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A Designed β-Hairpin Containing a Natural Hydrophobic Cluster**

Juan F. Espinosa and Samuel H. Gellman*

β-Sheets are a very common substructure in folded proteins,[1] and intermolecular sheet-type interactions play a crucial role in protein–protein recognition[2] and in pathological protein aggregation.[3] Therefore, understanding the balance of noncovalent forces that controls β-sheet formation is a goal of fundamental importance. Recently it has become possible to probe the origins of antiparallel β-sheet stability with short, designed peptides that fold autonomously in aqueous solution.[4] These model systems complement the more traditional approach of examining β-sheets that are embedded within a particular tertiary fold;[5] short peptides allow one to explore small increments of β-sheet in the absence of a specific structural context. Here, we describe a peptide in which a cluster of hydrophobic sidechains from the protein GB1 has been grafted onto a designed sequence. Thermodynamic analysis of folding provides insight on the origins of β-sheet stability.

The "β-hairpin" architectural motif, comprised of two antiparallel strands and a short connecting loop, is essential for creation of short peptides that display antiparallel β-sheet folding in water.[4] β-Hairpins are common in proteins,[6] but natural β-hairpin sequences seldom fold in water when extracted from their native protein context.[4] An exception is a 16-residue segment of the protein GB1, **1**, which displays partial population of a native-like β-hairpin conformation.[7] Peptide **1** differs from other autonomously folding β-hairpins

Gly-Ile-Trp-Thr-Tyr-Asp-Asp-Ala-Thr-Lys-Thr-Phe-Thr-Val-Thr-Glu

1

in the unusually large six-residue loop (Asp-Asp-Ala-Thr-Lys-Thr) that connects the two strand segments; other autonomously folding β-hairpins contain loops of two to four residues.[4, 8] Interstrand interactions within the folded conformation of **1** are limited to residues near the termini.[7] Clustering among the hydrophobic sidechains of Trp 3, Tyr 5, Phe 12, and Val 14 presumably provides a drive for β-hairpin folding, which overcomes the entropic cost of ordering the loop segment.

In order to elucidate the contribution of interstrand sidechain interactions to β-sheet stability, we incorporated the residues of the GB1 cluster into a 12-residue sequence, **2**,[9] that was expected to adopt a more highly defined β-hairpin conformation than does **1**. The arrangement of the Trp, Tyr,

Arg-Trp-Gln-Tyr-Val-Xxx-Gly-Lys-Phe-Thr-Val-Gln-NH_2

2, Xxx = D-Pro
3, Xxx = L-Pro

Phe, and Val residues in **2** allows native-like sidechain juxtapositions if the peptide folds to a β-hairpin conformation with a tight two-residue loop; the D-Pro–Gly segment strongly promotes this type of β-hairpin.[8e, 8g, 10] Our design hypothesis was that the mutually reinforcing effects of the D-Pro–Gly loop and the GB1 cluster in **2** would produce a β-hairpin conformation well-defined over most residues and, therefore, suitable for thermodynamic analysis. Diastereomer **3**, with D-Pro replaced by L-Pro, was expected to serve as a negative control, since L-Pro-Gly discourages formation of tight β-hairpin conformations.[8, 10]

Peptide **2** displays numerous NOE interactions between residues that are not adjacent in sequence, and all of these nonadjacent effects are consistent with the β-hairpin conformation shown in Figure 1. The subset of interstrand NOE interactions involving backbone protons (NH and H_α; Figure 1a, b) verifies antiparallel β-sheet formation at the backbone level. Sidechain–sidechain NOE data (Figure 1c) reveal the anticipated clustering among the GB1 sidechains. No interactions between nonadjacent residues were observed for L-Pro diastereomer **3**, which demonstrates that changing the proline configuration constitutes an "on/off" switch for β-hairpin formation in aqueous solution. The solution structure of **2** was calculated with the program DYANA,[11] using 37 NOE measurements (including all effects between nonadjacent residues) as distance restraints. Among the 20 best structures, the root mean square deviation (RMSD) was 0.58 ± 0.16 Å for the backbone atoms and 1.25 ± 0.22 Å for all heavy atoms, over residues 2–11 (terminal residues 1 and 12 were highly disordered). These results demonstrate that the

