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Multivalent Interactions with gp120 Are Required for the Anti-HIV Activity of Cyanovirin

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Abstract

Cyanovirin-N (CV-N) is a cyanobacterial lectin that binds to specific oligomannoses on the surface of gp120, resulting in nanomolar antiviral activity against HIV. In its monomeric form, CV-N contains two functional carbohydrate-binding domains, A and B. When refolded at high concentration, the protein can form a domain-swapped dimer. To clarify the role of multiple-binding sites in CV-N, we previously designed a monomeric mutant, P51G-m4-CVN, in which the binding site on domain A was rendered ineffective by four mutations (m4); in addition, a hinge region mutation (P51G) hinders the formation of a domain swapped dimer. The protein bound gp120 with diminished affinity and was completely inactive against HIV. Here, we present two mutants, Q50-m4-CVN and S52P-m4-CVN, which fold exclusively as domain-swapped dimers while containing the four mutations that abolish domain A. The dimers contain two intact B domains, thus restoring multivalency. Q50-m4-CVN and S52P-m4-CVN bind gp120 at low-nanomolar concentrations and recover in part the antiviral activity of wt CV-N. These results indicate that the number of carbohydrate binding domains, rather than their identity, is crucial to CV-N functionality.

Keywords

Cyanovirin; lectin; antiviral protein; HIV; multivalency

INTRODUCTION

In recent years, several algal lectins have emerged as attractive candidates for anti-HIV therapy.^{1–3} Although the exact mechanism of action at the molecular level is still under investigation, it is clear that these proteins bind to the mannose-rich glycans attached to the envelope glycoprotein of HIV, gp120, and interfere with viral entry into target cells.^{4–6}

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One of these lectins, Cyanovirin-N (CV-N), has been extensively investigated with regard to its structure, specificity, and mechanism of action.^{3,7–13} The protein is composed of 101 amino acids, which define two carbohydrate binding domains: A (1–38, 90–101) and B (39–89). CV-N is monomeric, but it can form domain-swapped dimers in conditions such as those encountered in overexpression in *Escherichia coli*. In particular, all the X-ray crystal structures of wt CV-N are dimeric.^{13,14} The sequence of the hinge region (residues 50–54), which adopts an α -helical turn in the monomer and an extended conformation in the dimer, is crucial in determining the relative population of the two forms. Mutations that augment the flexibility of the hinge region, such as P51G, result in a stabilization of the monomer form.¹⁵ Mutations that introduce further constraints, such as S52P, Q50, and P51S-S52P, result in almost exclusive formation of the domain-swapped dimer.^{16–20} Although slight differences between domain A and B exist, CV-N is extremely specific in binding linear oligomannosides containing a Man α (1–2)Man α linkage.^{7,21,22} In solution, each domain, A and B, binds di- and tri-mannose independently with dissociation constants in the micromolar range. In contrast, CV-N is functional at much lower concentrations: the protein binds gp120 and prevents HIV cell entry in in vitro experiments with nanomolar EC₅₀.^{10,18,23} Taken together with the presence of two-binding domains in CV-N and the further possibility of augmenting the valency through the formation of domain-swapped dimers, these data suggest that more than one site may be involved in binding. In apparent contradiction with this hypothesis, a mutant in which domain A had been inactivated by four mutations (m4-CVN, containing mutations Lys3Asn, Thr7Ala, Glu23Ile, Asn93Ala) inhibits HIV-1 envelope-mediated fusion with activity indistinguishable from wt CV-N.²⁴ However, m4-CVN contains a wild-type hinge region and thus can readily form domain-swapped dimers.

