Elongation of the BH8 β -hairpin peptide: Electrostatic interactions in β -hairpin formation and stability

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Abstract

An elongated version of the de novo designed β -hairpin peptide, BH8, has allowed us to gain insight into the role of electrostatic interactions in β -hairpin stability. A Lys–Glu electrostatic pair has been introduced by adding a residue at the beginning and at the end of the N-terminal and C-terminal strands, respectively, of the β -hairpin structure, in both orientations. The two resulting peptides and controls having Ala residues at these positions and different combinations of Ala with Lys, or Glu residues, have been analyzed by nuclear magnetic resonance (NMR), under different pH and ionic strength conditions. All of the NMR parameters, in particular the conformational shift analysis of C α protons and the coupling constants, $^3J_{\text{HN}\alpha}$, correlate well and the population estimates are in reasonable agreement among the different methods used. In the most structured peptides, we find an extension of the β -hairpin structure comprising the two extra residues. Analysis of the pH and salt dependence shows that ionic pairs contribute to β -hairpin stability. The interaction is electrostatic in nature and can be screened by salt. There is also an important salt-independent contribution of negatively charged groups to the stability of this family of β -hairpin peptides.

Keywords: NMR; β -hairpin; secondary structure; protein folding; peptides

The simplest β -sheet units are called β -hairpins and are composed of two antiparallel β -strands connected by a β -turn or short loop. To understand the formation of β -hairpins, it is necessary to analyze the energy contribution of the intrinsic secondary structure propensities of the different amino acids as well as of interstrand side-chain–side-chain interactions, in two structurally different regions of this secondary structure, the β -strands and the turn region. Site-directed mutagenized proteins have been the standard systems of choice to characterize and quantify the free energy contribution of noncovalent interactions. In protein models, it has been found that intrinsic residue preferences effects of

a β -sheet formation, modulated by context effects, can contribute significantly to protein stability (Minor and Kim 1994a,b; Smith et al. 1994; Smith and Regan 1995). The importance of side-chain–side-chain interactions on β -sheet stability has been analyzed by L. Regan's group on the protein G B1 domain (Smith and Regan 1995), by use of the double-mutant cycle approach (Carter et al. 1984). In these studies, a pairwise interaction is analyzed by performing a thermodynamic cycle in which the changes in free energy of unfolding are measured for the single and double mutant proteins. An alternative complementary approach is the use of synthetic model peptides. Such systems have been successfully used to dissect the contribution of local interactions to α -helix (Chakrabarty and Baldwin 1995; Muñoz and Serrano 1995, 1997; Lacroix et al. 1998). Both proteins and peptides have been used extensively to study local interactions and have provided valuable information on the thermodynamics of local interactions.

In the case of β -hairpins and β -sheets, there were not simple model peptide systems that could be used to dissect the different energy contributions to their stability. However, in recent years this has changed. Several peptides

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based on protein fragments, or designed sequences, with natural amino acids (for review, see Gellman 1998; Ramírez-Alvarado et al. 1999), fold as β -hairpins structures in aqueous solution and/or in water/alcohol mixtures. Some of these studies have found that intrinsic residue preferences, side-chain–side-chain interactions at the β -strands and the β -turn play an important role in β -hairpin formation in linear peptides (for review, see Gellman 1998; Ramírez-Alvarado et al. 1999).

The purpose of this work was to gain insight into the role of electrostatic interactions in β -hairpin stability. We have approached this objective by extending our previously de novo- designed eight-residue β -hairpin (BH8; Ramírez-Alvarado et al. 1996), up to 10 residues, by introducing two oppositely charged residues at the beginning and end of the BH8 strands. The elongated peptides with the electrostatic pair and the respective controls have been analyzed by nuclear magnetic resonance (NMR) in aqueous and 40% trifluoroethanol (TFE) solutions.

Results

Peptide design and sequences

The sequence of the BH8 peptide is as follows:

	1	2	3	4	5	6	7	8	9	10	11	12
BH8	Arg-Gly-	Ile-Thr-	Val-Asn-	Gly-Lys-	Thr-Tyr-	-Gly-Arg						

The two new residues have been introduced between positions 2 and 3, and between residues 10 and 11. Glutamate was chosen because it has a larger extended secondary structure propensity than Asp (Minor and Kim 1994a,b; Smith et al 1994). In the case of the positively charged residue, Lys was selected to avoid signal overlapping with the two Arg residues. Because Smith and Regan (1995) found that the orientation of the pairs in some cases results in different interaction energies, we placed the ionic pair in opposite directions (BH-EK and BH-KE). The final designed model sequences are as follows:

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
BHKE	Arg-Gly-	Lys-Ile-	Thr-Val-	Asn-Gly-	Lys-Thr-	Tyr-Glu-	Gly-Arg							
BHEK	Arg-Gly-	Glu-Ile-	Thr-Val-	Asn-Gly-	Lys-Thr-	Tyr-Lys-	Gly-Arg							

As shown in the following paragraphs, both peptides form the same 2:2 β -hairpin as BH8, but elongated in one residue at each strand. However, whereas peptide BH-KE is more structured than peptide BH8, the hairpin population of peptide BH-EK is similar to that of BH8. Therefore, we decided to do a double-mutant cycle to characterize the putative favorable electrostatic interaction of BH-KE. For this purpose, we designed three more peptides:

