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Design of Highly Stabilized β -Hairpin Peptides through Cation- π interactions of Lysine and N-Methyl Lysine with an Aromatic Pocket[‡]

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Abstract

Two tryptophan residues were incorporated on one face of a β -hairpin peptide to form an aromatic pocket that interacts with a lysine or N-methylated lysine via cation- π interactions. The two tryptophan residues were found to pack against the lysine side chain forming an aromatic pocket similar to those observed in trimethylated lysine receptor proteins. Thermal analysis of methylated lysine variant hairpin peptides revealed an increase in thermal stability as the degree of methylation was increased resulting in the most thermally stable β -hairpin reported to date.

Cation- π interactions play a vital role in biomolecular recognition. Interactions between basic and aromatic sidechains have been shown to contribute to folding in natural and de novo designed proteins as well as small structured peptides.^{1–6} Interactions between small molecules and proteins have also been shown to be mediated by cation- π interactions.⁷ More recently, protein-protein interactions that control gene expression have been shown to be dictated by a cation- π interaction between trimethyllysine (KMe3, where methylation is at the ϵ -amino group) and an aromatic pocket.^{8–11} Incorporation of methylated lysine residues at specific locations of the histone tails results in recruitment of various chromatin remodeling proteins which regulate chromatin condensation and gene expression.^{8,9,10} Recent crystallographic data has shown that proteins involved in chromatin remodeling, including chromodomains, PHD domains, and Tudor domains, recognize and bind trimethylated lysine of histone tails with an aromatic cage made up of three or four aromatic rings.^{9,10} The stabilizing forces between the methylated lysine and its aromatic binding pocket are driven by cation- π and van der waals interactions (Figure 1).^{9–11} In each case, two of the aromatic residues reside within a beta-sheet, forming a cleft.

Previous work in model β -hairpin systems has shown that lysine and N-methylated lysine packs against a tryptophan positioned diagonally cross strand to form a cation- π interaction (Figure 1c), simulating the interaction between KMe3 and one of the aromatic residues in the beta-sheet of the HP1 chromodomain or the BPTF PHD domain.^{1,2,3} Methylation of lysine in this hairpin greatly increases the hairpin stability due to an enhanced interaction with Trp.² Moreover, comparison of KMe3 to its neutral analog in which nitrogen is replaced by carbon results in loss of the interaction with Trp in the peptide model system as well as loss of the binding of the modified histone tail to the aromatic pocket in the HP1 chromodomain.¹¹ To

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Supporting Information Available. NOEs common to all peptides, CD of Trp Pocket peptides, thermal denaturation of Trp KMe3 pocket, double mutant cycles, chemical shift assignments of all peptides. This data can be obtained free of charge via the intranet at <http://pubs.acs.org>.

further investigate the interaction between methylated lysine and aromatic binding pockets we report the design of β -hairpin that contains an aromatic pocket similar to one observed in chromatin remodeling proteins (Figure 1a and b) that results in a highly stable β -hairpin containing a lysine positioned to interact with an aromatic pocket.

Results

Peptide Design

The Trp Pocket series of peptides were synthesized to investigate the effects of placing lysine and its varying methylation states cross-strand from a ditryptophan cleft on a β -hairpin. The sequence Ac-RWVWVNGOKMe_nILQ-NH₂, where $n = 0 - 3$, was used to create an aromatic pocket on the non-hydrogen bonded (NHB) face of the peptide with which KMe_n can interact (Figure 2). The NHB face consists of sidechains on all residues in the NHB positions of the beta-strand, which alternate with hydrogen bonding (HB) positions (Figure 2). This sequence was adapted from previously designed β -hairpins used to study cation- π interactions between lysine and tryptophan.^{1,2} This design assumes an interdigitated arrangement of sidechains 2, 4, 9, and 11 resulting from the twist in the β -sheet as has been shown in related peptide sequences.^{3,12} Thus residue 9 is oriented to pack between the two tryptophan side chains at positions 2 and 4, whereas residue 11 packs against the outer face of Trp2. Residues Asn6 and Gly7 were included to facilitate a type I' turn which have been shown to be favorable in β -hairpin formation.^{13,14,15} Cyclic peptides were synthesized as fully folded controls for each of the Trp Pocket series β -hairpins (Figure 2b). Cyclization was achieved by a disulfide bond between cysteine residues at the N and C-termini of the peptides. Unfolded control peptides consisting of either the N-terminal arm (residues 1-7) or the C-terminal arm (residues 6-12) were used to obtain random coil chemical shifts (Figure 2c).

