Peptide Folding

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Impact of Strand Number on Parallel β-Sheet Stability

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Abstract: *We have examined whether parallel β-sheet secondary structure becomes more stable as the number of β-strands increases, via comparisons among peptides designed to adopt two- or three-stranded parallel β-sheet conformations in aqueous solution. Our three-strand design is the first experimental model of a triple-stranded parallel β-sheet. Analysis of the designed peptides by nuclear magnetic resonance (NMR) and circular dichroism (CD) spectroscopy supports the hypothesis that increasing the number of β-strands, from two to three, increases the stability of the parallel β-sheet. We present the first experimental evidence for cooperativity in the folding of a triple-stranded parallel β-sheet, and we show how minimal model systems may enable experimental documentation of characteristic properties, such as CD spectra, of parallel β-sheets.*

Understanding protein conformational propensities is a major aim in protein science, and approaching this aim for a particular secondary structure is facilitated by peptides that adopt that structure in aqueous solution in the absence of a tertiary structural context. Despite the myriad of folded shapes adopted by natural proteins, only a handful of regular secondary structures are observed, and they can be classified as either helices or sheets. Design principles that reliably generate short, isolated α-helices were developed in the 1980s,[1,2] and comparable principles for small antiparallel β-sheets were identified in the 1990s;[3–7] however, guidelines for parallel β-sheet design have been slower to emerge. β-Sheets with any strand orientation are notoriously prone to aggregation, making them difficult to study.[3] An added challenge in the design and synthesis of parallel β-sheet model systems is that non-peptidic turn units must be employed to promote folding in the absence of a stabilizing, tertiary framework.[8–12] In contrast, short, autonomously-folding α-helices and antiparallel β-sheets can be generated from oligomers composed entirely from α-amino acid residues.

Here we report the first triple-stranded parallel β-sheet that folds autonomously in aqueous solution. We use the new design to ask whether a parallel β-sheet becomes more stable as strands are added. It has long been known that the α-helix grows more stable with increasing length along the helical axis.[13–15] The size of a β-sheet can be increased in either of two dimensions, along the strand direction (by adding residues to each strand) or perpendicular to the strand direction (by introducing new strands). Available evidence indicates that antiparallel β-sheet secondary structure becomes more stable as strands are added, at least up to four strands.[16–18] In contrast, there appears to be a limit to length-dependent stabilization of antiparallel β-sheet along the strand direction.[19,20] Parallel β-sheet secondary can be stabilized by lengthening the strands, without any limit yet detected, if the added residues have sufficient β-sheet propensity.[21] The effect of increasing parallel β-sheet size perpendicular to the strand direction has not previously been addressed. One could not assume that the size-stability relationship for parallel β-sheets was identical to that of antiparallel β-sheets, given the differential effects of lengthening along the strand direction for parallel versus antiparallel sheets.[19–21] Furthermore, the impact of strand addition in parallel β-sheets is particularly important from a biomedical perspective, because amyloid structures are generally dominated by parallel β-sheet interactions.[22,23]

We began by designing a peptide that would adopt a triple-stranded parallel β-sheet conformation in aqueous solution (**A**). This design contains three segments intended to form β-strands, along with two specialized linking units that connect pairs of strands in parallel orientation. Our laboratory previously developed the linking units, which promote, but do not enforce, formation of β-sheet interactions between attached strands: a diamine formed from D-proline and 2-methylpropane-1,2-diamine (= 1,2-diamino-1,1-dimethylethane, or DADME), for linking peptide strands via their C-termini, and a diacid formed from (1*R*,2*S*)-1,2-cyclohexanedicarboxylic acid (CHDA) and glycine, for linking peptide strands via their N-termini.[10,11] Compared to the longer, peptidic segments that link parallel β-strands in proteins, our linking units are likely less flexible, and are therefore expected to favor parallel β-sheet interactions entropically. Moreover, natural linking segments can engage in tertiary interactions, whereas our linking segments enable the study of secondary interactions in isolation.

