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Transmembrane Pore Structures of β -hairpin Antimicrobial Peptides by All-Atom Simulations

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Abstract

Protegrin-1 is an 18-residue β -hairpin antimicrobial peptide (AMP) that has been suggested to form transmembrane β -barrels in biological membranes. However, alternative structures have also been proposed. Here, we performed multimicrosecond, all-atom molecular dynamics simulations of various protegrin-1 oligomers on the membrane surface and in transmembrane topologies. The membrane surface simulations indicated that protegrin dimers are stable, while trimers and tetramers break down. Tetrameric arcs remained stably inserted in lipid membranes, but the pore water was displaced by lipid molecules. Unsheared protegrin β -barrels opened into β -sheets that surrounded stable aqueous pores, whereas tilted barrels with sheared hydrogen bonding patterns were stable in most topologies. A third type of observed pore consisted of multiple small oligomers surrounding a small, partially lipidic pore. We also considered the β -hairpin AMP tachyplesin, which showed less tendency to oligomerize than protegrin: the octameric bundle resulted in small pores surrounded by 6 peptides as monomers and dimers, with some peptides returning to the membrane surface. The results imply that multiple configurations of protegrin oligomers may produce aqueous pores and illustrate the relationship between topology and putative steps in protegrin-1's pore formation. However, the long-term stability of these structures needs to be assessed further.

Graphical Abstract

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Introduction

Protegrin-1 (hereafter called protegrin¹) is the best-studied member of the β -hairpin family of antimicrobial peptides (AMPs), which are small, usually cationic peptides that provide defenses against several classes of microbial agents^{2,3}. Because AMPs permeabilize lipid bilayers *in vitro*^{4–6} and *in vivo*^{7–9}, the lethal event is thought to be disruption of bacterial membranes by either detergent-like membrane dissolution (i.e., the carpet mechanism;¹⁰) or formation of pores¹¹, which may vary from cylindrical (lined by peptides¹²) to toroidal (lined by lipid headgroups¹³) to intermediate forms¹⁴. AMPs' cationic charge imparts selectivity for negatively charged bacterial membranes¹⁵. Although alternative mechanisms of AMP action have been proposed^{16,17}, including clustering of anionic lipids¹⁸ and targeting of intracellular molecules, such as DNA^{19–21}, the overall evidence for AMP-induced membrane pores is strong (e.g.,²²).

Protegrin's interaction with biological membranes has been studied with a variety of biophysical techniques. Neutron diffraction experiments suggested toroidal pores²³, and oriented CD showed the transition between surface and inserted states²⁴. Solid-state NMR provided some evidence that protegrin may form β -barrels containing 8–10 monomers in NCCN parallel topology²⁵ (see *Topology* below). However, solution NMR in detergents showed NCCN antiparallel topology for protegrin-1²⁶, protegrin-3²⁷, and protegrin-5²⁸. The same solid-state NMR study also showed that 75% of the hairpins have homodimerized N and C strands in an anionic membrane, implying that the β -barrel state is the dominant component of a heterogeneous mixture. Polyethylene glycol molecules with hydrodynamic radii up to 9.4 Å allowed membrane permeabilization, whereas ones with radii of 10.5 Å blocked permeabilization²⁵; therefore, protegrin's pore diameter was below 21 Å. However, another study that investigated protegrin-induced dextran leakage suggested a pore diameter of at least 3–4 nm at high salt concentrations²⁹.

An atomic force microscopy study of protegrin-1 and other AMPs in large unilamellar vesicles and solid-supported phospholipid bilayers showed that protegrin acts as a line-active agent, lowering interfacial bilayer tensions and promoting changes in membrane morphology³⁰. A more recent study of 13 AMPs found that line activity correlates with an imperfectly amphipathic secondary structure that positions positively charged arginine sidechains near the membrane interface^{14,31,32}. A solid-state NMR study of protegrin-1 in 12-carbon 1,2-dilauroyl-sn-glycero-3-phosphatidylcholine (DLPC) bilayers observed a tilt angle of 55°, also noting that positively charged side chains tilted into close proximity to membrane lipid headgroups³³.

Protegrin has been the subject of numerous computational studies; for reviews, see³⁴ and³⁵. Simulations have been performed of monomers and dimers^{36–39} and β -barrel models of octamers^{40–43} and decamers⁴⁴. To illustrate potential mechanistic steps, previous all-atom simulations provided potentials of mean force of protegrin monomer insertion into a lipid bilayer⁴⁵ and dimerization in different environments⁴⁶.

Previous work from our laboratory included both all-atom and implicit membrane simulations. Our thermodynamic investigation of several octameric protegrin-1 β -barrel

topologies in pre-formed implicit pores indicated maximal favorability of the NCNC parallel topology⁴², which allows the more hydrophobic face of each peptide to contact the membrane. Other simulations showed that the NCNC parallel tetramer has intrinsic curvature compatible with tilted arcs⁴⁷, which may form or contribute to membrane pores. A 300-ns all-atom simulation of a NCNC parallel tetrameric arc showed a stable aqueous pore⁴⁷. Pre-formed β -barrels of several similar β -hairpin AMPs in NCNC parallel topology were found to be much less stable than those of protegrin⁴³. We also studied protegrin NCNC parallel β -barrels of 6–14 peptides: although the nonamer had the lowest energy, a range of pore sizes 7–13 peptides had relatively consistent favorability⁴⁸. Those results were consistent with the predictions of the solid-state NMR study²⁵, which suggested an octamer, but inconsistent with²⁹, which suggested a larger pore.

Despite the large amount of computational work on protegrin, both the final pore state and the pathway to that state remain uncertain. This paper describes all-atom molecular dynamics (MD) simulations of protegrin oligomers performed using the Anton 2 supercomputer. We also check the results against those for β -hairpin AMP tachyplesin⁴⁹, whose β -barrel formation was assessed as unstable (in contrast to that of protegrin) in a previous implicit membrane investigation⁴³. To examine whether and how protegrin oligomerizes on membrane surfaces en route to pore formation, we performed one group of simulations of protegrin oligomers on 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC):1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (POPG) bilayer surfaces. For these, we simulated dimers, trimers, and tetramers in the NCNC parallel, NCCN antiparallel, and mixed topologies (for a guide to topology, see the *Methods* below). To examine how oligomeric multiplicity, topology, and peptide configuration may be associated with pore formation and stability inside the membrane, we also studied several protegrin systems inside all-atom membranes: tetrameric and octameric protegrin bundles (see *Topology* below), octameric and decameric β -barrels, and one or two tetrameric arcs. The octameric β -barrel was simulated in NCNC parallel and mixed topologies; the single tetrameric arcs were in NCNC parallel, NCCN antiparallel, or mixed topologies; and the systems with two tetrameric arcs were NCNC parallel or NCCN antiparallel. We also generated β -barrels with sheared, tilted peptides (NCNC parallel, NCCN antiparallel, NCCN parallel, and mixed topologies) and a system with four tilted dimers arranged close together. The results provide insights into the putative steps of protegrin's pore formation and show their relationship with topology, yielding information on the likelihood of possible pore formation pathways.

