Published in final edited form as:

Peptides. 2011 January ; 32(1): 188–201. doi:10.1016/j.peptides.2010.10.006.

Computational studies of protegrin antimicrobial peptides: a review

Dan S. Bolintineanu^a and Yiannis N. Kaznessis^{a,*}

^a Department of Chemical Engineering and Materials Science, University of Minnesota, 421 Washington Ave SE, Minneapolis MN 55455

Abstract

Antimicrobial peptides (AMPs) are small, naturally-occurring peptides that exhibit strong antibacterial properties generally believed to be a result of selective bacterial membrane disruption. As a result, there has been significant interest in the development of therapeutic antibiotics based on AMPs; however, the poor understanding of the fundamental mechanism of action of these peptides has largely hampered such efforts. We present a summary of computational and theoretical investigations of protegrin, a particularly potent peptide that is both an excellent model for the mechanism of action of AMPs and a promising therapeutic candidate. Experimental investigations have shed light on many of the key steps in the action of protegrin: protegrin monomers are known to dimerize in various lipid environments; protegrin peptides interact strongly with lipid bilayer membranes, particularly anionic lipids; protegrins have been shown to form pores in lipid bilayers, which results in uncontrolled ion transport and may be a key factor in bacterial death. In this work, we present a comprehensive review of the computational and theoretical studies that have complemented and extended the information obtained from experimental work with protegrins, as well as a brief survey of the experimental biophysical studies that are most pertinent to such computational work. We show that a consistent, mechanistic description of the bactericidal mechanism of action of protegrins is emerging, and briefly outline areas where the current understanding is deficient. We hope that the research reviewed herein offers compelling evidence of the benefits of computational investigations of protegrins and other AMPs, as well as providing a useful guide to future work in this area.

Keywords

Protegrin; antimicrobial peptides; molecular dynamics

1. Introduction

Antimicrobial peptides (AMPs) are important components of the innate immune systems of countless animal species, where they act as an effective, largely non-discriminatory first line of defense against invading pathogens [9,21,47,110–111]. Spurred by the proliferation of bacterial strains that exhibit resistance to conventional antibiotics, the last two decades have seen a flurry of research activities aimed at developing novel therapeutic antibiotics based on AMPs. While these efforts have met with some success [81], the full potential of AMPs

*Corresponding author: yiannis@cems.umn.edu, Tel: 612 624 4945, Fax: 612-626-7246.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

for therapeutic use is yet to be unlocked. A significant bottleneck is posed by the high levels of toxicity exhibited by most AMPs at the concentration levels required to treat severe infections. In order to rationally design peptides that are not toxic to host cells but retain their natural antibiotic activity, it is vital to understand their fundamental mechanism of action and identify the mechanistic steps and structural features that are most amenable to engineering. Given the tremendous diversity in the amino acid sequences and secondary structural features of these peptides – including α -helical, β -sheet, unstructured and loop-structured peptides [32,107] – it is no surprise that no single mechanism of action appears to fit all observed AMP behaviors [21]. However, a large body of evidence supports the notion that many AMPs act primarily by targeting and disrupting the cell membranes of bacteria [4,9,17,57,76,79,105,111]. Because of this highly nonspecific target, the hope is that AMP-based antibiotics can provide a long-term solution to antibiotic-resistant pathogens [32].

Research efforts to date have focused on several model AMPs, all of which show promising therapeutic potential. The most studied peptides include alamethicin, magainin, melittin and ovipirin from the α -helical structural class, defensins and protegrins from the β -sheet class, and indolicidin from the unstructured class. For broad reviews of AMPs across all three major structural classes, the reader is referred to [9,21,32,47,110–111]; this review focuses on protegrins [54], with a view to simulation and modeling efforts. The common theme among experimental and computational biophysical studies of protegrins (and indeed of all of the peptides mentioned above) is the interaction of these peptides with lipid bilayers and other membrane-mimicking environments. Although some studies suggest that AMPs may in fact act at an intracellular level [31,33,107], the bulk of the evidence points to the bacterial membrane as the primary target. This is especially true of protegrin, for which it has been shown that membrane disruption correlates with antimicrobial activity [57,76]; that substitution of D-amino acids causes no loss of activity, ruling out specific intracellular targets [17]; and that exposure to protegrins causes uncontrolled potassium leakage consistent with membrane permeabilization prior to cell death [5]. As such, protegrins have emerged as one of the leading model peptides for both experiments and simulations on account of their antibiotic efficacy, the wealth of biophysical data available, and their amenability to computer simulations due to their small size and rigid structure. Given the breadth of computational studies of protegrin to date, we believe a complete review of these efforts is essential in summarizing and guiding future work in this area, as well as in compiling and highlighting the main insights gained from computational approaches thus far. We do not discuss computational work with other peptide systems except where it pertains directly to protegrins; for reviews of simulation efforts relating to other AMPs, the reader is referred to [8,77,99]. We first review some of the key biological and biophysical characteristics of protegrins, including a summary of their hypothesized mechanism of action. The remainder of the review is organized according to various aspects of this mechanism of action, with modeling work generally discussed in order of increasing complexity.

Protegrins are a family of peptides originally isolated from porcine leukocytes [54]. Currently, there are five known naturally occurring members of this family, and dozens of mutants have been synthesized [17,26,80]. Protegrins have shown a broad spectrum of activity against both Gram-positive and Gram-negative bacteria, including *E. coli*, *P. aeruginosa*, and *N. gonorrhoeae* [54,82], as well as the fungus *C. albicans* and the HIV-1 virus. The minimum inhibitory concentration (MIC) of protegrin-1 (PG-1), the most common form, is on the order of 1 $\mu\text{g/mL}$ against several bacterial strains [80]. However, the potent naturally occurring peptides also exhibit high levels of toxicity against human epithelial cells and red blood cells. A larger data set presented in Table 1 of reference [80] includes more detailed activity/toxicity measurements for numerous protegrin mutants.

The naturally occurring protegrins are between 16 and 18 amino acids long, and are rich in positively charged arginine residues. Their defining feature is an anti-parallel β -hairpin structure, stabilized by two cysteine-cysteine disulfide bonds between the two strands of the hairpin. The solution structure of the most common naturally occurring protegrin variant, denoted PG-1 (amino acid sequence RGGRLCYCRRRFCVVCVGR-NH₂), is shown in figure 1. This structure is based on the NMR study conducted by Fahrner et al. [22], and is available in the protein data bank [3] (PDB code 1PG1). The disulfide bonds spanning the β -hairpin impose rigid constraints on the peptide structure. This has the effect of confining the positively charged, highly hydrophilic arginine residues in distinct spatial regions – the two termini, and the β -hairpin turn – while the remaining structure consists largely of hydrophobic residues (figure 1). Its rigid structure thus gives PG-1 its amphipathic nature, common to many other AMPs. Due to the highly constrained hairpin structure, the structure of PG-1 is not expected to undergo significant changes upon association with membranes or upon oligomerization, which is particularly fortuitous for simulation efforts, where conformational sampling is often a limiting factor. The highly cationic charge of protegrins (and many other AMPs) is hypothesized to be essential to their ability to bind more strongly to bacterial cell membranes, which contain a significant portion of anionic lipids, as opposed to mammalian membranes, which are primarily composed of zwitterionic lipids [110].

The currently accepted model of protegrin action against bacteria involves the electrostatic attraction and adsorption of cationic protegrin peptides to anionic bacterial membranes [28,30,41,73], followed by dimerization of the peptides [12–13,75], insertion into the hydrophobic membrane core [35–36,108], formation of higher aggregates (4–5 dimers) [74,95–97], and eventually formation of transmembrane pores that cause an unrestricted, lethal flux of ions and other molecules from the cytoplasm [5,72,92,109] (see figure 2). The precise order of these steps, the molecular interactions responsible for them, the thermodynamic driving forces and the kinetics involved in all of these steps are largely unknown. Given the extremely fast dynamics, small scales and complex solution-state nature of these processes, computer simulations can provide an excellent complement to experimental investigations and greatly enhance the level of detail attainable experimentally. Computational work to date relating to these key aspects of the mechanism of action of protegrin is reviewed herein, along with brief descriptions of pertinent experimental data.

The majority of computational investigations of biomolecular systems are performed using some form of molecular dynamics (MD) simulations. In fully atomistic MD simulations, all atoms, including the oxygen and hydrogen atoms of water molecules, are modeled explicitly and assigned fixed masses and charges; in coarse-grained simulations, an interaction site can consist of several atoms, or the solvent may be represented based on a continuum theory such as the Poisson-Boltzmann theory, as discussed below. In both types of MD simulations, atoms or sites are allowed to interact via Coulombic and van der Waals forces, as well as short-range bonded interactions. Both nonbonded and bonded interactions are computed based on a self-consistent, empirically-derived force field (e.g. CHARMM [71], GROMOS [100], AMBER [19]). Based on these interactions, forces are computed on each atom, and the equations of motion are solved numerically to propagate the system forward in time. Classical MD simulations, as opposed to more rigorous, quantum mechanical simulations, maintain a tractable balance between computational cost and modeling detail. Fully atomistic MD simulations currently represent the most rigorous and detailed simulations of antimicrobial peptides; such a high level of detail is often indispensable in order to capture many of the key structural features and interactions of antimicrobial peptides such as protegrin.

2. Molecular dynamics simulations of peptides interacting with micelles

The first fully atomistic MD simulations of protegrin peptides were performed using zwitterionic dodecylphosphocholine (DPC) and anionic sodium dodecylsulfate (SDS) micelles as mammalian and bacterial membrane mimics, respectively [6,60–61,63]. Although both micelles differ from cell membranes in shape, size and the chemistry of their constituent lipid molecules, it was hoped that the fundamental similarity of having a hydrophobic core surrounded by a flexible, hydrophilic interface is sufficient to capture some of the features of peptide-membrane interactions, and that the negatively charged SDS head group and the zwitterionic DPC head group can approximately model anionic bacterial and zwitterionic mammalian cell membranes, respectively. In particular, these simulations aimed to identify which residues have the highest affinity for the two different membrane environments, and consequently suggest mutations that would maintain or improve activity (i.e. strengthen interactions with SDS micelles) and reduce toxicity (i.e. weaken interactions with DPC micelles). The primary advantage of using micelles as membrane mimics in MD simulations is the much shorter time scale of lipid relaxation as compared to lipid bilayers (0.5–2ns in micelles as compared to 20 ns in lipid bilayers [52]). Additionally, peptide-micelle simulations contain a smaller number of atoms than lipid bilayer simulations, and are not plagued by the issues surrounding simulations of lipid bilayers (correct choice of ensemble, advance knowledge of the area per lipid, choice of surface tension value) [2,18,23–24,42,46]. The ability to run smaller and shorter simulations is essential for the simulation of multiple protegrin mutants, which would otherwise represent a daunting computational challenge for lipid bilayer systems. As far as we are aware, the only MD simulation studies to date involving multiple protegrin mutants have been carried out in lipid micelles. A review of some of our simulation work with several classes of AMPs using micelles as membrane mimics is available [52].

Langham and coworkers carried out a 13 ns MD simulation of PG-1 interacting with an SDS micelle and compared the results to a 40 ns simulation of PG-1 interacting with a DPC micelle [61]. In both cases, the peptide was initially placed at the centre of the micelle in order to avoid any bias toward a particular orientation, and the simulation was considered equilibrated when the peptide position with respect to the micelle had stabilized. The peptide was found to diffuse towards the surface of both micelles, and converged to the same location as in simulations that were started with the peptide outside of the micelles. At its equilibrium location, PG-1 was more deeply inserted into the DPC micelle, but showed a preference for the surface of the SDS micelle. The equilibrium orientations of the peptide in the two simulations were found to be reversed, in that strand 1 (RGGRLCYCRR) was closest to the center of the SDS micelle, while strand 2 (RFCVCVGR), which is more hydrophobic, was closer to the center of the DPC micelle. This led the authors to suggest that strand 1 is more important for bactericidal action, while strand 2 is more important for toxicity; therefore, mutations of strand 2 could reduce toxicity without having an adverse effect on activity. In a subsequent study, the authors carried out simulations of three PG-1 mutants in DPC and SDS micelles [60]. The three mutations consisted of replacing the final three residues of strand 2 in PG1 (VGR) by a single residue (T, I or E for the three different mutants). It was found that while they interacted with SDS micelles differently, all three mutants inserted to an equal depth in these micelles, which was consistent with experimental measurements that show their antibacterial activity to be equivalent. On the other hand, the differences in toxicity of the three peptides were found to correlate well with differences in the interactions of the three mutants with DPC micelles. This led the authors to conclude that experimentally measured toxicity can indeed be predicted and explained to some extent by simulations with DPC micelles.

