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ARTICLE *in* BIOPOLYMERS · SEPTEMBER 2008

Impact Factor: 2.39 · DOI: 10.1002/bip.21006 · Source: PubMed

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Folding Propensity and Biological Activity of Peptides: The Effect of a Single Stereochemical Isomerization on the Conformational Properties of Bombinins in Aqueous Solution

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Received 18 March 2008; revised 16 April 2008; accepted 16 April 2008
Published online 5 May 2008 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/bip.21006

ABSTRACT:

Folding propensities of bombinins H2 and H4, two members of amphibian bombinins H, a family of 17–20 residue α-helical peptides, have been investigated by means of circular dichroism (CD) measurements and molecular dynamics (MD) simulations. The two peptides, with primary structure IIGPVLGLVGSALGGLLKKI-NH2 and differing only for the configuration of the second aminoacid (an L-isoleucine in H2 and a D-alloisoleucine in H4) behave rather differently in solution. In particular both CD measurements and MD simulations indicate that bombinin H2 shows a markedly higher tendency to fold. From a careful inspection of MD trajectories it emerges that the stereochemical isomerization mutation of residue 2 to D-alloisoleucine in H4 peptide, drastically decreases its ability to form intrapeptide contacts. MD simulations also indicate that the conformational sampling in both systems derives from a subtle combination of energetic and entropic effects both involving the peptide itself and the solvent. The present results have been finally paralleled with preliminary information on bombinins H2 and H4 biological activity, i.e. interaction with membrane, supporting the hypothesis of an "already folded" conformation in water rather than interfacial folding tenet. © 2008 Wiley Periodicals, Inc. Biopolymers 89: 769–778, 2008.

Keywords: *antimicrobial peptides; frog skin; circular dichroism; molecular dynamics (MD); free energy*

This article was originally published online as an accepted preprint. The "Published Online" date corresponds to the preprint version. You can request a copy of the preprint by emailing the Biopolymers editorial office at biopolymers@wiley.com

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Contract grant sponsor: Italian Ministry of Education, University and Research
Contract grant numbers: PRIN 2004, 2005

INTRODUCTION

Cationic antimicrobial peptides (AMPs) are effector molecules of the ancient host defense system against microbial infections, known as innate immunity.[1,2] They are produced by all living organisms and in contrast with the clonal acquired immunity, which is present only in higher vertebrates, AMPs represent a fast and energy-effective mechanism that initially plays a key role in counteracting invading pathogens.[3–7] Despite differences in their size and secondary structure, most AMPs display a net

positive charge at neutral pH and the tendency to form amphipathic α-helix or β-sheet structures upon interaction with the cell membrane, which is believed to be the main target for their mechanism of action.[8,9] Among the natural sources for such molecules, the skin of a wide range of Anuran amphibians, particularly those belonging to the family Ranidae and Discoglossidae, has proved to be a remarkably rich storehouse.[10,11] In these animals, AMPs are produced and stored within granular glands, controlled by sympathetic nerves and released into the skin secretion upon stress, injury or contact with microorganisms.[10] Differently from classical antibiotics, produced by bacteria and fungi and synthesized step-wise by several enzymes, AMPs are encoded by genes and translated by ribosomes into prepropeptides, which are further processed to the active form.

An intriguing feature of their biosynthetic pathways is the occurrence of post-traslational modifications, which are often difficult to detect. Beside amidation of the C-terminal residue to prevent the cleavage by carboxypeptidases,[12] an interesting type of post-translational processing is given by the conversion of an L-aminoacid into its D-isomer.[13] Concerning this aspect, the amphibian bombinins H, a family of 17–20 residue α-helical peptides, represent a unique example.[14,15] Bombinins H1, H2, H3, and H4, isolated from the skin secretion of members of the *Bombina* genus are constituted of 20 amino acids, have a low net positive charge because of two lysine residues at their carboxyl end (+3), and differ by only one or two amino acid substitutions.[16] The most attractive feature of these molecules is the presence of a D-alloisoleucine at the second N-terminal position in some of them. It derives from a post-traslational epimerization of the pre-existing L-isoleucine, which undergoes a change in the chirality of its α-carbon.[13] The enzyme responsible for this L- to D- isomerization has been recently purified from skin secretions of *Bombina variegata*.[16] A D-leucine is instead found at the second N-terminal position of the 17-residues long bombinin H7.[17]

The existence of a D-aminoacid in ribosomally synthesized peptides of animal origin was first detected in the opioid dermorphins and delthorphins from the skin of South American frogs *Phyllomedusa sauvagei* and *Phyllomedusa bicolour*[18–21] and later in other neuropeptides and toxins from invertebrates.[22–25] More recently, D-aminoacids were also discovered in AMPs of bacterial origin (i.e. lantibiotics from gram-positive strains).[25] However, bombinins H are the first example of natural AMPs containing a single D-aminoacid which is the result of a post-translational change of configuration of the corresponding L-residue. Differently, in the case of lantibiotics, a two step reaction occurs, where the gene-encoded L-serine is converted to D-alanine via dehydratation and subsequent hydrogenation of the resulting dehydroalanine.[26–28]

Interestingly, at difference with opiate-like peptides, where the all L-isoform is completely inactive and apparently not detectable as a mature peptide in the skin secretion, the all-L bombinins H do occur naturally and are endowed with biological activity, alghough with a different efficacy and target-cell selectivity from that of the corresponding D-aminoacid-containing isoforms. One example of this is given by the pair bombinin H2 (IIGPVLGLVGSALGGLLKKI-NH2) and H4 differing by only the configuration of the second aminoacid (an L-isoleucine in H2 and a D-alloisoleucine in H4).

