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α-Helix Mimicry with α/β-Peptides

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Abstract

We describe a general strategy for creating peptidic oligomers that have unnatural backbones but nevertheless adopt a conformation very similar to the α-helix. These oligomers contain both α- and β-amino acid residues (α/β-peptides). If the β content reaches 25–30% of the residue total, and the β residues are evenly distributed along the backbone, then substantial resistance to proteolytic degradation is often observed. These α/β-peptides can mimic the informational properties of α-helices involved in protein–protein recognition events, as documented in numerous crystal structures. Thus, these unnatural oligomers can be a source of antagonists of undesirable protein–protein interactions that are mediated by natural α-helices, or agonists of receptors for which the natural polypeptide ligands are α-helical. Successes include mimicry of BH3 domains found in proapoptotic proteins, which leads to ligands for anti-apoptotic Bcl-2 family proteins, and mimicry of the gp41 CHR domain, which leads to inhibition of HIV infection in cell-based assays.

1. INTRODUCTION

Proteins evolve to display a specific set of properties that are advantageous to the organism in which they are produced. Scientists often seek molecules that mimic only a subset among the properties of a particular protein. Such mimics can be used as research tools, diagnostic agents, or medicines; some applications require the introduction of properties that are not manifested by the original protein.

Starting from a prototype protein, researchers have traditionally had access to only a few types of modification. (1) *Mutation*: the side chain can be altered at one position, or multiple side chains can be altered. (2) *Truncation*: portions of the polypeptide that are not necessary for the properties of interest can be removed. (3) *Augmentation*: polypeptide segments can be grafted onto the prototype protein to confer or enhance characteristics that are necessary for the intended application. (4) *Decoration*: nonpeptide moieties (e.g., a carbohydrate, a fluorophore, a synthetic polymer) can be attached to side chains or termini. These different modification strategies can be implemented in tandem.

Here, we focus on a different type of modification strategy, in which the polypeptide backbone is altered. This approach can be implemented in a way that does not affect the identity or sequence of side chains relative to the prototype. Alternatively, one can take advantage of unique property-modification opportunities that become available when α-amino acid residues are replaced with other subunits; however, this version of the strategy usually involves loss of native side chains. The backbone modification strategy can provide protein mimics that manifest unusual property profiles. Maintaining the recognition properties of a prototype polypeptide while diminishing or abolishing the susceptibility to degradation by proteases has been of particular interest (Seebach et al., 1998). We show how

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this goal has been achieved with oligomers that contain both α- and β-amino acid residues (α/β-peptides).

It should be noted that the use of subunits other than those derived from α-amino acids necessitates chemical synthesis of polypeptides at present. One can hope, however, that modification of the biosynthetic machinery responsible for protein synthesis (e.g., the ribosome, tRNA synthetases) will ultimately allow incorporation of building blocks beyond α-amino acids via RNA-templated synthesis. Hybrid approaches, such as using expressed protein ligation to connect a synthetic peptide that contains β-amino acid residues to a larger biosynthetic poly-α-peptide, offer considerable latitude for current efforts (Arnold et al., 2002; David et al., 2008).

2. HELICAL SECONDARY STRUCTURES FROM β-PEPTIDES AND α/β-PEPTIDES

Fundamental studies in several laboratories over the past two decades have revealed that oligomers of β-amino acids (β-peptides) can adopt a variety of helical secondary structures (Cheng, Gellman, & DeGrado, 2001; Gellman, 1998; Martinek & Fulop, 2003; Seebach & Matthews, 1997). β-Amino acids have two carbon atoms between the carboxyl and amino groups, and both carbons can bear side chains. Altering β-amino acid substitution patterns enables considerable variation in the nature and the extent of the secondary structure propensity. In this regard, β-amino acid residues are more versatile than α-amino acid residues, that is, there appears to be a larger number of easily accessed regular secondary structures among β-peptides than among α-peptides (Cheng et al., 2001; Gellman, 1998; Seebach & Matthews, 1997). β-Peptides constructed entirely from β^3-homoamino acid residues (Fig. 19.1E) generally seem to favor a helical secondary structure that contains a C=O(*i*)–N–H(*i–2*) H-bonding pattern among backbone amide groups. As these H-bonds occur in 14-atom rings, this conformation is sometimes referred to as the "14-helix" (Fig. 19.2) (Gellman, 1998; Seebach & Matthews, 1997; Seebach et al., 1996). The intrinsic 14-helical propensity of β^3 residues seems comparable to the α-helical propensity of most α-amino acid residues (Cheng & DeGrado, 2001), but the 14-helical propensity can be dramatically increased by using the cyclic β residue derived from *trans*-2-aminocyclohexanecarboxylic acid (ACHC) (Fig. 19.1A) (Appella, Barchi, Durell, & Gellman, 1999; Appella, Christianson, Karle, Powell, & Gellman, 1999; Lee et al., 2007; Vaz, Pomerantz, Geyer, Gellman, & Brunsveld, 2008). The nature of β-peptide helical propensity can be fundamentally altered by using a different ring constraint, as found in *trans*-2-aminocyclopentanecarboxylic acid (ACPC) (Appella et al., 1997; Appella, Christianson, Klein, et al., 1999; Lee, Syud, Wang, & Gellman, 2001), which promotes a secondary structure that contains C==O(*i*)–H—N(*i*+3) H-bonds ("12-helix"; Fig. 19.1B). The strong conformation-directing effects of ring-containing β-amino acid residues have no parallel among α-amino acid residues because in the latter case one cannot use a cyclic constraint to influence the torsional preferences of a backbone bond without removing an H-bonding site, as illustrated by proline.