In further work with two decapeptide PG-1 mutants, Langham and coworkers [63] performed MD simulations along with fluorescence spectroscopy, Fourier transform infrared spectroscopy (FTIR) and deuterium exchange experiments to ascertain the validity of simulations with SDS and DPC micelles. Simulations and FTIR experiments showed strong agreement with respect to peptide structural motifs (β -sheet vs. unstructured vs. loop-turn vs. helical), particularly in the case of DPC; deuterium exchange data suggested that in all cases, peptides are surface-bound and equally exposed to solvent, which likewise agrees with simulation results; fluorescence spectroscopy showed that tyrosine residues interact primarily with solvent, which was visually confirmed by simulations. In a follow-up study, Bolintineanu and coworkers compared MD simulations of PG-1 and two additional mutants in DPC micelles [6]. One of these mutants (PC9) entailed the removal one of the disulfide bridges in PG-1, which reduces its β -sheet character, while the other mutant (PC13) entailed the removal of the last two residues (GR), thereby reducing the total charge by one. Simulations with DPC micelles showed that the removal of the disulfide bond in PC9 caused significant distortion of its β -sheet motif, although interactions with the micelle were stronger as a result, while removal of a charged residue caused a significant reorientation of PC-13 in its membrane-bound state as compared to PG-1. A comparison of these findings with the known toxicity profiles of these mutants suggests that the β -sheet motif is a more critical feature for toxicity than the total charge of a peptide or its strength of interaction with DPC micelles.

Although simulations of monomeric protegrins in the presence of micelles have yielded useful structural information and have isolated some of the features of lipid-protegrin interactions that may be involved in toxicity and activity, more realistic membrane mimics are required for a complete, mechanistic understanding of these phenomena. In recent years, simulations of protegrin peptides have increasingly progressed towards the use of lipid bilayers with realistic bacterial membrane-like compositions, and have included simulations of protegrin monomers, dimers and transmembrane pore aggregates.

3. Molecular dynamics simulations of protegrin monomers in lipid bilayer membranes

Since protegrin peptides have been shown to exist primarily as monomers in solution [1,22], the first step in their mechanism of action is indisputably their association with the cell membranes of bacteria. Numerous experimental studies have investigated the interactions of protegrins with various membrane mimics. Lee and coworkers have conducted a series of experiments using lipid monolayers of assorted compositions as bacterial and mammalian membrane mimics. Their findings confirm that PG-1 readily inserts into anionic films and films composed of lipid A, a primary component in the bacterial outer membrane, but does not readily interact with zwitterionic lipids [28]. They have also been able to distinguish a higher affinity of PG-1 for phosphoethanolamine (PE) monolayers over phosphocholine (PC) monolayers, both of which are zwitterionic, but the former is more abundant in ruminants than in humans [41]. These results emphasize the importance of membrane lipid composition in the action of protegrin. Work from the same group using atomic force microscopy to investigate the effects of PG-1 on lipid bilayers has provided direct evidence of increased bilayer disruption by PG-1 with increased peptide concentration [58]. The selectivity of PG-1 towards anionic phosphoglycerol lipids over zwitterionic phosphocholine lipids was also confirmed through NMR investigations by Mani et al. [73]. From their NMR studies of protegrin interacting with lipid bilayers, Hong and coworkers have established that PG-1 causes disruption of the order of longer-tailed, unsaturated lipids such as POPC (1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphocholine) with tails 16 and 18 carbon atoms long, but does not disrupt thinner DLPC bilayers (1,2-Dilauroyl-sn-Glycero-3-Phosphocholine), with tails containing 12 carbon atoms [108]. The preservation of order in

the thinner bilayers allowed Yamaguchi et al. [108] to carry out further NMR experiments to determine that PG-1 was fully inserted in a DLPC bilayer, where it adopts an orientation in which its long axis is tilted approximately 55° from the bilayer normal (this is discussed in greater detail below). The insertion and tilt of PG-1 in DLPC bilayers was confirmed by Buffy et al. using paramagnetic Mn^{2+} NMR experiments [11]. These authors additionally estimated that DLPC bilayers are thinned by as much as 8–10 Å in the presence of PG-1. Heller and coworkers identified two concentration-, lipid- and hydration-dependent states of membrane-bound protegrin peptides using oriented circular dichroism (OCD) [36]. These states were hypothesized to correspond to the surface-adsorbed and membrane-inserted states of PG-1, although other differences, such as changes in hydrogen bonding patterns likely differentiate the two states. A subsequent study by Heller et al. [35] using X-ray diffraction in a 1,2-Diphytanoylphosphatidylcholine (DPhPC) bilayer showed that the surface-adsorbed state corresponds to membrane thinning, which ultimately drives the change in the state of PG-1 at higher peptide concentrations.

Several groups have studied the interactions of protegrin monomers with lipid bilayers using computational methods, primarily fully atomistic molecular dynamics simulations. Jang and coworkers [44] performed MD simulations of the PG-1 monomer interacting with zwitterionic palmitoyl-oleoyl-phosphatidylcholine (POPC) bilayers as well as 4:1 POPC/POPG (palmitoyl-oleoyl-phosphatidylglycerol) bilayers, which more closely represent the anionic character of bacterial membranes. Both simulations were started with the peptide placed on the surface of the lipid bilayer, and production runs of 10 ns were carried out using the CHARMM force field [71]. The authors observed a bend in the β -hairpin motif in simulations of PG-1 in water compared to a more stable, planar β -sheet structure in the presence of lipid bilayers. The PG-1 monomer was observed to reside closer to the bilayer surface in the case of the mixed POPC/POPG anionic lipid bilayer, which also exhibited a greater degree of thinning as compared to the zwitterionic POPC bilayer. The authors suggest that the weaker interaction with the zwitterionic bilayer is a result of screening of arginine-phosphate interactions by chloride ions; in the case of the anionic bilayer, chloride ions are electrostatically repelled by the anionic lipids, which allows cationic arginine groups to interact more freely with the lipid phosphate groups. Furthermore, the surface-adsorbed peptide was found to cause local deficiencies of lipids near its β -hairpin turn in the case of the anionic lipid bilayer. The authors refer to this effect as bilayer thinning, but it should be noted that this describes a different phenomenon than what is typically meant by lipid bilayer thinning, namely a decrease in the thickness along the bilayer normal direction. The latter was also observed in the case of the mixed anionic bilayer, and referred to as bending of the bilayer by these authors. Quantitative measures of the extent of this bending effect were not reported.

Khandelia and Kaznessis reported simulations of PG-1 in zwitterionic DLPC bilayers using the CHARMM force field [71], with the peptide initially placed in two different transmembrane orientations [50–51]. Both simulations converged to the same orientation within 20 ns. The resulting tilt and rotation angles (see figure 3) were in reasonable agreement with values computed based on solid-state NMR (ssNMR) measurements of the same systems: average values from the simulation place the range of the tilt angle τ between 20° and 50° , with an average around 30° [51], while experimental data suggest values between 50° and 73° , with an average around 55° [108]; the rotation angle ϕ was calculated to be around 60° based on simulations, whereas experimental measurements estimate the angle as $48 \pm 5^\circ$. Significant lipid bilayer thinning was observed, with an average decrease of 6 Å in the lipid bilayer thickness near the peptide as compared to the thickness far away from the peptide, in agreement with experimental estimates of 8–10 Å [11]. Furthermore, the authors reported a novel, stable “kick” conformation for the inserted PG-1 monomer, in which Gly-17 forms a persistent hydrogen bond with the hydroxyl group of Tyr-7, causing

the C-terminal strand to bend towards the bilayer center, forming a kick-shaped bend in the β -hairpin backbone [50]. The formation of such intramolecular hydrogen bonds would alleviate the energetic penalty of inserting these moieties into the hydrophobic core of the membrane, and may represent the types of changes posited for the inserted state of PG-1 by Heller and coworkers [36] based on their oriented circular dichroism measurements (see above). The “kick” conformation was only observed in one of the two simulations.

A subsequent study was conducted by Kandasamy and Larson using the GROMOS [100] force field, wherein PG-1 was initially placed in several transmembrane orientations and conformations in the interior of membranes of varying thickness (DLPC, DMPC, DPPC and POPC) [49]. MD simulations ranging from 30 to 100 ns were carried out for a total of 16 combinations of initial conditions and lipid bilayers. Across all bilayer types, the authors identified two predominant transmembrane binding modes of PG-1, distinguished by whether Arg-1 binds to the same leaflet as Arg-4 and Arg-18 (binding mode 1) or to the opposing leaflet, along with Arg-9, 10 and 11 (binding mode 2). The observed binding modes were found to be sensitive to the initial conditions of the simulation (peptide conformation and orientation); the variation was attributed to the flexibility of the RGGR domain, which allows Arg-1 sufficient mobility to interact with both lipid bilayer leaflets, even in bilayers as thick as POPC. The authors also performed simulations of spontaneous self-assembly, wherein POPC lipid and water molecules were initially placed randomly around a PG-1 peptide [49]. After 200ns of simulation, the lipids were observed to organize into a lipid bilayer spanned by a peptide-stabilized water pore. In all cases, the authors noted only a small degree of bilayer thinning (<2 Å), which was generally larger for longer lipid types and for binding mode 2 (thinning of DLPC bilayers was less than 1 Å). Tilt and rotation angles of PG-1 were not reported.

In a similar vein, Rui et al. [87] performed extensive MD simulations of PG-1 monomers and dimers inserted into DLPC and POPC lipid bilayers using the CHARMM force field [71]. In the case of monomers, three 80 ns-long simulations starting from different lipid packing arrangements were completed. The authors explored the issue of peptide tilting in unprecedented detail, and showed that the experimental estimate of the tilt angle of $55 \pm 5^\circ$, which arises from a rigid-body search using five fixed NMR structures (from PDB entry 1PG1[22]) to fit ^{15}N and ^{13}CO chemical shifts of Val¹⁶, may not be entirely accurate, as it fails to account for peptide flexibility and conformational changes caused by the lipid bilayer environment. Indeed, the root mean square deviation between ^{15}N and ^{13}CO chemical shifts calculated from the simulations and those measured experimentally is smallest for MD structures with tilt angles between 10 and 40° , somewhat smaller than the reported value of 55° . Nonetheless, the authors note that large fluctuations and significant discrepancies do exist between the ensemble-averaged chemical shifts and the experimental values. The rotation angle, which is defined differently than in [50,108](see figure 3), is found to converge to a value close to -120° for all three monomer simulations (for this alternate definition of the rotation angle, see [67,86–87]). This corresponds to an orientation in which the peptide’s disulfide bonds face down with respect to the plane of the β -hairpin, and the authors show that this rotation can also fit the experimentally measured chemical shift data. Membrane thinning for DLPC was found to be in the range of 3.3 to 4.0 Å, and for POPC in the range of 3.7 to 4.5 Å [87]. Strong interactions between the guanidinium groups of the arginine residues and lipid phosphate groups are hypothesized to be responsible for this membrane thinning. Furthermore, the authors present detailed analyses of hydrogen bonding patterns within the PG-1 monomer, between the peptides and lipid bilayers, and between the two monomers that constitute a PG-1 dimer (discussed in more detail below). In the DLPC bilayer/PG-1 monomer simulations, residues in the middle of the β -hairpin are able to bond lipid head groups, whereas in the POPC bilayer, only residues near the hairpin turn and the terminal regions are involved in hydrogen bonds with lipids.

This suggests an important reason for the tendency of PG-1 to oligomerize in thicker bilayers, but not in thinner DLPC bilayers. With regard to intrapeptide hydrogen bonds, the authors report that the C-terminal strand is more likely to form hydrogen bonds with the N-terminal strand in a DLPC bilayer than in a POPC bilayer.