Several considerations guided the design of the β-strand sequences. First, strand sequences were chosen to promote parallel β-sheet folding, based on intrinsic β-strand propensities of residues,[24,25] pairing preferences between residues in adjacent strands,[26] and published sequences for peptide model systems that fold in aqueous solution.[11,21] Second, charged residues were incorporated to promote water-solubility and prevent aggregation; positively charged residues were favored over negatively charged residues because the former have slightly higher β-strand propensities.[3,25] Third, given the challenge of synthesis of parallel β-sheet model

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peptides with more than two strands (Supporting Information (SI), Figure S3), amino acids that are known to add difficulty to peptide syntheses (e.g., Asp)[27] were avoided, as long as this avoidance did not significantly conflict with other design criteria. Fourth, the peptide included as much residue diversity as was allowed by the aforementioned considerations, to facilitate NMR data interpretation. Two-stranded peptides **B** and **C**, containing strands 1 and 2 or strands 2 and 3 of **A**, respectively, were prepared for spectroscopic comparisons (Figure 1).

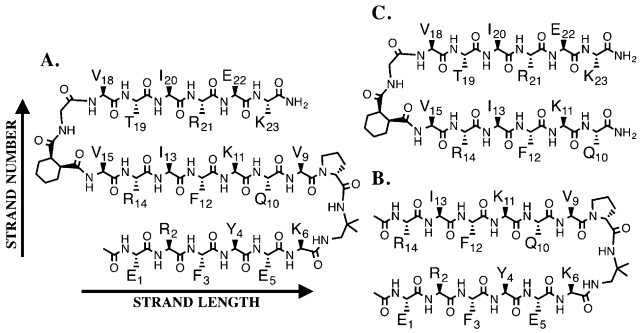

Figure 1. De novo design of peptide **A**, devised as described in the text to adopt a three-stranded parallel β-sheet conformation. Peptides **B** and **C** are truncated derivatives of peptide **A** that could adopt two-stranded parallel β-sheet conformations.

The extent of β-sheet formation was monitored by nuclear magnetic resonance (NMR) and circular dichroism (CD) spectroscopy. Sedimentation equilibrium analytical ultracentrifugation (AUC) (SI Figure S9 A and S10, Table S3), NMR diffusion (SI Table S4), and variable concentration CD and NMR experiments (SI Figure S13–S15) suggest that peptides **A**–**C** do not aggregate under the aqueous solution conditions used to acquire the spectroscopic data.

Triple-strand design **A** displays a CD signal minimum of modest intensity at 214 nm (Figure 2), which is consistent with significant population of β-sheet secondary structure. Double-strand design **B** displays a weaker minimum in the same region, which suggests a smaller population of β-sheet. Double-strand design **C** displays negative intensity but no defined minimum in this region, a CD signature that might be consistent with a small population of β-sheet secondary structure. Addition of 50 % (v/v) 2,2,2-trifluoroethanol (TFE) cosolvent enhanced the minimum near 214 nm for all three peptides (Figure 2 dashed lines), consistent with TFE-mediated promotion of secondary structure.[28] For purely aqueous conditions and with TFE cosolvent, comparison of the CD data for the three peptides suggests that the residue-normalized population of β-sheet structure is greater in three-stranded design **A** relative to either of the two-stranded designs, **B** or **C**. Thus, the CD data suggest that parallel β-sheet secondary structure becomes more favorable as the number of strands is increased. This presentation of CD wavelength scans (Figure 2 and S16) of parallel β-sheets with defined parameters (e.g., strand number, strand twist (Figure 3), aromatic residue composition) also demonstrates

