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Introduction of Cyclically Constrained γ-Residues Stabilizes an α-Peptide Hairpin in Aqueous Solution

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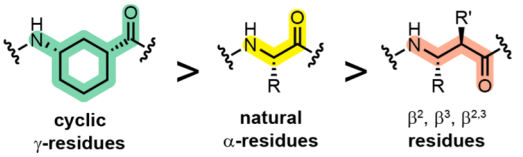
ABSTRACT

Sheet folding propensity:



The synthesis and structural characterization of hybrid α/γ-peptides resulting from a 1:1 α→γ residue substitution at cross-strand positions in a hairpin-forming α-peptide sequence are described. Cyclically constrained γ-residues based on 1,3-substituted cyclohexane or benzene scaffolds support a native-like hairpin fold in aqueous solution, and the unnatural residues stabilize the folded state by ∼0.2 kcal/mol per α→γ substitution.

Unnatural-backbone oligomers that fold like proteins, coined "foldamers,"[1] are of interest as therapeutic agents due to their ability to combine protein-like biological effects with stability to degradation by proteases.[2] One method for the design of a desired folding pattern and function in an unnatural oligomer is to systematically alter the backbone of a natural peptide or protein sequence to generate an analogue with similar folding behavior. To date, this sequence-based approach has been applied to generate unnatural-backbone mimics of peptides with helical,[3] sheet,[4] and nonregular[5] secondary structure as well as peptide/polymer chimeras based on a larger tertiary fold.[6]

As part of a program aimed at the mimicry of a protein tertiary structure by unnatural backbones, we have focused efforts recently on hairpin and sheet secondary structures.[4] β-Sheets and hairpins are essential components of natural tertiary folds,[7] and their mimics have potential use in diverse biomedical applications.[8] One method to generate unnatural-backbone sheet mimics is to introduce β-amino acid residues in an α-peptide context to produce heterogeneous-backbone α/β-peptides.[9] Application of 1:1 α→β residue substitution at matched positions in each strand of an α-peptide hairpin can lead to α/β-peptide analogues that fold in organic solvent[10] or aqueous solution;[4a] however, β-residue incorporation leads to an altered display of side chains relative to a

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natural backbone. Sequence-guided αα→β or αα→$\beta\beta$ residue replacement can restore a native-like side-chain display, but at a thermodynamic cost to folded stability.[4b]

We considered the possibility that a strategy for modification of an α-peptide sheet that made use of alternate unnatural building blocks besides β-residues might lead to an oligomer with both a native-like fold and enhanced aqueous folded stability relative to the natural prototype. Here, we show that the incorporation of cyclically constrained γ-amino acid residues[11] in each strand of a protein-derived hairpin generates an α/γ-peptide analogue with native-like folding behavior and improved aqueous folded stability relative to the natural sequence.

Simple acyclic γ-residues have been shown to be too flexible to be accommodated into a folded hairpin or sheet.[12] Replacement of one of the backbone C−C single bonds in a γ-residue with a double bond restricts torsional freedom and can lead to hairpin formation in organic solvent.[13] An alternate method to impart backbone conformational rigidity is to employ cyclically constrained γ-residues (Figure 1A).[14−17] Designed hairpins containing *m*-aminobenzoic acid (*m*ABA, **X**) can fold in organic solvent,[15] and substituted derivatives have been incorporated into protein-like sheets that fold in water, blocking interchain association in the process.[16] A saturated analogue of *m*ABA, (1*R*,3*S*)-3-aminocyclohexanecarboxylic acid (ACC, **Z**), has also found use as a component of sheet-like structures. Cyclic oligomers in which ACC is alternated with D-α-residues can stack via intermolecular backbone hydrogen bonding to form nanotubular assemblies.[17] These nanotubes, analogous to those that form from the self-assembly of cyclic D,L-α-peptides,[18] are essentially cylindrical sheets.

Given its behavior in a cyclic peptide context,[17] we hypothesized that pairing the (1*R*,3*S*) enantiomer of ACC with L-α-residues would generate a peptide chain with a high propensity to adopt an extended strand conformation. We further reasoned that such a strand might be used as a component of a protein sheet mimetic. In order to test these hypotheses, we introduced ACC residues into an α-peptide hairpin model system derived from a bacterial protein. We incorporated the related aromatic γ-amino acid *m*ABA into the same sequence as a point of comparison and determined the folded structure and thermodynamic folded stability of the resulting α/γ-peptides by multidimensional NMR in aqueous buffer. Collectively, the results obtained show that both *m*ABA and ACC stabilize the hairpin fold of the parent α-peptide without compromising the native-like display of side chains near the unnatural residues.

