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Very Short Peptides with Stable Folds:

Building on the Inter-relationship of Trp/Trp, Trp/cation, and Trp/backbone-amide Interaction Geometries

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Abstract

By combining a favorable turn sequence with a turn flanking Trp/Trp interaction and a C-terminal H-bonding interaction between a backbone amide and an *i* - 2 Trp ring, a particularly stable ($\Delta G_U > 7$ kJ/mol) truncated hairpin, Ac-WI-(D-Pro-D-Asn)-KWTG-NH₂, results. In this construct and others with a W-(4-residue turn)-W motif in severely truncated hairpins, the C-terminal Trp is the edge residue in a well-defined face-to-edge (FtE) aryl/aryl interaction. Longer hairpins and those with six-residue turns retain the reversed “edge-to-face” Trp/Trp geometry first observed for the trpzip peptides. Mutational studies suggest that the W-(4-residue turn)-W interaction provides at least 3 kJ/mol of stabilization in excess of that due to the greater β -propensity of Trp. The β -propensity of Trp is context dependent; but, for the systems studied, always greater than that of Thr (by 0.4 - 4.7 kJ/mol). At non-H-bonded positions remote from the turn, two alternative edge-to-face geometries are observed and there is no evidence of additional stabilization due to the Trp/Trp interaction. The NMR structuring shift diagnostics of edge-to-face Trp/Trp, Trp/Lys π -cation, and Trp/Gly-H_N interactions have been defined. The latter can give rise to > 3 ppm upfield shifts for the Gly-H_N in -WX_nG- units both in turns ($n = 2$) and at the C-termini ($n = 1$) of hairpins. Terminal YTG units result in somewhat smaller shifts (extrapolated to 2 ppm for 100% folding). In peptides with both the EtF and FtE W/W interaction geometries, Trp to Tyr mutations indicate that Trp is the preferred “face” residue in aryl/aryl pairings, presumably due to its greater π basicity.

Introduction

Aromatic residues are typically buried in the hydrophobic core of a protein; when exposed they are usually “hot spots” involved in biorecognition phenomena^{1–3}; this also appears to be the case for β -hairpins⁴ that have receptor properties. The significance of interactions involving aromatic sidechains in stabilizing protein structure is well accepted, while the geometry and specificity of these interactions are more elusive. These interactions fall into at least three categories: cation- π interactions^{5,6}, aryl-ring/backbone contacts (including amide-NH aromatic “H-bonding” interactions, *vide infra*), and hydrophobic clustering⁷. The last of these can be further distinguished as aryl/aliphatic and aryl/aryl interactions, with the aryl/aryl interactions displaying two dominant geometries - edge-to-face (EtF) and parallel displaced (PD) stacking.^{8,9} The most common aryl/aryl interaction pair, F/F, appears to display a wide variance in interaction geometries; but instances in which one ring displays a far upfield para-H are relatively common^{10,11}. aryl/aryl interactions including Tyr or Trp appear to more consistently favor EtF geometries.¹² These aryl/aryl ring interactions are not viewed exclusively as dispersive hydrophobic interactions.^{9,13} β -Hairpin peptides have provided scaffolds for probing most of these interactions, including the effects of turn type

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and propensities.¹³⁻³⁴ Statistical studies of residue pairings in protein β sheets provide additional insights.³⁴⁻⁴¹ Despite these studies, significant questions remain and the limit of Trp interactions to impose structure in short peptide sequences has not been examined.

Statistical analyses of the Protein Data Base (PDB) have revealed that aryl/aliphatic interactions are over-represented at H-bonded cross-strand pairs ($S \pm$ odd positions in Figure 1); while only two reports³⁶⁻³⁷ find that cross-strand aryl/aryl pairings occur predominantly at non-H-bonded sites ($S \pm$ even positions in Figure 1). Similar studies have also revealed that Gly residues at H-bonding β -strand positions are almost always aligned with cross-strand aryl residues; this effect has been designated as “Glyrescue”³⁶⁻³⁸. Another category of aromatic ring/backbone interactions has been viewed as an amide-NH to aryl H-bonding interaction⁴²⁻⁴⁵ and has been noted for both Tyr and Trp residues. This interaction, also observed in peptides and as a residual structuring feature in denatured proteins⁴²⁻⁴⁴ and random coil controls⁴⁶, is most commonly observed for a Gly- H_N . When it occurs in folded proteins it produces large upfield shifts for the Gly- H_N ; a search of the Madison Biological Magnetic Resonance Data Bank (BMRB) (<http://www.bmrbl.wisc.edu/>) reveals that more than 75% of the instances in which Gly- H_N appears upfield of 6.0 ppm in proteins represent aryl-X-G sequences: the occurrences of Gly- H_N shifts upfield of 6 ppm represent the most extreme 0.42% of the Gly- H_N observations recorded in the BMRB.

Turning to studies of cross-strand interactions in β -hairpin models (see Figure 1 for the hairpin position nomenclature employed herein), Lys/Trp, Phe and Arg/Trp, Phe cation- π interactions have been examined in a number of hairpin systems^{13-15, 18-9}. The largest hairpin stabilization reported for the natural amino acids has been on the order of 1 kJ/mol.

The association of β -strand aromatic sidechains in hydrophobic clusters has been extensively examined in hairpins and is a design basis for a number of hairpin models^{13-15, 20-22, 27-29} of β -sheets. The C-terminal hairpin (GB1p) of the B1 domain of Protein G has been a key inspiration in the development of designed β -hairpin peptides. This hairpin utilizes a direct cross-strand Tyr/Phe pair flanking the turn ($S \pm 2$) and a Trp/Val $S \pm 4$ pairing, and is sufficiently stable to maintain a measurable fold population (Table I) when excised from the protein.²⁵⁻²⁶ The hairpin stability of the wild-type GB1p sequence is marginal²⁷⁻²⁸ but without the aromatic cross-strand pair hairpin formation is abolished²⁸⁻²⁹.

GB1p and its optimized derivatives have been used in numerous studies of the both the thermodynamics and kinetics⁴⁷⁻⁵⁰ of β -hairpin formation. Both trpzip⁴²⁷ and peptide HP5W⁴²⁸, with the non-Trp residues of GB1p at $S \pm 2$ and $S \pm 4$ replaced by Trp, form particularly stable hairpins (Table I). Molecular Dynamics simulations of trpzip2 performed to assess the energetics of Trp side-chain interaction geometries suggested that an EtF W/W interaction provides significantly more stabilization than the PD (Parallel Displaced) conformation.⁹ The NMR structure ensemble generated for HP5W4 has only the single EtF Trp/Trp pair (at $S \pm 2$); which appears to be required for both hairpin stabilization and the generation of a diagnostic CD exciton couplet.¹³ The identical EtF geometry, with the N-terminal S-2 Trp displaying an upfield $H_{\alpha 3}$ (a ring current shift as large as 2.6 ppm) and a large diastereotopic ring current shift for the β -methylene ($\Delta\delta_{CH_2}$) characteristic of the edge aromatic side-chain in EtF interactions, is observed in HP7, HP6V and is retained in truncated peptides with the WNPATGKW sequence¹³; Table I. A stable 10-mer hairpin, chignolin (GY-DPETGT-WG), utilizes a similarly optimized turn and a flanking Y/W pair³⁰.

Despite the dramatic structure-stabilizing potential suggested by the MD simulations,⁹ Trp/Trp interactions are (with one possible exception³⁷) not significantly over-represented in β sheet proteins after taking into account the low frequency occurrence of tryptophan,⁷ and

there has only been a single measure (2.3 kJ/mol)⁵¹ of the cross-strand W/W interaction energy. The stability of HP7 and its truncated version,¹³ as well as chignolin,³⁰ suggest that an EtF aryl/aryl interaction immediately flanking a turn sequence is particularly stabilizing. One aim of the present study was to obtain quantitative measures of the extent to which cross-strand W/W pairs contribute to hairpin stability.

