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Interplay Between Hydrophobic Cluster and Loop Propensity in β-Hairpin Formation

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Autonomously folding β-hairpins have recently emerged as powerful tools for elucidating the origins of antiparallel β-sheet folding preferences. Analysis of such model systems has suggested four potential sources of β-sheet stability: (1) the conformational propensity of the loop segment that connects adjacent strands; (2) favorable contacts between side-chains on adjacent strands; (3) interstrand hydrogen bonds; and (4) the intrinsic β-sheet propensities of the strand residues. We describe the design and analysis of a series of isomeric 20 residue peptides in which factors (1)-(4) are identical. Differences in β-hairpin formation within this series demonstrate that these four factors, individually, are not sufficient to explain β-sheet stability. In agreement with the prediction of a simple statistical mechanical model for β-hairpin formation, our results show that the separation between the loop segment and an interstrand cluster of hydrophobic side-chains strongly influences β-hairpin size and stability, with a smaller separation leading to greater stability.

Keywords: β-hairpin; β-sheet; protein stability; secondary structure

A β-hairpin, two strands linked by a short loop, is the smallest increment of antiparallel β-sheet secondary structure. Several groups have shown that short peptides can fold to β-hairpin conformations in aqueous solution (Blanco *et al*., 1998; DeGrado *et al*., 1999; Gellman, 1998). Small designed β-hairpin peptides are proving to be very useful for analysis of the thermodynamics (Andersen *et al*., 1999; Honda *et al*., 2000; Searle *et al*., 1999; Syud *et al*., 1999) and kinetics (Muñoz *et al*., 1997, 1998) of β-sheet formation. The β-hairpin approach is complementary to the study of antiparallel β-sheets within folded proteins (Mayo & Ilyina, 1998; Otzen & Fersht, 1995; Smith & Regan, 1997) because tertiary context effects can influence results obtained with the latter but not the former. Use of β-hairpins to probe fundamental aspects of β-sheet folding mirrors the extensive use of autonomously folding helical peptide model systems (Baldwin & Rose, 1999; Bolin & Millhauser, 1999; DeGrado *et al*., 1999; Dyson & Wright, 1991).

Isomeric 20 residue peptides **1-3** were designed to probe the effect on β-hairpin formation of the spacing between the loop and a well-defined interstrand cluster of non-polar side-chains. The loop in each peptide is formed by the central D-Pro-Gly segment, which is a strong promoter of β-hairpin formation (Haque *et al*., 1996; Ragothama *et al*., 1998; Stanger & Gellman, 1998). D-Pro-Gly favors type I′ and II′ β-turn conformations, which are optimal for a two-residue β-hairpin loop (Gunasekaran *et al*., 1997; Sibanda & Thornton, 1985; Sibanda *et al*., 1989). Peptides **1-3** also contain a set of four residues, Trp and Tyr on the N-terminal strand and Phe and Val on the C-terminal strand, that are intended to form a hydrophobic cluster. The intrastrand neighbors have i, $i+2$ spacing, and if the intended β-hairpin conformations are adopted, the Trp/Val and Tyr/Phe interstrand pairings occur at non-hydrogen bonded positions in the two-stranded sheet. This arrangement of Trp, Tyr, Phe and Val matches that found in the C-terminal β-hairpin (residues 41-56) of the protein GB1; a 16 residue peptide corresponding to residues 41-56 of GB1 was the first example of a natural sequence that displays autonomous β-hairpin formation in aqueous solution (Blanco *et al*., 1994). This 16-mer has a wide loop, with interstrand interactions confined to residues near the termini. Mutational analysis of this β-hairpin in the context of GB1 indicates that variation in loop size and sequence are tolerated, although these variations lead to decreased tertiary

