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In vitro pore-forming activity of the lantibiotic nisin

Role of protonmotive force and lipid composition

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Nisin is a lantibiotic produced by some strains of *Lactococcus lactis* subsp. *lactis*. The target for nisin action is the cytoplasmic membrane of Gram-positive bacteria. Nisin dissipates the membrane potential ($\Delta\psi$) and induces efflux of low-molecular-mass compounds. Evidence has been presented that a $\Delta\psi$ is needed for nisin action. The *in vitro* action of nisin was studied on liposomes loaded with the fluorophore carboxyfluorescein. Nisin-induced efflux of carboxyfluorescein was observed in the absence of a $\Delta\psi$ from liposomes composed of *Escherichia coli* lipids or dioleoylglycerophosphocholine ($Ole_2GroPCho$) at low nisin/lipid ratios. The initial rate of carboxyfluorescein efflux is dependent on the nisin/lipid ratio and saturates at high ratios. Both $\Delta\psi$ (inside negative) and ΔpH (inside alkaline) enhance the action of nisin, while nisin is more potent at acidic external pH values. Efficient carboxyfluorescein efflux is observed with the zwitterionic phospholipid $Ole_2GroPCho$ or mixtures of $Ole_2GroPCho$ with dioleoylglycerophosphoethanolamine and neutral glycolipids, while anionic phospholipids are strongly inhibitory. It is concluded that a $\Delta\psi$ is not essential, but that the total protonmotive force stimulates the action of nisin.

Nisin is a lantibiotic of 34 amino acids (Fig. 1) produced by certain strains of *Lactococcus lactis* subsp. *lactis*. It has a broad spectrum of action against Gram-positive bacteria. Nisin has a negligible toxicity for humans and has been accepted as a very suitable food preservative in dairy industry and canned foods. Although nisin was the first lantibiotic discovered and the first whose structure in solution was determined, its mechanism of action is still poorly understood. Treatment of sensitive cells with nisin results in the release of cytoplasmic material and nisin was assumed to act as a cationic surface-active detergent [1]. The 2,3-didehydroamino acid residues of the nisin molecule are thought to play an important role in the mechanism of its action by interaction with thiol groups of cysteine residues in ger minated bacterial spores [2, 3]. Ruhr and Sahl [4] observed efflux of $^{86}Rb^+$ and accumulated amino acids from various Gram-positive bacteria when treated with nisin. Similar effects were observed when cells and cytoplasmic membrane vesicles were treated with Pep-5, another lantibiotic very similar to nisin in structure and action [5]. As a consequence, nisin causes a rapid dissipation of $\Delta\psi$ [4, 6, 7] and ΔpH [6] in intact cells, cytoplasmic membrane vesicles and liposomes. It has been suggested that membranes have to be in an energized state in order to allow nisin action [6, 8]. These data suggest that the primary target for nisin is the cytoplasmic membrane [4]. *Escherichia coli* [9] and other Gram-negative bacteria [10] are only affected by nisin when the outer membrane is disrupted.

In order to obtain more information about the effect of nisin on membranes, we have chosen for a simple model system: liposomes loaded with the fluorophore carboxyfluorescein to allow the detection of nisin-induced leakage of small-molecular-mass compounds. It is concluded that an energized membrane is not essential for nisin action, but that the total protonmotive force enhances its effect. Further studies on the interaction of nisin with liposomes composed of various phospholipids demonstrate that anionic phospholipids are strongly inhibitory. A tentative working model for the pore-forming action of nisin on membranes is presented.

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Abbreviations. $\Delta\psi$, membrane potential; ΔpH, transmembrane pH gradient; $Ole_2GroPCho$, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; $Ole_2GroPGro$, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; $Ole_2GroPEtn$, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; $acyl_2GalGro$, diacylgalactosylglycerol; $acyl_2Gal_2Gro$, diacylgalactosylglycerol.

