ARTICLE IN PRESS

Journal of Electroanalytical Chemistry xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Journal of Electroanalytical Chemistry

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

Channel-forming activity of nisin in two mercury-supported biomimetic membranes☆

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A R T I C L E I N F O

Keywords:
Antimicrobial peptide
Tethered bilayer lipid membrane
Voltage-gated ion channel
Ohmic ion channel
Cyclic voltammetry
Electrochemical impedance spectroscopy

A B S T R A C T

Nisin, the most prominent lantibiotic used as a food preservative, due to its high potency against certain Gram-positive bacteria, was investigated in mercury-supported lipid monolayers and bilayers, with the distal monolayer consisting of a dioleoylphosphatidylcholine (DOPC) or dioleoylphosphatidylserine (DOPS) monolayer. The voltage-gated behavior of the cyclic voltammogram (CV) of nisin at DPTL/DOPC tBLM in a pH 5.4 unbuffered solution of 0.1 M KCl is converted into an ohmic behavior upon addition of the glucopeptide CSF114(Glc), under favorable experimental conditions. This indicates that nisin targets Gram-positive bacteria mainly at the NHAc-substituted α-D-glucose ring, which is present both in the peptidoglycan polymer forming their cell walls and in CSF114(Glc). While the β-D-glucose and β-D-galactose rings of the glycosphingolipid GM1 are ineffective, addition of α-D-glucose, which interacts attractively with the GM1 sugar chain, has a moderate but significant enhancing effect on the nisin CV. This suggests a synergic effect of α-D-glucose and NHAc substitution in creating a target for nisin attack. Conversely, CSF114(Glc) has no appreciable effect on the DOPS distal monolayer at both pH values 5.4 and 6.8 and does not induce ohmic behavior. More precisely, at pH 6.8 the DOPS polar heads are negatively charged and recruit the positively charged nisin molecules, preventing their penetration into the hydrocarbon tail region. This penetration becomes possible at pH 5.4, where the DOPS distal monolayer is neutral, allowing the formation of an ion channel that yields a voltage-gated CV.

1. Introduction

Nisin is an antimicrobial peptide produced by numerous strains *of Lactococcus lactis*, which inhibits a broad range of Gram-positive bacteria [1,2]. It contains several post-translationally modified aminoacids, among which lanthionine and 3-methyl-lanthionine; these two residues introduce thioether bridges at various locations of the molecule, resulting in a series of five cyclic units. The first three rings (A, B and C) form the N-terminal domain of the peptide, whereas the C-terminal domain is made of the last two intertwined rings (D and E) [3]. Both domains are amphipathic in character [4]. Having a negligible toxicity for humans, nisin has been widely used as a suitable food preservative in dairy industry and canned foods. Nevertheless, its extensive use has reduced its antibacterial efficiency [5], such that attempts to minimize the resistance of bacteria to nisin are now made to synergistically combine it with other antibacterial agents [6]. Two naturally occurring nisin variants, nisin A and nisin Z, have been found in lactococcal strains [7,8]. Nisin A differs from nisin Z for having a histidine residue in place of an asparagine one at position 27. Nisin is positively charged due to the presence of three lysine residues; one (for nisin Z) or two (for nisin A) histidine residues are also positively charged at pH < 6.

The nisin activity against growing bacterial cells is primarily thought to arise from pore formation in their cytoplasmic membrane. It has been suggested that a sufficiently negative transmembrane potential, ϕ_m, on the intracellular side of a membrane is required for nisin action [9,10]. This has led to the conclusion that the primary target for nisin is the cytoplasmic membrane [11]. However, fluorescence measurements on liposomes have shown that a negative ϕ_m value is not essential for nisin action, even though it may enhance it [12]. A transmembrane pH gradient ΔpH (inside alkaline) enhances the nisin action, with nisin being more potent at acidic external pH values. Nisin pore formation causes a rapid dissipation of ϕ_m [9,11] and ΔpH [9,12]

☆ Dedicated to Professor Renata Bilewicz on the occasion of her 65th birthday and in recognition of her contributions to electrochemistry.
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https://doi.org/10.1016/j.jelechem.2017.11.061
Received 2 October 2017; Received in revised form 18 November 2017; Accepted 22 November 2017
1572-6657/ © 2017 Published by Elsevier B.V.

