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The Role of an Aliphatic–Aromatic Interaction in the Stabilization of a Model β-Hairpin Peptide

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Apart from with the conventional motivation of understanding the rules that govern protein folding, the determinant factors responsible for the stabilization of β structures have acquired special interest since this conformation is involved in amyloid structure.[1] Thus, studies directed to determining the factors that regulate the folding and solubility of β-structured peptides and proteins would be helpful in understanding the mechanism of amyloid formation and developing successful therapeutic strategies. Furthermore, the amyloid structure has also recently inspired the development of new nanomaterials[2,3] with potential applications in technologic fields that would also benefit from these studies.

The β-hairpin motif, composed of two antiparallel strands connected by a turn, is the smallest β structure. We have reported the use of a β-hairpin conformationally defined peptide library (CDL) for the design of well-folded β-hairpin peptides.[4,5] From the structural screening of the library, a set of 36 β-hairpin peptides was defined, and structural characterization showed that at least two peptides, MBH12 and MBH36, exhibited an average β-hairpin conformation higher than 60%. These two peptides contain aromatic residues in three out of four combinatorialized positions that were subjected to optimization by the screening procedure (−B3, −B1 and +B3) whereas at the remaining position (+B1) the selected amino acid was Ile. The calculated structure from the NOE pattern found in the ^{1}H 2D NMR spectrum of MBH12 showed that the three aromatic residues pack together, while the aliphatic residue closes a small hydrophobic minicore.

In this study, the MBH12 peptide was used as a model system to evaluate the contribution to stability of aliphatic and aromatic residues at the B+1 position in the β-hairpin motif. The advantage of using well-folded peptides as model systems is their small size and structural simplicity; these allow the contribution of each individual amino acid towards the stabilization of a defined structure to be identified. We replaced the original Ile residue at B+1 position in MBH12 by the hydrophobic β-branched amino acid Val and by the aromatic residues Phe, Tyr, and Trp (Table 1). The structural examination of this new set of peptides allowed the analysis of the contribution to the stability of the β-hairpin of the cross-strand Ile–Tyr, Val–Tyr, Trp–Tyr, Tyr–Tyr, or Phe–Tyr pairs in the hydrogen bonding (HB) sites defined by residues placed at B−1 and B+1 as well as the importance of a well-packed hydrophobic minicore.

The peptide MBH12 contains residues Arg1–Gly2 and Gly13–Arg14, which improve solubility, and was shown to be monomeric at the concentrations used for its characterization.[4] Although it is expected that a single amino acid replacement should have little influence on the physical properties of the peptide, an initial step previous to the conformational study was the characterization of the aggregation state of the new set of peptides. The peptides were analyzed by far-UV CD spectroscopy at two different concentrations (25 and 100 μM), and the spectra corresponding to each peptide were found to be identical. Furthermore, the 1D ^{1}H NMR spectra in H_2O/D_2O (90:10; pH 5) at 283 K of each peptide at 100 μM and 1.5 mM showed identical signal line widths and chemical shifts. These data suggest that all four peptides are monomeric in the concentration range from 25 μM to 1.5 mM.

β-Hairpin conformations render a characteristic far-UV CD signal, with a relative minimum ellipticity at 217 nm and a positive ellipticity band close to 200 nm. In contrast, random-coil ensembles show a minimum at around 202 nm. The concentration-independent ratio $\theta_{217}/\theta_{202}$ has been used for the quantitative analysis of the β-hairpin conformation.[4] The four peptides exhibit far-UV CD spectra in aqueous solution that are characteristic of folded β-hairpins in equilibrium with random-coil conformations (Figure 1 A). Initial examination of both the CD spectra and the $\theta_{217}/\theta_{202}$ ratio (Table 1) suggests that all four substitutions at position 9 are detrimental to the β-hairpin structure of the parental MBH12. However, peptides containing aromatic residues have unusual CD spectra with intense bands at 215 and 229 nm; this suggests the presence of interactions between the aromatic chromophores.[6,7] Therefore, only peptides containing the same aromatic residues in the same positions are suitable for comparative analysis of their CD spectra. Thus, it is not possible to establish a ranking of the structural content of the designed peptides since they have different aromatics in +B1, with the exception of the peptides MBH12 and MBH-V. The comparative analysis of the far-UV CD spectra (Figure 1 A) and $\theta_{217}/\theta_{202}$ ratio (Table 1) suggests that MBH12 has a higher percentage of β-hairpin structure than MBH-V. This is reinforced by analysis of the near-UV CD spectra (Figure 1 B), which show the adoption of a dichroic environment for the ar-

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Table 1. Structural parameters and β-hairpin population of the peptides.

