Interactions of the designed antimicrobial peptide MB21 and truncated dermaseptin S3 with lipid bilayers: molecular-dynamics simulations

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Molecular-dynamics simulations covering 30 ns of both a natural and a synthetic antimicrobial peptide in the presence of a zwitterionic lipid bilayer were performed. In both simulations, copies of the peptides were placed in an α -helical conformation on either side of the bilayer about 10 Å (1 Å = 0.1 nm) from the interface, with either the hydrophobic or the positively charged face of the helix directed toward the bilayer surface. The degree of peptide–lipid interaction was dependent on the starting configuration: surface binding and subsequent penetration of the bilayer was observed for the hydrophobically oriented peptides, while the charge-oriented peptides demonstrated at most partial surface binding. Aromatic residues near the N-termini of the peptides appear to play an important role in driving peptide–lipid interactions. A correlation between the extent of peptide–lipid interactions and helical stability was observed in

the simulations. Insertion of the peptides into the bilayer caused a dramatic increase in the lateral area per lipid and decrease in the bilayer thickness, resulting in substantial disordering of the lipid chains. Results from the simulations are consistent with early stages of proposed mechanisms for the lytic activity of antimicrobial peptides. In addition to these ‘free’ simulations, 25 ns simulations were carried out with the peptides constrained at three different distances relative to the bilayer interface. The constraint forces are in agreement with the extent of peptide–bilayer insertion observed in the free simulations.

Key words: carpet model, magainin, membrane lysis, membrane-binding peptides, molecular-dynamics simulation, peptide–bilayer interactions.

INTRODUCTION

Many cationic/hydrophobic peptides produced naturally by a wide variety of higher organisms possess broad-spectrum antimicrobial activity [1,2]. According to the popular Shai–Matsuzaki–Huang (SMH) model [2–5], these peptides first bind to the membrane surface up to a critical concentration, then penetrate the membrane interior. The thinning and strain of the membrane caused by the peptide inclusions leads to transient pore formation and eventual collapse of the membrane [3–5]. The physical nature of this mechanism prevents target organisms from developing resistance to the threat [2]. Consequently much attention has recently been focused on the development of antimicrobial peptides for clinical use [2,6].

Despite the inherent difficulties associated with structural studies on bilayers, substantial progress has been made in the description of peptide–bilayer systems using several experimental techniques, including solution [7,8] and solid-state NMR [9–11], X-ray diffraction [5,12], Fourier-transform IR spectroscopy [13,14] and CD spectroscopy combined with calorimetry [15]. Despite the utility of these methods, however, atomic-level interpretation of experimental results can be greatly enhanced through computer-simulation techniques. Molecular-dynamics (MD) simulations have proven particularly useful in describing phenomena relevant to various stages of the SMH model, including the binding of peptides to bilayer surfaces and properties of the surface-bound complexes [16–18], peptide insertion [19], structure and dynamics of individual transmembrane (TM) helices [20–22] and the properties of model pores constructed from aggregates of TM helices [23–25].

Recently, the mechanisms of membrane permeabilization for a designed, synthetic antifungal peptide known as Ac-MB21-NH₂ (Ac-FASLLGKALKALAKQ-NH₂, where Ac is acetyl) and a 16-residue truncation of dermaseptin S3 from frog skin, S3(1–16)-NH₂ (ALWKNMLKGIGKLAGK-NH₂), were investigated by measuring the interaction of these peptides with liposomes composed of either anionic (1,2-dioleoyl-*sn*-glycero-3-phosphatidylglycerol, DOPG) or zwitterionic (1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine, DOPC) lipids [26]. Helical-wheel plots for both peptides are shown in Figure 1. Fluorescence-emission and quenching measurements (a variant of the Ac-MB21-NH₂ peptide with the phenylalanine residue replaced by a tryptophan residue was used) indicated an immersion of aromatic residues into anionic (DOPG) liposomes. In general, the lytic ability of both peptides showed specificity for anionic liposomes, with Ac-MB21-NH₂ having higher activity. Both peptides interacted with zwitterionic liposomes to a lesser extent [26]. Quenching of fluorescence labels located at two different locations on the lipid chains indicated that the peptides insert at the liposome surface near the top of the hydrocarbon tails, but are probably too short to adopt a TM orientation. While the peptides lack significant secondary structure in aqueous solution, CD spectra of the peptides bound to DOPG liposomes show significant α -helical content for both S3(1–16)-NH₂ and Ac-MB21-NH₂ [26].

