Folding Propensity and Biological Activity of Peptides: New Insights from Conformational Properties of a Novel Peptide Derived from *Vitreoscilla* Haemoglobin

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ABSTRACT:

The synthetic peptide Vitr-p-13 (YPIVGQELLGAIK-NH_2), derived from the bacterial dimeric Vitreoscilla *haemoglobin (VHb) in the position 95–107, is characterized by a pre-eminent "statistical coil" conformation in water as demonstrated by CD experiments and long time-scale MD simulations. In particular, Vitr-p-13 does not spontaneously adopt an alpha-helix folding in water, but it is rather preferentially found in beta-hairpin-like conformations. Long time-scale MD simulations have also shown that Vitr-p-13 displays a "topological-trigger" which initiates alpha-helix folding within residues 7–10, exactly like seen in the temporins, a group of linear, membrane-active antimicrobial peptides of similar length. At variance with temporins, in Vitr-p-13 such a process is energetically very demanding (+ 10 kJ/mol) in water at 300 K, and the peptide was found to be unable to bind model membranes in vitro and was devoid of antimicrobial activity. The present results, compared with previous studies on similar systems, strengthen the hypothesis of the requirement of a partial folding when still in aqueous environment to allow a peptide to interact with cell-membranes and eventually exert membrane perturbation-related antibiotic effects on target microbial cells.* © *2007 Wiley Periodicals, Inc. Biopolymers 87: 85–92, 2007.*

Keywords: molecular dynamics; free energy; peptides; circular dichroism; Vitreoscilla *Hb*

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INTRODUCTION

In a recent study, we have quantitatively analysed by means of either molecular dynamics (MD) simulations and circular dichroism (CD) spectra, the behaviour of two peptides of potential pharmacological interest: temporin A and L.[1] Results obtained showed, for both pepti-

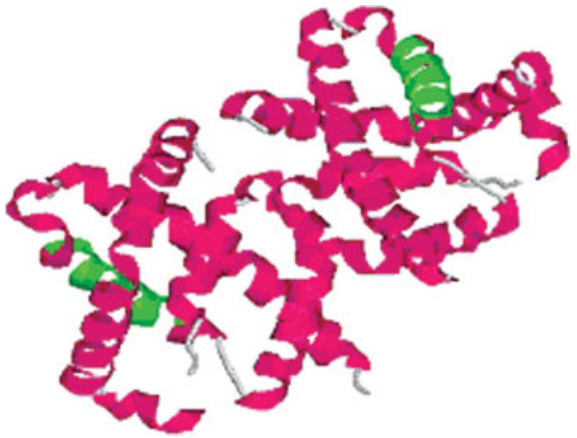

FIGURE 1 Schematic view of Vitreoscilla haemoglobin dimeric form. Position 95–107 are shown in green.

des, a strict correlation between their propensity to adopt alpha-helical structure and germicidal activity against Gram-positive/negative bacteria. In particular temporin L, which was previously proved to display higher affinity for binding to lipopolysaccharide for interfering with its biological activity in two rat models of Gram-negative septic shock,[2,3] was found from MD simulations to display a higher propensity (negative free energy of folding) to form alpha-helix in water solution. This observation has suggested the presence of a plausible correlation between the conformation adopted by the peptide in solution, i.e. *before* the interaction with the bacterial lipid membrane, and its pharmacological activity. Stimulated by these results, we decided to extend our studies to other peptides in order to acquire additional data aimed at assessing the above mentioned hypothesis. Recently we have thus focussed our attention on a novel 13-amino-acids peptide, hereafter termed as Vitr-p-13 (YPIVGQELLGAIK-NH_2), derived from the position 95–107 of the bacterial dimeric haemoglobin from *Vitreoscilla* (VHb; PDB ID: 1VHB)[4] (depicted in Figure 1).

