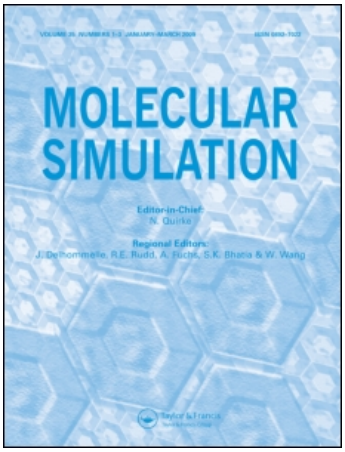

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Effects of mutations on the C-terminus of protegrin-1: a molecular dynamics simulation study

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Effects of mutations on the C-terminus of protegrin-1: a molecular dynamics simulation study

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In this work the effects of the charge of the C-terminus of protegrin-like peptides on activity and toxicity are examined by molecular dynamics simulations. Simulations are done in sodium dodecylsulphate and dodecylphosphocholine micelles, bacterial and mammalian membrane mimics, respectively. Three protegrin mutants are examined and it is found that while the peptides interact in different ways, the peptides all insert into the SDS micelles equally as deep, in agreement with their equal activities as determined by previous experimental work. There are clear differences in the interactions with the DPC micelles and it is demonstrated that simulations with DPC micelles can predict levels of toxicity of such peptides. We also see that removing the positive charge from the sequence of protegrin-1 does not have positive effects on the resulting peptide's toxicity, but that replacing the positive charge with a negative charge reduces the toxicity.

Keywords: Protegrin; Molecular dynamics simulations; Antimicrobial peptides; SDS micelles; DPC micelles

1. Introduction

In the two decades since Michael Zasloff first discovered the antimicrobial peptide (AMP) magainin, hundreds of other such peptides have been discovered from nearly all types of living organisms [1,2]. However, though we know now that AMPs kill microbial cells by disruption of the cell membrane lipid bilayer, our knowledge and understanding of exactly how this occurs is still not at the level necessary to successfully design new peptides for use as replacements for traditional antibiotics in humans.

Even though AMPs differ greatly in structure, sequence and functional activity, it is known that most are cationic and amphipathic [3]. Arginine and lysine have been shown to bind to the anionic bacterial cell envelopes and hydrophobic residues have been shown to be important in the interaction with the hydrophobic core [4]. Because many AMPs are not only active against microbial species but also toxic to humans, there have been attempts to modify active peptides to reduce host-toxicity levels and improve specificity toward pathogenic cells. In general, increasing positive charge and lowering the hydrophobic content, within reasonable limits, improves the specificity of peptides towards bacterial species. Magainin is one example of a peptide that has been the subject of such studies [5–7]. Unfortunately, the success of such approaches has been limited, mainly because the underlying molecular mechanism of cell lysis is still not fully understood. Understanding the interactions between AMPs and the membrane surface is of significant importance in characterising their mode of action. From examining the interactions of specific residues with micelles, we hope to determine which regions of the sequence contribute to the activity or toxicity of the peptide, and will then apply this information to the design of new AMPs.

In this study, we use molecular dynamics (MD) simulations to determine the effects caused by mutations on the sequence of protegrin-1 at a molecular level. Protegrins, a family of five potent β-hairpin peptides, were originally isolated from porcine leukocytes. Their structure is held in place by two disulfide bonds. Protegrin-1 (PG-1, RGGR LCYC RRRF CVCV GR) can launch a rapid response to infection by diverse bacterial species [8] such as *Escherichia coli*, *Candida albicans* and *Listeria monocytogenes* [9]. A long-term goal is the design of a protegrin-like peptide suitable for prevention of the transmission of sexually transmitted diseases [10], specifically those caused

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by *Neisseria gonorrhoeae* [11], *Chlamydia trachomatis* [12], and HIV type-1 virions [11,13]. In order to design such a peptide, there is a need to understand the sequence or structural components of the protegrins that are responsible for the hemolytic and cytotoxic properties. The three peptides that we are investigating in this work are related analogues of PG-1. The three peptides are equally active against Gram negative bacteria, but have different levels of toxicity (table 1) (R.I. Lehrer, personal communication with Y.N.K.).