Here we describe results from 30 ns ‘free’ MD simulations of both these peptides in the presence of a zwitterionic [POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine)] bilayer. The aim of these simulations is to examine early events in the SMH mechanism, including surface binding and insertion of

Abbreviations used: DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylglycerol; MD, molecular dynamics; PC, phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine; RMSF, root-mean-square fluctuation; SMH model, Shai–Matsuzaki–Huang model; TM, transmembrane.

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Figure 1 Helical-wheel diagrams for the two peptides, showing the amphipathic nature of the sequences

Hydrophobic residues are shaded in grey.

peptides, as well as the subsequent disordering effects of the peptides on the membrane. We will also present results from separate, 25 ns 'constraint' simulations, in which the centres of mass of the peptides are kept constrained to specific distances from the bilayer centre of mass. Three separate constraint simulations were carried out on the same systems used for the free simulations. Analysis of constraint forces from these simulations provides quantitative information on the preferred location of the peptide with respect to the bilayer interface.

SIMULATION DETAILS

The simulation systems for both S3(1-16)-NH₂ and Ac-MB21-NH₂ contained a pre-equilibrated bilayer of 128 POPC lipids (64 per leaflet). Using the program XPLOR [27], the peptides were built in a canonical α -helical conformation. The backbone atoms were then restrained and a simulated annealing protocol applied to the side-chain atoms to relieve bad contacts. For both the free and constraint simulations two copies of the peptide of interest were placed on either side of the bilayer, with their long axes parallel with the bilayer surface. In each system, one peptide was placed with its hydrophobic face toward the bilayer surface (referred to as the 'upper' peptide). The peptide on the other side of the bilayer (the 'lower' peptide) was oriented with its polar (charged) face toward the bilayer surface. The peptide-bilayer systems were placed in rectangular boxes of dimensions 60 Å × 60 Å × 85 Å, with the bilayer normal parallel with the *z*-axis, with

the peptides far from the bilayer surface at the edges of the box in the *z*-dimension. The system was then solvated with simple-point-charge ('SPC') [28] water as described previously [16]. Water molecules placed in void volumes of the bilayer hydrocarbon core were removed. Additional water molecules [eight and four for the S3(1-16)-NH₂ and Ac-MB21-NH₂ systems respectively] were replaced by chloride ions in order to render each system electrostatically neutral. The starting structures for the simulations were generated using the 'pull code' of the GROMACS simulation package [29] to pull each peptide toward the bilayer at a rate of 0.0001 Å per simulation step, with a step length of 2 fs. The configuration having a peptide-bilayer centre of mass distance of 30 Å was used for the equilibration of the 'free' simulations. Three 25 ns constraint simulations were carried out for both the S3(1-16)-NH₂ and Ac-MB21-NH₂ systems, corresponding to centre-of-mass distances of 15, 20 and 25 Å respectively. The 'pull' simulation employed a twin-range cut-off of 10/18 Å for electrostatic interactions, with no switch or shift function. Lennard-Jones interactions were truncated at 10 Å. Constant temperature and pressure were maintained at 300 K and 100 kPa (1.0 bar) using the weak coupling technique [30] and relaxation parameters of 0.1 and 1.0 ps respectively. The bilayer, solvent and peptides were separately coupled to the temperature and pressure baths. For each simulation, a 5 ns equilibration run was performed using the same parameters to allow relaxation of the lipids. As changes in the orientation and structure of the peptides occurred during these simulation runs, ideal α -helical peptide structures were least-squares-fitted on to those from the final configuration. In the case of the free simulations, the position of the peptide was also allowed to change, such that the distance between the peptide and bilayer surface was about 10 Å at the end of the equilibration in all cases. These configurations served as the starting structures for 500 ps position-restraint simulations, in which an isotropic force constant of 1000 kJ · mol⁻¹ · nm⁻¹ (100 kJ · mol⁻¹ · Å⁻¹) was applied to all peptide atoms, allowing further equilibration of the bilayer and solvent. Simulation parameters for the position restraint runs were identical with those of the pull simulation, except that the Particle-Mesh Ewald technique [31,32] was used for treatment of electrostatics, with a cut-off of 9 Å for the real part of the sum, a Fourier grid spacing of 1.2 Å and interpolation to fourth order. The final configurations from the position-restraint simulations served as the starting configurations for the production runs, which are 30 and 25 ns in length for the free and constraint runs respectively. To constrain the peptides in the constraint run, the pull code simply resets the co-ordinates of each peptide centre of mass so that the distance between it and the centre of mass of the bilayer remains constant and identical with that of the starting configuration. This method of constraint requires that the simulations be run using double precision. The GROMACS software package [29] version 2.0 was used for all simulations, with protein-force-field parameters taken from the GROMOS-87 parameter set with slight modifications. Lipid parameters were taken from Berger et al. [33], with the double bond in the oleoyl chain from GROMOS-87.

