Bioorganic & Medicinal Chemistry 19 (2011) 7008–7022

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

The effect of the placement and total charge of the basic amino acid clusters on antibacterial organism selectivity and potency

Amanda L. Russell [a], Anne M. Spuches [a], Brittany C. Williams [a], Divakaramenon Venugopal [a,†], David Klapper [b], Antoine H. Srouji [c], Rickey P. Hicks [a,*]

[a] Department of Chemistry, East Carolina University, Science and Technology, Suite 300, Greenville, NC 27858, USA
[b] Peptide Core Facility, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA
[c] Synthetic Proteomics, Carlsbad, CA, USA

ARTICLE INFO

Article history:
Received 5 August 2011
Revised 28 September 2011
Accepted 5 October 2011
Available online 18 October 2011

Keywords:
Unnatural amino acids
Antimicrobial peptides
CD spectroscopy
Isothermal titration calorimetry
Fluorescence spectroscopy
Calcein leakage

ABSTRACT

Extensive circular dichroism, isothermal titration calorimetry and induced calcein leakage studies were conducted on a series of antimicrobial peptides (AMPs), with a varying number of Lys residues located at either the C-terminus or the N-terminus to gain insight into their effect on the mechanisms of binding with zwitterionic and anionic membrane model systems. Different CD spectra were observed for these AMPs in the presence of zwitterionic DPC and anionic SDS micelles indicating that they adopt different conformations on binding to the surfaces of zwitterionic and anionic membrane models. Different CD spectra were observed for these AMPs in the presence of zwitterionic POPC and anionic mixed 4:1 POPC/POPG LUVs and SUVs, indicating that they adopt very different conformations on interaction with these two types of LUVs and SUVs. In addition, ITC and calcein leakage data indicated that all the AMPs studied interact via very different mechanisms with anionic and zwitterionic LUVs. ITC data suggest these peptides interact primarily with the surface of zwitterionic LUVs while they insert into and form pores in anionic LUVs. CD studies indicated that these compounds adopt different conformations depending on the ratio of POPC to POPG lipids present in the liposome. There are detectable spectroscopic and thermodynamic differences between how each of these AMPs interacts with membranes, that is position and total charge density defines how these AMPs interact with specific membrane models and thus partially explain the resulting diversity of antibacterial activity of these compounds.

1. Introduction

The crisis is caused by the dramatic and continued evolution of drug resistant strains of bacteria[1,2] has stimulated an extensive world-wide research effort to develop new classes of compounds that exhibit novel mechanisms of antibacterial activity.[1,3–8] Natural and synthetic, antimicrobial peptides (AMP) offer promise as new therapeutic agents because of their novel mechanisms of antibiotic activity.[4,6,9–12] In our laboratory we developed a series of novel AMPs incorporating unnatural amino acids into the primary sequence of the peptide. These compounds have shown low μM to nM in vitro efficacy and selectivity against several bacteria strains including Gram positive, Gram negative and mycobacterium.[13,14] The major advantage of incorporating unnatural amino acids in the primary sequence of peptide therapeutics is the increased metabolic stability[11,15–18] thus increasing the therapeutic potential of peptide based drugs.

It is generally accepted that electrostatic interactions are the first step in the process of an AMPs binding to the surface of a cell membrane. All AMPs exhibit a high net positive charge[19] while most bacterial cell membranes contain a relatively high percentage of negatively charged phospholipids when compared to mammalian cell membranes.[20] The resulting difference in the electronic character of the cell membranes thus explains the inherent selectivity of AMPs for bacteria over mammalian membranes.[5]

It is well documented in the literature that the selectivity and potency of an AMP against a particular organism is defined in large measure by the complementary nature of the physicochemical surface properties of the cell's membrane and the interacting AMP.[5,6,8,21–24] The chemical composition of bacterial membranes

Abbreviations: Ahx, 6-aminohexanoic acid; AMP, antimicrobial peptide; βAla, beta alanine; CD, circular dichroism; Gaba, gamma aminobutyric acid; ITC, isothermal titration calorimetry; LUV, large unilamellar vesicles; MIC, minimum inhibitory concentration; MR, *Mycobacterium ranae*; Oic, octahydroindolecarboxylic acid; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt); QSAR, quantitative structure–activity relationship; Tic, tetrahydroisoquinolinecarboxylic acid; SA, *Staphylococcus aureus*; ST, *Salmonella typhimurium*; SUV, small unilamellar

* Corresponding author. Tel.: +1 252 328 9702; fax: +1 252 3286210.
E-mail address: hicksr@ecu.edu (R.P. Hicks).
† Present address: HiTech Analytical and Diagnostic Solutions, 17B First Field Road, Suite 207, Gaitherburg, MD 20878, USA.

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doi:10.1016/j.bmc.2011.10.033

varies greatly between different strains, therefore the interaction of a particular AMP will also vary between different strains of bacteria.[19,23,24] The hypothesis upon which our work is based is that the 3D-physicochemical surface properties of a cell's membrane (bacterial or mammalian) interact with those of the approaching AMP in a very specific way, thus defining the resulting organism selectivity and potency. These AMPs exhibited different in vitro activity against *Staphylococcus aureus* and *Mycobacterium ranae* bacteria which have chemically very different cell membranes. Therefore, we hypothesized that the differences in the observed biological activity was a direct manifestation of the different physicochemical interactions that occur between these peptides and the cell membranes of the *S. aureus* and *M. ranae* bacteria.[25] For this hypothesis to be correct different physicochemical descriptors must correlate with the anti-bacterial activity of these compounds against *S. aureus* and *M. ranae* bacteria. Two 3D-QSAR (quantitative structure–activity relationship) models defining the physicochemical properties of these AMPs required for activity against *S. aureus* and *M. ranae* bacteria were developed. It was determined that there are five physicochemical descriptors necessary to define the activity of these AMPs against *S. aureus* and that five different physicochemical descriptors are necessary to define the activity of these AMPs against *M. ranae*.[25] These results support the hypothesis that for any particular AMP, organism selectivity and potency are controlled by the chemical composition of the target cell membrane.

