Published in final edited form as:

J Phys Chem B. 2011 February 10; 115(5): 1188–1198. doi:10.1021/jp107763b.

Molecular Dynamics Studies of Transportan 10 (Tp10) Interacting with a POPC Lipid Bilayer

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Abstract

We performed a series of molecular dynamics simulations to study the nature of interactions between transportan 10 (tp10) and a zwitterionic POPC bilayer. Tp10 is an amphipathic cell-penetrating peptide with a net positive charge of +5 and is known to adopt an α -helical secondary structure on the surface of POPC membranes. The study showed that tp10 preferentially binds to the membrane surface with its hydrophobic side facing the hydrophobic lipid core. Such orientation allows Lys residues, with positively charged long side chains, to stay in the polar environment during the insertion process. The simulations revealed that the Lys–phosphate salt bridge is a key factor in determining the orientation of the peptide in the interfacial region as well as in stabilizing the peptide-membrane interaction. The electrostatic attraction between Lys and phosphate groups is also believed to be the main bottleneck for the translocation of tp10 across the membrane.

I. INTRODUCTION

Cell-penetrating peptides (CPPs), short peptides of less than 30 amino acids, have gained significant attention in recent years because of their ability to transport cargo across cell membranes.¹ Therefore, CPPs have been considered excellent candidates for drug delivering vehicles. Most CPPs are highly cationic in neutral solutions; the best-known examples are penetratin,^{2,3} a 16-residue peptide from the Antennapedia homeodomain of *Drosophila*, the HIV-1 TAT peptide,^{4–7} and homopolypeptides of Lys or Arg, of which nonarginine (Arg9) is particularly active. With the exception of penetratin, none of these CPPs is α -helical even on the membrane surface. TAT and heptaarginine-tryptophan (Acetyl-R7W-amide) have no regular structure in water or on membranes,^{6–8} and the same probably applies to nonarginine. The sequence of penetratin contains hydrophilic and hydrophobic amino acids, but the helix formed is not amphipathic.^{3,6,9} Although many different classes of CPPs have been studied, the mechanism of membrane translocation of CPPs is still debated and not fully understood,¹ but it appears that, at some point, CPPs must cross bilayers passively.

Transportan 10 (tp10, AGYLLGKINLKALAALAKKIL-amide) is one of the so-called chimeric CPPs constructed by linking together, through an additional Lys residue, a 6-residue segment from the neuropeptide galanin and the 14-residue sequence of mastoparan, from the wasp *Vespula lewisii*.^{10,11} Transportan,¹² which is a longer version of tp10, has been used to transport even large molecules, such as DNA or proteins, into cells. Neither tp10 nor transportan binds to the galanin receptor,¹⁰ suggesting a direct interaction with the lipid bilayer. Unlike the typical arginine-rich CPPs mentioned above, transportan¹³ and the related mastoparans^{14–17} are amphipathic and all form α -helices on membranes. The helix

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content of tp10 on zwitterionic lipid bilayers is about 60%.¹⁸ The amphipathic α -helix of tp10, however, does not exhibit such a clean segregation of hydrophilic and hydrophobic residues as normally occurs in antimicrobial peptides.¹⁹

A recent investigation of the kinetics of interaction of the tp10 with model membranes was consistent with peptide permeation through the lipid bilayer.²⁰ A model was proposed in which accumulation of tp10 on the membrane surface creates a mass imbalance across the bilayer; this imbalance produces a perturbation that enables tp10 monomers to translocate to the inner surface of the membrane. The peptides remain at all times oriented parallel to the membrane surface, and sink deeper into the bilayer as the perturbation occurs, very much like in the “sinking raft” model proposed for the cytolytic peptide δ -lysin.²¹⁻²² In the case of tp10, however, the kinetics were consistent with the involvement of only monomers, without any peptide oligomerization on the membrane surface.

Although there is a lot to be learned from kinetic studies, it is not straightforward to investigate experimentally the behavior of an individual peptide penetrating the membrane. Complementary to experimental findings, molecular dynamics (MD) simulations have been used with great success to provide detailed structural and dynamical information on peptide-membrane systems. Most MD simulations, however, have focused on antimicrobial peptides and only a handful of simulations have been performed with CPPs.²³⁻²⁵ For example, Herce and Garcia²⁴ reported that HIV-1 TAT peptide induces pore formation at very high concentration or at elevated temperature, and the peptides translocate across a dioleoylphosphatidyl choline (DOPC) bilayer, in the same way as the antimicrobial peptide magainin-2.²⁶ The simulations indicated that Arg residues play a major role in peptide binding and destabilizing bilayer, leading to eventual pore formation. On the contrary, pore formation was not observed for TAT peptide, or penetratin, in more recent simulations by Mark and coworkers.²⁵ It was argued that the relevant time scale for the translocation of CPPs should be much longer than that of antimicrobial peptides, which renders the translocation process extremely rare for CPPs. Therefore, it is not expected to observe spontaneous translocation events in MD simulations within a few 100 ns. In fact, pore formation and peptide translocation may not be necessary all together for TAT peptide to disrupt the membrane. Instead, a process similar to micropinocytosis was proposed as a possible mechanism for TAT peptides to be encapsulated by the membrane.²⁵

Here, we present the results of molecular dynamics simulations performed in an attempt to better understand the nature of interaction between tp10 and a zwitterionic membrane, and to assess the plausibility of the sinking raft model. As implied by the two conflicting reports discussed above for the HIV-1 TAT peptide, the action of CPPs in a membrane environment has not been fully understood and could vary widely depending on the type of CPPs. Unlike penetratin and TAT peptides, tp10 is a lysine-rich CPP and an amphipathic α -helical peptide on the surface of zwitterionic membranes,²⁰ which resembles many antimicrobial peptides, including the well-known magainin-2. It has been suggested that arginine-rich CPPs, such as TAT peptide, cross cell membrane very efficiently because of their capability to form bidentate hydrogen bondings with the lipid headgroups, which is responsible for the stable peptide binding to the membrane⁸ and efficient pore formation.²⁷ It was also argued that random coil structure of TAT peptide facilitates translocation process because it reduces the hydrophobic surface.⁸ In this sense, lysine is often regarded as less effective a residue for cell translocation,^{8,27-29} but more detailed molecular level understanding of lysine-rich CPPs is clearly needed. Therefore, examining the interaction between lipid and, the lysine-rich, amphipathic tp10 at the molecular level, through MD simulations may provide new insight into the mechanism of membrane disruption for amphipathic CPPs in general.

