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Molecular details on the intermediate states of melittin action on a cell membrane

Jiaojiao Liu[a], Shufeng Xiao[a], Jingliang Li[b], Bing Yuan[a,*], Kai Yang[a,*], Yuqiang Ma[c,a]

[a] Center for Soft Condensed Matter Physics and Interdisciplinary Research, Soochow University, Suzhou 215006, PR China
[b] Institute for Frontier Materials, Deakin University, Geelong, Australia
[c] National Laboratory of Solid State Microstructures, Department of Physics, Nanjing University, Nanjing 210093, PR China

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ABSTRACT

Antimicrobial peptides (AMPs) provide a promising solution to the serious threat of multidrug-resistant bacteria or superbugs to public healthcare, due to their unique disruption to bacterial membrane such as perforation. Unfortunately, the underlying action mechanism of AMPs, especially the possible transition between the membrane binding and perforation states of peptides (i.e., the classical two-state model), is still largely unknown. Herein, by combining experimental techniques with pertinent membrane models and molecular dynamic (MD) simulations, new insights into the intermediate states of the AMP melittin-membrane interaction process are obtained. Specifically, it is demonstrated that, after the initial binding, the accumulated melittin on the bilayer triggers vigorous fluctuation of the membrane and even extracts some lipid molecules exclusively from the deformed outer leaflet of the bilayer. Such a distinctive mass removal manner and the resultant local asymmetry in lipid number between the two leaflets change the mechanical status of the membrane and in turn reduce the free energy barrier for the melittin insertion. Finally, the formation of the transmembrane pores is facilitated significantly. These findings provide new insights into the complicated antimicrobial mechanisms of AMPs.

1. Introduction

Development of novel antimicrobials becomes significantly urgent due to the devastating threat of multidrug-resistant bacteria and superbugs. Antimicrobial peptides (AMPs)-based drug design promises a fundamental solution to this serious issue [1–3]. As an effective defense line in nature for billions of years, AMPs have exceptional broad-spectrum activities against microorganisms including Gram-positive and Gram-negative bacteria as well as fungi and viruses [4]. Most conventional antibiotics kill bacteria by inhibiting enzymatic functions, which would be bypassed via genetic adaption; in contrast, AMPs work by directly disrupting the bacterial membranes, which are thought as the “Achilles heel” of bacteria [2]. Thus AMPs could avoid the development of microbial resistance to the greatest extent possible [5].

Melittin, composed of 26 amino acid residues with 6 positive charges, is one of the most representative AMPs [6,7]. Substantial interest has been attracted on this membrane-active peptide due to its potent antimicrobial activity and even tumor cell killing and HIV destroying abilities [8]. In general, the action of melittin follows the classical two-state model (i.e. membrane perforation after binding); as a result, the bacteria are killed due to leakage of contents through the formed pores [9,10]. Much effort is made on this event in the past decades, which mostly focuses on the structure of the transmembrane pore (e.g., barrel-stave or toroidal style) or membrane lysis by carpet-like patches of peptides [1,8,11,12]. Influence from peptide-to-lipid ratio (which is particularly used in computer simulations to accurately describe the peptide concentration) or membrane property (e.g., electrical, elastic and/or phase nature) on the melittin-induced poration and leakage behaviors is widely investigated [13,14]. Translocation and redistribution of peptide on the two sides of membrane are supposed to occur before formation of a stable pore [7]. Meanwhile, about the transition of peptides from a membrane surface-binding state to a transmembrane-inserting state, it is presumed that the internal membrane tension caused by peptide interaction, reflected by the membrane structural changes (e.g., membrane area expansion and thinning), might help create the pore [7,15,16]. However, little is known about the molecular details of this phase transition, which is actually most crucial for membrane perforation.

In this paper, the intermediate transition between the initial peptide binding and final insertion states during melittin-membrane interaction

* Corresponding authors.
E-mail addresses: yuanbing@suda.edu.cn (B. Yuan), yangkai@suda.edu.cn (K. Yang).

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process is investigated, by a combination of experiments with pertinent membrane models [17–20] and molecular dynamics (MD) simulations. Based on the comprehensive experiments with supported lipid bilayers (SLBs), giant unilamellar vesicles (GUVs) and silica-sphere supported multi-lamellar membranes (SMMs), as well as the coarse-grained lipid bilayers in Silico, it is demonstrated that melittin is able to trigger fluctuations and local deformations of the membrane when above a critical peptide concentration. Meanwhile, lipid extraction exclusively in the curved outer leaflet and/or asymmetric mass removal from the two leaflets, is surprisingly found. These phenomena are proved by combining MD simulations, dissipative quartz crystal microbalance (QCM-D) monitoring and fluorescence-release test based on bilayers with different NBD-label conditions. As a result, the bilayer becomes locally asymmetric in lipid number between the two leaflets, which changes the mechanical status of the membrane and lowers the free energy barrier of transmembrane insertion of melittin; consequently, the membrane perforation is facilitated greatly. These results provide a deep and systematic insight into the molecular mechanism of antimicrobial activity of melittin.

2. Experimental section

2.1. Optical observations

Optical observation was performed on an inverted confocal laser scanning microscope (Zeiss, LSM 710, Germany) equipped with a 100× oil objective. RhB-labeled lipids were excited by a HeNe laser (EX 543 nm) and the fluorescence was observed through filter set 20 (EM BP 575–640 nm). NBD-conjugated lipids and calcein were excited by an argon ion laser (EX 488 nm) and their fluorescence was observed through filter set 44 (EM BP 530/50 nm). The transmission channel illuminated with a Halogen lamp was acquired meanwhile.

