The HIV-1 envelope glycoprotein gp120 is heavily glycosylated and bears numerous high mannose sugars. These sugars can serve as targets for HIV-inactivating compounds, such as antibodies and lectins, which bind to the glycans and interfere with viral entry into the target cell. We determined the 1.6 Å x-ray structure of Cyt-CVNH, a recently identified lectin from the cyanobacterium *Cyanothece 7424*, and elucidated its glycan specificity by NMR. The Cyt-CVNH structure and glycan recognition profile are similar to those of other CVNH proteins, with each domain specifically binding to Manα(1–2)Manα units on the D1 and D3 arms of high mannose glycans. However, in contrast to CV-N, no cross-linking and precipitation of the cross-linked species in solution was observed upon Man-9 binding, allowing, for the first time, investigation of the interaction of Man-9 with a member of the CVNH family by NMR. HIV assays showed that Cyt-CVNH is able to inhibit HIV-1 with ~4-fold higher potency than CV-N<sup>51G</sup>, a stabilized version of wild type CV-N. Therefore, Cyt-CVNH may qualify as a valuable lectin for potential microbicidal use.

Despite the effective use of anti-retroviral therapies as a means to treat HIV infection and prolong the lifespan of those affected by AIDS, the number of HIV infections worldwide continues to grow. Unfortunately, at present, no vaccine is available to protect against HIV, creating the need to develop safe, effective, and acceptable prevention strategies that will help halt the spread of HIV infection globally. Promising candidates for inclusion into microbicides are lectins, which are carbohydrate-binding proteins that are present in a variety of plant, fungal, and cyanobacterial species. Over the last two decades, several lectins have been identified that potently block viral infection, being active against HIV and influenza virus (1–4). One such example is the extensively explored cyanobacterial lectin cyanovirin-N (CV-N),<sup>2</sup> which possesses virucidal properties against HIV types I and II, simian immunodeficiency virus, and other enveloped viruses like Ebola and influenza at nanomolar concentrations (5–7). Its anti-HIV activity is mediated by recognizing and interacting with high mannose glycans (Man-8, Man-9) that are present on the envelope glycoprotein gp120. Indeed, HIV-1 gp120 is highly glycosylated, and N-linked glycans account for approximately half of its molecular mass (8, 9). CV-N specifically binds the terminal Manα(1–2)Manα epitopes on the D1 and D3 arms of Man-8 and Man-9 glycans (10–14). The mechanism of action for mannos-binding lectins is assumed to involve the inability of the lectin-bound gp120 to productively engage the host cell CD4 and CCR5 receptors, effectively preventing the necessary conformational changes required for membrane fusion and viral entry into the host cell. At present, efforts to develop lectins such as CV-N for therapeutic use are focused on topical applications in microbicides. All known anti-HIV lectins vary in their degrees of potency and some were found to have mitogenic activity (15). Therefore, the continued search for and characterization of novel HIV inhibitory lectins are important for moving microbicide development forward (16–18). For CV-N, the presence of two binding sites and the multivalency of the carbohydrate have been implicated as important factors for its anti-HIV activity (6). Unfortunately, cross-linking-mediated aggregation and precipitation have hampered studies of CV-N/Man-9 interactions at the atomic level in *vitro* (7, 19, 20) and have precluded unambiguous determination of whether multivalent and multisite sugar/protein interactions are a necessary prerequisite for the antiviral activity of cyanovirin-N homolog (CVNH) proteins. However, in contrast, considerable atomic level information is available on CV-N binding to substructures of Man-8 and Man-9.

To elucidate the structural and mechanistic basis for the difference between CV-N and Cyt-CVNH, we determined the Cyt-CVNH crystal structure and assessed Man-2, Man-3, and...
Man-9 binding by solution NMR. The structure of Cyt-CVNH is similar to that of other members of the CVNH family, also possessing two carbohydrate-binding sites, one per domain. However, in contrast to CV-N, no cross-linking and aggregation is observed in the interaction with Man-9, permitting, for the first time, determination of accurate affinities for Man-9 binding to a CVNH lectin.