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structural stability (Zhou *et al*., 1996). We have recently incorporated the hydrophobic residues of the GB1 cluster into a designed sequence that has a central D-Pro-Gly segment; the resulting 12-mer ($RWQYV^{D}PGKFTVQ\text{-}NH_2$) shows a well-defined β-hairpin conformation in aqueous solution, with the expected two residue loop and extensive interstrand interactions among the side-chains of Trp2, Tyr4, Phe9 and Val11 (Espinosa & Gellman, 2000). Peptide **1** is an extension of this 12-mer, with Ser-Thr-Ser-Lys added to the N terminus and Thr-Ser-Thr-Ser added to the C terminus. These extensions were designed to have high β-sheet propensity and to promote aqueous solubility (Thr and Ser display the highest β-sheet propensities in an experimental scale for strands at the outer edge of a β-sheet (Minor & Kim, 1994)). Peptides **2** and **3** are permutations of **1** in which the Trp/Tyr/Phe/Val cluster is shifted progressively toward the termini. The high β-sheet propensities of the segments between the loop and the hydrophobic cluster in **2** and **3** should cause these intervening residues to become incorporated into the strand portions of the β-hairpins rather than becoming incorporated into expanded loops. The design goals were achieved, as shown by the data presented below.

Our design renders **1-3** identical in terms of the four sources of antiparallel β-sheet stability that have been identified in earlier work: (1) the conformational propensity of the loop segment that connects adjacent strands (de Alba *et al*., 1997a,b; Ramírez-Alvarado *et al*., 1997; Stanger & Gellman, 1998; Syud *et al*., 1999); (2) favorable contacts between side-chains on adjacent strands (Andersen *et al*., 1999; Espinosa & Gellman, 2000; Honda *et al*., 2000; Maynard *et al*., 1998; Searle *et al*., 1999); (3) interstrand hydrogen bonds (Constantine *et al*., 1995); and (4) the intrinsic β-sheet propensities of the strand residues (de Alba *et al*., 1997b; Muñoz & Serrano, 1994; Smith & Regan, 1997). Specifically, (1) the two residue loop segments are identical among **1-3**, (2) the hydrophobic cluster resulting from Trp/Tyr/Phe/Val interactions should be identical, (3) all three peptides have the potential to form the same number of interstrand hydrogen bonds, and (4) the amino acid compositions are identical. The key difference among **1-3** is the separation between the D-Pro-Gly loop and the GB1-derived hydrophobic cluster.

ROESY (Bothner-by *et al*., 1984) and NOESY (Macura & Ernst, 1980) data for **1-3** showed that a β-hairpin conformation containing the expected Trp/Tyr/Phe/Val side-chain cluster forms in each case (Figure 1). Each peptide displayed only one strong NH-NH NOE between adjacent residues, Gly11-Lys12, which is consistent with the expected two residue loop across the D-Pro-Gly segment (some additional weak NH-NH NOEs between adjacent residues were observed, mostly near the termini of **1-3**). The population of the β-hairpin conformation decreases as the cluster is shifted toward the termini, as shown by the decrease in the number and intensity of the long-range interstrand NOEs along the series, **1** > **2** > **3**. Each peptide displayed a small (ca 10%) population of the *cis* Val9-Pro10 rotamer. Analytical ultracentrifugation showed that **1-3** are monomeric under conditions employed for NMR analysis.

