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Differential Effects of β^3- vs. β^2-Amino Acid Residues on the Helicity and Recognition Properties of Bim BH3-Derived α/β-Peptides

Geoffrey A. Eddinger and Samuel H. Gellman*

Abstract: Oligomers containing α- and β-amino acid residues ("α/β-peptides") have been shown to mimic the α-helical conformation of conventional peptides when the unnatural residues are derived from β^3-amino acids or cyclic β-amino acids, but the impact of incorporating β^2 residues has received little attention. We have investigated the effects of β^2 residues on the conformation and recognition behavior of α/β-peptides that mimic an isolated α-helix. This effort has focused on 26-mers based on the Bim BH3 domain; we have compared a set of isomers with identical α/β backbones that differ only in the placement of certain side chains along the backbone (β^3 vs. β^2 substitution). Circular dichroism data suggest that β^2 residues can be helix-destabilizing relative to β^3 residues, although the size of this effect seems to depend on side chain identity. Binding data show that $\beta^3 \rightarrow \beta^2$ substitution at sites that contact a partner protein, Bcl-x_L, can significantly influence affinity in a way that transcends effects on helicity. Overall, these results suggest that β^2-amino acids are useful complements to isomeric β^3-amino acids for tuning the recognition properties of α/β-peptides.

Mimicking the information encoded in the surface of a folded polypeptide with an unnatural molecule represents a substantial design challenge. Such mimics may antagonize specific protein-protein or protein-nucleic acid interactions or engage polypeptide-activated receptors.[1–3] These functional goals are often achieved with engineered peptides or proteins based on a conventional poly-α-amino acid backbone, but peptides are susceptible to proteolysis and adverse immunological recognition, which can limit utility *in vivo*.[4] This situation has inspired many efforts to develop unnatural oligomeric scaffolds that can mimic informational surfaces displayed by poly-α-peptides.[5–8] The regularity of α-helical secondary structure and the frequency with which α-helices are found at protein-protein interfaces[9] have engendered diverse strategies for α-helix mimicry,[10–18] many involving unnatural oligomeric backbones.[19–26] One approach involves the use of β-amino acids as building blocks for α-helix mimics, either exclusively or in combination with other types of amino acids.[26–29]

Our group has developed strategies for arranging α and βresidues in patterns that enable the resulting oligomers ("α/β-peptides") to mimic the structure and function of specific α-helices.[29] To date, these efforts have focused on oligomers containing β^3-amino acid residues (Figure 1) and/or cyclic β-amino acid residues. β^2-Amino acid residues, which differ from β^3 residues only in the placement of the side chain (Figure 1), have received little attention.[30–32] This disparity arises because many protected β^3-amino acids are commercially available, but most protected β^2-amino acids must be synthesized.[33] We envisioned that β^2 residues might be worthy of consideration, as alternatives to isomeric β^3 residues, at positions that make direct contact with a binding partner. In such situations, the difference in side chain positioning for β^2 vs. β^3 residues might significantly influence α/β-peptide recognition properties.

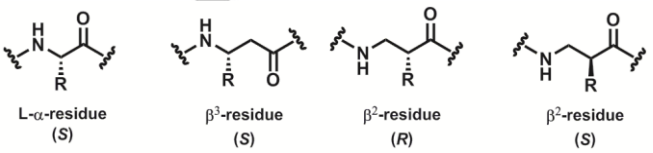

Figure 1. Generic structures of β-amino acid residues employed in this work. A generic L-α residue is shown for comparison.

