Structural basis for the enhanced activity of cyclic antimicrobial peptides: The case of BPC194

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A B S T R A C T

We report the molecular basis for the differences in activity of cyclic and linear antimicrobial peptides. We iteratively performed atomistic molecular dynamics simulations and biophysical measurements to probe the interaction of a cyclic antimicrobial peptide and its inactive linear analogue with model membranes. We establish that, relative to the linear peptide, the cyclic one binds stronger to negatively charged membranes. We show that only the cyclic peptide folds at the membrane interface and adopts a β-sheet structure characterised by two turns. Subsequently, the cyclic peptide penetrates deeper into the bilayer while the linear peptide remains essentially at the surface. Finally, based on our comparative study, we propose a model characterising the mode of action of cyclic antimicrobial peptides. The results provide a chemical rationale for enhanced activity in certain cyclic antimicrobial peptides and can be used as a guideline for design of novel antimicrobial peptides.

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1. Introduction

Antimicrobial peptides (AMPs) are currently in the spotlight as potential candidates to overcome bacterial resistance to conventional antibiotics. These peptides are natural weapons produced by a variety of organisms, including insects, animals and plants [1,2]. While displaying a wide spread in primary structures, most antimicrobial peptides exhibit common basic features [3–5]. The linear α-helical peptides have been shown to be unstructured in water and adopt a secondary structure upon association with the membrane [6,7]. Although acquiring structure upon membrane binding is an important feature of these AMPs, short peptides lacking a well-defined secondary structure are also active [8]. Cyclic AMPs are amongst the most effective antimicrobial agents [9,10] and some members of the family have been shown to adopt a structure even in water [11]. One of the best studied examples is gramicidin S, whose structure in aqueous media, an anti-parallel β-sheet characterised by two turns, is important for its activity [12]. Comparative studies between related peptides with differing activities, although not yet been undertaken, would help us rationalise the essential structural properties vital for function and will help us design more potent antimicrobial peptides.

Besides acquiring structure at the membrane interface, the partitioning behaviour of the peptides, i.e. the membrane–aqueous medium partition coefficient and the membrane penetration depth also contribute to their efficacy and action and selectivity [13]. In fact, the local concentrations of AMPs in the membrane can be up to 10,000 times higher than in the aqueous phase [14]; therefore the understanding of partitioning behaviour is of importance to unravel mechanisms of action of these peptides. Again, undertaking comparative studies would help us distinguish between essential and non-essential differences in partitioning and help to extract only the critical features.

The focus of our work is a cyclic peptide, BPC194 [c(KKLKFKKQL)], which, from a library of de novo designed cyclic decapeptides, proved most active against plant pathogens such as Erwinia amylovora, Pseudomonas syringae and Xanthomonas vexaticatoria [15,16]; its linear analogue is poorly active. Here, we present a combined Molecular Dynamics (MD) and biophysical study of the cyclic peptide interacting with anionic lipid bilayers to understand its mechanism of action. The linear analogue, BPC193 has been studied to contrast the behaviour of the two related peptides and extract the molecular basis for antimicrobial activity. We show how the cyclic and linear peptides differ both in
their partitioning behaviour and their folding state upon interaction with membranes. We speculate that these differences are related to differences in pore forming activity.

2. Materials and methods

2.1. Biophysical characterisation

2.1.1. Materials

The 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) and 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris–HCl) were from Roche Diagnostics GmbH (Germany), sodium chloride, sodium fluoride and Triton-X100 were from Merck (Germany). Phospholipids 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphatidylglycerol (DOPG) and 1,2-dioleoyl-sn-glycero-3-phosphatidyl-ethanolamine (DOPE) were from Avanti Polar Lipids.

2.1.2. Peptide synthesis

Decapeptides investigated in this study (Table 1) varied in the type of aromatic amino acid at position 6 and included cyclic analogues: BPC194, BPC294 and BPC418, together with their linear counterparts, BPC193, BPC293 and BPC417. All of them were synthesised by solid phase peptide synthesis as described in Supplementary Data (1. Materials and Methods).