Methods

Topology

Dimers of a β -hairpin can combine in six different topologies, depending on which β -strands (N or C) associate and the relative positioning of the turns. These topologies are NCNC, NCCN, and CNNC, each of which can have the peptides' turn region and ends oriented parallel or antiparallel to each other. In a closed β -barrel, the NCCN and CNNC topologies are identical. A characteristic that affects membrane binding ability is whether the topology allows the hydrophobic sides of all hairpins (i.e., the side opposite the disulfide bridges) to

point in the same direction. The NCNC parallel and NCCN antiparallel topologies satisfy this requirement, whereas NCCN parallel does not⁴². This study investigates up to decamers in NCNC parallel, NCCN parallel, NCCN antiparallel, and mixed topologies. The mixed topology was inspired by a recent crystallization study of a θ -defensin AMP, which found a mixed-topology trimer wherein one dimer had NCNC parallel topology and the other NCCN antiparallel topology (Fig. 1)⁵⁰.

We also distinguish our AMP systems inside lipid bilayers according to their initial hydrogen bonding pattern. “Transmembrane bundles” are peptide assemblies placed closely together but without initial hydrogen bonds imposed between peptides (Fig. 2a). The peptides in those systems could associate freely during all-atom MD simulations. Second, we simulated “unsheared” β -barrels (Fig. 2b), in which the peptides began all-atom MD with hydrogen bonds imposed in fully transmembrane orientation, flush with one another. In a third type of system, sheared β -barrels (Fig. 2c), the initially imposed β -sheet hydrogen bonds between peptides were shifted by two residues per dimer, tilting the β -barrel’s peptides. A hydrogen bonding shift of two residues was only employed once per dimer because preliminary implicit membrane MD simulations indicated that β -barrels with shearing every monomer were highly unstable. Further, our simulations indicated that units of unsheared dimers were stable, whereas sheared dimers dissociated rapidly (see *Results* below).

Preliminary Implicit Solvent Simulations

Preliminary implicit solvent simulations were conducted using Effective Energy Function 1 (EEF1;⁵¹) and Implicit Membrane Model 1 (IMM1;⁵²) using CHARMM version c41a1⁵³. The membranes with anionic pores were set up using mBuild, an in-house utility that constructs anionic implicit membranes at 5 different focusing levels with a final resolution of $0.5 \times 0.5 \times 0.5 \text{ \AA}^3$ ⁵⁴. The lipid bilayer’s dielectric properties were represented so that ϵ_{memb} , ϵ_{head} , and ϵ_{water} (the dielectric constant inside the membrane, the interfacial region, and water) were 2, 10, and 80, respectively⁵⁴. Headgroup width D was set to $3.0 \text{ \AA}$ to place the boundary around the phosphate group. The ion accessibility factor was set so that the ions, which were taken as monovalent with radius $2.0 \text{ \AA}$, could not penetrate below the phosphate groups. The positive and negative charge layers were separated by $1.0 \text{ \AA}$, hydrocarbon core thickness was set to $26 \text{ \AA}$, and area per lipid was set to $68 \text{ \AA}^2$. An anionic fraction of 30% was used, approximating a typical bacterial membrane⁵⁵. The membranes were embedded in cubic boxes filled with implicit 0.1-M aqueous salt solution.

A description of the simulated systems is presented in Table 1. The protegrin oligomers on the membrane surface and all of the transmembrane bundles were assembled geometrically from the coordinate files for protegrin-1 and tachyplesin, which were downloaded from the Protein Data Bank (PDB) (protegrin: 1PG1¹, sequence RGGRLCYCRRRFCVCVGR-NH₂; tachyplesin: 1WO0, sequence KWCFRVCYRGICYRRCR-NH₂ [a tachyplesin sequence without C-terminal amidation was also investigated]) and imported into CHARMM with all charged residues in ionization states corresponding with pH ~ 7 and all disulfide bonds patched. Copies of the peptides were oriented about the origin and then subjected to translations and rotations until they reached the appropriate configurations. The sheared

dimer and tetramer had the peptides offset by two residues along the hairpin axis to form an appropriately shifted hydrogen bonding pattern. The mixed topology for trimers and tetramers both consisted of one dimer in NCNC parallel and one dimer in NCCN antiparallel topology: NCNCCN(anti) (trimer), NCNCNCCN(anti) (tetramer). Then, without constraints in water, the peptides' energy was minimized using the adopted basis Newton–Raphson algorithm (used for all implicit minimizations for 300 steps), but no dynamics were applied. The configuration was then imported into the membrane builder feature of CHARMM-GUI⁵⁶ to add lipids, water molecules, and ions for all-atom simulations, as described below.

For the systems with β -barrels and one or two tetramer arcs, the production runs of which were run inside explicit membranes, the first step was implicit MD equilibration of a protegrin monomer in water. After importing the PDB file for protegrin-1 into CHARMM, the peptide's energy was minimized in water, followed by 200 ps of MD simulation with the Verlet integrator (time step: 2 fs, temperature: 298.5 K; used for all implicit simulations, following⁴³). Subsequent energy minimization then yielded the monomeric structure used for oligomerization.

We then constructed a single tetrameric arc or a decameric or octameric β -barrel by geometric transformation to space the copies approximately 10 Å apart and application of potential wells to enforce hydrogen bonding, followed by further equilibration MD of the tetramers in water. During equilibration, we imposed intermolecular β -sheet hydrogen bonds using CHARMM's NOE facility, following⁴³. To generate the tilted structure of the sheared β -barrels, the hydrogen bonding pattern was shifted by two residues only once for each pair of peptides, and the other half of the hydrogen bonding junctions remained as in an unsheared β -sheet.

After imposition of the β -sheet hydrogen bonds, further equilibration MD was run for 200 ps, followed by minimization. The NOE constraints were removed, and then the individual tetramers and octamers were placed with the more hydrophobic side facing an anionic implicit pore with radius $R_o = 12$ Å and curvature $k = 15$ Å, the optimal pore geometry for protegrin octamers found in⁴⁸. The decamer barrel employed the optimal decameric implicit pore geometry of $R_o = 16$ Å and $k = 20$ Å⁴⁸. Implicit MD simulations were then run for 2 ns. A final minimization then yielded the tetrameric arc and β -barrel structures imported into the membrane builder feature of CHARMM-GUI. For the systems containing two tetrameric arcs, a single tetramer was oriented about the origin, and then two diametrically opposed copies were translated 10 Å away from each other with both hydrophobic faces outward.

To assemble the system with four tilted protegrin dimers, the final configuration of the 5- μ s production run of the NCNC parallel dimer on the membrane surface was imported into CHARMM, and we tilted the dimer's major axis 30° from the vertical axis, translated the entire dimer 10 Å horizontally so that the more hydrophobic side faced outward, and rotated three copies by 90°, 180°, and 270° about the vertical axis to distribute all four evenly around the pore edge. The coordinates were then imported into CHARMM-GUI to add lipids, water molecules, and ions for all-atom simulations, as described below.