Cyt-CVNH inhibits HIV-1 in the low nanomolar concentration range and possesses 4-fold higher potency than CV-N. Based on these structural and functional results, we suggest that Cyt-CVNH holds significant promise for future clinical applications.

Results

Crystal Structure of Cyt-CVNH—Here, we report the crystal structure of a new CVNH, Cyt-CVNH, a recently identified lectin from the cyanobacterium Cyanothece 7424 (21), which comprises two tandem sequence repeats and exhibits ~43% identity with CV-N. Cyanothece sp. PCC 7424 is a unicellular cyanobacterium isolated from rice fields in Senegal. The genome shows that these cells have the ability to store the products of both photosynthesis (glycogen) and nitrogen fixation (cyanophycin) as intracellular inclusion bodies (21). Beyond these basic findings, however, the precise role of Cyt-CVNH within the host is unknown at present.

The structure of Cyt-CVNH was solved at 1.6 Å resolution by molecular replacement for orthorhombic crystals in space group P2₁2₁2₁ with cell dimensions a = 93.8 Å, b = 74.4 Å, c = 36.5 Å and two molecules in the asymmetric unit (ASU) (Fig. 1A, left panel). The Mathews coefficient Vₘₙ for the two molecules is 2.67 Å³/Da. The NMR solution structure of wild type CV-N (PDB accession code 3EZM) (5) was used as the search model. All pertinent crystallographic statistics are provided in Table 1. Two independent monomers in close proximity were selected as the ASU and are shown in Fig. 1A. Each independent monomer in the ASU is related to a copy of the other by pseudotranslational non-crystallographic symmetry. This relation, however, cannot become crystallographic by any alternative unit cell/symmetry.
definition. The current structure of Cyt-CVNH is similar to previously determined crystal structures of monomeric, non-domain-swapped CV-N variants (22, 23), in contrast to the domain-swapped wild type CV-N structures (13, 24, 25). We ascertained that the x-ray data were incompatible with a domain-swapped dimer structure by omitting the hinge-loop region (Trp⁴⁹—Asn⁵³) in the model employed for molecular replacement. After the first refinement step, the electron density map clearly showed strong density for the omitted region, connecting the two separate starting segments in a contiguous polypeptide chain (Fig. 1A, middle panel).

Within the asymmetric unit, the monomers are oriented with respect to each other by an angle of ~104° between the long axes of the two domains (AB’, A’B), using the S atoms of the cysteines in the two disulfide bonds (Cys8/Cys⁵⁹; Cys⁵⁹/Cys⁸⁸) to define the axes. This spatial arrangement is opposite in orientation to what was previously observed between the two halves of the domain-swapped CV-N structures (~101°) in the trigonal crystal (P3₂2₁; PDB accession code 3EZM; Fig. 1A, right panel).

A surface view of the two molecules in the ASU is provided in Fig. 1B (left panel). The monomer-monomer interface involves a different region, with Phe⁶⁹ of one monomeric unit engaged in a crystal contact with Trp⁵⁰ of the adjacent monomeric unit (Fig. 1B, middle panel). The side chain of Asp⁴⁵ in monomer 1 hydrogen bonds to the side chain of Lys⁶ in monomer 2, and similarly, the backbone carbonyl oxygen of Thr⁴⁷ in monomer 1 hydrogen bonds with the side chain amide group of Gln³ in monomer 2 (Fig. 1B, right panel).

Sugar Binding—The carbohydrate binding sites of Cyt-CVNH were mapped by monitoring chemical shift changes in the ¹H-¹⁵N HSQC spectrum of uniformly ¹⁵N-labeled protein as a function of sugar addition. Titration experiments were carried out with Man-2, Man-3, and Man-9. Interestingly, binding is in slow exchange on the chemical shift scale at 298 K, which is different from what was observed with wild type CV-N and Man2/Mαn3, where binding was in the fast exchange regime. Spectra of Cyt-CVNH in the absence and presence of Man-2, Man-3, and Man-9, respectively, are provided in Fig. 2. Mapping of the affected amide resonances clearly revealed that for all three sugars two binding sites exist: one on domain A and one on domain B.

