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The defensin–lipid interaction: Insights on the binding states of the human antimicrobial peptide HNP-1 to model bacterial membranes

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ABSTRACT

Antimicrobial peptides are an important component of innate immunity and have generated considerable interest as a new potential class of natural antibiotics. The biological activity of antimicrobial peptides is strongly influenced by peptide–membrane interactions. Human Neutrophil Peptide 1 (HNP-1) is a 30 aminoacid peptide, belonging to the class of α-defensins. Many biophysical studies have been performed on this peptide to define its mechanism of action. Combining spectroscopic and thermodynamic analysis, insights on the interaction of the α-defensin with POPE:POPG:CL negative charged bilayers are given. The binding states of the peptide below and above the threshold concentration have been analyzed showing that the interaction with lipid bilayers is dependent by peptide concentration. These novel results that indicate how affinity and biological activities of natural antibiotics are depending by their concentration, might open new way of investigation of the antimicrobial mode of action.

1. Introduction

A wide variety of organisms produces antimicrobial peptides (AMPs) as part of their first line of defence [1]. AMPs are typically relatively short (12 to 100 amino acids), positively charged (net charge ranging from +2 to +9, with +4 to +6 being the most common) and contain about ~50–70% of hydrophobic amino acids [1–5]. AMPs display a broad spectrum of antimicrobial activity against both Gram-positive and Gram-negative bacteria, fungi, yeast and enveloped viruses [6].

It is believed that the mechanism of antimicrobial action is related to the permeabilization of the microbial cell membrane [6,7]. In this context the lipid composition is a key factor to better understand the peptide:lipid interaction in model membranes [5–7]. Several models have been proposed to describe the molecular events involved in the AMP-mediated membrane disruption, including the formation of barrel-stave peptide channels, induction of peptide–lipid toroidal pores and a detergent-like mechanism [2–8]. The threshold concentration of AMPs is an important factor to understand their mechanism of action. Generally, the interaction between peptides and lipids takes place in two steps: deposition on the membrane surface and insertion into the bilayer. At exceeding threshold concentrations, all peptides are able to destabilize the phospholipid bilayer and cause membrane disruption [7,9–13]. For this reason, it is likely that some results for putative AMPs permeabilizing vesicles are essentially artefacts that arise from extremely high peptide:lipid ratios used. In general, any membrane-interacting molecule can disrupt membranes at very high concentrations.

Human Neutrophil Peptide 1 (HNP-1) is a peptide belonging to the class of α-defensins, which are produced in azurophil granules of neutrophils [14]. This 30 amino acid peptide is present as a dimer in solution. Each monomer unit is composed of three anti-parallel β-sheet and a β-hairpin. The peptide structure is strongly stabilized by three cysteine disulfide bridges (C_2–C_{30}, C_4–C_{19}, C_9–C_{29}) interconnecting each β-sheet and a salt bridge between Arg-5 and Glu-13 [15]. HNP-1 has a positive net charge equal to +3, conferred by four cationic arginine residues and the single anionic glutamic acid residue [16–18]. The general mechanism for its antimicrobial action relies on the permeabilization of the microbial cell membrane [19,20] and the importance of Arg and Trp residues of HNP-1 for this membrane interaction is now clear [21–24]. A "dimer pore" model for HNP-1 has been suggested on the basis of a crystal structure study [25], while a multimeric pore model was proposed on the basis of vesicle leakage and dextran permeability experiments [26].

Abbreviations: AMP, antimicrobial peptide; HNP-1, human neutrophil peptide 1; EPR, Electron Paramagnetic Resonance; CD, Circular Dichroism; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphatidylglycerol; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphatidylethanolamine, CL, 1,1′,2,2′-tetramyristoyl cardiolipin ammonium salt; MOPS, 3-(N-morpholino) propanesulfonic acid; LUV, Large Unilamellar Vesicle; PCSL, phosphatidylcholine spin-labels; DMPC:DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine:1,2-dimyristoyl- *sn*-glycero-3-phosphatidylglycerol

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In this study, a combined approach using spectroscopic (EPR, CD and Fluorescence) and thermodynamic techniques have been used to analyze the binding states of the HNP-1 peptide with POPE:POPG:CL liposomes as it accumulates on the bilayer surface. The peptide:lipid 1:20 molar ratio has been determined as the threshold concentration at which the peptide penetrates into the bilayer. When the α-defensin:lipid molar ratio is above or below the threshold concentration, the peptide tends to accumulate on the membrane surface with a different mechanism of action.

2. Material and methods

The HNP-1 peptide [ACYCRIPACIAGERRYGTCIYQGRLWAFCC] was obtained from Bachem Peptide (Bubendorf, CH) and used without further purification. The phospholipids for vesicle preparation, 1-palmitoyl-2oleoyl-*sn*-glycerol-3-phosphoglycerol (POPG), 1-palmitoyl-2oleoyl-*sn*-glycerol-3-phosphoethanolamine(POPE), 1,1′,2,2′-tetramyristoyl cardiolipin ammonium salt (CL), and spin-labeled phosphatidylcholine (1-acyl-2-[*n*-(4,4dimethyloxazolidinyl-N-oxyl)]stearoyl-*sn*-glycero-3-phosphocholine, *n*-PCSL, with n = 5, 7, 12) were obtained from Avanti Polar Lipids (Alabaster, AL). The buffer solution (pH 6.8) used in the experiments contains 50 mM of 3-(N-morpholino) propanesulfonic acid (MOPS) (Sigma Aldrich). MOPS is a buffer largely used for biological sample preparation due to its low salt content [27].

