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ORIGINAL PAPER

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Role of hydrophobic interactions and salt-bridges in β-hairpin folding

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Abstract β-Hairpins are the simplest form of β-sheets which, due to the presence of long-range interactions, can be considered as tertiary structures. Molecular dynamics simulation is a powerful tool that can unravel whole pathways of protein folding/unfolding at atomic resolution. We have performed several molecular dynamics simulations, to a total of over 250 ns, of a β-hairpin peptide in water using GROMACS. We show that hydrophobic interactions are necessary for initiating the folding of the peptide. Once formed, the peptide is stabilized by hydrogen bonds and disruption of hydrophobic interactions in the folded peptide does not denature the structure. In the absence of hydrophobic interactions, the peptide fails to fold. However, the introduction of a salt-bridge compensates for the loss of hydrophobic interactions to a certain extent.

Keywords β-Hairpin · Molecular dynamics · Hydrophobic interactions · Salt-bridge interactions · Peptide folding

Introduction

Understanding the mechanisms behind the folding of proteins, which is one of the most fundamental biochemical processes, is proving to be a challenging task to biochemists and biophysicists. Recent developments in instrumentation and methodology have enabled us to take major steps forward in comprehending the dynamics of proteins and peptides at the molecular level. Protein-engineering methods such as the Phi-value analysis [1] and various spectroscopic techniques such as NMR have brought down the enormity of this task.

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Proteins are mainly composed of two major secondary structural features: α-helices and β-sheets. It is observed that 40–70% of secondary structure in proteins falls inside these two elements [2]. As a result, these structures have been studied extensively using both theoretical and experimental approaches.

The α-helix is a very fundamental structural element. It is highly ordered and is stabilized by local interactions. In particular, backbone hydrogen bonds between the ith and the $i+4$th residues are responsible for the integrity of an α-helix.

β-sheets are more complex than α-helices as long-range interactions play an important role in their formation. Such interactions make one consider them as tertiary structures [3]. The interest in these structures is enhanced by the fact that they are involved in the formation of amyloid fibrils [4, 5]. This interest is further augmented by reproducible observations that all proteins can form fibrils with the same cross-β structure [6].

β-hairpins—two β-strands that are anti-parallel and hydrogen bonded together—are the simplest of β-structures. The simplicity of these structures and the surprising finding that they form stable structures in aqueous solutions [7], unlike isolated α-helices, makes them amenable to high-resolution studies. Experimental approaches to studying these structures include NMR [8–10] infrared, [11] CD [12, 13] and VCD [14]. These experimental techniques have been more than complemented by computational methods, of which molecular dynamics simulations are much favored [15–18]. The use of computational approaches in studying β-sheets becomes more important in light of their tendency to aggregate [19].

Molecular dynamics simulations, which make use of classical Newton mechanics to generate trajectories, are playing an ever-expanding role in biochemistry and biophysics because of substantial increases in computational power and concomitant improvements in force fields. In particular the contribution of such studies to protein folding is immense. As pointed out by Fersht

and Dagget [1], molecular dynamics simulations are capable of unraveling whole protein folding/unfolding pathways. Indeed, simulation techniques have been used widely for studying α-helices. Today, the focus has shifted to studies of β-structures for the aforementioned reasons. Several computational studies have concentrated on the mechanism of the folding and unfolding of β-hairpins in particular, since realistic atom-scale simulations of fully fledged β-sheets requires enormous computational power and have been achieved only with large parallel computers.

While it is not possible to elucidate all available computational data on β-hairpin folding, we intend to highlight a few contributions made using molecular dynamics. Pande's group at Stanford, using a huge parallel distributed computing system, have simulated a total of 38,000 ns folding time of a β-hairpin, and implicate a three-state folding mechanism. They show that partial hydrophobic core formation takes precedence over hydrogen bonds in initiating folding [15]. Zhou et al. [17] show that the folding-energy landscape of a β-hairpin peptide is rugged at 300 K but becomes smooth at 360 K. They also note that hydrophobic cluster and inter-strand hydrogen bonds form at about the same time [17]. Lee and Shin examine the contributions of different energy terms to the folding process. They illustrate that, while the protein–protein interaction energy favors the folded state, the protein–solvent interaction energy favors the unfolded state. While the electrostatic components of these energy terms correlate with the loss or formation of hydrogen bonds, the van der Waals terms fit well with the disruption of the hydrophobic core of a protein [18].