Structure Determination and Characterization

NMR was used to probe the interaction between the ditryptophan pocket and lysine in its various methylated forms. Downfield shifting of ≥ 0.1 ppm of the α hydrogen protons (H_α) along the peptide backbone relative to unfolded values indicates a β -sheet conformation.¹⁶ All of the Trp Pocket series exhibited highly folded β -hairpin structure (Figure 3). Residues KMe_n 9 and Leu 11 exhibit H_α upfield shifting relative to the unfolded control due to the electron shielding caused by their proximity to the aromatic indole rings of the tryptophans directly cross strand from these residues. The Asn 7 H_α is shifted upfield because this residue adopts a turn conformation in the β -hairpin. Minimal shifting of Gln12 H_α is due to fraying of the terminus.

The extent of folding to a β -hairpin by the Trp Pocket series of peptides was quantified using two methods. The first method utilizes the extent of H_α downfield shifting of residues in the HB sites relative to random coil controls and fully folded control as previously described (See Experimental Section and Supporting Information).¹⁷ The second method utilizes the extent of the distereotopic glycine H_α splitting located in the turn of the hairpin relative to glycine H_α splitting observed in the fully folded control.¹⁸ Both these methods showed that all of the peptides in the Trp Pocket series are all $\geq 90\%$ folded. However, due to error associated with a small change in chemical shift at this range in folding, it is difficult to determine which of the series is the most well folded (Table 1).

Direct interaction between the lysine sidechain and tryptophan pocket was also determined by NMR. The chemical shift of the side chain protons are affected by the surrounding environment, in particular the ring current effects arising from nearby aromatic rings. The degree of upfield shifting of the lysine side chain protons in this peptide series relative to the unfolded control is indicative of interaction of the lysine with the tryptophan pocket (Figure 4). All of the hairpins

show extensive upfield shifting indicating that the lysine residues are in close proximity to the face of the indole rings. The γ , δ and ϵ methylene groups of the lysine are shifted the greatest extent. An increase in upfield shifting upon methylation of the lysine is observed, indicating that the methyl groups are providing additional favorable interactions with the Trp pocket. This has been observed in other reported β -hairpins containing a single Trp residue.^{2,3} However in the Trp Pocket peptides the upfield shifting is more dramatic with the additional tryptophan sidechain.

NOEs were obtained for the Trp Pocket series to confirm that these β -hairpins are forming in the correct register and to investigate how the lysine and its methylated analogs interact with the two tryptophans on the opposite strand. NOE's were observed between the H_α of Trp2 and Leu11, and between the H_α of Trp4 and Lys9 for all of the peptides in the Trp Pocket series indicating that a β -hairpin is forming in the correct register. More NOE crosspeaks between cross-strand residues are observed along the entire strand as the number of methyl groups on Lys increases, indicating an increase in folding upon methylation (Figure 5; See Supporting information). Interestingly, two unique methyl groups are observed for dimethyllysine in Trp KMe2 (Figure 4). NOEs indicate that they are oriented towards specific tryptophan indole rings suggesting that KMe2 has a specific orientation within the tryptophan pocket (Figure 5c).

Nature of the Trp pocket

Comparison of the H_α chemical shifts and NOE data for the Trp pocket series of peptides and their cyclic controls suggests that changing the methylation state of the peptide results in subtle changes in the entire peptide structure and in particular the aromatic pocket. Comparison of The H_α chemical shifts in the Trp pocket series to the cyclic controls indicates that some of the variation in chemical shifts is due to the methylation state of Lys rather than the extent of folding (Figures 4a and 4b). For example, same trend in chemical shifts is observed from Trp K pocket to Trp KMe3 pocket for residues Orn 8 through Leu 11 in the cyclic and acyclic peptides. This is significant because the C-terminal strand is the one that interacts with the Trp pocket on the N-terminal strand.