In depth studies on the antimicrobial activity of these peptides and the underlying mode/s of action have shown that both peptides displayed the same antimicrobial spectrum and were able to perturb the membrane of target cells, causing leakage of large cytosolic components (e.g. proteins) or diffusion of smaller molecules through local membrane disruptions.[16] However, a stronger potency and faster killing kinetic was displayed by the D-aminoacid bearing bombinin H4,which was also found to have a higher haemolytic capacity on human erythrocytes.[15,28]

To get further insight into the mechanism underlying the quantitative difference of the biological activities of these two diastereomers and the eventual role played by the D-aminoacid, we focused our studies on circular dichroism (CD) spectroscopy and on molecular dynamics (MD) of both H2 and H4 in aqueous solution. Although more quantitative information should be certainly obtained by considering also simulations in lipid membrane, an interesting relationship between biological activity and intrinsic folding propensity in water has emerged during these investigations paralleling previous results on different peptides.[29,30]

EXPERIMENTAL AND COMPUTATIONAL METHODS

CD Measurements

CD spectra were carried out with a Jasco J710 spectropolarimeter, equipped with a DP 520 processor, using quartz cell with an optical path length of 1 mm. The peptide samples (80 μM) were prepared in H_2O-trifluoroethanol (TFE) solutions (0–80% TFE, by vol.) All spectra were recorded at 25°C. The spectra were measured in the wavelength interval from 190 to 250 nm with a 0.5-nm step resolution and a 2 nm bandwidth. The scanning rate was 20 nm/min with 0.25 s response time. The signal to noise ratio was improved by accumulating at least five scans. Data processing was carried out using the J-710 software package. The blank spectra of the pure subphase (water or TFE solution) were subtracted.

MD Simulations

MD simulations of H2 and H4 were performed in the NVT ensemble. For both the systems, and in line with our previous

investigations,[29,30] the simulations started with the peptide folded in α-helix. After an initial mechanical relaxation of the peptide and a subsequent solvent equilibration, we carried out a slow heating of the system (using short trajectories of 50 ps length from 50 to 300 K). After a few ns of equilibration (see RESULTS section), 194 ns of simulation were produced for both systems at 300 K and used for the analysis. A time step of 2 fs was adopted and the roto-translational motion was removed,[31] the temperature was kept fixed at 300 K by the isokinetic temperature coupling[32] and the long-range electrostatics was treated by means of the Particle Mesh Ewald method.[33] Single-Point-Charge (SPC) model[34] of water was used at the typical liquid density (55.32 mol/l) for a total of 2696 water molecules. The simulations were performed adopting a modified version of the Gromacs 3.0 software package[35] with the Gromos96 force field.[36]

Essential Dynamics analysis

The essential dynamics (ED) analysis is described in detail elsewhere.[37,38] Briefly, by diagonalizing positional fluctuations covariance matrix, as provided by the MD simulation, we obtain a set of eigenvectors and eigenvalues. The eigenvectors represent the directions in configurational space and the eigenvalues indicate the mean square fluctuations along these axes. Sorting the eigenvectors by the size of the corresponding eigenvalues, the configurational space can be divided in a low dimensional (essential) subspace in which most of the positional fluctuations are confined and a high dimensional subspace in which merely small vibrations occur. In particular we characterized the thermodynamics as a function of the position in the backbone essential plane spanned by the first and second eigenvectors (see RESULTS section).

Thermodynamic Analysis

The free energy change for any transition from a reference state ref to a generic state i, at constant volume and temperature (Helmholtz free energy, ΔA_i), can be calculated from the probabilities p (obtained by the MD simulation) of finding the system in both states i and ref

$$\Delta A_i = -RT \ln \frac{p_i}{p_{\text{ref}}} \tag{1}$$

where R is the ideal gas constant and T the (absolute) temperature.

The corresponding internal energy and entropy change

$$\Delta U_i = U_i - U_{\text{ref}} \tag{2}$$

$$\Delta S_i = (\Delta U_i - \Delta A_i)/T \tag{3}$$

can be obtained by averaging the potential energy over the NVT MD frames associated to the i and ref states. In this article, Eqs. (1), (2) and (3) have been used for evaluating the thermodynamics in the conformational space (hereafter called essential plane) defined by the first two essential eigenvectors obtained by the ED analysis as previously reported. In other words the generic state i, as well as the ref state, were identified by using the free energy basins on the essential plane. The same equations also provided the evaluation of whatever thermodynamic observable, as obtained by MD simulation data. On the purpose we evaluated the global thermodynamic changes due to the transitions from completely unfolded condition to all the conformational states defined by an increasing number n of residues forming whatever conformation, i.e. α-helix, β-sheet, β-bridge, or turn. Note that the peptide was considered as completely unfolded when in the absence of any of the above conformations. All these analyses were performed dividing the essential plane in a 30×30 grid.

