β-hairpin that extends into the membrane to form a β-barrel pore (Song et al., 1996). The core of VCC contains similar scaffold domains, but also includes two unique domains that resemble plant lectins (the β-trefoil and β-prism domains), and a cleavable prodomain that replaces the amino latch of α-hemolysin and LukF (Olson and Gouaux, 2005). These additional domains significantly alter the cellular specificity of VCC.

**Pore Formation Mechanism**

Upon binding the target membrane, the soluble VCC monomer oligomerizes into a heptameric pre-pore. When all seven identical subunits have come together, each monomer extends a β-hairpin into the target membrane in a concerted manner to form a stable 14-stranded β-barrel that has a 1-2 nm diameter and is slightly anion selective (Lohner et al., 2009; Pantano and Montecucco, 2006). In order for pore formation to occur, VCC must undergo several major structural rearrangements. CryoEM studies of the assembled pore reveal that the β-trefoil domain has collapsed towards the center of the pore by 75° and the β-prism has rotated nearly 180° compared to its position in the monomer structure. The latter rearrangement is necessary for oligomerization to occur (De and Olson, 2011; He and Olson, 2010).
Figure 1: Crystal structure of the VCC monomer with β-Ocetyl Glucoside bound. The cytolysin domain is marked in blue, the prodomain in red, the pre-stem in yellow, the β-trefoil domain in purple, and the β-prism domain in green. (PDB: 1XEZ)

Figure 2: Crystal structure of the VCC heptameric pore. The β-trefoil domain is marked in magenta, the β-prism domain is green, the cytolysin domain is blue, the rim region of the cytolysin domain is red, and the pore is yellow. The pore is inserted into the membrane up to the residues marked in cyan. (PDB: 3O44)
Cytolysin domain

The cytolysin domain of VCC has the highest degree of structural similarity to α-hemolysin and LukF (Olson and Gouaux, 2005; Olson et al., 1999; Song et al., 1996). This domain is split into two regions; the β-sandwich region, which serves as the central scaffold of the protein, and the protruding rim region, which is adjacent to the membrane in the fully assembled VCC pore (Olson and Gouaux, 2005). α-hemolysin contains a PC binding pocket in its rim region (Galdiero and Gouaux, 2004; Olson et al., 1999). However, the loops involved in coordinating PC are absent in VCC, whose rim loops protrude even further into space (Olson and Gouaux, 2005). It is likely that in the fully membrane inserted form, these highly flexible rim loops either extend into the target membrane in a conformation very similar to the crystal structure, or splay out along the membrane surface (He and Olson, 2010).

Prodomain

The soluble monomeric form of VCC contains a prodomain in a position similar to that occupied by the amino latch of α-hemolysin and LukF (Olson and Gouaux, 2005; Olson et al., 1999; Song et al., 1996). This domain is connected to the rest of the protein by a flexible linker that is highly susceptible to proteolysis (Hall and Drasar, 1990; Nagamune et al., 1996). When intact, the prodomain acts as a physical block to oligomerization and extension of the pre-stem into a functional pore (Olson and Gouaux, 2005).

Lectin domains

VCC also contains two domains that resemble plant lectins, one with a β-trefoil fold and another with a β-prism fold. The former shares similarities with ricin,
a sugar binding A-B toxin present in castor beans (Olsnes and Kozlov, 2001; Olson and Gouaux, 2003), and the human mannose receptor (Levan et al., 2013; Liu et al., 2000). Despite these similarities, this domain appears to have no extant sugar binding activity (Mazumdar et al., 2011; Olson and Gouaux, 2005; Rai et al., 2013), and may instead remain as a vestigial reminder of VCC’s evolutionary origins (Levan et al., 2013).

The β-prism domain was crystallized with a molecule of β-octyl glucoside (βOG) bound, which suggests that its sugar binding activity is intact (Olson and Gouaux, 2005). This domain shares structural homology to plant jacalins (Olson and Gouaux, 2005; Sankaranarayanan et al., 1996), artocarpin (Jeyaprakash et al., 2004), griffithsin (Ziolkowska et al., 2006), and agglutinin (Lee et al., 1998). Truncation of this domain severely impacts the binding abilities of VCC, suggesting that it plays an important role in toxin specificity and binding (Mazumdar et al., 2011; Rai et al., 2013).

1.1.3 Carbohydrate Binding Activity of VCC

VCC’s carbohydrate binding activity plays a major role in its ability to associate with and recognize specific types of cells. The pattern of weak VCC binding to human erythrocytes suggests the presence of a high number of low affinity sites, while the 7-fold stronger binding to rabbit erythrocytes occurs on a saturable number of high affinity binding sites (Zitzer et al., 1997). Truncation of the β-prism domain causes VCC to lose its ability to bind the high affinity sites on rabbit erythrocytes. But truncated VCC has approximately the same degree of activity against liposomes composed solely of PC and cholesterol as full length VCC does (Mazumdar et al.,
This evidence suggests that carbohydrate binding is mediated through the β-prism domain.

Figure 3: Binding of $^{125}$I labeled VCC to human and rabbit erythrocytes reveals a higher toxin affinity for rabbit erythrocytes over human cells. This suggests that rabbit erythrocytes contain a high affinity VCC receptor that human erythrocytes lack. Adapted from (Zitzer et al., 1997).

A pocket in the β-prism domain is primarily involved in coordinating the bound sugar (Olson and Gouaux, 2005). The ligand associates with two tyrosine residues and an essential aspartic acid residue (Figure 4) (Rai et al., 2013). Knocking out the sugar binding activity of this pocket does yield functional pores, albeit with a much lower affinity for rabbit erythrocytes (Levan et al., 2013). A glycan screen of potential sugar binders to the β-prism domain reveals that this domain prefers sugars with an NGA2 core. This complex two-tailed N-glycan is found specifically on mammalian cells and the highest affinity binder (an NGA2 core with additional Lewis-x sugars) is a marker of human neutrophils. Such specificity suggests that VCC may be targeting neutrophils (Levan et al., 2013). This data would be in agreement with previous studies that propose that VCC plays a role in early *V. cholerae* infection by preventing neutrophils from clearing low-density bacterial
colonies before they may establish themselves (Olivier et al., 2007; Olivier et al., 2009; Queen and Satchell, 2012).

![Figure 4: The sugar binding pocket in the β-prism domain of VCC](image)

*Figure 4: The sugar binding pocket in the β-prism domain of VCC* from the crystal structure of the VCC monomer with βOG bound. The sugar is primarily coordinated by two conserved tyrosine residues (Y654 and Y679) and an essential aspartic acid (D617). Adapted from (Olson and Gouaux, 2005).

### 1.1.4 Potential Cholesterol Binding Activity of VCC

Although there is ample evidence that VCC binds to cell surface glycans with a high and specific affinity, the toxin’s activity on PC-cholesterol liposomes appears intact, albeit diminished (Zitzer et al., 1999). This suggests that sugar binding is not essential for pore formation. While VCC is capable of binding and forming functional pores on PC-cholesterol liposomes, apparently it may bind to but not form functional pores on PC liposomes completely lacking cholesterol (Zitzer et al., 1999). This suggests that membrane cholesterol may play an important role in the mechanism of VCC membrane permeabilization and could be the low affinity binding site observed on human and rabbit erythrocytes. Yet, despite some promising evidence, a specific cholesterol binding site on VCC has yet to be identified.
Potential nonspecific interaction

The presence of a large number of low affinity binding sites on human and rabbit erythrocytes has some parallels to the affinity that α-hemolysin displays for clustered PC moieties (Watanabe et al., 1987). The α-hemolysin model of membrane binding posits that this toxin does not have a sufficiently high affinity for individual PC moieties to form a classical receptor-ligand association, but PC molecules can take on the role of a high affinity binding site when clustered together, hence concentrating toxin monomers to a small area and facilitating oligomerization (Valeva et al., 2006). Applying such a model to VCC binding seems attractive, particularly since no classical lipid and cholesterol binding interactions have been identified for VCC to date. The high degree of structural similarity between the two toxins also may suggest a similar mode of membrane binding in the cytolysin core (Olson and Gouaux, 2003), as do the comparable binding curves observed on rabbit and human erythrocytes for both proteins (Valeva et al., 2006). However, there remain sufficient differences between the ways VCC and α-hemolysin interact with membranes to cast doubt on a theory of nonspecific interaction.

Role of lipid head groups

Liposomes consisting of lipids containing ceramide moieties and other small head groups are more susceptible to VCC intoxication, while PC with its relatively large head group has an inhibitory effect on the toxin (Zitzer et al., 2001). The stereochemistry of the ceramide group does not appear to affect VCC pore formation, which suggests that the size of the head group is a primary driver of VCC sensitivity. By contrast, α-hemolysin does not display such an association, in keeping with its
specificity for the PC moiety, while the cholesterol-dependent cytolyisin (CDC) Streptolysin O does (Zitzer et al., 2001). This suggests that the larger head groups may inhibit VCC activity by shielding other membrane components like cholesterol from interaction with the toxin.

**Sensitivity to membrane cholesterol**

Depletion of membrane cholesterol is directly linked to decreased membrane sensitivity to VCC intoxication (Zitzer et al., 2000). The rate of VCC pore formation is proportional to the concentration of cholesterol in the membrane, although the mathematical relationship between cholesterol concentration and maximal pore formation suggests that VCC may interact with more than one cholesterol molecule at a time (Krasilnikov et al., 2007). Additionally, VCC is apparently sensitive to cholesterol on both membrane leaflets, as pore formation is limited by the leaflet with the lowest cholesterol content, regardless of whether it is cis or trans to the toxin binding site (Krasilnikov et al., 2007). These results support the idea that cholesterol may be important for VCC pore formation, rather than initial membrane targeting.

Based on their results, Krasilnikov et al also propose a potential pore formation mechanism in which the VCC monomer binds the membrane and interacts with cholesterol in the cis leaflet, oligomerization occurs, then the oligomer interacts with cholesterol in the trans leaflet, allowing it to form the membrane spanning β-barrel pore (Krasilnikov et al., 2007).

**Effects of modulating membrane fluidity**

Cholesterol is important for maintaining membrane fluidity (Ikonen, 2008). A membrane with high cholesterol content would be rendered more rigid and
susceptible to pore insertion without requiring any sort of specific protein interaction (Gilbert, 2002). Yet VCC pore formation does not appear to depend on membrane fluidity, since a decrease in temperature negatively impacts VCC pore formation on membranes independent of their fluidity (Zitzer et al., 2000).

**VCC and lipid rafts**

A notable characteristic of cholesterol is its role in the formation of membrane microdomains of distinctive lipid and protein content known as lipid rafts (Lingwood and Simons, 2010). These cholesterol and sphingolipid enriched domains allow for a differential distribution of lipids and proteins that can cause certain membrane components to cluster together in a manner that would greatly facilitate toxin oligomerization (Lafont et al., 2004). Such a lipid raft-dependent oligomerization mechanism has already been described for α-hemolysin (Valeva et al., 2006), and implicated in the pore formation mechanism of several CDCs (Ohno-Iwashita et al., 2010; Waheed et al., 2001). Although there is little direct evidence of VCC interacting with lipid rafts, its requirement for both cholesterol and sphingomyelin suggests that VCC may preferentially bind raft domains (Lafont et al., 2004).

**Specific cholesterol interaction**

All of this evidence suggests that VCC interacts directly with membrane cholesterol in a specific manner. Yet it remains to be seen exactly what aspects of the cholesterol molecule VCC recognizes and how conserved these elements must be. The specificity of the VCC-cholesterol interaction seems to be confirmed by the fact that replacing cholesterol with its enantiomer abolishes the beneficial effect that cholesterol had on pore formation (Zitzer et al., 2003). The enantiomer should have
an identical effect on membrane fluidity and distribution (Mannock et al., 2003), thereby ruling out nonspecific associations between VCC and cholesterol (Palmer, 2004). VCC tends to oligomerize on fluid microcrystals composed of pure cholesterol, though modification of certain cholesterol side chains disrupts this phenomenon. In particular, modification of the isoocetyl chain to alter its length and branching pattern adversely affected oligomerization, although deletion of this chain had no impact on pore formation (Harris et al., 2002). Only the most extreme changes to the 3β-hydroxyl group affected VCC interaction (Zitzer et al., 2003). These results stand in marked contrast with the strong specificity that most CDCs display towards the hydroxyl group of cholesterol and their inability to distinguish between cholesterol enantiomers (Harris et al., 2002; Zitzer et al., 2003). Hence, even if VCC interacts directly with cholesterol as the CDCs do, the details of this interaction are likely to be quite different between the two types of pore forming toxins.

