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Design of a bivalent peptide with two independent elements of secondary structure able to fold autonomously

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Abstract: This article describes a strategy to develop, starting from a *de novo* design, bivalent peptides containing two different (α-helix and β-hairpin) and independent secondary-structure elements. The design was based on the use of conformationally restricted peptide libraries. Structural characterization by NMR revealed that the peptides were stable and did not show any long-range NOE interactions between the *N*-terminal β-hairpin and the *C*-terminal α-helix. These results suggest that the two elements of secondary structure are stable and well folded. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

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Keywords: bivalent peptides; conformationally defined; peptide design; peptide libraries; NMR spectroscopy

INTRODUCTION

For several applications of synthetic peptides, a well-defined conformation is required. Considerable effort has been devoted to developing stably folded peptides that would constitute the basis for the design of miniature proteins, short polypeptides typically having fewer than 40 residues. These molecules would bring opportunities to study the fundamental forces behind protein folding, protein–peptide, and protein–protein interactions because of the decreased complexity of these systems when compared to larger proteins [1]. Of particular interest in this regard are small proteins that do not require disulfide bonds or cofactor binding to fold. The design and study of oligomeric α-helical peptides [2–4] have contributed to a good understanding of the forces that control interhelical associations. However, the understanding of β-sheet structure lags behind that of α-helices due to the scarcity of simplified models [5–8]. The next degree of complexity is the design and study of mixed α/β structures. Only a few of them have been successfully designed based on the stabilization of the structure by domain–domain tertiary interactions [9–14]. We are interested in the design of polypeptides with independent motifs able to fold autonomously that could be used as templates to design more complex model systems for the study of protein–protein interactions. In particular, it would be interesting to have peptides available with mixed α/β structures containing a well-defined β-hairpin domain and a monomeric α-helical domain, folded in the absence of tertiary interactions. These models will offer an attractive bivalent surface with different features that could be useful both for potential applications and for basic studies. In fact, properly folded structurally bivalent peptides would be able to contact a protein surface at different places simultaneously or bring together in space two proteins by independent binding of the two domains [15,16]. In basic studies, these simplified peptides could represent an attractive model to further design a mini-protein counterpart that would recognize and bind to the initial bivalent peptide. Until now, most of the multivalent peptide approaches [17,18] have focused on the modulation of protein interactions by increasing the biochemical binding affinity. However, little attention has been paid to the fact that structurally stable motifs, β-strands and/or α-helices, are critical in sustaining protein–protein interactions and molecular recognition [19]. In an attempt to address both issues, the multivalency and the need for structurally stable motifs, we report the rationally guided combinatorial design and structural characterization by NMR of a monomeric, bivalent peptide consisting of two independent elements of secondary structure, a β-hairpin and an α-helix, tethered by a Gly linker.

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MATERIALS AND METHODS

Conformationally Defined Peptide Library and Individual Peptide Synthesis

The library and individual peptides were prepared by simultaneous multiple-peptide synthesis, using Fmoc chemistry as described elsewhere [20–22]. The mixture ('X') positions were incorporated by coupling a mixture of 19 L-amino acids (cysteine was omitted) with a relative ratio suitably adjusted to yield close to equimolar incorporation. The quality of the synthesized peptide mixtures was validated by mass spectrometry. Individual peptides were purified by preparative RP-HPLC, and peptide identity was confirmed by laser desorption-TOF MS

Circular Dichroism Spectroscopy (CD)

Spectra were acquired on a Jasco J-810 CD spectropolarimeter. Far-UV CD spectra of the peptide mixtures were the average of a series of 20 scans recorded at peptide concentrations of 0.025 and 0.1 mM at 5 °C in 5 mM MOPS buffer, pH 7. The far-UV CD spectra of peptides were acquired at peptide concentrations of 0.025 and 0.1 mM, at different pH values, buffers, and temperatures (see Results and Discussion section and figure legends for details). Peptide concentrations were determined spectrophotometrically as described [22].

Peptide Aggregation

Different approaches were applied to determine the monomeric state of the peptides. First, the CD spectra of each peptide at different concentrations (0.025 and 0.1 mM) in buffer and in the presence of 30% or 50% (vol/vol) of TFE were analyzed. Second, the signal line widths and chemical shifts from the 1D ^{1}H NMR spectra (90% H_2O/10% D_2O) at peptide concentrations from 0.1 to 1.5 mM were compared. Third, some selected peptides (see Results and Discussion section) were further evaluated by sedimentation equilibrium experiments on dilute (0.1 mM) and concentrated samples (1 mM).