Oligomers that contain both α- and β-amino acid residues display distinctive helical secondary structures (Choi, et al., 2008; Choi, Guzei, Spencer, & Gellman, 2009; De Pol, Zorn, Klein, Zerbe, & Reiser, 2004; Hayen, Schmitt, Ngassa, Thomasson, & Gellman, 2004). The heterogeneous backbone of α/β-peptides introduces a new parameter of structural variation: in addition to selecting the side-chain identities and the nature and extent of conformational preorganization (for the β residues), one can choose from among diverse α/β backbone patterns. For oligomers with 1:1 α:β alternation, the nature of the helix can be changed by altering the cyclic constraint embedded in the β residues and other structural parameters. Two helices that are favored by ACPC residues in β sites are shown in

Fig. 19.1C and D. Varying the α/β pattern, beyond 1:1, leads to additional helical secondary structures (Berlicki et al., 2012; Choi et al., 2009).

3. BIOLOGICAL FUNCTION FROM HELICAL β-PEPTIDES

As the rules governing helix formation have been elucidated for β-peptides and α/β-peptides, efforts to parlay this folding behavior into biological function have been pursued in a number of laboratories. Initial studies focused on functions that depend on general structural features of natural α-helical prototypes rather than on a highly specific three-dimensional arrangement of side chains projected from an α-helical scaffold. The first example involved inhibition of cholesterol absorption by intestinal cells. This activity was known to be manifested by α-peptides that form a globally amphiphilic α-helix (Boffelli et al., 1997) (i.e., a helix that projects lipophilic side chains along one side and hydrophilic side chains along the other), but the activity does not depend on a specific amino-acid sequence. Seebach et al. showed that β-peptides that form a short, globally amphiphilic 14-helix (Fig. 19.2) display weak inhibition of cholesteryl ester uptake in a cell-based assay (Werder, Hauser, Abele, & Seebach, 1999).

Globally, amphiphilic helical β-peptides were used by Hamuro, Schneider, and DeGrado, (1999) and Porter, Wang, Lee, Weisblum, and Gellman (2000) to achieve functional mimicry of α-helical host-defense peptides, which constitute part of the innate immune response to bacterial infection (Hancock & Sahl, 2006). The membrane-disruption activity of these peptides does not depend on a specific amino acid sequence or on absolute configuration (Wade et al., 1990). β-Peptides were shown to inhibit bacterial growth if the sequence pattern of lipophilic and cationic residues allowed formation of a globally amphiphilic 12- or 14-helix (Godballe, Nilsson, Petersen, & Jenssen, 2011). Comparable behavior was subsequently demonstrated for α/β-peptides, although in some cases, selective antibacterial activity did not seem to depend upon the ability to form an amphiphilic helix (Schmitt, Weisblum, & Gellman, 2004, 2007). Ultimately, it was found that relatively small molecules (Doerksen et al., 2004) displaying globally amphiphilic conformations could manifest comparable antibacterial activity, as could sequence-random poly-β-peptides (Mowery et al., 2007) and other polymers (Kenawy, Worley, & Broughton, 2007).

Subsequent β-peptide work focused on more specific functions that nevertheless may not require a particular spatial arrangement of side chains, as illustrated by the inhibition of γ-secretase (Imbimbo & Giardina, 2011). This membrane-embedded protease processes amyloid precursor protein to generate Aβ40 and Aβ42, the peptides that form plaques associated with Alzheimer's disease. Hydrophobic α-helix-forming α-peptides can inhibit γ-secretase, and the enantiomers of these peptides (containing D-rather than L-α-amino acid residues) are very active (Das et al., 2003). Imamura et al. (2009) then found that 12-helical β-peptides, oligomers of ACPC, are potent γ-secretase inhibitors.

In another example that does not seem to require a specific three-dimensional arrangement of side chains, Beck-Sickinger et al. have examined analogues of interleukin-8 (IL-8) in which the C-terminal α-helix is replaced by a β-peptide segment intended to form a globally amphiphilic 14-helix four to five turns in length (David et al., 2008). These chimeric polypeptides contained the human IL-8 sequence through residue 60, which forms a β-sheet. The C-terminal α-helix packs against one face of the β-sheet, thereby stabilizing the entire tertiary structure. Signaling through the CXCR1 and CXCR2 receptors relies mainly on contacts with the N-terminus of IL-8, but IL-8 must be properly folded in order for signal transduction to occur. Beck-Sickinger et al. found that signaling activity via CXCR1 was largely retained when the native C-terminal segment was replaced with a completely different α-peptide sequence known to form a globally amphiphilic α-helix. Reduced

activity was observed for an IL-8 chimera containing a C-terminal β-peptide segment designed to adopt a globally amphiphilic 14-helical conformation. This level of success in α-helix mimicry may reflect the system's ability to tolerate considerable sequence variation in the C-terminal segment, which suggests that a signaling-competent IL-8 fold does not require a precise arrangement of specific side chains within the helical portion.

Several groups have explored the use of β-peptides to mimic α-helices that are crucial for specific protein–protein recognition events. Success has been reported for the N-terminal domain of the tumor suppressor p53, which forms a short, distorted α-helix that binds to a cleft on DM2. This interaction centers on three side chains (Phe, Trp, and Leu) aligned along one side of the p53 helix (*i*, *i*+4, *i*+7 sequence relationship) (Kussie et al., 1996). β-Peptides that project these three side chains along one side of a 14-helix (*i*, *i*+3, *i*+6 sequence relationship) can bind to the DM2 cleft as well (Kritzer, Lear, Hodsdon, & Schepartz, 2004), even though the β-peptide 14-helix and the α-helix differ significantly as scaffolds for side chain arrangement. The recognition cleft of DM2 appears to be somewhat tolerant in terms of the geometry of the three side chains on the binding partner, because even macrocyclic α-peptides that contain these side chains but adopt a β-sheet conformation can bind to DM2 (Fasan et al., 2004). In addition, the DM2 cleft can be effectively targeted by small molecules (Zhao et al., 2002), including the nutlins (Vassilev et al., 2004) and oligomers engineered to display protein-like side chains in an α-helix-mimetic fashion (Desai, Pfeiffer, & Boger, 2003; Hara, Durell, Myers, & Appella, 2006; Plante et al., 2009; Yin et al., 2005).

