Insight into the impact of environments on structure of chimera C3 of human β-defensins 2 and 3 from molecular dynamics simulations

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C3 is a chimera from human β-defensins 2 and 3 and possesses higher antimicrobial activity compared with its parental molecules, so it is an attractive candidate for clinical application of antimicrobial peptides. In continuation with the previous studies, molecular dynamics (MD) simulations were carried out for further investigating the effect of ambient environments (temperature and bacterial membrane) on C3 dynamics. Our results reveal that C3 has higher flexibility, larger intensity of motion, and more relevant secondary structural changes at 363 K to adapt the high temperature and maintain its antimicrobial activity, comparison with it at 293 K; when C3 molecule associates with the bacterial membrane, it slightly fluctuates and undergoes local conformational changes; in summary, C3 molecule demonstrates stable conformations under these environments. Furthermore, MD results analysis show that the hydrophobic contacts, the hydrogen bonds, and disulfide bonds in the peptide are responsible for maintaining its stable conformation. In addition, our simulation shows that C3 peptides can make anionic lipids clustered in the bacterial membrane; it means that positive charges and pronounced regional cationic charge density of C3 are most key factors for its antimicrobial activity.

Keywords: chimera C3; molecular dynamics; ambient environments; structural adjustments; electrostatic interaction

1. Introduction

In a multitude of organisms, antimicrobial peptides (APMs) secreted by epithelial cells actively take part in the first line of preventing the pathogens from penetrating into the host cell (Hancock & Lehrer, 1998; Izadpanah & Gallo, 2005; Jenssen, Hamill, & Hancock, 2006; Spudy, Sonnichsen, Waetzig, Grotzinger, & Jung, 2012). In the APMs family, defensins are characterized by six cysteine residues distributed in the form of three intramolecular disulfide bonds and richness in cationic amino acids (Ganz et al., 1985; Selsted, Brown, DeLange, Harwig, & Lehrer, 1985; Selsted, Harwig, Granz, Schilling, & Lehrer, 1985; Selsted & Ouellette, 2005; Tang et al., 1999). Also defensins can be fallen into three subfamilies, referred to as α-, β-, and θ-defensins which distinguish in localization and pairing of six cysteine residues (Ganz, 2003; Selsted et al., 1996).

Within a few past years, the structures of human β-defensins 2 (HBD2) and human β-defensins 3 (HBD3) were solved by Hoover et al. (2000) and Harder, Bartels, Christophers and Schröder (2001), respectively. Although the two peptides are very similar in structure and protein sequences (Hoover et al., 2000; Schibli et al., 2002), but remarkable divergence in antimicrobial activity against Escherichia coli and Staphylococcus aureus had been verified. Both of them display the equal activity toward E. coli, nonetheless, HBD3 kills S. aureus with four to eight times more efficiency compared to HBD2; it was also elucidated that both the N-terminal region of HBD3 and the middle region of HBD2 are the important determinants for their antimicrobial activities (Jung et al., 2011; Spudy et al., 2012). Chen, Yoon, Leong and Kwak (2013) theoretically researched the interactions between phosphatidylglycerol (POPG) membrane and dimers of HBD2 and HBD3 and emphasized the importance of cationicity and oligomeric ability for antimicrobial behavior of HBDs (Chen et al., 2013).

Recently, Jung et al. designed six chimeric peptides of HBD2 and HBD3 based on their three-dimensional structures and tested the antimicrobial activity of these chimeras. Herein, as a prototypical chimera, C3 (shown in Figure 1(a)) which contains N-terminal of HBD3 and middle region of HBD2 exhibits a strongly enhanced antimicrobial activity against E. coli and S. aureus in the presence of high salt concentration, in comparison with their parental peptides (Jung et al., 2011). The positive charges play an essential role in antimicrobial activity for defensin family. More important for antimicrobial activity is the charge distribution or regional charge density. It was reported that the regional cationic charge density on C3

