Assembly and Stability of Nisin−Lipid II Pores[†]

Hester Emilie Hasper,* Ben de Kruijff, and Eefjan Breukink

Center of Biomembranes and Lipid Enzymology, Department of Biochemistry of Membranes, Institute for Biomembranes, Utrecht University, Padualaan 8 3584 CH Utrecht, The Netherlands

Received March 16, 2004; Revised Manuscript Received June 17, 2004

ABSTRACT: The peptide antibiotic nisin was the first reported example of an antibiotic that kills bacteria via targeted pore formation. The specific target of nisin is Lipid II, an essential intermediate in the bacterial cell-wall synthesis. High-affinity binding of the antibiotic to Lipid II is followed by rapid permeabilization of the membrane. Here, we investigated the assembly and stability of nisin−Lipid II pore complexes by means of pyrene fluorescence and circular dichroism. We demonstrated that nisin uses all available Lipid II molecules in the membrane to form pore complexes. The pore complexes have a uniform structure and consist of 8 nisin and 4 Lipid II molecules. Moreover, the pores displayed a remarkable stability, because they were able to resist the solubilization of the membrane environment by mild detergents. Similar experiments with [N20P/M21P]nisin showed that the hinge region is essential for the assembly into stable pore complexes. The new insights were used to propose a refined model for nisin pore formation.

Nisin is an antimicrobial peptide produced by certain strains of *Lactococus lactis*, which very effectively kills a broad range of Gram-positive bacteria (*1*−*4*). Because of its stability in different environments and low toxicity toward humans, nisin has been used for several decades as a food preservative (*5*, *6*).

The peptide has an overall positive charge and contains 34 amino acids of which 13 are involved in post-translational modification reactions. As a consequence, five (*β*-methyl)-lanthionine rings are formed, which give nisin its unique structure (Figure 1A).

Nisin is the first known pore forming peptide that uses a specific target for its antimicrobial activity, namely, Lipid II. This lipid is present in all eubacterial membranes (*7*, *8*), where it is involved in transporting peptidoglycan subunits (*9*) (Figure 1B). When nisin is bound to Lipid II, nisin efficiently permeabilizes the membrane via the formation of pores, which causes a collapse of the vital ion gradients across the membrane. Additionally, the binding to Lipid II as such will interfere with cell-wall synthesis (*10*).

Site-directed mutagenesis studies as well as high-resolution nuclear magnetic resonance (NMR)[1] spectroscopy showed that the N terminus of nisin is involved in the interaction with Lipid II (*10, 11*). The binding involves the residues in rings A−C of nisin and the *N*-acetylmuramic acid (MurNAc)-pentapeptide moiety as well as the pyrophosphate of Lipid II (*11*) (Bonev, B. B., personal communication). Besides the binding of nisin, Lipid II was also found to enable nisin to insert into bilayers in a perpendicular (*12*) instead of parallel orientation with respect to the membrane surface (*13*). For the membrane permeabilizing activity of nisin especially, the flexible hinge region between rings C and D (Figure 1) was found to be important. This was concluded from site-directed mutagenesis studies, which revealed that mutations in the hinge region led to a 3−8-fold reduction in the antimicrobial activity but a far larger inhibition of the ability of nisin to permeabilize the membrane. Hence, the formation of pores is not essential for nisin to act as an antibiotic, but it allows the peptide to kill bacteria in a more efficient and direct way (*10*).

Recently, we developed a new semisynthetic route for the production of Lipid II, which also allows the synthesis of a pyrene-labeled analogue (Figure 1B). When the characteristic pyrene excimer fluorescence was explored, Lipid II was shown to be an integral part of the nisin pore (*14*). This technology opened up the possibility to study the stoichiometry, size, and stability of the nisin−Lipid II pore complex, which is reported here. Additionally, circular dichroism (CD) was used to characterize the effect on the nisin conformation of the pore-complex formation with Lipid II. Nisin was found to form pores that have a uniform and highly stable structure and that contain 4 Lipid II and 8 nisin molecules. In contrast, the hinge-region mutant [N20P/M21P]nisin does not assemble Lipid II in pore complexes, demonstrating that a flexible hinge region is essential for pore assembly.

EXPERIMENTAL PROCEDURES

Materials. Nisin A and [N20P/M21P]nisin A were produced by batch fermentation, isolated, and purified as described (*15*). Stock solutions of the peptides varied between 0.25 and 0.80 mM in 0.05% acetic acid and were stored at

[†] This work was funded by NWO-STW Grant 349-5257.

* To whom correspondence should be addressed: Department of Biochemistry of Membranes, Institute for Biomembranes, Hugo R. Kruytbuilding, Utrecht University, Room N.603, Padualaan 8 3584 CH Utrecht, The Netherlands. Telephone: +31-30-2533553. Fax: +31-30-2533969. E-mail: h.e.hasper@chem.uu.nl.

[1] Abbreviations: AMP, antimicrobial peptide; CD, circular dichroism; CF, carboxyfluorescein; Dha, dehydroalanine; Dhb, dehydrobutyrine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; GlcNAc, *N*-acetylglucosamine; LUV, large unilamellar vesicle; MES, 2-morpholinoethanesulfonic acid; MIC, minimal inhibitory concentration; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; MurNAc, *N*-acetylmuramic acid; NMR, nuclear magnetic resonance; OG, *n*-octyl-*β*-D-glucopyranoside; OPOE, *n*-octyl-polyoxyethylene; SDS, sodium dodecyl sulfate; SUV, small unilamellar vesicle.