All-Atom Simulations

We imported the systems generated above into CHARMM-GUI's membrane builder with the goal of employing 180 total lipids and approximately 50,000 total atoms, configuring the water thickness above and below the membrane accordingly (overall system size: approximately $81 \times 81 \times 78 \text{ \AA}^3$), except that during some protegrin arc and bundle simulations, the lipid composition and number of lipids varied as noted in Table 1. The systems with 100% POPC were preliminary runs, for which the membrane was conceived as a mammalian mimetic. The system with POPE:POPG was a preliminary run for which the membrane was conceived as a bacterial mimetic. Cholesterol is an important component of mammalian membranes and was simulated as 30% of the membrane in one preliminary simulation. We simulated the POPE:POPG:CL system on Anton 2 as a bacterial membrane mimetic because CL is a component of many bacterial membranes. 0.15 M potassium chloride was added to neutralize excess charges. All-atom simulations employed the CHARMM C36 force field⁵⁷ and the TIP3P water model.

The equilibrations were performed using NAMD 2.11⁵⁸ with an initial time step of 1 fs. After harmonic constraints ($k = 1 \text{ kcal/mol/\AA}^2$) were applied to the water atoms, ions, phosphorous atoms, and peptide backbone atoms, we conducted 20,000 steps of energy minimization followed by 7 ps of heating to 303 K. The constraints on the lipids were released in another 100-ps equilibration step, followed by a 500-ps one in which the constraints on waters and ions were also released. Then, we introduced pressure control using the modified Nosé-Hoover barostat with Langevin dynamics, and the backbone constraints were scaled down in increments of 0.2 for 200 ps. Then, we ran a 1-ns unconstrained equilibration with a time step of 1 fs, followed by a final 5-ns initial production step (also in NAMD) with a time step of 2 fs. Representative configurations after equilibration are shown with transparent lipid surfaces in the Supplementary Information (Figs. S1–S10).

The production simulations were carried out on the Anton 2 supercomputer⁵⁹ at the Pittsburgh Supercomputing Center using the Multigrator integration framework⁶⁰. We used VMD to generate Anton 2 input files from the equilibrated NAMD output and parameter files, and then we used the viparr program to add the force field information to the Anton input files. Particle motions, thermostat updates, and the Martyna Tuckerman and Klein (MTK) barostat were applied using Multigrator every 1, 24, and 240 steps, respectively. Long-range electrostatics were calculated using the Gaussian Split Ewald method⁶¹, and the cutoff values for electrostatic interactions were automatically calculated by the Anton setup procedure. We ran the full production simulations for the lengths indicated in Table 1 with a 2-fs time step, saving coordinates every 1.08 ns.

VMD 1.9.2⁶² was used for trajectory visualization and to wrap the trajectories according to periodic boundary conditions so that all peptides appeared in the same image. CHARMM⁵³ was used to calculate the number of lipid headgroups near the center of the bilayer, number of water molecules inside the pore, and interaction energy between peptides over the last 1 μ s. The numbers of lipid headgroups and water molecules inside the bilayer were found as those in the volumes within 5 and 10 $\text{\AA}$ of the membrane center, respectively. Secondary structure content was calculated with the Digital Shape Sampling and Processing tool, which

is available through the CPPTRAJ program⁶³. Pore radius values were calculated for consecutive Z axis slices (1 Å thick) between the upper and lower headgroups region of the bilayer. The COOR SEARCH command counted the water molecules in the channel, and pore radius was calculated from the cross-sectional area of the effective cylinder that contained these waters. We used VMD⁶² and PyMol⁶⁴ to produce molecular graphics of the resulting configurations.

Results

Protegrin oligomers on the membrane surface

To determine likely early intermediates of protegrin oligomerization, we simulated protegrin dimers, trimers, and tetramers on the membrane surface. The results of these simulations could yield connections between the surface oligomeric structures and the membrane-embedded arcs and β -barrels. If the oligomers spontaneously penetrate the membrane surface and reorient to transmembrane orientation, then the results would directly illustrate key steps in the putative pore formation pathway. However, even without such spontaneous entry of peptides into the membrane, the surface oligomer simulations show which oligomeric multiplicities and topologies are most stable when peptides first begin to aggregate on the membrane surface. This provides information about which smaller oligomers will combine later to form pores.

Two types of dimers are distinguished: flush (completely aligned) and sheared (with the shifted hydrogen bonding pattern). Both of the flush dimers (NCNC parallel [Fig. 3a] and NCCN antiparallel topologies) were stable on the membrane surface for 5 μ s. No significant events were recorded in either of the trajectories, and the peptides did not insert below the level of the membrane surface in any of the dimer (or other oligomer) simulations. The NCNC parallel sheared dimer system started with the hydrogen bonds between peptides shifted by two residues from a flush configuration. About 550 ns into the simulation, the two peptides separated from each other on the membrane surface, not rejoining for the remainder of the trajectory. Thus, in contrast to the flush dimers, the sheared dimer is unstable on the membrane surface.

All trimers and tetramers were unstable on the membrane surface. In the NCNC parallel trimer, one monomer flipped over, leaving the disulfide bonds of two adjacent monomers facing each other, while the remaining dimer was stable. In the mixed topology trimer on the membrane surface, one monomer dissociated from the end and separated at about 2.9 μ s of simulation time, whereas the remaining NCNC parallel dimer was stable. The NCNC parallel tetramer simulation was stopped after 1 μ s when the monomers on the ends dissociated within 100 ns, the central dimer remaining stable. In the mixed topology tetramer on the membrane surface, one monomer dissociated within 100 ns, whereas the fourth monomer dissociated from the central dimer at about 2.2 μ s. The central dimer again remained stable throughout the production phase. This pattern also held for the sheared NCNC parallel tetramer, which broke down in a similar fashion within 650 ns (Fig. 3b). These results indicate that surface trimers and tetramers were unstable because their apparent tendency to curve and twist would have moved the edge peptides' surfaces away from the membrane surface.

No production simulation of the NCCN antiparallel trimer and tetramer was performed because the oligomers broke apart on the membrane surface during equilibration despite repeated attempts to create a stable structure. In these structures, the N-terminal side of one peptide is close to the N-terminal side of another, bringing two tyrosine residues together. (In contrast, the NCCN antiparallel sheared β -barrel, in which this problem may exist but is mitigated by the shearing, was quite stable.)

Protegrin β -barrels

Unsheared (i.e., untilted, with hydrogen bonds aligned) β -barrels have been the most commonly proposed final pore structure for protegrin (e.g.,^{25,42}) and were investigated first. We investigated mostly octamers, but we also investigated a decameric unsheared β -barrel. Our previous implicit membrane studies on protegrin β -barrels^{43,48} had verified the stability of both octamers and decamers and predicted a range of approximately 7–13 peptides in which the pore would have approximately equal energetic favorability. Our expectation was that the present octameric and decameric systems would form fundamentally the same type of pore.