MATERIALS AND METHODS

Preparation of carboxyfluorescein-loaded liposomes

Large, unilamellar vesicles were formed by extrusion from multilamellar vesicles as described by Goessens et al. [11]. Lipids dissolved in $CHCl_3$/MeOH (9:1) were thoroughly dried under vacuum for 1 h. Traces of solvent were removed under a stream of N_2, and the dry lipid film was suspended in 50 mM 5(6)-carboxyfluorescein, 50 mM potassium 4-morpholineethanesulfonate (K/Mes) pH 6.0 to a final concentration of 14 mg/ml. At 50 mM, the fluorescence of carboxyfluorescein is almost completely self-quenched. The lipids were dispersed by ultrasonic irradiation using a bath sonicator (Sonicor, Sonicor Instr., New York), and subsequently subjected to five cycles of freezing (in liquid nitrogen) and thawing (in water at room temperature) to form large multilamellar vesicles. These were sized by extrusion through 400-nm, 200-nm and finally twice through 100-nm polycarbonate filters (Nucleopore Co., Pleasanton, CA) using an extrusion device (Lipex Biomembranes, Vancouver BC).

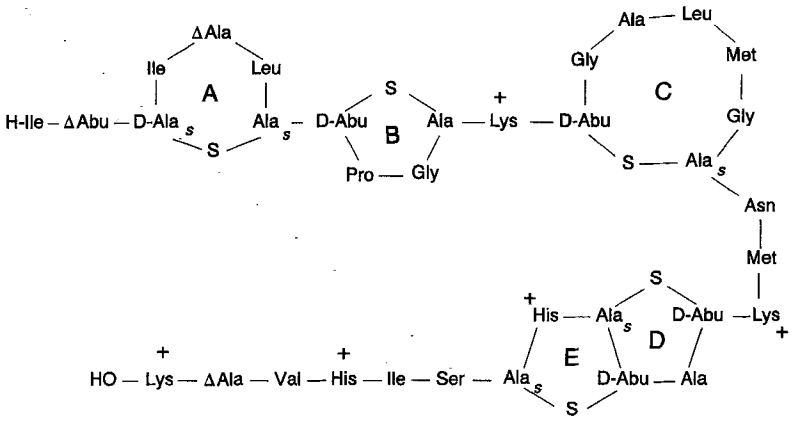

Fig. 1. Structure of nisin. Abbreviations: ΔAbu, dehydrobutyrine, ΔAla, dehydroalanine, D-Abu, 2-aminobutyric acid moiety of 3-methyllanthionine; Ala_s, alanine moiety of lanthionine or 3-methyllanthionine. Ring structures are labelled from A to E.

Non-encapsulated carboxyfluorescein was removed from the liposomes by gel filtration on Sephadex G-75 in 50 mM K/Mes pH 6.0. Liposomes produced by extrusion are homogeneous in diameter close to the pore size of the filters used to extrude them [12]. Liposomes composed of *E. coli* lipid and extruded through 0.2-μm polycarbonate filters had an averaged diameter of 220 nm (SD 56.5 nm) as determined by photon correlation spectroscopy (M. G. L. Elferink, unpublished results).

Carboxyfluorescein fluorescence

Release of liposome-encapsulated carboxyfluorescein results in the relief of fluorescence self-quenching. Fluorescence measurements were performed with a Perkin-Elmer spectrofluorimeter (LS 50), equipped with a thermostat and a continuous stirrer using excitation and emission wavelengths of 430 nm and 520 nm, respectively. Excitation and emission slit widths were 2.5 nm and 5.0 nm, respectively. Experiments were performed at a constant temperature of 25 °C. Liposomes were suspended in 50 mM K/Mes pH 6.0 or in 50 mM K/Mes/Mops/Hepes at the desired pH 5.5, 6.0, 7.0 or 8.0 at a final concentration of 70 μg lipid/ml. In experiments in which a membrane potential ($\Delta\psi$) was imposed, potassium buffers were replaced by sodium buffers. Generation of a $\Delta\Psi$ was initiated by the addition of the K^+ ionophore valinomycin (0.5 μM for *E. coli* liposomes, 0.1 μM for $Ole_2GroPCho$ liposomes). The 100% fluorescence was obtained by addition of Triton X-100 (0.2% by vol.) to the liposome suspension. The initial rate of carboxyfluorescein efflux was calculated as the slope of the tangent to the efflux curve at the point of nisin addition.

