

# Spontaneous Unfolding and Refolding of Plantaricin $\alpha$ -Helix in Molecular Dynamics Simulation

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**How to cite this paper:** Yan, S.M. and Wu, G. (2019) Spontaneous Unfolding and Refolding of Plantaricin  $\alpha$ -Helix in Molecular Dynamics Simulation. *Computational Molecular Bioscience*, 9, 27-39.

<https://doi.org/10.4236/cmb.2019.91003>

**Received:** January 29, 2019

**Accepted:** March 25, 2019

**Published:** March 28, 2019

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## Abstract

Antimicrobial peptides are promising therapeutic agents in view of increasing resistance to conventional antibiotics. Antimicrobial peptides usually fold in  $\alpha$ -helical,  $\beta$ -sheet, and extended/random-coil structures. The  $\alpha$ -helical antimicrobial peptides are often unstructured in aqueous solution but become structured on bacterial membrane. The  $\alpha$ -helical structure allows the partitioning into bacterial membrane. Therefore it is important to understand the mechanism of unfolding and refolding of  $\alpha$ -helical structure in antimicrobial peptides. It is not very easy to observe and study the process of unfolding and refolding of  $\alpha$ -helical antimicrobial peptides because of their rapidity. Therefore, molecular simulation provides a way to observe and explain this phenomenon. Plantaricin A is a 26 amino-acid antimicrobial pheromone peptide and can spontaneously unfold and refold under physiological condition. This study demonstrated the unfolding and refolding of plantaricin A by means of molecular simulation, and its mechanism was discussed with its implication to the Levinthal paradox.

## Keywords

Alpha-Helix, Antimicrobial Peptides, Protein Folding, Plantaricin A

## 1. Introduction

Antimicrobial peptides are promising therapeutic agents, not only because they can act on Gram-positive/negative bacteria, protozoa, yeast, fungi, viruses, etc., but also because they come from almost all organisms [1] [2]. Besides having a great diversity in their function, antimicrobial peptides also have a great diversity

ty in their structures including  $\alpha$ -helical,  $\beta$ -sheet, extended/random-coil structure [3] [4] [5].

Of three thousands of antimicrobial peptides [6],  $\alpha$ -helical structure accounts 14.88%,  $\beta$ -sheet structure accounts 2.69%, whereas the majority (58.85%) are unknown structures and the rest are mixed structures. Clearly,  $\alpha$ -helix is the most important structure in antimicrobial peptides. Although the net charge, amphipathicity and hydrophobicity, is the most important factor in antimicrobial peptides for their activity [3], the  $\alpha$ -helical structure produces distinct membrane-bound amphipathic conformation [7] and thus allows its partitioning into bacterial membrane.

Curiously, the  $\alpha$ -helical antimicrobial peptides are often unstructured in aqueous solution but become structured on bacterial membrane. Basically, the alternation between unfolding and refolding is not limited to  $\alpha$ -helical antimicrobial peptides because this feature is observed in diseases. The famous example is amyloid  $\beta$  ( $A\beta$ ) peptide, whose  $\alpha$ -helix spontaneously unfolds under the physiological condition and the unfolding of amyloid  $\beta$  is one of the causes for Alzheimer's disease [8]. Also, the unfolding and refolding are important in biotechnological settings, where microorganisms produce recombinant enzymes and biopharmaceutical proteins. For example, the refolding of secreted amylase in *Bacillus subtilis* is extremely important for a profitable production [9] [10].

Plantaricin A is an antimicrobial pheromone peptide produced by *Lactobacillus plantarum* and is composed of 26 amino acids. Plantaricin A has an  $\alpha$ -helix from position 11 to position 22, and this  $\alpha$ -helical structure can spontaneously unfold and refold under the physiological condition, because it is unstructured in water but becomes partly structured upon the exposure to micelles and fully structured under the physiological condition [11].

