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Membrane selectivity and biophysical studies of the antimicrobial peptide GL13K

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ABSTRACT

GL13K is a short (13 amino acid) antimicrobial peptide derived from the parotid secretory protein. GL13K has been found to exhibit anti-inflammatory and antibacterial activities in physiological salt conditions. We investigated the mechanism of interaction of GL13K, with model membranes comprising 1, 2-dioleoylphosphatidylcholine (DOPC) and 1, 2-dioleoylphosphatidylglycerol (DOPG) using various biophysical and imaging techniques. Circular dichroism studies showed that GL13K adopts a β-sheet structure in the presence of negatively charged DOPG liposomes while it retains its random coil structure with zwitterionic DOPC liposomes. GL13K did not cause any fusion of these liposomes but was able to selectively disrupt the negatively charged membranes of DOPG leading to vesicular leakage. There was no or minimal evidence of GL13K interaction with DOPC liposomes, however an analysis of supported lipid bilayers (SLBs) using atomic force microscopic (AFM) imaging and dual polarization interferometry (DPI) suggested that GL13K can interact with the surface of a DOPC planar bilayer. In the case of DOPG bilayers, AFM and DPI clearly showed membrane thinned regions where a portion of lipid molecules has been removed. These results suggest that the mechanism of GL13K action on bacterial membranes involves localized removal of lipid from the membrane via peptide-induced micellization.

1. Introduction

The discovery of penicillin in the 1940s led to the beginning of an “antibiotic era” enabling treatment of life threatening infections which was not possible before. Since then, the development of new and effective antibiotics made it possible to cure many fatal bacterial or microbial diseases. However, in recent decades the extensive and improper use of antibiotics has resulted in the emergence of new highly resistant bacterial strains and thus leading towards a “post antibiotic era” where we will have only few antibiotics left to combat multidrug resistant bacterial strains [1–3]. According to a recent report published by the World Health Organization [4], in this post-antibiotic era, the development of new effective antibiotic drugs has been diminished which has increased and prolonged illnesses leading to increased mortality rates and socio-economic burden on societies especially in developing nations. The most severe forms of bacterial infections are caused by Gram-negative bacteria and their biofilms, including *Pseudomonas* and *Acinetobacter* [5,6]. Bacterial biofilms are several folds more resistant to antibiotics compared to their planktonic (floating) state [7–9].

Antimicrobial peptides (AMPs) seem to be potent antibiotic candidates. AMPs are part of the innate immune system in most organisms (plant/animal kingdoms) [10]. Various bacteria, despite encountering these AMPs for millions of years, have not been able to develop resistance [11,12]. AMPs do not act via a stereospecific protein receptor mediated mechanism but rather target the fundamental difference in membrane composition between the host and the pathogens [12,13]. Bacteria are less able to develop resistance against AMPs by redesigning their membrane since changing membrane composition and/or lipid organization is not metabolically favorable due to limited membrane lipid synthesis capabilities [1]. This has attracted many researchers to exploit these AMPs and their synthetic analogs as novel antibiotics and an extensive database of almost 2000 known and potential AMPs (antibacterial, antiviral, antifungal and antitumor) has been established [14].

AMPs are reported to act mainly by causing membrane lysis either by barrel stave, toroidal pore or carpet mechanisms though various other mechanisms are also observed [10,15,16]. No single mechanism can be defined for all peptides [17]. Furthermore, membrane disruption mechanisms for a given peptide can vary depending on lipid composition or other environmental conditions, for example melittin and aurein were found to act by three different mechanisms (barrel stave, toroidal pore and carpet) depending on the conditions used [18–21]. The interaction of AMPs with membranes can involve a variety of processes including the disruption of peptide aggregates, binding to the membrane surface, secondary structure transformation, reorientation into the membrane,

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Abbreviations: AMPs, Antimicrobial peptides; DPI, Dual polarization interferometry; h-PSP, Human parotid secretory protein; P/L, Peptide/lipid ratio; TM, Transverse magnetic phase; TE, Transverse electric phase

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and aggregation within the membrane, disruption of membrane integrity and eventually lysis of the membrane.

It is well accepted that electrostatic interactions can play a major role in the activity and specificity of AMPs notwithstanding the significant roles of hydrophobic forces, membrane curvature and mechanical properties. Despite differences in mechanisms, it can be concluded that AMPs act mainly by binding to membranes and kill bacteria by disrupting membrane packing and organization causing defects in the membrane with the consequent destruction of transmembrane potential and leakage of important cellular contents [12,22].

GL13K is a small cationic AMP, which was designed by a modification of a peptide sequence derived from human parotid secretory protein (hPSP) [23]. PSP is predicted to be structurally similar to bactericidal/permeability-increasing protein and lipopolysaccharide-binding protein [24]. This predicted structural similarity of PSP was used to identify potential antimicrobial peptides in the PSP sequence [23,25]. One of the resulting peptides, GL13NH2, induced bacterial agglutination but was not bactericidal [25]. To produce GL13K, the GL13NH2 peptide was modified by introducing three lysine residues, which switched the activity from agglutinating to bactericidal [25]. Both peptides exhibit anti-lipopolysaccharide activity [23,26,27]. The modified peptide, GL13K, comprises 13 amino acids (GKIIKLKASLKLL-CONH2) and carries a net charge of +5 at pH-7 as calculated using the Innovagen online peptide property calculator [28]. GL13K has strong anti-inflammatory and antibacterial activities against both Gram-negative and biofilm forming bacteria but exhibits low hemolytic and cytotoxic activities. Also, like polymyxin B, GL13K binds lipopolysaccharide [26], a component of the outer bacterial membrane. Of particular note, GL13K has been found to be effective against the Gram-negative bacterium *Pseudomonas aeruginosa*. These opportunistic pathogens are associated with infections and biofilm formation in susceptible individuals, including nosocomial infections and cystic fibrosis patients [29].