For experiments at different pH values, liposomes were prepared in the same way as described above but using 50 mM K/Mes/Mops/Hepes at the desired pH (5.5, 6.0 or 7.0). Excess of carboxyfluorescein was removed from the liposomes as described above using the liposomes loading buffer as eluent. Liposomes were treated with nisin (1.4 μg/mg lipid) in the presence and in the absence of a $\Delta\psi$.

Determination of efflux of high-molecular-mass compounds

Dry *E. coli* lipids were suspended (6 mg/ml) in 50 mM K/Mes pH 6.0 supplemented with 0.5 mM fluorescein isothiocyanate dextrans (Sigma Chem. Co. St Louis, Mo) of molecular mass 4.4 kDa or 9.4 kDa. Non-encapsulated dextran was removed from liposomes by gel filtration on Sephadex G-100 in 50 mM K/Mes pH 6.0. Release of dextrans was determined by quenching the fluorescence of the fluorescein isothiocyanate group with anti-(fluorescein rabbit IgG) (Molecular Probes, Inc. Eugene, OR). Complete release of the encapsulated dextran was obtained by addition of 0.2% (by vol.) Triton X-100. Excitation and emission wavelengths of 490 nm and 515 nm were used using slid widths of 10.0 nm.

Reagents

Highly purified nisin was purchased from NBS Biologicals (North Mymms, Hatfield, Herts, UK). Nisin (10 mg/ml) was stored at −20 °C in acetic acid solution at pH 3.0 in the dark for 1 month without any noticeable inactivation. Convenient dilutions were prepared in the buffers used for each experiment just before use. All the manipulations were performed in wrapped vessels to exclude pernicious light effects on the lantibiotic [13]. Carboxyfluorescein was purchased from Eastman Kodak Co. (Rochester, NY) and purified as described [14] using 50 mM K/Mes pH 6.5 as eluent. Synthetic phospolipids were obtained from Avanti Polar Lipids, Inc. (Birmingham, AL, USA). Crude *E. coli* phospholipid was obtained from Sigma Chem. Co. (St Louis, MO) and acetone/ether washed. Wheat diacylgalactosylglycerol and diacyldigalactosylglycerol were obtained from Sigma. Bovine brain phosphatidylserine and phosphatidylinositol were a gift of Dr J. Wilschut (Department of Physiological Chemistry, University of Groningen, The Netherlands).

Other procedures

The concentration of the liposome preparations was determined by phosphate analysis [15].

RESULTS

Effect of $\Delta\psi$ on nisin-induced carboxyfluorescein efflux from liposomes composed of *E. coli* lipids

At a concentration of 1.4 μg/mg lipid, nisin induced a rapid efflux of the low-molecular-mass compound carboxy-

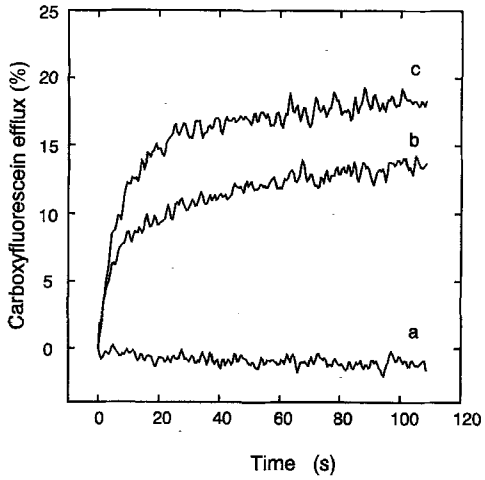

Fig. 2. Nisin-induced carboxyfluorescein efflux from liposomes composed of *E. coli* phospholipids. (a) No additions; (b) nisin in the absence of an imposed $\Delta\psi$; (c) nisin in the presence of an imposed $\Delta\psi$, inside negative. In each of the experiments, valinomycin was present at a concentration of 0.5 μM.