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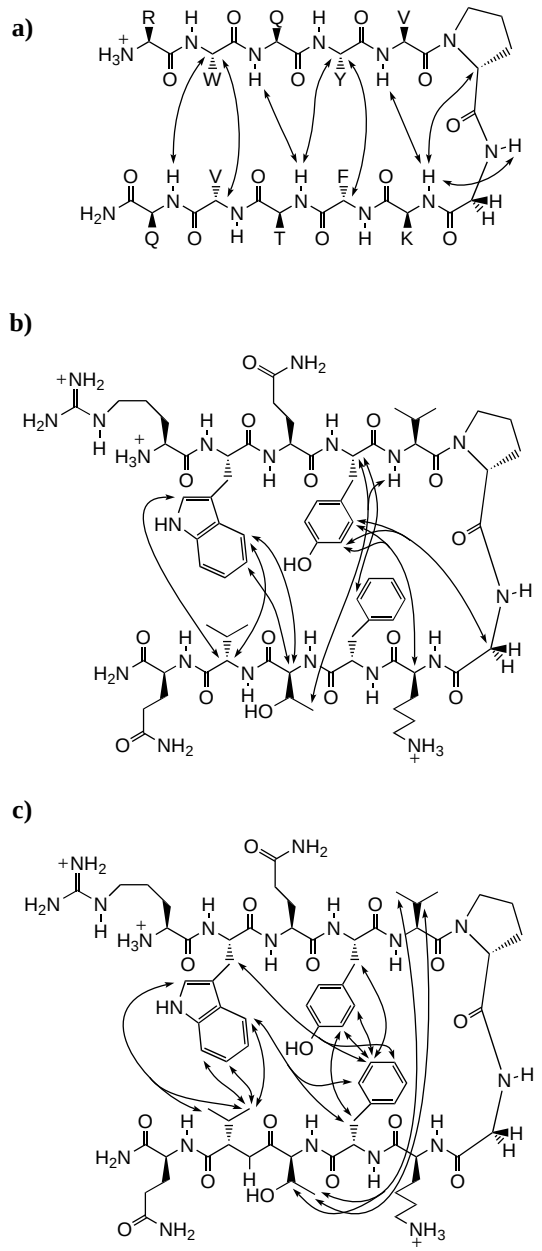


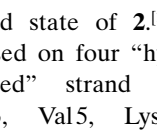
Figure 1. Summary of long-range NOE interactions observed in ROESY analysis for **2** (1.6 mM) in aqueous sodium deuteroacetate buffer (100 mM; H_2O:D_2O 9:1 or D_2O), at pH 3.8 (uncorrected) and 276 K. a) Backbone–backbone NOE interactions (NH–NH, NH–H_α, and H_α–H_α). b) Backbone–sidechain NOE interactions. c) Sidechain–sidechain NOE interactions. Resonance assignments were obtained from a combination of COSY, TOCSY, NOESY, and ROESY data at 500 MHz. Additional ROESY data were obtained at 750 MHz. Analytical ultracentrifugation showed that **2** does not self-associate under the conditions used for NMR structural analysis.

unnatural D-proline residue in the loop promotes native-like pairing of antiparallel β-strands.

NMR data indicate that the extent of β-hairpin folding is comparable at several different sites within **2**, and that the β-hairpin population changes in a coordinated way as a function of temperature throughout the peptide. Thus, β-hairpin formation appears to be cooperative, and we can use a two-state model (unfolded versus β-hairpin) to analyze thermal effects on folding.

