Turn Residue Sequence Determines β-Hairpin Conformation in Designed Peptides

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Abstract: A series of linear peptides designed to fold into different β-hairpin conformations in aqueous solution has been studied by ^{1}H NMR with the aim of understanding the role played by the turn residue sequence in defining β-hairpin structure. The designed peptides differ only in the amino acid sequence of the putative turn region and have identical strand residues. Our results clearly demonstrate that the turn residue sequence determines the turn conformation and, thereby, other features of the β-hairpin conformation, such as the pattern of interstrand residue pairing and the type of hydrogen-bonding register between β-strand backbone atoms. Furthermore, two key structural factors responsible for the stability of different types of turns were identified. Thus, a side-chain−side-chain interaction between Asn at position *i* and Thr at position *i* + 4 stabilizes five-residue turns, whereas a four-residue turn is stabilized when the first residue of the turn has high tendency to populate the α_R region of the Ramachandran map. Our results highlight the relevance of turn structures in the early events of protein folding.

Introduction

One of the proposed mechanisms by which a polypeptide chain folds into its native three-dimensional structure includes secondary structure formation in the early steps of the folding reaction.[1] It is therefore important to know the factors which stabilize the different elements of secondary structure in order to gain insights into the protein folding problem. The conformational study of designed peptides or protein fragments, where tertiary interactions are absent, has been exploited to obtain information on monomeric α-helix formation and stability.[2] However, similar data related to monomeric β-sheet or β-hairpin formation are scarce because β-sheet-forming peptides often have a strong tendency to aggregate. The search for models of β-sheet or β-hairpin formation led to peptides containing nonnatural amino acids at the turn region[3] or to nonpeptide scaffolds that bring the β-strands together.[4] Examples of linear peptides that contain only natural L-amino acids in their sequence and that fold into monomeric β-hairpin conformations in aqueous solution have been only very recently reported. Apart from the one described by Blanco et al.[5] which is a fragment of a native protein, the others are designed peptides.[6−10]

A β-hairpin is the simplest form of an antiparallel β-sheet conformation and is defined by a loop region flanked by two β-strands hydrogen-bonded by their corresponding backbone CO and NH groups. β-Hairpins are classified as hairpins *X*:*Y* according to the following: *X* and *Y* are the number of residues at the loop and *X* equals *Y* when the distal strand residues form two backbone hydrogen bonds, whereas *Y* = *X* + 2 if they form only one.[11a] On the other hand, in relation to the backbone dihedral angles, turn conformations are referred to by numbered types I to VI[11b] and their corresponding mirror images denoted by primed Roman numbers. It is important to note that changes of the loop length are mirrored in changes of β-sheet registration and that only certain types of turn are compatible with each β-hairpin conformation. From an experimental point of view, it is also important to consider that main experimental evidence to characterize a given β-hairpin conformation is the NOE cross-correlation between the CαH protons in interior face-to-face residues. It is the strong turn-sheet correlation which makes it possible to determine the actual turn conformation.

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(11) (a) The different types of β-hairpin conformations and turns connecting the two strands in each β-hairpin were named according to the classification proposed by: Sibanda, B. L.; Thornton, J. M. *Methods Enzymol.* **1991**, *202*, 59−82. Sibanda, B. L.; Blundell, T. L.; Thornton, J. M. *J. Mol. Biol.* **1989**, *206*, 759−777. The β-hairpin shown in Figure 2a is a β-hairpin 2:2 because it contains two residues at the loop region, and the distal strand residues, S17 and Y20, have two hydrogen bonds, while the β-hairpin shown in Figure 2b is a β-hairpin 3:5 because it has three residues at the loop region and the distal strand residues, N17 and S21, have only one hydrogen bond, and the β-hairpin shown in Figure 2c is a β-hairpin 4:4 since it contains four residues at the loop region and the distal strand residues, S3 and X8, have two hydrogen bonds. (b) Classification of β-turn conformations according to the backbone dihedral angles is given in Richardson, J. S. *Adv. Protein Chem.* **1981**, *34*, 167−339. Wilmot, C. M.; Thornton, J. M. *J. Mol. Biol.* **1988**, *203*, 221−232.

Tendamistat 15-23		Y	Q	**S**	**W**	**R**	**Y**	S	Q	A
Peptide 1		Y	Q	**N**	**P**	**D**	**G**	S	Q	A
Peptide 2	I	Y	S	**N**	**P**	**D**	**G**	T	W	T
Peptide 3	I	Y	S	**N**	**S**	**D**	**G**	T	W	T
Peptide 4	I	Y	S	**N**	**S**	**D**	**G**	V	W	T
Peptide 5	I	Y	S	**A**	**P**	**D**	**G**	T	W	T
Peptide 6	I	Y	S	**A**	**K**	**A**	**G**	T	W	T
Peptide 7	I	Y	S	**Y**	**N**	**G**	**K**	T	W	T

Figure 1. Peptide sequences. Turn residues are shown in bold. The segment 15−23 of Tendamistat is a β-hairpin 2:2 with a type I β-turn in the native protein. Peptides 1−3 were reported to adopt β-hairpin conformations in aqueous solution.[6,8,10] Peptides 4 and 5 present point mutations relative to peptides 3 and 2, respectively. The complete β-turn sequence was substituted by a natural type I β-turn sequence in peptide 6 and by a sequence designed to form a type I′ β-turn in peptide 7.

The first reported β-hairpin-forming peptide[6,10] (peptide 1 in Figure 1) conserves the β-strand residues of the native β-hairpin formed by the 15−23 segment of the protein Tendamistat,[12] while the chain-bend residues, NPDG, were selected to have maximum probability of being in each one of the four positions of a type I β-turn,[13,14] which is the one adopted in the native structure. By analysis of NMR data we were able to show that peptide 1 forms a β-hairpin 3:5 containing a five-residue type I + G1 bulge turn (Figure 2b)[11] instead of the native β-hairpin 2:2 with a type I β-turn (Figure 2a). As a consequence, the pattern of facing residues in the designed β-hairpin does not coincide with the one in the native structure (Figure 2a and b). Peptide 1 has been used by other authors as a test peptide to develop methods of analyzing dynamically averaged structures by using ensemble-averaged constraints.[15,16] Similar shiftings in the β-strand register arising from changes in the turn type have been observed in other designed peptide, a 16-residue fragment of the N-terminal sequence of ubiquitin, in which the native five-residue turn, being a type I + G1 bulge, was substituted by the sequence NPDG with the aim of obtaining a type I β-turn conformation.[7] The NMR data revealed that as occurred in peptide 1 the chain-bend region adopts a new type I + G1 bulge turn, which leads to a β-sheet registration different from the native one.[7] We had also shown that the fully designed peptides 2 and 3[8] (Figure 1), differing only in the residue at the second position of the turn, folded as two β-hairpin structures co-existing in fast equilibrium, with the fraction of the β-hairpin 3:5[11] predominating over the β-hairpin 4:4 in peptide 2 and the two β-hairpin structures being equally populated in peptide 3 (Table 1). As seen in Figure 2c and d, β-hairpin 4:4 and 3:5 differ in both the turn conformation and the β-sheet registration. The P5S substitution appears to provide sufficient conformational freedom to the chain-bend region to stabilize two turn conformations, a four-residue type I β-turn in a β-hairpin 4:4 and a five-residue type I + G1 bulge turn in a β-hairpin 3:5.