II. COMPUTATIONAL DETAILS

All molecular dynamics simulations reported in this work were performed with the Gromacs 4.0 package.³⁰ The GROMACS (ffgm) force field was used to describe the peptides, whereas the lipids were modeled with the parameters developed by Berger *et al.*³¹ The α -helical structure of tp10 was built with the C-terminus capped with an amide group (see Fig. 1 for the helical wheel diagram). The bilayer of 128 POPC molecules were taken from Tieleman.³² All simulations were conducted under periodic boundary conditions at constant temperature (298, 353 and 453 K) and pressure (1 atm). Temperature control was achieved by using Nose-Hoover thermostat with 0.1 ps coupling constant, whereas semi-isotropic Parrinello-Rahman scheme was used to maintain the pressure with the coupling constant of 2.0 ps. Electrostatic interactions were treated by particle-mesh-Ewald (PME) method (0.12 nm grid spacing) with a real-space cut-off of 1.0 nm. The Van der Waals interactions were also cut-off at 1.0 nm. Bond lengths were constrained via the LINCS algorithm.³³ A time step of 2 fs was employed, with the center of mass removal in every step, and neighbor list update every 10 steps. All simulations were conducted under electrostatically neutral environment with an appropriate number of Cl^- counterions.

For the peptide-only simulation, a tp10 molecule with α -helical initial structure was placed in a rectangular box and solvated by 5900 SPC34 water molecules. Since tp10 has a net positive charge of +5, counterions were added to neutralize the system. The system was first energy minimized for 2000 steps, followed by 1 ns position-restrained simulation for water molecules to settle around the peptide. The constraints were then removed and a simulation was performed for 50 ns under NPT (constant pressure and temperature) condition.

The peptide-bilayer systems were set up by placing one or two tp10 molecules at least 12 Å above the bilayer. Initial orientations of peptides were parallel to the bilayer surface, with either the hydrophobic or the hydrophilic side of tp10 facing the bilayer surface. Since the time scale (~200 ns) of the simulations performed in the present work is expected to be too short to observe spontaneous folding of unstructured peptides into an α -helical form on the membrane surface, the peptides had α -helical structure from the beginning. The peptide-bilayer systems were solvated by ~7400 SPC water molecules with counterions. We also performed a series of simulations starting with a tp10 molecule completely inserted into the bilayer core, either vertically or horizontally. The starting configurations of such peptide-bilayer systems were obtained by using InflateGRO method of Tieleman.³⁵ Each system went through 2000 step energy minimization and 1–2 ns position-restrained simulation before the production run.

We note in passing that the simulation protocol used in the present work is consistent with the recently suggested parameter sets³⁶ based on the convergence tests of lipid simulations. Although a patch of 128 POPC molecules is relatively small and is expected to be constrained by periodic boundary condition to some degree, it has been shown to reproduce a reasonably accurate area per lipid molecule with the present simulation protocol,³⁷ which is of particular importance in lipid simulations.³⁶ The same lipid bilayer size was also used in recent MD simulations of antimicrobial or cell penetrating peptides interacting with a lipid bilayer.^{24,26,38,39}

III. RESULTS

A. Transportan 10 (tp10) in water

We performed a 50 ns simulation of α -helical tp10 in water to investigate the stability of tp10 in an aqueous environment. Experimentally, it has been found that tp10 is largely unstructured in water, but the peptides spontaneously fold into α -helical structure upon

binding to a POPC bilayer.^{18,20,40} Since there is no available NMR or X-ray crystallographic data, the initial configuration was constructed assuming α -helical secondary structure.

Figure 2 shows the structure of tp10 after a 50 ns NPT simulation. The peptide, which had initially α -helical conformation, is partially unfolded and only the middle part of the peptide retains helical structure. The system was found to be quite stable for the first 10 ns, but started to lose helicity thereafter. The partially helical structure shown in Fig. 2 was established after 30 ns and was stable for the rest of the simulation. Although 50 ns is too short to reach the true equilibrium structure, the present results suggest that the α -helical structure of tp10 is not stable in solution and the helical content of tp10 continues to decrease as the simulation proceeds, which is consistent with the experimental finding that tp10 is not helical in solution.^{18,20,40} It also needs to be pointed out that the partially helical structure shown in Fig. 2 is not necessarily α -helical. The secondary structure profile of tp10 (as implemented in DSSP,⁴¹ not shown here) in solution shows significant contributions from a π -helical structure. A π -helical structure is often found in molecular dynamics simulations of antimicrobial peptides,^{42–45} even though it is not considered to be a typical structure found in peptides and proteins. In some cases, the occurrence of π -helix in MD simulations was attributed to a force field artifact.⁴⁶ However, the identification of π -helical structure may depend on the secondary structure definition and the existence of π -helix could be more prevalent than typically assumed.^{47–49}

B. Tp10-POPC bilayer association

As outlined in Sec. II, simulations of tp10-POPC bilayer system start with one or two α -helical peptides in water positioned parallel to the bilayer surface. In order to provide enough space for the peptides to freely rotate and adjust their orientations, peptides were initially located 12–15 Å above the bilayer surface. This initial peptide-bilayer distance is small enough that most peptides retain helical conformation until they reach the surface of the bilayer and start to interact with the POPC headgroups as helical peptides.

