# The C-Terminal Region of Nisin Is Responsible for the Initial Interaction of Nisin with the Target Membrane†

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ABSTRACT: The interaction of nisin Z and a nisin Z mutant carrying a negative charge in the C-terminus ([Glu-32]-nisin Z) with anionic lipids was characterized in model membrane systems, and bacterial membrane systems. We focused on three possible steps in the mode of action of nisin, i.e., binding, insertion, and pore formation of nisin Z. Increasing amounts of anionic lipids in both model and natural membranes were found to strongly enhance the interaction of nisin Z with the membranes at all stages. The results reveal a good correlation between the anionic lipid dependency of the three stages of interaction, of which the increased binding is probably the major determinant for antimicrobial activity. Maximal nisin Z activity could be observed for negatively charged lipid concentrations exceeding 50−60%, both in model membrane systems as well as in bacterial membrane systems. We propose that the amount of negatively charged lipids of the bacterial target membrane is a major determinant for the sensitivity of the organism for nisin. Nisin Z induced leakage of the anionic carboxyfluorescein was more efficient as compared to the leakage of the potassium cation. This lead to the conclusion that an anion-selective pore is formed. In contrast to the results obtained for nisin Z, the binding of [Glu-32]-nisin Z to vesicles remained low even in the presence of high amounts of negatively charged lipids. The insertion and pore-forming ability of [Glu-32]-nisin Z were also decreased. These results demonstrate that the C-terminus of nisin is responsible for the initial interaction of nisin, i.e., binding to the target membrane.

The antimicrobial peptide nisin belongs to the family of lantibiotics, a group of small peptides (<4 kDa) containing ($\beta$-methyl)lanthionine residues. It has antimicrobial activity against a broad spectrum of Gram-positive bacteria, and is widely used in the food industry as a safe and natural preservative (Delves-Broughton *et al.*, 1996). Two naturally occurring nisin variants, nisin A and nisin Z, have been found in lactococcal strains (Buchman *et al.*, 1988; Mulders *et al.*, 1991). Nisin Z (Figure 1) contains 34 amino acid residues of which 13 have been posttranslationally modified. These modifications include the dehydration of serine and threonine, resulting in three dehydroalanine and five dehydrobutyrine residues. Five of these dehydro residues are subsequently linked to the sulfhydryl groups of the five cysteine residues present in pre-nisin Z, resulting in the thioether bond of the characteristic ($\beta$-methyl)lanthionine rings (Gross & Morell, 1971; Jung, 1991). Nisin A differs from nisin Z in a single amino acid residue at position 27, being a histidine in nisin A and an asparagine in nisin Z (Mulders *et al.*, 1991). Another property of nisin Z is its positively charged nature due to the presence of three lysine residues (at positions 12, 22, and 34) and one histidine at position 31. The NMR structure of the nisin molecule contains two well-defined domains which are amphipatic in character (Van den Hooven *et al.*, 1996). The N-terminal domain encompasses residues 1−19 and comprises the first three lanthionine rings (A, B, and C). The other domain is formed by residues 23−28 and consists of the intertwined rings D and E, followed by a flexible stretch of the 6 C-terminal residues.

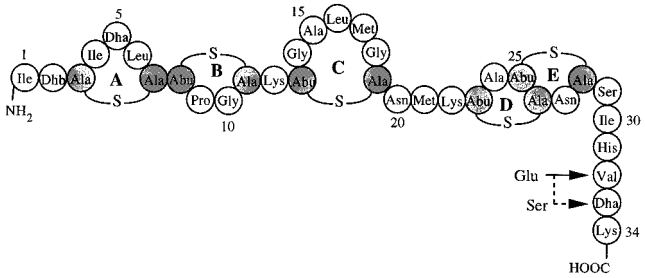


FIGURE 1: Primary structure of nisin Z. The modification at position 32 and the resulting lack of dehydration of Ser 33 are indicated by arrows. Dha = dehydroalanine, Dhb = dehydrobutyrine, Ala-S-Ala = lanthionine, Abu-S-Ala = $\beta$-methyllanthionine.

The dehydro residues have been suggested to play a role in the antimicrobial activity of nisin by reacting with the sulfhydryl groups of enzymes responsible for cell wall synthesis (Gross & Morell, 1971; Reisinger *et al.*, 1980). However, experiments with intact bacterial cells and cytoplasmic membrane vesicles showed that upon nisin addition the cells and membrane vesicles released small solutes in a trans-membrane potential dependent way (Ruhr & Sahl, 1985; Sahl *et al.*, 1987). Therefore, the nisin activity is primary thought to arise from pore formation in the

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cytoplasmic membrane of the target organism, resulting in dissipation of its vital ion gradients, loss of metabolites, and eventually cell death (Sahl, 1991). Black-lipid membrane studies have confirmed that nisin was capable of channel formation in lipid bilayers when a threshold potential of 100 mV was present (Sahl *et al.*, 1987). The mode of action of nisin is proposed to follow the barrel-stave model (Sahl, 1991), in which nisin first binds to the membrane by electrostatic interactions. This is then followed by formation of preaggregates, and upon energization of the membrane, pore formation is induced.

Liposomes have been used to study the lipid dependency of the nisin−membrane interaction (Abee *et al.*, 1991; Driessen *et al.*, 1995; Kordel *et al.*, 1989). Some of these studies documented that nisin preferably interacts with negatively charged lipids (Driessen *et al.*, 1995; Kordel *et al.*, 1989), as expected from the positive net charge of the peptide. Recent monolayer studies confirmed and extended the preferential interaction of nisin with anionic lipids, and in addition established a correlation between the antimicrobial activity of nisin Z and mutant forms of the peptide and their ability to insert in an anionic lipid dependent way into the lipid monolayer (Demel *et al.*, 1996).

Little is known about the initial interaction of nisin with the membrane. Since the C-terminal domain contains the major part of the positive charge of the nisin molecule, it is conceivable that this domain is responsible for the initial interaction of nisin with the membrane surface. To test this hypothesis, we studied in detail the anionic lipid dependency of several aspects of the membrane interaction of nisin Z, and a mutant containing a negatively charged glutamic acid residue in the C-terminus at position 32 ([Glu-32]-nisin Z). Both model membranes and *Escherichia coli* derived membrane systems containing varying concentrations of negatively charged lipids were used. It is shown that the presence of negatively charged lipids is essential for efficient binding, insertion, and membrane disruptive activity of nisin Z. Furthermore, it is shown that the presence of a negative charge in the C-terminus abolishes the negatively charged lipid dependency of the interaction. Especially the nisin−membrane binding is affected, demonstrating that the C-terminus is responsible for the initial interaction of nisin with the membrane.

