Solution Structure of a Hydrocarbon Stapled Peptide Inhibitor in Complex with Monomeric C-terminal Domain of HIV-1 Capsid

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The on-line version of this article (available at http://www.jbc.org) contains three supplemental figures and two supplemental tables.

The atomic coordinates and structure factors (code 2K1C) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

The on-line version of this article (available at http://www.jbc.org) contains three supplemental figures and two supplemental tables.

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2 The abbreviations used are: HIV-1, human immunodeficiency virus type 1; CA, capsid; CA-CTD, wild type C-terminal domain of capsid; mCA-CTD, monomeric and mutant form (W184A/M185A) of C-terminal domain of HIV-1 capsid protein; EIAV, equine infectious anemia virus; HSQC, hetero-nuclear single-quantum coherence; Lys, lysine-analog residue; MHR, major homology region; NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; PDB, Protein Data Bank; r.m.s.d., root mean square deviation.

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The human immunodeficiency virus type 1 (HIV-1) capsid protein plays a critical role in virus core particle assembly and is an important target for novel therapeutic strategies. In a previous study, we characterized the binding affinity of a hydrocarbon stapled helical peptide, NYAD-1, for the capsid protein \( K_D \approx 1 \mu M \) and demonstrated its ability to penetrate the cell membrane (Zhang, H., Zhao, Q., Bhattacharya, S., Waheed, A. A., Tong, X., Hong, A., Heck, S., Goger, M., Cowburn, D., Freed, E. O., and Debnath, A. K. (2008) J. Mol. Biol. 378, 565–580). In cell-based assays, NYAD-1 colocalized with the Gag polyprotein during traffic to the plasma membrane and disrupted the formation of mature and immature virus particles in vitro systems. Here, we complement the cellular and biochemical data with structural characterization of the interactions between the capsid and a soluble peptide analogue, NYAD-13. Solution NMR methods were used to determine a high resolution structure of the complex between the inhibitor and a monomeric form of the C-terminal domain of the capsid protein (mCA-CTD). The intermolecular interactions are mediated by the packing of hydrophobic side chains at the buried interface and unperturbed by the presence of the olefinic chain on the solvent-exposed surface of the peptide. The results of the structural analysis provide valuable insight into the determinants for high affinity and selective inhibitors for HIV-1 particle assembly.

Worldwide 30 million people are infected with human immunodeficiency virus type 1 (HIV-1), and it has claimed more lives than some of the deadliest epidemics in human history. HIV-1 belongs to the retroviral family, and significant progress made in understanding its life cycle has fueled the development of diverse therapeutic strategies. Important targets for intervention include inhibiting the fusion of the virus at the surface of CD4+ T cells, reverse transcription of the viral RNA, and processing of the gag polyprotein by the HIV-1 protease (1). However, none of these treatment strategies has proven to be fully effective against the rapid emergence of drug-resistant variants of the virus.

As we gain further insight into the structural biology of the virus particle itself and the mechanism of its assembly from the gag polyprotein, the latter has emerged as an important new target for drug development (2). The newly synthesized gag protein migrates to the inner surface of the cell membrane, where it buds into an immature particle that encapsulates the genomic material and important viral proteins. Subsequent proteolytic cleavage of the gag protein into the matrix protein, capsid, and nucleocapsid protein lead to repacking of the core and the formation of the mature virus particle. The interactions involving the capsid protein play a crucial role in the formation of the virus core particle with the correct morphology and are an important target for disrupting the assembly step.

The dimeric capsid protein consists of N- and C-terminal domains that are flexibly linked in each monomer, and dimerization is mediated by the C-terminal domain. In mature particles, the capsid protein assembles into a fullerene-like structure with densely packed hexamers of the capsid protein. The formation of the hexamer is mediated by the N-terminal domain of the capsid, and the hexameric rings are bridged by the C-terminal dimerization domain (3, 4). The first target for disrupting the CA assembly was the identification of small molecules that could bind to the N-terminal domain of the capsid protein (5, 6). Subsequently, a short peptide (CAI) bound to the C-terminal dimerization domain of the capsid protein was shown to disrupt the formation of the budding virus particle in vitro (7, 8). The possible clinical application of these compounds is limited by weak binding affinity, toxicity and, in the case of CAI, low membrane permeability.