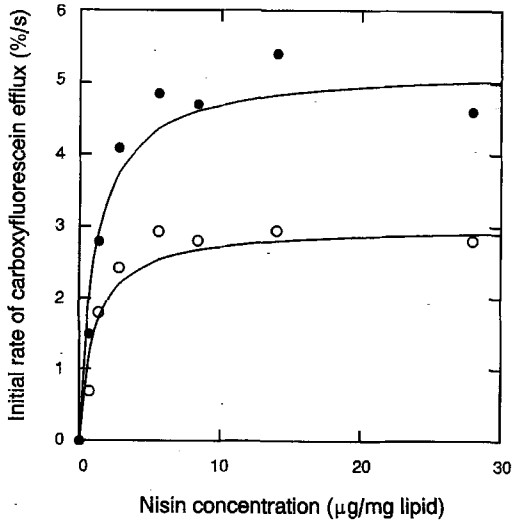

Fig. 3. Concentration dependency of nisin-induced carboxyfluorescein efflux from liposomes composed of *E. coli* phospholipids. Nisin-induced carboxyfluorescein efflux was studied in the absence (○) and presence (●) of an imposed $\Delta\psi$, inside negative.

fluorescein (molecular mass 376.32 Da) from liposomes composed of *E. coli* phospholipids (Fig. 2). The initial rate of carboxyfluorescein efflux in this experiment was 2.6%/s. When a $\Delta\psi$, interior negative (theoretically −120 mV), was created by imposition of a valinomycin-mediated outwardly-directed potassium diffusion gradient, the initial rate of carboxyfluorescein efflux was enhanced almost twofold, i. e. 4.1%/s while the final extent of carboxyfluorescein release remained the same (data not shown). Even at higher nisin concentration, complete leakiness (100%) of the encapsulated carboxyfluorescein was never detected. Successive additions of nisin did not produce further leakage of carboxyfluorescein from liposomes once treated with nisin.

The initial rate of carboxyfluorescein efflux was dependent on the concentra tion of nisin, and saturated at a nisin concentration of 5 μg/mg lipid (equivalent to approximately 100 molecules nisin/liposome with an average diameter of 100 nm assuming a phospholipid surface area of 7200 nm^2/molecule [16]) (Fig. 3). These results suggest the presence of a liposome population insensitive to nisin. At each nisin concentration, the presence of an imposed $\Delta\psi$ enhanced the initial rate of carboxyfluorescein efflux approximately 1.5-fold. Saturation was achieved at an identical nisin concentration. Similar behaviour has also been observed in the case of colicin A [17].

Effect of external pH and ΔpH on nisin-induced carboxyfluorescein efflux from liposomes composed of *E. coli* lipids

To obtain more information about the effect of pH and ΔpH on nisin-dependent carboxyfluorescein efflux, liposomes loaded with K/Mes/Mops/Hepes at different pH values (5.5, 6.0 and 7.0) were suspended in the same buffer adjusted at different pH values (5.5, 6.0, 7.0 and 8.0). The additional effect of a $\Delta\psi$, inside negative, on this nisin-dependent carboxyfluorescein-efflux was studied by diluting the potassium-loaded liposomes in sodium buffer at pH 5.5, 6.0 and 7.0 in the presence of valinomycin (0.5 μM). Rates of carboxyfluorescein efflux from liposomes were corrected for pH-dependent and nisin-independent leakage due to an increased protonation of the molecule at low pH [18] and the decreased fluorescence quantum yield of carboxyfluorescein at lower pH values. The data presented in Fig. 4 shows the rate of carboxyfluorescein efflux as a function of the external and internal pH in the absence (A) and presence (B) of a $\Delta\psi$, inside negative. In the absence of an imposed ΔpH, nisin is most active at acidic external pH. A ΔpH, inside alkaline, enhanced the action of nisin, both in the presence and in the absence of a $\Delta\psi$. This effect is more dramatic at larger ΔpH values. On the other hand, a ΔpH, inside acidic, inhibits the nisin-dependent leakage of carboxyfluorescein. A $\Delta\psi$, inside negative, is most effective at low external pH values (i. e. compare pH 5.5 with 8), while the impact of a $\Delta\psi$ is less pronounced in the presence of a ΔpH, inside alkaline. It is concluded that nisin is most effective at low pH values. These data further suggest that, in addition to $\Delta\psi$, the transmembrane pH gradient, ΔpH, is a important influencing factor of the action of nisin on liposomes.