The MD simulation studies summarized above confirm, complement and extend much of the experimental data compiled to date. The most important issues that have emerged as common threads throughout these studies are the orientation of PG-1 monomers in lipid bilayers of different thicknesses, the effect of PG-1 on different bilayers (particularly lipid bilayer thinning), the stability of the peptide structure and interactions between the peptide and the lipids. The interest in the orientation of PG-1 in lipid bilayers, particularly in the case of DLPC bilayers, stems from the ssNMR data reported by Yamaguchi and coworkers [108], which offers an excellent point of comparison for simulation efforts. Several simulation studies report somewhat smaller tilt angles than the value calculated by Yamaguchi et al.; however, in a lucid analysis of NMR chemical shift data, Rui et al. point out that this may be the results of inaccuracies in the way that tilt angles were computed from chemical shift data in the original experimental work, particularly the use of limited rigid peptide structures [87]. This point was further explored in subsequent studies [67,86], discussed below. With regards to bilayer thinning, the values reported by different simulation studies for the thinning of DLPC bilayers are less than 1 Å [49], 4 Å [87] and 6 Å [50]. Experimental data in DPhPC lipid bilayers suggests a thinning of less than 1 Å [35], while data for DLPC bilayers places the extent of thinning between 8 and 10 Å [11]. Clearly this is a point on which simulations and experiments do not converge, but from the limited data available, it appears that simulations using the CHARMM force field [71] fare somewhat better than those performed with the GROMOS force field [100]. The extent of bilayer thinning is important, as it may result in tension on the lipid bilayers that acts as a key driving force for peptide oligomerization and pore formation [38]. Furthermore, simulations appear to be in general agreement that the β -sheet motif of PG-1 monomers is stabilized by persistent intrapeptide hydrogen bonds in the lipid bilayer environment, which corroborates experimental findings showing that the β -sheet motif is one of the most relevant features of protegrins for pore formation [72] and biological activity [34,57]. Finally, simulations have been able to describe the interactions between peptides and lipid bilayers with an impressive level of detail. Not surprisingly, the guanidinium groups of the arginine side chains interact strongly with lipid phosphate groups [48,50,87], the positively charged PG-1 monomer adsorbs more strongly onto anionic lipid bilayers [44], and persistent hydrogen bonds form between peptide residues in close proximity to the bilayer surface and the lipid head groups [87]. Kandasamy and Larson have provided an astute analysis of the binding of PG-1 to lipid bilayers of varying thickness, and identified two distinct binding modes [49]. Overall, molecular dynamics simulations of PG-1 monomers in lipid bilayers show reasonable agreement with currently available experimental data, and shed additional light on molecular details that are difficult to access experimentally.

4. Thermodynamic calculations and potentials of mean force

Although a wealth of structural data is available from NMR experiments, the details of the thermodynamic driving forces that govern the interaction of peptides with membranes are difficult to measure experimentally. While typical methods such as surface plasmon resonance and titration calorimetry can measure free energy changes of peptide-membrane association, they cannot distinguish between different types of association processes (e.g. surface adsorption, membrane insertion, oligomerization), and as such lack the resolution required to identify and quantify the key steps in the various processes involved in the action of protegrin. Computational approaches, which are not limited by the small length scales and fast dynamics of these processes, can isolate particular details of peptide-membrane

interactions that experiments cannot, and provide pertinent thermodynamic data with far greater detail than experiments. Such data are extremely useful in identifying bottlenecks in the bactericidal and toxic mechanisms of action of protegrin, and engineering peptides accordingly in order to optimize their therapeutic profiles.

The most useful thermodynamic measure for a particular biomolecular process is the potential of mean force (PMF), which essentially describes the free energy of the system as a function of a particular reaction coordinate. Examples of such a reaction coordinate include the distance between a PG-1 monomer and a lipid bilayer surface, or the angles that describe PG-1 insertion into the lipid bilayer core. The potential of mean force, which is a function of one or more reaction coordinates, contains all the information required to assess equilibrium distributions of various states in the process, as well as some information regarding possible pathways from one state to another (e.g. surface-adsorbed peptide to membrane-inserted peptide) and the associated kinetics. A general discussion of the principles of the potential of mean force is omitted here; the interested reader is referred to [37,85].

In order to provide further insight into the tilt and rotation of PG-1 in DLPC lipid bilayers, Sayyed-Ahmad and Kaznessis implemented a computationally inexpensive approach to calculating an approximate, PMF-like quantity that describes these degrees of freedom [88]. In their approach, the electrostatic free energy for a particular orientation and location of a PG-1 monomer was found from a numerical solution to the Poisson-Boltzmann (PB) equation [39,90], where the lipid bilayer is represented by several low-dielectric regions (a “three-dielectric” [98] model, corresponding to a dielectric constant of 2 for the aliphatic tail region and a dielectric constant of 10 for the two lipid head group regions), while water is represented as a high-dielectric region. Although this grossly simplifies the structure and chemistry of both water and lipid molecules, it captures what is probably the most important electrostatic feature of the system, the large dielectric heterogeneity. The non-electrostatic component of the free energy was estimated based on a linear relationship to the exposed molecular surface area (SA) [91], where the proportionality coefficient is a function of the bilayer insertion depth [98]. This approach is often referred to as the PB/SA method [55]. The free energy profile of a PG-1 monomer was computed as a function of tilt and rotation angles (defined the same way as in [50,108]; see fig. 3). The membrane model was assigned hydrophobic and head group dimensions roughly corresponding to a DLPC bilayer. The resulting two-dimensional potential was found to have a well-defined minimum at a tilt angle near 20° and a rotation angle of 30°. In light of the analyses conducted by Rui and coworkers [86–87] that suggest experimental data overestimates the tilt angle (see discussion above), the results of Sayyed-Ahmad and Kaznessis are in good agreement with the experimental values of 55° and 48°. Considering the inherent approximations in the Poisson-Boltzmann formalism, as well as the fact that a single rigid peptide structure was used in this work (namely, the final structure of a previous 23 ns-long MD simulation[50]), the results are surprisingly good. This suggests that continuum-level electrostatic interactions are some of the most important effects with regards to peptide-membrane interactions. Free energies of insertion were not explicitly computed, but the variation in the reported PMF spanned a range of approximately 80 kcal/mol across a full sampling of tilt and rotation angles.

To explore some of these same issues via PMF calculations, Lee and coworkers [67] developed a set of restraining potentials specifically for β -hairpin peptides and implemented these in the CHARMM simulation program [10]. Their potentials allow for simulations with combinations of constraints on the principal axis of the β -hairpin, the rotation of the β -hairpin (as defined in [67,86–87]), and the distance separating two β -hairpin structures. The use of constraints is essential in computing potentials of mean force by methods such as

umbrella sampling and constraint force integration [85,104]. The constraints were tested by computing PMFs for the tilt and rotation of PG-1 monomers using the EEF1.1/IMM1 force field [64–65], which uses a distance-dependent dielectric constant for interatomic interactions and an empirical self-energy term based on the extent of exposure of a particular atom to solvent and its depth of insertion into the membrane. As such, the IMM1 force field is a largely empirical implicit membrane model; of the common implicit membrane models, it is one of the fastest and is easily amenable to dynamics, but lacks a rigorous physical basis and is only as accurate as the underlying empirical parameters. Lee and coworkers computed PMFs for two values of the hydrophobic thickness in the IMM1 model, namely 17.5 Å and 10 Å, corresponding to an unperturbed DLPC bilayer and a DLPC bilayer thinned by the presence of PG1, respectively [67]. The PMFs they obtained show a thermally accessible range for the tilt angle in the 17.5 Å bilayer between 3 and 20° (with the minimum at 10°), while the 10 Å bilayer has a range of thermally accessible tilt angles between 7 and 31° (with the minimum at 22°). The authors hypothesize the discrepancy from the experimental value of 55° [108] to be a result of inaccuracies due to the approximate nature of the IMM1 force field, as well as the possible overestimation of the tilt angle in the experimental work caused by the neglect of peptide flexibility (see earlier discussion). Interestingly, the authors found that both lipid bilayer thickness values gave the same minimum of –90° in the PMF of the rotation angle (again, see [67,86–87] for definition). The thermally accessible range of this angle is between –70 and –130°, which is in good agreement with the authors' previous all-atom molecular dynamics study [87] discussed above, where the predominant rotation angle was ~–120°.

In a subsequent study from the same group, Rui and Im [86] used the β -hairpin restraining potentials developed by Lee et al. [67] to compute PMFs along the tilt angle of a PG-1 monomer in fully atomistic DLPC and POPC bilayers at four different rotation angles. Once again, the minimum tilt angle in the PMF was found to be larger in the thinner DLPC bilayer (37°) as compared to the thicker POPC bilayer (20.9°), likely due to the peptide needing to tilt more in order to achieve favorable hydrophobic contact within the thinner bilayer [86]. These minima correspond to a rotation angle of –90°, consistent with the authors' previous findings [67]. The authors suggest that the faces of the peptide display different hydrophobicities, which explains the preference for a particular rotation angle. In the region of the PMFs corresponding to the thermally accessible tilt angles, notable membrane thinning was reported (2 Å in DLPC and 5 Å in POPC), hypothesized to be a result of hydrophobic interactions and strong arginine-phosphate interactions. Interestingly, different surface tension values had no significant effects in any of the fully atomistic MD simulations. Based on the all-atom PMFs, the authors calibrated the hydrophobic thickness parameter in the IMM1 force field and computed a two-dimensional PMF for rotation and tilt angles, which showed reasonable agreement with fully atomistic simulations. Finally, the authors also show that the chemical shift data obtained from NMR experiments can be closely matched by their simulations if PMF-weighted conformations from the entire orientational space are used (although some variation exists depending on the chemical shift tensor definition).

The studies discussed so far pertaining to PMF calculations have focused on the rotation and tilt angles of PG-1 monomers in various models of lipid bilayers. The goal of these works has been to explore the properties of the membrane-inserted state of PG-1 monomers, in particular to verify and understand the experimentally measured tilt and rotational preferences of these peptides in DLPC bilayers and compare them to behavior in thicker, more biologically relevant POPC bilayers. However, before they can insert into the membrane core, protegrin peptides must adsorb to the membrane surface, a process that can also readily be quantified by PMF calculations. Recently, Vivcharuk and Kaznessis have reported a study of the adsorption of PG-1 monomers and dimers in a particular orientation

to the surface of 3:1 POPE:POPG lipid bilayers in terms of the relevant potential of mean force [102]. Their calculations are based on fully atomistic MD simulations combined with a force constraint integration method [104]. Their results show a favorable free energy of adsorption of -2.4 ± 0.8 kcal/mol and -3.5 ± 1.1 kcal/mol for the monomer and dimer, respectively, and no significant energy barrier to adsorption for either. This confirms the affinity of protegrin peptides for anionic lipid bilayers, and suggests that adsorption is a diffusion-limited process. The authors were able to closely match the PMFs obtained from fully atomistic simulations with a simple Poisson-Boltzmann/Gouy-Chapman theory [101], and showed that the adsorption is driven to a large extent by the entropy gain associated with the release of membrane-adsorbed counterions [102].