Nuclear Magnetic Resonance (NMR) Spectroscopy

All NMR experiments were recorded at 283 K on a Bruker AV 600 MHz equipped with cryoprobe on peptide samples of 1.5 mM concentration in 90% H_2O/10% D_2O and 100% D_2O solutions at pH 5. Chemical shifts were internally referenced to the water signal, set at 4.8 ppm and ^{13}C chemical shifts were referenced indirectly using the gyromagnetic ratio ^{13}C : ^{1}H [23]. Phase-sensitive TOCSY spectrum and NOESY spectrum were performed using the time proportional phase incrementation mode and a heteronuclear single quantum coherence (^{13}C-HSQC) spectrum (natural abundance) was performed using echo/antiecho gradient selection. An 80-ms MLEV17 spin-lock sequence was used for the TOCSY experiment, and a 200-ms mixing time for the NOESY experiment. Solvent suppression was achieved by selective presaturation of the water signal during the relaxation delay (1.2 s) or by field-gradient pulses. Two dimensional acquisition data matrices for the TOCSY and the NOESY experiments consisted of 2048 × 512 data points in the t2 and t1 dimensions, respectively, and for the ^{13}C-HSQC was 4096 × 512 data points in t2 (^{1}H) and t1 (^{13}C) dimension. Spectra were processed with the Bruker TOPSPIN software and transferred into the NMR View program [24] for data analysis. Conformational shifts for ^{1}Hα($\Delta\delta_{1H\alpha}$) and ^{13}Cα($\Delta\delta_{13X\alpha}$) resonances for secondary structure evaluation were calculated with the equation $\Delta\delta_X = \delta_X^{obs} - \delta_X^{rc}$, where δ_X^{obs} is the observed chemical shift of a particular ^{1}Hα or ^{13}Cα resonance and δ_X^{rc} is the corresponding random-coil value, taken from Merutka *et al.* [25] and Wishart *et al.* [23] for ^{1}Hα and ^{13}Cα respectively. Structures were calculated using a simulated-annealing protocol based on molecular dynamics in torsion-angle space as implemented in the program CYANA [26,27], PROCHECK-NMR [28], and MOLMOL [29] were used to validate and to visualize the final structures, respectively.

RESULTS

The first challenge in developing a polypeptide with independent protein motifs able to fold autonomously was to select the two structural domains. These domains should be well folded and monomeric in a wide interval of concentrations at biological conditions of ionic strength, pH, and temperature. For the β-structure domain, we selected a 2 : 2 monomeric β-hairpin peptide with a type I′ β-turn previously designed in our laboratory [7,8]. For the α-structure, it was more difficult to make a selection and we found stability problems and a high tendency to self-aggregation in the first series of polyalanine-based peptides. Although Ala has been described as an α-helical stabilizing amino acid [30,31], when designing putative monomeric helical peptides it should be taken into account that polyalanine-based peptides have a high tendency to form helical bundles [32], can aggregate into β-pleated sheet complexes [33], may form toxic aggregates [34–36], and are unstable in aqueous solution at room temperature, and charged or neutral polar residues are needed to have access to α-helical conformations in mild solvents [37,38]. Based on the successful design of our previous β-hairpin peptide from a conformationally restricted peptide library (CRL), we decided to explore an α-helical peptide library (α-CRL) previously used in our laboratory for the identification of bioactive peptides [39,40]. In the present study we analyzed the library in order to explore the selection of both monomeric and oligomeric α-helical peptides. The α-CRL was based on a polyalanine scaffold that incorporates positive elements of α-helix design. The library was designed in a positional scanning format [22,41] with four mixture positions in one face of the helix. The scaffold peptide named HA1 and the library mixtures (Table 1) contained 11 and 7 Ala residues, respectively, out of 17 amino acids (see below). Ser was selected as the *N*-terminal residue because its side chain allows the formation of an extra hydrogen bond with the amino acid at the $i + 3$ position. At that position, we selected Glu since the Ser-Xaa-Xaa-Glu sequence, where Xaa is any amino acid residue, has been reported previously as a favorable *N*-terminal 'capping box' [42]. The *C*-terminal position was always occupied by Gly due to its

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Table 1 Peptide sequences and structural characterization of the MHA peptides