## MATERIALS AND METHODS

### *Materials*

Nisin Z was produced by batch fermentation and purified as previously described (Kuipers *et al.*, 1992). The nisin mutant [Glu-32]-nisin Z was obtained by site-directed mutagenesis and purified and characterized (van Kraaij *et al.*, unpublished observations). 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC)[1] and 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG) were purchased from Avanti Polar Lipids, Inc. Carboxyfluorescein was purchased from Kodak, and purified as described (Ralston *et al.*, 1981). All other chemicals were of analytical grade or better. Protein concentration was determined using the bicinchoninic acid protein assay reagent (Pierce Chemical Corp.) with BSA as a standard.

### *Methods*

*Growth of Bacteria and Preparation of Inner Membrane Vesicles.* *Escherichia coli* strain MRE600 (Cammack & Wade, 1965), possessing a wild-type phospholipid composition of 20% phosphatidylglycerol (PG), 5% cardiolipin (CL), and 75% phosphatidylethanolamine (PE), was grown at 37 °C in Luria broth (LB: 10 g/L bacto-tryptone, 5 g/L yeast extract, and 5 g/L NaCl). Strain HDL11 (Kusters *et al.*, 1991), in which the gene encoding phosphatidylglycerol phosphate synthase is under control of the *lac* promoter, was grown at 37 °C in LB supplemented with the antibiotics chloramphenicol (20 $\mu$g/mL), kanamycin (50 $\mu$g/mL), and tetracycline (10 $\mu$g/mL). The strain was grown in the presence of 100 $\mu$M IPTG, resulting in a wild-type phospholipid composition, and in the absence of IPTG, resulting in a membrane composition of only 8% negatively charged lipids (mainly phosphatidic acid, PA) and 92% PE (Kusters *et al.*, 1991). Strain AD93 (DeChavigny *et al.*, 1991), deficient in the gene encoding for phosphatidylserine synthase (*pss*) and thus unable to synthesize PE, was grown at 37 °C in LB supplemented with 50 mM $MgCl_2$, resulting in a membrane with only negatively charged lipids of composition 46% PG, 50% CL, and 4% PA (Rietveld *et al.*, 1993). Strain AD93, harboring plasmid pDD72 (AD93/pDD72) which contains a functional copy of the *pss* gene under control of the *lac* promoter, has a wild-type lipid composition. This strain was grown at 43 °C for 10 generations in LB to cure it from its plasmid (DeChavigny *et al.*, 1991), which results in a decrease in the PE content of the membrane to 30%, and a concomitant increase in the acidic lipid content to 70% (PG and CL). *Micrococcus flavus* strain DSM 1790 was grown at 30 °C in MF broth (0.1% sucrose, 1% peptone, 0.3% meat extract, 0.2% NaCl, and 0.15% yeast extract, pH 7.0). Right-side-out inner membrane vesicles of *E. coli* were prepared and isolated as described (Kaback, 1970). The phospholipid content of the vesicles was determined after extraction of the lipids (Bligh & Dyer, 1959) and determination of the $P_i$ content (Rouser *et al.*, 1970). The phospholipid composition of the vesicles was determined after extraction of the lipids, followed by one-dimensional high-performance thin-layer chromatography (Kieselgel 60, Merck) with chloroform/methanol/acetic acid (65:25:10, v/v/v). Spots were visualized with $I_2$ and excised for $P_i$ quantification.

*Radioactive Labeling of Nisin.* Nisin was $^{14}$C-labeled by reductive methylation of the $\epsilon$-amino group of the lysine residues, according to Dottavio-Martin and Ravel (1978). The modified lysyl groups retain their positive charge after the reaction. Five hundred micrograms of peptide was dissolved in a total volume of 500 $\mu$L of 50 mM Mes−KOH, pH 6.5, 100 mM $K_2SO_4$, 20 mM $NaCNBH_3$, and [$^{14}$C]formaldehyde (58 Ci/mol, 2:1 molar ratio with respect to nisin). After 60 min incubation at room temperature with continuous stirring, the labeled peptide was separated from unbound label on a 1.0 × 30 cm Sephadex G-10 column coupled to a Pharmacia FPLC system, equilibrated in 0.05% (v/v) acetic acid. The resulting nisin Z and [Glu-32]-nisin Z preparations had specific radioactivities of $1.36 \times 10^5$ and $1.15 \times 10^5$ dpm/nmol, respectively, which corresponds to an average of 1.05

[1] Abbreviations: DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; BSA, bovine serum albumin; diS-$C_2$-(5), 3,3′-diethylthiadicarbocyanine iodide; CF, 5-(and 6-)carboxyfluorescein; MIC, minimal inhibitory concentration; IPTG, isopropyl 1-thio-$\beta$-D-galactopyranoside.

and 0.89 methylated amino groups per peptide, respectively. The radiolabeled nisin Z and [Glu-32]-nisin Z were stored at a concentration of 0.2 mg/mL in 0.05% (v/v) acetic acid at −80 °C. This labeling procedure did not change the antibacterial activity toward *Micrococcus flavus* DSM1790 as determined in an agar diffusion assay nor did the labeling affect the lipid interaction monitored in the monolayer system described previously (Demel *et al.*, 1996) (data not shown).

*Preparation of Large Unilamellar Vesicles and Generation of Ion Gradients.* Dry lipid films of DOPC−DOPG mixtures were hydrated in 50 mM Mes−KOH, pH 6.0, 100 mM $K_2SO_4$ (buffer $K^+$) by vortexing. The dispersions were frozen and thawed 10 times and then extruded through 400 nm pore size polycarbonate filters as described (Hope *et al.*, 1985). Transmembrane $K^+_{in}/Na^+_{out}$ chemical gradients were obtained by a 100 times dilution of the vesicles in 50 mM Mes−NaOH, pH 6.0, 100 mM $Na_2SO_4$ (buffer $Na^+$). A trans-membrane potential (negative inside) was induced by adding valinomycin (1 $\mu$g/mL in ethanol) in a 1:$10^4$ molar ratio with respect to phospholipid. Phospholipid was determined as inorganic phosphate, after destruction with perchloric acid (Rouser *et al.*, 1970). The trapped volumes were determined to be 2.0 and 3.0 $\mu$L/$\mu$mol lipid for DOPC vesicles as assessed for carboxyfluorescein and $K^+$ enclosure, respectively. For DOPG vesicles, the values were 0.5 and 4.5 $\mu$L/$\mu$mol. These differences reflect the influence of electrostatic interactions on the enclosure of these solutes in the negatively charged vesicles.

*Binding Experiments.* The nisin Z−vesicle association was determined by two methods. In the first method, binding was determined under equilibrium conditions. Vesicles (150 $\mu$M, on a phosphorus basis) were incubated at room temperature with a mixture of [$^{14}$C]nisin Z and unlabeled nisin Z in 0.5 mL of 25 mM Mes, pH 6.0, 50 mM $K_2SO_4$ for 5 min. The mixture was then centrifuged at 430000*g* for 1 h to spin down the vesicles and the bound nisin Z, and the amount of nisin in the supernatant was determined by scintillation counting. Under these conditions, less than 5% of the phospholipids remained in the supernatant. In the absence of lipid, more than 90% of the nisin was recovered.