The unsheared octameric β -barrel in NCNC parallel topology first tore into an open octameric β -sheet by breaking apart at a single junction point within 150 ns. The sheet continued to tilt and twist until the monomers on the edges of the sheet were almost parallel to the membrane surface, coming into contact with the lipid headgroup region. Then, at about 4.7 μ s, a single monomer separated from the other seven and dissociated from the pore. That monomer had zero interaction energies with the other seven peptides throughout the last 1 μ s of simulation time (Table S1). However, an open pore surrounded by seven peptides remained through the end of the 10- μ s production phase (Fig. 4a).

The mixed-topology octameric β -barrel also broke open into an octameric β -sheet at 2 μ s. No other significant events occurred until the simulation was stopped at 4 μ s (Fig. 4b). The pore remained open and surrounded by eight peptides. Similarly, the mixed-topology decameric barrel sheared apart at a single junction point within the first 25 ns. Then, at about 900 ns, the decamer broke apart into a heptameric sheet and trimeric bundle (Fig. 5). These two structures surrounded a large, partially lipidic pore, which remained open until the end of the simulation. The cause of the unsheared β -barrels' breakage seems to involve the oligomers' tendency to bend and twist, which is not facilitated by the unsheared β -barrel configuration.

Seeing that the unsheared protegrin transmembrane β -barrels all broke apart into twisted β -sheets during the long production simulations, we designed the sheared octameric β -barrel systems to achieve a tilted peptide configuration that would accommodate the β -sheet's natural tendency to twist. In preliminary implicit solvent simulations, we attempted to impose four shears by shifting the hydrogen bonds by two residues once for each pair of peptides, but the NCNC parallel, mixed, and NCCN antiparallel topologies spontaneously settled with only three total shears, yielding a configuration of dimer $\rightarrow$ shear $\rightarrow$ dimer $\rightarrow$ shear $\rightarrow$ tetramer $\rightarrow$ shear $\rightarrow$. The NCCN parallel topology settled with four shears.

The NCNC parallel sheared β -barrel remained stable with an open pore for 5 μ s (Fig. 6a). There was no sign of breaking during the simulation and the interaction energies between all adjacent monomers were -57.61 ± 6.01 kcal/mol (mean $\pm$ SD) over the last 1 μ s (Table S1). The mixed topology sheared β -barrel showed similar results to the NCNC parallel one, with the pore remaining open and the barrel intact for 5 μ s (Fig. 6b). The interaction energies between adjacent monomers were -47.54 ± 7.18 kcal/mol over the last 1 μ s, indicating a stable β -barrel at the end of the simulation (Table S1).

The NCCN antiparallel sheared β -barrel also remained intact for 5 μ s (Fig. 6c). The interaction energies between adjacent monomers were -62.00 ± 7.83 kcal/mol over the last 1 μ s, indicating β -barrel stability (Table S1). These results indicate that the insertion of shears into the β -barrel structure satisfies the peptides' intrinsic tendency to bend and twist in a way that the unsheared β -barrel structure does not.

The octameric NCCN parallel sheared β -barrel, in which the hydrophobic clusters of the β -hairpins point in alternating directions, was not stable as the other sheared β -barrels in our simulations. The barrel gradually collapsed and the pore fully closed within 5 μ s, with the monomers remaining close together in transmembrane orientation (Fig. 6d). This difference in results from the other topologies can be attributed to the fact that the peptide's hydrophobic side faces the membrane in all topologies except NCCN parallel.

Protegrin tetramer arcs in the membrane

Previous studies in our laboratory had indicated that protegrin arcs may form stable pores⁴⁷, but the short length of those simulations did not allow definitive conclusions. The present simulations of systems containing protegrin NCNC parallel tetramer arcs were conducted in POPC (Fig. 7a), POPE/PG (Fig. 7b), and POPC/cholesterol (Fig. 7c) to determine whether such arcs can surround stable aqueous pores that are partially lined by lipids. The arcs remained inserted in the membrane, but the arcs in POPE/PG and POPC/cholesterol deformed slightly, with three peptides forming a β -sheet and the fourth reorienting to face the other three. None of those systems generated stable, open pores: the simulations ended with lipids occupying space inside the pore lumen instead of an aqueous channel (lipid tails shown for selected systems in Fig. S11). We also conducted simulations of NCCN antiparallel (Fig. 7d) and mixed topology (Fig. 7e) tetramer arcs in POPC/PG, whose configurations remained stable within the membrane throughout production phases of 2 and 5 μ s, respectively, but as above, no aqueous pore formed for either system. The above results indicate that a single protegrin tetrameric arc is insufficient to support a stable pore: although the arcs themselves are relatively stable, the presence of lipids in the pore region prevents stable aqueous conduction (Fig. S11). The results did not change according to membrane composition.

We then examined the possibility that the arcs are intermediates en route to β -barrel formation. Therefore, we ran systems containing two transmembrane tetramer arcs (NCNC parallel [Fig. 8a] or NCCN antiparallel [Fig. 8b]) placed opposite to each other with their centers of mass separated by 20 Å and water molecules (but no lipids) initially filling the space between them. The arcs diffused in the membrane throughout the trajectory without forming hydrogen bonds between the two tetramers. Although very small aqueous channels

remained until the simulations ended, no β -barrel formation was observed. This indicates that the pairs of protegrin arcs face a kinetic barrier against combining into a complete β -barrel.

Protegrin bundles

We simulated transmembrane protegrin bundles without imposition of initial hydrogen bonding to allow the peptides to associate freely and examine the possibility of more classical toroidal pore structures observed with helical AMPs^{13,23,65}. This provides a more unbiased look at oligomerization than the systems beginning with β -barrels or arcs, as the bundle simulations started farther from the putative final state. The protegrin bundles began as tightly packed assemblies of monomers rotated with the more hydrophobic faces outward but with no hydrogen bonding imposed.

We simulated tetrameric protegrin bundles in both pure POPC and POPC/PG membranes. In POPC, three peptides came together without extensive hydrogen bonding between them, and the fourth monomer sandwiched behind the third (Fig. 9a). Table S1 shows that the peptides' intermolecular interaction energies were as strong as -34.00 kcal/mol (SD: 17.5); this indicates that the peptides interacted moderately strongly despite the absence of a pore. In POPC/PG, the results were similar: the four peptides rapidly associated into a trimeric arc and a monomer facing the opposite direction (Fig. 9b). There was no aqueous pore in either simulation, although the peptides' geometries outlined small pores of a few Å in diameter (Table S1). Therefore, whether the initial structure was a bundle or arc, four peptides were insufficient to stabilize aqueous channels for long durations. Membrane composition did not seem to affect the results in any case.

The octameric protegrin bundle associated into an NCNC parallel hexameric β -sheet and an opposing, loosely associated dimer within 300 ns. The hexamer was curved and twisted, and it and the dimer surrounded a stable, partially lipidic pore (Fig. 10). The results were in accordance with those of the unsheared β -barrels and indicate that eight peptides are sufficient to stabilize an open pore, whereas four peptides are insufficient.

