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Role of Transmembrane pH Gradient and Membrane Binding in Nisin Pore Formation

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Nisin is a cationic antimicrobial peptide that belongs to the group of lantibiotics. It is thought to form oligomeric pores in the target membrane by a mechanism that requires the transmembrane electrical potential (ΔΨ) and that involves local perturbation of the lipid bilayer structure. Here we show that nisin does not form exclusively voltage-dependent pores: even in the absence of a ΔΨ, nisin is able to dissipate the transmembrane pH gradient (ΔpH) in sensitive Lactococcus lactis cells and proteoliposomes. The rate of dissipation increases with the magnitude of the ΔpH. Nisin forms pores only when the ΔpH is inside alkaline. The efficiency of ΔΨ-induced pore formation is strongly affected by the external pH, whereas ΔpH-induced pore formation is rather insensitive to the external pH. Nisin1–12, an amino-terminal fragment of nisin, and (des-ΔAla6)-nisin1–32 amide have a strongly reduced capacity to dissipate the ΔΨ and ΔpH in cytochrome c oxidase proteoliposomes and L. lactis cells. Both variants bind with reduced efficiency to liposomes containing negatively charged phospholipids, suggesting that both ring A and rings C to E play a role in membrane binding. Nisin1–12 competes with nisin for membrane binding and antagonizes pore formation. These findings are consistent with the wedge model of nisin-induced pore formation (A. J. M. Driessen et al., Biochemistry 34:1606–1614, 1995).

Lantibiotics are posttranslationally modified peptide antibiotics that owe their name to the presence of cyclic structures formed by lanthionines or 3-methyllanthionine residues. The lantibiotic nisin is produced by Lactococcus lactis subsp. lactis and has five such intramolecular rings, termed A, B, C, D, and E, in a total of 34 amino acids. Because of its bactericidal activity against a broad range of gram-positive bacteria, nisin is exploited as a food preservative. Its genetics (23) as well as the regulation of its synthesis (22) are known, and several mutants have been generated (24–26).

Nisin forms nonselective, transient, multistate pores in membranes (38–40). In addition to having bactericidal activity, it inhibits the outgrowth of spores (11, 18, 29, 33) and interferences with the activity of biosynthetic (36) and autolytic (2–5) enzymes. Nisin is able to form pores in cells, proteoliposomes, liposomes, and black lipid membranes. It has been reported that the pores are formed only when the transmembrane electrical potential (ΔΨ), negative inside, is sufficiently high (15, 38, 39). In cells, pore formation induces the release of ions, amino acids, and ATP and causes the collapse of the proton motive force (ΔpH) (7, 16, 21, 35, 37, 39, 40). Nisin requires anionic phospholipids for membrane binding and pore formation (12, 15), while in the absence of anionic phospholipids, it acts as an anion carrier (15, 17).

The structure of nisin in solution (28, 44) and in the bound state to micelles of dodecylphosphocholine and of sodium dodecyl sulfate (42, 43) has been studied by nuclear magnetic resonance (NMR) analysis. Nisin is a flexible molecule in solution but contains two relatively structured domains: an amino-terminal domain (residues 3 to 19) containing rings A, B, and C, in which rings A and B are particularly well structured; and a carboxyl-terminal domain (residues 22 to 28) containing rings D and E. Both domains are amphipathic, a property consistent with the ability of nisin to interact with phospholipid membranes. Based on structural and functional studies, we have proposed a wedge model for pore formation by nisin (15, 32a). A wedge-like pore composed of multiple nisin molecules may form once the ΔΨ drives the membrane insertion of the phospholipid surface-bound molecules. In this model, the structure of nisin changes only in the putative hinge region between rings C and D/E, while the orientation of the molecule relative to the lipid headgroups remains unchanged. The carboxyl-terminal residues, which have a net positive charge, together with the bound lipids, are drawn across the membrane in response to the ΔΨ, causing the formation of a structural defect in the phospholipid bilayer.