Other potential membrane targets

There are comparatively few studies that have explored the possibility that VCC cytolysin domain interacts with a target other than cholesterol, although the alternative is worth considering. A number of related pore forming toxins have demonstrated affinities for lipid or protein receptors in the membrane. LukF and α-hemolysin both contain PC binding sites within their rim loops, although this binding site is absent in VCC (Olson et al., 1999; Song et al., 1996). α-hemolysin was also co-localized with the disintegrin and metalloprotease ADAM10, suggesting that this protein may act as an additional receptor for the toxin (Wilke and Bubeck Wardenburg, 2010). Anthrax toxin, an A-B toxin that uses its β-barrel pore to
translocate toxic enzymes into the target cell has a high affinity for the receptor proteins CGM2 and TEM8. The crystal structure of the protective antigen (PA) subunit of anthrax toxin in complex with CGM2 reveals that this interaction mimics the binding of integrins to CGM2 (Santelli et al., 2004). Such associations reveal a rich variety of potential pore forming toxin targets beyond cholesterol.

**Continuing issues**

Evidence suggests that VCC specifically interacts with membrane cholesterol. Although a definite mechanism and relationship have yet to be confirmed, it might prove useful to draw some parallels between VCC and the CDCs, a class of pore forming toxins known to rely heavily on the presence of membrane cholesterol. For the CDCs, the presence of cholesterol is an absolute requirement for pore formation (Gilbert, 2002). This relationship is less stringent in VCC’s case and has not been explored as fully. The greatest obstacle that remains in unanimously confirming the interaction between VCC and cholesterol is the identification of an as-yet elusive cholesterol binding site on the toxin. To further explore the likelihood of such an interaction, I will examine the role of cholesterol in the membrane, and the relationship between cholesterol and the CDCs as a model for how pore forming toxins have evolved to interact with sterols.

**1.2 Cholesterol in the Membrane**

Cholesterol is a sterol with a planar conjugated ring system, an iso-octyl hydrocarbon chain, and a single hydroxyl group at the 3β position that accounts for its amphipathic character (Figure 5). It is an essential component of the eukaryotic cell membrane and as such the human body has the capability to both take up dietary
cholesterol and synthesize it \textit{de novo} using acetyl CoA (Ikonen, 2008). Cholesterol is a precursor of many important biological molecules such as steroid hormones and bile acids. It also plays a key role in regulating membrane rigidity and thickness by inducing the ordered packing of phospholipid acyl chains, while also generating a semipermeable barrier. Cholesterol in membranes helps drive the lateral organization of lipids into distinct domains and this molecule modulates the activity of many membrane associated proteins through direct and indirect means (Gimpl, 2010).

![Figure 5: Structure of cholesterol.](image)

Cholesterol’s relatively small polar head group and large, rigid hydrophobic ring system account for its unique physical properties in the membrane (Palmer, 2004). Its single hydroxyl group is not sufficient to shield the remainder of the molecule from solution when placed into a polar environment, so it does not preferably exist as an exposed component of the membrane. There are a number of models that describe the different physical forces that drive the organization of cholesterol in the membrane (Ikonen, 2008).

**Umbrella model**

The umbrella model posits that hydrophobic forces are the primary driver of cholesterol organization in the membrane. Since cholesterol’s small head group is not sufficient to shield the entire molecule from solution, it preferably partitions beneath
the large head groups of certain phospholipids such as PC. Such lipids provide ample shielding for a certain number of cholesterol molecules. However, as the concentration of cholesterol is further increased, these molecules begin to crowd together beneath the polar phospholipid head groups and cause an overall decrease in membrane fluidity. Eventually, the solubility limit of cholesterol is reached, and any molecules that do not fit beneath the shielding head groups will begin to precipitate out of the membrane (Huang and Feigenson, 1999).

**Condensed complex model**

Studies of model membranes reveal that a mixture of cholesterol and other lipids yields a condensing effect in which both components take up a smaller surface area than would be expected from a pure mixing effect (Leathes, 1925). This observation underlies the condensed complex model of cholesterol distribution. According to this model, cholesterol forms preferential interactions with certain lipids such as sphingolipids that contain a long, unsaturated acyl chain (McConnell and Radhakrishnan, 2003). The interaction forces the acyl chain into an extended all trans conformation, increasing the thickness of the membrane (Brown, 1998). Such interactions between cholesterol and neighboring lipids are highly localized and transient, allowing subsequent complexes to form and break apart rapidly. Yet, when certain proteins begin to interact preferentially with complexes, they may stabilize them into larger domains known as lipid rafts (McConnell and Radhakrishnan, 2003).

**Lipid Rafts**

Lipid rafts are detergent insoluble complexes enriched in cholesterol, sphingolipids, lipids with long, unsaturated acyl chains, and certain proteins such as
caveolin and flotillin (Epand, 2008). These domains are larger and less transient than condensed complexes, although they are still thought to be quite dynamic. The higher packing density of lipid rafts is generally unfavorable for membrane protein insertion, so many proteins will localize to cholesterol-depleted regions of the membrane, thus driving up cholesterol content elsewhere (Epand, 2008). However, there are many proteins that preferentially associate with rafts. These proteins are involved in a wide variety of cellular processes such as cell signaling (Cherezov et al., 2007; Pankov et al., 2005) and the pathology of a diverse assortment of diseases (Mahfoud et al., 2002). Rafts’ ability to cluster together and organize membrane components can create high affinity regions on the membrane that leads to more efficient signaling and ligand binding.

Protein associations with lipid rafts

Proteins that preferentially partition into lipid rafts share a certain subset of features that make this sort of interaction possible. Many are covalently linked to or specifically bind raft-associated lipids such as cholesterol or sphingolipids (Epand, 2006). Other proteins will localize with raft-associated proteins such as flotillin or caveolin. Although the dense packing of lipids in these domains tends to be unfavorable for the insertion of protein transmembrane domains, certain proteins containing transmembrane domains with a smooth contour may also partition into the raft (Epand, 2008).

1.2.1 Protein Interactions with Cholesterol

Cholesterol is an attractive binding target for many membrane associated proteins due to the distinct polarity of its single hydroxyl group relative to
neighboring lipids (Epand, 2008). There are a number of different motifs and binding sites that have been identified in cholesterol binding proteins. These vary from deep hydrophobic pockets, to transmembrane helices and individual amino acid sequences inserted into the membrane (Epand, 2006).

**START domain**

The START domain is found in many proteins involved in binding and transferring lipids such as cholesterol, PC, phosphatidylethanolamine (PE), and ceramides, particularly members of the StAR family. The domain consists of a deep hydrophobic pocket that sequesters cholesterol away from solution with a tight fitting lid. Binding of cholesterol often requires major conformational changes in the protein leading to partial unfolding, but this allows for very high affinity binding and transport of the hydrophobic ligand even through a polar environment (Alpy and Tomasetto, 2005; Epand, 2006).

![Image of START domain](image)

**Figure 6:** Cutaway of the START domain of PCTP (Human phosphatidycholine transfer protein) in complex with PC. The START domain is a deep, hydrophobic pocket that extends into the core of the protein to securely bind and sequester cholesterol for transport. (PDB: 1LN1)
Sterol Sensing Domain

The sterol sensing domain (SSD), another common cholesterol binding motif, is frequently found in proteins involved in regulating cholesterol homeostasis such as Hmg-CoA reductase. The motif is typically found at the tip of a transmembrane helix and often contains a highly conserved YIYF sequence that coordinates cholesterol (Epand et al., 2010; Kuwabara and Labouesse, 2002).

CRAC motif

The CRAC motif is a consensus sequence common to many proteins known to bind cholesterol, including caveolin, the Gp41 fusogenic protein of HIV, and the peripheral-type benzodiazepine receptor. This sequence can be anywhere from five to thirteen amino acids in length with the overall consensus sequence of \((-\text{L/V})(\text{X})_{1-5}-\text{Y-} (\text{X})_{1-5}-\text{R/K}-\)). The bolded residues are highly conserved and necessary to the motif. This peptide sequence wraps partially around cholesterol and undergoes a number of pi stacking and hydrogen bonding interactions with cholesterol’s hydroxyl group, particularly via tyrosine (Epand, 2006; Epand, 2008; Li and Papadopoulos, 1998).

![Figure 7: Model of a putative CRAC motif](Image)

Figure 7: Model of a putative CRAC motif in the human pro-apoptotic protein BAX. The protein wraps partially around cholesterol, and primarily coordinates the sterol via Y115 and L113. Adapted from (Martinez-Abundis et al., 2011).
Identification of a cholesterol interaction

There are a number of experimental techniques that can be useful in determining whether a given protein interacts with cholesterol or lipid rafts, including fluorescence binding assays (Cheruku et al., 2006; Liou et al., 2006), affinity labeling, competition assays with another cholesterol binding protein, and binding experiments using $^{3}$H labeled cholesterol (Infante et al., 2008; Okamura et al., 1999). In general, a protein is identified as a cholesterol binder if it preferentially localizes to lipid rafts, if the presence of cholesterol alters its function or structure in some way, if replacing cholesterol with another sterol affects its function, or if a cholesterol binding domain such as the previously described START, SSD, or CRAC motifs is found to be present and functional on the protein (Gimpl, 2010).

1.2.2 Bacterial Targeting of Cholesterol

Since cholesterol is almost exclusively present on eukaryotic plasma membranes, it makes an attractive target for bacteria and their associated proteins to specifically bind their eukaryotic hosts (Palmer, 2004). Bacteria may take advantage of lipid raft cholesterol clusters as high affinity binding sites or concentration platforms, thus allowing for the localization of many binding units and more efficient infection of cells. Some bacteria are thought to use lipid rafts as a site for endocytotic cell entry instead of the traditional clathrin-mediated mechanism in order to evade the host’s immune response because many of their signaling targets are found on lipid rafts (Lafont et al., 2004). Pore forming toxins have been documented to take advantage of the heterogeneous distribution of membrane cholesterol to efficiently cluster monomers and facilitate rapid oligomerization (Laurence Abrami, 1999). The
cholesterol-dependent cytolysins (CDCs) are a family of pore forming toxins with a well-characterized absolute cholesterol requirement (Heuck et al., 2010; Soltani et al., 2007) that may be used as a model for how and why pore forming toxins like VCC could interact with cholesterol.

1.3 VCC and the Cholesterol Dependent Cytolysins

Structural comparison of the soluble monomers of the prototypical CDC, Perfringolysin O (PFO) and VCC reveals very low structural similarity, apart from the tendency to form a β-barrel pore (Figure 8). PFO and other CDCs oligomerize into large complexes with more than 30 subunits that form massive pores over 200Å in diameter (Heuck et al., 2010). On the other hand, VCC consistently forms a heptameric pore around 20Å in diameter that is only wide enough accommodate ions.

Despite these structural differences, both classes of pore forming toxins contain a similar kind of β-sandwich fold with long loops that insert into the target membrane and are key to the initial binding interaction. In PFO, these loops contain conserved tryptophan-rich undecapeptide and T-L motifs that are involved in cholesterol binding (Farrand et al., 2010; Soltani et al., 2007). The rim loops of VCC are similarly rich in aromatic peptides that are good candidates for sterol interactions. Given this similarity and the well characterized relationship that the CDCs have with cholesterol, it may be possible to use this family of toxins as a model for potential ways that VCC could interact with sterol and membrane lipids via its rim domain.
Figure 8: Comparison of the VCC and PFO monomers. VCC (A) contains a central cytolysin domain (blue) onto which extra domains have been added, while PFO (B) consists of four domains of similar size. Domain 4 of PFO (blue) contains putative cholesterol binding sites including the undecapeptide loop (cyan), and has a β-sandwich motif comparable to the lower part of the VCC cytolysin domain (blue).

**CDC interaction with cholesterol**

The observation of high and low affinity binding sites on PFO target cells could explain this toxin’s apparent specificity for both raft and free cholesterol (Nollmann et al., 2004). There are a limited number of high affinity binding sites, but binding to these sites alone is probably strong enough to elicit cytolysis (Ohno-Iwashita et al., 1988). On the other hand, the low affinity sites are abundant, but not as accessible for protein binding (Ohno-Iwashita et al., 1988). Similarly, lipid rafts are fewer in number but have high cholesterol content, while free cholesterol is
distributed throughout the membrane but is generally too shielded by neighboring phospholipids to be available for binding.

