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Aggregates of nisin with various bactoprenol-containing cell wall precursors differ in size and membrane permeation capacity

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ABSTRACT

Many lantibiotics use the membrane bound cell wall precursor Lipid II as a specific target for killing Gram-positive bacteria. Binding of Lipid II usually impedes cell wall biosynthesis, however, some elongated lantibiotics such as nisin, use Lipid II also as a docking molecule for pore formation in bacterial membranes. Although the unique nisin pore formation can be analyzed in Lipid II-doped vesicles, mechanistic details remain elusive. We used optical sectioning microscopy to directly visualize the interaction of fluorescently labeled nisin with membranes of giant unilamellar vesicles containing Lipid II and its various bactoprenol precursors. We quantitatively analyzed the binding and permeation capacity of nisin when applied at nanomolar concentrations. Specific interactions with Lipid I, Lipid II and bactoprenol-diphosphate (C_{55}-PP), but not bactoprenol-phosphate (C_{55}-P), resulted in the formation of large molecular aggregates. For Lipid II, we demonstrated the presence of both nisin and Lipid II in these aggregates. Membrane permeation induced by nisin was observed in the presence of Lipid I and Lipid II, but not in the presence of C_{55}-PP. Notably, the size of the C_{55}-PP–nisin aggregates was significantly smaller than that of the aggregates formed with Lipid I and Lipid II. We conclude that the membrane permeation capacity of nisin is determined by the size of the bactoprenol-containing aggregates in the membrane. Notably, transmitted light images indicated that the formation of large aggregates led to a pinch-off of small vesicles, a mechanism, which probably limits the growth of aggregates and induces membrane leakage.

1. Introduction

The antimicrobial peptide nisin is produced by many strains of *Lactococcus lactis* and is active against a broad range of Gram-positive bacteria [1,2]. Nisin belongs to the group of lantibiotics, which are characterized by intramolecular rings formed by the thioether amino acids lanthionine and 3-methyllanthionine [3,4]. The peptide is a member of the subgroup of type-A lantibiotics consisting of elongated, screw-shaped peptides with a positive net charge.

Positively charged, antimicrobial peptides like nisin have the capacity to adopt an amphipathic structure upon interaction with membranes [5], and it has been suggested that nisin kills bacteria by disturbing the integrity of the cell membrane [6]. Thus, it was shown in model membrane studies that nisin destroys the membrane of small unilamellar vesicles (SUVs) at micromolecular concentrations [7]. However, when applied against Gram-positive bacteria, nisin shows minimal inhibitory concentrations (MICs) in the nanomolar range [8]. This observation argues against a simple membrane perforation mechanism as primary biological effect. In further studies the cell wall precursor Lipid II was identified as a docking molecule. Evidence that this interaction is crucial for killing bacteria was provided when it was shown that the specific interaction of nisin and Lipid II leads to pore formation [4]. *In vitro* studies revealed that nanomolar concentrations of nisin were sufficient to cause membrane perforation in Lipid II-doped vesicles [7].

For bacteria, Lipid II is an indispensable constituent of the cell membrane because it delivers the monomeric peptidoglycan units for the cell wall biosynthesis. This makes it a prominent target for a number

Abbreviations: AF647, Alexa Fluor 647 N-hydroxysuccinimide hydrazide; Atto488-NHS, Atto 488 N-hydroxysuccinimide; CF, 5(6)-carboxyfluorescein; CLSM, confocal laser scanning microscopy; C_{55}-PP, undecaprenyl-diphosphate (bactoprenol-diphosphate); C_{55}-P, undecaprenyl-phosphate (bactoprenol-phosphate); DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) (sodium salt); EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; GlcNAc, N-acetylglucosamine; GUV, giant unilamellar vesicle; LI, Lipid I; LII, Lipid II; LSM, laser scanning microscope; LUV, large unilamellar vesicle; LY, Lucifer yellow; MES, 2-(N-morpholino)ethanesulfonic acid; MIC, minimal inhibitory concentration; MurNAc, N-acetylmuramic acid; nisin–AF647, nisin coupled to Alexa Fluor 647; SUV, small unilamellar vesicle; TFA, trifluoroacetic acid; UDP, uridine diphosphate