Tp10 is an amphipathic peptide with distinct hydrophilic and hydrophobic faces (See Fig. 1). Therefore, it is important to understand the role of the amphipathic nature of the peptide upon binding to the bilayer surface. To investigate the preferential orientation of the tp10 molecule bound to the surface of POPC bilayer, we initially positioned each peptide with either the hydrophobic or hydrophilic side facing the bilayer and monitored the change in peptide orientation when the peptides approach the lipid headgroup region. We performed a total of six simulations, four simulations with two tp10, and two simulations with one tp10 molecule. The peptide concentration used in our previous experimental studies²⁰ of tp10 with POPC is close to the situation with two tp10 and 128 POPC molecules (P/L = 1/64). We also studied the influence of temperature by performing simulations at three different temperatures, 298, 323, and 423 K. An overview of all the simulations performed in the present work is given in Table I. The six simulations with peptides placed above the bilayer surface are labeled as S1–S6. Most analyses reported in Sec. IIIB and C were performed for S3 and S4, with two tp10 molecules.

Figure 3 shows the z -coordinates of tp10 molecules in S3 and S4, measured from the center of the lipid bilayer as a function of simulation time. Note that in S3, the hydrophilic sides (Lys groups) of both peptides were initially oriented toward the bilayer, whereas, in S4, the two peptides have opposite orientations. In both simulations, the peptides rapidly approach the bilayer surface as the distances between the headgroups (phosphorous in Fig. 3) and the peptides are reduced to a couple of Å within 20 ns. It takes roughly 25–50 ns for the peptides to pass the headgroup region. This time scale is somewhat longer than what was observed for some antimicrobial peptides interacting with POPC,^{43,44} but it is about the

same as for penetratin.²³ Two out of four peptides shown in Fig. 3 eventually reach the lipid carbonyl group within 200 ns. One exception to the trend described above is the second peptide in S4 (P2 in Fig. 3), which stayed in the aqueous phase much longer. The average insertion depth, along with the maximum depth, obtained from the last 50 ns of simulations S1–S6 are included in Table I. As a representative example, we show snapshots from S3 in Fig. 4 for the initial association of tp10 with the bilayer surface.

In most of our simulations reported in Table I (S1–S6), the peptides approach the interface at an angle (sometimes perpendicularly) and either the C- or N-terminus makes the initial contact. However, all peptides eventually become parallel to the bilayer surface before they are inserted into the bilayer. This behavior we observed for tp10 is somewhat different from that of penetratin, which is a 16-residue cell-penetrating peptide with four Lys and three Arg. It was shown from an MD simulation that penetratin remains parallel to the membrane with the *hydrophilic* residues facing the bilayer during initial approach.²³ However, with tp10, the opposite is usually observed: The peptides approach the bilayer with their *hydrophobic* side facing the membrane. As shown in Fig. 4(a), P2 (the peptide on the right) has the hydrophilic side facing the bilayer initially, but changes its orientation while approaching the lipid bilayer so that the *hydrophobic* side faces the bilayer. As shown in Fig. 4(c), all the Lys side chains from P2 are pointing toward the water phase after 20 ns.

To characterize the peptide orientation without ambiguity, the *z*-coordinates of the center of mass of Lys and Leu side chains were calculated as a function of simulation time. These two residues, Lys and Leu, are representatives of hydrophilic and hydrophobic residues, respectively, and they are located at the opposite faces of the helix (Fig. 1). The results are shown in Fig. 5 for S3 as an example, which allows us to clearly distinguish the difference in orientation between two peptides when they are approaching the bilayer surface. We first observe that, for P1, the insertion of Lys7 and Lys11 is much faster than the Leu side chains. The first contact between Lys7/Lys11 side chains and the headgroups occurs around 10 ns, followed by Lys19-headgroup interaction at 25 ns. In case of Leu side chains, Leu16 passes the headgroup region around 20 ns and continues to drift toward the carbonyl group of the lipids. But other Leu side chains do not even pass the headgroup region until 50 ns. On the other hand, P2 shows a markedly different behavior. As shown in Fig. 5(d), four out of six Leu side chains pass or are near the lipid headgroup region within 10 ns, whereas Lys side chains do not pass the headgroup region until 40 ns. This clearly indicates that the orientation of P2 has been changed while it approaches the interface from the water phase. The orientations of the peptides are generally maintained while they insert into the lipid (see Sec. IIID), although the amphipathic nature of peptides becomes less clear due to the loss of helicity. It should also be noted that Fig. 5 shows the center-of-mass of only the side chain of each residue. For the Lys residue, which has a very long and flexible side chain, the location of the backbone can be several angstroms away from the side chain. This, along with the loss of helicity, makes it less obvious to identify the peptide orientation later in the simulation, when the peptides bound to the membrane have significant interactions with lipid headgroups (see Sec. IIID). For example, the side chain of Lys19 in Fig. 5(c) seems to be inserted deeper into the lipid core than those of Leu4 and Leu16 in Fig. 5(d) as the simulations approach the end. However, the backbone of Lys19 is actually further away from the lipid core compared to those of Leu4 and Leu16 in S3.