In order to elucidate the structural or sequence basis for the varying levels of toxicity in these three protegrins, we have simulated their interactions with dodecylphosphocholine (DPC) micelles and sodium dodecylsulphate (SDS) micelles, mammalian and bacterial membrane mimics, respectively. Like lipid bilayers, micelles possess a well-defined hydrophobic core and a flexible, hydrophilic interface and are commonly used in place of monolayers or bilayers in experimental methods such as NMR spectroscopy [14–17]. MD studies of micelles have been performed for many types of micelles, including anionic micelles such as SDS [18–22]; zwitterionic micelles such as DPC [23,24]; and a few mixed composition micelles [25,26]. DPC micelles are considered to be good models of eukaryotic cell membranes, which are generally rich in zwitterionic phospholipids. SDS is used as a mimic for the negatively charged molecules found in bacterial membranes [27], because it possesses an anionic exterior and a hydrophobic interior [22,28–31]. The differences between the SDS micelle and the DPC micelle provide a basis for the study of the activity and toxicity of AMPs.

MD simulations offer a molecular level picture of peptide-membrane interactions that is necessary to supplement the limited experimental information available. Most of the previous MD simulation studies of peptides, antimicrobial and otherwise, have focused on using lipid bilayers as a membrane mimic (for reviews on lipid bilayer simulations please see [32–34]). However, just as micelles are used in NMR for practical reasons, there are several advantages for the use of micelles over lipid bilayers in simulations. One of the main concerns in MD simulations is the constraints on current computational capabilities, which generally restrict large simulations to the order of tens of nanoseconds. The micelle–peptide–water system contains approximately half the number of atoms of a typical 128-molecule lipid bilayer–peptide–water simulation. This permits the fully atomistic micelle system to be simulated for a significantly longer time, allowing for monitoring of biological phenomena over longer time scales using the same computational resources. Additionally, the time scales of lipid motion must be considered. The time scales of motion are significantly faster for micelles than for lipid bilayers. Micelle relaxation times have been shown, through experiment [35] and simulation, to be on the order of 500–1000 ps [23,36–38], in contrast with lipid bilayers, which require tens of nanoseconds [32]. For a study such as this one, involving simulations of three peptides in two different systems, it would not be feasible to simulate six systems for the hundreds of nanoseconds required by lipid bilayers.

Another problem with lipid bilayers lies in the difficulty in initialising the system. Clearly we cannot simulate long enough to sample the entire configurational phase space of a system. Lipid bilayer simulations are usually initialised based on prior knowledge of the orientation and position of the peptide with respect to the interface; however, all-atom MD simulations of lipid bilayers where the starting conformation is far from the experimentally observed conformation may not converge to the correct equilibrium state in a feasible length simulation. La Rocca [39] noted that one of the main problems with AMP–lipid bilayer simulations lies in the initial set up of the system. This need for prior knowledge of the binding conformation, and the restrictions on the accessible time scales has hindered the predictive ability of lipid bilayer simulations. Rarely is specific information available about the location of the peptide in a lipid bilayer and so one has to assume a starting orientation and position for the peptide and that choice has an influence on the behaviour of the peptide and on the conformation of the system at the end of the simulation. This problem is overcome in micelles, in part, due to their spherical symmetry and also by starting with the peptide inside the micelle. Additionally, for micelles we have completed simulations of PG-1 with starting configurations both inside and outside of the micelle and shown that they converge to the same conformation [40].

There have been many successful studies in which micelle membrane mimics have been used to provide a detailed picture of the interaction with a peptide [24,37,41,42]. Using NMR techniques, van den Hooven and coworkers studied nisin in both DPC and SDS micelles in order to determine its structure–function relationship [31]. In addition to our work using MD simulations to examine the interactions of β-sheet and α-helical AMPs in micelles [40,43–46], there have been several other studies using micellar membrane mimics, directly comparing the results to simulations in bilayers. Bond and coworkers have examined OmpA in SDS

Table 1. Activity and toxicity of the three protegrins studied and protegrin-1. Hemolysis is given by the percent of red blood cells killed at 80 μg/ml of the peptide. Cytotoxicity is measured as the concentration of peptide required to kill 50% of human cervical epithelial cells.

Name	*Sequence*	*E. coli activity (μg/ml)*	*P. aeruginosa activity (μg/ml)*	*Hemolysis (%)*	*Cytotoxicity (μg/ml)*
PG-1	RGGRLCYCRRRFCVCVGR	0.9	0.9	>50	25–50
PC101	RGGRLCYCRRRFCVCT	0.7	1.4	12–25	100–200
PC104	RGGRLCYCRRRFCVCI	0.6	1	>50	25–50
PC107	RGGRLCYCRRRFCVCE	0.6	0.5	0–3	100–200

micelles and a DMPC bilayer. They found in this study, and in previous studies using only micelles, that the micelle environment is a suitable membrane mimic [18,19,47]. Lague and coworkers performed a similar study of the influenza hemagglutinin fusion peptide in DPC micelle and a POPC bilayer, and reached similar conclusions that though there are some differences between the two environments, specifically an increase in the solvation and rigidity of the peptide in the micelle, micelles are valid surrogates for bilayers [48]. We have also previously studied the HIV-1 fusion peptide FP-1 in an SDS micelle, verifying the equilibrium conformation and location with respect to the membrane mimic [45,49].