In the second method, binding was determined under nonequilibrium conditions in an assay employing mini columns loaded with 1.5 mL of Sephadex G50 (Pharmacia). A mixture of radiolabeled and nonradiolabeled nisin Z or [Glu-32]-nisin Z at the given concentration range was added to vesicles (0.5 mM final concentration, on phosphorus basis) in the presence or absence of a membrane potential. The amount of radiolabeled nisin corresponding to $3 \times 10^4$ dpm was kept constant. After 2 min of incubation at room temperature, 150 $\mu$L aliquots were withdrawn and within 10 s centrifuged through the mini columns (1 min, 300*g*). The mini columns were preequilibrated using the same buffer as used in the nisin−vesicle incubation. The amount of nisin associated to the vesicles was quantified by analysis of the filtrates for phospholipid by phosphorus determination, and for nisin by scintillation counting, and corrected for the amount of nisin which eluted through the mini columns in the absence of vesicles (less than 7%). At least 75% of the vesicles was recovered from the mini columns in all cases.

*Monolayer Experiments.* Monolayer surface pressure was measured by the (platinum) Wilhelmy plate method (Demel, 1994) at room temperature, using a Cahn D-202 microbalance. The subphase was continuously stirred with a magnetic bar. Nisin Z was added to the subphase through a hole in the edge of the dish. The pressure changes were followed until the surface pressure increase had reached a maximal value. The monomolecular lipid layers were spread from a chloroform/methanol 75:25 (v/v) solution to give an initial surface pressure of 25 mN/m. A subphase of buffer $K^+$ was used at room temperature. The Teflon dish had a volume of 5 mL and a surface area of 8.81 cm$^2$. The results were obtained using saturating amounts of nisin Z or [Glu-32]-nisin Z (2 $\mu$g/mL).

*Carboxyfluorescein (CF) Leakage Experiments.* Carboxyfluorescein-loaded vesicles were made according to the protocol described above in the presence of 50 mM CF. The nonentrapped CF was removed by gel filtration on a 0.5 cm × 30 cm Sephadex G50 column, equilibrated with buffer $K^+$. The CF-loaded vesicles (20.8 $\mu$M on a phosphorus basis) were diluted in 1.2 mL of buffer $K^+$ (or buffer $Na^+$ if a membrane potential was required) followed by the addition of nisin (nisin Z or [Glu-32]-nisin Z). The nisin-induced leakage of CF from the vesicles was monitored by measuring the increase in fluorescence intensity at 515 nm (excitation at 492 nm) on a SPF 500 C spectrophotometer (SLM instruments Inc., USA) at 20 °C.

*Potassium Leakage Experiments.* Nisin-induced potassium leakage from lipid vesicles was monitored with a potassium selective electrode (15-K, Philips) and a reference electrode (R44/2, Philips). Vesicles prepared in 30 mM Mes, 20 mM Tris, pH 6.5, and 100 mM $K_2SO_4$ were subjected to a gel filtration step using a Sephadex G50 column (1 cm × 30 cm) equilibrated in 30 mM Mes, 20 mM Tris, pH 6.5, and 150 mM choline chloride (buffer C). The potassium-loaded vesicles (30 $\mu$M on a phosphorus basis) were added to 5 mL of buffer C in a thermostated cuvette (20 °C). Nisin Z or [Glu-32]-nisin Z was added, and the nisin-induced potassium leakage was followed. The amount of potassium released after nisin addition was expressed relative to the total amount of potassium released after lysis of the vesicles by the addition of 20 $\mu$L of a 20% solution of the nonionic detergent *N*,*N*-dimethyldodecylamine *N*-oxide (Fluka). If required, a membrane potential was generated by adding valinomycin to a molar ratio of 1:7000 with respect to phospholipid. The electrodes were calibrated by titrating known amounts of potassium to the buffer solution.

*Bacterial Membrane Vesicle Experiments.* A membrane potential was generated with the bacterial membrane vesicles using the artificial electron donor system ascorbate−phenazine methosulfate (PMS) (Konings *et al.*, 1971). The action of nisin on the membrane potential was monitored with the membrane potential sensitive probe 3,3′-diethylthiodicarbocyanine iodide [diS-$C_2$-(5)] (Sims *et al.*, 1974). Vesicles (25 nmol of lipid−$P_i$) stored in 50 mM $KP_i$, pH 7.0, were diluted in 25 mM Mes−KOH, pH 6.0, 50 mM $K_2SO_4$, followed by the addition of 1 $\mu$M diS-$C_2$-(5), 4 $\mu$M PMS, and 0.1 $\mu$M nigericin. A membrane potential was applied upon the addition of 10 mM ascorbate. Nisin was added, and the nisin-induced decrease in the membrane potential was followed in time. After 4 min, gramicidin D was added in a 1:400 molar ratio over lipid−$P_i$ to completely abolish the remaining membrane potential. The final fluorescence intensity after the gramicidin addition was taken as the 100% leakage value. The activity of nisin in this assay was

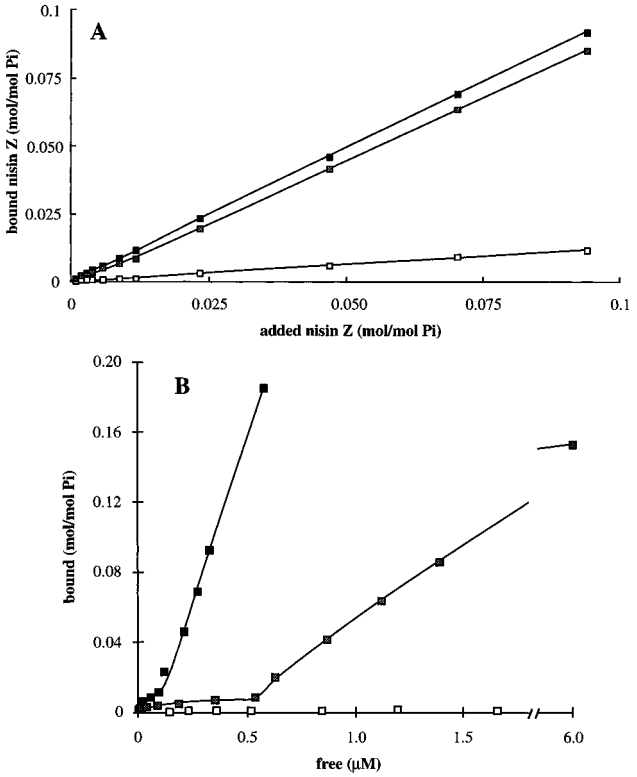


FIGURE 2: (A) Binding of nisin Z to DOPG vesicles (■), DOPG/DOPC (1:1) vesicles (cross-hatched square), and DOPC vesicles (□). Binding was determined under equilibrium conditions. (B) Binding isotherms of nisin Z to vesicles of different lipid composition. DOPG vesicles (■), DOPG/DOPC (1:1) vesicles (cross-hatched square), and DOPC vesicles (□).

expressed as percent inhibition, and was calculated with the equation:

$$\% \text{ inhibition} = 100[(F_n - F_0)/(F_g - F_0)]$$

in which $F_0$ is the stable fluorescence value after the onset of the membrane potential by addition of ascorbate, $F_n$ is the fluorescence value 2 min after addition of nisin, and $F_g$ is the fluorescence value after the addition of gramicidin D.