BHKA	Arg-Gly-	Lys-Ile-	Thr-Val-	Asn-Gly-	Lys-Thr-	Tyr-Ala-	Gly-							
Arg														

BHAE	Arg-Gly-	Ala-Ile-	Thr-Val-	Asn-Gly-	Lys-Thr-	Tyr-Glu-	Gly-							
Arg														
BHAA	Arg-Gly-	Ala-Ile-	Thr-Val-	Asn-Gly-	Lys-Thr-	Tyr-Ala-	Gly-							
Arg														

Aggregation tests

The peptide ^1H signals were sharp and concentration independent within the range 10 μM –10 mM. The chemical shifts were also unchanged over the same concentration range. Similar results were observed in circular dichroism spectra recorded at 5 μM and 1 mM (data not shown). This behavior is observed consistently in the BH peptide series (Ramírez-Alvarado et al. 1996, 1997) and indicates that the peptides do not aggregate in the conditions used in the current report. Finally, peptide BHKE has been analyzed by analytical ultracentrifugation (T.K. Kortemme and L.S. Serrano, in prep.) and found to be monomeric under the conditions used in this study.

NMR structural analysis in aqueous solution

The NMR analysis confirms that all of the peptides adopt a β -hairpin structure in equilibrium with random-coil conformations. The observed nonsequential NOEs, together with their relative intensities, are summarized in Table 1, and a spectrum region with some of the NOEs defining the β -hairpin structure in peptide BH-KE is shown in Figure 1A. Although some of the NOEs are not found in all of the peptides (in some cases due to signal overlapping), the NOEs observed in the six peptides are consistent with the same β -hairpin structure. In particular, the diagnostic $\alpha\alpha$ (i,i + 5) between Thr 5 and Thr 10, characteristic of the β -hairpin 2:2 structure (following the nomenclature proposed by Sibanda et al. 1989), is found in aqueous solution in all peptides, the only exception being peptide BH-AA, in which it overlaps with other signals. We find in aqueous solution, a new $\alpha\alpha$ NOE between residues 3 and 12 in peptides BH-KE and BH-AE, which is stronger in peptide BHKE (see Fig. 1B and Table 1). In the case of peptides BH-EK and BH-AE, the NOE could not be found because of signal overlapping. Its absence in peptide BH-AA could be due to a lower population or to the fraying at the end of the β -hairpin.

Another NMR parameter that is sensitive to the presence of folded structures is the conformational shift of the C α protons (differences between a random-coil reference and the experimental values). Downfield chemical shifts are associated to extended populations, whereas upfield values are indicative of helical or turn regions (Williamson 1990). Figure 2A shows the values for the different peptides in aqueous solution. The conformational shifts are downfield shifted (positive) on the β -strand residues equivalent to those in BH8 (Ile 4, Thr 5, Val 6, Lys 9, Thr 10, and Tyr 11)

Table 1. Non sequential NOEs in NOESY and ROESY spectra of the different peptides in aqueous solution pH = 5.0 (H), in 40% TFE at pH = 5.0 (T)

NOE type	Residues	P E P T I D E S									
		BHEK		BHKE		BHAE		BHKA		BHAA	
		H	T	H	T	H	T	H	T	H	T
α , N(i, i + 2)	N7-K9	w	*	w	*	w	*	w	*	w	w
G8-K10		—	—	—	—	—	—	w	*	—	—
N, N(i, i + 3) ^a	V6-K9	w	w	w	m	m	m	w	w	w	w
N, N(i, i + 7) ^a	I4-Y11	*	w	*	w	m	w	*	*	—	w
α , α (i, i + 5) ^a	T5-T10	m	*	w	s**	m	*	w	*	D	*
α , α (i, i + 9) ^a	X3-X12	*	*	m	s**	w	*	*	w	—	—
β , N(i, i + 2)	N7-K9	—	—	—	—	w	—	w	w	—	—
	X3-T5	w	—	—	—	—	—	—	—	—	—
γ , N(i, i + 2)	V6-G8	—	—	—	—	—	—	—	—	—	w
γ , N(i, i + 3)	V6-K9	w	m	—	—	w	w	w	w	—	w
γ , N(i, i + 4)	T5-K9	w	w	—	w	m	w	m	m	—	w
γ , N(i, i + 5)	V6-Y11	—	—	w	—	m	w	—	—	—	—
γ , N(i, i + 6)	T5-Y11	—	—	—	—	—	—	—	—	—	w
β , δ (i, i + 5)	V6-Y11	w	w	—	—	m	—	*	w	—	w
β , ϵ (i, i + 5)	V6-Y11	m	m	—	m	w	w	w	m	w	m
γ , δ (1, 1 + 5)	V6-Y11	m	*	m	—	m	—	m	m	m	m
γ , ϵ (i, i + 5)	V6-Y11	m	*	m	s	m	m	w	*	w	m
γ H ₃ , ϵ (i, i + 7)	I4-Y11	—	w	w	s	m	m	*	*	—	w
δ , δ (i, i + 7)	I4-Y11	*	w	w	w	w	—	w	*	—	w
γ , ϵ (i, i + 7)	I4-Y11	—	—	—	—	—	w	*	w	—	—
δ , ϵ (i, i + 7)	I4-Y11	*	w	w	w	w	w	w	m	—	—
β , ϵ (i, i + 2)	K9-Y11	m	m	—	w	m	w	w	m	—	m
δ , ϵ (i, i + 2)	K9-Y11	m	m	—	—	m	—	w	—	—	w
γ , ϵ (i, i + 2)	K9-Y11	m	m	—	—	w	—	w	*	—	w
γ , δ (i, i + 6)	T5-Y11	—	—	—	—	m	—	—	—	—	—
γ , ϵ (i, i + 6)	T5-Y11	—	—	—	w	m	m	—	—	—	—
β , ϵ (i, i + 6)	T5-Y11	—	—	—	—	—	—	—	m	—	—
δ , β (i, i + 7)	I4-Y11	—	—	w	w	w	w	—	—	—	—
γ , γ (i, i + 2)	T10-X12	—	—	w	—	—	—	—	—	—	—
β , γ (i, i + 7)	X3-T10	—	—	w	—	—	—	—	—	—	—
δ , γ (i, i + 7)	X3-T10	—	—	w	—	—	—	—	—	—	—
γ , β (i, i + 7)	I4-Y11	—	—	—	—	w	—	—	—	—	—
δ , β (i, i + 7)	I4-Y11	—	—	—	—	w	w	—	—	—	—
γ H ₃ , $\beta\beta'$ (i, i + 7)	I4-Y11	—	—	—	—	m/m	—/w	—	—	—	—
γ , $\gamma\gamma'$ (i, i + 7)	T5-X12	—	—	—	—	m/w	w/—	—	—	—	—
β , $\gamma\gamma'$ (i, i + 9)	X3-X12	—	—	—	—	w	—	—	—	—	—
α , $\gamma\gamma'$ (i, i + 9)	X3-X12	—	—	—	—	—	w	—	—	—	—
β , γ (i, i + 9)	X3-X12	—	—	—	—	—	w	—	—	—	—
γ , γ (i, i + 2)	X3-T5	w	*	—	—	—	—	—	—	—	—
γ , γ' (i, i + 7)	X3-T10	w	*	—	—	—	—	—	—	—	—

