JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Communication
pubs.acs.org/JACS

Enhancement of α-Helix Mimicry by an α/β-Peptide Foldamer via Incorporation of a Dense Ionic Side-Chain Array

Lisa M. Johnson,[†] David E. Mortenson,[†] Hyun Gi Yun,[†] W. Seth Horne,[†] Thomas J. Ketas,[‡] Min Lu,[§] John P. Moore,[‡] and Samuel H. Gellman*[,†]

[†]Department of Chemistry, University of Wisconsin, 1101 University Avenue, Madison, Wisconsin 53706, United States

[‡]Department of Microbiology and Immunology, Weill Medical College of Cornell University, New York, New York 10021, United States

[§]Public Health Research Institute Center, Department of Microbial Biology and Molecular Genetics, UMDNJ—New Jersey Medical School, Newark, New Jersey 07103, United States

Supporting Information

ABSTRACT: We report a new method for preorganization of α/β-peptide helices, based on the use of a dense array of acidic and basic side chains. Previously we have used cyclically constrained β residues to promote α/β-peptide helicity; here we show that an engineered ion pair array can be comparably effective, as indicated by mimicry of the CHR domain of HIV protein gp41. The new design is effective in biochemical and cell-based infectivity assays; however, the resulting α/β-peptide is susceptible to proteolysis. This susceptibility was addressed via introduction of a few cyclic β residues near the cleavage site, to produce the most stable, effective α/β-peptide gp41 CHR analogue identified. Crystal structures of an α- and α/β-peptide (each involved in a gp41-mimetic helix bundle) that contain the dense acid/base residue array manifest disorder in the ionic side chains, but there is little side-chain disorder in analogous α- and α/β-peptide structures with a sparser ionic side-chain array. These observations suggest that dense arrays of complementary acidic and basic residues can provide conformational stabilization via Coulombic attractions that do not require entropically costly ordering of side chains.

Considerable effort has been devoted to the development of oligoamides that are partially or totally comprised of non-proteinogenic subunits but nevertheless mimic the informational properties of α-helices.[1,2] Such compounds can be useful for blocking protein—protein interactions that depend upon α-helix recognition while avoiding the rapid proteolysis that limits biological applications of conventional peptides. Despite recent progress, a universally accepted design protocol has not yet emerged, and it remains important to explore new strategies. Our previous work in this area has focused on oligomers that contain α- and β-amino acid residues ("α/β-peptides"); for example, we have reported α/β-peptides that mimic the long α-helix formed by the C-terminal heptad-repeat (CHR) domain of HIV protein gp41.[3] These α/β-peptides inhibit the gp41-mediated HIV entry process, presumably by interfering with formation of a critical six-helix bundle intermediate that drives fusion of the viral envelope and the target cell membrane.[4] This intermediate comprises three CHR segments and three N-terminal heptad-repeat (NHR) segments of gp41.[5] Here we use gp41 CHR mimicry to evaluate the interplay between two strategies for optimization of α/β-peptide helicity.

Our original CHR mimics were based on α-peptide T-2635 (38 residues),[6] a very potent HIV fusion inhibitor. In the first of two design steps,[3a] $\alpha \rightarrow \beta^3$ replacements were made systematically to generate an ααβααaβ backbone pattern. The resulting α/β-peptide (**1α/β**; Figure 1) bears the same side-chain sequence as T-2635, but the backbone contains additional CH_2 units. When **1α/β** adopts an α-helix-like conformation, the β residues are aligned along one side, and the "β-stripe" is oriented toward the solvent upon six-helix bundle formation.

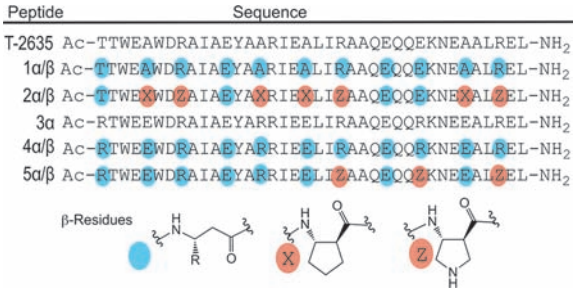

Figure 1. Sequences of α- and α/β-peptides derived from the CHR domain of the HIV protein gp41. Blue and tan circles indicate β^3 and cyclically constrained β residues, as illustrated at the bottom.

