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Hairpin Folding Behavior of Mixed α/β-Peptides in Aqueous Solution

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Supporting Information

ABSTRACT: The invention of new strategies for the design of protein-mimetic oligomers that manifest the folding encoded in natural amino acid sequences is a significant challenge. In contrast to the α-helix, mimicry of protein β-sheets is less understood. We report here the aqueous folding behavior of a prototype α-peptide hairpin model sequence varied at cross-strand positions by incorporation of 16 different β-amino acid monomers. Our results provide a folding propensity scale for β-residues in a protein β-sheet context as well as high-resolution structures of several mixed-backbone α/β-peptide hairpins in water.

The development of unnatural-backbone oligomers capable of protein-like folding is the focus of intensive research efforts,[1] due to the prospect of these molecules to deliver protein-like functions (e.g., biomolecular recognition, catalysis) on protease-resistant backbones. A fundamental challenge in the design of unnatural species that fold in defined ways is the same faced in the de novo design of folded proteins:[2] the generation of a sequence of monomer building blocks that will manifest the correct fold.

Early efforts to develop folded oligomers with tailored functions relied primarily on structure-based engineering of known scaffolds to display functional groups in defined arrangements.[1,3] Recent work has demonstrated an alternative approach in which natural protein sequences can serve as blueprints for the design of unnatural mimics. Specifically, the display of natural side-chain sequences on heterogeneous backbones comprising mixtures of α- and β-amino acid residues can generate "α/β-peptides"[4] that adopt folds similar to those of the α-peptide prototypes.[5] Termed "sequence-based design," this strategy has produced protein-like helix-bundle quaternary structures[5a,c,e] and protease-resistant inhibitors of biomedically important protein−protein-binding interactions.[5b,d]

Applications of sequence-based design to date have focused exclusively on α-helical prototype sequences.[5] This method has the potential to provide analogues of more complex protein tertiary structures; however, an unmet need for the realization of this potential is the development of complementary strategies for the mimicry of β-sheets. Here, we explore the ability of α/β-peptides to adopt a sequence-encoded protein β-sheet fold in aqueous solution.

Compared to helical folding, sheet structures in unnatural oligomers have received considerably less attention. An interesting parallel exists in the study of folding in natural proteins where the development of peptides that form discrete β-sheets in water[6] lagged behind α-helical model systems[7] by more than two decades. Sheet folding has been demonstrated in pure β-peptides, however, only in organic solvent.[8] In mixed backbones, it has been shown that one class of β-residue (β^3) can form sheet structures in organic solvent, in the solid state, and in two-dimensional (2D) crystals at an air/water interface.[9] These prior studies showed that β-peptides tend to form sheets with a different hydrogen-bonding pattern than that of α-peptide β-sheets due to inversion of hydrogen bonding at the unnatural residue.[8,9] These precedents leave several open questions that we sought to address: (1) Can a mixed backbone α/β-peptide adopt a sequence-encoded sheet fold in aqueous solution?[10,11] (2) What is the relationship between the structure of a β-residue and its propensity to be accommodated into a protein sheet? (3) Can an appropriately structured β-residue promote a protein-like hydrogen-bonding pattern?

The minimal β-sheet model is a two-stranded antiparallel β-hairpin. The first reported sequence to form a discrete hairpin fold in water was the C-terminal segment of the *Streptococcal* protein GB1.[6a] We employed a known analogue of this GB1 sequence, 12-residue peptide **1a** (Figure 1),[12] as a model system

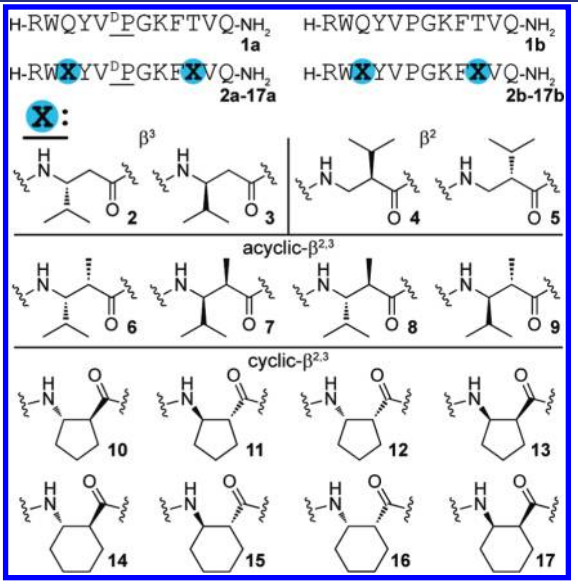

Figure 1. Structures of the peptides synthesized and characterized in the present study. α-Peptides **1a** and **1b** served as prototype sequences for incorporation of matched β-residue ("X") pairs at sequence positions 3 and 10 to produce α/β-peptides **2a**−**17a** and **2b**−**17b**. "^{D}P" denotes D-Pro.

