Interaction of aurein 1.2 and its analogue with DPPC lipid bilayer

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Abstract Antibacterial peptides have potential as novel therapeutic agents for bacterial infections. Aurein 1.2 is one of the smallest antibacterial peptides extracted from an anuran. LLAA is a more active analogue of aurein 1.2. Antibacterial peptides usually accomplish their function by interacting with bacterial membrane selectively. In this study, we tried to find the reasons for the stronger antibacterial activity of LLAA compared with aurein 1.2. For this purpose, the interaction of aurein 1.2 and LLAA with dipalmitoylphosphatidylcholine (DPPC) was investigated by molecular dynamics (MD) simulation. In addition, the structure of peptides and their antibacterial activity were investigated by circular dichroism (CD) and dilution test method, respectively. MD results showed that LLAA is more flexible compared with aurein 1.2. Furthermore, LLAA loses its structure more than aurein 1.2 in the DPPC bilayer. A higher amount of water molecules penetrate into bilayer in the presence of LLAA relative to aurein 1.2. According to the antibacterial result that indicated LLAA is remarkably more active than aurein 1.2, it can be concluded that flexibility of the peptide is a determining factor in antibacterial activity. Probably, flexibility of the peptides facilitates formation of effective pores in the lipid bilayer.

Keywords Antibacterial peptides · Aurein 1.2 · LLAA · DPPC bilayer · Molecular dynamics simulation

1 Introduction

The quick increase in drug-resistant infections has introduced a critical challenge to antimicrobial therapies. The failure of the most powerful antibiotics to kill superbugs emphasizes the immediate need to develop other control factors [1]. Antimicrobial peptides (AMPs) have been introduced as new candidates for the fight against microbial infections.

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AMPs have an important role in the innate immune systems of a wide variety of organisms, from humans to amphibians to insects. Besides their natural roles in innate immunity, AMPs are also being studied as possible therapeutics for conditions such as drug-resistant infections [2, 3] and cancers [4, 5].

AMPs are generally amphipathic and cationic in nature [6]. They accomplish their antibacterial function using these features, which allow them to interact with the bacterial membrane selectively. Most AMPs attach to and insert into membrane bilayers to form pores by ‘barrel-stave’, ‘carpet’ or ‘toroidal-pore’ mechanisms [1]. Therefore, most studies on the mechanism of action of AMPs have been focused on their interaction with lipid bilayers, either as their direct mechanism of killing or as means of getting inside the cell to disrupt intracellular targets [7–9].

Aurein 1.2 is one of the smallest (13 amino acids) anticancer and antibiotic active peptides extracted from an anuran. In the granular dorsal gland secretions of bell frogs *Litoria aurea* and *L. raniformis*, there exist 17 and 16 aurein peptides, respectively. Thirteen of these aurein peptides exhibit broad-spectrum antibiotic and anticancer function. According to their sequences, these peptides are categorized in three groups (aureins 1–3) and each group contains some members. Aurein 1.2 (GLFDIHKKIAESF-NH₂) is the most active peptide in the first group [10]. It exhibits high activity versus Gram-positive bacteria, presumably by disrupting the integrity of cell lipid bilayers [11].

The solution structure of aurein 1.2 in membrane-mimetic micelles has been determined by NMR spectroscopy. It forms an amphipathic α -helix with well-known hydrophobic and hydrophilic regions in sodium dodecyl sulfate (SDS) [12].

Aurein 1.2 has sequence homology to a section of the peptide sequence of human LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLPRTES). The reverse of its sequence between F17 and R29 results in a peptide that is similar to that of aurein 1.2 and is called LLAA [13]. LLAA with the sequence of “RLFDKIRQVIRKF-NH₂” is considered as an analogue of aurein 1.2. The structure of LLAA in SDS micelles has also been determined to be an amphipathic α -helix, like aurein 1.2 [14]. Li et al. have shown experimentally that antibacterial activity of LLAA is considerably greater than aurein 1.2 [14].