These results suggest that the turn amino acid sequence affects the turn conformation and consequently the β-sheet registration

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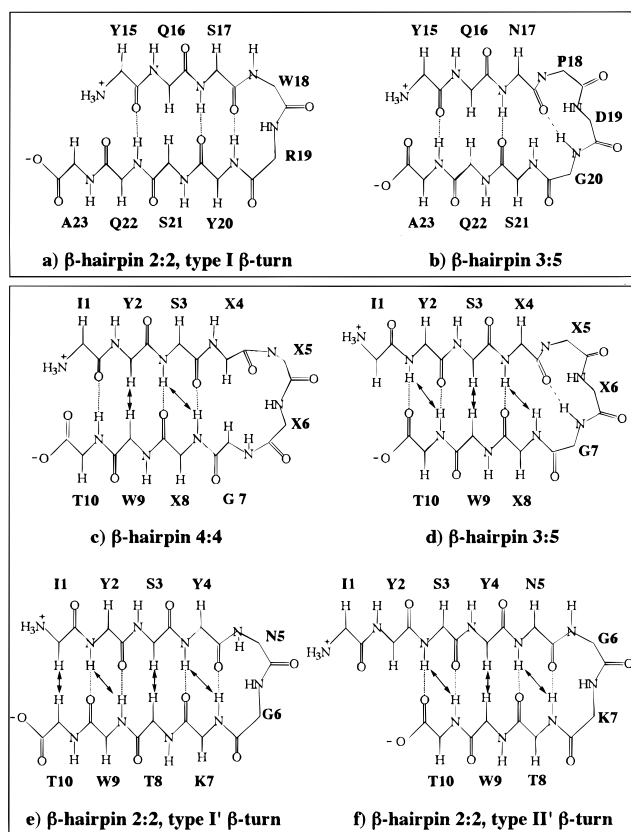
Peptide	X4	X5	X6	X8
2	N	P	D	G	T
3	N	S	D	G	T
4	N	S	D	G	V
5	A	P	D	G	T
6	A	K	A	G	T

Figure 2. Schematic representation of the peptide backbone conformations: (a) the β-hairpin 2:2 with a type I β-turn corresponding to region 15−23 of native Tendamistat.[12] (b) β-hairpin 3:5 with a type I β-turn + G1 bulge adopted by peptide 1 in aqueous solution.[6] (c) β-hairpin 4:4 formed by peptides 2,[8] 3,[8] 4, 5, and 6. (d) β-hairpin 3:5 adopted by peptides 2,[8] 3,[8] 4, and 5. (e) β-hairpin 2:2 with a type I′ β-turn adopted by peptide 7. (f) β-hairpin 2:2 with a type II′ β-turn formed by peptide 7. The amino acids corresponding to X in peptides 2−6 are given in the inset. The dotted lines indicate the β-sheet hydrogen bonds and the black arrows the normally observed long-range NOEs. Note that in the top frame the sequences of peptides in the left and right side are different.

of the hairpin, overriding even the eventual stabilization provided by favorable interstrand side-chain interactions present in alternative conformations. With the aim of checking if this hypothesis is well-founded and of establishing the effect of turn residue sequence on the final β-hairpin conformation, we describe in this ^{1}H NMR study the conformational properties of a series of peptides (peptides 4 to 7 in Figure 1), having the same β-strand residues as peptides 2 and 3, but with partial or completely different residue sequences in the turn region. Our results underline the importance of turn residue sequence in defining the β-hairpin conformation and provide the basis to identify key structural factors leading to the different β-hairpin conformations.

Materials and Methods

Peptide Synthesis and Purification. Peptides were chemically synthesized by stepwise solid-phase procedures using pentafluorophenyl esters of fluorenylmethoxycarbonyl amino acids and 1-hydroxybenzotriazole as catalyst for the coupling reaction.[17] Peptides were purified

Table 1. Populations[a] of the β-Hairpin Conformations Formed by Peptides 2−7 in D_2O Samples at pH 6.3 and 2 °C and Intensities[b] for the Backbone NOEs Characteristic of β-hairpin 3:5, β-Hairpin 4:4, and β-Hairpin 2:2 Conformations Observed in D_2O Samples at pH 6.3 and 2 °C and in Aqueous Solution at pH 4.3 and 5 °C for NOEs Involving Amide Protons

	peptide					
estimated population[a]	2[c]	3[c]	4	5	6	7
% β-hairpin 3:5	moderate	moderate	low	low	−	−
% β-hairpin 4:4	barely detected	moderate	moderate	low	moderate	−
% β-hairpin 2:2 type I′ turn	−	−	−	−	−	relatively high
% β-hairpin 2:2 type II′ turn	−	−	−	−	−	relatively high
NOEs characteristic of						
β-hairpin 3:5 (Figure 2d)						
NH2−NH 10	m	m	−	w	−	−
CαH3−CαH 9	m	m	w-m	w-m	−	−
NH4−NH 8	m	m	−	−[d]	−	−
β-hairpin 4:4 (Figure 2c)						
CαH2−CαH 9	vw	m	m	w-m	m	−
NH3−NH 8	−[d]	w	−	−	w-m	−
β-hairpin 2:2 type I′ turn (Figure 2e)						
CαH1−CαH 10	−	−	−	−	−	m
NH2−NH 9	−	−	−	−	−	w-m
CαH3−CαH 8	−	−	−	−	−	m
NH4−NH 7	−	−	−	−	−	s
β-hairpin 2:2 type II′ turn (Figure 2f)						
NH3−NH 10	−	−	−	−	−	m
CαH4−CαH 9	−	−	−	−	−	s

[a] The populations were estimated as explained in text[7−9] and classified into the following qualitative ranges: barely detected <5%; low, 5−20%; moderate, 20−35%; relatively high, 35−50%. Nondetected β-hairpins are indicated by a bar. [b] NOE intensities are classified as strong, s; medium, m; intermediate between weak and medium, w-m; weak, w; and very weak, vw. A bar indicates an unobserved NOE. [c] Data taken from de Alba et al.[8] [d] Overlaps with other signals.

by reverse phase fast protein liquid chromatography (FPLC) with gradients of acetonitrile containing 0.1% trifluoroacetic acid. Peptide purity and identity were checked by FPLC and by the complete assignment of the ¹H NMR spectra, respectively.