NOE data provide two independent estimates for the β-hairpin population of **2**. The intensities of interstrand H_α–H_α NOE interactions between laterally aligned residues[12] can be used to estimate β-hairpin population if one assumes that there is a fixed H_α–H_α separation in the folded state (2.3 Å) and no contribution to NOE intensity from the unfolded state.[13] We calculate that, at 3 °C, **2** has 54% β-hairpin population based on the $H_\alpha4$–$H_\alpha9$ NOE measurement and 76% based on the $H_\alpha2$–$H_\alpha11$ NOE data. These two populations are in reasonably good agreement, given the intrinsic limitations of the NOE-based approach. (Small differences between assumed and actual H–H separations in the folded state lead to large errors because the NOE intensity varies as the inverse sixth power of H–H separation.[12]) The fact that the NOE near the termini is slightly more intense than the NOE near the turn indicates that the hairpin is well-defined over most of its length.

Hα chemical shifts ($\delta_{H\alpha}$) offer an independent basis for population quantification[4, 14] because $\delta_{H\alpha}$ values are highly sensitive to the local secondary structure.[15] This approach requires $\delta_{H\alpha}$ data for each of the two limiting states of **2**, unfolded and β-hairpin, since equilibration is rapid on the NMR time scale. The L-Pro diastereomer **3** provides $\delta_{H\alpha}$ data for the fully unfolded state of **2**, and cyclic peptide **4** serves as a model for the completely folded state of **2**.[14, 16] We focused on four "hydrogen-bonded" strand residues, Gln3, Val5, Lys8, and Thr10. Populations deduced at these four sites[17] vary in a coordinated way as a function of temperature (Figure 2). The α-protons of these residues point away from the opposite



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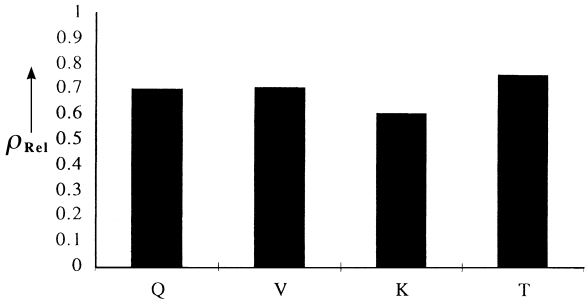

Figure 2. Relative population of the β-hairpin conformation (ρ_{Rel})of **2** at 321 K versus 276 K, as determined from $\delta_{H\alpha}$ data at four hydrogen bonded strand residues, Gln3 (Q), Val5 (V), Lys8 (K), and Thr10 (T).

strand in the β-sheet conformation, and these $\delta_{H\alpha}$ values should therefore be less susceptible than $\delta_{H\alpha}$ values of the "non-hydrogen-bonded" strand residues to secondary effects from the aromatic sidechains on the adjacent strand. Indeed, the $\delta_{H\alpha}$ values of Phe9 and Val11 display an upfield shift relative to unfolded reference **3**, rather than the expected downfield shift,[15] as a result of anisotropic effects of the Trp2 and Tyr4 sidechains that are brought into proximity by β-hairpin formation.[18, 19]

ΔH°, ΔS°, and ΔC_p° for β-hairpin formation by **2** were estimated from $\delta_{H\alpha}$ data obtained between 275 K and 315 K (Figure 3). An average β-hairpin population was determined