To clarify these issues, we previously designed a mutant, P51G-m4-CVN,^{6,15} which like m4-CVN contains the four mutations that abolish binding in domain A,²⁴ and an additional mutation in the hinge region, P51G, which stabilizes the monomeric form.²⁰ The high-resolution X-ray structures of the free and dimannose bound forms of P51G-m4-CVN confirmed the presence of a single-functional carbohydrate-binding domain (Figure 1A) and showed changes in the hinge region that explained the observed stabilization of the monomeric form.^{6,15} P51G-m4-CVN bound gp120 with affinity almost three orders of magnitude weaker than CV-N. Furthermore, the mutant was inactive against HIV at concentrations up to 1 μ M. The domain-swapped dimer of P51G-m4-CVN can be obtained in 50% yields by partial unfolding in 2M guanidinium and slow refolding by dialysis at millimolar concentrations; once separated from the monomer by gel filtration, the dimer can be kept as metastable form at low temperatures. The dimer restored binding to gp120 to levels comparable with wt CV-N. However, the fast interconversion of the dimer back to the stable monomeric form at 38°C prevented a direct comparison of the antiviral activity of the two forms.⁶

The critical role of multivalency was supported by independent and complementary work on a monomeric domain B knockout mutant, CV-N^{mutDB}, which contains several mutations that abolish binding in domain B as well as the P51G mutation that stabilizes the monomeric form. CV-N^{mutDB} demonstrated complete loss of activity in several antiviral assays.²⁵ Initially, the results on CV-N^{mutDB} were interpreted as proof that a functional domain B is

necessary for activity. In light of our results, it appears that two or more domains, regardless of their identity, are necessary for activity.

In this work, we introduced mutations to the hinge region designed to restore multivalency to the P51G-m4-CVN construct through the formation of an obligated domain-swapped dimer. The constructs, S52P-m4-CVN and Q50-m4-CVN, contain two copies of domain B presented on the same side of the dimer (Figure 1B). We show that both constructs bind gp120 with low-nanomolar affinity and restore some of the antiviral activity of wt CVN.

RESULTS AND DISCUSSION

S52P-m4-CVN and Q50-m4-CVN are Dimers in Solution

The hinge region of CV-N plays a critical role in determining the oligomerization state of the protein. Wt CV-N is a monomer in physiological conditions, but overexpression in *E. coli* and purification from inclusion bodies yields a mixture of monomer, dimer, and higher-order aggregates (~ 60%: 35%: 5%, respectively). Each form can be separated by gel filtration chromatography; the stable form of wt CV-N is monomeric, while the dimer reverts to monomer at high temperatures.²⁰ The domain-swapped form of CV-N, which is a metastable state in wild type, is stabilized relative to the monomer by mutations S52P and Q50: hinge region mutants S52P-CVN and Q50-CVN result in the formation of obligate domain-swapped dimers, as verified by NMR.^{16–20,26} In contrast, mutating a proline in the hinge region to glycine results in monomers, as seen in P51G-m4-CVN and [P51G]CV-N.^{6,20}

We introduced S52P and Q50 on m4-CVN to restore multivalency to the construct. The oligomerization states of recombinant S52P-m4-CVN and Q50-m4-CVN in solution were evaluated by size-exclusion chromatography. The elution profile of the proteins purified in similar conditions exhibits a single peak, which accounts for 95% of the total protein; the remaining mass balance corresponds to higher order aggregates. Co-injection experiments with monomeric and dimeric wt CV-N, separated by gel filtration chromatography, demonstrated that S52P-m4-CVN and Q50-m4-CVN coeluted with the dimeric form of wt CV-N, and are distinct from the monomeric form (data not shown). As in wt CV-N, the dimer does not revert to monomer simply by dilution.

On the basis of the evidence presented here, we concluded that the hinge region mutations S52P and Q50 affect the oligomerization state consistently on the background of wt CV-N and on m4-CVN, resulting in the formation of domain-swapped dimers.

Secondary Structure and Thermodynamic Stability

The circular dichroism (CD) spectra of S52P-m4-CVN and Q50-m4-CVN each display a minimum at 206 nm and maximum at 192 nm. The spectra resemble closely that of the domain-swapped dimer of CV-N²⁷ (see Supporting Information Material), but show a higher random coil component.