We have simulated a 13-residue peptide (WRYYESSLEPYPD)—henceforth called peptide X—whose hairpin structure in a toxin-bound form has been elucidated using NMR [20]. In the simulations described here, we show that peptide X folds from an extended conformation, almost immediately, into a hairpin and after 20 ns bends at its ends. The simulation was continued up to 100 ns and the structure was found to be stable. We also investigate the temperature stability of the peptide. To investigate the folding process further we make use of rational in-silico mutants. Using these we study the role of hydrophobic interactions both in the formation and stability of the hairpin. We also go further by asking whether salt-bridges can replace hydrophobic interactions as initiators of the peptide-folding process.

Materials and methods

Generation of starting structures and mutant peptides**

All starting structures, including the mutant peptides, were generated using Hyperchem on an SGI-Fuel system running IRIX. The starting structures were modeled in their fully extended and *trans* conformations. This means that all the ω and ϕ angles were set at 180° and the ω angle, which specifies the geometric isomer about the rotationally rigid peptide bond, was also set to 180°. The structures were written out in PDB format. Mutations on the folded peptide were carried out using Swiss PDB Viewer (http://www.expasy.ch/spdbv).

Simulation details

All simulations were carried out using GROMACS 3.2 running on a single 2.8 GHz Pentium IV IBM machine with 512 MB RAM and running Fedora Core 2 Linux. The GROMACS force field [21, 22] including all hydrogens was used. The peptide was solvated in a box containing approximately 1,000 water molecules [23]. Periodic boundary conditions were employed to eliminate surface effects. Energy minimization with a tolerance of 2,000 kJ mol^{-1} n^{-1} m^{-1} was carried out using the steepest–descent method. All bonds were constrained using LINCS. The system was loosely coupled to a temperature bath using Berendsen's method [24]. Berendsen's pressure coupling was used. Long-range electrostatics were handled using the PME method. All potential cutoffs were set at 1 nm. The final MD simulations were carried out with a time-step of 2 fs and without any position restraints.

Analysis

All analyses were carried out using programs built within GROMACS. The RMSD values were obtained from the least square fit of the respective backbones. The radius of gyration was also calculated for the whole backbone indicating the compactness of the overall structure. For calculating hydrogen bonds, the cutoff distance was fixed at 0.35 nm. Formation of the hydrophobic cluster was assessed by distances between the aromatic portions of the side-chains concerned. Compiled DSSP [25], which was downloaded separately and run from GROMACS, was used to calculate secondary-structure formation.

Results and discussion

Peptide X and its variants

The peptide X, which forms a hairpin in a toxin-bound form [20], has several hydrophobic residues that can be expected to interact in the event of hairpin formation. In this work, we have used this peptide and some suitable variants in order to study some interactions that may be essential for peptide folding. A list of in-silico peptides used in this study is given in Table 1. This table also gives some information on the rationale behind the choice of these peptides.

Table 1 Table showing the peptides that were used in this study of β-Hairpin folding

Peptide sequence	Simulation time	Comments
WRYYESSLEPYPD	100 ns Temperature scan: 10 ns at four elevated temperatures each	The wild-type peptide X
WRYYESSLEPAPD	35 ns to show the role of hydrophobic interactions in initiating the folding process 20 ns to show that hydrogen bonds stabilize the folded structure once formed	Peptide to show the importance of hydrophobic interactions to the formation and stability of the peptide
WRYYESSLEPEPD	50 ns	Peptide to show the role of salt-bridges in the folding of the peptide

