Chapter 1

Introduction

Viruses are subcellular agents that rely on the molecular machinery of a host cell to replicate. They have evolved to infect almost every organism and are increasingly being employed in scientific research. Although viruses contain very small genomes encoding only a few necessary proteins, they have adapted to evade immune systems and transmit efficiently from host to host.[1,2] While viruses come in different shapes, sizes, and types of genomic information, here, we are specifically interested in enveloped viruses. Enveloped viruses are a class of viruses that bud from the plasma or internal membrane of plants or animals during their replication.[2] The newly budded viral particle contains the genomic material inside a protein capsid, which in turn is surrounded by membrane from the host and envelope proteins. Envelope proteins are often heavily glycosylated by the host machinery, and therefore are often not immediately recognized by the immune system. The envelope glycoproteins are usually responsible for interactions with cellular receptors on target host cells, triggering membrane fusion and infection.

Enveloped viruses cause many well known diseases, including influenza, Ebola, chicken pox, SARS (severe acute respiratory syndrome), small pox, and AIDS.[1,2] Human immunodeficiency virus (HIV), the virus that causes AIDS, affects approximately 33 million people throughout the world and causes approximately 2 million HIV-related deaths per year.[3] While current retroviral therapies have extended the length and quality of life of those infected with HIV, resistant strains are becoming increasingly common, and additional treatments and a broad vaccine are necessary to prevent additional infections. Although influenza does not typically cause the mortality of HIV, it is a highly contagious virus that can be lethal, usually in the very young and very old and in those with immune deficiencies. Moreover, influenza pandemics, such as the one in 1918 when

an estimated 40 million people worldwide were killed, are capable of causing a significant number of deaths, including in healthy young adults.[2,4]

Current treatment for viral infection varies widely for different viruses. Effective vaccines are available for smallpox, measles, hepatitis, and varicella-zoster (chicken pox) viruses, among others.[2] Influenza vaccines are typically effective against the strains included in the vaccine, but must be readministered every year due to the rapid mutation rate of the virus.[5] For some enveloped viruses, however, there is no vaccine and therefore treatment of the infection is the primary clinical goal. There are currently no vaccines available for Ebola virus, herpesviruses, hanta viruses, HIV, and many other potentially deadly viruses. For many of these diseases, treatment is administered to make the patient more comfortable, provide symptom relief, or decrease the viral load to allow the immune system to more easily fight off the infection.[2,6,7]

Efforts to develop a vaccine for HIV have been met with limited success, with promising laboratory results thus far leading only to failures in clinical trials.[8-10] Although an effective vaccine has not yet been developed, researchers have isolated some broadly neutralizing anti-HIV antibodies from non-progressing patients, leading to hope that a vaccine may be possible.[11-14] While research continues on developing an effective and cross-reactive vaccine, patients currently rely on antiviral drugs to decrease their viral load and prolong their lives. HIV antiviral therapy usually consists of three or more antiretroviral drugs from at least two inhibitory classes in a therapeutic regimen known as highly active antiretroviral therapy (HAART).[1,2,10] As of 2008, 32 antivirals have been approved by the FDA for treatment of HIV-1 infections. These mostly small molecule drugs can be divided into six categories: nucleoside reverse transcriptase inhibitors

(NRTIs), nonnucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors, fusion inhibitors, entry inhibitors, and integrase inhibitors. Although HAART has been quite successful at reducing the viral load of patients, the rapid mutation rate of HIV often eventually leads to drug resistant strains, rendering antiviral treatment ineffective.[15] New and improved small molecule and protein-based therapeutics that limit the development of drug resistant strains, along with the creation of effective vaccines, will greatly improve the outlook for currently infected patients as well as those at high risk for becoming infected.

Unlike for HIV, there is a very effective vaccine for influenza.[2,5] However, the inconvenience and cost of yearly immunization, as well as the unpredictable mutation of influenza, means that millions of people are susceptible to infection every year.[16,17] In addition, the recent emergence of a highly lethal H5N1 strain ("bird flu") has led to concerns that this strain could become easily transmittable from human to human or weaponized, creating a massive influenza pandemic.[18-21] Influenza A, including H5N1 strains, can be treated with antiviral medications, including oseltamivir (Tamiflu) and zanamivir (Relenza),[1,22,23] although resistance to oseltamivir and other common influenza antivirals have already been reported in H5N1 cases.[24-27] Additional anti-influenza therapeutics and prophylactics would be beneficial in the case of a pandemic, especially for medical personnel and first-line defenders.