It has recently been reported that helical β-peptides can be designed to interact with α-helical partners embedded in a membrane, specifically a transmembrane helix of the integrin $\alpha_{IIb}\beta^3$ (Shandler et al., 2011). Many aspects of this example are intriguing, including the observation that some all-β^3 sequences can apparently adopt 12-helical conformations rather than 14-helical conformations (Korendovych, Shandler, Montalvo, & DeGrado, 2011; Raguse, Porter, Weisblum, & Gellman, 2002). The helix–helix interaction mode in this system features a relatively large crossing angle, which means that the helices make contact over only a relatively small region (in contrast to a coiled-coil dimer, in which the helices are nearly parallel).

As the α-helix to be mimicked grows longer, and the number of side chains arrayed along one side of that helix and recognized by a binding partner grows larger, the negative impact of deviations from the α-helical spacing among side chains is likely to increase. This observation may explain why nearly all attempts so far to mimic informational α-helices with β-peptides, peptoids, or aryl oligomers have been limited to examples involving just three side chains (typically displaying an *i*, *i*+4, *i*+7 sequence relationship in the natural prototype), as summarized above. We devoted considerable effort to trying to mimic the arrangement of *four* aligned hydrophobic side chains that is characteristic of BH3 domain α-helices (*i*, *i*+4, *i*+7, *i*+11 sequence relationship) (Chen et al., 2005; Lessene, Czabotar, & Colman, 2008; Petros, Olejniczak, & Fesik, 2004), but we found that neither the β-peptide 14-helix nor the β-peptide 12-helix was an effective scaffold for this purpose (Sadowsky et al., 2007, 2005). Also unsuccessful was the 11-helical scaffold formed by α/β-peptides with a 1:1 α:β backbone pattern. The 14/15-helix formed by 1:1 α/β-peptides was somewhat more effective but still imperfect for BH3 domain α-helix mimicry. Part of the challenge in this system is that a properly folded BH3 domain displays a side-chain carboxylate on the opposite side of the α-helix relative to the linear array of four hydrophobic side chains (Asp at position *i*+9). Thus, in this case, and many others, successful mimicry of an α-helical "message" requires more than recreating an isolated side-chain stripe. Unnatural scaffolds will be of greatest use for α-helix mimicry if they allow recapitulation of the complete three-

dimensional arrangement of groups that make energetically important contacts with the partner protein.

4. α-HELIX MIMICRY WITH α/β-PEPTIDES

4.1. Sequence-based design

Our failure to identify effective BH3 domain mimics via structure-based design, starting from established β-peptide or 1:1 α/β-peptide helices, led us to explore a new approach to α-helix mimicry that has proven to be very fruitful. This approach begins with "sequence-based design," in which a subset of the α residues in a prototype sequence is replaced with homologous β^3 residues. α→β Replacements are made throughout the sequence according to simple patterns that result in 25–33% β residue incorporation. At this stage, β residue choice is "automatic" because the original side chain is retained, that is, leucine is replaced with β^3-homoleucine (β^3-hLeu), serine is replaced with β^3-hSer, and so on. In a second step, which is necessary in only some cases, the helix-forming propensity of the α/β-peptide is enhanced by $\beta^3\rightarrow$ cyclic β replacements. Fundamental α/β-peptide structural studies showed that incorporation of the C_α—C_β bond into a five-membered ring, with *trans* disposition of the amino and carboxyl groups, promotes a local conformation consistent with α-helix-like secondary structure (Choi et al., 2008; Horne, Price, & Gellman, 2008; Price, Horne, & Gellman, 2010). Therefore, residues derived from the β-amino acids ACPC and APC (Fig. 19.1E) are useful for residue-based preorganization of α-helix-mimetic α/β-peptides.

Initial evaluation of the sequence-based design approach involved self-recognizing α-helices based on the dimerization domain of yeast transcriptional regulator GCN4. GCN4-pLI is a designed variant that forms a parallel helix-bundle tetramer (Harbury, Zhang, Kim, & Alber, 1993). Figure 19.3 compares the crystal structure of GCN4-pLI with those of analogues containing α→β^3 replacements in three regular patterns, ααβααaβ, ααβ, and αααβ (Horne, Price, et al., 2008). Each of the α/β-peptides retains the side-chain sequence of the α-peptide prototype because for each α→β replacement, the β^3 residue is homologous to the original α residue. All three α/β-peptides adopt conformations very similar to the α-helix. Because the ααβααaβ pattern is tailored to the heptad residue repeat characteristic of the α-helix, in this case, the β^3 residues are aligned along one side of the helix. By design, this "β-stripe" is diametrically opposed to the hydrophobic side-chain stripe that provides the basis for self-assembly; thus, the β^3 residues reside exclusively on the exterior of the four-helix bundle for the ααβααaβ version. In contrast, the ααβ or αααβ patterns cause the β residues to spiral around the helix periphery. Two of the β^3 side chains in each case form part of the tetramer core (Horne, Price, et al., 2008).

The α/β-peptide helix-bundle crystal structures reveal that the ααβ, αααβ, and ααβααaβ backbones all adopt conformations that adhere closely to the α-helical prototype over eight helical turns, despite the presence of approximately one extra backbone carbon atom per turn in the α/β-peptides. Accommodation of these extra atoms appears to be smoothly distributed along the entire backbone (Horne, Price, et al., 2008).

The excellent structural mimicry of α-helical GCN4-pLI displayed by α/β-peptide homologues containing α→β^3 replacements in various periodic patterns was accompanied by destabilization of the tetrameric quaternary structure. We hypothesize that the lower stability of the α/β-peptide helix bundles relative to the α-peptide helix bundle results from conformational entropy. Each α→β^3 replacement introduces an "extra" flexible bond into the peptidic backbone, and there are 8–11 such replacements among the α/β-peptide homologues of GCN4-pLI. Thus, these α/β-peptides must suffer a greater loss of conformational entropy upon helical folding than does the α-peptide (Horne, Price, et al., 2008).