In the following, we describe the simulation method used, present the results and discuss the hypotheses we can generate about the effects of charge on the C-terminus of protegrin-like peptides.

2. Methods

The simulations of PC101, PC104, and PC107 in SDS and DPC micelles were carried out using the same methods described in our previous work with HIV-1 fusion peptide, protegrin-1, PC72 and PC73 [40,45,46]. Peptides with mutations at the C-terminus were chosen based on the availability of experimental toxicity and activity data for the chosen peptides. For a more complete review of our simulation method please refer to [40]. Structures for PC101, PC104, and PC107 were created using homology modeling as described in [50], a reliable method given the level of sequence similarity and the highly constrained nature of the β-sheet structure of all four peptides. The starting coordinates of the SDS micelle–water complex were obtained from simulations carried out by MacKerell [21], and the DPC micelle–water complex coordinates were obtained from Wong and coworkers [37]. The system is composed of a 60-molecule micelle, 4375 water molecules (4377 for the DPC system), 0.15 mM NaCl electrolyte, a single peptide and appropriate counterions. Water is modeled using the TIP3P potential in a 54.14 Å cube [51].

All simulations were started with the peptide placed in the micelle with the micelle and peptide centres of mass overlapping. Due to the spherical symmetry of the micelle, the orientation of the peptide in the interior is unimportant. Though this choice of starting configuration will prohibit the ability to use the simulation to determine the mechanism by which an AMP enters a membrane, the final conformation can be used to determine the specific interactions between residues and the micelle that are responsible for the activity or toxicity of the peptide. The system was minimised to remove initial bad contacts between the peptide and micelle for a total of 40,000 steps as described in our previous work [40,45,46]. The system was then heated to 303.15 K and after 500 ps of equilibration the assembly was subjected to NPT dynamics in the constant pressure–temperature module of CHARMM at a constant temperature of 303.15 K and a constant pressure of 1 atm. The leap-frog integrator with 2 femtosecond time step was used. The CHARMM program, its force field and parameters are described in detail by both Brooks [52] and MacKerell *et al.* [53]

Simulations were stopped approximately eight to ten nanoseconds after the point at which the peptide shows no change in its location from the centre of the micelle, a minimum of 25 ns for each system.

3. Results

In the following discussion, the β-hairpin structure of the peptide will be alluded to by references to the first and second strands. The first strand includes the first nine residues, Arg-10 and Arg-11 make up the turn region, and residues twelve through sixteen compose the second strand.

Shown in figure 1 are final views of the systems. The peptides move to the interface of the micelle and water in both SDS and DPC micelles. There are distinct differences in the orientation of the peptide, which will be explored further in subsequent sections. In SDS, PC101 and PC104 take similar positions with respect to the micelle surface, inserting sideways. PC107 interacts differently, positioning itself parallel to the micelle surface. In DPC, PC101 appears to be most deeply inserted into the micelle, while PC107 has little interaction with this micelle.

3.1 Centre of mass distances

To quantify the positions shown in figure 1, we calculated the distance between the micelle centre of mass and the peptide centre of mass. In SDS, the bacterial membrane mimic, all three peptides converge to the same relative location with respect to the micelle core. These results are in agreement with the experimental results that show all three peptides being equally as active. In DPC, there is a difference between the toxic and non-toxic peptides. PC101 and PC104 are toxic, and remain near the DPC micelle–water interface. PC107 is non-hemolytic and only slightly cytotoxic, and we see that it moves from the DPC micelle out into the bulk water. These results show that we can differentiate between toxic and non-toxic peptides through our simulations (figure 2).

