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Effect of dimerization on the mechanism of action of aurein 1.2

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ABSTRACT

The mechanism of action of antimicrobial peptides depends on physicochemical properties such as structure, concentration, and oligomerization. Here, we focused on the effect of dimerization on the mechanism of action of aurein 1.2 (AU). We designed a lysine-linked AU dimer, $(AU)_2K$, and its interaction with membrane mimetics was studied using four biophysical techniques and molecular dynamics simulations. Circular dichroism and molecular dynamics studies showed that AU displayed a typical spectrum for disordered structures in aqueous solution whereas $(AU)_2K$ exhibited the typical spectrum of α-helices in a coiled-coil conformation, wherein helices are wrapped around each other. With the addition of large unilamellar vesicles (LUVs), AU adopted an α-helix structure whereas the coiled-coil structure of $(AU)_2K$ assumed an extended conformation. Carboxyfluorescein release experiments with LUVs showed that both peptides were able to permeabilize vesicles although the leakage response to increases in peptide concentration differed. Optical microscopy experiments showed that both peptides induced pore opening and the dimer eventually caused the vesicles to burst. Finally, calorimetric traces determined by isothermal titration calorimetry on the LUVs also showed significant differences in peptide–membrane interactions. Together, the results of our study demonstrated that dimerization changes the mechanism of action of AU.

1. Introduction

Antimicrobial peptides (AMPs) are promising candidates for the development of novel molecules against pathogenic microorganisms [1]. These molecules have a broad-spectrum of activity, act rapidly, rarely develop drug resistance, and also exhibit anti-biofilm activity. AMPs comprise a large group of toxins used in the defense systems of almost all organisms [2–4]. Although some AMPs have specific cellular targets and others lead to apoptosis [5,6], most reported AMPs utilize the membrane of microorganisms as their main target. Membrane disruption is accepted to occur primarily via one of three mechanisms. According to the "detergent mechanism", the peptides remain tightly bound to the membrane interface up to a threshold concentration, beyond which bilayer solubilization occurs. In contrast, in the "toroidal pore" model, peptides form transmembrane pores in which the peptides are tightly bound to the polar lipid groups of the membrane, thus promoting a local curvature of the membrane. In the "barrel-stave" model, an alternative pore-model, the peptides aggregate into a barrel-like structure instead [7,8]. The mechanism of action of a particular bioactive molecule is always an essential issue to explain their activity and to consider in the design of new molecules. In order to gain information about the peptide–lipid interactions, different experimental and theoretical techniques have been used, including electron paramagnetic resonance, quartz crystal microbalance, nuclear magnetic resonance (NMR), isothermal titration calorimetry (ITC), carboxyfluorescein (CF) release from large unilamellar vesicles (LUVs), circular dichroism (CD), light scattering, and molecular dynamics (MD) [9–13]. These are usually combined in the biophysical studies of membrane interacting peptides, since they provide complementary information [14]. However, it is important to note that different conclusions may be derived according to the specific methods used [15]. The difficultly in correlating the results obtained from various methods can be attributed to the different membrane mimetics used and their composition. For example, whereas some methodologies use supported lipid bilayers, others use micelles or vesicles of different sizes and compositions. Furthermore, some experiments monitor the initial steps involved in membrane binding while others monitor the final steps.

Since the discovery of magainins from the skin secretions of *Xenopus*, amphibians have been one of the most abundant sources of AMPs [16]. Among these molecules, aureins, originally isolated from the Australian frogs *Litoria aurea* and *Litoria raniformis*, have been extensively studied [17–20]. One of the most active peptides of the aurein family is aurein 1.2 (AU), a short 13-residue peptide with a molecular mass of 1480 g mol^{-1}. This peptide is active against several microorganisms and some tumor cells, whereas it has low toxicity against red blood cells [21,22]. In terms of conformation, aurein 1.2 has no defined

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secondary structure in aqueous solutions but adopts a well-defined helical structure in the presence of structurant solvents, micelles, or vesicles [23–25].

The "detergent-like" or "carpet-like" model has been proposed for the action of AU [25–28]. However, the exact mode of action of this peptide is still not fully established, potentially owing to the fact that the mechanism by which an AMP executes its function depends on both membrane composition and peptide concentration [21,29–32]. In our previous research, we found differences in the concentration/activity relationship between AU and its C-terminal dimeric version, suggesting that a change in the mechanism of action might be imposed by dimerization [33]. In this study, we examined the effects of dimerization on the mechanism of action of AU using four biophysical techniques (ITC, CD, CF release from LUVs, and phase contrast microscopy) in conjunction with atomistic MD simulations [34,35].

2. Materials and methods

2.1. Materials

The phospholipids 1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine (SOPC) and 1-stearoyl-2-oleoyl-sn-glycero-3-phospho-(1′-rac-glycerolsodium salt (SOPG) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). The fluorescent probe 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (Dil C18) was from Life Technologies (Carlsbad, CA, USA). All other chemicals were from Sigma Aldrich (St. Louis, MO, USA).

2.2. Peptide synthesis

AU (GLFDIIKKIAESF-NH_2) and $(AU)_2K$ were manually synthesized by solid phase peptide synthesis using standard Fmoc (9-fluorenylmethyloxycarbonyl) protocols on a Rink MBHA resin, as described in Lorenzón et al. [33]. The peptide homogeneity was checked by analytical HPLC on a Shimadzu system and the identity of the peptide was confirmed by mass spectrometry in positive ion mode ESI on a Bruker model apparatus (Supplementary data).

