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Published in:
Biochemistry

DOI:
10.1021/bi980931b

Publication date:
1998

Pore Formation by Nisin Involves Translocation of Its C-Terminal Part across the Membrane†

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Received April 24, 1998; Revised Manuscript Received July 22, 1998

ABSTRACT: Nisin is an amphiphilic peptide with a strong antimicrobial activity against various Gram-positive bacteria. Its activity results from permeabilization of bacterial membranes, causing efflux of cytoplasmic compounds. To get information on the molecular mechanism of membrane permeabilization, a mutant of nisin Z containing the C-terminal extension Asp-$(His)_6$ was produced. The biological and anionic lipid-dependent membrane activity of this peptide was very similar to that of nisin Z. Analysis of the pH dependence of model membrane interactions with the elongated peptide indicated the importance of electrostatic interactions of the C-terminus with the target membrane for membrane permeabilization. Most importantly, the membrane topology of the C-terminus of the molecule could be determined by trypsin digestion experiments, in which trypsin was encapsulated in the lumen of large unilamellar vesicles. The results show that the C-terminal part of the peptide translocates across model membranes. The pH and anionic lipid dependence of translocation closely paralleled the results of membrane permeabilization studies. Binding of nickel ions to the histidines blocked translocation of the C-terminus and concomitantly resulted in a 4-fold reduced capacity to induce K^+ leakage. The results demonstrate for the first time that pore formation of nisin involves translocation of the C-terminal region of the molecule across the membrane.

Nisin, a 34-residue posttranslationally modified bacteriocin, is produced by various strains of *Lactococcus lactis* (*1*). It has antimicrobial activity against a broad range of Gram-positive bacteria, and it is widely used as a safe preservative in the food industry (*2*). As a member of the family of lantibiotics, the structure of nisin is characterized by the occurrence of uncommon amino acid residues, such as dehydroalanine, dehydrobutyrine, and (β-methyl)lanthionine residues (*3*). Several studies have been performed to gain insight in the molecular mechanism of action of nisin, and these have shown that the site of action is primarily at the cytoplasmic membrane (*4*−*7*). Nisin destabilizes the phospholipid bilayer causing rapid efflux of cytoplasmic solutes and dissipation of the membrane potential (*4*−*7*). The initial interaction between nisin and the target membrane is mediated by electrostatic interactions between the cationic peptide and anionic membrane lipids (*8*). It is not known yet how nisin inserts into a lipid bilayer and what kind of structure it forms in the hydrophobic part of the membrane to allow efflux of cytoplasmic solutes. The 'barrel-stave' model of poration suggests that nisin molecules insert into the lipid bilayer and take an orientation perpendicular to the membrane to form a membrane-spanning pore (*4*, *5*). Another mechanism proposed for the membrane perturbing activity of nisin is that surface-bound peptides insert together with phospholipids giving rise to wedge-like pores (*6*). The topology of membrane insertion of nisin is completely unknown.

It was recently shown that nisin most efficiently interacts with anionic membrane lipids (*8*−*11*) and that positive charges in the C-terminal domain of nisin are important for this interaction (*8*). To obtain further insight into the importance of the C-terminal region of nisin for its membrane activity, a nisin Z variant, named His-tag-nisin Z, was produced containing the C-terminal extension Asp-$(His)_6$. This peptide could readily be isolated by use of immobilized metal affinity chromatography (IMAC)[1] (*12*). It was investigated how the activity of this nisin Z variant in model membrane systems was affected by the anionic lipid content of the membranes and by the charge of the C-terminus, which could be modulated by changes in the pH of the buffer. His-tag-nisin Z contains a cleavage site for trypsin behind residue Lys-34, which allows the determination of the membrane topology of the peptide. To elucidate the topology of the C-terminal part of the peptide in the membrane, it was

† This work was supported by The Netherlands Foundation for Chemical Research (SON) with financial aid of The Netherlands Foundation of Scientific Research (NWO) and the Foundation of Applied Science (STW).

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[1] Abbreviations: IMAC, immobilized metal affinity chromatography; MIC, minimal inhibitory concentration; ESI−MS, electrospray ionization−mass spectroscopy; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; MES, 2-(*N*-morpholino)ethanesulfonic acid; LUV, large unilamellar vesicle; PMSF, phenylmethanesulfonyl fluoride; Dha, dehydroalanine; Dhb, dehydrobutyrine.

10.1021/bi980931b CCC: $15.00
Published on Web 10/29/1998

investigated whether this cleavage site was accessible for trypsin encapsulated in the lumen of large unilamellar vesicles. The results demonstrate that the C-terminal part of nisin translocates across the membrane and that this event is coupled to the pore-forming activity. On the basis of these observations, the mode of action of nisin is discussed.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids. The plasmid pNZ9013 (*13*) containing the *nisZ* gene was used for site-directed mutagenesis and cloning experiments. The strain *Lactococcus lactis* NZ9800 (*14*) was used for production of His-tag-nisin Z. As a host for cloning experiments, *Escherichia coli* MC1061 (*15*) was used, which was cultivated with aeration in TY broth at 37 °C. *L. lactis* strains were grown at 30 °C without aeration in M17 broth (Difco) containing 0.5% (wt/vol) sucrose, supplemented with 10 μg/mL chloramphenicol when appropriate. *Bacillus cereus* P7 and *Micrococcus flavus* DSM 1790 were used as target organisms in MIC assays as previously described (*13*).

The *Eschericha coli* strains HDL11, MRE600, AD75, and AD93, each displaying a well-defined phospholipid composition of their inner membranes, were grown as previously described (*8*).