3.2 Electron density profiles

Electron density profiles were calculated for each major functional group in the system and for each residue of the peptide. Electron density profiles were calculated based on the radial distance from the centre of the micelle and averaged over the last 5 ns of each simulation. In each differential element of the sphere, the number of each type of atom or group of atoms was calculated. These data are then converted to electron density by multiplying by the number of electrons for the atom or molecule and dividing by the volume of the spherical shell. Electron densities can be used to determine the positions of residues relative to

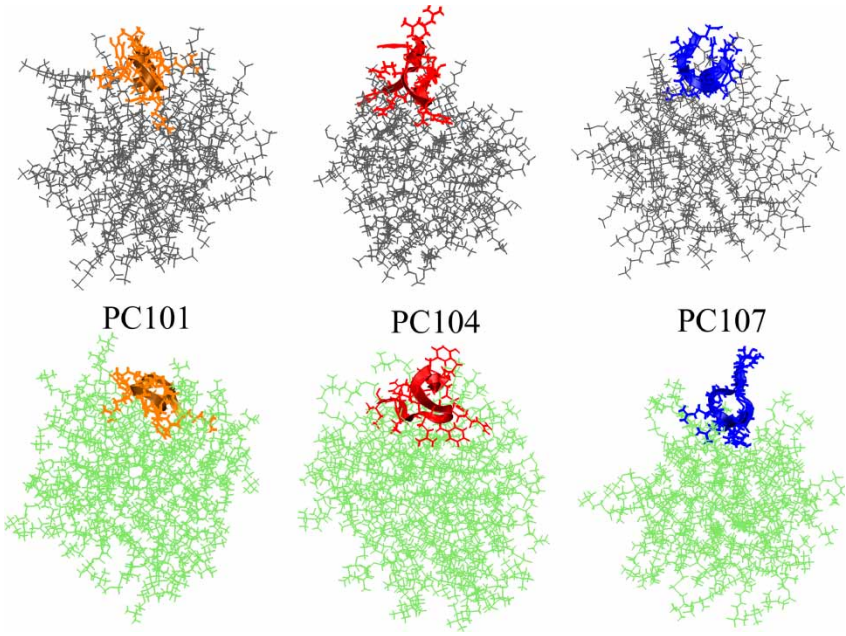

Figure 1. Views of the six systems at the end of each simulation. SDS is shown in gray and DPC in green. PC101 and PC104 exhibit similar conformations in SDS and DPC. PC107 is seen to have few interactions with the DPC micelle. See online journal for colour figure.

the micelle and in our case we can compare the relative positions of the residues.

From the electron density profiles for SDS, we can quantify the differences that are visible in the interactions of the peptides with this micelle seen in figure 1. The residue of most interest when interacting with SDS micelles is Leu-5. We have previously seen that leucine is responsible for the antimicrobial activity of PG-1 [40,46], as in simulations of both peptides the leucine residue inserts most deeply into the SDS micelle, and we would have expected to see this residue located farthest inside of the SDS micelle for all three of the peptides we are studying here. In fact, Leu-5 is farthest inside for PC107, but the leucine residues on PC101 and PC104, though interacting with the micelle core, are located more near the micelle–water interface. PC101 and PC104 insert more completely on the second strand of the β-hairpin, as evidenced by the locations of Phe-12, Val-14, and Thr-16 or Ile16, which are located well inside the micelle. These same residues on PC107 are located much father out in the micelle–water interface region. This results in the tilt of PC101 and PC104 with respect to the micelle surface visible in figure 1, where the C-terminal strand of these hairpins is inserted into the micelle (figure 3).

The peptides orient themselves differently in the DPC micelles than they do in the SDS micelles. As seen in figure 4, Leu-5 on PC107 is clearly not interacting with the DPC micelle, though it was shown from the electron density profiles that this residue inserts deeply into the SDS micelle