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of antibiotics [9]. The molecule consists of a membrane-anchoring hydrocarbon chain, undecaprenyl-phosphate (bactoprenol-phosphate), covalently coupled to the monomeric peptidoglycan unit through a pyrophosphate linker. This unit is the basic building block of the cell wall and consists of the two amino sugars N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc), with a pentapeptide bound to the latter. Lipid II is assembled on the cytosolic face of the membrane by successive enzymatic reactions (Fig. 1). The thereby generated intermediate is named Lipid I, which is composed of undecaprenyl-diphosphate covalently bound to MurNAc. Lipid II then translocates from the cytoplasmic to the external side of the membrane. The mechanism of translocation is not fully understood, but it is known that the integral membrane protein FtsW, an essential cell division protein, induces the trans-bilayer movement of Lipid II in model membranes [10]. On the external face of the membrane the insertion of peptidoglycan-units into the cell wall is catalyzed. The remaining lipid anchor carrying the pyrophosphate, C_{55}-PP, is dephosphorylated to the monophosphate, C_{55}-P, and shuttled back to the cytosolic side of the membrane to start a new synthesis cycle.

The incubation of Gram-positive bacteria with nisin induces membrane perforation, which leads to cell lysis [11]. *In vitro* studies showed that stable membrane gaps with inner diameters of 2–2.5 nm can occur [12]. At saturation the ratio of nisin molecules per Lipid II was proposed to be 2:1 [13]. The specific interaction of nisin and Lipid II leads to the inhibition of cell wall biosynthesis as Lipid II is bound by nisin and subsequently removed from the reaction cycle. Remarkably, when the interaction of nisin with the bacterial membrane was directly observed by using fluorescently labeled nisin, the peptides were not homogeneously distributed on the bacterial membrane, but clustered in large aggregates [14]. Clustering was also observed by direct imaging of fluorescent nisin on Lipid II containing giant unilamellar vesicles (GUVs) [14]. The pyrophosphate unit of Lipid II was identified as a possible structural binding motif for nisin, around which two lanthionine rings of the peptide might form a cage structure [15]. Accordingly, binding of nisin to C_{55}-PP was observed, however no nisin-induced membrane permeabilization in C_{55}-PP-doped SUVs did occur [15]. Here, the question remains why the specific binding of nisin to the pyrophosphate does not lead to membrane destabilization.

To gain a deeper insight into the effect of nisin on Lipid II and its bactoprenol precursors we used confocal laser scanning microscopy (CLSM) to directly visualize the interaction of fluorescently labeled nisin with respective GUV membranes. We analyzed quantitatively the binding behavior and permeation capacity of nisin when applied in the MIC range. We discriminated between unspecific and specific interactions, the first driven by electrostatic forces and the latter influenced by the binding sites that were offered to nisin. We found that nisin formed large-scale aggregates when C_{55}-PP, Lipid I or Lipid