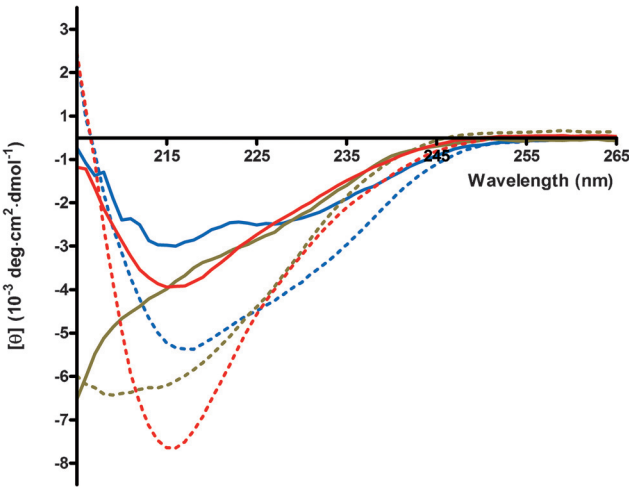

Figure 2. Mean residue ellipticity of peptides **A** (red), **B** (blue), and **C** (yellow) at 20.0 °C (293.2 K). Spectra are reported for 200 μM peptide samples in 10 mM sodium acetate buffer, pH 3.8, without (solid lines) or with (dashed lines) addition of 50 % (v/v) TFE.

Figure 3. Superposition of aqueous solution state structures of peptides **A** (magenta), **B** (cyan), and **C** (yellow). Each structure represents the average of the 10 lowest energy structures (out of 100) calculated from NMR ROE restraints. To facilitate visual comparison, residues 1–7, 15–9, and 18–23 of all peptides are depicted using cartoon ribbon diagrams, representing backbone atom positions, but not necessarily canonical β-strand structure.

how model systems such as ours may be employed to experimentally probe computational predictions of the CD spectra of parallel β-sheets.[29,30]

NMR data provided further insight on the folding behavior of peptides **A**–**C** in aqueous solution. Two-dimensional COSY, TOCSY, and ROESY spectra obtained for each peptide at 2 mM in aqueous solution (9:1 H_2O:D_2O, 2.5 mM sodium [D_3]acetate, pH 3.8) at 4 °C were sufficient for assigning almost all protons (SI Table S1). ROE peak intensities (SI Figure S6) were translated into a continuous distribution of interproton distance restraints, and these restraints were used to carry out simulated annealing of each peptide, as described in the Supplemental Information. The average of the 10 lowest energy (out of 100 calculated) structures for each peptide is shown in Figure 3. These NMR structures show a parallel β-sheet conformation for peptides **A** and **B**, and a less ordered fold or more strand fraying for peptide **C**.

Chemical shifts of α-protons ($\delta_{\alpha H}$) are sensitive to secondary structure, with residues in β-sheet or extended

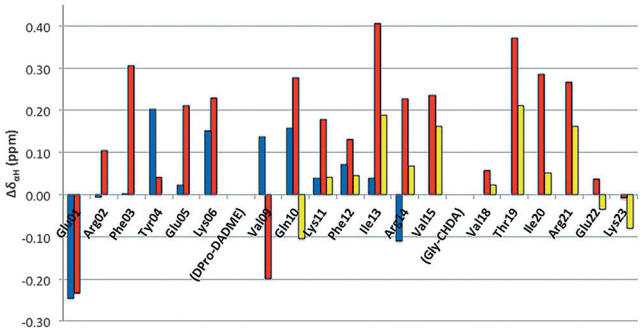

Figure 4. Secondary α-proton chemical shifts of peptides **A** (red), **B** (blue), and **C** (yellow). Random coil values were calculated using CSDb[2].