10.1021/bi049476b CCC: $27.50
Published on Web 08/18/2004

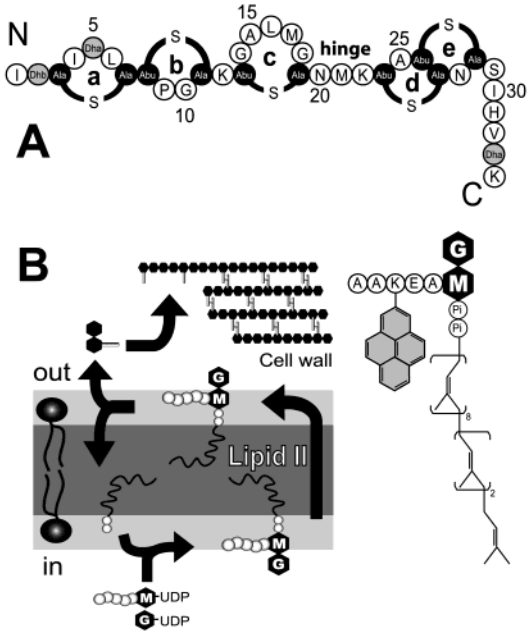

FIGURE 1: Structure of nisin and Lipid II. (A) Schematic representation of the amino acid structure of the lantbiotic nisin. The residues that are shown in gray are serines and threonines, which have become dehydrated during post-translational modification to dehydroalanine (Dha) and dehydrobutyrine (Dhb), respectively. Ala-S-Ala and Abu-S-Ala are lanthionine and β-methyllanthionine bonds that are formed because of enzymatic coupling of Dha or Dhb to cystein residues. The residues that participate in the formation of the five (methyl)lanthionine rings (a−e) are shown in black. (B) Essential role of Lipid II as an intermediate in the peptidoglycan synthesis is shown in the cartoon on the left side. Lipid II is assembled at the inner leaflet of the bacterial membrane by sequential addition of the UDP-activated sugars MurNAc and GlcNAc (black hexagons M and G) to the bactoprenol phosphate molecule. To the first sugar, a linear peptide of five amino acids is covalently bound (white circles). The sugar−peptide product is transported over the membrane to the outside of the bacterium where the headgroup is used to further elongate the peptidoglycan polymer, the main constituent of the cell wall. The remaining bactoprenol pyrophosphate flips back to the cytosolic leaflet of the membrane and is dephosphorylated for the next synthesis cycle. On the right, the structure of Lipid II with the pyrene label is shown. The fluorophore (shown in gray) is attached to the ϵ-NH2 group of the lysine residue of the pentapeptide.

−20 °C until further use. Lipid II with and without the pyrene label was synthesized and purified as published recently (*14*). Labeled and unlabeled Lipid II were dissolved in chloroform/methanol (1:1, by volume) at a concentration of 0.27 mM and 1.0 mM, respectively, and both were stored under nitrogen at −20 °C.

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was purchased from Avanti polar lipids, and carboxyfluorescein (CF) and pyrene-sulfonyl-chloride were obtained from Molecular Probes. G-50 Sephadex Fine was purchased from Amersham Pharmacia Biotech AB, and the detergents were obtained from different manufacturers, namely, *n*-octyl-β-D-glucopyranoside (OG) from ANATRACE, sodium dodecyl sulfate (SDS) from Brunschwig Chemie, Tween-20 from BioRad Laboratories, and *n*-octyl-polyoxyethylene (OPOE) from Bachem. All other chemicals were of analytical grade or better.

General Procedures. The concentrations of Lipid II and phospholipids stock solutions in chloroform/methanol (1:1, by volume) and vesicle preparations were determined by the inorganic phosphate-based procedure described by Rouser (*16*). The nisin concentration was determined using a bicinchoninic acid protein assay (Pierce Chemical Corp.), with bovine serum albumin as a standard (*17*). All experiments were performed using a 50 mM 2-morpholinoethanesulfonic acid (MES) buffer of pH 6 with 100 mM Na_2SO_4, unless stated otherwise.

Membranes: Large Unilamellar Vesicles (LUVs). The experiments with fluorescence spectroscopy were performed with LUVs of DOPC containing a varying Lipid II content. The LUVs were prepared from mixed lipid films, unless indicated otherwise. Dried lipid films were obtained by evaporating the organic solvents under a continuous flow of nitrogen gas, followed by storage for 2 h under vacuum to remove traces of chloroform and methanol. The lipid films were hydrated, followed by 10 cycles of freezing and thawing of the suspensions and 10 times manual extrusion through disposable 200-nm membrane filters (Anotop 10, Whatman, Maidstone, U.K.) (*18*).

To incorporate pyrene-labeled Lipid II asymmetrically, a small aliquot of an ethanol solution of pyrene-labeled Lipid II at submillimolar concentrations was rapidly mixed with at least 12 times the volume of a millimolar DOPC LUV preparation. After incubation for 2 h at room temperature, the vesicles were separated from the nonincorporated pyrene-labeled Lipid II using a G-50 Sephadex Fine spin column. The percentage of pyrene-labeled Lipid II in the eluted LUVs was determined by comparing the fluorescence intensity at 380 nm to DOPC LUVs containing 0.5 mol % pyrene-labeled Lipid II.

Small Unilamellar Vesicles (SUVs). CD measurements were performed with SUVs. A Branson 250-tip sonifier was used to obtain SUVs from DOPC LUVs containing 4 mol % unlabeled Lipid II in 10 mM sodium phosphate buffer at pH 7 with 40 mM Na_2SO_4. During the process of sonication, the lipid suspension was kept on ice and under a continuous nitrogen flow. After the vesicle suspension became clear, residual metal particles were removed by centrifugation in an Eppendorf Tabletop centrifuge at 14 000 rpm for 20 min at 4 °C.

All LUV and SUV suspensions were stored under nitrogen at 4 °C and used within 48 h.

Fluorescence Spectroscopy. The fluorescence of pyrene-labeled Lipid II was used to measure the assembly and stability of nisin−Lipid II pores, as essentially described in ref *14*. Emission spectra as well as single wavelength recordings of excimer and monomer fluorescence were used to determine the properties of the pore complex in a quantitative way. All fluorescence measurements were performed on an SLM-Aminco SPF-500 C fluorimeter. Samples (1.2 mL) were measured in a 10 × 4-mm quartz cuvette, which was stirred continuously and kept at 20 °C using a water bath with continuous circulation.

Emission spectra were recorded between 360 and 550 nm. The emission at 380 and 495 nm was recorded and averaged over 50 s, to obtain the values for the monomer and excimer intensity, respectively, for quantitative analysis purposes. In both the measurements of the spectra and the single wavelength recordings, the excitation wavelength was 350 nm and the bandwidth for emission and excitation was set to 5 nm.

When appropriate, fluorescence was corrected for inner-filter effect contributions and background scattering of fluorescence. The inner-filter effect contribution was calculated from the absorbance values between 345 and 550 nm of each individual sample and affected the signal to a maximum of 5% (*19*). Blank scattering, which was determined by recording signals arising from nonfluorescent samples of identical composition, contributed to less than 10% of the recorded signal.

Nisin−Lipid II Titration Experiment. The concentration dependency of the nisin−Lipid II pore assembly was measured in DOPC LUVs with 0.1, 0.5, and 1.0 mol % pyrene-labeled Lipid II, and vesicles to which pyrene labeled Lipid II were externally added. Titration experiments were carried out in samples (1.2 mL) containing 0.25 μM pyrene-labeled Lipid II to which varying aliquots of a nisin stock solution were added. For each data point, a fresh sample was taken.

The same method was used to perform experiments with [N20P/M21P]nisin, with the modification that DOPC vesicles were used, that contained 0.17, 0.44, and 1.0 mol % pyrene-labeled Lipid II. The final concentrations of Lipid II varied between 0.15 and 0.20 μM.

Number of Lipid II Molecules Pro Pore. Nisin was added to DOPC LUVs containing 1.0 mol % Lipid II with a varying fraction of pyrene-labeled Lipid II, ranging from 0.03 to 1. The total Lipid II concentration within each experiment was 0.50 μM. A total of 5 min after the addition of nisin (1.51 μM), the monomer (M) and excimer (E) intensity were recorded to calculate the excimer over monomer ratio (E/M).