4.2. BH3 domain mimicry

Successful structural mimicry of self-recognizing α-helices by GCN4-inspired α/β-peptides that contain periodic, side chain-preserving $\alpha \rightarrow \beta^3$ replacements led us to explore comparable approaches for mimicry of α-helical "messages" that are read out by complementary proteins. BH3 domain α-helices represented an interesting starting point for such studies since we had previously encountered difficulties in mimicking BH3 domains with β-peptide or 1:1 α/β-peptide helices (Sadowsky et al., 2007, 2005). Initial sequence-based efforts focused on the Puma BH3 domain, which binds tightly to many antiapoptotic members of the Bcl-2 family (Chen et al., 2005). There are seven ways to incorporate the ααβααaβ pattern into a given amino acid sequence, which give rise to seven different positions of the β residue stripe on the helix (Fig. 19.4B) (Lee et al., 2011). We evaluated all seven of these $\alpha \rightarrow \beta^3$ replacement registers in the context of a Puma-derived 26-mer; binding to Bcl-x_L and to Mcl-1 was assessed for each α/β-peptide with a competition fluorescence polarization (FP) assay (Horne, Boersma, et al., 2008). Although these seven α/β-peptides are isomers or nearly isomers of one another (some have one more CH_2 unit thanothers, because some have eight β^3 residues while others have seven), and they all contain the same sequence of side chains, their affinities for each protein spanned several orders of magnitude (as measured by K_i). One ααβααaβ registry (**1**) showed the tightest binding to both Bcl-x_L and Mcl-1; if we use the standard *abcdefg* parlance of the coiled-coil field to define positions in each sequence heptad, and designate the positions of the four key hydrophobic residues as *a* and *d*, then the β residue positions in the tightest-binding α/β-peptide are *c* and *g*. For Bcl-x_L, α/β-peptide **1** bound too tightly to be quantified, as was true of the Puma BH3 α-peptide (K_i <1 n*M* in each case). For Mcl-1, however, α/β-peptide **1** bound at least 15-fold less tightly than the Puma α-peptide (K_i =150 n*M* vs. K_i <10 n*M*).

In light of the conformational entropy hypothesis offered above, it is intriguing that an α/β-peptide such as **1** could match a homologous α-peptide in binding to a partner proteins (Bcl-x_L) when the former has seven additional flexible backbone bonds relative to the latter. A crystal structure of Bcl-x_L complexed with α/β-peptide **2**, a slightly truncated analogue of **1**, showed that the stripe of β^3 residues is largely oriented toward solvent, as expected, but that some of the β^3 residues make direct contact with the protein (Fig. 19.4D) (Lee et al., 2011). Thus, it is possible that the α/β-peptide could serendipitously form improved contacts with the complementary Bcl-x_L cleft relative to the original BH3 domain. As the best α/β-peptide is substantially inferior to the BH3 domain α-peptide in binding to Mcl-1, this hypothetical serendipity would be protein-specific.

Our interest in mimicking BH3 domain α-helices is twofold: we seek to recapitulate the features required for tight binding to the BH3-recognition clefts of antiapoptotic proteins in the Bcl-2 family, but we want to *discourage* recognition by proteases. One well-appreciated problem associated with the use of peptides as drugs is the very high susceptibility of α-amino acid-based oligomers to proteolytic degradation *in vivo* (Lee, 1988). Replacing a single α residue with a β residue has long been known to inhibit proteolytic cleavage at nearby amide bonds, presumably because the β residue disrupts recognition of the peptide backbone by the protease active site (Steer, Lew, Perlmutter, Smith, & Aguilar, 2002). We wondered whether an α/β-peptide such as **1** has a sufficient β residue density to provide significant resistance across its entire length to the action of an aggressive protease.

We used proteinase K, a relatively nonspecific enzyme, to assess proteolytic susceptibility of α/β-peptide **1** (Wu & Kim, 1997). Although **1** contains mostly α residues (19 of 26), the half-life for cleavage of this α/β-peptide by proteinase K is >4000-fold longer than the half-life for cleavage of the corresponding α-peptide (Puma BH3 domain). This striking level of protection is not universal among oligomers with the ααβααaβ backbone pattern; for another member of this series, the half-life in the presence of proteinase K is only ~240-fold

longer than that of the α-peptide. Thus, the precise positioning of α→β replacements is important with regard to proteolytic susceptibility. However, these results and others discussed below suggest that incorporation of 25–30% β residues, with even distribution along the sequence, often delivers significant improvements in half-life in the presence of proteinase K.

In order to determine whether sequence-based design is broadly useful for generating BH3 domain mimics that resist proteolysis, we turned to the Bim BH3 domain, which binds to all antiapoptotic Bcl-2 family members (Chen et al., 2005). Starting from a Bim BH3 18-mer α-peptide, we examined all seven α/β-peptide homologues with the ααβααβ backbone pattern as well as all three with the ααβ pattern and all four with the αααβ pattern for binding to Bcl-x_L and Mcl-1 via competition FP assays. This set of 14 α/β-peptides displayed a large range of K_i values for each protein. Among the ααβααβ set, the one with the highest affinity for Mcl-1 (**3a**) had the same α→β registry that led to the highest Mcl-1 affinity among Puma-based α/β-peptides (i.e., β residues at heptad positions *c* and *g*). However, in contrast to the Puma series, a different α→β registry (**3b**; heptad positions *d* and *g*) provided the highest Bcl-x_L affinity among the Bim-derived oligomers with the ααβααβ pattern. α/β-Peptide **3a** bound to both proteins examined, that is, to Bcl-x_L as well as Mcl-1, but **3b** was highly selective for Bcl-x_L (Boersma et al., 2012).