2.3. CD spectra

CD spectra were obtained between 195 and 250 nm in a JASCO J-815 CD spectrophotometer (Tokyo, Japan) using nitrogen flushing and 1 mm path length quartz cuvettes at 25 °C. The peptide concentration was 30 μmol/L and the buffer used was HEPES pH 7.4 (10 mmol/L). Different amounts (from 0 to 20 times the peptide concentration) of LUVs, prepared as described above, were added to investigate the conformational changes imposed by membrane mimetics. CD spectra were typically recorded as an average of six scans that were obtained in millidegrees and converted to molar residue ellipticity $[\theta]_{mrw}$ (in deg cm^2 $dmol^{-1}$).

2.4. MD setup and simulation

Classical MD simulations were used to investigate the conformations of AU and $(AU)_2K$ in solution and in a membrane mimetic environment composed of 1:1 molar ratio 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine/1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPC/POPG). The membrane mimetic environment was constructed using the software Packmol version 14.225 [36]. The internal layer was built by mixing 40 units of POPC and 40 units of POPG to create a small sphere with a radius of 14 Å. An external layer of 47 Å was placed surrounding this internal sphere so that the headgroups covered the external and internal surfaces of the membrane. The external layer was composed of 150 POPC and POPG units. Each membrane was filled with explicit water molecules and immersed in a 100 mmol/L NaCl solution. The atomic coordinates for the initial conformation of the AU peptide were taken from previous NMR spectroscopy measurements (PDB ID: 1VM5) [37], in which the peptide adopted a fully helical conformation. The structure of $(AU)_2K$ was constructed by connecting two copies of the AU peptide through a lysine residue to create an isopeptide bond between its side chain and the C-terminus of the second AU chain. The GROMOS force field was employed to describe the potential energy of the system [38]. The parameters for proteins and phospholipids were taken from the GROMOS54a7 parameter set and the parameters for ions were taken from the GROMOS53a6 set [39,40]. All simulations were performed using the GROMACS package for a time length of 100 ns [41,42]. Periodic boundary conditions were applied to an orthogonal box large enough to ensure a minimum distance of 2 nm among the periodic images. After initial geometry optimization, the MD protocol was carried out as follows: 1 ns at 300 K and substance/volume/temperature (NVT) conditions were used for equilibration, 1 ns at 300 K and substance/pressure/temperature (NPT) conditions for pressure (1 atm) and density equilibration, and 100 ns for the production phase, for each simulation. The simple point charge water model was used [43], along with the Nosé–Hoover thermostat algorithm [44,45] to maintain the systems at the temperature of 300 K by separately coupling the temperatures of the membrane bilayer, peptide, and the solvent with a time constant of 0.1 ps. The pressure was maintained by weakly coupling the particle coordinates and box dimensions in the x, y, and z axes separately to a pressure bath at 1.0 bar by means of isotropic coordinate scaling with a relaxation time of 5.0 ps and a compressibility of 4.5×10^{-5} $(kJ\ mol^{-1}\ nm^{-3})^{-1}$, as appropriate for water [46]. The bond lengths between hydrogen and heavy atoms and the geometry of the water molecules were constrained using the linear constraint solver algorithm with a tolerance of 10^{-4} [41]. The particle mesh Ewald approach was used to treat long range electrostatics [47]. In all cases, the pair list for short-range non-bonded and long-range electrostatic interactions was updated with a frequency of 10 fs. The trajectory configurations were recorded every 2 ps. Defined secondary structure of protein (DSSP) software was used [48] to assign the secondary structure of the peptides over time.

2.5. Preparation of LUVs

LUVs composed of SOPC/SOPG (50/50 molar ratio) were prepared by mixing the appropriate amounts of lipid in a 4:1 (v/v) chloroform:methanol mixture in a round-bottom flask. The solvents were evaporated using nitrogen gas. A buffer solution containing 30 mmol/L HEPES, pH 7.4, was added and multilamellar vesicles were formed by mechanical agitation. This suspension was extruded 41 times through two stacked nucleopore polycarbonate filters (100 nm pore size) using an extruder system from Avanti Polar Lipids (Alabaster, AL, USA). In all experiments, the phospholipid concentration was measured by indirect determination of the phosphorus content, according to the methodology described by Rouser et al. [49].

2.6. CF release from LUVs

LUVs were prepared as described above with a buffer solution of HEPES pH 7.4 (10 mmol/L) with 50 mmol/L CF. The vesicles were separated from the non-encapsulated CF by gel filtration on a Sephadex G-50 column using HEPES pH 7.4 (10 mmol/L) with 115 mmol/L glucose for elution. The release of CF from LUVs was measured by the fluorescence intensity at a wavelength of 517 nm (492 nm excitation wavelength) after the addition of different concentrations of peptides. At the end of each experiment (9 min), Triton X-100 (1%) was added to promote full CF leakage. The percentage of CF leakage was given by $100(Ft - Fo) / (Fmax - Fo)$, where Ft is the fluorescence at 9 min, Fo is the initial fluorescence (before peptide addition), and Fmax is the maximum fluorescence obtained after the addition of Triton X-100. Data were acquired using a fluorescence spectrophotometer (Cary Eclipse, Varian, Inc., Palo Alto, CA, USA). The experiments were performed at room temperature.