Sedimentation Equilibrium. Sedimentation equilibrium experiments were performed to obtain the average molecular weight of peptide samples at the concentrations used in the NMR experiments apart from peptide 7 for which a 5 mM sample was used. Peptide samples (30 μL) were centrifuged at 40 000 rpm at 278 K in 4-mm double-sector Epon charcoal centerpieces, using a Beckman Optima XL-A ultracentrifuge with a Ti60 rotor. Radial scans were taken at different wavelengths every 2 h until equilibrium conditions were reached. The data were analyzed using the program XLAEQ from Beckman. The partial specific volumes of the peptides at 5 °C were calculated on the basis of their amino acid composition and corrected for temperature.[18] They were 0.714 mL/g for peptide 4, 0.719 mL/g for peptide 5, 0.741 mL/g for peptide 6, and 0.722 mL/g for peptide 7.

¹H NMR Spectra. Peptide concentrations for NMR experiments were 2 mM for peptide 4, 5 mM for peptides 5 and 6, and 15 mM for peptide 7 in 0.5 mL of H_2O/D_2O (9:1 ratio by volume). pH values were measured with a glass microelectrode and were not corrected for isotope effects. The temperature of the NMR probe was calibrated using a methanol sample. Sodium [3-trimethylsilyl-*2,2,3,3-²H*]propionate (TSP) was used as an internal reference. The ¹H NMR spectra were acquired on a Bruker AMX-600 pulse spectrometer operating at a proton frequency of 600.13 MHz. One-dimensional spectra were acquired using 32K data points, which were zero-filled to 64K data points before performing the Fourier transformation. Phase-sensitive two-dimensional correlated spectroscopy[19] (COSY), total correlated spectroscopy[20] (TOCSY), nuclear Overhauser enhancement spectroscopy[21,22] (NOESY), and rotating frame nuclear Overhauser effect

spectroscopy[23,24] (ROESY) spectra were recorded by standard techniques using presaturation of the water signal and the time-proportional phase incrementation mode. A mixing time of 200 ms was used for NOESY and ROESY spectra. TOCSY spectra were recorded using an 80-ms MLEV 17 spin-lock sequence.[25] Additional NOESY and ROESY spectra were recorded on peptide samples in pure D_2O to facilitate the observation of the CαH−CαH NOE crosspeaks close to the water signal. Acquisition data matrices were defined by 2018 × 512 points in t_2 and t_1, respectively. Data were processed using the standard UXNMR Bruker programs on a Silicon Graphics computer. The 2D data matrix was multiplied by a square-sine-bell window function with the corresponding shift optimized for every spectrum and zero-filled to a 4K × 2K complex matrix prior to Fourier transformation. Base-line correction was applied in both dimensions.

Estimation of β-hairpin population was performed from the intensity of the CαH−CαH NOE characteristic of each β-hairpin, taking as reference the intensity of an intraresidue CαH−Cα′H Gly NOE as previously described.[7−9] NOE intensities were measured by integration in ROESY spectra (100-ms mixing time) recorded in pure D_2O samples. In peptides 4 and 6 where integration of the CαH−Cα′H Gly NOE crosspeak was hindered by its closeness to the diagonal, the intraresidue CαH−CβH NOE crosspeak of Trp9 was used instead. Its intensity was calibrated according to the population obtained for peptide 3 using the intraresidue CαH−Cα′H Gly NOE.

Structure Calculations. Because of the presence of conformational averaging in linear peptides, the use of intraresidue and sequential NOEs can complicate the calculation of singular structures existing in the ensemble. For this reason, only medium- and long-range NOE crosspeaks were considered in the calculation of β-hairpin conformations. Their intensities were evaluated in a qualitative way as strong, medium, weak, and very weak, and used to obtain upper limit distance constraints. Pseudoatom corrections were added where necessary. φ angles were constrained to the range 0° to −180° except for Gly and Asn. For those residues with $^3J_{CαH−NH}$ coupling constants greater than 8 Hz, φ angles were further restricted to the range −160 to −80.

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Structures were calculated on a Silicon Graphics Indigo computer using the program DIANA.[26]

Results

Peptide Design. The sequences of peptides 4−7, shown in Figure 1, were designed to investigate the effect of turn residue sequence on the β-hairpin forming properties of peptides. The general design criterion was that of changing the turn residues while retaining the β-strand residues of the already reported peptides 2 and 3.[8]

The change of T8 to V in peptide 4 relative to peptide 3 was performed to destabilize the β-hairpin 3:5. Peptide 3 forms two different, nearly equally populated β-hairpin structures, one having a five-residue type I + G1 bulge turn (β-hairpin 3:5, Figure 2d) and the other having a four-residue type I turn (β-hairpin 4:4, Figure 2c). One factor responsible for the stabilization of the type I + G1 bulge turn may be a hydrogen bond between the side chains of residues N4 and T8, which are in close proximity in the calculated model structures of peptide 2.[8] Furthermore, statistical data indicate that such an interaction is very favorable in an antiparallel β-sheet.[27] Removal of this interaction by a T8 to V substitution would be expected to destabilize the type I + G1 bulge turn and consequently the β-hairpin 3:5. We chose Val because of its high β-strand propensity[28−31] and because it lacks any hydrogen bond forming group in its side chain, while retaining the size and topology of Thr.

The substitution of N4 to A in peptide 5 relative to peptide 2 was selected to stabilize the β-hairpin 4:4 structure. According to Sibanda and Thornton,[11a] the first residue in a type I + G1 bulge turn of a β-hairpin 3:5 is in the β region of the Ramachandran map, whereas the same residue in a type I turn of a β-hairpin 4:4 is in the α_R region. Thus, a residue with α_R propensity, such as Ala,[32] should favor a β-hairpin 4:4. This N4 to A change also eliminates the N4−T8 interaction which could stabilize the type I + G1 bulge turn (see above). Thus, the sequence of peptide 5 contains two features potentially destabilizing the type I + G1 bulge turn and one that may favor the type I turn required for β-hairpin 4:4. Peptide 2 was selected as the reference basis for the N4 to A substitution because this peptide is the one forming the lowest population of β-hairpin 4:4[8] (Table 1), so that a better control of the population change of that species could be performed, should our hypothesis prove correct.