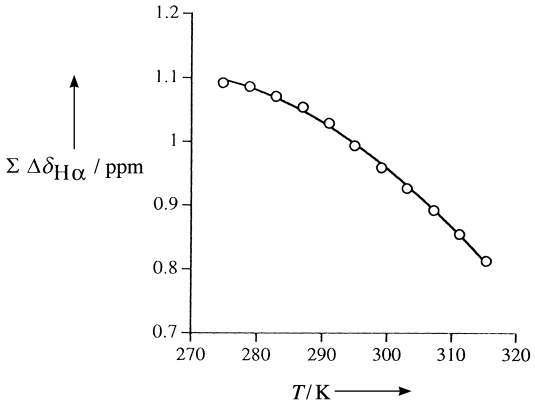

Figure 3. Use of variable-temperature NMR chemical shift data ($\delta_{\alpha H}$) to estimate folding thermodynamics for β-hairpin **2**. $\Sigma\Delta\delta_{H\alpha}$ is the sum of the α-proton chemical shifts for four strand residues in hydrogen-bonded positions, Gln3, Val5, Lys8, and Thr10, for **2**, minus the analogous sum for unfolded reference **3**, at the indicated temperature. These data were fitted to the equation $\Sigma\Delta\delta_{H\alpha} = \Sigma\Delta\delta_{H\alpha limit}[\exp(x/RT)]/[1+\exp(x/RT)$, where $x = [T(\Delta S^\circ_{298} + \Delta C^\circ_p \ln(T/298)) - (\Delta H^\circ_{298} + \Delta C^\circ_p(T-298)]$ and $\Sigma\Delta\delta_{H\alpha limit} = \Sigma\Delta\delta_{H\alpha}(\mathbf{4}) - \Sigma\Delta\delta_{H\alpha}(\mathbf{3})$. This analysis is based on Ref. [20]. The resulting ΔH°, ΔS°, and ΔC°_p values are given in the text.

at each temperature by using the sum of $\delta_{H\alpha}$ values for Gln3, Val5, Lys8, and Thr10 in **2** and in reference peptides **3** and **4**.[17–19] The β-hairpin population was calculated to be 61% at 275K (consistent with NOE-based populations discussed above) and 45% at 315K. A previously reported nonlinear fitting method[20] was used to estimate thermodynamic parameters for the two-state equilibrium (unfolded versus β-hairpin) from the temperature-dependent $\delta_{H\alpha}$ data. This analysis gave $\Delta H^\circ = -3.2 \pm 0.1$ kcal mol^{-1}, $\Delta S^\circ = -10.2 \pm 0.2$ cal K^{-1} mol^{-1}, and $\Delta C^\circ_p = -98 \pm 8$ cal K^{-1} mol^{-1} (the uncertainties arise from the fitting).[21]

The thermodynamic profile deduced for the folding of **2** differs qualitatively from that reported for **5**, a designed peptide that adopts a β-hairpin conformation with a two-residue loop at Asn–Gly.[20] At 298K, the folding of **5** in

Lys-Lys-Tyr-Thr-Val-Ser-Ile-Asn-Gly-Lys-Lys-Ile-Thr-Val-Ser-Ile

5

aqueous solution is enthalpically unfavorable ($\Delta H^\circ = +1.7$ kcal mol^{-1}) and entropically favorable ($\Delta S^\circ = +5.5$ cal K^{-1} mol^{-1}), and formation of this β-hairpin involves a large negative change in heat capacity ($\Delta C^\circ_p = -330$ cal K^{-1} mol^{-1}). This thermodynamic profile is consistent with a classical hydrophobic driving force for β-hairpin formation.[20, 22] In contrast, the thermodynamic profile of β-hairpin folding for **2** does not conform to expectations for a purely hydrophobic driving force, since the process is enthalpically favorable and entropically unfavorable at 298K. The negative ΔC°_p for **2**, however, suggests a classical hydrophobic component to β-hairpin stabilization.[22]

Many lines of evidence indicate that dehydration of hydrophobic surfaces in the folded state provides a major source of protein conformational stability.[22, 23] The data for **2** are interesting because the thermodynamic profile suggests that classical hydrophobic interactions alone are not the major driving force for folding, despite the clustering of the four GB1 nonpolar sidechains. Interstrand hydrogen bonds formed in the β-hairpin state of **2** might explain the ΔH° and ΔS° data for the folding of this peptide,[24] but these contributions, if dominant, should have been evident in the folding of β-hairpin **5** as well.[20, 25] The thermodynamic differences between β-hairpin formation by **2** and **5** can be reconciled through a hypothesis of Diederich et al.,[26] who proposed that tight and loose interactions between nonpolar entities in aqueous solution lead to qualitatively different thermodynamic profiles. The classical hydrophobic signature, involving an entropic driving force at 298K, was proposed to correspond to a loose interaction, while an enthalpic driving force at 298K was proposed to result from a tight interaction.[26] Thus, it is possible that the folded state of **2** allows tighter association among nonpolar sidechains than does the folded state of **5**. In this regard, the higher proportion of aromatic sidechains in **2** relative to **5** may be significant, since it has been argued that aromatic groups have a greater intrinsic affinity for other nonpolar groups than do aliphatic groups.[27]