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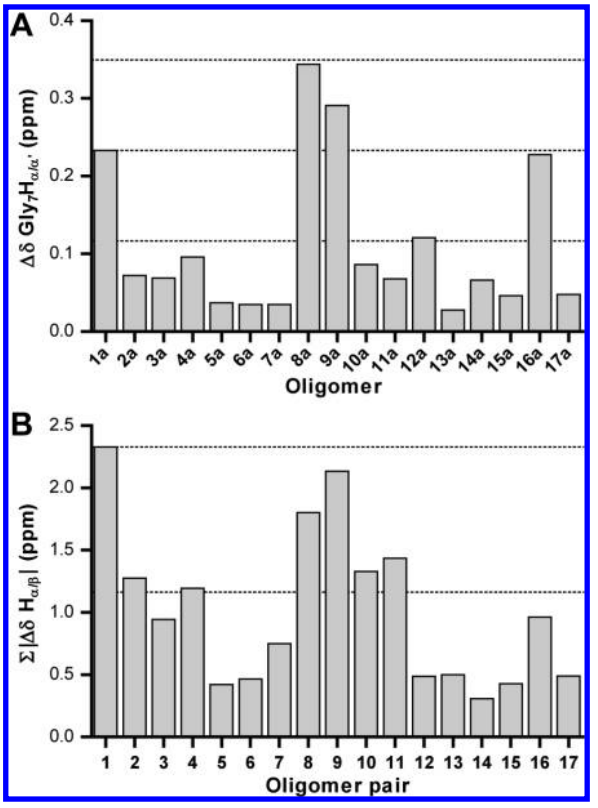

Figure 2. Summary of NMR chemical shift data for oligomer pairs **1**−**17**. (A) Separation ($\Delta\delta$) of the diastereotopic Gly_7 H_α signals in **1a**−**17a**. (B) Sum of the absolute values of the chemical shift deviations ($\Sigma|\Delta\delta|$) of backbone C−H signals with mutation of D-Pro_6 (**1a**−**17a**) to L-Pro (**1b**−**17b**); turn residues 6−7 were excluded from the analysis. Spectra were acquired at 4 °C in 100 mM NaOAc-d_3 buffer, 9:1 H_2O/D_2O, pH 3.8. Dashed lines indicate 50% increments of the bar height for the α-peptide.