Peptide		$[\theta]_{222\ nm}$ [b]		α-helix [c]		Oligomeric state analysis		Agadir [f]
Name	Sequence [a]	Buffer	TFE	Buffer	TFE	A.U. [d]	NMR [e]	
HA1	SAAEAAAKAAAEAAAKG	−18 599	−20 700	57	64	n.d.	n.d.	75
	Positional scanning library sequences							
	SAAEA**O**$_{6}$AK**X**$_{9}$**X**$_{10}$AEA**X**$_{14}$AKG							
	SAAEA**X**$_{6}$AK**O**$_{9}$**X**$_{10}$AEA**X**$_{14}$AKG							
	SAAEA**X**$_{6}$AK**X**$_{9}$**O**$_{10}$AEA**X**$_{14}$AKG							
	SAAEA**X**$_{6}$AK**X**$_{9}$**X**$_{10}$AEA**O**$_{14}$AKG							
MHA1	SAAEA**Y**AK**EI**AEA**L**AKG	−22 086	−16 727	68	52	M—Olg	Olg	54
MHA2	SAAEA**Y**AK**EI**AEA**M**AKG	−8 395	−14 557	27	45	n.d.	n.d.	44
MHA3	SAAEA**Y**AK**EL**AEA**L**AKG	−16 350	−20 020	51	62	n.d.	n.d.	65
MHA4	SAAEA**Y**AK**EL**AEA**M**AKG	12 774	−11 910	40	38	n.d.	n.d.	54
MHA5	SAAEA**Y**AK**RI**AEA**L**AKG	−36 651	−13 499	111	42	n.d.	n.d.	62
MHA6	SAAEA**Y**AK**RI**AEA**M**AKG	−16 976	−19 488	53	60	M—M	M	54
MHA7	SAAEA**Y**AK**RL**AEA**L**AKG	−18 404	−17 439	57	54	n.d.	n.d.	71
MHA8	SAAEA**Y**AK**RL**AEA**M**AKG	u.a.				n.d.	n.d.	67
MHA9	SAAEA**Y**AK**WI**AEA**L**AKG	u.a.				Olg—Olg	n.d.	66
MHA10	SAAEA**Y**AK**WI**AEA**M**AKG	−19 525	−28 466	60	87	M—Olg	n.d.	54
MHA11	SAAEA**Y**AK**WL**AEA**L**AKG	−18 841	−25 904	58	79	n.d.	n.d.	71
MHA12	SAAEA**Y**AK**WL**AEA**M**AKG	−48 376	−22 540	147	69	n.d.	Olg	62

[a] *N*-terminal acetylated and *C*-terminal amidated peptides.
[b] $[\theta]_{222\ nm}$ is the mean residue molar ellipticity in MOPS buffer pH 7 or in the presence of 50% TFE. The CD spectra were acquired at 100 μM at 5 °C.
[c] The helical content was calculated by $FH = ([\theta]_{222\ nm} - [\theta]^{0\%}_{222\ nm}) \times 100/([\theta]^{100\%}_{222\ nm} - [\theta]^{0\%}_{222\ nm})$ where $[\theta]^{0\%}_{222\ nm}$ is 640 and $[\theta]^{100\%}_{222\ nm}$ based on a theoretical value of 100% helix ($[\theta]^{100\%}_{222\ nm} = -40\,000\ (1 - k/n)$ where the wavelength-dependent constant, k, is 2.5; and n is the number of helical residues) for a 17-residue peptide is $-34\,120$ deg cm^2 dmol^{-1}.
[d] Analytical ultracentrifugation analysis at two different peptide concentrations. The left entry is the oligomeric state found at 0.1 mM peptide concentration, while the right entry is at 1 mM peptide concentration. The entries are M for monomeric or O for oligomerized.
[e] One-dimensional NMR analysis at two different peptide concentrations to evaluate the oligomerization state of the peptides in water at pH 5. A peptide was considered monomeric (labeled M) if there was no significant change in the line width and chemical shifts of ^{1}H 1D NMR resonances of spectra measured at 0.1 mM and 1.5 mM concentrations. Otherwise, the peptide was considered oligomeric (labeled Olg).
[f] Percentage of helical content as determined by the software Agadir (http://www.embl-heidelberg.de/Services/serrano/agadir/agadir-start.html).
n.d, Not determined.
u.a., The peptide precipitated at 100-μM peptide concentration.

high frequency at the end of helices found in natural proteins [43] and model peptide systems [44]. The *N*- and *C*-terminal amino acids were further acetylated and amidated, respectively, to minimize charge repulsion with the helix dipole. Two Glu/Lys pairs were inserted at positions i, $i + 4$ in contiguous helix turns (pairs at positions 4, 8 and 12, 16) as stabilizing ionic interactions. Ala residues at positions 5, 11, and 15 completed the helix stabilizing face. The number and place for the library positions were carefully selected in order to maximize as possible their distribution along the peptide sequence and to minimize any interference with the residues in the helix stabilizing face. The library face was then defined by Ala residues at positions 2, 3, 7, 13, and four X positions (6, 9, 10, and 14 – Table 1), defined by a close to equimolar mixture of the 19 natural amino acids (cysteine was excluded to avoid the formation of intermolecular disulfide bridges).

Structural Screening of the α-CRL

A CD spectroscopy-based structural deconvolution was applied to the α-CRL with the aim of identifying particular amino acids that would favor the formation of monomeric α-helices. For our purposes, the selection strategy should contemplate the ability of the particular amino acid that defines each peptide mixture to stabilize the helix and the analysis of parameters that could differentiate a tendency to get monomeric helices (as opposed to aggregates or helix bundles). To this aim one should take into account that the characteristic far-UV CD spectrum of an α-helical polypeptide shows a positive band at 190 nm and two negative bands at 208 and 222 nm, respectively. Thus, it has been reported that peptides that populate single stranded α-helical conformations have $[\theta]_{222}/[\theta]_{208}$ ratio (q) smaller than 1, whereas α-helical coiled-coils show