Molecular dynamics simulations of peptide X

The peptide X was modeled in its fully extended *trans* conformation ($\psi = 180$, $\phi = 180$, $\omega = 180$). This peptide was solvated in an aqueous environment, energy minimized and unrestrained molecular dynamics simulations using the GROMACS force field to a time-scale of 100 ns was carried out. Structures were written to the trajectory every 0.5 ps. We have calculated the following time-dependent parameters, apart from the coordinates, for detailed analysis: Backbone root mean square deviation (RMSD) with respect to the starting, extended conformation and also to the experimental NMR structure, the radius of gyration, solvent accessible surface area, backbone hydrogen-bond patterns and distances between rationally chosen groups. Lee and Shin [18], in their extensive MD calculations of a hairpin-forming peptide, have used the radius of gyration and the number of hydrogen bonds as order parameters to measure the compactness of the structure. In order to test the equilibration of the system, we calculated the time evolution of box volume and temperature. The volume of the system remains constant throughout and the temperature relaxes inside a few ps. The energy of the system oscillates about the local energy minimum. The secondary structures in the peptide were calculated using the DSSP program [25].

It could be seen that the extended conformation folds into a hairpin inside 2 ns (Structure RH). This structure remains stable for another 18 ns and at about 20 ns undergoes a conformational change that leads it to be bent at the ends (structure BH) (Fig. 1a). The RMSD (Fig. 1b) with respect to the starting structure increases rapidly until it stabilizes at 2 ns. The bending of the hairpin corresponds to a fall in the RMSD. The compact nature of the folded structure is seen by a plot of the radius of gyration (Fig. 1c), which falls from 1.2 nm for the starting structure to 0.5 nm for the final structure, with respect to time. The same figure also includes the representative images of the folding simulation. It might be worth noting that the structure BH is more compact than RH. The time evolution of the number of residues involved in β-structures, as seen using DSSP (Fig. 1d), shows that there is reversible formation of secondary structures during the simulation time.

The folding process inside the first 20 ns is accompanied by a remarkable fall in the inter-strand hydrophobic residue distances (W1–Y11, Y3–Y11 and Y4–Y11) (Fig. 2a–c). By inter-strand hydrophobic residue distances we mean distances between the side-chains of the residues concerned. The bending of the hairpin structure, which occurs after 20 ns, is accompanied by a further fall in the Y3–Y11 distance. However, this is also accompanied by a slight increase in the W1–Y11 distance. There is no appreciable change in the Y4–Y11 distance, though. The hairpin structure is stabilized by backbone inter-strand hydrogen bonds. The bent structure is further characterized by the formation of additional backbone hydrogen bonds between Y3 and Y11 (Fig. 2d).

The results of this preliminary simulation led us to ask the following questions: (1) How stable is the folded structure of X? (2) What is the role of hydrophobic interactions in the folding process concerned? (3) Are hydrophobic interactions essential only for the formation of the hairpin but not for its stability once it is formed? (4) What role do salt-bridges play in folding a peptide?

We sought to answer the above questions by altering the simulation temperature or by generating rational in-silico mutants of peptide X and simulating these peptides under the same conditions as X for reasonable time durations. All further simulations were carried out using the GROMACS force field so that approximations and errors inherent to this force field would be carried over to all our simulation systems. These results are summarized below.

Stability of the folded structure to temperature variations

In order to study the stability of the hairpin to temperature, we simulated the stable structure in water at different temperatures −350, 400, 450, and 500 K—for 10 ns each. The hairpin structure formed appears to be exceptionally stable. During the course of these 10 ns simulations at elevated temperatures, the hairpin maintains its structure and the RMSD of the final structure with respect to the starting structure is in the range of 0.15 nm. However, we could observe a surge in the