In this study, we evaluated the antibacterial activity of both aurein 1.2 and LLAA using experimental methods. Here, the interaction of aurein 1.2 and LLAA with dipalmitoylphosphatidylcholine (DPPC) was also investigated by molecular dynamics simulation to find the reasons for the stronger antibacterial activity of LLAA compared with aurein 1.2, in spite of the fact that they are analogues. In addition, the secondary structure of two peptides was investigated by circular dichroism spectroscopy. We evaluated the effect of two peptides on the model DPPC membrane and also the structure and flexibility of the peptides in the model membrane. Our goal was to compare the two peptides and understand more about their antibacterial mechanism.

2 Materials and methods

2.1 Molecular dynamics simulation

The initial structures of the two peptides (aurein 1.2 and its analogue, LLAA) were downloaded from the Protein Data Bank (PDB Ids: 1VM5 for aurein 1.2 and 2F3A for

LLAA). Both peptides were amidated with an NH_2 group in their C-terminus [12–14]. We consider a cubic box of side lengths 6.41840, 6.44350, and 6.59650 nm. In the middle of the box, in the x - y plane, we put a lipid bilayer composed of 128 DPPC molecules taken from [15]. To have the peptide vertically inside the bilayer, it should be rotated in the z -direction and then placed in the middle of the bilayer (Fig. 2a, b for aurein 1.2 and LLAA, respectively).

To avoid the probable overlaps between the lipids and the peptide, the INFLATEGRO program from P. Tieleman's group [16] was used to scale the whole system by a factor of 4. In this step, the overlapped phospholipids were deleted. Then, in 26 iterations, the system was scaled down by a factor of 0.95 until reaching the desired area per lipid. In each step, a short energy minimization was performed.

The SPC water model was used to model the solvent [17]. Water molecules were added to the box randomly; 2507 and 2535 water molecules were added respectively to the simulation of Aurein 1.2 and LLAA. Sufficient Cl^- counter-ions were also added to neutralize the overall charge of the system: one Cl^- ion in the case of aurein 1.2 and five ions for LLAA.

All simulations were carried out under periodic boundary conditions with GROMACS simulation package version 4.5 [18]. To simulate our heterogeneous system, which has lipids and peptide together, we made a combined force field by appending the essential contents of lipid force field file (from the so-called “Berger lipids”, derived by Berger et al. [19]) into the *gromos53a6.ff* force field [20].

A time step of 2 fs was used in all the simulations. The LINCS algorithm was employed to constrain the covalent bonds between hydrogen atoms and any heavy atoms [19]. To calculate long-range electrostatic interactions, the particle-mesh Ewald (PME) algorithm with Fourier spacing of 0.16 nm was used [21]. A cut-off distance of 1.2 nm was employed for both the real-space electrostatic and the LJ interactions. Equilibration was conducted in two steps: (1) 100 ps with NVT ensemble, and (2) 1000 ps with NPT ensemble while restraining the position of all the peptide atoms. Then restraints on peptide were removed and all the simulations were run for about 350 ns in the isothermal-isobaric (NPT) ensemble. The Nose–Hoover thermostat was used to keep the temperature constant at our desired temperature of 323 K [22, 23]. Also, to have a constant pressure of 1 atm, the Parrinello–Rahman coupling method was employed with a compressibility of $4.5 \times 10^{-5} \text{ bar}^{-1}$ [24].

In total, two simulations with different random series were performed for each peptide. The initial configurations for simulations of aurein 1.2 and LLAA are the same. Result analyses were accomplished using Gromacs softwares and our own code. RMSD was calculated using the *g_rms* tool included in GROMACS to study the stability of the peptides while they were inside the bilayer. RMSF was also calculated using *g_rmsf* to study the flexibility of the two peptides during equilibrium. Tilt angles of the two peptides inside the lipid bilayers were calculated using the *g_bundle* tool. To study the time evolution of the secondary structure of the peptide during simulation and to calculate the percentage of time each peptide takes on the α -helical and coil conformations, we utilized DSSP (Define Secondary Structure of Proteins) software [25, 26]. The number of water molecules penetrating into the membrane was also calculated using our own code to compare the effectiveness of each peptide in the perturbing lipid bilayer. The number of hydrogen bonds formed between the peptide and lipids was calculated using *g_hbond* for comparing the strength of interaction between the two peptides and the lipid bilayer.