Cyanovirin-N (CVN), a potent antiviral lectin, is uniquely positioned to become a novel therapeutic and prophylactic for enveloped virus treatment and prevention. CVN is a small 11-kDa protein that was originally isolated from the cyanobacterium *Nostoc ellipsosporum* during a high-throughput screen intended to discover novel anti-HIV

activities. CVN was shown to be active against various strains of HIV, including primary isolates of HIV-1, T-lymphocyte-tropic strains, macrophage-tropic strains, and HIV-2.[28] This study also showed that CVN specifically interacts with the HIV envelope glycoprotein, gp120. Because of the great potential of CVN as an antiviral therapeutic, a number of additional studies quickly elucidated that CVN is a lectin with two carbohydrate binding sites that specifically bind to α(1-2) linked oligomannose moieties within Man-8 or Man-9 glycosylation sites.[29-32] Interestingly, high mannose glycosylation is very uncommon in mammalian oligosaccharides, but is often seen on the surface of viruses and microorganisms, creating an important distinction between the recognition of pathogens during potential treatment with CVN.[33] The two carbohydrate binding sites in CVN show distinct affinities for Man-9: the "high affinity" binding site has a K_a of 7.2 x 10^6 M, and the "low affinity" binding site has an approximately 10-fold lower affinity.[30] Later studies confirmed that both binding sites are important for HIV neutralization, and the destruction of either site renders the CVN variant inactive.[34-36] These two binding sites provide a mechanism for high affinity and high avidity interactions with glycosylated envelope proteins on viruses.

In addition to its potent activity against HIV,[28,37-39] CVN has also been shown to effectively neutralize influenza,[40,41] Ebola,[42,43] hepatitis C,[44] herpesvirus 6, and measles virus.[45] In each case, CVN binds specifically to high mannose glycosylation sites on envelope glycoproteins and inhibits vital interactions between the virus and the host cell. To date, CVN has shown no antiviral activity against any non-enveloped viruses, including rhinoviruses and enteric viruses,[40] and also appears to be inactive against some enveloped viruses, including vaccinia.[45]

In the case of influenza, CVN interacts with glycosylation sites on hemagglutinin, one of the two surface glycoproteins expressed on influenza particles. CVN showed highly potent antiviral activity against strains of influenza A, including H1N1 and N3N2, exhibited moderate neutralization against influenza B strains,[40,41] and was able to protect mice from a highly fatal strain of influenza when administered before infection.[46] Unfortunately, there was no apparent activity against H5N1 strains ("bird flu").[41] However, with increased understanding of the specific interactions between CVN and hemagglutinin, engineered variants may provide increased neutralization of H5 and other strains, allowing a broad and potentially successful method for preventing infection in the case of an influenza outbreak in the absence of an effective vaccine.

Similarly to the mechanism for influenza neutralization, CVN inhibits HIV by binding to glycosylated surface proteins. In this case, CVN binds specifically and with high affinity to glycosylated gp120[28] and with significantly lower affinity to gp41.[47] CVN binds with approximately 5:1 stoichiometery to soluble gp120, indicating that there are not only multiple sites of glycosylation to which CVN can bind, but that avidity may also play a significant role in the neutralization of HIV.[47] Additionally, studies have shown that CVN does not bind to a single glycan on gp120, but instead three to five separate N-linked glycosylation sites must be mutated before CVN resistance is incurred.[39,48,49] Although CVN-treated gp120 can still bind to soluble CD4,[50] membrane-bound CD4 binding is inhibited, probably due to steric constraints.[45,50] CVN also blocks the interaction between gp120 and the CCR5 coreceptor, adding a secondary inhibitory effect.[45] These two mechanisms together make CVN an efficient inhibitor of the pre-membrane fusion event of HIV infection.

The structure of CVN has been extensively studied to attempt to elucidate a mechanism for its broad antiviral activity. CVN exists in solution mainly as a monomer, but a trapped, metastable domain-swapped dimer can be formed.[51] In crystal form, however, wild-type (WT) CVN is only seen as a domain-swapped dimer (Figure 1-1). The monomer contains two pseudo domains that display high sequence homology. Interestingly, however, the gene does not appear to result from a simple gene duplication since the first domain contains residues 1-39 and 90-101, and the second domain contains residues 39-89.[52] Instead, there was probably a gene duplication then rearrangement or an uneven recombination event that resulted in the CVN gene. Monomeric CVN also contains two native disulfide bridges: between residues 8 and 22, and between residues 58 and 73. These two disulfide bridges are located near each end of the molecule and anchor the secondary structure. The dimer contains the same topology, but is domain-swapped at residues 51-53.[53] In the dimeric structure, the first domain of one chain (A) forms a "monomer-like" structure with the second domain of the other chain (B') in an almost symmetric domain swapping (Figure 1-1B). The two quasi-monomers can sample different orientations relative to each other due to the flexibility of the domain-swapped region, and the orientation appears to be pH dependent in crystal structures.[51,53,54]