A second aim was to utilize the full range of interactions that the indole ring of Trp offers to prepare very short peptides with hyperstable folds. In the course of these studies, amide-NH/indole and π -cation interactions were observed and these appear to modulate the geometric preference observed for Trp/Trp interactions. The observation that AW-SNGK-WT was, based on a CD melt of the W/W exciton couplet, more stable than Ac-WNPATGKW-NH₂ even though the NMR assignment (*vide infra*) indicated that the W/W interaction was flipped face-to-edge (FtE) (with the N-terminal Trp as the face aromatic, rather than the classic EtF geometry observed in all previous trpzip analogs) raised additional questions. What caused this change in orientation; could it be utilized to create other stable truncated hairpins?

Results

Folding quantitation and the intrinsic β -strand propensities of Trp and Thr

Determining the fold population of peptide hairpins is somewhat problematic since it is difficult to determine the 100% folded values of spectroscopic parameters.^{31,50} It was apparent that the intended study of Trp effects on hairpin stability would require accurate measures of hairpin fold populations (f_{HP} , the equilibrium fractional population of the folded state) for quantitating the energetic contributions of Trp interactions. We also wanted to distinguish hairpin-favoring effects of Trp insertion due to its intrinsic β -strand propensities from those arising from Trp-specific cross-strand interactions. Protein β -strand occurrence statistics^{39,40} generally suggest the following order of β -strand propensities - V > I > F/Y $\geq$ W > T, with studies specific to antiparallel alignments indicating V/Y > I > F/W > T⁴¹. The classic experimental study⁵² using the B1 domain of protein G (GB1) indicated T > I > Y > F/V > S > W/L. These studies also suggested that specific cross-strand hydrophobic interactions were particularly favorable. In contrast, data from the Cochran laboratory^{53,54}, including phage display studies of GB1 mutants⁵⁵, indicate that cross-strand interactions are less important and that the intrinsic β propensity series is W/Y/F $\geq$ V > T > I. A recent study⁵¹ adding additional Trp residues to a WW domain also indicated a substantial hairpin stabilization (0.9 kJ/mol versus Val) from a single Trp insertion, and is in accord with the β -propensity series immediately above..

NMR structures of trpzip4, HP5W4 and HP7 show a W-loop-W motif, with a single orientation of the indole rings; this appeared to apply to HP6V and a NPATGK to INGK turn mutant of HP7 as well¹³, suggesting a W/W interaction that was independent of turn type. This geometry gives rise to a CD exciton couplet and large upfield chemical shifts for the H ϵ 3 proton of the Trp at the N-terminus of the reversing loop. Prior studies suggested that molar ellipticities (deg cm² dmol⁻¹) on order of +490,000° at 228 nm and H ϵ 3 shifts of *circa* -2.25 ppm represented 100% folded values for these peptides. Melting curves based on these spectroscopic probes are used throughout this study. However there is no compelling basis for assuming that these values hold for all possible W-loop-W geometries and quite different CD couplet ellipticities and ring current shifts might apply for other cross-strand Trp/Trp pairings. As a result, we wanted to be able to utilize other measures of hairpin fold population based on chemical shift deviations (CSDs) such as the shift diastereotopicity ($\Delta\delta_{Ha}$) at the glycine in NG β -turn loci,^{16,33,34} the downfield shifts of cross-strand H-bonding H_N sites, and the similar downfield shift of H α resonances in non-H-bonded strand sites¹⁶ as additional measures of fold population changes.

When aromatic residues are present in a peptide sequence, substantial ring current shifts are expected in the folded state and a prior study of GGXGG systems⁴⁶ suggests some caution in applying statistical coil norms for calculating CSDs. In order to have the best possible 'coil' values for determining CSDs, an additional set of small peptides including Trp and Tyr residues was prepared using a -KAXAA- context: the results appear in the Supporting Information.

With one exception, at the $i+1$ H_N site, the upfield shifts we observed were significantly smaller than those observed by Schwarzsinger et al.; it appears that amino acid sidechains prevent a close spatial association between the backbone and the Trp indole ring. The large $i+2$ H_N effect (-0.52 ppm) noted by Schwarzsinger et al. does, however, reappear in our peptides when the $i+2$ residue is Gly: *e.g.* -0.9 ppm in Ac-RKAWAGK, and an even larger shift (-1.2 ppm) appears at this site in a tripeptide model (Ac-WTG).

We selected MrH4a (KKLTVS-INGK-KITVSA) and its more stable N8 to D-Pro mutant (MrH4b), which are known to be monomeric in aqueous solution at NMR concentrations^{16,22}, as the test system for examining the intrinsic β propensities of Thr and Trp. Cross-strand W/W pairs were inserted at the $S\pm 2$ and $S\pm 4$ positions and the effects of A to T and A to W mutations were examined in the presence and absence of a cross-strand partner.

The initial effort focused on evaluating the β propensity and cross-strand interaction energy for T4/T13 and the corresponding W/W pairing. An N8 to Aib mutation⁵⁶ provided the best folded system (MrH4e). To maximize the drive for folding we added a fluoroalcohol cosolvent^{14,16,21,26,31}, the CSDs observed (see Supporting Information) for MrH4e in 20 vol-% hexafluoro-isopropanol (HFIP) were assumed to correspond to $f_{HP} = 0.96$ and were employed to evaluate f_{HP} for all species lacking aromatic sidechains. The introduction of Trp residues did produce significant changes in some of these values; these, as well as the probes selected for measuring f_{HP} for each series, are presented and justified in the Supporting Information. NMR melts (to 320K) were performed for all species and those containing W/W pairs were also examined by CD melts.

Studies of T to A substitutions at the $S\pm 4$ position employed MrH4b, in order to have a quantifiable fold population for the T4A/T13A mutant. Selected results appear in Table II. The addition of HFIP to the medium produced well-folded ($f_{HP}^{280} \geq 0.7$) hairpins for all of the species examined: even the T4A,T13A double mutant was 82% folded at 280K in the HFIP-containing medium. A standard mutational cycle analysis indicates that the reinsertion of single Thr residues in (T4A,T13A)-MrH4b has a more favorable fold-enhancing effect in the N-terminal strand (1.92 kJ/mol), *versus* 1.05 kJ/mol for A13T. An additional 0.84 kJ/mol of stabilization in MrH4b appears to be due to a cross-strand T/T interaction.

A similar study of Trp insertions at the $S\pm 4$ positions, which were fold stabilizing, employed the less folded MrH4a series. The resulting stability series, $(S-4/S+4) W/W > W/A > A/W > T/W > W/G \geq T/T > T/A > A/A$, indicates that a Trp insertion is more stabilizing when there is a small residue (Gly or Ala) in the cross-strand position. In this system, T $\rightarrow$ W mutations were fold-stabilizing by 0.4 - 4.7 kJ/mol. The added stabilization, if any, associated with the S4 W/W pairing is modest (< 1.5 kJ/mol) and does not produce in a distinct geometric preference for the Trp sidechains. Even though NMR measures of strand association for the (T4W,T13W)-mutant of MrH4a indicated $f_{HP} = 0.67 \pm 0.05$ in water, the CD exciton couplet (as measured at the ellipticity maximum, $\lambda = 228$ nm) was only 35% that of $S\pm 2$ W/W HP peptides. The indole aryl-H shifts were also unusual, modest upfield shifts (≤ 0.6 ppm) were observed at the $H\epsilon 3$ site of *both* Trp residues. While NMR structuring shifts reflecting β -strand association indicated increased hairpin populations upon addition of

HFIP and also for the Asn8 to D-Pro turn mutation, both of these changes decreased the structuring shifts at the Trp H ϵ 3 sites *and* the amplitude of the Trp/Trp exciton couplet.