Construction of His-tag-nisin Z. Site-directed mutagenesis was performed as described previously (*13*). The following primers were used for PCR (mutations are underlined and restriction enzymes used for cloning experiments are in parentheses): 5′-GATTAAATTCTGCAGTTTGTTAG-3′ (*Pst*I), 5′-CCCTAAAAAGCTTATAAAAATAGG-3′ (*Hin*dIII), 5′-CACGTAAGCAAAGATCTAAATCAAAG-GATAGTATTTTG-3′ (*Bgl*II). By introducing a *Bgl*II site, the stop codon in the *nisZ* gene was removed. The fragment *Bgl*II−*Hin*dIII was replaced by a DNA linker of the following primers: 5′-GATCACCACCACCACCACCACTA-3′ and 5′-AGCTTAGTGGTGGTGGTGGTGGT-3′. The resulting plasmid, named pNZhistag, encodes a nisin Z variant containing the extension Asp-$(His)_6$. Introduction of a *Bgl*II site offers the advantage that the *Bgl*II−*Hin*dIII fragment can be replaced by any DNA linker. As a consequence, the codon for an Asp residue is introduced. DNA manipulations were carried out according to Sambrook et al. (*16*). DNA-modifying enzymes were purchased from Gibco/BRL Life Technologies, and oligonucleotides were purchased from Pharmacia. Plasmid DNA was introduced into *L. lactis* strains by electroporation (*17*). The modifications in the plasmid were confirmed by restriction analyses and DNA sequencing, using the dideoxy chain-termination method (*18*).

Purification and Characterization of His-tag-nisin Z. The Histrap kit (Pharmacia) was used for small-scale purification of His-tag-nisin Z. His-tag-nisin Z was purified on large scale from 10 L of culture supernatant of *L. lactis* NZ9800 harboring pNZhistag as described previously (*13*). To enhance mutant nisin production, induction with nisin A (0.03 μg/mL) was performed (*19*, *20*). Absolute amounts of nisin species were estimated by using the bicinchoninic acid protein assay reagent (Pierce Chemical Corporation). His-tag-nisin Z was characterized by ^{1}H NMR spectroscopy using a Bruker AM-400 spectrometer as described previously (*13*) and by electron spray ionization mass spectrometry (ESI-MS). For ESI-MS, the nisin Z analog was dissolved in 50% CH_3CN/0.5% HCOOH to a concentration of 20 ng/mL. Mass spectra were recorded on a VG Quattro II mass spectrometer (Fisons Instruments). His-tag-nisin Z was ^{14}C-labeled by reductive methylation of the ϵ-amino group of the lysyl residues, as described previously (*8*). Nisin samples were stored at −20 °C in 0.05% HAc at 1 mg/mL.

Model Membrane and Bacterial Membrane Experiments. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG) were purchased from Avanti Polar Lipids, Inc. To measure the capacity of nisin species to induce leakage of carboxyfluorescein, 1 μM nisin was added to carboxyfluorescein-loaded vesicles (25 μM lipid-Pi), and leakage 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) after 2 min at pH 6.5, at 20 °C, as described previously (*8*). Preparation of right-side-out vesicles of different mutant *E. coli* strains and generation of an artificial membrane potential were performed as described previously (*8*). The decrease of the membrane potential upon addition of 1 nmol of nisin to the vesicles (25 nmol of lipid-Pi) was monitored by use of the membrane potential sensitive probe 3,3′-diethylthiodicarbocyanine iodide. The activity of nisin was expressed as the percentage inhibition after 2 min and was determined by measuring the nisin-induced decrease in the membrane potential relative to the gramicidin-induced total collapse of the membrane potential (*8*). Monolayer experiments were performed as described (*8*). The following buffers were used: at pH 5.5 and 6.5, 50 mM MES−KOH and 100 mM K_2SO_4; at pH 7.5, 25 mM MES/25 mM Tris and 100 mM K_2SO_4. Large unilamellar vesicles (LUVs) were prepared according to the extrusion procedure of Hope et al. (*21*) using 10 mM Pipes/50 mM K_2SO_4, pH 7.0, as a buffer. The phospholipid concentration was determined by phosphorus analyses (*22*). To measure the capacity of His-tag-nisin Z to induce K^+ leakage from lipid vesicles, 1 μM His-tag-nisin Z was added to potassium-loaded vesicles (25 μM lipid-Pi). Leakage of K^+ was determined as previously described (*8*) using the following buffers: at pH 5.5 and 6.5, 25 mM Tris/MES and 150 mM choline chloride; at pH 7.5, 50 mM Tris-HCl and 150 mM choline chloride.

Translocation Experiments. Phospholipid vesicles were prepared in 10 mM Pipes/50 mM K_2SO_4, pH 7.0, in the presence of 5 mg/mL trypsin according to the extrusion procedure (*21*). However, to reduce trypsin inactivation, the freeze/thaw cycles were reduced to 5 times. Removal of external trypsin was performed by three washing steps, each consisting of pelleting the vesicles at 37000*g* for 25 min at 4 °C and resuspending them in 10 mM Pipes/50 mM K_2SO_4, pH 7.0. A typical translocation experiment was performed in a total volume of 75 μL. Prior to the addition of His-tag-nisin Z, vesicles (50 nmol of lipid-Pi) were incubated in the presence of 0.5 mg/mL of the trypsin inhibitor aprotinin (Boehringer) in the appropriate buffer at 0 °C for 10 min. Upon addition of 1 nmol of ^{14}C-labeled His-tag-nisin Z, the mixtures were incubated at 30 °C for 45 min. Reactions were stopped by addition of 0.5 μL of 100 μM PMSF to completely block trypsin activity. Samples were analyzed by tricine−SDS−PAGE (*23*), and the amount of radiolabeled protein was quantified by use of a phosphor-

Table 1: Comparison of the Activity of Nisin Z and His-tag-nisin Z[a]

	MIC (μg/L)		% CF leakage		% inhibition of $\Delta\psi$ of RSO vesicles			
	M. flavus	*B. cereus*	DOPC	DOPG	HDL11	MRE600	AD75	AD93
nisin Z	11	150	0	23	0	5	46	59
His-tag-nisin Z	33	280	0	30	0	12	83	49

[a] The table compares the capacity of both peptides to inhibit growth of bacteria, to permeabilize model vesicles enclosed with carboxyfluorescein, and to dissipate the membrane potential of right-side-out (RSO) membrane vesicles derived from mutant strains of *Escherichia coli*, with well-defined phospholipid composition. For details, see Experimental Procedures.