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molecule’s surface was more pronounced than HBD2 and HBD3; this character made C3 more active than its parental molecules (Spudy et al., 2012) and attracted onto the bacterial membrane surface via electrostatic interaction with the negative-charges of phospholipids (Jung et al., 2011; Spudy et al., 2012). These interactions had already resulted in the disruption or perforation of the bacterial membrane and eventually led to membrane permeabilization and cell death in defensins (Harder et al., 2001; Hoover et al., 2000; Wu et al., 2003). Meanwhile, structural features of C3 are different from those of HBD2 and HBD3 in several aspects, i.e. the orientation and shorten length of N-terminal α-helix, the replacement of the third β-sheet by a less-defined loop, and a far more striking amphipathic trait (Spudy et al., 2012). Besides, Spudy et al. also highlighted that C3 possessed thermal stability by stepwise increasing the temperature from 20 to 90 °C and underwent conformational changes in the presence of model bacterial membrane (it is composed of pure POPG lipids) (Spudy et al., 2012). However, how the structure or conformation is influenced by these conditions and the antimicrobial mechanism still remain ambiguous. Thereby, it is necessary to further investigate the effect of these conditions on the dynamics of C3 molecule and characterize the mechanism.

Here, in our contribution, dynamics behaviors of C3 are studied in order to dissect in detail the effect of ambient environments (temperature and bacterial membrane) and explore the intrinsic factors for retaining stable structure of the peptide by the means of molecular dynamics (MD) simulations. The results show that C3 has higher flexibility, more flexible regions, and more local conformational adjustments at 363 K than at 293 K. The root mean square inner product (RMSIP) reflects that C3 has a large degree in similarity of motion modes at different temperatures. It indicates that the overall stable conformation is retained from low to high temperature. Meanwhile, C3 is stabilized and only has minute conformational changes in bacterial membrane environment; the existence of the bacterial membrane restricts the internal motions of C3, thus favors the stable conformation of C3. Our simulation also shows that a certain number of C3 peptides can make anionic lipids clustered in the bacterial membrane by electrostatic interaction, which connotes that positive charges and regional charge density of C3 are most pivotal factors for its antimicrobial activity. Furthermore, the vital aspects relating to hydrogen bonds (HBs), disulfide bonds, hydrophobic contacts in the peptide are advantageous to maintain conformational stability under the two conditions. In conclusion, stable conformation in ambient environments is a precondition for further functional research and makes C3 become a more promising candidate with improved potency for clinical application and manageability with reference to storage and fabrication.

2. Materials and methods
2.1. Simulation system
We choose the crystal structure from the Protein Data Bank (PDB ID: 2LXO) (Spudy et al., 2012) as the simulation model. The remaining force field parameters including bond, angle, torsional angle, and VdW radii were taken from the GROMOS96 53a6 force field (Billeter et al., 1996). Two systems under 293 and 363 K were solvated in a bath of simple point charge (SPC) (Berendsen, Grigera, & Straatsma, 1987) water molecules and placed in a cubic box of 6.22824 × 6.22824 × 6.22824 nm³ in size with 7667 water molecules, respectively. Sodium and chloride ions were subsequently added to systems so as to keep charge neutrality. Besides, one and six C3 peptides were randomly placed above the x–y plane of the bacterial membrane (Murzyn & Pasenkiewicz-Gierula, 1999), respectively; the shortest distance between peptides and membrane is 5.5 nm. The bacterial membrane was composed of phosphatidylethanolamine (POPE) and POPG lipids with a molar rate of 3:1. The system totaled 512 lipids, split and distributed evenly between the two leaflets. Then two systems containing one and six C3 peptides
were solvated in a bath of SPC water molecules and placed in box of 12.24272 × 12.09021 × 11.80000 nm² in size with 37,597 and 36,540 water molecules, respectively. In order to keep charge neutrality, sodium ions were also added.

