The geometry and efficacy of cation– π interactions in a diagonal position of a designed β -hairpin

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Abstract

Cation– π interactions are common in proteins, but their contribution to the stability and specificity of protein structure has not been well established. In this study, we examined the impact of cation– π interactions in a diagonal position of a β -hairpin peptide through comparison of the interaction of Phe or Trp with Lys or Arg. The diagonal interactions ranged from -0.20 to -0.48 kcal/mole. Our experimental values for the diagonal cation– π interactions are similar to those found in α -helical studies. Upfield shifting of the Lys and Arg side chains indicates that the geometries of cation– π interactions adopted in the 12-residue β -hairpin are comparable to those found in protein structures. The Lys was found to interact through the polarized C ϵ , and the Arg is stacked against the aromatic ring of Phe or Trp. Folding of these peptides was found to be enthalpically favorable ($\Delta H^\circ \sim -3$ kcal/mole) and entropically unfavorable ($\Delta S^\circ \sim -8$ cal mole $^{-1}$ K $^{-1}$).

Keywords: Cation– π ; diagonal interaction; β -hairpin peptide; peptide secondary structure; stability

Supplemental material: See www.proteinscience.org

Protein folding to the single correct structure requires contributions from many noncovalent interactions. A typical protein is stable only by ~ 5 to 10 kcal/mole. Hence, small differences in energy between a myriad of possible noncovalent interactions are summed up to provide one unique folded structure. The relevance of cation– π interactions in biological structure have only been recognized in recent years. Cation– π interactions have been observed in a number of systems, including protein structure (Dougherty 1996; Gallivan and Dougherty 1999b), particularly the nicotinic-acetylcholine receptor (Dougherty and Stauffer 1990; Nowak et al. 1995), protein–DNA binding (Wintjens et al. 2000), ion channels (Kumpf and Dougherty 1993), catalysis (Gruber et al. 1999), and molecular recognition (Kearney et al. 1993; Forman et al. 1995). Understanding of the cation– π interaction comes principally from small molecule

cyclophane receptors, protein structures, and theoretical studies. Computational methods have implicated charge-quadrupole, charge-dipole, charge-induced dipole, charge transfer, dispersion forces, and hydrophobic packing as components of cation– π interactions (Duffy et al. 1993; Caldwell and Kollman 1995; Mecozzi et al. 1996; Cubero et al. 1998). This interaction has also been shown to stabilize α -helices (Andrew et al. 2002; Shi et al. 2002; Tsou et al. 2002). Studies in proteins and cyclophanes have defined geometries, distances, and strengths of interactions. Although widespread, the contribution of the cation– π interaction to protein stability and specificity has not been well established. Here, we describe the use of a β -hairpin peptide as a model system to probe the stability and geometry of cation– π interactions in a biologically relevant environment in the absence of tertiary interactions.

Many components of β -hairpin structure have been shown to impact stability, including cross-strand interactions (Smith and Regan 1995; Russell and Cochran 2000; Syud et al. 2001), turn propensity (Chung et al. 1998; Stanger and Gellman 1998; Fisk et al. 2000), β -sheet pro-

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pensity (Distefano et al. 2002), and strand length (Stanger et al. 2001). Recently, diagonal interactions have been implicated in β -hairpin stability (Syud et al. 2001). This initial study indicates that diagonal interactions may be as stabilizing as cross-strand interactions. Geometric descriptions of β -sheet structure derived from the Protein Data Bank (PDB) indicate that diagonal side chains are in close proximity. Each residue side chain is defined as a shell from the geometric mean of side-chain atoms from coordinates in the PDB. Similar side-chain separation is observed for diagonal residues in the i and $j - 2$ positions (3.0 Å) as for cross-strand residues (2.8 Å; Cootes et al. 1998), supporting the observation of diagonal interactions. In contrast, the side-chain separation for the i and $j + 2$ side chains is 4.5 Å, which is too great a distance for any natural side chain to span.