The strongest binding to both Bcl-x_L and Mcl-1 among the 14 Bim-derived α/β-peptides was observed for **4**, which has the αααβ backbone pattern. α/β-Peptide **4** bound almost as tightly to Bcl-x_L as did the corresponding Bim BH3 α-peptide, but the affinity of **4** for Mcl-1 was considerably reduced relative to the α-peptide. Two of the four key hydrophobic side chains in the Bim BH3 sequence are contributed by β^3 residues in **4**, and a crystal structure of **4** bound to Bcl-x_L confirmed that these β^3 side chains make intimate contacts with the protein (Fig. 19.4E). Most studies of the Bim BH3 domain have involved 26-residue α-peptides rather than the 18-mer we used as a basis for the α/β-peptide Bim BH3 homologues. We therefore prepared **5**, the 26-mer that maintains the α/β backbone pattern of **4**. Comparison of **5** with the Bim BH3 26-mer α-peptide via competition surface plasmon resonance assays revealed that these two oligomers have nearly identical affinities for Bcl-x_L, Bcl-w, and Mcl-1, and that the α/β-peptide binds significantly more tightly than the α-peptide to Bcl-2. The half-life of α/β-peptide **5** in the presence of proteinase K is >180-fold longer than the half-life of the Bim BH3 α-peptide (Boersma et al., 2012).

Overall, our findings in the Bim system show that it is not always necessary to avoid α→β replacement at sites that contribute key side chains to a protein-recognition interface, as β residues contribute key side chains to the interface between Bcl-x_L and α/β-peptide **4**. In addition, these findings raise the possibility that α→β replacements need not be limited to the simple backbone patterns we have explored so far. These patterns are helpful, however, in allowing a researcher to examine diverse α+β arrangements in small oligomer libraries (e.g., the set of 14 from which **3a**, **3b**, and **4** were obtained) while ensuring a sufficient proportion and distribution of β residues to inhibit protease action.

4.3. Mimicry of the gp41 CHR domain: Inhibitors of HIV infection

We turned to mimicry of a long α-helix that is essential for infection of cells by HIV in order to assess the versatility of our α/β-peptide design strategy. The protein gp41 occurs on the surface of the HIV particle; after the virus has recognized a target cell, gp41 orchestrates the fusion of the viral envelope with the cell membrane (Eckert & Kim, 2001). This fusion process is believed to require formation of six-helix bundle from three gp41 molecules, each of which contributes one C-terminal heptad repeat (CHR) and one N-terminal heptad repeat (NHR) segment (Chan, Fass, Berger, & Kim, 1997). The CHR segment forms an α-helix

containing >10 turns. Long α-peptides derived from either the CHR or NHR domain of gp41 inhibit HIV infection in cell-based assays (Sodroski, 1999), and one such peptide, the 36-mer enfuvirtide, is an FDA-approved drug for AIDS patients. Enfuvirtide has a half-life of just a few hours in the bloodstream, and the daily dose is two 90 mg injections (Matthews et al., 2004).

Our efforts began with the 38-residue α-peptide T-2635 (Fig. 19.5), developed by researchers at Trimeris (Dwyer et al., 2007). T-2635 contains many mutations relative to the native CHR sequence that are intended to enhance α-helical propensity, including replacement of native residues with Ala and introduction of acid/base residue pairs intended to form salt bridges in the α-helical conformation (*i*, *i*+4 spacing). These mutations were introduced in positions that do not form interhelical contacts upon six-helix bundle formation. Figure 19.5B shows the crystal structure of the six-helix assembly that forms when T-2635 is mixed with the NHR-derived peptide N36 (Horne et al., 2009).

We used designed protein gp41-5 to compare the recognition properties of α-peptide T-2635 with α/β-peptide homologues. This protein contains five of the six α-helices of the six-helix bundle, with short, flexible linkers between each pair of helix-forming segments (Frey et al., 2006). The crystal structure of gp41-5 (Fig. 19.5A) shows that this protein adopts a five-helix bundle tertiary structure that displays a long groove into which a CHR helix can bind (Johnson, Horne, & Gellman, 2011). gp41-5 provides the basis for a competition FP assay that can be used for initial assessment of α/β-peptides intended to serve as CHR mimics.

The CHR helix is significantly longer than BH3 domain helices; therefore, CHR domain mimicry is more challenging than BH3 domain mimicry, because any small mismatch between a prototype α-helix and the similar helix formed by an α/β-peptide will be magnified as length increases. This difference in helix length presumably underlies an important functional difference between these two systems: small molecules can serve as potent inhibitors of BH3 domain recognition (Desai et al., 2003; Hara et al., 2006; Plante et al., 2009; Vassilev et al., 2004; Yin et al., 2005; Zhao et al., 2002); however, extensive efforts directed toward blocking gp41-mediated HIV infection with small molecules (including short α-peptides or β-peptides) have met with limited success so far (Frey et al., 2006; Johnson et al., 2011).

The length of the gp41 CHR domain discouraged us from exploring all of the diverse patterns of α→β replacement that had been examined in the context of BH3 domain mimicry. We focused on the ααβααααβ pattern in a registry that would orient the β residue stripe away from the surfaces of partner helices in the six-helix bundle, resulting in α/β-peptide **6**. This T-2635 homologue displays much lower affinity for gp41-5 than does T-2635 itself (K_i =3800 n*M* vs. K_i <2 n*M*, in the competition FP assay) (Horne et al., 2009). This profound drop in affinity is tentatively ascribed to the conformational entropy associated with the 11 additional flexible bonds in the backbone of **6** relative to T-2635. This hypothesis is supported by the crystal structure of **6** bound to gp41-5 (Fig. 19.5C), which does not reveal any obvious mismatch at the interface between the α/β-peptide and the complementary cleft on the protein. The conformational entropy hypothesis led us to examine analogues of **6** in which flexible β^3 residues were replaced with appropriate ring-constrained residues. "Appropriate" in this context means that the constraint is provided by incorporation of the C_α—C_β bond into a five-membered ring (as discussed earlier), and that the chemical properties of the ring reflect those of the side chain that is replaced. Thus, **7** was generated by replacing the four β^3-hAla residues in **6** with ACPC (hydrophobicity maintained) and the three β^3-hArg residues in **6** with APC (positive charge maintained). Preorganized α/β-peptide **7** showed considerable improvement in binding to gp41-5 (K_i =9 n*M*) relative to flexible α/β-peptide **6**, although **7** did not match the affinity of T-2635. A