We have shown that transplantation of a nonpolar sidechain cluster from a β-sheet in a folded protein to a short designed peptide (**2**) generates a well-defined β-hairpin conformation as judged by both backbone and sidechain NMR indicators. These results demonstrate that the tight β-hairpin backbone conformation induced by the D-Pro-Gly segment is compatible with the well-defined cluster of hydrophobic sidechains observed in the protein GB1. Further analysis of the differences between **2** and previously reported β-hairpin **5**[20] should shed additional light on the origins of β-sheet stability; the differences themselves underscore the importance of studying multiple model β-sheet systems.

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[17] The β-hairpin population of **2** was estimated at each indicator residue and temperature by interpolating the $\delta_{H\alpha}$ value for **2** between the $\delta_{H\alpha}$ value for the corresponding residue in unfolded reference **3** at that temperature and the $\delta_{H\alpha}$ value for folded reference **4** at 276 K. (We assume that **4** is maximally folded at low temperature.)

[18] Attempted population analysis based on $\delta_{H\alpha}$ data for the non-hydrogen-bonded strand residues of **2** (Trp 2, Tyr 4, Phe 9, and Val 11) and reference peptides **3** and **4** provides nonsensical results. For example, at 275 K, $\delta_{H\alpha}$ data for Trp 2 imply 180 % β-hairpin population for **2**, while $\delta_{H\alpha}$ data for Phe 9 imply < 0 % β-hairpin population for **2**. We suspect that subtle differences in aromatic sidechain packing between cyclic peptide **4** and the fully folded state of **3** are responsible for these observations. (This hypothesis requires differences of < 0.3 ppm in non-hydrogen-bonded residue $\delta_{H\alpha}$ values between these two systems.) Although **4** appears not to be a fully accurate model for interstrand sidechain packing in the β-hairpin conformation of **3**, we believe that **4** is a good model for the backbone in the β-hairpin conformation of **3** because of the good agreement among data for the four hydrogen-bonded strand residues (Gln 3, Val 5, Lys 8, and Thr 10) and for the Gly residue in the turn (see Ref. [19]).

[19] The folding equilibrium of a designed β-hairpin has been monitored by the chemical shift separation ($\Delta\delta_{H\alpha}$) between the geminal α-protons of a glycine residue in the loop (loop sequence = Asn–Gly): M. S. Searle, S. R. Griffiths-Jones, H. Skinner-Smith, *J. Am. Chem. Soc.* **1999**, *121*, 11615. We estimated the β-hairpin population of **2** by interpolating between $\Delta\delta_{H\alpha}$ values for reference peptides **3** and **4**. This analysis indicated 66 % β-hairpin population for **2** at 275 K, and 56 % at 315 K; these estimates agree well with those obtained from strand residue $\delta_{H\alpha}$ data.

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Complimentary Polytopic Interactions**

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Substantially enhanced mesophase ranges can be obtained by mixing the discotic liquid crystal **1a** with one equivalent of the "larger core" polynuclear aromatic compounds **2a** or **3a** (Scheme 1). The special stability of these novel π-stacked systems is not the result of either charge-transfer or (net) quadrupolar interactions but instead arises from a complimentary polytopic interaction (CPI).

Chemical doping of discotic liquid crystals is well known and, in some cases, it produces enhanced mesophase ranges.[1] Hence, mixtures of the discotic liquid crystal **1a** with 2,4,7-trinitrofluoren-9-one (TNF) have been extensively studied.[2] Although charge-transfer bands are observed in the UV/Vis spectrum of this mixture, they are weak. It is now believed

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