We selected binding to the protein Bcl-x_L as a model system for evaluating the impact of interchanging β^3 and β^2 residues on the affinity of α/β-peptides for a partner. Bcl-x_L is an anti-apoptotic member of the Bcl-2 family, which includes Bcl-2 itself, Mcl-1, and other members.[34] The natural ligands for Bcl-x_L are pro-apoptotic proteins that contain a Bcl-2 homology 3 (BH3) domain, which adopts an α-helical conformation upon binding to an anti-apoptotic partner protein. Identification of ligands for these binding sites has been widely pursued for medicinal purposes;[35] a small-molecule antagonist of BH3 domain binding to Bcl-x_L is an anti-cancer drug.[36] Insights and techniques that have emerged from extensive studies in this area make binding to anti-apoptotic Bcl-2 family members a useful model system for evaluation of α-helix mimicry strategies.[10,16,37,38] The α/β-peptides discussed here are based on previously reported 26-mer α-peptide **1** (Figure 2),[39] which encompasses the BH3 domain of the pro-apoptotic protein Bim. Peptide **1** contains four key hydrophobic residues (designated h1-h4) that are buried upon binding to the BH3-recognition cleft of Bcl-x_L. α/β-Peptide **2** is an analogue that retains the side chain sequence of **1** but features an ααβααβ pattern in the backbone. All four of the key hydrophobic side chains (h1-h4) are contributed by β^3 residues in **2**. α/β-Peptide **2** displays modest affinity for Bcl-x_L according to a competition fluorescence polarization (FP) assay[40] (K_i = 190 nM for **2** vs. K_i < 0.7 nM for **1**). This situation is ideal for comparing isomeric α/β-peptides in which some or all side chains buried against Bcl-x_L (h1-h4) are projected by β^2 rather than β^3 residues, because the competition FP assay can detect increases or decreases in affinity relative to that manifested by **2**.

α/β-Peptides **3-10** are isomers of **2** in which a subset of the original β^3 residues at positions h1-h4 has been replaced with isomeric β^2 residues. One of the protected β^2-amino acids required for these peptides, Fmoc-β^2-hIle, is not commercially

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available and was prepared by a previously described synthetic method that features an asymmetric Mannich reaction.[41] Competition FP data for binding to Bcl-x_L revealed that K_i varied by more than 100-fold among **2-10** (Figure 2); thus, this interaction is quite sensitive to the difference in side chain position between β^3 and β^2 residues. Single $\beta^3 \rightarrow \beta^2$ substitution at h1 or h2 (**3** or **4**) caused little change in affinity, but substitution at h3 or h4 (**5** or **6**) diminished affinity. We explored combinations of $\beta^3 \rightarrow \beta^2$ substitutions among h1-h4 (**7-10**) to determine whether the impact of these changes would be additive. Placing β^2 residues at h1 and h2 (**7**) resulted in a ~6-fold increase in affinity relative to **2**; **7** displayed the highest affinity for Bcl-x_L among the α/β-peptides in this series. Placing β^2 residues at h1 and h3 (**8**) caused a 17-fold decrease in affinity relative to the all-β^3 case (**2**). α/β-Peptide **9** contains β^2 residues at h1, h2, and h3 and displayed an affinity for Bcl-x_L indistinguishable from that of **2**, while inclusion of the fourth β^2 residue at h4 (**10**) eroded affinity. These findings suggest that the effects of multiple $\beta^3 \rightarrow \beta^2$ substitutions on affinity for Bcl-x_L are roughly additive. The Bim BH3 domain (**1**) binds tightly to Mcl-1, but α/β-peptide **2** does not bind detectably to Mcl-1 (Figure S6). α/β-Peptides **3**-**10** also did not bind to Mcl-1 (Figure S6).

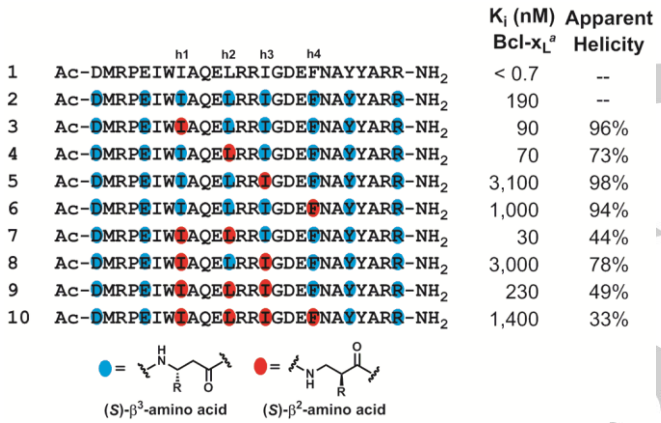

Figure 2. Sequences of Bim BH3-derived α- and α/β-peptides with their corresponding K_i values and apparent helicity. [a]K_i values obtained from competition FP assays are the average of at least three independent experiments. Maximum experimental error is approximately 2-fold.