crystal structure of **7** bound to gp41-5 shows that the rigidified β residues are well accommodated within a long α-helix-like conformation (Fig. 19.5D). Rigidified α/β-peptide **7** was quite resistant to degradation by proteinase K: the half-life for **7** was >280-fold longer than for T-2635 (in contrast, the half-life for flexible α/β-peptide **6** was only 20-fold longer than for T-2635). α/β-Peptide **7** is nearly as potent as T-2635 at inhibiting HIV infection in cell-based assays (Horne et al., 2009).

Recent efforts based on T-2635 have identified a new strategy, based on engineered ion pairs, for improving functional α-helix mimicry that is complementary to sequence-based design and the use of preorganized β residues. α/β-Peptide **8** has the same α→β replacement sites as **6** or **7**, but the sequence of **8** has been altered to maximize the number of possible intrahelical side chain ion pairs (*i*, *i*+3 and/or *i*, *i*+4). Despite the fact that **8** contains only flexible β^3 residues, this α/β-peptide matches preorganized α/β-peptide **7** in terms of affinity for gp41-5. In addition, **7** and **8** are equipotent in terms of inhibiting HIV infection in cell-based assays (Johnson et al., 2012).

The half-life of α/β-peptide **8** in the presence of proteinase K is quite similar to that of the corresponding α-peptide, which was surprising in light of the resistance to proteolysis of other α/β-peptides with comparable β residue proportion and distribution. Mass spectrometric analysis indicated that there are four major cleavage sites in the α-peptide, three of which are suppressed in the α/β-peptide, but that the remaining cleavage site in α/β-peptide **8** (after Ala-23) is very susceptible. We were pleased to find that replacement of three β^3-hArg residues near the cleavage site in **8** with ring-preorganized APC residues, to generate **9**, delivered an α/β-peptide with extraordinary resistance to proteinase K action (half-life ~2.5 days). Moreover, **9** is the tightest-binding α/β-peptide ligand for gp41-5 found to date (K_i <0.2 n*M*) (Johnson et al., 2012). Overall, the results obtained with **8** and **9** show that conformation-specific ion pairing and β residue preorganization can be employed in a coordinated manner to produce very effective α-helix mimics.

5. TOWARD A GENERAL APPROACH FOR α-HELIX MIMICRY WITH PROTEASE-RESISTANT α/β-PEPTIDES

The results summarized above suggest a straightforward strategy for developing α/β-peptides that mimic the recognition properties of an α-helical prototype. This strategy begins with the preparation of a small library of α/β-peptides in which subsets of α-amino acid residues are replaced with homologous β^3-amino acid residues (i.e., each α residue is replaced with the β^3 residue bearing the same side chain). These initial oligomers can have the ααβ, αααβ, or ααβααβ backbone patterns discussed earlier; however, it is possible that other β residue arrangements, not necessarily regular, will produce superior properties. One key goal for achieving biological activity is to have a density and specific placement of β residues that inhibit the action of proteases encountered by the α/β-peptide *in vivo*. Peptides containing a combination of α- and β^3-amino acid residues can be prepared via conventional stepwise solid-phase methodology, in either manual or automated mode. We have found that microwave irradiation enhances reaction rates of difficult couplings, especially with cyclic β residues. β^3-Amino acid building blocks that contain nearly all of the proteinogenic side chains are commercially available in protected forms amenable for Fmoc-based solid-phase synthesis.

If the α/β^3 set does not produce oligomers with sufficient efficacy, then one can modify the best member(s) of this set by replacing flexible β^3 residues with ring-preorganized residues. Ideally, these replacements would be made in a way that preserves the chemical character of the original side chain. The Fmoc-protected derivative of ACPC is commercially available (the (*S*,*S*) configuration is necessary to mimic the right-handed α-helix formed by $_L$-α-amino

acid residues), and this building block can be used to replace hydrophobic β^3 residues. Unfortunately, protected APC derivatives cannot currently be purchased. Recent results suggest that engineering of intrahelical ion pairs into solvent-oriented positions can be useful as well (Johnson et al., 2012).

We continue to refine strategies for the mimicry of signal-bearing α-helices with unnatural peptidic oligomers. Topics of ongoing interest include the following: (1) Development of ring-rigidified β-amino acids that provide acidic or neutral-polar side chains (for replacing residues such as β^3-hGlu or β^3-hGln). (2) Exploration of β^2-amino acid residues, particularly at positions that tolerate $\alpha \rightarrow \beta^3$ replacement and contribute a side chain to the interface between the prototype α-helix and the partner protein. β^2-Amino acids have the side chain adjacent to the carbonyl; therefore, replacing a β^3 residue with the isomeric β^2 residue causes a subtle shift in the spatial position of the side chain. Unfortunately, few β^2-amino acid building blocks are commercially available at present. (3) Incorporation of γ-amino acid residues (Guo et al., 2009). This prospect is attractive because an αβαaγα hexad contains the same number of backbone atoms as an ααααααα heptad, and we recently found that use of properly preorganized β and γ residues leads to formation of a helix very similar to the α-helix (Sawada & Gellman, 2011). (4) Extension of α/β- or α/β/γ-peptide design strategies to mimic poly-α-peptide conformations other than the α-helix so that this approach can be used to develop antagonists for a wider range of protein–protein interactions (Haase et al., 2012). Although improvements in protein surface mimicry should emerge from these ongoing efforts, current strategies for α-helix mimicry with α/β-peptides seem sufficiently mature for broad application.