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typically q ratios close to or larger than 1. These spectra are concentration-independent for monomeric α-helices and concentration-dependent for peptides with a tendency to form helix bundles. Furthermore, the presence of TFE disrupts the quaternary structure of oligomeric helices rendering single-stranded α-helical species [45], a process that can be followed by CD as an apparent loss of helical content due to the reduction in the ellipticity value at 222 nm. Therefore, the deconvolution procedure was based on the following criteria: (i) the dependence of the ellipticity with the concentration of the peptide mixture; (ii) the q ratio, and (iii) the behavior of the peptide mixtures in the presence of TFE. All 80 peptide mixtures that defined the positional scanning library were analyzed in buffer solutions at two different peptide concentrations (25 and 100 μM) and in the presence of 30% and 50% TFE. The starting criterion used to select the most suitable amino acids at each position was that the calculated percentage of α-helical content of the peptide mixture should be higher than 10% (Figure 1(a)). Although relatively redundant, the screening allowed the selection of defined amino acids at each position. For the first position (O_6), Trp and Tyr were chosen; for the second position (O_9) Glu, Lys, Arg, Ser, Thr, Trp, and Tyr; for the third position (O_{10}), Ala, Glu, Ile, Leu, Met, Arg, Val, and Trp; and for the fourth position (O_{14}), Ala, Ile, Lys, Leu, and Met. The peptide mixture defined by Pro at position O_9 showed an unexpected apparently high content of α-helical conformation (Figure 1(a)). However, Pro was not selected because of its well-known role as α-helix breaker [46]. To further refine the amino acid selection procedure, the most α-helical mixtures were evaluated by means of the parameter $F = [(\text{relative } \alpha\text{-helical content in each series})/q]$. Then, according to the data in Figure 1(b), Tyr was selected at position 6 and Glu, Lys, Arg, Trp, and Tyr at position 9. However, at this stage the pairs Lys/Arg, and Trp/Tyr can be seen as chemically redundant, and in order to decrease the final number of peptides, Lys and Tyr were excluded from selection for position 9. Ile and Leu were selected at position 10, and Leu and Met at position 14. With the amino acids chosen for each position a total of 12 ($1 \times 3 \times 2 \times 2$) individual peptides, representing all possible combinations, were designed and synthesized (MHA peptides – Table 1).

Structural Characterization of the MHA Peptides

The initial structural characterization of the MHA peptides was performed by CD (Table 1) following the

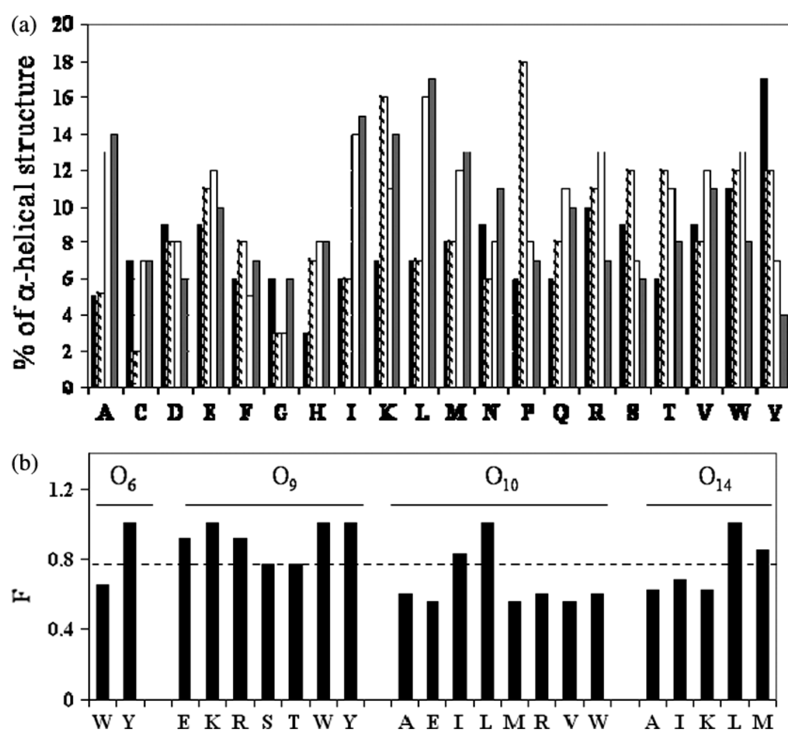

Figure 1 Structural screening. (a) Percentage of helical structure for the peptide mixtures calculated as described in footnote c of Table 1. The panel represents a set of 20 mixtures and each bar represents the percentage of α-helical structure of each peptide mixture defined by each amino acid in the four different sublibraries that complete the positional scanning α-helical CRL. Ac-SAAEA**O**$^{\mathbf{6}}$AK**XX**AEA**X**AKG-NH_2 (black bars), Ac-SAAEA**X**AK**O**$^{\mathbf{9}}$**X**AEA**X**AKG-NH_2 (black and white bars), Ac-SAAEA**X**AK**XO**$^{\mathbf{10}}$AEA**X**AKG-NH_2 (white bars) and Ac-SAAEA**X**AK**XX**AEA**O**$^{\mathbf{14}}$AKG-NH_2 (gray bars). (b) To further refine the amino acid selection procedure, the most helical peptide mixtures were evaluated by means of the parameter F (y axis – see the Results and Discussion section for a definition of F). In the x axis, the amino acids that define the most α-helical mixtures from each library are listed.