Because of importance of  $\alpha$ -helical structure in antimicrobial peptides and proteins, it is necessary to study the unfolding and refolding process in order to understand the underlying mechanism. Although it is not so easy to observe and study this process at small time scale, molecular dynamic simulation could provide a way to observe and explain this phenomenon. In this study, the unfolding and refolding of plantaricin A was found in molecular dynamic simulation and an attempt was made to discuss its mechanism.

## 2. Materials and Methods

### 2.1. Data

The 3D structure of plantaricin A is available at Protein Data Bank [12] identifier 1YTR, which contains 20 best structures from 100 calculated structures measured by NMR at 25°C, pH 4, zero ionic strength and ambient pressure. The selection of these 20 best structures was based on their lowest energy [13].

### 2.2. Molecular Dynamics Simulation

Of 20 best structures of 1YTR, the 20th structure was used for the molecular dy-

dynamic simulation because it is also the one shown in various 3D visualization programs. Hydrogen atoms that did not appear in NMR measurement were added to plantaricin A using the Visual Molecular Dynamics Program [14].

The NAMD2 2.9 [15] was used to perform all the simulations with the all-atom force field CHARMM (v. 27) [16]. In simulations, the electrostatic interactions, the short-range non-bonded electrostatic and van der Waals interactions, and the long-range interactions were computed every 1 fs, 2 fs, and 4 fs; the particle-mesh Ewald was used with grid points no more than  $\sim 1$  Å apart; the Langevin dynamics was coupled to all atoms except for the hydrogens with a  $5 \text{ ps}^{-1}$  damping coefficient; the Nose-Hoover Langevin piston was used with a decay period of 100 fs and a damping time of 50 fs under 1 atm; a constant temperature was maintained; and 9 Å was chosen for the distance between ions [17]. The final system size was 24,661 atoms. Each simulation was continued until the time that no change was observed in plantaricin A structures, usually 15 ns.

Plantaricin A became unfolded in solution with ionic strengths of 150 mM NaCl and 1.5 mM  $\text{CaCl}_2$  at pH 7.4 and  $37^\circ\text{C}$  with the random seed of 1420883531.

Root mean square deviation (RMSD), which characterizes the amount of simulated molecule deviates from their defined positions in space, is calculated by program using the equation:

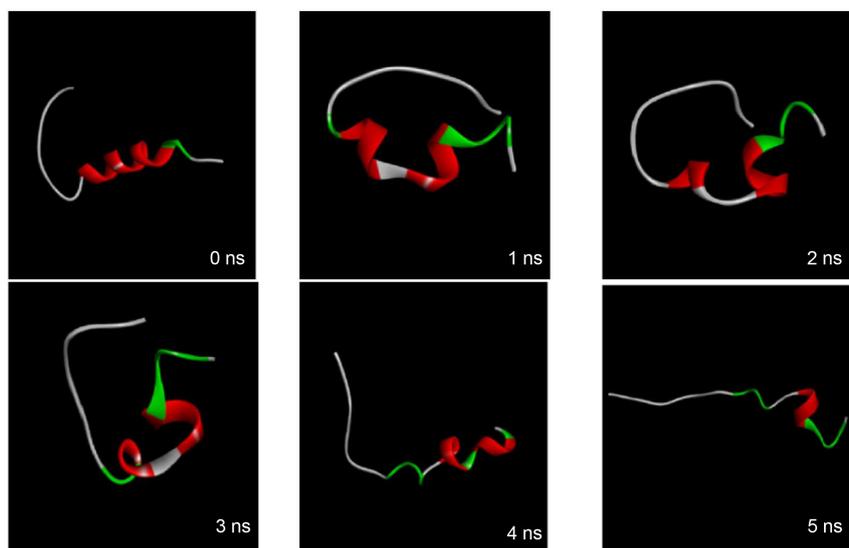
$$\text{RMSD}_a(t_j) = \sqrt{\frac{\sum_{a=1}^{N_a} (r_a(t_j) - r_a)^2}{N_a}} \quad (1)$$

where  $N_a$  is the number of atoms whose positions are compared,  $t_j$  is time,  $r_a(t_j)$  is the position of atom  $a$  at time  $t_j$ ,  $\langle r_a \rangle$  is the average value of the position of atom  $a$ , to which the position  $r_a(t_j)$  is compared [18].