RESULTS

Peptide-bilayer insertion

Figure 2 shows the difference in the *z*-co-ordinate of the peptide centres of mass and that of the choline nitrogen atoms in the nearest leaflet in the free simulations. For reference, Figure 3 shows snapshots taken every 10 ns throughout the simulations. In the simulation of S3(1-16)-NH₂, the peptide oriented with its

Figure 2 Distance between the centres of mass of the peptides and that of the headgroup nitrogens in the nearest bilayer leaflet

Upper panel: free S3(1–16)-NH₂ simulation; lower panel: free Ac-MB21-NH₂ simulation. A running average over 25 ps is shown for clarity.

hydrophobic face towards the bilayer surface (upper peptide) adsorbs to the lipid interface within the first 10 ns. Within roughly the same time frame, the lower peptide also binds to the bilayer interface, although the contacts are limited to the N-terminal region (see Figure 3). Between 10 and 20 ns the centre of mass distance for the upper peptide decreases almost linearly, indicating a steady insertion of the peptide into the bilayer core. The degree of interaction between the lower peptide and the bilayer clearly increases relative to the first 10 ns, but these interactions remain limited to the N-terminal region of the peptide, and the centre of mass distance achieves an effectively constant, stable value after about 12 ns. In contrast, the distance for the upper peptide continues to decrease steadily until about 22.5 ns, when it reaches an apparently stable value of 6 Å below the choline nitrogen atoms (approx. 10 Å from the centre of the bilayer).

In the case of Ac-MB21-NH₂, the behaviour of the upper peptide resembles that of the upper peptide in the S3(1–16)-NH₂ simulation. For approximately the first 10 ns, this peptide resides at a stable distance 10 Å from the upper-leaflet choline nitrogen atoms. After this point, the peptide then inserts gradually into the bilayer. After approx. 25 ns, the depth of the peptide within the bilayer is stable and roughly 5 Å below that of the headgroup

nitrogen atoms. In stark contrast, the lower peptide remains in the aqueous phase for the entire simulation and even rotates about its long axis to nearly perpendicular orientations with the bilayer surface (see Figure 3). Interestingly, these rotations are such that the N-terminal aromatic residue (Phe¹) is distal to the bilayer interface. In all other cases, the N-terminal aromatic residues (shown in space-filling mode in Figure 3) of the peptides seem to interact strongly with the lipids. A closer view of the interaction between the Trp³ of the upper S3(1–16)-NH₂ peptide and nearby lipids is shown in Figure 4. In an analogous simulation of the same peptides using an 'octane slab' to approximate the bilayer, the peptides showed similar behaviour: the hydrophobic sides of the amphipathic helices inserted into the slab [34]. In contrast with the present simulation, however, this behaviour was independent of starting orientation: the peptides oriented with the charged surface toward the slab rotated during the course of the simulation to expose hydrophobic groups to the octane. Compared with phospholipid/water interfaces, the time-scale of peptide motions in octane/water interfaces is much shorter, such that converged results can be obtained within a few hundred picoseconds; unfortunately, important information on the interactions between the peptide and lipid headgroups is lost.

Foreshadowing results to be discussed below, the snapshots of Figure 3 provide some qualitative information on the disruption of the bilayer caused by peptide penetration: an overall thinning of the bilayer along the bilayer normal and a general decrease in lipid order can be clearly seen as the simulations progress.