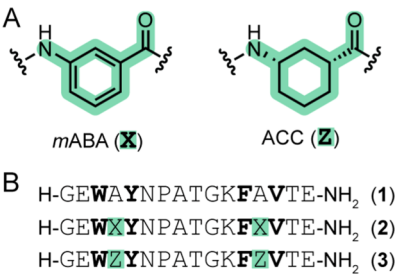

Figure 1. (A) Structures of γ-amino acid residues *m*ABA (**X**) and ACC (**Z**). (B) Sequences of α-peptide **1** and α/γ-peptide analogues **2** and **3**; hydrophobic residues key to hairpin folding are shown in bold.

We chose α-peptide **1** (Figure 1B),[4b,19] a mutant of the C-terminal hairpin from the *Streptococcal* protein GB1,[20] as a model system to examine the folding propensity of ACC and *m*ABA in a protein sheet context. Two α/γ-peptide variants of **1** were synthesized, incorporating *m*ABA (peptide **2**) or ACC (peptide **3**) in place of Ala residues at cross-strand positions 4 and 13. This substitution pattern maintains the hydrophobic side-chain cluster of Trp_3, Tyr_5, Phe_{12}, and Val_{14}, which is essential for hairpin folding of **1** in water.

We synthesized the Fmoc-protected derivatives of ACC and *m*ABA for use in solid-phase peptide synthesis (SPPS). Commercially available *m*-aminobenzoic acid was Fmoc protected using standard methods. Enantiomerically pure Boc-ACC was obtained by recrystallization of racemic material derivatized as the (*R*)-1-phenethylamine salt[21] and converted to Fmoc-ACC by treatment with TFA followed by Fmoc protection. Peptides **2** and **3** were prepared using standard SPPS techniques, purified by reversed-phase HPLC, and their identities were confirmed using MALDI-TOF MS. All samples used for biophysical analysis were >95% pure by analytical HPLC. Full experimental details can be found in the Supporting Information (SI).

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Peptides **2** and **3** were analyzed by NMR at 278 K in 50 mM phosphate buffer at pH 6.3 (9:1 H_2O/D_2O, pH uncorrected for the presence of D_2O). Structural analysis in the demanding environment of aqueous buffer at near-physiological pH provides an important test as to the potential applicability of the α→γ substitutions examined in a biological context.[22] Moreover, these specific conditions allow direct comparison with data we have previously obtained for α-peptide **1** and α/β-peptide analogues derived from sequence-guided α→β residue replacement.[4b]

Homonuclear $^1H-^1H$ TOCSY, NOESY, and COSY experiments enabled unambiguous assignment of all backbone N−H and C−H resonances as well as most side chain resonances in α/γ-peptides **2** and **3** (Tables S1, S2). Comparison of these data with results reported for parent α-peptide **1** under identical conditions[4b] allowed us to ascertain the impact of γ-residue incorporation on hairpin thermodynamic stability.

We used NMR chemical shift data for Gly_{10} to quantify the folded population of α/γ-peptides **2** and **3**, by analogy to methods previously reported for α-peptide **1**.[4b] The chemical shift separation between diastereotopic Gly H_α protons ($\Delta\delta$) is diagnostic of folding in hairpin peptides; the magnitude of $\Delta\delta$ increases with folded population.[23] We used data from a variant of **1** cyclized via a disulfide bridge between N- and C-terminal Cys residues to determine $\Delta\delta$ for the fully folded state.[4b] In prior work, we showed the $\Delta\delta$ value for the fully folded state does not vary among this cyclic variant of **1** and several analogues with different unnatural backbone compositions.[4b]

Folded populations determined by NMR were used to calculate the equilibrium constant for hairpin formation and, in turn, the folding free energy (ΔG_{fold}) of each peptide. Comparison of the thermodynamic data for the folding equilibria of **1**−**3** (Table 1) yields two noteworthy observations. First, the folded populations of the two α/γ-peptides are similar, although the oligomer with the ACC residues appears to be slightly more stable. Second, both α/γ-peptides are actually more folded than parent α-peptide **1** under the conditions of the experiment. This gain in thermodynamic folded stability is a surprising finding, since analogous sequence-based α→β residue substitution is accompanied by a loss in stability to the folded hairpin. The α/γ-peptide hairpins are stabilized by ~0.2 kcal/mol per α→γ residue replacement. This value is comparable in magnitude to that observed for mutation of Ala to Ile at a hydrophilic position in a β-sheet.[24] Considering that isoleucine has the highest sheet propensity among ribosomally encoded α-residues, a similar stabilization from incorporation of an unnatural residue is a significant finding.