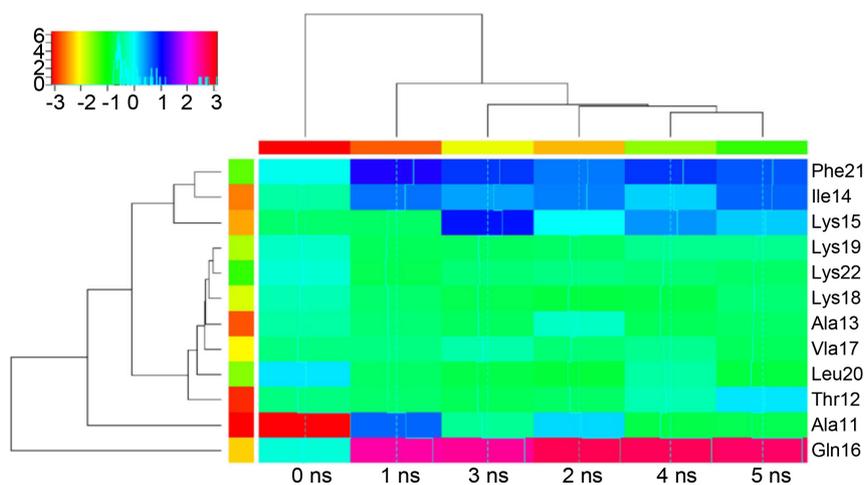
### 3. Results

It is important to study the mechanism of how  $\alpha$ -helical structure can spontaneously unfold and refold, considering its significance in clinical and biotechnical settings. In this study, the unfolding and refolding of plantaricin A were conducted in molecular dynamics simulation, because of its ready switch between folded and unfolded  $\alpha$ -helical structures.

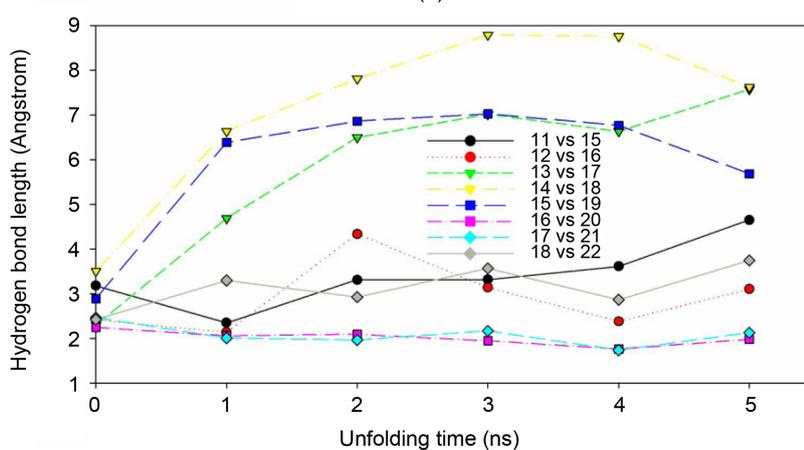
**Figure 1(a)** demonstrates the unfolding process of  $\alpha$ -helix at 1 ns interval. As can be seen, the unfolding of  $\alpha$ -helix is not monotonic since it progresses faster between 0 and 1 ns, and between 4 and 5 ns than other intervals. It is not surprising that the unfolding is faster between 0 and 1 ns because many studies showed the great role of the initial phase in molecular dynamics simulations. This is due to the change from the measured structure under a particular experimental condition to the condition of molecular dynamics simulation [19]. In addition, the unfolding did not begin at the termini of  $\alpha$ -helix, but began in its middle as seen in panels 0 ns and 1 ns.