Please cite this article as: Becucci, L., Journal of Electroanalytical Chemistry (2017), https://doi.org/10.1016/j.jelechem.2017.11.061

in intact cells, cytoplasmic membrane vesicles and liposomes. Nisin is soluble in aqueous solution at pH 2, but at high pH values it forms oligomers and is inactivated [13].

The role of membrane lipid composition on nisin activity is a subject of controversy. A strong electrostatic interaction of the positively charged nisin with phosphatidylglycerol and cardiolipin, the predominant anionic lipids of Gram-positive bacteria, is undisputed. This is supported by a surface pressure increase at negatively charged lipid monolayers upon injection of nisin to the subphase [14] and by an (anionic lipid)-dependent translocation of the C-terminal part of a nisin mutant containing the C-terminal extension Asp-$(His)_6$ across a model membrane [15]. However, these measurements, carried out by the Utrecht group [14–16], do not provide clear evidence that such an interaction between nisin and anionic lipids actually favors pore formation and ion translocation across membranes, apart from a report of a higher release of K^+ and carboxyfluorescein ions from dioleoylphosphatidylglycerol (DOPG) vesicles than from dioleoylphosphatidylcholine (DOPC) ones [17].

Fluorescence spectroscopy measurements on nisin mutants with Trp residues on positions 1, 17 and 32 point to an overall parallel orientation with respect to the membrane surface, with the N-terminus more deeply inserted in the membrane than the C-terminus [16], as also supported by a Monte Carlo simulation [18]. It was, therefore, assumed that the membrane-spanning orientation of the peptides is only transient, such that the peptides might rapidly flip back to the original parallel orientation in the outer leaflet [14]. Nisin was reported to induce efflux of the fluorescent dye carboxyfluorescein from liposomes composed of the zwitterionic phosphatidylcholine (PC), whereas it was strongly inhibited by anionic phospholipids in the liposomes [12]. Analogously, it was found to be prevented from transporting anionic fluorophores in phosphatidylglycerol (PG) liposomes, presumably because it remains bound as a partially neutralized complex at the liposome surface [19]. Conversely, planar lipid bilayer measurements demonstrated that a negative ϕ_m on the opposite side of the bilayer with respect to that (the *cis* side) where nisin is added is required for pore formation, with anionic phospholipids lowering the threshold requirement for ϕ_m [10,20]. Thus, a nisin-doped DOPC/phosphatidylserine (PS) (4:1) BLM causes the current to increase exponentially when the *trans*-negative ϕ_m is made progressively more negative than -100 mV and to decrease more slowly when moving in the opposite direction [10]. This large hysteresis is typical of voltage-gated channels. Single-channel recordings yield short-lived pulse-like fluctuations stable in the millisecond range, whose maximum amplitude points to a pore diameter of about 0.9 nm.

Nisin is the first known pore-forming peptide that exerts its antimicrobial activity by specifically targeting a Gram-positive cell wall precursor, namely, lipid II, and by using it as a docking molecule for pore formation [21–23]. Pyrene excimer fluorescence and circular dichroism investigations demonstrated that lipid II is an integral part of the nisin pore [24], which has a uniform and highly stable structure and contains 4 lipid II and 8 nisin molecules [25]. The binding between nisin and lipid II involves the residues in rings A to C of nisin and the *N*-acetylmuramic acid (MurNAc)-pentapeptide moiety of lipid II [26,27]. The specificity of the nisin-lipid II interaction results in an enhancement of nisin pore-formation activity by three orders of magnitude toward Gram-positive bacteria [23]. When nisin is bound to lipid II, it is induced to change its orientation from parallel to perpendicular to the membrane plane [28], and the resulting pore causes a collapse of the vital ion gradients across the membrane. The hinge region of nisin interposed between the three rings A, B and C and the last two intertwined rings C and D is most important for pore formation. If this flexible segment is shorted by site-directed mutagenesis, the pore formation activity of nisin is lost, whereas its capacitance for binding lipid II is only slightly affected [23]. Consequently, nisin retains its activity to prevent lipid II incorporation into polymeric peptidoglycan, which forms the cell wall of Gram-positive bacteria, severely interfering with cell-wall synthesis. In this respect, wild type nisin exerts a dual antimicrobial activity against Gram-positive bacteria, by dissipating transmembrane potential and pH gradients across the bacterial membrane via pore formation and by opposing cell-wall synthesis via recruitment of lipid II molecules. Addition of nisin to the *cis* side of a planar lipid bilayer made of diphytanoylphosphatidylcholine causes the current to increase with respect to the background level only upon applying *trans*-negative potentials. Addition of lipid II converts the resulting current-voltage curve, which exhibits voltage-gated behavior, into one exhibiting ohmic behavior. Thus, the current increases linearly with voltage, intersecting the zero current axis at zero transmembrane potential [29]. Moreover, single-channel experiments provide prominent conductance levels pointing to an average pore diameter of 2 to 2.5 nm and a pore lifetime of about 6 s, to be compared with a pore size of 1 nm and a pore lifetime in the millisecond range in the absence of lipid II. This confirms the direct participation of lipid II in the formation of the nisin pore.

