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Optimizing the Fold Stability of Miniprotein Sequences

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Introduction

Peptides (< 25 residues) rarely have well-defined, deep energy wells for folding with the possible exception of a reported few hairpin and miniprotein structures. However, these systems display relatively low melting temperatures and broad thermal transitions for unfolding. Our lab has recently employed several strategies that provide exceptional stability for both types of folds.

In the case of the Trp-cage motif, combining helix stabilizing mutations with one other feature provided three mutants that display T_M values greater than 75°C, with two-state folding (Fig. 1). In the hairpin, Trp/Trp pairings flanking a turn have been shown to greatly stabilize folds [1]. These residue pairs adopt an edge-to-face (Etf) geometry, as evidenced by circular dichroism (CD) exciton couplets and an upfield Trp H 3 proton (−2.2 ppm). Adding a backbone-NH/inhole H-bonding interaction to a turn motif stabilized by and Etf Trp/Trp interaction, produced a 10-mer hairpin that is greater than 85% folded at 25°C ($T_M > 60^\circ\text{C}$).

Methods

Linear peptides were synthesized using standard Fmoc solid-state chemistry and purified reverse-phase HPLC as previously described [1, 2]. The cyclized peptide was synthesized employing a 4-sulfamylbenzoyl AM "safety catch" resin, which produces a C-terminus thioester when cleaved by a mercaptan. Under aqueous conditions that favor Trp-cage folding, the termini are spatially near resulting in the efficient attack of the N-terminal amide on the thioester producing the cyclic peptide. Backbone-amide H/D exchange, NMR and CD data collection, as well as the methods for calculating T_M and G_U , have previously described, [1–3]

Results and Discussion

To date, two strategies have been reported for stabilizing the Trp-cage fold, helix stabilization by alanine insertion [2] and substitutions at Pro 12 [4–5]. Bunagan *et al.* [5] reported that a P12W mutation results in enhanced stability ($T_M = 15^\circ\text{C}$, compared to TC5b) and faster folding, and also noted that an Etf Trp-Trp interaction could be observed. We now establish that the P12W mutation ($T_M = 19^\circ\text{C}$) combines synergistically with helix stabilization. Trp2-cage provided the details of the Etf W/W interaction: upfield shifts at the H 2 and H 2 protons of W6 and a modest exciton couplet in the CD spectra; in addition, the P18 H 3 showed the largest upfield chemical shift difference (−2.7 ppm) for a Trp-cage to date. The NMR structure ensemble established that the P12W mutation does not change the backbone torsion angles of the Trp-cage motif (distant from the site of mutation).

Two residues (Y3, P19) positioned close to the termini of the Trp-cage motif have been shown to interact strongly: mutating either to Ala decreases stability by 16 kJ/mol [6]. As a result, we predicted that cyclizing the Trp-cage should provide additional stabilization

without disrupting the basic structure, A GG linker, with a stabilizing K8A mutation that also removes a potential competitor in the cyclization reaction, afforded cycloTC which showed a dramatic increase in melting temperature of 40°C and G_U of 6.4kJ/mol (Fig. 1) [7]. We attribute the stabilization to the decreased contact order for the stabilizing Y3/P19 hydrophobic interaction combined with a smaller entropic associated with the cyclization. This stabilization cannot exclusively be described by the decrease in entropy change from the unfolded to folded state as a cyclic 22-mer can adopt a large number of configurations.

Fold stability can also be enhanced by decreasing S_U by mutating glycines adopting positive angles to D-alanine [8]. For the Trp-cage miniprotein, G10 and G15 / values are *circa* 83/22 86/14, respectively [6]. Combining L-alanine mutations in both the α -helix and 3_{10} helix with D-alanine at positions 10 and 15 provided a gain of 31°C in T_M and 4.1 kJ/mol in G_U .

It is now established that a variety of fold stabilizing strategies combine synergistically with hydrophobic core formation in the Trp-cage system and that 20-mers with truly remarkable stability result. The stability has been enhanced to the extent that even a sidechain hydroxy group (Ser14) displays a protected OH signal in water at pH 7. This feature, rare even in large proteins, has now been observed in a decapeptide, WP2 (see fig. 1) WP2 is an optimized version of an EtF cross-strand W/W stabilized β -hairpin motif. WP2 was derived from Ac-WINGKWTG-NH₂, which displayed in the diagnostics of a flipped EtF W/W [3] and an upfield CSD (−2.71 ppm) for G8H_N that suggested that an additional interaction involving the NH and the indole ring of W6 was present. Turn stabilization (N3 D-Pro), improvement of the hydrophobic cluster (K5L), and a C-terminal tag (TG-NH₂ TGPS) increased stability: the G8H_N upfield CSD increased to −3.40 ppm. In WP2, the hydroxyl group of T7 is evident in NMR spectra (presumably due to H-bonding with the acetyl carbonyl) and displays both inter and intra-residue NOE_s. The lower size limit for observing protein like fold stability has now been reduced to the 10-mer level.

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WP2	Ac-WIpGLWTGPS	>85% folded @ 25°C	
TC10b	DAYAQWLKDGGPSSGRPPPS	$T_M = 56^\circ\text{C}$	$\Delta G_U = 12.2 \text{ kJ/mol}$
CycloTC	(DAYAQWLADGGPSSGRPPPSGG)	$T_M = 96^\circ\text{C}$	$\Delta G_U = 18.6 \text{ kJ/mol}$
TC16b	DAYAQWLADaGPASaRPPPS	$T_M = 87^\circ\text{C}$	$\Delta G_U = 16.3 \text{ kJ/mol}$
Trp-2 cage	DAYAQWLADGGWASGRPPPS	$T_M \geq 75^\circ\text{C}$	$\Delta G_U = \sim 15 \text{ kJ/mol}^*$

Fig. 1.

Peptides sequences, lower case amino acid abbreviations indicate D-AA. The melting temperatures, defined at 50% folded, were obtained from the temperature dependence of the CD. The ΔG values for unfolding (280K) were calculated from H/D exchange protection factors of backbone NH_α , except, which was estimated from T_M .