The bacterial haemoglobin of *Vitreoscilla* represents an interesting tool for many whole cell biotechnological applications.[5] In fact, heterologous expression of VHb inside bacteria, yeasts, fungi, and eukaryotic plant cells has been performed to improve either host cell growth and synthesis of selected enzymes.[6,7] Valuable intermediate metabolites, especially under oxygen-limiting conditions, have also been obtained.[8] Interesting examples of the in vivo effects of VHb are the enhancement of α-amylase production in *Escherichia coli* and cephalosporin C in *Acremonium chrysogenum*, the degradation of toxic wastes, and the improved activity of chimeric VHb-amino acid oxidases.[9–13] Recently, the intracellular coexpression in *Pichia pastoris* of methionine adenosyltransferase gene from *Streptomyces spectabilis* and *Vitreoscilla* hemoglobin gene has been reported as an efficient way to produce S-adenosylmethionine (SAM).[14] In this case, the presence of VHb might improve ATP synthesis rate and thus stimulate cell growth and SAM production in the recombinant yeast.

Such a surprisingly large array of biotechnological applications of VHb is not in line with the scarce understanding of its biochemical reactivity towards heme ligands and its physiological role. At the moment, the following two general hypotheses have been postulated: (i) VHb behaves like a scavenger of oxygen or NO radical species,[15] or (ii) VHb acts as an oxygen-delivering protein (myoglobin-like) that favours the oxygen diffusion towards terminal oxidases.[16,17] This latter hypothesis implies that the presence of VHb within the respiratory chain enhances the oxygen flux to one or both terminal oxidases, namely cytochromes *bo* and *bd* in *E. coli*, under hypoxic conditions. Indeed, the observation that exogenously added VHb stimulates the ubiquinol oxidase activity of either the respiratory chain and the cytochrome *bo* proteoliposomes, indicates that the interaction between VHb and cytochrome *bo* is physiologically relevant.[17] Therefore, the interaction of VHb with the phospholipid membrane of *E. coli* appears to play a pivotal physiological role and thus requires further investigation. In analogy with a flavohemoglobin (HMP) isolated from the same microorganism, VHb has been reported to deeply penetrate the phospholipid bilayer, and HMP has been also demonstrated to bind phospholipids within the active site.[16,18] The lipid binding properties, together with alkylhydroperoxide reductase activity, suggested a peculiar role for HMP in the bacterial response to oxidative/nitrosative stress. The structural similarity of VHb and HMP is limited to the first shell of aa residues that form the envelope of the heme pocket,[4,19,20] while significant differences between the two proteins are evident in the neighbouring regions, especially within the solvent-exposed CE moiety that limits the external surface of the active site. These findings suggest that the two proteins could display a similar, although not identical, behaviour with respect to lipid-binding properties, protein-membrane interactions, and oxygen-binding ability. More recently, VHb was shown to reversibly bind cyclopropanated fatty acids and phospholipids within the active site, a property that probably modulates the protein's oxygen-binding activity, and to readily interact with lipid monolayers.[21] Another very recent study indicated that VHb exhibits peroxidase activity, a finding in line with the hypothesis that VHb has cellular functions beyond the role as an oxygen carrier.[22]

The aim of the present work is to investigate the intrinsic propensity of peptide Vitr-p-13 to form alpha-helix in water

by using long time-scale MD simulations and CD measurements. This peptide, identified upon molecular modelling of VHb (it corresponds to helix G, according to the globin nomenclature), was selected on the basis of its length, its partial sequence homology with temporin L (FVQWFSKFLGRIL-NH_2), and for its position on the surface of the protein, which would ideally enable it to interact with the bacterial membrane. The results of the present study will be correlated to the previous ones,[1] in order to provide further insights towards identifying a quantitative correlation between folding propensity of peptides in dilute solution and their biological/pharmacological activities.

EXPERIMENTAL AND COMPUTATIONAL METHODS

CD Measurements

Synthetic Vitr-p-13 was purchased from New England Peptide (Gardner, MA). The purity of the peptide (higher than 95%), its sequence and concentration were determined as previously described.[23] CD spectra were carried out with a Jasco J710 spectropolarimeter, equipped with a DP 520 processor, at 25°C, using a quartz cell of 1-mm pathlength. The peptide samples (71 μM Vitr-p-13) were prepared in H_2O–trifluoroethanol (TFE) solutions (0–80% TFE, by vol). For each sample five spectra were recorded at the scan rate of 20 nm/min and averaged.