α/β-Peptides **2-10** share the same backbone and contain the same complement and sequence of side chains; therefore, it is noteworthy that these α/β-peptides display >100-fold variation in affinity for Bcl-x_L. α/β-Peptide **7** displays the highest selectivity for Bcl-x_L over Mcl-1 (>300-fold) among all Bim BH3-derived α/β-peptides reported to date.[38,42] Recognition surfaces that contain β-residues allow the position of a side chain to be shifted by the length of a single carbon-carbon bond, via interconversion of β^3 and β^2 residues, and the affinity range manifested among **2-10** shows that this variation in side chain position can exert a substantial impact on recognition properties. Conventional peptides, comprised entirely of α-amino acids, do not allow for comparably subtle alterations in side chain arrangement.

Binding of α/β-peptides to Bcl-x_L and related proteins requires that these oligomers adopt an α-helix-like conformation, as documented in multiple co-crystal structures involving BH3-mimetic peptides that contain β^3 residues.[38,43–46] We wondered whether isomeric β^2 and β^3 residues differ in helix-forming propensity, because this factor could influence affinity for Bcl-x_L among **2-10**. The conformational behavior of α/β-peptides that contain β^2 residues has received little attention to date. In the two pertinent examples, Tavenor et al. and Fisher et al. measured the helix propensity of β^3 and β^2 residues using the GB1 tertiary motif and a parallel coiled-coil thioester exchange system, respectively.[31,47] Tavenor et al. concluded that there was no significant difference in helix propensity between the isomeric β^3 and β^2 residues they studied, while Fisher et al. measured a small decline in helix propensity for β^2 residues relative to their β^3 analogues. The Bim BH3 sequence we have studied forms an isolated α-helix, allowing us to address the impact of $\beta^3 \rightarrow \beta^2$ substitution on helicity in the absence of any tertiary context.

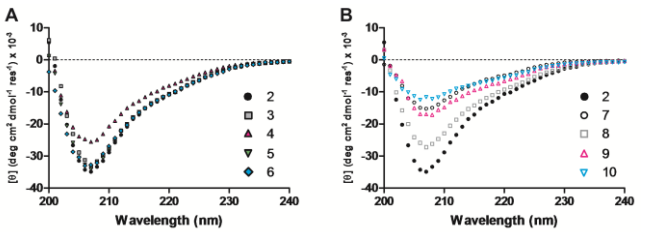

Figure 3. Far-UV CD spectra of α/β-peptides **2**-**10** in 50% MeOH, 50% 10 mM TBS, pH 7.5 (v/v). Peptide concentrations range from 75 to 95 μM. CD signal is corrected for sequence length and concentration (i.e., the vertical axis units are mean residue ellipticity, [θ]). α/β-Peptides containing single β^2 residues (A) or multiple β^2 residues (B) are compared with parent α/β-peptide **2**.