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same criteria applied for the library deconvolution (see above). In general terms, the 12 defined MHA peptides, selected from an initial pool of close to 140 000 sequences, fold predominantly in an α-helical conformation, and in most cases we obtain a good correlation with the percentage of α-helix predicted by semiempirical algorithms like Agadir (Table 1). Furthermore, in MOPS buffer at pH 7 and 5 °C, the far-UV spectra of all peptides, but MHA8 and MHA9, were independent of peptide concentration in the 25–100 μM range. The latter two peptides, containing residues Tyr, Arg, Leu, Met, and Tyr, Trp, Ile, Leu, at positions 6, 9, 10, and 14, respectively (Table 1), showed CD spectra typical of α-helix at 25 μM, but precipitated at 100 μM peptide concentrations. The CD spectra were then also acquired in the presence of 30 and 50% of TFE. For all peptides, the α-helix content was higher in 30% TFE than in plain buffer, but in 50% TFE they showed two different behaviors. A set made of MHA peptides 2, 3, 6, 10, and 11 rendered approximately the same percentage of helical structure in the presence of 30% and 50% TFE, which is indicative of monomeric state. However, a second set, including MHA peptides 1, 4, 5, 7, and 12, showed an apparent loss of helix conformation at high TFE concentration suggesting a loss of quaternary structure. From these results, two groups of peptides can be established depending on the amino acid at positions 10 and 14, regardless of the residues at positions 6 and 9. The first group is composed by peptides containing the pairs Ile^{10}–Met^{14} or Leu^{10}–Leu^{14} that showed a tendency to adopt monomeric structures: MHA peptides 2, 3, 6, 10, and 11, with the exception of MHA7. The second includes peptides with the pairs Ile^{10}–Leu^{14} or Leu^{10}–Met^{14} that tend to adopt oligomeric structures: MHA peptides 1, 4, 5, 8, 9, 12. Favorable interactions to stabilize monomeric helices can be deduced from the sequence and structure of peptides within the first group. The structural content ranking obtained from this analysis is MHA10 > MHA11 > MHA6 ∼ MHA3 > MHA2. Peptides MHA10, MHA6, and MHA2 have the presence of Ile^{10} and Met^{14} residues in common. Thus, their different structural content must be related to the type of amino acid at positions 6 and 9. Peptides MHA10, MHA6, and MHA2 contain Tyr^{6}–Trp^{9}, Tyr^{6}–Arg^{9}, and Tyr^{6}–Glu^{9}, respectively. Therefore, in our model peptide system, $i - i + 3$ aromatic–aromatic interactions appear to be more helix stabilizing than aromatic-positive (cation–π) or aromatic-negative charge (anion–π), although the presence of Glu12 could induce an additional repulsive effect on Glu^{9}. For the other two peptides of the monomeric set, having in common Leu^{10} and Leu^{14}, the rank order also suggests that the $i - i + 3$ aromatic–aromatic interactions (peptide MHA11) are more stabilizing than anion–π interactions (peptide MHA3).

The influence of a partial neutralization of the negative charge of the Glu residues on the conformation of the peptides was evaluated from the CD spectra recorded in MOPS buffer at pH 5 and 5 °C. In general, and regardless of the number of Glu residues in the sequence, peptides with a tendency to populate oligomeric states were found to be more sensitive to variation of pH than those adopting monomeric α-helices. As representative examples, the observed percentage of helical content for monomeric peptides MHA6 and MHA10 were 53/40 and 60/55 (helical content at pH 7/helical content at pH5), respectively, while for oligomeric peptides MHA1 and MHA5 we obtained values of 57/27 and 111/30, respectively. Thus, the loss of helicity at acidic pH is most likely related to a loss of oligomerization, which indicates the importance of electrostatic interactions for the stabilization of helix bundles. In contrast, the temperature dependent loss of the secondary structure was more uniform and all peptides showed a reduction (20–30%) of α-helical conformation at 25 °C with respect to the values obtained at 5 °C.

Further examination of the oligomerization state of selected MHA peptides was performed by analytical ultracentrifugation and NMR. According to the ultracentrifugation analysis, peptides MHA 1 and 10 were monomeric at 0.1 mM but oligomeric at 1 mM concentration, while MHA9 was oligomeric at the two concentrations analyzed. In contrast, the peptide MHA6 was monomeric at both 0.1 and 1 mM. The analysis of 1D NMR spectra supported the monomeric state of the MHA6 peptide as there was no significant change in the line width and chemical shifts of ^{1}H NMR resonances of spectra measured at 0.1 and 1.5 mM concentrations. Therefore MHA6 was chosen for further structural analysis by NMR.