Molecular Dynamics Simulations

We performed MD simulation of Vitr-p-13 in the NVT ensemble. 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 1 ns of equilibration, 400 ns of simulation were produced and used for the analysis. A 2-fs time step was adopted and the rototranslational motion was removed,[24] the temperature was kept fixed at 300 K by the isokinetic temperature coupling[25] and the long-range electrostatics was treated by means of Particle Mesh Ewald method.[26] Single-Point-Charge (SPC) model[27] of water was used at the typical liquid water density (55.32 mol/l). The simulation was performed adopting a modified version of the Gromacs software package[28] with the Gromos96 force field. The peptide was initially put in the original (alpha-helix) conformation[4] which turned out to be lost after a few ns of simulation.

Essential Dynamics Analysis

The Essential Dynamics analysis is described in detail elsewhere.[29] Briefly, by diagonalizing all-atoms 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 essential plane spanned by the first and second eigenvectors.

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

$$\Delta A_i = -RT \ln \frac{p_i}{p_{\text{ref}}} \qquad (I)$$

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

The corresponding internal energy change

$$\Delta U_i = U_i - U_{\text{ref}} \qquad (II)$$

can be obtained by averaging over the MD frames associated to the i and ref states. In this study, Eqs. (I) and (II) 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. The same equations also provided the folding thermodynamics as obtained by MD simulation data. On the purpose we evaluated the global thermodynamic changes due to the transitions from completely unfolded (reference) condition to all the conformational states defined by an increasing number n of residues forming whatever conformation, i.e. alpha helix, beta-sheet, beta-bridge or turn. Note that the peptide was considered as completely unfolded in the absence of any of the above conformations. All these analyses were performed dividing the essential plane in a 25 × 25 grid

RESULTS

Circular Dichroism

CD spectroscopy was used to determine the conformation of synthetic peptide Vitr-p-13 in solution by recording the spectra in water and after addition of increasing amounts of TFE. The spectra were comparable to those obtained for Temporin A under similar conditions and published elsewhere,[1,30] and were in accordance with the structural studies performed on Temporin A analogues.[31,32] A detailed analysis of CD experiments with peptide Vitr-p-13 in water indicate a large "statistical-coil" conformation. Addition of TFE, the most commonly used agent for stabilizing the alpha-helix structure in peptides, induced Vitr-p-13 to adopt partially helical structure (Figure 2).

However a comparison between the spectra in aqueous and TFE solution shows a transition to more ordered conformations and this effect is complete at about 30% TFE. This

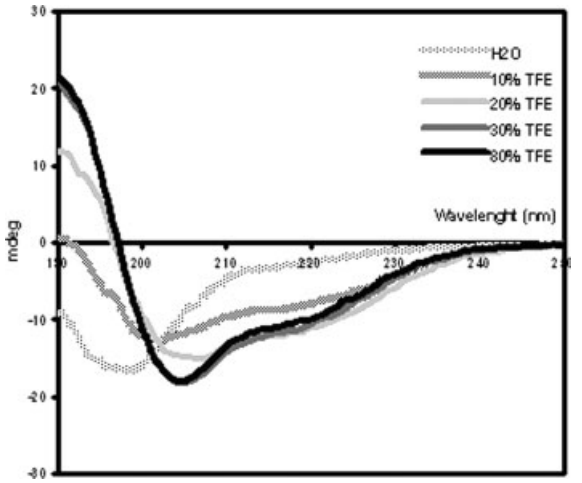

FIGURE 2 CD spectrum of Vitr-p-13.