A number of groups have attempted to modulate the domain swapping of CVN to determine whether this property is a crystallographic artifact or a biologically relevant state. Because the domain-swapped dimer of WT CVN is metastable at physiological temperatures, purified dimer quickly converts to monomer during the course of a neutralization assay.[51] Variants have been generated that stabilize both the monomeric state[51] and the dimeric state.[55,56] However, the effect of dimerization remains unclear,

since some groups have concluded that the dimeric state is more active than monomeric WT CVN[55] and others have shown that monomeric and dimeric variants have the same antiviral activity.[56]

CVN has great potential therapeutic value both as a prophylactic as well as a treatment for viral infection. In fact, CVN is currently in clinical trials as a prophylatic gel (Cellegy Pharmaceuticals, Inc.) and has been shown to be effective against both rectal and vaginal SIV/HIV-1 transmission in non-human primate studies when used as a topical microbicide.[37,38] Additionally, it has been shown that CVN has limited toxicity in tissue culture,[28,45,57] in mice,[43,46,58,59] and in non-human primate models,[37,38] although a recent study indicates that CVN can increase the levels of chemokines in treated cells and potentially allow much higher susceptibility for viral replication after CVN is removed.[60] Additionally, CVN can be prepared in large quantities, is stable for long periods of time, and is extremely resistant to degradation.[28,61-63] It was also demonstrated that WT CVN can be specifically PEGylated to increase the serum half-life while retaining most of the anti-HIV activity.[59]

Although the viral neutralization activity of CVN is important in the prevention of infection, this function may prove even more beneficial as a potential therapeutic. Because CVN specifically targets glycosylation on viral envelopes, escape variants will likely appear rapidly upon treatment with this lectin. Under evolutionary pressure by CVN[39,41,48,49] and other carbohydrate-binding proteins,[64-66] HIV and influenza have both been shown to eliminate N-linked glycosylation sites on their envelope proteins to escape neutralization. However, HIV-1 and other viruses use glycosylation to prevent recognition by the innate and adaptive immune systems.[67] With the removal of

glycosylation and the exposure of antigen, these viruses may become more sensitive to neutralization and clearance by the immune system.[68-70] In fact, Reitter *et al*. found this to be true when rhesus monkeys were infected with SIV (simian immunodeficiency virus, an HIV homolog) lacking various glycosylation sites. In this case, the viruses were significantly more susceptible to antibody neutralization.[71] Additionally, glycosylation of these viral proteins is often necessary for their proper folding and function, and therefore treatment with CVN or other lectins may decrease their viability.[60,72,73]

Various attempts to increase the HIV neutralization of CVN have met with some success. Mori *et al*. showed that a chimera of CVN and an exotoxin from *Pseudomonas* had enhanced cytotoxicity to HIV-infected cells.[74] Another chimera between CVN and an allosteric peptide inhibitor of HIV-1 fusion also showed synergy between the two components, creating a more effective compound against HIV.[75] Attempts to engineer CVN itself, however, have not resulted in variants with increased potency.

Although the Mayo lab has typically used computational methods to study and engineer proteins, for the last few years I have worked on a non-computational project involving engineering CVN to create variants with increased antiviral potency. As described above, the information we gathered through characterizing these variants has led us to believe that they have potential therapeutic as well as scientific value. In Chapter 2 of this thesis, I describe the generation and characterization of dimeric and trimeric CVN variants that display increased HIV neutralization activity. Although we showed that these variants exhibit increased potency, a mechanism was elusive. We therefore solved four crystal structures of three CVN_2 variants to determine whether

structural changes or differences in domain swapping could account for the increase in activity. These data and results are presented in Chapter 3.

In Chapter 4 I discuss a chimeric CVN-Fc construct, which we have termed a "lectibody" because it is a fusion of a lectin (CVN) and the constant region of an antibody (Fc). This construct may have therapeutic value as a molecule that not only neutralizes free viral particles through the CVN domain, but also functions as part of the human immune system through the Fc, inducing immune response against infected cells which are budding new virus.

While the vast amount of research has been focused on CVN projects, I also had the opportunity to work on some computational protein design projects that are presented as chapters in the Appendix of this thesis. One project involved designing calmodulin (CaM) to optimize the protein-peptide surface and provide specificity between two high-affinity native CaM peptide partners (Appendix A). While this research did not ultimately result in a variant that displayed increased specificity, the groundwork was set for future CaM designs and experiments that may prove more successful.

