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Biochimica et Biophysica Acta 1778 (2008) 983–996
Biochimica et Biophysica Acta BBA
www.elsevier.com/locate/bbamem

Binding of LL-37 to model biomembranes: Insight into target *vs* host cell recognition

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Received 17 August 2007; received in revised form 20 November 2007; accepted 26 November 2007
Available online 14 December 2007

Abstract

Pursuing the molecular mechanisms of the concentration dependent cytotoxic and hemolytic effects of the human antimicrobial peptide LL-37 on cells, we investigated the interactions of this peptide with lipids using different model membranes, together with fluorescence spectroscopy for the Trp-containing mutant LL-37(F27W). Minimum concentrations inhibiting bacterial growth and lipid interactions assessed by dynamic light scattering and monolayer penetration revealed the mutant to retain the characteristics of native LL-37. Although both LL-37 and the mutant intercalated effectively into zwitterionic phosphatidylcholine membranes the presence of acidic phospholipids caused augmented membrane binding. Interestingly, strongly attenuated intercalation of LL-37 into membranes containing both cholesterol and sphingomyelin (both at $X=0.3$) was observed. Accordingly, the distinction between target and host cells by LL-37 is likely to derive from *i)* acidic phospholipids causing enhanced association with the former cells as well as *ii)* from attenuated interactions with the outer surface of the plasma membrane of the peptide secreting host, imposed by its high content of cholesterol and sphingomyelin. Our results further suggest that LL-37 may exert its antimicrobial effects by compromising the membrane barrier properties of the target microbes by a mechanism involving cytotoxic oligomers, similarly to other peptides forming amyloid-like fibers in the presence of acidic phospholipids.

Keywords: LL-37(F27W); Fluorescence spectroscopy; Acidic phospholipid; Phase separation; Cytotoxic oligomer; Amyloid-like fiber

Abbreviations: AMPs, antimicrobial peptides; Chol, cholesterol; CD, circular dichroism; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)]; DPPDns, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamino *N*-(5-dimethylaminonaphthalene-1-sulfonyl)triethylammonium salt; ET, energy transfer efficiencies; EDTA, ethylenediaminetetraacetic acid; FRET, fluorescence resonance energy transfer; K_{SV}, Stern–Volmer quenching constant; k_q, bimolecular quenching constant; K_d, apparent dissociation constant; LL-37, native LL-37; LL-37(F27W) with Phe27 replaced by Trp; LUV, large unilamellar vesicles; L/P, lipid to peptide ratio; LB, Luria–Bertani; MIC, minimal inhibitory concentration; NBD-PC, 1-oleoyl-2-[6-[7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-rac-glycerol; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine; (6,7)-Br_2-PC, 1-palmitoyl-2-(6,7-dibromostearoyl)phosphocholine; (9,10)-Br_2-PC, 1-palmitoyl-2-(9,10-dibromostearoyl)phosphocholine; (11,12)-Br_2-PC,-palmitoyl-2-(11,12-dibromostearoyl)phosphocholine; PC, phosphatidylcholine; PG, phosphatidylglycerol; PS, phosphatidylserine; P/L, peptide to lipid ratio; Q, quencher; RT, room temperature; Spm, sphingomyelin; SLBs, supported lipid bilayers; SOPC, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; λ_{max}, fluorescence emission maximum; τ_f, fluorescence lifetime; ζ, zeta potential

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0005-2736/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2007.11.016

1. Introduction

A variety of antimicrobial peptides (AMPs) constitute in the human body its first line of defense against microorganisms, including bacteria, fungi, and enveloped viruses [1–3]. These peptides have recently gained increasing interest because of their potential for treating infections by bacteria resistant to conventional antibiotics. Cathelicidins constitute a group of structurally diverse AMPs found only in mammals [4–7], and derived their name because of the similarity of their conserved N-terminal proregions to cathelin [8–10], a porcine leukocyte inhibitor of cathepsin L [3]. So far the only known human cathelicidin is LL-37 [11], which was identified as a putative AMP in a cDNA clone isolated from a human bone marrow library [4]. The mature LL-37 peptide (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) was first isolated from granulocytes [12] and is also expressed in mast cells, neutrophils, as well as epithelial tissues, including skin, salivary glands, lung, urogenital and gastrointestinal tract. It is secreted into wounds, sweat, and airway surface fluids and is considered to play an important role against both local infections and systemic invasion of pathogens at sites of inflammation and damaged skin [13]. LL-37 is not as selective as some other α-helical, amphipathic AMPs, with MICs ranging from 1 to 10 μM for a variety of Gram-positive and Gram-negative bacteria, with eukaryotic cytotoxicity *in vitro* observed at 13–25 μM [14–16]. The antimicrobial activity of LL-37 has been confirmed also *in vivo* [17]. Intriguingly, in addition to its antimicrobial activity and stimulation of the innate immune system, LL-37 has been shown to be involved also in reproduction [18] and differentiation [19].