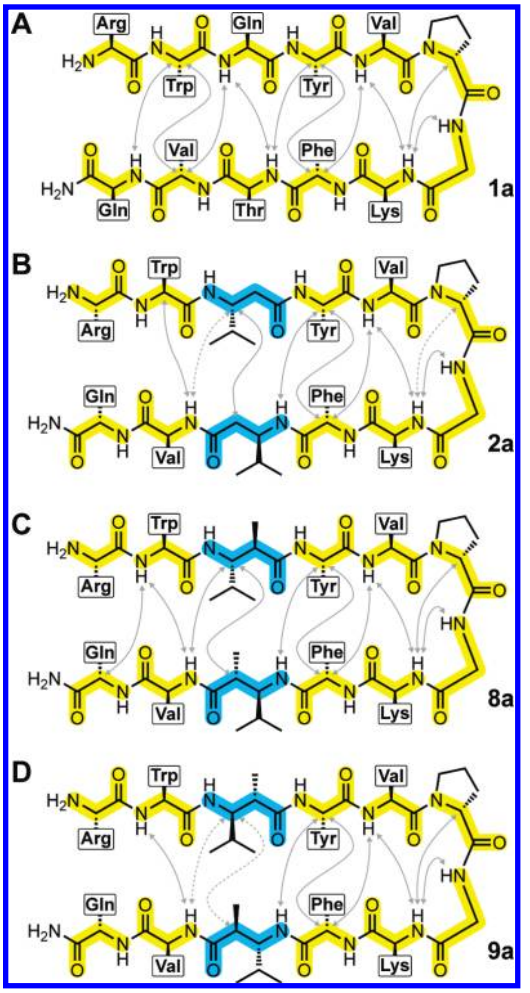

Figure 3. Select backbone NOEs observed for α-peptide hairpin **1a** and α/β-peptide analogues **2a**, **8a**, and **9a**. Spectra were acquired at 4 °C in 100 mM NaOAc-d_3 buffer, 9:1 H_2O/D_2O, pH 3.8. Dashed lines indicate cases where overlap prevented an unambiguous assignment.

to examine the folding of α/β-peptide hairpins in aqueous solution. Peptide **1a**, which contains a four-residue hydrophobic cluster from GB1 and a turn-promoting D-Pro-Gly segment,[13] is on the razor's edge of conformational stability with less than 0.5 kcal/mol separating the folded and unfolded states.[12] The instability of **1a** makes it and related hairpin sequences highly sensitive probes for detecting small energetic contributions to protein folding.[14]

We introduced 16 different β-amino acid residues at cross-strand sequence positions 3 and 10 midway along the hairpin formed by α-peptide **1a** to generate α/β-peptide derivatives **2a**−**17a** (Figure 1). Identical substitutions were made in unfolded control sequence **1b** to generate **2b**−**17b**. Three monomer structural variables were considered: backbone substitution pattern (β^2 vs β^3 vs $\beta^{2,3}$), backbone stereochemistry, and constraint of the C_α−C_β torsion by incorporation into a ring. Collectively, the compounds examined were intended to systematically probe the impact of these β-residue structural properties on the ability of oligomers to incorporate that residue into the hairpin fold encoded by parent α-peptide sequence **1a**.

Oligomers **2**−**5** incorporate either enantiomer of a β^3-residue (**2**, **3**) or β^2-residue (**4**, **5**) bearing an isopropyl side chain.[15] Oligomers **6**−**9** are substituted with all four possible stereoisomers of an acyclic disubstituted $\beta^{2,3}$-residue bearing an isopropyl group at the β^3-position and a methyl group at the β^2-position. Oligomers **10**−**17** probe the role of backbone preorganization through incorporation of a 5-membered (**10**−**13**) or 6-membered (**14**−**17**) ring constraint in all possible stereochemical configurations. An assortment of known routes were employed for the asymmetric synthesis of the different protected β-amino acid monomers.[16] Peptides were prepared by microwave-assisted Fmoc solid-phase peptide synthesis, purified by reverse-phase HPLC, and the identity of each confirmed by mass spectrometry.

We compared the folding of α-peptide **1a** and α/β-peptides **2a**−**17a** in aqueous solution using NMR spectroscopy. Spectra of each oligomer were acquired in deuterated acetate buffer (pH 3.8) at a temperature of 4 °C (the use of an acidic medium disfavors aggregation in this sequence[12]). A series of solvent-suppressed 1D and 2D homonuclear experiments (TOCSY, COSY, NOESY) allowed complete assignment of all backbone resonances.

Separation of the diastereotopic H_α atoms in turn residue Gly_7 is diagnostic of folded population in **1a** and related hairpins.[14] These two protons have significantly different chemical shifts in well-folded structures, but tend to coalesce into a single peak in random coils. We compared the separation ($\Delta\delta$) of H_α resonances in Gly_7 of prototype α-peptide **1a** to the separation observed in α/β-peptide derivatives **2a**−**17a** (Figure 2A). Among the α/β-peptides, only four (**8a**, **9a**, **12a**, and **16a**) showed separation of at