Inspection of the KMe_n — Trp NOEs in Figure 5 indicates that methylation results in an increase in the NOEs between H2 of Trp2 and the KMe_n sidechain, as well as an overall increase in NOEs between the KMe_n sidechain and Trp4. This also suggests a subtle change in the Trp pocket upon methylation.

Comparison of the CD spectra for the four peptides also suggests a change in the β -hairpin structure upon methylation. As shown in Figure 6, the minimum at 215 nm is consistent with β -sheet structure, but is less intense for the peptides containing a methylated Lys sidechain than for the parent Trp K pocket. This may arise from distortion of the β — hairpin structure to optimize the Trp- KMe_n interaction.

Thermal Denaturation Studies

To determine the thermodynamic parameters for folding of the Trp Pocket series of peptides, thermal denaturations were performed by following the change in Gly splitting with temperature (Figure 7a). For comparison, the thermal denaturation was also followed by the H_α chemical shifts of the residues in the hydrogen bonded position to investigate any differences between denaturation at turn and strand residues (Figure 7b). The hydrogen bonded positions were selected because they have been shown to provide a more reliable measure of fraction folded than the non-hydrogen bonded sites.¹⁹ There are some gaps in the data for the H_α thermal denaturation due to overlap of the peak of interest with the water peak. The thermal denaturations exhibit the same trend in stability for the Trp pocket series. The extent of folding is in reasonably good agreement up to approximately 40 °C, but the peptides appear to unfold

to a greater extent at higher temperatures as measured by the average of the H_α chemical shifts. Inspection of individual residues indicates that Val 5 uniformly tracks closely with the Gly splitting, whereas Val 3, Orn 8, and Ile 10 unfold to a greater extent (Figure 7c and Supporting Information).

All of the hairpins are well folded even at 72-73 °C showing that these peptides are very thermally stable (Figure 6). Nonetheless, folding is still fast on the NMR timescale. **Trp KMe3 Pocket** is the most thermally stable of all the variants and is still ~96% folded at 72 °C based on the Gly splitting (80% folded based on the average H_α chemical shifts). A thermal melt of **Trp KMe3 Pocket** using Circular Dichroism (CD) in 4M urea was also performed to further investigate the high stability of this peptide (see Supporting Information). The use of a fairly high concentration of chemical denaturant in conjunction with increasing temperature was required to observe unfolding of this peptide, supporting the fact that this sequence produces an extremely stable folded structure.

Thermodynamic parameters for folding were obtained for **Trp KMe Pocket** and **Trp K Pocket** by nonlinear fitting of the data derived from the Gly splitting (see Experimental Section) and are given in Table 2. Since **Trp KMe2 Pocket** and **Trp KMe3 Pocket** are well folded even at high temperatures we were unable to fit the thermal denaturation data. **Trp K Pocket** has a highly favorable enthalpic component for folding but also has a fairly high entropic penalty for folding as compared to the previously reported **WK** peptide, which has only a single Trp-Lys interaction.¹ **Trp KMe Pocket** has a weaker enthalpic driving force for folding than **Trp K Pocket** but also has smaller entropic penalty. The same trend was observed in the **WKMe_n** series of peptides reported previously.^{2,3} The reduced enthalpic driving force upon methylation has been explained by the distribution of positive charge on the methylated lysine over a larger surface area, whereas the decreased entropic penalty resulting from Lys methylation has been attributed to the increased hydrophobicity of the methylated Lys, which results in an decreased entropic penalty when it is removed from aqueous solution upon folding.

Contributions of Trp2 and Trp4

Because tryptophan has the most favorable β -sheet propensity of any of the twenty natural amino acids,²⁰ the enhanced stability of the tryptophan pocket hairpins relative to those with a single tryptophan cannot solely be attributed to increased favorable side chain-side chain interactions. To assess whether the interaction of lysine 9 with tryptophan at position 2 and position 4 was significantly contributing to the high stability of the Trp Pocket peptides, individual double mutant cycles were performed.¹ Double mutant cycles isolate the interaction energy of two residues by mutating each individual residue, but also accounting for unintended changes to stability by comparison to the double mutant, yielding the ΔG for the interaction of interest. For the interaction between lateral cross strand residues, tryptophan 4 was replaced with alanine and lysine 9 was replaced with serine. The cross-strand Trp4-Lys9 interaction was calculated to be -1.1 kcal/mol (See Supporting Information). For the diagonal lysine-tryptophan interaction, tryptophan 2 was replaced with alanine and lysine 9 with serine. The diagonal interaction was also determined to be -1.0 kcal/mol. Because Trp K pocket is so stable, there is an inherently large error in calculating its free energy of folding (ie, 99% folded results in a stability of -2.67 kcal/mol, whereas 96% folded gives -1.85 kcal/mol). Thus, the absolute values from the double mutant cycles are not meaningful. Nonetheless, because the error in the energetic stability of **Trp K pocket** is common to both double mutant cycles, the relative energies of the lateral and diagonal Trp-Lys interactions are meaningful. These results indicate that the interaction energy is equivalent despite differences in geometry.