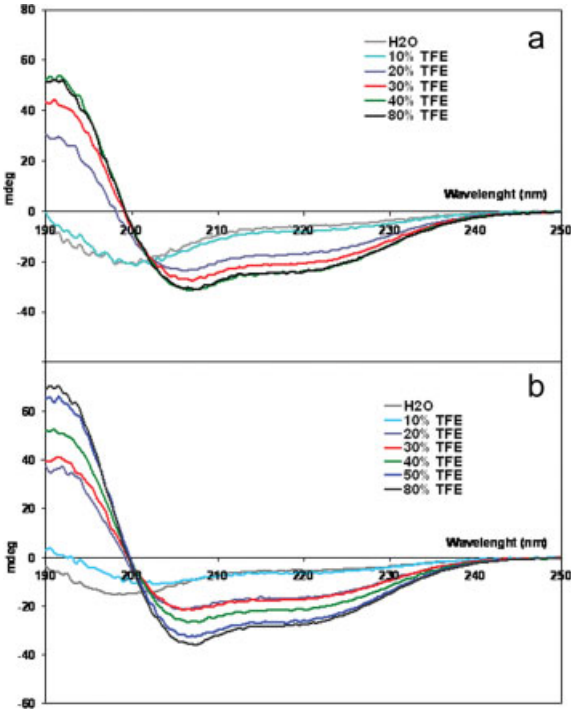

FIGURE 1 CD spectra of bombinins H2 (a) and H4 (b) in H_2O and increasing concentrations of TFE.

RESULTS

CD

The secondary structure of H2 and H4 peptides in water and after addition of TFE was determined by CD experiments. CD spectra of both peptide in water is typical for a random coil conformation (minimum at 198 nm). CD measurements were also performed with peptides in TFE solutions, to evaluate the propensity of H2 and H4 peptide to adopt an α-helical conformation. Addition of fluoroalcohols, such as TFE, promotes the formation of α-helical structures. Both peptide display a predominant α-helical structure in the presence of TFE (see Figure 1).

A comparison between the spectra in aqueous and increasing TFE solution shows a transition to more ordered conformations and this effect is complete at about 40% TFE for H2 peptide. In the case of H4 peptide, the effect was

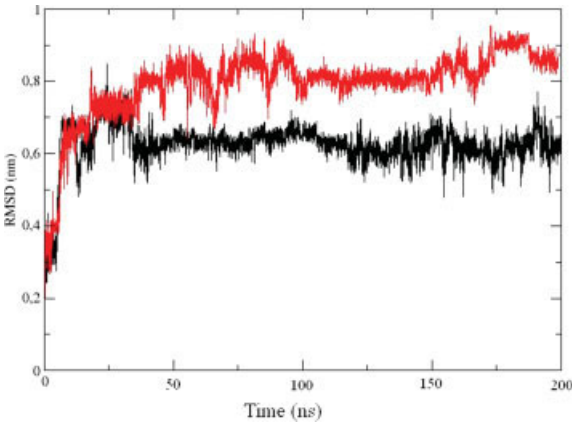

FIGURE 2 Backbone root mean square deviation of bombinins H2 (black) and H4 (red), with respect to their (common) C-alpha initial structure.

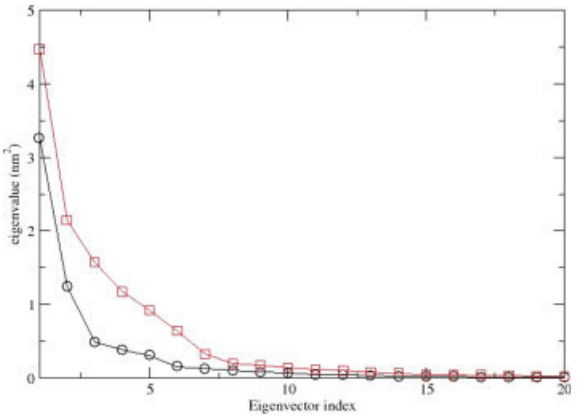

FIGURE 3 Eigenvalues of backbone covariance matrix for bombinins H2 (black) and H4 (red).

almost complete at about 50% TFE. This different propensity to adopt an ordered conformation even in a relatively poor hydrophobic solvent prompted us to investigate the thermodynamics as well as the energetics associated to the dynamics of H2 and H4 peptides in solution. In particular, MD simulations have been used in order to evaluate the "intrinsic" ability of these peptides in forming α-helix, and their folding propensity in the complete absence of TFE.

Conformational Analyses from MD Simulations

As a preliminary analysis of the systems under investigation, it was first necessary to evaluate the portion of the trajectory actually spanning the meaningful portion of the configurational space. To this end we calculated the C-alpha root mean square deviation (root mean square deviation [RMSD]), for both bombinins H2 and H4, with respect to the initial structure which, we wish to further remark, is exactly the same with the only difference of the absolute configuration of the second residue. The result is reported in Figure 2.

RMSD time course shows that both systems reach a plateau after 6 ns of trajectory. A further check of the convergence has been carried out by considering the fluctuation of the free energy along the trajectory (see Tables II and III in the next subsection). For all the analyses reported in this study, we therefore used 194 ns for both systems. Interestingly, the structure of bombinin H2 shows an RMSD markedly lower than that of bombinin H4. This finding, even though preliminarily, already provided a clear indication of some different structural repertoire of the two peptides, which had already emerged by previous CD spectra analyses.