Effect of lipid composition on the action of nisin

Carboxyfluorescein-loaded liposomes composed of the zwitterionic $Ole_2GroPCho$ were highly sensitive to the action of nisin. A saturation curve was obtained at different nisin concentrations using $Ole_2GroPCho$ liposomes (Fig. 5). As observed with liposomes composed of *E. coli* lipids, saturation is reached at the same nisin concentrations irrespective of the presence or absence of $\Delta\psi$. With $Ole_2GroPCho$, saturation is reached at higher nisin concentrations compared to *E. coli* lipids (compare Figs 5 and 3). When liposomes were used composed of a mixture of $Ole_2GroPCho$ and the anionic phospholipids, such as dioleoylglycerophosphoglycerol ($Ole_2GroPGro$), bovine brain phosphatidylserine (PtdSer) or phosphatidylinositol (PtdIns), nisin was nearly completely ineffective in provoking carboxyfluorescein efflux (Table 1). Even at high nisin concentrations (Table 1) or upon imposition of a $\Delta\psi$ (data not shown), hardly any carboxyfluorescein efflux was observed. In contrast, when $Ole_2GroPCho$ was mixed with $Ole_2GroPEtn$ or neutral glycolipids such as diacylgalactosylglycerol ($acyl_2GalGro$) or diacyldigalactosyl-

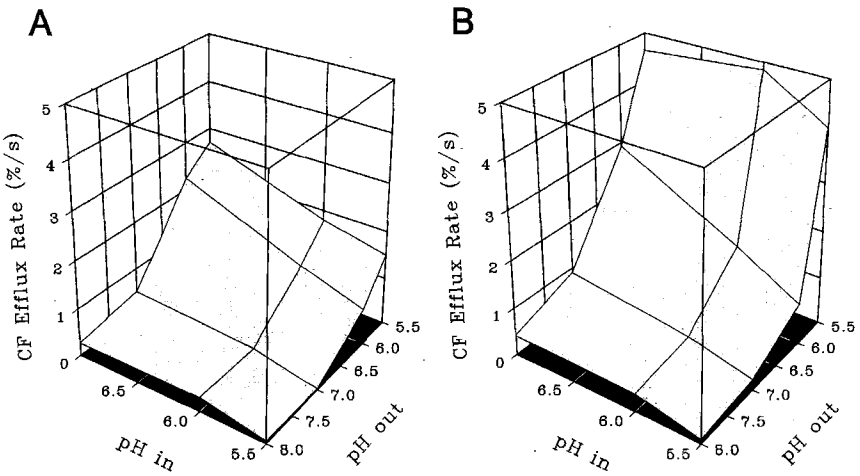

Fig. 4. Effect of the internal and external pH on nisin-induced carboxyfluorescein efflux from liposomes composed of *E. coli* phospholipids. Nisin-induced carboxyfluorescein efflux at different internal and external pH values was studied in the absence (A) and presence (B) of an imposed $\Delta\psi$, inside negative. CF, carboxyfluorescein.