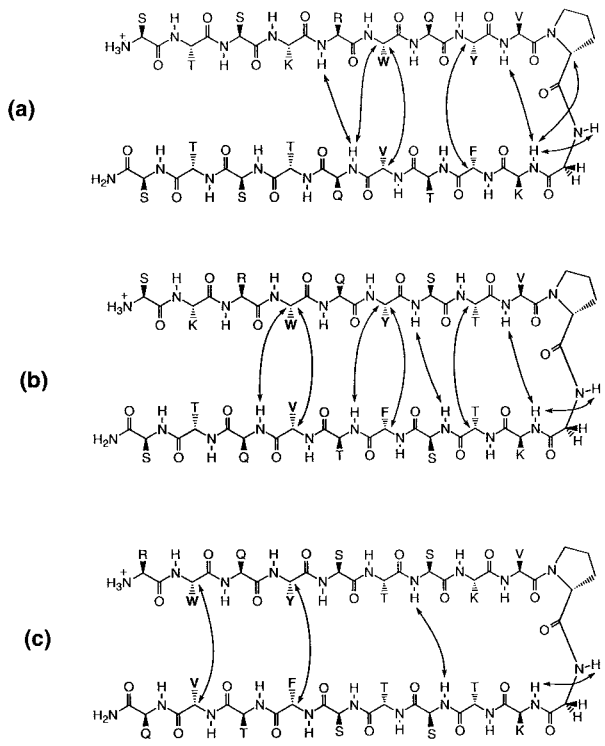

Figure 1. Selected NOEs, including all backbone-backbone NOEs between NH and/or H^{α} on non-adjacent residues and the one strong NH-NH NOE between adjacent residues (Gly11-Lys12): (a) peptide 1; (b) peptide 2; (c) peptide 3. The NOEs were observed in NOESY and ROESY spectra at 500 MHz and/or in ROESY spectra at 750 MHz. Samples contained ca 3 mM peptide in 9:1 (v/v) $H_2O/^2H_2O$ with 100 mM sodium deuterioacetate buffer (pH 3.8 uncorrected) at 3 °C (500 MHz) or 5 °C (750 MHz). H^{α}-H^{α} NOEs were observed in ^2H_2O solution. Resonance assigments were obtained from a combination of COSY, TOCSY and ROESY experiments.

Peptide **1** displayed a total of 39 NOEs between non-adjacent residues, and all of these are consistent with the expected β-hairpin conformation for the inner portion of the peptide, residues Arg5 to Gln16. NOEs involving backbone protons (H^{α} and/or NH) verified the formation of β-sheet secondary structure (Figure 1(a)) (Wüthrich, 1986). A network of side-chain/side-chain NOEs demonstrated the expected clustering of the four GB1-derived side-chains; such NOEs were observed between lateral interstrand neighbors Trp6 and Val15, and between Tyr8 and Phe13. In addition, ''diagonal'' NOEs were observed between Trp6 and Phe13, but not between Tyr8 and Val15, which indicates that the β-hairpin conformation adopted by **1** has the right-hand twist commonly observed for β-sheets embedded in proteins (Chothia, 1973).

No long-range NOEs were observed for the terminal residues of **1**, Ser1 to Lys4 or Thr17 to Ser20. Thus, these outer residues appear not to participate significantly in the β-hairpin conformation. The non-adjacent NOEs for the inner residues were used to determine the solution structure with the program DYANA (Guntert *et al.*, 1997). The ten best structures resulting from this analysis corresponded to a β-hairpin conformation with a two-residue loop at D-Pro-Gly (Figure 2(a)). For these ten structures, the RMSD among backbone atoms (residues 5 to 16) was 0.85(±0.25) Å, and the RMSD among all heavy-atoms was 1.82(±0.31) Å. There was no NOE violation among these structures.

NOE data for peptide **2** show that moving the hydrophobic cluster toward the termini, relative to **1**, increases the length of the β-hairpin but diminishes NOE intensities. This behavior suggests that the β-hairpin population is lower in **2** than in **1**. Peptide **2** displayed a total of 24 NOEs between non-adjacent residues, and all of these are consistent with the expected β-hairpin conformation for residues Trp4 to Val17. NOEs involving backbone protons show that the correct interstrand register extends from the two-residue loop to the GB1 side-chain cluster (Figure 1(b)). In addition, NOEs involving side-chains were observed for lateral interstrand neighbors Trp4/Val17 and Tyr6/Phe15. No long-range NOE was were observed for terminal residues 1-3 or 19-20. DYANA analysis was carried out for residues 4-17 of **2** using the non-adjacent NOEs; the ten best structures corresponded to a β-hairpin conformation with a two residue loop at the D-Pro-Gly segment (Figure 2(b)). The RMSD for backbone atoms among these ten structures was 1.38(±0.60) Å, and the RMSD for all heavy-atoms was 2.30(±0.69) Å. There was no NOE violation.