It is likely that cholesterol plays a far greater role in promoting the CDC pore formation mechanism beyond acting as a binding target (Rosado et al., 2008). Depleting free cholesterol in the membrane causes PFO to lose pore formation capacity without affecting toxin binding to the membrane (Giddings et al., 2003). Pure cholesterol aggregates are also sufficient to induce PFO oligomerization and pore formation in the absence of any other membrane components (Heuck et al., 2007). Membrane cholesterol may facilitate pore formation by forming a tight hydrophobic seal around the β-barrel pore, thereby stabilizing it (Tilley et al., 2005). Cholesterol may also shield polar trans-membrane residues from the highly hydrophobic bilayer core during pore formation, thus allowing their insertion to become more thermodynamically favorable (Rossjohn et al., 1997). Since cholesterol acts as a cone shaped lipid in the bilayer, high levels of sterol promote the formation of an inverted hexagonal lipid phase. A bilayer thus destabilized is more amenable to pore insertion (Gilbert, 2002).

Comparison to VCC

While the CDCs have an absolute threshold requirement for cholesterol, VCC expresses a constant linear cholesterol-dependence curve (Figure 9). Although the presence of cholesterol in the membrane facilitates VCC pore formation, VCC does not require cholesterol as stringently as the CDCs do. Perhaps in VCC’s case cholesterol is modulating pore formation rather than acting as a specific binding
target. This may explain why a cholesterol binding site on VCC remains so elusive despite such overwhelming evidence in support of a cholesterol interaction.

**Figure 9: The cholesterol dependence of VCC and PFO.** The rate of VCC pore formation on DOPE liposomes (A) and Bovine brain lipid liposomes (B) increases linearly as the concentration of cholesterol is raised. This is in contrast with the distinct transition observed for PFO, from no binding to significant pore formation, at a threshold concentration of cholesterol (C). Adapted from (Krasilnikov et al., 2007; Ohno-Iwashita et al., 2010).

### 1.4 Goals of this Study

Although there is a substantial body of evidence that suggests that VCC interacts with other membrane components in addition to its well characterized association with glycans, little work has been done to identify exactly which aspects of VCC structure are involved in these interactions. The rim region of the cytolysin domain seems a particularly good candidate for further investigation given its putative membrane inserted location. Structurally similar pore forming toxins also contain various types of membrane binding sites within their rim domain loops. This
study aims to systematically characterize the contributions of each residue in three protruding loops of the rim region and relate them to the binding activity of VCC to rabbit erythrocytes.

Alanine scanning mutagenesis was used to mutate individual side chains in the rim loops to alanine, the most functionally neutral amino acid. The pore forming activity of these mutants on rabbit erythrocytes was determined using a hemolysis assay to generate dose-dependent cytolysis curves. The structural integrity of each mutant was further assessed using proteolytic cleavage and size exclusion chromatography (SEC) to ensure homogenous, monodisperse protein samples. Isothermal titration calorimetry was used to confirm that the most inactive rim mutants had unaffected sugar binding activity. The pore forming activity of these mutants was also tested on liposomes consisting of asolectin lipids and cholesterol to determine the contributions of these membrane components to the VCC cytotoxic mechanism. Overall, these experiments reveal a number of functionally essential rim region residues that may be involved in a ligand binding site.
Chapter 2
Methods
2.1 Methods in Theory

2.1.1 Alanine Scanning Mutagenesis

Alanine scanning mutagenesis is a biochemical technique used to identify functionally important amino acids in proteins with regions already known to have some mechanistic importance. This method has been used to identify the specific points of interaction between proteins and small molecule or polypeptide ligands in the absence of detailed structural data (Wells, 1991). The technique involves replacing single amino acids with alanine and using a protein-specific assay to determine the effects that such substitutions have on protein function. Changes in mutant function ranging from 2 to 100 fold less than wild type have been recorded, even as a result of altering just one amino acid in the protein of interest (Wells, 1991).

The overarching philosophy of alanine scanning relies on replacing the functional side-chain of a given amino acid with a neutral group that would knock out this function without otherwise disrupting protein folding or backbone dynamics. Alanine consists of a single methyl group that is not sterically bulky and has no hydrogen bonding potential nor hydrophobic character, allowing it to exist in both core and solvent exposed positions in a protein (Figure 10). This is the most common amino acid and hence is considered the “default” side chain without any notable functions or interactions (Wells, 1991).

![Structure of Alanine](image)

Figure 10: Structure of Alanine
Many alanine-scanning studies have used homolog scanning mutagenesis, a complementary technique involving replacement of a putative functional sequence of a protein with the sequence from an inactive homolog, in order to identify the general region on which to focus more intently. From here, alanine scanning may be used to generate a detailed functional map of the region and identify particular binding pockets or points of interest (Cunningham and Wells, 1989). Hydrophobic and polar amino acids serve as particularly good targets for mutagenesis, since they are often involved in mediating protein interactions with some ligand (Ashkenazi et al., 1990).

Following functional assessment of alanine mutants, it is important to confirm that the changes in protein activity observed are due to functional effects rather than structural perturbations. Such assessment has been done using a number of techniques, including circular dichroism (CD), nuclear magnetic resonance (NMR), binding of antibodies specific to protein tertiary structure, and intrinsic tryptophan fluorescence (Cunningham and Wells, 1989; Farrand et al., 2010; Wells, 1991). Certain mutations may result in poorly expressed species that are typically assumed to be misfolded species that are proteolytically cleaved in vivo (Wells, 1991). If a mutant passes these tests, it is highly likely that the residue in question plays some vital role in the specific protein function that is being assessed.

There are some limitations to alanine scanning. Since only the side chain has been replaced by mutagenesis, it is not possible to probe the role of main chain carboxyl groups in protein function. In such cases, introducing glycine or proline mutations to increase or decrease backbone flexibility could provide information on main chain contributions to function. Additionally, even if a residue has been
identified as essential to a protein’s function, there is no way to determine what aspects of side chain structure are most important to that activity. Subsequent, conservative mutations that only alter portions of the side chain would be required to reach a more detailed level of structural understanding. In other cases where a side chain plays a role in protein folding and is highly sensitive to perturbation, more conservative mutations would also be required to generate testable protein.

Regardless, alanine-scanning mutagenesis is a useful technique for the identification of residues that are crucial for protein interactions and functions, which may be carried out effectively in the absence of detailed structural data. Many studies have used this approach to characterize interactions between targets such as the human growth hormone and its receptor (Bass et al., 1991; Cunningham and Wells, 1989), the CD4 receptor and an HIV viral envelope glycoprotein (Ashkenazi et al., 1990), and the cholesterol dependent cytolysin PFO and cholesterol (Farrand et al., 2010).

### 2.1.2 Hemolysis Assay

VCC was first identified as a hemolysin in crude cell extracts due to its ability to lyse various species’ erythrocytes (Yamamoto et al., 1984). This function lends itself well to a hemolytic assay for the determination of VCC activity, and so it has become the procedure of choice for many researchers investigating this toxin.

When erythrocytes undergo lysis, the leakage of hemoglobin and other cell contents causes a marked decrease in sample turbidity, which can easily be measured as a factor of light scattering by any spectrophotometer (Jackson et al., 1970). In the case of pore forming toxins like VCC, the formation of only a handful of pores on a
single cell open up an aqueous channel that eventually causes the cell to burst as osmotic pressure builds (Zitzer et al., 1997b). This property of VCC can be used to generate a dose response curve, in which different concentrations of toxin are incubated with erythrocytes for a set period of time, after which hemolysis may be calculated as a factor of the change in turbidity of the sample. This yields a sigmoidal curve, indicating a cooperative binding process. From that curve, an HD$_{50}$ value, the concentration of VCC required to achieve 50% sample hemolysis after a given time period, may be calculated and compared to the HD$_{50}$ of other samples to determine if activity has been lost or gained.

2.1.3 Size Exclusion Chromatography

Size exclusion chromatography (SEC) is an HPLC method that separates molecules according to size and shape. It is commonly used in chemistry to study polymers, but also has applications in studies of proteins and other biomolecules. SEC has been used to assess the folding and aggregation of various proteins, and as an alternative to dialysis when changing a protein buffer (Barth et al., 1994).

An SEC column consists of a matrix of cross-linked gel beads, often dextran based, through which a solution containing the solute of interest is passed. The beads in the column contain small pores into which smaller molecules in the mobile solvent phase may diffuse. However, larger molecules that cannot fit into these pores are relegated to the interstitial solute and thus elute rapidly in the void volume of the column. The size of a solute dictates how long it will take to pass through the column. Smaller solutes may pass more freely into the bead pores and thereby access a greater
proportion of the total column volume, which causes them to travel through the column more slowly (Mori and Barth, 1999).

![Schematic of SEC separation of solutes by size](image)

**Figure 11: Schematic of SEC separation of solutes by size.** Adapted from (Striegel, 2009)

As a solute passes through the column, it remains in equilibrium between the interstitial solvent and pore spaces. The concentration gradient formed between these areas makes up the driving force that sends small molecules through the bead pores (Striegel, 2009). Species with a smaller molecular weight tend to travel through the column more slowly than larger counterparts, but the shape of a given molecule also plays a role in its rate of elution. For instance, denatured protein will travel through the column more slowly than its globular counterpart since its flexible and narrow shape allows it to sample more bead pores, despite both species having the same exact molecular weight (Mori and Barth, 1999). Thus, the utility of SEC in
determining precise molecular weights is limited; yet, this technique is still quite useful for assessing qualitative aspects of protein size and shape while yielding a purified sample.

2.1.4 Isothermal Titration Calorimetry

When two molecules associate with one another, they can form both covalent and noncovalent interactions, such as hydrogen bonds and salt bridges. The subsequent breakage and formation of these bonds and interactions will either consume or release energy in the form of heat. Reactions in which the products contain less energy than the reactants will radiate off the excess energy and therefore are classified as exothermic. Reactions in which the products are of a higher energy will take up this energy from their surroundings, resulting in an endothermic drop in ambient temperature.

The energy absorbed or released during a chemical reaction is known as a change in enthalpy (ΔH), which is positive in endothermic reactions and negative in exothermic reactions. Enthalpy is related to ambient pressure and the internal energy of a molecule. Equation 1 indicates the relationship between the change in enthalpy of a reaction (ΔH), to heat leaving or entering the system (q), the volume of the system (V), and the change in pressure (Δp) that may occur as a result of the reaction (Sheehan, 2009).

\[
\Delta H = q - (V \Delta p)
\]

Equation 1

At standard conditions when the pressure is held constant, this portion of the equation can be cancelled out and the enthalpy of a reaction may be directly measured by the change in temperature of the products compared to reactants:
\[ \Delta H = q \]

**Equation 2**

Isothermal titration calorimetry (ITC) measures the change in enthalpy resulting from the interaction of a protein and ligand, which can then be used to extrapolate the entropy and Gibbs free energy of the reaction. This is done by carefully measuring the change in heat caused by the addition of ligand to a protein sample. An ITC machine is set up such that a well filled with buffer is maintained at a reference temperature while precise amounts of ligand are injected at regular intervals into a second well containing protein. The machine detects the minute changes in temperature that are caused by the association of protein and ligand and adjusts the temperature of the protein well to match the reference well accordingly. The input energy required to maintain a constant temperature in the protein well is then converted into a measure of the heat released or absorbed during binding.

![Figure 12: Schematic of an isothermal titration calorimeter. Adapted from (Sheehan, 2009).](image)

During an ITC experiment, there are repeated injections of ligand into the protein sample. If the protein contains a saturable ligand binding site, then the heat change of reaction will be relatively high during the first few injections as ligand is binding to protein; but over time as the sites are saturated by the addition of more and
more ligand, the changes in heat will decrease. $\Delta H$ is calculated from the maximum change in heat observed after an injection of ligand into the sample. This data of heat changes is converted into a Wiseman plot, which can be used to calculate the binding constant ($K$) of a ligand to the protein of interest as a factor of the rate of binding saturation (Figure 13).

![Wiseman plot]

**Figure 13:** A sample set of ITC data (top graph) and its corresponding Wiseman plot (bottom graph). $\Delta H$ is calculated as the maximum change in temperature observed during an injection while $K_D$ is measured as the concentration of ligand at which half of binding sites are saturated. Adapted from (García-Fuentes et al., 2011).