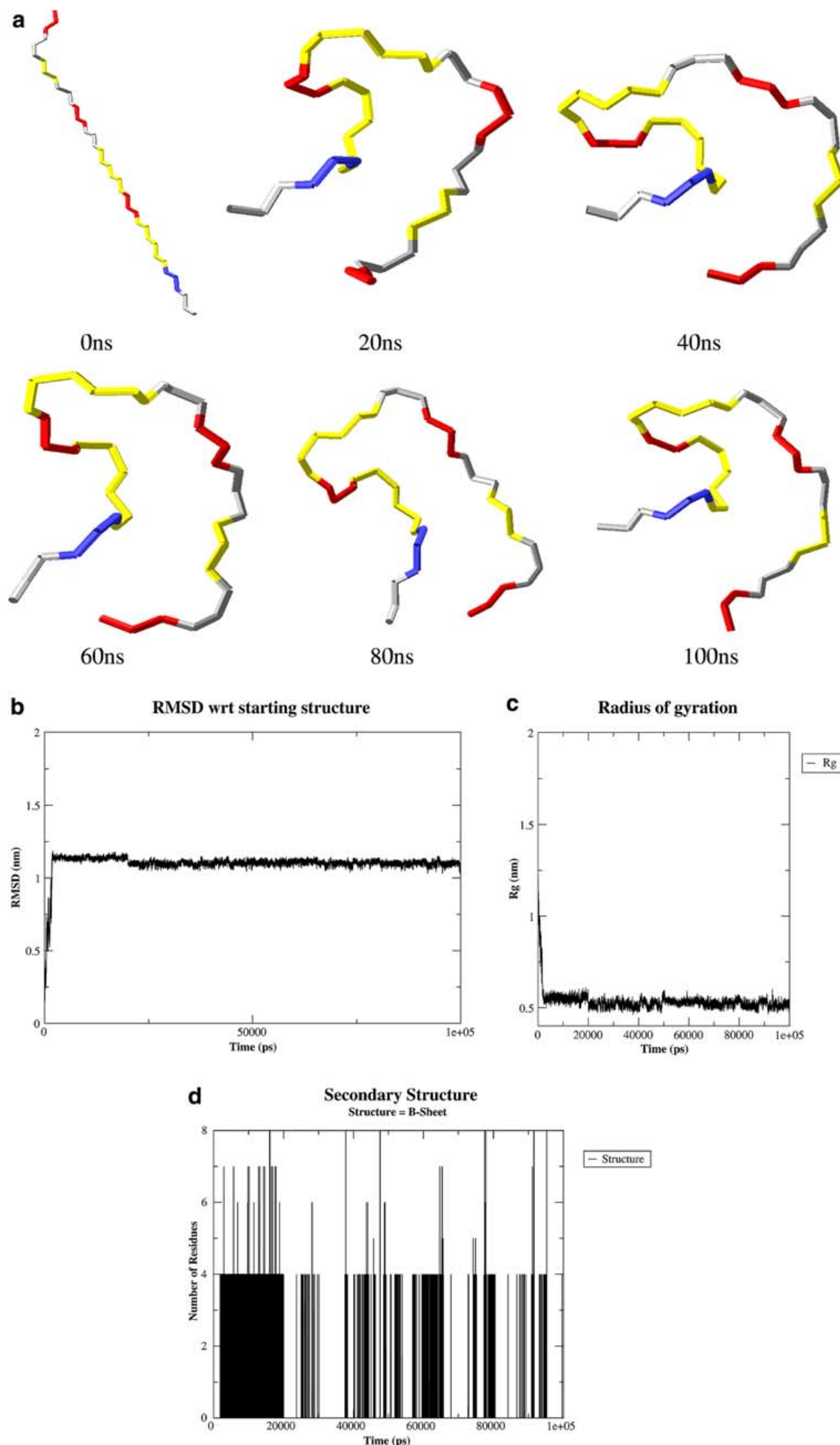


Fig. 1 **a** Structures generated after every 20 ns (20 ns structure corresponds to RH and the subsequent structures represent BH), from the stating conformation, are shown. *x*-Axis label is time in ps while the parameter is on the *y*-axis. **b** Time evolution of RMSD (**a**) of peptide X during the course of the 100 ns simulation in water. *x*-Axis label is time in ps while the parameter is on the *y*-axis. **c** Time evolution of radius of gyration and pictures depicting representative structures from the simulation of peptide X in water for 100 ns. **d** Plot of the number of residues in peptide X falling in *β*-sheets (*y*-axis) against time (*x*-axis in ps)