Stability of the Nisin−Lipid II Complex. Two experimental approaches were used to get insight in the stability of the nisin−Lipid II pore complex. First, the possible dissociation of the pore complexes was tested by addition of an excess of unlabeled Lipid-II-containing membranes. Pyrene fluorescence spectra of the samples containing nisin (0.50 μM) and DOPC membranes with 0.5 mol % pyrene-labeled Lipid II (0.25 μM) were recorded 5 min after incubation. Second, DOPC LUVs containing 0.5 mol % unlabeled Lipid II were added, resulting in a total Lipid II concentration of 4.5 μM. The fluorescence spectrum of the incubation mixture was recorded after an incubation period of 1 h at 20 °C.

Second, we investigated the stability of nisin−Lipid II complexes upon detergent-induced solubilization of the membrane. First, nisin−Lipid II pores were assembled by adding nisin up to 0.75 μM to samples containing 50 μM (total Lipid−P_i) DOPC LUVs with 0.5 mol % pyrene-labeled Lipid II. Subsequently, the membranes were dissolved by adding various detergents. The concentrations, which were required to solubilize the membranes, were determined as described in ref *20* with the exception that DOPC LUVs of 200 nm were used and the turbidity was measured at 375 nm. Pyrene fluorescence spectra were recorded after 1 h and overnight incubation at 20 °C.

The stability of the assembled complexes with [N20P/M21P]nisin toward detergent solubilization was tested according to the above protocol, with slightly different concentrations of pyrene-labeled Lipid II (0.17 μM, 0.44 mol % in DOPC vesicles) and nisin (0.38 μM).

CD Measurements. CD spectra were measured on a Jasco-810 spectropolarimeter in a quartz cuvette with a 2-mm light path, which was kept at 20 °C by a Jasco Peltier CDF 426S. The ellipticity was recorded between 190 and 260 nm at a 0.2-nm step size with 2-s response time. The spectra were averaged over 5 recordings. Samples contained 20 μM nisin, and for the preparation and dilution, 10 mM sodium phosphate buffer at pH 7 and 40 mM Na_2SO_4 were used.

The same buffer containing 40 mM SDS was used to hydrate a dried lipid film of pure Lipid II to prepare Lipid-II-containing SDS micelles. The DOPC SUVs containing 4 mol % Lipid II and the Lipid-II-containing SDS micelles were added to a final Lipid II concentration of 20 μM. The effect of Lipid II on the nisin structure was evaluated by comparison of the data with reference spectra obtained in buffer and in the presence of SDS micelles and DOPC SUVs without Lipid II. Blank spectra were subtracted from the nisin spectra, and the CD signal at 340 nm was taken as a reference to correct for baseline shifts originating from differential scattering (*21*).

RESULTS

Pyrene fluorescence was found to be an attractive method to investigate the distance between components in several biological systems (*22*−*25*), because close proximity of two pyrene fluorophores will result in clearly detectable excimer formation (*26*). In addition, labeling Lipid II with pyrene hardly affected nisin in its ability to form pores (*14*). These properties make pyrene Lipid II an ideal probe to get more insight in the assembly of the nisin−Lipid II complex and its stability in different conditions. In addition, CD was used to follow the changes in secondary structure of nisin upon formation of a complex with Lipid II as well as to probe the stability of this conformation.

Effect of Lipid II Location and Concentration on the Assembly of Nisin Pores. When pyrene-labeled Lipid II is present at a low concentration in DOPC membranes (0.5 mol %), the Lipid II molecules are too far apart to allow the formation of pyrene excimers (*14*). As a consequence, the fluorescence spectrum contains only three maxima, namely, at 380, 399, and 419 nm (thin black tracing in Figure 2A). A 3-fold molar excess of nisin was added, and the overall intensity of the monomer spectrum drastically decreased. Concomitantly, a broad fluorescence band appeared between 440 and 550 nm, which is the characteristic excimer fluorescence (thick black tracing). Both phenomena are indicative for complex formation and suggested that multiple Lipid II molecules were assembled into a nisin pore of which Lipid II is a structural part (*14*).

It should be noted that fluorescence decreased more than 50% by addition of an excess of nisin (Figure 2A). The vesicles used within this experiment are expected to have their Lipid II evenly distributed over the two bilayer leaflets, as found with dithionite-quenching experiments (*27*) with NBD-labeled Lipid-II-containing vesicles (Breukink, E., unpublished results). This implies that also the pyrene-labeled Lipid II molecules at the inner leaflet participate in the formation of nisin−Lipid II complexes, for which either Lipid II has to flip across the membrane to appear on the cis side or nisin traverses the membrane to reach the trans side. Spontaneous Lipid II flip-flop does not occur (unpublished observations), but the reorganization in the membrane, which accompanies pore formation, might stimulate it,