5. Protegrin dimerization in lipid bilayers

Both experiments and simulations suggest that PG-1 monomers cannot act independently to cause the type of membrane disruption that leads to cell death. The current leading hypothesis involves the dimerization of protegrin, followed by subsequent aggregation of dimeric units to form transmembrane pores that cause uncontrolled leakage of ions and other cell contents, leading to cell death [74]. The dimerization of protegrin monomers was initially a problematic proposal, as it places highly charged arginine residues on the two monomers in close proximity, which would result in electrostatic repulsions and dimer destabilization [22]. Indeed, experimental evidence suggests that PG-1 exists primarily as a monomer in aqueous solution and in DMSO [1,22]. However, a number of NMR investigations have shown that in certain lipid environments, hydrogen bonding along the backbone of the two monomers is sufficiently strong to overcome the electrostatic repulsion between the arginine residues. Roumestand et al. [84] measured nuclear Overhauser effects (NOEs) in NMR spectra of PG-1 interacting with DPC micelles, and found that their data was most readily explained by a peptide dimer structure in which the two protegrin monomers align in an NCCN anti-parallel fashion, with the C-terminal strands interacting through backbone hydrogen bonds (see figure 4B). A parallel NCCN structure (see fig. 4A) was ruled out due to strong $d_{\alpha\alpha}$ NOE effects between residues R-18 and F-12, as well as V-14 and V-16, which are in close proximity only for the anti-parallel NCCN motif [84]. In contrast, a solid-state NMR study conducted by Tang et al. found that PG-1 forms large oligomeric aggregates in a phosphate buffer saline solution, with ^{13}C and ^1H spin diffusion data indicating a parallel orientation of the monomers [94]. Further NMR investigations by Buffy and coworkers have shown that PG-1 is immobilized in POPC bilayers to a much greater degree than in DLPC bilayers [13]. The authors suggest this immobilization to be a result of peptides oligomerizing in the thicker, more biologically relevant POPC bilayers, but not the thinner DLPC bilayers. Using ^{19}F spin diffusion NMR experiments, Buffy and coworkers were able to show that with increasing peptide concentration, PG-1 peptides primarily exists as dimers in POPC lipid bilayers [12]. The authors estimated a free energy of dimerization of -10.2 ± 2.3 kJ/mol (or -2.44 ± 0.55 kcal/mol), but did not report details of the dimer structure. A subsequent study by Mani et al. using the rotational-echo double-resonance (REDOR) ssNMR technique strongly corroborated the parallel NCCN dimer motif, and the three-dimensional structure of the NCCN parallel dimer was resolved (PDB entry 1ZY6) [75]. An additional REDOR ssNMR experiment carried out by the same group suggests that in anionic lipid bilayers, PG-1 dimers in a parallel, NCCN arrangement aggregate to form transmembrane pores, most likely composed of 4 or 5 dimer units [74]. In zwitterionic membranes that contain cholesterol, the same dimers are hypothesized to bind to the surface of the membrane [74]. Overall, the available experimental data suggest that dimerization is an essential first step in the mechanism of action of protegrin, and that PG-1 dimers are the predominant form of protegrin in biologically relevant lipid bilayers. The NCCN parallel dimer structure is the only configuration that has been observed in lipid bilayers, while the NCCN anti-parallel motif has only been observed in DPC micelles.

A number of molecular simulation studies have explored the dimerization of PG-1 in order to explain some of the experimental findings discussed above. The first fully atomistic simulations of PG-1 dimers in lipid bilayers were carried out by Jang and coworkers [45], who simulated both parallel and antiparallel NCCN dimers on the surface of POPC lipid bilayers as well as in water. In all cases, the dimer structure was preserved for the duration of the simulations (20 ns in the case of the bilayer simulations, 10 ns in the case of the water simulations). However, a notable difference was observed in the overall stability of the β -sheet motif in the two different dimers, as measured by fluctuations in the fraction of possible hydrogen bonds: in water, only two hydrogen bonds between the C-terminal strands hold the dimers together, and the β -sheet structure is only somewhat preserved; in lipid bilayers, the β -sheet structure is well-preserved, and the C-terminal strands of the antiparallel dimer were more tightly bound, suggesting that this arrangement is more stable. Despite their somewhat lower stability, the parallel dimers were found to have stronger interactions and a more disruptive effect on the lipid bilayers, leading the authors to conclude that the parallel motif is the more biologically relevant of the two, as suggested by experimental evidence [12–13,74–75].

As part of a study mentioned earlier in the context of PG-1 monomers, Rui and coworkers also conducted a series of simulations of parallel NCCN dimers interacting with DLPC and POPC lipid bilayers [87] (as noted above, the NCCN parallel dimer is the only PG-1 dimer configuration experimentally observed in lipid bilayers). In their work, the dimers were initially placed in a transmembrane orientation, and a total of 180 ns of MD simulation were performed for each of the two bilayer types. Based on a hydrogen bond analysis, the dimer structure was found to be more stable in the thicker POPC bilayer, in agreement with experimental evidence that suggests that PG-1 does not dimerize in DLPC, but exists primarily as a dimer in POPC [13]. Tilt angles of the PG-1 dimer were measured to be between 0 and 48.9° in DLPC bilayers, and between 0 and 34.9° in POPC bilayers, hypothesized once again to be a result of the peptides tilting to achieve a favorable hydrophobic match with the lipid bilayer core. Based on their simulations, the authors suggest that the preference for PG-1 to dimerize in thicker POPC bilayers is related to the higher availability of hydrogen bond acceptors in the lipid head group region in thinner DLPC bilayers, which essentially replaces hydrogen bonding between PG-1 monomers. Due to the longer tails of POPC bilayers, the backbone amide hydrogen atoms of PG-1 are unable to form hydrogen bonds with the lipid head group atoms, which causes them to form hydrogen bonds with another PG-1 peptide in order to minimize the energetic penalty associated with inserting these otherwise exposed polar groups in the aliphatic membrane core.

In another study from the same group, the process of PG-1 dimerization was investigated from a thermodynamic point of view. Lee and coworkers performed MD simulations using the EEF1/IMM1 force field along with umbrella sampling and weighted histogram analysis method (WHAM) calculations to compute the PMF associated with PG-1 dimerization [67]. Their simulations started with PG-1 monomers in transmembrane orientations (with the long axis oriented parallel to the bilayer normal), and PMFs were computed along a reaction coordinate corresponding to the minimum distance between arbitrary points on the β -hairpin principal axes of the two monomers. Because of the computational savings associated with using the IMM1 force field, the authors were able to compute PMFs for all six possible dimerization arrangements (NCCN, NCNC, CNNC, each in a parallel or antiparallel arrangement). The dimerization PMFs show very large, favorable free energies, with PMF minima in all cases around -40 kcal/mol, much larger than the experimental estimate of -2.4 kcal/mol [12]; no statistically significant difference was discernible in the values of the PMF minima for the six possible configurations. The authors attribute these shortcomings largely to the inaccuracies inherent in the EEF1/IMM1 force field.

This last issue was addressed by fully atomistic MD simulations recently carried out by Vivcharuk and Kaznessis, who used a combination of MD and the constraint force integration method to compute the PMFs of PG-1 dimerization in three different environments: in bulk water, on the membrane surface and inside the membrane core [103]. Simulations were performed with parallel and anti-parallel dimers in the presence of 3:1 POPE:POPG anionic lipid bilayers. In all cases, dimerization was found to be a thermodynamically favorable process, with the parallel motif preferred in all cases except on the surface of the membrane. The free energies of dimerization, as measured by differences in the PMF between the bound and unbound state, varied significantly with both the dimer configuration and the environment: in bulk water, the free energies of dimerization were -17.5 kcal/mol and -4.8 kcal/mol for the parallel and anti-parallel dimers, respectively; on the membrane surface, they were -20.4 and -23.2 kcal/mol; in the membrane core, -8.1 and -3.8 kcal/mol. Although these values differ significantly from the experimental estimate of -2.4 kcal/mol [12], a direct comparison may not be warranted, as the experimental measurement was conducted in zwitterionic POPC lipid bilayers, while the simulations were carried out in the presence of an anionic 3:1 POPE:POPG lipid bilayer. Furthermore, the experimental result corresponds to the overall process of dimerization, rather than dimerization in a particular environment. More importantly, the simulation results suggest that dimerization is most favorable on the surface of the membrane, which indicates that it likely follows monomer adsorption to the surface and precedes dimer insertion into the membrane core. The authors also present analyses of hydrogen bonding patterns and salt bridges, and conclude that the latter, in the form of bridges formed by chloride ions between arginine residues of the two constituent monomer peptides are the dominant factor in dimer stability.

6. Molecular dynamics simulations of protegrin pores

We have so far discussed two important processes that are precursors to the bactericidal mechanism of PG-1: monomers interacting with model membranes and monomers coming together to form dimers. We now come to what is both the most difficult and the most important aspect of the problem – the formation of transmembrane pores in the bacterial membrane. Early evidence of pore formation by protegrins came from electrophysiological studies, which showed that PG-1 forms anion channels in *Xenopus laevis* oocytes [72], and that PG-1 and PG-3 (a naturally-occurring analogue of PG-1) cause an increase in the conductivity of lipid bilayers consistent with the formation of ion channels [92]. Based on neutron diffraction data, Yang and coworkers were able to crystallize membrane pore structures for protegrins and magainins [109]. More recently, Mani et al. reported solid state NMR (ssNMR) data suggesting that PG-1 forms transmembrane, NCCN multimers in 3:1 POPE:POPG lipid bilayers, where the repeat unit is an NCCN parallel dimer [74]. Combining this with the fact that membranes treated with PG-1 allowed the permeation of polyethyleneglycol beads with a hydrodynamic radius up to $10.5\text{\AA}$, the authors concluded that PG-1 forms transmembrane pores composed of four or five dimer repeat units, creating a water-filled pore with an inner diameter around $21\text{\AA}$ [74]. In contrast, they found that PG-1 dimers adsorb to the surface of mammalian-mimicking 1:1 POPC:cholesterol lipid bilayers, where they likely form tetrameric β -sheet clusters. Subsequent work from the same group using ssNMR measured distances between several ^{13}C atoms of PG-1 and ^{31}P atoms in anionic POPE/POPG lipid bilayers, from which they concluded that lipids surrounding the PG-1 pore most likely adopt a toroidal conformation (see fig. 5B) [95]. Further ssNMR experiments revealed that Arg-11 is more mobile than Arg-4 and Leu-5 in the transmembrane pore state [96], that dimethylation of the PG-1 arginine groups results in a peptide that does not aggregate or insert in a transmembrane orientation [97] and that the arginine side chains of PG-1 bind both water and lipid head groups [69]. Additionally, Wi and Kim have reported ^{31}P and ^2H ssNMR experiments that yield anisotropic line shapes

consistent with toroidal pore formation and bilayer thinning in the binding of oriented lipids with PG-1 [106].

In order to simulate the PG-1 pore structure, a realistic initial conformation must be constructed; on the simulation time scales currently accessible to molecular dynamics simulations, one cannot expect to observe spontaneous peptide insertion, aggregation or pore formation. Based on the experimental data available to date, pores are formed by four or five transmembrane, parallel NCCN PG-1 dimer units [74], and the balance of the evidence [69,95,106] suggests that the lipids surrounding the pore most likely adopt a toroidal conformation (see fig. 5B). An additional problem for simulations of a flexible pore structure embedded in a small patch of a lipid membrane is the choice of simulation ensemble [2,18,23–24,42,46]. With the traditional CHARMM force fields [70–71], most lipid bilayer simulations discussed so far have been performed in the NP_zAT ensemble, where the pressure component perpendicular to the bilayer plane, the temperature and the lateral area of the bilayer patch are kept constant, or the $NP_z\gamma T$ ensemble, where the area of the system is allowed to fluctuate, but a constant surface tension γ is applied that restrains the area. Simulations in the isotropic NPT ensemble, where the area is not constrained to a fixed value (as in NP_zAT simulations) or partially controlled with an applied surface tension (as in the $NP_z\gamma T$ ensemble) have been shown to yield unrealistic compression of the lipids [16,24,93]. The exact cause of this is a matter of some debate, which we will not delve into here [2,18,23–24,42,46]. In the case of pore simulations, the NP_zAT ensemble may suffer from compression of the lipids surrounding the pore, leading to an unrealistic pore expansion, while simulations in the NPT ensemble would result in compression of the lipids towards the pore, which could yield an unrealistic area per lipid. Simulations in the $NP_z\gamma T$ ensemble may be a good compromise between these alternatives, but no clear physical basis exists for the selection of the surface tension value γ . Very recently, a modified version of the CHARMM lipid force field has been published, which purportedly does not suffer from any of these drawbacks, and permits simulations in the NPT ensemble [53]. This should provide a significant benefit to all lipid bilayer simulations using the CHARMM force field, and particularly to simulations of flexible, membrane-inserted structures such as protegrin pores.