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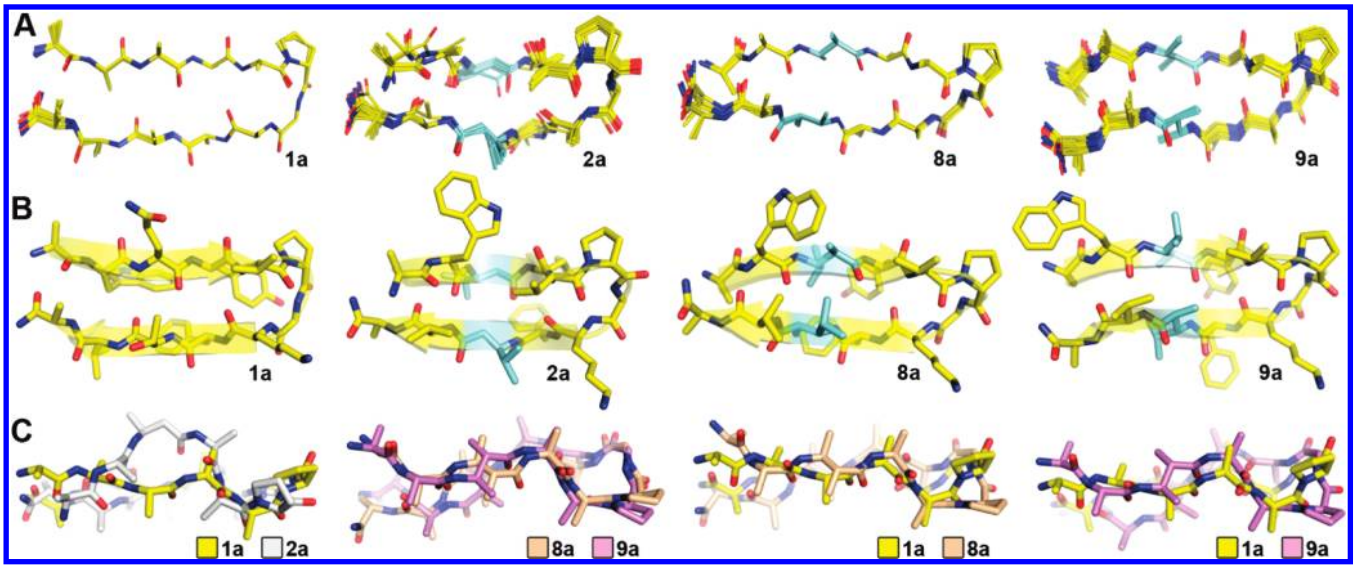

Figure 4. NMR solution structures of α-peptide **1a** and α/β-peptides **2a**, **8a**, and **9a**. (A) Overlay of the backbone coordinates for the 20 lowest-energy structures resulting from simulated annealing and (B) minimized average coordinates; carbon atoms are colored cyan in β-residues. (C) Overlays of **1a**/**2a**, **8a**/**9a**, **1a**/**8a**, and **1a**/**9a**. Some side chains are omitted for clarity.

least 50% that of α-peptide **1a**. Of note, incorporation of a *trans*-substituted acyclic $\beta^{2,3}$-residue (**8a** or **9a**) led to greater-magnitude $\Delta\delta$ than the parent sequence; this observation may indicate that the *trans*-$\beta^{2,3}$-residue promotes hairpin folding more strongly than the native α-peptide backbone. The folding behavior of **8a** and **9a** is consistent with the high sheet propensity of *trans*-$\beta^{2,3}$-residues in organic solvent,[8a,b,e] presumably due to a strong preference for an *anti* relationship between the two continuing peptide chains about the central C−C bond.

Mutation of the turn-promoting D-Pro_6 in **1a** to L-Pro (peptide **1b**) abolishes folding.[12] A significant change in backbone ^{1}H chemical shifts upon the mutation of D-Pro_6 to L-Pro in otherwise identical sequences can be indicative of hairpin folding; the magnitude of these deviations is often used to estimate folded population.[12,17] We acquired NMR data for control peptides **1b**−**17b** under the same conditions described above for **1a**−**17a**. For each oligomer pair (i.e., **1a**/**1b**, **2a**/**2b**, etc.), we determined the difference in chemical shifts ($\Delta\delta$) between corresponding protons in the D- and L-Pro mutants. We summed the absolute values of $\Delta\delta$ for backbone C−H groups adjacent to nitrogen (excluding turn residues) for each pair (Figure 2B). Six of the 16 α/β-peptides examined showed backbone chemical shift deviations at least 50% of that observed for prototype α-peptide **1a**. Among the α/β-peptides, **8a** and **9a** showed backbone chemical shift deviations closest in magnitude to the natural backbone **1a**, similar to the results of Gly H_α separation analysis.