The intensity of the NOEs is described as follows: w = weak, m = medium, s = strong. D = Only observed in D₂O. ^aNOEs which are critical in defining the backbone hydrogen bonding register in the β -hairpin.

*Denotes an NOE that could not be observed due to signal overlapping.

**NOEs overlapped in H₂O-40% TFE spectra, only observed in D₂O-40% TFE spectra.

and downfield for the β -turn residues (Asn 7 and Gly 8). In addition to the previously included β -strand residues, the residues added at positions 3 and 12 also exhibit downfield shifts. The conformational shift values for Arg 1, Gly 2, Gly 13, and Arg 14 are almost identical in all of the peptides within the experimental error (Fig. 2A). In some cases, there

are some changes on Gly 2/Gly 13 conformation shift values, probably due to local effects produced by the mutation at positions 3 and/or 12. The peptide that exhibits the higher difference with respect to random-coil values is BH-KE.

The ³J_{NH α} coupling constants are related to the ϕ dihedral angle (Karplus 1959) and therefore should also be sensitive

Fig. 1. (A) Selected region of the 500 MHz NOESY spectrum of BH-KE peptide (10 mM, 278 K, pH 5.0, aqueous solution). The protons involved in each NOE are labeled as: N=NH, α =C α H, with the corresponding residue numbers. The important long-range NOEs are boxed. (Inset) The two $\alpha\alpha$ NOEs that define the β -hairpin. (B) Diagram of peptide BH-KE showing a summary of the NOEs found in 40% TFE and presented in Table 1. (Thick lines) Main-chain-main-chain NOEs. (Arrows) Side-chain-side-chain and side-chain-main-chain NOEs.

to the formation of a β -hairpin structure. The experimental $^3J_{\text{NH}\alpha}$ coupling constants values in the β -strand regions are generally higher than in the random-coil conformation (Serrano 1995; Smith et al. 1996) and also higher than in reference unstructured peptides (BH1, RGATANGATAGR; BH3, RGATANGKTYGR; and BH4, RGITVNGATAGR) (Ramírez-Alvarado et al. 1996), as expected for a preferred extended conformation (Table 2). In general, the $^3J_{\text{NH}\alpha}$ coupling constant values are higher for peptides BH-KE and BH-AE.

All of these results indicate that the β -hairpin structure is restricted to residues 3–12, at least for two of the peptides, extending the previous BH8 β -hairpin in one residue at each β -strand.

Fig. 2. C α H conformational shifts of the different BH-peptides. The conformational shifts of the C α H protons are obtained by subtracting the random-coil values (Merutka et al. 1995) from the experimental values. (A) Aqueous solution. (B) 40% TFE.