The chain-bend region in peptide 6 is taken from the type I β-turn formed in the 18−35 fragment of Bovine Pancreatic Trypsin Inhibitor (BPTI), a four-residue turn β-hairpin (β-hairpin 4:4), while the previously β-strand residues are retained. The purpose of this substitution is to get a peptide that adopts uniquely a β-hairpin 4:4 conformation (Figure 2c). This native turn was selected because the corresponding β-hairpin of BPTI is the only secondary structure that persists in all the interconverting molten globule states formed by a variant of BPTI.[33] Furthermore, the N−T interaction is no longer possible in this peptide, and Ala, the residue in the first position of the turn,

has a high tendency to populate the α_R region of the Ramachandran map, factors that may destabilize the β-hairpin 3:5 and stabilize the β-hairpin 4:4, respectively. In addition, it contains a Gly in the position $i + 3$, which is present in most β-hairpin 4:4 conformations with a type I β-turn.[11] The strand sequences of peptide 6 are compatible with various patterns of interstrand side-chain interactions, and so they can accommodate different types of turn conformations.[8] Thus, if we find that peptide 6 folds into a unique β-hairpin 4:4 conformation (Figure 2c), we could plausibly conclude that one unique β-hairpin conformation is formed owing to the preference of the chain-bend sequence.

With the objective of obtaining a type I′ turn (the one most commonly found in β-hairpin 2:2 structures in proteins[11a]), the amino acids in the chain-bend sequence of peptide 7 were selected according to the simplest approach: that of having the highest individual probability for being in each position of a type I′ turn structure.[14] It is to be noted that the four selected amino acids have high statistically significant probability.[14] The β-hairpin 2:2 structure (Figure 2e) differs in side-chain interaction pattern and backbone hydrogen bond register from β-hairpin 3:5, and has a similar interstrand side-chain−side-chain interaction pattern but a totally different hydrogen bond register from that in β-hairpin 4:4 (Figure 2c). Therefore, if peptide 7 forms the expected β-hairpin 2:2 conformation, it will provide us the clearest evidence showing the importance of the turn conformation in dictating β-hairpin structure and that the interstrand side-chain−side-chain interactions do not have a major influence in β-hairpin backbone conformation.

Aggregation Test. One-dimensional ^{1}H NMR spectra were recorded for a very dilute sample (about 0.1 mM peptide concentration) and at the peptide concentration used for the subsequent NMR analysis (2−15 mM depending on the peptide solubility). The absence of any appreciable change in either line widths or chemical shifts implies the absence of aggregation in peptides 4−7.

To further ensure that monomeric states of the peptides were being examined, sedimentation equilibrium experiments were performed at the peptide concentration used for the NMR study, except for peptide 7 for which sedimentation equilibrium samples were 5 mM and NMR samples 15 mM. The average molecular weight obtained for peptides 4−7 correspond to monomeric peptides.[34]

^{1}H **NMR Analysis.** The ^{1}H NMR spectra of peptides 4−7 in aqueous solution at various pH values were assigned using the standard sequential assignment procedure.[35,36] The chemical shifts of the proton resonances for the four peptides in aqueous solution at pH 4.3 and 5 °C are available as Supporting Information (Tables SM1−4).

Evidence for the β-hairpin structures adopted by each peptide was provided by several NMR parameters, such as long-range nuclear Overhauser effects (NOE) and CαH conformational shifts (deviation of the chemical shift values with respect to those in random coil peptides). NOE data, which greatly depend on interproton distances, give the most sound and straightforward structural information. Since the different classes of β-hairpin differ in the type of loop and in the β-sheet registration, and the changes in β-sheet registration lead to different patterns of main-chain NOE connectivities, the NOE pattern allows the

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discrimination among the various classes of β-hairpin. The most characteristic main-chain NOEs for each β-hairpin conformation are shown in Figure 2. Thus, β-hairpin 4:4 (Figure 2c) is the only one that has an CαH2−CαH9 NOE, β-hairpin 3:5 (Figure 2d) the only one with an CαH3−CαH9, β-hairpin 2:2 (Figure 2e) the only one with CαH1−CαH10 and CαH3−CαH8 NOEs, and β-hairpin 2:2 (Figure 2f) the only one with an CαH4−CαH9 NOE. The NH*i*−NH*j* NOE connectivities also discriminate among the different β-hairpins (Figure 2 and Table 1). Once the class of β-hairpin conformation is identified, only a limited number of turn conformations is possible. The differences on the pattern of NOE restrictions that allow discrimination between different types of β-turn are quite subtle. So it is not surprising that the experimental NOE restrictions in the turn region are not enough to unambiguously define the β-turn type, a problem which is also found in structures of proteins in solution. Therefore, the assignment to a given turn type is based on the class of β-hairpin, which limits the number of possible turn conformations, and on the most probable turn sequence.[11a] The backbone conformation also influences the chemical shift value, in particular, the CαH conformational shifts which are negative for residues adopting angles characteristic of the α_R conformation (turns and helices) and positive for those located in β-strands.[37,38] Nevertheless, the interpretation of conformational shifts in terms of secondary structure must be considered with caution since ring current effects from aromatic residues can mask the conformational shifts.

Once the existence of a given β-hairpin conformation is demonstrated, the population of such β-hairpin structure is estimated from the NOE intensities of the interstrand Cα*i*H−Cα*j*H NOEs, using the CαH−Cα′H NOE of Gly as a reference.[7−9] Although these estimates are approximate because they involve several assumptions, such as equal correlation times for the peptide in any conformational state and equal interstrand proton distances for the different β-hairpins, we have used that approach since there is no other well-established method. In spite of the many caveats of this method, we think that the use of these estimates for the comparison of the populations of β-hairpins formed by a series of peptides of the same size and differing in a maximum of four residues is reasonable, at least from a qualitative point of view.

Conformational Behavior of Peptide 4. Peptide 4 forms the same two β-hairpin structures as peptide 3, as indicated by its NOE pattern (Table 1 and Table SM5 as Supporting Information), which is similar to the one observed in peptide 3[8] except for differences in the intensities of crucial NOE crosspeaks. One of the CαH−CαH NOE crosspeaks observed could not be unambiguously assigned because of the coincidence in the δ-values of the CαH of residues Y2 and N4 under all the experimental conditions tested (various temperatures and pH values, various percentages of trifluoroethanol, and 0.5 M urea). Therefore, the assignment to CαHY2−CαHW9 NOE, characteristic of the β-hairpin 4:4 (Figure 2c), was based on the equivalent unambiguously assigned NOE in peptides 2, 3, and 5. Even if this assumption were not correct, our conclusions would remain valid since they are based on the fact that β-hairpin 3:5 is destabilized by the T8V mutation, and there is no ambiguity on the assignment of the CαHS3−CαHW9, characteristic of the β-hairpin 3:5 (Figure 2d). In peptide 4 the CαHS3−CαHW9 is significantly weaker than the CαHY2−CαHW9 NOE, whereas these two NOE crosspeaks have nearly equal intensities in peptide 3 (Table 1 and Figure 3). Further-