2.1.3. Liposome preparation

2.1.3.1. Liposomes for calcine dequenching. Liposomes were prepared as described previously [17]. Briefly, liposomes at a final concentration of 1 mM of lipid molecules were prepared by rehydration of a dried chloride ions. Furthermore, liposomes were extruded through a 200 nm polycarbonate filter (Whatman International Ltd, UK), instead of 200 nm, which is caused by surface activities. Liposomes were prepared by rehydration of DOPG phospholipid (including DOPG and salt) and 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris-BPC294 c(KKLKKYKKLQ) BPC293 H-KKLKKYKKLQ-OH 106% Release = \( \frac{I_{\text{calc}} - I_0}{I_{100} - I_0} \times 100 \),

where \( I_0 \) is the measured fluorescence intensity at a given time, \( I_0 \) is the initial background fluorescence intensity, and \( I_{100} \) is the fluorescence intensity upon complete lysis of the liposomes, which was elicited by adding Triton X-100 (0.06% final concentration).

2.1.4. Calcein dequenching

The calcein dequenching assay was performed as reported previously [17]. A 20 μl sample of liposomes (1 mM of lipid), obtained as described in Section 2.1.3.1 of Materials and methods, was placed in 3 ml of buffer A, which yielded a final concentration of 6.7 μM of lipid in the cuvette. The membrane permeabilizing activity of the peptide was followed by measuring the increase in fluorescence (calcine dequenching), resulting from vesicle leakage after peptide addition (0–52 μM). Fluorescence emission was monitored at 520 ± 5 nm (excitation at 485 ± 2 nm) in a Cary Eclipse Fluorescence Spectrophotometer (Varian Inc.). The release of the calcein was calculated as a percentage of the total amount present in the liposomes:

\[ \text{% Release} = \frac{I - I_0}{I_{100} - I_0} \times 100 \]

where \( I \) is the measured fluorescence intensity at a given time, \( I_0 \) is the initial background fluorescence intensity and \( I_{100} \) is the fluorescence intensity upon complete lysis of the liposomes, which was elicited by adding Triton X-100 (0.06% final concentration).

2.1.5. Circular dichroism

Far-UV circular dichroism spectra were recorded on a Jasco J-815 CD spectrometer (Jasco, UK) between 190 and 250 nm at room temperature and scanning every 1 nm, using a quartz cell of 1 mm path length. The peptide at 0.15 mM was titrated with small aliquots (1 to 10 μl) of DOPG liposomes at a concentration of 25 mM. 2,2,2-Trifluoroethanol (TFE, Janssen Chimica) was used as a secondary structure inducer. The spectra were corrected for the absorption of buffer and vesicles.

2.1.6. Peptide environment probed with tryptophan fluorescence

The tryptophan analogues (BPC417 and BPC418) were placed in a quartz cuvette in buffer A at 2 μM and their fluorescence was monitored with a Fluorolog-3 (Jobin Yvon) spectrophotometer at an excitation wavelength of 285 ± 5 nm and emission scanning from 295 to 400 nm. Different amounts of DOPG vesicles were introduced into the cuvette (at final lipid concentrations of 2.28, 28 and 275 μM, corresponding to P:L ratios of 0.88, 0.071 and 0.0073, respectively). Subsequently, NaCl was titrated into the cuvette to a final concentration of 350 mM. The emission spectra were corrected with the corresponding buffer spectra (including DOPG and salt) and fitted to a skewed Gaussian curve [18] to obtain the fluorescence emission maximum, λ_{max}.

2.1.7. Peptide binding by ultracentrifugation

Increasing amounts of DOPG liposomes (0–753 μM) were added to 0.5 ml of 6 μM peptide in buffer A up to a P:L ratio of 1:114 and incubated for 20 min at room temperature. The samples were spun down by ultracentrifugation (1 h at 270,000g) and supernatants were separated from the pellets. A control experiment with an encapsulated dye in the same vesicle was carried out and under those conditions the vesicles were quantitatively spun down. To correct for the differences in quantum yield of the tryptophan fluorescence in different environments (hydrophilic buffer A vs. hydrophobic membrane environment), 2% (w/v) of sodium dodecyl sulphate (SDS) was added to the supernatant fraction and the fluorescence was measured. A correction was made for the background signal using a reference solution of buffer A, liposomes and SDS without the peptide. The percentage of binding was calculated using the equation:

\[ \text{% Binding} = 100 - \left( \frac{I_0}{I} \right) \times 100 \]

where \( I_0 \) is the fluorescence intensity of the supernatant sample with peptide without liposomes and \( I \) is the fluorescence intensity of the samples with liposomes. The obtained data were fitted to a general binding equation to obtain the dissociation constant (K_d):

\[ \text{% Binding} = \left[ \frac{B_{\text{max}} \times C_{\text{lipid}}}{K_d + C_{\text{lipid}}} \right] \]
where, $C_{lipid}$ is the concentration of lipid and $B_{max}$ is the maximum value for binding. For this purpose a simple binding equilibrium was assumed where peptide interacts [P] with the membrane [M] to form a peptide-membrane complex [PM]. This approach is equivalent to what was described by Matos and coworkers [19]. The affinity of peptide membrane interaction can also be expressed as partitioning coefficients [20]. The relationship between the two models is discussed in the work of Matos et al [19].