To provide a deeper structural insight, and even to illustrate the overall conformational features, we analyzed in detail the internal backbone fluctuations of both peptides by carrying out ED analysis as described in the methodological section. In Figure 3 we report the eigenvalues, i.e. the extent of the fluctuations along their specific eigenvectors, obtained from the diagonalization of backbone covariance matrix. Result clearly indicates that, similarly to other peptides, both H2 and H4 backbone fluctuation can be significantly

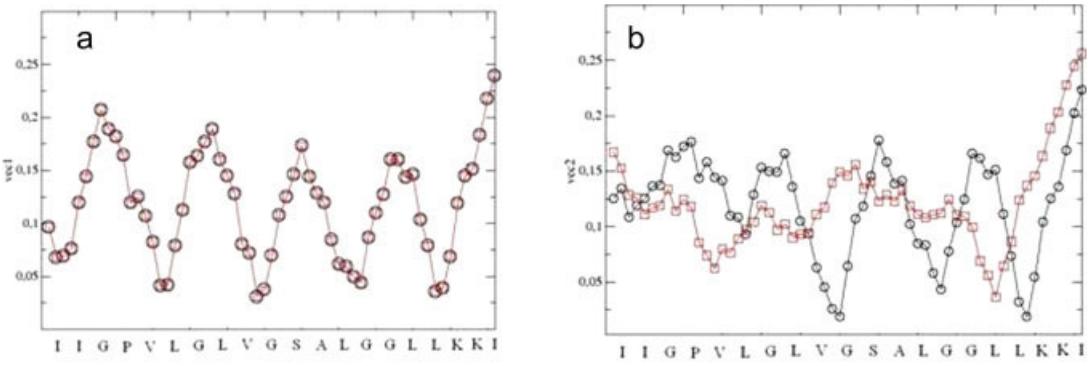

FIGURE 4 Square of the atomic composition of the first (a) and second (b) eigenvector obtained from bombinins H2 (black circles) and H4 (red squares) backbone covariance matrix.

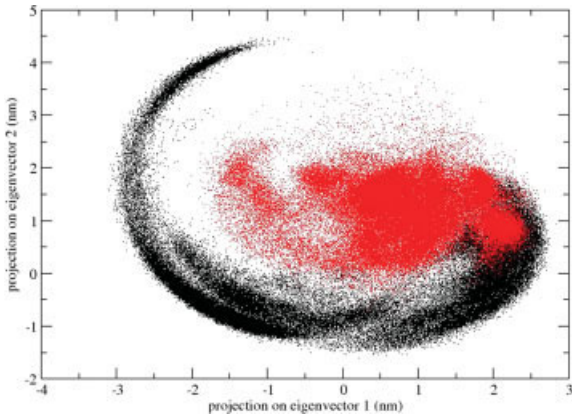

FIGURE 5 Projection of H2 (black) and H4 (red) trajectory onto the bombinin H2 essential plane.

described by using only two eigendirections—hereafter termed as essential eigenvectors—along the configurational space. It is also worth to notice the drastic reduction of fluctuation, i.e. decrease of the trace of covariance matrix, when going from H4 (12.642 nm^2) to H2 (6.654 nm^2). This result, paralleling the structural differences outlined from Figure 2, also suggests a sharp alteration of the internal flexibility induced by the stereochemical isomerization in the second position. To better visualize the internal motion of the two peptides we also report, in Figures 4a and 4b, the square of the atomic composition of the first two (see Figure 3), i.e. essential, eigenvectors.

A careful inspection of the figures qualitatively indicates a surprisingly similar (basically equal) composition of the first eigenvector and a rather different pattern for the second one. It is interesting to underline that the inner product between bombinins H2 and H4 eigenvector turns out to be lower than 0.75. This finding indicates that the motion of the two peptides is characterized by an equal "fingerprint", i.e. the eigenvector with largest eigenvalue, with quantitatively less important but not negligible differences in the second eigenvector.

Such a finding may be better figured out by projecting H2 and H4 trajectory over the (essential) plane defined by the first two eigenvectors from the diagonalization of H2 backbone covariance matrix, as reported in Figure 5. Note that the same result was obtained by using the H4 essential plane.