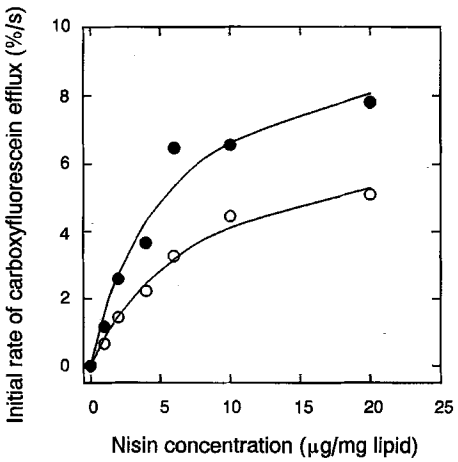

Fig. 5. Concentration dependency of nisin-induced carboxyfluorescein efflux from liposomes composed of $Ole_2GroPCho$. Carboxyfluorescein efflux in the absence (○) and presence (●) of an imposed $\Delta\psi$, inside negative. Valinomycin was present at a final concentration of 0.1 μM.

Table 1. Effect of the lipid composition of liposomes on nisin-induced carboxyfluorescein efflux. A dash (−) indicates no detectable leakage.

Lipid composition	Initial rate of carboxyfluorescein efflux at nisin concn (μg/mg lipid) of	
	2	20
	%/s	
$Ole_2GroPCho$	1.36	5.30
$Ole_2GroPCho/Ole_2GroPGro$ (3:1)	−	0.14
$Ole_2GroPCho/Ole_2GroPGro$ (1:1)	−	0.07
$Ole_2GroPCho/Ole_2GroPGro$ (1:3)	−	0.08
$Ole_2GroPGro$	−	−
$Ole_2GroPGro/Ole_2GroPEtn$ (1:1)	−	−
$Ole_2GroPCho/Ole_2GroPEtn$ (1:1)	0.35	1.26
$Ole_2GroPCho$/PtdSer (1:3)	−	0.03
$Ole_2GroPCho$/PtdIns (1:3)	0.07	0.34
$Ole_2GroPCho/acyl_2GalGro$ (1:1)	0.18	0.71
$Ole_2GroPCho/acyl_2Gal_2Gro$ (1:1)	0.91	3.30

glycerol ($Acyl_2Gal_2Gro$), the liposomes remained sensitive to nisin (Table 1).

Nisin bears a net positive charge at physiological pH values. In order to test the ability of nisin to bind to anionic lipids, we made use of an indirect assay. $Ole_2GroPGro$ liposomes were treated with nisin at a concentration of 1.4 μg/mg lipid, and subsequently carboxyfluorescein-loaded $Ole_2GroPCho$ liposomes were added to test whether the nisin was still available for interaction with these liposomes. When nisin was preincubated with buffer in the absence of liposomes, it retained the ability to effect carboxyfluorescein leakage from the $Ole_2GroPCho$ liposomes (Fig. 6, a). Only a low level of carboxyfluorescein efflux was observed when the carboxyfluorescein-loaded $Ole_2GroPCho$ liposomes were added to $Ole_2GroPGro$ (Fig. 6, b) or $Ole_2GroPCho$ (Fig. 6, c) liposomes pretreated with nisin. This result suggests that $Ole_2GroPGro$ liposomes interact with the lantibiotic but that this interaction does not lead to carboxyfluorescein leakage.

Efflux of high-molecular-mass compounds from *E. coli* liposomes

To determine whether nisin provokes a general disruption of the liposomes, nisin-induced leakage of high-molecular-mass compounds was investigated. Liposomes were prepared with fluorescein-isothiocyanate-labelled dextrans with an average molecular mass of 4.4 kDa or 9.4 kDa. Loaded liposomes were diluted into a buffer containing anti-fluorescein antibody which quenches the fluorescence of fluorescein-isothiocyanate dextrans when released into the medium. When the liposomes loaded with fluorescein-isothiocyanate dextran were treated with Triton X-100, complete quenching of the fluorescence was observed. Liposomes were treated with two different concentrations of nisin (2.8 μg/mg lipid and 28 μg/mg lipid) in the presence or absence of $\Delta\psi$. In none of the experiments was nisin-induced leakage of the fluorescein isothiocyanate dextrans (not shown) detected, suggesting that the putative pores have a defined size.