The change in peptide orientation from the hydrophilic side to the hydrophobic side facing the bilayer is not uncommon. As shown in Table I, eight out of ten peptides in S1–S6 have the hydrophobic side directed toward the bilayer when binding, even though only three peptides had such orientation initially. The first contact point between the peptide and the bilayer does not seem to have any correlation with the final orientations of the peptides, given that roughly half the peptides from S1–S6 have the N-terminus as the first contact

point, and the other half have C-terminus as the first contact point. The fact that each simulation produces different peptide behavior implies that our simulations are not fully converged, although the simulation protocol used in the present work, including the length of simulation, is a typical choice for simulations of peptides interacting with a lipid bilayer. However, at the same time, it suggests that running multiple MD trajectories is crucial to understand the average behavior of a peptide in a lipid bilayer environment. In typical MD studies of large biomolecular systems, a very limited number of simulations is usually carried out. But, it is possible that a single simulation, regardless of its duration, can lead to a wrong conclusion for the mechanism of peptide binding to a lipid bilayer. Although ten peptides is definitely too small a number to draw any statistically meaningful conclusion, the present results show that tp10 clearly has a tendency to orient itself so that the hydrophobic side of the peptide makes the initial contact with the POPC headgroup. As mentioned above, this is not always the case for cell-penetrating peptides, and penetratin seems to prefer to have the hydrophilic residues interacting with the lipid surface first when it approaches the interfacial region.²³ Comparing the insertion depths of four peptides from S3 and S4, the peptides with hydrophobic side directed toward the bilayer surface (P2 in S3 and P1 in S4) have higher insertion depth than the other (P1 in S3). P2 in S4 stayed in the water phase for the first 100 ns and, as a result, the insertion depth is significantly smaller, even though it is the hydrophobic side that eventually makes the first contact with the lipid headgroup region.

There are a few factors that might lead to the observed orientation of tp10. First, the Lys side chains (hydrophilic) should prefer to face the water phase, rather than the headgroups. In addition, the choline groups, which occupy the outermost layer of the membrane, are positively charged, and, therefore, should repel the Lys residues with the same charge. As will be shown in the next section, the peptide orientation with the hydrophobic side facing the membrane also allows the peptides to avoid unfavorable electrostatic interaction on initial contact, and penetrate deeply into the bilayer interior.

C. Peptide penetration and bilayer disordering

The preferred orientation of tp10 discussed above is generally maintained while the peptide inserts into the POPC bilayer. As a representative example, the snapshots taken from S4 are shown in Fig. 6, which describes the initial insertion process of the peptide. Just like simulation S3 (Fig. 4), one of the tp10 molecules (P1) quickly turned around and oriented itself with the hydrophobic side facing the bilayer before it is inserted. In S4, the other peptide (P2) remains in the water phase vertically for more than 100 ns and, therefore, it is highly unlikely that there exists any cooperative effect between two peptides during the initial insertion of P1. In fact, dimerization of tp10 was not observed in any of our simulations. Although the insertion process described below focuses on P1 in S4, we observed similar behavior in general for the most of the peptides reported in Table I.

During insertion, the Lys side chains of P1 maintain their orientations and point toward the water phase. However, as the peptide passes through the headgroup region, the NH_3^+ of Lys residues become tightly bound to the headgroup PO_4^- . We analyzed the $\text{NH}_3^+ \cdots \text{PO}_4^-$ interaction by computing the minimum distance between two groups as a function of time (Fig. 7). Fig. 7(c) shows that three out of four Lys NH_3^+ of P1 in S4 form salt bridges with PO_4^- around $t = 25$ ns. This is about the time when P1 passes the top layer of the interface and positions itself under the headgroup region (Fig. 3(b)). As indicated by very little fluctuation in $\text{NH}_3^+ - \text{PO}_4^-$ distance, the salt bridge between NH_3^+ and PO_4^- is very stable. Once it is formed, it is rarely broken for the remainder of each simulation.

The snapshots in Fig. 6 also indicate that the PO_4^- groups of POPC molecules around the peptide are dragged into the bilayer interior at the beginning of the insertion process (Fig. 6(b)), which causes significant disturbance to the membrane structure. This occurs even before the salt bridges between NH_3^+ and PO_4^- are formed. The peptides are simply pushing the headgroup toward the bilayer core. The integrity of the lipid headgroup is somewhat restored later, once P1 is located below the headgroup region (Fig. 6(c)). However, all Lys side chains are anchored on PO_4^- and no significant change in the insertion depth was observed after 160 ns. This is mainly because of the strikingly stable salt bridge formed between Lys and the headgroup PO_4^- . Note that two Lys residues at the C-terminus of P1 (Lys18, Lys19) in S4 established the salt bridges with PO_4^- early in the simulation and maintained strong interaction until the end of simulation. The center-of-mass of other two Lys (Lys7, Lys11), located in the middle part of the peptide, are found around the lipid tail after $t = 120$ ns. However, because the charged NH_3^+ group is at the end of long Lys side chain, it can easily grab one of the headgroup PO_4^- a few Å away and maintain a stable salt bridge. Such peptide-lipid configuration effectively allows the charged side chains (Lys) to interact with the headgroups while the hydrophobic side of peptide is deeply inserted and interacts with the lipid tails. It is worthwhile to note that the above-mentioned structure of tp10 inserted into the POPC bilayer bears close resemblance to the “snorkeling” hypothesis proposed for lysine-rich peptides.^{50–52} In fact, it was suggested that snorkeling leads to higher lipid affinity of amphipathic peptides.⁵⁰