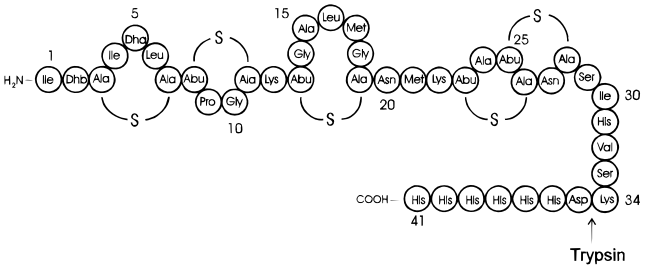

FIGURE 1: Primary structure of His-tag-nisin Z. The cleavage site for trypsin is indicated by an arrow. His-tag-nisin Z contains an unmodified Ser residue on position 33 instead of the usual Dha. Dha, dehydroalanine; Dhb, dehydrobutyrine; Ala-S-Ala, lanthionine; Abu-S-Ala, β-methyllanthionine.

imaging system (Molecular Dynamics). The pH dependence was tested by using the following buffers: at pH 5.5 and 6.5, 25 mM MES−KOH and 50 mM K_2SO_4; at pH 7.5, 25 mM Tris-HCl and 50 mM K_2SO_4. To test whether the amount of aprotinin externally added to the vesicles was sufficient to inactivate remaining traces of extravesicular trypsin, we prepared control vesicles without entrapped trypsin. To the external medium of these vesicles, 5 mg/mL trypsin was added, and the usual wash steps were carried out. The translocation experiments with these vesicles were performed as described above. To test the general barrier function of the vesicles, trypsin-loaded vesicles (50 nmol) were added to a 1-mL cuvette, containing 0.25 mM chromogenic substrate Chromozym Try (carbobenzoxy-Val-Gly-Arg-4-nitranilide acetate, Boehringer). Proteolysis of the substrate was measured at 405 nm.

RESULTS

Production and Characterization of His-tag-nisin Z. A modified *nisZ* gene was constructed encoding a nisin Z variant with the C-terminal extension Asp-$(His)_6$ (Figure 1). It was established that this peptide can be purified directly from the culture supernatant by using IMAC (*12*). This procedure enables specific purification of His-tag-nisin Z and is less laborious than the usual purification procedure, which is based on hydrophobic interaction chromatography. It should be noted, however, that the production level of His-tag-nisin Z by the lactococcal expression strain was approximately 10-fold reduced as compared to the production of nisin Z. Isolated His-tag-nisin Z (purity >96%) was characterized by mass spectrometry and ^{1}H NMR analysis. The NMR spectrum of His-tag-nisin Z demonstrated resonances of additional His residues and showed that His-tag-nisin Z harbors an unmodified Ser-33 instead of the usual Dha (data not shown). Lack of dehydration of Ser-33 was previously found in several other C-terminal nisin mutants and did not affect the biological activity of nisin (*20*). The remainder of the NMR spectrum of His-tag-nisin Z was found to be very similar to the one of nisin Z. Mass spectrometry demonstrated that the mass of the mutant was 4286.64 ± 0.64 Da, which is expected for [Ser33]nisin Z-Asp-$(His)_6$ (theoretical value, 4287.14).

His-tag-nisin Z displayed 2- and 3-fold lower antimicrobial activity than nisin Z against the organisms *Bacillus cereus* and *Micrococcus flavus*, respectively (Table 1), indicating that the Asp-$(His)_6$ extension at the C-terminus does not severely affect the bacteriocidal activity of nisin. The activity of His-tag-nisin Z and wild-type nisin Z was further compared by measuring the capacity of both peptides to induce leakage of vesicle-enclosed carboxyfluorescein from vesicles composed of DOPC (a zwitterionic lipid) or DOPG (PG is an abundant anionic lipid in Gram-positive bacteria). Similar to wild-type nisin Z, His-tag-nisin Z efficiently permeabilized vesicles composed of DOPG and was inactive against DOPC vesicles (Table 1). Also the activity of both peptides toward bacterial membrane vesicles containing different amounts of anionic lipids was tested. For this purpose, right-side-out membrane vesicles of three different mutant *Escherichia coli* strains of known lipid composition were prepared (*8*), and the nisin-induced dissipation of an artificially applied membrane potential was monitored. Both peptides showed no activity toward vesicles derived from *E. coli* strain HDL11 containing only 8% anionic lipids and little activity against MRE600 containing 25% anionic lipids. The membrane potential of vesicles derived from the strains AD75 and AD93 containing 70% and 100% anionic lipids, respectively, was efficiently abolished. Apparently, the interaction of His-tag-nisin Z with bacterial and model membranes is very efficient, dependent on the anionic lipid content of the membrane, and in this respect similar to that of wild-type nisin Z.