Herein we present the biophysical studies to understand the membrane disruption mechanism for GL13K. Bacterial membranes are highly anionic in nature whereas outer leaflets of eukaryotic membranes tend to be neutral [30–32]. Therefore, we chose zwitterionic DOPC and anionic DOPG lipids as models for eukaryotic and bacterial membranes, respectively, to elucidate the effect of electrostatic interactions on the activity and specificity of GL13K.

2. Materials and methods

2.1. Materials

Lipids were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). 5-(and-6)-Carboxyfluorescein (CF) mixed isomers were supplied by Molecular Probes (Eugene, OR, USA) and used as the sodium salt of CF, obtained by neutralization with two molar sodium hydroxide. The peptide GL13K was obtained from the University of Minnesota peptide synthesis facility at better than 95% purity as evidenced by HPLC and mass spectrometry [26]. The peptides were supplied as lyophilized trifluoroacetate salts. Stock solutions were prepared in water and then diluted in appropriate buffer. Sephadex G-50 (fine with a bead size of 20–80 μm, ACS grade), sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$), sodium hydrogen phosphate heptahydrate ($Na_2HPO_4 \cdot 7H_2O$), 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), sodium hydroxide (NaOH) and sodium chloride (NaCl) were obtained from Sigma-Aldrich. Unless otherwise stated, all experiments were conducted in 10 mM sodium phosphate buffer at pH 7.4 ("sodium phosphate") in this report prepared from $NaH_2PO_4 \cdot H_2O$ and $NaH_2PO_4 \cdot 7H_2O$. HEPES buffer was prepared by dissolving the desired amount of HEPES in water and adjusting the pH to 7.4 using NaOH. In all cases ultrapure water (18.2 MΩ cm resistivity) was used.

2.2. Liposome preparation

Liposomes were prepared by extrusion as described by Mayer et al. [33] using a mini-extruder supplied by Avanti Polar Lipids Inc. (Alabaster, AL, USA). The desired lipid was dissolved in chloroform and the solvent removed under vacuum for 40 min to yield a thin film of lipid. The dried films were hydrated using the desired buffer at room temperature for 2 h and then vortexed at high speed for 10 min resulting in the formation of multi lamellar vesicles. These vesicles were then extruded 41 times at room temperature through 0.1 μm size nucleopore polycarbonate membranes mounted in a mini-extruder in order to ensure liposomes of low polydispersity.

2.3. Isothermal titration calorimetry (ITC)

The thermodynamic parameters for the interaction of peptide with DOPG and DOPC liposomes were studied using a VP-ITC Microcalorimeter (MicroCal Inc., Northampton, MA, USA). All solutions were degassed for 30 min under a vacuum with continuous stirring before loading the reaction chamber and syringe. In one set of experiments, 4 μl of 1 mM DOPC or DOPG liposomes were injected sequentially into 0.1 mM GL13K contained in the reaction cell. A stirring speed of 300 rpm with injection periods of 8 s were chosen with an equilibration time of 4 min between each injection to ensure proper mixing and a stable baseline. In another set of experiments, 3 μl of 1 mM GL13K were injected sequentially into 0.1 mM DOPG liposomes contained in the reaction cell while all other parameters remained the same. These experiments were carried out in sodium phosphate buffer in the presence or absence of 107 mM NaCl (as indicated) to study the effect of charge shielding on GL13K binding parameters. All experiments were performed at 22 °C. Data analysis was performed using MicroCal Origin® 5.0 software. Diminished heat signals observed after the system reached saturation were used to correct for the heat of dilution. The binding site model was chosen based on best mathematical fit rather than a mechanistic model as the binding of AMPs with membranes is not due to a specific binding/receptor site.

2.4. Circular dichroism (CD)

CD spectra were recorded using a J-815 spectrometer (Jasco Corporation, Essex, UK) as an average of 5 scans obtained using a 0.2 cm path length quartz cuvette at 22 °C from 200 nm to 260 nm with data pitch 0.2 nm, scan speed 20 nm/min and a response time of 1 s. All spectra were corrected by subtraction of the buffer spectra. We were not able to record below 200 nm due to high tension voltage value going above 600 V leading to low signal to noise ratio. Spectra for GL13K in the presence or absence of liposomes were obtained at a fixed peptide concentration of 60 μM with varying lipid concentrations to obtain peptide/lipid ratios (P/L) ranging from 1/15 to 1/2.5. All spectra were corrected for path length and concentration to give the mean residue ellipticity.

2.5. Carboxyfluorescein (CF) release assay

Liposomal leakage caused by GL13K was studied by following the release of CF encapsulated at high concentration in the core of liposomes [34,35]. CF is self-quenched when entrapped in the liposome core [36]. Membrane disruption leading to the release of CF yields an increase in fluorescence intensity. Liposomes for CF assays were prepared by hydrating the dried film with 50 mM CF in 10 mM HEPES buffer at pH-7.4 and extruded as explained in Section 2.2. Non-encapsulated CF was separated from CF encapsulating liposomes using a Sephadex G-50 column with 10 mM HEPES, 107 mM NaCl at pH-7.4 as an eluent. The lipid concentration was determined using phosphate analysis based on the method by Bartlett et al. [37]. CF leakage experiments were recorded using a Varian Cary Eclipse spectrophotometer (Agilent Technologies Inc., Mississauga, ON, Canada) equipped with a Varian Cary