The aim of this work was to obtain more insight into the mechanism of pore formation and to study the role of the structural domains of nisin in this process. For this purpose, we studied the role of the transmembrane pH gradient (ΔpH) in pore formation and the characteristics of two nisin variants: nisin1–12, a fragment of nisin containing only rings A and B (10), and (des-ΔAla6)-nisin1–32 amide (8), a form of nisin in which ring A is opened. The results demonstrate that nisin is not a voltage-dependent bacteriocin per se, but that it can be activated by the ΔpH as well. A possible mechanism of the ΔpH-induced pore formation is discussed.

MATERIALS AND METHODS

Materials. Nisin was obtained as a gift of Aplin & Barrett. Dioleoylphosphatidylethanolamine (DOPE), egg phosphatidylcholine (PC), N-(7-nitro-2, 1,3-benzoxadiazol-4-yl) (NBD)-labelled phosphatidylethanolamine (PE), and PE from Escherichia coli were purchased from Avanti Polar Lipids Inc. (Birmingham, Ala.). 9-Amino-6-chloro-2-methoxyacridine (ACMA) was obtained from Molecular Probes. 13C-penicillanic acid (22 μCi/mmol) was purchased from NEN. (des-ΔAla6)-nisin1–32 amide and nisin1–12 were obtained as described previously (8, 9).
Preparation of liposomes. Liposomes were made by injection of phospholipid-containing ethanol into buffer (1).

Binding of peptides to liposomes. Binding of nisin and the variants was measured by a fluorometric assay that is based on the increase in the fluorescence quantum yield of NBD-PE (15). Experiments were performed in 50 mM KPi (pH 7.0) at 30°C. Fluorescence was measured with a Perkin-Elmer LS-50 spectrophotometer, using excitation and emission wavelengths of 460 and 536 nm, respectively. The slit widths were set to 4 nm.

Growth inhibition assay. Half-maximal inhibitory concentrations were determined as previously described (34), except that Tween 80 was excluded, using a microtiter assay system. Experiments were started at a cell density of 20 μg of cellular protein/ml.

Cytochrome c oxidase vesicles. Beef heart cytochrome c oxidase was reconstituted into liposomes composed of ether-acetone-washed E. coli lipids as described previously (14). After dialysis, proteoliposomes were subjected to five subsequent freeze-thaw cycles, by freezing them in liquid nitrogen and thawing them slowly. Thereafter they were extruded 11 times through 400-nm and subsequently 11 times through 200-nm pore-size polycarbonate filters. Proteoliposomes (0.4 mg of lipid/ml) were energized with the electron donor cytochrome c (10 μM)-N,N′-N′′-N′′′-tetramethylphenylenediamine (TMPD) (10 μM)-ascorbate (10 mM). The K⁺/H⁺ antipporter nigericin (0.25 μM) prevented the generation of a ΔpH, or alternatively, the K⁺ carrier valinomycin (0.25 μM) prevented the generation of a Δψ.

Bacteriorhodopsin vesicles and measurement of a ΔpH, inside cells. Bacteriorhodopsin was reconstituted into lipid vesicles (E. coli lipids/egg PC lipids, 3:1) as described previously (14) in 50 mM KPi, Bacteriorhodopsin-mediated proton pumping was initiated by illumination with white light (Xenon lamp, 450 W), filtered through a 495-nm-pore-size high-pass filter. Changes in the ΔpH were measured by recording the fluorescence of ACMA at 2.2 μM, using excitation and emission wavelengths of 409 and 474 nm, respectively.

Measurements of proton motive force. The Δψ, inside negative, was calculated from the distribution of tetraphenylphosphonium ion (TPP⁺) as measured with a TPP⁺-selective electrode (20). Cells were used at a concentration of 2.1 mg of protein/ml. The magnitude of the Δψ was calculated according to the Nernst equation. A correction for TPP⁺ binding to the cells and proteoliposomes according to reference 30 was made.

The internal pH of cytochrome c oxidase vesicles was determined from the fluorescence of pyranine (excitation and emission wavelengths of 450 and 508 nm, respectively) as described previously (16). Entrapment was achieved by adding 1 mM pyranine to the vesicles before the sonication step. External pyranine was removed by passing the vesicles through a Sephadex G-50-medium column (1 by 20 cm).