Our initial interest into diagonal cation- π interactions arose from an unexpected finding in a related system. The two β -hairpin peptides Ac-RFVEVNGOKI(Cha)Q-NH₂ and Ac-R(Cha)VEVNGOKIFQ-NH₂, where Cha is cyclohexylalanine, were investigated (C.D. Takto and M.L. Waters, unpubl.). Each peptide has the same 12 residues, although in different orientations, but the degree that they are folded differs, at 49% and 38%, respectively. As these peptides have the same 12 residues, the total β -sheet propensity of each peptide is identical, and all lateral interactions are preserved. Seemingly, the only difference is that one peptide has the potential for a diagonal cation- π interaction, whereas the other does not. The determination of the difference in stability of 11% or 0.3 kcal/mole led us to the current investigation.

The present study investigates the influence of cation- π interactions between two diagonal residues on β -hairpin stability. We have used double-mutant cycles to determine the interaction of residues in diagonal positions. We have also obtained information on the geometry of the interaction through probing side-chain chemical shifts. Our results indicate that cation- π interactions provide stabilization of the hairpin through a diagonal interaction. Moreover, the geometries observed for cation- π interactions in this minimalist system correspond to those observed in proteins. Thermal denaturation studies of these peptides indicate that folding is enthalpically favorable and entropically unfavorable, indicating a driving force other than the classical hydrophobic effect. Taken together, these results provide insight into the driving force and significance of cation- π interactions to protein structure and stability.

Results

β -Hairpin design

The general sequence design was based on a previously reported β -hairpin from the Gellman Laboratory (Syud et al.

2001), in which X₁ is an aromatic residue (Phe or Trp) and X₂ is a cationic residue (Fig. 1). The overall charge of the peptides was +2 to increase solubility and prevent aggregation. Concentration studies indicate that these peptides are monomeric under the conditions used in this study. An Asn-Gly turn was incorporated as it has been shown to promote a type I' turn (Griffiths-Jones et al. 1998). Similar peptides have been shown to be ~50% folded, allowing for significant (de)stabilization to easily be observed (Tatko and Waters 2002).

The geometry of a β -hairpin creates two faces from the alternating direction of the side chains. The nonhydrogen-bonded (NHB) face contains the diagonal interaction and is composed of four residues involving positions 2, 4, 9, and 11. Positions 2 and 9 were mutated, whereas positions 4 and 11 were held constant. Leucine was selected for position 11, and glutamic acid was selected for position 4 to maximize the stability of the β -hairpin.

The cation- π interactions between Phe or Trp and Lys or Arg were investigated. Tyr was not included as it has been shown to behave similarly to Phe, and the additional hy-

Figure 1. (A) The model peptide system (Ac-RX₁VEVNGOX₂ILQ-NH₂) used to probe the cation- π diagonal interaction. Peptides are referred to by the diagonal residues in the text (X₁X₂). HB-site indicates hydrogen-bonded site; NHB-site, nonhydrogen-bonded site. (B) The disulfide-linked cyclic control peptides. Cyclic peptides are referred to as CX₁X₂C in the text.

droxyl may complicate the interpretation. His is capable of interacting as either a cation or π -system, so it was also omitted in this study.

To characterize the structure of the 12-residue peptides by NMR, we compared them to the seven-residue peptides representing the N- and C-terminal β -strands and the disulfide-bonded 14-residue cyclic peptides (Fig. 1). The seven-residue peptides are models for the random coil state, and the cyclic peptides are models for the fully folded state. Disulfide-linked cyclic peptides are commonly used to represent the fully folded state of a β -hairpin peptide. Nonetheless, in each case, this assumption must be confirmed. We have done this by analyzing the downfield shifting of the $H\alpha$ protons and the cross-strand nuclear Overhauser effect crosspeaks (NOEs) of the cyclic peptides. Residues in a β -sheet display downfield shifting of their $H\alpha$ protons, whereas residues in the turn display upfield shifting. Downfield shifting of ≥ 0.1 ppm is typically taken to indicate significant β -sheet population. The $H\alpha$ shifts of the cyclic peptides relative to random coil chemical shifts indicate that the peptide is in a β -hairpin conformation (see Supplemental Material). Cross-strand NOEs also support a β -hairpin conformation (*vide infra*).