(a)



(b)



(c)

**Figure 1.** The unfolding of  $\alpha$ -helix of plantaricin A at 1 ns interval (a), the distance of the residues from position 11 to position 22 to the ideal  $\alpha$ -helical center  $-60^\circ$  in  $\phi$ -axis and  $-50^\circ$  in  $\psi$ -axis and their dendrogram produced by the cluster analysis during unfolding process (b), and the length of hydrogen bond between the residues  $i$  and  $i + 4$  (c).

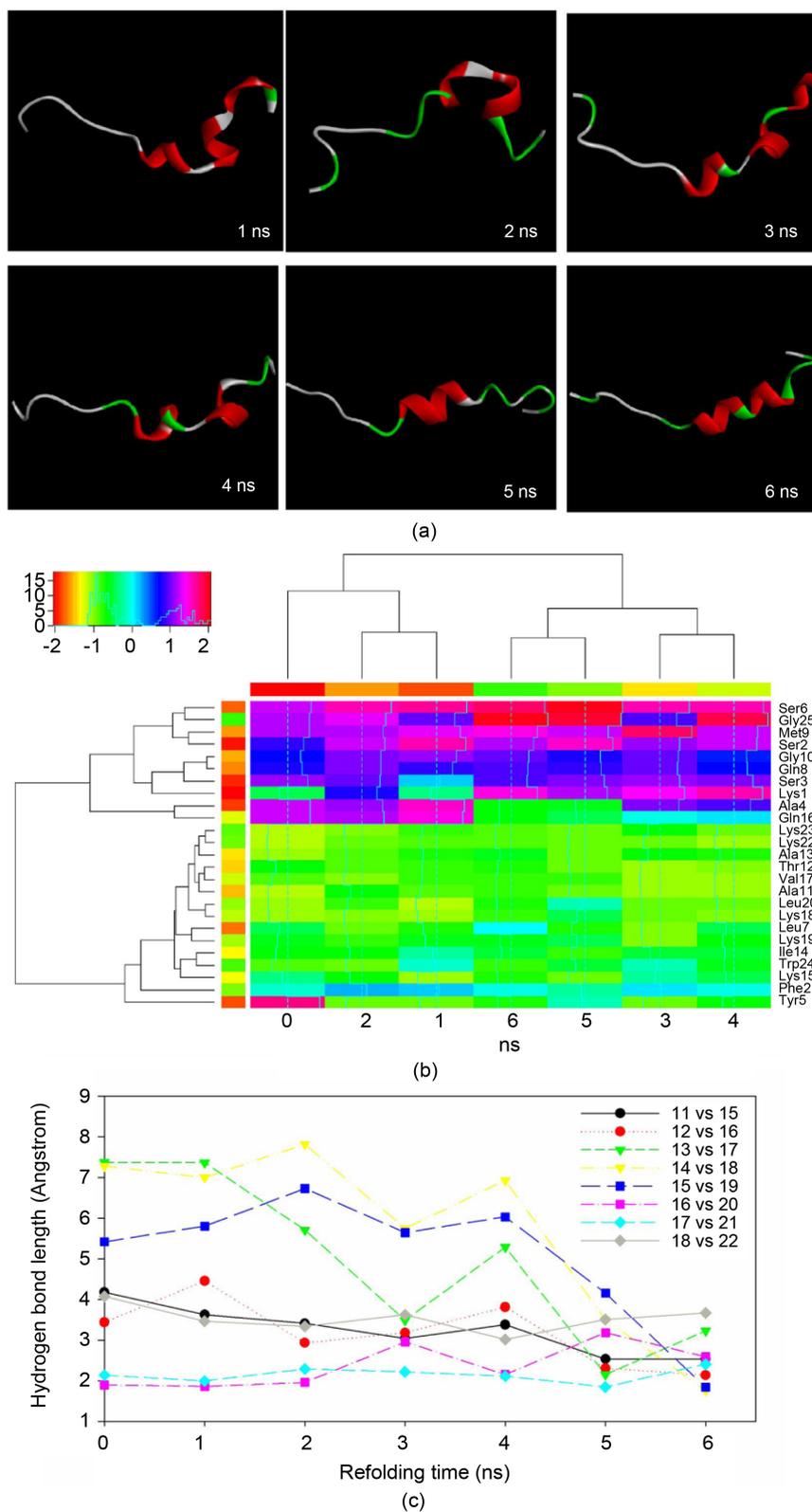
In **Figure 1(b)**, different colors illustrate how far away each residue moved away from the reference point, which is the ideal  $\alpha$ -helical center  $-60^\circ$  in  $\phi$ -axis and  $-50^\circ$  in  $\psi$ -axis in terms of the Ramachandran notation. As can be seen, the residues with green color did not have great movements from the reference point because their color did not change over 5 ns, so these residues could not lead the  $\alpha$ -helix to unfold. On the contrary, the residues on the top and the bottom in **Figure 1(b)** moved away from the reference point because of changes in their colors, especially the glutamine at position 16. Meanwhile, the dendrogram produced by the cluster analysis suggests that this residue was the main force for the unfolding of the  $\alpha$ -helix. Moreover the alanine at position 11 appeared the main force to maintain the  $\alpha$ -helix because it moved towards the reference point over the time. This is reasonable because alanine is too short to effectively mediate its degradation [4]. Still, **Figure 1(b)** indicates that the turning point of unfolding of  $\alpha$ -helix is the time between 3 ns and 4 ns, because most residues actually move back to the reference point at 3 ns. In effect, the  $\alpha$ -helix will be re-folded if such a tendency holds on.