### 2.2. Molecular dynamics simulations

#### 2.2.1. System set-up

Simulations were performed with BPC194 [c(KKLKKFKKLQ)], and the linear analogue BPC193 [H-KKLKKFKKLQ-OH]. For the cyclic peptide, a peptide bond was created between the N-terminal lysine residue and the C-terminal glutamine residue. The initial structure of the peptides was modelled using the leap module of AMBER 9 [21].

#### 2.2.2. Peptide in aqueous environment

A number of systems were prepared with either one cyclic or one linear peptide solvated by water. The cyclic peptide was initially unstructured in all simulations but different starting conformations of the linear analogue were tested: $\alpha$-helix, bend and random-coil. An overview of all simulations performed is shown in Table 2 (Wa-Wk), and the details are presented in Table S1. All systems were simulated in a cubic box of length 4.5−5 nm and about 3000 water molecules. In a few simulations, counterions ($Cl^−$) were added to neutralise the system.

#### 2.2.3. Peptide-membrane systems

A number of simulations were performed with peptides in a solvated DPPG (1,2-dipalmitoyl-sn-glycero-3-phosphatidylglycerol) membrane system. Simulations were performed with molar peptide-lipid (P:L) ratios of 1:128, 2:60, 4:128 and 9:128 (and 6000−8000 water molecules) for both BPC194 and BPC193 (see Table 2 for an overview and Table S1 for details). Initially, the peptides were placed in the water phase close to the membrane surface, with distances between the peptide and membrane ranging from 1.5 to 2.3 nm. The cyclic peptide was initially unstructured and different conformations were tested for the linear peptide. Multiple simulations were run from different initial random velocity distributions for each of the P:L ratios. The simulations were carried out in the presence and absence of counter ions, and using different electrostatic schemes. No significant differences were observed with respect to these details of the simulations, however.

#### 2.2.4. Simulations parameters

The GROMACS software package [22] was used to perform all MD simulations. The GROMOS force-field 43a2 [23] was used to describe the peptide and peptide−solvent interactions. Simulations in water were also run using GROMOS force-field 53a6 [24]. The force-field for DPPG lipids was optimised from DPPC [25] and POPG [26] lipids, compatible with the GROMOS96 parameters. The choline head-groups were replaced by glycerol from the POPG force-field. The tail parameters were taken from the DPPC force-field and Table S1 for details). Initially, the peptides were placed in the water phase close to the membrane surface, with distances between the peptide and membrane ranging from 1.5 to 2.3 nm. The cyclic peptide was initially unstructured and different conformations were tested for the linear peptide. Multiple simulations were run from different initial random velocity distributions for each of the P:L ratios. The simulations were carried out in the presence and absence of counter ions, and using different electrostatic schemes. No significant differences were observed with respect to these details of the simulations, however.