Upon peptide insertion, the helicities of peptides change rather significantly and the peptides sample a variety of structures. A typical evolution of peptide secondary structure is shown in Fig. 8 (a) as an example. This is for P1 in S4, corresponding to the snapshots in Fig. 6. The peptide maintains α -helical structure for the most part during the initial stage of insertion. The second half of the peptide (C-terminus side) remains helical for more than 70 ns, even though the insertion starts at about $t = 25$ ns from the N-terminus side. However, between $t = 70$ and 170 ns when the insertion is actively taking place, the helicity of the peptide is mostly lost except in the middle part of the peptide. Toward the end of simulation, the N-terminus side is deeply inserted into the bilayer and the helicity of the inserted part seems to be partially restored. Loss of helicity during peptide-lipid simulations is common. For instance, recent MD simulation of magainin-2 interacting with a DPPC bilayer showed significant loss of helicity, while the peptides were inserting into the bilayer, and forming disordered toroidal pores.²⁶ In our simulations, the average helicity varies from peptide to peptide as shown in Fig. 8 (c) and (d), with lower and higher helicity, respectively, than in Fig. 8(a). Experimentally, the average helicity of tp10 on the POPC bilayer is about 60%,¹⁸ which is comparable with the results of present MD simulations given that the temperature in MD simulations is higher. In one case, we observed a π -helix throughout the simulation (Fig. 8(b)). Interestingly, this particular peptide has the “wrong” orientation (hydrophilic side facing the bilayer), and its insertion depth is smaller than those of other peptides. It should be noted that a π -helix was also observed during the simulation of tp10 in solution (Sec. IIIA). This suggests that the interaction between POPC bilayer and tp10 with its hydrophobic side facing the surface leads to further stabilization of the α -helical structure.

The effect of peptide insertion on the structure of the lipid bilayer was also examined by the density profile across the bilayer normal. Fig. 9 shows the density profiles of various parts in the system obtained from simulations S4, S5 and S6: water, phosphorous, carbonyl oxygens, lipid tails, and peptide. The density profiles were calculated from the last 40–50 ns of each simulation, during which the locations of the peptides do not change significantly. As demonstrated above, in most cases, tp10 molecules occupy the region a few Å below the headgroup, close to the carbonyl groups of the lipid. This orientation allows the hydrophobic side of tp10 to face the lipid core, whereas the hydrophilic Lys residues interact with the

headgroup by stretching their long side chains toward the phosphate. This picture is consistent with the density profile shown in Fig. 9 (a).

Although the membrane thickness and the area per lipid molecule hardly change (within 2% of that of pure POPC) upon peptide insertion, the headgroup region of the bilayer is significantly perturbed. The POPC molecules around the peptides are dragged toward the core, causing local bilayer thinning. (Figs. 6 and 9(a)). This is demonstrated by the noticeable bump on the density distribution of the phosphorous and carbonyl oxygens of the upper leaflet, which interacts with the peptides. The overall shape of the density distribution of the upper leaflet is also much broader than that of lower leaflet. Previous MD simulations of antimicrobial peptides interacting with lipid bilayers showed mixed observations, depending on the peptides. For example, Shepherd *et al.* reported a significant decrease in lipid thickness (up to 16% decrease in 30 ns) when a synthetic and a natural antimicrobial peptide interacts with a POPC bilayer.⁴³ On the other hand, Leontiadou *et al.* found no evidence of lipid thinning in the simulation of magainin-2 interacting with a DPPC bilayer.²⁶

D. Effect of simulation temperature

The results described above indicate that spontaneous translocation of tp10 across the POPC bilayer is not likely to happen within the time frame of the current simulation (~200 ns), with the peptide concentration (P/L = 1/128 or 1/64) studied in this work. In order to speed up conformational sampling, a simulation was performed at elevated temperature (423K, S5). A simulation at lower temperature (298K, S6) was also performed, where the temperature is more comparable to the experimental condition.

As expected, the peptide penetration into the bilayer core is, indeed, accelerated at higher temperature as shown in the density profile (Fig. 9(c)). One of the peptides is now located below the carbonyl group after 200 ns of simulation, and the density profile of this peptide is completely overlapping with that of the lipid tails. The peptide is 12Å away from the center of bilayer, which is a much smaller distance than observed in S3 and S4 at 323 K (See Table I for insertion depths). The bilayer thickness is also considerably smaller at higher temperature, and the peak-to-peak distance of phosphorous density is reduced by more than 2Å compared to that of S3 (Fig. 9(a)). The impact of temperature is also evident when the temperature is lowered (Fig. 9(b)). At 298K, neither peptide even passed the headgroup region after 200 ns. One of the peptides simply sits on the very top layer of the bilayer and stays in such configuration for the entire duration of the simulation. The bilayer thickness also increases by 2Å compared to that of S3 (Fig. 9(a)) and the α -helical nature of the peptide was maintained much better in S6 than in S3/S4.

Interestingly, no drastic change in the way the peptides penetrate into the bilayer was observed at the higher or lower temperature, at the current peptide concentration. Bilayer thinning probably contributed to the accelerated insertion of peptide at higher temperature, but a complete insertion of tp10 into the bilayer core was not observed even at 423K. The barrier to be overcome for the tp10 translocation across a POPC bilayer is expected to be in the order of 10–20 kcal/mol,²⁰ based on the White-Wimley hydrophobicity scale.⁵³ This implies that 200 ns is still too short to observe spontaneous translocation of tp10 at $T = 423\text{K}$. In fact, the time scale for the translocation of tp10 across the POPC bilayer was measured to be on the order of minutes at room temperature.²⁰ Although the elevated temperature enhanced the insertion of peptide, it also destroyed the helicities of both peptides. At 423K, both peptides in S5 completely lose their helicities and become random coils with a few turns while interacting with the headgroups of POPC. In MD simulations of antimicrobial peptides, it is often observed that the insertion and translocation of peptides occur with unfolded conformations.^{54,55} Therefore, at higher temperature, we may be able

to capture the same physics in a shorter period of time that is involved in the translocation of unfolded tp10 at lower temperature. Experimentally, however, it is not entirely clear yet whether a helical form of tp10 is required for the translocation of peptide, although peptides are known to have a helical conformation at the interface.^{18,20}

E. Inserted mode of tp10

As described above, none of the simulations with the peptides initially placed in the water phase (S1-S6), led to a configuration with tp10 completely inserted into the bilayer core. This is due to the strong electrostatic interactions between Lys residues and lipid headgroups (in particular, $\text{NH}_3^+ \cdots \text{PO}_4^-$ interaction). After 200 ns simulations of tp10-POPC system (P/L = 1/64, or 1/128), the majority of peptides is located between the headgroup and the acyl chain with the hydrophobic residues facing the bilayer core, and the Lys side chains are stretched toward the interface to associate with the phosphate groups. In order for tp10 to translocate across the POPC bilayer, as suggested in experiment,²⁰ Lys-phosphate salt bridges should be broken (stochastic events). Some Lys-phosphate interactions are weaker than others and these salt bridges can be broken temporarily as shown in Fig. 7. However, the lengths of present simulations (200 ns) are simply too short to observe complete insertion, not to mention translocation.