Peptide structure and flexibility

Figure 5 shows the secondary structure of the peptides throughout the free simulations. For both the upper and lower S3(1–16)-NH₂ peptides, the initial α -helical structure remains stable for only the first nanosecond of the simulations. Following this, there is some turn formation in the middle of the upper peptide and near the C-terminal end of the lower peptide. Aside from the period between roughly 9 and 12 ns, during which there is an extensive amount of π -helical conformation, the structure of the upper peptide consists of two stable α -helical segments separated by a turn. Likewise, the α -helical structure near the N-terminus of the peptide remains essentially stable throughout the simulation, while the C-terminal region samples a variety of secondary structure types, including turn, bend and even some minor β -sheet hydrogen-bonding. Overall, the α -helical structure seems to be more stable in the upper peptide. This, coupled with the retention of helical structure in the N-terminus of the lower peptide, implies a correlation between helical stability and the extent of peptide–bilayer interactions. This correlation is also seen in the Ac-MB21-NH₂ simulations, with the upper peptide remaining in a remarkably stable α -helix for the entire 30 ns. In contrast, the α -helical structure in the lower peptide quickly breaks down into a primarily π -helical conformation.

To further examine the apparent relationship between peptide–bilayer interactions and helical stability, we have analysed the flexibility of the peptides throughout the simulations. Figure 6 shows the root-mean-square fluctuations (RMSF) in the backbone atom co-ordinates of the peptide, calculated in windows of 10 ns. Lowering of the backbone RMSF values with penetration of the peptide into the bilayer indicates a rigidifying effect of the lipids on the peptides, which will stabilize helical structure in the latter. In the cases of the upper peptides from both simulations, the flexibility of all residues decrease gradually as the simulations progress and the peptides embed themselves in the lipids. This trend is also observed for the lower S3(1–16)-NH₂

Figure 3 Snapshots from the free simulations

Structures from the S3(1–16)-NH₂ simulation are shown on the left, those from the Ac-MB21-NH₂ are on the right. In both cases the upper and lower peptides are shown as black and grey ribbons respectively. Trp³ of S3(1–16)-NH₂ and Phe¹ of Ac-MB21-NH₂ are shown in space-filling mode. The labels indicate simulation time. Snapshots were made with the program VMD ('Visual Molecular Dynamics') [51].

Figure 4 Interaction between the Trp³ residue of the upper peptide in the free S3(1–16)-NH₂ simulation and nearby lipids

Any lipid which formed one or more contacts (as defined by an interatomic distance ≤ 6 Å) with the tryptophan side chain in at least half of the simulation frames are shown. For the lipids, carbon, phosphorus, oxygen and nitrogen atoms are coloured in progressively darker shades of grey. The tryptophan side chain is shown in space-filling mode with hydrogen atoms, carbon atoms and the nitrogen atom in progressively darker shades of grey. The Figure was made with the program VMD [51].

peptide, in that the overall backbone RMSF values tend to be lower at later stages in the simulations. However, there are exceptions for specific residues which have more flexibility between 20 and 30 ns than between 10 and 20 ns, for example. Notably, most of these residues are located in the C-terminal portion of the peptide, which does not interact significantly with the lipids. The flexibility of residues in the lower Ac-MB21-NH₂ peptide, which remains in an aqueous environment for most of the simulation, does not follow any particular trend: much higher RMSF values are observed for the entire chain between 10 and 20 ns compared with the other time windows.

Density profiles and lipid disordering

Figure 7 shows density profiles for the aromatic peptide residues and various lipid groups during the first and last 10 ns of the S3(1–16)-NH₂ simulation. As might be expected from Figures 2 and 3, the density of the Trp³ side chain in the upper S3(1–16)-NH₂ peptide shows some overlap with the hydrocarbon core of the bilayer within the first 10 ns. The same side chain in the lower peptide shows substantial overlap with the headgroup/phosphate regions of the lipids. In the last 10 ns, the tryptophan side-chain density for the upper peptide has shifted even closer to the hydrocarbon core. The tryptophan side chain of the lower peptide, however, remains in essentially the same position. The location of this residue is similar to that observed in neutron-diffraction experiments on a small peptide with a ²H-labelled tryptophan residue in multilamellar bilayer samples [35]. It is noteworthy that the density of the Trp³ side chain in the upper peptide is bimodal, which is another feature observed from these experiments [35].

Figure 8 shows the same information for the Ac-MB21-NH₂ simulation. Aside from the fact that it is not bimodal, the relative position of the Phe¹ side-chain density for the upper Ac-MB21-

NH₂ peptide behaves very much the same as that of Trp³ in the upper S3(1–16)-NH₂ peptide: between the first and last 10 ns of the simulation, this side chain moves from a location near the headgroups to a significant depth within the hydrocarbon core. Some small degree of overlap occurs between the Phe¹ side-chain density of the lower Ac-MB21-NH₂ peptide and the choline headgroups during the first 10 ns of the simulation. During the last 10 ns, however, this side chain is located almost entirely in the aqueous phase.