### Table 2

Overview of secondary structure and binding characteristics in all simulations performed on the cyclic (BPC194) and linear (BPC193) peptides. Results are averaged over all peptides present in the system, and over all independent simulations. The average binding depth as well as the maximum binding depth, with respect to the average position of the phosphorous atoms in the outer leaflet, observed for a particular peptide is given. The standard errors reported are calculated from the standard deviation, between all peptides in all simulations.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>P:L ratio</th>
<th>Code</th>
<th>Nr. of Sim.</th>
<th>%Non-structured</th>
<th>%P-sheet</th>
<th>Peptide depth$^a$</th>
<th>Phe depth$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Av. Max</td>
<td>Av. Max</td>
</tr>
<tr>
<td>Cyclic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>Wa-Wc</td>
<td>3</td>
<td>87±5</td>
<td>13±5</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>1:128</td>
<td>1Ca-1Cg</td>
<td>7</td>
<td>79±3</td>
<td>21±3</td>
<td>−0.34±0.38</td>
<td>1.16</td>
<td>−0.52±0.65</td>
</tr>
<tr>
<td>4:128</td>
<td>4Ca-4Cc</td>
<td>3</td>
<td>67±4</td>
<td>33±4</td>
<td>−0.08±0.23</td>
<td>−0.99</td>
<td>−0.03±0.29</td>
</tr>
<tr>
<td>9:128</td>
<td>9Ca-9Cg</td>
<td>10</td>
<td>68±2</td>
<td>32±2</td>
<td>−0.24±0.25</td>
<td>1.2</td>
<td>−0.57±0.20</td>
</tr>
<tr>
<td>Linear</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>Wa-Wk</td>
<td>8</td>
<td>84±4</td>
<td>16±4</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>1:128</td>
<td>1La-1Lb</td>
<td>2</td>
<td>100±0</td>
<td>0±0</td>
<td>−0.18±0.25</td>
<td>−0.78</td>
<td>−0.10±0.41</td>
</tr>
<tr>
<td>2:60</td>
<td>2La-2Lb</td>
<td>2</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>4:128</td>
<td>4La-4Lb</td>
<td>2</td>
<td>100±0</td>
<td>0±0</td>
<td>−0.10±0.18</td>
<td>−0.75</td>
<td>0.50±0.23</td>
</tr>
<tr>
<td>9:128</td>
<td>9La-9Lg</td>
<td>7</td>
<td>99±0</td>
<td>1±0</td>
<td>0.41±0.19</td>
<td>−0.62</td>
<td>0.53±0.19</td>
</tr>
</tbody>
</table>

$^a$ Centre of mass of the peptide.

$^b$ Centre of mass of the phenyl ring of the phenylalanine residue.
previous studies of peptide-membrane interactions. For a general review on MD studies of peptide-membrane interactions see the work of Mátyus et al.

2.2.5. Analysis
The secondary structure of the peptides was calculated with the DSSP code. For the cyclic peptide, the bonded N-terminus (K1) and C-terminus (Q10), present in the turn region, were neglected while calculating the secondary structure. The structural properties were then calculated from the average number of residues involved in each secondary structural feature along the simulation. The total "β-structure" is reported as the sum of β-sheet and β-bridge (one hydrogen bond less than β-sheet) and the "non-structured," as the sum of coil, bend and turn.

To analyse the alignment of the lysine residues, the distance between the planes formed by the Cα, Cβ, and Nζ atoms of the spatially symmetric lysine residues K1-K8, K2-K7 and K4-K5 were calculated.

3. Results
3.1. The cyclic peptide permeabilizes the membrane and has a preference for anionic lipids
The cyclic peptide BPC194 has a high antimicrobial activity (MIC values ranged from 3.1 to 12 μM) and low hemolytic activity (not exceeding 17% at 375 μM). A calcein dequenching assay was performed to determine the membrane permeabilizing activity of the cyclic peptide, BPC194, two cyclic analogues (BPC294 with Tyr and BPC418 with Trp instead of Phe at position 6) and the corresponding linear analogues BPC193, BPC293, BPC417 (see Table 1 for peptide names and compositions). Fig. 1 summarises the results obtained for the cyclic analogue BPC194 and shows that it caused the leakage of calcein from vesicles comprising of anionic lipids. The leakage reached 100% in DOPG membranes though it did not exceed 5% in DOPC, even at high P:L ratios (Fig. 1A). Experiments with liposomes composed of mixtures of DOPG, DOPC and/or DOPE confirmed that the cyclic peptide (BPC194) has a preference for anionic lipids since the leakage increases with the content of DOPG lipids in the vesicles (Fig. 1B). All three cyclic analogues caused the leakage of calcein for anionic membranes (Fig. S1), independent of the size of the vesicles (Fig. S1). These results are line with the in vivo data and are consistent with the fact that bacterial membranes are generally more rich in negatively charged lipids than the outer leaflet of the plasma membrane of higher eukaryotes, e.g. mammalian cells. The calcein leakage from vesicles was negligible for the linear analogues, irrespective of the lipid composition. The low antimicrobial activity of the linear peptide is consistent with the lack of membrane permeation in model membrane systems. Below, we focus on the interaction of the cyclic and linear analogues with anionic lipids to understand the remarkable differences in their membrane permeabilizing properties.