2.2 Experiments

2.2.1 Antibacterial susceptibility testing

Antibacterial activity of the peptides was investigated using the dilution test method [27]. *E. coli* bacteria were grown in Mueller–Hinton broth (MHB) medium at 37 °C until the turbidity of 0.5 McFarland was achieved. Then, the peptide solution with desired concentration, the diluted McFarland standards (1:1000), and MHB medium were poured into sterile tubes. Finally, the tubes were incubated overnight (12–16 h) at 37 °C. The lowest concentration of the peptide displaying no visible growth in the tubes was determined as minimal inhibitory concentration (MIC).

2.2.2 Circular dichroism

Circular dichroism measurements were performed on an Aviv Model 215 Circular dichroism Spectrometer (Lakewood, NJ) at room temperature. Far-UV circular dichroism spectra were recorded in a quartz cell of 1-mm path length. The molar ellipticity was determined as [28]:

$$[\theta] = \frac{\theta \times 100MRW}{cl}$$

where c is the enzyme concentration in milligrams per milliliter, l is the light path length in centimeters, MRW is the mean residue weight of the peptide, and θ is the observed ellipticity in degrees.

2.2.3 Preparation of multilayer DPPC

For preparation of vesicles, DPPC was dissolved in chloroform and added to an indium tin oxide (ITO)-coated coverslip. The coated coverslip was put under nitrogen gas for several minutes and then placed into a vacuum chamber. The coated area was filled with swelling solution and then AC electric voltage was applied.

3 Results

3.1 Molecular dynamics simulation

3.1.1 RMSD, RMSF, and tilt angle

To investigate the flexibility of the peptides in the DPPC bilayer, the root-mean-square deviation (RMSD) of the α -carbon atoms of one of our simulation runs for each peptide is presented in Fig. 1a. The RMSD shows the mean spatial deviation of the peptide α -carbon atoms relative to the initial conformation of the peptides. In Fig. 1a, it is also observed that for the last 150 ns, fluctuations are more obvious for LLAA relative to aurein 1.2. The mean value of RMSD and its error bar over the last 150 ns of the simulations are 0.29 and 0.021 nm, respectively, for aurein 1.2. The same parameters for LLAA are 0.24 and 0.037 nm, respectively. The larger error bar for LLAA confirms its higher flexibility.

Fig. 1 **a** Time-dependent change of RMSD for α -carbon of aurein 1.2 and LLAA. **b** RMSF for the α -carbon of aurein 1.2 and LLAA averaged over the last equilibrated states of MD simulation. The error bars are calculated over two simulation runs

The root-mean-square fluctuation (RMSF) of each residue is the time average of its spatial fluctuations relative to a reference conformation. RMSF is a criterion that shows a measure for the structural flexibility of the peptide. RMSF diagrams for α -carbon atoms of both aurein 1.2 and LLAA averaged over the equilibrated states of our two simulation runs are plotted in Fig. 1b. The error bars are representative of the standard deviation averaged over two simulation runs. It can be concluded that LLAA shows more flexibility compared to aurein 1.2. This observation is also in agreement with the RMSD result (Fig. 1a).

We also calculated the tilt angles of the two peptides inside the bilayer (data not shown), which has an almost constant value, i.e., in the range of 20–30 and 40–50 degrees, respectively, for aurein 1.2 and LLAA. The larger value for the tilt angle of LLAA with respect to aurein 1.2 implies more accessible orientational configurations and thus its higher entropy.

3.1.2 VMD snapshots and DSSP

The final snapshots of aurein 1.2 and LLAA were plotted using VMD software (Fig. 2c, d). It was observed that while the initial structure of both peptides are α -helix, throughout the simulation, they lose their initial α -helical conformation to a different extent. Based on our observation of the time evolution of the systems, aurein 1.2 maintains its α -helical structure more than LLAA.

To study the time-dependent structural changes of the peptides during simulation, we exploited the DSSP tool of the GROMACS package. DSSP for both aurein 1.2 and LLAA embedded in the bilayer is shown in Fig. 3.