We used far-UV circular dichroism (CD) to probe for differences in helical propensity among α/β-peptides **2-10**. These studies were conducted in a 1:1 mixture of 20 mM aqueous Tris buffer (TBS), pH 7.5, and methanol. Water-alcohol mixtures promote helicity among conventional peptides and α/β-peptides;[48] in addition, the mixed solvent prevented α/β-peptide aggregation in the 10-100 μM range used for our measurements. The CD comparison revealed significant variation in the extent of helix formation among the α/β-peptides (Figure 3) In the TBS/methanol solvent, α-peptide **1** displayed strong minima near 220 and 208 nm, characteristic of α-helix formation (Figure S10). α/β-Peptide **2** displayed a single strong minimum near 207 nm in this solvent (Figure 3A), which is consistent with previous observations for α/β-peptides that contain similar β residue distributions and adopt an α-helix-like conformation.[38,42,46]

We established an *apparent* helicity scale for comparisons among **2-10** based on mean residue ellipticity at 207 nm ($[\theta]_{207}$) normalized to $[\theta]_{207}$ for **2**, which contains exclusively β^3 residues and displayed the strongest minimum (Figure 3). We cannot quantify the extent of helix formation for **2** based on the CD data, but the apparent helicity scale provides qualitative insight on helicity differences within the series. α/β-Peptides **3**, **5**, and **6** each contain a single $\beta^3 \rightarrow \beta^2$ substitution, and each is

indistinguishable from **2** (Figure 3A). In contrast, **4**, which also contains just one $\beta^3 \rightarrow \beta^2$ substitution, displays a significantly less intense minimum relative to **2** and therefore appears to be less helical than **2**. α/β-Peptide **8** contains two $\beta^3 \rightarrow \beta^2$ substitutions that do not individually affect helicity, but **8** appears to be moderately less helical relative to **2** (Figure 3B). A more substantial decline is observed for **7**, which also contains two $\beta^3 \rightarrow \beta^2$ substitutions. α/β-Peptides **9** and **10**, with three and four $\beta^3 \rightarrow \beta^2$ substitutions, respectively, are significantly less helical than **2**, but **9** and **10** are comparable to **7**.

Taken together, the CD data suggest that β^2 residues have a slightly smaller propensity than β^3 residues for participation in an α-helix-like conformation The data also raise the possibility that side chain identity affects the extent to which $\beta^3 \rightarrow \beta^2$ substitution influences helical propensity. This effect is illustrated by the lower apparent helicity of **4** relative to **3**, **5**, and **6** (each of these four α/β-peptides contains just one $\beta^3 \rightarrow \beta^2$ substitution), and by the lower apparent helicity of **7** relative to **8** (each contains two $\beta^3 \rightarrow \beta^2$ substitutions). The variations in helical propensity revealed by the CD data do not correlate directly with variations in affinity for Bcl-x_L, as illustrated most clearly by the behavior of α/β-peptide **7**, which displays the strongest affinity for Bcl-x_L among **2**-**10** but manifests one of the lowest extents of helix formation according to the CD data.

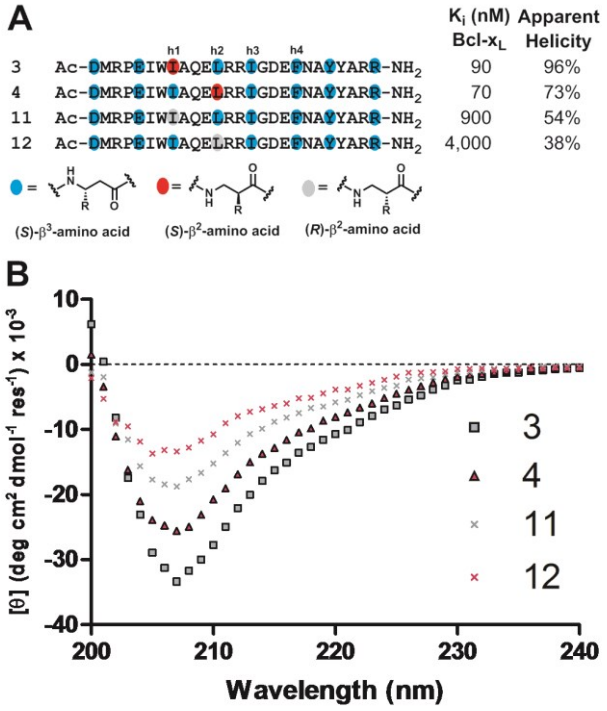

Figure 4. Effects of (*S*)-$\beta^2 \rightarrow$ (*R*)-β^2 substitution on the affinity and helicity of Bim BH3-derived α/β-peptides. The sequences of the two pairs of diastereomers, **3** + **11** and **4** + **12,** along with their K_i values and apparent helicities are shown (A). The peptides containing single (*R*)- β^2 residues bind significantly less tightly to Bcl-x_L (A) and are noticeably less helical (B)