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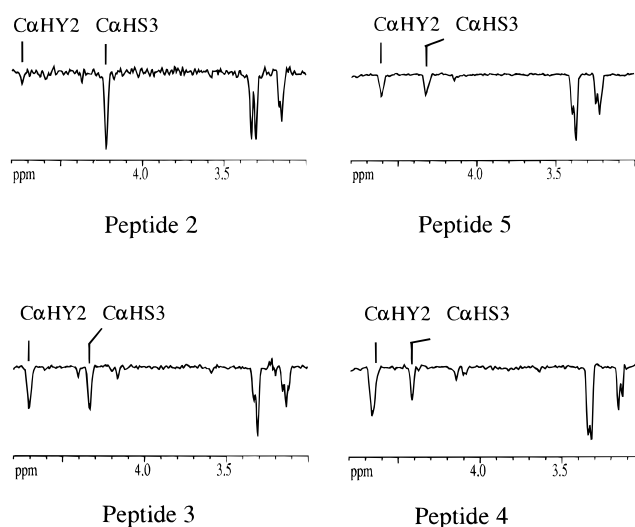(38) Case, D. A.; Dyson, H. J.; Wright, P. E. *Methods Enzymol.* **1994**, *239*, 392−416.

Figure 3. ROESY rows at the δ corresponding to the CαH proton of residue W9 where the relative intensities of their NOEs with the CαH proton of residues Y2 and S3 can be observed for peptides 2, 3, 4, and 5 (100-ms mixing time; D_2O samples at pH 6.3 and 2 °C).

more, the NOE between the amide protons of residues Y2-T10 (characteristic of the β-hairpin 3:5) of medium intensity in peptide 3[8] is not detected for peptide 4 (Table 1). The intense NOEs between the side chains of residues I1 and W9 observed in peptide 3[8], a consequence of the proximity of these side chains in the β-hairpin 3:5, are extremely weak in the NMR spectra of peptide 4 (Table SM5, Supporting Information). In brief, the set of NOE data observed for peptide 4 clearly indicates a loss of population of the β-hairpin 3:5 relative to peptide 3. The profile of CαH conformational shifts of peptide 4 (data not shown) is similar to that of peptide 3, but with smaller absolute values as expected for a less populated structure. The presence of a very weak NOE between the CαH of residues S3 and V8 (data not shown) may indicate the existence of a very minor third β-hairpin conformation which could correspond to a β-hairpin 2:2, probably with a type I turn formed by residues NSDG, (similar to the one represented in Figure 2a), but we have no other evidence for it.

Conformational Behavior of Peptide 5. Both peptides 2 and 5 have similar sets of NOE connectivities, except for the lower intensities of many medium- and long-range NOE connectivities and the absence of some of them in peptide 5 (Table 1 and Table SM6, Supporting Information). All the NOE connectivities characteristic of the β-hairpin 3:5, i.e., the NHY2−NHT10 and the CαHS3−CαHW9 NOE crosspeaks (see Figures 2, c and d) as well as those involving the side-chain protons of residues I1 and W9, decrease in intensity or disappear completely in peptide 5 (Table 1, Figure 3, and Table SM6, Supporting Information). This indicates a loss in the β-hairpin 3:5 population in peptide 5 relative to peptide 2. On the other hand, the β-hairpin 4:4 population increases greatly in peptide 5 as shown by the observation of a CαHY2−CαHW9 NOE of medium intensity, characteristic for this conformation, which is nearly at the noise level for peptide 2[8] (Figure 3). The profile of CαH conformational shifts (data not shown) is very similar to that of peptide 2, but those residues with positive CαH conformational shifts values, in particular Trp 9 which shows the largest CαH conformational shift,[8] decrease significantly, which again indicates a lower global population of β-hairpin conformations. The fact that the substitution of N4 to A involves the disappearance of the stabilizing hydrogen bonding interaction between the side chains of N4 and D6, identified in
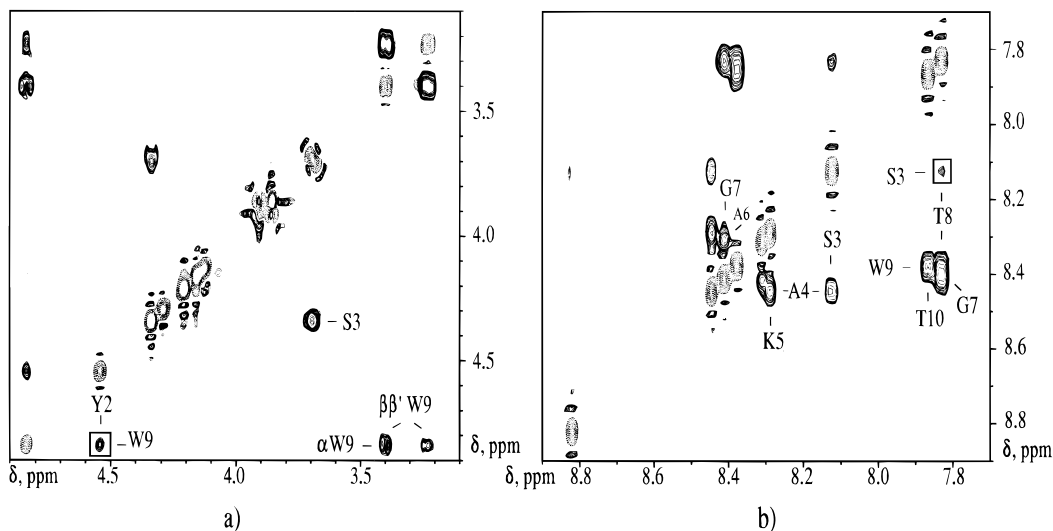
Figure 4. Selected regions of ROESY spectra of peptide 6. Experimental conditions were 5 mM, H_2O/D_2O 9:1, pH 4.3 and 5 °C, and 200-ms mixing time.
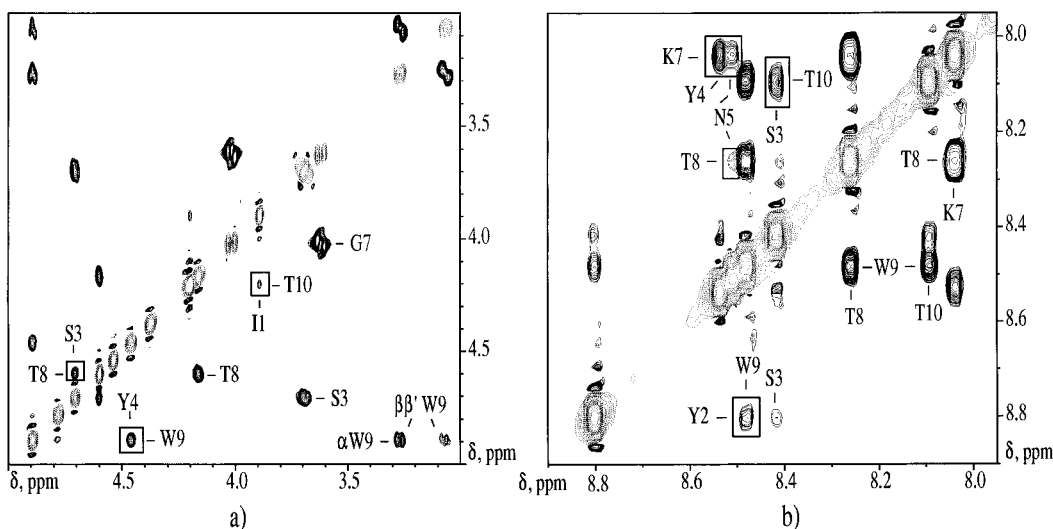