According to this figure, the most obvious structural change is a decrease in the helical content of both peptides, aurein 1.2 and LLAA. This change occurred in the N- and C-terminal in which the helical structure is transformed to a random coil, while in the middle of the peptide chains, α -helical conformation is conserved throughout the simulation. As shown in Fig. 3, for the last 150 ns that the variation in RMSD decreases, the structure of LLAA changes between helix and turn, but structural transformation of aurein 1.2 is negligible. To have a more quantitative conclusion on this, the time average percentage of α -structure is calculated, which is 40 and 44% in the case of LLAA and aurein 1.2, respectively. This observation is in agreement with the RMSF results in which LLAA fluctuation is on average greater than that of aurein 1.2.

Fig. 2 VMD snapshots. Initial configurations of simulations for **a** aurein 1.2 and **b** LLAA. Final configurations of simulations for **c** aurein 1.2 and **d** LLAA

3.1.3 Number of water molecules penetrating into the lipid bilayer and number of hydrogen bonds

A useful measure for comparing the antibacterial efficiency of the two peptides in the simulation is the number of water molecules that penetrate into the lipid bilayer. To this end, the number of water molecules present within 0.5 nm around the peptide was calculated (Fig. 4). It was observed that both peptides allow some water molecules to penetrate into the lipid bilayer, but LLAA leads to a greater penetration of water. The average number of water molecules penetrating the bilayer and its standard deviation have been calculated to be 1.5 and 1.4, respectively, for aurein 1.2, but for LLAA the mean and the standard deviation are 4.8 and 2.1, respectively. It can be concluded that LLAA leads to more disturbance of the lipid bilayer.

The number of water molecules interacting with each amino acid separately has also been calculated (data not shown). It is found that the residues close to the membrane interface have no considerable contributions to the total number of water molecules but embedded amino acids such as F3, K7, and K8 in aurein 1.2 and R1, R7, and R11 in LLAA have the most prominent effect in absorbing water molecules into the membrane.

Fig. 3 DSSP time-dependent change of **a** aurein 1.2 and **b** LLAA

We also calculated the number of hydrogen bonds formed between peptide and lipids. The average number of hydrogen bonds and its standard deviation are 13.59 and 1.53 for aurein 1.2 and the same parameters for LLAA are 23.25 and 3.05. These values show that the number of hydrogen bonds which LLAA formed with lipids is considerably higher than aurein 1.2 and the interaction between LLAA and lipid bilayer is stronger. This interaction is a key factor in the antibacterial activity of the peptide.

Fig. 4 Number of water molecules penetrating inside the lipid bilayer during simulation of aurein 1.2 and LLAA

Fig. 5 Order parameters of **a** first, and **b** second acyl chains of DPPC in the presence of the two peptides

3.1.4 Order parameter and density profile

To compare the disordering effect of the two peptides on the lipid bilayer, the order parameters of DPPC acyl chains are calculated using *g_order*. The results for the order parameters in the presence of the two peptides are shown in Fig. 5a, b, respectively, for the first and second acyl chains of the DPPC molecules.

The figure shows that the order parameter is slightly smaller in the presence of aurein 1.2 relative to LLAA, which implies that inside the lipid bilayer, aurein 1.2 has a more disordering effect on the arrangement of acyl chains, but since this difference is not too considerable, more investigations are needed to be done in the future to present a more definitive conclusion.

Moreover, the density distribution of both peptides and DPPC in the two simulations are calculated using *g_density* and shown in Fig. 6. The density profiles demonstrate an interesting asymmetric distribution for LLA in contrast to aurein 1.2. At high concentration of the peptide, this observed effect may induce some side effects.

According to previous studies, it has been shown that, at high AMP concentration, human cells can also become permeable to antibacterial peptides. The higher density of peptides on one side of the bilayer may lead to bilayer instability on one side and to leaflet fragmentation [29]. On the other hand, the higher density of LLAA on one side of the bilayer may imply that

Fig. 6 Density profile of DPPC and peptides in the simulation of **a** aurein 1.2 and **b** LLAA

Fig. 7 Circular dichroism spectra of aurein and LLAA in phosphate buffer and in DPPC phospholipid

it forms long-lived contacts with one lipid leaflet. In such a case, the stronger interaction may affect lipid dynamics and organization of the lipid-water interface. However, more detailed investigations have to be carried out to study the importance of this phenomenon.