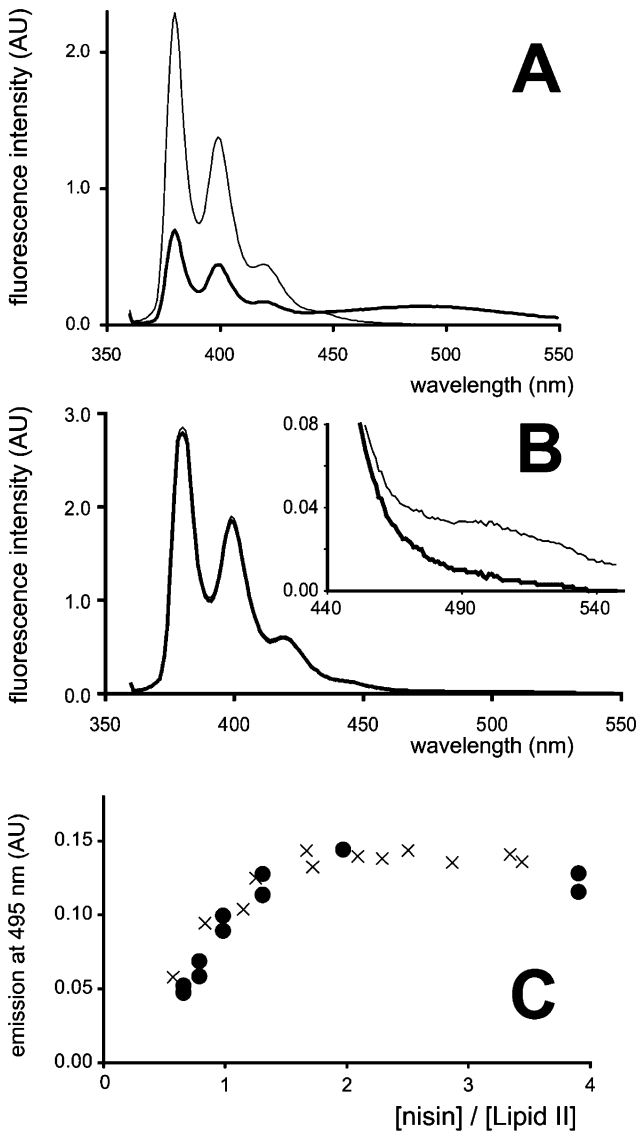

FIGURE 2: Nisin uses all Lipid II in the membrane to form pores. (A) Fluorescence spectrum of 0.5 mol % pyrene Lipid II in DOPC LUVs in the absence (thin black tracing) and in the presence (thick black tracing) of nisin. The nisin and Lipid II concentrations were 0.75 and 0.25 μM, respectively, and the total lipid concentration was 50 μM. (B) Fluorescence spectrum and enlarged view of the excimer part (inset) of pyrene-labeled Lipid II that was externally added to DOPC LUVs. The thin black tracing is the spectrum of the incubation mixture before gel-filtration chromatography, and the thick black tracing represents the spectrum of the eluate containing the vesicles. To record the spectra, LUVs were added to obtain a concentration of 50 μM total lipid$-P_i$. (C) Effect of increasing nisin concentrations on the fluorescence intensity at 495 nm, for DOPC LUVs containing 0.45 mol % pyrene Lipid II located exclusively at the outer leaflet of the membrane (●) and DOPC LUVs, which contain symmetrically distributed pyrene Lipid II at 0.5 mol % (×). For a quantitative comparison of the excimer intensities, identical pyrene-labeled Lipid II concentrations were used (0.25 μM).

similar to the effect of transmembrane peptides on phospholipid flip-flop (*23*). The possibility that nisin traverses the membrane is supported by earlier observations that the peptide is able to translocate over membranes that contain a substantial amount of negatively charged lipids (*26*). Although it is not known whether this also happens within the Lipid-II-containing membranes that were used in our experiments, it is conceivable that it might occur.

To investigate more directly the role of Lipid II on the trans side in the formation of nisin pores, we studied the formation of nisin−Lipid II complexes with vesicles containing the pyrene-labeled cell-wall precursor exclusively on the outside of the membrane. The vesicles with the asymmetric Lipid II content were prepared by incorporation of pyrene-labeled Lipid II into DOPC vesicles in a similar way as described previously for other lipids (*28*). Figure 2B shows that a small excimer band was found after the addition of the pyrene-labeled Lipid-II-containing ethanol solution (thin black tracing), which disappeared after a gel-filtration chromatography step (thick black tracing). Because the amount of Lipid II added is too small to give rise to an excimer signal upon complete incorporation, this suggested that a small part of the Lipid II added from the ethanol solution self-organizes in structures where excimer formation is very well possible, such as Lipid II micelles. Because the excimer band was found to disappear after the gel-filtration step, we concluded that the final preparation contained only vesicles with pyrene-labeled Lipid II inserted in the lipid bilayer (inset of Figure 2B). Approximately half of the pyrene-labeled Lipid II was found to incorporate in the DOPC bilayer. The pyrene-labeled Lipid II molecules are likely to remain only in the outer leaflet after they have become incorpated, because dithionite-quenching experiments with similarly prepared NBD Lipid-II-containing vesicles indicated that Lipid II does not spontaneously flip across a model membrane (Breukink, E., unpublished results).

When different amounts of nisin were added to these asymmetric 0.45 mol % containing pyrene-labeled Lipid-II-containing vesicles, the excimer fluorescence linearly increased up to a nisin−Lipid II ratio of approximately 2:1 (●, Figure 2C). Thereafter, the excimer intensity did not increase further. Interestingly, when the titration was performed with symmetric vesicles (×) at similar Lipid II and nisin concentrations, the increase of the excimer fluorescence and the final saturation level were comparable with that of the asymmetric vesicles (●). Apparently, the addition of nisin to both symmetric and asymmetric Lipid-II-containing vesicles lead in both cases to the formation of excimers, showing that the formation of nisin−Lipid II complexes does not require Lipid II on the trans side of the bilayer and that irrespective of their initial location all Lipid II molecules are involved in the formation of complexes with a uniform structure.

To test whether pore assembly is related to the membrane concentration of Lipid II molecules, nisin was titrated to DOPC LUVs with varying Lipid II content. In this titration experiment, DOPC LUVs with 0.1, 0.5, or 1.0 mol % pyrene Lipid II were used and the excimer intensities were plotted against the nisin−Lipid II ratio (Figure 3A). The excimer intensity increased to a similar extent for all vesicle types (◆, ○, and ▲) until a saturation level, corresponding to a nisin−Lipid II ratio of approximately 2:1, and remained unchanged thereafter. The resemblance between the three titration curves suggest that a uniform complex is formed independent of the surface concentration of Lipid II.

The ability of nisin to form pores containing pyrene-labeled as well as unlabeled (wild-type) Lipid II molecules was used to determine the number of Lipid II molecules pro pore. When pores contain only one pyrene-labeled Lipid II molecule pro pore, no excimers can be formed, and as a

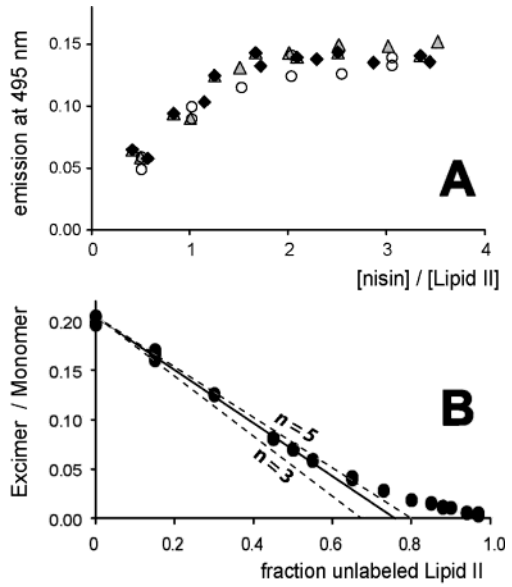

FIGURE 3: Nisin forms a defined pore structure containing 4 Lipid II molecules. (A) Excimer formation in DOPC LUVs with 0.1 mol % (▲), 0.5 mol % (◆), and 1.0 mol % (○) measured with fluorescence recordings at 495 nm using similar fluorimeter settings and a pyrene-labeled Lipid II concentration of 0.25 μM. A fresh batch of lipid vesicles was used for the addition of varying nisin concentrations. (B) Saturation values for the E/M ratio, as calculated from emission recordings at 495 nm (excimer) and 380 nm (monomer). Nisin was added in a 3-fold excess to DOPC LUVs that contained 1 mol % Lipid II with different fractions of unlabeled Lipid II. The Lipid II concentration (unlabeled plus labeled) was 0.50 μM for all of the samples. The E/M data obtained for samples with a fraction of unlabeled Lipid II up to 0.55 were used to obtain a linear equation by least-squares fitting (—). The extrapolation to zero E/M was performed to obtain the number of Lipid II molecules that are required to form a pore. The two dashed lines are shown to illustrate what would be the outcome of performing a linear fit to data obtained for pores containing either $n = 3$ or 5 molecules of Lipid II (See the text for details).

