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Structural diversity and mode of action on lipid membranes of three lactoferrin candidacidal peptides

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ABSTRACT

The structure and membrane interactions of three antimicrobial peptides from the lactoferrin family were investigated through different techniques. Circular dichroism shows that the peptides adopt a secondary structure in the presence of DMPC/DMPG, and DSC reveals that they all interact with these membranes, albeit differently, whereas only LFchimera has an effect in pure zwitterionic membranes of DMPC. DSC further shows that membrane action is weakest for LFcin17-30, increases for LFampin265-284 and is largest for LFchimera. These differences are clearly reflected in a different structure upon interaction, as revealed by SAX. This technique shows that LFcin17-30 only induces membrane segregation (two lamellar phases are apparent upon cooling from fluid phase), whereas LFampin265-284 induces micellization of the membrane with structure compatible to a micellar cubic phase of space group Pm3n, and LFchimera leads to membrane destruction through the formation of two cubic phases, Pn3m and Im3m. These structural results show a remarkable parallel with the ones obtained previously by freeze fracture microscopy of the effect of these peptides against *Candida albicans*.

1. Introduction

We continue to face a reemergence of infectious diseases, mainly due to the increasing resistance of the pathogens to current therapies and the lack of new and more effective antimicrobial drugs. One potential and interesting alternative to conventional antibiotics is the use of antimicrobial peptides (AMPs). Natural AMPs are present in almost all living organisms as a primary defense mechanism against invading pathogens, with remarkably different structures and bioactivity profiles [1].

AMPs are considered membrane-active agents leading to cell death by acting on the phospholipid membrane [2]. Within this broad umbrella, it is recognized today that they do not act through a universal mechanism. Different mechanisms have been proposed, consistent with experimental results, providing possible ways for the peptides to disrupt the membrane, leading to cell death. All rely on the same main factor for initial action — adsorption of AMPs onto the membrane due to electrostatic interactions between the cationic peptides and the headgroups of anionic phospholipids. Thereafter, accumulation and positional change eventually lead to the formation of pores, membrane permeabilization or membrane micellization [1,3–7]. In some cases internal targets have also been described [5,8–11].

Independently of the details of the mechanism of action the interaction must be as selective as possible regarding the distinction between mammalian cells (higher eukaryotes) and pathogen cells, such as bacteria (prokaryotic cells) or lower eukaryotes as fungi and protozoan. Cytoplasmic membranes of mammalian cells expose predominantly zwitterionic phosphatidylcholine (PC) and sphingomyelin to the extracellular side [1]. On the other hand, cytoplasmic bacterial membranes are mainly composed of zwitterionic phosphatidylethanolamine (PE) and negatively charged phosphatidylglycerol (PG) conferring an overall

Abbreviations: AMPs, antimicrobial peptides; CD, circular dichroism; DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; DSC, differential scanning calorimetry; IMPs, intra-membranous particles; LC_{50}, lethal concentration that causes 50% of cell death; LFampin265-284, lactoferrampin 265–284; LFchimera, LFcin17-30-K-LFampin265-284; LFcin17-30, lactoferricin 17–30; LUVs, large unilamellar vesicles; MLVs, multilamellar vesicles; P:L, peptide-to-lipid molar ratio; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; SAXD, small angle X-ray diffraction; WAXD, wide angle X-ray diffraction

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negative charge to the membrane [1,12]. Lower eukaryotes, such as fungi and protozoa also have PC, but they have higher amounts of exposed anionic phospholipids, like phosphatidylserine, than mammalian cells [1,13,14]. Indeed it is this differential composition that justifies the unifying electrostatic character of the initial interaction, as well as the ability of the AMPs to act preferentially against pathogens.

Biophysical studies can provide important information on the details of AMPs interaction with the membranes and thus help to unravel their mechanism of action, by providing insight into the effects of the peptides on the membrane structure and information on peptide location. Different techniques have been employed, such as calorimetry, spectroscopy, X-ray diffraction and others [3,12,15–28].

X-ray diffraction studies can give quantitative information on the effects of AMPs on membrane structure, namely if they are capable of altering the phospholipid structure and organization, as well as phase behavior. This information thus allows correlation of these possible changes in lipid polymorphism with models for the mechanism of action of AMPs [12,22,23,28]. Growing evidence shows that lipid cubic phases are ubiquitous in the biological world as they have been detected in the plasma membrane of archaebacteria, as well as in the endoplasmic reticulum and mitochondria of mammalian cells. These phases are also involved in biological processes such as membrane fusion, fat digestion and in the reorganization of cell membrane composition [1,29–32]. In the AMP research area, some reports started to appear in the literature indicating the ability of AMPs to induce cubic phases. So far most studies have revealed the existence of bicontinuous (single or double) cubic phases [12,19,24–28,33–35] and a recent one reported a micellar cubic phase [36].