RMSD towards the very end of the simulation at 500 K. While it cannot be stated that the structure does not denature at elevated temperatures, it can be affirmed that during the course of a significantly long simulation period, the structure remains stable. And at temperatures less that 500 K, there is no indication that the

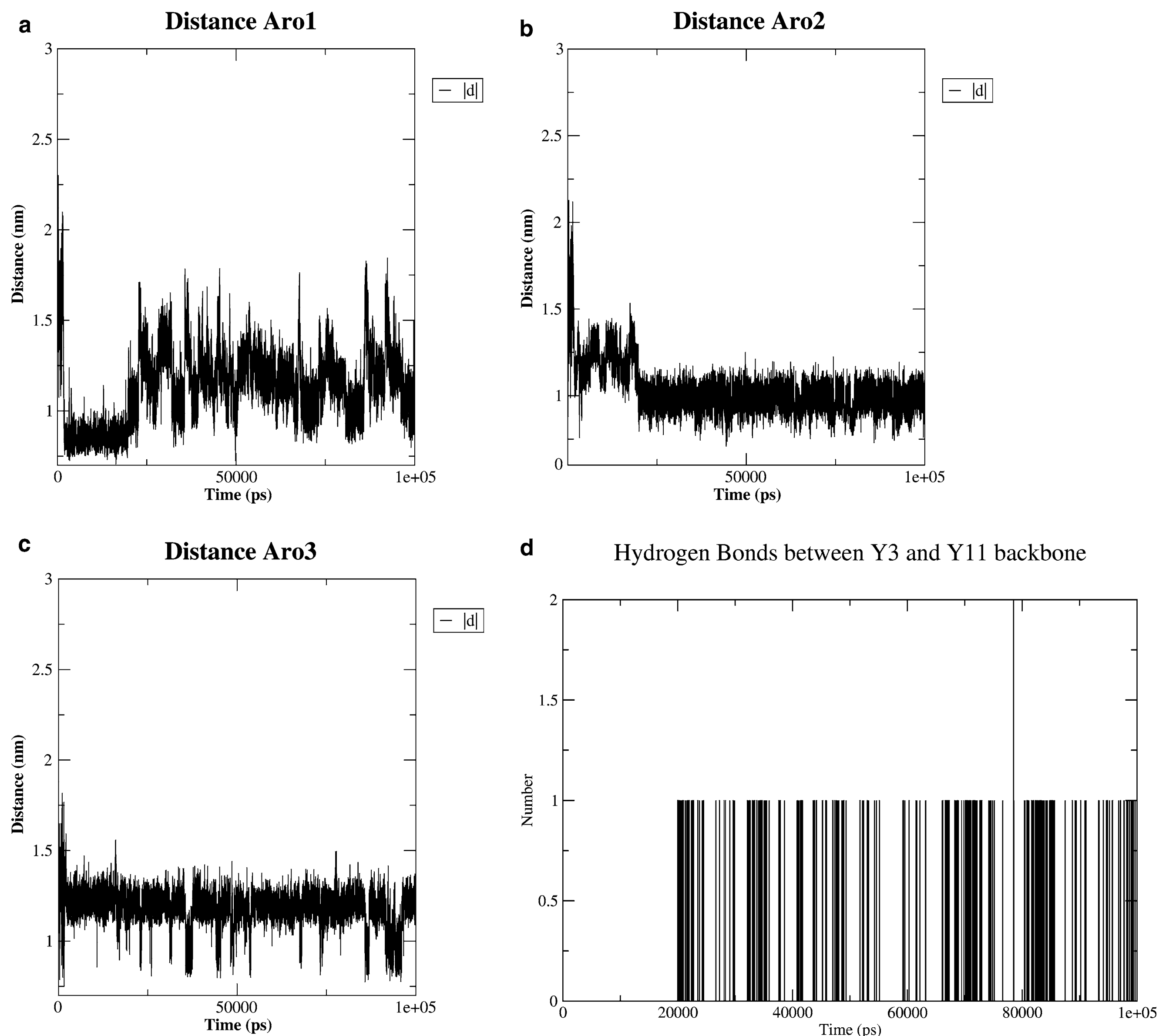


Fig. 2 Time evolution of distances between the side-chains of **a** W1–Y11, **b** Y3–Y11, **c** Y4–Y11, and **d** number of hydrogen bonds between the backbones of Y3 and Y11. The distances (nm) and the number of hydrogen bonds are plotted along the *y*-axis while the time (ps) is along the *x*-axis

structure would denature at any time point in the near vicinity and greater than 10 ns (as seen from the RMSD trends in Fig. 3). This might mean that there exists a high-energy barrier for the process of denaturation, which is much higher than that for folding.

The role of hydrophobic interactions in the folding process

Role of hydrophobic interactions in hairpin folding has been investigated by several groups [26]. From our simulations of peptide X, we observed that Y11 side-chain forms favorable contacts with W1, Y3, Y4, thus forming an aromatic-hydrophobic core involving both strands of the hairpin. In order to study the importance of this in the folding process, we generated the Y11A mutant in-silico. This peptide was simulated in water for 35 ns. It was found that this peptide fails to fold into any structure even remotely resembling a hairpin. The high conformational flexibility of this peptide can be seen from the wild fluctuations observed in the time-dependent parameters such as RMSD and radius of gyration. A remarkable fall in the radius of gyration towards the end of the simulation time does not correlate with the formation of any folded conformation. It is interesting to note that a folded structure does not form despite the formation of a turn-like conformation at early time