As reported above, protegrin NCNC parallel dimers were stable on the membrane surface. However, trimers and tetramers were not and sheared β -barrels were stable, whereas unsheared ones broke open. Therefore, we arranged four tilted dimers around an aqueous pore to see how they would associate and whether they would support an open pore. The trajectory showed one dimer splitting from the other three and returning to the membrane surface at about 2.3 μ s. At about 4.3 μ s, one of the three dimers remaining in the pore split up, with one monomer joining another dimer to form a trimer and the other monomer returning to the membrane surface. A dimer and a trimer were then left on opposite sides of a small, stable pentameric pore. Although this situation continued until the end of the simulation (Fig. 11), the pore continued to shrink until the end of the production phase, indicating that the pore may have been in the process of breaking down and destined to close (see *Pore Statistics* below).

Tachyplesin bundles

Although tachyplesin has a similar β -hairpin AMP structure to protegrin, our previous implicit simulations indicated that it is less stable as a β -barrel than protegrin⁴³. We investigated bundles of tachyplesin for comparison to the data on protegrin. The reference structure of tachyplesin contains C-terminal amidation, but we also investigated a structure without C-terminal amidation to determine the effect of this modification on pore formation. The tachyplesin bundle with C-terminal amidation exhibited some hydrogen bonding between dimers, but no larger oligomers formed. The peptides' intramolecular hydrogen bonding structure remained intact, and at the end of 5 μ s, an aqueous pore remained open (Fig. 12a). However, three peptides had reoriented to the membrane surface, and four monomers in the pore all convened in one lipid leaflet, giving the impression that the pore may be en route to breaking down. (Pore size vs. simulation time for this system is also plotted in *Pore Statistics* below.) The dimer in the opposite lipid leaflet remained associated with the pore region but parallel to the membrane surface.

The tachyplesin bundle without C-terminal amidation showed similar results to the amidated one. The bundle associated only into monomers and dimers, which only loosely associated with each other, and multiple peptides reoriented parallel to the membrane surface, possibly indicating pore breakdown in progress. However, the peptides that remained inside the pore still surrounded an aqueous channel at the end of the simulation time (Fig. 12b). The similarity of these results indicates that amidation does not significantly affect tachyplesin's pore formation ability.

Pore statistics, interactions, and β -sheet content

The pore radius, number of water molecules in the pore, and number of lipid headgroups vertically within 5 Å of the membrane center for applicable systems with stable pores are shown over the last 1 μ s of the production phase in Table 2. Peptide-peptide interaction energies over the last 1 μ s of simulation [kcal/mol] are shown for the indicated systems in Table S1.

For the protegrin system with four tilted dimers and the amidated tachyplesin octameric bundle, which had aqueous channels without the presence of larger oligomers at the end of production, we plotted pore radius vs. simulation time to visualize their pore stability (Fig. 13). For both, pore radius showed a decreasing tendency throughout the simulation, indicating that the pores may be en route to breaking down. However, the pore radius of the amidated tachyplesin bundle seems to have stabilized over the last 1.8 μ s, and aqueous channels remained in these systems for the duration of our simulations, so longer simulations are needed to confirm whether this type of pore is stable.

We also determined the interaction energies between positively charged arginine side chains and negatively charged PG headgroups for both the sheared and unsheared mixed topology octameric β -barrels. Maximization of these interactions may account for unsheared β -barrels' tendency to break apart and tilt, while the sheared β -barrels were stable. We analyzed 921 observations per trajectory (i.e., the last 1 μ s) using CHARMM to examine the energies when the peptides had stabilized. The overall standard deviation for each trajectory

was found by the pooled variance method using all eight peptides. The interaction energies per peptide averaged -48.25 kcal/mol (SD: 23.0) and -45.38 kcal/mol (SD: 23.3) for the unsheared and sheared β -barrels, respectively. No significant differences were found between these two systems' interaction energies between arginine side chains and PG headgroups, indicating that the movements during the trajectories yielded interactions of similar strength.

We examined the β -sheet content of the peptides in selected systems throughout the last 1 μ s of simulation time to examine trends in the systems' β -hairpin structure (Table 3). Throughout the simulations, most peptides in β -sheets in both the protegrin and tachyplesin systems maintained their β -hairpin structure at levels only slightly below those of the native PDB structures. However, some peptides on the edges of β -sheets had lower β content (e.g., peptide F from the NCNC parallel octamer barrel), and those that ended the simulations separated from any β -sheets had almost no β content (e.g., peptide H from the NCNC parallel octamer barrel). The peptides' β -structure seems to be positively associated with their continued oligomerization throughout the simulations. When there were average differences between systems' overall β -sheet content levels, the differences seemed driven by the few peptides with very low β content.

Discussion

In this study, we performed μ s-scale all-atom simulations of protegrin oligomers in lipid bilayers and observed the relationship between oligomeric multiplicity, topology, and pore stability. The results provide insights regarding the stability of putative pore structures and the feasibility of certain pore formation pathways but leave important questions unanswered.

We observed three types of pores that are stable on a time scale of 5–10 μ s. The first involves monomers and small oligomers surrounding a partially lipidic pore. These pores tended to shrink throughout the simulation and may have closed if the simulation time had been extended. Nevertheless, even five copies of protegrin (a trimeric arc and an opposing dimer) were sufficient to support a substantial aqueous channel for 5 μ s (Fig. 10). The bundles of tachyplesin, which showed lower tendency to aggregate than protegrin, resulted in pores of this type (Fig. 11). A second type of stable pore consisted of a twisted β -sheet plus smaller oligomers surrounding a partially lipidic aqueous channel (Fig. 4, 5). The third type observed was a completely proteinaceous pore comprised by a sheared protegrin β -barrel (Fig. 6). Longer simulations are necessary to verify the longer-term stability of these pores.

It is also instructive to examine the systems that did not generate stable pores. The systems with transmembrane tetrameric arcs did not show stable pores, in contrast to previous shorter simulations⁴⁷. In addition, tetrameric bundles failed to adopt a configuration that would support an open pore. The present results indicate that more than four copies of protegrin are needed to support a stable pore and that a single tetrameric arc is insufficient to maintain a substantial aqueous channel. Further, two tetrameric arcs placed in proximity to each other failed to form a β -barrel. Therefore, protegrin β -barrel formation seems to face a substantial kinetic barrier.

Whereas the sheared β -barrels were stable, the unsheared β -barrels broke open and twisted, in contrast to our previous shorter simulations⁴². Sheared barrels have not been previously proposed for protegrin, but they would result in similar NMR signals to those that have been previously observed (e.g.,^{25,33}, and result in pore radii within the experimentally observed range (Table 2)^{25,29}. The unsheared β -barrels showed a similar propensity to break open, while still supporting a stable pore, whether 8 or 10 monomers were used. In both cases, the barrels broke into a β -sheet and some smaller units, the latter of which also seemed to support pore stability. These results support our earlier conclusion that protegrin β -barrel pores with 7–13 peptides have approximately equal energetic favorability⁴⁸.