**Figure 1(c)** demonstrated the length of hydrogen bonds between the residues  $i$  and  $i + 4$  of  $\alpha$ -helix. As can be seen, the hydrogen bonds between residues 14 and 18, between residues 15 and 19, and between residues 13 and 17 increased more than others. These residues, isoleucine, lysine, glutamine, valine, lysine and lysine, have similar helix propensities except for valine [20]. Just two hydrogen bonds, between residues 16 and 20, between residues 17 and 21, hold their length around 2 Å, which is the typical length of a hydrogen bond [21].

The simulation in at 0 ns and 5 in **Figure 1(a)** demonstrates the difference between folded and unfolded  $\alpha$ -helix of plantaricin A, and this difference marks the opening of  $\alpha$ -helical structure.

Because the start of refolding of plantaricin A was different in each simulation, we set the refolding time scale from 0 to 5 ns (**Figure 2**) in order to be identical with time scale in unfolding (**Figure 1**). **Figure 2(a)** depicts the refolding process of  $\alpha$ -helix of plantaricin A at 1 ns interval, where the refolding began from a very short  $\alpha$ -helix, which is generally considered as the folding nuclei [22]. The same explanation in **Figure 1(b)** and **Figure 1(c)** can be applied to **Figure 2(b)** and **Figure 2(c)**. Collectively, alanine is the initial residue for refolding as it has showed to be the main force to maintain the  $\alpha$ -helix in **Figure 1(b)**.

Misfolded plantaricin A, which can be eliminated by DnaK and GroEL in bacteria [23], suggests the difficulty in folding of native structure, because each residue has several pathways to be folded. When a plantaricin A can find a correct pathway to fold, it reached its native structure in 6 ns (**Figure 2(a)**). This is an extremely short period of time if we consider the number of pathways that a protein can fold itself. According to HP model [24] [25] [26] [27], where a protein folds in a lattice, the number of possible folding pathways for 26-residue sequence in 3D HP model is  $6 \times 5^{25}$  to find its native state in the worst case.