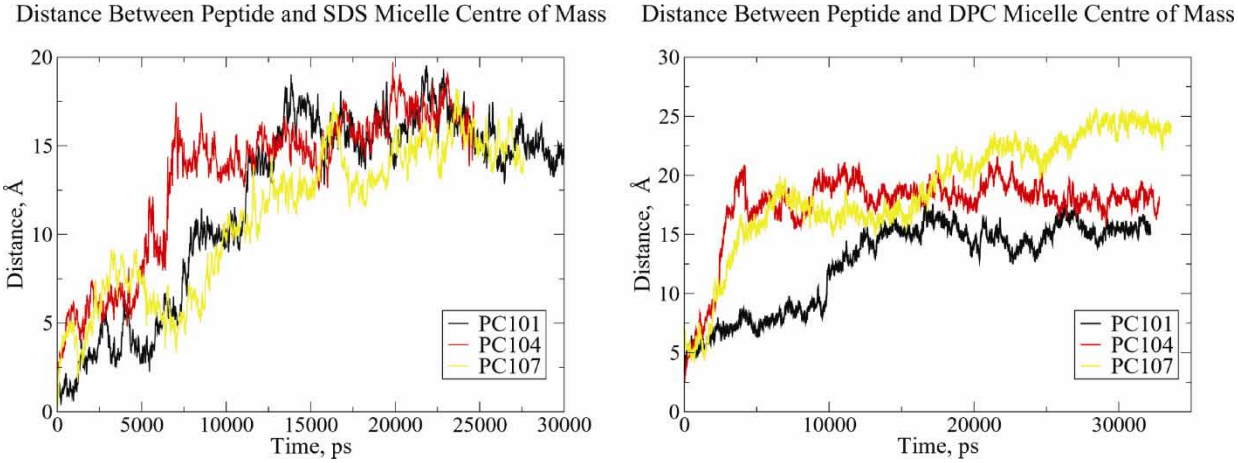

Figure 2. Distance between the peptide and micelle centres of mass. Though all three peptides converge to a similar distance from the micelle centre in SDS, PC107 clearly exits from the micelle–water interface region in DPC. These plots demonstrate the predictive ability of the simulations with regard to toxicity.

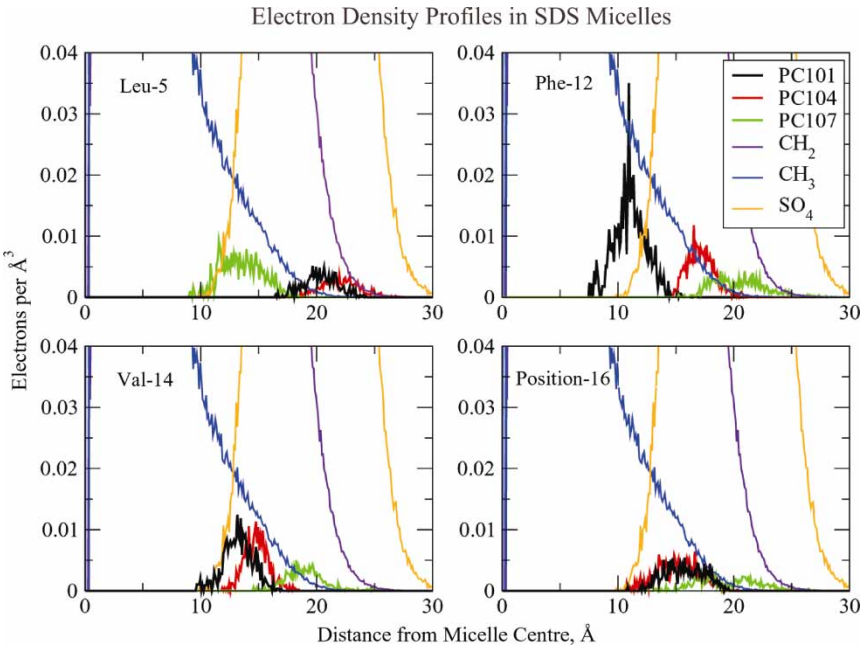

Figure 3. Electron density profiles for three hydrophobic residues and position 16 for each peptide. Position 16 is a threonine residue on PC101, isoleucine on PC104 and glutamate on PC107. From these plots we can compare the insertion of different residues into the SDS micelle. Leu-5 on PC107 inserts most deeply into this micelle. Phe-12 plays an important role in disruption of the SDS micelle for PC101.

core. The residues on PC107 that are interacting most strongly with the DPC micelle core are the two hydrophobic residues on the second strand of the peptide, Phe-12 and Val-14, but even these do not insert to the same degree as on the other peptides examined here. This is in agreement with the low level of toxicity of PC107. We can also see from figure 4 that for both PC101 and PC104 there are several hydrophobic residues that insert into the micelle core. On PC101, Val-14 inserts most deeply into the micelle. The threonine residue, Thr-16, is located in the region near the phosphate head groups and separate calculations (data not shown) show that this residue forms a hydrogen bond with a phosphate oxygen. We see also, for PC104, that the hydrophobic residues on the second strand, Phe-12, Val-14, and Ile-16, insert into the micelle, explaining this peptide's high level of toxicity.