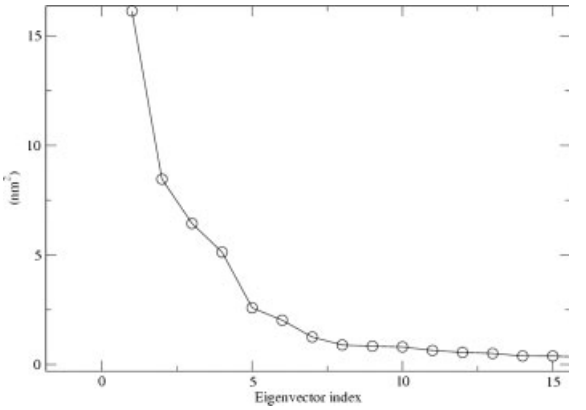

FIGURE 3 Eigenvalues of all-atoms covariance matrix of Vitr-p-13.

behaviour closely resembles the CD profile displayed by temporin A but greately differs from that exibited by temporin L in the same experimental conditions.[1] This scarce propensity to adopt an ordered conformation even in a relatively poor hydrophobic solvent, which clearly represents an intriguing difference between temporin L and Vitr-p-13, prompted us to investigate the thermodynamics as well as the energetics associated to the dynamics of the peptide in solution. In particular, MD simulations have been used in order to evaluate the "intrinsic" ability of Vitr-p-13 in forming alpha-helix, and its folding propensity in the complete absence of TFE.

Structural Motions

We first characterized Vitr-p-13 by a mechanical point of view. On the purpose we used ED analysis as described in the methodological section.

In Figure 3, we report the eigenvalues obtained from the diagonalization of Vitr-p-13 all-atom covariance matrix from which it emerges that, only a small fraction of the eigenvectors of all-atoms covariance matrix is actually associated with significant internal motions of the system, i.e. the corresponding eigenvalues are significantly different from zero. The squared atom composition of the first two eigenvectors (hereafter termed as essential eigenvectors) are reported in Figure 4, in order to better figure out the mechanical features of the peptide.

For comparison, we also constructed and diagonalized the C-alpha covariance matrix. The atom composition of the first eigenvector, shown on the top of Figure 5, interestingly resembles the corresponding curve depicted on the top of Figure 4 clearly suggesting that the most relevant conformational fluctuations of Vitr-p-13 are basically dominated by C-alpha large-amplitude motions with terminal (Y1-P2 and I12-K13) and central (Q6-E7) residues mainly involved and, at the same time, with side-chains basically undergoing conformationally irrelevant small amplitude vibrations. On the other hand the second eigenvector (also reported in the bottom of Figures 4 and 5), is characterized by a more variegated C-alpha/backbone/side-chains combination. This first analysis resulted in a sharp difference with that relevant to temporins A and L both characterized by essential eigenvectors with side chains systematically providing a relevant contribution.[1]

Thermodynamics

By using Eq. (I), we then calculated the 300 K global formation free energy for different kinds of conformations (beta-

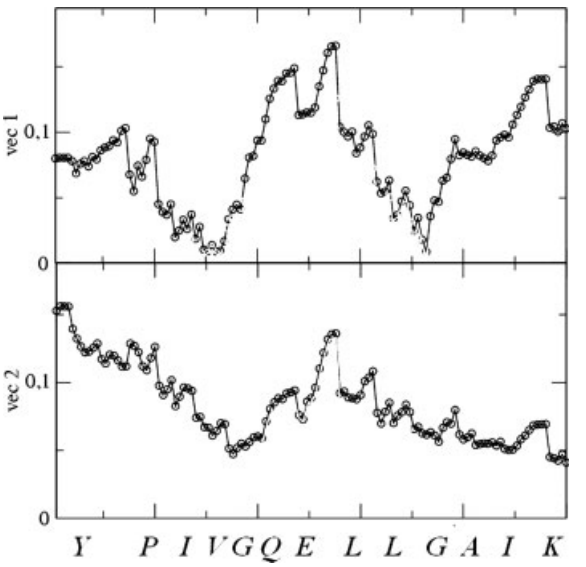

FIGURE 4 Squared atom composition of the firt two eigenvectors of all-atom covariance matrix of Vitr-p-13. In the *x*-axis we show the position of the C-alpha.