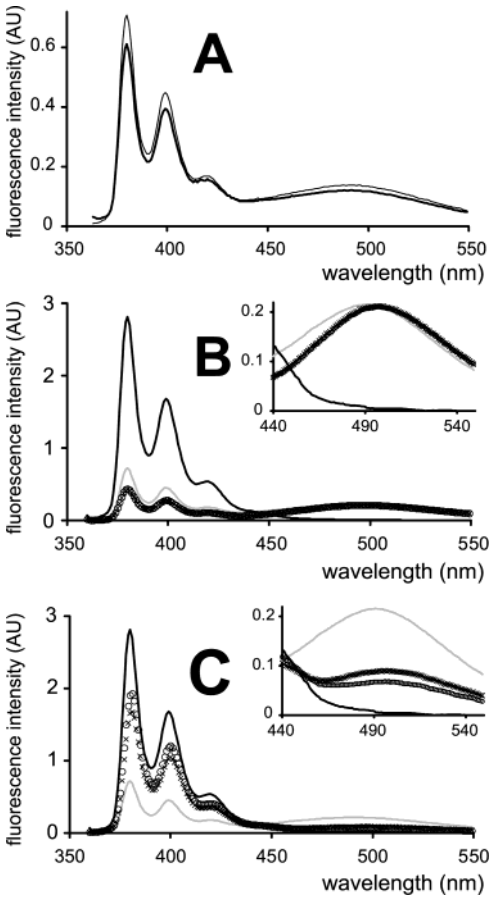

FIGURE 4: Stability of assembled complexes. (A) Fluorescence spectrum of DOPC LUVs with 0.5% pyrene Lipid II in the presence of nisin, before (thin black tracing) and 15 min after (thick black tracing) the addition of unlabeled Lipid II (0.5 mol % in DOPC) up to a final concentration of 4.5 μM. (B) Pyrene fluorescence spectrum of DOPC LUVs with 0.5% pyrene Lipid II (0.25 μM) before (thin tracing) and 5 min after the addition of excess of nisin (0.75 μM) (thick tracing). OPOE was added up to a concentration of 1%. The fluorescence was measured after 1 h (×) in the presence of the detergent and after overnight incubation (○). (C) Fluorescence spectrum of DOPC LUVs with 0.5% pyrene Lipid II (0.25 μM) before (thin tracing) and 5 min after the addition of excess of nisin (0.75 μM) (thick tracing). The fluorescence was measured after 1 h (×) and overnight (○) incubation in the presence of 4 mM SDS. The insets in parts B and C show enlarged views of the excimer emission part of the spectra.

result, the excimer fluorescence is zero. Thus, by determining the fraction at which the excimer fluorescence is zero, we are able to estimate the number of Lipid II molecules pro pore.

Figure 3B shows that the nisin-induced excimer formation (expressed as the E/M ratio) indeed decreased with an increase in the fraction of unlabeled Lipid II molecules. The linear decrease indeed demonstrates that nisin does not discriminate between labeled and unlabeled Lipid II to form a pore, which is a prerequisite for this approach. Above a fraction of 0.55 unlabeled Lipid II, a positive deviation from linearity is observed, which most likely is the result of a nonideal distribution of the pyrene labels over the pore complexes and possible contribution from small variations in the pore stoichiometry. The fraction of unlabeled Lipid II molecules at which the excimer fluorescence was zero was determined by linear extrapolation with a least-squares fit using E/M values for 0.55 or lower (—, Figure 3B). The intercept with the x axis was found to be 0.76 ± 0.02. This corresponds to 4.2 ± 0.3 molecules of Lipid II pro pore.

In Figure 3B, two dashed lines illustrate in which way the obtained intercept value reflects the number of Lipid II molecules pro pore. One line has an intercept at 0.67, corresponding to $n = 3$ Lipid II molecules pro pores, and the other line has an intercept at 0.8, which corresponds to 5 Lipid II molecules pro pore. The intercept from our own extrapolation resides between these values and was best consistent with the value of 0.75, from which we conclude that the most probable number of Lipid II molecules pro pore is 4.

Stability of Nisin−Lipid II Complexes. To gain insight into the stability of the nisin−Lipid II pore complexes, we challenged the assembled pores containing pyrene-labeled Lipid II and nisin with a large excess of unlabeled Lipid II in DOPC LUVs. We used the excimer fluorescence of pyrene-labeled Lipid II as a probe to detect whether the pore complexes remained intact. Any decrease of pyrene excimer fluorescence and concomitant increase of the monomer fluorescence would indicate disassembly of pore complexes. After the addition of unlabeled Lipid II in membranes to preassembled nisin−Lipid II complexes with pyrene label, only a minor change of the overall fluorescence intensity was observed that did not coincide with an increase in monomer fluorescence (hick black tracing of Figure 4A).

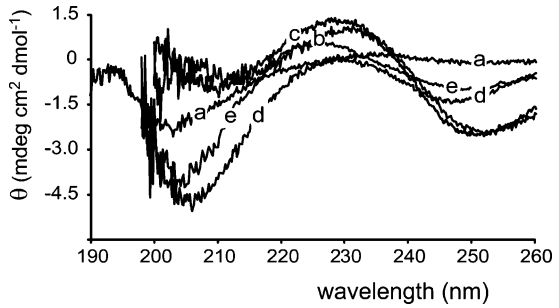

FIGURE 5: Lipid-II-induced secondary structure of nisin and the effect of detergents on stability. CD spectra recorded for 20 μM nisin in 10 mM sodium phosphate buffer at pH 7 with 40 mM Na_2SO_4 (tracing a) and in the presence of 4 mol % Lipid II in DOPC SUVs (b), which was added to obtain a final Lipid II concentration of 20 μM. The effects of 4% OPOE (c) and 20 mM SDS (d) on the secondary structure of nisin in the complex are shown as well. Spectrum e was recorded in the presence of 20 mM SDS micelles with 20 μM Lipid II.

This implicates that the major part of the nisin molecules remained stably integrated in the nisin−Lipid II pore complex and is not able to dissociate from the complex.

The high stability of the pore complex prompted us to test if the pore complex would withstand the solubilization of the bilayer by detergents. Nisin was added first to DOPC vesicles containing 0.5 mol % pyrene-labeled Lipid II to allow pore formation. The incubation mixtures contained 0.75 μM nisin, and the total phospholipid concentration was 50 μM, hence the pyrene-labeled Lipid II concentration was 0.25 μM. Second, detergents were added to dissolve the membranes, and after 1 h and overnight incubation, the pyrene fluorescence was recorded.