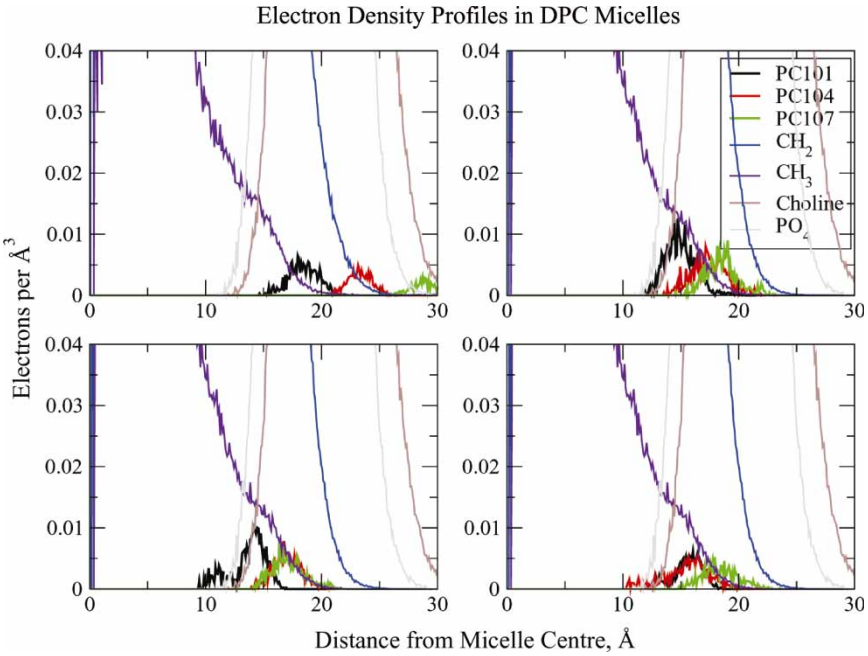

Figure 4. Electron density profiles for three hydrophobic residues and position 16 for each residue. Position 16 is a threonine residue on PC101, an isoleucine on PC104 and a glutamate on PC107.

3.3 Radial distribution functions

Radial distribution functions were calculated for each residue side chain with the SDS and DPC micelle cores. Radial distribution functions tell how likely it is that a certain type of atom will be found in a given distance from another specific atom or atom group, thus giving relative affinities between sets of atoms in the system.

In figure 5, the radial distribution functions for the three peptides with the carbon chains of the SDS micelle are shown. As was apparent from the electron density profile data, Leu-5 on PC107 interacts strongly with the SDS micelle core and is thus most likely responsible for this peptide's activity. The residues nearby, Cys-6, Tyr-7 and Cys-8, also appear to play a role in the disruption of this micelle likely due to their proximity to Leu-5. PC101 and PC104 insert into the SDS core with a different interaction pattern. The C-terminal strands of these peptides insert to allow for strong interactions between Phe-12, Val-14, and Thr-16/Ile-16 with the SDS micelle core. There is less interaction between Thr-16 on PC101 and the micelle core as for Ile-16 on PC104, as would be expected for the hydrophobic residue as compared to the slightly polar threonine residue.

Radial distribution functions with the DPC micelle core provide insight as to which residues are most likely responsible for the toxicity of the peptides. PC104 is highly toxic, PC101 is moderately toxic and PC107 is relatively nontoxic and we see different levels of interaction in figure 6. First note that PC107 has significantly lower interactions with the DPC core than the other two peptides, in agreement with its low level of toxicity. There are a few substantial peaks for this peptide, particularly the one for Glu-16 with DPC. This residue interacts more strongly with DPC than SDS due to the fact that the DPC micelle is zwitterionic as opposed to anionic so this negatively charged residue is somewhat attracted to the positively charged choline groups, instead of repelled as it is from the sulphate groups on SDS, allowing it to remain close to the micelle. In figure 4 it is apparent that this residue is not completely inserted into the micelle core, but is near the head group region. The interactions for PC101 and PC104 appear to be similar to each other. There are strong interactions with the hydrophobic residues Leu-5, Phe-12, and Ile-16 (PC104). Several of the other residues that would not be expected to have strong interactions with the hydrophobic micelle interior, such as Arg-9 through Arg-11 likely exhibit large peaks due to the overall hydrophobicity of PC104 and PC101, which encourages these peptides to remain in the core of the micelle.

4. Conclusions

In this work we examine the interactions of three protegrin mutants with mammalian and bacterial membrane