2.2. **MD simulations**

The simulations were run in the NPT (i.e. constant number of atoms N, pressure P and temperature T) ensemble utilizing GROMACS 4.5.3 package (Abraham & Gready, 2011; Hess, Kutzner, van der Spoel, & Lindahl, 2008) with GROMOS96 53a6 parameters for force field. Four molecular simulations were put forward, short for C3-293, C3-363, C3-m1, and C3-m6. C3-293 and C3-363 represented that one C3 molecule was simulated in water solution at 293 and 363 K, respectively; C3-m1 and C3-m6 represented that one and six C3-molecules were, respectively, simulated in membrane environment at room temperature. For C3-293 and C3-363, the temperatures were coupled by the Nosé–Hoover thermostat (Hoover, 1985; Hunenberger, 2005; Nosé, 1984) at 293 and 363 K with a coupling time of \( T = 0.1 \) ps, respectively; the pressures were kept constant at 1 bar by a compressibility of \( 4.5 \times 10^{-5} \text{ bar}^{-1} \) and coupling to a Berendsen barostat with \( p = 1.0 \) bar (Berendsen, Postma, van Gunsteren, DiNola, & Haak, 1984). For C3-m1 and C3-m6, the temperatures of protein, lipid, and solvent (waters and ions) were separately coupled by the Nosé–Hoover thermostat at 300 K with a coupling time of \( T = 0.1 \) ps; the pressures were semi-isotropically coupled at 1 bar by the Parrinello–Rahman barostat, via a coupling constant of \( p = 1.0 \) ps and a compressibility of \( 4.5 \times 10^{-5} \text{ bar}^{-1} \) (Aoki & Yonezawa, 1992; Parrinello & Rahman, 1981). All bonds were constrained using the linear constraint solver for molecular simulations algorithm (Hess, Bekker, Berendsen, & Fraaije, 1997). An integration step of 2 fs was harnessed. Long-range electrostatic interactions were calculated by particle mesh Ewald summation (Darden, York, & Pedersen, 1993; Essmann et al., 1995) with a Fourier spacing of 0.16 Å with a fourth-order cubic interpolation. After 1000 steps of steepest descent energy minimization removing all short contacts, the MD simulations were followed. The solvent and ions were equilibrated during a .5 ns MD simulation with the protein heavy atoms subjected to harmonically constraints with a force field constant of \( k = 1000 \text{ kJ mol}^{-1} \text{ nm}^{-2} \). Then, an additional 1.0 ns simulation was carried out for equilibration of all systems, respectively. Eventually, MD simulations of C3-293, C3-363, and C3-m1 were run for 100 ns; simulation of C3-m6 was performed for 120 ns. The coordinates of all the atoms and velocities were stored at every picosecond for further analysis.

2.3. **Principal components analysis (PCA)**

We utilized PCA (van Aalten, Findlay, Amadei, & Berendsen, 1995; van Aalten et al., 1999) to verify the principal modes of motion of C3 molecule in MD ensemble. To begin with, the covariance is established on the basis of three-dimensional positional fluctuations of Ca atoms from their ensemble average position. Subsequently, diagonalization of the covariance matrix forms a series of eigenvectors and their corresponding eigenvalues which together denote the direction and amplitude of motion. The first eigenvector mostly contributes the total fluctuation of system through ranking eigenvectors with decreasing the eigenvalues. The structures extracted from respective ensemble can also be projected onto each eigenvector and shifted back into Cartesian coordinates. As a result, the collective motions can provide valuable insight into the function of the protein. Moreover, the similar degree of correlated motion of the protein at different temperatures was evaluated by comparing the principal subspace (first ten eigenvectors) of each trajectory using the RMSIP (Amadei et al., 1999) between the first 10 eigenvectors. The value of RMSIP close to zero elucidates that the correlated motions of the protein under different conditions are totally different. On the contrary, the value equal to one means that the correlated motions are exactly the same. The g covar and g anaeig commands were used to calculate the two-dimensional projection. Then the two-dimensional projection was converted to probability distribution file; the free energy landscape was plotted from the probability distribution file.