The stability of the proteins was assessed by thermal denaturation, monitoring the loss of secondary structure at increasing temperatures from 5 to 95°C by CD at 200 nm; thermal denaturation profiles are shown in Figure 2, and the corresponding melting points (T_m) are

reported in Table I. Compared with wt CV-N, m4-CVN is significantly less stable: the melting temperature is lowered by about 12°C from 58°C for wt CV-N to 46°C for m4-CV-N.⁶ Additional S52P or Q50 mutations to m4-CVN further destabilize the protein to 39.5°C and 32.5°C, respectively. The extent of destabilization observed follows the same trend observed for S52P and AQ50 on the background of wt CV-N, with T_m s of $\sim -7^\circ\text{C}$ and -11°C , respectively.¹⁹ Mutations in the hinge region and in domain A are additive, resulting in a significant destabilization of S52P-m4-CVN and Q50-m4-CVN compared to wt CV-N.

Binding to gp120 Is Modulated by the Oligomerization State

Binding of S52P-m4-CVN and Q50-m4-CVN to its viral target, gp120, was measured in an ELISA experiment (see Figure 3). Both mutants bind gp120 at low-nanomolar concentrations; for comparison, the EC_{50} of relevant CV-N mutants are compared in Table II.

The binding observed differs between two groups of CV-N analogs: a first group, comprising wt CV-N, m4-CVN, and dimeric P51G-m4-CVN, which can form multidentate interactions with gp120 through different mechanisms, bind gp120 with subnanomolar EC_{50} . A second group, formed by P51G-m4-CVN⁶ and CVN^{MutDB},²⁵ can only form one interaction with gp120 per molecule. Each contains a single carbohydrate-binding domain (B and A, respectively) and the P51G mutation in the hinge region, resulting in a higher barrier for conversion to dimer. These mutants bind gp120 with much higher EC_{50} , reflecting weaker interactions (~ 180 nM for P51G-m4-CVN,⁶ above $1 \mu\text{M}$ for CVN^{MutDB}).²⁵

The results obtained for S52P-m4-CVN and Q50-m4-CVN are intermittent between these two groups, suggesting a multivalent effect. A diminished binding compared with the first group of mutants described above might be a consequence of the limited thermal stability of the mutants, which might cause partial unfolding in the conditions of the experiment. Differences in binding between the domain-swapped dimer forms of m4-CVN could also arise in part from differences in the relative orientation and distance between domains B and B', which could result in preferential binding to gp120 for one form versus the others.

Antiviral Activity of Hinge Mutants

The antiviral activity of CV-N and its mutants follows a pattern similar to what observed for binding to gp120 (Table III). Q50-m4-CVN displays high-nanomolar antiviral activity comparable to m4-CVN, but much weaker than wt CV-N. Weak activity was observed at the highest concentration tested ($1 \mu\text{M}$) for S52P-m4-CVN, but the EC_{50} value could not be determined. For comparison, monomeric P51G-m4-CVN is completely inactive at the concentration range tested (0.01 nM- $1 \mu\text{M}$); note that loss of activity was observed at concentrations higher than those conducive to binding of gp120.⁶ In a similar antiviral assay, the complementary monovalent, monomeric mutant CVN^{MutDB} is completely inactive.²⁵ Note that P51G-m4-CVN and CVN^{MutDB} contain different intact domains (B and A, respectively), yet display similar impairment of function. These data support our hypothesis

that the presence of two or more domains, regardless of their identity, is necessary for activity.⁶

In contrast with wt CV-N, the obligate domain-swapped dimer m4-CVN mutants presented here are active at concentrations higher than the observed EC₅₀s for binding to gp120. However, one should keep in mind that Q50-m4-CVN and S52P-m4-CVN exhibit thermal unfolding profiles with midpoints close to 38°C. Loss of activity for m4-CVN has been previously attributed to its diminished thermostability compared to wt CV-N.⁶ The cellular assay used in this experiment requires incubation for several days at 38°C, which could lead to partial denaturation of the protein. In support of this interpretation, it has been reported that m4-CVN inhibits HIV-1 envelope-mediated cell fusion²⁸ with activity nearly identical to monomeric wt CV-N²⁴; this assay requires short incubation of only 1–2 h. Thus, the difference in activity relative to wt CV-N could be due to partial unfolding of Q50-m4-CVN and S52P-m4-CVN in the conditions of the experiments.