Fig. 3 Comparison of RMSD (nm, *y*-axis) vs time (ps, *x*-axis) of the folded peptide at different temperatures **a** 350 K, **b** 400 K, **c** 450 K, and **d** 500 K

points. This is because, the N-terminal strand, due to the absence of an aromatic-hydrophobic partner on the other strand, finds no reason to approach it and subsequently forms a H-bond stabilized structure. Some representative structures from this simulation are shown in Fig. 4.

In order to test whether hydrophobic interactions are essential for the stability of the hairpin after it is formed, the in-silico Y11A mutation was carried out on the 80 ns wild-type structure. This mutant peptide was simulated under identical conditions for another 20 ns, during which time the structure remained stable. The RMSD between the starting structure and the structure generated after 20 ns was only 0.09 nm. A least-square fit image of the simulated structure on the starting structure is shown in Fig. 5a.

These results imply that strong hydrophobic interactions are a prerequisite for the folding of the extended peptide into a hairpin conformation but are not essential for its stability once formed. An alternative explanation may be ascribed to a possible high-energy barrier for the unfolding process. The structure is clearly stabilized by hydrogen bonds. This is seen from a comparison of the dependence of the number of main-chain hydrogen bonds on time in the mutant structure (Y11A from 80 ns wild-type; 0–20 ns) and the wild-type structure (from 80 to 100 ns). This is illustrated in Fig. 5b.