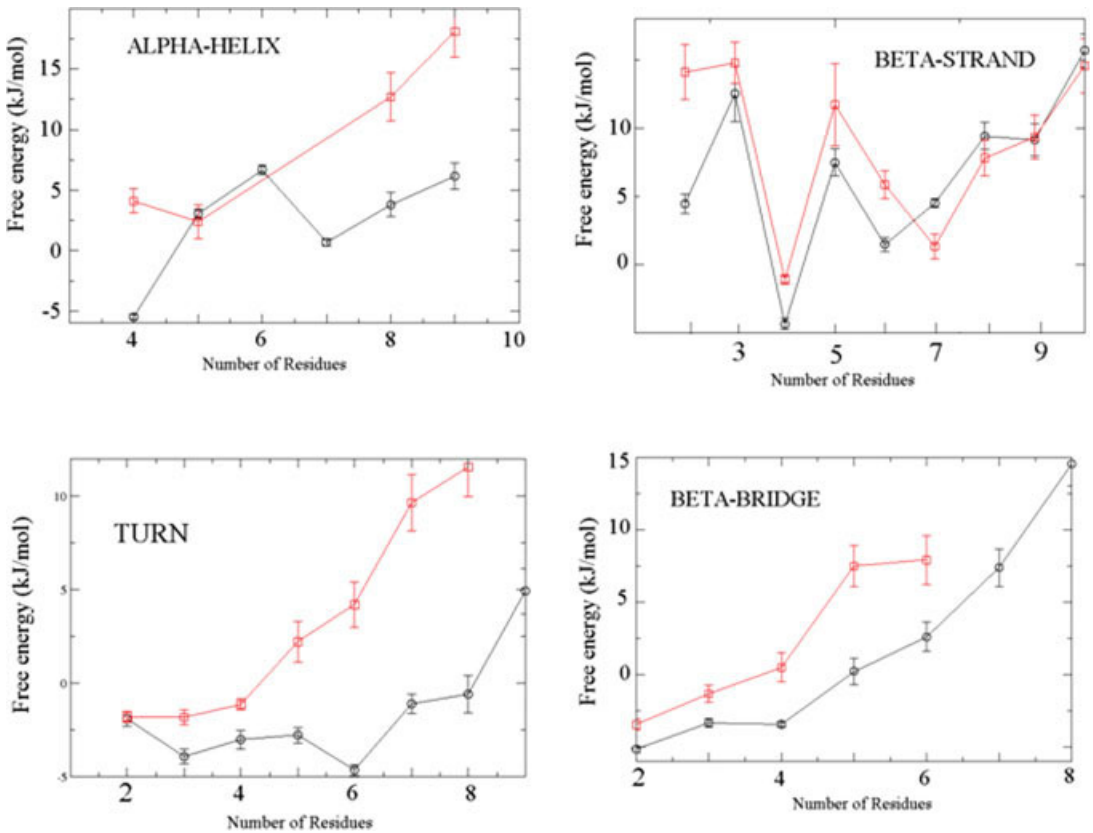

FIGURE 6 300 K Helmholtz free energy of formation (ΔA) of secondary structure elements as a function of number of involved residues (NR) for bombinins H2 (black circles) and H4 (red squares).

Table I Occurrence of Turn Along H2 Sequence (see also Figure 6)

Sequence	%
*IIGPVLGLVGS**AL**GGLLKKI*	41
*IIGPVLGLVGSAL**GG**LLKKI*	48
*IIG**PV**LGLVGSALGGLLKKI*	11
*IIGPVLGLVGSA**LGG**LLKKI*	72
*IIGPVLGLVG**SAL**GGLLKKI*	25
*IIGPVLGLVGS**ALG**GLLKKI*	3
*IIGPVLGLVGS**ALGG**LLKKI*	25
*IIGPVLGLV**GSAL**GGLLKKI*	19
*IIGPVLGLVG**SA**L**GG**LLKKI*	48
*IIGPV**LG**LV**GS**ALGGLLKKI*	8
*IIGPVLGLV**GSA**L**GG**LLKKI*	47
*IIGPVLGL**VGS**AL**GG**LLKKI*	10
*IIGPVLGL**VGSAL**GGLLKKI*	9
*IIGPVLGL**VGS**AL**GG**LLKKI*	20
*IIGPV**LG**LV**GSA**LGGLLKKI*	14
*IIGPVLGLV**GSALGG**LLKKI*	3
*IIGPV**LG**LVGS**ALGG**LLKKI*	75
*IIGP**VL**GLVGS**ALGG**LLKKI*	4
*IIGPVLGL**VGSA**L**GG**LLKKI*	15
*IIGPVLGL**VGS**A**LGG**LLKKI*	3
*IIGPVLGL**VGSALGG**LLKKI*	18
*IIGPVLG**LVGSA**L**GG**LLKKI*	28
*IIGPV**LGL**V**GSAL**GGLLKKI*	54

The two projected trajectories overlap in a limited portion of the configurational space thus confirming that the stereochemical isomerization alters the amount and, although to a lower extent, the character itself of the fluctuation.

At this point it would be interesting to understand the actual origin (or origins) of such a behavior. This is the ultimate goal of the next subsection.

Thermodynamic View of Bombinins H2 and H4 Structural Features

By using Eq. (1) we calculated the 300 K global formation free energy for different secondary structure elements (sse) such as β-sheet, β-bridge, turn, and α-helix, for an increasing number of residues (from 2 to 20) with respect to the completely unfolded structure (taken as the ref condition). Note that we considered as completely unfolded whatever MD configuration not showing any of the above sse.

The results shown in Figure 6 suggest, again, strong differences between the two peptides. Bombinin H2 appears to be basically confined in a permanent "turn-like" condition. In fact we always observe a negative free energy of "turn" formation up to eight residues. In this respect we have analyzed which are the residues more involved in "turn" formation. The result, reported in Table I shows a relevant occurrence concentrated in the central moieties of the peptide with a large involvement of Gly10, Ser11, Ala12, Leu13, Gly14, and Gly15 Consistently, other sse are also easily accessible to H2, i.e. endowed with negative free energy of formation. It is interesting to remark the negative free energy of formation of α-helical structures involving four and seven residues of H2, confirming the strong folding propensity of this peptide as already emerged by CD measurements.

The situation appears rather different for bombinin H4. First of all spontaneous formation of "turn" is observed only

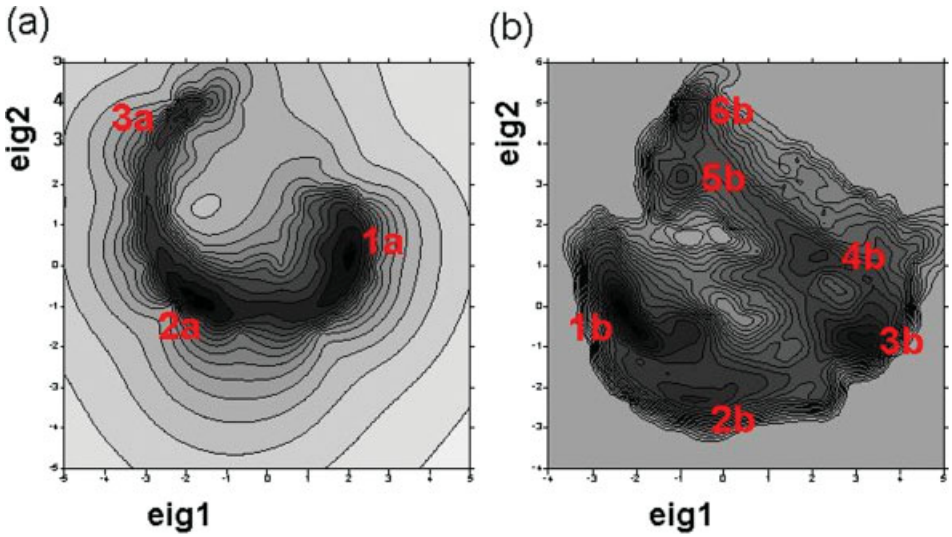

FIGURE 7 300 K Helmholtz free energy minima as a function of the essential coordinates for bombinins H2 (a) and H4 (b). The axes (in nm) correspond to the essential (first two) eigenvectors from diagonalization of backbone covariance matrix.