The higher the rate of binding site saturation, the greater the binding constant, which corresponds to a strong and long-lived interaction between protein and ligand. The dissociation constant, $K_D$ is a ratio of the products of a reaction, the protein-ligand complex [PL], to the reactants, the unassociated protein and ligand [P][L], calculated according to the following equation:
\[
K_D = \frac{[P][L]}{[PL]}
\]

Equation 3

\(K_D\) can subsequently be used to calculate the Gibbs free energy (\(\Delta G\)) of a reaction according to the equation:

\[\Delta G = -RT\ln k_D\]

Equation 4

Combining the calculated \(\Delta G\) with a measured \(\Delta H\) and temperature (T) yields a value for the change in entropy of the reaction (\(\Delta S\)), calculated according to equation 5, providing a clearer picture of the thermodynamics of ligand binding.

\[\Delta G = \Delta H - T\Delta S\]

Equation 5

2.1.5 Liposome Leakage Assay

Due to their amphipathic nature, phospholipids in a polar solution spontaneously form vesicles, in which the lipids arrange themselves into a bilayer structure that curls around into a closed sphere such that the polar head groups shield all hydrophobic tails from solution. These structures form the basis of cell membranes and thus are useful tools for investigations on membrane dynamics and associated proteins. Such artificial vesicles are known as liposomes and generally come in three varieties: multilamellar vesicles (MLV), small unilamellar vesicles (SUV), and large unilamellar vesicles (LUV) (Lasic, 1988).

MLV are the most energetically stable liposome forms and consist of many bilayers enveloped around one another. These structures form spontaneously when a dry film of phospholipids is suspended in an excess of water or buffer by swirling or
vortexing. SUV consist of single bilayers encapsulating solvent ranging between 20 and 100 nm in diameter. These structures are typically formed by sonicating or passing an MLV sample through a Millipore extruder. LUV are larger unilamellar vesicles ranging from 0.1 to 1 μm in diameter and may be generated using detergent or solvent depletion techniques (Figure 14) (Lasic, 1988). These are the least energetically stable vesicles and tend to break apart into more stable SUV and MLV over time.

![Figure 14: Different types of liposomes and how they are formed. Adapted from (Pinheiro et al., 2011).](image)

Liposomes are a useful in studying membrane physiology and membrane associated proteins because their lipid and sterol contents can be carefully regulated. Furthermore, it is possible to load liposomes with any number of dyes and reporter molecules and measure fusion or permeabilization as a factor of dye leakage (Tokunaga et al., 1979). Liposome leakage assays have been used to probe the membrane permeabilization properties of various pore forming toxins, including α-hemolysin (Ikigai and Nakae, 1987) and VCC (Ikigai et al., 1996).

In a liposome leakage assay, vesicles are loaded with a fluorescence reporter molecule that emits strongly when encapsulated in a small space, while the external
solution contains a quencher molecule, which will eradicate the fluorescent properties of escaped dye. Thus, the rate of dye leakage can be measured as a factor of reduction in fluorescence. The maximal degree of leakage is generally taken as the change in fluorescence observed upon addition of a detergent such as Triton-x, which will break up larger lipid structures.

In this study, terbium (Tb) and dipicolinic acid (DPA) were used as a fluorescent donor/acceptor pair and EDTA as an external quencher. Terbium is a rare earth metal that forms a complex with three DPA molecules that fluoresces robustly at ranges over 470 nm when excited at 276 nm (Figure 15) (Glaser and Gross, 1994). EDTA acts as a Tb chelator that sequesters the metal away from DPA, thus quenching the fluorescent pair (Figure 16) (Wilschut et al., 1980). The reduction in fluorescence can thus be related to the degree of Tb and DPA leakage from vesicles into an EDTA solution as the vesicles are permeabilized by VCC.

![Figure 15: Terbium and DPA form a Tb(DPA)3 complex with fluorescent properties.](image1)

![Figure 16: EDTA in complex with Tb. EDTA has a higher affinity for Tb than DPA, so it will chelate Tb away from DPA, thus acting as a fluorescence quencher.](image2)
2.2 Methods in Practice

2.2.1 Generating Mutant VCC

I designed complementary 5’ and 3’ VCC primers containing the mutations of interest that were 39 to 45 bases long for each of the 29 selected mutations. These were sent to IDT (Integrated DNA Technologies) for synthesis. The primers were cloned into a vector containing the full-length VCC gene, pHis-Parallel2, using a quickchange site-directed mutagenesis kit from agilent and PfuUltra DNA polymerase. The reaction was confirmed by gel electrophoresis and products were transformed into *Escherichia coli* DH5α competent cells and cultured overnight at 37°C on ampicillin (AMP) plates. Single colonies were taken from these plates and grown in LB culture overnight at 37°C. The cultures were mini-prepped according to the AxyPrep plasmid miniprep vacuum protocol using an AxyPrep plasmid miniprep kit. Purified plasmid was then sent to the Science Hill DNA Sequencing Center at Yale for sequencing.

The presence of each mutation within the VCC gene was confirmed using ApE plasmid editor. Subsequently, plasmids were transformed into *E. coli* T7 shuffle competent cells and incubated overnight at 37°C on AMP plates. Single colonies from these plates were cultured in LB overnight at 37°C and saved as 50% glycerol stocks for future experiments.

2.2.2 Small Scale Expression of VCC

T7 shuffle competent cells containing the VCC plasmid of interest were cultured in 5 ml LB/AMP overnight at 37°C. This overnight was added to 50 ml LB/AMP culture and incubated with constant shaking at 37°C until the OD₆₀₀ had
reached approximately 0.6 (~4 hours), then 1 mM IPTG was added to induce VCC expression, and the culture was incubated with shaking for another 3 hours at 30° C. Following expression, samples were spun down in a swinging bucket centrifuge at 3900 g for 20 minutes and the pellets were resuspended in 1 ml TBS buffer (20 mM TRIS pH 7.5 150 mM NaCl).

2.2.3 Western Blotting of VCC

The 1 ml cell suspension was sonicated for 3 minutes (30 seconds on, 15 seconds off) using a Fisher 550 sonic dismembrator to lyse the cells. This sample was then spun down in a Sorvall rotor at 3900 g for 40 minutes at 10° C to pellet the lysate. 100 µl of supernatant was collected and 5 µl of this was run on an SDS-PAGE gel with a stained protein standard for reference. The protein bands were transferred to blotting paper and blocked in 10% milk powder overnight. This was washed in PBST buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Sodium Phosphate dibasic, 2mM Potassium Phosphate monobasic, 0.1% Tween-20, pH 7.4) several times before incubating in a solution of 4 ml PBST, 40 mg BSA, and 2µL anti-polyHistidine mouse antibody for 1 hour. Following more PBST washes, the blotting paper was incubated in a solution of PBST, BSA, and goat anti-mouse antibody for 1 hour and washed in PBST. Colorimetric detection of the goat antibody was performed using a Biorad Opti-4CN kit.

2.2.4 Purification of VCC

VCC was expressed in T7 shuffle competent cells as previously described with a 10 ml overnight for each 500 ml LB/AMP culture, and cells were resuspended in 5 ml TBS. To the sample were added 1 mg DNAase, 1 mM PMSF, 2.5 mM CaCl₂,
0.5 mM MgCl$_2$, and 10 mM imidazole, and it was passed through an Emulsiflex C5 High Pressure Homogenizer 3 times to lyse the cells. Lysate was centrifuged at 17,000 g for 40 minutes at 10° C in a Sorvall Rotor to pellet cell debris.

Following centrifugation, supernatant was collected and purified using the GE Healthcare AKTA purification system. 5 ml of sample was run over a 1 ml Ni-chelating column and washed with 7 ml TBS and 7 ml TBS with 40 mM imidazole. Purified VCC was eluted by a 7 ml wash of TBS with 250 mM imidazole. Protein integrity and purity was confirmed by SDS-PAGE gel.

### 2.2.5 Proteolytic Cleavage of VCC

VCC was proteolytically activated by incubating each sample at room temperature for 30 minutes in 10 mM CaCl$_2$ and 1:350 4 mg/ml chymotrypsin by volume. The reaction was terminated by the addition of an excess of 20 mM EDTA and 10 mM PMSF. Cleavage was confirmed by SDS-PAGE gel.

### 2.2.6 Hemolysis of VCC

A triplicate serial dilution of proteolytically activated VCC was incubated in a solution of 0.5% rabbit erythrocytes in blood dilution buffer (20 mM NaH$_2$PO$_4$, 150 mM NaCl, 1 mg/ml BSA, pH 7.4) in a 96-well clear bottom plate at room temperature (25°C). Light scattering data was collected from an iMark microplate reader at 595 nm every 15 seconds for 60 minutes, shaking for 1 second between measurements. Data at t=0 and t=30 minutes was extracted for analysis (Table 1, 2).
Table 1: Raw hemolysis data at t=0s

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<tr>
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<tr>
<td>A</td>
<td>0.44</td>
<td>0.432</td>
<td>0.427</td>
</tr>
<tr>
<td>B</td>
<td>0.398</td>
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<td>0.398</td>
</tr>
<tr>
<td>C</td>
<td>0.424</td>
<td>0.413</td>
<td>0.418</td>
</tr>
<tr>
<td>D</td>
<td>0.42</td>
<td>0.409</td>
<td>0.409</td>
</tr>
<tr>
<td>E</td>
<td>0.421</td>
<td>0.415</td>
<td>0.419</td>
</tr>
<tr>
<td>F</td>
<td>0.403</td>
<td>0.399</td>
<td>0.398</td>
</tr>
<tr>
<td>G</td>
<td>0.423</td>
<td>0.424</td>
<td>0.42</td>
</tr>
<tr>
<td>H</td>
<td>0.431</td>
<td>0.434</td>
<td>0.413</td>
</tr>
</tbody>
</table>

Table 2: Raw hemolysis data at t=1799s (30 min)

<table>
<thead>
<tr>
<th></th>
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<th>WT</th>
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<tbody>
<tr>
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<td>0.07</td>
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<tr>
<td>B</td>
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<td>0.056</td>
<td>0.062</td>
</tr>
<tr>
<td>C</td>
<td>0.119</td>
<td>0.098</td>
<td>0.118</td>
</tr>
<tr>
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<td>0.238</td>
<td>0.302</td>
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<tr>
<td>E</td>
<td>0.403</td>
<td>0.425</td>
<td>0.4</td>
</tr>
<tr>
<td>F</td>
<td>0.353</td>
<td>0.402</td>
<td>0.397</td>
</tr>
<tr>
<td>G</td>
<td>0.421</td>
<td>0.408</td>
<td>0.415</td>
</tr>
<tr>
<td>H</td>
<td>0.431</td>
<td>0.418</td>
<td>0.409</td>
</tr>
</tbody>
</table>

A measure of percent lysis after 30 minutes was calculated from the data at the two time points using Equation 6.

\[
\frac{T_0}{T_0} \frac{T_{30}}{100} = \% \text{ lysis (raw)}
\]

Equation 6

The raw percent hemolysis value was normalized to a maximum value of 100% using Equation 7.

\[
\frac{\text{max \% lysis}}{100} T_{30} = \% \text{ lysis (normalized)}
\]

Equation 7

The average percent lysis at each concentration of VCC in the triplicate experiment was plotted and fit to a sigmoidal function for calculating \( \text{HD}_{50} \) (concentration of VCC required to achieve 50% hemolysis after 30 minutes) using Kaleidagraph.
Figure 17: Representative hemolysis curve of wild type VCC. The formula for a sigmoidal curve fit is in the insert and is used to calculate the HD50 of the toxin, which is 0.453 nM in this case.

2.2.7 Size Exclusion Chromatography of VCC

VCC that had been purified over a Ni-chelating column was run through an S200 10/300 superdex size exclusion column on the GE healthcare AKTA purification system. Approximately 1 mg of protein was used for each run, and it was eluted in TBS buffer. As this was an analytical run, no fractions were collected, but the elution volume of each mutant VCC was determined from the UV absorbance of the eluent at 280 nm.

2.2.8 ITC of VCC

Purified VCC was run over an S200 16 60 size exclusion column on the AKTA purification system to extract a homogenous protein sample. Following elution of the protein, sample was concentrated to 3 mg/ml using Millipore Americon
Ultra Concentrators with a 50 kDa cutoff. 10 mg of the product was dialyzed for 24 hours in 20 mM TRIS pH 7.5 150 mM NaCl using SnakeSkin Dialysis Tubing with a 10 kDa cutoff.