In order to understand the stability and conformation of tp10 inside the POPC bilayer, we performed a few simulations with a tp10 already inserted into the bilayer core. Different orientations (vertical insertion and parallel insertion) and conformations (α -helix and random coil) of tp10 were studied (S7–S11 in Table I). All simulations were preceded by a few ns of position-restrained run so that water and POPC molecules are settled around the peptide. The simulation protocol used in these simulations is identical to that used in S1 and S2.

Figure 10 shows the snapshots taken at $t = 0$ (left column) and $t = 200$ ns (right column) from simulations S7, S8, S9 and S10 (from top to bottom). The conformations at $t = 0$ refer to the final configurations of position-restrained runs. When tp10 is inserted vertically across the bilayer (Fig. 10(a)), phosphate groups are attracted to the Lys side chain and protrude into the bilayer core during the position-restrained run. Water molecules follow the phosphate groups and surround the Lys side chains as well. Overall, the vertically inserted α -helical tp10 seems to be relatively stable inside of the POPC bilayer. No drastic change in the conformation was observed during a 200 ns NPT simulation, except that the helix becomes a little curved at the N-terminus. This configuration allows all Lys side chains to interact with the headgroups and the peptide to be strongly associated with both leaflets. Similar behavior was observed when a tp10 with random coil configuration was vertically inserted (Fig. 10(b)). In both cases, the bilayer integrity is mostly restored by the end of 200 ns simulation. However, given the time scale of our simulations, vertically inserted tp10 should be regarded as a metastable state at best. The peptide is most likely trapped in a local free energy minimum, which is caused by the need to break $\text{NH}_3^+ \cdots \text{PO}_4^-$ salt bridges on either side of the peptide. It is unlikely that the actual translocation process takes place with the peptide oriented vertically as it is contradictory to the mechanism of initial insertion process discussed in Sec. IIIB and C.

When a tp10 is inserted into the core of the POPC bilayer in a parallel orientation, noticeable changes occur during the simulation. In case of parallel insertion of a long random coil (Fig. 10(c)), two Lys side chains at the C-terminus are fully stretched at the end of a position-restrained run with significant intrusion of phosphate groups and water molecules. Again, this configuration prevents positively charged Lys groups from being exposed to the hydrophobic bilayer core. At the end of a 200 ns simulation, the peptide becomes more

compact, although spontaneous folding into a α -helical structure was not observed within our time scale. Thermodynamically, it is expected that the stabilization of the helical structure over the unfolded form in a hydrophobic environment is on the order of ~ 4 kcal/mol per residue.^{56,57} Therefore, the helical structure should be favored within the lipid bilayer, but the system seems to be kinetically trapped in the unfolded structure in simulation S9 at 323K. In fact, interconversions between unfolded and folded conformations of small peptides, such as for the WALP/DPPC system, were observed at elevated temperatures in recent molecular dynamics simulations.⁵⁵

In parallel insertion of the peptide, it is also found that penetration of phosphate groups into the bilayer core is more significant than what is seen in the vertical insertion case. This allows some water molecules to pass through the bilayer, although the extent of water transport across the bilayer was observed to be low. In fact, the configuration shown in Fig. 10(c) (right) resembles the disordered toroidal pore model proposed for the antimicrobial peptide magainin-2 in a DPPC bilayer.²⁶ In the case of magainin-2 in a DPPC bilayer, a stable toroidal pore was formed when the peptide density is larger than $P/L=32$. It was demonstrated that a high peptide density is needed to create a pore as well as to stabilize it. However, the highly disordered structure shown in Fig. 10(c) seems to be quite stable as well (for more than 100 ns), even though the tp10 concentration in our simulation is much lower than what was employed in the MD study of magainin-2.²⁶

In the case of a helical peptide inserted into the bilayer core in parallel (Fig. 10(d)), it is clear from the figure that the phosphate groups and water molecules were attracted only to the hydrophilic face of tp10 during the position-restrained run. As in the random coil case, significant penetration of phosphate groups was observed, which leads to local thinning of the bilayer. Note that only the Lys side chain closest to the C-terminus (Lys19) is pointing toward the upper leaflet and the remaining Lys side chains toward the lower leaflet at the beginning of S10 (Fig. 10(d), left). During a 200 ns MD simulation, the peptide moves to the lower leaflet quickly and the integrity of the lower leaflet is restored to some degree. However, due to the strong interaction between Lys and the headgroup phosphate, the Lys side chain close to the C-terminus (Lys19) is still anchored on the upper leaflet (Fig. 10(d), right), which prevents the peptide from being completely shifted toward the bottom leaflet. Since there are more Lys-phosphate interactions at the bottom leaflet, the peptide is being pulled in that direction, but even a single Lys-phosphate bond at the upper leaflet is strong enough to maintain the current configuration, with significant bilayer disruption at the upper leaflet, for more than 100 ns. As observed in the case of vertical insertion, helical content of the peptide parallel to the membrane surface hardly changed within the POPC bilayer.