Anionic Lipid and pH Dependence of the Membrane Interaction of His-tag-nisin Z. To obtain more detailed insight into the effect of the membrane lipid composition and the charge of the C-terminus on the membrane activity of His-tag-nisin Z, we investigated the activity of the peptide in model membranes composed of DOPC, DOPG, or an equimolar mixture of both lipids at pH 5.5, 6.5, and 7.5. The capacity of the peptide to insert into a lipid monolayer was determined by measuring the surface pressure increase induced by the peptide. Peptide-induced pressure changes are interpreted as penetration of the peptides into the lipid phase, whereas only binding of a peptide to the lipid polar headgroup will not affect the surface pressure of the monolayer (*24*). Insertion of His-tag-nisin Z was stimulated by the presence of DOPG (Figure 2), a phenomenon that is very similar to insertion of nisin Z (data not shown, refs *8* and *11*). Interestingly, insertion of His-tag-nisin Z was nearly independent of the pH, despite the expected charge differences in the peptide. The net charge of His-tag-nisin Z will

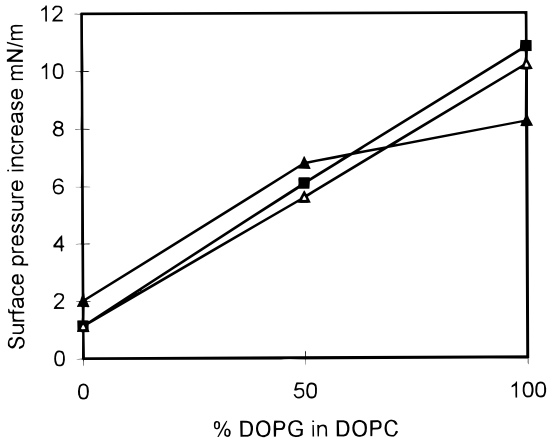

FIGURE 2: Monolayer insertion of His-tag-nisin Z. Monolayers were composed of DOPC, DOPG, or an equimolar mixture of both lipids.The pH of the buffer was as follows: (■) pH 5.5, (△) pH 6.5, and (▲) pH 7.5. The experiments were performed at room temperature at an initial monolayer pressure of 25 mN/m. The results were obtained using saturating amounts of His-tag-nisin Z (3 μg/mL).

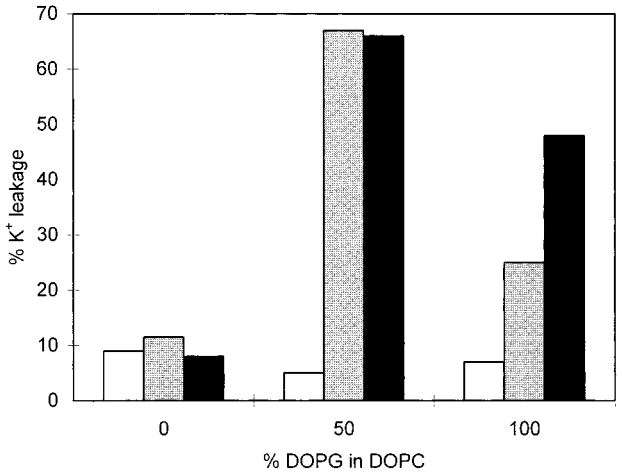

FIGURE 3: Lipid and pH dependence of His-tag-nisin Z-induced K^+ leakage; (□) pH 5.5, (gray box) pH 6.5, (■) pH 7.5. The amount of K^+ was determined 4 min after addition of His-tag-nisin Z. The average value of three individual experiments are shown. The error is in the order of 10%. The percentage leakage was expressed relative to the amount of released potassium after lysis of the vesicles by addition of the detergent *N*,*N*-dimethyldodecylamine-*N*-oxide.

be +8.4 at pH 5.5, +5.5 at pH 6.5, and +2.6 at pH 7.5, assuming a p*K* value of 6.5 for the histidine residues.

The anionic lipid and pH dependence of the capacity of His-tag-nisin Z to permeabilize a bilayer was assayed via K^+ efflux measurements using LUVs composed of DOPG and DOPC containing enclosed K^+ (Figure 3). The results show the importance of negatively charged lipids for His-tag-nisin Z to induce leakage. Optimal activity was observed when vesicles composed of an equimolar mixture of DOPC and DOPG were used. Interestingly, the capacity of His-tag-nisin Z to induce K^+ release was much lower at pH 5.5 than at pH 6.5 and pH 7.5. Most likely, this strong pH dependence of His-tag-nisin Z results from the charge differences brought about by protonation of the histidine residues. Apparently, a highly charged His-tag has little effect on insertion of the peptide into a lipid monolayer but significantly affects the capacity of the peptide to permeabilize a bilayer.

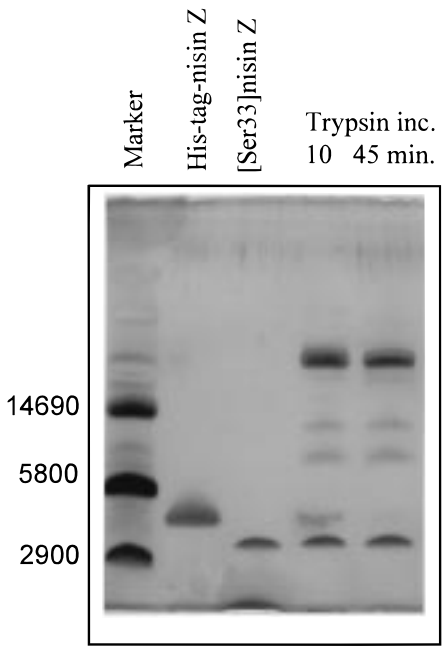

FIGURE 4: Tricine−SDS−polyacrylamide gel stained for protein, showing the cleavage of His-tag-nisin Z by trypsin. His-tag-nisin Z was incubated for 10 or 45 min of incubation with 1 mg/mL trypsin. The molecular mass of some of the marker proteins (in Da) is shown on the left. His-tag-nisin Z and [Ser33]nisin Z run with apparent molecular masses of 4.3 and 3.3 kDa, respectively. [Ser33]nisin Z was isolated as previously described (*20*).