A superposition of the spectra of Cyt-CVNH in the absence (black contours) and presence (cyan contours) of 15 molar equivalents of Man-2 are provided in Fig. 2A. Likewise, superposition of free Cyt-CVNH (black contours) and protein in the presence of 6 molar equivalents of Man-3 or 2 molar equivalents of Man-9 (magenta) are provided in Fig. 2 (B and C, respectively). The extracted binding isotherms derived from the intensity changes of sugar-bound amide resonances of residues from domain A and B are depicted in the right panels. The dissociation constants for Man-2 binding to the sites on domain A and B are 53.2 ± 8.7 and 48.4 ± 9.5 μM, respectively. The equivalent Kᵢ values for Man-3 are 7.5 ± 1.2 and 9.4 ± 0.8 μM, respectively.

In the past, attempts to structurally monitor Man-9 binding to CV-N by NMR were hampered by extreme line broadening and ultimately disappearance of resonances in the ¹H-¹⁵N HSQC spectra, accompanied by precipitation of the sugar-protein caused by multisite/multivalent cross-linking (7, 19, 20). In contrast to the findings with CV-N, no aggregation or precipitation was observed for Man-9 binding to Cyt-CVNH. Therefore, it was possible to identify those amide resonances that were affected by Man-9 binding. Again, two binding sites are present, and Kᵢ values of ~500 nM were obtained for domain A and B. Thus, domain A and domain B of Cyt-CVNH possess essentially the same affinities for Man-9. The same holds for Man-2 or Man-3.

Because the D1 and D3 arms of Man-9 contain α1→2-linked mannoses, a single molecule of Man-9 can interact with more than one lectin molecule that recognizes Manα1(1-2)Manα units. The glycan binding site of CV-N in domain A exhibits a slight preference for Man-3, whereas domain B preferentially binds Man-2, resulting in cross-linking when interacting with Man-9 (7, 19, 20). Thus, Man-9 interacts with CV-N with the D1 arm, engaging domain A, and the D3 arm, binding to domain B. For Cyt-CVNH, we noticed in the titration that Man-9 binding elicited changes in the ¹H-¹⁵N HSQC spectrum very similar to those of Man-3 and somewhat different from Man-2 (Fig. 3). For instance, at a 0.5:1 Man-2:Cyt-CVNH molar ratio the Thr⁵⁸ resonance (domain B) is shifted, whereas the Thr⁷ resonance (domain A) is not affected (Fig. 3). This is different from what was observed with wild type CV-N and Man2/Mαn3, where binding was in the fast exchange regime. Spectra of Cyt-CVNH in the absence and presence of Man-2, Man-3, and Man-9, respectively, are provided in Fig. 2. Mapping of the affected amide resonances clearly revealed that for all three sugars two binding sites exist: one on domain A and one on domain B.
FIGURE 2. Carbohydrate binding by Cyt-CVNH. A, superposition of the $^1$H-$^15$N HSQC spectra of free Cyt-CVNH (150 μM; black) and in the presence of 15-fold molar excess of Man$_1$–Man$_2$ (cyan). B, free Cyt-CVNH (50 μM; black) and in the presence of 6-fold molar excess Man$_1$–Man$_2$Man$_2$ (magenta). C, free Cyt-CVNH (10 μM; black) and in the presence of 2-fold molar excess Man$_9$GlcNAc$_2$ (magenta). Resonances of residues that undergo chemical shift changes upon carbohydrate binding are labeled in A–C. For Man$_1$–Man$_2$, Man$_1$–Man$_2$Man$_2$Man$_2$, and Man$_9$GlcNAc$_2$ binding to Cyt-CVNH the sugar-bound and free Cyt-CVNH resonances are in slow exchange. In the right panels of A and B, the binding isotherms (bound state signal intensity versus ligand/protein molar ratio) for each domain are shown. In the right panel of C, the chemical structure of Man$_9$GlcNAc$_2$ is shown. The D1 arm (magenta) contains the Man$_1$–Man$_2$ trimannoside, whereas the D3 arm (cyan) contains the Man$_1$–Man$_2$Man$_2$ dimannoside.
This similarity between the Man-3 and Man-9 effects is observed throughout the entire titration, for resonances belonging to both domains A and B (supplemental Figs. S1 and S2). For instance, at a 1:1 sugar-protein molar ratio, the Thr$_{86}$ resonance (domain B) clearly shows a smaller chemical shift difference between free and sugar-bound protein for Man-2 binding, compared with Man-3 or Man-9. In addition, Man-3 exhibits ~6-fold higher affinity compared with Man-2, for both binding sites. These suggest that Man-3 and Man-9 interact in similar fashion with domain A and domain B of Cyt-CVNH.