A MicroCal VP-ITC from GE Healthcare was used to collect ITC data. 23 μM dialyzed VCC was added to the sample chamber, and 120 μM asialofetuin that had been dialyzed in 20 mM TRIS pH 7.5 150 mM NaCl buffer was added to the injections chamber. The experiment was run at 25°C using 50 injections of 5 μl asialofetuin. Data was analyzed using Nitpic (Keller et al., 2012), fitted using Sedphat, and the curves were generated using Gussi (Houtman et al., 2007).

2.2.9 Liposome Leakage Assay of VCC

Large unilamellar vesicles containing soybean asolectin (mixture of lecithin, cephalin, phosphotidylinositol, and other trace phospholipids) and 20 mol% cholesterol were made by dissolving 5 mg/ml asolectin and 20% cholesterol in butanol and drying overnight under vacuum in a desiccator until the lipids formed a thin film. The film was resuspended in TBS with 3 mM terbium, 3 mM sodium citrate, and 9 mM DPA. The sample underwent 15 freeze/thaw cycles before filtering through a 15 ml sepharose CL-6B column with 20 mM HEPES, 150 mM NaCl buffer to extract free Tb and DPA.

A triplicate serial dilution of proteolytically cleaved VCC was added to a 1:1 dilution of liposomes and TBS, along with 5 mM EDTA in a 96-well clear bottom plate. Fluorescence data was collected from a Spectramax M5 plate reader using an excitation wavelength of 276 nm and collecting emissions at 490 nm for 30 minutes at 25°C. Following data collection, liposomes were lysed with 1% Triton-x and the
fluorescence data was collected using the same parameters. Leakage of liposomes as a result of VCC pore formation was measured as a factor of decrease in fluorescence, with the 100% leakage taken as the fluorescence of the sample in 1% Triton-x in the same way that hemolysis data was processed.

2.2.10 Native Non-Denaturing PAGE

VCC WT, S357A, and Y421A were activated by proteolysis as previously described. Samples were run over a 10% PAGE gel in nondenaturing buffer (40 mM glycine, 5 mM TRIS, pH 8.3). After staining the gel with bromophenol blue, the location of each mutant VCC band was compared to WT VCC.
Chapter 3
Results
3.1 Selection of Residues

I selected residues of interest in the rim domain based on their degree of protrusion into solution on the monomeric and oligomeric crystal structures of VCC (De and Olson, 2011; Olson and Gouaux, 2005). This domain contains three loops (residues 234-243, 356-365, 417-430) that are of particular interest. Since these loops extend beyond the membrane insertion point of the $\beta$-barrel pore of the VCC heptamer crystal structure, they would either have to insert into the target membrane or splay out along its surface. Hence the loops are likely to come into contact with the membrane in some way (Figure 18). This study focuses on residues in these loops, chosen on the basis of surface exposure, charge, and polarity (Table 3).

<table>
<thead>
<tr>
<th>List of Mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>S234A</td>
</tr>
<tr>
<td>Y235A</td>
</tr>
<tr>
<td>T236A</td>
</tr>
<tr>
<td>L238V</td>
</tr>
<tr>
<td>D239A</td>
</tr>
<tr>
<td>F242A</td>
</tr>
<tr>
<td>R243A</td>
</tr>
<tr>
<td>R356A</td>
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<td>S357A</td>
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<tr>
<td>T358A</td>
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<tr>
<td>D359A</td>
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<td>T365A</td>
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<td>H419A</td>
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</tr>
<tr>
<td>V423A</td>
</tr>
<tr>
<td>H426A</td>
</tr>
<tr>
<td>Q427A</td>
</tr>
<tr>
<td>S428A</td>
</tr>
<tr>
<td>Y429A</td>
</tr>
<tr>
<td>H430A</td>
</tr>
<tr>
<td>L238A</td>
</tr>
</tbody>
</table>

Table 3: List of Mutations
**Figure 18:** VCC pore with rim region loops of interest in orange. These extend below the membrane insertion line (cyan) on the pore (yellow) and thus may interact with the target membrane during pore formation. I selected residues in these loops for mutagenesis.

**Figure 19:** Sequence of the VCC cytolsin domain. The cytolsin residues are marked in blue, the stem is yellow and the mutated residues are marked in red. Grey residues were not tested, either because they were already alanine or glycine (A240, A360, G424, A425), had been tested in a previous study to no effect (T237A), or could not be expressed in time (W362A).
3.2 Overview of Experimental Results

I sought to systematically assess the structure and activity of each of the selected mutants using a multistep approach. First, I expressed small volumes of each mutant and measured the levels of soluble protein by western blot. Then, larger volumes of protein were expressed and purified using nickel affinity chromatography. Following purification, the function of each mutant was tested using a hemolysis assay. Finally, I ran protein samples through a size exclusion column to assess the homogeneity and structural integrity of each mutant. Functionally impaired mutants were further characterized by ITC, liposome leakage assay, and non denaturing PAGE. An overview of these results is pictured (Table 4), followed by detailed descriptions of each step.
Table 4: Overview of mutations and data (+: good result, o: poor result, -: no result/expression)

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Western expression</th>
<th>Ni column</th>
<th>HD_{50} (nM)</th>
<th>StDev HD_{50}</th>
<th>x/WT</th>
<th>StDev x/WT</th>
<th>Cleavage</th>
<th>SEC (mL)</th>
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<tbody>
<tr>
<td>S234A</td>
<td>+</td>
<td>+</td>
<td>0.44</td>
<td>0.27</td>
<td>2.00</td>
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<td>27.92</td>
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</tr>
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<td>+</td>
<td>0.21</td>
<td>0.04</td>
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<td>0.20</td>
<td>+</td>
<td>30.99</td>
</tr>
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<td>3.94</td>
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<td>151</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
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<tr>
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<td>0.00</td>
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<td>0.08</td>
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<td>0.04</td>
<td>1.78</td>
<td>0.26</td>
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<td>H430A</td>
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<td>0.00</td>
<td>1.77</td>
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<td>17.61</td>
<td>2.05</td>
<td>+</td>
<td>24.53</td>
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</tbody>
</table>
3.3 Cloning of VCC Mutants

I designed primers containing the mutations of interest and sent them to be synthesized at IDT. These primers were then cloned into the pHis-Parallel2 plasmid using quickchange site-directed mutagenesis to replace the wild type VCC genomic sequence with the mutated primer. Results of the PCR were confirmed on a DNA agarose gel (Figure 20) to ensure that full-length plasmid was present in the product. The PCR product was then transformed into a line of DH5α E. coli competent cells, mini prepped to extract the plasmid, and sequenced to confirm the successful introduction of each mutation. Extracted and purified plasmid was transformed into a line of T7 shuffle E. coli competent cells for growth and expression of mutant VCC.

Figure 20: Agarose gel of quick-change PCR product. A number of quickchange PCR reactions (lanes 3-12) incorporated the new primers into plasmid template. Successful PCR reactions contain plasmid product that is approximately the same size as the template (lane 2), which is about 8000 bp. The few extra bands around 2000 bp are byproducts that did not affect cloning.

3.4 Expression of Mutant VCC

Prior to commencing large-scale expression and purification, I aimed to determine the expression levels of soluble protein for each mutant of VCC to confirm the viability of the protein. Improperly folded mutants should be structurally unsound and form aggregates that crash out of solution. Hence, the amount of soluble protein serves as a preliminary indicator of robust folding. To do this, I expressed the VCC mutants in T7 shuffle E. coli and ran the soluble cell lysate on a western blot of the
His-tagged protein. The presence of a dark band around 80 kD indicates the presence of soluble VCC (Figure 21).

Results of the western blots are listed in the appendix (Figures 49-52). Most of the mutants expressed soluble protein, with a few notable exceptions. The initial blotting of N364A did not reveal any soluble protein, although a small but distinct band of protein was present in a repeated experiment. Y417A and K418A both had exceptionally low levels of soluble protein on the western blot. This suggests that these mutations are producing inherently unstable protein that may aggregate in the lysate pellet or be cleaved by proteolysis in vivo.

![Image of western blot](image)

**Figure 21: Representative western blot of soluble VCC.** *E. coli* expressing VCC was lysed and the supernatant of the lysate run over an SDS-page gel and transferred to a western blot to stain for his-tagged protein. The presence of strong bands around 80kD indicates that these cell lines expressed significant, albeit variable levels of soluble VCC. For comprehensive western blotting data, see appendix, figures 49-52.

### 3.5 Purification of VCC Mutants

Following this preliminary confirmation that the VCC mutants could be expressed as soluble protein, I grew, expressed, and purified each mutant in a high throughput manner. The T7 shuffle *E. coli* containing each mutant plasmid were grown and expressed to a total volume of 500 ml. Each resuspended pellet was lysed using a cell homogenizer and centrifuged at high speed to pellet lysate, which was run over a nickel-affinity column and eluted in 250 mM imidazole tris buffer. A sample run is shown in Figure 22.
Figure 22: Ni affinity column run of WT VCC. VCC eluted as a robust peak around 22 mL after washing the column with 250mM imidazole tris buffer.

Most of the mutants had a reasonably high yield of protein from the nickel affinity purification. However, a number of mutants were poorly expressed but eventually yielded testable amounts of protein. These included N364A, Y417A, Q427A, and Y429A. Examination of the results of the western blots reveals that these mutants also had fairly low levels of soluble protein in the unpurified lysate. The only mutant that did not express at any appreciable level through nickel affinity chromatography was K418A. So, it was not possible to assay the activity of this mutant, although these issues with purification suggest that the mutant is probably structurally destabilized.

3.6 Activation of VCC by Proteolytic Cleavage

In order to test the function of each mutant VCC, the pro-domain of the protein had to be cleaved by chymotrypsin to yield a functional monomer. The proteolysis product was run over an SDS-PAGE gel to confirm that the cleavage was
successful. Uncut VCC appears as a band around 80 kD, while cleaved VCC shifts to approximately 60 kD. In a successful proteolysis reaction, all of the protein present in the purified sample should shift to 60 kD, indicating the presence of fully activated protein without any impurities (Figure 23).

**Figure 23: Representative proteolysis of VCC.** Uncut VCC appears as a strong band around 80 kD, as evidenced by uncut R243A, uncut F242A, and WT VCC (lanes 2, 4, and 6). Upon incubation with chymotrypsin, this band shifts to around 60 kD, as seen in the cut R243A and cut WT samples (lanes 1 and 5). However, the cut F242A (lane 3) appears as three smaller bands that indicate that this mutant is being overcut and may be structurally unstable. For comprehensive data, see appendix figures 53-62.

Most of the mutants underwent successful and efficient cleavage by chymotrypsin (Figures 53-62). Notably, F242A exhibits an unusual cleavage pattern. Uncut F242A occurs as a strong band around 80 kD, as would be expected for full length VCC. However, upon incubation with chymotrypsin, this band is fragmented into a number of faint proteolysis products that indicate that the protein is being excessively cleaved. Such a result suggests that this mutant is structurally unstable and hence more susceptible to promiscuous proteolysis by chymotrypsin.

3.7 Functional Assessment of VCC by Hemolysis Assay

A hemolysis assay was used to assess the function of each mutant version of VCC against rabbit erythrocytes. A serial dilution of cleaved VCC was incubated with 0.5% rabbit blood for 30 minutes and the light scattering of the sample was converted to percent lysis. The results were plotted on a sigmoidal curve of [VCC] versus percent hemolysis and the HD$_{50}$ of lysis was used as a measure of toxin.
efficacy by comparing it to the HD$_{50}$ of wild type VCC (Figure 24, appendix figures 63-68). The loss of function of each mutant is plotted in Figure 25.

**Figure 24: Hemolysis curve of WT VCC and selected mutants.** The concentration of VCC required to achieve 50% lysis has been shifted to higher values for the mutants S357A, Y420A, and Y421A, indicating a loss of toxin efficacy. In this case, the over 100-fold shift indicates that these mutations may play an important role in VCC function. For comprehensive data, see appendix figures 63-68.
Figure 25: Loss of function of each mutant tested. The HD$_{50}$ of each mutant was compared to that of WT VCC to determine the loss of function. K418A expressed poorly, and thus was untested. Most residues had a modest change in function compared to WT, with notable exceptions in S357A, Y420A, Y421A, Q427A, and a few others.

Most of the mutants in the first loop (residues 234 to 243) did not display a loss of function in excess of 10-fold, aside from F242A, suggesting a relatively unimportant role for this region. More dramatic losses in function occur in the second loop (residues 356 to 365). In particular, S357A showed an approximately 150-fold loss of function, while D359A and N364A had more modest yet still significant 25-fold and 35-fold losses of function, respectively. The third loop tested (residues 417 to 430) contained the greatest reductions in function. In particular, Y420A and Y421A had losses of function in excess of 200-fold, while H426A and Q427A were
also impacted 45-fold and 80-fold, respectively. A closer examination of the structure of the rim loops of the VCC heptamer (Figure 26) reveals that this loop protrudes especially far into solution, and hence may be inserted into the membrane during pore formation.