1α/β is significantly less effective than homologous α-peptide T-2635 in terms of participating in six-helix bundle assembly or inhibiting HIV infection of cells.[3a] At least two factors (potentially working in tandem) could underlie this difference. First, each of the 11 $\alpha \rightarrow \beta^3$ replacements in **1α/β** introduces a new, flexible C—C bond into the backbone relative to α-peptide homologue T-2635; these extra bonds should increase the conformational entropic penalty associated with helical folding of **1α/β** relative to T-2635. Second, although the helix formed by the ααβααaβ backbone of **1α/β** is very similar

Received: March 13, 2012
Published: April 23, 2012

   dx.doi.org/10.1021/ja302428d | J. Am. Chem. Soc. 2012, 134, 7317—7320

to an α-helix, the backbone change may nevertheless cause subtle geometrical alteration of the side-chain array at the packing interfaces of the six-helix bundle. Evidence for the importance of backbone flexibility in the poor performance of **1α/β** comes from the finding that helical assembly and inhibition of HIV fusion were considerably improved when 7 of the 11 β^3 residues were replaced with ring-rigidified β residues, to generate **2α/β**. Periodic β residue incorporation discourages protease activity: preorganized α/β-peptide **2α/β** was ~300-fold more resistant to degradation by proteinase K than was α-peptide T-2635.[3a]

We began the new studies by asking whether an increase in the number of side-chain ion pairing opportunities within the α/β-peptide helix could provide an alternative to use of ring-constrained β residues for enhancing helical propensity. Side-chain ion pairs have been widely employed to stabilize helical conformations of α-[7,8] and β-peptides.[9] Starting from C34, a potent α-peptide inhibitor of HIV fusion, Otaka et al. previously showed that the introduction of acidic and basic residues in a manner intended to promote multiple intrahelical i,i+4 ion pairs stabilizes the α-helical conformation and enhances helix bundle formation.[8] This work constitutes an important precedent for our effort to improve α/β-peptide design via ion-pair-based engineering. Indeed, a key feature of the subsequent design of α-peptide T-2635 was the introduction of seven acid/base residue pairs (mostly Glu/Arg) with i,i+4 spacing, to promote α-helicity via ion pairing[6] (Figure 2a). The engineered residue pairs were placed so that

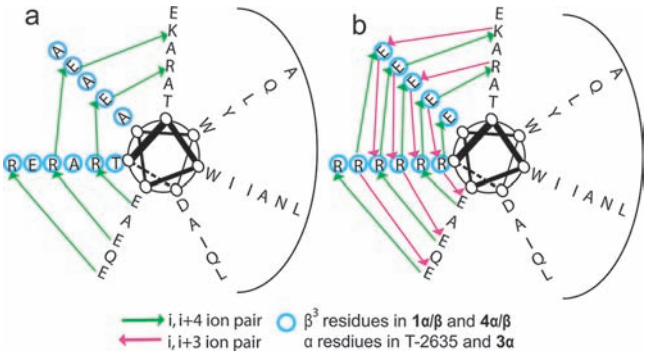

Figure 2. Helical wheel diagram of potential ion pairs for α/β-peptides (a) **1α/β** and (b) **4α/β** when blue circles are β^3 residues and for α-peptides (a) T-2635 and (b) **3α** when blue circles are α residues. The curved black lines indicate the surface engaged upon helix-bundle formation.

their side chains would be oriented toward the solvent, rather than buried, when T-2635 participates in six-helix bundle assembly. In addition to new acidic and basic residues, the sequence of T-2635 contains many mutations that introduce non-charged residues with high α-helical propensity (mostly Ala) relative to the wild-type gp41 CHR domain.[6]