At a 1:1 molar ratio of sugar:protein, amide resonances of the majority of residues in the binding site of domain A, including Gly$_2$, Gln$_3$, Thr$_7$, Thr$_{29}$, Leu$_{30}$, Gln$_{23}$, Asp$_{102}$, Gly$_{103}$, and Thr$_{104}$ undergo chemical shift changes only upon Man-3 and Man-9 binding, whereas two residues, Phe$_4$ and Lys$_{24}$, exhibit perturbed resonances for all three sugars (Man-2, Man-3, and Man-9). Similarly, resonances of residues in domain B, such as Gly$_{46}$, Arg$_{81}$, Asp$_{85}$, and Thr$_{86}$ undergo chemical shift changes upon Man-3 and Man-9 binding at 1:1 molar ratio of sugar:protein, whereas Gly$_{42}$, Leu$_{44}$, Gly$_{45}$, Leu$_{46}$, Trp$_{50}$, and His$_{55}$ are affected by Man-2, Man-3, and Man-9 binding, with Asp$_{52}$, Asp$_{54}$, and Phe$_{55}$ affected only by Man-2 binding.

This is illustrated diagrammatically in the scheme of Man-9 interacting with domains A and B of Cyt-CVNH (Fig. 3D). In this scheme, Man-9 interacts with domains A and B of Cyt-CVNH through its D1 arm only (Fig. 3D and supplemental Figs. S3 and S4A), and it is likely that this type of recognition, which is different from what is observed for CV-N (supplemental Fig. S4B), prevents cross-linking of Cyt-CVNH by Man-9.

To corroborate that indeed the Man9-Cyt-CVNH complex comprises one protein and two sugars, we carried out NMR relaxation measurements. Heteronuclear $T_2$ values for Cyt-CVNH and the Man-9-Cyt-CVNH complex were determined, yielding average $T_2$ values of 106 ± 4.5 and 71 ± 3.7 ms, respectively. These values are consistent with the expected mass of ~12 kDa for the protein and with the ~16 kDa mass matching the 2:1 Man-9-Cyt-CVNH complex (26).

**HIV Assays**—To determine the antiviral potency of the new lectin, Cyt-CVNH was assessed in parallel with monomeric...
CV-NP51G, a stabilized version of wild type CV-N (25), in a single-cycle luciferase reporter assay. This assay relies on completion of the early steps in infection, including entry, reverse transcription, integration, and expression of the viral Tat protein.

Normalized inhibition curves for concentrations up to 100 nM are provided in Fig. 4. An IC_{50} value of ~0.175 ± 0.01 nM was extracted for Cyt-CVNH, compared with an IC_{50} value of ~0.7 ± 0.02 nM for CV-NP51G. Thus, Cyt-CVNH exhibits ~4-fold higher activity than CV-NP51G.