NMR Studies on MHA6

Assignment of backbone proton and carbon resonances of MHA6 was complete. Further analysis of the TOCSY and NOESY experiments allowed a nearly complete ^{1}H side chain assignment, except for H^{ε} of Met^{14} and H^{δ} of Lys^{16}. NMR chemical shifts were assigned and deposited at the BMRB databank by combining information extracted from 2D experiments using the classical strategy [47]. The α-helical conformation of MHA6 was confirmed by $^{1}H_{\alpha}$ conformational chemical shifts, the intensity and type of backbone sequential connections, as well as short-range NOEs. An example of the quality of the spectra obtained for MHA6 is shown in Figure 2, where the $^{1}H_{N}$–$^{1}H_{\alpha}$ region used for sequential assignment and the $^{1}H_{N}$–$^{1}H_{N}$ region of the NOESY spectrum confirming the existence of an α-helical structure for MHA6 are displayed. Moreover, MHA6 structure displays many of the expected hydrogen bonds between the CO groups of residues i and the NH groups of residues

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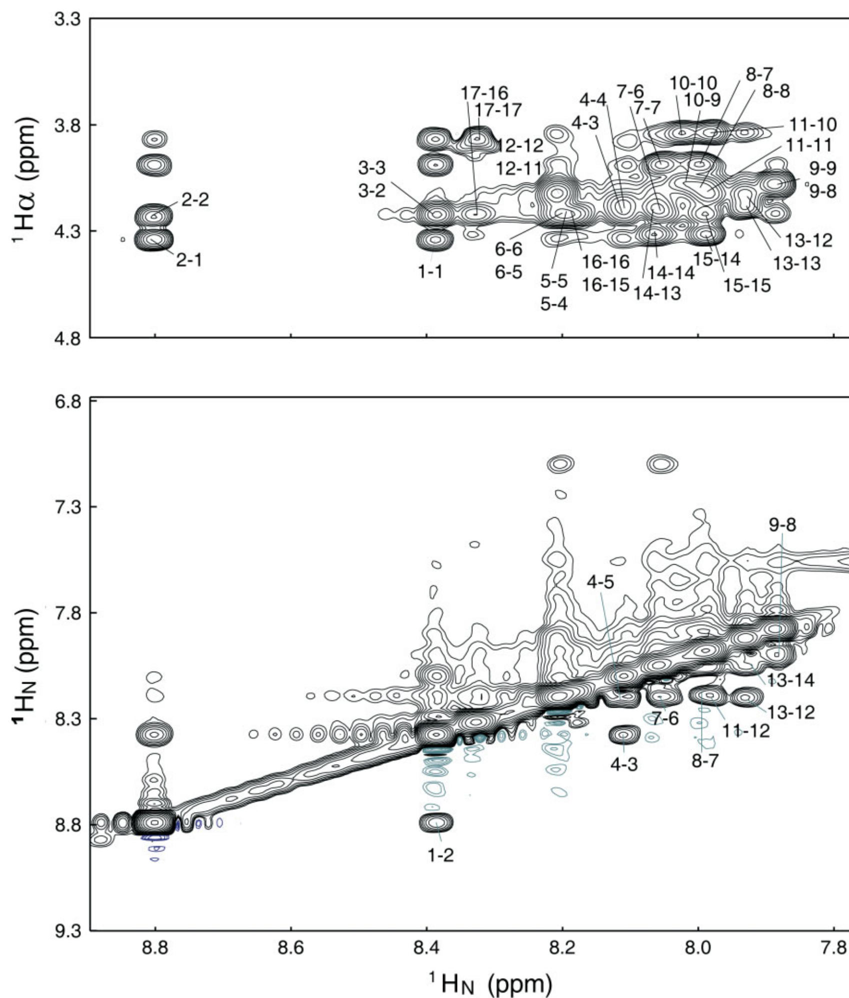

Figure 2 2D NOESY spectrum of MHA6. $^1H_\alpha-^1H_N$ region (a) and $^1H_N-^1H_N$ region (b) of the 2D NOESY spectrum of MHA6. Cross peaks are labeled with their residue numbers (Y-axis – X-axis).

$i+4$ (Ala^2–Tyr^6; Tyr^6–Ile^{10}; Ala^7–Ala^{11}; Lys^8–Glu^{12}; Arg^9–Ala^{13}, and Ile^{10}–Met^{14}). Detailed structural information was obtained by standard 2D NMR methods (Table S1, Supplementary information). From a structural point of view, MHA6 structure is characterized by an amphipathic four-turn α-helix with a hydrophobic face exposing residues Ala^2, Tyr^6, Ile^{10}, and Met^{14}.

Synthesis and Structural Characterization of the Mini-Protein with Two Structurally Independent Domains

Once stable monomeric conformations were independently obtained for the peptides, they were linked (*N*-terminal domain: β-hairpin; *C*-terminal domain: α-helix) using a string of Gly residues, since it was not expected that this amino acid would be involved in tertiary interactions. Two different lengths for the linker were chosen according to previously reported results for a Zn-finger motif [48] (4 Gly, MHB4A) and based on our own computational studies (8 Gly, MHB8A). The idea was to give the system enough freedom, so it could explore the possibility of any interaction between the β-hairpin and the α-helix.