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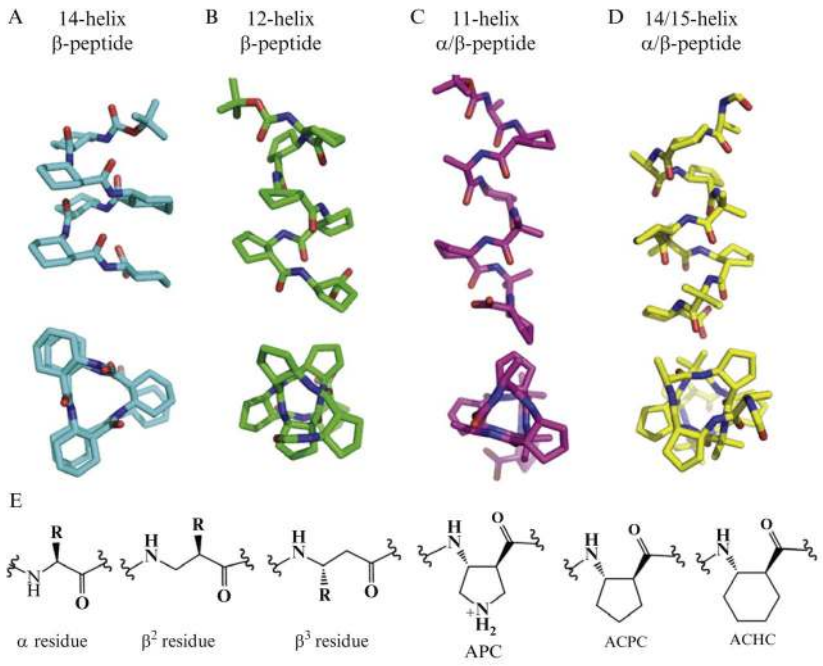

Figure 19.1.

Crystal structures: (A) the β-peptide 14-helix (ACHC hexamer; CSD ID: REF-PUN01; Appella, Christianson, Karle, et al., 1999), (B) the β-peptide 12-helix (ACPC hexamer; CSD ID: WELMAB; Appella, Christianson, Klein, et al., 1999), (C) the α/β-peptide 11-helix (1:1 α/β octamer; alternation of ACPC and Ala or Aib residues; CSD ID: OGATUM; Choi, Guzei, Spencer, & Gellman, 2008), (D) the α/β-peptide 14/15-helix (1:1 α/β decamer; alternation of ACPC and Ala or Aib residues; CSD ID: OGAVEY; Choi et al., 2008), and (E) α-Amino acid residue and several types of β-amino acid residues.

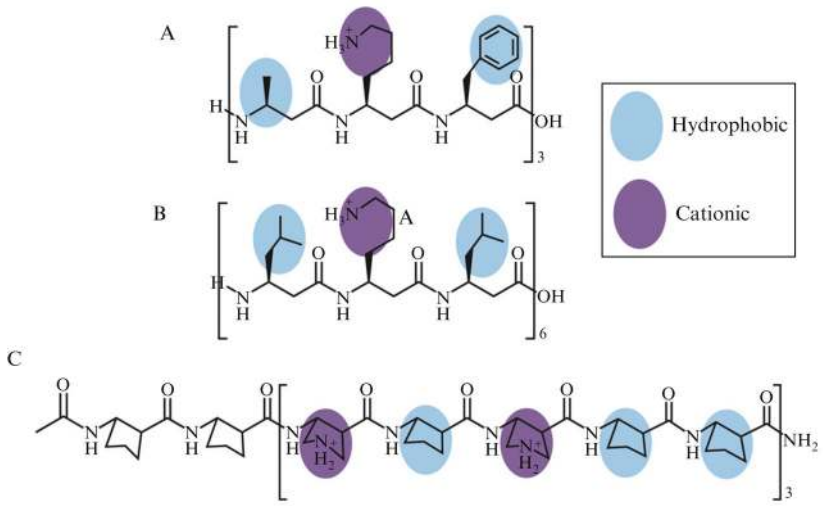

Figure 19.2.
Three examples of β-peptides designed to form globally amphiphilic helices: (A) 14-helical (3 residues per turn) cholesterol uptake inhibitor (Werder et al., 1999), (B) 14-helical antimicrobial peptide (Hamuro et al., 1999), and (C) 12-helical (2.5 residues per turn) antimicrobial peptide (Porter et al., 2000).

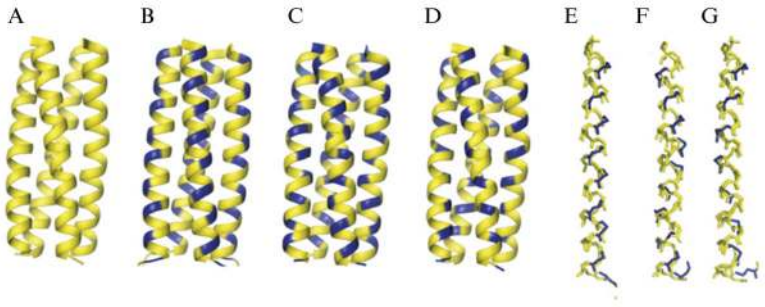

Figure 19.3.
Helix bundles formed by α-peptide GCN4-pLI (A) (PDB ID: 1GCL; Harbury et al., 1993) and three α/β-peptide homologues with varying backbone patterns: (B) ααβαααβ (PDB ID: 2OXK), (C) ααβ (PDB ID: 3C3G), and (D) αααβ (PDB ID: 3C3F). Each image is based on a crystal structure. α Residues are shown in yellow, and β^3 residues are shown in blue. Backbone overlays between the α peptide GCN4-pLI and (E) ααβαααβ, (F) ααβ, and (G) αααβ homologues (Horne, Price, et al., 2008).