The first fully atomistic MD simulation of the PG-1 pore was reported by Langham and coworkers, who simulated an octameric PG-1 pore (four dimers) embedded in a 3:1 POPE:POPG anionic lipid bilayer for a total of more than 150 ns[59]. In order to circumvent some of the issues of lipid bilayer simulations described above, the authors ran their simulations in the NP_zAT ensemble for 50 ns, followed by 100 ns of NPT simulation, which yielded a reasonable value for the equilibrium area per lipid. The simulation showed that the structure proposed by NMR experiments[74] is indeed stable over a significant time scale; that the pore opening is large enough to readily conduct anions, but rarely allows cations to pass through due to the large positive charges of the peptides; and that lipids initially arranged in a barrel-stave conformation (see figure 5A) tilt towards the peptides to form what was dubbed a semi-toroidal structure (figure 5C). A comparison between this structure and the structures resulting from simulations started from fully toroidal pore conformations is currently in preparation. The authors also reported the water, sodium and chloride potentials of mean force through the pore based on the observed probability densities, and conjectured that protegrin pores can effectively eliminate the cell's ability to regulate its transmembrane potential, which could cause the failure of multiple vital membrane functions, and ultimately lead to cell death. This point was explored further in subsequent work by Bolintineanu and coworkers [7], discussed at the end of this section.

Additional MD simulations of PG-1 pore structures were reported by Jang et al. [45] and Capone et al. [14], the latter as part of a combined atomic force microscopy (AFM),

electrical conductance and MD study. Jang et al. describe the results of MD simulations of barrel stave and toroidal PG-1 pores consisting of both parallel and antiparallel NCCN oligomers in zwitterionic POPC lipid bilayers as well as anionic 4:1 POPC:POPG lipid bilayers, for a total of eight simulations. The authors used both the $NP_{\gamma}AT$ ensemble and the $NP_{\gamma}T$ ensemble with $\gamma=0$ (completely equivalent to the NPT ensemble), and noted no significant differences on the timescales of their simulations (~ 30 ns)[43]. A slight increase in the outer diameter was observed in all cases, which was slightly larger in the case of the toroidal pore simulations, while a decrease in the inner pore diameter was observed in all cases. The toroidal pores were found to be stable throughout the simulations, while the barrel-stave pores adopted a semitoroidal configuration, in which lipids near the peptide pore tilted towards the peptides, but did not result in a complete merging of the two leaflets. No clear differences were noted between the zwitterionic and anionic lipid bilayers, but the authors point out that these may play a more important role at the peptide insertion stage than in the stability of an already formed pore. In all cases, the initial pore structure was built from eight individual monomers that were not initially arranged in dimer subunits, but as the simulations progressed, interactions between monomers resulted in the formation of dimers (primarily of the NCCN variety in the antiparallel case, and CNNC in the case of the parallel arrangements). The authors note a striking similarity between PG-1 pores composed of β -strand-turn- β -strand subunits and β -Amyloid ion channels associated with Alzheimer's disease.

More recently, work from the same group combined AFM, electrical conductance measurements and MD simulations to investigate the nature of PG-1 pores [14]. The MD simulations were constructed by inserting ten PG-1 monomers in an annular arrangement in an anionic lipid bilayer composed of dioleoyl phosphatidylserine (DOPS) and palmitoyloleoyl phosphatidylethanolamine (POPE) in a 1:2 ratio of DOPS:POPE. Simulations were performed for both parallel NCCN and antiparallel NCCN motifs, with the lipids in both cases initially placed in a barrel-stave configuration. The antiparallel β -barrels were observed to organize into four subunits, while the parallel subunits were observed to organize into five subunits. Chloride ions were able to pass through the pore readily, while Na^+ and Mg^{2+} ions were not. AFM measurements confirmed the existence of channel structures only in anionic DOPS/POPE bilayers, but not in DOPC bilayers. The outer diameters of the channel structures as measured by AFM were found to be in good agreement with the results of MD simulations, and the majority of the channels detected by AFM were found to consist of three or four subunits. Electrical conductance measurements indicated stable ion channels at low PG-1 concentrations in DOPS/DOPE and DOPS/POPE anionic lipid bilayers, as well as in zwitterionic DOPC/DOPE bilayers, albeit at a higher PG-1 concentration. Bilayer membranes composed only of PC lipids showed little electrical activity. This study provided a multi-faceted analysis of PG-1 pore formation, and confirmed the strong dependence of PG-1 pore formation on membrane composition.

7. Modeling the conductance and biological effects of protegrin pores

The molecular dynamics studies just described point towards an extremely large increase in the ion conductance of membranes as a result of PG-1 channel formation, which may have crucial implications for biological activity. Modeling ion conduction through PG-1 pores is not a straightforward task. Ideally, fully atomistic MD simulations would be performed with an applied electric field, and the ionic current would be measured for different electric field strengths. However, the simulation time required to obtain a statistically meaningful value of the ionic current render this approach computationally intractable. We do note that the conductance of the classic potassium channel KcsA (conductance about 100 pS) has been simulated by this method [20] using a modified Kubo algorithm to extend the linear response (Ohm's law) regime, and it may be possible to do something similar for protegrin

pores (conductance about 200 pS). A variety of more approximate methods have been discussed for similar systems [15,40,56,78]. Bolintineanu and coworkers [7] have presented a modeling effort based on the Poisson-Nernst-Planck (PNP)[15,56] system of equations, which describes all of the ionic species as continuum concentrations (or equivalently, probability densities), while maintaining atomistic detail for the peptides and lipids. In steady-state PNP theory, the flux of each ionic species is described by a transport equation - essentially a differential mass balance - that includes a Fickian diffusion term and a drift term due to the presence of the surrounding electric field:

$$\nabla \cdot \left(D_i \nabla c_i + \frac{D_i q_i e}{k_B T} c_i \nabla \phi \right) = 0 \quad (1)$$

Where c_i , D_i and q_i are the concentration, diffusion coefficient and valence of each ionic species, ϕ is the electrostatic potential, and e , k_B and T are the elementary charge, Boltzmann's constant and temperature, respectively. The electrostatic potential is given by the Poisson equation of electrostatics, where the charge density is a sum of the fixed charge density due to the presence of partial charges in the peptides and lipids and an additional term due to the presence of the mobile ionic species:

$$\nabla \cdot (\epsilon \nabla \phi) = -\rho_f - \sum_{i=1}^N q_i c_i \quad (2)$$

Here, ϵ is the space-dependent dielectric constant, and ρ_f is the fixed charge density, determined by the positions and partial charges of all peptide and lipid atoms. The summation is performed over all ionic species, in this case only potassium and chloride. Regions that are occupied by peptide and lipid atoms are assigned as inaccessible to ions, and a no-flux boundary condition is imposed on this bounding surface. The top and bottom surfaces (above and below the lipid bilayer) are assigned concentration values corresponding to experimental conditions, and electric potential values corresponding to the magnitude of the applied electric field. Two equations corresponding to equation 1 for potassium and chloride, coupled with equation 2 were solved numerically, using a variety of pore structures obtained from earlier MD simulations [59] to define the domain and boundaries. The net ionic current was then obtained from the resulting three-dimensional concentration profiles and electrostatic potential profile by integrating the flux over any x-y plane perpendicular to the pore axis, and adding the fluxes for potassium and chloride:

$$I_z = \sum_{i=1}^N q_i \int_{x,y} \left(D_i \frac{dc_i}{dz} + \frac{D_i q_i e}{k_B T} c_i \frac{d\phi}{dz} \right) dx dy \quad (3)$$

The procedure described above was repeated for multiple values of the applied electric field in order to construct a current-voltage (I-V) relationship for the protegrin pore for each of the conformations extracted from the MD simulations. In all cases, the I-V relationship was completely linear and symmetric about the zero-voltage point, which is not surprising considering the rigid pore structure approximation inherent in the PNP model. The net conductance value was found to be in agreement with an earlier experimental study by Sokolov et al [92]. These authors found that beyond certain applied voltage values, the current dropped off significantly. Our work suggests that this apparent voltage-gating is a result of voltage-dependent pore formation; so long as the pore is formed and open, our results of 280–430 picoSiemens (pS) for the total pore conductance are in good agreement

with the experimental values of 40–360 pS in the I-V regime where current is indeed measured. The variation in the computed values is a result of using a series of different snapshots extracted from MD simulations. The only empirical adjustment made to the model is setting the diffusion coefficients of both ionic species to 10% of their bulk solution values inside the peptide pore. The same scaling was used by Kurnikova et al. [56] and Furini et al. [27] in their PNP modeling of gramicidin-S and KcSA, respectively, and was found to yield good agreement with experimental results.

Most importantly, this approach provides a quantitative link between the pore structure suggested by NMR experiments[74] and refined by MD simulations[59] and the conductance properties determined by electrophysiological methods[92]. By comparing the currents of the two ionic species, we also found that the pore is highly anion selective, with the anionic Cl^- current typically exceeding the cationic K^+ current by a factor of around 1,000. This is not surprising given the highly cationic nature of the pore, and is in agreement with the MD simulation studies discussed above [14,43,59]; however, it somewhat contradicts the experimental findings of Sokolov et al.[92], who report only a slight anion selectivity, and occasional cation selectivity depending on lipid compositions. We additionally show that the charges of the arginine residues are by far the most important factor determining the conduction properties of these pores; their removal results in a ten-fold reduction in the net conductance, and a shift from strong anion selectivity to cation selectivity.

Encouraged by our success with modeling conduction through a single PG-1 pore, we set out to model the effects of a large number of pores on an entire bacterial cell in terms of ion transport. Based on the 3D-PNP results obtained for a single PG-1 pore [7], we estimated the overall permeabilities of each ionic species through a protegrin pore in the context of a one-dimensional integrated form of the PNP equation (this is closely related to the famous Goldman-Hodgkin-Katz equation [29]). Using these single-pore permeability values, we were then able to compute the overall permeability of the membrane to each ionic species as a function of the number of pores, which was used as an input to a set of transient mass balances on all ionic species:

$$\frac{dn_i}{dt} = \frac{FV}{RT} P_i q_i A \frac{c_i^o - c_i^i e^{q_i FV/RT}}{1 - e^{q_i FV/RT}} \quad (4)$$

Here, n_i is the number of moles of ionic species i inside the cell (in this case, Na^+ , K^+ or Cl^-), P_i is the permeability of the relevant ion through the membrane (calculated based on the 3D-PNP results, and a function of the number of pores), q_i is the valence of each ionic species, A is the cell surface area, V is the transmembrane voltage, c_i^o and c_i^i are the ion concentrations inside and outside of the cell, and F , R and T are the Faraday constant, the gas constant and the temperature, respectively. The transmembrane voltage is found by requiring that the total charge inside the cell does not change with time (thereby maintaining charge neutrality), which acts as a constraint to the set of equations 4 (there are multiple such equations, one for each of the three ionic species). Our model additionally allows for changes in the cell volume and surface area, potassium flux due to active transport, and flow of water due to changes in osmolarity. The model treats the bacterial cell as a well-mixed volume with respect to ion concentrations, which is not expected to introduce any significant errors, since ion diffusion through the membrane is many orders of magnitude smaller than ion diffusion through the interior of bacteria.

The main result of this work was an estimate of the time-dependent ion concentration in the bacterial cells as a result of PG-1 pore formation. By adjusting only the number of pores in

our model, we were able to match experimentally measured potassium leakage data from live *E. coli*, which appears to correlate with the bactericidal action of protegrin [5]. We were thus able to provide the first estimate of the number of pores required to kill an *E. Coli* cell – approximately one hundred, provided they are all stable and open for several minutes. However, we were not able to match experimental data of the dynamics of optical density changes (which presumably correlate with cell volume changes), which suggests that osmolytes other than ions are involved. This model, despite many approximations and limitations inherent in the underlying theories, represents a first attempt to bridge the gap between the molecular pore structure, ion transport at the level of a single pore, and ion transport from an entire cell, which may be a key element in the protegrin bactericidal mechanism of action.