Peptide	Sequence	Ellipticity $\theta_{217}/\theta_{202}$ ratio water vs. 40% MeOH	$\Delta\delta$ $C_{\alpha H}$ [ppm] (+B2)	Thr (−B2)	Asn (L1)	Average % of population[a]
MBH12	RGKWTYNG**I**TYEGR	−8.50 vs. −1.64	0.67	0.21	−0.36	66
MBH-W	RGKWTYNG**W**TYEGR	0.75 vs. 0.98	0.18	0.00	−0.26	30
MBH -Y	RGKWTYNG**Y**TYEGR	0.32 vs. 0.42	0.35	0.09	−0.22	36
MBH-F	RGKWTYNG**F**TYEGR	0.20 vs. 0.52	0.38	0.11	−0.24	40
MBH-V	RGKWTYNG**V**TYEGR	1.10 vs. −4.01	0.61	0.20	−0.33	60

[a] Ranking of β-sheet population from H^{α} conformational chemical shifts, calculated by using the linear peptides that correspond to the two strands of the β-hairpin as reference for the chemical shifts of the unfolded state.[4] As a reference for 100%-folded peptide, we used the data obtained from a mutated version of the spectrin SH3 domain, which contained the sequence RGKITVNGKTYEGR inserted as an elongation of a protein β-hairpin.[18] The population of the β-hairpin state for each peptide was calculated at each indicator residue from $\Delta\delta_{H\alpha}$ data by using Equation (1)

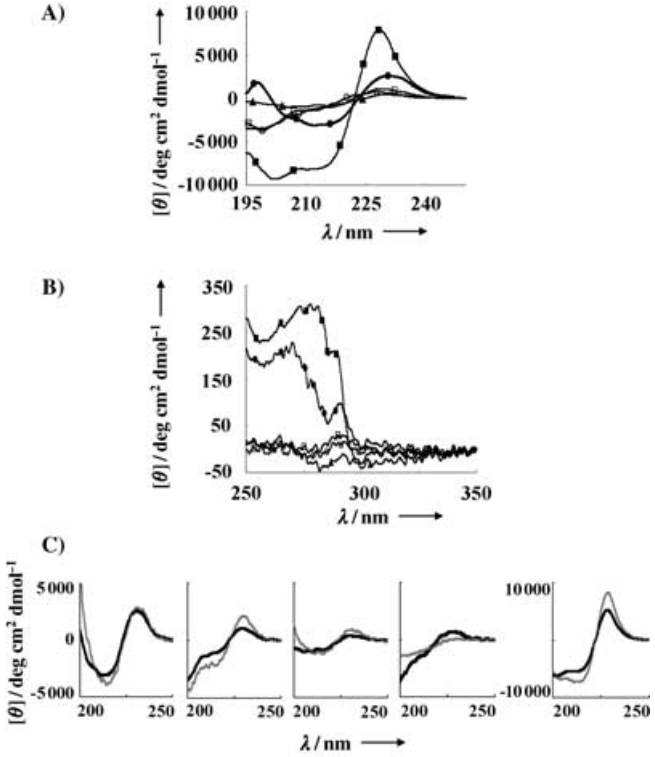

Figure 1. A) Far- and B) near-UV CD spectra of peptides MBH 12 (●), MBH-F (○), MBH-V (▲), MBH-W (■), and MBH-Y (□) acquired at 5 °C in 5 mM acetate buffer, pH 5. C) Comparison of the far-UV CD spectra of peptides in buffer (black line) or in the presence of 40% MeOH (grey line); from the left, MBH 12, MBH-Y, MBH-V, MBH-F, and MBH-W.

omatic residues of peptides MBH12 and MBH-W, while these residues should be less conformationally restricted in the peptides MBH-F, MBH-V, and MBH-Y.

CD analysis of the peptides was also performed in the presence of 40% MeOH (Figure 1C). This solvent is a stabilizing agent that increases β-hairpin population relative to that existing in water.[4] The β-hairpin characteristics of the CD spectra increase on going from 0 to 40% MeOH for all five peptides (Figure 1C). This indicates that the hairpin conformation increases although it has to be noted that minor changes are obtained for MBH12 in the overall β-hairpin characteristics (negative values for the $\theta_{217}/\theta_{202}$ ratio in water and in the presence of MeOH; Table 1) while the peptide MBH-V increases its conformation noticeably (the ratio $\theta_{217}/\theta_{202}$ is positive in water and negative in MeOH).