There may be two reasons for the instability of unsheared barrels: first, β -sheets have a natural tendency to twist, which cannot be accommodated by unsheared barrels⁶⁶. Indeed, in all known β -barrel membrane proteins, the β -strands exhibit a substantial tilt angle⁶⁷. A second possible reason is that tilting may improve the interaction of the positively charged arginine side chains at the turn region and termini with the lipid headgroups. The peptide's overall length, which is larger than the thickness of the POPC:POPG membranes, necessitates a tilt to optimize these interactions. Large experimental tilt angles of 48°–55° for 12-carbon DLPC lipids³³ can be compared with computational results indicating tilt angles of up to 34.9° for 16–18-carbon POPC lipids³⁹. After breaking, the arginine residues in the unsheared β -barrel systems interacted with PG headgroups with approximately the same strength as those in the sheared β -barrels, supporting this conclusion.

Simulations of oligomers on the membrane surface were performed to determine the stability of putative early intermediates in the pore formation pathway. Protegrin unsheared dimers on the membrane surface were stable, but trimers, tetramers, and sheared dimers were not, and none of the surface oligomer systems resulted in membrane insertion of the peptides. This indicates that a larger number of protegrin copies must accumulate on the membrane to initiate the poration event. These results also lead us to propose that the unsheared dimer could function as a basic building block of larger protegrin oligomers. The sheared β -barrels were comprised by building block units of unsheared dimers, and most topologies of sheared β -barrels were quite stable. Sheared β -barrels' stability when composed of dimeric units matches with unsheared protegrin dimers' observed stability on the membrane surface. This is in accordance with previously obtained potentials of mean force of protegrin dimerization, which showed the favorability of dimerization in multiple environments⁴⁶.

To test the hypothesis of a dimer as a building block, we performed a simulation starting from four tilted protegrin dimers. Contrary to expectations, we observed that two NCNC parallel dimers interacted to form an NCNC parallel trimer and a monomer. After the single peptide split from its partner and joined the other dimer (as a trimeric β -sheet), the abandoned monomer left the pore region and returned to the membrane surface. This makes the first type of pore described above (i.e., one comprised by smaller arcs and oligomers surrounding a partially lipidic pore) seem like it could be an intermediate towards the formation of other pore states in the presence of larger numbers of smaller protegrin oligomers. However, in this study, we did not observe any events of protegrin oligomers coming together to form tilted β -barrels inside the pore, and even though we witnessed the

spontaneous formation of several unsheared β -sheets during our simulations, unsheared β -barrels always broke down. Further, whereas sheared barrels are stable in our simulations, we have been unable to observe the formation of such a barrel. Alternatively, protegrin may form unstructured pores comprised by multiple non-interacting small oligomers supporting a toroidal channel, analogous to our previous Anton results on melittin⁶⁸. This could hint at commonalities between the mechanisms of different AMPs.

The present results confirm the importance of topology to protegrin's pore formation. The NCCN parallel topology was unstable even as a sheared barrel, in contrast to solid-state NMR suggestions of a pore with that topology²⁵, but in accordance with previous computational studies by our laboratory⁴². The NCCN parallel sheared β -barrel is the only topology with the peptides' hydrophobic faces pointing in alternate directions. The other two topologies, in which all of the peptides' hydrophobic sides point outward towards the membrane, sustained aqueous pores as both unsheared and sheared β -barrels, even after the unsheared ones broke open into β -sheets. In addition, when eight protegrin peptides were placed as a bundle, six associated into an NCNC parallel β -sheet. These observations indicate that the hydrophobic faces of all protegrin monomers must face the membrane to participate in poration.

We also noticed distinctions in pore size between the topologies. Pore radius and number of water molecules inside the pore were measured for systems that supported stable aqueous pores (Table 2). These measures were both closely associated with the number of peptides comprising the pore, which ranged from 5 to 10. The observed pore radii for pores comprised by eight peptides generally agree with previous estimates of protegrin's pore radius^(25; but the pore size is smaller than stated by 29). Among both sheared and unsheared β -barrels comprised by eight peptides, the mixed topology resulted in relatively larger pores than NCNC parallel. This difference is likely due to the increased twist of the NCNC parallel topology. The protegrin system with four tilted dimers, which formed a pore comprised by five peptides, was similar in size to the tachyplesin pore (Table 2).

Even though all lipid headgroups began the simulations near the plane of the membrane surface, most systems with stable pores had 3–5 lipid headgroups within 5 Å of the membrane center by the end of the simulation, whereas all sheared β -barrels had 0 (Table 2). This indicates that the protegrin transmembrane bundles, unsheared β -barrels, and tilted dimers caused the pore to become more toroidal than the sheared β -barrels did (the sheared β -barrels supported mostly cylindrical pores). Neutron diffraction experiments have indicated that protegrin forms toroidal pores²³. To reconcile this with the presence of sheared barrels, which did not generate toroidal pores in the current investigation, further studies might assess sheared protegrin β -barrels' ability to introduce membrane defects³¹.

The tachyplesin octameric bundle simulations yielded similar results with and without C-terminal amidation. Further, tachyplesin showed lower overall tendency towards mutual interaction than protegrin did: larger oligomers than dimers of tachyplesin did not form at all. This result verifies our previous implicit membrane simulations showing that tachyplesin is unstable as a β -barrel⁴³. In contrast, protegrin (which had the ability to form stable β -

barrels in ⁴³) showed the ability to form new associations between peptides within the pore (i.e., the trimer in Fig. 10).

These differences between protegrin and tachyplesin can also be understood in terms of structural distinctions between the peptides. Our previous structural comparison ⁴³ indicated the differences in hydrophobic residue packing between these two peptides' putative pore structures. Although both peptides show "imperfect amphipathicity," which concentrates the hydrophobic side chains on one face, the central region of the hairpin between the two disulfide bonds is longer in tachyplesin than protegrin, and this lengthened portion in tachyplesin contains two arginine side chains (R5 and R14) that face the same direction as both disulfide bonds (i.e., into the pore lumen). In contrast, protegrin's fewer residues between the two disulfide bonds contain no side chains facing the pore lumen. Because a relatively hydrophilic peptide face is thought to stabilize the pore lumen, the presence of two arginine side chains per peptide in the center of the pore lumen could contribute to tachyplesin pores' lack of observed stability. Because these extra arginine side chains pack closely together in tachyplesin β -barrel structures, their bulk could cause steric hindrance that reduces the stability of higher-order oligomers.

Further simulations are necessary to determine whether the observed tachyplesin pore will remain stable or disintegrate in additional time. The tachyplesin pores were similar in size and constitution to the pore formed by the system beginning with four tilted protegrin dimers (Table 2); longer simulations are necessary to determine whether each represents a breakdown product (Fig. 12).

These results can be analyzed in terms of the investigated peptides' experimentally observed pore induction. Voltage clamp experiments on protegrin-infused planar lipid bilayers and liposome studies revealed that protegrin forms weakly anion-selective channels in lipid bilayers and induces potassium leakage from liposomes ⁶⁹. A study of tachyplesin's interaction with liposomes and planar lipid bilayers also indicated the formation of anion-selective pores that allowed peptide translocation across the lipid bilayer ⁷⁰, in accordance with the movement of tachyplesin peptides from the present study's pores back to the membrane surface. A solid-state NMR study of tachyplesin indicated that the peptide's orientation was parallel to the membrane surface, in contrast to that of protegrin ⁷¹. Dye leakage from liposomes has been measured for both protegrin ^{69,72-74} and tachyplesin ⁷⁵⁻⁷⁷, but a quantitative comparison between the two peptides' ability to induce dye leakage has been lacking.