Comparing the lipid group densities in the first and last 10 ns of both simulations respectively, the bilayer-thinning effect mentioned above is observed in the density profiles as a sharpening and compression of the lipid group peaks along the bilayer normal.

Lipid disordering

Figure 9 shows lipid order parameters from the S3(1–16)-NH₂ simulation. In all cases, the overall shape of the order parameter profiles calculated in the first 10 ns of the simulations resemble those which are obtained from ²H-NMR experiments on aligned samples of fully hydrated bilayers [36–38], with a plateau near the top of the chains and a gradual decrease toward the methyl termini. The zig-zag appearance of the order parameter for alternating carbon atoms along the unsaturated oleoyl chain is reproduced, as is the sharp dip due to the geometry of the double bond [37]. The magnitude of the values in the first 10 ns, which have a maximum near 0.2, are somewhat smaller than that observed in a simulation of a pure POPC bilayer at a constant area per lipid of 65.5 Å² [39], particularly near the top of the chains. This is to be expected, as the average area per lipid during this time in both the S3(1–16)-NH₂ and Ac-MB21-NH₂ simulations is closer to 70 Å² [see Figure 11 and Table 1 (below)].

Figure 5 Secondary structure of the peptides in the free simulations

The residue numbers run along the ordinate and simulation time along the abscissa. Secondary structure was calculated with the DSSP ('Definition of Secondary Structure of Proteins') program [52] and shaded according to the key given. As the most important features of the plot are deviations from ideal α -helical structure, bend and turn conformations have been grouped together, as have β -sheet and β -bridge conformations. For both peptides the upper and lower panels correspond to the upper and lower peptides respectively.

Figure 6 RMSF of the peptide backbone for each residue in the free simulations

The backbone RMSF was calculated in 10 ns time windows. Continuous line, 0–10 ns; broken line, 10–20 ns; dotted line, 20–30 ns.

Comparing these profiles with those calculated from the time periods between 10 and 20 and 20 and 30 ns respectively clearly shows the decrease in lipid order resulting from peptide insertion. In the S3(1–16)-NH₂ simulation, a drastic decrease in lipid order occurs between 10 and 20 ns. This decrease is approximately the same for each atom in the respective chains, with the profiles more or less maintaining their overall shape. Also, the extent of

the effect is roughly the same for both the oleoyl and palmitoyl chains. In the time period between 20 to 30 ns, precisely the same trend is observed, although the overall magnitude of the effect is not as great. While only the upper peptide inserts into the core of the bilayer, order parameter profiles calculated separately for each leaflet are nearly identical with those in the Figure (results not shown).

Order parameters from the Ac-MB21-NH₂ simulation are shown in Figure 10. In this case, the difference in the order parameters for the first two windows are relatively small compared with the drastic lowering that occurs in the last 10 ns. This is consistent with the fact that the upper peptide does not penetrate the bilayer surface substantially until approx. 15 ns, compared with 10 ns in the S3(1–16)-NH₂ simulation. Comparing the first and last 10 ns windows, the overall lowering of the order parameters in the Ac-MB21-NH₂ simulation is somewhat less than that observed in the S3(1–16)-NH₂ simulations.

Table 1 summarizes the changes in gross bilayer structure during the simulations as bilayer thickness and area per lipid values calculated in 10 ns time windows. Figure 11 shows the area per lipid from both of the free simulations as a function of simulation time. The values for bilayer thickness confirm the thinning effect seen in both the simulation snapshots and density profiles: during the first 10 ns of both simulations, the values are within the 35–41 Å range determined from experiment [40], but continue to decrease rapidly thereafter. In the case of the lipid surface areas, substantial increases are observed even during the first 10 ns of the runs, with values near 70 Å². These values are significantly higher than that of the starting configurations (approx. 60 Å²) prior to the 5 ns equilibration run (see the Simulation details section above) and the ranges obtained from

Figure 7 Density profile for aromatic peptide residues, lipid components and water in the first (upper panel) and last (lower panel) 10 ns of the free S3(1–16)-NH₂ simulation

Lipid component nomenclature is based on the pseudo-molecular model of King and White [53]. PO₄*Chol consists of the entire choline headgroup, the phosphate group and the methylene group on C-3 of the glycerol backbone. C=O*Glyc consists of the remainder of the glycerol backbone and the ester linkages of the backbone to the fatty acid chains.