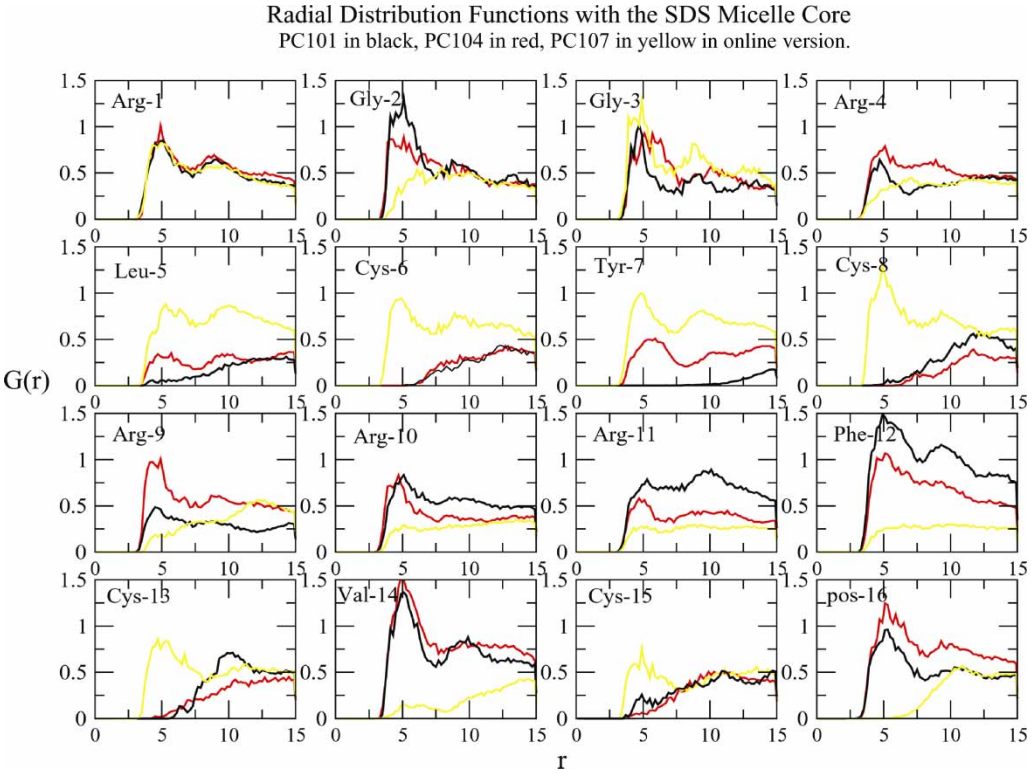

Figure 5. Radial distribution functions for each residue on PC101, PC104, and PC107 with the SDS micelle core. As with the electron density profiles, we see a stronger interaction for Leu-5 on PC107 than the other peptides examined. We also see strong interactions for PC101 and PC104 at Phe-12, Val-14, and position 16 (Thr-16 on PC101 and Ile-16 on PC104).