Recent studies on LL-37 have been mainly focused on its antimicrobial and cytotoxic activities and factors, which can inhibit its latter activity, such as truncation [20], and the presence of serum [21], or plasma [22]. LL-37 binds human plasma apolipoprotein A-I, which may act as a scavenger for this peptide [23]. It also associates with both plasma low density and very low density lipoproteins [22]. LL-37 is the first amphipathic α-helical AMP isolated from human, with 16 charged residues and a net charge of +6 at neutral pH [24]. Recent studies have shown that the amphipathic helix of LL-37 lies parallel to the membrane surface [21]. Neville et al. [25] observed lipid headgroup discrimination by LL-37. More specifically, LL-37 in low concentrations penetrates parallel to the plane into dipalmitoylphosphatiglycerol monolayers and resides in the phospholipid headgroup region. However, dipalmitoyl-phosphocholine and -phosphoethanolamine monolayers remained virtually unaffected. Oren et al. using fluorescence spectroscopy [21] and Henzler-Wildman et al. using solid state nuclear magnetic resonance and differential scanning calorimetery (DSC) [26] came to the conclusion that LL-37 forms "carpets" on the surface of both zwitterionic PC and negatively charged vesicles containing phosphatidylserine. NMR and DSC experiments revealed that LL-37 induces positive curvature strain but does not break membrane into smaller fragments or micelles [26–28]. More detailed understanding of the LL-37-lipid interactions, including structural changes that occur within the phospholipid bilayer, could aid the rational design of novel antibiotics.

In the present study we characterized the interactions of LL-37 with model biomembranes using Langmuir balance, dynamic light scattering, and circular dichroism. In order to employ fluorescence spectroscopy we constructed a mutant LL-37(F27W) with Phe27 replaced by Trp.

2. Experimental

2.1. Materials and methods

Hepes, EDTA, acryl amide, and Congo red were from Sigma. 1-Stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-*rac*-glycerol (POPG), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS), 1-palmitoyl-2-(6,7-dibromostearoyl)-*sn*-glycero-3-phosphocholine [(6,7)-Br_2-PC], 1-palmitoyl-2-(9,10-dibromostearoyl)-*sn*-glycero-3-phosphocholine [(9,10)-Br_2-PC], 1-palmitoyl-2-(11,12-dibromostearoyl)-*sn*-glycero-3-phosphocholine [(11,12)-Br_2-PC], 2-decanoyl-1-{*O*-[11-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-sindacene-3-ropionyl)amino]undecyl}-*sn*-glycero-3-phosphol-choline (Bodipy-PC), sphingomyelin, and cholesterol were from Avanti Polar Lipids (Alabaster, AL). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanol amino *N*-(5-dimethylaminonaphthalene-1-sulfonyl), triethylammonium salt (DPPDns) and 1-oleoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-*sn*-glycero-3-phosphocholine (NBD-PC) were from Molecular Probes (Eugene, OR). The purity of lipids was checked by thin-layer chromatography on silicic acid coated plates (Merck, Darmstadt, Germany) developed with a chloroform/methanol/water mixture (65:25:4, v/v/v). Examination of the plates after iodine staining, and when appropriate, upon UV illumination revealed no impurities. The concentrations of the lipids were determined gravimetrically with a high precision electrobalance (Cahn, Cerritos, CA) and lipid stock solutions were made in chloroform. Concentrations of fluorescent lipids DPPDns and NBD-PC were determined spectrophotometrically using their molar absorptivities of 21,000 (in CH_3OH) and 19,000 M^{-1} cm^{-1} (in C_2H_5OH) at 463 and 465, respectively. Large unilamellar vesicles (LUVs, with diameters between 111 and 117 nm) were prepared by extrusion (for cholesterol/sphingomyelin mixtures at 50 °C) through a 100 nm pore size (Millipore, Bedford, MA) filters essentially as described previously [29]. LL-37 and its F27W mutant were synthesized by Synpep (Dublin, CA) and their purities (>98%) verified by HPLC and mass spectrometry. All other chemicals were of analytical grade and from standard sources. Unless otherwise indicated the experiments were conducted using 5 mM Hepes, 0.1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.0 buffer.

2.2. Antimicrobial activity of LL-37 and LL-37(F27W)

Bacillus subtilis strain 168 and *Staphylococcus aureus* strain Newman were used to determine minimal inhibitory concentrations (MIC) for LL-37 and LL-37(F27W). *B. subtilis* was grown in Luria–Bertani (LB) and modified Spizizen's minimal salts media (BFA, Anagnostopoulos and Spizizen, 1961), and *S. aureus* in BHI medium in shake flasks at 37 °C with vigorous shaking up to cell densities yielding $OD_{600}=1$. Approximately 10^5 cells of these cultures were used to inoculate 150 μl of growth media in honeycomb two plate wells for the determination of MIC values by plate reader (Bioscreen C Microbiology, Growth Curves Ltd, UK). The peptides were added to each well in two-fold dilution series from 0.5 μM to 16 μM, with three separate measurements for each peptide concentration. The MIC was the lowest concentration at which no bacterial growth was detected.

2.3. Circular dichroic (CD) spectra

UV-CD spectra from 250 to 190 nm were recorded with a CD spectrophotometer (Olis RSF 1000F, On-line Instrument Systems Inc., Bogart, GA) with cuvette temperature maintained at 25 °C with a circulating water bath. A 1-mm path length quartz cell was used with final concentrations of 20 μM and 3 mM of peptide and liposomes, respectively, in buffer. Interference by circular differential scattering by liposomes was eliminated by subtracting CD spectra for liposomes from those recorded in the presence of peptide. Data are shown as mean residue molar ellipticity (deg cm^2 $dmol^{-1}$) and represent the averages of seven scans. The