## RESULTS

*Binding of Nisin to Model Membranes.* The first step in the membrane action of nisin will be binding to the target membrane. We mimicked this process using large unilamellar vesicles composed of DOPC (a zwitterionic lipid which forms stable bilayers) and DOPG (as a model for an abundant bacterial anionic lipid). Radioactively labeled nisin was used to allow sensitive detection of binding over a wide range of conditions. Nisin Z binding was first studied under equilibrium conditions.

Figure 2A shows that virtually all nisin Z added binds to the vesicles when they are composed of DOPG. In strong contrast, very little nisin binds to DOPC vesicles. In mixed DOPG/DOPC (1:1) vesicles, efficient binding is observed. When the data are replotted as a binding isotherm (Figure 2B), two different binding stages can be described for the DOPG-containing vesicles. Above a certain concentration of free nisin (~0.1 and 0.5 $\mu$M for DOPG and DOPG/DOPC 1:1, respectively), the affinity of the peptide for the bilayer strongly increases, suggesting a cooperative interaction.

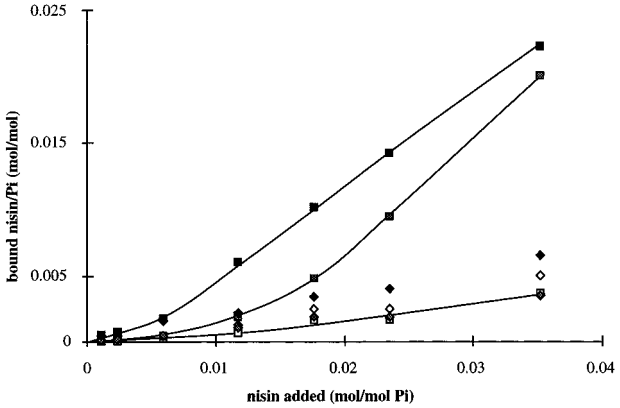


FIGURE 3: Binding of nisin Z and [Glu-32]-nisin Z to vesicles determined using the minicolumn centrifugation assay. Nisin Z: DOPG (■), DOPG/DOPC (1:1) (cross-hatched square), and DOPC (□). [Glu-32]-nisin Z: DOPG (◆), DOPG/DOPC (1:1) (cross-hatched diamond), and DOPC (◇).

To get insight into the stability of the nisin−vesicle interaction, the amount of nisin bound to the vesicles was also determined using a protocol in which bound nisin and free nisin are separated on mini columns (Figure 3). Qualitatively, a similar result was obtained, i.e., efficient nisin binding to the DOPG vesicles and much less to the DOPC vesicles. Intermediate behavior was observed for the mixed DOPC/DOPG (1:1) system. However, quantitative differences can be noticed. Binding of nisin Z to the DOPG-containing vesicles is reduced as compared to the equilibrium binding experiments (compare Figures 2A and 3). The strongest differences are observed for the mixed system at lower nisin concentrations. This strongly suggests that part of the nisin detected in association with the vesicles under equilibrium conditions is loosely associated and is released from the vesicles upon separation of the vesicles from the medium on the mini columns. Thus, the data shown in Figure 3 reflect the more stable association of nisin to the vesicles.

The behavior of [Glu-32]-nisin Z in this system differed markedly from the nisin Z binding especially in the presence of acidic phospholipids. The binding of [Glu-32]-nisin Z to DOPC vesicles (open diamonds) was very similar to the nisin Z−DOPC binding. However, in the mixed PC/PG system and in the PG system, the binding of [Glu-32]-nisin Z was greatly reduced (Figure 3). Moreover, the reduction was such that the amount of vesicle-bound [Glu-32]-nisin Z in the presence of negatively charged lipids was comparable to the amount bound to PC vesicles.

The DOPG dependency of nisin Z and [Glu-32]-nisin Z binding was examined in more detail at a fixed initial nisin:phospholipid ratio of 1:25 (Figure 4). When the percentage of DOPG is increased in the vesicles, the amount of stable associated nisin Z increases, with a steep increase above 40% DOPG (Figure 4, open squares). The binding levels off at DOPG concentrations above 60%. The amount of binding of [Glu-32]-nisin Z to vesicles with a PG content below 20% was equal to the amount of bound nisin Z. Above this percentage, the binding of [Glu-32]-nisin Z was significantly lower, and showed hardly any dependence on the presence of DOPG (Figure 4, open circles). Only a slight increase in binding could be observed above 60% PG.

We tested whether the presence of a membrane potential had an effect on the amount of stable associated nisin Z. It

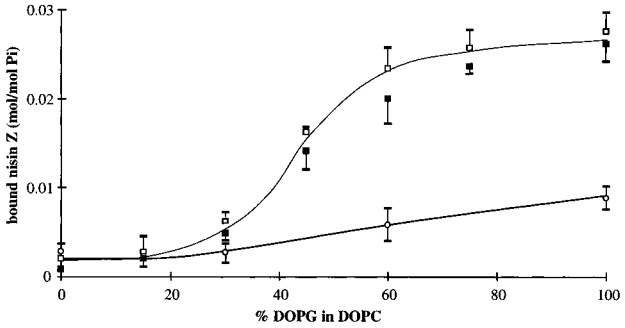


FIGURE 4: Binding of nisin Z and [Glu-32]-nisin Z to lipid vesicles with different DOPG:DOPC ratios at a constant nisin:phospholipid ratio of 1:25. Binding studies of nisin Z were performed in the presence (■) or absence (□) and for [Glu-32]-nisin Z in the absence (○) of a membrane potential, using the mini column centrifugation assay.