We mutated six residues of T-2635 to generate peptide **3α**. As shown in Figure 2b (blue circles correspond to α residues for **3α**), these changes create the possibility of many new side-chain interactions: three new i,i+4 Arg-Glu ion pairs and nine new i,i+3 ion pairs involving Glu and Arg or Lys. Each of these interactions should stabilize the α-helical conformation of **3α** relative to that of T-2635; however, it is not clear whether all of these interactions could occur simultaneously, or whether cooperative or anti-cooperative effects should be expected within such a complex ion pair network.[7] T-2635 co-assembles with gp41-derived NHR peptide N36 to form a 3+3 α-helical hexamer that was previously characterized via crystallography (PDB 3F4Y).[3a] Peptide **3α** undergoes comparable self-assembly with N36, as detected via circular dichroism (CD). Variable-temperature CD measurements suggest that the **3α**+N36 helix-bundle is more stable than the T-2635+N36 helix-bundle (apparent T_m 91 vs 78 °C; see Supporting Information (SI)), which is consistent with our hypothesis that α-helix stabilization via enhanced i,i+3 and/or i,i+4 ion pairing in **3α** would promote helical assembly.

The ultimate goal of this research is to improve α/β-peptide mimicry of an information-bearing α-helix, so we prepared **4α/β**, the homologue of **3α** with an $\alpha\alpha\beta\alpha\alpha\alpha\beta$ backbone pattern[3,10] and locations of $\alpha\rightarrow\beta$ replacement sites matching those of **1α/β** and **2α/β**. To assess α-helix mimicry, we used a competition fluorescence polarization (FP) assay to quantify binding of **4α/β** to designed protein gp41-5.[3a,11] This protein contains three NHR segments and two CHR segments linked by flexible loops. Protein gp41-5 adopts a five-helix-bundle tertiary structure that displays a binding site for a CHR helix;[3b] the gp41-5+CHR complex mimics the putative six-helix bundle formed by a gp41 trimer during the HIV fusion process. As previously observed for T-2635,[3a] **3α** binds too tightly to gp41-5 for quantitative evaluation (K_i < 0.2 nM). In contrast, this system is suitable for comparing **1α/β** (K_i = 3800 nM) with new analogue **4α/β** (K_i = 11 nM). These data reveal that the additional ion-pairing potential in **4α/β** leads to a substantial improvement in functional α-helix mimicry: the affinity for gp41-5 of **4α/β**, which contains only flexible β^3 residues, is indistinguishable from the affinity of **2α/β**, in which 7 of the 11 β residues are preorganized with cyclic constraints.

In an effort to gain further insight on the role of side-chain ion pairing in promoting α- and α/β-peptide helicity, we co-crystallized the three complexes formed by protein gp41-5 with **3α**, **1α/β**, or **4α/β** and solved the structures based on X-ray diffraction data (Figure 3). In each case, the peptidic ligand completes the six-helix-bundle assembly, with intimate contacts

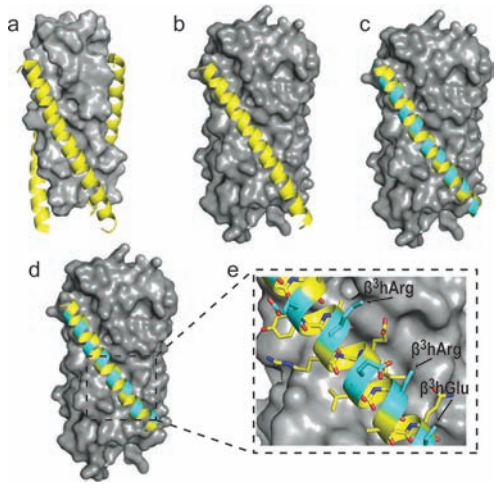

Figure 3. (a) Structure of T-2635+N36 (PDB 3F4Y; resolution = 2.0 Å).[3a] N36 is the gray surface, and T-2635 is the yellow ribbon. (b) Structure of gp41-5+**3α** (PDB 4DZU; resolution = 2.1 Å). (c) Structure of gp41-5+**1α/β** (PDB 3O42; resolution = 3.0 Å). (d) Structure of gp41-5+**4α/β** (PDB 4DZV; resolution = 2.1 Å). (e) Enlarged view highlighting missing side chains of **4α/β**. For parts (b–e) the gray surface is gp41-5, the yellow ribbon designates α residues, and the cyan ribbon designates β^3 residues.