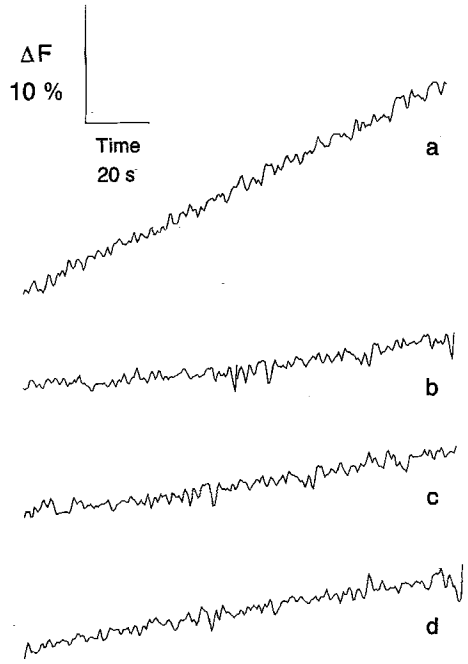

Fig. 6. Effect of precubation of nisin with liposomes on carboxyfluorescein efflux from $Ole_2GroPCho$ liposomes. Nisin was diluted into buffer without (a) or with $Ole_2GroPGro$ (b) or $Ole_2GroPCho$ (c) liposomes at a concentration of 70 μg lipid/ml and 98 ng nisin/ml. After a 1-min incubation, carboxyfluorescein-loaded $Ole_2GroPCho$ liposomes (indicator liposomes) were added to the cuvette (70 μg phospholipid/ml), and the release of carboxyfluorescein (ΔF) was monitored. (d) A control of carboxyfluorescein-loaded $Ole_2GroPCho$ liposomes incubated for the same time in the absence of nisin.

DISCUSSION

Although nisin was the first lantibiotic discovered, its mode of action is still poorly understood. It has been suggested that the cytoplasmic membrane is the target for nisin action [4]. In the present study, we have shown that nisin is able to interact with liposomes producing discrete-size pores that allow the efflux of molecules of low molecular mass. Sahl et al. [8] observed that nisin produced transient multistate pores in black lipid membranes with a predicted diameter in the range of 0.2−1 nm which allows the efflux of hydrophilic solutes with molecular masses up to 500 Da. These putative pore are too small to allow the passage of large compounds such as dextrans with a molecular mass of 4.4 kDa and higher.

Previous studies [4] suggested that a preformed $\Delta\psi$ is needed for nisin on whole cells. Kordel et al. [19] proposed that in the absence of a $\Delta\psi$, lantibiotics will not be able to span the membrane of non-energized liposomes. These studies were performed at pH 7.0−7.5. Our data demonstrates that nisin is already effective in the absence of a $\Delta\psi$, although $\Delta\psi$ enhances the rate of nisin-induced carboxyfluorescein efflux. As previously suggested [8, 20], the threshold $\Delta\psi$ necessary for nisin action may be lower at acidic pH implying a stronger dependency at higher pH values.