Table II Relative Thermodynamic Quantities, Solvent Accessibile Surface (SAS), Root Mean Square Fluctuations, Number of Solute–Solvent Contacts and Radius of Gyration Evaluated for Each Free-Energy Basin of H2

Free Energy Basin	ΔA[a] (kJ/mol)	SAS (nm^2)	ΔU (kJ/mol)	TΔS (kJ/mole)	RMSF (nm)	Solute–Solvent Contacts	Radius of Gyration (nm)
1a	0	11.93 ± 0.04	12 ± 4	12 ± 4	0.028	22 ± 1	0.648 ± 0.002
2a	0	11.92 ± 0.03	0.0	0.0	0.014	24 ± 1	0.653 ± 0.001
3a	+7 ± 2	12.00 ± 0.07	0.0 ± 2	−7 ± 4	0.018	24 ± 1	0.682 ± 0.002

[a] Error estimation was obtained by dividing the overall trajectory in three portions. Numbers without errors indicate reference states.

for a limited number of residues, i.e. from two to four. Similar analysis carried out for H2 shows that residues 10–15 are mainly involved in the case of H4. Secondly, the free energy of formation of whatever sse is always higher than H2. It follows that H4 is a less constrained, more flexible structure with a reduced, although not completely inexistent, propensity to form α-helix.

Such a picture, qualitatively not inconsistent with CD data, can be further enriched by specifically extracting the actual representative structures of H2 and H4 peptides in aqueous solution. To this purpose, as already described in the methodological section, we evaluated the free energy of formation of a given state "*i*" defined onto the essential plane. This analysis, carried out separately for bombinins H2 and H4, provided the results outlined in Figure 7. To evaluate the internal consistency of our results we carried out a further analysis of the convergence. On the purpose we divided the trajectory in three parts and evaluated the free energy of formation for each of the species onto the essential plane. The resulting picture, reported in Tables II and III, indicates a rather low fluctuation of formation free energy indicating that the system, at least from 6 to 194 ns of trajectory, does not undergo any sort of relevant drift. Of course such a result does not ensure about the actual equilibration of the system. However it provides a good indication about the internal consistency of the result. Consistently with previous analysis of the fluctuations, bombinins H2 and H4 show three and six different free energy minima, respectively. The representative structures and related properties are reported in Figures 8 and 9 and Tables II and III. Note that for each of the above structures, extracted using a cluster analysis carried out in the centre of the related basin, i.e. excluding all the structures with formation free energy higher than 2.5 kJ/mol. All of the representative structures of bombinin H2 (see Figure 8) appear folded with a variegated combination of α-helix/β-strands elements. At the same time (see Figure 9), bombinin H4 shows a much larger structural repertoire. It is important to remark that in the case of the peptide H2 residue 2 is strongly involved (two out of three structures) in forming intrapeptide contacts (see Figure 8). On the other hand, the involvement of this residue in bombinin H4 is statistically much less relevant. To put the above observation on more quantitative ground we calculated [again by using Eq. (1)] the free-energy of formation of *i*-2 contact with *i* indicating whatever residue found at a distance closer than 0.6 nm taken as the ref condition. The results, outlined in Figures 10a and 10b confirm that in the case of H2 peptide, residue 2 (L-Ile) is thermodynamically allowed (free energy of formation not higher than thermal energy), to form contact essentially with residues 9, and10. On the other hand, in the case of H4 peptide, residue 2 (D-allo-Ile), experiences very strong thermodynamic difficulties to bind to other residues. In conclusion, because of the favorable combination of position and absolute configuration, residue 2 shows a good propensity to

Table III Relative Thermodynamic Quantities, Solvent Accessibile Surface (SAS), Root Mean Square Fluctuations, Number of Solute–Solvent Contacts and Radius of Gyration Evaluated for Each Free-Energy Basin of H4

Free Energy Basin	ΔA[a] (kJ/mol)	SAS (nm^2)	ΔU (kJ/mol)	TΔS (kJ/mole)	RMSF (nm)	Solute–Solvent contacts	Radius of Gyration (nm)
1b	0	11.13 ± 0.04	0	0	0.019	26 ± 1	0.646 ± 0.001
2b	+2 ± 1	11.10 ± 0.10	9 ± 5	7 ± 6	0.092	27 ± 1	0.671 ± 0.004
3b	+2 ± 1	10.94 ± 0.08	15 ± 5	13 ± 6	0.050	28 ± 1	0.682 ± 0.002
4b	+5 ± 1	11.50 ± 0.10	0 ± 1	−5 ± 1	0.020	28 ± 1	0.685 ± 0.001
5b	+7 ± 1	11.84 ± 0.02	15 ± 8	10 ± 9	0.092	26 ± 1	0.683 ± 0.005
6b	+7 ± 2	11.32 ± 0.01	20 ± 8	13 ± 10	0.092	27 ± 1	0.694 ± 0.005

[a] Error estimation was obtained by dividing the overall trajectory in three portions. Numbers without errors indicate reference states.