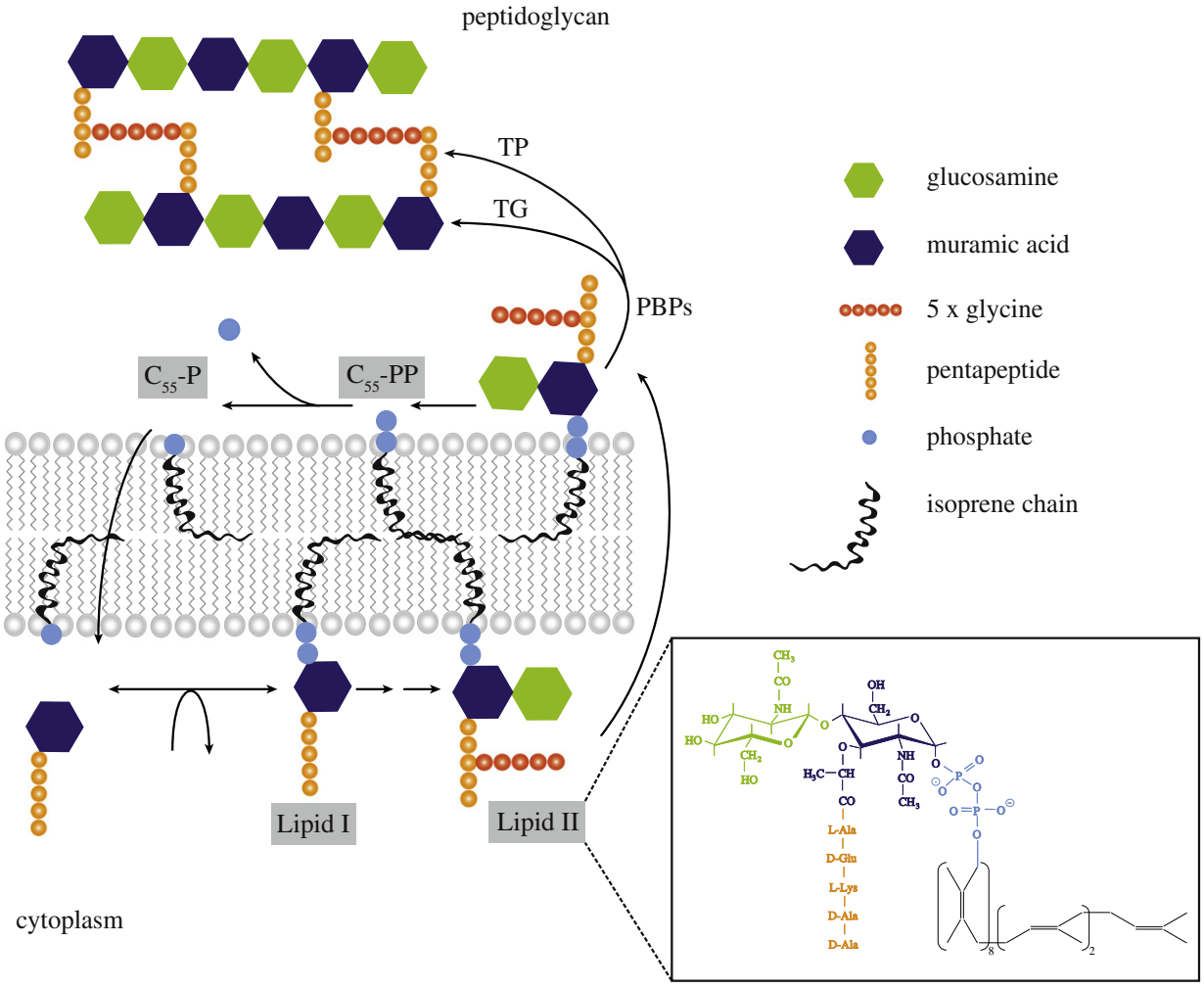

Fig. 1. Cell wall biosynthesis cycle in *S. aureus*. In the first membrane-linked step Lipid I is formed at the inner face of the membrane by coupling the UDP-MurNAc-pentapeptide to the lipid carrier C_{55}-P. Then, through linkage of UDP-GlcNAc to Lipid I, Lipid II is produced. Lipid II translocates across the membrane mediated by the membrane protein FtsW [10]. On the outside, the peptidoglycan unit is incorporated into the peptidoglycan network through the activity of penicillin-binding proteins (PBPs) by transglycosylation (TG) and transpeptidation (TP) reactions. This leaves C_{55}-PP, which is recycled through dephosphorylation, and the retrieved C_{55}-P is available for the next biosynthesis cycle.
Figure modified from [9].