The efflux of carboxyfluorescein from liposomes is highly dependent on the number of molecules nisin/liposome, and saturates at approximately 100 molecules nisin/liposome. Nisin may belong to the group of cytolytic pore-forming proteins [21] which function through a so-called 'barrel-stave' mechanism. Three discrete steps can be discriminated: (a) water-soluble monomers bind to the target membrane, (b) insert into the membrane, and (c) aggregrate like barrel staves surrounding a central, water-filled pore that increases in diameter through the progressive recruitment of additional monomers. Such channels are usually large enough to allow the passage of ions and small solutes across the membrane, but too small to allow the passage of cytoplasmic proteins. Consequently, an ionic imbalance is elicted which leads to osmotic lysis. Alternatively, monomers could oligomerize before inserting into the membrane. We do not yet know for certain whether nisin inserts into the membrane as a monomer and then self-assembles into a oligomer to form the water-filled pore, or whether this aggregration event precedes membrane binding or insertion. However, the observation that nisin aggregated at pH values above 7−7.5 [13] is not capable of inducing pores, suggests that insertion has to precede aggregation for activity. Since a nisin molecule contains only 34 amino acids, it seems unlikely that it functions as a monomer to form an aqueous channel [8], unless it provokes the formation of nonbilayer structures. Nisin acts efficiently on $Ole_2GroPCho$ membranes. However, biophysical studies utilizing high lantibiotic/lipid ratios reveal only a minimal interaction between nisin and closely related lantibiotics with $Ole_2GroPCho$ [19] (see also below). No information about the discrete number of monomers participating in a possible nisin complex is available. Heterogeneous pores with varying diameters are to be expected when variable numbers of nisin monomers constitute the pore. $\Delta\psi$ enhances the rate of carboxyfluorescein efflux, while the affinity of nisin for the membrane is not affected by $\Delta\psi$ (Figs 3 and 6). A $\Delta\psi$, inside negative, may accelerate insertion, promote oligomerization or modulate the opening and/or size of the pore. In this respect it will be important to evaluate the impact of membrane fluidity and phospholipid acyl chain composition on the $\Delta\psi$ dependency of nisin-induced carboxyfluorescein efflux.

Experiments carried out at different pH values show that an acidic external pH and a ΔpH (alkaline inside) enhance the action of nisin on *E. coli* liposomes, whereas a ΔpH (acidic inside) dramatically inhibits the action of nisin (Fig. 4). The need for an acidic pH for optimal activity was also observed for colicin A [22] and several other toxins [23, 24]; it is thought to be required to provide a more unfolded state of the protein. Merrill et al. [25] proposed for colicin E1 that at acidic pH the peptide assumes a net hydrophobic character. Nisin becomes more soluble and stable at low pH values [13]; at high pH values, it undergoes a reversible modification by which the molecule is inactivated. Under these conditions aggregration can occur. The nature of this modification is unknown. At this stage, the mechanism by which ΔpH, inside alkaline, promotes the action of nisin remains obscure. ΔpH may facilitate the deprotonation of the imidazole rings of the two histidine residues (Fig. 1) in nisin. This, in turn, may affect the stability of nisin in a membrane-spanning conformation.

The phospholipid composition of liposomes often affects the action of peptide toxins and antibiotics [26]. Previous studies have demonstrated that the phospholipid composition can affect the interaction of nisin with the membrane [6, 19]. This study shows that anionic phospholipids strongly inhibit the action of nisin in a liposomal system. Indirect evidence suggests that, at high concentration, nisin binds to $Ole_2GroPGro$ liposomes or is inactivated possibly by aggregration outside the membrane. The first option would be in line with previous observations that nisin reduces the fluidity of PtdSer liposomes [19]. On the other hand, nisin was nearly without

effect on the fluidity of $Ole_2GroPCho$ vesicles. Thus, the interaction of nisin with anionic lipids seems to restrict the mobility of the phospholipid acyl chains. Since anionic phospholipids inhibit nisin function with no apparent specificity for the phospholipid polar headgroup, it seems likely that the cationic nisin electrostatically interacts with the anionic phospholipids. This electrostatic interaction may render the molecule inactive possibly by preventing insertion of nisin into the membrane or by formation of aggregrates outside the membrane. Benz et al. [27] observed that in $Ole_2GroPCho$ black lipid membranes the presence of 50% PtdSer decreased the threshold potential for the initiation of voltage-dependent membrane conductance, although the efficiency of membrane insertion was not addressed in this study. Our results showed only a very slight enhancement of carboxyfluorescein efflux in $Ole_2GroPCho/Ole_2GroPGro$ liposomes by $\Delta\psi$ (unpublished data).

In conclusion, our results further suggest that the cytoplasmic membrane is the target for nisin action and that the bacteriocidal effect is due to the generation of discrete size pores by a process that is facilitated by the protonmotive force.

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