NMR structural analysis in trifluoroethanol solution

We have shown previously that a plateau in the β -hairpin population was found at 40% TFE for the BH8 series (Ramírez-Alvarado et al. 1997;). In these conditions, the conformational shift of the C α protons are close to that found in β -sheets of proteins, and a NOE-based calculation yielded hairpin population of 115% (Ramírez-Alvarado et al. 1997), indicating a population close to 100%. To compare the results obtained with BH8, we have used the same TFE concentration here. The NOE pattern of all of the peptides in both aqueous solution and 40% TFE is qualitatively the same,

Table 2. $^3J_{\text{NH}\alpha}$ of the BH system peptides (in Hz) in H_2O

Residue	BH-EK	BH-KE	BH-AE	BH-KA	BH-AA	Random
Arg1						
H ₂ O	—	—	—	—	—	6.5
Gly2						
H ₂ O	5.8	6.0, 5.8	5.8, 5.8	6.3, 6.0	6.1, 6.0	5.8
Glu3	5.8					6.1
Lys3		6.61		*		6.4
Ala3			*		*	5.8
Ile4						
H ₂ O	7.8	9.0	8.0	7.9	7.9	7.2
Thr5						
H ₂ O	8.2	9.0	*	8.5	*	7.5
Val6						
H ₂ O	7.3	8.1	8.1	*	*	7.4
Asn7	7.0	7.0	7.1	7.6	7.2	7.0
Gly8						
H ₂ O	5.8,*	5.7,*	6.1, 5.9	6.0, 6.6	6.2, 6.1	5.8
Lys9						
H ₂ O	7.4	7.6	7.6	7.5	7.5	6.4
Thr10						
H ₂ O	7.8	7.7	7.7	7.7	8.0	7.5
TFE						
Tyr11						
H ₂ O	7.2	7.6	7.6	*	7.2	7.2
Lys12	7.1					6.4
Glu12		7.8	7.3			6.1
Ala 12				7.5	*	5.8
Gly13						
H ₂ O	*	5.9, 5.8	5.9, 5.9	5.9, 6.1	5.4, 5.6	5.8
Arg14						
H ₂ O	7.4	7.5	7.6	7.9	7.7	6.5

Experimental conditions: 278 K, 10 mM, pH = 5.0, $\text{H}_2\text{O}/^2\text{H}_2\text{O}$ (9:1 v/v). The $^3J_{\text{NH}\alpha}$ values have an estimated error of ± 0.2 Hz.

* Not determined due to signal overlapping.

with differences due to changes in signal overlapping and a general increase in the NOE intensities (Table 1). The conformational shifts of the C α protons in 40% TFE (Fig. 2B) are larger than in aqueous solution (Fig. 2A) indicating an increase of the structured population. As in aqueous solution, peptide BH-KE is the one with the highest population. Its conformational C α H shifts are large, with similar values as found in β -sheets in proteins. Furthermore, it shows a pattern of up–down values for the strand residues that is predicted from chemical shift calculations for residues in β -sheets (Ösapay and Case 1994). This pattern is not recognized in BH8 because of its short strands and is not usually seen in protein because of ring currents and tertiary shifts. It is seen, for example, in the second β -strand of protein G B1 domain (Gronenborn et al. 1991; Blanco et al. 1994;). Determination of the population for this peptide

using the NOE intensities between the C α protons of residues 5–10 and 3–12, shows that it is close to 100% folded (104 ± 7 and 76 ± 1 , respectively). These observations indicate that the population of BH-KE is close to 100% in TFE, although there could be some fraying at the beginning and end of the two- β -strands.

Structure calculation

NOEs found in both aqueous and TFE experiments were included as distance restraints in the calculation of an ensemble of structures that represent the hairpin structure of BH-KE. All NOEs are compatible with essentially the same β -hairpin structure. This structure is similar to that calculated for the BH8 peptide in our previous report (Ramírez-Alvarado et al. 1996), only two residues longer (data not shown). The backbone dihedral angles are, within the standard deviation, the same as in the 10 best structures from BH8 peptide, but now with 4 residues per strand. Furthermore, these new structures can explain all of the observed NOEs without predicting undetected backbone–backbone NOEs. Therefore, the experimental data do not support the existence of alternative β -hairpin structures, but is consistent with equilibrium between a β -hairpin 2:2 structure and random-coil conformations in water.

β -hairpin population in water solution

We have calculated the β -hairpin population in these peptides assuming a two-state model in which the β -hairpin is in rapid equilibrium with random-coil conformations (Ramírez-Alvarado et al. 1997). The calculation has been done by use of different criteria, namely C α H conformational shift values, intensity of the NOEs between the C α protons, and the $^3J_{\text{NH}\alpha}$ coupling constants (see Materials and Methods for details). The summary of these calculations is shown in Table 3. In general, there is a reasonable agreement between the different methods used. The population ranking is BH-KE > BH-AE > BH-EK $\geq$ BH-KA $\sim$ BH-AA. The conformational shift profiles shown in Figure 2 suggest that there might be some fraying for residues 3 and 12. In fact, in all cases, the values for these positions are separated from the mean by >1 SD (data not shown). One possibility to explain this behavior could be that the Gly–Gly–X–Gly–Gly reference values (Merutka et al. 1995) used for these two residues for the random-coil state are not correct, due to local effects. However, the fact that we found similar behaviors for Lys, Glu, and Ala residues indicates that the low population for these positions cannot only be due to an incorrect reference state, but rather reflects fraying at the β -hairpin ends.

pH and ionic strength effect on the β -hairpin population

To discern the relative importance of the electrostatic interactions to the differences in the structured populations, we