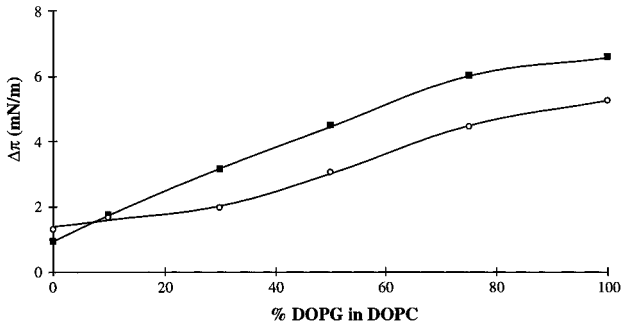


FIGURE 5: Insertion of nisin Z (■) and [Glu-32]-nisin Z (○) into monolayers composed of different DOPC:DOPG ratios. The initial surface pressure of the monolayer was 25 mN/m. The peptide concentration was 0.6 $\mu$M.

had no effect on the binding of nisin Z to the phospholipid vesicles (Figure 4, filled squares). Control experiments, using the membrane potential sensitive probe diS-$C_2$-(5) (Sims *et al.*, 1974), showed that after elution of the vesicles from the spin columns in the absence of nisin Z, the membrane potential remained intact (not shown).

*Membrane Insertion of Nisin Z and [Glu-32]-Nisin Z.* To analyze the ability of nisin Z to insert into the lipid part of a membrane, we examined the increase in surface pressure induced by the peptide when injected underneath monolayers made of DOPC/DOPG mixtures. Figure 5 clearly shows that the presence of DOPG in the monolayer is essential for efficient insertion and that the ability of nisin Z to insert in the monolayer levels off above 70 mol % DOPG. Since it was observed that [Glu-32]-nisin Z binding to vesicles containing more than 20% DOPG was significantly lower as compared to nisin Z, this should also affect the monolayer insertion of [Glu-32]-nisin Z. Indeed, [Glu-32]-nisin Z inserted less efficiently in monolayers containing more than 20% DOPG, although the insertion remained high in view of the low amount of stable bound [Glu-32]-nisin Z. In the presence of lower amounts of PG, the insertion of [Glu-32]-nisin Z was comparable to nisin Z. These results demonstrate that the anionic lipid-dependent insertion of nisin Z not only occurs in mixtures of phosphatidylethanolamine and cardiolipin (Demel *et al.*, 1996) but also is a more general property of the peptide.

*Nisin Z and [Glu-32]-Nisin Z Induced Release of Anionic and Cationic Solutes.* The increased binding of nisin Z and the low binding of [Glu-32]-nisin Z in the presence of anionic phospholipids could lead to different membrane-disruptive

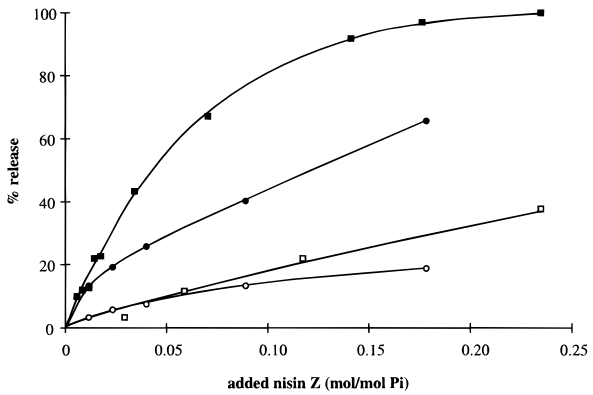


FIGURE 6: Nisin Z induced leakage of carboxyfluorescein from DOPC vesicles (□) and DOPG vesicles (■) and of potassium from DOPC vesicles (○) and DOPG vesicles (●). The extent of CF release was determined 5 min after the addition of nisin. The vesicle concentration was 20.8 $\mu$M on a $P_i$ basis. The extent of $K^+$ release was determined 13 min after addition of nisin. The vesicle concentration was 30 $\mu$M on a $P_i$ basis.

activity. Therefore, we investigated the anionic lipid dependency of the nisin Z and [Glu-32]-nisin Z induced release of vesicle-entrapped solutes. Both the nisin-induced leakage of the anionic carboxyfluorescein (CF) and the potassium cation were investigated.

*CF Leakage.* The CF-release experiments showed an initial fast increase of CF fluorescence in the first minute which leveled off in the next 4 min, but continued to slowly increase in time (not shown). To quantify the leakage, the amount of CF released after 5 min was determined. When nisin Z was titrated to DOPG vesicles, efficient release of the enclosed CF was observed already at low nisin Z concentrations (Figure 6, filled squares). Adding more than 1 nisin per 10 DOPG molecules even results in complete release. Much less release of CF was observed for the DOPC vesicles. Even at the highest nisin Z:DOPC ratio (1:2) tested, no more than 47% release of CF was observed (not shown). At a nisin Z:phospholipid ratio of 1:25, the percentage release of CF from the DOPG vesicles is about 10 times higher as compared to the CF release from the DOPC vesicles, which is comparable to the difference in nisin Z binding to the two vesicle systems (compare Figure 4).

The anionic lipid dependency of the CF leakage was examined in more detail using vesicles with varying DOPC:DOPG ratios and a nisin Z ([Glu-32]-nisin Z):phospholipid ratio of 1:25. An increase in the anionic phospholipid content of the membrane resulted in an increase of the nisin Z induced initial leakage rate (Figure 7, open squares). The strongest effect is observed in the 40−75% DOPG range. At higher DOPG concentrations, the rate of nisin Z induced CF release leveled off. The extent of induced release of CF followed a similar DOPG-dependency as the initial leakage rate (not shown). Strikingly, [Glu-32]-nisin Z hardly induced leakage of CF at all (open circles). CF leakage could only be observed at 10-fold higher concentrations of [Glu-32]-nisin Z (data not shown).

The presence of a membrane potential (negative inside) increased the initial rate of nisin Z induced CF leakage from all the vesicles (Figure 7, closed squares). Especially in the 30−60% DOPG concentration range the leakage rate was affected, with a maximal effect at 50% DOPG. However, the total extent of leakage was hardly affected. Only at DOPG concentrations below 30%, an increase of about 10−

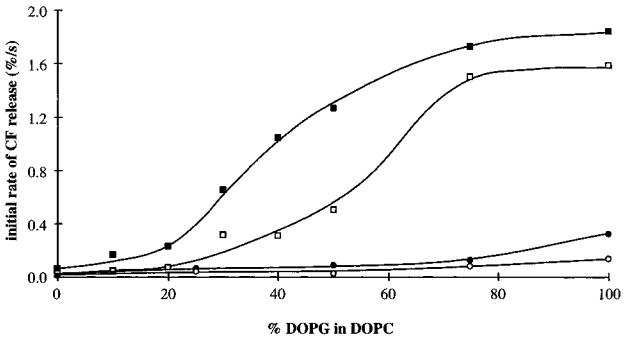


FIGURE 7: Initial rate of nisin-induced carboxyfluorescein leakage from vesicles with different DOPC:DOPG ratios at a constant nisin:phospholipid ratio of 1:25. Nisin Z in the presence (■) or absence (□) and [Glu-32]-nisin Z in the presence (●) or absence (○) of a membrane potential. The vesicle concentration was 20.8 $\mu$M on a $P_i$ basis.