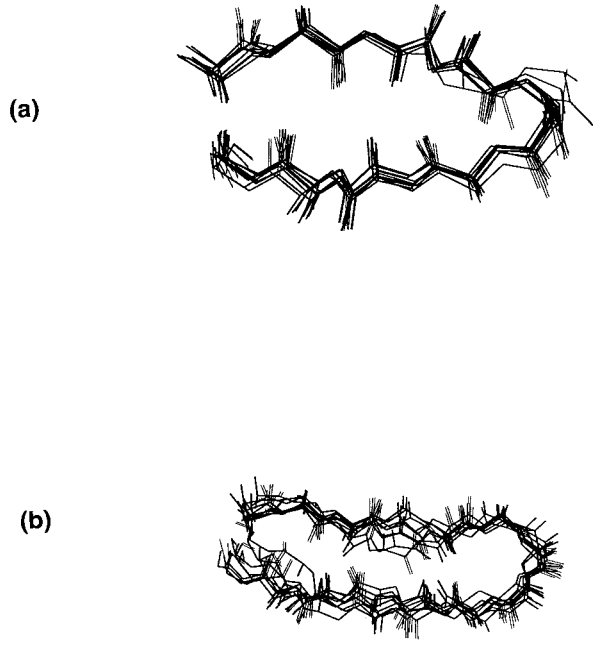

Figure 2. Superposition of the ten best calculated structures from DYANA analysis of NMR data. Only backbone atoms are shown: (a) peptide **1**, residues 5-16; (b) peptide **2**, residues 4-17. Structure calculations were not performed for peptide **3** because of the small number of NOEs.

Peptide **3**, with the GB1-derived residues closest to the termini, showed only ten NOEs between non-adjacent residues, and all were weak or very weak (Figure 1(c)). Each of these NOEs is consistent with the expected β-hairpin conformation, which appeared to be only modestly populated. As seen for **1** and **2**, H^{α}-H^{α} NOEs were observed between the two pairs of GB1 residues that are expected to be lateral neighbors in non-hydrogen bonded positions, Trp2/Val19 and Tyr4/Phe17. These lateral pairings were further indicated by NOEs involving the side-chains Trp2, Tyr4, Phe17 and Val19. A third backbone-backbone NOE, between Ser7 NH and Ser14 NH, suggests that the β-hairpin conformation extends back from the GB1 side-chain cluster toward the loop. (The chemical shift dispersion was dramatically lower for **3** than for **1** or **2**; therefore, additional NOEs may have been present but not detected for **3**. We have previously observed that low chemical shift dispersion is correlated with low population of folded states in designed peptides.)

α-Proton chemical shift ($\delta_{H\alpha}$) data for peptides **1-3** provided independent support for the principal conclusions derived from the NOE data, i.e. that the length of the β-hairpin conformation increases but the β-hairpin population decreases as the GB1-derived hydrophobic cluster is moved away from the loop. $\delta_{H\alpha}$ values are very sensitive to secondary structure, with residues in β-sheet displaying downfield shifts and residues in α-helix displaying upfield shifts relative to random coil $\delta_{H\alpha}$ values (Wishart *et al.*, 1991). Figure 3 shows $\Delta\delta_{H\alpha}$ values ($\delta_{H\alpha}$ (folded peptide) - $\delta_{H\alpha}$ (unfolded reference)) for the strand residues of peptides **1-3** (residue 1 is omitted in each case because the N terminus is not acylated). The $\delta_{H\alpha}$ (unfolded reference) values were obtained from the diastereomers of **1-3** in which D-Pro10 is replaced with L-Pro. Data from several designed β-hairpin systems show that switching from D-Pro to L-Pro completely abolishes β-hairpin folding (Espinosa & Gellman, 2000; Haque & Gellman, 1997; Ragothama *et al.*, 1998; Stanger & Gellman, 1998). Similar behavior is observed for **1-3**: none of the L-Pro diastereomers shows any NOE between non-adjacent residues. In addition, $\delta_{H\alpha}$ data for the L-Pro diastereomers of **1-3** display small but significant deviations from tabulated ''random coil'' values (Wishart *et al.*, 1991; Wüthrich, 1986), which presumably result from sequence context effects (data not shown). The L-Pro diastereomers are therefore ideal sources of unfolded $\delta_{H\alpha}$ values for the strand residues of **1-3**. As previously observed for other L-Pro peptides (Haque & Gellman, 1997; Stanger & Gellman, 1998), there is no systematic trend in the $\delta_{H\alpha}$ data