The lack of insight into the mechanism of inhibition by these small molecules was remedied by the recently published cryo-electron microscopy image reconstruction of the capsid assembly (4). We now know that both CAP-1 and CAI bind onto a surface that could potentially disrupt the interactions between the N- and C-terminal domains of two different CA molecules packed in the hexameric rings of the lattice (4, 6, 9).

In a previous study starting with the sequence of CAI, we presented the structural basis for a redesigned peptide NYAD-1 that is cell-permeable and has a 10-fold higher affinity for CA-
C-terminal domain (Kd ~ 1 μM) (10). In the x-ray structure of CAI in complex with CA-CTD, the peptide forms an amphipathic helix with the hydrophobic surface buried at the complex interface (8). The location of charged residues Asp-4 and Glu-8 on the solvent-exposed surface of the peptide was ideal for chemical modification by hydrocarbon stapling, a well known technique for stabilizing the secondary structure of helical peptides by cross-linking the i and i + 4 positions with an olefinic chain via β-alanine analogues (13). The olefinic chain is also known to enhance cell permeability of peptides and has been used quite successfully for designing an activator from the BID (Bcl-2 interacting protein) (11) and reactivation of p53 tumor suppressor pathway successfully for designing an activator from the BID (Bcl-2 interacting protein) (11) and reactivation of p53 tumor suppressor pathway (12). Residues Asp-4 and Glu-8 from CAI were replaced with a non-standard amino acid (S)-2-(2′-pentenyl) alanine, is indicated by X in the amino acid sequence of the peptide.

SCHEME 1. Schematic representation of the NYAD-13 peptide structure. The non-standard amino acid, (S)-2-(2′-pentenyl) alanine, is indicated by X in the amino acid sequence of the peptide.

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ACCELERATED PUBLICATION: Structure of Complex of HIV-1 CA-CTD

**EXPERIMENTAL PROCEDURES**

**Peptide Synthesis**—The NYAD-13 peptide was synthesized by replacing the fourth (i) and eight (i + 4) residues of the linear peptide CAI ITFEDLLDYGGKKK by the olefin-bearing unnatural amino acid (S)-2-(2′-pentenyl) alanines stapled by the olefin metathesis using the Grubbs catalyst (13). The helical conformation of the free peptide was confirmed (data not shown) from circular dichroism (10). In the NMR assignment and structure, the peptide is numbered 1–14.

**NMR Samples**—Uniformly 15N/13C-enriched protein samples of mCA-CTD were produced by expressing the pET14b plasmid encoding the gene in Escherichia coli BL21 (DE3) cells cultured in M9-minimal medium containing 15NH4Cl (Cambridge Isotope Laboratories) and [13C6]glucose as sole nitrogen and carbon source, respectively. Recombinant proteins were isolated from bacteria as described previously (10), and the integrity of the samples was confirmed by mass spectrometry. NMR samples were prepared from U-15N,13C-labeled C-CA sample complexed with unlabeled NYAD-13 peptide dissolved in 100 mM NH4Ac buffer, 95% H2O, 5% D2O, and 2 mM dithiothreitol at pH 7.0. The molecular concentrations were estimated from the UV absorbance at 280 nm with extinction coefficients of 2,980 m-1 cm-1 (peptide) and 3,105 m-1 cm-1 (protein), respectively. Resonance assignments were made on a sample containing 400 μl of 1.9 mM U-15N,13C-labeled mCA-CTD complexed with 1.8 mM unlabeled NYAD-13 in the acetate buffer. NOE data were collected on two samples containing 600 μM U-15N,13C-labeled mCA-CTD complexed with 900 μM unlabeled peptide in 90% H2O, 10% D2O, and 100% D2O. In the NMR assignment and structure, the CA protein is numbered from the natural sequence, 147–231 Gl: 2801 504.

**NMR Resonance Assignments**—NMR data were acquired at 25 °C on Bruker AVANCE spectrometers equipped with Z axis gradient triple axis CryoProbes. A standard suite of backbone (HNCA, HN(CO)CA, HN(CA)CO, HN(CC)CO, HN(CAC)CO) and side-chain experiments (HCCCONH, HCH-TOCSY, HCH-COSY) was acquired at 500 MHz for chemical shift assignments (14, 15). Distance restraints were obtained from 13C-edited three-dimensional NOESY-HSQC (100-ms mixing time) optimized for aliphatic and aromatic resonances and acquired on both H2O and D2O samples. Information for the amide region was obtained from a 15N-edited three-dimensional NOESY-HSQC acquired at 800 MHz.