Figure 26: A closer look at the mutated loops of the rim domain. Loop 1 (residues 234 to 243) is marked in yellow, loop 2 (residues 356 to 365) is marked in green, and loop 3 (residues 419 to 430) is marked in red. All three loops protrude into space from the main body of the rim region, but loop 3 is the longest and most exposed of these regions and coincidentally also contains the highest number of extremely impacted mutants.

When these mutants are mapped to the crystal structure of the VCC pore and colored according to their loss of function (Figure 27), a number of salient features become apparent. There is a patch of functionally important residues in the middle of loop 3, which includes Y420A and Y421A, among others. Due to its position on a highly protruding loop and the hydrophobic nature of the impacted residues in this area, it is possible that this region is inserted into the bilayer during pore formation. The aforementioned pair of highly impacted tyrosines in particular could exist comfortably near the surface of the bilayer due to these residues’ aromatic yet slightly polar character.

Another highly impacted mutant, S357A, resides in a different part of this region. It is located midway up loop 1 and does not protrude into solution in a manner
that would favor membrane insertion, particularly since other residues located further down this loop are not as functionally impacted. Due to its polar nature, serine does not typically partition into membranes; yet, this residue may be involved in coordinating some as-yet unidentified ligand.

**Figure 27: Loss of function of VCC residue mutants.** The hemolysis data was plotted to a surface representation of the crystal structure of VCC and colored according to the loss of function such that red residues have the greatest loss of activity, while white residues have the same level of activity as WT and grey residues were not able to be tested.

### 3.8 Assessment VCC Homogeneity by Size Exclusion Chromatography

In order to confirm that the losses in activity measured by hemolysis assay were due to changes in the functionality of VCC rather than its overall folding and structural integrity, I ran each sample through a size exclusion column. This chromatography technique separates proteins by their apparent size to yield a more homogenous sample than may be collected from nickel affinity chromatography. Importantly, well-folded, homogenous samples will elute as a single, well-defined peak while misfolded and aggregated proteins will elute as a collection of smaller, polydisperse peaks. Thus, this technique serves as a good high-throughput preliminary assay of mutant structural integrity.
Each VCC sample was concentrated to its solubility limit of 3 mg/ml, 1 ml of this solution was loaded into an S200 superdex size exclusion column, and the column was washed with TRIS buffer to elute the protein. UV absorbance spectra of the eluted solution reveals the presence of protein as a sharp peak in absorbance. Due to its glycan binding activity, WT VCC interacts with the sugars in the column matrix and elutes around 28 ml rather than 15 ml, as it would be expected to elute according to its molecular weight in the absence of sugar binding activity. The majority of the mutants also eluted as single, well defined peaks (Figure 28, appendix figures 69-75), which suggests that their structural integrity has not been compromised. Notable exceptions included Y417A and K418A, which had polydisperse spectra (Figures 29 and 30). Since these proteins were poorly expressed in the western blot, it is likely that these mutations yielded unstable protein.

![Figure 28: Monodisperse SEC result for Y235A.](image)

The protein elutes from SEC as a single, sharp peak around 24 ml, with no significant byproducts. This is the hallmark of a well folded, homogenenous sample.
Figure 29: Polydisperse SEC result for Y417A. The large number of overlapping peaks in the spectrum suggests that this protein is probably aggregated and/or misfolded.

Figure 30: Polydisperse SEC result for K418A. The SEC spectrum for K418A, much like that of Y417A, contains many overlapping peaks instead of a single, sharp peak, suggesting protein aggregation.

Although most of the mutants eluted as single, well-defined peaks, there is a wide range of variability in the elution volume of these peaks. Some mutants eluted as much as 10 ml earlier than WT, although they still appeared as single, well defined peaks and an SDS-page gel confirmed that these peaks are indeed full length VCC.
On the other hand, other mutants eluted exceptionally late, yet still appeared to contain structurally homogenous protein (Figure 31).

![SEC of Selected VCC Mutants](image)

**Figure 31: Wide range of SEC elution volumes.** SEC spectra of WT VCC and several mutants reveal a wide variation in elution volumes. WT VCC (green) elutes relatively late given its molecular weight since it is known to interact with the column matrix. Most of the mutants, such as S234A (red) eluted around the same location as WT, indicating no real change in the overall shape and structure. However, some mutants eluted exceptionally early, like Y421A (blue), while others eluted even later than WT, like T358A (purple).

I mapped the change in elution volume to the crystal structure of VCC to see if there were any trends apparent in which mutants much eluted earlier or later than WT (Figure 32). Notably, a large patch of early eluting mutants occurs on loop 3, an area of the rim domain that also contained the highest number of functionally impacted mutants, while all of the threonine mutants tested eluted exceptionally late. Although many of the mutants that eluted early appeared to be functionally unaffected, all of the most highly impacted mutants eluted exceptionally early on the
SEC. This suggests that there may be some kind of association between elution volume and activity, although this is probably not a direct correlation.

Figure 32: Mutated residues colored by SEC elution volume (surface representations). Mutation of residues colored in red resulted in protein that eluted much earlier than WT, purple residue mutants eluted around the same time as WT, and blue residue mutants eluted later than WT.

Given the apparent connection between the hemolysis and SEC results, I sought to conduct further tests to assess the liposome permeabilization activity, sugar binding activity, and oligomerization state of a subset of the rim mutants. The mutants selected for detailed analysis were a representative assortment of the variation in SEC and hemolysis results that passed the aforementioned quality control tests. So far, only the highly inactive and early eluting mutants S357A and Y421A have been tested using a liposome binding assay, ITC, and non-denaturing PAGE. These are part of a larger planned set, which includes mutants that are active on hemolysis but elute early on SEC, mutants that elute late on SEC, and mutants that elute around the same time as WT on SEC but are inactive by hemolysis.
3.8 Liposome Leakage Assay Results

Inactive mutants Y421A and S357A were tested on 20% cholesterol asolectin liposomes filled with fluorescent reporters terbium and DPA in a solution containing EDTA. The degree of liposome leakage was measured as a factor of the decrease in fluorescence (Figure 33). A control of the liposome assay was performed using a variant of VCC with an engineered disulfide bond blocking pore formation that had no effect on liposome sample fluorescence. Reducing these bonds allows VCC to rapidly form functional pores on the liposomes, resulting in a decrease in sample fluorescence, therefore proving that changes in liposome fluorescence observed are caused by VCC pore formation (Figure 34). Although the liposome experiment was performed on soybean asolectin liposomes rather than the same bovine brain lipids as the control experiment, a subsequent liposome assay using mutant VCC was performed using brain lipids with the same result.

Although Y421A and S357A retained less than 1% of WT activity on rabbit erythrocytes, they appear to have the same degree of permeabilizing activity as WT VCC on the liposomes, though much higher concentrations of toxin were required to achieve appreciable leakage. This suggests that the mutations do not disrupt VCC’s ability to form functional pores and are not involved in directly binding lipids or cholesterol in the membrane.
Figure 33: Liposome assay of VCC WT, S357A, and Y421A. While these mutants retain less than 1% WT activity on rabbit erythrocytes, they appear to have the same degree of activity on asolectin/cholesterol liposomes as WT.

Figure 34: Liposome assay control experiment. C3C7 VCC with disulfide bonds blocking pore formation does not cause liposome leakage (red), but reducing these bonds with DTT causes a rapid decrease in fluorescence, indicating pore formation and liposome leakage (blue). Data collected by Swastik De.
3.10 Assessment of VCC Mutant Sugar Binding by ITC

The SEC profile of WT VCC is complicated by how the toxin’s carbohydrate binding activity causes it to interact with the column sugars and elute later than predicted by the molecular weight standard of the column. Given the unusual shifts in SEC elution volume observed among some of the VCC mutants tested, it is possible that the sugar binding ability of these mutants had been compromised in some way. In order to examine this possibility, S357A and Y421A were tested by ITC to assess their binding affinity for the glycoprotein asialofetuin. WT VCC binds asialofetuin with a $K_D$ of approximately 329 nM (236 to 448 nM range, $p=0.95$) using a 1:1 stoichiometry model. When the mutants S357A and Y421A, which had less than 1% WT activity in a hemolysis assay and eluted earlier than WT on SEC, were tested using ITC, they displayed similar affinities for asialofetuin as WT VCC, with a $K_D$ of 236 nM (104 to 455 nM range, $p=0.95$) and 1.13 μM (1.04 to 1.24 μM range, $p=0.95$), respectively (Figure 35).

The relatively minor 4-fold change in sugar binding activity is far out of proportion with the greater than 100-fold changes in hemolytic activity. This suggests that the changes in hemolytic activity caused by mutations in the rim domain are not the result of impaired carbohydrate binding and that the mutants are able to interact with the SEC column matrix as effectively as WT.
Figure 35: ITC of WT VCC, S357A, and Y421A binding to asialofetuin. WT binds the glycans on this protein with a $K_D$ of 329 nM, S357A binds with a $K_D$ of 236 nM, and Y421A binds with a $K_D$ of 1.13 μM, according to a 1:1 binding stoichiometry model. Data collected by Swastik De.

3.11 Non-denaturing PAGE of Mutant VCC

Following confirmation of the sugar binding activity of Y421A and S357A, the oligomerization state of each mutant was probed using non-denaturing PAGE gel. Running protein under these conditions should maintain any oligomeric structures that form, unlike a typical denaturing SDS-PAGE experiment. Y421A, S357A, and WT VCC were activated by proteolysis and run over a non-denaturing PAGE. All samples ran to about the same location on the gel, which suggests that neither mutant has changed shape or size in a significant manner (Figure 36). There is a second band that appears in uncut S357A and Y421A. But since this disappears when the samples undergo proteolysis, it is possible that the band corresponds to VCC with its prodomain intact and that the WT sample does not have that band because it has already undergone spontaneous prodomain proteolysis prior to chymotrypsin cleavage.
Figure 36: Native PAGE of WT VCC, Y421A, and S357A. All protein samples appear as equivalent bands, suggesting that the overall structure and oligomerization state of Y421A and S357A are unchanged. Data collected by Jinsol Hyun and Swastik De.
Chapter 4
Discussion
The rim loops of VCC are a prime candidate for mediating toxin-membrane interactions due to their location adjacent to the pore stem in the crystal structure of the VCC heptamer. Since VCC is known to be sensitive to levels of cholesterol in the membrane, it is possible that these rim loops contain a cholesterol binding site. This study was aimed at characterizing the role of the VCC rim region in membrane binding and pore formation in the hopes of identifying a specific binding site for cholesterol or a new ligand. Such information would shed further light on the targeting specificity of VCC and potentially reveal the mechanisms that regulate the monomer to pore conversion of VCC and related pore forming toxins.

Alanine scanning mutagenesis reveals a number of residues in the rim loops of VCC that severely impact hemolytic activity when mutated, although expression, proteolysis, and SEC results suggest that their overall structure has not changed. Furthermore, SEC experiments suggest a correlation between loss of function and early elution volume compared to wild type toxin. Based on this data, it is possible that mutations in the rim region prevent VCC from interacting with the sugars in the column the way WT does, or that these mutations are triggering oligomerization in solution. Additionally, many of the functionally essential mutants are localized in a potential ligand binding surface, which could be involved in binding lipid, sterol, or polypeptide targets. Overall, the data suggests that the rim region of VCC plays a key role in mediating membrane interactions of the toxin, although the specifics of that role are up for speculation.
4.1 Mapping of Hemolytically Deficient VCC Mutants

Mapping the changes in function of the mutants tested to the crystal structure of VCC reveals that the majority of these residues are not functionally essential (Figure 37). Sixteen of the mutations tested caused a less than 10-fold loss of function. This constitutes the majority of the list, and although these changes in function may have greater implications in VCC’s mechanism of activity, they seem relatively modest compared to the remainder of the data set. On the other hand, a few mutational “hot spots” appear, in which mutating single residues dramatically reduced VCC activity. Six mutations caused a greater than 50-fold loss of function, resulting in toxin that retained less than 2% of wild type hemolytic activity. This dramatic loss of activity suggests that these residues play some vital role in VCC structure, folding, or function.