Discussion

The envelope glycoprotein gp120 of HIV-1 mediates host cell entry, which is initiated by engaging the host cell CD4 receptor. This causes a conformational change in gp120, resulting in coreceptor (CCR5 or CXCR4) binding and ultimately fusion of the viral and cellular membranes (27–29). Gp120 is highly glycosylated, containing a large number of high mannose sugars (8, 9, 30, 31), and the glycosylation sites are well conserved (32). A number of broadly neutralizing antibodies (Abs) target glycans on gp120 (33–35) and potentially could be used to combat HIV-1 infection. 2G12 was one of the first such antibodies described (36–38), followed more recently by a collection of Abs, including PGT121, PGT122, PGT128, and PGT135, that recognize dual protein–glycan epitopes, especially involving the sugars on Asn156/Asn173, and Asn137 (44).

A stable glycosylation site on Asn332 is one of the most conserved among HIV isolates. The crystal structure of the gp120 trimer clearly delineates the Asn332 glycan as a key element in PGT122 recognition (39, 44).

Akin to mannose-targeting Abs, lectins also interact with sugars, and several mannose-targeting lectins have been shown to possess virucidal properties against HIV. CV-N, discovered as one of the first HIV-1-inactivating lectins, is active against HIV-1 and -2, simian immunodeficiency virus, and other enveloped viruses like Ebola and Influenza (5) at nanomolar concentrations (6). Previous biochemical and biophysical studies revealed that two binding sites for Manα(1–2)Manα-containing sugars are located on CV-N: one each on domains A and B. It was shown that CV-N recognizes the terminal Manα(1–2)Manα units of both the D1 and the D3 arms of Man-8 and Man-9 as the primary target (10–14). Likewise, members of the CVNH family from Tuber borchii (TbCVNH), Ceratopteris richardii (CrCVNH), Neurospora crassa (NcCVNH), and Gibberella zeae (GzCVNH) also recognize Manα(1–2)Man disaccharides, but with lower affinity than CV-N (45, 46). Each of these proteins exhibits carbohydrate binding sites that are different in number and location. CrCVNH possesses two sites; TbCVNH possesses a single binding site on domain A, whereas GzCVNH and NcCVNH have one site only on domain B. With the exception of GzCVNH, carbohydrate binding specificities are distinct as well, and no potent HIV inactivation was observed with any of these proteins (45, 46).

Although in principle very powerful for delineating binding sites on proteins, using ^1H–^15N HSQC spectroscopy for following Man-9 binding to CV-N, even at low concentration, was impossible because precipitation of the sugar-protein complex occurred, caused by multisite/multivalent cross-linking (7, 19, 20). Here, by contrast, for Cyt-CVNH, no aggregation or precipitation was seen for the Man9-Cyt-CVNH complex. In addition, based on the patterns observed throughout the titrations with Man-2, Man-3, and Man-9, it is evident that Man-9 binds both sites on Cyt-CVNH through its D1 arm only (Fig. 3D). Thus, unlike for CV-N, where 1:1 binding between Man-9 and protein is causing precipitation, for Cyt-CVNH a 2:1 sugar-protein complex is formed, without any cross-linked higher molecular species noted.

Based on all available data, we developed an interaction model for CVNH lectins and glycosylated gp120, assuming that the interaction involves the glycan on Asn332. As the starting model, we used the crystal structure of the HIV-1 Env trimer in complex with the antibody PGT122 (blue; PDB accession code 4NCO; the antibody coordinates are omitted from one of the gp120 monomeric units). Onto the trimer, we superimposed the monomeric gp120 core structure (orange) in complex with CD4 (magenta; PDB accession code 3JWD) (Fig. 5, top panel). As can be appreciated, the Env trimer structure contains a large number of sugar molecules. In addition to Asn332, which bears Man8/Man9, the PGT122 Ab interacts with the base of the V1 and V3 loops on the protein and three more glycans on Asn301, Asn156/Asn173, and Asn332 (44).

