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Engineering an Obligate Domain-Swapped Dimer of Cyanovirin-N with Enhanced Anti-HIV Activity

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Three-dimensional (3D) domain swapping refers to the event wherein one protein molecule exchanges a structural domain with the same of a second identical molecule to form an intertwined dimer or oligomer.[1] Structural domains that have been observed to undergo domain swapping range from single β-strands,[2a] α-helices,[2b] and loops,[2c] to larger structural domains.[1,2d,e] Domain exchange occurs about a so-called "hinge-loop", or linker, which separates the two regions of each monomer of an intertwined dimer. Thus, the 3D structures of each half of a domain-swapped dimer are nearly identical to the parent monomer, with the exception of the linker which becomes extended to accommodate domain exchange.

The anti-HIV cyanobacterial protein cyanovirin-N (CVN) can undergo domain-swapping under nonphysiological conditions,[2e] demonstrated structurally by NMR[3a,b] and X-ray crystallography.[2e] Specifically, following purification by reversed-phase HPLC at low pH in the presence of organic solvents and lyopholization, the resolubilized protein is present as a mixture of approximately 70:25:5 monomer:dimer:oligomer (Supporting Information). However, the domain-swapped dimeric form is not favored at neutral pH and converts back into a monomer upon titration to pH > 5.0 to yield samples comprising only 5−10% dimeric CVN.[2e]

Interest in CVN arises from its ability (at nM concentrations) to potently block viral entry by human immunodeficiency viruses (HIV) through highly avid interactions with the viral envelope glycoprotein gp120.[4a,b] CVN:gp120 interactions are governed by high affinity binding of CVN to the D1 and D3 arms of oligomannose-8 (Man_8) D1D3 and oligomannose-9 (Man_9),[5] mammalian oligosaccharides that are abundant on the viral surface. This unprecedented specificity arises from the presence of two extensive carbohydrate binding pockets that are specific for the disaccharide Manα(1−2)Manα, which represents the termini of the more accessible D1 and D3 arms of Man_8 and Man_9.[6]

While structural studies of domain-swapped dimers are fairly plentiful, corresponding functional studies are lacking. This may be attributed to the fact that the domain-swapped dimeric or oligomeric forms of various proteins observed in the crystal lattice are insufficiently stable at low concentrations to study function.[7] Given that CVN blocks viral entry through carbohydrate-mediated interactions[5,6,8] and that increasing valency in protein−carbohydrate interactions is known to decrease apparent equilibrium dissociation (K_D) constants,[9] we supposed that the domain-swapped dimeric form of CVN, which possesses four carbohydrate binding sites, should be twice as potent as the naturally occurring monomer which possesses two carbohydrate binding sites.[5,6] To test this hypothesis rigorously,[10] we have engineered an obligate domain-swapped dimeric form of CVN as described below.

CVN has a pseudosymmetrical 3D structure comprising two adjacent triple-stranded antiparallel β-sheets in the back of the protein (as viewed in Figure 1a), and two oppositely placed

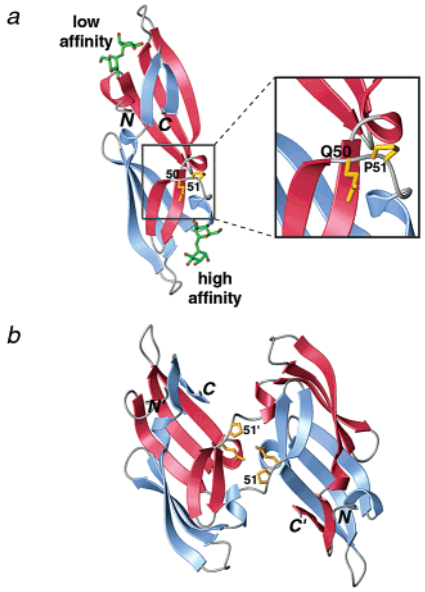

Figure 1. Solution structures of (a) monomeric wtCVN in complex with 2 equiv of the disaccharide Manα(1−2)Manα (PDB accession code 1IIY[6]) and (b) the domain-swapped wtCVN dimer (1J4V).[3b] In (a), the two sequential domains are colored red and blue, respectively; the expansion highlights the hinge-linker about which domain-swapping occurs. In (b) the two intertwined monomers appear as red and blue ribbons. The figure was generated using the program MolMol.[18]

β-hairpins on the front of the protein, each of which is preceded by a single 3_{10} helical turn.[11] The homologous sequence repeats (residues 1−50 and 51−101) are separated by a central linker (comprising Gln50-Pro51-Ser52-Asn53) that precisely crosses over β-strand 4,[11] and facilitates domain swapping.[2e] On the basis of this structure, we predicted that a mutant bearing a shortened hinge linker would be restricted to form a domain-swapped dimer due to unfavorable steric interactions that would occur between the shortened linker and the underlying β-hairpin if the monomer fold was assumed. Since the presence of proline in hinge linkers correlates with domain-swapping[12] and since Ser52 and Asn 53 participate in carbohydrate binding,[6] we chose to delete Gln50 from the linker when engineering an obligate dimer.