Translocation of the C-Terminal Part across the Membrane. The His-tag extension allows for an assay to study possible translocation of the C-terminal part because trypsin is expected to cleave His-tag-nisin Z specifically behind Lys-34. This is illustrated in Figure 4, which shows a tricine−SDS−polyacrylamide gel stained for protein. The appearance of the lower band after incubation with trypsin demonstrates that His-tag-nisin Z was hydrolyzed. By mass spectrometry the cleavage product could be identified as [Ser33]nisin Z (determined mass, 3349.22 ± 0.38; theoretical value, 3349.08). Cleavage products with a molecular mass corresponding to nisin Z(1−12) or nisin Z(1−22) were not detected, which confirmed the results of previous studies (*25*, *26*), in which it was shown that the peptide bonds between the residues Lys12-Abu13 and Lys22-Abu23 are highly resistant to trypsin cleavage. As shown in Figure 4, 45 min incubation of His-tag-nisin Z with 1 mg/mL trypsin resulted in >95% conversion of the peptide into [Ser33]nisin Z.

To elucidate the location of the C-terminal part of nisin relative to the membrane phospholipids, trypsin was enclosed in DOPC/DOPG vesicles at a concentration of 5 mg/mL. The majority of nonenclosed trypsin was removed by washing steps. To block the activity of residual extravesicular trypsin, an excess of the trypsin inhibitor aprotinine was added. His-tag-nisin Z was added to the mixture (His-tag-nisin:phospholipid molar ratio of 1:50), and after 45 min of incubation the samples were analyzed by tricine−SDS−PAGE. After protein staining of the gel, the appearance of a band on the position of [Ser33]nisin Z could be detected, indicating that the C-terminal extension had been cleaved off (not shown). However, the results of this experiment could not be quantitatively analyzed because autodigestion products of trypsin overlap the band of His-tag-nisin Z on the gel. To obtain better interpretable results, His-tag-nisin

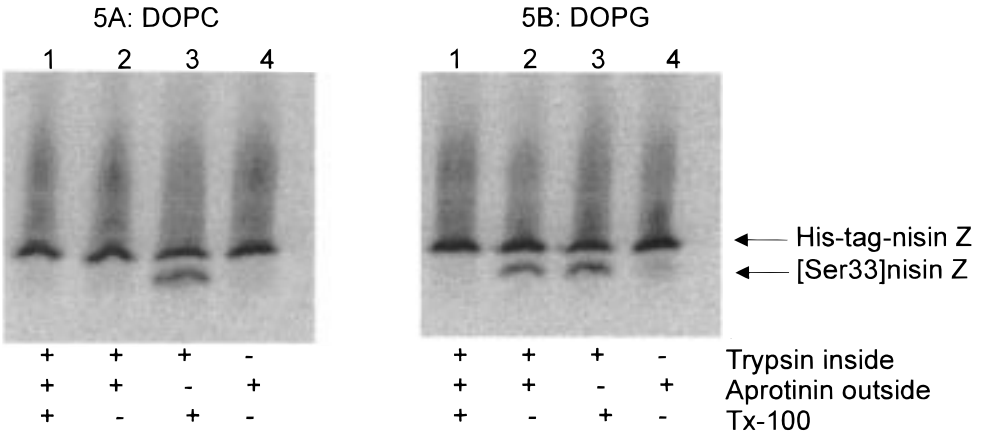

FIGURE 5: Translocation of His-tag-nisin Z across the membrane. ^{14}C-Labeled His-tag-nisin Z was added to vesicles composed of DOPC (A) or DOPG (B) in which 5 mg/mL trypsin was enclosed. Gels were analyzed by phosphorimaging, showing only radioactive molecules. Lanes 2: the results of the translocation experiment. Lanes 1, 3, and 4 are control experiments. Lanes 1: vesicles were lysed by Tx-100 under translocation conditions. Lanes 3: vesicles were lysed by Tx-100 but in the absence of aprotinin. Lanes 4: the experiment in which empty vesicles were prepared and trypsin was added to the outside. After the usual washing steps, the vesicles were preincubated with aprotinin and used in the translocation experiment.

Z was ^{14}C-labeled by reductive methylation, allowing analysis by phosphorimaging of the gels, thereby only visualizing radiolabeled molecules. Labeling of the molecule by reductive methylation decreased the susceptibility of the molecule toward trypsin. Nevertheless, 1 mg/mL of trypsin caused digestion of at least 80% of the labeled peptide in 45 min, which is sufficiently sensitive for detection of possible translocation.

Figure 5 shows the results of the translocation assay using ^{14}C-labeled His-tag-nisin Z and trypsin-enclosed vesicles composed of DOPC (Figure 5A) or DOPG (Figure 5B). The appearance of the lower band in lane 2 of Figure 5B clearly demonstrates that ^{14}C-labeled His-tag-nisin Z, like the unlabeled peptide, was cleaved after incubation with trypsin-enclosed vesicles composed of DOPG. Because trypsin cleavage can only occur when the C-terminal part of His-tag-nisin Z reaches the lumen of the vesicle, the results indicate that the C-terminal domain of these molecules had crossed the membrane, such that the Lys-34-Asp-35 peptide bond becomes accessible for the enclosed trypsin. When DOPC vesicles were used (Figure 5A, lane 2), His-tag-nisin Z remained nearly intact, demonstrating a strongly reduced ability of the C-terminus to translocate across the bilayer.