^{1}H NMR resonances for the two peptides were assigned following the same strategy described previously. Most of the ^{1}H NMR assignments were similar to those previously reported for the isolated peptides. The main difference, from the NMR point of view, between the isolated and the Gly-tethered peptides is the existence of a duplicate set of signals for some residues located at the *N*-terminal ends of the α-helices in the bivalent peptide. This phenomenon could be explained by local structural impositions induced, by the β-hairpin and/or the Gly linker, on some residues located at the beginning of the α-helix.

Figure 3 summarizes the ^{1}Hα conformational shifts and short-range NOEs for both peptides and the comparison with their isolated constituents. The data support the presence of a β-hairpin and an α-helix in MHB4A and MHB8A, and the absence of important differences between the two bivalent peptides (Tables S2 and S3, Supplementary information). A comparison of the isolated β-hairpin and those of MHB4A and MHB8A

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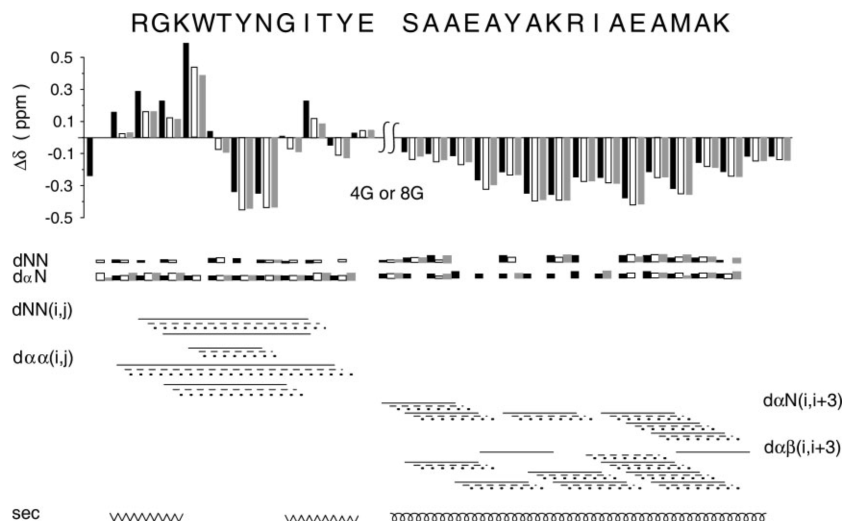

Figure 3 Summary of $^{1}H_{\alpha}$ conformational chemical shifts and short-range NOEs. A comparative summary of $^{1}H_{\alpha}$ conformational chemical shifts and short-range NOEs calculated for the isolated peptides (black boxes and solid lines), MHB4A (white boxes and dashed lines), and MHB8A (gray boxes and dotted lines).

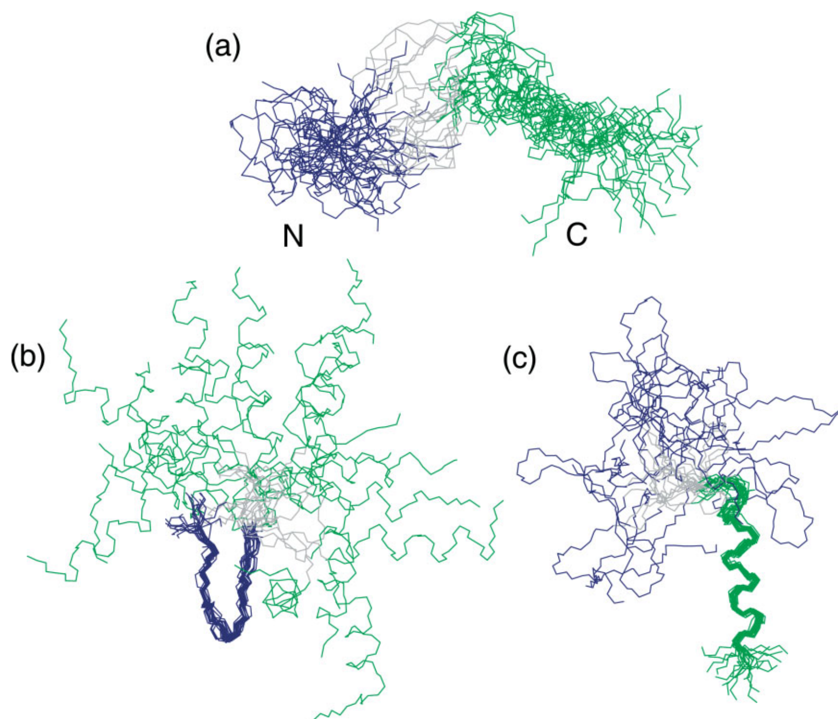

Figure 4 Calculated structures of the peptides. Superposition of the 20 best CYANA structures obtained for (a) all the residues of MHB4A, (b) residues 2–12 (β-hairpin moiety, in blue) and (c) residues 18–32 (α-helical moiety, in green). The linker has been represented by a gray line.

provided RMSD values for the Cα atoms of 0.732 and 0.836 ´Å, respectively. The values for the α-helix comparison were of 0.773 and 0.740 ´Å, respectively.