We analyzed the NOESY spectra of peptides **1a**−**17a** to ascertain basic information about their folded structures in solution. α-Peptide **1a** showed long-range NOE correlations consistent with the expected β-hairpin fold (Figure 3A). Among the α/β-peptide derivatives, some showed no NOE correlations, consistent with a nativelike hairpin (**5a**, **12a**, **15a**−**17a**); others (**3a**, **4a**, **6a**, **7a**, **10a**, **11a**, **13a**, **14a**) showed NOEs in the turn region but no correlations past the unnatural residue. Only three of the 16 α/β-peptides examined showed evidence of hairpin folding along the entire length of the chain (Figure 3B−D): one β^3-residue enantiomer (**2a**) and each enantiomer of the *trans*-$\beta^{2,3}$-monomer (**8a**, **9a**). Contrary to the expectation that one enantiomer in the *trans*-$\beta^{2,3}$-residue pair might be better matched to the chiral L-α-peptide backbone, the absolute stereochemical configuration of the β-residue had a minimal impact on the pattern of NOEs observed or the extent of folding evident from chemical shift analysis. Of note, the hydrogen-bonding pattern is inverted by each β-residue substitution (Figure S1, Supporting Information [SI]) with an accompanying change in side-chain display along the strand.[8a,9a]

We performed simulated annealing with NOE-derived distance restraints to determine the solution structures of α-peptide **1a** and the best folded α/β-peptide analogues **2a**, **8a**, and **9a** (Figure 4A,B). The ensemble of structures resulting from each calculation showed good internal agreement (Figures 4A and S2 [SI]). The folded conformation of β^3-residue-containing oligomer **2a**, although reminiscent of α-peptide **1a**, showed significant deviations from a canonical β-sheet fold. The two oligomers with *trans*-$\beta^{2,3}$-residues (**8a**, **9a**) exhibited folded conformations very similar to one another (Figure 4C); despite some differences around the unnatural residue, both were also similar to the fold of α-peptide **1a**. Among all the oligomers examined, α/β-peptide **8a** showed the fold closest to that of the native backbone hairpin.

A single α→β residue substitution in each strand of an α-peptide hairpin results in a change in the display of side chains as a result of inversion of backbone hydrogen-bonding at the position of the unnatural monomer (Figure S1 [SI]).[8a,9a] This difference is subtle in the context of folding in a model β-hairpin, but would be disastrous in the sequence-based modification of a β-sheet in a protein tertiary structure. Our initial goal was to try to restore a native hydrogen-bonding pattern with an appropriately structured β-residue; however, none of the 16 monomers we examined achieved this. Nevertheless, our results suggest to us a strategy that might turn the natural conformational predisposition of β-residues in sheets from a problem into an advantage. We have demonstrated here that *trans*-$\beta^{2,3}$ residues maintain high sheet-folding propensity in aqueous solution, and the recently developed synthetic method[16d] we employed for their preparation allows introduction of diverse side chains at either backbone carbon. We hypothesize that substitution of two

adjacent α-residues in a protein sheet by a single *trans*-$\beta^{2,3}$-residue with side chains derived from the parent dipeptide sequence will generate a mixed α/β-peptide analogue with native-like display of side chains (Figure S3 [SI]). We are currently working to test this hypothesis and related strategies in a larger β-sheet prototype.

In summary, we have shown that the ability of a mixed-backbone α/β-peptide to manifest the sheet fold encoded by a prototype α-peptide sequence depends critically on the structure of the β-residues employed. Quantitative comparison of folded populations is precluded here by the lack of a fully folded control sequence for each monomer examined; however, qualitative analysis of NMR chemical shift data, observed long-range NOEs, and the resulting NMR-derived solution structures leads to some general conclusions. An α→β^3 replacement is tolerated, while α→*trans*-$\beta^{2,3}$ substitution leads to an oligomer that folds as well or better than the native α-peptide. Backbone torsional constraints in the form of 5- or 6-membered rings do not promote folding in a protein sheet context. Our results provide a family of unnatural scaffolds for the mimicry of bioactive β-hairpins.[18] Moreover, the insights we have obtained into the sequence-based mimicry of protein β-sheets, combined with earlier studies on the α-helix,[5] deliver a testable strategy for the sequence-based design of unnatural-backbone oligomers that fold like a natural protein tertiary structure.

■ ASSOCIATED CONTENT

Supporting Information. Complete ref 18b, Figures S1−S3, Tables S1−S7, coordinates for NMR-derived solution structures, and experimental methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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