To test whether sufficient aprotinin was present to immediately block the total activity of trypsin present in the samples, the vesicles were lysed by the addition of a detergent (Figure 5, panels A and B, lanes 1). The absence of [Ser33]nisin Z in these samples demonstrates that the activity of trypsin is completely inhibited, from which it can be inferred that any residual activity of trypsin outside the vesicles is efficiently blocked by the inhibitor. When aprotinin is left out and the vesicles are lysed, the released trypsin digests a large part of His-tag-nisin Z (Figure 5, panels A and B, lanes 3), irrespective of the lipid type. The observed incomplete cleavage is most likely the result of the strong dilution of trypsin upon lysis of the vesicles (750-fold dilution, assuming an enclosed volume of 2 μL/μmol phosphate). In an additional control experiment, it was confirmed that aprotinin sufficiently blocked the activity of possible trypsin adhering to the surface of vesicles. For this purpose, 5 mg/mL trypsin was added to empty LUVs. The usual washing steps were performed, and the vesicles were preincubated with aprotinin. His-tag-nisin Z was added, and it was observed that it remained intact, demonstrating that any remaining external trypsin is completely inactivated (Figure 5, panels A and B, lanes 4).

The general barrier function of the vesicles under translocation conditions was studied by measuring the possible hydrolysis of a tripeptide chromogenic substrate by trypsin enclosed in DOPC/DOPG (ratio 1:1) vesicles (see Experimental Section). In the absence of His-tag-nisin Z almost no hydrolysis of the peptide was observed (data not shown), demonstrating that the peptide cannot cross the bilayer. Also upon addition of His-tag-nisin Z to the mixture very little hydrolysis of the tripeptide analogue was observed, demonstrating that the vesicles remain impermeable for the small substrate. Lysis of the vesicles by the detergent TritonX-100 in the absence of the inhibitor confirmed that the substrate was rapidly digested by trypsin (data not shown).

The combined results show that the conversion of His-tag-nisin Z in the translocation assay can be fully assigned to the activity of the trypsin present in the vesicle lumen and that it reflects the appearance of the C-terminus of nisin in the vesicle lumen.

Membrane Permeabilization by His-tag-nisin Z Is Correlated to Translocation. To obtain more insight into the mechanism of translocation and its relation to the pore-forming activity of His-tag-nisin Z, we systematically changed the phospholipid composition of the vesicles and the pH of the buffer in the translocation experiment. Trypsin-loaded vesicles composed of DOPC and DOPG were prepared in buffer of pH 7.0, while the pH of the outside buffer was 5.5, 6.5, or 7.5.

In Figure 6, the cleavage of His-tag-nisin Z by vesicle-enclosed trypsin under various conditions is shown. The percentage of cleaved peptides was calculated from the intensity of the [Ser33]nisin Z protein band relative to the total amount of radioactive peptide. The control experiments, in which Tx-100 was added, confirmed that trypsin was exclusively active inside the vesicles in all conditions. Furthermore, it was established that after release of enclosed trypsin by addition of Tx-100, His-tag-nisin Z was digested to the same extent, irrespective of the pH of the reaction

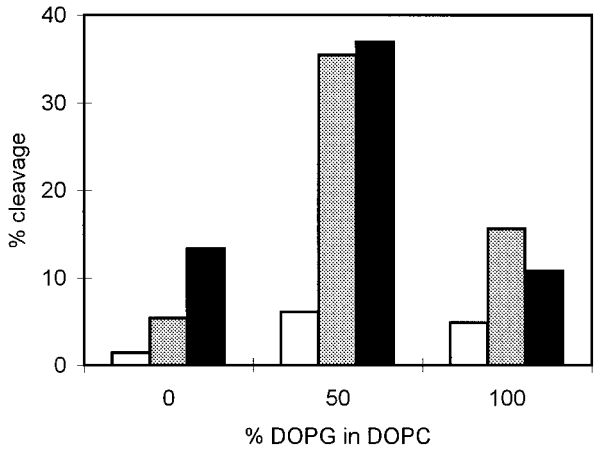

FIGURE 6: Lipid and pH dependence of His-tag-nisin Z translocation.The lipid composition of the trypsin-containing vesicles and the pH of the outer buffer was varied in translocation experiments; (□) pH 5.5, (gray box) pH 6.5, (■) pH 7.5. The results shown are the average of three individual experiments. The error was in the range of 15%. The percentage of His-tag-nisin Z that was converted to [Ser33]nisin Z by enclosed trypsin was determined by phosphorimaging analyses.

mixture or the phospholipid composition (not shown). Therefore, the extent of cleavage of His-tag-nisin Z under these different conditions by vesicle-enclosed trypsin can be directly compared. His-tag-nisin Z was cleaved most efficiently when vesicles composed of an equimolar mixture of DOPC and DOPG were used. With all tested lipid compositions, the percentage of proteolyzed peptides was lowest at pH 5.5. This indicates that at low pH, when the C-terminal histidine residues are positively charged, translocation across the bilayer is hampered.

A comparison of the lipid and pH dependence observed in the translocation experiments (Figure 6) and the K^+ release assays (Figure 3) indicates that translocation of the C-terminus is correlated to the pore-forming activity. Slight differences in the results might be explained by the different setup of the experiments. In the leakage experiments, the nisin-induced potassium leakage was measured directly by using a potassium electrode. In the translocation experiments, the percentage of translocated peptides was measured indirectly by calculating the peptides that were cleaved by enclosed trypsin. The final result (percentage cleavage) depends on the efficiency and kinetics of cleavage by trypsin. It was confirmed that the presence of trypsin in the vesicles did not interfere with the permeabilizing activity of the peptide by measuring the His-tag-nisin Z-induced K^+ release from vesicles containing trypsin as well as K^+ ions in the lumen (not shown).