Characterization of β -hairpin structure and side-chain-side-chain interactions

NOE assignment

To determine the structure of the investigated peptides, NOE assignments were made. There are numerous nonadjacent NOEs in peptides FK, FR, WK, and WR that are consistent with a β -hairpin formation (see Supplemental Material). These NOE interactions were found throughout the length of the strands between residues that are up to 12 residues apart linearly. The long-distance NOEs observed are uniquely related to β -hairpin structure.

The NOEs also provide evidence for a diagonal interaction between residues 2 and 9. Diagonal interactions have only recently been noted for their contribution to β -hairpin stability but have been observed in a number of peptides (Espinosa and Gellman 2000; Syud et al. 2001; Espinosa et al. 2002). There are many NOEs among the NHB face of peptides FK, FR, WK, and WR, indicating a bundle of residues reminiscent of the hydrophobic cluster in the 41- to 56-amino-acid region of Protein G (Blanco et al. 1994; Blanco and Serrano 1995). To account for any disparities in NOEs arising from different hairpin populations, we compared the side-chain-side-chain NOEs of the cyclic control peptides CWRC and CWKC. Figure 2 shows selected NOEs from the cyclic WR and WK peptides. For cyclic WR, there are numerous diagonal NOEs between the Trp and Arg with each methylene, $H\beta$, $H\gamma$, and $H\delta$, having a resonance to the Trp. The diagonal NOEs in cyclic WK

involve the δ -methylene and ϵ -methylene of the Lys side chain, near the polarizing amine.

$H\alpha$ chemical shifts

β -Sheet conformation induces systematic shifts of a number of resonances of the peptide. These changes offer information to confirm β -hairpin structure and can be quantified to assess the degree of folding. In a β -sheet conformation, the $H\alpha$ resonances are downfield-shifted relative to a random coil. This arises from increased cross-strand hydrogen bonding, which deshields the $H\alpha$. Multiple adjacent shifts of >0.1 ppm relative to random coil values are typically taken to indicate β -sheet structure (Wishart et al. 1991, 1992). The investigated peptides demonstrate quite dramatic deviations from random coil chemical shifts. Shifts of up to 0.6 ppm from random coil are observed, and as many as four adjacent residues were shifted >0.1 ppm, with fraying at the N and C termini. This type of fraying is typically observed for the terminal residues in a β -hairpin. Figure 3 shows the shift of these representative peptides, indicating that both FK and WK take on a β -hairpin structure, but WK is clearly more structured than FK. The control peptide, VK, is also shown for reference.

Amide chemical shifts

Amide hydrogens in hydrogen-bonding sites of a β -hairpin experience significant downfield shifting in the NMR spectrum, whereas the NH resonances of NHB sites are not significantly shifted. The resulting pattern of amide shifts indicates the geometry of the β -hairpin. The most significantly shifted amides in the peptides studied are positions Val3, Val5, and Ile10 (Fig. 4). The hydrogen-bonded amides are shifted up to 0.9 ppm, whereas the NHB amides are shifted <0.2 ppm, relative to random coil, with the exception of Orn8. Although Orn8 is in a hydrogen-bonded position, it is upfield-shifted. This is likely due to its proximity to the turn, which influences its geometry. Thus, the observed amide shifts match the expected hydrogen-bonding pattern for a β -hairpin peptide.

Turn residue amide couplings

The incorporation of an Asn-Gly turn typically promotes the formation of a type I' turn. We investigated the J_{NH} amide couplings to characterize the turn geometry in the peptides studied here. Type I' turns have J_{NH} couplings of 7 and 5 Hz for the $i + 1$ and $i + 2$ positions. The investigated peptide series has amide couplings of 7.0 ± 0.1 Hz and 5.0 ± 0.7 Hz. No other turn geometry has a similar coupling pattern, providing good evidence for the formation of a type I' turn.