While the S $\pm$ 4 W/W pairing failed to display a distinct preference for an EtF vs FtE indole/indole geometry, the turn flanking S $\pm$ 2 pairing gave results consistent with prior studies of W-loop-W systems. In water at 280K, the (S6W,K11W)-mutant of MrH4a displayed a distinct CD exciton couplet ($[\theta]_{228} = +264,000^\circ$) and the N-terminal Trp residue displayed the ring current shifts ($\Delta\delta_{\text{C}\beta\text{H}_2} = 1.16$ ppm, with H ϵ 3 upfield by 2.1 ppm) similar to those in the trpzip peptides²⁷ and their truncated analogs¹³. However, the extent of fold stabilization associated with cross-strand pairing could not be quantitated since there was no firm basis for assigning precise 100% folded values. The contribution of individual Trp introductions at the S $\pm$ 2 position was *ca.* 5 kJ/mol based on the fold stability increments associated with adding each Trp to (S6A,K11A)-MrH4a. While other cross-strand interactions (*vide infra*) could be contributing to the fold stabilization associated with Ala to Trp mutations, these studies do indicate that Trp has a greater β propensity than Thr at all non-H-bonded positions examined.

Truncating HP6V and (S6W,K11W)-MrH4

The MrH4 mutations confirmed the stabilizing effect of a turn flanking W/W pair utilizing an IZGK turn (Z = N and D-Pro) and the retention of the same geometry observed about SNGK (HP6V) and NPATGK (HP7) loops. When HP6V was minimized to a β -turn with only two cross-strand pairs (HP6-tr = AWSNGKWT), a large positive CD exciton couplet was observed (Figure 2) even though the orientation of the indole rings had flipped from 'EtF' to 'FtE' with the C-terminal Trp displaying the upfield ring current shifts of the 'edge' ring (Figure 3). The key NOEs that defined which set of Trp resonances represented W2 and W7 (including connections between the aromatic and aliphatic spin systems) appear in the Supporting Information. The effects of additional mutations on HP6V are illustrated by the CD melts that appear in Figure 2B. NOEs established that all species other than HP6-tr had the N-terminal Trp as the 'edge' species in the indole/indole interaction. These include an S5I mutation which, based on both backbone proton CSDs (see Supporting Information) and the amplitude of the CD exciton couplet, increases the hairpin population. However, the increased CD amplitude for (S5I)-HP6V was not coupled with a similar increase in the upfield shift of W4H ϵ 3 5.71 ppm vs 5.57 ppm for HP6V. The Y2A mutation establishes that Y2 in HP6V is not essential for hairpin stabilization; the diminished exciton couplet magnitude reflects a small decrease in fold population (rather than a Tyr sidechain chromophore contribution). The decreased fold population is also reflected in the backbone proton CSDs (Supporting figure S2) and is attributed to the lower β propensity for Ala versus Tyr.

From panel B of figure 2, it is apparent that WXZGKW (Z = D-Pro or Asn) loops in hairpins can give rise to large exciton couplets ($[\theta]_{228} \geq +400,000^\circ$) for both X = Ile and Ser. This stands in some contrast to the results for (S6W,K11W)-MrH4a (maximal $[\theta]_{228} = +264,000^\circ$) even though all series displayed S-2 WH ϵ 3 shifts of *circa* - 2.1 ppm. Apparently, subtle geometry changes produce larger changes in the exciton couplet magnitude without influencing the ring current shifts. The reversed "FtE" Trp/Trp geometry can also yield the larger exciton couplet magnitude (HP6-tr in Figure 2). The G5A mutation in HP6-tr establishes that this interaction also requires a turn favoring sequence.

The hairpin stability data for the MrH4a mutants, as well as the S5I mutation data for HP6V suggested that in a truncated species such as Ac-WINGKW-NH₂, the favorable W/W pair would allow for a significant fold population, what remained unclear was which conformations the Trp side-chains would adopt. A series of peptides was made to investigate the fold propensities and conformations of 6 - 9 residue peptides containing the -

WXZGKW- (X=Ser, Ile and **Z** = Asn, D-Pro, or L-Pro.) motif. The **Z** = L-Pro species were intended to serve as unfolded controls; and did, indeed, display no signs of folding either as backbone-H CSDs, or as ring current or CD indicators of an EtF W/W interaction. With **Z** = Asn or D-Pro there were still significant variations in fold populations but it was apparent that all species preferred the reversed “FtE” interaction geometry and form hairpins with the same strand register, evidenced by the appearance in the W-(S/I)ZGK-WX sequences of a common set of cross-strand NOEs -- W1 α /W6 α , W1 α /X7H_N, and (S/I)2H_N/K5H_N. The turn glycine could be mutated to D-residues (for both **Z** = Asn and D-Pro) with little change in hairpin fold population.

One peptide (with no ‘unnatural’ residues) from this series displayed a large exciton couplet ($[\theta]_{228} = +370,000^\circ$) and NMR features suggesting enhanced hairpin formation: Ac-WINGKWT-NH₂ ($f_{HP}^{280K} = 0.77$ versus ~ 0.4 for Ac-WINGKW-NH₂). As to *a priori* rationales for the increased stability associated with the C-terminal Thr-insertion, the only ones we can suggest are: a) that the β -branched neighbor decreases the flexibility of the Trp residue strengthening the indole/indole interaction, and b) that the T7H_N is a better cross-strand H-bonding partner for the acetyl carbonyl than the E-amide NH of a terminal W-CONH₂ group. Subsequent studies (to be reported elsewhere) have established that a T7H γ 1(sidechain OH) $\rightarrow$ O=C-Me H-bond contributes; but at this point in our studies, the increased stability of Ac-WINGKWT-NH₂ was attributed to an amide-indole interaction between the S+2 Trp and the terminal amide, functioning as a hairpin C-cap. The amide’s proximity to an aromatic ring was established by the observation of a 2.7 ppm upfield CSD for the E-amide proton. NOEs suggested a geometry similar to that found in *i*/*i*+2 aryl-X-Gly interactions.⁴⁴⁻⁵⁷ In proteins aryl-TG units are a common feature at the ends of β -strands⁵⁷ and are also observed to have residual structuring⁴²⁻⁴³⁻⁴⁶ in small peptides. This prompted the synthesis of Ac-WINGKWTG-NH₂ for further NMR studies. In addition, the C α H₂ of the added Gly was expected to provide useful NOEs for a structure calculation. In 20 vol-% glycol at 270K, the W6H ϵ 3 ring current shift increased to -1.87 ppm with a further increase to -2.02 ppm under these conditions for the N3p turn mutant. The unusual pattern of shifts for the C-terminal Trp and Gly⁸-H_N, which corresponds to the position of terminal E-amide proton of Ac-WINGKWT-NH₂, are illustrated in Figure 4.

NOESY data for Ac-WINGKWTG-NH₂ (with 20 vol-% d₆-ethylene glycol added to increase viscosity and thus NOE intensities) were used to derive an NMR structure, segments of the NOESY spectrum at 270K appear in the Supporting Information. The co-solvent did not change the CSDs other than as would result from a slight increase in fold population associated with the lower temperatures. For the large ¹³C structuring shifts, cooling and glycol addition increased the CSDs only by 8% (the ¹³Ca CSDs, based on a literature method³¹, indicated 85% hairpin formation at 280K, see Supporting Information). For comparison with the Ac-WINGKWTG-NH₂ structure, an NOE-based structure was also derived for (S5I)-HP6V, KYVWINGKWTVE. When the two structures are overlaid (Figure 5), the divergence of the tryptophan ring orientations is apparent. Also noteworthy is the bent orientation of the C-terminal region of Ac-WINGKWTG-NH₂; all of the structures in the NMR ensemble place G8H_N directly in the shielding cone of the indole ring with geometries that would be consistent with a backbone-NH to indole ring H-bond⁴⁴ and which predict large upfield ring current shifts.