3. **Results and discussion**

3.1. **The effect of temperature on C3 dynamics**

3.1.1. **Simulation equilibrium and overall structural stability**

The root mean square deviations (RMSD) of the protein backbones calculated after a mass weighted superposition with the initiate structure are shown in Figure 2(a). It can be clearly seen that equilibrations are detected for C3-293, C3-363 after 20 and 10 ns simulations, respectively. The RMSD value increases rapidly from .2 to .8 nm during the unstable stage for different temperatures. Among the equilibrium phase, RMSD values of both C3-293 and C3-363 fluctuate between .3 and .9 nm and more fluctuations are monitored for C3-363. These reflect that there may be some obvious conformational changes for protein while heating the system. In addition, as the sufficient reflections of structural flexibility, root mean square fluctuations (RMSF) (shown in Figure 2(b)) of Ca of protein are compared during the equilibrations. Generally speaking, the flexibility
changing in C3-293 is very similar with that in C3-363. Moreover, the protein has higher RMSF values at 363 K overall essentially caused by thermal stress. Obviously, the β-sheets composing the central core region are less susceptible and show low mobility, which is in agreement with the characteristic of peptide containing β-sheets (Spudy et al., 2012). The higher RMSF values are corresponded to the structurally less-defined regions including LpNt, α-helix, Lp1, and LpCt. The relative small motion range of Lp2 is favorable to the stabilized features of β-sheets. Besides, the reference structures of b-factors elucidate the larger flexibility of N-terminal from HBD3 for both temperatures (Figure 3). Regarding the solvent accessible surface area (SASA) in Figure 2(c), both curves show more fluctuations in the first 25 ns. Since then, they are converged and slightly fluctuated around 40 nm². The average SASA values (listed in Table 1) are nearly unchanged; it means that almost no obvious changes occurring at the protein surface. Taking the radius of gyration (Rg) into consideration, the Rg values display different fluctuant phases for C3-293 and C3-363, respectively (as Figure 2(d)); at 363 K, the maximum value of 1.4 nm is evidence of higher flexibility of the whole protein. Nonetheless, the average values are much closer to each other (shown in Table 1), which reveal the similar structural compactness.

In addition, hydrogen bonds (HBs) were calculated using a maximum cut-off distance between the donor and acceptor as .35 nm and the donor-hydrogen-acceptor angle to be ≥ 120°. The number of HBs varies unnoticeably during entire simulation time and is centered on 15 (shown in Figure 4(a) and (b)). Then the average
numbers of HBs are computed and listed in Table 1. Seven HBs are formed in the crystal structure of C3 (Figure 5(a)). By contrast, the growing trends of the average number of HBs are visible in comparison with that of the crystal structure so as to keep the whole structure stable. Then, the HBs of C3-293 and C3-363 are also depicted in Figure 5(b) and (c), respectively; the important residues involved HBs are distributed throughout the protein. As shown in Table 2, the existence time expressed in percentage which is calculated based on the total number of times of each HB divided by the total number of frames are computed. It is suggested that the HBs with the existence time below 50% (considerably short-lived) can be considered unstable. Clearly, two HBs formed by Ile30-Cys40 and Gly31-Cys40 are maintained during more than 90% time, and may give important contribution to the stability of the two β-sheets. Additionally, most of the stable HBs are positioned in loop regions, which restrict the motion intensity of these flexible regions to a certain degree. Although an increment in the number of time averaged HBs, a lower number of long-lived (existence time over 50%) HBs are formed at 363 K than at 293 K on account of increased flexibility. But to some extent, the exceptional short-lived HBs may also actually contribute to the stability of the peptide at 363 K. By contrast, there is one more HB existed between Gly31 and Cys40 for C3-293, which is further beneficial to keep the two β-sheets stable. Thereby, the analysis reveals that the HB networks favor the structural stability of C3 under different temperatures.

It had been proposed that the role of disulfide bonds in defensins was to bolster antimicrobial activity by allowing the peptides to remain stable in a diverse range of environments (Northfield et al., 2014). During the courses of the two simulations, the distance of sulfur atoms of Cys23 and Cys41 sometimes became larger, while the atoms were still linked for most of time. But the structure is not substantially influenced due to the persistence of the rest two disulfide bonds. Aside from the above analysis, the gains in the total number of Cα contacts and unique hydrophobic atoms (such as C, Cα, Cβ, Cγ, Cδ, Cε, Cζ, Cη) contacts with short distance (0.4–0.8 nm) cut-off are calculated and listed in Table S1. It is noted that the number of contacts increase with the increasing distance cut-off. The Cα contacts gained at 363 K for all cut-offs are larger than at 293 K. This observation is supported by the greater flexibility attained at 363 K. Similarly, a larger number of hydrophobic contacts are detected at high temperature. It was established that a higher number of hydrophobic contacts stabilized the protein (Pace et al., 2011). Thereby, hydrophobic contacts may be another major factor for the stability in our present study. Taken together, HBs network, hydrophobic interactions, and disulfide bonds not only maintain the protein scaffold but also prevent its unfolding or denaturation at high temperature.