dx.doi.org/10.1021/ja302428d | J. Am. Chem. Soc. 2012, 134, 7317–7320

between many peptide side chains and a long complementary groove on gp41-5. The helical surfaces containing residues that can potentially form ion pairs are oriented away from gp41-5 in all three complexes, as expected and as previously observed in the T-2635+N36 structure (PDB 34FY).[3a]

For the CHR-derived α- and α/β-peptides with an augmented set of acidic and basic residues, **3α** and **4α/β**, most of the putative ion pairing side chains are insufficiently ordered to be included in the structural model. In contrast, most of the acidic and basic side chains can be modeled into the electron density for T-2635 and homologue **1α/β**, despite the fact that the resolution of the latter structure compared to the others is significantly poorer. This trend raises the interesting possibility that the conformation-stabilizing effect of a dense network of acidic and basic residues, as found in **3α** and **4α/β** but not in **1α** and **2α/β**, results from a net Coulombic attraction that can be achieved via multiple alternative sets of side-chain conformations. (See the SI for a discussion of crystal packing differences among the structures.) If this hypothesis is correct, then the side-chain array may tolerate considerable disorder without sacrificing Coulombic stabilization, which could minimize the entropic cost that would arise from formation of specific side chain−side chain contacts. The high degree of apparent disorder in these side chains in the crystalline state is consistent with the proposal that the ion pairing networks in **3α** and **4α/β** bolster helical secondary structure despite the apparent population of multiple side-chain conformations. This conformational stabilization could underlie the high affinity of **4α/β** for gp41-5 and other favorable properties of **4α/β** and **5α/β** discussed below.

Our hypothesis regarding conformation-stabilizing effects of dense ionic side-chain arrays in **3α** and **4α/β** is distinct from and complementary to previous proposals based on experimental assessment of ionic interactions in peptides and proteins; however, a related hypothesis has been offered by Missimer et al. based on molecular dynamics simulations of the assembly of a designed peptide.[12] Geometric features of side-chain salt bridges have been explored via statistical surveys of the protein crystal structure database,[13] but this approach necessarily misses the phenomena operative in polypeptides such as **3α** and **4α/β**, for which side chains cannot be reliably modeled based on observed electron density. Several groups have analyzed so-called "complex salt bridges", formed by sets of three ionized side chains.[14] Both cooperativity and anti-cooperativity have been documented, relative to the component binary ion pairs, but the origin for these variations has not yet been fully explained. Clusters of acidic and basic side chains occur in many protein structures,[15] and it is therefore possible that the factors at work within the "delocalized" side-chain clusters in **3α** and **4α/β** are relevant to natural proteins. From a design perspective, our findings suggest that dense networks of complementary acidic and basic residues can be used as a rational strategy to enhance polypeptide conformational stability.[6,8]

The ability of **4α/β** to inhibit HIV infection of TZM-bl cells (Table 1)[16] was assessed to determine whether the high propensity for helical assembly, as manifested by strong binding to gp41-5, would result in disruption of the crucial gp41-mediated membrane fusion process. Across five HIV strains, **4α/β** displayed IC_{50} values for infection inhibition that are comparable to or better than those of T-20 (enfuvirtide),[17] an FDA-approved fusion inhibitor. The activity of **4α/β** was indistinguishable from that of **2α/β**, which contains many ring-constrained β residues. This similarity in inhibitory potency matches the similarity in affinity for gp41-5 of **4α/β** and **2α/β**.

Table 1. Inhibition of Infectivity of Different Viral Strains of HIV-1 by 4α/β, 2α/β, 3α, and Drug T-20[a]

Virus	Clade, Co-receptor	IC_{50} (St Dev) in nM 4α/β	2α/β	3α	T-20
DJ258	A , R5	78(25)	42(13)	26(2)	200(60)
JRFL	B , R5	153(61)	131(48)	105(56)	180(116)
NL4-3	B″, X4	8(5)	8(5)	3(2)	170(112)
CC 7/86	B , DM	49(14)	47(22)	63(63)	140(109)
UG270	D , X4	56(43)	39(24)	25(18)	110(91)

[a]T-cell-line-adapted strain of clade B.