Table 3. Determination of the β -hairpin population

Method	Position	BHKE	BHAE	BHEK	BHKA	BHAA
C α H ^a	3*	30*	20*	13*	15*	13*
	4	54	35	38	31	23
	5	49	41	35	28	32
	6	40	40	33	33	27
	9	64	55	45	36	36
	10	48	32	28	21	22
	11	56	50	44	25	25
	12*	24*	16*	4*	8*	4*
C α H _{av} ^b		52 $\pm$ 8	42 $\pm$ 9	37 $\pm$ 7	29 $\pm$ 7	28 $\pm$ 7
C α H _{av} ^c (1M NaCl)		21 $\pm$ 10	48 $\pm$ 13	18 $\pm$ 7	29 $\pm$ 9	28 $\pm$ 5
NOE ^d ² H ₂ O		49 $\pm$ 3	40 $\pm$ 2	40 $\pm$ 8	35 $\pm$ 3	38 $\pm$ 1
$\alpha\alpha$ 5-10						
NOE ^d ² TFE		104 $\pm$ 7				
$\alpha\alpha$ 5-10						
NOE ^d ² H ₂ O		45 $\pm$ 4	29 $\pm$ 2**	**	**	—
$\alpha\alpha$ 3-12						
NOE ^d ² TFE ^e		76 $\pm$ 1				
$\alpha\alpha$ 3-12						

All the peptides have been analyzed at pH 5.0.

^a The percentage of β -hairpin population was estimated assuming that the C α H chemical shifts of peptide BH1 (12) represents the random coil value and that of peptide BH-KE in 40% TFE the 100% population. The population has been calculated at a residue level using the six amino acids in the two strands (see Materials and Methods).

^b Average values obtained from the individual populations. The errors are the standard deviations.

^c Average values obtained from the six selected strand residues in 1 M NaCl. The errors are the standard deviations.

^d The absence of the $\alpha\alpha$ NOE is considered the 0% reference.

* These values have been calculated using as reference for 0% population a conformational shift of 0, instead of the value found in a random-coil peptide with a similar sequence.

** Not determined due to signal overlapping. Not reliable since it is too close to the diagonal.

^e The population estimate in 40% TFE was done by analyzing peptide BH-KE in D₂O – 40% TFE. The error is the result of measuring the NOE intensity on both sides of the diagonal.

(—) Absent.

analyzed the effects produced by changing the pH or increasing the ionic strength.

Addition of 1 M NaCl produces a change in the chemical shift C α H values of the β -turn for all peptides (Fig. 3). Regarding the β -strands, addition of salt significantly decreases the β -hairpin population of peptides BH-KE and BH-EK as shown by the upfield shift of all β -strand C α protons (Fig. 3). In contrast, the control peptides showed no changes within the experimental error ($\pm$ 0.02 ppm). Structural content of peptides BHKE and BHEK in 1 M NaCl is similar to that of the control peptide BH-AA (Table 3). This indicates that there is a favorable electrostatic interaction between Glu and Lys screened by salt. Interestingly, peptide BH-KA and especially peptide BH-AE are more structured than peptides BH-KE and BH-EK at 1M NaCl (Table 3). This could suggest that the favorable electrostatic interaction is in part counterbalanced by an unfavorable salt-independent interaction of the two residues.

The peptides analyzed in this work contain five groups that can be pH titrated: Glu, Lys, Arg, Tyr side chains, and the C-terminal carboxyl group. We analyzed the peptides between pH 2.5 and pH 6.5. to follow the titration of the Glu side chain and of the free C-terminal carboxyl group (pK_a ~4.5 for Glu and ~3.6 for the C-terminal carboxyl group, depending on the surrounding chemical environment), without titrating Lys, Tyr, and Arg residues. Comparison of the C α proton conformational shifts in aqueous solution and TFE indicates that the two Thr residues of our peptides are the most sensitive to changes in population, in agreement with our previous results (Ramírez-Alvarado et al. 1996, 1997). Therefore, we decided to follow the changes in population upon pH titration by plotting the C α proton values of Thr 5 and Thr 10 (Fig. 4). As a control, we pH titrated peptide BH1 (RGATANGATAGR; Ramírez-Alvarado et al. 1996) that is not folded in aqueous solution. In this peptide, there is an apparent pH transition, corresponding to titration

Fig. 3. Ionic strength effect on the C α H conformational shifts of the different BH-peptides. (●) Aqueous solution. (□) 1 M NaCl. The size of the symbols corresponds to the error in the measurement of the conformational shifts (± 0.02 ppm).

of sodium 3-trimethylsilyl (2,2,3,3- $^2\text{H}_4$) propionate (TSP) used to calibrate the NMR spectra. This transition is independent of the changes observed in the titrable groups of the peptides, and we have taken it into account in our fitting (de Marco 1977).

All of the peptides, except BH1, are more structured at higher pH values as shown by the downfield shift of the C α H of the two Thr residues (Fig. 4). Similar results are found by use of the amide groups of the strand residues as probes (data not shown). In the case of peptides BH-AA and BH-KA, the downfield shift (~ 0.06 ppm) can only be attributed to the pH titration of the C-terminal group, thus suggesting a stabilizing effect of the charged terminal carboxyl group. Similar results, but smaller in magnitude, are found for the other strand residues, except for the titrating Glu that is affected by the ionization state of its side chain

(data not shown).