Magnitude of the diagonal interaction

The stabilization of β -hairpin secondary structures occurs through the interplay of many weak noncovalent interac-

Figure 2. The NOEs observed in the cyclic Trp series. (A, C) The lateral NOEs between positions 2 and 4 or positions 9 and 11. (B, D) The diagonal and $i-i+2$ NOEs. All others are excluded for clarity.

tions. Double-mutant cycles have been used to measure noncovalent interactions in proteins (Horovitz and Fersht 1990; Serrano et al. 1991; Schreiber and Fersht 1995), peptides (Blanco and Serrano 1995; Sharman and Searle 1998; Searle et al. 1999; Shi et al. 2002), and organic intermolecular systems (Carver et al. 2001, 2002). Double-mutant cycles are capable of identifying interaction energies <0.5 kcal/mole, which makes the method well suited to determine the magnitude of the diagonal interactions in this

study. In a double-mutant cycle, the two interacting residues are mutated to noninteracting residues (Fig. 5). A single mutation to give B or C disrupts the diagonal interaction of interest but may cause other changes to stability, such as a change in the overall sheet propensity. The double mutant, D, corrects for all changes other than the noncovalent interaction being investigated. Hence, the energy of the isolated diagonal interaction can be determined from the equation in Figure 5.

We have incorporated Ser and Val as the control residues for the double-mutant cycle. Ser is substituted in position 9 for the cationic residues because the small polar side chain of Ser has been shown not to interact diagonally (Carver et al. 2001; Syud et al. 2001; Espinosa et al. 2002). Val was selected to replace the aromatic residues in position 2 because hydrophobic β -branched residues stabilize the hairpin, but it does not have aromatic character. Ser and Val have been shown, from protein β -sheet propensity scales, to have a high β -sheet propensity that minimizes net loss of β -hairpin stability, so the side-chain interactions should account for the majority of the stability lost (Minor and Kim 1994; Smith et al. 1994). β -Sheet propensity scales from peptides indicate that Ser has a low β -sheet propensity. From the peptide variants investigated, the order of sheet

Figure 3. The difference in β -hairpin and random coil shifts. Asn is shifted upfield, as is expected for the turn residue. The Gly H α is the difference in chemical shift of the diastereotopic H α s. Residues Arg1 and Gln12 are frayed and show little shifting relative to the random coil values.

Figure 4. The downfield chemical shift of the amide resonances relative to random coil. Positions Val3, Val5, Orn8, and Ile10 are cross-strand hydrogen-bonded. The upfield shift of the Orn8 is caused by the turn geometry.

propensities for the residues in this study are Trp > Phe > Val and Arg > Lys > Ser, which agrees with reported scales in β -hairpin peptides (Russell and Cochran 2000).

Table 1 shows that the stability of the peptides investigated ranges from 29% to 84% folded, indicating that the model system was adept at measuring perturbations in stability. The largest increase in stability gained from a single mutation relative to VS was WS, giving 0.76 kcal/mole.

From the double-mutant cycle, these peptides were found to have modest diagonal interaction energies, ranging from -0.2 to -0.5 kcal/mole (Table 2). Nonetheless, some general trends are observed. The Trp...Arg interaction provides the greatest stability. For both Trp and Phe, the diagonal interaction is more significant with Arg than with Lys. This supports findings from statistical analyses of protein structures and may be attributed to the increased van der Waals interactions due to the stacked geometry in WR and FR that is not possible in WK and FK (vide infra). Trp was found to form stronger diagonal interactions with both Lys and Arg

Figure 5. The double-mutant cycle varies X and Z individually and jointly. The diagonal interaction between X and Z can be determined by subtracting the stability of B and C from A and D.

than does Phe, in qualitative agreement with the findings of Gallivan and Dougherty (1999).

Geometries of the diagonal interactions

The chemical shift perturbation of residue 9 arising from its proximity to an aromatic residue at position 2 provides information regarding the geometry and prevalence of the diagonal interaction. Thus, we compared the upfield shifting of Lys and Arg at position 9 when paired with Phe or Trp in position 2. To account for any differences in the folded population, we also investigated the upfield shifting of residue 9 in the cyclic peptides, CWKC and CWRC.

Table 1. The stabilities of all investigated peptides

	$\Delta\delta$ (ppm) ^a	Fraction folded ^b	ΔG^c
FK	0.230	0.51	-0.03
FR	0.274	0.61	-0.27
WK	0.346	0.77	-0.72
WR	0.378	0.84	-1.00
VK	0.152	0.35	0.36
VR	0.177	0.41	0.21
FS	0.161	0.38	0.29
WS	0.253	0.60	-0.23
VS	0.124	0.29	0.53

^a $\Delta\delta$ is the glycine H α chemical shift difference at 298 K. Error is ± 0.005 ppm.