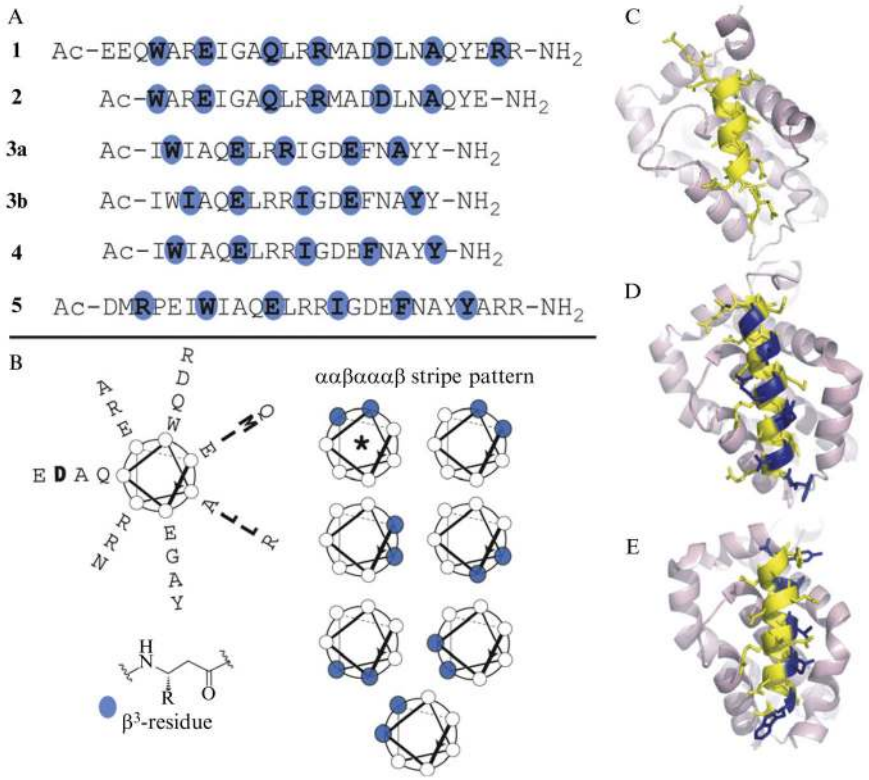

Figure 19.4.

(A) α/β-Peptides with side chain sequences derived from the Puma BH3 domain (**1** and **2**) or the Bim BH3 domain (**3a-b**, **4**, and **5**). The standard single-letter code is used; letters covered with a blue dot indicate β^3-homologues of the α residue designated by the letter. Compounds **1**, **2**, and **3a-b** feature the ααβαααβ backbone repeat, while **4** and **5** have the αααβ backbone repeat. (B) Helical wheel diagram of the Puma BH3 domain with the four key hydrophobic residues and the key Asp in bold. The smaller helix-wheel diagrams show how the stripe of β^3 residues shifts incrementally around the helix periphery among the seven different versions of the ααβαααβ backbone repeat (Horne, Boersma, Windsor, & Gellman, 2008); the asterisk (*) indicates the α/β registry found in Puma-derived α/β-peptides **1** and **2**. (C–E) Images illustrating the complexes between the BH3 domain-derived α- or α/β-peptides (α-residues are yellow, β residues are blue) and Bcl-x_L (light purple): (C) Bak BH3 domain (NMR structure; PDB ID: 1BLX; Sattler et al., 1997); (D) α/β-peptide **2** (crystal structure; PDB ID: 2YJ1; Lee et al., 2011); (E) α/β-peptide **4** (crystal structure; PDB ID: 4A1W; Boersma et al., 2012).

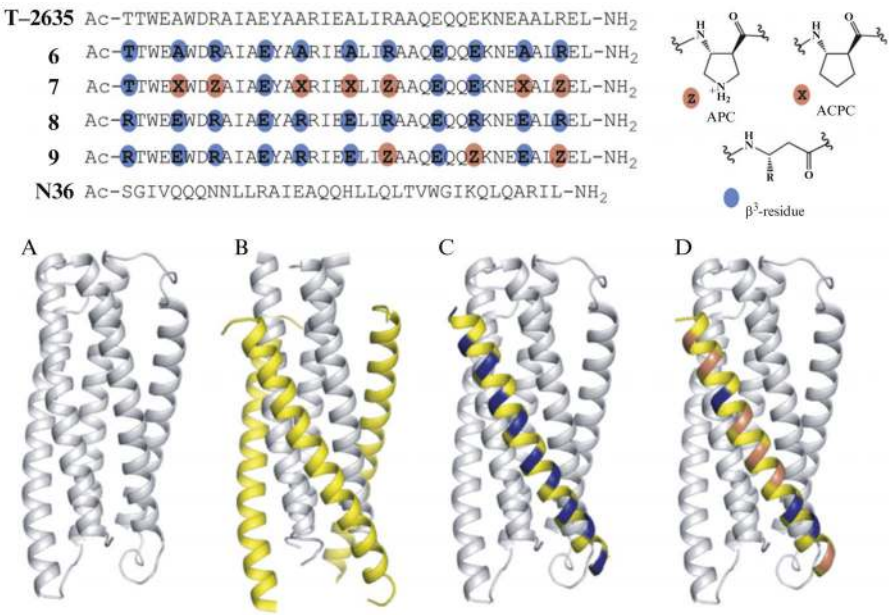

Figure 19.5.

α- and α/β-Peptides related to gp41. The color scheme key for the β residues in the sequences is shown at the right: blue for β^3 residues and peach for cyclic β residues. Crystal structures: (A) gp41-5 shown in gray ribbon cartoon (PDB ID: 3O3X; Johnson et al., 2011); (B) 6-helix bundle formed by α-peptides T-2635 (yellow) and N36 (gray) (PDB ID: 3F4Y; Horne et al., 2009); (C) α/β-peptide **6** (yellow for α residues, blue for β residues) bound to gp41-5 (gray) (PDB ID: 3O42; Johnson et al., 2012); (D) α/β-peptide **7** (yellow for α residues, blue and peach for β residues) bound to gp41-5 (gray) (PDB ID: 3O43; Johnson et al., 2011).