8. Alternative computational approaches: QSAR studies

All of the computational studies discussed so far have been aimed at trying to describe and explain the mechanism of action of protegrins within a physics-based framework. An altogether different approach consists of models relying largely on statistical correlations rather than purely on physical descriptions. The most common such methods are based on quantitative structure-activity relationships, or QSARs[66]. The QSAR method requires defining a set of physicochemical or structural properties among a class of proteins with similar sequences (e.g. protegrin mutants) and finding a statistical correlation between some mathematical function of these properties and a desired biological behavior (e.g. activity against bacteria or toxicity to human cells). Early work that led directly to the development and testing of a protegrin-based pharmaceutical product was based in part on the use of structure-activity relationships, and showed that the toxicity of various protegrin mutants was dependent upon the preservation of the β -sheet structure, the amphiphilicity of the peptide, and the cationic charge [17]. Even though these properties were not treated quantitatively, the resulting work led to the development and testing of a protegrin mutant dubbed IB-367 for the treatment and prevention of oral mucositis. A later study by Ostberg and Kaznessis took a more quantitative approach, and attempted to quantify a number of features of protegrin mutants from single rigid structures constructed by homology modeling based on the known structure of PG-1 [80]. Various quantities describing peptide hydrophobicity, shape and electrostatic properties were shown to be relevant in the statistical prediction of the activity, toxicity and hemolysis properties of sixty protegrin mutants against *E. coli*, with typical correlation coefficients ranging from $R^2 = 0.5$ to 0.7 . One of the likely shortcomings of this approach comes from the use of rigid structures for all mutants, which was partially addressed in a later study by Langham and coworkers, in which physicochemical properties were computed based on MD trajectories of the same sixty mutant peptides simulated in water [62]. Their QSAR model included properties such as peptide-water interaction energies, average peptide length, number of hydrogen bond acceptors and solvent accessibility of the peptide, and was created using the correlations of these and other properties with measured hemolysis and cytotoxicity values. Reported correlation coefficients for the set of sixty peptides ranged between 0.7 and 0.8 , and the model was able to correctly rank the toxicities of four out of five synthetic peptides in a blind test.

Other efforts in this area have focused on finding improved descriptors as well as improved selection and fitting procedures. Fernandez and Caballero have reported a novel method of assigning amino acid sequence descriptors which accounts for the proximity of amino acids in a sequence by assigning mathematically smoothed properties to each residue that can be weighted and added to the properties of its neighboring residues [25]. They applied their methods in the context of a QSAR study based on multiple linear regression and a genetic algorithm applied to the same protegrin mutant dataset used by Ostberg and Kaznessis [80].

Their results show that altogether different properties are required to capture activity against different microbial species, leading them to conclude that protegrins act by fundamentally different mechanisms against different microbial species. Freceer [26] analyzed a dataset of protegrin-derived cyclic peptides synthesized by Robinson et al. [83]. Based on a QSAR model constructed with a genetic function approximation algorithm, he found that antimicrobial properties of these peptides correlate well with charge and amphipathicity, while hemolytic properties correlate with lipophilicity. While QSAR studies in general do not offer the same level of physical, quantitative insight as molecular dynamics or other physics-based models, they can clearly provide strong guidelines for peptide engineering, as well as pointing towards physical properties that are most relevant to biological functions.

9. Summary and future directions

The work described herein spans a variety of computational studies aimed at understanding the mechanism of action of protegrin peptides and ultimately engineering novel therapeutics based on protegrin templates. The earliest MD simulations of protegrin peptides were conducted in lipid micelles [6,52,60–61,63], partially because of the limitations of computational power at the time, but also because this was and arguably still remains the most computationally feasible way to study multiple protegrin mutants on relevant time scales. Simulations with micelles are likely to be useful in cases where peptide conformations in a lipid environment are of crucial importance, such as the folding of otherwise linear peptides into α -helices in the presence of a hydrophobic environment; however, given the rigid nature of protegrin, only minor conformational changes are likely to be observed in micelles, and peptide-lipid interactions are better captured with more realistic membrane mimics, such as lipid bilayers. Subsequent studies have focused exclusively on lipid bilayer models, and have provided helpful verification, explanation and guidance for experimental work. For instance, MD simulations have shown the importance of peptide flexibility in evaluating the orientation of PG-1 monomers in lipid bilayers using chemical shift data [67,86–87], provided measures of peptide-induced lipid bilayer thinning [49–50,87], identified important hydrogen bonding sites, peptide conformations and lipid bilayer binding modes, demonstrated the importance of guanidinium-phosphate interactions and clarified the driving forces for protegrin dimerization in terms of hydrogen bonding, lipid bilayer thickness and salt bridges [44–45,49–50,86–87]. More sophisticated simulations that couple MD with PMF calculations have evaluated free energy profiles (PMFs) that quantify peptide orientation in a lipid bilayer [67,86], adsorption [102] and dimerization [103]. The properties of oligomeric protegrin pores have also been analyzed by several MD simulation studies [14,43,59], which confirmed their stability, demonstrated their ability to cause uncontrolled ion transport and showed a tendency for pore-lining lipids to tilt towards the peptides, favoring a toroidal or semi-toroidal arrangement. Finally, multiscale modeling of ion conductance through PG-1 pores has shown good agreement with experimental measurements [7], and has provided a quantitative, physics-based model for the effects of protegrin pores on entire bacterial cells, thereby bridging the gaps between molecular structure, ion conductance properties and cellular-level effects [5].

With computational capabilities continually improving, the future of computer modeling of antimicrobial peptides such as protegrins looks bright. In terms of MD simulations, work in all of the areas described would indisputably benefit from longer simulations with larger lipid bilayers and more realistic membrane-mimicking lipid compositions; we will not focus on such particular details in suggesting future studies, but instead outline four main areas that could yield useful and interesting results: simulations of protegrin mutants, modeling of peptide insertion, modeling of pore formation, and modeling of toxicity mechanisms. Simulations of the types discussed above with protegrin mutants are essential for identifying the effects of mutations on the biophysical behavior of protegrins – in particular,

comparisons between the wild-type PG-1 peptide and choice mutants with respect to membrane adsorption, insertion and dimerization would be of interest. Several studies have presented large datasets of protegrin mutants and their therapeutic profiles [17,80,83]. Given the highly constrained nature of protegrin peptides due to the presence of disulfide bonds, homology models based on the known structure of PG-1 are almost certain to be more than adequate as starting points for molecular dynamics simulations (provided, of course, that the mutation does not entail the removal of the disulfide bonds). Although PG-1 has been simulated in both the surface-bound and the membrane-inserted state, the transition between these two states has yet to be investigated. PMF calculations of the types presented by Vivcharuk and Kaznessis [102–103] or Rui and Im [86] would be of particular interest, as they would provide both structural and thermodynamic data for this process. Similarly, although membrane-inserted PG-1 dimers as well as transmembrane pores have been studied separately, the process of higher oligomerization and water permeation remains unclear. Longer simulations of higher oligomers (octamers and decamers) embedded in lipid bilayers may provide clues to the events responsible for water permeation, lipid tilting into the toroidal configuration and ultimately pore formation. Such simulations have been reported for melittin [89] and the magainin MH-2 peptide [68], albeit using a coarse-grained force field in the latter case.

Finally, one of the most obviously deficient areas of knowledge about PG-1 relates to its mechanism of toxicity. This is true for most other AMPs as well, both on the experimental and the computational side. NMR investigations discussed earlier have shown that PG-1 binds in fundamentally different ways to 3:1 POPE:POPG bacterial membrane mimicking lipid bilayers as compared to 1:1 POPC:cholesterol mammalian-mimicking lipid bilayers [74]. This may represent a fortuitous finding in terms of the potential to engineer peptides that retain their natural efficacy against bacterial cells and exhibit much lower toxicity towards mammalian cells, but, as far as we are aware, no other major findings on the toxicity mechanism of protegrins have been reported. A simple starting point for simulations in this area may be the comparison of membrane properties from simulations of a 1:1 POPC:cholesterol lipid bilayer to simulations that include membrane-bound PG-1 dimers. Additionally, dimerization and higher oligomerization on the surface of these bilayers may represent a key step of the toxicity mechanism, and is highly amenable to study by molecular simulations of the types described herein (ranging from regular MD simulations of surface-bound dimers to PMF calculations relating to membrane adsorption and oligomerization in the presence of these bilayers). In all cases, the recent release of the updated CHARMM c36 lipid force field [53] provides an exciting opportunity for more accurate simulations of peptide-lipid systems, while circumventing the issues associated with the choice of ensembles. It is hoped that the research reviewed herein offers a comprehensive view of computational investigations of PG-1 to date, and thereby provides a useful guide to future work in this area.

Research Highlights

- Computational studies augment experimental investigations of antimicrobial peptides.
- Simulations explain the biophysical interactions that underlie AMP biological activity.
- Simulations have promoted a consistent, mechanistic description of AMP action.
- Peptide-micelle simulations reveal correlations between structural features and function.

- Improved understanding is available with simulations of peptides in lipid bilayers.
- A combination of atomistic and continuum models provides improved insight.

Acknowledgments

This work was supported by a grant from NIH (GM 070989). Computational support from the Minnesota Supercomputing Institute (MSI) is gratefully acknowledged. This work was also partially supported by National Computational Science Alliance under TG-MCA04N033 and utilized the TeraGrid Cluster.

References

1. Aumelas A, Mangoni M, Roumestand C, Chiche L, Despaux E, Grassy G, et al. Synthesis and solution structure of the antimicrobial peptide protegrin-1. *Eur J Biochem* 1996;237:575–83. [PubMed: 8647100]
2. Berger O, Edholm O, Jahnig F. Molecular dynamics simulations of a fluid bilayer of dipalmitoylphosphatidylcholine at full hydration, constant pressure, and constant temperature. *Biophysical Journal* 1997;72:2002–13. [PubMed: 9129804]
3. Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, et al. The Protein Data Bank. *Nucleic Acids Res* 2000;28:235–42. [PubMed: 10592235]
4. Bessalle R, Kapitkovsky A, Gorea A, Shalit I, Fridkin M. All-D-magainin: chirality, antimicrobial activity and proteolytic resistance. *FEBS Lett* 1990;274:151–5. [PubMed: 2253768]
5. Bolintineanu D, Hazrati E, Davis HT, Lehrer RI, Kaznessis YN. Antimicrobial mechanism of pore-forming protegrin peptides: 100 pores to kill *E. coli*. *Peptides* 2010;31:1–8. [PubMed: 19931583]
6. Bolintineanu DS, Langham A, Davis HT, Kaznessis Y. Molecular dynamics simulations of three protegrin-type anti-microbial peptides: interplay between charges at the termini, β -sheet structure and amphiphilic interactions. *Molecular Simulation* 2007;33:809–19. [PubMed: 21113420]
7. Bolintineanu DS, Sayyed-Ahmad A, Davis HT, Kaznessis YN. Poisson-Nernst-Planck Models of Nonequilibrium Ion Electrodifffusion through a Protegrin Transmembrane Pore. *PLoS Comput Biol* 2009;5:e1000277. [PubMed: 19180178]
8. Bond PJ, Khalid S. Antimicrobial and Cell-Penetrating Peptides: Structure, Assembly and Mechanisms of Membrane Lysis via Atomistic and Coarse-Grained Molecular Dynamics Simulations. *Protein Pept Lett*. 2010
9. Brogden KA. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nat Rev Microbiol* 2005;3:238–50. [PubMed: 15703760]
10. Brooks BR, Brooks CL 3rd, Mackerell AD Jr, Nilsson L, Petrella RJ, Roux B, et al. CHARMM: the biomolecular simulation program. *J Comput Chem* 2009;30:1545–614. [PubMed: 19444816]
11. Buffy JJ, Hong T, Yamaguchi S, Waring AJ, Lehrer RI, Hong M. Solid-state NMR investigation of the depth of insertion of protegrin-1 in lipid bilayers using paramagnetic Mn²⁺. *Biophysical Journal* 2003;85:2363–73. [PubMed: 14507700]
12. Buffy JJ, Waring AJ, Hong M. Determination of peptide oligomerization in lipid bilayers using ¹⁹F spin diffusion NMR. *J Am Chem Soc* 2005;127:4477–83. [PubMed: 15783230]
13. Buffy JJ, Waring AJ, Lehrer RI, Hong M. Immobilization and aggregation of the antimicrobial peptide protegrin-1 in lipid bilayers investigated by solid-state NMR. *Biochemistry* 2003;42:13725–34. [PubMed: 14622019]
14. Capone R, Mustata M, Jang H, Arce FT, Nussinov R, Lal R. Antimicrobial protegrin-1 forms ion channels: molecular dynamic simulation, atomic force microscopy, and electrical conductance studies. *Biophys J* 2010;98:2644–52. [PubMed: 20513409]
15. Cardenas AE, Coalson RD, Kurnikova MG. Three-dimensional Poisson-Nernst-Planck theory studies: Influence of membrane electrostatics on gramicidin A channel conductance. *Biophysical Journal* 2000;79:80–93. [PubMed: 10866939]