The NMR data did not reveal any long-range NOE interactions between the *N*-terminal β-hairpin and the *C*-terminal α-helix. This fact evidenced that MHB4A and MHB8A consisted of two independent well-defined elements of secondary structure (Figure 4).

DISCUSSION

The stability of both MHB4A and MHB8A peptides is a direct result of the stability of the isolated domains that define these structural bivalent peptides. In the present study, the connecting loop is just a passive element of secondary structure. This work illustrates the possibility of designing structurally

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stable bivalent peptides by a combination of rational and diversity-based approaches. The stability and native-like properties of these peptides are similar to previously reported designs based on the optimization of protein fragments [9,11–13,49]. The novelty of our approach is that the primary structure is entirely derived from a *de novo* design. The quality of the designed β-hairpin was earlier discussed in terms of quality of the template sequence and the method of screening [7,8]. The success of the design of the monomeric α-helix and by extension, the MHB4A and MHB8A peptides can be discussed in the same terms. The design of the α-CRL provides a constrained scaffold shared by all peptide sequences in the library that allows the selection of amino acids, fulfilling the restrictions imposed in the structural screening procedure. The 12 defined MHA peptides, selected from an initial pool of close to 140 000 sequences, fold predominantly into an α-helical conformation. Furthermore, from our results, it appears that $i - i + 3$ aromatic–aromatic interactions close to the middle of the helix (between amino acid residues at positions 6 and 9) are more stabilizing than aromatic-positive charge (cation–π) or aromatic-negative charge (anion–π) interactions. However, the MHA peptides adopt sequence-dependent mono- or oligomeric states that in principle could not be easily predicted. Notably, in some cases, this different behavior can be attributed to changes of only one residue, even when the change does not involve large variations in the side-chain properties. As an example, peptides MHA5 and MHA6 only differ in the amino acid at position 14, which is Leu for the former and Met for the latter, and yet MHA6 was classified as monomeric according to all the different criteria used in this study, while MHA5 showed a tendency to adopt oligomeric structures. A similar observation can be made from the comparison of peptides MHA6 and MHA8. The two peptides only differ in the amino acid at position 10 (Ile in the case of MHA6 and Leu in and the case of MHA8); however, MHA8 showed nonspecific peptide aggregation in buffer solutions. Additionally, the dependence of structure on pH shows the influence of electrostatic forces in the stabilization of oligomers. Nevertheless, the MHA6 was studied in more detail, and was found to be monomeric and α-helical at all experimental conditions assayed, providing the peptide sequence required for the synthesis of the bivalent peptides.

The balance of hydrophobicity and hydrophilicity is probably critical to favor the stabilization of monomeric α-helical and β-hairpin conformations in water solution. In our structure-directed *in vitro* evolution approach, this optimized equilibrium has been achieved by selecting amphipathic aromatic amino acids in the hydrophobic face of the β-hairpin and a balance between hydrophobic (Tyr^6, Ile^{10}, and Met^{14}) and positively charged (Arg^9) in one face of the helix. Interestingly, the folding pathway selected for the bivalent peptide was the one that allowed the self-stabilization of the two elements of secondary structure. It suggests that this process is energetically more favorable than the hydrophobic collapse that could be envisioned when two hydrophobic faces (one from each structural element) are set free to interact.

Taken together, these data show that it is possible to design bivalent peptides by linking using a string of Gly residues, two peptides able to fold into stable secondary structures without the need of stabilizing long-range interactions. Thus, biopharmaceutical applications where small peptides with well-defined structures are required could greatly benefit from this work. A very recent example of a simplified version of this approach has been reported by Paduch *et al.* [50]. In this case, symmetric and bivalent parallel heptapeptides linked by poly(ethylene glycol) were used to study protein–protein interactions mediated by PDZ domains. Furthermore, the value of the β-hairpin peptide previously designed in our laboratory [8] has been recently reported by Carotenuto *et al.* [19] for the optimization of an antigenic probe for multiple sclerosis. Future work in our lab includes the introduction of bi-functionality into the structural bivalent peptides. Also, although the Gly linker was probably the simplest available, we would like to explore more sophisticated peptide sequences. The addition of tunable interactions in the linker, together with acceptor–donor fluorescence pairs at the ends of the peptides could provide access to a diverse range of 'environment-sensing peptides'. Ongoing work in our laboratory is directed to further developing this class of peptides in an effort to satisfy the increased demand of well-folded functional peptides for applications ranging from novel drugs to minimized models of complex protein–protein interactions.

Supplementary Material

Supplementary electronic material for this paper is available in Wiley InterScience at: http://www.interscience.wiley.com/jpages/1075-2617/suppmat/

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