We anticipated that β^2 residues with *S* absolute configuration would be stereochemically compatible with L-α-amino acid residues based on precedent.[31,47] We tested this hypothesis by synthesizing and evaluating α/β-peptides **11** and **12**, which are diastereomers of **3** and **4**, respectively, that contain single (*S*)-$\beta^2 \rightarrow$(*R*)-β^2 substitutions (Figure 4A). CD data obtained in 1:1 TBS/methanol show that **11** and **12** are significantly less helical than **3** or **4** (Figure 4B). These results indicate that even a single (*R*)-β^2 substitution is detrimental to right-handed helix formation. Both of the (*S*)-$\beta^2 \rightarrow$(*R*)-β^2 substitutions led to substantial declines in affinity for Bcl-x_L (Figure 4A), further supporting the notion that the *S* absolute configuration of β^2 residues is the correct choice for mimicry of a natural α-helix.

We have used the binding of α/β-peptide variants to a specific protein partner to ask whether the choice between isomeric β^3- and β^2-amino acid residues, which corresponds to a small difference in side chain placement along the α/β-peptide backbone, exerts a significant impact on the recognition properties of these compounds. The association between α/β-peptide **2** and Bcl-x_L provided an excellent opportunity to address this question, because four of the β^3 residue side chains from **2** are expected to make intimate contacts with Bcl-x_L. Comparisons involving α/β-peptides **3-10**, which are isomers of **2** containing $\beta^3 \rightarrow \beta^2$ substitution at one or more of the contact positions, show that subtle variation in side chain arrangement leads to >100-fold variation in affinity for Bcl-x_L. One of the new α/β-peptides, containing two $\beta^3 \rightarrow \beta^2$ substitutions, binds to Bcl-x_L with six-fold higher affinity relative to all-β^3 prototype **2**. Although CD measurements suggest that $\beta^3 \rightarrow \beta^2$ substitution may modestly diminish the propensity to adopt the helical conformation necessary for binding, this factor does not appear to be decisive in terms of α/β-peptide affinity for Bcl-x_L. These findings suggest that comparing β^3 vs. β^2 substitution in other systems should be useful for optimizing α/β-peptide binding to specific macromolecular partners.

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Conflict of Interest

S. H. G. is a cofounder of Longevity Biotech, Inc., which is pursuing biomedical applications for α/β-peptides.

Keywords: α-helix mimicry • β^2-amino acids • circular dichroism • peptides • protein-protein interactions

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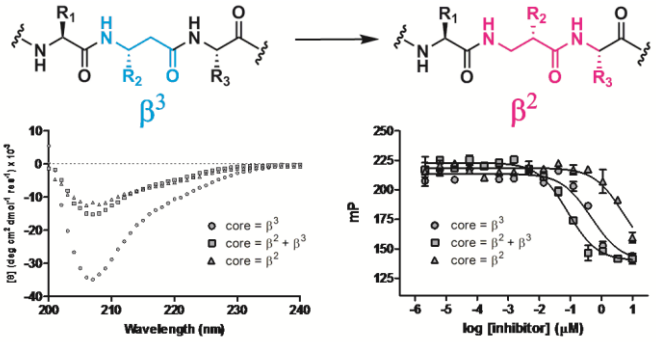

Isomeric β^3-to-β^2-amino acid substitution within a helical α/β-peptide exerts significant influence on the peptide's properties. β^2 residues can be helix-destabilizing, but they are also capable of increasing the peptide's affinity for a protein binding partner.

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