Comparison to KMe3 Recognition Domains

The β -hairpin model system here has structural similarities and differences to the aromatic cages found in the chromodomains and PHD domains. The aromatic cages in these domains are made up of three or four aromatic residues, two of which are in a diagonal orientation of two strands of a β -sheet, whereas the other one or two aromatic residues come from a nearby loop. In both protein domains, the two residues in the β -sheet make contacts with both the KMe3 alkyl sidechain and the methyl groups, whereas the loop residues form the base of the aromatic cage and only interact with the methyl groups. The model system provides an aromatic cleft within a two-stranded β -sheet, but the two aromatic residues are in the same strand, rather than in neighboring strands. Nonetheless, the Trp pocket appears to provide a similar cleft for binding to both the alkyl chain of KMe3 and the methyl groups, as is found in the native proteins. Because of the extensive contacts with the alkyl chain, even the unmethylated Lys binds well in this pocket and provides a well-folded system. To gain greater selectivity for a methylated Lys over the unmethylated Lys, an additional aromatic residue would be needed to act as the base of the cage, in a third β -strand, for example.

Comparison to Other Well Folded β -Hairpins

The stability of the Trp Pocket peptides are comparable to other highly stabilized hairpins such as trpzip4¹², HP5W4²², and WKMe3², which utilize a variety of stabilizing interactions. The trpzip4 peptide designed by Cochran and coworkers is stabilized by a four-tryptophan cluster on the NHB face of the hairpin and is 92% folded in aqueous solvent which is similar to the Trp Pocket peptides. However, trpzip4 denatures at a much lower temperature than the Trp Pocket peptides. The NMR structure of trpzip reveals that these tryptophans interact in an edge-to-face orientation of their indole rings cross-strand from each other, which is common in proteins.¹²

The HP5W4 peptide designed by Andersen and coworkers resulted in a β -hairpin that is at least 96% folded at ambient temperature in aqueous solution²². This hairpin was based on the trpzip peptide containing 4 tryptophan residues on the NHB face but increased the thermal stability by optimizing the turn sequence. The turn sequence of the hairpin helps to promote and stabilize the formation of β -hairpins by adopting a favorable orientation that properly positions the strands to form an anti-parallel backbone hydrogen bonding pattern of a β -sheet with concomitant sidechain-sidechain interactions.

The WKMe3 peptide designed by Hughes and Waters resulted in a β -hairpin that is more stable than both trpzip4 and HP5W4². This hairpin utilized a cation- π interaction between trimethylated lysine and tryptophan as a stabilizing factor that gives a hairpin that is about 70% folded at 75° C,² whereas trpzip4 is 40% folded¹² and HP5W4 is 60% folded²². Interestingly, this hairpin did not require the hydrophobic cluster of four tryptophan residues as are found in both trpzip4 and HP5W4². The Trp Pocket peptides are based on WKMe3 and have improved upon stability by the addition of another tryptophan which increases both the β -sheet propensity and favorable cation- π contacts, mimicking the binding pockets of histone binding proteins. The unmethylated **Trp K Pocket** hairpin is 78% folded at 73° C which is as stable than WKMe3, but without the methylated Lys. Addition of KMe3 to the Trp pocket results in a peptide that is still at least 80% folded at 72° C, based on H_α chemical shifts. Thus, using high β -sheet propensity Trp residues coupled with highly favorable cation- π interactions provides an optimal combination for stabilizing β -hairpin structure.