3.2. Both the linear and cyclic peptides bind to PG membranes
To test whether the low activity of the linear peptides stems from their inability to bind to anionic membranes, we analysed their membrane binding properties by MD simulations and fluorescence experiments. In the MD simulations, the peptides (BPC193 and BPC194) were placed in the water phase close to the surface of a DPPG bilayer at P:L ratios 1:128, 4:128 and 9:128. Two peptides bound to the...
membrane rather fast and on similar (nanosecond) time scales (Fig. 2A and B). To validate the simulation data, the fluorescence of the tryptophan in BPC417 and BPC418 was used to monitor the interaction of the linear and cyclic peptides with the membranes. BPC417 and BPC418 bound to DOPG membranes albeit with different affinities (Fig. 2C). BPC418 bound to the membrane with a $K_d$ of $7 \pm 2 \mu\text{M}$ (under these conditions the overall $P:L$ ratio = 0.84). BPC417 bound with a $K_d$ for DOPG of $96 \pm 10 \mu\text{M}$ ($P:L$ ratio = 0.0625). From the data presented in Fig. 2C, one can derive $P:L$ ratios at which the amount of cyclic and linear peptide bound is equal and compare these conditions with those in Fig. 1A. Even at equal amounts of peptide interacting with the membrane, the cyclic peptide is orders of magnitude more effective in membrane permeabilization than the linear one. For instance, when the cyclic and the linear peptide are $-60\%$ bound ($P:L$ ratio of around 0.6 and 0.06, respectively) the cyclic peptide causes $-50\%$ of dye leakage, while the linear analogue is not active.

3.3. Both linear and cyclic peptides are structure-less in aqueous solution

To analyse the structure of the peptides in aqueous solution, atomistic MD simulations as well as CD measurements were performed. In the simulations, the cyclic and the linear peptide showed no defined secondary structure in aqueous solution and appeared mainly in a non-structured conformation (Fig. 3A and B). On average, only 13 and 16% $\beta$-structure was adopted by the cyclic and linear peptide, respectively (Table 2). Although we found that 50 ns of simulation were not sufficient for the peptides to completely sample their conformational space, the simulation data taken as a whole points to an unstructured conformation for the peptides to completely sample their conformational space (Fig. 3A and B). On average, only 13 and 16% of the membrane was con

3.4. Cyclic peptide adopts $\beta$-structure upon binding to membranes

Upon interaction of BPC194 with the DPPG membrane surface, a secondary structure was induced within nanoseconds in the simulations (Fig. 3C–E). The average fraction of $\beta$-structure increased from 13% to 32% (Table 2). The increase in $\beta$-structure was most pronounced at the higher $P:L$ ratios of 4:128 and 9:128. Visual inspection of the MD simulations of BPC194 showed that the $\beta$-structure formation gives rise to an amphipathic-like structure with a spatially symmetric arrangement of two pairs of lysine residues: $K^1-K^8$ and $K^2-K^7$ on opposite strands. When the peptide adopted a $\beta$-structure, these lysine pairs aligned in a parallel manner and gave rise to a high charge density (Fig. 4A and B). When the intra-strand hydrogen bonds were weakened and the $\beta$-structure was lost, the lysine residues could point away from each other (Fig. 4C). On average, the distances between the lysine pairs at the membrane interface were reduced from $1 \text{ nm}$ (in aqueous medium) to about $0.7 \text{ nm}$. Upon addition of DOPG membranes, a substantial change in ellipticity of BPC194 was observed (Fig. 3I); similar behaviour was seen for the tryptophan (BPC418) and tyrosine containing (BPC294) cyclic peptides but not for the linear analogues (Fig. S3). The CD spectra of the cyclic peptides in the presence of DOPG are reminiscent of $\beta$-sheet and $\beta$-turn structures reported for other (cyclic) peptides [37–39]. However a quantitative deconvolution of the spectra is difficult as CD spectra simulators are designed for large proteins rather than cyclic small peptides. Unfortunately, it is not possible to perform solution-state NMR measurements on the membrane-associated states of the peptides, since the molecular mass of the vesicles to which the peptides are associated is so large that any NMR signal of the peptide would be broadened beyond detection.