Previously, Bolscher et al. [37,38] have obtained freeze-fracture results on the action of peptides of the lactoferrin family against *Candida albicans*, showing that the peptides have a quite different effect on the membrane of this pathogen. In the present work we studied the action of these peptides on model membranes of DMPC/DMPG (3:1), considered to be a good model system for *C. albicans*, by a variety of biophysical techniques. We found that lactoferricin 17–30 (LFcin17-30) induces phase segregation and is the peptide with lowest membrane activity, lactoferrampin 265–284 (LFampin265-284) induces a micellar cubic phase (Pm3n) [36], which to the best of our knowledge is the first experimental evidence of such phase in the context of antimicrobial peptide/membrane interaction. Finally LFchimera, a hybrid peptide between the first two [16], induces two cubic phases of Pn3m and Im3m symmetry. These results parallel their effect on *C. albicans* as derived from freeze-fracture electron microscopy, indicating a remarkable agreement between simple model systems and living organisms.

2. Materials and methods

2.1. Peptide synthesis, purification and characterization

LFcin17-30, LFampin265-284 and LFchimera were synthesized by solid phase peptide synthesis using Fmoc-protected amino acids (Orpegen Pharma GmbH, Heidelberg, Germany) in a Syro II synthesizer (Biotage, Uppsala Sweden) as described previously [16]. The chimerical peptide comprises a single C-terminal amidated lysine substituted at the α- and ε-amino groups with the two peptides via the C-terminal site and leaving two N-termini as free ends. Peptides were purified to a purity of at least 95% by semipreparative RP-HPLC (Jasco Corporation Tokyo, Japan) on a Vydac C18-column (218MS510; Vydac, Hesperia, CA, USA) and the authenticity of the peptides was confirmed by MALDI-TOF mass spectrometry on a Microflex LRF mass spectrometer equipped with an additional gridless reflectron (Bruker Daltonik, Bremen, Germany) as described previously [39]. In Table 1 we provide basic information of the peptides, together with their LC_{50} and their ultrastructural effects against *C. albicans*, as obtained previously by Bolscher et al. [37,38].

2.2. Preparation of liposomes

1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) was dissolved in chloroform, and its mixture with 1,2-dimyristoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (DMPG) at a molar ratio of 3:1 was dissolved in chloroform/methanol (3:1 (v/v)). Both lipids were from Avanti Polar Lipids, Alabama, USA. A film was prepared thereafter in round bottom flasks by drying the sample under a stream of nitrogen, and was kept under vacuum for 3 h to remove all traces of organic solvents. After drying, the lipid film was first warmed for 30 min at *ca.* 10 °C above the temperature of the gel-to-liquid crystalline phase transition (T_m) in a thermostated water bath, and afterwards hydrated with buffer, either HEPES (10 mM HEPES, 100 mM NaCl, pH 7.4) or PBS (9.3 mM, 154 mM NaCl, pH 7.2), kept at the same temperature. The multilamellar vesicles (MLVs) were obtained by alternating gentle vortex with short periods in the thermostated water bath at ~35 °C. After this the MLVs were frozen in liquid nitrogen and thawed in a water bath at 35 °C, and this process was repeated 5 times.

Large unilamellar vesicles (LUVs) were obtained from the MLVs by extrusion in a 10 ml stainless steel extruder (Lipex Biomembranes, Vancouver, BC, Canada), inserted in a thermostated cell with a re-circulating water bath, at 35 °C. The samples were passed several times through polycarbonate filters (Nucleopore, Pleasanton, CA, USA) of decreasing pore size (600, 200 and 100 nm; 5, 5 and 10 times, respectively), under inert (N_2) atmosphere.

Size distribution of extruded vesicles was determined by *qels* analysis (Malvern Zeta Sizer 5000, Malvern Instruments, Malvern, Worcestershire,UK) using a helium-neon laser (633 nm) as a source of incident light, and operating at a scattering angle of 90° and at 37 °C. Mean particle size was thus determined as being of 106 ± 4 nm (average and standard deviation of 6 independent measurements). The phospholipid concentration was determined by the phosphomolibdate method [40].

2.3. Differential scanning calorimetry

Differential scanning calorimetry (DSC) was performed in a Micro-DSCIII microcalorimeter (SETARAM, Caluire, France) essentially as described previously [41]. In brief, samples were run against HEPES buffer in the reference cell, and blank experiments with HEPES buffer in both cells were also performed for subsequent blank correction. The solution or suspension volume used in each cell was of around 0.8 ml, and the masses of solution in sample and reference cells were subsequently matched by weighing ±0.00005 g. Two successive heating and cooling scans were performed for each sample, the heating scan at a scanning rate of 0.5 °C/min and the cooling scan at 3 °C/min, over the temperature range of 10–35 °C. The results provided here always refer to the second heating scan, as we have observed that small differences can exist between first and second scans, but not thereafter. The sample mixtures were prepared immediately before the DSC run, by adding the desired amount of peptide (LFcin17-30, LFampin265–284 or LFchimera) stock solution (in HEPES buffer) to the LUVs suspension of DMPC or DMPC/DMPG (3:1). Samples with peptide-to-lipid molar ratios (P:L) from 1:197 to 1:29 were used. All procedures regarding sample preparation and handling (lag time at low temperature, time between mixtures, and start of the experiment) were kept constant in all experiments, to ensure that all samples had the same thermal history. The instrument was electrically calibrated for temperature and the scan rate with the SETARAM Calibration Unit. The Micro-DSCIII software was used for blank subtraction (run with buffer solution on both cells (sample and reference)). T_m and the $\Delta_{trans}H$ were calculated by integration of the heat capacity versus temperature curve (*Cp* versus Temperature). A linear baseline was used to calculate the integral areas under the curves [41].

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