Conclusions

We have mimicked a binding motif found in a native protein-protein interaction to design an extremely well folded β -hairpin. This system exploits the high β -sheet propensity of Trp in

conjunction with favorable and selective cation- π interactions between Trp and Lys or methylated Lys to give the most thermally stable designed hairpin peptides currently reported. The interaction between the lysine and the tryptophan pocket is comparable to previously reported tryptophan-lysine and N-methylated lysine interactions in β -hairpins and a similar trend is observed with an increased stability as N-methylation is increased. The high stability of the Trp pocket peptides may be amenable to other applications such as peptide antibiotics where highly structured β -hairpin peptides are required and are usually cyclized to stabilize the hairpin structure.²³ Work is currently underway involving incorporation of Trp Pocket motif in other designed β -hairpin as a stabilizing structural element.

Experimental Section

Synthesis and purification of peptides

Peptides were synthesized by automated solid phase peptide synthesis on an Applied Biosystems Pioneer Peptide Synthesizer Fmoc protected amino acids on a PEG-PAL-PS resin. Mono and dimethylated Fmoc-protected lysine were purchased from AnaSpec. A 5-fold excess of amino acid was used for each coupling step. Activation of amino acids was performed with HBTU, HOBT in the presence of DIPEA in DMF. Deprotections were carried out in 2% DBU (1,8 diazabicyclo[5.4.0]undec-7-ene), 2% piperidine in DMF for approximately 10 minutes. Extended cycles (75 minutes) were used for each amino acid coupling step. All peptides were acetylated at the N-terminus with 5% acetic anhydride, 6% lutidine in DMF for 30 mins. Cleavage of the peptide from the resin was performed in 95:2.5:2.5 Trifluoroacetic acid (TFA): Ethane dithiol or Triisopropylsilane (TIPS): water for 3 hours. Ethane dithiol was used as a scavenger for sulfur containing peptides. TFA was evaporated and cleavage products were precipitated with cold diethyl ether. The peptide was extracted into water and lyophilized. It was then purified by reverse phase HPLC, using a Vydac C-18 semipreparative column and a gradient of 0 to 100% B over 40 minutes, where solvent A was 95:5 water:acetonitrile, 0.1% TFA and solvent B was 95:5 acetonitrile:water, 0.1% TFA. After purification, the peptide was lyophilized to a powder and identified with ESI-TOF mass spectroscopy.

Cyclization of Cyclic peptides

Cyclic control peptides were cyclized by oxidizing the cysteine residues at the ends of the peptide by stirring in a 10 mM phosphate buffer (pH 7.5) in 1% DMSO solution for 9 to 12 hours. The solution was lyophilized to a powder and purified with HPLC using previously described method.

Methylation of dimethyl lysine

Peptides containing dimethyl lysine were methylated to trimethyl lysine on resin by reacting with 8 μ l 1,3,4,6,7,8-Hexahydro-1-methyl-2H-pyrimido[1,2-*a*] pyrimidine and 62 μ l methyl iodide brought up to 5 ml in DMF. Reaction mixture was agitated by nitrogen bubbling under a vented septum for 5 hours. Resin was washed with DMF 3x and then washed with 3x dichloromethane.

NMR Spectroscopy

NMR samples were made to a concentration of 1 mM in D₂O buffered to pD 4.0 (uncorrected) with 50 mM NaOAc-d₃, 24 mM AcOH-d₄, 0.5 mM DSS. Samples were analyzed on a Varian Inova 600-MHz instrument. One dimensional spectra were collected by using 32-K data points and between 8 to 128 scans using 1.5 s presaturation. Two dimensional total correlation spectroscopy (TOCSY)²⁴ and nuclear overhauser spectroscopy (NOESY)²⁵ experiments were carried out with a 7248.3 Hz window and States-TPPI was used for quadrature detection in the indirect dimension. TOCSY experiments were run with a 60ms mixing time, a 2.0 s relaxation

delay and 128-512 free induction decay (FID) increments of 16-32 transients each (corresponding to 2048-4096 data points in the f2). NOESY experiments were run with 200 to 500 ms mixing time, a 1.5 s relaxation delay, and 256-512 FID increments of 32 transients (corresponding to 4096-8192 data points in the f2). Varian VNMR software was used to analyze all spectra using standard window functions (sinbell and Gaussian with shifting). Presaturation was used to suppress the water resonance. Assignments were made by using standard methods as described by Wüthrich²⁶. All experiments were run at 298.15 K.

