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Selective Aromatic Interactions in β-Hairpin Peptides

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Determining the fundamental forces that specify peptide secondary structure is principle in understanding protein folding. Recent studies of β-sheets in proteins and β-hairpin peptides have suggested that cross-strand interactions are important for stability of this secondary structure.[1] A common requirement for stability of β-hairpins is a hydrophobic cluster.[2] However, specific preferences between aromatic and aliphatic residues in the clusters have not been delineated. Aromatic interactions have been implicated in the structure and stability of proteins[3] but the origin of this stability, which may be dependent on hydrophobicity, electrostatic interactions, and van der Waals forces, is still a matter of debate.[4] To determine selectivities in a hydrophobic cluster, we have examined the effect of varying a single cross-strand pair of residues in a 12-residue β-hairpin and determined the interaction preferences between aromatic and aliphatic residues (Figure 1). In this system we have found that there is a significant preference for *self-association* among aromatic residues and that the unique nature of the aromatic interaction appears to be the source of this selectivity.

Peptides **1**−**4** were investigated, in which the cross-strand pair at positions 2 and 11 was varied and the effect on β-hairpin stability was determined (Figure 1). These sequences, which are modified from a β-hairpin developed by Gellman and co-workers, have a net charge of +2 to promote solubility and prevent aggregation.[5] The peptides also include an Asn-Gly turn which has been shown to promote hairpin formation via a type I′ turn.[6] The peptides were synthesized by standard FMOC solid-phase peptide synthesis and characterized by Maldi mass spectrometry and NMR. NOEs between each of the cross-strand pairs were observed, with the exception of the terminal Arg-Gln pair, consistent with β-hairpin formation.[7] Moreover, no NOEs were observed between the sites of mutation and diagonal residues, confirming that positions 2 and 11 provide an isolated site for study of cross-strand interactions.[1b]

Like other β-hairpins, these peptides interconvert between folded and unfolded conformations rapidly on the NMR time scale, such that the chemical shifts represent an average of folded and unfolded states. Both the downfield shifting of the β-sheet H_α resonances and the separation of the Gly H_α resonances have been shown to correlate with the extent of folding in β-hairpins.[2b,8] We quantified the fraction folded from both the H_α chemical shifts and the glycine splitting (Figure 2) as compared to fully folded and random coil control compounds **7**, **8**, and **9** (Figure 1) using eq 1.[1b,9] The thermodynamic profile was also determined as described by Searle to give ΔH°_{298}, ΔS°_{298}, and $\Delta C_p{}^\circ$.[2b,7]

$$\text{fraction folded} = [\delta_{obs} - \delta_0]/[\delta_{100} - \delta_0] \qquad (1)$$

Peptide **1** offers a good starting point as it is approximately 50% folded so that small changes in stability result in measurable perturbations in the fraction folded. We compared Phe to cyclohexylalanine (Cha) in peptides **1**−**4**. Phenyl and cyclohexyl groups have similar facial solvent accessible surface areas, but electronically they are quite different, allowing us to probe the contribution of the hydrophobic and electronic components to aromatic and aliphatic interactions.[10] We also studied the control peptides **5** and **6**, in which Phe or Cha is cross-strand from Ala, to determine the relative sheet propensities for Phe and Cha. Peptide **6** is about 7% more folded than peptide **5** at 283 K (25 and 18%, respectively), indicating that Cha has a larger β-sheet propensity than Phe.

Comparison of **1** and **4** indicates that they are of nearly equal stability (Table 1), which taken alone may suggest that the similar surface area of the two rings leads to similar stabilities or that they have equivalent β-sheet propensities. However, the lower stabilities of the mixed pairs in **2** and **3** are not consistent with simple burial of surface area as the main contributor to stability, since the surface area of residues X_1 and X_2 are similar for all four peptides. In fact, the greater stability of **1** over **2** and **3** indicates a preference for *self-association* among aromatics and indicates a significant interaction energy.[11]

(a) Ac-Arg-**X_1**-Val-Orn-Val-Asn-Gly-Lys-Glu-Ile-**X_2**-Gln-NH_2

1 X_1 = Phe, X_2 = Phe
2 X_1 = Phe, X_2 = Cha
3 X_1 = Cha, X_2 = Phe
4 X_1 = Cha, X_2 = Cha
5 X_1 = Phe, X_2 = Ala
6 X_1 = Cha, X_2 = Ala

(b) **7** Ac-Cys-Arg-Phe-Val-Orn-Val-Asn-Gly-Lys-Glu-Ile-Phe-Gln-Cys-NH_2

(c) **8** Ac-Arg-Phe-Val-Orn-Val-Asn-Gly-NH_2

9 Ac-Asn-Gly-Lys-Glu-Ile-Phe-Gln-NH_2

Figure 1. (a) β-Hairpin peptides studied; Cha = cyclohexylalanine. (b) Control peptide for the fully folded state with a disulfide bond between the two Cys residues. (c) Control peptides for the unfolded states.

Table 1. β-Hairpin Stabilities in Water at 283 K Determined from Glycine Chemical Shifts and H_α Chemical Shifts

peptide	X_1, X_2	$\Delta\delta$ Gly ppm	% folded (Gly)	% folded (H_α)[b]
1	Phe, Phe	0.289	52	53
2	Phe, Cha	0.204	37	34
3	Cha, Phe	0.243	44	43
4	Cha, Cha	0.295	53	52

[a] Variation in % folded from Gly and H_α provide a reasonable estimate of the error in these measurements. [b] Values are the average of Val3, Orn4, Val5, Lys8, Glu9, and Ile10.