![Figure 37: Hemolytic data mapped to the crystal structure of VCC rim, surface (A) and side chain representations (B). A deeper red color denotes a greater loss of function, grey residues were not tested. Most residues have relatively minor changes in function, but a few yielded dramatic losses in function when mutated, most notably S357, Y420, and Y421. Y417A and K418A expressed poorly in western blots and nickel-affinity purifications and had polydisperse SEC profiles. F242A underwent excessive](image-url)

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proteolysis during activation of the toxin, though it expressed well on a western blot and had a monodisperse SEC profile. These three mutants retained less than 5% of WT activity, but since they did not pass all of the quality control assessments it is likely that Y417, K418 and F242 play an important role in mediating the structural integrity of the rim region or folding of VCC, rather than participating in a binding site or the pore formation mechanism. A closer look at their location in the crystal structure of the VCC heptamer reveals that these residues face towards the interior of the protein, particularly into the cavity formed between loops 1, 2, and 3 (Figure 38).

Figure 38: Poorly expressed hemolytically impacted mutants (grey) tend to face towards the cleft formed between loops 1, 2, and 3 of the rim region (orange). F242A, Y417A, and K418A retain less than 5% of WT activity, but also display aberrant expression and cleavage profiles. This suggests that they may play a role in stabilizing the rim domain loop conformation.

Of the remaining mutants, S357A, D359A, N364A Y420A, Y421A, H426A, and Q427A had dramatic reductions in function, retaining less than 5% of WT activity in a hemolysis assay. Q427 and N364 are positioned towards the core of the rim region, much like Y417, K418 and F242. Although mutating these residues did
not compromise the structural integrity of VCC, their location in the crystal structure suggests that they may play a role in stabilizing the conformation of the rim loops, but perhaps one that is not essential for producing well-folded protein overall (Figure 39).

A closer look at S357, D359, Y420, Y421, and H426 on the crystal structure of the VCC heptamer places them all in fairly solvent exposed conformations that are much less likely to be involved in stabilizing rim domain structure. Thus, these mutants are particularly good candidates as functionally important residues. It is interesting to note that all of these residues all face towards the β-barrel pore in a vaguely defined line (Figure 40).

Figure 39: N364 and Q427 face towards the core of the rim region, much like the structurally important residues Y417, K418, and F242.
Figure 40: Well expressed but inactive mutants (red) are all solvent exposed and positioned in a line facing the β-barrel pore (yellow) around or below the aromatic ring of putative membrane inserted residues (cyan). S357A, D359A, Y420, Y421A, and H426A have less than 5% WT activity, yet are well expressed, undergo efficient proteolysis, and elute on SEC as single peaks. S357A, Y420A, and Y421A are particularly inactive mutants with less than 1% WT activity that seem to play a vital role in VCC pore formation.

Given the distinctive locations of the most functionally impacted mutants, S357, D359, Y420, Y421, and H426 may constitute a ligand-binding surface or otherwise stabilize membrane insertion of the rim domain prior to pore formation. Y420, Y421, and S357 appear to be particularly important for VCC function, as mutating each of these residues produces a mutant with less than 1% the activity of WT.

4.2 SEC Results

While assessing the structural homogeneity of each mutant VCC via SEC, it was interesting to note the wide variation in elution volumes for each protein. Although their molecular weights were essentially unchanged, a number of mutants eluted as much as 10 ml earlier than WT VCC, while others eluted even later than WT. Notably, many of the most hemolytically inactive mutants, including S357A,
Y420A, and Y421A eluted exceptionally early on the SEC column. Plotting the change in activity against the SEC elution volume of each mutant reveals this correlation (Figure 41).

![SEC vs Hemolysis of VCC mutants](image)

**Figure 41: Comparison of SEC elution volume and loss of hemolytic function** of rim region mutants reveals a correlation. The most inactive mutants elute almost exclusively early, while mostly active mutants have a much wider range of elution volumes. Shown for comparison are elution volumes for WT VCC and D617K, a mutant VCC that loses glycan binding affinity.

Given the very minor changes in the composition of each mutant, such dramatic shifts in behavior are quite unexpected and suggest that the rim region may play a far more complex role in VCC pore formation than postulated. Since sizing columns separate molecules based on size, samples that elute early are doing so because they exist as larger species, have a shape that does not fit though the pores in the bead matrix as freely, or do not bind to the column as well. The carbohydrate binding activity of VCC complicates SEC experiments, since the toxin binds to the dextran-based column beads and elutes at 28 ml rather than at 15 ml, where it would be predicted to come out based on the molecular weight standard for the column used.
The mutants that elute earlier than WT VCC thus actually come much closer to the predicted elution volume that discounts any additional matrix interactions. So, it is possible that mutations in the rim region have somehow compromised VCC’s ability to bind carbohydrates.

ITC experiments measuring the affinity that VCC has for the glycoprotein asialofetuin reveal that the functionally impacted and early eluting mutants S357A and Y421A have not lost sugar binding activity relative to WT protein. This implies that the reason these mutants elute early is not due to defects in sugar binding. Since the carbohydrate binding activity of VCC is localized to the β-prism domain, it seems highly unlikely that a single point mutation in a completely different part of the protein could affect the affinity of a binding pocket in this domain. It is possible that there may be a second, lower affinity binding site in the rim region that interacts with the sugar-based SEC column and that the activity of this hypothetical site could be masked on ITC by robust glycan binding in the β-prism domain. But since truncating the β-prism domain causes VCC to lose all sugar binding activity on a glycan screen and ITC and elute much earlier on SEC, it is unlikely that the rim region contains a binding site specific to carbohydrates (Levan et al., 2013).

Another reason that mutant VCC runs through the SEC column faster could be because its size or shape have changed dramatically. The simplest explanation for this would be that the mutations are causing VCC to misfold into a larger, more loosely packed species. However, misfolded proteins will generally occur as heterogeneous species or aggregate. Such phenomena would yield polydisperse SEC profiles or large peaks of aggregated protein in the void volume. The SEC profiles of mutants that
elute before WT VCC are still monodisperse peaks that are indicative of homogeneous and soluble samples. Additionally, many of these mutants still retain normal function, which would probably be disrupted if some misfolding had occurred.

Alternatively, it is possible that residues in the rim region contain a trigger for oligomerization that is linked to membrane binding. Thus, mutants may elute early because they exist as larger multimers in solution. Figure 41 reveals that there are distinctive clusters of mutants that all elute around the same volume on SEC, including 4 mutants around 19 ml, 6 mutants around 21 ml, 9 mutants around 24 ml, 3 mutants around 28 ml, and 2 mutants around 31 ml. It is possible that the mutants in each of these clusters may affect the oligomerization state of soluble VCC to a different degree.

To test this, Y421A and S237A were run over a nondenaturing PAGE to compare their apparent size and charge to WT. The mutants appear as bands at the same location as WT, which suggests that their oligomerization state and overall structural properties remain unchanged. However, it is possible that the mutants may be in some sort of equilibrium between monomeric and oligomeric states that would not appear on a native PAGE. Furthermore, the high salt buffers used for the experiment could cause loosely interacting multimers to dissociate. Thus, it would be prudent to collect more accurate data on the oligomerization state of each mutant using analytical ultracentrifugation that can probe oligomers formed in solution rather than in a PAGE gel matrix.

Although there exist a number of logical explanations for the reason that some proteins may elute earlier than expected on a sizing column, perhaps the most
puzzling result is that some VCC mutants elute even later than wild type protein. This would imply that modifying certain side chains creates a species that can interact with the column more effectively in some way; yet these mutants all appear perfectly functional on a hemolytic assay. Replacing threonines in the rim loops with alanine in particular seems to yield mutants that elute later on SEC. Perhaps there is something about removing the polar group of threonine that changes the way VCC interacts with SEC.

The three residues that cause VCC to elute after WT have solvent exposed side chains. This suggests that these residues do not play a major role in stabilizing the conformation of the rim loops (Figure 42). Furthermore, the mutation T358A causes VCC to elute exceptionally late on SEC at around 35 ml, and since this residue lies within the putative ligand binding surface of VCC, it is possible that it may be involved in stabilizing this site. Alternatively, if this surface contains an oligomerization trigger, the T358A mutation might keep VCC from forming oligomers in solution.

Figure 42: Location of mutants that elute late on SEC. T236, D239, and T358 (red) all elute after WT on SEC. Interestingly T358A, the latest eluting mutant, is directly adjacent to S357A, one of the most functionally impaired mutants.
4.3 Nonspecific Membrane Insertion

Although VCC shares a common central fold with α-hemolysin, the loops in its rim region are unusually long and extend beyond the putative membrane insertion point of the β-barrel pore, which is defined by the ring of aromatic residues including E289 and Y313. A comparison to the crystal structure of α-hemolysin yields little structural homology in the rim region loops (Figure 43). In the case of α-hemolysin, its rim domain loops project far enough into solution to potentially interact with the surface of the target membrane. On the other hand, VCC’s rim loops protrude enough that they could penetrate deep into the membrane, and the large number of aromatic residues in this region would exist most comfortably at the surface of the bilayer (De and Olson, 2011).

![Figure 43: Comparison of the rim domain (red) of α-hemolysin (A) and VCC (B). Although the overall cytolysin domain fold of both toxins is homologous, the rim domain loops of VCC extend far below the membrane insertion point of the pore (cyan) than those of α-hemolysin and into the membrane (grey). Note, the β-prism and β-trefoil domains of VCC are not pictured.](image)

Given their location on the toxin heptamer, it is highly possible that the rim region loops of VCC may interact with the target membrane in a nonspecific manner.
Many of the residues in this region found to be important for VCC function are tryptophans, which due to their partially polar and aromatic character preferentially exist at the surface of the cell membrane (Figure 44). These residues could play a key role in stabilizing the membrane-inserted form of the rim domain, which could explain why many of the mutations tested in the rim region caused modest, yet noticeable, decreases in toxin hemolytic activity.

![Aromatic residues](image)

**Figure 44: Energetics of membrane insertion of aromatic amino acids** reveals their preference for membrane interfaces. However, there is a large penalty for inserting all of these side chains, except for phenylalanine, into the hydrophobic core of the membrane. Adapted from (von Heijne, 2006).

On the other hand, the highly dramatic changes in function that result from mutating certain single residues seem to be out of proportion with a nonspecific model of membrane interaction. A serine residue, S357, is among the most functionally important residues in the rim; but its polar character means that it is unlikely to insert into a lipid bilayer favorably. It is uncertain what role this residue could possibly play in nonspecific membrane insertion.

A comparison of the change in partitioning coefficient incurred by mutating each amino acid in the rim region to alanine with their hemolytic function and SEC elution volume yield apparently random distributions (Figures 45 and 46).
Furthermore, the most functionally impacted residues incurred very minimal changes in side chain hydrophobicity as a result of mutation, suggesting that this nonspecific effect does not play a major role in the dramatic changes in function observed. All of the most impacted mutants tend to have slightly negative partitioning coefficients, meaning that they would likely exist close to the membrane interface. This trend suggests that whatever function these residues participate in is localized to the surface of the target membrane.

Although there is no distinctive correlation between side chain hydrophobicity and changes in function, the possibility of a largely nonspecific model of membrane interaction would be difficult to rule out by its very nature. The crystal structure of the VCC heptamer was solved in the presence of detergent solubilized micelles, so it is not certain whether the conformation of the rim domain loops observed in this structure reflects the *in vivo* state of VCC.

![Function vs Change in Hydrophobicity](image)

**Figure 45: Function versus change in hydrophobicity.** Plotting the change in hemolytic function of VCC mutants against ΔG of membrane insertion incurred by an alanine mutation does not yield any particular correlation. ΔG values come from (Wimley and White, 1996).
Figure 46: SEC versus change in hydrophobicity. Plotting the various SEC elution volumes of each mutant against the ΔΔG of membrane insertion incurred by an alanine mutation similarly does not yield any visible correlation.

4.4 Ligand Binding Sites

The most functionally important residues in the rim region of VCC, S357, D359, Y420, Y421, and H426, are localized in such a way that they might constitute a ligand-binding surface. This theory is not without precedent, since binding sites have been identified in the rim regions of several other β-pore forming toxins, including leukocidin (Olson et al., 1999), NetB (Savva et al., 2013), and α-hemolysin (Galdiero and Gouaux, 2004). Leukocidin F and α-hemolysin were crystallized in the presence of PC and clearly reveal a PC binding pocket within the rim domain (Galdiero and Gouaux, 2004; Olson et al., 1999). In these cases, the lipid is coordinated by conserved tryptophan and arginine residues (Figure 47). However, the primary loop involved in coordinating the PC moiety is absent in VCC (Figure 48).
Furthermore, the presence of PC inhibits VCC pore formation (Zitzer et al., 2001), so it is unlikely that the essential residues identified in the rim region of VCC are involved in a PC binding site.