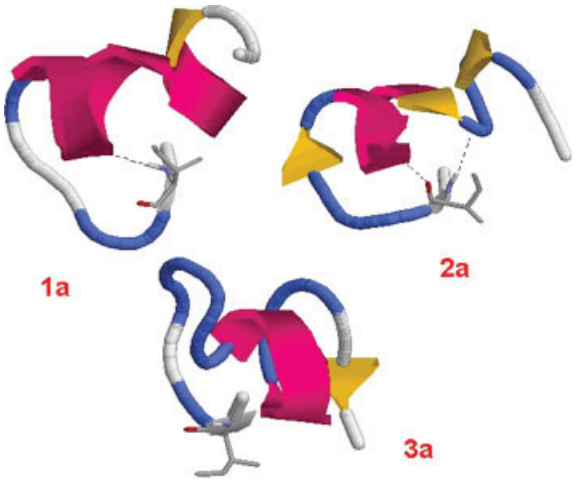

FIGURE 8 Cartoon-like representation of 300 K Helmholtz free energy minima of bombinin H2 (see Figure 7a). Interactions between residue 2 and the rest of the peptide are also indicated.

form intramolecular contact in bombinin H2 but not in bombinin H4. This finding may partially explain the strong conformational differences observed in our previous analysis and CD measurements, as reported above.

To somewhat explain the observed equilibration of the system, we evaluated the lifetime for each of the above conformers. On the purpose we calculated the residence time in each free energy basin. From this analysis we could estimate average lifetimes of 15 ps for the deepest minima, i.e. **1a** and **2a**, and shorter values for all the other species. Consequently the different basins are sampled several times along the 194 ns of trajectory.

Finally, in order to attempt a thermodynamic characterization, the different free energy minima have been extensively analyzed and the resulting picture summarized in Tables II and III.

Noteworthy, in both peptides all the basins show a very similar radii of gyration and, most interestingly very similar Surface Accessible Area (SAS). This latter finding, in slight disagreement with our previous study on Vitr-p13, a peptide derived from the sequence of the *Vitreoscilla* bacterial hemoglobin,[30] suggests that in the present case solvation effects presumably do not dominate the conformational sampling. According to the data collected in Table II, bombinin H2 degenerated minima, i.e. **1a** and **2a**, correspond to an entropy maximum and a potential energy minimum, respectively. Although both **1a** and **2a** are characterized by a well organized (ordered) central α-helix moiety, the first one shows an increased fluctuation which is probably responsible, at least partially, of the entropy increase. The third minimum **3a**, with the same internal energy as **1a**, shows a reduction of entropy partially ascribed both to the relatively low fluctuation and to the reduced number of free water molecules upon its formation. Concerning bombinin H4, we could

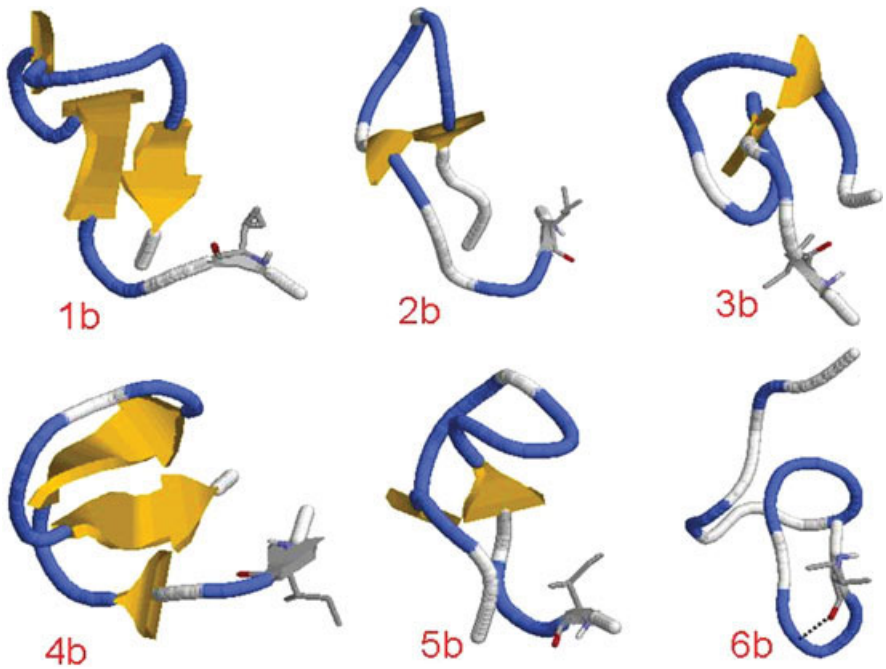

FIGURE 9 Cartoon-like representation of 300 K Helmholtz free energy minima of bombinin H4 (see Figure 7b). Interactions between residue 2 and the rest of the peptide are also indicated.