RESULTS

Dissipation of the ΔpH and Δψ by nisin in intact cells. Previously it has been shown that in the absence of a Δψ, nisin is able to dissipate the ΔpH in cytochrome c oxidase vesicles composed of E. coli lipids and egg PC (9:1) (16). To determine if this activity of nisin is also relevant for intact cells of L. lactis, ΔpH measurements were performed with cells loaded with the fluorescent pH indicator BCECF (31). The presence of valinomycin and potassium ions prevented the generation of a Δψ, as evident from measurements of the distribution of TPP⁺ (data not shown). At a concentration of 42 nM, nisin completely collapsed the ΔpH in L. lactis cells (Fig. 1A, a). Similar results were obtained when the ΔpH measurements were performed by monitoring the distribution of radiolabelled benzoic acid (Fig. 1B). This concentration is about 250-fold lower than that needed to dissipate the ΔpH in proteoliposomes (see Fig. 3 and reference 16). These data demonstrate that nisin is not a voltage-dependent bacteriocin per se, but that a ΔpH suffices as well.

The efficiency of nisin-induced ΔpH and Δψ dissipation was studied as a function of the external pH in cells energized with glucose. In the presence of valinomycin, the efficiency of ΔpH
dissipation by nisin is hardly affected by the external pH within the range pH 6.0 to 7.0 (Fig. 1B). In contrast, Δψ measurements in the presence of nigericin show a striking pH dependence of the nisin-induced Δψ dissipation (Fig. 1C). At a low pH, nisin is much less effective than at the higher pHs. Comparison of the dose-response curves shows that at pH 7.0, nisin is equally effective in dissipating the ΔpH and Δψ. However, at pH 6.0, the ΔpH is more effectively dissipated than the Δψ, implying an important role of ΔpH in nisin pore formation.

When nisin was added to the cells prior to energization, 400 nM nisin (Fig. 2, d) was needed to prevent the generation of a ΔpH upon the addition of glucose. This concentration is nearly 10-fold higher than needed to dissipate a preexisting ΔpH, i.e., when nisin is added after energization with glucose (Fig. 1A, a). Moreover, especially at the lower concentrations of nisin, the generation of a ΔpH appeared to be transient (Fig. 2, b and c), suggesting that in the absence of a Δψ, a ΔpH is required for pore formation.

Dissipation of the ΔpH in proteoliposomes. It was previously shown that nisin dissipates only a Δψ inside negative (15, 38, 39). To establish whether a similar polarity effect exists with the ΔpH, nisin was added to proteoliposomes containing cytochrome c oxidase or bacteriochlorophyll. In the presence of reduced cytochrome c, cytochrome c oxidase liposomes generated a ΔpH, inside alkaline (14). On the other hand, upon illumination, bacteriochlorophyll liposomes generate a ΔpH, inside acid (14). Valinomycin was added to prevent the formation of a Δψ. Nisin was much more effective in dissipating a ΔpH, inside alkaline, than a ΔpH, inside acid (Fig. 3). Only at very high concentrations (around 46 μM) of nisin, dissipation of the ΔpH, inside acid, occurred (data not shown). Addition of 0.2 μM nigericin caused in both cases complete dissipation of the ΔpH (data not shown). These data demonstrate that nisin dissipates the ΔpH only in an oriented manner.

Dissipation of the ΔpH and Δψ by nisin derivatives. Nisin1-12 is an amino-terminal fragment derived from nisin upon chymotrypsin cleavage (10), and (des-ΔAla5)-nisin1-32amide is a form of nisin with an open ring A resulting from the hydrolysis of the dehydroalanine at position 5 (10). Both derivatives exhibit a strongly reduced bactericidal activity with intact cells (8–10) (Table 1). To establish if the reduced bactericidal activity of nisin1-12 and (des-ΔAla5)-nisin1-32amide correlates with their pore-forming capacity, their effects on the ΔpH in cells and cytochrome c oxidase vesicles were determined. Both de-