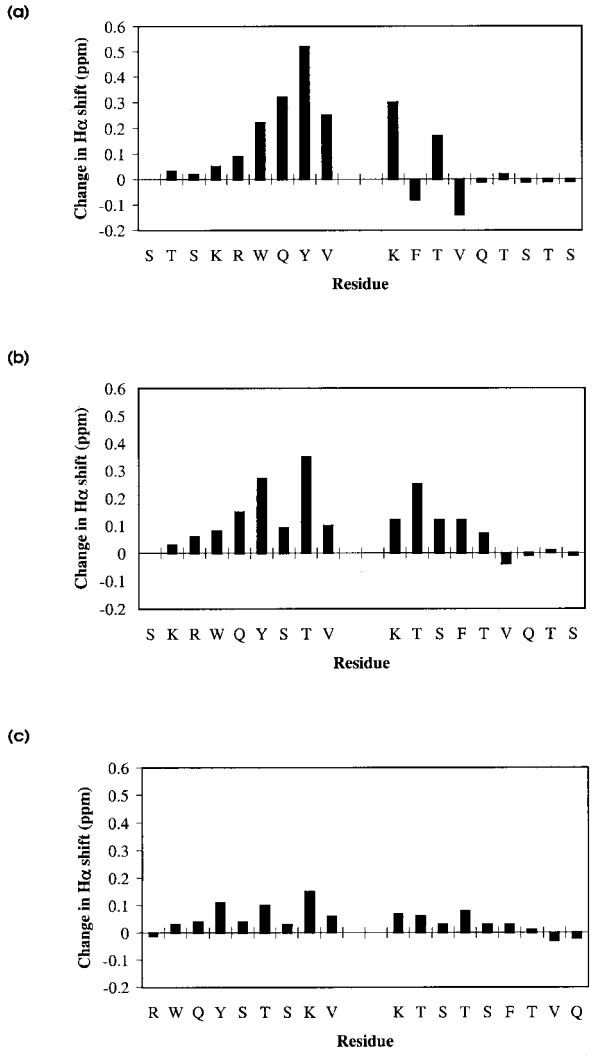

Figure 3. $\Delta\delta_{H\alpha}$ values ($\delta_{H\alpha}$ (folded peptide) $-$ $\delta_{H\alpha}$ (unfolded reference)) for the strand residues of peptides **1-3**. Data are omitted for residue 1 in each case because the N terminus is not acylated, and for the turn residues. Unfolded reference $\delta_{H\alpha}$ values were obtained from the diastereomers of **1-3** in which D-Pro10 is replaced with L-Pro: (a) peptide **1**; (b) peptide **2**; (c) peptide **3**. Conditions: ca 3 mM peptide in ^2H_2O, 100 mM sodium deuterioacetate buffer (pH 3.8 uncorrected) at 3 °C.

for the L-Pro diastereomers of **1-3**, which indicates that extended β-strand conformations are not adopted by the C-terminal or N-terminal portions in the absence of the interstrand interactions made possible by the D-Pro-Gly loop.