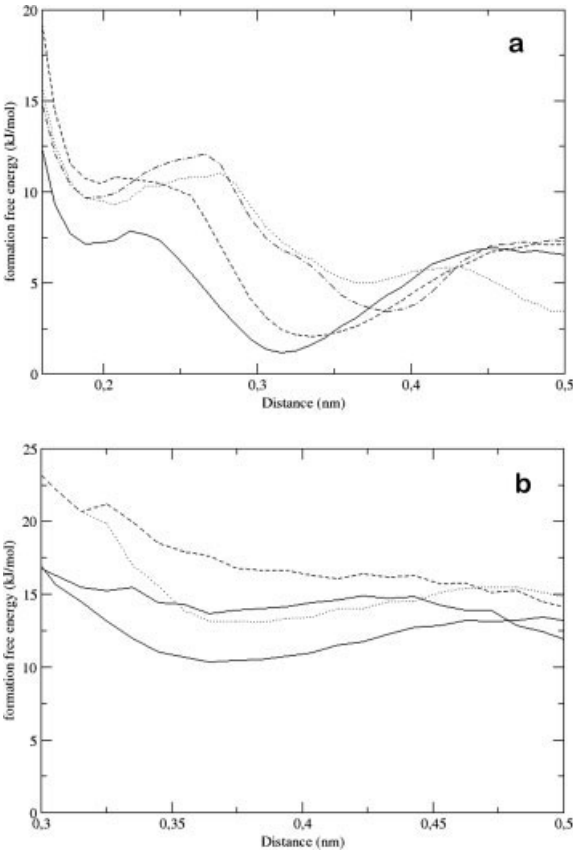

FIGURE 10 (a) Free energy of contact formation (see text) between residue 2 and residue 9 (continuous line), 10 (dashed line), 13 (dotted line), and 14 (dashed-dotted line) in bombinin H2. (b) Free energy of contact formation (see text) between residue 2 and residue 9 (continuous line), 10 (dashed line), 13 (dotted line), and 14 (dashed-dotted line) in bombinin H4.

identify one absolute free energy minimum, **1b**, corresponding to an internal energy minimum with low fluctuation. Similarly, **4b**, structurally resembling **1b**, shows the same internal energy but a reduced entropy probably related to the increased number of bound solvent molecules. For the other basins displayed in Table III, we observe an increased fluctuation with respect to **1b**, probably responsible for the observed higher entropy, but also a rise of internal energy in itself very difficult to explain.

DISCUSSION

The majority of AMPs physically permeate the cell membrane, causing damage which is hard to fix, rather than acting through recognition of specific receptors, as in the case of commonly used drugs, which in the latter case makes it relatively easy for the pathogens to acquire resistance.[39,40] To face the challenge of the increasing resistance of microbes to the available drugs, the discovery of new antibiotics with a new mode of action is urgently needed, and AMPs represent promising candidates for the development of new anti-infective agents.[39–41] In this context, frog skin has proved to be a rich source of AMPs endowed with intriguing features, such as bombinins H, which could well serve as candidate antimicrobial drugs, either in their naturally occurring forms or as templates for the rational design of improved biomimetic synthetic peptides.

It has been previously demonstrated the importance of a single D-aminoacid substitution as a new approach developed by nature not only to protect AMPs from proteolytic degradation but also to modulate their antimicrobial activity.[42] As reported earlier, a higher antimicrobial activity with a faster killing kinetic was displayed by H4,[43] but the biophysical explanation for such differences was not clear. Therefore, to investigate the role played by a D-alloisoleucine, (at the second N-terminal position) in such functions, CD and long time-scale MD simulations were carried out on the two enantiomer peptides bombinin H2 and bombinin H4.

An apparently irrelevant alteration of the primary structure, i.e. inversion of the configuration of isoleucine in position 2, induces a drastic alteration in peptide conformational properties. In particular, bombinin H2 shows a markedly higher tendency to fold in aqueous environment. A careful inspection of MD trajectories indicated that the stereochemical isomerization mutation of Ile residue 2 to D-alloisoleucine in H4 peptide, drastically decreases its ability to form intrapeptide contacts.

Similarly to our previous studies,[29,30] also in the case of bombinins, the conformational sampling in aqueous solution results from a subtle combination of energetic and entropic effects both involving the peptide itself and the solvent. The present study also further confirms the importance of simulations at atomistic details in order to avoid loss of information which may substantially alter the thermodynamic landscape. In our previous MD simulation studies on comparable peptide systems,[29,30] we have systematically observed a strict correlation between intrinsic folding propensity of the peptide (negative free energy of folding in dilute water solution) and its biological activity. In other words, conceivably assuming a strict relationship between interaction with cell membranes and biological activity, the presence of prefolded structures in peptides when still outside the membrane appears a very likely situation, as such extending and completing the generally accepted concept that AMPs get structured upon interaction with cytoplasmic membranes and/or with components of the outer bacterial membranes, a process sometimes referred to as interfacial folding. Indeed, our results on amphibian peptides temporins already provided some sort of evidence supporting the hypothesis of an

"already folded" conformation in water rather than interfacial folding tenet.[29,30]

Present results seem to validate such a hypothesis also in the case of bombinins H, even though further experimental evidences are probably necessary, in particular to highlight, if any, differences between H2 and H4 membrane activity. In this respect, although both peptides show a rather good tendency to fold, nevertheless the configurational restriction shown for H2 could theoretically alter its ability in interacting with lipid membranes to some extent. Intriguingly, what is currently known of the biological activity of bombinins H2 and H4 confirms this possibility, as the D-amino acid-containing peptide (H4) displays a stronger antibiotic activity and a higher rate of killing than the corresponding L-isomer against all the bacterial and fungal strains tested—with a recorded 1.4-3 fold lower value of lethal concentration[43]—and also against the protozoan pathogen *Leishmania.*[44]

MA thanks Dr. Andrea Amadei (Universita' di Roma 'Tor Vergata') for very helpful comments and suggestions.

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Reviewing Editor: J. McCammon