The CVN Gln50 deletion mutant (ΔQ50-CVN) was constructed by site-directed mutagenesis (Supporting Information), and uniformly labeled ^{15}N-ΔQ50-CVN was overexpressed as described previously.[11] The recombinant protein was purified from a crude cell lysate (50% aq CH_3CN) in a single step by reversed-phase HPLC. The presence and relative abundance of monomeric and dimeric wildtype CVN (wtCVN) can be readily assessed from a ^{1}H−^{15}N single quantum coherence correlation spectrum (HSQC) which shows doubling of 18 resolved signals.[3a] ^{1}H−^{15}N HSQC spectra of NMR samples of ΔQ50-CVN (10% D_2O) prepared with and without adjusting the pH (measured pH values of 6.4 and 2.3,

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Table 1. Relaxation Parameters

protein	^1H_N T_2[a] (ms)	^{15}N T_2[a] (ms)	τ_c[b] (ns)
ΔQ50-CVN	~22	93 ± 4	9.7
dimeric CVN	~20	95 ± 6	9.6
monomeric CVN	~40	167 ± 14	4.5

[a] ^1H_N T_2 and ^{15}N T_2 values were measured as described in ref 13. [b] τ_c values were calculated from ^{15}N T_1/T_2 ratios as described in ref 14.

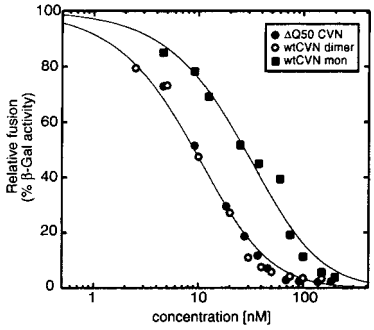

Figure 2. Inhibition of HIV-1 envelope-mediated cell fusion by ΔQ50-CVN (●), dimeric wtCVN (○) and monomeric wtCVN (■) as determined in a quantitative cell fusion assay.[15] Assays were conducted in triplicate on at least three separate occasions as described in ref 5; errors were not greater than 10% for any given point. The solid lines represent a best fit to a two-independent site model with a stoichiometry of two molecules of CVN per one molecule of fusogenic gp120 (% fusion = $100/(1 + 2[CVN]/K_D + [CVN]^2/K_D^2)$.[5]

respectively) were recorded. Unlike wtCVN, which shows the presence of ~25% domain-swapped dimer upon dissolution (pH ≈ 2.3−3.0), the $^1H-^{15}N$ HSQC spectrum of ΔQ50-CVN revealed the presence of a single species, regardless of pH (Supporting Information). NMR relaxation measurements were carried out to determine whether this single species was monomeric or dimeric. At 35 °C, samples of ΔQ50-CVN had average ^1H_N and ^{15}N T_2 values[13] of 22 ms and 93 ms, respectively, and a rotational correlation time[14] t_c of 9.7 ns (Table 1), values that are only consistent with a dimer of ~22 kDa. (See Table 1 for comparison of values for monomeric and dimeric wtCVN.) In addition, equilibrium sedimentation measurements for ΔQ50-CVN yielded an average molecular mass of 24 (±0.9) kDa, further confirming that ΔQ50-CVN is an obligate dimer.

CVN potently inhibits viral entry by HIV.[4a,b,5] To determine the efficacy of ΔQ50-CVN, we tested in parallel ΔQ50-CVN, dimeric wtCVN, and monomeric wtCVN (obtained after gel filtration chromatography) in a quantitative vaccinia virus-based HIV-1 fusion assay.[15] ΔQ50-CVN and dimeric wtCVN are more potent inhibitors of HIV-1 fusion than monomeric wtCVN (Figure 2), as one would predict given that CVN binds to gp120 via protein−carbohydrate interactions. Nonlinear least-squares best fitting of the titration data to a 2-independent site model[5] for ΔQ50-CVN, dimeric wtCVN and monomeric wtCVN yields average K_D's of 22 nM, 21 nM, and 67 nM, respectively, with corresponding IC_{50} values[16] of 9 nM, 9 nM, and 32 nM.

We had anticipated a 2-fold decrease for the K_D's and IC_{50}'s of dimeric wtCVN and ΔQ50-CVN relative to those for monomeric wtCVN, but observe instead 3.5-fold decreases for these values. Others have observed greater than additive decreases in K_D's for carbohydrate binding proteins binding to multivalent presentations of their carbohydrate ligands, presumably arising from lower entropic costs of binding.[9] For ΔQ50-CVN, an analogous model likely applies since deletion models indicate that all four carbohydrate binding sites are preserved, which would easily allow for simultaneous binding of the dimer to two gp120 monomers.[6] In this study, by constructing an obligate dimer of CVN, we have created a new tetravalent carbohydrate binding protein[17] that forms a pure domain-swapped species that is stable at pH values ranging from at least 2.3 to 8.0. When overexpressed in *Escherichia coli*, this obligate dimer can be obtained in >98% purity in a single chromatographic step. Last, the ΔQ50-CVN dimer is a more potent inhibitor of HIV-1 fusion than the wtCVN monomer. Thus, enhancements in affinity for carbohydrate binding proteins can be realized by increasing valency of the protein as well as of the ligand.

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Supporting Information Available: Chromatograms of analytical gel filtration for RP-HPLC purified-samples of wtCVN and ΔQ50-CVN, equilibrium sedimentation data, and overlays of $^1H-^{15}N$ HSQC spectra of ΔQ50-CVN and (i) monomeric wtCVN, and (ii) dimeric wtCVN (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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