Figure 3B shows that dissolving the bilayer with OPOE had hardly any effect on the pyrene fluorescence. The monomer fluorescence dropped slightly, and a small red shift of the excimer band was observed without affecting the intensity (×). Prolonged incubation overnight in the presence of OPOE did not result in further effects on the fluorescence spectrum of pyrene-labeled Lipid II (●). Very similar results were obtained with OG and Tween-20. This suggests that nisin−Lipid II complexes are still intact after the solubilization of the membrane with these detergents.

The stability of the complex was not infinite, as demonstrated by membrane solubilization with a stronger detergent, SDS. Figure 4C shows the pyrene fluorescence of nisin−Lipid II pore complexes after 1 h (○) and overnight (×) incubation in the presence of a membrane-solubilizing concentration of SDS. The decrease of the excimer and concomitant increase of the monomer fluorescence indicates that SDS induces the dissociation of the nisin−Lipid II pore complexes.

CD. CD was used to characterize the effects of pore formation on the conformation of nisin and additionally used as a tool to investigate the effects of solubilization of the pore complex on the secondary structure of nisin. The secondary structure of nisin was remarkably changed upon addition of Lipid-II-containing vesicles as indicated by the large changes in the CD characteristics (see Figure 5) compared to the situation in the buffer (tracing a). The CD spectrum in the presence of Lipid-II-containing membranes has a maximum at 230 nm flanked by two minima, at 211 and 254 nm (b). The observed effects were caused by the presence of Lipid II in the membrane, because the addition of pure DOPC liposomes hardly induced any change in the CD spectrum of nisin (not shown). Because nisin contains many unusual amino acids and lanthionine rings (see Figure 1A), quantitative structural analysis of the CD spectra, as done for proteins, is not feasible (*29*). Therefore, the CD data were only used as fingerprints to monitor the stability of the nisin conformation after the solubilization of membranes containing preformed nisin−Lipid II pore complexes with detergents.

After dissolving the membranes with OPOE, the CD spectrum of nisin did not undergo significant changes (see tracing c of Figure 5). Similar results were found for treatment with OG and Tween-20. Thus, the structure of nisin remains intact upon solubilization of the pore complex from the membrane. This makes OG, Tween-20, and OPOE suitable candidates for isolation of the pores for future experiments focused on structure determination of the nisin−Lipid II pore complex.

On the contrary, solubilization of the membranes by SDS resulted in a clear change of the CD characteristics of nisin. There are still two minima present (206 and 247 nm), but their intensities are clearly different (d). The CD spectrum of nisin was also recorded in the presence of SDS micelles containing Lipid II (e). The observed changes were specific for the presence of Lipid II, because pure SDS micelles only induced minor changes in the secondary structure of nisin (not shown). The pyrene fluorescence data in Figure 4C and the observed similarities in Figure 5 between d and e indicate that nisin−Lipid II complexes are still present but with a different secondary structure.

Role of the Flexible Hinge Region in Pore Formation. To investigate the importance of the hinge region of the nisin molecule for the assembly of stable nisin pores, similar experiments were performed with [N20P/M21P]nisin. Compared to the wild type, this mutant has a 3−8-fold higher MIC value, but its ability to permeabilize the membrane is severely hampered (*10*).

The ability to induce excimer fluorescence was found to be strongly reduced for [N20P/M21P]nisin, which suggests that the assembly is inhibited compared to the wild-type peptide (Figure 6). However, a small increase of the E/M ratio was observed, which reached a saturation level at higher peptide concentrations. This indicates that [N20P/M21P]nisin was still able to assemble into complexes containing multiple Lipid II molecules. Because this mutant did not induce CF leakage under the same conditions, these complexes are not expected to be pores but probably the result of pore assembly becoming trapped in prepore complexes. The different saturation levels that were reached for vesicles containing Lipid II only at the cis side (closed symbols) and at both sides of the membrane (open symbols) indicate that [N20P/M21P]nisin forms these complexes only with the Lipid II molecules at the cis side of the membrane. Apparently, only when nisin−Lipid II pore complexes are formed, the Lipid II molecules at the trans side of symmetric membranes become accessible (Figure 2C).

As observed for wild-type nisin, the E/M ratio saturated at a value independent of the initial Lipid II content in the membrane (open symbols of Figure 6A). This indicates that the prepore complex is organized in a defined structure. Moreover, these results demonstrate that modifications in

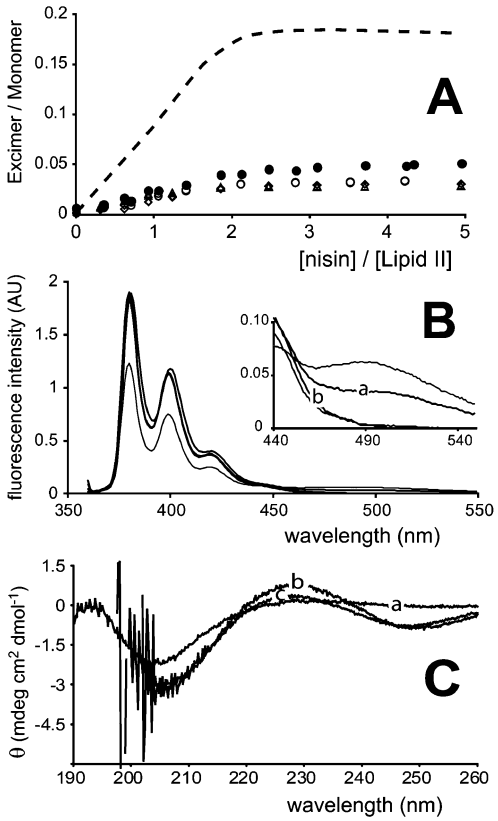

FIGURE 6: Interaction between hinge-region mutant [N20P/M21P]-nisin and Lipid-II-containing membranes. (A) Effect of [N20P/M21P]nisin on the E/M ratio obtained for DOPC LUVs containing pyrene Lipid II located exclusively at the outer leaflet of the membrane (●) and DOPC LUVs with symmetrically distributed pyrene Lipid II at different concentrations, namely, 0.17 mol % (◇), 0.44 mol % (○), and 1.0 mol % (△). To correct for the variations in the measured excimer intensity because of variations in the pyrene-labeled Lipid II concentration (0.15−0.20 μM), the E/M ratio was plotted. The dashed line shows the trend in E/M ratio as it was found for the interaction between wild-type nisin and different types of pyrene-labeled Lipid-II-containing vesicles. (B) Fluorescence spectra of DOPC LUVs with 0.44 mol % pyrene Lipid II at a concentration of 0.17 μM, incubated overnight at 20 °C in buffer (thin black tracing) and in the presence of 0.36 μM [N20P/M21P]nisin (thick gray tracing). The inset shows an enlarged view of the excimer emission part of the spectra. The thick black tracings show the effect of dissolving the bilayers of samples containing nisin and Lipid II at similar concentrations as mentioned above, either with 2% OPOE (tracing a) or with 2 mM SDS (tracing b). Complex formation was allowed for 5 min, and samples were incubated overnight as well. (C) CD spectra recorded for 24 μM [N20P/M21P]nisin in 10 mM sodium phosphate buffer at pH 7 with 40 mM Na_2SO_4 (tracing a) and in the presence of 4 mol % Lipid II in DOPC SUVs (b), which was added to obtain a final Lipid II concentration of 24 μM. Tracing c was recorded in the presence of 4% OPOE.

the hinge region do not affect the affinity for Lipid II, as also indicated by the moderate increase of the MIC value (*10*).