To investigate the effect of peptide orientation at the bilayer core, we performed an additional simulation (S11), with a tp10 fully inserted into the core in a parallel orientation to the surface (Fig. 11). In this simulation, the peptide was initially oriented in such a way that no Lys side chain points toward the bottom leaflet. This peptide orientation should be compared to that of S10, where one Lys side chain points toward the upper leaflet and the remaining three Lys chains toward the bottom leaflet. As shown in Fig. 11, only the phosphate groups at the upper leaflet are affected and penetrate into the core after the position restrained simulation. Once the MD simulation started, the peptide turned around slightly so that all Lys side chains point toward the upper leaflet. Then, the entire peptide moved toward the upper leaflet. Eventually, all Lys side chains are connected to the phosphate groups at the upper leaflet, and the hydrophobic side of the peptide points to the bilayer core. The peptide is now located right below the headgroups with the Lys side chains stretched toward the headgroups. Such orientation and location of the peptide are consistent with the initial insertion process observed for the majority of peptides in simulations S1–S6: *i.e.* the peptides approaching the bilayer surface from the aqueous phase, with the

hydrophobic side facing the bilayer, and the Lys side chains pointing away from the bilayer. The insertion process is quite slow as described earlier, but after a 200 ns simulation, the average location of the peptide is below the headgroup region with the long Lys chains coupled to the phosphate groups (Fig. 6). Although the insertion depths (Table I) found in simulation S1–S6 are not as great as seen in Fig. 11, due to the limited simulation length, these simulations strongly suggest that peptide insertion shown in Fig. 6, from the aqueous phase into the POPC bilayer core, continues until the peptide reaches the point depicted in Fig. 11(d) (right).

IV. DISCUSSION

In the present work, we performed a series of MD simulations to investigate the nature of the interactions of tp10, an α -helical amphipathic CPP, with POPC, a zwitterionic lipid. Multiple trajectories obtained under similar conditions showed that tp10 preferentially binds to the POPC membrane in a parallel orientation, with its hydrophobic side facing the bilayer core. Although the insertion process is rather slow, the hydrophobic side of the peptide is inserted fairly deeply, even below the carbonyl group in some cases, within a 200 ns simulation time. The hydrophilic side, which contains five positively charged Lys residues, with long and flexible side chains, points toward the water phase, in general, during the insertion process. It is understandable that such orientation is energetically favorable, because it allows the hydrophobic face to be in a nonpolar environment, while the charged groups remain in a polar environment, interacting with the lipid headgroups. Therefore, the amphipathic nature of tp10 is essential for the efficient peptide binding. This observation clearly distinguishes tp10 from other classes of CPPs, such as arginine-rich HIV-1 TAT peptide, which adopts mostly an unstructured conformation at the interfacial region to facilitate peptide binding and insertion.⁸ Although we were not able to observe complete insertion of tp10, due to the limited length of MD simulations, we expect that the peptide insertion proceeds until all Lys residues pass the headgroup regions and the peptide resembles the structure described in Fig. 11(d). It should be noted that the peptide orientation and insertion process observed for tp10 is consistent with the “snorkeling” effect of lysine-rich peptides.⁵⁰

The MD simulations also strongly suggest that the salt bridges formed between the Lys residues of tp10 and the phosphate groups of the lipids play an important role in peptide binding, and especially in determining its orientation in the interfacial region of the membrane. The importance of Arg-phosphate salt bridges was recently emphasized in connection with the structure of the HIV-1 TAT peptide, whose structure on the membrane interface determined by NMR.⁸ In tp10, because the Lys residues are distributed along the sequence on the same side of the peptide, a parallel orientation to the membrane surface is favored, in which the Lys side chains establish salt bridges with the lipid headgroup phosphates, while the hydrophobic side chains penetrate significantly into the bilayer core. In fact, short polylysines, which are not amphipathic, do not easily penetrate the polar headgroup region,⁵⁸ and the peptide-lipid interaction is predominantly electrostatic.^{58,59} The prevalence of Lys–phosphate salt-bridges in the simulations suggests that the orientation of amphipathic peptides on membranes may be determined by the distribution of basic residues, Lys and Arg, along the peptide sequence. For example, if Lys residues were clustered at one end, the peptide may be prone to adopt a tilted orientation relative to the bilayer normal.

The peptide orientation, parallel to the membrane, was a feature of the sinking raft model proposed to describe the translocation mechanism of tp10,²⁰ and is consistent with the present MD simulations. In the MD simulations, however, no translocation is observed. Experimentally, tp10 is not very efficient in membrane permeabilization, which is believed

to happen concomitant with translocation.²⁰ Therefore, it is perhaps not surprising that tp10 translocation is not observed in 200 ns MD simulations. The expected free energy barrier of translocation is on the order of 10–20 kcal/mol for tp10 according to the White-Wimley hydrophobicity scale.⁵³ It is possible to induce more significant peptide penetration or membrane disruption by further increasing either peptide density or temperature.^{24–26} However, this would make it more difficult to compare the results from MD simulations and those from experiments.

Although translocation was not observed, we may speculate on the possible translocation mechanism of tp10 based on the results of MD simulations with tp10 initially placed in the membrane hydrophobic interior. After the initial insertion process (Fig. 4 and 6), the peptide is expected to be located right below the headgroup as depicted in Fig. 11(d). Further insertion of tp10 requires breaking of tightly bound Lys-phosphate interaction, which is energetically unfavorable. Therefore, the important step for tp10 to translocate across the membrane is probably the breaking of the salt bridges between Lys residues and phosphate groups. This is a stochastic process initiated by the mass imbalance across the bilayer, the time scale of which is closely tied to the free energy of peptide translocation. Once the Lys side chains become free, they should be connected to the opposite side of the membrane and establish new Lys-phosphate interactions (Fig. 10(d)). As shown in Fig. 10(c) and (d), the membrane disordering can be significant upon peptide translocation, with water and phosphate groups deeply penetrating into the bilayer core. In fact, the bilayer perturbation proposed in the sinking raft model is reminiscent of the structures observed in these MD simulations, which resembles a disordered toroidal pore.²⁶ However, the size of pore and the extent of membrane disruption are much smaller in the present MD simulations, due to the lower peptide density.