Figure 47: Crystal structure of Leukocidin F in complex with PC. The lipid is coordinated primarily by a conserved tryptophan and arginine (shown in red).

Figure 48: Superposition of VCC (blue and green) with LukF (red and orange) reveals a homologous cytolysin fold, but VCC is missing the rim loops that are required for lipid binding (LBP) in LukF. Adapted from (Olson and Gouaux, 2005).
A number of studies have linked VCC activity to cholesterol and sphingolipids in the membrane (Lafont et al., 2004). The rate of VCC pore formation has been shown to be correlated to the concentration of cholesterol in the membrane (Krasilnikov et al., 2007). Furthermore, VCC displays specificity for cholesterol over its enantiomers, a finding that strongly supports the notion of a specific cholesterol binding site (Zitzer et al., 2003). However, unlike the cholesterol-dependent cytolysins, VCC does not have an absolute threshold requirement for cholesterol, and instead follows a linear dependence curve (Krasilnikov et al., 2007). This observation casts some doubt on the theory that VCC directly binds cholesterol, since cholesterol in the membrane is typically well shielded and would need to reach a threshold concentration before becoming sufficiently exposed for protein binding (Huang and Feigenson, 1999).

One potential protein-cholesterol interaction sequence is the CRAC motif, a consensus sequence first identified in the peripheral-type benzodiazepine receptor consisting of the pattern \(-L/V-X_{1.5}-Y-X_{1.5}-R/K-\) (Li and Papadopoulos, 1998). This is a fairly loose definition, discovered primarily through sequence analysis of several cholesterol-binding proteins. However, VCC does contain a few sequences in the rim loops that satisfy the criteria for a CRAC motif, which were investigated further.

The first of these sequences is a LDAYFR motif stretching from residues 239 to 243. I tested the possibility that this may be a CRAC motif by mutating the central tyrosine, Y241, to proline to change the backbone structure and potentially disrupt any cholesterol binding activity this site could have. However, the mutation Y241P yielded hemolytically active protein and further analysis of this stretch reveals that it
is fairly insensitive to mutation. So, it is unlikely that this constitutes an active CRAC motif.

The sequence KHYYVV from residues 418 to 423 also satisfies the criteria for a CRAC motif and contains two functionally important tyrosine residues (Y420 and Y421) identified by alanine scanning mutagenesis. This seems promising, given the fact that tyrosine plays the most critical role in coordinating cholesterol in a CRAC binding site. However, Y421A, along with S357A, appears to be as active as WT VCC on liposomes composed of soy asolectin and 20 mol% cholesterol.

The results of the liposome assay suggest that the functional changes observed for Y421A and S357A on rabbit erythrocytes are not caused by defects in binding cholesterol or other lipids in the asolectin, including lecithin, cephalin, phosphatidylinositol. However, it does not rule out the possibility that VCC contains a binding site specific to some lipid present exclusively in rabbit erythrocyte membranes, or of a binding site that recognizes a combination of cholesterol and other membrane components. Furthermore, although no difference was observed in the amount of wild type and mutant VCC to permeabilize vesicles, the HD50 of WT VCC on these membranes increased dramatically from about 0.1 nM on rabbit erythrocytes to 5 μM on liposomes. It is possible that the pore formation observed on the liposomes could be caused by VCC interacting with the membrane in a nonspecific manner at high concentrations in the absence of a high affinity binding site. This suggests that the change in hemolytic activity observed for S357A and Y421A has not impacted the intrinsic pore forming capabilities of VCC, only removed its ability to associate with high affinity binding sites on rabbit erythrocytes.
The liposome assay suggests that Y421 and S357, along with the other functionally important residues nearby, are involved in binding some ligand that is present on rabbit erythrocytes but not liposomes, such as some previously unknown protein receptor. Given the wide variety of possible protein targets and binding sites, it would be difficult to predict what sort of target the VCC rim domain may bind to in the absence of additional co-localization data. However, the possibility of protein binding might explain the extensive evidence that suggests a connection between VCC and cholesterol and sphingomyelin, in the absence of a direct lipid or sterol binding site.

For instance, *S. aureus* α-hemolysin is sensitive to cholesterol and sphingomyelin enriched domains in the membrane, yet evidence for a direct binding site to these membrane components proved elusive (Valeva et al., 2006). A recently identified protein target for α-hemolysin, the ADAM10 disintegrin and metalloprotease, may mediate this connection by partitioning preferentially in cholesterol/sphingomyelin enriched domains. This would increase the local concentration of α-hemolysin monomers and facilitate oligomerization (Wilke and Bubeck Wardenburg, 2010). The studies that suggest that VCC requires cholesterol and sphingomyelin in the membrane were conducted on protein-free liposomes, so it seems that VCC’s sterol dependence does not require any extraneous proteins. However, it is possible that VCC recognizes sterols in complex with some protein, but has a lower affinity for each component alone. Such complexes would presumably exist primarily in lipid raft domains, increasing the local concentration of membrane bound VCC and facilitating rapid and efficient oligomerization.
4.5 Summary

It is clear that there are a number of residues in the rim region of VCC that are essential for its hemolytic activity and yet do not seem to affect the structural integrity or intrinsic pore formation capabilities of VCC. These results support our hypothesis that the rim region plays a role in VCC membrane binding, possibly by recognizing a protein ligand or a more complex combination of targets. However, it is not certain what type of ligand the rim region recognizes and how the unusual SEC results contribute to this model. Thus, future investigations will focus primarily on identifying this potential ligand and reconciling the variation in SEC with functional data.
Chapter 5
Conclusions
Current literature on the pore forming toxin *Vibrio cholerae* cytolysin does not provide a satisfactory explanation for the apparent specificity that VCC has for cholesterol and sphingomyelin rich membranes, which appears to play a role in pore formation even in the absence of glycan binding activity. This study investigated the role of the VCC rim region in membrane binding and pore formation through systematic alanine scanning mutagenesis of selected residues in protruding loops of this domain. The location of the rim region directly adjacent to the stem domain of VCC makes it a prime candidate for membrane insertion, thus I sought to identify residues that are important in the VCC membrane interaction.

A hemolytic assessment of rim domain mutants reveals a number of residues with dramatically reduced function that otherwise appear structurally homogeneous. Three mutations in particular, S357A, Y420A, and Y421A, retained less than 1% wild type activity on rabbit erythrocytes. Mapping the most functionally important residues revealed a putative binding region facing towards the stem domain. Yet, testing the activity of hemolytically impaired mutants on liposomes consisting of asolectin lipids and cholesterol yielded no shift in activity compared to WT, suggesting that these residues are not involved in binding to lipids or sterols. This result strongly implies the existence of a novel protein or specialized lipid receptor present on rabbit erythrocytes.

Surprisingly, there appears to be a correlation between the loss of hemolytic activity of the VCC mutants and their elution volume on a size exclusion column. Although these mutants eluted as monodisperse peaks, the most inactive mutants eluted far earlier than WT VCC. Yet, not all of the mutants that shifted on SEC lost
hemolytic activity. Since VCC has glycan binding activity, interacts with the sugars in the column matrix, and thus elutes late, it was hypothesized that the mutations may cause defects in sugar binding activity would prevent toxin interaction with the matrix. However, ITC binding experiments reveal that selected VCC mutants have the same affinity for asialofetuin as WT.

Although these results do not definitively explain the rim region’s role in VCC pore formation, the identification of a number of crucial residues does confirm the importance of this domain to VCC toxicity. Ongoing experiments are aimed towards identifying a protein receptor on rabbit erythrocytes that binds to the rim region. The SEC data also introduces the possibility that these mutations may be triggering premature oligomerization of VCC in solution, causing it to elute earlier on the column. Thus, another subset of ongoing experiments seeks to determine the oligomerization state of several early eluting mutants using analytical ultracentrifugation. Together, these results should be able to paint a detailed portrait of the rim region’s role in VCC toxicity and further characterize this toxin’s exquisite specificity for certain types of cells.
Chapter 6
Appendix
6.1 Expression of VCC Mutants

Figures 49-52: Western blots of supernatants showing VCC mutant expression bands at 60 kD. N364A, Y417A, and K418A yielded poor expression levels of soluble protein, suggesting structural instability and premature proteolysis.

![Figure 49: Western blot of WT, S234A, Y235A, T236A, and L238A](image)

N364A, Y417A, and K418A yielded poor expression levels of soluble protein, suggesting structural instability and premature proteolysis.

![Figure 50: Western blot of V363A, D359A, T358A, R356A, R243A, F242A, D239A, and T236A](image)

Figure 50: Western blot of V363A, D359A, T358A, R356A, R243A, F242A, D239A, and T236A

![Figure 51: Western blot of V422A, Y421A, Y420A, H419A, K418A, Y417A, T365A, N364A, and T236A](image)

Figure 51: Western blot of V422A, Y421A, Y420A, H419A, K418A, Y417A, T365A, N364A, and T236A

![Figure 52: Western blot of V423A, H426A, Q427A, S428A, Y429A, and H430A](image)

Figure 52: Western blot of V423A, H426A, Q427A, S428A, Y429A, and H430A

6.2 Proteolytic Activation of VCC

VCC was incubated with chymotrypsin for 30 minutes at room temperature to cleave the prodomain and activate the toxin. 5 µg of each product was run over SDS-PAGE gel to confirm this cleavage. Uncleaved VCC monomer runs at approximately 80 kD, while cleaved VCC appears as a band around 60 kD. Over time, VCC undergoes spontaneous cleavage of
the prodomain, but this does not affect hemolytic activity. Most mutants underwent clean and efficient cleavage. A few exceptions include F242A, which was overcut, and Y417A and K418A, which were poorly expressed.


Figure 53: SDS/PAGE of WT, S234A, Y235A, and T236A proteolysis

Figure 54: SDS/PAGE of L238A and D239A proteolysis

Figure 55: SDS/PAGE of R356A, R243A, and F242A proteolysis

Figure 56: SDS/PAGE of D359A and T358A proteolysis
Figure 57: SDS/PAGE of T365A, N364A, and V363A proteolysis. Slightly less cut N364A was used in this case.

Figure 58: SDS/PAGE of N364A, Y417A, and K418A expression

Figure 59: SDS/PAGE of Y421A and Y420A proteolysis

Figure 60: SDS/PAGE of V422A and V423A proteolysis

Figure 61: SDS/PAGE of H430A, S428A, and H426A proteolysis
Figure 62: SDS/PAGE of WT, Q427A and Y429A proteolysis. These samples had already undergone spontaneous proteolysis of the prodomain while in storage, but further addition to chymotrypsin did not cause excessive proteolysis.

6.3 Hemolysis Assay of VCC

VCC follows a sigmoidal hemolytic curve against rabbit erythrocytes, requiring about 0.1 nM of wild type toxin to lyse 50% of the sample after 30 minutes at room temperature. Many of the mutants tested have hemolytic curves that closely match that of WT, but a number of curves are shifted to the right. This shift indicates a loss of toxin function, as higher concentrations of VCC are required to achieve the same degree of erythrocyte lysis.

Figure 63: Hemolysis of S234A, Y235A, T236A, L238V, and D239A.
Figure 64: Hemolysis of R243A, R356A, T358A, D359A, and F242A.

Figure 65: Hemolysis of V363A, N364A, and T365A.
Hemolysis of VCC Mutants

Figure 66: Hemolysis of Y420A, Y421A, H419A, V422A, and V423A.

Hemolysis of VCC Mutants

Figure 67: Hemolysis of H426A, S428A, Y429A, H430A, and Q427A
Figure 68: Hemolysis of L238A, L361A, and Y241P
6.4 SEC of VCC

WT VCC elutes from the column around 28 ml as a single, well-defined peak. While the mutants also mostly eluted as single peaks, indicating the presence of a single homogeneous species, there was a wide variation in the location of these peaks.

Figures 69-75: SEC profiles of VCC mutants.
Figure 71: SEC of D359A, T365A, V363A, and R243A

Figure 72: SEC of T358A, H419A, and N364A
Figure 73: SEC of Y421A, Y429A, Q427A, S428A, and Y420A

Figure 74: SEC of V422A, V423A, and H430A
Figure 75: SEC of L238A, and L361A