Determination of Fraction Folded

To determine the unfolded chemical shifts, 7-mers were synthesized as unstructured controls and cyclic peptides were synthesized for fully folded. The chemical shifts for residues in the strand and one turn residue were obtained from each 7-mer peptide. The chemical shifts of the fully folded state were taken from the cyclic peptides. The fraction folded on a per residue bases was determined from equation 1.

$$\text{Fraction Folded} = [\delta_{\text{obs}} - \delta 0] / [\delta_{100} - \delta 0], \quad [1]$$

where δ_{obs} is the observed C α H chemical shift, δ_{100} is the C α H chemical shift of the cyclic peptides, and $\delta 0$ is the C α H chemical shift of the unfolded 7-mers. The overall fraction folded for the entire peptide was obtained by averaging the fraction folded of residues Val3, Orn8, and Ile10. These residues are in hydrogen bonded positions have been shown to be the most reliable in determining fraction folded¹⁷. The overall fraction fold was also determined using the extent of C α H glycine splitting observed in the turn residue Gly10 given in equation 2.

$$\text{Fraction Folded} = \left[\Delta\delta_{\text{Gly Obs}} \right] / \left[\Delta\delta_{\text{Gly 100}} \right], \quad [2]$$

where $\Delta\delta_{\text{Gly Obs}}$ is the difference in the glycine C α H chemical shifts of the observed, and $\Delta\delta_{\text{Gly 100}}$ is the difference in the glycine C α H chemical shifts of the cyclic peptides.

Determination of thermodynamic parameters

Variable temperature NMR was used in order to determine the thermodynamic parameters of the peptide folding. A temperature range of 275 to 351 K was explored in five-degree increments using a Varian Inova 600-MHz spectrometer. Temperature calibration was performed with ethylene glycol and methanol²⁷ standards by using standard macros in Varian software. The change in glycine chemical shift difference was followed with temperature. The fraction folded of the peptide was plotted against temperature using Origin 7.5 software²⁸, and the thermodynamic data was obtained by using a nonlinear least-squares fitting algorithm to equation 3²¹:

$$\text{Fraction folded} = (\exp [x/RT]) / (1 + \exp [x/RT]) \quad [3]$$

Where

$$x = \left(T \left[\Delta S_{298}^{\circ} + \Delta C_p^{\circ} \ln \{T/298\} \right] - \left[\Delta H_{298}^{\circ} + \Delta C_p^{\circ} \{T - 298\} \right] \right)$$

CD Spectroscopy Temperature Melt

CD spectroscopy was performed on an Applied photophysics Pistar-180 Circular Dichroism spectrophotometer. Spectra were collected from 260 to 185nm at 25°C with every 1 nm per

0.3 sec scanning for standard scans and at 215nm from 20°C to 90°C with 1 sec scanning for thermal melts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(a) Structure of the aromatic binding pocket of the HP1 chromodomain bound to a histone peptide containing trimethyllysine (green); β -sheet structure is shown in blue (pdb: 1KNE). (b) Structure of the aromatic binding pocket of the BPTF PHD domain bound to a histone peptide containing trimethyllysine (green); β -sheet structure is shown in blue (pdb: 2FUU). (c) NMR structure of the beta-hairpin peptide WKMe3 indicating the cation— π interaction between Trp and trimethyllysine (green); β -sheet structure is shown in blue.³

(b) Ac-Cys-Arg-Trp-Val-Trp-Val-Asn-Gly-Orn-**KMe_n**-Ile-Leu-Gln-Cys-NH₂

(c) Ac-Arg-Trp-Val-Trp-Val-Asn-Gly-NH₂

Ac-Asn-Gly-Orn-**KMe_n**-Ile-Leu-Gln-NH₂

n = 0 - 3

Figure 2.

(a) Trp Pocket series of peptides containing Trp at positions 2 and 4 and Lys, mono-, di-, or trimethyl Lys at position 9 (R = H or Me, accordingly). HB = hydrogen bonded site; NHB = non-hydrogen bonded site. (b) Sequence of control peptides for the fully folded state cyclized with a Cys-Cys disulfide bond between the underlined residues. (c) Sequence of control peptides for the unfolded state.

(a)

(b)

Figure 3.