3.1.2. Conformational changes under different temperatures

Subsequently, the conformational changes of C3-293 and C3-363 are comparatively analyzed by the free energy landscape and secondary structure evolution. Firstly, different free energy changes are observed shown in Figure 6. C3-293 and C3-363 have one and two conformational regions, respectively. At the same time, we compare their metastable conformational states (Stock, Jain, & Riccardi, 2012) in the conformational regions with the crystal structure. A few differences are found in the regions such as residues 1–10 of C3-293, residues 1–18 of C3-363, but most of residues and overall structure have no obvious differences. Then, secondary structure changes with the proceeding of simulations are evaluated and depicted in Figure 7(a) and (b). It can be
clearly seen that the original β-sheets appear to be remarkably stable for both the two cases. At 293 K, α-helix is absolutely absent while it is maintained intermittently at 363 K. It seems that the α-helix is more adaptive to high temperature. Furthermore, residues 9–12 in Lp1 of C3-363 enter into β-sheets. In Figure 1(b) and (c), the average structures of C3-293 and C3-363 further confirm the secondary structural changes. The residues involved conformational changes are from the N-terminal part of HBD3. These results suggest that even if C3 has structural deviations and local adjustments, its overall structure does not undergo remarkable changes.

The protein internal motions are characterized by PCA analysis with the first four eigenvectors and their eigenvalues are listed in Table 3. The Ca collective motions along with the first four eigenvectors are depicted in Figure 8. At 293 K, the main motion mode is observed in the regions, including LpNt, α-helix, and residues 12–17 in Lp1 for the first eigenvector; the motion is mainly concentrated on LpNt for the next three eigenvectors. However, for C3-363, the overall intensity of these movements is particularly strong especially in the three aforementioned regions; this result is in accordance with the above RMSF analysis. Although the overall motions have been continually reduced for the next three eigenvectors, the protein still retains stronger motions, especially residues 12–17 and LpNt for the third and fourth eigenvectors, respectively. Besides, the motion similarity is assessed utilizing the first ten eigenvectors through calculating the RMSIP as an indicator of the similar in the motion direction. Here, the RMSIP between the two different temperatures is .8195. The result indicates the degree of similarity in motions is high and the overall stable structure is not severely

Figure 4. The number of protein HBs as a function of simulation time: (a) C3-293; (b) C3-363; (c) C3-m1.
fluenced by temperatures. Simultaneously, the trajectories of C3-293 and C3-363 are projected onto the first two eigenvectors demonstrated in Figure 9. Larger conformational space is explored at 363 K and has a good overlay with that of 239 K. The result is consistent with the above RMSIP and flexibility analysis. Based on the above analysis, we can conclude that C3 possesses thermal stability which is coincided with the previous experimental results (Spudy et al., 2012).

![Figure 5: Snapshots of protein average structures under different conditions: (a) C3-crystal, (b) C3-293, (c) C3-363, and (d) C3-m1. Important residues involved HBs are labeled. All the molecules are shown in stick representations. The carbon, hydrogen, nitrogen, oxygen, and sulfur atoms are colored in cyan, white, blue, red, and orange, respectively.](image)