2.2. QCM-D measurements

QCM-D measurements were performed on a Q-sense E1 instrument (Sweden) [21,22]. A SiO_2-coated quartz crystal with a fundamental frequency of 5 MHz was used. A flow-through system, at 50 μL min^{-1}, was applied for the successive application of sample fluids to the sensor surface. The changes of resonance frequency (Δf) and energy dissipation (ΔD) during experiments were monitored simultaneously at five different overtones (from 3rd to 11th, i.e., 15–55 MHz). Multiple measurements for each condition were conducted for repeatability. Before each measurement, the crystal was immersed in a 2 wt% SDS for 30 min, rinsed with abundant distilled water, dried under N_2 flow, and treated with UV/ozone for 5 min prior to being mounted inside the flow module. Buffer solution was injected into the module for ~30 min for stabilization. A baseline was established before tests. All the experiments were carried out at room temperature of 25 °C.

2.3. Electro-formation of calcein-encapsulated GUVs

GUVs were prepared following the conventional electro-formation method [23]. Briefly, a solution of lipids (DOPC containing 1 mol% RhB-PE for fluorescence labeling; 60 μL × 2.0 mg mL^{-1} in chloroform) was deposited onto two ITO-coated glass slides and dried under vacuum overnight. The dry film was transferred in a homemade electro-formation chamber (with the two glass slides as electrodes), and rehydrated in 0.1 M sucrose buffer containing 0.2 mg mL^{-1} calcein. Alternating voltages were applied (0.5 V × 20 min, 1.0 V × 20 min and 1.5 V × 3 h). The obtained vesicles were washed three times via centrifugation (8000 rpm × 20 min). Well dispersed GUVs with a size distribution of 8–30 μm and encapsulated calcein were collected (~0.02 mg lipid mL^{-1}) and transferred to a home-made chamber cell for observation.

2.4. Fabrication of silica sphere-supported multi-lamellar membrane

The silica-supported lipid membrane, i.e., membrane@silica composite sphere, was fabricated following the traditional solvent-exchange method [24,25]. 0.2 mg of DOPC lipid, containing 2 mol% NBD-PE for fluorescence labeling, was dissolved in 100 μL chloroform and dried overnight in vacuum. After that, 100 μL silica sphere dispersion (40 μL silica dispersion in ethanol, containing ~10^{12} spheres, premixed with 60 μL distilled water) was added to rehydrate the dry lipid membrane. 1 mL distilled water was then added to the mixture. A micelle-to-bilayer transition and deposition process was supposed to occur on the silica sphere surface during this solvent-exchange process. The bulk solution was centrifuged at 6000 rpm for 10 min. The wash and centrifugation treatment was repeated three times to remove the excessive lipids. The precipitates were re-suspended in 500 μL distilled water for use.

2.5. Construction of SLB and real-time monitoring of melittin-SLB interactions under QCM-D

SLB was fabricated in situ on the surface of QCM-D crystal by the normal vesicle fusion method [26]. A solution of monodisperse unilamellar lipid vesicles was firstly prepared by a conventional extrusion technique. Lipids were dissolved in chloroform, dried under vacuum overnight, and rehydrated in Tris buffer to 2.0 mg mL^{-1}. The suspensions were sonicated for 20 min, and then extruded 21 times (at 50 °C) through a membrane with a pore size of 100 nm (Avanti Polar Lipids). The obtained vesicle suspension (with a size of ~110 ± 15 nm) was diluted to a lipid concentration of 0.1 mg mL^{-1} and used immediately after preparation.

During SLB preparation, the vesicle suspension was injected into the QCM-D module after a baseline was acquired. A flow-through system was employed and the parameters, f and D, were monitored simultaneously. Characteristic changes in f and D demonstrate the dynamic process of vesicles' deposition, rupture, and the formation of a SLB on the sensor surface. The ultimate shifts in f and D were −25 ± 0.2 Hz and 0×10^{-6}, respectively, indicating the formation of a high quality SLB [27]. After that, the vesicle suspension was replaced with buffer solution. A new baseline was acquired for the following introduction of melittin. During the interactions between SLB and melittin, changes in f and D values at different harmonics were monitored.

2.6. PL analyzing the lipids released from SLB by melittin

A high quality SLB (DOPC labeled with 2.5 mol% NBD-PE) was prepared with QCM-D and immersed in 1 mL refreshed buffer solution. After incubation at room temperature for 40 min without or with melittin addition (at 0.35 μM), the incubating solution (containing the peptides and lipids removed from the SLB) was extracted carefully with pippet for PL test (λex = 466 nm). Thus, quantity of the lipids disassociated from both leaflets of the bilayer would be determined from the PL intensity. In another system, the NBD-labeled SLB was pretreated with 100 μL $Na_2S_2O_4$ (at 1 M) for 5 min, before being incubated with fresh buffer without/with melittin for the following test. Such $Na_2S_2O_4$ treatment is supposed to thoroughly quench the fluorescence from the outer leaflet without disturbing the inner one of a bilayer. In this case, the measured PL intensity would reflect only the lipids disassociated from the inner leaflet. A normalization procedure was applied to the PL intensity at 539 nm under all conditions for comparison.

In control experiments, BSA and polyelectrolytes were used respectively, to pre-decorate the substrate before SLB deposition [24]. In brief, for BSA coating, the substrate was immersed in BSA solution (1 wt %), incubated for 10 min, and washed with distilled water carefully before SLB fabrication. For polymer coating, four layers consisting of positively charged poly(diallyl dimethyl ammonium chloride) (PDDA, Mw 100,000) and negatively charged poly(sodium 4-styrenesulfonate) (PSS, Mw 100,000, 1 wt% aqueous solution for use) were deposited on

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