Both NMR ensembles were well-converged with intra-ensemble backbone RMSDs of 0.37 ± 0.20 (Ac-WINGKWTG-NH₂) and 0.40 ± 0.16 Å (KYVWINGKWTVE). The full statistics for the structure elucidations appear in the Methods section with the complete set of constraints in the Supporting Information. The Trp/Trp geometries were particularly well-determined in both structures with all torsion angles (ϕ/ψ as well as χ^1 and χ^2) displaying less than $\pm 7^\circ$ variance.

The turn geometries deserve some comment. The turn for the (S5I)-HP6V ensemble (ϕ/ψ T1 and T2 = +55/+59, +146/-34°) diverges significantly from the norms (+60/+30 and +90/0°) for a type I' hairpin turn. This likely reflects the effect of W4 which lies directly on top of the glycine and the G-K amide unit in the turn. In the case of Ac-WINGKWTG-NH₂, a nearly ideal type I' turn (+57/37 and +82/-3°) was observed within the structure ensemble. A wide variety of turn mutants of Ac-WINGKWTG-NH₂ were examined. The order of fold stability (based on both hairpin backbone CSDs, and the magnitudes of the upfield ring current shifts at both W6H ϵ 3 proton and the Gly⁸-H_N) was: **pn** > **pa** / **pG** / **pA** / **pq** > **pl** > **Nn** > **NG** >> **NN** >> **PG**. In water at 280K, the largest upfield Gly⁸-H_N shift (CSD = -3.2 ppm) and W6H ϵ 3 CSD (-1.93 ppm) were observed for the **pn** turn species. Throughout, the Trp/Trp geometry was the reversed 'FtE' type: apparently this interaction geometry is allowed for both type I' and type II' turns. Less stable species, for example the **NN** turn mutant, displayed an enhanced tendency toward aggregation. The **NA** turn mutant was also prepared, but no measures of fold population could be obtained since this species forms a hydrogel even at the concentrations that apply during HPLC elution and CD measurements.

Amide-Indole interactions

Upfield Gly-H_N CSDs have been observed in peptides designed as random coil controls when an aromatic residue occurs at the i-2 position.⁴⁶ These CSDs are typically in the 0.5 - 1.3 ppm range, and may represent population of conformers with stereochemical features like those seen at the C-terminus of the NMR structure of Ac-WINGKWTG-NH₂. Ac-WTG-NH₂, Ac-WTG-, and +WTG-prepared for the present study display Gly-H_N CSDs of -1.26, -1.22, and -0.73 ppm, respectively at 280K. Based on a search of the BMRB, when similar aryl-X-G interaction geometries appear in the folded states of proteins, shifts as large as 4 ppm are observed. We have now observed similarly large shifts (at the bold **G**) in both Ac-W**I**pGKWTG-NH₂ (in water, 20% glycol, and 30% TFE) and KKLT**V**W-I**p**GK-WIT**V**SA (in 20% HFIP).

Until the present study, this amide-aryl interaction had not been fully realized in designed peptides. The requisites for this interaction (and the extent to which it provides fold stabilization) were probed by mutational studies. In the case of Ac-WTG-NH₂, the upfield CSD at the (i+2)-H_N is reduced from 1.2 to < 0.2 ppm when Gly is replaced by either L- or D-Ala: the Gly residue is essential, mirroring the strong preference for Gly seen for peptide bond n→(n-2) π -hydrogen bonds⁴⁴ in proteins. For species containing the -WIXZKWTG-sequence, XZ = PG (which prohibits hairpin turn formation), serves as a 'statistical coil' control, displaying a Gly-H_N CSD of -1.08 ppm. C-terminal extension of the -WIXZKWTG- motif was well-tolerated, with the upfield CSDs of both Trp-H ϵ 3 and Gly-H_N retained (data not shown). N-terminal extension gave different results. The N-acetyl group appears to be particularly favorable. When Ac- is deleted, or replaced by T-, Ac-T- or AT-, the Gly-H_N shift is greatly reduced (see Table III). The most dramatic effect was due to deletion of the acetyl, which reduces all of the CSD measures of hairpin and turn formation, as well as the W6H ϵ 3 CSD, to less than 25% of their values in the acetylated species, with G8H_N retaining its 'statistical coil' WTG value. In the case of TWINGKWTG, the Gly-H_N CSD was further reduced (to -0.48 ppm) even though the upfield shift of Trp⁷-H ϵ 3 actually increased to -1.92 ppm. The TWINGKWTG and Ac-TWINGKWTG-NH₂ mutants also retained the CSDs that reflect hairpin formation. Melting studies for TWINGKWTG provided additional evidence for a significant local W/G interaction in -WTG units (Fig. 6).

At first glance, the CSD melt of TWINGKWTG appears to represent a case of non-2-state behavior, with the Gly⁹-H_N displaying an increase in its CSD while all the other diagnostic structuring CSDs decrease in concert. This is, instead, interpreted as warming-induced population of non-hairpin states allowing the *local* aromatic-amide interaction to "melt in," heading toward convergence with the CSD melt for the analogous tripeptide control.

The insertion of a Thr-Gly unit after the C-terminal Trp of Ac-WNPATGKW-NH₂ provided additional insights. With the optimized NPATGK loop there appears to be a strict requirement for the C-terminal Trp indole ring to be the face unit of the EtF Trp/Trp interaction. The fold population of Ac-WNPATGKW-NH₂ ($f_{HP} = 0.5$) was unaltered by a Thr-Gly insertion at the C-terminus, and G10H_N CSD was -0.58 ppm, reflecting formation of the local WTG interaction only in the unfolded state.

Mapping the Transition Between the Two Edge-to-Face Orientations

N-terminally extended peptides Ac-TWINGKWTG-NH₂ and TWINGKWTG still exhibit very large CSDs for the C-terminal Trp-H ϵ 3, indicating that the Trp/Trp interaction remains flipped ('FtE') even though the large upfield shift associated with an optimized WTG interaction is absent. However, a smaller, but significant upfield CSD for the *N-terminal* Trp-H ϵ 3 is also present. Further addition of one alanine residue to the N-terminus appeared to return the W/W EtF interaction geometry predominantly to the orientation observed for the HP, MrH4 and trpzip hairpins, with the larger CSD seen on the N-terminal Trp. The H ϵ 3 CSD comparisons appear in Table III along with other indicators of structuring. Finally, with one additional N-terminal strand extension, and replacing the C-terminal Gly with a dipeptide unit (VE) with a significant β propensity, only a single unflipped EtF geometry is present.

From Table III it is apparent that the shielding at the S+4 H_N, the upfield shift of Trp H ϵ 3 sites and hairpin populations do not correlate through the series. Fraction folded measures cannot be derived from the S+4 H_N shifts, because values of the S+4 H_N CSD on the order of -1 ppm can represent the residual local WTG interaction in an otherwise unfolded peptide. The migration toward positive S+4 H_N CSDs for the longer sequences represents a hairpin (β configuration) related effect.

Examining W/Y pairings (and the Tyr-X-G interaction)

Based on literature data for chignolin, a well-folded ten-residue hairpin with YDPETGTW as the aryl-loop-aryl unit, the EtF Y/W interaction in this peptide appears nearly as stabilizing as the comparable W-loop-W interactions. We therefore examined the effect of replacing each of the Trp residues of KAVWINGKWTVE and Ac-WIpGKWTG-NH₂ with Tyr (Table IV). The first system has the normal EtF geometry: the second system has the reversed "FtE" geometry with the C-terminal aryl involved in a strong aryl-X-G interaction. The backbone proton CSDs used for the f_{HP} estimates in Table III were also used here as a qualitative measure of hairpin formation. These, as well as the aryl sidechain and S+4 H_N shifts, are expected to vary due to the different ring current effects of Tyr versus Trp. Nonetheless, some qualitative conclusions emerge.

In the case of Ac-WIpGKWTG-NH₂, substituting either Trp with Tyr appears not to alter the "FtE" geometric preference; the appropriate S+2 aryl-H is further upfield in all cases. The hairpin strand CSDs suggest that W to Y mutation at the S-2 position is the more destabilizing one. Both mutations decrease the upfield shift at the S+4 H_N to a comparable degree, but probably for different reasons. For the W6Y mutant this may represent a smaller ring current shift from Tyr rather than Trp. For the S-2 W to Y mutation, the decrease is attributed to a reduced hairpin fold population since the peptide still has the C-terminal WTG sequence.