Conclusions

The present all-atom simulations revealed different types of pores induced by β -hairpin AMPs. First, unsheared β -barrels of protegrin were not stable for long periods, in contrast to previous contentions ^{25,42}. Such barrels opened into twisted β -sheets that surrounded stable, partially lipidic pores. Second, sheared β -barrels of protegrin were stable, and created completely proteinaceous pores, in all topologies that allow the peptide's hydrophobic side to face the membrane. Although these pores represent a relatively ordered state that may face an entropic barrier to formation, they were stable for the length of our simulations.

Third, tachyplesin and the bundle of protegrin dimers formed small pores that were lined by only a few peptides, with other peptides seeming to support the pores from the membrane surface. This leads to the overall conclusion that protegrin can form a diverse set of pore states depending on conditions. Additional simulations of dimers, trimers, and tetramers on the membrane surface showed only the unsheared dimers to be viable as early intermediates in pore formation.

This work leaves open several important questions. More simulations are necessary to determine how various conditions can lead to the formation of these various pore types. Other factors that could be manipulated more comprehensively include salt concentration, peptide concentration, and membrane composition. In addition, the present simulations were set up in a biased fashion that encouraged the formation of the desired final states. Eventually, it would be more compelling to illustrate the formation of a stable pore from peptides in solution or on a membrane surface. Sheared β -barrels are stable once formed, but a study showing the formation of a complete β -barrel from smaller oligomers is highly desirable.

Several of these questions could be straightforwardly addressed by longer conventional MD simulations. However, we also need to determine the relative favorability of these various states, for which free energy calculations may also be useful. Our laboratory is also investigating other computational approaches, such as simulated tempering⁷⁸, which may provide additional information on β -barrel closure with our current computing power. Other simulation methods like reaction path annealing⁷⁹ may also provide information about the critical peptide aggregation steps.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Supporting Information Available

Snapshots of representative systems at the beginning of the production phase of simulation; post-simulation snapshots of selected tetramer arc systems with lipid tails inside pore shown; peptide-peptide interaction energies of selected protegrin systems during the last 1 μ s of simulation time. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Fig. 1. Possible topologies of protegrin dimerization. A) NCNC parallel; B) NCCN antiparallel; C) NCCN parallel. Brackets outline the boundaries of mixed topology trimer and tetramer. Yellow bridges represent disulfide bonds. Arrows point N→C.

Fig. 2.

Initial hydrogen bonding patterns of protegrin octamers. A) Bundle, front half: no initial hydrogen bonding between peptides; B) Unsheared β -barrel, front half: initial hydrogen bonding between flush transmembrane peptides; C) Sheared β -barrel, front half: hydrogen bonding shifted by two residues per pair of peptides, introducing a tilt. Sidechains omitted for clarity. Where applicable, topology is NCNC parallel. NH atoms, CO atoms, and β -barrel hydrogen bonds shown in white, red, and magenta, respectively.

Fig. 3. Snapshots of representative protegrin oligomer systems on the membrane surface at the end of the production phase of simulation, top views. (A) Dimer, NCNC parallel topology; (B) Tetramer, NCNC parallel topology. (*Rainbow cartoons*) peptides; (*Orange spheres*) lipid headgroup phosphates within 5 Å of peptides. Lipid tails omitted for clarity.

Fig. 4. Snapshots of protegrin octameric unsheared β -barrel systems at the end of the production phase of simulation. (A) NCNC parallel topology; (B) Mixed topology. (*Rainbow cartoons*) peptides; (*Orange spheres*) lipid headgroup phosphates within 5 Å of peptides; (*Red and white lines*) water. Lipid tails omitted for clarity.

Fig. 5. Snapshots of protegrin mixed topology decameric unsheared β -barrel system at the end of the production phase of simulation. (*Rainbow cartoons*) peptides; (*Orange spheres*) lipid headgroup phosphates within 5 Å of peptides; (*Red and white lines*) water. Lipid tails omitted for clarity.

Fig. 6. Snapshots of protegrin sheared octameric β -barrel systems inside the membrane at the end of the production phase of simulation. (A) NCNC parallel; (B) Mixed topology; (C) NCCN antiparallel; (D) NCCN parallel. (*Rainbow cartoons*) peptides; (*Orange spheres*) lipid headgroup phosphates within 5 Å of peptides; (*Red and white lines*) water. Lipid tails omitted for clarity.

Fig. 7. Snapshots of tetramer arc systems inside the membrane at the end of the production phase of simulation. (A) NCNC parallel, POPC; (B) NCNC parallel, POPE/PG; (C) NCNC parallel, POPC/cholesterol; (D) NCCN antiparallel, POPC/PG; (E) Mixed topology, POPC/PG. (*Rainbow cartoons*) peptides; (*Orange spheres*) lipid headgroup phosphates within 5 Å of peptides; (*Red and white lines*) water. Lipid tails omitted for clarity.

Fig. 8. Snapshots of systems with two tetramer arcs inside the membrane at the end of the production phase of simulation. (A) NCNC parallel, POPC/PG; (B) NCCN antiparallel, POPC/PG. (*Rainbow cartoons*) peptides; (*Orange spheres*) lipid headgroup phosphates within 5 Å of peptides; (*Red and white lines*) water. Lipid tails omitted for clarity.

Fig. 9. Snapshots of protegrin tetrameric transmembrane bundle systems inside the membrane at the end of the production phase of simulation. (A) POPC; (B) POPC/PG. (*Rainbow cartoons*) peptides; (*Orange spheres*) lipid headgroup phosphates within 5 Å of peptides; (*Red and white lines*) water. Lipid tails omitted for clarity.

Fig. 10. Snapshots of protegrin octameric transmembrane bundle inside the membrane at the end of the production phase of simulation. (*Rainbow cartoons*) peptides; (*Orange spheres*) lipid headgroup phosphates within 5 Å of peptides; (*Red and white lines*) water. Lipid tails omitted for clarity.

Fig. 11.

Snapshot of octameric protegrin system with four tilted dimers inside the membrane at the end of the production phase of simulation. (*Rainbow cartoons*) peptides; (*Orange spheres*) lipid headgroup phosphates within 5 Å of peptides; (*Red and white lines*) water. Lipid tails omitted for clarity.

Fig. 12.

Snapshots of octameric transmembrane bundle systems of β -hairpin antimicrobial peptide tachyplesin inside the membrane at the end of the production phase of simulation. (A) With C-terminal amidation; (B) Without C-terminal amidation. (*Rainbow cartoons*) peptides; (*Orange spheres*) lipid headgroup phosphates within 5 Å of peptides; (*Red and white lines*) water. Lipid tails omitted for clarity.