The $\Delta\delta_{H\alpha}$ data for **1** are consistent with the NOE data in suggesting that the inner residues form a well-populated β-hairpin conformation, and that the hairpin does not extend to the final four or five residues in either strand. The observation that $\Delta\delta_{H\alpha} < 0$ for Phe13 and Val15 presumably results from proximity of the aromatic side-chains of Trp6 and Tyr8 in the folded conformation; indeed, NOEs were observed from the side-chain of Trp6 to H^{α} of Val15 and from the side-chain of Tyr8 to H^{α} of Phe13. The $\Delta\delta_{H\alpha}$ data for **2** are consistent with a well-formed β-hairpin in the central residues that dissipates toward the termini. The $\Delta\delta_{H\alpha}$ data for **3** confirm that placing the hydrophobic cluster farthest from the D-Pro-Gly loop leads to the lowest β-hairpin population.

A simple model to describe the thermodynamics and kinetics of β-hairpin formation has been described recently (Muñoz *et al.*, 1997, 1998). The model was capable of describing quantitatively the folding behavior of the 41-56 GB1 β-hairpin peptide (Muñoz *et al.*, 1997). This model predicts that the separation between a stabilizing cluster of side-chains and the connecting loop strongly influences both the overall stability of the β-hairpin and the fraying of its ends (Muñoz *et al.*, 1998). To test this prediction in more depth, we have used this model

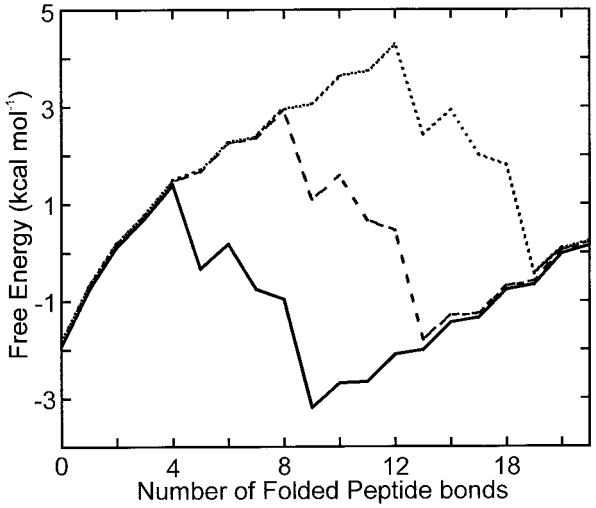

Figure 4. Free-energy profiles for peptides **1** (continuous), **2** (broken) and **3** (dotted) calculated with a simple statistical mechanical model of β-hairpin formation (Muñoz *et al.*, 1997, 1998). This model defines hairpin structure in terms of the individual peptide bonds (specifically, the dihedral angle ψ of preceding residue and dihedral angle ϕ of following residue), rather than in terms of residues. Therefore, a stretch of $n-1$ peptide bonds in the model is equivalent to a stretch of n residues, including the residues that flank the first and the last peptide bond. The calculations were carried out using the same pattern of interactions and the parameters previously obtained from the thermodynamic and kinetic analysis of the GB1 β-hairpin peptide (Muñoz *et al.*, 1997). The parameters were: hydrogen bond free energy ($\Delta G_{hb} = -1.1$ kcal mol^{-1}); hydrophobic interaction free energy ($\Delta G_{sc} = -2.1$ kcal mol^{-1}); entropic cost of fixing a residue in native conformation ($\Delta S_{conf} = 3.2$ cal mol^{-1} K^{-1}). To account for the special β-turn-inducing properties of D-Pro, we used a $\Delta S_{conf} = 1.6$ cal mol^{-1} K^{-1} (half the value of a normal residue) for the peptide bond between D-Pro and Gly. In these peptides, there are up to nine possible hydrogen bonds and up to three possible hydrophobic interactions (Y-F, W-V and W-F) when the β-hairpin spans the entire peptide.