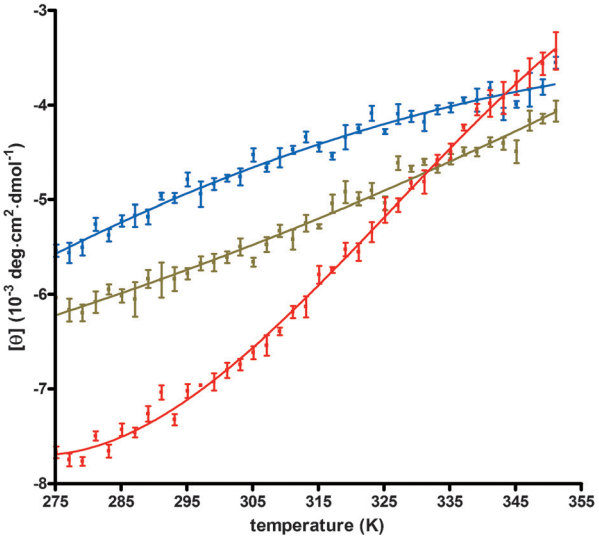

Figure 5. Mean residue ellipticity of peptides **A** (red), **B** (blue), and **C** (yellow) as a function of temperature. Solutions consisted of 200 μM peptide in 50% (v/v) aqueous buffer (10 mM sodium acetate, pH 3.8) and 50% (v/v) TFE. Points represent mean values ($n=3$), with accompanying standard error of the mean. Lines represent best fits of the data to polynomial models, which are intended to facilitate visualization of the data, and have no physical meaning.

conformations generally occurring downfield of those in unfolded or "random coil" regions, and residues in helices generally occurring upfield of random coil positions.[31] A gross pattern of downfield α-proton chemical shifts was observed for peptides **A**–**C** (Figure 4). Observation of $\Delta\delta_{\alpha H}$ $(=\delta_{\alpha H}(\text{observed})-\delta_{\alpha H}(\text{random coil}))>0.1$ ppm for three consecutive residues can be evidence of β-strand structure at these positions.[32] By this criterion, peptide **A** shows strong evidence for formation of strand 2 (residues Gln 10 to Val 15) and strand 3 (residues Thr 19 to Arg 21). The segment corresponding to strand 1 shows substantial $\Delta\delta_{\alpha H}$ values for residues Arg 2, Phe 3, Glu 5, and Lys 6, but not for Tyr 4; it is possible that a nearby aromatic ring shields the α-proton of Tyr 4. For two-stranded peptides **B** and **C**, $\Delta\delta_{\alpha H}$ at nearly every strand residue is less than $\Delta\delta_{\alpha H}$ for the corresponding residue in **A**. This trend suggests that the β-sheet population is higher for peptide **A** than for two-stranded peptides **B** or **C**. In other words, the presence of strand 1 increases the extent to which strand 3 in **A** populates the β-sheet state relative to strand 3 in **C**, and the presence of strand 3 increases the extent to which strand 1 in **A** populates the β-sheet state relative to strand 1 in **B**. Thus, the $\Delta\delta_{\alpha H}$ comparisons imply that the extent of β-sheet formation becomes larger as strand number is increased from two to three.

For each of the three peptides in the presence of a TFE co-solvent, the CD signal at approximately 214 nm was monitored as a function of temperature for thermal denaturation experiments. Thermal melt curves for samples with TFE are reported in Figure 5. Similar transitions were observed in the absence of TFE, but signal intensities were smaller in aqueous buffer (SI Figure S8). A sigmoidal transition was observed for the three-stranded peptide, but not for either of its two-stranded counterparts. This observation is the first experimental evidence of cooperative parallel β-sheet growth in the perpendicular direction.

Elucidating the impact of size on the stability of secondary structures found within proteins is fundamental to understanding the folding preferences of specific amino acid sequences. Parallel β-sheets of fewer than five strands are rarely found among natural proteins, whereas antiparallel β-sheets of just two strands are common.[33] This observation may suggest a particularly robust correlation between strand number and stability for parallel β-sheets, or an energetically favorable propagation of parallel β-sheet structure beyond two strands. The propagation of parallel β-sheet structure is of great interest because this motif is found in many pathological amyloids.[34,35]

Here we present a unique peptide system that includes a three-stranded parallel β-sheet that folds autonomously in aqueous solution. We find that increasing strand number from two to three increases parallel β-sheet population. We observe evidence for cooperative folding of the three-stranded peptide, but not for either of its two-stranded counterparts. Cooperative growth of the β-sheet perpendicular to the strand direction has been predicted by computational studies to arise from electrostatic attractions among amide dipoles.[36] Another explanation for increased parallel β-sheet stability with increasing strand number is based on entropy: formation of the two-stranded sheet preorganizes the system for β-sheet interactions with a third strand.

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