Turning to the longer hairpin system, the backbone CSDs and the turn Gly- $\Delta\delta H_{\alpha}$ values provide guidance regarding relative fold stability. Of the two Tyr mutants, the peptide with Y at the S-2 position is significantly more stable. Complete backbone CSD comparisons appear in a supporting figure. Furthermore, the less-stable peptide hairpin with Y at the S+2

position appears to populate both EtF orientations; both the S-2 Trp H ϵ 3 and the S+2 Tyr H δ protons have significant upfield CSDs. CD spectroscopy provided confirmatory data for the conclusion that the hairpin of the S+2 Tyr mutant is less stable. Exciton couplets similar to (but smaller than) those seen in W/W EtF containing peptides were seen for W/Y peptides, but only when tryptophan was at the site with the preference for the “face” orientation.

Thus, in both peptide series, replacing the Trp that was the ‘face’ of the EtF interaction resulted in substantial fold destabilization, *e.g.* the S-2 W to Y mutation in Ac-WIpGKWTG-NH₂ is the most destabilizing one as it forces the tyrosine to act as the “face” of the EtF interaction, while the alternative W to Y mutation was well tolerated. The simplest rationale for this observation is a preference for Trp to be the “face” residue of EtF aryl/aryl interactions.

Discussion

The present study has provided additional measures of both the stabilizing effect of HFIP and of Asn to D-Pro mutations at the turn locus. The full-length hairpin systems all displayed an increase in f_{HP} that corresponds to $\Delta\Delta G_U = 5.2 \pm 1.2$ kJ/mol upon addition of HFIP (to 20 vol-%) to the medium. In both the full-length and severely truncated systems, Asn to D-Pro mutations at the turn locus are uniformly stabilizing by 2.5 - 2.9 kJ/mol. However, for some of the strand sequences with cross-strand W/W interactions, the increase in hairpin population (due to both Asn to D-Pro mutations and addition of HFIP) actually decrease the W/W exciton couplet magnitude (see Supporting Information).

The primary goal of this study was to investigate the role of aromatic side-chains, especially tryptophan, in stabilizing short β -hairpin peptides. The constructs examined displayed, in addition to W/W interactions, Lys/Trp cation- π interactions and Trp/backbone-NH interactions; the latter gave rise to upfield shifts at the NH as large as 3.42 ppm (for Ac-WIpGKWTG-NH₂ in 20% glycol at 270K). Amide NH shifts greater than 2.7 ppm were observed for constructs with -WIpG-, -WT-NH₂, and -WTG-sequences. In proteins, upfield shifts for amides i+3 to Trp are rarer than their i+2 counterparts (<http://www.bmrb.wisc.edu>); herein, we observed a 3.46 ppm upfield shift at Gly-H_N in a hairpin with a -WIpGKW- turn region in 20 % HFIP medium. This is not a typical feature of this sequence or more generally for -IpGK- turns: PWIpGKW-NH₂ and Ac-WIpGKWTG-NH₂ both display small downfield shifts (0.1 - 0.4 ppm) for the T2 Gly as do well-folded -IpGK- hairpins lacking turn-flanking aromatic residues.¹⁶ A full rationale of this unusual observation will require additional model systems.

The Lys/Trp cation- π interactions and Trp/Trp interactions also give rise to diagnostic ring current shifts. Upfield shifts at Lys H δ and H ϵ sites as large as 0.49 and 0.74 ppm have been reported previously¹³; comparable shifts were observed for constructs reported herein. Cation- π interactions require an indole ring plane oriented perpendicular to the hairpin backbone plane. Most edge-to face Trp/Trp interactions give rise to ≥ 2 ppm upfield shifts for one of the H ϵ 3 resonances, but we have also observed an alternative geometry (Figure 9C, *vide infra*) in which the H δ 1, rather than H ϵ 3, displays an upfield shift do to its proximity to the face of the other Trp ring. Thus all of the Trp interactions that occur in hairpins can be recognized by distinct chemical shift criteria. In addition the cross-strand W/W and W/Y interaction usually result in large exciton couplets in the CD spectrum.

Aryl/aryl ring exciton couplets have been recognized as contributors to the CD spectra of proteins since at least 1994.⁵⁸ Aryl/aryl geometries in proteins are of long-standing⁷ and continuing⁵⁷ interest. In both a 1999 survey¹² and a more extensive recent survey⁵⁷ of aryl/aryl interaction in proteins, Chakrabarti and co-workers, noted that EtF Trp/Trp interactions

are the rule, greatly outnumbering stacking interactions. The same survey suggested that Tyr has a strong preference for the face location in EtF F/Y interactions and modest similar preference in W/Y interactions. A W/Y EtF geometry, giving rise to an exciton couplets and a ~ 0.8 ppm upfield shift at H ϵ 3, has also been observed for a hairpin structure studied in MeOH.⁵⁹ These observations (a W/Y EtF preference) are contrary to the results obtained in the present study and to studies^{44,60,61} that indicate that Trp is a better π base than Tyr. In the protein occurrence most analogous to our HP6V W $\rightarrow$ Y mutations, the RWKYVNGRWVPG sequence in T domain of the *Brachyury* transcription factor⁶², the bold Trp is the face aryl in a near perfect EtF interaction. Cross-strand aryl/aryl interactions have also figured in hairpin design essentially from the onset; in the early work of de Alba et al.^{14,63}, which aimed to prepare hairpins with a type I β -turn; instead, 3:5 and 4:4 hairpin structures were observed, including structures with a cross-strand Tyr/Trp at non-H-bonded sites. This pairing, however, did not give rise to the diagnostic EtF pairing ring current shifts. To our knowledge, the first W/W interaction in a designed hairpin which displayed large H ϵ 3 shifts (1.6 ppm, with dimethylsulfoxide as the solvent) is due to Favre et al.⁶⁴ This W/W interaction, and those in the trpzip peptides²⁷ and other GB1 hairpin analogs^{13,28}, immediately flanks the turn of a β hairpins.

All of the highly stabilized structures prepared in the present study contain a turn-flanking Trp-Trp pair in one or another edge-to-face interaction geometry. Since there has been some disagreement with regard to the intrinsic β -propensity of Trp (and whether this alone accounts for the enhanced hairpin stability of Trp-containing hairpins), we examined the stabilizing effects of individual Trp insertions. The effects of Ala, Thr and Trp at non-H-bonding strand positions (S $\pm$ 4 and S $\pm$ 2) were compared. Thr at the non-H-bonded S $\pm$ 4 sites was found to have a β -propensity circa 1.5 kJ/mol greater than that of alanine. The cation- π interactions between the W introduced at S $\pm$ 4 and cross-strand lysines make it difficult to give a precise β -propensity value for Trp. Lys sidechain CSDs of KKLWVSINGKKIWVSA reflecting shielding due to the indole rings were observed: K2 δ (-0.36), K2 ϵ (-0.44), K11 δ (-0.54) and K11 ϵ (-0.72 ppm). The bold sites all are on the same face of the hairpin. While in-strand interactions may account for a small fraction of the shielding observed, W to A mutation effects on the shifts suggest that (S-4/S+2)-W4/K11 is the dominant interaction with less contribution from the diagonal (S-6/S+4)-K2/W13 interaction and in-strand interactions. Of these the (S-4/S+2)- π /cation interaction is the one that has appeared in numerous designed hairpins^{17,18}; however, a (S-6/S+4)-K/W interaction giving rise to 0.32 and 0.46 ppm upfield shifts at Lys H δ and H ϵ , respectively, has been reported¹². The appearance of both cation- π interactions in the S $\pm$ 4 W/W construct suggests contributions from two folded conformations in which one or the other indole rings are perpendicular to the hairpin backbone plane. The CSDs for the Trp aryl signals also reflect a mixture of EtF states with upfield H ϵ 3 shifts observed at both tryptophans. Given that individual Lys/Trp cation- π interactions appear to account for only 1 kJ/mol of hairpin stabilization in other systems, we conclude, in agreement with Cochran, that the β -propensity of Trp is larger than that of Thr by 1 - 3 kJ/mol.