experiment (63–66 Å²) [41,42]. However, the area per lipid values are stable for approximately the first 7 ns of both simulations, as can be seen in Figure 11. This is a fairly long time scale for conventional MD simulations on bilayer systems and indicates that the bilayer dimensions have become stable prior to subsequent changes in bilayer structure brought about by insertion of the peptides, which are quite dramatic. After the 10 ns mark, the area in the S3(1–16)-NH₂ simulation increases significantly and in a nearly linear manner. This is in direct correlation with the insertion of the upper peptide in to the bilayer phase (see

Figure 8 Density profile for aromatic peptide residues, lipid components and water in the first (upper panel) and last (lower panel) 10 ns of the Ac-MB21-NH₂ simulation

The lipid component nomenclature is based on the pseudo-molecular model of King and White [53].

Figure 2). A similar correlation is seen with Ac-MB21-NH₂. While the area per lipid settles to a fairly stable value at around 22 ns in the S3(1–16)-NH₂ simulation, this is not the case in the Ac-MB21-NH₂ run.

Constraint forces

We have carried out three additional simulations for each peptide in which the peptide centres of mass have been constrained to specific distances from the bilayer centre of mass (see the

Figure 9 Order parameters for both chains in both leaflets of the free S3(1-16)-NH₂ simulation

Order parameters were calculated in 10 ns windows. Continuous line, 0–10 ns; broken line, 10–20 ns; dotted line, 20–30 ns. Carbon atoms are numbered starting at the first aliphatic carbon atom of the chain. $-S_{CD}$ is the negative value of the deuterium (²H) order parameter.

Simulation details section above). This was done in the hope that the resulting constraint forces (which are recorded every time step) would provide quantitative information on the preferred location of the peptides with respect to the bilayer surface. The distances of 25, 20 and 15 Å were chosen in an effort to examine a relatively large distance, a reasonably favourable distance, and a distance which might represent too deep a penetration of the bilayer respectively.

An analysis of the constraint forces displayed an interesting agreement with the results of the free simulations. A cumulative average (i.e., every data point is an average over all preceding points) of the constraint force from a simulation will have converged if this cumulative average settles to a stable value. This was observed for peptides of both orientations in all constraint simulations. Taking the data from the upper peptide of the S3(1-16)-NH₂ constraint simulations as an example, an average of the constraint forces over the last 2.5 ns of the simulations resulted in values (means ± S.D.) of -0.1662 ± 0.275 , 4.222 ± 0.14 and 6.046 ± 0.24 kJ · mol⁻¹ · Å⁻¹ for the distances of 25, 20 and 15 Å respectively.

For the largest constraint distance, the stable value is close to zero, indicating that the peptide exerts very little force on the bilayer and vice versa. The final values for the two closer constraint distances are larger and increase with decreasing distance. Since the constraint force is opposite in sign to the

Figure 10 Order parameters for both chains in both leaflets of the free Ac-MB21-NH₂ simulation

Order parameters were calculated in 10 ns windows. Continuous line, 0–10 ns; broken line, 10–20 ns; dotted line, 20–30 ns. Carbon atoms are numbered starting at the first aliphatic carbon atom of the chain. $-S_{CD}$ is the negative value of the deuterium (²H) order parameter.

Figure 11 Area per lipid as a function of time for the free S3(1-16)-NH₂ and Ac-MB21-NH₂ simulations

forces occurring between chemical groups in the simulation, there is then an *attractive* force between the peptide and bilayer at the smaller distances. Notably, this is true even for a centre-of-mass distance of 15 Å, which, *a priori*, was thought to be 'too deep'. This is consistent with the fact that the upper S3(1-16)-

Table 1 Bilayer thickness and area per lipid in 10 ns time windows for the free simulations

Results are means ± S.D. (*n* = 10 000).

Time (ns)	Peptide ...	S3(1-16)-NH ₂		Ac-MB21-NH ₂	
		Thickness (Å)	Area/lipid (Å ²)	Thickness (Å)	Area/lipid (Å ²)
0-10		37.3 ± 0.20	69.7 ± 0.05	37.4 ± 0.19	69.6 ± 0.04
10-20		32.8 ± 0.08	74.3 ± 0.03	36.6 ± 0.20	73.6 ± 0.05
20-30		31.3 ± 0.03	78.9 ± 0.01	34.0 ± 0.10	75.5 ± 0.03

NH₂ peptide inserts itself to an even smaller centre-of-mass distance (10 Å) in the free simulation and indicates a consistency between the results from the free and constraint simulations.