**Table 2. The existence time of each HB with the evolution of simulation time.**

| Species<sup>a</sup> (C3-293) | Occupied<sup>b</sup> (%) | Species<sup>a</sup> (C3-363) | Occupied<sup>b</sup> (%) | Species<sup>a</sup> (C3-m1) | Occupied<sup>b</sup> (%) |
|-----------------------------|---------------------------|-----------------------------|---------------------------|-----------------------------|---------------------------|
| L6 H<sup>•••</sup>O N4      | 18.24                     | L6 H<sup>•••</sup>O N4      | 41.85                     | L6 H<sup>•••</sup>O N4      | 22.18                     |
| C11 H<sup>•••</sup>O K8     | 0.36                      | T5 HG1<sup>•••</sup>O T5    | 11.38                     | T5 G1H<sup>•••</sup>O T5    | 16.81                     |
| R14 H<sup>•••</sup>O R12    | 70.80                     | T5 HG1<sup>•••</sup>OH Y10   | 24                        | T5 H<sup>•••</sup>O Q7      | 0.03                      |
| H19 H<sup>•••</sup>O K39    | 20.31                     | C11 H<sup>•••</sup>O K8     | 5.028                     | C11 H<sup>•••</sup>O K8     | 5.4                       |
| H19 HD1<sup>•••</sup>O P20  | 100                       | H19 HD1<sup>•••</sup>O K39   | 37.26                     | R14 H<sup>•••</sup>O R12    | 40.58                     |
| F22 H<sup>•••</sup>O P20    | 99.99                     | H19 HD1<sup>•••</sup>O F22   | 38.96                     | R12 H<sup>•••</sup>O Y10    | 91.13                     |
| Y27 H<sup>•••</sup>O R25    | 94.93                     | F22 H<sup>•••</sup>O P20     | 33.69                     | H19 HD1<sup>•••</sup>O P20   | 3.57                      |
| I30 H<sup>•••</sup>O C40    | 96.99                     | Y27 H<sup>•••</sup>O R25     | 96.11                     | H19 HD1<sup>•••</sup>O F22   | 7.13                      |
| G31 H<sup>•••</sup>O C40    | 98.77                     | I30 H<sup>•••</sup>O C40     | 99.75                     | F22 H<sup>•••</sup>O P20     | 47.65                     |
| C40 H<sup>•••</sup>O G31    | 99.82                     | C40 H<sup>•••</sup>O G31     | 90.46                     | Y27 H<sup>•••</sup>O R25     | 57.12                     |
| K39 HZ2<sup>••</sup>O Y27   | 28                        | G37 H<sup>•••</sup>O L35     | 25.14                     | K42 H<sup>•••</sup>O K28     | 82.36                     |
|                            |                           | K39 HZ2<sup>••</sup>OH Y27   | 13.57                     | I30 H<sup>•••</sup>O C40     | 99.99                     |

<sup>a</sup>The hydrogens and acceptors from the key residues forming HBs.
<sup>b</sup>The existence time of HBs throughout the simulation process.

influenced by temperatures. Simultaneously, the trajectories of C3-293 and C3-363 are projected onto the first two eigenvectors demonstrated in Figure 9. Larger conformational space is explored at 363 K and has a good overlay with that of 239 K. The result
3.2. The effect of bacterial membrane environment on the C3 dynamics

3.2.1. Simulation equilibrium and overall structural stability

As shown in Figure 2(a), RMSD value keeps constant after 10 ns, which indicates the slight fluctuation of C3 peptide. The peaks in the RMSF values correspond to the structurally less-defined regions including LpNt, Lp1, and LpCt. The β-sheets show low mobility. Less flexibility is shown in α-helix region, which confirms the reported investigations (Spudy et al., 2012) that this part became stable when exposed to lipid environment. The higher flexibility of C3 N-terminal may be helpful to mediate the pathogen specificity against S. aureus and E. coli inferred from the research by Jung et al. (2011). The SASA value is slightly bounded around 38 nm² in the entire duration of simulation, which means almost no remarkable changes occurring at the protein surface. Regarding the Rg value, it marginally fluctuates between 1.05 and 1.10 nm, which implies structural compactness and no apparent volume expansions. Moreover, the average values of SASA and Rg are listed in Table 1. All the basic features discussed above reveal the stability of C3 is also high in the bacterial membrane environment.

The number of HBs keeps mainly constant during the entire simulation (as Figure 4(c)); 16 HBs are formed and the essential residues involved in HBs are widespread in the whole protein (Table 1 and Figure 5(d)). As shown in Table 2, two long-lived HBs formed by Ile30-Cys40 and Gly31-Cys40 favor the stability of the two β-sheets. The rest HBs are mostly positioned in loop regions, which limit the motion amplitude of these flexible regions to a certain degree. The weak flexibility also causes a number of contacts of Cα atoms and hydrophobic atoms (Table S1). These hydrophobic contacts may be conducive to the overall stable conformation. The existence of the intrinsic disulfide bonds is another important factor for maintaining the protein stability. Therefore, HBs network, hydrophobic contacts, and disulfide bonds also lend strong support to the stable conformation of C3 peptide on the bacterial membrane.