<table>
<thead>
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<th>Lantibiotic</th>
<th>IC50 (μM)</th>
<th>Δψ</th>
<th>Membrane binding (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nisin</td>
<td>0.0086 ± 0.0016</td>
<td>0.94 ± 0.06</td>
<td>100.0 ± 1.7</td>
</tr>
<tr>
<td>(des-ΔAla5)-nisin1-32amide</td>
<td>&gt;30.0</td>
<td>&gt;30.0</td>
<td>74.0 ± 1.9</td>
</tr>
<tr>
<td>Nisin1-12</td>
<td>15.3 ± 1.4</td>
<td>&gt;18.0</td>
<td>17.0 ± 0.8</td>
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* IC50 represents the concentration of nisin or derivative needed for half-maximal killing of the indicator strain or dissipation of the Δψ. Binding experiments were performed at 10.0 μM nisin or nisin variant with DOPG vesicles (220 μM lipid) containing 2 mol% NBD-PE. The relative levels of binding were estimated from the NBD fluorescence increase, with the value for nisin set to 100%.
rivatives showed a dramatically reduced ability to dissipate the ΔpH (Fig. 1A) and Δψ (Table 1) in cells. In cytochrome c oxidase vesicles, both nisin 1-12 and (des-DAla5)-nisin1-32amide were nearly inactive in eliminating the ΔpH and Δψ (data not shown). On the other hand, increasing concentrations of nisin 1-12 reduce the rate and extent of the nisin-induced Δψ dissipation in cytochrome c oxidase vesicles (Fig. 4). This antagonistic effect of nisin 1-12 on the nisin activity was stronger on the initial rate of Δψ dissipation (Fig. 4) than on the steady-state level of the Δψ (Fig. 4, inset). These data suggest that nisin 1-12 interferes with the formation of a functional nisin pore.

Membrane binding of nisin derivatives. The antagonistic effect of nisin 1-12 on the action of nisin could be due to competition for membrane binding sites, to the formation of hetero-oligomeric pore complexes, or both. The ability of the nisin variants to interact with the membrane was analyzed by the use of phosphatidylglycerol liposomes containing 2 mol% NBD-PE (15). Binding of nisin to these membranes results in an increase in the fluorescence quantum yield of NBD, and this effect has been attributed to shielding of NBD from the aqueous environment (15). Nisin 1-12 but more notably (des-DAla5)-nisin 1-32-amide retained the ability to bind to phosphatidylglycerol liposomes, but the binding activity was lower than that of nisin (Table 1). The membrane-bound nisin 1-12 rapidly dissociates from the liposomes, as the addition of a 30-fold excess of phosphatidylglycerol liposomes without NBD-PE resulted in a complete loss of NBD fluorescence at a rate of about 50%/s, while only a very slow release was observed with nisin (0.8%/s). These results demonstrate that truncation of the carboxyl-terminal domain of nisin severely interferes with binding to phosphatidylglycerol.

A binding experiment was performed with increasing concentrations of nisin 1-12 followed by addition of a fixed but a suboptimal amount of nisin (Fig. 5). The addition of increasing amounts of nisin 1-12 results in a gradual increase in NBD fluorescence, but identical final fluorescence levels were obtained after the subsequent addition of a fixed amount of nisin. These results demonstrate that nisin and nisin 1-12 compete for the same binding sites, which can, at least in part, explain the antagonism of nisin 1-12 on nisin action.

**DISCUSSION**

The present results show that even in the absence of a Δψ, nisin is able to effectively dissipate the ΔpH in _L. lactis_ cells. This finding demonstrates that, in contrast to previous suggestions, nisin does not obligatorily require a Δψ, but that this function can also be fulfilled by the ΔpH. The characterization of nisin pores as voltage-gated channels is therefore not correct. The efficiency with which nisin dissipates the Δψ decreases markedly when the pH is lowered from pH 7.0 to 6.0. In contrast, the efficiency by which nisin collapses the ΔpH is only marginally lower at pH 6.0 than at pH 7.0.