The prepore complexes partially dissociated in the presence of OPOE (Figure 6B), while the stronger detergent SDS induced complete dissociation of the prepore complexes formed by [N20P/M21P]nisin and Lipid II. Thus, the prepore complexes are much less stable than the pore complexes formed by wild-type nisin. Similar to wild-type nisin, we used CD spectroscopy to get more insight into the structure of [N20P/M21P]nisin in the presence of Lipid II. In solution, [N20P/M21P]nisin shows similar CD characteristics as that of the wild-type peptide (compare tracings a in Figure 5 and 6C, respectively). As was found with wild-type nisin, only the presence of Lipid-II-containing DOPC bilayers induced a change of the CD spectrum, because no effect of pure DOPC liposomes could be observed (not shown). However, the changes in the CD spectrum of [N20P/M21P]nisin that were induced by Lipid-II-containing DOPC bilayers, differed from the wild-type peptide (tracings b in Figure 5 and 6C, respectively). The maximum at 230 nm was flanked by two minima, at 206 and 250 nm, which are slightly lower than the 211 and 254 nm found for wild-type nisin. Mostly, the relative intensity of these minima differed. While the minimum at 250 nm was less negative, the minimum at 206 nm had the most negative ellipticity. In fact, the spectrum of [N20P/M21P]nisin in the presence of Lipid-II-containing bilayers resembled to a large extent the spectrum of wild-type nisin in the presence of Lipid-II-containing SDS micelles (compare tracing e in Figure 5 with tracing b in Figure 6C). Although about half of the prepore complexes dissociated as a consequence of the solubilization of the membranes by OPOE (Figure 6B), this resulted in only a minor change of the CD spectrum (tracings b and c of Figure 6C). Hence, the formation of prepore complexes by [N20P/M21P]nisin and complexes with nisin and Lipid II in SDS micelles induce comparable conformational changes of the peptide structure. This implies that after initial binding to Lipid II, only a minimal change of the nisin structure accompanies the formation of a prepore complex or its detergent-induced dissociation. The properties of the hinge region determine whether nisin is able to undergo the drastic conformational changes that are characteristic for the nisin−Lipid II pore complex.

DISCUSSION

In this study, we analyzed the assembly, structure, and stability of nisin pores using pyrene fluorescence spectroscopy and CD. The interaction between nisin and its membrane-anchored target Lipid II leads to the assembly of pores with a defined number of Lipid II and nisin molecules. Furthermore, nisin−Lipid II pore complexes were found to be very stable and require a flexible hinge region in the molecule.

Our experiments were performed at conditions where Lipid II induces a transmembrane orientation (*12*) and membranes become permeabilized (*8*); therefore, the appearance of the excimer fluorescence of pyrene-labeled Lipid II upon addition of nisin was considered to be a reflection of pore formation. These pores have a uniform structure, containing defined amounts of nisin and Lipid II as demonstrated by the observation that the maximum intensity of the excimer fluorescence was reached irrespective of the distribution or membrane concentration of Lipid II. These observations also imply that the excimer signal is obtained from an interaction between two pyrene-labeled Lipid II molecules within one pore complex. Because nisin did not discriminate between pyrene-labeled or unlabeled Lipid II in pore formation, we could establish for the first time that a pore complex contains four molecules of Lipid II. The linear extrapolation to zero excimer fluorescence in Figure 3B yielded an intercept of 0.76 ± 0.02, corresponding to 4.2 ± 0.3 Lipid II molecules. The uncertainty in this value comes from experimental variations and a possible variation in the number of Lipid II

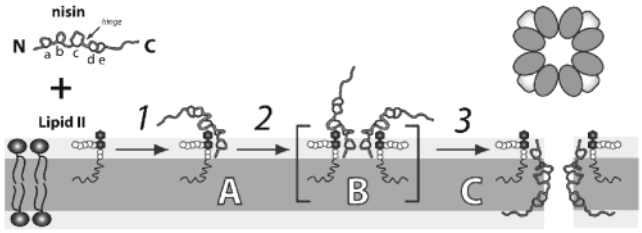

FIGURE 7: Model for the formation of nisin−Lipid II pores in lipid bilayers. The polypeptide antibiotic nisin is presented here in a very simplified way, showing only the main structural properties such as the five thioether rings (a−e) and the position of the hinge region (top left). We propose that the formation of the nisin−Lipid II pore (C) occurs in 3 steps, namely, via the formation of two intermediate structures (A and B) (for details, see the text). The topview of complex C (top right) shows the proposed arrangement of the molecules in a pore complex composed of 8 nisin (grey ellipse) and 4 Lipid II (white triangle) molecules.

molecules pro pores (estimated to be maximally 8%). The deviation from linearity at lower pyrene-labeled Lipid II fractions is most likely caused by statistical spreading of the pyrene labels over the nisin−Lipid II pore complexes. Because the saturation of excimer fluorescence occurred at a 2:1 nisin−Lipid II ratio, we concluded that the pore most likely consists of 8 nisin and 4 Lipid II molecules. Course modeling of the nisin−Lipid II complex yielded the model of the pore, which is shown in topview in the forthcoming Figure 7. The size of the hole, which is formed by the 8 nisin and 4 Lipid II molecules in our model, is consistent with recently obtained information on the size of the nisin pore in the Lipid-II-containing membrane (2−2.5 nm, ref *30*).

The nisin−Lipid II pore complexes are very stable, because no drop in excimer fluorescence was found when Lipid-II-containing membranes were added to membranes containing pore complexes formed with pyrene-labeled Lipid II. Even the addition of mild detergents such as OPOE that completely dissolved the membranes could not disrupt the pore complex. This was confirmed by CD measurements, which showed that the conformation in the pore complex did not change upon solubilization. Therefore, we conclude that dissolving the membrane by mild detergents leaves the pore complex intact and makes OPOE, OG, or Tween-20 suitable candidates for isolation and further characterization of the nisin pore.