The C-terminal Arg residues should be in a random conformation, as attested by the small changes in the C α H chemical shift values upon changes in structured population (Fig. 2A,B). Thus, pH titration of its C α H chemical shift value should provide a good estimate for the pK_a of the C-terminal group, whereas Arg 1 is a control (Fig. 5). We found a similar pK_a value for all peptides (3.1 ± 0.2) (data not shown). Using this value, we can fit the titration curves shown in Figure 4 (see Materials and Methods). For those peptides having a Glu residue, BH-EK, BH-AE, and BH-KE, we found a pK_a value between 4.0 and 4.5 for the Glu side chain (Table 4). In all cases, the charged Glu residue contributed favorably to β -hairpin stability, but the magnitude of the change for peptide BH-KE is significantly larger.

Fig. 4. pH titration of the C α protons of Thr 5, Thr 10, and Glu 12 in the different BH-peptides. We show the error bars for all points (± 0.02 ppm).

Fig. 5. pH titration of the C α protons of Arg 1 and Arg 14 in BH-AA peptide. We show the error bars for all points (± 0.02 ppm).

Discussion

We have studied the role of electrostatics interactions on β -hairpin stability by adding different amino acid pairs (Lys–Glu, Glu–Lys, Ala–Glu, Lys–Ala, Ala–Ala) at the termini of the sequence of the de novo-designed β -hairpin peptide BH-8. Addition of the ionic pair Lys–Glu resulted in the formation of a more stable β -hairpin motif having four-residues-per-strand. When looking at the individual residues, it seems that, similarly to what happens in α -helices, there is some fraying at the first and last positions of the

β -hairpin (positions 3 and 12). This fraying was not observed in the shortest peptide BH8 (Ramírez-Alvarado et al. 1997). Fraying at the ends of a β -hairpin has also been observed in a four-residue β -hairpin analyzed by Rico and coworkers (de Alba et al. 1996). The fact that the first and last residues in a four-residue-per-strand β -hairpin are not involved in main chain–main chain hydrogen bonds, as it happens in a three-residue-per-strand β -hairpin, could explain these results. Analysis of peptide BH-KE in 40% TFE supports this hypothesis, because the population estimate with the α - α NOE intensity between residues 3 and 12 seems to be lower than that using residues 5–10.

Ionic pairs in β -hairpin stability

Introduction of an ionic pair in our β -hairpin system results in a more stable structure when compared with the control peptide BH-AA. However, one orientation seems to be more favorable, with peptide BH-KE being more stable than BH-EK. This effect is partly due to a stabilizing effect of Glu at the end of the second β -strand, as shown by peptide BH-AE being more stable than peptide BH-AA. In addition, there is a favorable electrostatic interaction that is screened by 1 M NaCl. In previous work, the electrostatic interaction of ionic pairs in a zinc finger (Blasie and Berg 1997) and in the B1 domain of protein G (Smith and Regan 1995) have been determined. In both cases, a favorable contribution was found, but the magnitudes were different. In the first case, the value was <0.2 kcal/mol, whereas Regan and coworkers (Smith and Regan 1995) found values close to 1 kcal/mol. This difference could be due to the orientation of the ionic Lys–Glu pairs as follows: KE in the zinc-finger and EK in the B1 domain of protein G. Moreover, in the first case, the two residues were not in a main-chain hydrogen-bonded site, whereas they are in the B1 domain. In principle, we could determine the contribution to β -hairpin stability of the ionic pairs by performing a double-mutant cycle, as has been done for α -helices in proteins (Serrano et al. 1990). In the absence of a theoretical formalism with reasonable parameters, as for α -helices (Chakrabarty and Baldwin 1995; Muñoz and Serrano 1995, 1997; Lacroix et al. 1998), we need to assume that formation of the β -hairpin structure is an all or nothing event. This results in an apparent lower-limit estimate for the free-energy contribution of the Lys–Glu interaction in peptide BH-KE of approximately -0.3 kcal/mol. This value is similar to that measured in a hairpin peptide derived from the sequence of Tendami-stat, with modified residues at the turn, which forms a 3:5 β -hairpin (de Alba et al. 1995). In this peptide, a pH-dependent interaction between the side chains of Asp (the second residue of the turn) and Asn (the last residue of the first strand) contributes -0.3 kcal/mol to the free energy of hairpin formation. In another model, peptide (with a 2:2 β -hairpin structure) electrostatic interactions were also

Table 4. pH titration of the Glu containing peptides using the C α H groups of Thr5 and 10 as reporters

Peptide	Residue	Amplitude ^a ppm	Glu (pKa) ^b
BH-AE	Thr 5	0.05 ± 0.01	4.0 ± 0.2
	Thr 10	0.02 ± 0.01	4.0 ± 0.2
BH-KE	Thr 5	0.15 ± 0.02	4.0 ± 0.1
	Thr 10	0.13 ± 0.01	4.0 ± 0.2
BH-EK	Thr 5	0.03 ± 0.01	4.5 ± 0.4
	Thr 10	0.02 ± 0.01	4.3 ± 1.0

In all the peptides, the largest effect on the conformational shift of the C α proton upon pH titration is observed for the C-terminal Arg residue. We have used the pH titration curve of this proton to determine the pKa of the C-terminal carbonyl group, which is the same in all peptides within the error (3.0 ± 0.2). The dependence of the chemical shift of TSP with pH has been taken into consideration when fitting the data shown in Figure 5.