3.2 Experiments

Circular dichroism (CD) spectra of aurein and LLAA were obtained in aqueous buffer, 30% trifluoroethanol (TFE) and in the presence of DPPC bilayer. TFE enhances polar interactions and stabilizes the extended helical structure [30]. As shown in Fig. 7, both peptides have a negative peak around 200 nm, indicating formation of irregular structure in aqueous buffer. The negative peak disappears and two intense negative peaks appear around 208 and 222 nm in the presence of TFE. As is well known, α -helical proteins have negative peaks at 208 and 222 nm, so it can be concluded that aurein and LLAA have a propensity to form a helical structure [27].

Circular dichroism spectra of the peptides in the presence of multilayer DPPC were also obtained (Fig. 7). Results indicate that CD spectra of both peptides have negative peaks at about 200 nm. Weak negative CD spectra were also observed at 220–240 nm. This observation shows that the peptides have predominantly irregular structures, but a small section of the peptides is regular. As shown in Fig. 7, the intensity of the negative peak at 200 nm for LLAA is obviously less than that for aurein. This result can be related to the greater flexibility of LLAA compared with aurein [27]. CD results are in agreement with the results of molecular dynamics simulation.

Antibacterial assay was also carried out to compare antibacterial activity of LLAA and aurein. The result implied that MIC for LLAA is much less than aurein (data not shown). This result is consistent with the previous one.

4 Discussion

In previous investigations, antibacterial activity of the two peptides, aurein 1.2 and LLAA, was compared [12] and their structures were determined in SDS (sodium dodecyl sulfate) using an NMR technique. To the best of our knowledge, no computer simulation has been carried out in DPPC membrane for comparing the two peptides.

In this investigation, structure and dynamics of both peptides in DPPC lipid bilayer were evaluated by circular dichroism and molecular dynamics simulation. We tried to find determining factors of the antibacterial activity of the peptides. To do this, their secondary structure and flexibility were studied in the DPPC membrane. Previous studies showed that both peptides have a predominantly regular helical structure in SDS and our results demonstrated a transition from the regular to irregular structures.

Comparing the results of simulation and CD method for the two peptides indicated that LLAA has a greater tendency for losing the helical structure inside the lipid bilayer. It also has higher flexibility with respect to aurein 1.2, especially in the middle amino acids. Our results also showed a larger value for the tilt angle of LLAA with respect to aurein 1.2, which implies more accessible orientational configurations and higher entropy while it is inside the membrane.

Our results showed that the average number of water molecules penetrating inside the lipid bilayer in the case of LLAA is almost three times larger than aurein 1.2. Therefore, it can be concluded that the effect of LLAA in water penetration (and thus in membrane disruption) would be more pronounced with respect to aurein 1.2. These observations are in agreement with previous studies, which showed experimentally that the antibacterial activity of LLAA is greater than aurein 1.2. We also found that some embedded amino acids, such as F3, K7, and K8 in aurein 1.2 and R1, R7, and R11 in LLAA, have the most prominent effect in absorbing water molecules into the membrane.

It can be concluded that flexibility of the peptides and the number of water molecules penetrating into the membrane influence their antibacterial effect. Based on our results, although LLAA is slightly more flexible than aurein 1.2, its MIC value is remarkably higher than aurein. Although we need more information, we expect that flexibility of the peptides facilitates the formation of an efficient channel for penetration of water molecules and disintegration of the membrane.

As we know, the mechanism of action of AMPs is more complicated than the simplified model used in this paper. They have been proposed to kill bacteria through attraction, penetration, and lysis, but since all-atom simulations of the whole process would be too time-consuming, a solution might be to investigate each step separately. In this manuscript, we tried to understand the difference between the interactions of the two peptides with the lipid bilayer while they are inside the membrane to find the determining factor responsible for their different antibacterial activity. The first step in the mechanism of action of AMPs is to get inside the membrane. In our future research, we will also investigate the interaction of these peptides with the membrane surface.

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