CONCLUSIONS

The results presented here point to a crucial role of multivalent interactions between CV-N and gp120 as a prerequisite for antiviral activity.⁶ Mutants containing two or more binding sites, such as wt CV-N, S52P-CVN, and Q50-CVN, are functional at low-nanomolar concentrations.^{16,18,19} Monovalent, monomeric mutants of CV-N (P51G-m4-CVN and CVN^{MutDB}) are inactive in inhibiting viral entry in the range of concentrations tested and bind gp120 with greatly reduced affinity.^{6,25} Constructs containing a single carbohydrate-binding domain, but capable of forming domain swapped dimers either through a wild type hinge region, such as m4-CVN,^{6,24} or by specific hinge region mutations, such as S52P-m4-CVN and Q50-m4-CVN, are functional. The recovery of biological function observed in dimeric mutants compared to monomeric, monovalent ones, further corroborates the experimental evidence for a mechanism of action involving high-affinity binding through multivalent presentation of binding sites in CV-N and clustered oligomannosides on the surface of gp120. In this context, it should be noted that other antiviral lectins active in the nanomolar range, such as griffithsin,^{29,30} actinohivin,^{31,32} and MVL,³³ contain multiple carbohydrate-binding sites. Although the exact mechanism of action of antiviral lectins remains unclear, they might use multivalent interactions to crosslink glycans on gp120 and prevent cofactor interactions or gp120 rearrangement during virus-cell fusion.^{1,2,14,34,35}

MATERIALS AND METHODS

Construction of Q50-m4-CVN and S52P-m4-CVN Plasmids

The starting point of cloning Q50-m4-CVN and S52P-m4-CVN genes is m4-CVN plasmid, which was constructed by sequentially introducing mutations into the pET-26b(+) (wt CV-N) background (Novagen).⁶ The gene contains a C-terminal His tag (six histidines) separated by a two-residue spacer (Leu and Glu). To construct Q50-m4-CVN and S52P-m4-CVN plasmids, mutations were introduced into m4-CVN vector by using QuikChange II site-directed mutagenesis kit (Stratagene) with forward/reverse primers. Primers used in mutagenesis were ordered from GeneTech Biosciences, Tempe, AZ. The desired mutation sites are in the middle of the primers. Melting temperatures for Q50-m4-CVN and S52P-

m4-CVN primers are 73.8°C and 75°C, respectively. The forward/reverse primers for Q50 and S52P are as follows:

(Gln50)

[5'-GACGGTTCCTGAAATGGCCGTCCAAC TTCATCGAAACC]

[5'-GGTTTCGATGAAGTTGGACGGCCATTT CAGGGAACCGTC]

(Ser52Pro)

[5'-TCCCTGAAATGGCAGCCGCCGAAC TTCATCGAAACCTGC]

[5'-GCAGGTTTCGATGAAGTTCTGGCGGCTGCCATTT CAGGGA]

Expression and Purification of Q50-m4-CVN and S52P-m4-CVN

Q50-m4-CVN and S52P-m4-CVN plasmids were transformed by heat pulse into *E. coli* BL-21(DE3) (Stratagene) cells to express proteins. BL-21(DE3) cells containing the target plasmid were grown at 37°C in Luria Bertani (LB)-kanamycin (30 µg/ml) medium. Cell cultures were induced for protein expression at an OD₆₀₀ of 0.8 with 1 mM isopropyl β-D-thiogalactoside and harvested after 3–4 h by centrifugation. The expression of target proteins was assessed by analysis of total cell protein on a Tricine SDS-polyacrylamide gel (Invitrogen) followed by Coomassie blue staining.