^a Magnitude of the change upon titration of the Glu side chain. This is on top of the ~ 0.06 ppm change produced by titration of the carboxy terminal group.

^b pKa of the Glu side chain.

found to contribute to hairpin stability (Searle et al. 1999). In this peptide, the side chains of Lys 1 and Lys 2 interact with the charged C terminus carboxylate contributing approximately -0.25 and -0.1 kcal/mol, respectively. Thus, all of these studies indicate that ionic interactions can contribute significantly to β -hairpin stability, as they do in α -helices (Marqusee and Baldwin 1987).

Favorable contributions of negative charges at the C-terminal end of the β -hairpin

The pH titration experiments show a stabilization of the β -hairpin motif when Glu 12, or the C terminus are charged. In the β -hairpin derived from Tendamistat, it was found that the charged N and C termini, which were part of the structure, stabilized the β -hairpin (de Alba et al. 1995). In our case, the charged termini are separated from the structured region by two unstructured residues, and therefore, any interaction between them or with other charged residues should be relatively small. Moreover, as we showed in the Results section, addition of 1 M NaCl does not affect the β -hairpin population in those peptides that do not have an interstrand ionic pair. This means that the stabilizing effect of Glu 12 and of the C-terminal carboxyl group is not purely electrostatic. It is possible that the uncharged carboxyl group of Glu 12 and of the C terminus competes with the main-chain carbonyl groups involved in the β -hairpin structure, favoring some local non-hairpin turn-like structures. It is important to mention in this context that in α -helical peptides, the secondary structure propensity of Glu is pH dependent (Lacroix et al. 1998). Similarly, the charged termini can affect α -helix stability in a salt-independent manner (Lacroix et al. 1998). In any case, these effects should be taken into consideration when designing β -hairpin peptides, or when formulating models to explain β -hairpin formation in aqueous solution.

Materials and methods

Peptide synthesis

The peptides were synthesized by the EMBL peptide synthesis service using Fmoc chemistry and PyBOP activation at a 0.025 mmole scale. Peptide homogeneity and identity were analyzed by analytical high-performance liquid chromatography, amino-acid analysis, and matrix-assisted laser desorption time-of-flight mass spectrometry. The concentration of the peptide samples was determined by ultraviolet absorbance (Gill and Hippel 1989).

Nuclear magnetic resonance

NMR samples were prepared in H_2O with 10% $^2\text{H}_2\text{O}$ (by volume), or in $^2\text{H}_2\text{O}$, with milli Q water from a Millipore water system and $^2\text{H}_2\text{O}$ from Cambridge Isotope Chem., 40% aqueous perdeuterated trifluoroethanol ($\text{CF}_3\text{C}^2\text{H}_2\text{O}^2\text{H}$, Cambridge Isotope Chem.).

Minute amounts of HCl and NaOH were added to adjust the pH of the samples; this was measured with an Ingold combination electrode (Wilmad) inside the NMR tube, and isotope effects were not corrected. Sodium 3-trimethylsilyl (2,2,3,3- $^2\text{H}_4$) propionate (TSP) was used as an internal reference at 0.00 ppm. NMR experiments were performed on Bruker AMX-500 and AMX-600 spectrometers. The data were processed with the program XWINNMR from Bruker in an SGI computer. DQFCOSY (Piantini et al. 1982), NOESY (Kumar et al. 1980), and ROESY (Bothner-By et al. 1984) spectra were acquired by use of standard procedures. NOESY and ROESY spectra were recorded routinely for every peptide, and the spectra were jointly analyzed to discard artifactual NOEs as those arising from spin diffusion. The mixing times used were 200 and 100 ms for spectra recorded in water and 40% TFE, respectively. TOCSY (Bax and Davis 1985) and TOWNY (Kadkhodaei et al. 1993) spectra were acquired by use of the standard MLEV17 spin-lock sequence and 80-ms mixing time. The spectral width was 5555.55 and 6666.66 Hz, and the water signal was presaturated during the relaxation delay (1 sec) and also during the mixing time of NOESY spectra. The peptide ^1H -NMR spectra were assigned by the sequential assignment procedure (Wüthrich 1982). Crosspeak intensities were evaluated by visual inspection of the contour levels and by peak integration procedure. The conformational shifts (conformation-dependent chemical shifts dispersion) of the $\text{C}\alpha$ protons were obtained by subtracting the random-coil values (Merutka et al. 1995) from the measured ones for each residue.

The $^3\text{J}_{\text{HN}\alpha}$ values were measured in proton 1D spectra with high-digital resolution and signal-to-noise ratio. Resolution enhancement functions were used prior to Fourier transform, thus minimizing the distorting effect of the line width in some of the amide signals. The $^3\text{J}_{\text{HN}\alpha}$ values were also measured from 2D spectra by use of the program MEDEA (J. Santoro, unpubl.).

Structure calculations

Crosspeak intensities in the NOESY spectra of BH-KE in H_2O and 40% TFE were classified into four categories as follows: strong, medium, weak, and very weak. These categories were translated into upper limit distance constraints of 0.3, 0.35, 0.4, and 0.5 nm, respectively. One hundred structures were generated by use of a set of 22 sequential and 20 nonsequential distance restraints plus 10 ϕ angle restraints (for threonines, whose $^3\text{J}_{\text{HN}\alpha}$ were larger than 8 Hz, the applied restraints were $-160^\circ < \phi < -60^\circ$ and for the rest of the residues, the ϕ angle was constrained to be negative except for Asn 6 and Gly 7, in which no constraint was applied) with the distance geometry program DIANA (Güntert et al. 1991).