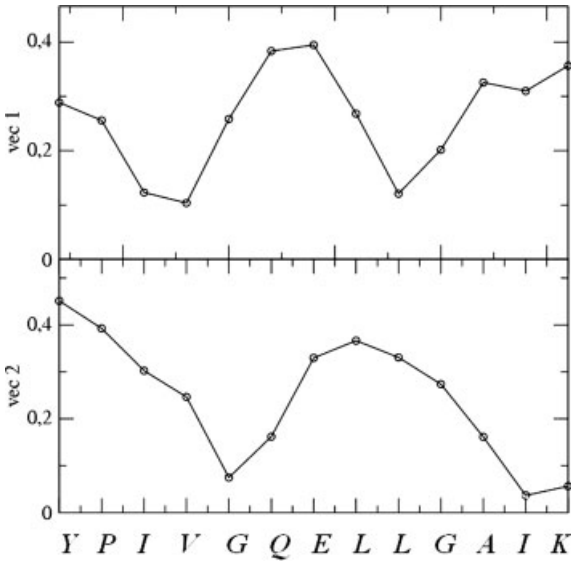

FIGURE 5 Squared atom composition of the firt two eigenvectors of C-alpha covariance matrix of Vitr-p-13.

sheet, beta-bridge, turn, alpha-helix), for an increasing number of residues (from 2 to 13) with respect to the one not showing any secondary structure element (taken as the reference condition). Note that we considered as completely unfolded whatever structure not showing any secondary structure element.

In Figure 7, we also report the global probability to find each combination of residues in the thermodynamically most relevant conformations of Figure 6.

First of all we observe, as expected by the previous CD measurement in section 3.1, that the formation of alpha-helix (only with four residues corresponding to positions 7–10) is rather endoergonic (+10.1 kJ/mol) at least under our conditions. On the other hand, Vitr-p-13 spontaneously adopts conformations characterized by two residues in beta-bridge conformations (free energy of formation equal to −1.9 kJ/mol), four residues in beta-sheets (free energy of formation of −1.8 kJ/mole) and three residues forming beta-turns (free energy of formation equal to −0.9 kJ/mol). In correspondence of residues E7-L8/A11-I12 and E7-L8-L9 we find the highest probability to find beta sheets and turns, respectively. From the same graphics we also note that two-residues turns may be spontaneously formed (free energy of formation equal to −0.4 kJ/mol), although their occurrence results rather distributed along different residues.

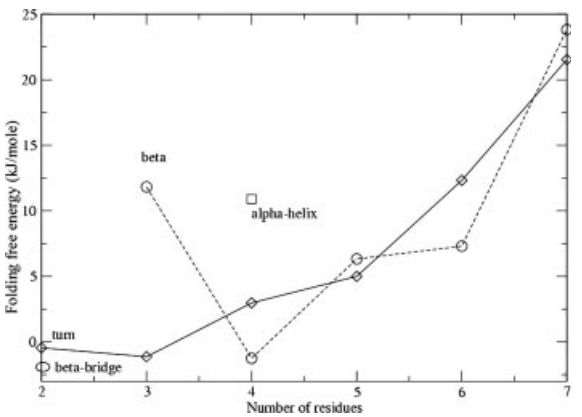

FIGURE 6 300 K Helmholtz free energy of formation of beta-strands (circles), alpha-helix (squares), turn (diamonds) and beta-bridge (ellipse) as a function of number of involved residues. Alpha-helix and beta-bridge were found to be formed only with combination of four and two residues, respectively.

The folding of any other number of residues in whatever combination is found to be an endoergonic process under our MD simulation conditions.

From the above analysis we like to enucleate the following interesting aspects:

i *differently* from temporins,[1] alpha-helix does not represent a thermodynamically accessible condition for Vitr-p-13.

ii *similarly* to temporins,[1] alpha-helix formation, despite its very low occurrence, does involve the same sequence, i.e. residues 7–10, found in the case of both temporins.[1,32]

This last observation further confirms that alpha-helix formation mechanism seems to be triggered in any cases by the same mechanical motion topologically concentrated in the same region of the peptide. Therefore, differences and analogies among different peptides concerning their propensity to form alpha-helix, relies on a combination of subtle effects governed by the specific residue sequence.

Let us now consider in more detail the actual nature of the conformations adopted by this peptide in our simulation conditions.