to calculate the thermodynamic properties of peptides **1-3**, using the parameters previously obtained from 41-56 GB1 (Figure 4). In these calculations, we have assumed for simplicity that in peptides **1-3** the hydrophobic cluster produces the same pattern of side-chain interactions (Tyr-Phe, Trp-Val and Trp-Phe) that was observed in 41-56 GB1. The calculations indicate that peptide **1** should have a very stable β-hairpin conformation spanning residues 6-15, and that both ends (residues 1-5 and 16-20) should be frayed. Thus, the predicted free energy minimum for **1** corresponds to a conformation with only the ten central residues in the folded state (nine central peptide bonds). Peptide **2** is predicted to have a less stable β-hairpin conformation, but the β-hairpin is predicted to be longer, spanning residues 4-17. Thus, the central 14 central residues of **2** are in the β-hairpin conformation at the predicted free energy minimum (13 central peptide bonds). Peptide **3** is predicted to display a β-hairpin conformation of marginal stability that spans residues 2-19, i.e. all but the C and N-terminal residues are predicted to be folded at the free energy minimum (17 central peptide bonds). These predictions agree well with NMR observations for peptides **1-3**.

Our results show that peptides **1-3** display quite different β-hairpin folding behavior (length and population of the β-hairpin) despite the fact that they contain the same residues, the same loop segment and the same lateral interstrand pairing of side-chains. Thus, the four factors to which β-hairpin stability is commonly attributed, loop conformational preference (de Alba *et al*., 1997a,b; Ramírez-Alvarado *et al*., 1997; Stanger & Gellman, 1998; Syud *et al*., 1999), strand residue propensities (de Alba *et al*., 1997b; Smith & Regan, 1997), interstrand hydrogen bonding (Constantine *et al*., 1995) and interstrand hydrophobic interactions (Andersen *et al*., 1999; Espinosa & Gellman, 2000; Honda *et al*., 2000; Maynard *et al*., 1998; Searle *et al*., 1999), cannot individually account for these differences. Our data suggest that favorable interstrand interactions among the side-chains of the four GB1-derived residues, Trp, Tyr, Phe and Val, provide a major driving force for β-hairpin formation, which is consistent with previous conclusions regarding hydrophobic stabilization of β-hairpins (Andersen *et al*., 1999; Espinosa & Gellman, 2000; Honda *et al*., 2000; Maynard *et al*., 1998; Searle *et al*., 1999). This driving force, however, is strongly modulated by the distance between the side-chain cluster and the hairpin-promoting loop segment. Our results corroborate the predictions of a simple statistical mechanical model for β-hairpin formation (Muñoz *et al*., 1998). The model indicates that the relationship between conformational stability and loop/hydrophobic cluster separation is a consequence of compensation between the cost in conformational entropy of forming the β-hairpin and the stabilization produced by interstrand interactions (backbone hydrogen bonds and hydrophobic side-chain contacts). Backbone hydrogen bonds, alone, are unable to stabilize the β-hairpin conformation, because the cost of fixing four residues in a hairpin conformation is higher than the gain in free energy from forming two hydrogen bonds (Muñoz *et al*., 1997). Interstrand clustering of hydrophobic side-chains can compensate for the entropic penalty associated with folding (Yang & Honig, 1995), but only if the cluster is sufficiently close to the connecting loop. Further evidence of the interplay between the side-chain cluster and the loop segment is seen in the difference between **3** and its L-Pro diastereomer: β-hairpin formation depends upon proline configuration even when the distance between cluster and loop is largest. The impact of proline configuration in this diastereomeric pair is particularly interesting in light of the wide loop in the β-hairpin conformation adopted by residues 41-56 of GB1 (Blanco *et al*., 1994). The ability of the D-Pro to L-Pro mutation to abolish β-hairpin formation in **3** highlights the cooperativity between the loop segment and the side-chain cluster in stabilizing the β-hairpin fold.

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