3.5. The inactive linear peptide remains structure-less at the membrane surface

In contrast to the cyclic peptide, the linear analogue remained fully unstructured (Fig. 3F–H) with no intra-molecular hydrogen bonding at the DPPG membrane interface at $P:L$ ratios of 1:128 and 4:128 (Table 2). At the highest $P:L$ ratios (9:128) a few intermolecular interactions were observed, resulting in peptide clusters. This gave rise to some $\beta$-sheet characteristics on the interacting peptides, originating mainly from interactions of the peptide backbone and the glutamine side-chain. We also performed a simulation with the linear peptide folded into an ideal $\alpha$-helix and an unfolding of the helix to random-coil conformation was observed (Table 1; 2La–2Lb). The CD measurements were entirely consistent with the MD simulations (Fig. 3J).

3.6. The cyclic and linear peptides differ in membrane penetration

BPC194 was able to penetrate deeper into the phospholipid membrane than its linear counterpart. In the MD simulations, the distances between the phosphorus atoms of the outer leaflet and the centre of mass of the peptide were calculated and are plotted in Fig. 5A–D. The cyclic peptide BPC194 was able to insert below the level of the phosphorus atoms (see Table 2). Although a single peptide ($P:L$ 1:128) appeared on average to bind deeper ($-0.34 \text{ nm}$) compared to systems with $P:L$ ratios of 4:128, 9:128 ($-0.08 \text{ nm}$ and $-0.24 \text{ nm}$), the maximum insertion for individual peptides was at $P:L$ ratio of

![Fig. 4. Different binding modes of the cyclic peptide. The lysine residues are depicted in orange, the head-group phosphorous atoms as yellow spheres and the acyl tails in grey. A–B. In certain binding modes during the simulation, the lysine residues aligned with inter-strand hydrogen bonds (depicted in red). C. In others, the lysines snorkelled out independently and the inter-strand distances increased.](image-url)
membranes were saturated with peptide, the backshift of resulting in a shift of the deeper insertion into the membrane. Increasing the ionic strength peptide points to a more hydrophobic environment and thus to essentially all peptides are bound). The larger blue-shift for the cyclic (to 350 mM. The peptide concentration was 2 μM; DOPG was added at different concentrations, yielding (E) P.I. = 0.0073, (F) P.I. = 0.071 and (G) P.I. = 0.88.

9:128 (−1.20 nm). Visual inspection of the trajectories showed that large values of insertion of the cyclic peptide were associated with high local perturbations, which led to poration of the membrane, as discussed elsewhere [40]. The fluctuations in the outer leaflet, calculated as the deviation of the phosphorous atoms of the head-groups from their centre of mass were ±0.44 nm for the cyclic peptide. In contrast to the cyclic peptide, the linear analogue BPC193 did not embed very deep. The maximum insertion observed was −0.62 nm below the level of the phosphorous atoms and was not accompanied by large bilayer perturbations. Furthermore, low perturbations were seen in comparison to the cyclic peptide and the outer-leaflet fluctuations were ±0.29 nm for the linear peptide.

Next, we examined the environment of the tryptophan from fluorescence emission spectra. The peptide that was initially in buffer was exposed to DOPG vesicles, which resulted in a blue-shift of the fluorescence emission peak (λmax) of ~14 and ~10 nm for the cyclic and linear peptides, respectively (see Fig. 5E and F; at these P.I. ratios essentially all peptides are bound). The larger blue-shift for the cyclic peptide points to a more hydrophobic environment and thus to a deeper insertion into the membrane. Increasing the ionic strength resulted in a shift of the λmax of the linear peptide back to what it was in the aqueous medium, which is indicative of reversible binding. The salt effect was not observed for the cyclic peptide, and even at the highest P.I. ratio of 0.88 (where about 50% was bound (Fig. 2C) and the membranes were saturated with peptide), the backshift of λmax was only marginal (Fig. 5G). Thus, the salt effects are also consistent with a deeper insertion of the cyclic peptide, BPC418, as compared to the linear one. It has been reported that certain peptides (e.g. pep-1) [41] can translocate through the membrane without causing leakage/poration. We believe that the shallow and reversible membrane-embedding of BPC193 (incl. MD simulations and much less pronounced fluorescence blue-shift of the linear peptide as compared to the cyclic counterpart) argue against such possibility for the linear peptides.