Acknowledgments

This work was supported in part by NIH grant GM072507 (PFA and AP) and a Cahill Award from UNCW (HSL).

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FIG. 1.
Helical wheel diagram of tp10. Each residue is categorized according to the following color scheme: dark gray (hydrophobic), light gray (hydrophilic and neutral), purple (aromatic), and blue (Lys)

FIG. 2. Final conformation of tp10 in water after 50 ns NPT simulation at 323K. The same coloring scheme was used as in Fig. 1 The section of peptide with α -helical structure is overlapped with ribbons.

FIG. 3.

The z -coordinates of the center of mass of two peptides (red for P1 and blue for P2), the phosphorous atoms (black) in the headgroup and the carbonyl oxygens (brown) in the tail obtained from (a) simulation S3 and (b) S4 (see Table I). The center of the lipid bilayer was considered to be $z = 0$. In S3, both peptides have the *hydrophilic* side facing the bilayer initially, whereas, in S4, they have opposite orientations with the *hydrophobic* side of P₂ facing the bilayer.

FIG. 4.

Snapshots representing the initial association of tp10 molecules with the POPC bilayer surface. They are taken from S3 with two tp10 molecules: (a) Initial conformation of the system, (b) first contact of tp10 with the bilayer interface, (c) snapshot after 21 ns. Both peptides are fully engaged in the interaction with the bilayer headgroup region. Small spheres represent the phosphorous atoms and the gray lines are for the rest of POPC bilayer. Water molecules are removed for clarity.

FIG. 5.

The z -coordinates of the center of mass for Lys side chains (K7 (red), K11 (green), K18 (blue) and K19 (magenta)) for (a) P1 (first peptide) and (c) P2 (second peptide) obtained from S3 are overlapped with that of phosphorous (black) and carbonyl oxygens (brown). The same quantities for Leu side chains (L4 (red), L5 (green), L10 (blue), L13 (magenta), L16 (orange), L21 (cyan)) are plotted in panel (b) and (d) for P1 and P2, respectively.

FIG. 6.

Snapshots representing the insertion of tp10 molecules into the POPC bilayer. They are taken from S4 with two tp10 molecules: (a) conformation after 4 ns, (b) after 30 ns, and (c) after 163 ns. Note that one of the peptides maintains a vertical orientation for more than 100 ns before it starts to interact with the headgroup region of the bilayer. Only the phosphorous atoms and peptides are included in the figure for clarity.

FIG. 7.

Minimal distance between NH_3^+ of Lys side chains and PO_4^- of lipid headgroup: (a) P1 in S3, (b) P2 in S3, (c) P1 in S4, and (d) P2 in S4. Color schemes are as follow: (black) K7, (red) K11, (green) K18, and (blue) K19.

FIG. 8. Evolution of secondary structure of tp10 as defined by DSSP:41 (a) P1 in S4, (b) P1 in S3, (c) P2 in S3, and (d) P1 in S1

FIG. 9.

Density profiles for various parts of the system as a function of the distance from the bilayer center ($z = 0$): (a) S3 (323K), (b) S6 (298K), and (c) S5 (423K). The peptides are placed above the upper leaflet ($z > 0$). Color schemes are as follow: (black) water, (red) phosphorous, (green) carbonyl oxygens, (blue) P1, (orange) P2, and (brown) lipid tail. For easier comparison, the density of carbonyl oxygens is multiplied by three and the densities of phosphorous atoms and two peptides by five.

FIG. 10. Snapshots representing a fully inserted tp10 molecule in the POPC bilayer. They are taken from simulations S7, S8, S9 and S10 (from top to bottom): (left column) Initial configuration after 2 ns position restrained simulation and (right column) final configuration after a 200 ns NPT simulation at 323 K.

FIG. 11.

Snapshots taken from S11 at (a) $t = 0$, (b) $t = 440$ ps, (c) $t = 13$ ns, and (d) $t = 200$ ns.

Initially, the α -helical peptide was located at the core of POPC bilayer in parallel orientation with respect to the bilayer surface. Lysine side chains were either pointing toward the upper leaflet or in parallel to the bilayer.

TABLE I

Overview of simulation set-up. For simulations S1–S6, the peptides are initially placed above the bilayer surface. Orientations of hydrophobic side chains with respect to the bilayer surface are denoted as P (parallel), U(up, facing away from the surface) and D (down, facing toward the surface). The average peptide distance from the bilayer core obtained from the last 50 ns of S1–S6 is shown in the last column. The numbers in parenthesis are the minimum distances. For simulations S7–S11, a peptide is pre-inserted into the POPC bilayer and placed at the core of bilayer. The orientation and structure of peptide are denoted by V (vertically inserted), Hx (α -helix), and Co (random coil).

simulation label	number of peptides	initial orientation	orientation on the membrane	simulation length (ns)	temperature (K)	insertion depth (nm)
S1	1	U	D	200	323	1.75(1.35)
S2	1	P	D	200	323	1.57(1.23)
S3	2	UU	UD	200	323	1.75(1.42),1.66(1.34)
S4	2	UD	DD	225	323	1.54(1.19),1.85(1.48)
S5	2	UD	DD	200	423	1.23(0.52),1.54(1.06)
S6	2	UD	DU	200	298	2.34(2.05),2.23(1.93)
S7	1	V-Hx	-	200	323	
S8	1	V-Co	-	200	323	
S9	1	P-Co	-	200	323	
S10	1	P-Hx	-	200	323	
S11	1	P-Hx	-	200	323	