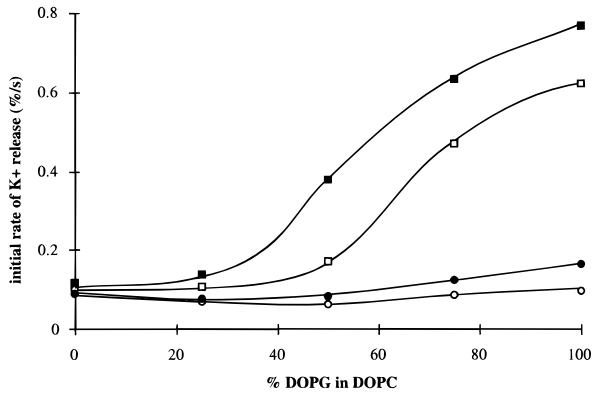


FIGURE 8: Rate of nisin-induced leakage of potassium from vesicles with different DOPG:DOPC ratios at a constant nisin:phospholipid ratio of 1:30. Nisin Z in the presence (■) or absence (□) and [Glu-32]-nisin Z in the presence (●) or absence (○) of a membrane potential. The vesicle concentration was 30 $\mu$M on a $P_i$ basis. The rate was calculated by determining the amount of released potassium 50 s after addition of nisin Z.

15% could be observed (not shown). The effect of a membrane potential on [Glu-32]-nisin Z induced CF leakage was also investigated. Only for 100% DOPG vesicles an increase in the [Glu-32]-nisin Z induced CF leakage could be observed (Figure 7, closed circles).

*$K^+$ Leakage.* Addition of nisin Z to DOPG vesicles also results in the release of entrapped potassium which levels off in time (not shown). Figure 6 shows that increasing amounts of nisin Z caused an increased loss of $K^+$, which was determined 13 min after the addition of nisin Z to the DOPG vesicles (filled squares). In contrast to the release of CF, no complete release of $K^+$ was observed at the highest concentration of nisin tested. Much less $K^+$ release was observed when nisin Z was titrated to DOPC vesicles (open squares).

The rate of nisin Z induced $K^+$ leakage also was DOPG-dependent (Figure 8, open squares), similar to the induced CF leakage (compare Figure 7). However, the nisin Z induced $K^+$ leakage was a factor of 2−2.5 lower as compared to the CF leakage, suggesting that the nisin Z pore is to some extent anion-selective. The total amount of $K^+$ release showed a similar DOPG dependency as the leakage rate (not shown). In contrast to what was observed for the CF leakage experiments, $K^+$ leakage induced by [Glu-32]-nisin Z *could* be observed at the concentrations used for nisin Z induced $K^+$ leakage (open circles). Leakage of $K^+$ from PC vesicles was equal for both peptides, while at PG concentrations above 20% the [Glu-32]-nisin Z induced $K^+$ leakage was significantly lower as compared to the nisin Z induced $K^+$ leakage. These results show that also the nisin-induced leakage of $K^+$ is directly correlated to the amount of nisin bound to the membrane.

Addition of valinomycin to generate a trans-membrane potential caused release of a small amount of $K^+$ (within 2−4%, not shown). Further leakage of potassium through valinomycin is inhibited due to the generation of a potential. When nisin is added to vesicles possessing a trans-membrane potential, at DOPG concentrations above 25% both the nisin-induced leakage rate increased (filled squares) and also the extent of leakage increased (by a factor of 1.5−2; not shown). Also the [Glu-32]-nisin Z induced $K^+$ leakage was increased in the presence of a membrane potential, albeit only at PG concentrations of 75 or 100% (Figure 8, closed circles). This increased $K^+$ loss could potentially be caused by nisin-induced influx of protons, thus relieving the inhibition of the valinomycin-mediated $K^+$ efflux by the trans-membrane potential. However, the nisin-induced leakage rate (and extent) was found to be independent of the valinomycin concentration (not shown). Thus, the observed leakage of $K^+$ is directly caused by the effect of nisin on the barrier function of the model membrane.

*Nisin Z and [Glu-32]-Nisin Z Activity toward Biological Membrane Vesicles.* We tested whether the observed acidic lipid dependent activity of nisin Z (and [Glu-32]-nisin Z) in systems containing model lipid vesicles can also be observed with biological membranes. The use of bacterial membranes derived from Gram-positive bacteria from different genetic backgrounds could lead to difficulties in interpretation of the data due to large differences in the membrane−lipid composition. Therefore, we used membranes derived from *E. coli* strains of well-defined lipid composition. The *E. coli* strains used carry mutations in genes which function in phospholipid biosynthesis. Due to these mutations, the membrane−lipid composition, with respect to acidic phospholipids, varies from 8% (HDL11, grown in the absence of IPTG) to 25% (HDL11 grown in the presence of IPTG and MRE600), 70% (AD93/pDD72), and 100% (AD93). Since *E. coli* is relatively resistant to the action of nisin, due to the presence of an outer membrane, right-side-out inner membrane vesicles were prepared, and the action of nisin on an artificially induced transmembrane potential was investigated using the membrane potential sensitive probe diS-$C_2$-(5). As can be seen in Figure 9, nisin Z shows little activity with inner membrane vesicles derived from strains HDL11 or MRE600, with an anionic lipid content lower than 30% (filled bars). However, nisin Z almost completely abolished the membrane potential of vesicles derived from AD93/pDD72 and AD93 with an anionic phospholipid composition of 70 and 100%, respectively. For a comparison, the activity of nisin Z against *M. flavus* vesicles containing 90% anionic lipids (O'Leary & Wilkinson, 1988) was in the same range as found for the *E. coli* vesicles with a high acidic phospholipid content. The activity of [Glu-32]-nisin Z in this system was very low. Therefore, a 4 times higher concentration of the peptide was used. At this concentration, still no activity of [Glu-32]-nisin Z could be detected with the HDL11- or MRE600-derived vesicles. [Glu-32]-nisin Z activity could be detected with the AD93/pDD72 and AD93 vesicles containing more than 50%

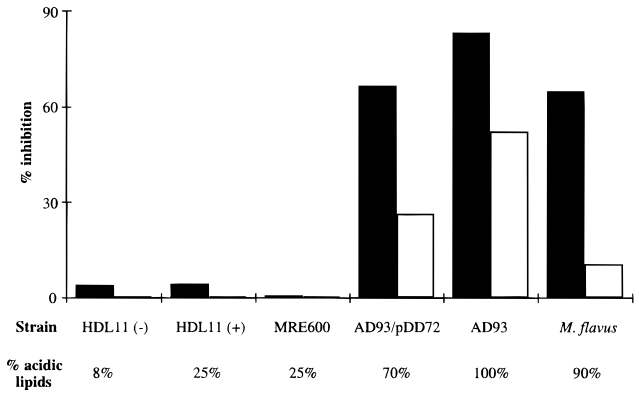


FIGURE 9: Nisin-induced dissipation of an artificially applied membrane potential in right-side-out membrane vesicles from *E. coli* strains and *M. flavus*. The closed bars represent the nisin Z activity and the open bars the [Glu-32]-nisin Z activity (at 4 times higher concentration), expressed as a percentage of inhibition (for details, see Methods).

negatively charged lipids. This was similar to the results obtained with the monolayer experiments (Figure 5) and the $K^+$ leakage experiments (Figure 8).