To carry out a reliable ranking of the structural content, the 2D ^1H spectra of the peptides at 1.5 mM were acquired in H_2O/D_2O (90:10; pH 5) at 283 K. The NMR analysis showed that all five peptides fold in a β-hairpin conformation, although they exhibited different structural content. The characteristic pattern of long-range nuclear Overhauser effect peaks (NOEs) for a β structure is defined by the presence of strong sequential H^{α}-N (*i*, *i*+1) and weak NN (*i*, *i*+1) NOEs that are present in the NMR spectra of all five peptides analyzed. The chemical-shift dispersion is remarkable and allows the unambiguous assignment of nearly all NOE peaks. However, the indubitable hallmark of secondary structure in peptides is the presence of nonsequential NOEs, such as the H^{α}–H^{α} NOE, between face to face residues in adjacent β-strands. Therefore, the β-hairpin structure is confirmed by the existence of the H^{α}–H^{α} NOE between Thr5 and Thr10 that is found in aqueous solution for peptides MBH12, MBH-F, MBH-V, and MBH-Y but not for MBH-W. The absence of this characteristic NOE suggests that MBH-W is probably the least structured peptide of the set. Furthermore, the presence of the H^{α}–H^{α} NOE between Thr5 and Thr10 suggests that the peptides adopt a 2:2 β-hairpin (following the nomenclature proposed by Sibanda et al.[8]). Also of interest for the qualitative conformational analysis is the H^{α}–H^{α} NOE between Lys3 and Glu12, the first and last residues, respectively, of the β-hairpin. Such a NOE is identified in the spectra of MBH12 and MBH-V, while it is absent in the spectra of MBH-W, MBH-Y, and MBH-F. Thus, qualitative NMR analysis suggests that peptides MBH12 and MBH-V have similar β-hairpin populations; peptides MBH-F and MBH-Y adopt the β-hairpin conformation, although the N and C termini are less structured; while MBH-W is the least-structured peptide. However, as shown above, CD analysis of this peptide could suggest the adoption of a defined secondary and tertiary structure.

$^1H_{\alpha}$ conformational shifts ($\Delta\delta_{C\alpha H}$) are good indicators of secondary-structure formation. This parameter is obtained from the difference between the observed $^1H_{\alpha}$ chemical shifts of the studied peptide ($\Delta\delta^{obs}_{C\alpha H}$) and those from random-coil reference ($\Delta\delta^{rd}_{C\alpha H}$). Since these chemical shifts are dependent on the chemical environment, we used the isolated strands of the β-hairpins as random-coil reference peptides.[4] Four peptides,

but not MBH-W, showed the expected $^1H_\alpha$ conformational-shift profile for a β-hairpin (Figure 2): that is, positive values for the strand residues and negative values for Asn7 and Gly8 at the turn. The first two and last two residues (Arg–Gly) do not form

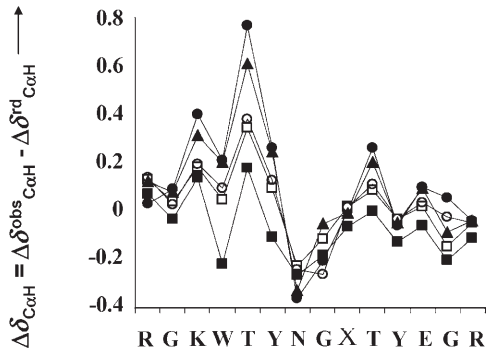

Figure 2. Observed conformational H^α chemical-shift increments ($\Delta\delta_{C\alpha H}$) for peptides MBH 12 (●), MBH-F (○), MBH-V (▲), MBH-W (■), and MBH-Y (□).

part of the hairpin, as expected from the original design. With all this information in hand, we can now proceed to a detailed calculation of β-sheet population. The ranking was derived from comparison of the conformational shifts of the CαH's of Thr5 (−B2), Thr10 (+B2), and Asn7 (L1) obtained for each peptide from the set.[4] These three residues are present in all five peptides and occupy key positions in the folded structure, that is, the threonines reside in the middle of the strands and the Asn resides in the turn. The structural-content ranking obtained is MBH-12 > MBH-V > MBH-F > MBH-Y > MBH-W (Table 1).