4. Discussion

4.1. Molecular mechanism of pore formation

Based on our results, we propose here a molecular-detailed model outlining the differences in mode of action of the cyclic and linear peptides (schematically presented in Fig. 6). Both peptides when present in the aqueous phase are mainly unstructured and have a propensity to bind to (anionic) membrane surfaces. From this point on, the behaviour of the two peptides diverges. The cyclic peptide has a larger tendency to fold, enabling it to insert deeper into the membrane interface. Through cooperative interactions, some of these membrane-embedded and folded cyclic peptides cause large perturbations in the bilayer. The linear peptide assumes a more extended conformation and is unable to perturb the membrane substantially. Finally, the cyclic peptide is able to porate the vesicles and cause dye leakage while the linear is unable to do so. The model highlights the structural and partitioning differences of two related peptides with the same sequence but varying antimicrobial activity and thereby point to the critical features required for high antimicrobial activity. The mechanistic model proposed here resembles the one described for gramicidin S in the sense that the cyclic peptide interacts with the membrane, adsorbs to the bilayer surface, folds and gets inserted into the hydrophobic core of the membrane [42]. However, for BPC194 the poration has been confirmed experimentally and evaluated with molecular detail by MD simulations [40]. The results of our study provide important clues for rational design of novel cyclic antimicrobial peptides (work in progress).
4.2. Folding and insertion of BPC194 at the membrane interface

The cyclization of the peptides that were studied is not linked to gain of structure in solution. However, upon membrane binding the cyclic peptide assumes a β-structure unlike the linear one. For BPC194, the β-structure gives rise to a spatially symmetric arrangement of the lysine pairs, resulting in an amphipathic-like structure, a conformation that was also seen for gramicidin S [43]. The linear peptide has larger conformational entropy, and folding of the backbone to a β-hairpin structure is less favourable than in the cyclic peptide. The structure formation and consequently a deeper insertion of the cyclic peptide were accompanied by local perturbations of the membrane-leaflet and could be linked to its higher charge density, compared to BPC193. The lower charge density of the linear peptide and the formation of peptide clusters may prevent the linear peptide from substantially perturbing or embedding into the membrane. A correlation between high charge density and poration has been previously seen for cyclic analogues of linear antimicrobial peptides [44].

4.3. Folding of BPC194 is reminiscent of folding in linear β-hairpin peptides

A very important aspect of this work is the prediction of the peptide folded states at the membrane interface by MD simulations and supported by CD studies. Predicting the correct folds and folding pathways of (poly)peptides, even short ones, remains a challenge. Folding of peptides into β-hairpins has been used as a paradigm for protein folding and a large number of pathways such as zip-in, zip-out and middle-out have been proposed [45–47]. Multiple, long simulations and enhanced sampling methods have often been used to probe the folding events. In contrast, in the simulations presented here, folding at the membrane surface is sampled within nanoseconds after the peptide binds to the membrane. While kinetics at membrane interfaces is usually slow, the ‘fast-folding’ observed here is due to the limited phase-space of the cyclic peptide. For BPC194, only three hydrogen bonds are present in the folded state and due to cyclization, the backbone atoms involved in these hydrogen bonds are close together even in the unfolded state. In water, the peptide is non-structured because the thermal fluctuations are enough to break these three backbone hydrogen bonds that are then compensated by the neighbouring water molecules. However, at the membrane interface the backbone atoms (that are always spatially close) form the three hydrogen bonds because of lack of other hydrogen-bond partners and the lower polarity of the membrane leading to the peptide adopting the folded state very fast. The folding is reminiscent of the zip-in model described for linear β-hairpins, whereby the ends first approach each other and then the hydrogen bonds are formed.

5. Conclusions

In conclusion, there is now compelling evidence that cyclization of certain sequences of membrane-active peptides enhances their antimicrobial performance. In this paper, we analyse the molecular basis for activity in a cyclic antimicrobial peptide, BPC194 by comparing the mechanism of action of the active and inactive analogues. We show that the molecular basis for the enhanced activity resides most likely in the restriction of the number of conformations in the cyclic peptide. We show that it can adopt a favourable orientation towards the membrane and acquire an ordered structure that allows a high charge density and amphipathic arrangement. The latter allows the cyclic peptide to locate itself deeper in the membrane as well as to perturb it more than its linear counterpart. Thus, the alignment of lysine residues on opposite strands leads to a high charge density and an amphipathic arrangement. We propose that the structural and partitioning behaviour determined here are related to the difference in poration propensity and thus the antimicrobial activity.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbamem.2011.05.001.

References