## DISCUSSION

The antibacterial activity of nisin is primarily thought to arise from pore formation in the cytoplasmic membrane of the target organism (Sahl, 1991). We examined the anionic lipid dependency of the interaction of nisin Z with model membranes, and focused on three possible steps in the mode of action of nisin. These steps are binding of nisin Z to the membrane, insertion of nisin Z into the lipid phase of the membrane, and finally pore formation. In addition to model membrane studies, the activity of nisin Z toward bacterial membranes of different phospholipid composition, especially with respect to the amount of negatively charged lipids, was studied. Furthermore, the effects of introducing a negative charge ([Glu-32]-nisin Z) in the C-terminal region were investigated. The results clearly demonstrate that the presence of the negatively charged phospholipid DOPG is essential for efficient binding, insertion, and pore formation. The results obtained with [Glu-32]-nisin Z suggest an important function of the C-terminus of nisin in the initial interaction with the target membrane.

The binding studies show that nisin Z has a much higher affinity for membranes containing the negatively charged lipid DOPG relative to membranes composed of the zwitterionic DOPC. For the DOPC/DOPG (1:1) vesicles, saturation of the amount of binding could be observed at free nisin concentrations around 6 $\mu$M (Figure 2B). This allowed us to determine the maximal number of binding sites, and from this value the stoichiometry of the nisin−lipid interaction. This resulted in a nisin:phospholipid ratio of 1:6 at saturation. Assuming that DOPC is not involved in the interaction, it means that nisin interacts with three DOPG molecules. This value is close to full charge stoichiometry, which implies that all the DOPG molecules in the vesicles are available for interaction with nisin Z, including those located in the inner monolayer. This could suggest that nisin can translocate over the bilayer, for instance via pore formation, to reach the DOPG molecules in the inner monolayer as has been reported for magainin (Matsuzaki *et al.*, 1995a,b). The number of phospholipids of area 0.70 $nm^2$ that is covered by the 10 $nm^2$ nisin (Goodman *et al.*, 1991) is equal to 14 (DOPC+DOPG). This is much higher than the observed 1:6 ratio; this is probably caused by aggregation of nisin at the membrane surface. In this respect, an important observation was that in the presence of negatively charged lipids, the binding isotherms of nisin Z showed a biphasic character (Figure 2B). Similar shaped isotherms were reported for pore-forming peptides such as the antimicrobial peptide alamethicin (Rizzo *et al.*, 1987), the neurotoxin paradaxin and derivatives (Sahl *et al.*, 1985), and others (Gazit & Shai, 1993a,b). This is indicative of a process whereby the peptides first incorporate into the membrane, and once inside the membrane aggregate to form a pore (Schwarz *et al.*, 1986, 1987). Recent studies with nisin mutants containing unique tryptophan residues confirmed that nisin aggregated in the membrane in the presence of negatively charged lipids (unpublished observations).

The differences between the results in Figures 2A and 3 suggested that part of the vesicle-associated nisin Z is loosely bound, while the other part has strongly interacted with the lipid vesicles. This latter pool could correspond to the nisin molecules inserted into the lipid phase, possibly in combination with translocation. Indeed, we showed that nisin Z is able to insert into the lipid phase of the membrane in an anionic lipid dependent way. Moreover, we observed for DOPG monolayers that 90% of the monolayer-associated nisin Z could be washed away with high salt concentrations while the surface pressure only decreased by a factor of 2 (not shown). Extrapolating this to the vesicle-system, this implies that part of the vesicle associated nisin Z is inserted into the lipid phase of the membrane. The other part is bound electrostatically to the membrane surface, and a fraction of this pool is lost on the minicolumn.

We studied the pore formation of nisin Z in model vesicles by following the nisin Z induced release of both the anionic CF and the cationic $K^+$. We are assuming that the leakage of both CF and $K^+$ is caused by actual pore formation in the membrane, also in the absence of a membrane potential. The leakage of both ions was strongly dependent on the presence of negatively charged lipids. Comparing the characteristics of the $K^+$ leakage and the CF leakage shows that indeed both the rate and the extent of leakage of $K^+$ are lower (compare Figures 6, 7, and 8). This strongly suggests that nisin Z forms an anion-selective pore. Therefore, when the nisin Z molecule is in the pore-forming state, the positively charged lysines are probably situated such that it results in anion selectivity.

Introduction of a negative charge in the C-terminus of nisin Z by replacing the valine residue at position 32 by a glutamic acid residue ([Glu-32]-nisin Z) resulted in a decrease in antimicrobial activity, as shown by an increase in the minimal inhibitory concentration for *Micrococcus flavus* and *Streptococcus thermophilus* by a factor of 3 and 6, respectively (van Kraaij *et al.*, unpublished observations). It was found that due to the mutation at position 32, the serine residue at position 33 was not dehydrated in the biosynthetic pathway (van Kraaij *et al.*, unpublished observations). It could be argued that the effects we have observed for introducing a negative charge at position 32 are actually caused by a loss of the dehydroalanine at position 33. However, earlier studies showed that loss of residues 33 and 34 due to chemical breakdown of nisin A, which resulted in [1−32]-nisin A and an amidated C-terminus, did not result in a

change in antimicrobial activity (Chan *et al.*, 1989). It should be kept in mind that this change does not affect the overall charge of the C-terminal part of nisin due to loss of a lysine residue since the C-termini of nisins A and Z are *not* blocked. Furthermore, it has been shown that the absence of Dha 33 in [Ser-33]-nisin A does not influence nisin activity (Rollema *et al.*, 1991, 1996). Therefore, we interpreted the results obtained with [Glu-32]-nisin Z as caused by the introduction of the negatively charged residue only. The binding studies of [Glu-32]-nisin Z showed that the mutation has caused almost a complete loss of the anionic lipid dependent binding to model vesicles. This was quite surprising since the peptide still possesses a net positive charge of +2. The N-terminal part of [Glu-32]-nisin Z remains the most positively charged part of the peptide with charges from the N-terminus itself and the lysine at position 12. Apparently these residues cannot compensate for the negative charge introduced in the C-terminus. Interestingly, very recent experiments with nisin [1−12], a fragment of nisin containing only the N-terminal 12 residues, showed that binding of this fragment to DOPG vesicles was strongly reduced (17% of the wild-type level) (Moll *et al.*, 1997). Therefore, we conclude that the C-terminus of nisin is involved in the initial interaction of nisin with the target membrane, and constitutes an important anionic phospholipid binding site.