In Figure 8(a) we report the 300 K Helmholtz free energy surface of Vitr-p-13, as a function of the position in the

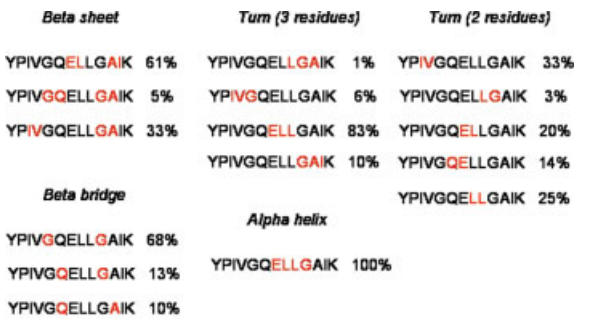

FIGURE 7 We report the folding free energy as a function of the number of involved residues in Figure 6. We also report the probability to find each combination of residues in Figure 7.

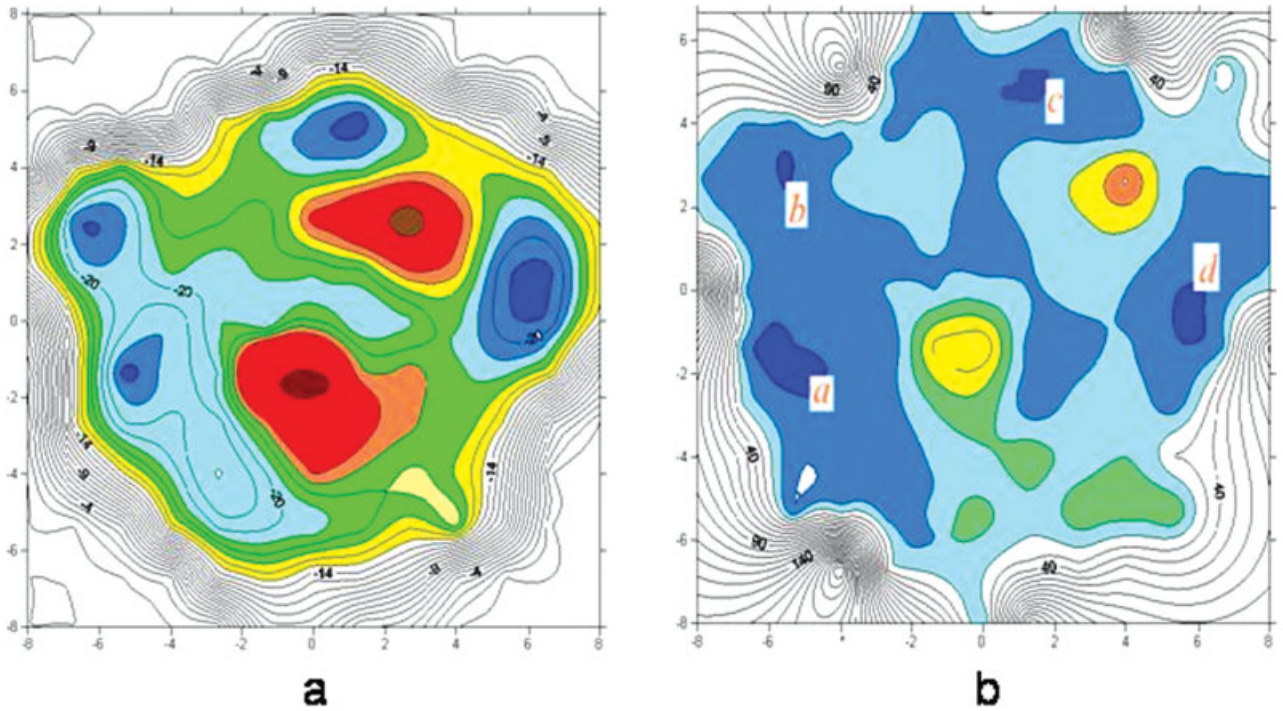

FIGURE 8 300 K Helmholtz free energy (a) and internal energy (b) maps onto the all-toms essential plane. Changes of color (from blue to red) indicate energy variations of 3.0 kJ/mole.

essential plane (defined by the first two eigenvectors of all-atoms covariance matrix). Note that such a figure was produced by the use of Eq. (I) in which the basin indicated with *a* in Figure 8(a), was adopted as reference (ref) state. Four almost degenerate basins, separated by free energy barriers of 6–9 kJ/mol, are shown in these figures. Their related structures are reported in Figure 9. As expected from the analysis outlined in Figures 6 and 7, none of the free energy minima actually corresponds to a alpha-helix conformation. Rather, all of them correspond to structures dominated by beta and turn regions.