2.1. Liposome preparation

Liposomes were prepared mixing 14 mM POPE, 12.9 mM POPG and 6.6 mM CL (POPE:POPG:CL 70:25:5 molar ratio), in agreement with the composition of Gram-negative bacterial inner membrane lipids [13,28].

Lipids at the desired molar ratio were dried down from chloroform stock solutions under a stream of nitrogen gas and then dried under vacuum for 1 h. The resulting lipid film was hydrated by adding 50 mM MOPS at pH 6.8 to reach a final concentration of about 50 mM phospholipids. Large unilamellar vesicles (LUVs) were prepared by freeze-thawing this lipid suspension five times followed by extrusion through 200 nm polycarbonate membrane filters using a miniextruder syringe device (Avanti Polar Lipids). Final concentration of LUVs was determined using the Stewart phospholipids assay [29]. LUVs containing 1 mol of 5, 7, or 12-PCSL were prepared as described above. Different peptide:lipid molar ratios were prepared (1:5, 1:10, 1:15, 1:20, 1:30, 1:50, 1:100, 1:250 and 1:350) where the HNP-1 peptide was incubated with LUVs in 50 mM MOPS buffer at pH 6.8 for 1 h.

2.2. Circular dichroism spectroscopy

CD experiments were performed at room temperature on a Jasco CD-J-815 spectropolarimeter using a quartz cuvette with a path length of 1 mm. Peptide was suspended at 0.02 mM concentration in 50 mM MOPS at pH 6.8 with a variable concentration of LUVs ranging from 0.1 to 7 mM. CD spectra were recorded from 190 to 250 nm and accumulated ten times to improve the signal-to-noise ratio. Baselines of either solvent or vesicular suspension without peptide were subtracted from each respective sample to calculate the peptide contribution [30].

2.3. Electron Paramagnetic Resonance spectroscopy

EPR spectra were recorded on a Bruker E500 ELEXSYS X-Band spectrometer equipped with a super-high-Q cavity. Samples prepared for EPR measurements contained 0.4 mM of *n*-PCSL LUVs and a variable concentration of peptide ranging from 0.02 to 0.08 mM. Spectra were recorded using the following instrumental settings: 120 G sweep width; 100 kHz modulation frequency; 1.0 G modulation amplitude; 40 ms time constant; 20 mW microwave power. Several scans, generally 15, were made to improve the signal-to-noise ratio. Values of the outer hyperfine splitting, $2A_{max}$, were determined by measuring the difference between the low-field maximum and minimum. Measurements were performed in triplicate and the reproducibility of $2A_{max}$ is typically ±0.02–0.07 G. The relative values of $2\Delta A_{max}$ were obtained by calculating the difference of outer hyperfine splitting constant for the spectra with or without HNP-1. To assess the rotational mobility of 12-PCSL, the apparent rotational correlation time (τ) was determined according to [13]:

$$\tau = \left(0.65 \cdot 10^{-9}\right) \Delta H_0 \left[\left(A_0/A_{-1}\right)^{1/2} - 1\right] \quad (1)$$

where ΔH_0 is the peak-to-peak width of the center line in gauss, A_0 is the amplitude of the center line, and A_{-1} is the amplitude of the high field line (see Fig. 2). The rotational correlation time is inversely related to the motional spin label rate such that an increase in τ indicates slower motion.

2.4. Fluorescence and thermodynamic measurements

Peptide–lipid interactions were studied by monitoring the change in the Trp-26 fluorescence emission spectra in the presence of LUVs. Samples prepared for fluorescence measurements contained 0.02 mM peptide and a variable phospholipids concentration ranging from 0.1 to 7 mM. Fluorescence measurements were performed on a Jasco FB-6500 spectrometer. The excitation wavelength was 280 nm, and emission spectra were recorded between 290 and 400 nm, with a 1 nm slit widths at room temperature [31,32]. A very small emission band in the range of 290–310 nm due to tyrosine residues contribution is present in some samples, but it does not influence the tryptophan fluorescence band [33].

Binding experiments of peptide to LUVs (0.02 mM HNP-1 in 50 mM MOPS buffer at pH 6.8) were performed with an ultrafiltration assay to separate lipid phase from the free peptide using Centricon-30 kDa cut-off filters (Millipore Inc.). The peptide was added to LUVs at a peptide:lipid molar ratio of 1:350, 1:250, 1:100, 1:50, 1:30, , 1:20, 1:15, 1:10, 1:5, incubated for 30 min and then centrifuged at 6000 × *g* for 1 h [36].

The free peptide concentration in the eluate has been determined through a calibration curve plotting known peptide concentration versus fluorescence intensity. The amount of the peptide bound to lipid was measured by subtracting the free peptide concentration from the total peptide concentration. Based on our CD studies, we found that α-defensin can form aggregates in solution at physiological pH at a concentration above 0.04 mM (data not shown). In all partition coefficient experiments, HNP-1 concentration has been maintained below 0.04 mM to avoid peptide aggregation process that could bring to a misleading determination of free protein concentration.

The mole fraction partition coefficient (K_p) was determined as following:

$$K_p = \left([P]_{bilayer}/[L]\right)/\left([P]_{wat}/[W]\right) \quad (2)$$

where [P] is the peptide concentration in the bilayer or water phase, [L] is the molar lipid concentration and [W] is the water molar concentration (55.3 M at 25 °C). The free energies of transfer, ΔG°, from water to lipid were calculated from:

$$\Delta G^\circ = -RT \ln K_p \quad (3)$$

where R is the gas constant and T is the temperature [34–36].

For quenching experiments, samples were prepared dissolving HNP-1 in 50 mM MOPS (pH = 6.8) at a final concentration of 0.02 mM. Vesicles were added in specific peptide:lipid molar ratio equal to 1:5,