Trp insertions at the S $\pm$ 2 position were even more favorable: each A to W mutation immediately flanking the INGK turn of KKLTVAINGKAITVSA was stabilizing by 5 kJ/mol (see Table II). Here also, due to the high and, thus, unquantifiable fold stability of the double mutant, it was not possible to obtain a value for the stabilization associated with the cross-strand interaction, but it was apparent that only a single EtF geometry was present, with the C-terminal Trp as the face residue. The robustness of the Trp-Trp interaction geometry made this an intriguing system to test the lower size limit of β -hairpin formation. The lower limit, greater than 75% folding for a seven residue peptide hairpin, was achieved in the case of Ac-WINGKWT-NH₂, but the truncated sequences displayed the reversed 'FtE' geometry. The strong preference for the 'FtE' orientation results in an extreme upfield

shift for H ϵ 3 of the C-terminal edge-Trp. Both the 'EtF' and FtE W/W interactions give rise to a positive CD exciton couplet. While the magnitude of the 100% folded edge-Trp H ϵ 3 CSD is relatively constant at *ca.* 2 ppm, the intensity of the exciton couplet maximum, $[\theta]_{228}$, varies widely. Both orientations can produce molar $[\theta]_{228}$ values in excess of +430,000° but in other systems our 100% folded $[\theta]_{228}$ extrapolations are as small as +290,000°. Apparently, rather subtle changes in indole ring geometry that still yield the near maximal upfield ring current shift can produce angular orientations of the transition moments that yield much smaller exciton couplets. As a result, we no longer view CD measurements alone as a basis for estimating fold populations.

Initially, we viewed the H-bonding interaction⁴⁴ of the *i*+2 amide NH with the indole ring as the primary reason for the stability of the flipped W/W interaction. However, both the observation of 'FtE' interactions in systems lacking this C-terminal H-bonding motif and the studies summarized in Table III suggest that strand length may be the primary determinant. All hairpins with two pairs of cross-strand hydrogen bonds have a strong preference for the EtF geometry originally seen in the trpzip peptides. One rationale for this would be that the normal twisted geometry of hairpins with the larger set of cross-strand H-bonds is incompatible with the FtE W/W geometry. It has been noted⁵⁴ that trpzip type systems are unusually twisted hairpins. The turn length and type are also important. In tr-HP7 (Ac-WNPATGKW-NH₂; NPATGK vs INGK turn) replacing -NH₂ with -TG-NH₂ did not change any shifts by > 0.05 ppm, the EtF Trp/Trp geometry was retained, and the terminal Gly-H_N CSD was consistent with the *i* to *i*+2 WTG interaction being present exclusively in the unfolded state.

The intermediate length hairpins in Table III and (T4W,T13W)-MrH4a, in which both Trp residues display upfield H ϵ 3 protons, present an interesting dynamic question. The observation of two upfield H ϵ 3 signals requires a folded state with both 'EtF' and 'FtE' interactions. In all such cases, one or both of the H ϵ 3 resonances is broadened significantly more than other peaks in the spectra which display net structuring shifts of similar magnitude. The exchange phenomenon responsible for the broadening is still rapid enough to yield population-weighted average δ values but the effective $\Delta\nu$ for each H ϵ 3 site is on the order of 1000 Hz (2 ppm), not the smaller value derived from the apparent CSDs. On this basis, the broadening observed suggests exchange time on the order of 2 μ s.⁵⁰ If all that was required for the EtF/FtE exchange was χ_1/χ_2 rotamer shift for the two Trp sidechains in the folded state, faster exchange would be expected. Therefore we suggest that, in intermediate-length peptides displaying evidence for both EtF and FtE interactions, the transition between the two states involves significant unfolding of the hairpin state: a process that is characterized^{48-50,65} as having low μ s time constants.

The reversal of the orientation of the tryptophan EtF interaction upon hairpin truncation shows that the aryl ring interactions responsible for stabilizing proteins and peptides are still somewhat unpredictable. Three geometries observed in the present study appear in Figure 7.

Returning to the diagnostic features of our new 'mini-hairpin' systems, Ac-WINGKWT-NH₂ and PWIpGKW-NH₂ are both 7 residue systems with C-terminal Trp H ϵ 3 CSD values in the -1.7 ppm range, with the latter owing a significant part of its stability to the pG turn locus. For Ac-WINGKWT-NH₂, Ac-WINGKWTG-NH₂ and Ac-WIpGKWTG-NH₂, the *i*/*i*-2 NH/indole-ring H-bond appears essential for hairpin folding. For Ac-WINGKWTG-NH₂ ($f_{HP} \approx 0.77$), we measured the destabilizing effects of both the W1T [$f_{HP} < 0.27$ (≈ 0.15)] and W6T ($f_{HP} < 0.19$ (≈ 0.09)) mutations (data not shown). The appearance of essentially statistical coil values for the chemical shifts of the W to T mutants was not entirely surprising. However, we also observed that the removal of the N-terminal acetyl moiety causes nearly a total loss of all indicators of folding: the CD exciton couplet, the H ϵ 3 shift,

and β -strand CSDs. This is, presumably, the result of losing at least one hydrogen bond, and the loss of the hydrophobic packing between the Ac methyl group and the indole rings. The Ac vs no-Ac effect is, in many respects, the most dramatic finding of the present study. The acetyl appears to be essential for an effective W/W interaction at the termini of the hairpin. This suggests the possibility that an Ac-W...WXG motif as a generally applicable cap for β hairpins. Efforts in this direction are in progress.

The Ac-XINGKWTG fold population data, X = Thr ($f_{HP} \approx 0.15$) versus W ($f_{HP} \approx 0.77$), also provides a measure of the stabilizing contribution of a W/W interaction. With our estimate of a 2 kJ/mol β propensity difference between Trp and Thr, an additional 4-5 kJ/mol stabilizing effect is required to rationalize the observed stability of Ac-WINGKWTG. A 4-5 kJ/mol stabilization from the favorable EtF pairing of two indole rings is line with other experimental determinations of aryl/aryl interaction energies: a mutational cycle for an S $\pm$ 2 cross-strand Trp/Trp pairing in the hPin1 WW domain revealed an additional 2.3 kJ/mol stabilization for a Trp/Trp interaction⁵¹, Serrano⁶⁶ reported a 5.4 kJ/mol stabilization of a helix by an EtF Tyr/Tyr interaction. Peptide and protein re-engineering using the cross-strand W/W interaction should be a powerful technique for producing stable structural scaffolds; the method has already been applied⁶⁷ to detect intracellular protein/protein association.

Materials and Methods

All peptides were synthesized using fast Fmoc chemistry on an ABI 433A peptide synthesizer, and were cleaved from the resin by shaking in excess TFA with 2.5% water and 2.5% triisopropylsilane for 1.5hrs. Acetylation, when required, was accomplished prior to cleavage by reacting the resin-bound peptide with 3% acetic anhydride and 4.3% triethylamine, in DMF. Peptides were precipitated and washed thrice in ether, and purified by reverse phase HPLC (C18 column) with a water (0.1% TFA)/acetonitrile (0.085% TFA) gradient. The identity and purity of the peptides were verified by positive ion mass spectrometry on a Bruker Esquire ion trap spectrometer. NMR ensured purity and proper connectivities by NOESY or ROESY spectra.