Fig. 13. Pore radius vs. simulation time for selected systems. (*Blue*) protegrin, four tilted dimers; (*Gray*) tachyplesin, octameric bundle.

Table 1

Systems simulated in all-atom studies. All membranes contained 180 lipids unless noted. Bundle: a tightly packed assembly of peptides with the hydrophobic face outwards but no imposed hydrogen bonding. Tilted: the dimer's major axis was reoriented 30° from transmembrane. POPC: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine. POPE: 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine. POPG: 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol). CL: cardiolipin. Chol: cholesterol. For a guide to topology, see *Methods*. Mixed topology: one dimer NCNC parallel and the other dimer NCCN antiparallel.

Peptides	Topology	Membrane composition	Time run
<u>Protegrin oligomers on membrane surface</u>			
Dimer	NCNC parallel	75% POPC:25% POPG	5 μ s
Dimer	NCCN antiparallel	75% POPC:25% POPG	5 μ s
Dimer (sheared)	NCNC parallel	75% POPC:25% POPG	5 μ s
Trimer	NCNC parallel	75% POPC:25% POPG	5 μ s
Trimer	Mixed	75% POPC:25% POPG	5 μ s
Tetramer	NCNC parallel	75% POPC:25% POPG	1 μ s
Tetramer	Mixed	75% POPC:25% POPG	5 μ s
Tetramer (sheared)	NCNC parallel	75% POPC:25% POPG	3 μ s
<u>Protegrin unsheared barrels</u>			
Octamer	NCNC parallel	75% POPC:25% POPG	10 μ s
Octamer	Mixed	75% POPC:25% POPG	4 μ s
Decamer	Mixed	75% POPC:25% POPG	3 μ s
<u>Protegrin sheared barrels</u>			
Octamer	NCNC parallel	75% POPC:25% POPG	5 μ s
Octamer	Mixed	75% POPC:25% POPG	5 μ s
Octamer	NCCN parallel	75% POPC:25% POPG	5 μ s
Octamer	NCCN antiparallel	75% POPC:25% POPG	5 μ s
<u>Protegrin arcs</u>			
Tetramer	NCNC parallel	100% POPC (294 lipids)	3 μ s
Tetramer	NCNC parallel	70% POPE:30% POPG (270 lipids)	2 μ s
Tetramer	NCNC parallel	70% POPC: 30% Chol (100 lipids)	2 μ s
Tetramer	NCCN antiparallel	75% POPC:25% POPG	2 μ s
Tetramer	Mixed	75% POPC:25% POPG	5 μ s
Two tetrameric arcs	NCNC parallel	75% POPC:25% POPG	8 μ s
Two tetrameric arcs	NCCN antiparallel	75% POPC:25% POPG	3 μ s
<u>Protegrin bundles</u>			
Tetramer	N/A	100% POPC (72 lipids)	9 μ s
Tetramer	N/A	70% POPE:22% POPG:8% CL	4 μ s
Octamer	N/A	75% POPC:25% POPG	5 μ s
Octamer (four tilted dimers)	NCNC parallel	75% POPC:25% POPG	5 μ s
<u>Tachyplesin bundles</u>			

Peptides	Topology	Membrane composition	Time run
Tachyplesin octamer (amidated)	N/A	75% POPC:25% POPG	5 μ s
Tachyplesin octamer (non-amidated)	N/A	75% POPC:25% POPG	5 μ s

Table 2

Pore radius, number of water molecules in pore, and number of lipid headgroups within 5 Å of the membrane center for systems with stable pores during the last 1 μs of simulation time. Bundle: a tightly packed assembly of peptides with the hydrophobic face outwards but no imposed hydrogen bonding. Tilted: the dimer's major axis was reoriented 30° from transmembrane. Pore radius and number of water molecules: determined for the last 1 μs of simulation, as per the *Methods*. Lipid headgroups near membrane center: number within 5 Å of the midline for the last 1 μs. N/A: Certain values not shown for systems without stable aqueous pores. For a guide to topology, see the *Methods*. Mixed topology: one dimer NCNC parallel and the other dimer NCCN antiparallel. N/A: For values in this study, not applicable. In ²⁹, the number of peptides in the pore was not measured.

Peptides	Topology	Pore radius (Å)	Water molecules in pore	Lipid headgroups near membrane center
<u>Protegrin unsheared barrels</u>				
Octamer	NCNC parallel	7.5 +/- 1.0	130 +/- 20	3 +/- 1
Octamer	Mixed	9.4 +/- 0.7	204 +/- 21	0 +/- 1
Decamer	Mixed	12 +/- 1.0	307 +/- 41	3 +/- 1
<u>Protegrin sheared barrels</u>				
Octamer	NCNC parallel	8.7 +/- 0.3	159 +/- 10	0
Octamer	Mixed	10.0 +/- 0.4	203 +/- 13	0
Octamer	NCCN antiparallel	9.0 +/- 0.3	167 +/- 9	0
<u>Protegrin arcs</u>				
Tetramer in POPC	NCNC parallel	5.0 +/- 1.5	N/A	N/A
Tetramer in POPC/Cholesterol	NCNC parallel	7.0 +/- 1.0	N/A	N/A
Tetramer in POPE/POPG	NCNC parallel	4.0 +/- 1.0	N/A	N/A
<u>Protegrin bundles</u>				
Tetramer	N/A	5.5 +/- 0.5	N/A	N/A
Octamer	N/A	10.5 +/- 1.0	225 +/- 34	3 +/- 1
Octamer (four tilted dimers)	NCNC parallel	7.0 +/- 1.0	125 +/- 27	4 +/- 1
<u>Other transmembrane bundles</u>				
Tachyplesin octamer (amidated)	N/A	7.0 +/- 1.0	99 +/- 19	5 +/- 1
<u>Published values</u>				
Octamer (25)	NCCN parallel	<10.5	N/A	N/A
N/A (29)	N/A	15–20	N/A	N/A

β-sheet content of peptides in selected systems throughout last 1 μs of simulation time (%). Values presented as averages for each system and separately for each monomer.

Table 3

System	A	B	C	D	E	F	G	H	I	J	Avg
Protegrin: Four tilted dimers	45	53	61	52	42	54	60	56			52.88
Protegrin: Octameric bundle	49	50	36	64	63	63	63	57			55.63
Protegrin: Mixed topology unsheared octameric barrel	52	60	40	55	51	54	48	65			53.13
Protegrin: mixed topology sheared octameric barrel	63	55	47	50	60	61	55	57			56.00
Protegrin: NCCN antiparallel sheared octameric barrel	56	56	55	59	62	57	50	53			56.00
Protegrin: NCNC parallel unsheared octameric barrel	52	52	56	54	62	20	33	1			41.25
Protegrin: NCNC parallel sheared octameric barrel	58	50	57	56	66	65	58	49			57.38
Protegrin: Mixed topology decameric barrel	57	60	54	53	59	37	38	3	49	37	44.70
Tachyplesin: Octameric bundle	47	47	59	78	60	35	46	55			53.38
PDB value: Protegrin (1PGI)											63
PDB value: Tachyplesin (1WO0)											55