The peptide NYAD-13 was assigned using two-dimensional 15N,13C f1,f2-filtered NOESY acquired at 900 MHz (16). Inter-molecular NOEs were obtained from two-dimensional 15N,13C f1-filtered NOESY at 900 MHz and three-dimensional 15N,13C f1-filtered 13C-edited NOESY-HSQC experiment recorded with 100-ms mixing time at 800 MHz on both H2O and D2O samples (17).

**Structure Calculations**—NMR data were processed in Topspin 1.3 from Bruker Biospin and analyzed using CARA1.5 (18). The structure of the peptide-bound complex was calculated in two stages using the programs CYANA 2.1 (19, 20) for the automatic assignment of the NOE peak lists, and a final water refine-
structures had a backbone r.m.s.d. of 0.36 Å. The new definitions of the bond lengths and angles were intro-
linker was generated and energy-minimized in PRODRG2 (24). The structure for the non-standard amino acid

dihedral violations greater than 5°. The CYANA-generated dis-
There were no distance violations greater than 0.2 Å and no
distance restraints were converted into the CNS format
(25). The structural statistics of the 20 best structures are
reported in Table S1.
The wild type C-terminal domain of the HIV-1 capsid pro-
protein forms a symmetric dimer with a $K_d \sim 18 \mu M$ (26). The
dimer interface is stabilized by hydrophobic interactions
involving the side chains of Trp-184 and Met-185 and salt
bridges (26). Dimerization of the capsid domain is completely
abolished by mutating either one of these sites (Trp-184 and
Met-185) to alanine, and the defective variants inhibited assem-
bling from conformational exchange, we decided to work with
the monomeric form of the mutant capsid protein.

Structure of NYAD-13 in Complex with mCA-CTD—The
monomeric form of CA-CTD as shown in Fig. 1 consists of a
four helix bundle (161–172, 179–192, 196–205, 211–217) sta-
bilized by the hydrophobic core of the protein. The N-terminal
region has a short $3\rightarrow 10$ helix (150–152) and a strand (153–158)
connected by a type 1 β turn (156–159) to helix I. The extreme
C-terminal residues 219–231 form an unstructured floppy tail.
The absence of residual structure at the C terminus was con-
firmed by backbone amide–proton relaxation experiments.

Helix I, which is also part of the highly conserved MHR motif,
plays a crucial role in stabilizing the helical scaffold of the
protein. Important hydrophobic contacts are manifested in a large
number of long range NOEs involving the aromatic rings of
Phe-161, Tyr-164, and Tyr-169 and the methyl groups of Val-
165 and Leu-172 (Fig. 1B). The tight packing of side chains in
the buried core of the protein is reinforced by the absence of
Tyr-164 ring flips on the fast NMR time scale. In the peptide
complexed structure of mCA-CTD, helix 2 is very well defined
with a kink starting at Thr-188, and this is in contrast to the
partial loss in structure reported in previous studies (28, 29).
Binding of the peptide thus appears to stabilize the secondary
structure of helix 2.

In the x-ray structure of CA-CTD (PDB code 1AUM), con-
served residues from the MHR motif, Arg-167, Glu-159, Gly-
156, and Arg-154, participate in a network of hydrogen bonds
vital to the stability of the capsid fold (26). Although we did not
see evidence of these hydrogen bonds in all our structures, we
did observe the following donor-acceptor pairs (Glu$^{\text{HN}}$–159 –
Gly$^{\text{CO}}$–156, Arg$^{\text{HH}2}$–167–Arg$^{\text{CO}}$–154) within hydrogen-bond-
ing distance in at least 40% of the ensemble.