CD Spectroscopy

CD stock solutions were made by dissolving weighed amounts of peptide in 10-20 mM pH 6 potassium phosphate buffer), and the concentrations of the stock solutions were measured by the combined expectation UV absorption of Trp ($\epsilon = 5580 \text{ M}^{-1} \text{ cm}^{-1}$ per Trp) and Tyr ($\epsilon = 1280 \text{ M}^{-1} \text{ cm}^{-1}$ per Tyr) at 279 ± 1 nm. Stock solutions were diluted with buffer to yield 30 μM solutions of peptide. Spectra were recorded on a Jasco J715 spectropolarimeter using 0.10 cm path length cells. CD data are reported in molar units ($\text{deg cm}^2 \text{ dmol}^{-1}$), shown as degrees molar ellipticity throughout, rather than residue-molar ellipticity units ($\text{deg cm}^2 \text{ res-dmol}^{-1}$) because the exciton couplet, which dominates the CD spectra for most peptides, is due to a single chromophore and not the backbone amide units. The exciton couplet has a maximum at 227-229 nm, and this maximum value was used (after subtraction of the coil control value of $-11,000^\circ$ for each Trp at 228 nm) to provide a folding measure that should approach zero when the fold population of each peptide approaches zero.

NMR Spectroscopy

NMR spectra were recorded on 500, 600 cryoprobe, and 750 MHz NMR instruments with Bruker DRX, AV, and DMX consoles respectively. Solvent suppression was accomplished in all cases by a WATERGATE⁶⁸ pulse train. TOCSY spectra employed a 60 ms MLEV-17 spinlock,⁶⁹ and NOESY spectra had a 140-150 ms mixing time. Aqueous samples were comprised of 1-2 mM peptide in 20-50 mM sodium phosphate buffer, pH 6-7, with 10%

D₂O and sodium 2,2-dimethyl -2-silapentane-5-sulfonate (DSS) as an internal standard. Glycol was added to some samples to increase the tumbling time of the smaller peptides and provide access to lower temperatures; these samples were identical to the aqueous samples except that they contained 20% deuterated ethylene glycol.

All resonances displayed sharp signals: line widths consistent with the tumbling times of monomeric peptides. NMR fold population measures are corroborated by CD fold population measures taken at 20 μM, additional support for monomeric behavior of all peptides discussed.

NMR Structure Ensemble Elucidation

NOESY spectra for NMR ensemble generation of (S5I)-HP6V was acquired on a 750 MHz NMR at 280K with a mixing time of 140ms. The Ac-WINGKWTG-NH₂ NOESY spectra were taken on a 500 MHz NMR with a mixing time of 150ms. A range of mixing times from 50-250ms was examined to investigate potential secondary NOEs. Ethylene glycol was added to reduce the tumbling time and access a lower temperature range. NOE intensities were converted to distant ranges using an in-house program which corrects for multiple chemically equivalent protons and sharp aromatic peaks.^{70,71}

NMR structure generation and acceptance criteria were described previously¹³. No structures with NOE constraint violations greater than 0.28 Å were included in the accepted ensembles. Ensemble statistics for both structures are listed in Table V with lists of the NOE constraints appearing in the Supporting Information. MolMol72 was used to calculate RMSD values. The CNS-generated structures were subjected to 500 steps of steepest-descent minimization using the SANDER application of AMBER6, since some bond lengths and angles are parameterized differently by CNS and the structure analysis algorithm used by the Research Collaboratory for Structural Bioinformatics (RCSB-PDB). This minimization has no significant effect on the NOE-constraint deviations.

When this manuscript was submitted, the PDB was not accepting structures of polypeptides less than 24 residues in length; the final structure ensembles (WP and (S5I)-HP6V) are hosted on our website at <http://andersenlab.chem.washington.edu/structures> and the full chemical shift assignments (including ¹³Cα and ¹³Cβ in the case of peptide WP) appear in the Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BMRB	Madison Biological Magnetic Resonance Data Bank
CD	circular dichroism
CSD	chemical shift deviation
CSDb	an in-house chemical shift data base

EtF (edge-to-face) and FtE (face-to-edge) refer to aromatic rings in a T relationship rather than parallel alignment	the first designation (E or F) refers to the aromatic ring plane of the residue with the lower sequence number
GB1p	the isolated peptide corresponding to the C-terminal hairpin of the B1 domain of protein G
HFIP	1,1,1,3,3,3-hexafluoro-2-propanol
HP5	HP6, HP7 designations for series of designed hairpin peptides
MrH (Met-repressor-inspired hairpin)	designed hairpins based on the β -sheet interface of the dimeric β -sheet motif of the Met repressor
NMR	nuclear magnetic resonance
PD (parallel displaced)	a stacking relationship between aromatic ring planes
PDB	protein data base.

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Figure 1.
Nomenclature for residue positions in a β -hairpin with a tight (4-residue) β -turn.

Figure 2.

A: the temperature dependent CD spectra of AWSNGKW. B: CD melting curves based on the observed ellipticity at 228 nm (the exciton couplet maximum): the site of mutation in HP6V and HP6-tr is shown on the figure. The G5A mutant of HP6-tr serves as nearly unfolded control.

Figure 3. Chemical shifts for the S-2 and S+2 tryptophans in HP6V and HP6-tr at 280K: upon truncation the upfield chemical shifts are seen at the S+2 site. NOESY connectivities that validate the sequential assignments of the Trp residues are given in the Supporting Information.

Figure 4.
TOCSY spectrum segments showing the upfield G8H_N (right panel) and S+2 WHe3 (left panel) signals, taken in this case from Ac-WI**p**GKWTG-NH₂, at 280K in H₂O.

Figure 5.

Overlay of NMR structures for peptides Ac-WINGKWTG-NH₂ (Blue) and KYVWINGKWTVE (Red), the C-terminus of each peptide is labeled. The degree of convergence within each ensemble is illustrated in panels A and B of Figures S5.

Supporting Information Available: Chemical shift deviations used for %-fold calibrations, NOE derived distance constraints, and peptide chemical shift assignments of structures described in this work, as well as supporting figures of CD melts and CSD distributions across sequences.

Figure 6.

Left scale - fractional CSD versus temperature for diagnostic chemical shifts in TWINKWTG, a well-folded peptide that displays apparent non 2-state behavior. For this plot, CSDs at 280K are set to 1. Right scale - the numerical values of the Gly-H_N CSD of TWINKWTG and Ac-WTG. On warming, the CSD of the G8 amide proton approaches the CSD of the G3 amide proton in the tripeptide Ac-WTG.

Figure 7.

A: tryptophan EtF interaction (red) and “flipped” FtE interaction (green). They represent simple χ_1 rotamer changes, and are essentially simple rotations of each other (as illustrated in figure B) though the orientation of the Trp indole rings in relation to the rest of the peptide changes dramatically (See Figure 5.) Panel C: the alternative “turned” EtF interaction seen in KKLTVW-IpGK-WITVSA is a χ^2 rotamer shift for the “edge” Trp residue. This results in large ring current shifts on the H δ 1 proton instead of the H ϵ 3 proton, and a complete loss of the exciton couplet at ~ 228 nm.

Table I

Representative sequences with turn flanking aryl/aryl interactions

name	sequence	T_m (°C)
trpzip2	SWTW- ENGK -WTWK-NH ₂	72 ^a
HP6V	KYVW- SNGK -WTVE	58 ^b
GB1p	GEWTY-DDATKT-FTVTE	1 - 9 ^c
trpzip4	GEWTW-DDATKT-WTWTE-NH ₂	70 ^{a,d}
HP5W4	KKWTW-NPATGK-WTWQE	85 ^b
HP7	KTW-NPATGK-WTE	66 ^b
tr-HP7	Ac-W-NPATGK-W-NH ₂	7 ^b

^aData reported by Cochran et al.²⁷^bData reported by Andersen et al.¹³^c T_m estimates using multiple NMR probes⁵⁰; the most recent estimate from another laboratory is 1.3 °C ³².^dA stabilizing role for W14 was suggested by a 26° drop in the T_m upon substitution by valine²⁷, a residue that is generally considered to have a similar or even greater β -propensity.