^b Fraction folded is determined from the Gly H α chemical shift difference (see Materials and Methods).

^c The ΔG reflects the stabilities of the β -hairpins and not the diagonal interaction.

Based on the chemical shift perturbation of the Lys side chain in FK, WK, and CWKC relative to random coil, the most prominent site of interaction of Phe or Trp with Lys is between C ϵ and the aromatic ring. The ϵ -methylene is shifted by 0.28 ppm for FK and 0.39 ppm for WK. The δ -CH₂ is also in close proximity to the aromatic ring, as indicated by shifting of 0.27 and 0.30 in FK and WK, respectively. In contrast, the ammonium group is shifted by only 0.1 and 0.24 ppm, respectively, indicating that interaction with the alkyl side chain is favored over the ammonium group. The cyclic peptide demonstrates the same trends as WK. The interaction of the ϵ -CH₂ group of lysine has also been observed in proteins and in isolated α -helices, and appears to be the favored geometry for cation- π interactions with Lys.

Figure 7 shows the possible geometries for the Aryl...Arg interaction (Gallivan and Dougherty 1999b). The stacked conformation is stabilized through the cation- π as well as additional nonelectrostatic effects such as van der Waals and π - π interactions. The splayed conformation is similar to the stacked conformation with the C δ interacting with the

Table 2. The diagonal interaction energies as determined from double-mutant cycles

Peptide	$\Delta\Delta G^a$
FK	-0.20
FR	-0.29
WK	-0.35
WR	-0.48

^a The error in the diagonal interaction energies is ± 0.05 kcal/mole.

aromatic ring, but with the guanidinium region weakly associated. The T-shaped geometry points one of the NH groups at the centroid of the ring. The upfield shifting of the Arg side chain with Phe or Trp provides information about the preferred geometry in this system (Fig. 6C). In peptide FR, the C δ is the most shifted methylene. The NH groups of the guanidinium are also shifted by 0.17 ppm, indicating some stacked or splayed character to the cation- π interaction. In WR, the guanidinium NH resonances are shifted more than the C δ : The C δ is shielded by 0.39 ppm, and the amines are shifted by 0.41 and 0.47 ppm. The shifting of both the C δ and NH groups is a strong indicator that the Trp...Arg interaction is in a stacked orientation.

NMR thermal denaturation was performed to determine the energetic contributions to folding (Fig. 8). Van't Hoff analysis of the resulting curves was accomplished with a nonlinear fitting (Maynard et al. 1998). This provides a measure of the enthalpic and entropic parameters of folding. Peptides containing Trp were analyzed in this way because of their higher extent of folding. Folding of both WR and WK was found to be enthalpically favorable and entropically unfavorable, indicating a driving force other than the classical hydrophobic effect (Table 3). The magnitude of the thermodynamic parameters is similar to that observed in peptides with hydrophobic clusters on the NHB face of the β -hairpin (Espinosa and Gellman 2000; Tatko and Waters 2002). To investigate the contribution of the salt bridge, we also performed the thermal denaturations at pH 1.5. Under these conditions, we observed no substantial difference relative to pH 4.2.

Discussion

We have exploited the NHB cluster on the face of a β -hairpin to investigate the nature of a diagonal interaction between an aromatic and cationic residue. The strength of noncovalent interactions involving Phe or Trp with Lys or Arg were assessed through double-mutant cycles. The diagonal interactions observed ranged from -0.20 to -0.48 kcal/mole, which are similar in magnitude to cross-strand interactions (Smith and Regan 1995; Merkel et al. 1999) and the stability gained from β -sheet propensities (Minor and Kim 1994; Smith et al. 1994).