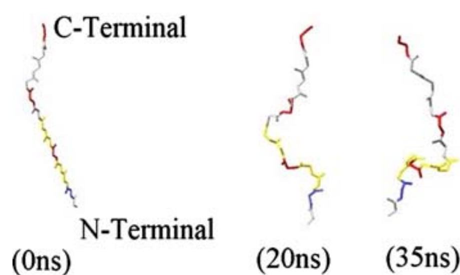
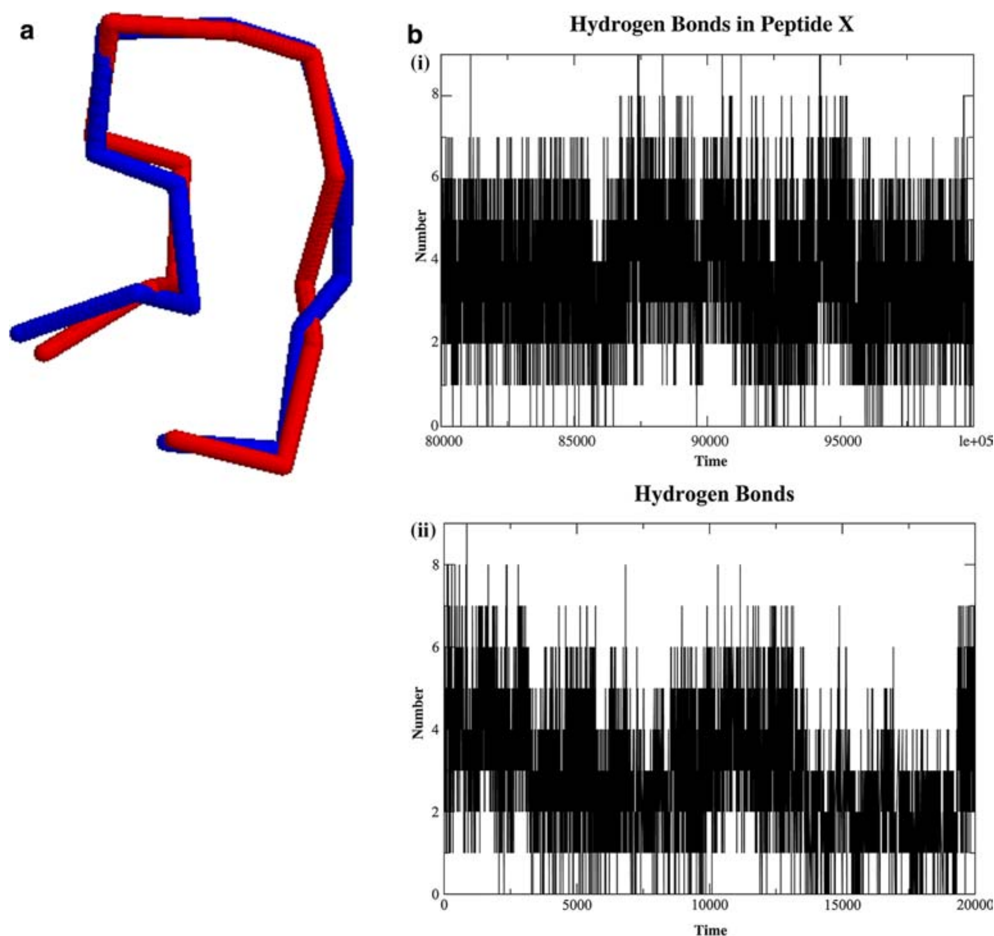
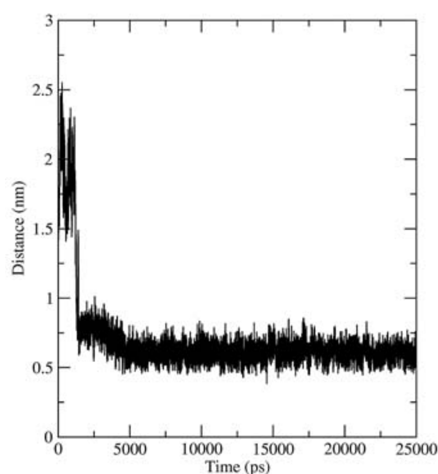


Fig. 4 Figure showing snapshots from the 35 ns simulation of the Y11A peptide. The starting structure along with the 20 and 35 ns structures are shown

Fig. 5 **a** Figure showing a least square fit between peptide X after 80 ns (*blue*) and the structure after 20 ns of its Y11A mutant (*yellow*). This shows that once the folded peptide is formed, disruption of hydrophobic interactions does not affect the structure. **b** Comparison of hydrogen bond numbers (*y*-axis) vs time (ps, *x*-axis) in (**i**) peptide X: 80–100 ns and (**ii**) peptide Y11A with starting structure generated from the folded peptide X





