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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 ($\Delta\psi$) 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 $\Delta\psi$, nisin is able to dissipate the transmembrane pH gradient ($\Delta\text{pH}$) in sensitive Lactococcus lactis cells and proteoliposomes. The rate of dissipation increases with the magnitude of the $\Delta\text{pH}$. Nisin forms pores only when the $\Delta\text{pH}$ is inside alkaline. The efficiency of $\Delta\psi$-induced pore formation is strongly affected by the external pH, whereas $\Delta\text{pH}$-induced pore formation is rather insensitive to the external pH. Nisin (11-22), an amino-terminal fragment of nisin, and (Des-$\Delta$Ala$_{10}$)-nisin (1-12) amide have a strongly reduced capacity to dissipate the $\Delta\psi$ and $\Delta\text{pH}$ 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. Nisin (1-22) 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).
FIG. 1. (A) Nisin dissipates the ΔpH in sensitive L. lactis cells. Logarithmically grown cells loaded with BCECF (17 μg of protein/ml) in 50 mM KP, (pH 7.0) were energized with 0.5% glucose (arrow 1). Valinomycin (0.25 μM; arrow 2) collapsed the total membrane potential and increased the pH gradient. Subsequently, either nisin (42 nM) (a), or (des-Akla)nisin-32amide (42 nM) (b), or nisin (42 nM) (c) was added (arrow 3). Nigericin (0.25 μM; arrow 4) dissipated the remaining pH gradient. (B) pH dependence of the ΔpH-induced pore formation. Logarithmically grown cells (2.1 mg of protein/ml) in 50 mM KP, at pH 7.0 (C), pH 6.5 (D), or pH 6.0 (E) were energized with 0.5% glucose and incubated with radiolabelled benzoic acid. After 4 min, 0.25 μM valinomycin was added; 1 min later, nisin was added. Cells were incubated for 15 min and separated from the medium by centrifugation through silicon oil (6, 31). (C) pH dependence of the ΔΔpH-induced pore formation. Logarithmically grown cells (2.1 mg of protein/ml) in 50 mM KP, at pH 7.0 (C), pH 6.5 (D), or pH 6.0 (E) were energized with 0.5% glucose and supplemented with nigericin (0.25 μM). The ΔΔpH was measured by using a TPP + electrode as described in Materials and Methods.

10). L. lactis IL 1403 was used as a sensitive strain and grown as described previously (32).

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 fluorimetric assay that is based on the increase in the fluorescence quantum yield of NBD when the molecules bind to the liposomes containing 2 mol% NBD-PE (15). Experiments were performed in 50 mM KP, (pH 7.0) at 30°C. Fluorescence was measured with a Perkin-Elmer LS-50 spectrofluorimeter, 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.

Cytchrome 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)–NADH (100 μM) or ascorbate (10 mM). The K + /H + antiporter 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 ΔΔpH, inside cells. Bacteriorhodopsin was reconstituted into lipid vesicles (E. coli lipids/egg PC lipids, 3:1) as described previously (14) in 50 mM KP, 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 tetraethylammonium 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 Nerst 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).

The internal pH of cells was measured with the fluorescent pH indicator 2',7'-bis-(2-carboxyethyl)-5(and-6)-carboxyfluorescein (BCECF) as described previously (31). In short, two suspensions of 20 μl of 50 mM KP, (pH 7.0) containing washed cells (100 μg of protein) on ice were supplemented with 0.5 mM BCECF. To one suspension 2.0 μl, and to the other 2.5 μl, of 0.5 M HCl was added. Suspensions were kept for exactly 5 min at room temperature. External BCECF was removed by five washes in 1 ml of 50 mM KP, (pH 7.0). Cells loaded under the lowest HCl concentration were used for experiments. Experiments were performed in 50 mM KP, at 30°C. Alternatively, the ΔpH was measured by monitoring the distribution of radiolabelled benzoic acid by the silicon oil centrifugation technique (6, 19).

RESULTS

Dissipation of the ΔpH and ΔΔpH 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 ΔΔpH 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, \( Dc \) measurements in the presence of nigericin show a striking pH dependence of the nisin-induced \( \Delta \psi \) 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 \( \Delta pH \) and \( \Delta \psi \). However, at pH 6.0, the \( \Delta pH \) is more effectively dissipated than the \( \Delta \psi \), implying an important role of \( \Delta pH \) 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 \( \Delta pH \) upon the addition of glucose. This concentration is nearly 10-fold higher than needed to dissipate a preexisting \( \Delta pH \), 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 \( \Delta pH \) appeared to be transient (Fig. 2, b and c), suggesting that in the absence of a \( \Delta \psi \), a \( \Delta pH \) is required for pore formation.

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

**Dissipation of the \( \Delta pH \) and \( \Delta \psi \) by nisin derivatives.** Nisin\(^{1-12}\) is an amino-terminal fragment derived from nisin upon chymotrypsin cleavage (10), and (des-\( \Delta Ala\))\(^{1-32}\)-amide 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 nisin\(^{1-12}\) and (des-\( \Delta Ala\))\(^{1-32}\)-amide correlates with their pore-forming capacity, their effects on the \( \Delta \psi \) in cells and cytochrome \( c \) oxidase vesicles were determined. Both de-

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**TABLE 1. Activity and membrane binding of nisin and variants\(^a\)**

<table>
<thead>
<tr>
<th>Lantibiotic</th>
<th>IC(_{50}) ((\mu)M)</th>
<th>(\Delta \psi)</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-( \Delta Ala))(^{1-32})-amide</td>
<td>&gt;30.0</td>
<td>&gt;30.0</td>
<td>74.0 ± 1.9</td>
</tr>
<tr>
<td>Nisin(^{1-12})</td>
<td>15.3 ± 1.4</td>
<td>&gt;18.0</td>
<td>17.0 ± 0.8</td>
</tr>
</tbody>
</table>

\(^a\) IC\(_{50}\) represents the concentration of nisin or derivative needed for half-maximal killing of the indicator strain or dissipation of the \( \Delta \psi \). Binding experiments were performed at 10.0 \( \mu \)M nisin or nisin variant with DOPG vesicles (220 \( \mu \)M 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%.
derivatives 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-ΔAla5)-nisin 1-32 amide 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-ΔAla5)-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 nisin induces a wedge-like pore. Bound, anionic phospholipids result 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, 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 nisin 1-29 shows only a 16-fold decrease in activity (10), and even nisin 1-20, 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 nisin 1-20 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 nisin 1-12 exhibits a strongly reduced membrane binding activity, while the opening of ring A in (des-ΔAla2)-nisin 1-32amide 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 nisin 1-12 and absence of the interaction of residues 33 and 34 in the case of (des-ΔAla2)-nisin 1-32amide might also contribute to the observed reduced membrane binding of both variants.

Both nisin 1-12 and (des-ΔAla2)-nisin 1-32amide 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-ΔAla2)-nisin 1-32 must be the consequence of the opening of the A ring, since nisin 1-7-amide, which also lacks the two carboxyl-terminal residues, and the ΔAla2-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 Δha5 (41, 43). Studies with a Dha5Dhb mutant (25) and with [α-OH-Ala2]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 > nisin Dha5Dhb > [α-OH-Ala2]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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