These AMPs were designed to systematically investigate the influence of small structural changes on the peptide's surface physicochemical properties, and then determine their effect on the binding to membrane model systems. As pointed out by several researchers in order to develop AMPs as potential therapeutic agents it is critical to develop an understanding of the basis for the different interactions that occur between the AMP and zwitterionic and anionic lipids in order to improve selectivity for bacterial cells.[11,26,27]

Micelles are excellent models to study the surface interactions that occur between peptides and membranes since micelles do not form bilayers and therefore, the peptides can't fully insert into the micelle to form pores as is the case with phospholipids. For this investigation, SDS micelles[28] were selected as a simple model for anionic lipids of prokaryotic cells and DPC micelles[29] as a simple model for zwitterionic lipids of eukaryotic cells. Large unilamellar vesicles (LUVs) and small unilamellar vesicles (SUVs) consisting of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were selected as a simple model for the zwitterionic membranes of mammalian cells and LUVs consisting of (4:1) 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)/1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt) (POPG) were selected as a simple model for the anionic membranes of bacterial cells to investigation the inserted state interaction associated with membrane binding.[30] The selection of mixed POPC/POPG lipids was not the best choice to mimic the lipid composition of bacteria membranes since POPC is normally not found in bacterial membranes, however it was selected to allow for comparison to previously reported investigations of AMP lipid interactions.[27,31–36] As pointed out by Huang and co-workers 'clarifying the pore forming mechanism' of a peptide 'will facilitate ... development of antimicrobial peptides as human therapeutics'.[37] This requires an understanding of the binding process to both zwitterionic and anionic membrane.

2. Materials and methods

Sodium dodecyl sulfate (SDS) and Bis–Tris buffer were purchased from Sigma–Aldrich. Monobasic and dibasic sodium phosphate, EDTA and NaCl were purchased from Fischer Scientific. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt) (POPG) and dodecylphosphocholine (DPC) were purchased from Avanti Polar Lipids. High purity calcein was purchased from Invitrogen. All chemicals were used without further purification.

2.1. Peptide synthesis

Peptide synthesis was performed either manually using *t*BOC chemistry or with an automated peptide synthesizer using FMOC chemistry[38,39] as previously reported.[13,14,40] All peptides were purified by Reverse Phase HPLC using an Agilent 1100 Series Preparative Instrument using a Vydac C18 Reverse Phase Preparative HPLC Column as previously reported.[13,14,40] All purified peptides were analyzed again by HPLC and Mass-Spec. Mass Spectral analyses using a Finnigan LTQ ESI-MS instrument running Xcalibur 1.4SR-1 or a Kratos PC Axima CFR Plus instrument (MALDI) running Kompact V2.4.1. ESI-MS showed multiply charged ions and the accurate mass was calculated. MALDI analyses were performed in reflectron mode.[14,40]

2.2. Preparation of POPC and POPC/POPG SUVs

The appropriate amount of dry POPC or 4:1 POPC/POPG (mol to mol) was weight out to yield a final lipid concentration of 35 mM. The lipid was dissolved in chloroform and vortexed for 3 min. The sample was dried under nitrogen for 4 h and under high vacuum overnight. The lipid was then hydrated with 2 mL of buffer (40 mM sodium phosphate, pH 6.8) and vortexed extensively. SUVs were prepared by sonication of the milky lipid suspension using a titanium tip ultra-sonicator (Qsonica Sonicators model Q55) for approximately 40 min in an ice bath until the solution became transparent. The titanium debris were removed by centrifugation at 14,000 rev./min for 10 min using an Eppendorf table top centrifuge.[41] Final lipid concentration used for CD studies was 1.75 mM.

2.3. Preparation of POPC and POPC/POPG LUVs

A defined amount of dried POPC or 4:1 POPC/POPG (mol to mol) was weight out and suspended in buffer (40 mM sodium phosphate, pH 6.8) and spun for 30 min resulting in a total lipid concentration of 1.75 mM for CD studies and 35 mM for ITC studies. Other concentrations used are noted in the text. Large unilamellar vesicles (LUVs) were prepared by extrusion using a Mini-Extruder (Avanti Polar Lipid Inc.).[42,43] The solution was extruded through a 100 nm pore size polycarbonate membrane 21 times. After extrusion the LUVs were allowed to 'rest' for at least 2 h before use to allow equilibration to occur. The final lipid concentration was calculated based on the weight of the dried lipid.[31,33,40,41,44,45] Previously Kennedy and co-workers reported the preparation of LUVs using this procedure resulted in a homogeneous population of LUVs with >95% of the particles falling into the particle size range of 70–100 nm.[46] Kennedy and co-workers have also previously shown by ^{31}P NMR that these LUVs are unilamellar.[46]

2.4. LUVs for dye release experiments

A defined amount of dried POPC or 4:1 POPC/POPG LUVs (mol to mol) was weighed and suspended in a calcein-containing buffer (70 mM calcein, 10 mM Bis–Tris, 150 mM NaCl, 1 mM EDTA, pH 7.1, the pH was corrected using 3 mM NaOH and the final lipid concentration was calculated based on dilution). The resulting solution was vortexed for 1 min (5 times). The calcein-encapsulated LUVs were extruded using the same technique as above. Following extrusion the unencapsulated calcein was removed by gel filtration