The distance between the two glycan binding sites on CVNH lectins is ~40 Å. If the D1 arm of Man-8/Man-9 on Asn332 is binding one of these two sites, a possible second sugar on Asn156 could interact with the second site. First, we placed the Cyt-CVNH x-ray structure onto the Asn332 glycosylation site. This allows Man8/Man-9 to interact with domain A. Next we rotated the Cyt-CVNH model around the Asn332 glycosylation site. This allows Man8/Man-9 to interact with domain B; this was the sugar on Asn156. Using the sugars on Asn156 and Asn332 (Fig. 5, bottom panel), the CVNH lectin is contacting approximately the same region as the PGT122 antibody.
Structure and Anti-HIV Activity of Cyt-CVNH

It is well established that interaction of CD4 with the HIV envelope causes conformational changes in the trimer, leading to a more open conformation that exposes the co-receptor binding site (47). Although steric occlusion of the CD4 binding site by PGT121 binding to gp120 does not seem to be the mechanism for competitive inhibition, allosteric effects that interfere with the CD4-induced conformational changes may play a role (40). Such mechanisms may also apply to glycan-binding lectins, although other mechanisms cannot be ruled out at present, and further studies are necessary to determine whether CVNH lectins are capable of preventing CD4 binding through an allosteric mechanism. Alternatively, they may exert their antiviral activity by inducing post-binding conformational effects that prevent CD4-bound gp120 from interacting with CCR5 or CXCR4 co-receptors.

Experimental Procedures

Protein Expression and Purification—The protein was expressed from a synthetic gene, using pET26b(+) (Novagen; Madison, WI) and *Escherichia coli* BL21(DE3) as expression vector and host strain, respectively. The cells were initially grown at 37 °C, induced with 1 mM isopropyl-β-D-thiogalactopyranoside at 16 °C, and grown for ~12 h at 16 °C for protein expression. Uniform 15N and 13C labeling of Cyt-CVNH was carried out by growth in minimal medium, using 15NH4Cl and 13C6-glucose as the sole nitrogen and carbon sources, respectively. The cells were harvested by centrifugation (4600 × g for 15 min at 4 °C), resuspended in 20 mM potassium phosphate buffer, pH 6.0, and lysed using a microfluidizer (MicroFluidics M-110Y, Hyland Scientific). Cell debris was removed by ultracentrifugation (120,000 × g), and the supernatant was fractionated by gel filtration on a Superdex 75 (HiLoad 2.6 × 60 cm; Amersham Biosciences) column, equilibrated in 20 mM sodium phosphate buffer, pH 6.0. Protein fractions containing Cyt-CVNH were collected and concentrated to 10 mg/ml using Centriprep devices (Millipore). Protein purity was estimated >99% by SDS-PAGE and mass spectrometry.

Crystalization and X-ray Data Collection—Cyt-CVNH protein was crystallized by sitting drop vapor diffusion from a 1.0 mM protein solution in 20 mM sodium phosphate buffer, 0.01% NaN3, pH 6.0. The best crystals were obtained at room temperature in 0.2 M magnesium chloride hexahydrate, 0.1 M Bis-Tris, pH 5.5, with 25% (w/v) PEG 3350 and 15% ethylene glycol as precipitants. Crystal growth took ~30 days, yielding crystals with dimensions of 0.20 × 0.30 × 0.70 mm. X-ray diffraction data were collected from a single flash-cooled crystal (~180 °C) at the Southeast Regional Collaborative Access Team facility sector 22-ID of the ADSC Photon Source (Argonne National Laboratory, Chicago, IL). 451,609 total observations were reduced to yield 34,474 unique reflections (98% complete), with a 13.1 redundancy, to 1.6 Å resolution, with an internal R factor (based on intensities) of 0.11. The data were processed and scaled with the HKL2000 package (48).

Crystal Structure Determination and Refinement—The crystal structure of Cyt-CVNH was solved by molecular replacement in Phenix (49), using the monomeric NMR structure of wild type CV-N (PDB accession code 2EZM) (5) as the search model. The initial model included two independent subunits of the chain, comprising residues Leu1–Lys48 and Phe54–Glu101, with the hinge-loop region (Trp49–Asn53) omitted. Iterative rigid body and simulated annealing refinement in Phenix was alternated with model building, including the hinge-loop region, in Coot (50). The final stages of refinement included periodic examinations of σA-weighted electron density (2mFo − DFo) and difference electron density (mFo − DFo) maps, as well as the introduction of water and several cryoprotectant molecules. Analysis of the final structural model was performed using PROCHECK (51). Approximately 98% of all residues reside in the favored region of the Ramachandran plot (52) with no residues in the disallowed regions. The final model was also validated by MolProbity (53), with an overall clash score of 0.31 and percentile of 100%. Atomic coordinates and structure factors have been deposited in the Research Collaboratory for Structural Bioinformatics Protein Data Bank under accession code 5K79. All structural figures were generated with PyMOL (54).