The actual thermodynamic origin of the above minima may be better envisaged by calculating the corresponding internal energy on the essential plane by using Eq. (II). Also in this case we adopted state ***a*** as the reference one. From the results, also reported in Figure 8(b), it is evident that all the free energy minima actually correspond to internal energy minima. In other words, entropy does not play any role in the conformational sampling of this peptide. This result (closely resembling the analogous findings obtained for temporins[1]) might be obviously related to the intrinsic features of the employed force field, but it can be also rationalized on the basis of the differential occurrence of intra-peptide and peptide-solvent contacts. In this respect a somewhat more accurate analysis can be carried out comparing, within and outside the free energy basins, the number of intrapeptide contacts, the water molecules actually coordinated to the peptide and the corresponding solvent accessible surface (SAS). The results are reported in Table I. Note that in order to avoid spurious effects due to an arbitrary choice of solvent-peptide maximum distance, we carried out this analysis both at 0.25 and 0.35 nm. It is very interesting to observe that when the aqueous peptide adopts a minimum free energy configuration it systematically enhances its SAS. At the same time we do not observe relevant differences between internal contacts. This means that, at least in the present case, the driving force governing the conformational transitions of the peptide, is not related to the formation of intra-peptide hydrogen bonds but, less intuitively, to the maximization of the interaction of the peptide with the solvent.

DISCUSSION

Both CD experiments and long time-scale MD simulations, carried out on Vitr-p-13, clearly indicate its scarce propensity to form alpha-helical structures in water. In particular, aqueous Vitr-p-13, does not spontaneously adopt a alpha-helix folding (which is completely lost within a few ns of simulation) but, rather, it is preferentially found in beta-hairpin-like conformations. Our MD long-time-scale simulation has also shown that Vitr-p-13 presents a "topological-trigger," characterized by residues 7–10, exactly like in temporins,[1,32]

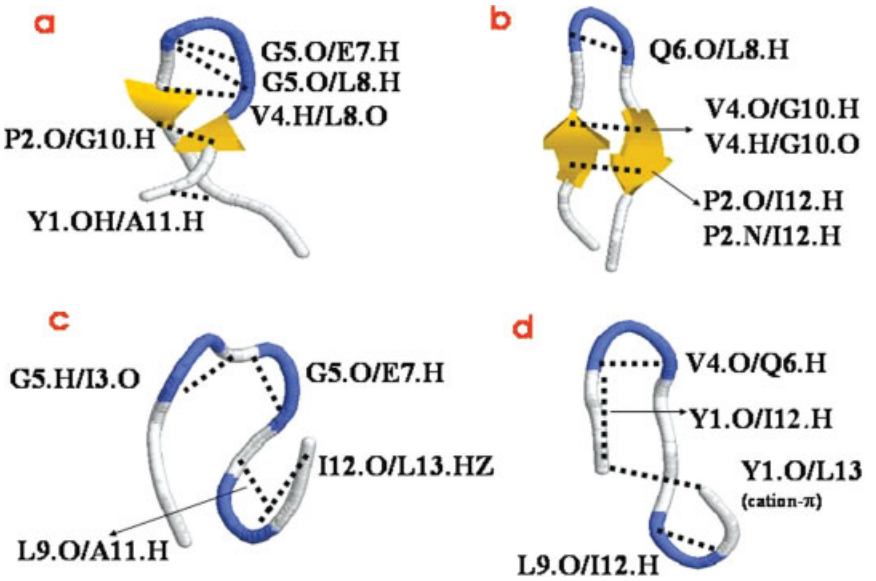

FIGURE 9 Cartoon-like view of the structures representing the free energy basins in Figures 8(a) and 8(b). The most relevant contacts are also indicated.

which initiates alpha-helix folding. The difference with temporins is that, in the present case, such a process in water at 300 K is energetically very demanding (+10 kJ/mole). This finding actually reveals that predicting the folding propensity of a peptide on the basis of its conformation within the protein matrix may be a very hard task due to the presence of subtle effects related to the specific residue sequence.