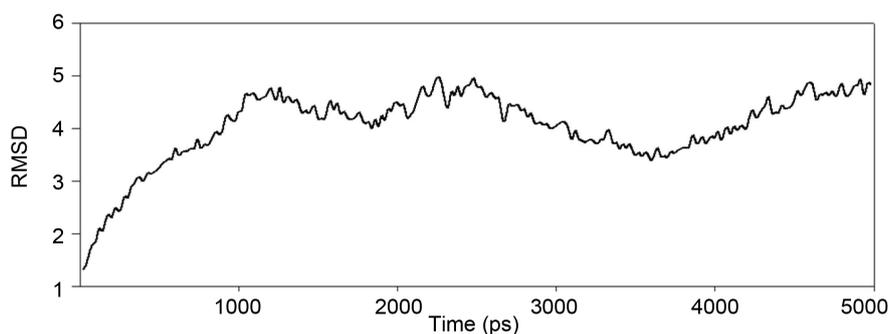


**Figure 2.** The refolding of  $\alpha$ -helix of plantaricin A at 1 ns interval (a), the distance of the residues from position 11 to position 22 to the ideal  $\alpha$ -helical center  $-60^\circ$  in  $\phi$ -axis and  $-50^\circ$  in  $\psi$ -axis and their dendrogram produced by the cluster analysis during refolding process (b), and the length of hydrogen bond between the residues  $i$  and  $i + 4$  (c).

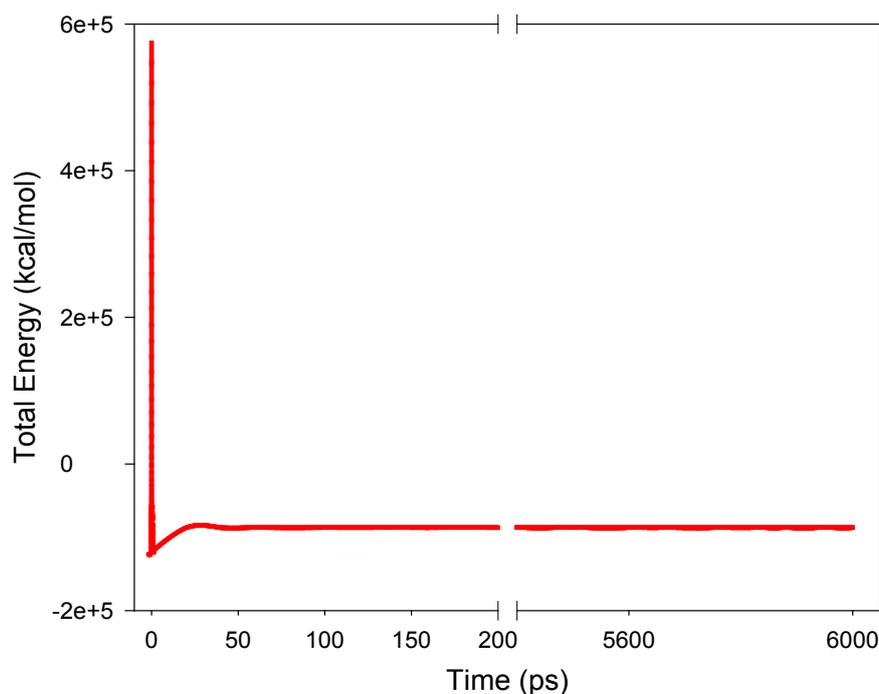
The simulation in at 1 ns and 6 in **Figure 2(a)** shows the difference between unfolded and folded  $\alpha$ -helix of plantaricin A, and this difference marks the forming of  $\alpha$ -helical structure.

The native state of a protein is associated with the minimal energy. Molecular dynamics simulation provides the trajectories of atomic positions and energy over time for equilibration and minimization. **Figure 3** reveals that plantaricin A needs roughly 5 ns to reach its equalization and minimization during refolding process. This period of time was used by plantaricin A to find the correct refolding pathway. It was suggested that the hydrophobic force is the main force determining the unique native state [24], while the percent of hydrophobic residues of majority of proteins is 40% - 50% [28]. The percent of hydrophobic residues in plantaricin A is 42%, so plantaricin A would follow this to fold itself.