Table IIMutational changes in ΔG_F (kJ/mol) for hairpin formation in MrH4b and MrH4a mutants

	ΔG_F^{280}	ΔG_F^{300}	$\Delta\Delta G_F^{280}$ measures for mutations
MrH4b (water)	0.18	-0.32	+2.76(T4A),+1.88(T13A),-2.8 (T4W,T13W), -2.6 (T4W,T13W) @300K
(20% HFIP)	-3.51	-4.27	+1.3 (T4A @300K)
T4A (water)	2.94	1.90	
T13A (water)	2.06	1.09	
T4A,T13A (water)	3.99	3.22	-1.92 (A4T), -1.05 (A13T)
T4A,T13A (20%HFIP)	-3.46	-3.22	
MrH4a (water)	2.81	2.18	+2.1(T4A),-0.3(T4V),+1.5(T13A),-0.5(T13W),-4.3(T4W,T13W),<-5.8(S6W,K11W)
MrH4a (8% HFIP)	-1.06	-2.9	
T4W,T13W (water)	-1.52	-1.76	+2.9 (W4A), +1.1 (W13A), +4.1 (W13G)
T4W,T13W(20%HFIP)		-4.3	
T13A (water)	4.34		-4.7 (T4W)
S6A,K11A (water)	5.0	4.25	-5.5(A6W),≈-5.2(A11W),<-8 (A6W,A11W)
(20% HFIP)		-2.7	

Table III

Effects of Termini Changes on the NMR Structuring Diagnostics of -WIZGKW-^a

Sequence	Turn G $\Delta\delta\text{H}\alpha$	Hairpin $\langle\text{frCSD}\rangle^b$	CSD (ppm)		
			S-2 W H α 3	S+2 W H α 3	S+4 HNg
Ac-WINGKW-NH ₂	0.287	≤ 0.45 (~ 0.42 CD)	-0.378	-0.738	n. a.
Ac-TWINGKW-NH ₂	0.278	≤ 0.30 (~ 0.1 CD)	-0.570	-0.384	n. a.
PWlpGKW-NH ₂	n.a. ^c	0.79 ± 0.11	-0.269	-1.856	n. a.
Ac-WINGKWT	0.284	≤ 0.45	-0.193	-0.603	n. a.
Ac-WINGKWT-NH ₂	0.393	0.77 ± 0.04	-0.380	-1.680	-2.7 ^d
Ac-WIPGKWTG-NH ₂	n.a. ^c	$< 0 >$	-0.05	0.05	-1.08
Ac-WlpGKWTG-NH ₂	n.a. ^c	0.95 ± 0.03	-0.25	-1.87	-3.06
Ac-WINGKWTG-NH ₂	0.404	0.78 ± 0.04	-0.379	-1.556	-2.71
WINGKWTG	0.079	0.15 ± 0.10	-0.05	-0.11	-1.11
TWINGKWTG	0.469	0.81 ± 0.10	-0.407	-1.923	-0.48
Ac-TWINGKWTG-NH ₂	0.454	0.69 ± 0.08	-0.528	-1.620	-1.05
ATWINGKWTG	0.519	$\cong 0.78$	-1.354	-0.758	+0.11
KAVWINGKWTVE	0.589	$\cong 0.85$	-1.922	-0.280	+0.55

^aCD melts for 7 of the peptides listed, including ATWINGKWTG (which has a mixed W/W geometry with the EtF form predominating) appear in a supporting Figure.

^bWe employ the CSDs at six backbone sites in the common WIZGKW sequence to estimate the hairpin population; the backbone chemical shifts are given in full in the Supporting Information. The reported $\langle\text{frCSD}\rangle$ values are the average (and standard error) of the fHP values derived from these sites assuming that Ac-WIPGKWTG-NH₂ represents zero folding and that the best folded species (Ac-WlpGKWTG-NH₂) represents fHP = 0.98. This measure does not apply to the longer hairpins in the last two entries

^cThe turn Gly $\Delta\delta\text{H}\alpha$ measure does not apply to Pro-Gly turns 16 and appear to be of diminished magnitude in NG species with the FIE W/W interaction. 100% folded values for Gly $\Delta\delta\text{H}\alpha$ of NG turn hairpins from the literature are 0.55-0.72 ppm. 16:27-33:34

^dCSD increases to -3.26 ppm at 270K in 20% glycol. with a proportionate increase in the S+2 H α 3 structuring shift.

Table IV

The Effects of Trp to Tyr Mutations

Sequence	Turn G	Hairpin	CSD (ppm)				
WP analogs	$\delta\Delta H\alpha$	f_{HP} based on $<CSD>$	S-2 W/Y H ϵ 3/H δ	S-2 H β 3	S+2 W/Y H ϵ /H δ	S+2 H β 3	S+4 H N_E
Ac-WIpGKWTG-NH ₂	n. a.	0.95 $\pm$ 0.03 ^a	-0.38	-0.15	-1.87	-1.44	-3.07
Ac-YIpGKWTG-NH ₂	n. a.	0.62 $\pm$ 0.06 ^a	-0.11 ^c	-0.31 ^c	-0.89	-0.26	-1.93
Ac-WIpGKYTG-NH ₂	n. a.	0.86 $\pm$ 0.05 ^a	-0.21	-0.20	-1.05 ^c	-1.79 ^c	-1.78
HP6V analogs							
KAVWINGKWTVE	0.589	0.85 ^b	-1.922	-1.54	-0.280	-0.26	+0.55
KAVYINGKWTVE	0.504	$\approx$ 0.76 ^b	-1.324 ^{c,d}	-2.05 ^c	-0.240	-0.14	+0.55
KAVWINGKYTVE	0.445	$\approx$ 0.33 ^b	-0.702	-0.14	-0.562 ^c	-0.95 ^c	+0.23

^a Fraction folded estimates for the WP peptide analogs are based on the fractional CSDs observed for the applicable subset of the 1/2/5/6 α and 2/6N backbone sites.

^b CSDs used for (Y2A,S5I)-HP6V series were those for the 2 α , 11 α , 3N, 8N, and 10N sites. Due to changes in ring current effects these are viewed as approximate values.

^c When the edge aryl is tyrosine, the beta proton shifts are expected to be proportionally greater than delta proton shifts. This is due to the averaging of the two δ sites, only one of which can be close to and in the shielding cone of the indole ring.

^d The 1.32 ppm ring current shift for the averaged Tyr H δ 's is remarkably large. It is accompanied by a very large upfield shift for one the Tyr H β resonances (H β 3). These values in themselves imply a large folded population.

Table V**NMR Structure Ensemble Statistics**

A. Structure Generation Summary			
		(S5I)-HP6V	WP
Distance Constraints			
	total	118	106
	intraresidue	54	52
	sequential	24	21
	$i/i+n$ ($n = 2-4$)	6	16
	$i/i+n$ ($n \geq 5$)	28	17
Ensemble Statistics (with standard deviation)			
	accepted/random starts	30 / 50	47 / 50
	NOE Distance viol RMSD	0.041±0.006	0.043±0.010
	E _{NOE} (kcal/mole)	7.03±2.63	18.71±2.00
	E _{LJ} (kcal/mole)	-26.46±3.41	-28.01±1.84
	Bond RMSD Å	0.003±0.000	0.005±0.000
	Angles RMSD (deg)	0.481±0.016	0.476±0.078
	Improper RMSD (deg)	0.201±0.027	0.337±0.080

B. Intra-ensemble RMSD Values (Å)		
	(S5I)-HP6V	WP
Backbone	0.40±0.16	0.17±0.07*
All heavy atoms	1.37±0.29	0.84±0.30
Turn backbone	0.11±0.10	0.07±0.04
Turn heavy atoms	0.60±0.34	0.11±0.06
Indole rings	0.13±0.07	0.06±0.03

* does not include the terminal Gly (G8) carbonyl carbon (with this atom: 0.37 ± 0.20).