The magnitude of the cation- π interaction in this system is significantly lower than that predicted by theory (Gallivan and Dougherty 2000), but the observed order of stabilities correlates well with that determined for proteins (Gallivan and Dougherty 1999a, 2000). In this system, we have found that Trp is the preferred aromatic group and Arg is the preferred cation for formation of a cation- π interaction, in agreement to the preferences found in proteins. The magnitudes of the interactions reported here are similar to those measured in α -helical model systems (Andrew et al. 2001; Olson et al. 2001; Shi et al. 2002; Tsou et al. 2002). These investigations found that both Orn-Phe and Arg-Trp interactions in the $i, i + 4$ positions of an α -helix yielded a stabilizing interaction (Andrew et al. 2002; Shi et al. 2002; Tsou et al. 2002). The magnitude of the cation- π interactions in α -helices varied but did not exceed -0.4 kcal/mole, compared with a range of -0.2 and -0.5 kcal/mole observed here.

Based on upfield shifting of the cationic side chain and NOE resonances, the favored geometries for FK, WK, and cyclic WK were found to direct the ϵ -methylene, and to a lesser extent the δ -methylene, of Lys at the aromatic ring. This orientation allows the protonated amine to remain solvated by water while packing the ϵ -methylene against the

Figure 6. The upfield shifting of the diagonal side-chains in position 9 relative to random coil values. (A) Atom labels for Lys, Arg, and Nle. (B) Lys side-chain resonances in FK, WK, and CWKC. (C) Arg side-chain resonances in FR, WR, and CWRC.

Figure 7. The stacked, splayed, and T-shaped geometries of the Phe-Arg interaction.

aromatic ring. Analysis of the PDB has shown that cation- π interactions with Lys typically involve the ϵ -methylene ($C\epsilon$). As many as 60% of all Lys residues in proteins interact through $C\epsilon$, as we have seen in our model system (Gallivan and Dougherty 1999b). The interaction of Lys via $C\epsilon$ likely reflects some combination of hydrophobic and electrostatic forces, because the methylene is highly polarized by the neighboring ammonium group yet poorly solvated by water (Reetz 1988). It is notable that NMR chemical shifts in the α -helical studies indicate an interaction with the Orn δ -methylene and Lys ϵ -methylene, indicating a similar type of geometry as observed here (Tsou et al. 2002).

In cation- π interactions with Arg, we have found that the guanidinium group orients itself parallel to the Trp ring, as evidenced by the side-chain chemical shifts. The FR peptide is less definitive, as the $C\delta$ is more shielded than are the amines, but the shifting of the amines implicates at least some parallel geometry. The stacking of Arg with Trp is also in accord with findings in protein structures. In the stacked geometry, the delocalized π system in the guanidinium group allows for favorable van der Waals contacts with the π system of the aromatic side chain as well as electrostatic interactions. In addition, Arg is less well solvated than is Lys so the desolvation penalty for interaction with the aromatic ring is reduced. Although the cation- π interaction in "T" geometry has been proposed to be stronger than in the parallel geometry, the nonelectrostatic contacts seem to favor the parallel orientation, as has been observed in proteins (Gallivan and Dougherty 1999b).

With regard to the aromatic residues, we have found that Trp interacts with a cation more favorably than does Phe,

although the observed differences in this system are modest. This trend is also seen in proteins, with a remarkable 24% of all Trp residues interacting with cationic residues in proteins, according to a study of the PDB (Gallivan and Dougherty 1999a).

Thermal denaturation studies have shown that folding of these peptides is enthalpically favorable and entropically unfavorable. Although there are still only a few β -hairpins for which thermodynamic parameters have been reported, most that are well folded also demonstrate enthalpically driven folding. Typically, this is associated with an aromatic (Espinosa and Gellman 2000; Cochran et al. 2001; Tatko and Waters 2002) or hydrophobic (Tatko and Waters 2002) cluster on the NHB face of the β -hairpin. Interestingly, those peptides with hydrophilic residues on the NHB face of the hairpin are the ones that demonstrate cold denaturation and a classical hydrophobic driving force (Ciani et al. 2003; Kiehna and Waters 2003). This may be a result of hydrophobic interactions on the HB face of these hairpins. Espinosa and Gellman (2000) has proposed that the enthalpic driving force in a peptide with an aromatic cluster may be the result of a "tight" interaction, as defined by Diederich et al. (Smithrud and Diederich 1990; Smithrud et al. 1991). In the system described here, a "tight" interaction may result from the contribution of an electrostatic component between the aromatic residue and the cationic residue, which may provide specificity to the interaction. Further studies are underway to explore this possibility.