3.2.2. C3 conformational changes on the bacterial membrane

To begin with, four conformational regions are shown in the free energy landscape (Figure 6(e)); their metastable conformational states in the conformational regions are

Figure 6. The free energy landscapes (in kJ mol⁻¹) of (a) C3-293, (c) C3-363, and (e) C3-m1. The energy is plotted in the plane of the first two principal components Vec1 and Vec2. And the overlap of the metastable conformational states: 1 (yellow), 2 (green), 3 (orange), 4 (cyan), 5 (hotpink), 6 (limon), and 7 (gray) and the corresponding crystal structure (pink). The structural change regions are marked in blue.
compared with the crystal structure (Figure 6(f)) in order to locate conformational changes. Although a few diversities are found in residues 12–17 and 33–38, the majority of residues and overall structures do not undergo obvious changes. Then, secondary structure evolution is plotted in Figure 7(c). The appearance of a short β-sheet (residues 6–7) instead of α-helix for C3-m1 somewhat contradicts the investigation (Spudy et al., 2012), where a higher α-helical content of C3 was induced by the interaction with the bacterial membrane. Furthermore, residues 10–12 and 19–21 in Lp1 enter into β-sheets; the regions including residues 6–7 and 10–12 are from the N-terminal of HBD3 and another region including residues 19–21 is from the middle part of HBD2. The structural changes can also be seen in Figure 1(d).

In order to further obtain information about flexibility and conformation, the collective motions along with the first four eigenvectors are shown in Figure 8. The motion regions mainly focus on LpNt, residues 12–17, 20–28 in Lp1 and LpCt, which is well correlated with flexibility analysis from RMSF result. Moreover, obvious movement is detected for residues 20–28 in the second eigenvector; these residues are from the middle part of HBD2 and have higher flexibility shown in RMSF result. Based on the above analysis, it is quite notable that the less-defined region (it is from the N-terminal of HBD3) between Cys11 and Cys18 which replaces the third β-sheet of HBD2 and HBD3 has markedly flexible motions. This finding validates the previous studies which indicated the flexibility in the non-cysteine form was increased; and Spudy et al. considered that the enhanced antimicrobial activity of C3 appeared to originate from the increased flexibility of this specific region (Liu et al., 2008; Spudy et al., 2012). Further, the smallest conformational space shown in Figure 9 also verifies that the protein is stabilized in the membrane environment.

3.2.3. Interaction between C3 and bacterial membrane

Finally, we analyze the interaction between C3 molecule and the bacterial membrane. As shown in Figure 10(a), the changes of the distance between basic residues of C3 and POPG bilayer are not obvious; it means that C3 can associate with lipid surface through electrostatic interaction.

### Table 3. The eigenvalues (nm) of the first five eigenvectors for C3-293, C3-363, and C3-m1.

| Species  | C3-293 | C3-363 | C3-m1  |
|----------|--------|--------|--------|
| Eigenvector 1 | 2.131200 | 2.849482 | .361436 |
| Eigenvector 2 | 1.089800 | 1.304380 | .198939 |
| Eigenvector 3 | .309391 | .755786 | .0836092 |
| Eigenvector 4 | .109801 | .228082 | .068852 |
| Eigenvector 5 | .00723084 | .167563 | .0306001 |