The monolayer experiments with [Glu-32]-nisin Z followed the same tendency as the binding experiments, i.e., decreased insertion in the presence of more than 20% acidic phospholipids as compared to nisin Z. However, the insertion of [Glu-32]-nisin Z was unexpectedly high in view of the results of the binding experiments. Apparently, the low amount of bound [Glu-32]-nisin Z is sufficient to cause a surface pressure increase, similar to the remaining 10% of nisin Z after washing with high salt, as mentioned above. A likely explanation for this finding is that the observed pressure increase in the monolayer system is dominated by the insertion of the N-terminal part of the peptide. This suggestion is supported by two considerations; i.e., the N-terminal part of the peptide is the most apolar part, and the insertion of this part of the peptide is less likely influenced by a mutation in the C-terminal part.

Our CF results directly contrast the results of another research group (Driessen *et al.*, 1995; Garcera *et al.*, 1993), who found no nisin A induced leakage of carboxyfluorescein from vesicles composed of pure DOPG or DOPC/DOPG (1:1). The reason for this discrepancy is not known to us. We did not detect any difference between nisin A and nisin Z in the CF leakage assays (not shown). Furthermore, our results with nisin Z are comparable to the anionic lipid dependency of other cationic pore-forming peptides such as magainins (Matsuzaki *et al.*, 1989) and defensins (Fujii *et al.*, 1993; Wimley *et al.*, 1994), in which case the activity of these peptides also increases in the presence of negatively charged lipids. These peptides were also active in the absence of a membrane potential in the model membrane systems. Recently, the nisin A induced leakage of carboxyfluorescein from membranes composed of lipid extracts from *Listeria monocytogenes* was investigated (Winkowski *et al.*, 1996). The *L. monocytogenes* vesicles, made by detergent dilution, were composed of at least 50% negatively charged lipids. Nisin A was able to induce CF leakage quite efficiently from these vesicles in the absence of a membrane potential, which supports our observation that nisin is able to induce leakage of CF from vesicles containing 50% anionic lipids. Also, very recent results obtained with calcein leakage which showed an increase in nisin-induced leakage in the presence of 50% cardiolipin, but in the absence of a membrane potential (Martin *et al.*, 1996), support our observations with CF leakage (see Note Added in Proof).

Several studies have shown that the membrane-disruptive activity of nisin both in model systems and in biological systems is increased in the presence of a membrane potential (Garcera *et al.*, 1993; Ruhr & Sahl, 1985; Sahl *et al.*, 1987). Our data confirm these observations since in the presence of a membrane potential an increase in both the CF and $K^+$ leakage was observed. The membrane potential could affect the nisin activity in at least two ways. First, it might increase the binding of nisin to the membrane and thereby stimulate pore formation. Second, it might act on membrane-associated nisin, for instance by changing the topology of nisin with respect to the membrane in such a way that pore formation is more favorable. Since no effect of the membrane potential could be observed for the binding of nisin Z, the latter possibility is more likely. Driessen et al. (1995) suggested for the mode of action of nisin a wedge model in which nisin pore formation is initiated by the action of the membrane potential on the orientation of nisin. It was furthermore suggested that the C-terminus of nisin inserts in the membrane upon pore formation, while the N-terminus remained at the surface of the membrane (Martin *et al.*, 1996). In view of this model, the membrane potential could have an electrophoretic effect on the positively charged C-terminus of wild-type nisin, thereby pulling the C-terminus into the bilayer and probably across the bilayer, thus inducing pore formation. The presence of a negative charge in the C-terminus should have a negative influence on such an electrophoretic effect. In our system, this would be reflected in a decrease in [Glu-32]-nisin Z induced $K^+$ or CF leakage in the presence of a membrane potential. However, both the [Glu-32]-nisin Z induced CF leakage as well as the $K^+$ leakage were enhanced in the presence of a membrane potential. These data suggest that the membrane potential acts on the N-terminal half of nisin by pulling it into the bilayer and thus inducing pore formation.

Interestingly, a fragment of nisin A composed of the first 12 residues (nisin [1−12]) was recently shown to inhibit the activity of full-length nisin A in an agar diffusion assay (Chan *et al.*, 1996). In view of our results which suggest a role for the C-terminus in binding of nisin Z to the target membrane and those of Moll *et al.* (1997) which showed that the binding of nisin [1−12] was decreased to 17% of the wild-type level, it seems unlikely that fragment 1−12 could influence the binding of the full-length nisin A. The results with the fragment could well be explained with an important role of the N-terminal part of nisin in the aggregation of the peptide at the membrane surface. This aggregation could be inhibited by the fragment, resulting in inhibition of the activity.

We showed that efficient leakage can only be observed for DOPG concentrations above 50%. Similarly, the binding as well as the insertion of nisin Z in the membrane was maximal at DOPG concentrations above 50−60%. This suggests that the negatively charged lipids (above a threshold concentration) increase the nisin Z binding and thereby stimulate pore formation. However, it cannot be excluded that the negatively charged lipids also play a direct role in

the insertion and pore formation of nisin Z. This also suggests that negatively charged lipids are an important determinant for the sensitivity of bacteria for nisin. Support for this suggestion comes from our observations that membrane vesicles derived from *E. coli* strains were sensitive to nisin Z only if the membranes were composed of 70 or 100% negatively charged phospholipids. Also vesicles derived from *M. flavus*, a nisin-sensitive bacterium (MIC = 11 $\mu$g/L) with a membrane composed of 90% negatively charged lipids (O'Leary & Wilkinson, 1988), showed high sensitivity toward nisin Z, thus supporting our suggestion. That other factors besides the lipid composition of the membrane could play a role in determining the sensitivity of a bacterium toward nisin can be seen in the case of *Listeria monocytogenes*. This is a relatively nisin-insensitive species (MIC = 200−1000 $\mu$g/L). However, the negatively charged lipid composition of the membrane is relatively high, i.e., reported to be 50−88% (O'Leary & Wilkinson, 1988; Winkowski *et al.*, 1996). This suggests that the negatively charged lipid content of the plasma membrane is not the only important factor in determining the sensitivity of the organism. The cell wall composition or thickness is another likely factor in determining the sensitivity of a target cell toward nisin.

## NOTE ADDED IN PROOF

Two very recent articles also showed that the binding (El-Jastimi & Lafleur, 1997) and membrane disruptive activity (Giffard *et al.*, 1997) of nisin are strongly dependent on the presence of anionic phospholipids.

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