The susceptibility of the new α/β-peptides to proteolysis was evaluated with proteinase K. As expected for a conventional α-peptide, **3α** was rapidly degraded: the half-life under our conditions was 2.2 min, and mass spectrometry indicated four major cleavage sites (Figure 4). Cleavage at three of these sites was suppressed for **4α/β**, but the fourth site remained susceptible, and **4α/β** displayed a half-life (3.5 min) similar to that of **3α**. This half-life is indistinguishable from that of **1α/β** (4.0 min), although **1α/β** is cleaved at two additional sites beside that observed for **4α/β**. Thus, the helical stabilization of **4α/β** relative to **1α/β** suggested by the dramatic difference in affinity for gp41-5 is not manifested at the level of proteolytic susceptibility.

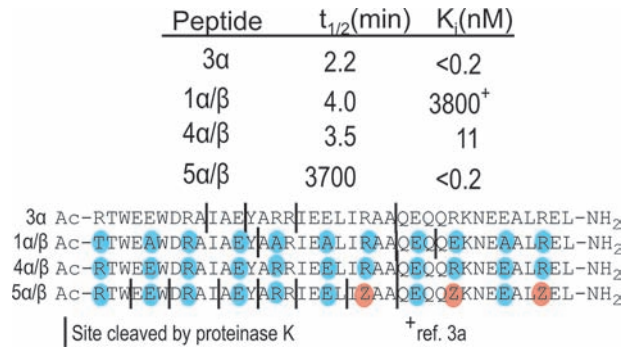

Figure 4. Comparison of half-life values from *in vitro* proteolysis with proteinase K, and deduced dissociation constants from a competition FP assay for binding to the protein gp41-5 (see SI for more detail). Sites cleaved by proteinase K were determined via mass spectrometry. Note that although many cleavage sites are detected for **5α/β**, this molecule is cleaved very slowly.

In an effort to enhance resistance to proteolysis, we prepared **5α/β**, an analogue of **4α/β** in which the flexible β^3-hArg residues near the lone cleavage site have been replaced with cyclically constrained APC residues (Z in Figure 1). This approach was very successful, leading to >1000-fold improvement in half-life (3700 min; i.e., ~2.5 days). FP analysis indicated that preorganized **5α/β** binds too tightly to gp41-5 for accurate measurement ($K_i \leq 0.2$ nM). Thus, partial preorganization via three $\beta^3 \rightarrow$cyclic replacements not only suppresses proteolytic degradation but also improves functional α-helix mimicry.

The results described here show that coordinated implementation of acid/base side-chain cluster engineering and β residue preorganization can lead to an improved activity profile for an α-helix-mimetic α/β-peptide. Although side-chain ion

dx.doi.org/10.1021/ja302428d | J. Am. Chem. Soc. 2012, 134, 7317−7320

pairs have previously been introduced in order to stabilize particular polypeptide conformations, the magnitude of the benefit has varied, and the origins of enhancements have been unclear.[6−9,14] In our case, augmenting the already substantial ion pair network of **1α/β** to generate **4α/β** provided a ∼350-fold improvement in binding to gp41-5 (∼3 kcal/mol). Moreover, our crystallographic analysis of α- and α/β-peptides bound to gp41-5 suggests a mechanism by which dense ionic side-chain clusters could contribute to the stability of a specific conformation while minimizing the entropic cost associated with structural ordering. It will be interesting to see whether these principles can be extended to other α-helical targets and to non-helical structures.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Detailed methods and crystallographic information. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

gellman@chem.wisc.edu

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the NIH (R01 GM056414 and U19 AI76982). L.M.J. was supported in part by a Chemistry-Biology Interface Training Grant (T32GM008505), D.E.M. was supported in part by a Molecular Biophysics Training Grant (T32GM008293), H.G.Y. was supported by a Hilldale Fellowship from the University of Wisconsin—Madison, and W.S.H. was supported in part by an NIH fellowship (CA119875). We are grateful to Katrina Forest and Kenneth Satyshur for technical advice and use of X-ray equipment. We thank I. Guzei for helpful comments. Use of the Advanced Photon Source was supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract No. DE-AC02-06CH11357.

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