16. Castro-Roman F, Benz RW, White SH, Tobias DJ. Investigation of finite system size effects in molecular dynamics simulations of lipid bilayers. *Journal of Physical Chemistry B* 2006;110:24157–64.
17. Chen J, Falla TJ, Liu H, Hurst MA, Fujii CA, Mosca DA, et al. Development of protegrins for the treatment and prevention of oral mucositis: structure-activity relationships of synthetic protegrin analogues. *Biopolymers* 2000;55:88–98. [PubMed: 10931444]
18. Chiu SW, Clark M, Balaji V, Subramaniam S, Scott HL, Jakobsson E. Incorporation of surface tension into molecular dynamics simulation of an interface: a fluid phase lipid bilayer membrane. *Biophys J* 1995;69:1230–45. [PubMed: 8534794]
19. Cornell WD, Cieplak P, Bayly CI, Gould IR, Merz KM, Ferguson DM, et al. A second generation force field for the simulation of proteins, nucleic acids, and organic molecules. 1995;117:5179. *Journal of the American Chemical Society* 1996;118:2309.
20. de Haan HW, Tolokh IS, Gray CG, Goldman S. Nonequilibrium molecular dynamics calculation of the conductance of the KcsA potassium ion channel. *Phys Rev E* 2006;74:030905.
21. Epand RM, Vogel HJ. Diversity of antimicrobial peptides and their mechanisms of action. *Biochim Biophys Acta* 1999;1462:11–28. [PubMed: 10590300]
22. Fahrner RL, Dieckmann T, Harwig SS, Lehrer RI, Eisenberg D, Feigon J. Solution structure of protegrin-1, a broad-spectrum antimicrobial peptide from porcine leukocytes. *Chem Biol* 1996;3:543–50. [PubMed: 8807886]
23. Feller SE, Pastor RW. On simulating lipid bilayers with an applied surface tension: periodic boundary conditions and undulations. *Biophys J* 1996;71:1350–5. [PubMed: 8874010]
24. Feller SE, Pastor RW. Constant surface tension simulations of lipid bilayers: The sensitivity of surface areas and compressibilities. *Journal of chemical physics* 1999;111:1281–7.
25. Fernandez M, Caballero J. Analysis of protegrin structure-activity relationships: the structural characteristics important for antimicrobial activity using smoothed amino acid sequence descriptors. *Molecular Simulation* 2007;33:689–702.
26. Frecer V. QSAR analysis of antimicrobial and haemolytic effects of cyclic cationic antimicrobial peptides derived from protegrin-1. *Bioorg Med Chem* 2006;14:6065–74. [PubMed: 16714114]
27. Furini S, Zerbetto F, Cavalcanti S. Application of the Poisson-Nernst-Planck theory with space-dependent diffusion coefficients to KcsA. *Biophys J* 2006;91:3162–9. [PubMed: 16877513]
28. Gidalevitz D, Ishitsuka Y, Muresan AS, Konovalov O, Waring AJ, Lehrer RI, et al. Interaction of antimicrobial peptide protegrin with biomembranes. *Proc Natl Acad Sci U S A* 2003;100:6302–7. [PubMed: 12738879]
29. Goldman DE. Potential, Impedance and Rectification in Membranes. *Journal of General Physiology* 1943;27:37–60. [PubMed: 19873371]
30. Gottler LM, Bea RD, Shelburne CE, Ramamoorthy A, Marsh ENG. Using fluoruous amino acids to probe the effects of changing hydrophobicity on the physical and biological properties of the beta-hairpin antimicrobial peptide protegrin-1. *Biochemistry* 2008;47:9243–50. [PubMed: 18693751]
31. Hale JD, Hancock RE. Alternative mechanisms of action of cationic antimicrobial peptides on bacteria. *Expert Rev Anti Infect Ther* 2007;5:951–9. [PubMed: 18039080]
32. Hancock RE, Lehrer R. Cationic peptides: a new source of antibiotics. *Trends Biotechnol* 1998;16:82–8. [PubMed: 9487736]
33. Hancock RE, Rozek A. Role of membranes in the activities of antimicrobial cationic peptides. *FEMS Microbiol Lett* 2002;206:143–9. [PubMed: 11814654]
34. Harwig SSL, Waring A, Yang HJ, Cho Y, Tan L, Lehrer RI. Intramolecular disulfide bonds enhance the antimicrobial and lytic activities of protegrins at physiological sodium chloride concentrations. *Eur J Biochem* 1996;240:352–7. [PubMed: 8841398]
35. Heller WT, Waring AJ, Lehrer RI, Harroun TA, Weiss TM, Yang L, et al. Membrane thinning effect of the beta-sheet antimicrobial protegrin. *Biochemistry* 2000;39:139–45. [PubMed: 10625488]
36. Heller WT, Waring AJ, Lehrer RI, Huang HW. Multiple states of beta-sheet peptide protegrin in lipid bilayers. *Biochemistry* 1998;37:17331–8. [PubMed: 9860847]
37. Hill, TL. *An Introduction to Statistical Thermodynamics*. New York: Dover; 1987.

38. Huang HW, Chen FY, Lee MT. Molecular mechanism of peptide-induced pores in membranes. *Phys Rev Lett* 2004;92.
39. Im W, Beglov D, Roux B. Continuum Solvation Model: computation of electrostatic forces from numerical solutions to the Poisson-Boltzmann equation. *Computer physics communications* 1998;111:59–75.
40. Im W, Roux B. Ion permeation and selectivity of OmpF porin: a theoretical study based on molecular dynamics, Brownian dynamics, and continuum electrodiffusion theory. *J Mol Biol* 2002;322:851–69. [PubMed: 12270719]
41. Ishitsuka Y, Pham DS, Waring AJ, Lehrer RI, Lee KY. Insertion selectivity of antimicrobial peptide protegrin-1 into lipid monolayers: effect of head group electrostatics and tail group packing. *Biochim Biophys Acta* 2006;1758:1450–60. [PubMed: 16989771]
42. Jahnig F. What is the surface tension of a lipid bilayer membrane? *Biophys J* 1996;71:1348–9. [PubMed: 8874009]
43. Jang H, Ma B, Lal R, Nussinov R. Models of toxic beta-sheet channels of protegrin-1 suggest a common subunit organization motif shared with toxic alzheimer beta-amyloid ion channels. *Biophys J* 2008;95:4631–42. [PubMed: 18708452]
44. Jang H, Ma B, Woolf TB, Nussinov R. Interaction of protegrin-1 with lipid bilayers: Membrane thinning effect. *Biophysical Journal* 2006;91:2848–59. [PubMed: 16861271]
45. Jang H, Ma BY, Nussinov R. Conformational study of the protegrin-I (PG-I) dimer interaction with lipid bilayers and its effect. *Bmc Struct Biol* 2007;7. [PubMed: 17313689]
46. Jensen MO, Mouritsen OG, Peters GH. Simulations of a membrane-anchored peptide: structure, dynamics, and influence on bilayer properties. *Biophys J* 2004;86:3556–75. [PubMed: 15189854]
47. Jenssen H, Hamill P, Hancock RE. Peptide antimicrobial agents. *Clin Microbiol Rev* 2006;19:491–511. [PubMed: 16847082]
48. Kandasamy SK, Larson RG. Binding and insertion of alpha-helical anti-microbial peptides in POPC bilayers studied by molecular dynamics simulations. *Chem Phys Lipids* 2004;132:113–32. [PubMed: 15530453]
49. Kandasamy SK, Larson RG. Binding modes of protegrin-1, a beta-strand antimicrobial peptide, in lipid bilayers. *Molecular Simulation* 2007;33:799–807.
50. Khandelia H, Kaznessis YN. Structure of the antimicrobial beta-hairpin peptide protegrin-1 in a DLPC lipid bilayer investigated by molecular dynamics simulation. *Biochim Biophys Acta* 2007;1768:509–20. [PubMed: 17254546]
51. Khandelia H, Kaznessis YN. Corrigendum to “Structure of the antimicrobial beta-hairpin peptide protegrin-1 in a DLPC lipid bilayer investigated by molecular dynamics simulation” [Biochim. Biophys. Acta 1768 (2007) 509–520]. *Biochim Biophys Acta* 2010;1798:1623.
52. Khandelia H, Langham AA, Kaznessis YN. Driving engineering of novel antimicrobial peptides from simulations of peptide-micelle interactions. *Biochim Biophys Acta* 2006;1758:1224–34. [PubMed: 16753133]
53. Klauda JB, Venable RM, Freites JA, O'Connor JW, Tobias DJ, Mondragon-Ramirez C, et al. Update of the CHARMM All-Atom Additive Force Field for Lipids: Validation on Six Lipid Types. *J Phys Chem B*. 2010
54. Kokryakov VN, Harwig SS, Panyutich EA, Shevchenko AA, Aleshina GM, Shamova OV, et al. Protegrins: leukocyte antimicrobial peptides that combine features of corticostatic defensins and tachyplesins. *FEBS Lett* 1993;327:231–6. [PubMed: 8335113]
55. Kollman PA, Massova I, Reyes C, Kuhn B, Huo SH, Chong L, et al. Calculating structures and free energies of complex molecules: Combining molecular mechanics and continuum models. *Accounts Chem Res* 2000;33:889–97.
56. Kurnikova MG, Coalson RD, Graf P, Nitzan A. A lattice relaxation algorithm for three-dimensional Poisson-Nernst-Planck theory with application to ion transport through the gramicidin A channel. *Biophys J* 1999;76:642–56. [PubMed: 9929470]
57. Lai JR, Epand RF, Weisblum B, Epand RM, Gellman SH. Roles of salt and conformation in the biological and physicochemical behavior of protegrin-1 and designed analogues: correlation of antimicrobial, hemolytic, and lipid bilayer-perturbing activities. *Biochemistry* 2006;45:15718–30. [PubMed: 17176094]