The present work aims at investigating the behavior of nisin in DOPC and dioleoylphosphatidylserine (DOPS) lipid environments bathed by aqueous 0.1 M KCl in pH 5.4 unbuffered solution and in pH 6.8 phosphate buffer. To this end, two mercury-supported biomimetic membranes were employed, namely a lipid self-assembled monolayer (SAM) and a tethered bilayer lipid membrane (tBLM). The latter biomimetic membrane consists of a monolayer of a thiolipid, called DPTL, with a phospholipid monolayer on top of it. The DPTL thiolipid consists of a tetraethyleneoxy hydrophilic chain terminated at one end with a lipoic acid residue for anchoring to the mercury surface, and covalently linked at the other end to two phytanyl chains mimicking the hydrocarbon tails of a lipid [30,31]. The hydrophobic interactions between the phytanyl chains and the overlying phospholipid monolayer give rise to a lipid bilayer interposed between the tetraethyleneoxy chain, called spacer, and the bulk aqueous solution. The spacer may accommodate a number of water molecules and ions, thus acting as an ionic reservoir. Mercury provides a defect free, fluid and readily renewable surface to the tBLM. Moreover, it imparts to the lipid molecules of the whole bilayer a lateral mobility comparable with that of biomembranes. Hg-supported tBLMs have been extensively employed in our laboratory for the investigation of ion channels [32–34].

2. Material and methods

Nisin was purchased from Merck (Darmstadt, Germany) and used without further purification. Merck suprapur® KCl was baked at 500 °C before use to remove any organic impurities. KH_2PO_4 and K_2HPO_4 from Merck were used without further purification. DOPC and DOPS were purchased in chloroform solution from Avanti Polar Lipids (Birmingham, AL, USA). The 2,3,di-O-phytanyl-sn-glycerol-1-tetraethylene-glycol-D,L-α lipoic acid ester thiolipid (DPTL) was provided by Prof. Adrian Schwan (Department of Chemistry, University of Guelph, Canada) [31]; stock solutions of this thiolipid were stored at -18 °C. Deionized water was distilled once and then redistilled from alkaline permanganate before its use. Stock solutions of 6 mg/ml nisin in water were stored at 4 °C. CSF114 and CSF114(Glc) peptide chains were independently synthesized in solid phase by Fmoc/tBu strategy and purified by RP-HPLC as previously described to a final purity $> 95\%$ [35]. All measurements were carried out with a home-made hanging mercury drop electrode (HMDE) described elsewhere [36]. A home-made glass capillary with a finely tapered tip was used. Capillary and mercury reservoir were thermostated at 25 ± 0.1 °C in a water-jacketed box to avoid any changes in drop area due to a change in temperature. The HMDE acted as the working electrode in a three-electrode system, with an Ag/AgCl (0.1 M KCl) reference electrode and a platinum coil counter electrode. The procedures for preparing mercury-supported lipid SAMs and DPTL/phospholipid tBLMs are thoroughly described in previous works [33,37]. Electrochemical impedance spectroscopy (EIS), potential-step chronocoulometry, AC and

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