At this stage, it is unclear why nisin becomes less effective in dissipating the Δψ when the pH is lowered. Proton conductance may involve protonable side chains in the nisin molecules. For instance, the histidine residues (His27 and His31) at the carboxyl-terminal part of nisin may play an essential role in the ΔpH-dissipating activity. In aqueous solution, both histidines have a pKa of about 6.5 (41), and thus a significant fraction will be in the protonated state around neutral pH. When bound to micelles of sodium dodecyl sulfate, the pKa increases to 7.5 (43); this phenomenon has been explained by the electrostatic effects of the adjacent negatively charged sulfate groups, implying that the histidine side chains reside at the micellar surface. A similar phenomenon may occur when nisin binds to liposomes containing acidic phospholipids. Under the influence of a pH gradient, the C-terminal part of nisin, which contains the protonated histidines, may insert into the membrane together with bound phospholipids, forming a wedged pore (15). The C-terminal domain harbors most of the cationic amino acid side chains that have been implicated in the Δψ-dependent formation of a nisin pore. It is important to note that nisin is highly active only against a ΔpH, inside alkaline, not a ΔpH, inside acid. This mechanism is consistent with the
membrane binding activity, while the opening of ring A in a wedge-like pore. Bound anionic phospholipids results in bending of the lipid surface, giving rise to a wedge-like pore.

The naturally occurring variant of nisin A, nisin Z, in which His27 has been replaced by Asp27 (24), has full bactericidal activity, as do the H27K (13) mutant of nisin A and H31K (26) mutant of nisin Z, showing that the histidine residues are apparently not essential for activity. Similarly, the fragment nisin1-29 shows only a 16-fold decrease in activity (10), and even nisin1-20, lacking the cationic C-terminal domain of the molecule, has detectable though low (~1%) activity (10). However, it is not clear if these variants are still able to form pores in the presence of only a ΔpH.

According to the wedge model of pore formation (Fig. 6), a hinge region may act as a twist that allows nisin to bend the lipid surface (15). Our model speculates that the ABC rings might bind to the membrane and remain surface bound, while the Δp drives the membrane insertion of the carboxyl-terminal domain containing the DE rings together with the bound lipids. The detectable activity of nisin1-20 and the importance of an intact ring C (10) suggest that ring C may also partly insert into the pore. NMR studies of nisin in solution (41, 44) indicate that the flexibility of the hinge between rings B and C is nearly as high as that of the hinge between rings C and D.

The wedge model predicts that contributions to membrane binding will not be confined to specific subdomains but will be found all along the nisin molecule. In line with this assumption is the observation that nisin1-12 exhibits a strongly reduced membrane binding activity, while the opening of ring A in (des-ΔAla3)-nisin1-32amide has a smaller effect on membrane binding. NMR studies on micelle-bound nisin are also consistent with the idea that several parts along the nisin molecule contribute to membrane binding, as both dehydroalanines (residues 5 and 33) as well as residues Ile30 to Lys34 are shielded to some extent from the solvent. Spin label experiments indicate that the interactions of residues Ser29 to Lys34 with the micelles are the strongest (43). Absence of the latter interactions in the case of nisin1-12 and absence of the interaction of residues 33 and 34 in the case of (des-ΔAla3)-nisin1-32amide might also contribute to the observed reduced membrane binding of both variants.

Both nisin1-12 and (des-ΔAla3)-nisin1-32amide are strongly defective in Δψ and ΔpH dissipation in intact cells and cytochrome c oxidase proteoliposomes. This finding parallels the observations that these variants are devoid of bactericidal activity. It should be stressed that the loss of activity of (des-ΔAla3)-nisin1-32 must be the consequence of the opening of the A ring, since nisin1-7-amide, which also lacks the two carboxyl-terminal residues, and the ΔAla3-Ala mutant have full bactericidal activity (8, 11). The largest conformational differences between soluble nisin and sodium dodecyl sulfate micelle-bound nisin are seen in ring A around Dha5 (41, 43). Studies with a Dha5Dhhb mutant (25) and with [α-OH-Ala3]nisin further confirm the importance of the A ring. Both the bactericidal activity and the extent of the structural change of nisin upon micelle binding decrease in the following order: nisin A > nisinDha5Dhb > [α-OH-Ala3]nisin (42). This finding suggests that the micelle-bound conformation of ring A might indeed mimic that of active membrane-bound nisin and confirms the importance of the conformation of this ring for activity.

In summary, these studies have established that nisin is not a Δψ-dependent pore per se, but that pore formation can also be affected by the ΔpH. At an acidic pH, nisin dissipates the ΔpH more effectively than the Δψ, indicating that under the conditions with which most bacterial fermentations in food manufacturing are performed, the ΔpH is the primary target for nisin action.

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