**Figure 4** represents the change in total energy of refolding of plantaricin A



**Figure 3.** Root mean square deviation of refolding process of plantaricin A over time.



**Figure 4.** Change in total energy of refolding of plantaricin A during molecular dynamics simulation.

during molecular dynamics simulation. At time 0, there should be 100% unfolded structure and the total energy is at maximum. From 0 ps to 20 ps, the total energy decreased from 574,306 kcal/mol to -120,017 kcal/mol, while from 20 ps to 6000 ps the total energy fluctuated between -120,000 and -80,000 kcal/mol. These rapid decline in total energy suggests an energy funnel for folding [29] [30].

#### 4. Discussion

Plantaricin A was chosen because it comes from *Lactobacillus plantarum*. It is known that bacteria evolved a mechanism to use proteins such as DnaK and GroEL to prevent the accumulation of unfolded and misfolded proteins [23]. Additionally, the folding of plantaricin A occurs at co-translational stage [31]. This is very delicate because the folding of proteins occurs either at the post-translational stage or at the co-translational stage with different requirement of time. Thus, a peptide sequence must fold itself into a well-defined functional structure within a reasonable period of time. Essentially, the signal peptide of protein has the information on timing of co-translational stage [32], which is the case for plantaricin A [31].

The most important observation in the unfolding of  $\alpha$ -helix of plantaricin A is that the  $\alpha$ -helix is unfolded from the middle of  $\alpha$ -helix rather than from its termini. Theoretically, the breaking of hydrogen bond requires 30 kJ/mol energy, which could be possible under the fluctuation in the system [33]. Hence the spontaneous unfolding of  $\alpha$ -helix is very probably to occur in vivo because molecular dynamics simulation with the artificial periodicity stabilizes a protein otherwise it would unfold quickly [34]. A study on the folding of DNA loop revealed that the required energy was roughly the same for misfolded and native loops [35].

For many proteins, their unfolding is irreversible, for example, the coiled-coil structure is irreversible in Hv1/VSOP [36]. Plantaricin A can refold itself again when exposing to micelles and under the physiological condition [11]. In molecular dynamic simulation, the refolding of plantaricin A is not easy with unspecific time of simulation, and the most important point is that most refolded structures are misfolding. A large number of misfoldings is understandable because the unfolding does not have many choices for each residue, but the folding of protein is far more complex, which is explained at least by three theories. 1) The hydrophobic-polar (HP) model [24] and the hydrophobic-hydrophilic-neutral (BPN) model [37] enumerate all the possible folding pathways in order to find out the optimal pathway to rapidly fold the native structure. 2) The theory focuses on folding intermediates, and analyzes the stability and activation barriers between folding intermediates with folding reaction [38]. 3) The theory concentrates on folding energy landscape and proposes a rugged funnel-like landscape biased toward the native structure [29] [30]. Over years, the first and third theories are becoming more and more complementary [39].

Generally, the folding is faster for  $\alpha$ -helix than for  $\alpha$ -sheet as well as the mixture of  $\alpha$ -helix and  $\alpha$ -sheet [40], and plantaricin A belongs to the small ultra-fast-folding proteins.

How a protein can find out its native state without globally exhaustive search is the Levinthal paradox [41], which extends beyond the protein folding into other combinatorial problems in biological fields such as protein-protein interaction [42] and biomolecular complex assembly [43].

In the past, molecular dynamics simulation was used to unfold proteins with external forces, including mechanic force [44], chemical force [45], high temperature [46], pressure [47], light/electromagnetic radiation [48], whereas the simulation of refolding of proteins was also studied with low temperature [49]. In this study, we simulated the spontaneously unfolding and refolding of plantaricin A, which is the advantage of our study because we did not apply any foreign force and environmental conditions. Molecular dynamics simulation demonstrates that plantaricin A unfolds its  $\alpha$ -helix from the middle residue, refolds its  $\alpha$ -helix from a terminal. Moreover, our study reveals that fluctuation of energy under the physiological condition is sufficient to initiate unfolding of plantaricin A.

## Fund

This study was supported by National Natural Science Foundation of China (31460296 and 31560315), Key Project of Guangxi Scientific Research and Technology Development Plan (AB17190534) and Special Funds for Building of Guangxi Talent Highland.

## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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