With thermodynamic evidence of folding in hand, we next analyzed long-range NOEs in α/γ-peptides **2** and **3** to determine their folded structure in solution. Both **2** and **3** showed interstrand contacts consistent with hairpin formation along the entire length of the backbone (Figure 2). Additionally, cross-strand NOEs involving backbone protons indicated a hydrogen-bond register where side chains flanking the γ-residue were juxtaposed on the same face of the sheet. This observation is in contrast to the side-chain inversion resulting from analogous 1:1 α→β residue replacement[4] and suggests the 1:1 α→γ residue substitution leads to a native-like sheet fold.

We employed NOE-derived distance restraints and simulated annealing to generate high-resolution NMR solution structures of **2** and **3**. The ensemble of low-energy structures obtained for each peptide (Figure S1) was consistent with the expected hairpin fold. Minimized average coordinates derived from each ensemble provide a snapshot of the solution structures (Figure 3A,B). In the case of **2**, a minor conformer with a horseshoe shape (Figure S1B) was observed in 2 of the 10 low energy structures; only the structures from the major conformer family were used when calculating the minimized average coordinates.

Table 1. Thermodynamic Analysis of Folding Equilibria for α-Peptide **1** and α/γ-Peptides **2** and **3**[a]

peptide	$\Delta\delta$ Gly_{10} $H_\alpha/H_{\alpha'}$ (ppm)[b]	fraction folded (%)	ΔG_{fold} (kcal/mol)[c]	$\Delta\Delta G_{fold}$ (kcal/mol)[d]
1	0.21 ± 0.01	67 ± 6	−0.4 ± 0.1	–
2	0.25 ± 0.01	79 ± 6	−0.7 ± 0.2	−0.3
3	0.26 ± 0.01	83 ± 6	−0.9 ± 0.2	−0.5

[a] NMR experiments were performed in 50 mM phosphate, 9:1 H_2O/D_2O, pH 6.3 at 278 K. [b] Chemical shift separation between diastereotopic H_α protons of Gly_{10}. [c] Free energy of folding. [d] Difference in ΔG_{fold} compared to α-peptide **1**.

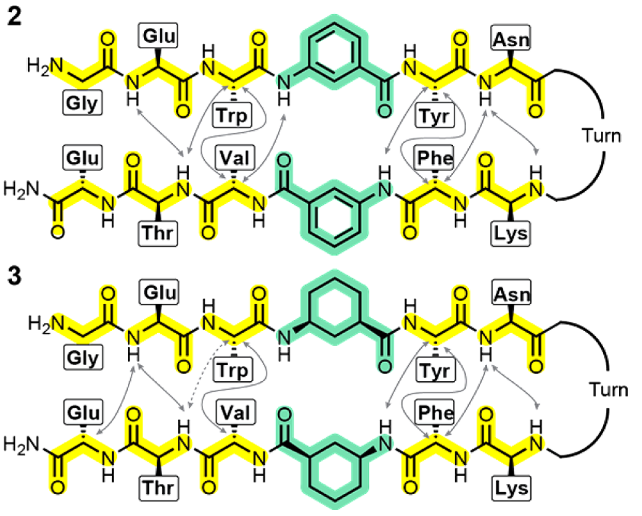

Figure 2. Select interstrand NOEs for α/γ-peptides **2** and **3**. *m*ABA and ACC residues are shown in green. Dashed lines indicate ambiguous NOE assignments. NMR experiments were performed in 50 mM phosphate, 9:1 H_2O/D_2O, pH 6.3 at 278 K.