Figure 5. Selected regions of ROESY spectra of peptide 7. Experimental conditions were 15 mM, H_2O/D_2O 9:1, pH 4.3 and 12 °C, and 200-ms mixing time for the amide proton region and 5 mM, D_2O, pH 6.3 and 2 °C and 100-ms mixing time for the CαH−CαH region.

peptides 1,[10] 2, and 3,[8] may account for the general loss of β-hairpin population found in peptide 5.

Conformational Behavior of Peptide 6. The presence of a single CαHi−CαHj NOE crosspeak, the CαHY2−CαHW9 (Figure 4), and the NHS3−NHT8 NOE connectivity clearly reveal the existence of a unique β-hairpin conformation, the β-hairpin 4:4 (Figure 2c). In addition, NOE connectivities involving the side chains of residues facing each other in the β-hairpin 4:4 are also observed (Table SM7, Supporting Information). A weak CαHK5−NHG7 NOE is indicative of the formation of the chain bend (Table SM7, Supporting Information), probably a type I β-turn. The absence of medium- or long-range range NOEs characteristic of β-hairpin 3:5 confirms that it is not formed (Table 1 and Figure 4).

Conformational Behavior of Peptide 7. The NOE connectivities most characteristic for the β-hairpin 2:2 with a type I′ turn formed by residues YNGK (Figure 2e) for which peptide 7 was designed includes the long-range NOEs between the CαH protons of residues I1−T10 and S3−T8 and between the amide protons of residues Y2−W9 and Y4−K7. Such long-range NOE connectivities are shown in the ROESY spectrum of peptide 7 (Figure 5). Other NOE correlations compatible with the pattern of facing residues in this β-hairpin 2:2 with YNGK

residues are also observed (Table SM8, Supporting Information). These include, for example, NOE correlations between side-chain protons of residues S3−T8, Y2−W9, and I1−T10. A CαHN5−NHK7 NOE of medium intensity and a weak CαHY4−NHK7 one together with the one between NHN5−NHK7 (Figure 5) evidence the formation of the β-turn.[36] The YNGK residues are probably forming a type I′ β-turn.

The NMR spectra of peptide 7 show some additional NOE connectivities which are not compatible with the β-hairpin 2:2 with residues YNGK forming the β-turn, as deduced from β-hairpin structure calculations (see below). These NOE connectivities involve the CαH protons of residues Y4−W9, the NH protons of residues S3−T10, and the NH protons of residues N5−T8 (Table 1 and Figure 5), which are the most characteristic NOE correlations that should be expected for the β-hairpin 2:2 with NGKT as the turn residue sequence (Figure 2f). Most likely these residues will form a type II′ β-turn since the sequence NGKT favors this turn type, specially residues G and T which have the highest probabilities of being at positions $i + 1$ and $i + 3$ in a type II′ turn, respectively.[14] The sequence of peptide 7 can support two different turn conformations, one being a type I′ β-turn formed by residues YNGK, and the other a type II′ β-turn (residues NGKT). Each gives rise to a different
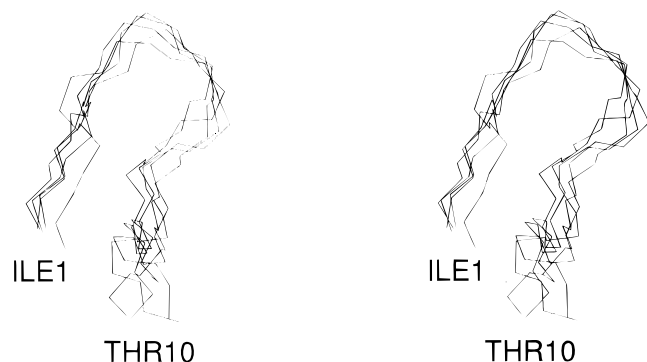
Figure 6. Stereoscopic view of the superposition of the backbone atoms of 5 calculated structures for peptide 6.

β-hairpin 2:2 conformation (Figure 2, e and f). The presence of a very weak CαHY2−CαHW9 NOE, which is not compatible with either β-hairpin 2:2 form, may correspond to a very minor third β-hairpin conformation, but we lack other evidence for it.

The β-hairpin 2:2 with YNGK turn residues formed by peptide 7 contains two CαH*i*−CαH*j* NOE connectivities, the CαHS3−CαHT8 and the CαHI1−CαHT10 NOE connectivity (Figure 2e), which can in principle be used for estimating the hairpin population as described above. The population estimated from the intensity of the CαHS3−CαHT8 and CαHI1−CαHT10 NOE crosspeaks differs notably, the former being three times greater than the one calculated from the latter. Such a discrepancy, large even considering the error sources concomitant to the method, probably arises from the dynamic behavior of the peptide which involves a larger flexibility of their chain termini, thus giving rise to an average interstrand CαHI1−CαHT10 distance larger than the inner one. This behavior of the β-strand ends would be analogous to the fraying observed in α-helical conformations.[2b]

Calculation of β-Hairpin Structures. Although the conformational averaging that usually occurs in peptides hinders a rigorous interpretation of NOE intensities in terms of a unique structure, it is useful to calculate a limited number of structures compatible with NOE constraints, which helps to visualize the conformational properties of the ensemble.

Structure calculations were not performed for peptides 4 and 5 because they fold into a mixture of two β-hairpins which makes still more difficult the structure calculation, and there exist available model structures for the types of β-hairpin adopted by these peptides. Thus, the structures calculated for peptide 2,[8] where the major population corresponds to the β-hairpin 3:5, and those calculated for peptide 6, which folds uniquely into β-hairpin 4:4, can be models for the β-hairpin 3:5 and β-hairpin 4:4, respectively. The structures calculated from the complete set of nonsequential NOE constraints observed for peptide 6 are not well-defined, as seen in the superposition of the five best calculated structures (Figure 6). The pairwise root mean square deviations for the backbone atoms, RMSDs, are 1.1 ± 0.3 Å.