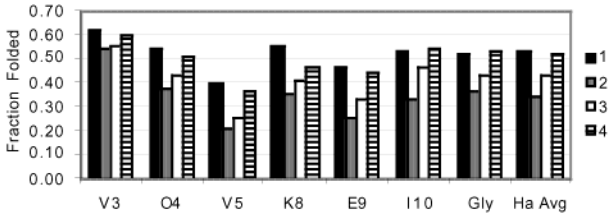

Figure 2. Fraction folded of peptides **1**−**4** as determined by individual H_α's, Gly, and the average Hα chemical shifts at 283 K.

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Table 2. Thermodynamic Parameters[a] for Folding at 298 K[13]

peptide	ΔH° (kcal/mol)	ΔS° (cal/mol K)	ΔC_p° (cal/mol K)
1	−4.4	−15.3	−87
2	−2.3	−9.1	−80
3	−3.2	−11.7	−97
4	−3.5	−11.9	−90

[a] Determined from the temperature dependence of the Gly chemical shift from 5 to 60 °C. Error is determined to be 6% from 95% confidence limits of the chemical shift data.

Thermal denaturation studies provide further insight into the preference for self-association in these cross-strand interactions (Table 2). The thermodynamic parameters, determined from fitting of the change in Gly and H_α chemical shift with temperature, suggest different properties for aliphatic and aromatic interactions. A comparison of **1** and **4** shows a greater enthalpic driving force for **1** as well as the greater entropic cost.[12] This is consistent with the fact that aromatics typically interact in a specific orientation (offset stacked or edge-face), which is expected to result in a higher entropic cost than for nonspecific hydrophobic packing. In addition, the greater enthalpic driving force in **1** suggests a greater contribution of van der Waals or electrostatic interactions or both, relative to hydrophobic interactions.

Evidence for a specific aromatic interaction in **1** was found in analyzing the aromatic region of the NMR spectrum. The ortho hydrogen of the Phe at position 11 of peptide **1** is shifted upfield by about 0.5 ppm at 283 K, relative to the control peptide **9**. There is no significant shifting of any of the peaks corresponding to Phe2 in this peptide, nor are there any upfield-shifted resonances in the other peptides. The shifting of a single hydrogen is consistent with an edge−face interaction;[14] if the rings were interacting in an offset stacked geometry, one hydrogen on each ring would be upfield-shifted. In addition to the upfield-shifting there are significant NOEs between the two ring systems in **1**, indicating a tight interaction. The fact that the rings interact in an edge−face geometry even though they are solvent-exposed implies that the interaction is not driven by the hydrophobic effect, which would favor the maximum burial of surface area. Edge−face interactions have been proposed to be driven by electronic or van der Waals interactions or both between the partial positive hydrogen on one ring and the π-cloud of the other ring.[4] This is consistent with the larger enthalpic term in peptide **1** relative to peptides **2**−**4**.

The thermodynamic cycle from peptides **1**−**4** gives a value for the preference for self-association of −0.55 kcal/mol.[15] This is similar in magnitude to that found in the cold shock protein CspA, in which Phe was mutated to Leu.[3c] The selectivity appears to originate from fundamental differences inherent in aromatic and aliphatic residues. The greater enthalpic and entropic properties of **1** over **4** in conjunction with the specific geometry of the Phe-Phe cross-strand pair suggest that the two aromatics form a specific interaction that is unique to aromatic moieties. This is in contrast to the findings of Russell and Cochran, which suggest that sheet propensities are more important than cross-strand interactions.[11]

These results suggest how Nature may use different hydrocarbon residues to obtain both stability and *specificity* in protein folding and may explain why aromatics often cluster in proteins.[3a,b] Moreover, the preferences for self-association should be useful in de novo protein design[3f−h] since aromatic interactions appear to provide the selectivity similar to that of a hydrogen bond while providing the stability of a hydrophobic interaction. Further investigation into the selectivity of aromatic interactions is in progress.

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Supporting Information Available: Synthesis, NMR analysis, and thermodynamic measurements of reported compounds (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(10) On the basis of log *P* values of cyclohexane (3.44) and benzene (2.13), cyclohexylalanine is expected to have a greater hydrophobic interaction than phenylalanine.

(11) Russell and Cochran have found a linear free-energy relationship between the residues in the cross-strand pair and the stability of the hairpin. Analysis of our data in this manner provides a negative slope, which indicates that there is a nonzero interaction energy between the two Phe residues in peptide **1**. See Russell, S. J.; Cochran, A. G. *J. Am. Chem. Soc.* **2000**, *122*, 12600−12601.

(12) Similar enthalpic and entropic differences between aliphatic and aromatic clusters have been observed in other hairpins, but direct comparison was difficult due to significant differences in the peptide sequences. See refs 2b and 2d.

(13) Values determined from H_α chemical shifts show the same trend as from the Gly splittings. See Supporting Information.

(14) For examples of crystal structures of cross-strand edge−face interactions between two Phe residues, see, for example: (a) Schindelin, H.; Jiang, W.; Inouye, M.; Heinemann, U. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 5119−5123. (b) Das, C.; Shankaramma, S. C.; Balaram, P. *Chem. Eur. J.* **2001**, *7*, 840−847.

(15) If one assumes no significant cross-strand interaction in peptides **2**−**4**, then this value represents the interaction energy for the Phe-Phe pair. We were unable to perform a thermodynamic cycle with Ala or Gly as the reference residue because the β-hairpins are too unstable.

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