Purification of both proteins follows the same procedures. The cell pellet is resuspended in cold 100 mM Tris-HCl, pH 7.0 wash buffer and lysed by sonication. After several wash steps with 100 mM Tris-HCl pH 7.0, 2 M urea washing buffer, the final cell pellet is dissolved in loading buffer (6 M GuHCl, 0.5 M NaCl, 20 mM imidazole, 20 mM Tris-HCl, and pH 7.5) and centrifuged. The supernatant is loaded on a Ni Sepharose fast-flow-binding column (Novagen), and proteins are eluted with 400 mM imidazole under denaturing conditions (6 M guanidine hydrochloride). The denatured proteins were treated with 5 mM DTT (final concentration) and refolded by dialysis against 2 M GdnHCl, 50 mM Tris-HCl, pH 8.0 at room temperature for 12 h and then against 10 mM Tris-HCl pH 8.0, 100 mM NaCl for 2 days at 4°C. Purified proteins were stored at 4°C. Molecular weights and purity were confirmed by mass spectrometry MALDI-TOF and 10–20% Tricine SDS-PAGE gel (Invitrogen). The constructs used in this study (S52P-m4-CVN, Q50-m4-CVN, and wt CV-N) contained a eight amino acid sequence attached to the C terminus (LEHHHHHH), which comprises the His-tag. Protein concentrations were calculated by measuring tryptophan absorbance at 280 nm using an extinction coefficient of 10,010 M⁻¹ cm⁻¹. Throughout the work, concentrations are expressed as monomer.

Oligomerization STATE of Q50-m4-CVN and S52P-m4-CVN

The oligomerization state of AQ50-m4-CVN and S52P-m4-CVN proteins was determined by size-exclusion chromatography on a Superdex™ peptide 10/300 GL high-performance column. Proteins were eluted in 10 mM potassium phosphate buffer, pH 7 at a flow rate of 0.5 ml/min at room temperature.

CD Spectroscopy

CD spectroscopy experiments were carried out on a Jasco J-1710 spectropolarimeter equipped with a thermostatic cell holder PTC 424S. CD spectra were recorded from 240 nm to 190 nm in 1 mm cells at protein concentrations of 10–15 μ M in 10 mM potassium phosphate, pH 7.0 buffer; the final spectra are the average of three data sets. Thermal unfolding curves were determined by monitoring the molar ellipticity at 200 nm; the sample was heated at rates of 15°C/h or 30°C/h with a data pitch of 0.5°C over temperature range of 10–85°C. As in wt CV-N, thermal unfolding is irreversible; thus, the curves could not be analyzed to obtain the thermodynamic parameters of folding.

Binding to gp120

Binding of wt CV-N, S52P-m4-CVN, and Q50-m4-CVN to glycosylated soluble gp120 was assessed by ELISA as previously described.¹⁰ The primary antibodies (rabbit polyclonal anti-CVN) were tested for binding to the mutants in a separate experiment. Briefly, each well of 96 well plates (Nunc, Maxisorp, Gaithersburg, MD) was incubated with 100 ng of gp120 (rgp 120 HIV-1 MN, Baculovirus, ImmunoDiagnostics, Inc.) in PBS at room temperature for 2 h. The plates were washed with PBS containing 0.1% Tween 20 (PBST) thrice and blocked with a solution of 3% bovine serum albumin (BSA) overnight at 4°C. A control plate was incubated with BSA only. After washing plates three times with PBST, 0.5- $\log_{10}$ serial dilutions of CV-N, m4-CVN, P51G-m4-CVN monomer and dimer to a final concentration in the 0.01 μ M to 1 iM range were added to triplicate wells (100 μ l/well) and incubated for 1 h. The plates were washed with PBST and incubated with anti-CV-N rabbit polyclonal antibody (1:1000 dilution; final concentration 10 μ g/ml) for 1 h, washed again with PBST, and incubated with secondary anti-rabbit IgG antibodies conjugated to horseradish peroxidase (Sigma, St. Louis, MO). After 1 h, the plates were washed with PBST, and 100 μ l of tetramethylbenzidine peroxidase substrate (Kir-kegaard & Perry Laboratories, Gaithersburg, MD) was added to each well. After incubating for ~ 2 min, the absorbance at 650 nm was recorded. The reaction was stopped with 50 μ l of 2M H₂SO₄, and the absorbance at 450 nm was read. The data were plotted in a linear-log graph and analyzed using commercial software; the EC₅₀ obtained had errors below 15%.