In conclusion, our study shows that the peptide MBH12, identified from the screening of a β-hairpin CDL, was truly optimized at the positions that were subjected to analysis,[4] as demonstrated when the initially unexpected Ile at position +B1 was replaced in a new set of peptides derived from MBH12 (Table 1). Structural analysis demonstrated that peptides with an aliphatic β-branched residue at position +B1 (MBH12 and MBH-V) showed a higher β-hairpin content than those with aromatic residues (MBH-F, MBH-W, and MBH-Y) at the same position. Thus an Ile/Val–Tyr cross-strand pair at the HB position closest to the turn contributes more to the hairpin stability than do the aromatic–aromatic pairs analyzed here (Phe–Tyr, peptide MBH-F; Trp–Tyr, peptide MBH-W, and Tyr–Tyr, peptide MBH-Y). This can be explained in terms of a combination of individual propensities to populate β-sheet structures, side-chain–side-chain interactions, and hydrophobic minicore stabilization. The particular backbone geometry adopted by MBH12 could have also a role. β-Branched aliphatic residues are preferred over aromatic residues in certain β-hairpin model peptides at HB strand positions,[9] while aromatic–aromatic interactions seem to be preferred at NHB positions in models with slightly different backbone geometry.[10,11] However, the stability rank order obtained here for the −B1–+B1 pair analyzed—(Ile or Val)–Tyr ≫ Phe–Tyr > Tyr–Tyr > Trp–Tyr—does not correlate with reported experimental β-sheet-propensity scales[12,13] or with observed residue pair frequencies in known β-sheets.[14–16] These propensity scales are usually derived from structural databases of folded proteins or from single mutations in fully folded proteins. When analyzing β-sheet short peptides in which significant side-chain hydrophobic surface will be exposed to solvent, it is still difficult to perform a rational design of fully folded monomeric peptides. However, combinatorial approaches based on CDL, as a design tool, then emerge as a useful methodology. In our previous work,[4] the structural screening of a CDL containing close to 140 000 peptide sequences was used for the identification of the MBH β-hairpin peptides. The combinatorial approach selected Trp4, Tyr6, Ile9, and Tyr11 at the nonpolar face of the MBH12 peptide. The results suggest that the β-branched side chains of Ile and Val adopt the appropriate orientation to pack and to stabilize the hydrophobic minicore defined by Trp4, Tyr6, and Tyr11. Thus, in de novo design of peptides and proteins with β-structure, the context plays an important role and it is difficult to evaluate the contribution of isolated amino acids or cross-strand pairs. In contrast, it is necessary to take into account the amino acid sequence of the two faces that define the hairpin.

Experimental Section

Peptide synthesis. Peptides were prepared by Fmoc-based solid-phase synthesis on a 433 A Applied Biosystems Peptide synthesiser. Peptides were purified by preparative reversed-phase HPLC, and their identity was confirmed by laser desorption time-of-flight mass spectrometry.

Circular dichroism measurements. Spectra were acquired on a Jasco J-810 CD spectropolarimeter and were the average of a series of twenty scans recorded at 5 °C in acetate buffer (5 mM, pH 5). Peptide concentrations were determined spectrophotometrically.

Nuclear magnetic resonance. All NMR experiments were performed on a Brucker DRX 500 spectrometer on peptide samples of ≈1–2 mM concentration in pure D_2O and H_2O/D_2O (90:10) at pH 5. All chemical shifts were internally referenced to the sodium salt of trimethylsilylpropionate. Phase-sensitive TOCSY (mixing time 50 ms) and NOESY (mixing times 150, 200, and 250 ms) experiments were performed at 283 K, collecting 2048 points in f2 and 512 points in f1. Solvent suppression was achieved by selective presaturation during the relaxation delay (1.2 s) or field-gradient pulses. The proton resonances were assigned by the sequential-assignment procedure.[17]

Calculation of the β-sheet population from H_α conformational chemical shifts. Ranking of the β-sheet population from CαH; conformational chemical shifts was calculated by using the linear peptides that correspond to the two strands of the β-hairpin as reference for the chemical shifts of the unfolded state.[4] The population of the β-hairpin state for each peptide was calculated at each indicator residue from $\Delta\delta_{H\alpha}$ data by using Equation (1):

$$\left[\frac{\delta_{obs}-\delta_U}{\delta_F-\delta_U}\right] \times 100 \qquad (1)$$

Experimental uncertainties reflect error propagation from the ±0.01 ppm uncertainty in the $\delta_{H\alpha}$ measurement.

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