A structure calculation performed with the complete set of experimental NOE constraints found for peptide 7 evidenced their incompatibility with a single structure. This was the expected result since the observed NOE connectivities can arise from either of the two β-hairpin structures adopted by peptide 7 in aqueous solution or from both. Nevertheless, the absence of model β-hairpin 2:2 structures in peptides prompted us to try the calculation of the structures for the two β-hairpin 2:2 formed by peptide 7. A classification of the NOE constraints into two groups is required prior to the structure calculations.

NOEs involving backbone protons were easily classified as belonging to β-hairpin 2:2 with YNGK turn or to β-hairpin 2:2 with NGKT turn on the basis of the schematic representations shown in Figure 2, e and f, respectively. For the side-chain−side-chain NOEs, the fact that they are more probable between residues facing each other in the β-hairpin was taken into account. Thus, NOEs involving side-chain protons of the residue pairs I1−T10, Y2−W9, and S3−T8 were included in the structure calculation for β-hairpin 2:2 with YNGK turn, and those involving side-chain protons of the residue pairs S3−T10 and Y4−W9 in the structure calculation for β-hairpin 2:2 with NGKT (Figure 2, e and f). NOEs between residues I1−T8 were assigned to β-hairpin 2:2 with YNGK turn where they are on the same side, but not in β-hairpin 2:2 with NGKT turn (Figure 2, e and f). In addition, the NOEs characteristic of the YNGK turn were excluded from the structure calculation for the β-hairpin 2:2 with NGKT turn. The remaining NOE restraints were initially introduced in both β-hairpin structure calculations, and the systematically violated NOE restraints in the calculation of one of the β-hairpin 2:2 structures were excluded from that one and included in the other and vice verse. Through an iterative procedure, we were able to obtain NOE sets compatible with each β-hairpin structure which accounted for all the experimental NOE restraints. This classification is available as Supporting Information (Table SM8). The final structures calculated from these two groups of NOE constraints are well-defined for both main-chain and side-chain atoms (Figure 7). RMSDs for the best 10 calculated structures for β-hairpin 2:2 with YNGK turn are 0.8 ± 0.2 Å and 1.7 ± 0.3 Å for the backbone and for all heavy atoms, respectively. RMSDs for the best 10 calculated structures for β-hairpin 2:2 with NGKT turn obtained considering only residues 3−10 are 0.3 ± 0.1 Å and 1.1 ± 0.2 Å for the backbone and for all heavy atoms, respectively. The first two residues were not considered for obtaining the RMSDs for β-hairpin 2:2 with NGKT turn because they were not ordered in it.

Discussion

The aim of this work was to investigate the role of turn residue sequence in determining β-hairpin structure. With this purpose we have analyzed by ^{1}H NMR the structure of four peptides designed to fold into different β-hairpin conformations in aqueous solution. We show that the conformational behavior of the peptides greatly depends on the sequence of the chain-bend region. The β-strand interresidue interactions seem to be of less importance in determining β-hairpin structure relative to the β-turn type preference, but a role of the side-chain interactions on the stability of β-hairpin structures is not discarded.[39] We address in this work the relative importance of factors such as interactions between turn residues, statistical preferences toward a particular β-turn type, and intrinsic tendencies of particular residues in the turn to be in different regions of the Ramachandran plot in determining the stability of different types of turns and β-hairpins.

The decrease in the population of the β-hairpin 3:5 (Figure 2d) in peptide 4 (with turn residues NSDGV) relative to peptide 3 (with turn residues NSDGT) evidences that the N−T interaction involving the first and the last residues of the type I + G1 bulge turn is responsible, at least partially, for the stability of this five-residue turn (Table 1). Furthermore, this suggests that the stability of the turn and not the pattern of interstrand side-chain−side-chain interactions or the backbone

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(39) Conformational properties of peptides with strand mutations is now in progress to better determine the importance of β-strand residues on the stability of β-hairpin structures.
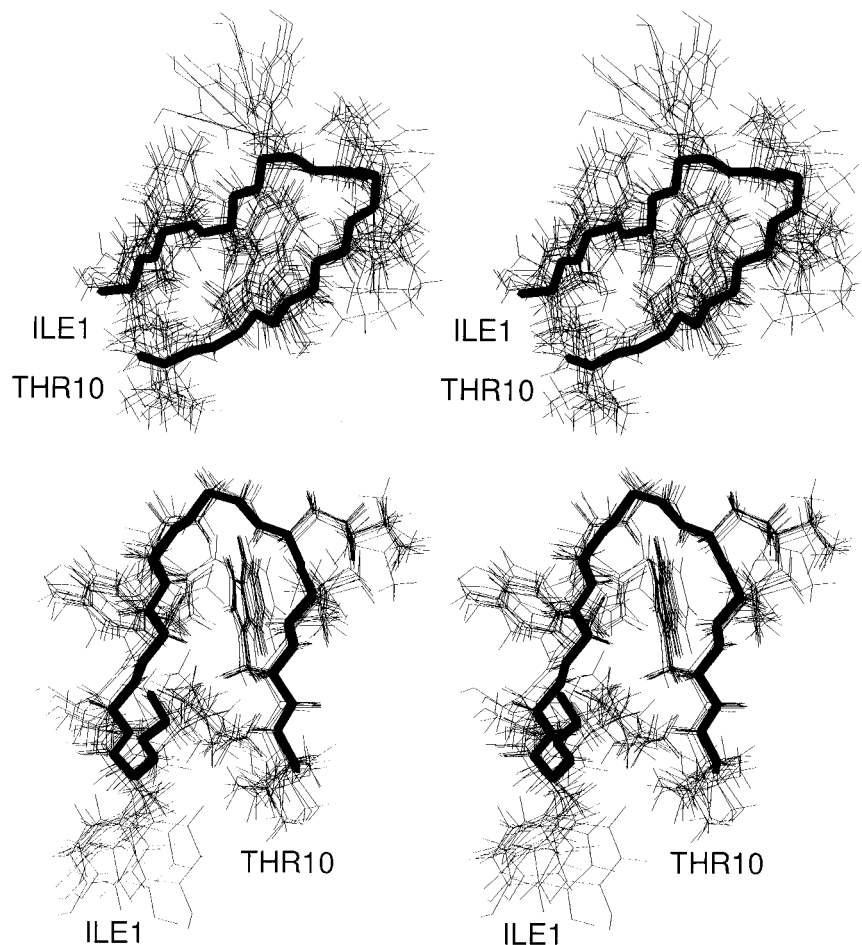
Figure 7. Stereoscopic view of the superposition of side-chain and backbone atoms of the best 10 calculated structures for (top) β-hairpin 2:2 with YNGK turn and (bottom) β-hairpin 2:2 with NGKT turn in peptide 7. The backbone atoms of the best structure are shown in bold.