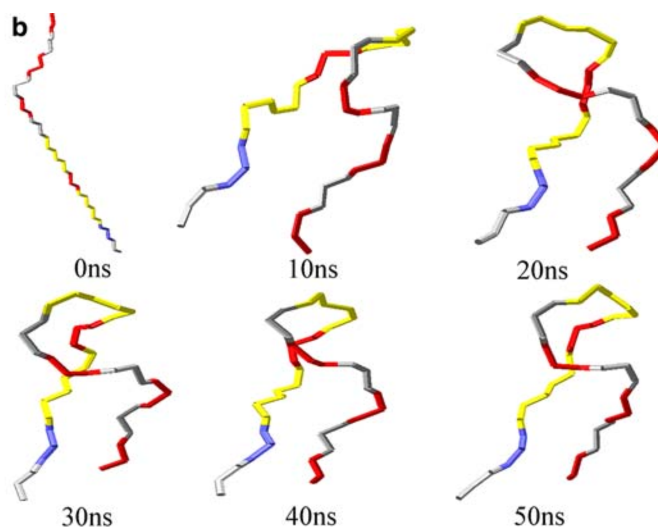


Fig. 6 a Distances between (**a**) R2–D13 and (**b**) R2–E11 in peptide Y11E. The starting conformation was in a fully extended conformation. The distances measured in nm are on the *y*-axis while time (ps) is along the *x*-axis. **b** Pictures depicting representative structures from the simulation of peptide Y11E in water for 50 ns. Structures generated after every 10 ns, from the starting extended conformation, are shown. The side-chains of the groups involved in salt-bridge interactions are also highlighted

Role of salt-bridges in the folding of the peptide

Salt-bridge interactions are known to play important roles in the stability of proteins. For example, archaeal proteins with the property of temperature stability have highly optimized salt-bridge interactions [27]. In order to investigate the role of these interactions in the folding of peptide X, we generated the computer mutant Y11E and simulated the extended conformation of this peptide in water for 50 ns. We found that β-structures form in 5 ns. While it is apparent that these structures are stable only for 10 ns, we were able to observe that the structure maintains a well-folded conformation. The folded conformation is such that it brings R2 close to E11 and consequently to D13 (Fig. 6a). That E11 is essential for this form of folding is apparent from the already explained observation that the peptide with A11 does not fold. Representative structures indicating the progress of the simulation are shown in Fig. 6b.

The formation of the hairpin

In a classic paper, Klimov and Thirumalai [28], making use of an off-lattice computational model (in which the amino-acid residues are represented as united side-chains around their centers of mass), proposed a mechanism for hairpin folding that begins with hydrophobic collapse leading to the formation of hydrogen bonds and subsequently arriving at the native state. We have extended these results through all-atom simulations of another hairpin peptide and proceeded further by integrating various other results. We implicate a role for salt-bridge formation in the folding process. Our results point out that a key hydrophobic interaction can be compensated for by a salt-bridge interaction, at least to the extent of forming a folded structure, if not a typical hairpin. We also show that once the native state is formed, annihilation of hydrophobic interactions fails to denature the peptide, whose conformation is stabilized by hydrogen bonds. Earlier, long-duration simulations of the atom–atom model of the peptide have been carried out in implicit solvent systems, which are not as accurate as the explicit representation [15]. Thus, integrating results from MD simulations of several mutant forms of a hairpin peptide, we provide a more comprehensive understanding of the folding process.

Conclusions

On a single IBM computer, we have simulated the folding of a 13-residue peptide to a total simulation time-scale of 250 ns. We establish the role of hydrophobic interactions in the formation of the hairpin peptide. We also go on to show that, once formed, the structure is stabilized by hydrogen bonds and that disruption of the hydrophobic core at this time fails to affect the folded conformation adversely. We also show that in the absence of a hydrophobic cluster, salt-bridges can promote the folding of the peptide into a compact structure. The wild-type hairpin is temperature-stable.

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