Figure 7. The evolution of the secondary structural elements of the protein in the MD simulations: (a) C3-293; (b) C3-363; (c) C3-m1.
Figure 10(b) shows that the side chains of most basic residues are much closer to the membrane surface; it results in the absorbance of C3 onto the membrane surface. Besides, the side chains of residue 6–7 and 10–12 even have direct contacts with negative-charged surface of POPG; these important interactions probably lead to conformational changes of the two aforementioned regions. Another region (residues 19–21) mainly composed of hydrophobic residues is far away from lipids and exposed in water solution. Slight conformational adjustment is adopted probably to avoid the disruption from hydrophilic solution. And these conformational changes in membrane environment are presumably correlated with the better association between peptide and lipids. During the simulation, the bacterial membrane is almost intact (data not shown). And neither the phenomenon that the peptide not only promotes the segregation and aggregation of one lipid from another but ultimately accelerates the membrane disruption (Epand, Maloy, Ramamoorthy, & Epand, 2010) nor the disruption of membrane by electrostatic interaction (Garcia et al., 2001; Hoover et al., 2000) is observed; it is likely due to the lower negative charges from only one peptide and the limited simulation time. For preliminarily confirming the C3 action mode on the bacterial membrane, a simulation containing six C3 peptides interacting with the bacterial membrane was run. The result shows that the POPG lipids are prone to be gradually aggregated to the space directly beneath the C3 peptides by electrostatic interaction; the peptides can not form oligomers and are represented by monomers (Figure 10(c)). In Figure 11, the deuterium order parameter (Marrink & Berendsen, 1994) of POPE/POPG membrane for sn-1 chain was calculated. Of particular note
is that the order parameter in the course of simulating process is very similar with and even a little higher than that of initial stage of simulation (for the first 1 ns). This elucidates that the mixed membrane still remains rigid and phase transition from liquid crystal to gel phase does not occur. The headgroup density of POPG is subsequently analyzed. In Figure 12, there is a remarkable increase in density during the whole process in one leaflet (Z > 6 nm), which is occupied by the six peptides, in comparison with the density value of the initial membrane structure. It indicates that the reorganization and clustering of POPG may appear while it connecting with C3 peptides. Besides, the thickness (Allen, Lemkul, & Bevan, 2008) of POPE/POPG displays a gradually increased trend (in Figure 13). This probably stems from the aggregation of POPG to the region below C3 peptides.

As analyzed above, it is predicted that the regions composed of pure POPG and POPE lipids will gradually be formed and some defects will appear on the border of the two kinds of regions; as a result, the membrane leakage will emerge. The predictive mechanism discussed above implies that the two classic antimicrobial mechanisms (Shai, 1999), known as “barrel-stave model” and “carpet-model”, are not suitable for C3 peptides. Further attempts are being made to thoroughly investigate the antimicrobial mechanism of C3 in our group.

To sum up, C3 shows lower mobility and stable conformational changes in bacterial membrane environment;
the overall stable structure is retained. The electrostatic interactions between cationic peptides and anionic lipids tend to disrupt the bacterial membrane.

4. Conclusions
In this work, an attempt is made to delineate the impact of ambient environments on the structure of chimera C3 through investigating the dynamic aspects at different temperatures and in bacterial membrane using MD simulations. The main purpose is concentrated on elucidating the structural dynamic properties, identifying the determinants for maintaining the overall structure under the influence of ambient environments, and studying the interaction between peptide and bacterial membrane.

For comparative analysis, at high temperature, the protein has higher flexibility, larger intensity of motion and more relevant secondary structural changes. Meanwhile, the increase in HBs, hydrophobic contacts, and persistence of disulfide bonds give an explanation for the relative thermal stability of C3 compared to 293 K. Additionally, the high degree of similarity in motions at two temperatures is supported by the RMSIP result.

Differently, when C3 is bound to the membrane surface, a stabilized state is reflected by the common features including RMSD, RMSF, SASA, and Rg which show no obvious fluctuations. The key factors including HBs, hydrophobic contacts, and persistence of disulfide bonds are helpful to retain the global structure of C3. Apart from those, the electrostatic interactions between C3 molecule and lipid bilayer may also contribute to limit the fluctuation of protein, thus favor the stable conformation of C3. The tiny intensity of motion and some local conformational adjustments of peptide are presumed to well associate with bacterial membrane. When six C3 peptides contact with bacterial membrane, the action mode of C3 is observed: POPG lipids are inclined to be gradually aggregated to the space directly beneath the C3 peptides and the membrane will be separated into two regions that contain pure POPE and POPG lipids; the possible antimicrobial mechanism of C3 peptides is different from the two classic ones.
To sum up, with the help of explicit MD simulations, the effect of environments on the structural dynamics of chimera C3 is well studied and some important factors and essential residues are considered important to retain the stable global structure. Thereby, we can conclude that C3 is stable under different environments. Furthermore, our work provides detailed insight into the poor understood structural features of chimera C3 at the atomic level, and should contribute to further understanding of the structures and dynamics of other chimeras; it also establishes a basis to absolutely comprehend the antimicrobial mechanism of C3 peptides.

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