NMR Spectroscopy—NMR spectra were recorded at 298 K on Bruker 600 MHz and 800 MHz AVANCE spectrometers,
equipped with 5 mm, triple resonance, three-axis gradient probes, or z axis gradient cryoprobes. For three-dimensional NMR experiments, the sample contained 0.5 mM protein in 20 mM sodium phosphate buffer, pH 6.0. For chemical shift assignments, a series of heteronuclear, multidimensional experiments, routinely used in our laboratory, were recorded (55–58). Complete 1H, 15N, and 13C backbone resonance assignments were obtained using NMR data obtained from two-dimensional 1H–15N HSQC, three-dimensional HNCACB, HN(CO)CACB, HNCA, and HN(CO)CA spectra.

**Binding Studies**—Binding of sugar to protein was assessed in titration experiments, using uniformly 15N-labeled Cyt-CVNH (150 μM) at 298 K in 20 mM sodium phosphate buffer, pH 6.0, 0.01% sodium azide, and 90% H2O/10% D2O, monitoring the chemical shift changes in 1H–15N HSQC spectra upon sugar addition. For fucamannose (Man-2) binding, aliquots of a 50 mM Man-2 stock solution were added to yield sugar/protein molar ratios of: 0, 0.5, 1, 2, 3, 4, 5, 7, 10, and 15. Analogous NMR titration experiments were performed with trimannose (Man-3), using aliquots of a 10 mM stock solution to yield sugar/protein molar ratios of: 0, 0.5, 1, 2, 3, 4, 5, and 6. The protein concentration in the titrations with oligomannose-9 (Man-9) was 10 μM, and aliquots from a 500 μM stock solution were added to yield sugar/protein molar ratios of: 0, 0.5, 1, and 2.

Free and sugar-bound protein resonances are in slow exchange on the chemical shift scale for all three sugars. The maximum sugar-bound protein signal intensity at the end of the titration is directly proportional to the bound protein signal at each point in the titration, and Kd values were obtained by nonlinear best fitting of the titration curves using KaleidaGraph (Synergy Software, Reading, PA) and the following equation:

\[
f_b = \frac{l_b}{l_{b \text{ max}}} = 0.5 \left( \frac{M + 1 + \frac{K_d}{[P]} \sqrt{M + 1 + \left( \frac{K_d}{[P]} \right)^2 - 4M}}{2M} \right)
\]

(Eq. 1)

**Anti-HIV Assay**—HIV-1 infectivity was assayed as described previously (59). For antiviral assays, recombinant proteins were serially diluted in sterile phosphate-buffered saline, and 5 μl were added to 500 μl of prediluted infectious HIV-1 (produced by transfection of 293T cells with the R9 molecular clone and incubated for 30 min at room temperature). Aliquots of the mixture (125 μl in duplicate) were added to cultures of TZM-bl cells (20,000 cells seeded per well the day before in a 48-well format), and after 2 days, cells were lysed and assayed for luciferase activity as previously described (60). IC_{50} values were determined by nonlinear best fitting of the normalized inhibition curves using KaleidaGraph (Synergy Software, Reading, PA). The results are representative of two independent experiments.

**Author Contributions**—E. M. and A. M. G. conceived and coordinated the study. R. B. performed NMR assignments and helped with NMR titrations and the preparation of figures. C. C. performed crystal optimization, and W. F. collected the diffraction data at the Advance Photon Source and was involved in x-ray data interpretation. J. S. and C. A. performed the HIV assays. E. M. and A. M. G. wrote the paper, and all authors approved its final version.

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