DISCUSSION

As mentioned in the Introduction, both the S3(1–16)-NH₂ and Ac-MB21-NH₂ peptides demonstrated significantly higher lytic activity toward vesicles composed of anionic (DOPG) rather than zwitterionic (DOPC) lipids. In fact, the S3(1–16)-NH₂ peptide was completely ineffectual in dissipating TM potential of DOPC vesicles [26]. Interactions between the cationic residues of antimicrobial peptides and anionic lipids in the outer membranes of bacteria and fungi is generally considered to be the most important determinant of specificity against invasive microbes, as the cells of higher animals do not display anionic lipids on the outer membrane leaflets and as such are not susceptible [2]. The fact that neither S3(1–16)-NH₂ nor Ac-MB21-NH₂ is haemolytic [43,44] demonstrates that they follow this paradigm. In light of this, the extensive peptide–lipid interactions and disordering of the POPC bilayer observed in our simulations may seem unexpected. However, it is important to note the difference in behaviour between the upper and lower peptides in the course of the simulations: strong bilayer binding and penetration was only observed for the cases in which the hydrophobic face of the helix was oriented toward the bilayer surface. In the case of S3(1–16)-NH₂ the peptide that started with its polar face toward the lipid bilayer bound to the bilayer surface but did not penetrate substantially. In the analogous case for Ac-MB21-NH₂, there was no substantial interaction between the peptide and the bilayer whatsoever. While the timescale of the simulations is fairly long by current standards, there is insufficient sampling to observe a convergence of behaviour for the peptides with different starting configurations. It is possible that, in longer simulations, perhaps starting with the peptides at a greater distance from the bilayer interface, rotation of the helices about their long axes might occur, and a favourable orientation at the bilayer interface could be adopted by peptides in both orientations. As it stands, our results seem to indicate that hydrophobic interactions are the major force driving the binding and penetration of the POPC bilayer in the case of the upper peptides. The importance of hydrophobic interactions in the partitioning of antimicrobial peptides into zwitterionic bilayers has been demonstrated in a number of studies examining the relative activity of various antimicrobial peptides towards both bacterial and mammalian cells and the folding energetics of melittin on the surface of a POPC bilayer [46]. While the partitioning of some antimicrobial peptides in zwitterionic membranes can be much lower than for negatively charged membranes, permeation of the zwitterionic membranes by these peptides can occur if a high enough concentration of peptide is used to ensure a sufficient number of bound peptides [45]. Also, it should be remembered that erythrocytes contain a highly negatively charged glycocalyx layer to which positively charged antimicrobial peptides can bind. If the binding of the peptides to the glycocalyx is stronger than the partitioning of the peptides into the lipid membranes, then the peptides will not be haemolytic, even if they can efficiently permeate zwitterionic bilayers. In the case of bilayers containing anionic lipids, surface binding of antimicrobial peptides would certainly be driven by electrostatic interactions between the negatively charged headgroups and the cationic face of the helix. It is likely that more drastic bilayer disordering effects than those presented here would occur in simulations using charged lipids. However, in the latter stages of the proposed SMH mechanism

[3–5] the peptides form channels in which the polar faces of the helix would face toward an aqueous pore, with the hydrophobic regions immersed in the lipids. The peptide–bilayer structures observed in the latter stages of our simulations may therefore be representative of natural events following surface binding but prior to pore formation.

Fluorescence measurements on the aromatic residues of S3(1–16)-NH₂ (Trp³) and [Phe¹]Ac-MB21-NH₂ indicate that these residues do not insert deeply into phosphatidylcholine (PC) liposomes [26]. Our simulations indicate that the side chains of these residues play an important role in peptide–bilayer association and can penetrate the bilayer to the depth of the hydrocarbon region. This is best demonstrated in the case of the lower S3(1–16)-NH₂ peptide, in which the tryptophan residue seems to drive the interaction between the N-terminal region and the lipids. The preference of tryptophan side chains for the interfacial region of PC lipids is well documented [50] and likely makes a significant contribution to the hydrophobic binding observed in our simulations. However, the N-terminal region of the lower Ac-MB21-NH₂ peptide spends most of its time in the aqueous phase, distant to the bilayer surface, which demonstrates the dependence of the simulation results on the starting orientation for the helices. This is not the case for simulations employing an octane slab as a bilayer mimetic [34]: in these simulations, convergence of behaviour is observed for both peptides, regardless of starting orientation. The octane simulations thus offer increased sampling efficiency; owing to the lack of complex interactions (such as hydrogen-bonding) in the interfacial region, the peptide is allowed to adopt a wider range of orientations and conformations within the time scale of the simulation. It is interesting that, despite the presence of a more complicated interfacial region in our simulations, hydrophobic interactions seem to dominate the peptide–lipid interactions, as they do in the octane/water system [34]. The density plots for the upper peptides in the last 10 ns of both simulations (see Figures 7 and 8) are very similar to those obtained from X-ray-diffraction measurements of an amphipathic, cationic model peptide in the interface of a zwitterionic (DOPC) bilayer [12]. Thus, the positions and orientations of the upper peptides relative to the bilayer surface in the simulations are in agreement with experimental results.