H_α chemical shift differences from random coil controls: (a) Trp Pocket series. (b) Trp pocket cyclic control peptides. The Gly bars reflect the H_α separation in the hairpin. Conditions: 293 K, 50 mM sodium acetate- d_4 , pH 4.0 (uncorrected), referenced to DSS.

Figure 4. Side-chain chemical shifts of lysine and methylated states of the Trp Pocket series relative to random coil values. Methyl shifts are indicated as M. Conditions: 293 K, 50 mM sodium acetate- d_4 , pH 4.0 (uncorrected), referenced to DSS.

Figure 5. NOEs of side-chain side-chain interactions between cross strand residues on the NHB face in (a) **Trp K Pocket**, (b) **Trp KMe Pocket**, (c) **Trp KMe2 Pocket**, and (d) **Trp KMe3 Pocket**. Red arrows indicate NOEs between KMe_n and Trp2; green arrows indicate NOEs between KMe_n and Trp4; cyan arrows indicate NOEs between KMe_n and Leu11.

Figure 6.
CD spectra of the Trp pocket series of peptides. Peptides were monitored 10mM sodium phosphate buffer pH 7.0 at 25°C.

(a)

Figure 7. Thermal Denaturation of Trp Pocket Series (a) fraction folded derived from the Gly splitting; (b) fraction folded derived from the average H_{α} chemical shift of Val3, Val5, Orn8, and Leu10; (c) individual thermal denaturation data for the Trp KMe3 Pocket. Values were determined from data obtained in 50 mM sodium acetate- d_4 , pH 4.0 (uncorrected), referenced to DSS.

Table 1

Fraction folded for Trp Pocket series peptides. Values calculated from data obtained at 293 K, 50 mM sodium acetate- d_4 , pH 4.0 (uncorrected), referenced to DSS.

Peptides	Fraction Folded (Gly) ^(a)	Fraction Folded (H α) ^(b)
Trp K Pocket	≥ 0.99 (± 0.02)	0.96 (± 0.09)
Trp KMe Pocket	≥ 0.99 (± 0.02)	0.9 (± 0.1)
Trp KMe2 Pocket	0.97 (± 0.02)	0.98 (± 0.01)
Trp KMe3 Pocket	≥ 0.99 (± 0.02)	0.94 (± 0.03)

^(a) Error determined by chemical shift accuracy on NMR spectrometer.

^(b) Average of the H α values from Val3, Val5, Orn8, and Ile10. The standard deviation is in parentheses.

Table 2

Thermodynamic Parameters for Folding at 298 K for **Trp K Pocket** and **Trp KMe Pocket** peptides. Conditions: 50 mM sodium acetate- d_4 , pH 4.0 (uncorrected), referenced to DSS.

Peptide	ΔH° kcal/mol	ΔS° cal/mol K	ΔC_p° cal/mol K	Source
Trp K Pocket	-10.9 (± 0.8)	-28 (± 3)	-100 (± 21)	this work
Trp KMe Pocket	-2.8 (± 0.2)	-1.6 (± 0.7)	-311 (± 8)	this work
WK	-2.8 (± 0.03)-6.8	(± 0.1)	-163 (± 3)	Ref 1
WKMe	-1.7 (± 0.1)-2.2	(± 0.3)-221	(± 33)	Ref 3

Table 3Comparison of highly stable β -hairpins.

Peptides	Sequence	Method	Fraction Folded at 25° C	Fraction Folded at 72-75° C
Trpzip4 ¹²	GEWTWDDATKTWTWTE	CD	92%	40%
HP5W4 ²²	KKWTWNPATGKWTWQE	CD and H _{α} shifting of Trp5 and Pro7	> 96%	60%
WKMe3 ²	RWVEVNGOKMe3ILQ	NMR (Gly splitting)	92%	72%
Trp K Pocket	RWVWVNGOKILQ	NMR (Gly and H _{α} splitting)	99% (93%) ^{a)}	78% (65%) ^{a)}
Trp KMe3 Pocket	RWVWVNGOKMe3ILQ	NMR (Gly and H _{α} splitting)	99% (95%) ^{a)}	96% (80%) ^{a)}

^{a)} The value in parentheses comes from the average H _{α} splitting of the residues in the hydrogen bonded sites.