It has previously been shown that translocation of the His_6-tagged protein pro-OmpA across inverted membrane vesicles of *E. coli* (*27*) and import of the modified chloroplast precursor protein $pSS(His)_6$ into *Chlamydomonas* chloroplasts (*28*) could be inhibited by the addition of nickel ions, which bind tightly to the imidazol groups of histidine residues. Therefore, it was tested how the presence of $NiCl_2$ affected membrane insertion, permeabilization, and translocation of His-tag-nisin Z. Prior to the addition of His-tag-nisin Z in the different experiments, 1 nmol of peptide was preincubated for 15 min in 100 mM Tris-HCl (pH 7.5) in the presence of 1 mM $NiCl_2$. It was established that preincubation of wild-type nisin Z with nickel ions did not affect the activity of the wild-type peptide. The effect of nickel on insertion of His-tag-nisin Z into equimolar DOPC/DOPG lipid monolayers was very low (not shown). In contrast, the capacity of His-tag-nisin Z to permeabilize K^+-enclosed vesicles (50% DOPC/DOPG) was 4-fold reduced by the presence of nickel (Figure 7A). In accordance, membrane translocation of His-tag-nisin Z was also significantly reduced in the presence of $NiCl_2$ (Figure 7B). It was confirmed by the various control experiments that the activity of trypsin and aprotinin in the translocation experiment were not affected by the presence of $NiCl_2$ (data not shown). These results demonstrate that binding of nickel ions to the His-tag inhibits both pore formation and translocation of the C-terminus of the peptide across the membrane.

DISCUSSION

In this study, the membrane interaction of nisin was analyzed using a variant containing the C-terminal extension $Asp\text{-}(His)_6$. His-tag-nisin Z showed antimicrobial and anionic lipid-dependent membrane activity similar to nisin Z and therefore is considered to be a reliable model for the wild-type peptide. With His-tag-nisin Z, new information on the importance of the C-terminus of nisin for its membrane activity was obtained. Furthermore, it could be demonstrated for the first time that translocation of the C-terminal part of the peptide occurs and that this process is related to the membrane permeabilizing effect of the peptide.

Membrane Insertion. Membrane insertion of nisin appears to be an essential step in the membrane-permeabilizing effect of the peptide. The monolayer data reflect the penetration of nisin into the outer leaflet of the membrane, while binding to the lipids will not affect the surface pressure of the monolayer (*24*). The monolayer experiments directly demonstrate that, like wild-type nisin (*8*, *11*), His-tag-nisin Z inserts efficiently into the lipid layer in an anionic lipid-dependent way. Strikingly, increasing the charge density at the C-terminal His_6 sequence by lowering the pH hardly had an effect on the insertion efficiency. Also complexing the His-tag with Ni^{2+} barely affected insertion into the lipid monolayer. In contrast, it has been reported that modifications at the N-terminus of nisin, including an N-terminal extension of IPTQ (*25*), significantly affected the insertion capacity, correlating with antimicrobial properties of these mutants (*11*). Therefore, it is concluded that the surface pressure increases in the monolayer experiments are largely due to insertion of the N-terminal part of the peptide, as was previously hypothesized by Breukink et al. (*8*).

Membrane Permeabilization. Both the percentage of negatively charged lipids in the membrane and the amount of C-terminal positive charges on His-tag-nisin Z were found to be key determinants in the capacity of the peptide to permeabilize a membrane. The results suggest that a certain amount of anionic lipids and C-terminal positive charges on the peptide is required for optimal activity. In the absence of negatively charged lipids a very low K^+ leakage is induced. In the presence of anionic lipids leak is strongly increased, but now an excess of positive charges at the C-terminus, as induced by lowering the pH to 5.5, is harmful. From the p*K* value of DOPG (29), it can be concluded that

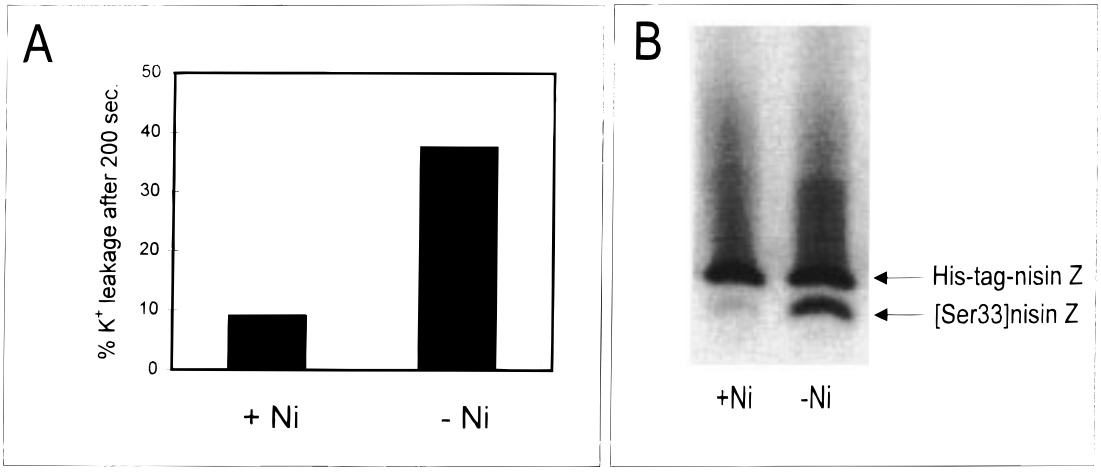

FIGURE 7: Effect of nickel on His-tag-nisin Z-induced K^+ leakage and translocation. His-tag-nisin Z was preincubated in 100 mM Tris-HCl, pH 7.5, with or without 1 mM $NiCl_2$ for 15 min on ice and subsequently used in the experiments. (A) Preincubated His-tag-nisin Z was added to K^+-loaded vesicles (50% DOPG/DOPC). The percentage of released K^+ was determined as described in the legend of Figure 3. (B) Preincubated ^{14}C-labeled His-tag-nisin Z was added to trypsin-loaded vesicles (50% DOPG/DOPC).

the charge of the lipid is only very slightly affected at this pH. The lowered membrane permeabilizing activity of His-tag-nisin Z upon increasing the anionic lipid content to 100% in both model and bacterial membrane vesicles might reflect a decreased surface pH that facilitates protonation of the His-tag and thereby inhibits the membrane permeabilizing activity of the peptide.