58. Lam KL, Ishitsuka Y, Cheng Y, Chien K, Waring AJ, Lehrer RI, et al. Mechanism of supported membrane disruption by antimicrobial peptide protegrin-1. *J Phys Chem B* 2006;110:21282–6. [PubMed: 17048957]
59. Langham AA, Ahmad AS, Kaznessis YN. On the nature of antimicrobial activity: a model for protegrin-1 pores. *J Am Chem Soc* 2008;130:4338–46. [PubMed: 18335931]
60. Langham AA, Kaznessis YN. Effects of mutations on the C-terminus of protegrin-1: a molecular dynamics simulation study. *Molecular Simulation* 2006;32:193–201.
61. Langham AA, Khandelia H, Kaznessis YN. How can a beta-sheet peptide be both a potent antimicrobial and harmfully toxic? Molecular dynamics simulations of protegrin-1 in micelles. *Biopolymers* 2006;84:219–31. [PubMed: 16235232]
62. Langham AA, Khandelia H, Schuster B, Waring AJ, Lehrer RI, Kaznessis YN. Correlation between simulated physicochemical properties and hemolysis of protegrin-like antimicrobial peptides: predicting experimental toxicity. *Peptides* 2008;29:1085–93. [PubMed: 18455267]
63. Langham AA, Waring AJ, Kaznessis YN. Comparison of interactions between beta-hairpin decapeptides and SDS/DPC micelles from experimental and simulation data. *BMC Biochem* 2007;8:11. [PubMed: 17634088]
64. Lazaridis T. Effective energy function for proteins in lipid membranes. *Proteins-Structure Function and Genetics* 2003;52:176–92.
65. Lazaridis T, Karplus M. Effective energy function for proteins in solution. *Proteins-Structure Function and Genetics* 1999;35:133–52.
66. Leach, AR. *Molecular modelling: principles and applications*. Englewood Cliffs, N.J.: Prentice Hall; 2001.
67. Lee J, Ham S, Im W. Beta-hairpin restraint potentials for calculations of potentials of mean force as a function of beta-hairpin tilt, rotation, and distance. *J Comput Chem* 2009;30:1334–43. [PubMed: 19009593]
68. Leontiadou H, Mark AE, Marrink SJ. Antimicrobial peptides in action. *J Am Chem Soc* 2006;128:12156–61. [PubMed: 16967965]
69. Li S, Su Y, Luo W, Hong M. Water-protein interactions of an arginine-rich membrane peptide in lipid bilayers investigated by solid-state nuclear magnetic resonance spectroscopy. *J Phys Chem B* 2010;114:4063–9. [PubMed: 20199036]
70. MacKerell AD Jr, Feig M, Brooks CL 3rd. Improved treatment of the protein backbone in empirical force fields. *J Am Chem Soc* 2004;126:698–9. [PubMed: 14733527]
71. MacKerell JAD, Bashford D, Bellott M, Dunbrack RL Jr, Evanseck JD, Field MJ, Fischer S, Gao J, Guo H, Ha S, Joseph-McCarthy D, Kuchnir L, Kucera K, Lau FTK, Mattos C, Michnick S, Ngo T, Nguyen DT, Prodhom B, Reiher WE III, Roux B, Schlenkrich M, Smith JC, Stote R, Straub J, Watanabe M, Wiorkiewicz-Kuczera J, Yin D, Karplus M. All-atom empirical potential for molecular modeling and dynamics Studies of proteins. *Journal of Physical Chemistry B* 1998;102:3586–616.
72. Mangoni ME, Aumelas A, Charnet P, Roumestand C, Chiche L, Despau E, et al. Change in membrane permeability induced by protegrin 1: implication of disulphide bridges for pore formation. *FEBS Lett* 1996;383:93–8. [PubMed: 8612801]
73. Mani R, Buffy JJ, Waring AJ, Lehrer RI, Hong M. Solid-state NMR investigation of the selective disruption of lipid membranes by protegrin-1. *Biochemistry* 2004;43:13839–48. [PubMed: 15504046]
74. Mani R, Cady SD, Tang M, Waring AJ, Lehrer RI, Hong M. Membrane-dependent oligomeric structure and pore formation of a beta-hairpin antimicrobial peptide in lipid bilayers from solid-state NMR. *Proc Natl Acad Sci U S A* 2006;103:16242–7. [PubMed: 17060626]
75. Mani R, Tang M, Wu X, Buffy JJ, Waring AJ, Sherman MA, et al. Membrane-bound dimer structure of a beta-hairpin antimicrobial peptide from rotational-echo double-resonance solid-state NMR. *Biochemistry* 2006;45:8341–9. [PubMed: 16819833]
76. Mani R, Waring AJ, Lehrer RI, Hong M. Membrane-disruptive abilities of beta-hairpin antimicrobial peptides correlate with conformation and activity: a ³¹P and ¹H NMR study. *Biochim Biophys Acta* 2005;1716:11–8. [PubMed: 16182236]

77. Matyus E, Kandt C, Tieleman DP. Computer simulation of antimicrobial peptides. *Curr Med Chem* 2007;14:2789–98. [PubMed: 18045125]
78. Noskov SY, Im W, Roux B. Ion permeation through the alpha-hemolysin channel: theoretical studies based on Brownian dynamics and Poisson-Nernst-Planck electrodiffusion theory. *Biophys J* 2004;87:2299–309. [PubMed: 15454431]
79. Oren Z, Shai Y. Mode of action of linear amphipathic alpha-helical antimicrobial peptides. *Biopolymers* 1998;47:451–63. [PubMed: 10333737]
80. Ostberg N, Kaznessis Y. Protegrin structure-activity relationships: using homology models of synthetic sequences to determine structural characteristics important for activity. *Peptides* 2005;26:297–306.
81. Pathan FK, Venkata DA, Panguluri SK. Recent patents on antimicrobial peptides. *Recent Pat DNA Gene Seq* 2010;4:10–6. [PubMed: 20218955]
82. Qu XD, Harwig SS, Shafer WM, Lehrer RI. Protegrin structure and activity against *Neisseria gonorrhoeae*. *Infect Immun* 1997;65:636–9. [PubMed: 9009324]
83. Robinson JA, Shankaramma SC, Jettera P, Kienzl U, Schwendener RA, Vrijbloed JW, et al. Properties and structure-activity studies of cyclic beta-hairpin peptidomimetics based on the cationic antimicrobial peptide protegrin I. *Bioorgan Med Chem* 2005;13:2055–64.
84. Roumestand C, Louis V, Aumelas A, Grassy G, Calas B, Chavanieu A. Oligomerization of protegrin-1 in the presence of DPC micelles. A proton high-resolution NMR study. *FEBS Lett* 1998;421:263–7. [PubMed: 9468319]
85. Roux B. The calculation of the potential of mean force using computer simulations. *Computer physics communications* 1995;91:275–82.
86. Rui H, Im W. Protegrin-1 orientation and physicochemical properties in membrane bilayers studied by potential of mean force calculations. *J Comput Chem*. 2010
87. Rui H, Lee J, Im W. Comparative Molecular Dynamics Simulation Studies of Protegrin-1 Monomer and Dimer in Two Different Lipid Bilayers. *Biophysical Journal* 2009;97:787–95. [PubMed: 19651037]
88. Sayyed-Ahmad A, Kaznessis YN. Determining the orientation of protegrin-1 in DLPC bilayers using an implicit solvent-membrane model. *PLoS One* 2009;4:e4799. [PubMed: 19277199]
89. Sengupta D, Leontiadou H, Mark AE, Marrink SJ. Toroidal pores formed by antimicrobial peptides show significant disorder. *Biochim Biophys Acta* 2008;1778:2308–17. [PubMed: 18602889]
90. Sharp KA, Honig B. Electrostatic Interactions in Macromolecules - Theory and Applications. *Annu Rev Biophys Bio* 1990;19:301–32.
91. Sitkoff D, BenTal N, Honig B. Calculation of alkane to water solvation free energies using continuum solvent models. *J Phys Chem-Us* 1996;100:2744–52.
92. Sokolov Y, Mirzabekov T, Martin DW, Lehrer RI, Kagan BL. Membrane channel formation by antimicrobial protegrins. *Biochim Biophys Acta* 1999;1420:23–9. [PubMed: 10446287]
93. Sonne J, Jensen MO, Hansen FY, Hemmingsen L, Peters GH. Reparameterization of all-atom dipalmitoylphosphatidylcholine lipid parameters enables simulation of fluid bilayers at zero tension. *Biophysical Journal* 2007;92:4157–67. [PubMed: 17400696]
94. Tang M, Waring AJ, Hong M. Intermolecular packing and alignment in an ordered beta-hairpin antimicrobial peptide aggregate from 2D solid-state NMR. *Journal of the American Chemical Society* 2005;127:13919–27. [PubMed: 16201813]
95. Tang M, Waring AJ, Hong M. Phosphate-mediated arginine insertion into lipid membranes and pore formation by a cationic membrane peptide from solid-state NMR. *J Am Chem Soc* 2007;129:11438–46. [PubMed: 17705480]
96. Tang M, Waring AJ, Hong M. Arginine dynamics in a membrane-bound cationic beta-hairpin peptide from solid-state NMR. *Chembiochem* 2008;9:1487–92. [PubMed: 18442147]
97. Tang M, Waring AJ, Lehrer RI, Hong M. Effects of guanidinium-phosphate hydrogen bonding on the membrane-bound structure and activity of an arginine-rich membrane peptide from solid-state NMR spectroscopy. *Angew Chem Int Ed Engl* 2008;47:3202–5. [PubMed: 18338418]

98. Tanizaki S, Feig M. A generalized Born formalism for heterogeneous dielectric environments: Application to the implicit modeling of biological membranes. *Journal of Chemical Physics* 2005;122.
99. Tieleman DP, Sansom MS. Molecular dynamics simulations of antimicrobial peptides: From membrane binding to trans-membrane channels. *Int J Quant Chem* 2001;83:166–79.
100. van Gunsteren, WF.; Billeter, SR.; Eising, AA.; Hünenberger, PH.; Krüger, P.; Mark, AE., et al. *Biomolecular Simulation: The GROMOS96 Manual and User Guide*. Zurich, Groningen: vdf Hochschulverlag AG an der ETH Zürich and BIOMOS b.v; 1996.
101. Verwey, EJW.; Overbeek, JTG. *Theory of the Stability of Lyophobic Colloids*. New York: Elsevier; 1948.
102. Vivcharuk V, Kaznessis Y. Free energy profile of the interaction between a monomer or a dimer of protegrin-1 in a specific binding orientation and a model lipid bilayer. *J Phys Chem B* 2010;114:2790–7. [PubMed: 20136112]
103. Vivcharuk V, Kaznessis YN. Dimerization of protegrin-1 peptides in different environments. *Int J Mol Sci*. 2010 Submitted June 2010.
104. Vivcharuk V, Tomberli B, Tolokh IS, Gray CG. Prediction of binding free energy for adsorption of antimicrobial peptide lactoferricin B on a POPC membrane. *Phys Rev E Stat Nonlin Soft Matter Phys* 2008;77:031913. [PubMed: 18517428]
105. Wade D, Boman A, Wahlin B, Drain CM, Andreu D, Boman HG, et al. All-D amino acid-containing channel-forming antibiotic peptides. *Proc Natl Acad Sci U S A* 1990;87:4761–5. [PubMed: 1693777]
106. Wi S, Kim C. Pore structure, thinning effect, and lateral diffusive dynamics of oriented lipid membranes interacting with antimicrobial peptide protegrin-1: 31P and 2H solid-state NMR study. *J Phys Chem B* 2008;112:11402–14. [PubMed: 18700738]
107. Wu M, Maier E, Benz R, Hancock RE. Mechanism of interaction of different classes of cationic antimicrobial peptides with planar bilayers and with the cytoplasmic membrane of *Escherichia coli*. *Biochemistry* 1999;38:7235–42. [PubMed: 10353835]
108. Yamaguchi S, Hong T, Waring A, Lehrer RI, Hong M. Solid-state NMR investigations of peptide-lipid interaction and orientation of a beta-sheet antimicrobial peptide, protegrin. *Biochemistry* 2002;41:9852–62. [PubMed: 12146951]
109. Yang L, Weiss TM, Lehrer RI, Huang HW. Crystallization of antimicrobial pores in membranes: magainin and protegrin. *Biophys J* 2000;79:2002–9. [PubMed: 11023904]
110. Yeaman MR, Yount NY. Mechanisms of antimicrobial peptide action and resistance. *Pharmacol Rev* 2003;55:27–55. [PubMed: 12615953]
111. Zasloff M. Antimicrobial peptides of multicellular organisms. *Nature* 2002;415:389–95. [PubMed: 11807545]

Figure 1.

The structure of PG-1, the most common naturally occurring protegrin variant. Arginine side chains are shown in blue ball-and-stick representation, cysteine-cysteine disulfide bonds are shown as yellow bonds, and the backbone is shown as a ribbon. The amphipathic nature of the molecule can be seen by the color-coding of the backbone: green corresponds to weakly hydrophilic, neutral residues; blue corresponds to charged, hydrophilic residues, and white corresponds to hydrophobic residues.

Figure 2.

The key steps in the mechanism of action of PG-1. A) Adsorption of PG-1 to the surface of anionic bacterial membranes. B) Insertion of peptides into the membrane core C) Dimerization of PG-1 (may occur prior to insertion). D) Oligomerization of dimers to form higher number aggregates and transmembrane pores that cause uncontrolled ion leakage (toroidal pore shown).

Figure 3. The definitions of the tilt angle (τ) and rotation angle (ϕ) as per references [50] and [108]. The tilt angle τ describes the angle between the peptide principal axis and the bilayer normal, while the rotation angle ϕ describes the angle between the normal of the least square fit plane to the peptide backbone and the bilayer normal.

Figure 4. The two experimentally observed modes of PG-1 dimerization. A. Parallel NCCN alignment, observed in phosphate-buffered saline solution and POPC lipid bilayers. B. Anti-parallel NCCN alignment, only observed in DPC micelles.

Figure 5.

The different models of lipid topologies surrounding the PG-1 transmembrane pore. A. The barrel-stave pore, where lipids retain their alignment with the bilayer normal. B. The toroidal pore, where lipids tilt fully towards the pore to create a continuous leaflet that completely lines the outside of the pore. C. The semi-toroidal pore, where lipids tilt partially towards the peptides, but do not form a continuous leaflet.

Figure 6.