Rather interestingly, the driving force governing Vitr-p-13 conformational sampling predominantly relies on its ability of forming hydrogen bonds with solvent. This finding, somewhat surprising considering our mental representation of conformational sampling as essentially driven by intra-molecular interactions, may also help in rationalizing the striking difference observed within protein environment (where Vitr-p-13 is in alpha-helix folding) and in water solution.

In our previous study, the intrinsic folding propensity of antimicrobial and membrane-binding peptides temporins was related to their activity against Gram-positive/negative bacteria.[1] It turned out that temporin L, showing the strongest activity among all temporins tested against several biological targets (including human erythrocytes, fungi and bacteria), exhibited in water a high propensity (negative Helmholtz free energy) to form 4/5 residues alpha-helical structures. Consistently, temporin A, possessing a lower tendency do adopt such a folding in water, was previously demonstrated to behave in a less efficient fashion against biological targets. The mode of action of these antimicrobial peptides is obviously to be ascribed in large part, if not uniquely, to their ability to interact with target's cellular membrane. Nevertheless, conclusive evidence is still lacking as to whether the peptide interacts with cell membrane in an "already-folded" conformation or, rather, the folding process takes place within the membrane. Our results on temporins provided some sort of partial evidence supporting the former hypothesis, i.e. larger is the fraction of peptide already folded outside the membrane higher is its affinity with the membrane, and thus, presumably, its antimicrobial activity. Interestingly, this finding could be of more general relevance, if one envisages that a certain degree of pre-organization could be necessary also for the binding of peptides to protein receptors, as some recent studies on the interaction of peptidic antagonists to heat shock protein 90 (Hsp90) seem to suggest.[33,34]

In the present case, Vitr-p-13, when tested in artificial membranes, was unable either to insert into phospholipids monolayers and to induce calcein leakage from calcein-loaded liposomes (data not shown). In addition, several in

Table I Average Number of Water Molecules at Distance Not Exceeding 0.25 nm (Column A), 0.35 nm (Column B). Solvent Accessible Surface (nm^2), Third Column. Number of Intrapeptide Contacts (distance lower than 0.3 nm)

State	A	B	SAS	Intrapeptide Contacts
Basin ***a***	118 ± 2	65 ± 2	13.8 ± 0.1	5 ± 1
Basin ***b***	118 ± 2	62 ± 2	13.8 ± 0.1	5 ± 1
Basin ***c***	113 ± 2	60 ± 2	13.3 ± 0.1	4 ± 1
Basin ***d***	115 ± 2	60 ± 2	13.5 ± 0.1	4 ± 1
No-minima	105 ± 1	56 ± 1	12.9 ± 0.1	4 ± 1

vitro antimicrobial assays with a number of different strains showed no activity toward either Gram +/− bacteria (data not shown). In view of our CD and MD results, indicating the extremely low alpha-helix propensity of Vitr-p-13 and is existence essentially in statistical-coil conformation in water, the hypothesis that interaction with lipid membranes is somehow precluded to those peptides unable to adopt a partially-folded structure when in aqueous solutions, is undoubtedly reinforced. In the case of Vitr-p-13, of course, it is not particularly surprising that the peptide does not display any antimicrobial activity. Rather it is of some biochemical significance that it does not fold into a structure able to interact with lipid membranes, although it lies exposed on the surface of a protein believed to bind phospholipids bilayers in the course of its physiological action. Further work will be needed to elucidate the role of this peptide, which is highly conserved among a number of bacterial hemoglobins and haemoglobin-like proteins (data not shown).

In conclusion, our data on Vitr-p-13 and temporins provide further insights into the working hypothesis of a strict correlation between folding propensity of peptides in solution and their pharmacological activity as antimicrobial agents.

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