hydrogen bonding register determines the formation of the β-hairpin 3:5 structure. In contrast, the population of the alternative conformation formed by this peptide (β-hairpin 4:4) is not affected by the mutation T8V (Table 1), in agreement with statistical data that consider the interstrand S3−T8 and S3−V8 interactions to be equally probable in a hydrogen bonded site of an antiparallel β-sheet.[27,40]

Relative to peptide 2, the population of β-hairpin 3:5 decreases in peptide 5 and that of β-hairpin 4:4 increases (Table 1). The substitution of N4 in peptide 2 by Ala in peptide 5 involves the loss of the N−T interaction, which accounts for the decrease of β-hairpin 3:5 population, as well as a change in the propensity of the first residue of the turn to populate angles from the β region to the α_R region of the Ramachandran map. If only the loss of the N−T interaction was responsible for the conformational change in peptide 5 relative to peptide 2, we would expect a decrease in the population of the β-hairpin 3:5, as is indeed observed (Table 1), without changing the population of the β-hairpin 4:4. However, the population of the β-hairpin 4:4 adopted by peptide 5 increases even with the presence of Pro which is unfavorable for the four-residue turn responsible for this type of β-hairpin.[8] Therefore, Ala in the first position of a turn remarkably stabilizes the four-residue type I turn, and so the population of the β-hairpin 4:4 increases. This evidences that the intrinsic tendency of the residue in the first position of

the turn to populate different regions of the Ramachandran map greatly influences the conformation adopted by the bend region and, thus, the nature of the β-hairpin. Summarizing, a residue-with tendency to populate the α_R region of the Ramachandran map favors the four residue type I turn in the β-hairpin 4:4 over the five residue type I + G1 bulge turn present in the β-hairpin 3:5.

Peptide 6 is the only peptide that forms a unique β-hairpin conformation, β-hairpin 4:4 (Figure 2c). The turn region of this peptide is the native type I β-turn present in the 18−35 β-hairpin of BPTI. A mutant of BPTI lacking two of the three disulfide bridges was shown to form a highly ordered β-sheet molten globule that interconverts with other conformations all having the native 18−35 β-hairpin 4:4 structure on the basis of NMR data.[33] Thus, even though the β-strand residues are completely different in BPTI and in peptide 6, the type of β-hairpin formed is the same. This observation again underlines the relevance of the turn conformation in determining β-hairpin structure, more so than the type of interstrand side-chain−side-chain interactions. The evidence is stronger when considering that the β-strand residues of peptide 6 (identical with those of peptides 2, 3, 4, and 5) can adopt at least two different β-sheet registrations corresponding to different β-hairpin conformations.[8] The residue Ala located in the first position of the turn and the absence of the N−T interaction (as in peptide 5) contribute to the stabilization of the β-hairpin 4:4 and the destabilization of the β-hairpin 3:5, respectively. It thus appears that the turn sequence AKAG (peptide 6) has a marked propensity to populate only one turn type and so a unique type of β-hairpin conformation, while the sequence NSDG (peptide 3) can

(40) In antiparallel β-sheets, a hydrogen bonded site corresponds to a pair of residues with their backbone atoms hydrogen bonded to each other and a non-hydrogen-bonded site to the pair where the backbone atoms of the residues are not hydrogen bonded.[27] For example, in the β-hairpin 3:5 shown in Figure 2d, the pair Y2−T10 is in a hydrogen-bonded site and the pair S3-W9 in a non-hydrogen-bonded site.

populate at least two turn types and therefore leads to two different β-hairpin conformations. Additional work is needed to clarify why no β-hairpin 3:5 population was detected in peptide 6.

Peptide 7, which has a chain-bend sequence (YNGK) with maximum statistical preference to adopt a type I' turn,[14] adopts the expected β-hairpin 2:2 conformation with turn residues YNGK, likely forming the type I' turn for which they were designed, and another β-hairpin 2:2 with turn residues NGKT, probably forming a type II' turn. In spite of the intrinsic tendencies of residues G and T to be located in positions $i + 1$ and $i + 3$, respectively,[14] of a type II' turn, the simultaneous formation of the β-hairpin 2:2 with NGKT turn residues by peptide 7 was unexpected because it implies the loss of a interstrand side-chain−side-chain interaction (Figure 2f) and also because the type I' turn (YNGK) is statistically favored over the type II' turn (NGKT) in β-hairpin 2:2 structures.[11] A plausible explanation is that the N5−T8 interaction, which can occur in β-hairpin 2:2 with the NGKT turn, but not in the one with YNGK turn, and the high propensity of G6 and T8 for being in the adopted positions of the type II' turn appear to compensate for the loss of the interstrand side-chain−side-chain interaction, so that the two different β-hairpin 2:2 structures have similar stabilities. Both β-hairpin 2:2 conformations have the same number of interstrand backbone hydrogen bonds (Figure 2, e and f). Even though the strand residues are the same as in peptides 2−6, peptide 7 forms two β-hairpin conformations completely different from the β-hairpin 3:5 and β-hairpin 4:4 conformations found in the others, differing both in interstrand side-chain−side-chain interactions and in the pattern of backbone hydrogen bonds.

It is also worth noting that the net population of the structure formed by peptide 7 increases with respect to the other peptides forming a type I and/or a type I + G1 bulge turn (Table 1). This agrees with the fact that type I' and II' turns are most favorable in general terms for the formation of a β-hairpin.[11] Thus, the turn sequence may determine both the type of the β-hairpin conformation and its stability.

The results presented here have important implications in protein folding because different types of β-hairpin structures may form in the initial stages of the folding pathway, and the selection of a final unique β-hairpin may depend on either local and/or nonlocal interactions. Probably, the coexistence of more than one possible β-hairpin conformation, and the flexibility derived from it, is convenient for the folding of most proteins in order to attain the most favorable conformation compatible with stabilizing tertiary contacts.

Conclusion

We have shown the key role played by the sequence of the turn region in determining the pattern of backbone hydrogen bonding and side-chain−side-chain interactions in a β-hairpin. In addition, we have identified some factors affecting the formation of β-hairpin conformations, such as the polar interaction between the side chains of Asn at position i and Thr at position $i + 4$ which stabilizes the five-residue type I + G1 bulge turn and thus the β-hairpin 3:5 conformation and the presence in the first position of the turn of a residue with high tendency to populate the α_R region which favors the formation of β-hairpin 4:4 conformations with a four-residue type I turn. Our results also evidence that a turn sequence favorable to adopt a β-turn with the appropriate geometry for the formation of a β-hairpin, such as a type I' or II' β-turn, increases the stability of the adopted β-hairpin.

Supporting Information Available: Four tables listing the δ values of peptides 4, 5, 6, and 7 in aqueous solution at 5 °C and pH 4.3, and four tables listing the medium- and long-range NOEs observed for peptides 4, 5, 6, and 7 in aqueous solution (10 pages). See any current masthead page for ordering and Internet access instructions.

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