The peptide binding site on the protein is a shallow hydro-
phobic pocket lined by residues from all four helices. The
bound peptide (residues 3–11) adopts a helical conformation
where the hydrophobic side chains make extensive intermolec-
ular contacts with specific direct experimental confirmation
from NOEs. The long axis of the peptide is perpendicular to
both helix I and helix II with the N terminus buried against helix

The absence of any change in line width between
the free and the peptide-bound forms of CA-CTD further sup-
ported this conclusion. To meet the immediate goal of this
study and circumvent the problem of differential line-broaden-

3 S. Bhattacharya, H. Zhang, A. K. Debnath, and D. Cowburn, unpublished results.
II and stabilized by the capping interactions involving Thr-2 and Asn-183 (8). The importance of Asn-183 at this position is supported by observing several NOEs between Asn-183 sidechain protons and Thr-2 and Phe-3 resonances. The peptide forms an amphipathic helix that buries several hydrophobic side chains of Phe-3, Leu-6, Tyr-9, and Tyr-10 at the interface of the complex. The most important residue appears to be the aromatic ring of Phe-3, which inserts itself partly between helix I and helix II of mCA-CTD (pink) and mCA-CTD (green) based on alignment generated from residues in helix I, helix III, and helix IV (r.m.s.d. = 0.8 Å). When helix II is included, the r.m.s.d. increases to 1.3 Å. Residues that are important for binding the target peptide and rearranged through the helix movement are indicated in the figure. The PDB code for CA-CTD structure used in the alignment is 1A8O. The figures were generated in MOLMOL 2.1 (26).

Acetylated PDB 2BUO (CA-CTD) and complex with NYAD-13 (2BUO) in the presence and absence of CAI (2BUO) are remarkably high (Fig. 2). The most significant differences between the three structures are observed in the relative position of helix II, which plays a critical role in dimer formation. The backbone Cα atom r.m.s.d. of all four helices when compared with CA-CTD in the absence of CAI is 0.8 Å, whereas the superposition of helix I (161–172), helix III (196–205), and helix IV (211–217) is better than 0.4 Å. When complexed with NYAD-13, the C terminus of helix II in mCA-CTD is displaced by 2.8 Å when compared with the free protein (Fig. 2D). This repositioning of helices I and II results in a much larger (6 Å) movement in the wild type CA-CTD complexed with CAI (8). This movement of helix II is also shown to be coupled to a decrease in the buried surface area at the dimer interface and predicted to destabilize the structure of the dimer (8).

In the complex of mCA-CTD with NYAD-13, the movement of helix II appears to be restricted and may simply reflect the need to preserve the packing between the two long helices in the absence of the stabilizing effect of the dimerization contacts. As such, the rotation of helix II does not trigger a major conformational change in the hydrophobic core of the protein, but the flipping of side chains essentially remolds the topology of the binding surface. In support of an induced fit mechanism, the coupled rearrangement of several side chains creates new cavities and provides access to buried residues. The importance of these changes is highlighted in Fig. 2D. The aromatic ring of Tyr-169 flips by 90°, which facilitates the docking of Phe-3 into a newly created cavity lined by Leu-172 and Phe-168. The lateral movement of helix II also removes Lys-182 from the N terminus of the peptide, and this position is now occupied by Asn-183 important for capping interactions.

The similarities in the packing of side chains at the intermolecular surface of the NYAD-13 and CAI complexes suggest a common mechanism of binding that relies on the recognition of hydrophobic side chains. The packing of the aromatic rings of Phe-3, Tyr-9, and Tyr-10 play a crucial role in anchoring the peptide to the protein. The relative positions and average ori-
entation of the rings is preserved in the two structures. We do see a difference in the stereochemistry of the methyl groups of the two leucines. The side chain of Leu-6 is completely buried at the complex interface and thus favors a single rotamer in NYAD-13 but is flipped when compared with CAI. This is in contrast to Leu-7, which is not constrained by interactions on the solvent-exposed surface of the peptide. In conclusion, the relative flexibility of the helix I/II interface and the floppiness of the solvent-exposed surface of the peptide play a critical role in eliminating steric clashes and maximizing the binding affinity without sacrificing some of its favorable drug like properties.

A strategic goal relevant to future design efforts is to understand the molecular basis for specificity of such peptide inhibitors against retroviruses in general. In the previous study, we have shown the peptide analogue NYAD-1 to be active only in inhibiting mature and immature HIV particle assembly and not against EIAV particles (10). In the sequence alignment of p24 domains (supplemental material), the highly conserved residues belong to the MHR motif, but elsewhere, there are few similarities. In the EIAV sequence, the two substitutions (Y169L and N183K) in the hydrophobic binding surface could dictate the ability to bind NYAD-1 with sufficient affinity. The conclusions drawn from the sequence analysis are highly speculative and do not include the effect of EIAV particle morphology. Structural details of the capsid formation in the presence and absence of inhibitors are crucial for addressing some of these issues.

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