Conclusions

In our study of cation- π interactions in a minimalist β -hairpin system, we have found the same geometric preferences for these interactions as have been observed in protein structures. Specifically, Lys prefers to interact via the ϵ -CH₂ group, and Arg prefers to stack with the aromatic ring. The energetic trends observed here agree with the predicted trends, although the magnitude of the interactions was significantly lower than predicted. Nonetheless, the interaction energies are in good agreement with those found in model α -helical systems. Trp was found to be the preferred aromatic residue, and Arg was the preferred cationic residue. Thermal denaturation studies indicate that folding of these peptides is enthalpically driven, similar to other β -hairpins containing aromatic or hydrophobic clusters.

Materials and methods

Materials

N-9-Fluorenylmethyloxycarbonyl amino acids and coupling reagents, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and *N*-hydroxybenzotriazole H₂O (HOBt), were obtained from Anaspec. PEG-PAL amide resin was

Table 3. Thermodynamic parameters from the thermal denaturation at pH 4.2

Peptide	ΔH°	ΔS°	ΔC_p°
WR	-3520 (4)	-8.4 (0.1)	-181 (5)
WK	-3080 (30)	-7.9 (0.1)	-145 (4)

Units are as follows: ΔH° , cal/mole; ΔS° , cal mole⁻¹ K⁻¹; and ΔC_p° , cal mole⁻¹ K⁻¹. All parameters are determined from the fitting to the van't Hoff equation as shown in Figure 8. The error is determined from the fitting.

Figure 8. Thermal denaturation of WR and WK at pH 4.2. The fraction folded is determined from reference compounds. The fitting of the curve was accomplished by using the equation: fraction folded = $(\exp[x/RT]) / (1 + \exp[x/RT])$, where $x = \{T(\Delta S^\circ_{298} + \Delta C_p^\circ \ln[T/298]) - (\Delta H^\circ_{298} + \Delta C_p^\circ [T - 298])\}$.

obtained from Applied Biosystems. N,N-dimethylformamide (DMF) was obtained from Fisher Scientific. Acetic anhydride, 2,6-lutidine, trifluoroacetic acid, and triisopropyl silane were obtained from ACROS. Water used for the high-performance liquid chromatography (HPLC) was from a Mili Q A10 purifier. HPLC grade acetonitrile was obtained from Fischer Scientific. The deuterated water for the NMR was obtained from Cambridge Isotope Laboratories, and the deuterated acetic acid was obtained from ACROS. 3-(Trimethylsilyl)-1-propanesulfonic acid sodium salt (DSS) was obtained from Fisher Scientific.

Peptide synthesis and purification

The synthesis of all peptides was performed on an Applied Biosystems Pioneer peptide synthesizer using PEG-PAL amide resin. Peptides were synthesized on 0.5-mmol scale with 60-min coupling times. All amino acids with functionality were protected during synthesis as follows: Arg(Pbf), Asn(trt), Lys(Boc), Orn(Boc), Gln(trt), and Glu(tBu). Coupling reagents were HBTU and HOBt in DMF. The C terminus was acylated for all peptides with a solution of 5% acetic anhydride and 6% 2,6-lutidine in DMF. Cleavage conditions removed all side-chain protection with a cocktail of 90% TFA/5% triisopropylsilane/5% H₂O. Peptides were purified by semipreparative RP-HPLC on a Vydac C₁₈ silica column (218TP510) at a flow rate of 5 mL/min. Peptide elution was monitored at 214 and 280 nm. Peptides were purified with a gradient of A and B (A, 95% H₂O/5% ACN with 0.1% TFA; B, 95% ACN/5% H₂O with 0.1% TFA) and typically eluted between 18% to 25% B. Once purified, peptides were lyophilized to powder and characterized by matrix-assisted laser desorption/ionization (MALDI) mass spectroscopy and NMR.

Concentration study

A concentration study was performed to determine if aggregates formed in the concentration range used for the NMR spectroscopy. A concentration range from 12 to 0.12 mM was investigated. The maximum observed change in chemical shift from 12 to 0.12 mM

was 0.006 ppm. Most of the change in chemical shift occurred between 12 and 6 mM. There was no observed change in chemical shift between 1 and 0.12 mM. The concentration of the NMR samples was ~1 mM, within the region where little or no chemical shift change is observed, indicating that the peptide is monomeric at the concentrations studied.