The importance of positive charges at the C-terminus for membrane activity is not restricted to His-tag-nisin Z. It was shown previously that C-terminal positive charges in nisin Z are important for membrane binding and the bilayer permeabilizing activity of the peptide (*8*). Therefore, the general picture that now emerges is that a certain electrostatic anchoring of the C-terminus to the membrane surface is essential for membrane-permeabilizing activity. However, too strong electrostatic interactions between the negatively charged phospholipids and the positive charges in the C-terminus of nisin are harmful because they trap the C-terminus at the interface allowing N-terminal insertion to occur but without giving rise to membrane leak. This leads to the suggestion that for membrane permeabilization by nisin the C-terminus has to move across the membrane.

Translocation of the C-Terminus of His-tag-nisin Z. The ability of the C-terminal part of His-tag-nisin Z to cross the membrane was tested using an experimental approach originally developed to study spontaneous translocation of the mitochondrial precursor protein apocytochrome *c* across model membranes (*30*). In this approach, trypsin is enclosed in lipid vesicles and translocation is assayed by monitoring proteolytic cleavage of the protein or peptide added to the outside of these vesicles. The results unambiguously showed that His-tag-nisin Z was cleaved by the enclosed trypsin, such that the C-terminal part actually crossed the phospholipid bilayer, thereby exposing the Lys34-Asp35 peptide bond to trypsin in the lumen of the vesicles. Since we have shown that His-tag-nisin Z is a good representative for the wild-type peptide, we consider that the translocation of the carboxyl-terminus is a general aspect of the interaction of nisin with a lipid bilayer.

Several experiments demonstrated a coupling between the ability of His-tag-nisin to permeabilize the bilayer and translocation of the C-terminal part. First, it was shown that both the anionic lipid content of the membrane and the charge of the C-terminus of His-tag-nisin Z affected the translocation process and the permeabilizing activity of the peptide in a very similar way. Second, the presence of nickel ions inhibited the translocation process as well as the permeabilizing activity. Most likely, this is caused by folding of the His_6 around the Ni^{2+} thereby sterically hindering translocation of the C-terminus across the membrane. An alternative explanation could be that an increased charge upon Ni^{2+} binding contributes to the inhibition of translocation. It should be noted that under similar conditions Ni^{2+} does not affect the membrane activity of wild-type nisin Z, excluding the possibility that Ni^{2+} interferes with the nisin−membrane interaction.

Given the kinetics of ^{14}C-labeled His-tag-nisin Z cleavage by trypsin and the kinetics of permeabilizing the membrane, it is most likely that cleavage by trypsin is the rate-limiting factor in the translocation experiment. Therefore, it is not possible to obtain information on the kinetics of the translocation process.

Mechanistic Implications. The conclusion that pore formation by nisin involves translocation of the C-terminal part across the membrane is of importance for understanding the mode of action of nisin. It can be excluded that the membrane-permeabilizing effect of nisin is induced by peptides acting solely at the *cis* site of the membrane−water interface, thereby disturbing the organization of the lipid layer, as has been proposed by some authors for the mode of action of melittin (*31*). In contrast, our results show that the C-terminal part of nisin actually reaches the inner leaflet of the membrane. After electrostatic binding of nisin molecules to the outer leaflet of the bilayer and insertion of the N-terminus into that layer, the peptides might flip into a membrane-spanning orientation, with the C-terminus of at least part of the peptides in the lumen of the vesicle (Figure 8).

Recently, it was demonstrated by fluorescence spectroscopy studies using nisin mutants with Trp residues on positions 1, 17, and 32 that nisin molecules adopt an overall parallel orientation with respect to the membrane surface (*32*). Therefore, it is assumed that the membrane-spanning orientation of the peptides is only transient, such that the

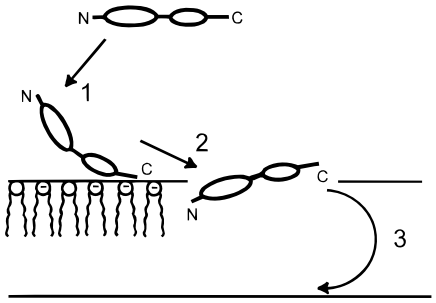

FIGURE 8: Model of the first steps of the interaction of nisin with a lipid bilayer. 1: Binding of nisin to the membrane. Electrostatic interactions between the positive residues of the peptide and acidic lipids play an important role. 2: Insertion of nisin into the membrane. The N-terminal part of nisin penetrates into the outer leaflet of the membrane. 3: Translocation of the C-terminal part of nisin across the membrane.

peptides might rapidly flip back to the original parallel orientation in the outer leaflet. An alternative mechanism worth considering is that peptides entirely translocate to the inner leaflet of the bilayer, as has been proposed for the mechanism of action of the α-helical peptides magainin 2 (*33*), mastoparan X (*34*), and melittin (*35*). Similar to the mode of action of these peptides, nisin molecules might entirely translocate across the membrane via formation of short-lived, transmembrane pores and take an orientation parallel to the plane of the membrane in both leaflets of the bilayer. Possibly, membrane translocation of amphiphilic peptides is a general mechanism by which these molecules can exert their membrane-permeabilizing activity.

ACKNOWLEDGMENT

We thank H. S. Rollema for recording ^{1}H NMR spectra and C. J. Slangen for mass spectrometrical analyses of His-tag-nisin Z.

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BI980931B