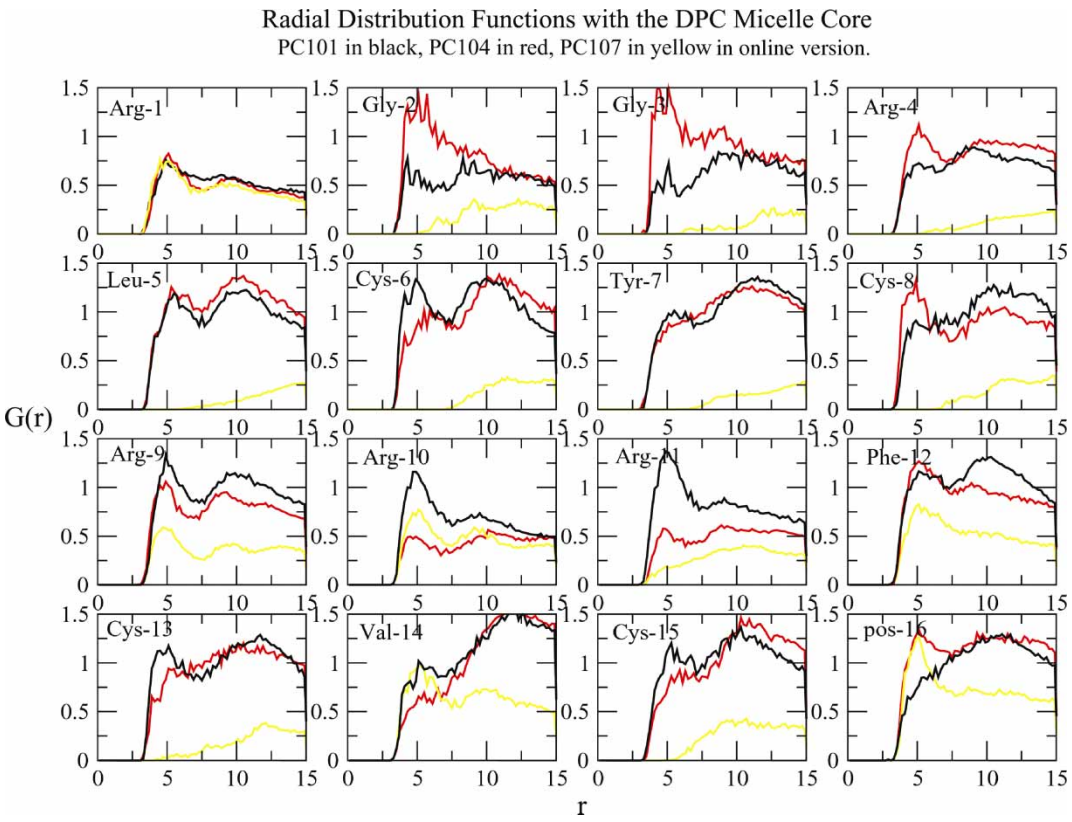

Figure 6. Radial distribution functions for PC101, PC104 and PC107 with the DPC micelle core. There are very few strong interactions for PC107 and the DPC micelle. There are large peaks at Arg-1 and Glu-16 for this peptide, likely due to the proximity of the charges on the head groups. PC101 and PC104 have several more strong interactions, particularly at Leu-5 and Phe-12.

mimics. All have had the three C-terminal residues of PG-1 removed and replaced with a hydrophobic residue (PC104), a polar residue (PC101), or a negatively charged residue (PC107). The resulting peptides have activity equal to PG-1 against gram negative bacteria but different degrees of toxicity.

We previously postulated, based on our work on PG-1 that Leu-5 is important for the activity of the peptide but does not play an important role in toxicity. This is evidenced by its deep insertion into the SDS micelles and also by its lack of insertion into DPC micelles. While this rule holds true for PC107, it does not for PC101 and PC104. The mutations at the C-termini of PC101 and PC104 have far reaching effects on the peptides' interactions with the micelles due to the reduction of charge. All three peptides studied here are equally as active as PG-1, but have different methods of inserting into the SDS micelle. For PG-1, Arg-18 acts as a tether, anchoring the peptide on the surface of a negatively charged micelle. Removing this residue allows the C-terminus to locate itself in differently, as seen in the simulations of PC101, PC104 and PC107. For PC104 and to an extent PC101, the C-terminus is more hydrophobic than PG-1 and also less positively charged. Removing the Arg-18 "tether" positions the C-terminal strand residues Phe-12, Val-14 and Ile-16/Thr-16 nearer to the micelle, allows these residues to move closer to the micelle and ultimately insert into the micelle core more easily, instead of being pulled to the micelle surface. This changes the angle of the peptide as it interacts with the micelle. We saw in our work with PG-1 that the peptide tilts to allow the first strand, mainly Leu-5, to insert into the SDS micelle [40]. For PC101 and PC104, we see the opposite occurring, with second strand free to insert into the anionic SDS micelle. For PC107, replacing the positively charged residue with a negatively charge glutamate residue at the new C-terminus causes this end of the peptide to be repelled from the micelle surface. PC107 exhibits behaviour similar to PG-1, where the peptide tilts to allow Leu-5 to insert in the micelle core while the C-terminal Glu-16 is repelled from the SDS head groups.

The mutations to create PC104 and PC101 provide no significant improvements in decreasing the toxicity. The removal of the positively charged arginine residue from the C-terminus that would normal pull the peptide to a position on the micelle surface allows this end of PC104 to be free to insert its hydrophobic residues Phe-12, Val-14, and Ile-16 into the DPC micelle core. Replacing the C-terminal VGR with threonine, as in PC101 creates a peptide with a similar sequence pattern as PG-1, where the threonine residue that replaces the VGR end of PG-1 has interactions with the head group of the DPC, between the hydroxyl group and the phosphate group. These interactions position the peptide favorably to allow for the hydrophobic residues of this peptide to insert into the micelles, thus resulting in a toxic peptide. The difference

in toxicity between PC101 and PG-1 is likely due to the reduction of the number of hydrophobic residues in PC101.

PC107 shows dramatic reduction in toxicity with only minor levels of toxicity in epithelial cells (table 1). The charge reversal caused by replacing VGR with Glu-16 directs the hydrophobic Val-14 away from the DPC micelle, however, there are still interactions between Phe-12 and the micelle core. In order to further improve this peptide and reduce its remaining toxicity to a level suitable for humans, we suggest the replacement of Phe-12 or Val-14, as we suggested for PG-1 [40]. This would leave us with a peptide that should have no region to insert deeply into a zwitterionic mammalian cell, but would still leave the leucine residue to retain the activity.

This study has demonstrated the usefulness of micelles as membrane mimics. To our knowledge, this is the first work to study the effects of mutations on several mutants using simulations, a task that would be impractical in lipid bilayers. Though bilayer simulations would be of interest, particularly in much larger simulations of several AMPs to examine the means of insertion, for the purpose of examining the effects of specific mutations on protegrin toxicity, micelles have shown to be a highly effective membrane mimic.

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