NMR spectroscopy

NMR samples were made to concentrations of 1 to 4 mM and analyzed on a Varian Inova 600-MHz instrument. Samples were dissolved in either 1 H₂O/1 D₂O or D₂O buffered to pH 3.9 (uncorrected) with 50 mM d₃-NaOAc (pH adjusted with HCl). One-dimensional NMR spectra were collected by using 32-K data points and between 8 and 128 scans using a 1- to 3-sec presaturation or solvent suppression. All two-dimensional NMR experiments used pulse sequences from the chempack software, including total correlation spectroscopy (TOCSY), double quantum filter correlation spectroscopy (DQCOASY), gradient correlation spectroscopy (gCOASY), two-dimensional nuclear Overhauser spectroscopy (NOESY), and rotating frame NOESY (ROESY). Two-dimensional NMR scans were taken with eight to 64 scans in the first dimension and 128 to 512 in the second dimension. All spectra were analyzed by using standard window functions (sinbell and gaussian with shifting). Presaturation is used to suppress the water resonance. Mixing times of 100 or 200 msec were used for the NOESY and ROESY spectra. TOCSY spectra were recorded with 80-msec spin-lock. Assignments were made by using standard methods as described by Wuthrich (1986). The temperature was calibrated by using MeOH and ethylene glycol standards.

Peptide stability

The stability of the peptides was determined by comparison of the NMR chemical shifts α -protons (Syud et al. 1999; Searle 2001; Sharman et al. 2001) and the diastereotopic glycine protons (Griffiths-Jones et al. 1999; Searle et al. 1999) of the peptide of interest relative to those of reference compounds representing the random coil and fully folded states by using equation 1. The seven-residue peptides representing the N- and C-terminal fragments of the parent peptides were used for the random coil reference state. A cyclic disulfide-linked dimer was used to represent the fully folded state. Characterization of these cyclic peptides by ROESY NMR indicates that they maintain a well-folded β -hairpin structure, confirming that they are reasonable models for the fully folded state.

$$\text{Fraction Folded} = \frac{(\delta_{\text{observed}} - \delta_{\text{random coil}})}{(\delta_{\text{fully folded}} - \delta_{\text{random coil}})} \quad (1)$$

In determining the fraction folded of the peptide, there is typically good agreement between the fraction folded determined by the H α resonances and the Gly chemical shift for peptides with Asn-Gly turns (Griffiths-Jones et al. 1999; Tatko and Waters 2002). With the mutation pattern of the present systems, discrepancies arise due to anisotropy and electrostatic differences of the mutated residues. Previous investigations have indicated that the α -hydrogens in H-bonded sites of the β -hairpin provide the best assessment of the fraction folded because those sites are less flexible than are the NHB sites. However, in the system studied here, the α -hydrogens in HB sites are perturbed to different extents by the proximity of the aromatic residues, resulting in inconsistent determination of the

fraction folded from these residues. In contrast, the glycine H α , H α' chemical shift difference is not perturbed by the aromatic substitutions. None of the adjacent residues are varied and because Gly occupies a turn position, ring current effects from the aromatic residues do not influence it. Hence, the glycine chemical shift difference was the primary method used to determine the extent of folding, but the agreement between H α and glycine was found to be good when not affected by extraneous factors.

The cross-strand hydrogen bonding also shifts the amides of the hydrogen-bonding site residues. Consequently, positions 3, 5, 8, and 10 were used to assess the fraction folded. Although there are small variations, the agreement between the glycine chemical shift and the amides is $\pm 3\%$ to 4% , which is within error. The extent of folding as determined by the amides are consistently less stable than based on glycine and may reflect the position of the glycine in the turn versus the amides in the strands, but the fraction folded trends are identical and the differences are within experimental error. As the glycine is distant from any side-chain influences, it was used as the prime determinant of stability.

Electronic supplemental material

The complete proton assignments and NOE data of all peptides used in this investigation are included.

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