I also had the opportunity to collaborate with Jonathan Kyle Lassila on the design and characterization of various chorismate mutase variants (Appendices B, C). The goal of this project was to investigate the role of secondary active site residues in an enzyme (these residues do not directly contact substrate but instead interact with active site residues). We used our protein design software (ORBIT) to generate a variant that showed a modest increase in catalytic efficiency and to identify other mutations that were consistent with activity. We also performed site-saturation mutagenesis on six secondary active site residues and characterized each active variant. This data allowed us to

determine the tolerance for mutation in a natural enzyme system and use that information for future computational studies involving functional proteins.

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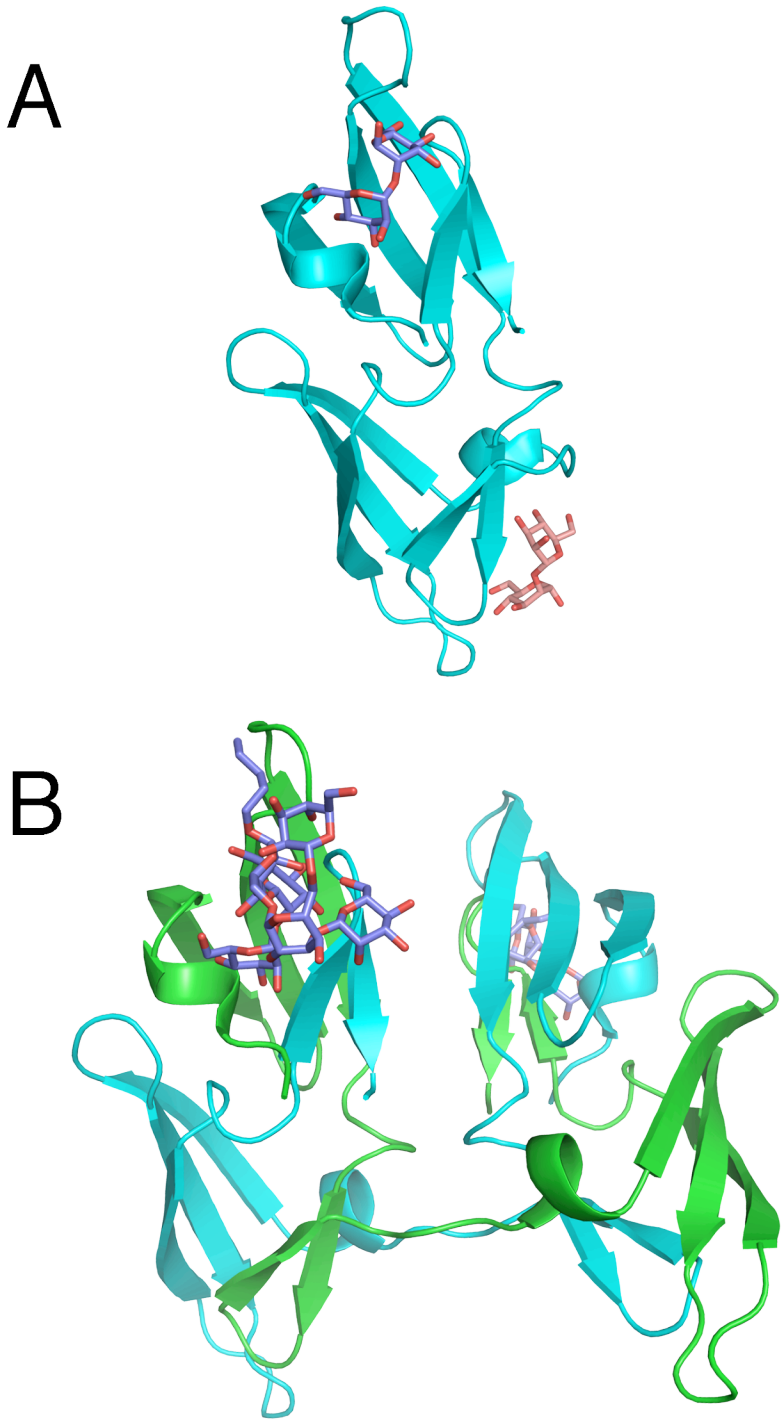

Figure 1-1. Wild-type (WT) CVN structures. In solution, WT CVN exists mainly as a monomer (A),[76] while it always crystallizes as a domain-swapped dimer (B).[77] CVN is shown in green and cyan ribbons to indicate protein chains. Carbohydrates bound in the high-affinity site are shown with orange carbons (present only in A), and carbohydrates bound in the low affinity site are shown with blue carbons (present in both A and B). The monomer and the left half of the dimer are in approximately the same orientation.