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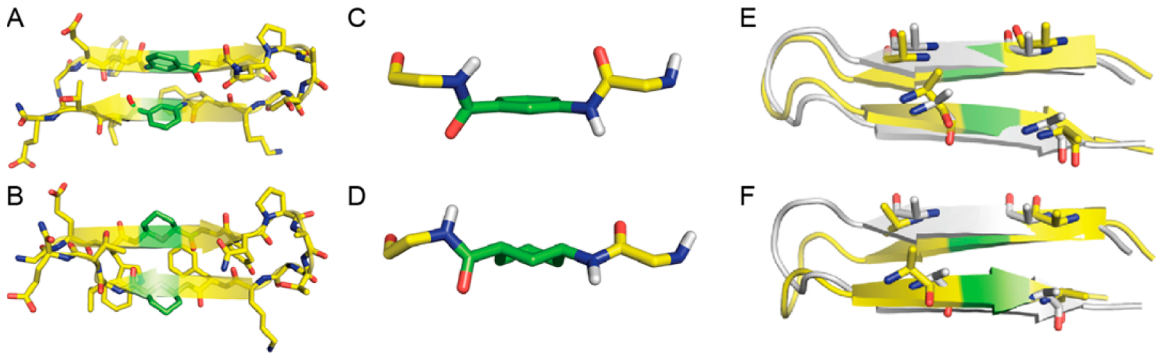

Figure 3. (A,B) Minimized average coordinates from the NMR solution structure of α/γ-peptide **2** (A) and **3** (B). (C,D) Side-on views of an *m*ABA residue from **2** (C) and an ACC residue from **3** (D). (E,F) Overlay of α/γ-peptide **2** (E) or **3** (F) with the C-terminal hairpin from protein GB1. The α/γ-peptides are shown in yellow, and the natural protein is in gray; the C, N, O, C_α, and C_β atoms of the four hydrophobic residues used in pair-fitting are shown as sticks. γ-Residues are colored green. NMR experiments were performed in 50 mM phosphate, 9:1 H_2O/D_2O, pH 6.3 at 278 K.

α/γ-Peptides **2** and **3** both show hairpin folds with the four key hydrophobic residues from **1** on the same face of the sheet (Figure 3A,B). Close inspection of the γ-residues in the structures of **2** and **3** show that the amides flanking the unnatural residues are poised to stack in an extended sheet without interference by the rings of the unnatural residues (Figure 3C,D).

In order to quantify the ability of α/γ-peptides **2** and **3** to display key hydrophobic side chains in a native-like arrangement, we compared their NMR structures with the corresponding hairpin segment from a crystal structure of full length GB1,[25] on which prototype sequence **1** is based (Figure 3E,F). The overlays and resulting root-mean-square deviation (RMSD) values were based on structural alignment of C_α and C_β atoms from residues Trp_3, Tyr_5, Phe_{12}, and Val_{14}. α/γ-Peptide **2** shows high structural homology to the full-length GB1 protein in its display of hydrophobic core residues. The RMSD for the overlay of **2** with the native protein (1.12 Å) is almost identical to that for α-peptide **1** (1.18 Å). α/γ-Peptide **3** had an even better agreement, with an RMSD of just 0.68 Å for the same overlay, suggesting that ACC residues may be a better mimic of natural structure than *m*ABA residues. In total, the above data suggest that the folded structures resulting from introduction of either *m*ABA or ACC γ-residues at cross-strand positions of an α-peptide are very similar to the natural backbone in how they display side chains flanking the points of α→γ substitution.

In summary, we have shown in this work that a 1:1 α→γ substitution at cross-strand positions in a prototype α-peptide hairpin sequence leads to an α/γ-peptide analogue that folds in neutral aqueous buffer. Thermodynamic analysis of folding equilibria reveals that introduction of the cyclically constrained γ-residue *m*ABA or ACC stabilizes the folded state on the order of 0.2 kcal/mol per residue replaced. NMR solution structures of the α/γ-peptides show that the display of hydrophobic core residues from the α-peptide prototype is maintained and that the γ-residues are oriented in a way that would allow stacking in a larger sheet. Both constrained γ-residues examined give a similar fold when introduced into the parent sequence; however, ACC showed a slightly more stable folded structure and a greater degree of structural similarity to the natural protein. These observations suggest ACC may be particularly amenable for use in the mimicry of multistranded sheets in larger protein tertiary structures.

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Supporting Information Available. Figure S1, Tables S1–S4, experimental methods, and NMR data for peptides **1**–**3**. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.

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