Estimation of the β -hairpin population

The chemical shifts are linear averages over the population of the different conformers. Although in Figure 2 we show the conformational shifts of the $\text{C}\alpha$ protons using as a reference the chemical shift values of Merutka et al. (1995), to calculate the populations we have used a different reference. The reason we do this is to eliminate possible local structural and chemical effects on the $\text{C}\alpha\text{H}$ chemical shifts for the 20 amino acids (Serrano 1995; Smith et al. 1996) that are independent of the formation of the β -hairpin. The reference values for the random-coil state in the case of the alpha proton conformational shifts were those derived from the unstructured peptides BH1, BH3, and BH4 (Ramírez-Alvarado et al. 1996). Peptide BH3 was used as a reference for Ile 4, Thr 5, and Val 6, whereas peptide BH4 was used as a reference for Lys 9, Thr

10, and Tyr 11. For positions 3 and 12 we followed Merutka et al (1995) as we did not have a reference peptide. The reference parameters for 100% β -hairpin population used were the C α H conformational shifts of peptide BH-KE in 40% TFE.

When using the intensity of the $\alpha\alpha(i, i + 5)$ NOE between Thr 5 and Thr 10 for population estimation, the average over the two states is nonlinear due to the r^{-6} dependence of the NOE intensity with interproton distance. We have considered that the contribution of the random-coil state to the intensity of this NOE is zero, as this distance is going to be very large, and the distance in the β -hairpin structure is 2.3 Å, the average distance in protein β -sheets (Wüthrich 1982). This method assumes that the overall correlation time of the molecule is the same in both states and requires an internal calibration for translating NOE crosspeak intensities to interproton distances. The intensity of the intraresidue NOE between the geminal C β protons of Tyr 10 was used for this purpose (distance = 1.75 Å), and the NOE intensity ratio $d_{\alpha\alpha}(T5-T10)/d_{\beta\beta}(Y10)$ was used to estimate the β -hairpin population as described in Bradley et al. (1990). In a previous work, we used the NOE intensities, measured by integration of the volume of the crosspeak, or the area below 1D cross-sections of 200 ms NOESY spectra recorded in pure $^2\text{H}_2\text{O}$. The measurements derived from the 1D cross-sections are more consistent, especially when one of the protons has a resonance frequency close to that of the remaining water protons, as the baseline correction procedures are more efficient. The values in Table 4 are those derived from the cross-sections and were obtained from experiments in $^2\text{H}_2\text{O}$ to eliminate the possible distortion due to the water signal.

pH titration

TOCSY spectra were recorded at different pH values ranging from 2 to 7. Three NOESY spectra were acquired at pH 2, pH 5, and pH 7.0 to confirm the assignment of the TOCSY crosspeaks. The changes in the chemical shifts of the C α and amide protons in the peptides that do not have a Glu residue were fitted with the following equation that takes into consideration the displacement of the TSP signal with pH (de Marco 1977).

$$\text{C}\alpha\text{H}_{\text{obs}} = \text{C}\alpha\text{H}_{\text{ref}} + (\text{Amp}_{\text{C}\alpha\text{H}} / (1 + 10^{(\text{Carb}_{\text{pKa}} - \text{pH})})) + (0.019 / (1 + 10^{(\text{TSP}_{\text{pKa}} - \text{pH})})) \quad (1)$$

where $\text{C}\alpha\text{H}_{\text{obs}}$ is the value we observe, $\text{C}\alpha\text{H}$ is the value of the corresponding proton at pH 2.0, $\text{Amp}_{\text{C}\alpha\text{H}}$ is the amplitude of the change induced by titration of the charged C-terminal-free carboxyl group, Carb_{pKa} is the pKa of this group, 0.019 is the amplitude of the change in the position of TSP, and TSP_{pKa} is the pKa of TSP (de Marco 1977). To obtain the pKa value of the C-terminal group, we followed the titration of the C α proton corresponding to the last residue in the peptide (Arg 14), that is in a random-coil conformation. This value is ~ 3.1 for all of the peptides analyzed. The amplitude of the changes in other protons induced by titration of the free C-terminal group is small, therefore, to diminish errors, we fixed its pKa value to 3.06 in the fitting.

In the case of peptides containing a Glu residue, we used the following equation:

$$\text{C}\alpha\text{H}_{\text{obs}} = \text{C}\alpha\text{H}_{\text{ref}} + (\text{Amp}_{\text{C}\alpha\text{H}} / (1 + 10^{(\text{Carb}_{\text{pKa}} - \text{pH})})) + (0.019 / (1 + 10^{(\text{TSP}_{\text{pKa}} - \text{pH})})) + \text{Amp}2_{\text{C}\alpha\text{H}} / (1 + 10^{(\text{Glu}_{\text{pKa}} - \text{pH})}) \quad (2)$$

where $\text{Amp}2_{\text{C}\alpha\text{H}}$ is the amplitude of the change produced on the particular proton being analyzed by the titration of the Glu residue and Glu_{pKa} is the pKa of Glu residue.

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