Complicated interfacial environments such as lipid bilayers present great obstacles to complete sampling of peptide conformations in MD simulations [47,48]. In this sense, it is not surprising that the helical starting conformations remain stable over the timescale of our simulations. However, the relative instability of helical structure in the lower peptides relative to the upper peptides in the simulations suggests a tempting correlation between the structural stability and degree of peptide–lipid interactions. While the CD measurements of Moll et al. [26] clearly show a random-coil-to-helix transition in the peptide upon binding to liposomes, the study of such ‘partitioning–folding coupling’ [49,50] using an MD approach would require prohibitively long simulations. Nevertheless, the local unwinding of the starting helical structures indicates that our simulations are sufficiently long to observe significant conformational changes in the peptides. The general rigidifying effect of the bilayer on the peptide (see Figure 6) provides further evidence that the helical stability observed in the simulations is a direct result of peptide–bilayer interactions. Similar effects have been observed in simulations of alamethicin binding to a PC bilayer surface [16]. These simulation results are particularly interesting in that the stabilization of helical structure seems to play an important role in the interaction of antimicrobial peptides with zwitterionic membranes [45]: incorporation of D-amino acids

into peptides active against mammalian cells drastically reduces the content of α -helical structure of the bound peptides and consequently reduces their activity. In comparison, diastereomeric variants of peptides specifically active towards negatively charged bacterial cells are often just as active as the natural peptides, indicating that the formation of helical structure at the membrane interface is relatively unimportant to the antimicrobial mechanism.

From the results of our simulations, it is clear that the penetration of the upper peptides into the bilayer has drastic effects on lipid order and dimensions of the bilayer. While decreases in lipid order parameters are known to result from the presence of surface-bound as well as TM helices, these are frequently significant only for certain lipids within a certain close distance to the peptide. We have not analysed subsets of lipids in our simulations, simply because the results show a significant and general disordering for both chain types. While only the upper peptides penetrate the bilayer to an appreciable extent, both leaflets are similarly affected (results not shown). This indicates that the natural process of peptide binding to the outer leaflet of pathogen membranes causes lipid disordering in both bilayer leaflets. The increase in lipid disorder of both leaflets is caused by a large increase in the lateral surface area per lipid (approx. $10 \text{ \AA}^2$ over the course of each simulation). This change is rather large, given that the surface area contributed by the peptide itself can be roughly estimated from the pitch and diameter of an ideal α -helical backbone as approximately $110 \text{ \AA}^2$ for peptides of this length. The effect of the S3(1–16)-NH₂ peptide on the bilayer seems to have stabilized toward the end of the simulation (see Figure 11). This is not the case for the Ac-MB21-NH₂ peptide, indicating that further membrane disruption could possibly occur in a longer simulation. However, lysis of the cell via the SMH mechanism requires the co-operative effect of many peptides binding to the surface of the membrane. In particular, the lytic activity of certain peptides towards the PC bilayers of mammalian cell membranes often depends on their oligomerization state at the bilayer surface [45]. The peptide/lipid ratio employed in our simulation (1:64) falls in the lower end of the range used in the experiments of Moll et al. [26]. Simulations involving several peptides would be required to study co-operative effects and later stages of bilayer disruption. Such large-scale simulations are currently being carried out on the insertion and aggregation of the pore-forming peptide alamethicin in bilayers, and will likely be applied to other systems in the near future (D. P. Tieleman, unpublished work).

The correspondence between the constraint force analysis and the degree of penetration observed in the free simulations gives us some confidence in the method. This technique could prove useful in determining roughly the preferred location of arbitrary solutes with respect to the bilayer interface.

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