Anti-HIV Activity Assay

The anti-HIV activity of Q50-m4-CVN and S52P-m4CVN was evaluated using a modified XTT assay,³⁶ using wt CV-N and P51G-m4-CVN as controls. Briefly, 100 μ l of serial dilutions of each protein was added to 50 μ l human lymphocyte CEM-SS (1×10^5 cells/ml) on 96 well plates. Final protein concentrations ranged between 2 nM and 8.5 μ M. Cells were infected with 50 μ l HIV-1_{RF} virus. Plates were incubated for 6 days at 37°C and stained with XTT. Metabolic reduction of tetrazolium salt XTT to colored formazan was measured at 450 nm to determine cellular viability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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FIGURE 1.

The putative position of the two carbohydrate-binding domains in S52P-m4-CVN and Q50-m4-CVN can be modeled from existing structures. Panel A shows the crystal structure of P51G-m4-CVN at 1.35 Å resolution (PDB ID: 2RDK).¹⁵ Domain A is depicted in blue, domain B in cyan, and dimannose is represented in sticks; amino acid involved in binding are highlighted in orange. Panel B shows a model of S52P-m4-CVN reconstructed from the domain-swapped dimer of wt CV-N (PDB ID: 1L5B)²⁰ and 2RDK. One of the monomers is depicted in grey, and the second is shown with the color scheme adopted in Panel A.

FIGURE 2.

Thermal denaturation profiles of Q50-m4-CVN (triangles), S52P-m4-CVN (circles), and wt CV-N (squares). The unfolding transitions were monitored at 200 nm in the 5–90°C range. All measurements were carried out at protein concentrations of 10–15 μ M in 10 mM potassium phosphate buffer, pH 7.0.

FIGURE 3.

ELISA of the binding of S52P-m4-CVN (triangles) and Q50-m4-CVN (circles) to gp120. Serial dilutions of proteins were added to plates coated with 100 ng/well of native gp120. In all cases, polyclonal rabbit anti-CVN antibodies were used to detect the bound proteins followed by incubation with anti rabbit IgG-Peroxidase antibodies; the peroxidase catalyzed oxidation of TMB was monitored at 450 nm.

Table I

Thermal Stability of CV-N Mutants

Mutant^a	<i>T</i>_m, °C
wt CV-N ^b	58
P51G-m4-CVN ^b	62
m4-CVN ^b	46
S52P-m4-CVN ^c	39.5
Q50-m4-CVN ^c	32.8

^aHis-tagged constructs.

^bFrom Ref. 6.

^cThis work.

Table II

Binding of CV-N Mutants to HIV gp120

Mutant	EC ₅₀ , nM
Q50-m4-CVN ^a	23
S52P-m4-CVN ^a	9.6
wt CV-N ^b	0.1
M4-CVN ^b	0.6
P51G-m4-CVN (Dimer) ^b	0.8
P51G-m4-CVN (Monomer) ^a	173
CVN ^{MutDB} ^c	No binding (up to 1 μ M)

^aThis work.^bFrom Ref. 6.^cFrom Ref. 25.

Table III

Antiviral Activity of CV-N Mutants

Mutant	EC ₅₀ , nM
Q50-m4-CVN ^a	320
S52P-m4-CVN ^a	≈3000
Wt CV-N ^a	0.9
M4-CVN ^b	240
P51G-m4-CVN (M) ^a	>>5000

^aThis work.^bFrom Ref. 6.