Our conclusions that nisin forms defined and stable complexes with Lipid II were recently supported by black lipid membrane experiments (*30*). In the presence of Lipid II, nisin formed uniformly sized pores, which exhibit dramatically increased lifetimes (6 s) compared with nisin pores in the absence of Lipid II (milliseconds). These properties of the nisin−Lipid II pores strongly contrasts the characteristics of pore-forming antimicrobial peptides (AMPs) that do not have a specific target. These AMPs show a far lower affinity for their target membranes, and upon binding of sufficient peptide molecules, they will form only transient pores, which are neither uniform nor stable [for example, magainin (*31*) and alamethicin (*32*)].

To investigate the role of the flexible hinge region in the mode of action of nisin, similar experiments were performed in parallel with [N20P/M21P]nisin. This nisin variant shows a considerable antimicrobial activity because it only has a 3-fold higher MIC value than the wild-type peptide, but its ability to permeabilize Lipid-II-containing membranes is severely impaired (*10*).

Our data showed that [N20P/M21P]nisin was severely hampered in the formation of stable pore complexes with Lipid II in the membrane, although the affinity for its membrane-anchored target was found to be comparable to that of the wild type. [N20P/M21P]nisin was still able to assemble into multiple Lipid-II-containing complexes with a defined structure, which were regarded as prepore complexes. The CD spectrum of the hinge-region mutant in these prepore complexes resembled that of wild-type nisin in complex with Lipid II in SDS micelles, while the CD fingerprint spectrum of the pore complex was quite different. It has been shown that in SDS micelles containing Lipid II only the N-terminal part of nisin is involved in the interaction (*11*). However, the formation of pore complexes is likely to induce a conformational change in the other parts of the peptide as well, as nisin becomes largely membrane-embedded because of the transmembrane orientation (*12*). In return, this will probably have large effects on the CD signal. Therefore, we propose that the assembly of prepore complexes takes place on the membrane surface, involving mainly the N terminus of nisin. Hence, the prepore complexes formed by [N20P/M21P]nisin and Lipid II are not membrane-embedded. Correct anchoring of the pore complex within the membrane has been shown to greatly affect the stability of the pore complex (*14*). Our data have shown that the pore complex formed by wild-type nisin in conjunction with Lipid II was very stable in the presence of mild detergents and moderately stable in the presence of SDS. However, the prepore complexes of the hinge-region mutant were largely unstable even in the presence of mild detergents. This supports our proposition that the prepore complexes are not membrane-embedded. In conclusion, the flexible characteristics of the hinge region in nisin are required for stable integration of pore complexes in the membrane.

Model. On the basis of the present data, we propose a refined model for nisin−Lipid II pore formation (*10*, *12*, *14*) (see Figure 7). When nisin encounters a Lipid-II-containing membrane, it will first form a complex with Lipid II (A). Previous experiments with Lipid-II-containing SDS micelles have shown that the stoichiometry for the interaction with nisin is 1:1 (*11*). These experiments were performed in the absence of phospholipid bilayers and may therefore reflect the formation of the initial binding complex A, which consists of a single nisin and a single Lipid II molecule. Within pyrene-labeled Lipid-II-containing membranes, the interaction with Lipid II will lead to the formation of a nisin−Lipid II pore complex consisting of 2 times more nisin than Lipid II molecules. This means that during the assembly process either more nisin molecules will bind or Lipid II molecules will dissociate. This latter possibility seems highly unlikely considering the high stability of the nisin−Lipid II interaction.

The formation of the pore most likely occurs via an intermediate state, the prepore complex B, in which multiple nisin and Lipid II molecules are assembled at the interface of the bilayer. The formation of the stable pore complex C is completed by the cooperative insertion of the nisin molecules into a perpendicular orientation with respect to the membrane surface. In this position, the nisin molecules are stabilized by the Lipid II molecules that act as mortar

between the nisin bricks (*12*). The flexibility in the hinge region of the nisin molecule is highly likely a prerequisite to undergo the rearrangements that accompany the transformation from complex B to complex C. The final pore complex that is formed is very stable and consists of 8 nisin and 4 Lipid II molecules, which align a water-filled pore with a diameter of 2−2.5 nm (*30*).

REFERENCES

1. Sahl, H. G., Jack, R. W., and Bierbaum, G. (1995) Biosynthesis and biological activities of lantibiotics with unique post-translational modifications, *Eur. J. Biochem. 230*, 827−853.
2. Jack, R. W., and Jung, G. (2000) Lantibiotics and microcins: Polypeptides with unusual chemical diversity, *Curr. Opin. Chem. Biol. 4*, 310−317.
3. Kupke, T., and Gotz, F. (1996) Post-translational modifications of lantibiotics, *Antonie van Leeuwenhoek 69*, 139−150.
4. Sahl, H. G., and Bierbaum, G. (1998) Lantibiotics: Biosynthesis and biological activities of uniquely modified peptides from Gram-positive bacteria, *Annu. Rev. Microbiol. 52*, 41−79.
5. Delves-Broughton, J., Blackburn, P., Evans, R. J., and Hugenholtz, J. (1996) Applications of the bacteriocin, nisin, *Antonie van Leeuwenhoek 69*, 193−202.
6. Hurst, A. (1981) Nisin, *Adv. Appl. Microbiol. 27*, 85−123.
7. Brotz, H., Josten, M., Wiedemann, I., Schneider, U., Gotz, F., Bierbaum, G., and Sahl, H. G. (1998) Role of lipid-bound peptidoglycan precursors in the formation of pores by nisin, epidermin, and other lantibiotics, *Mol. Microbiol. 30*, 317−327.
8. Breukink, E., Wiedemann, I., van Kraaij, C., Kuipers, O. P., Sahl, H., and de Kruijff, B. (1999) Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic, *Science 286*, 2361−2364.
9. van Heijenoort, J. (1994) in *Bacterial Cell Wall* (Hakenbeck, R., Ed.) pp 39−72, Elsevier, Amsterdam, The Netherlands.
10. Wiedemann, I., Breukink, E., van Kraaij, C., Kuipers, O. P., Bierbaum, G., de Kruijff, B., and Sahl, H. G. (2001) Specific binding of nisin to the peptidoglycan precursor lipid II combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity, *J. Biol. Chem. 276*, 1772−1779.
11. Hsu, S. T., Breukink, E., de Kruijff, B., Kaptein, R., Bonvin, A. M., and van Nuland, N. A. (2002) Mapping the targeted membrane pore formation mechanism by solution NMR: The nisin Z and lipid II interaction in SDS micelles, *Biochemistry 41*, 7670−7676.
12. Van Heusden, H. E., De Kruijff, B., and Breukink, E. (2002) Lipid II induces a transmembrane orientation of the pore-forming peptide lantibiotic nisin, *Biochemistry 41*, 12171−12178.
13. Breukink, E., van Kraaij, C., van Dalen, A., Demel, R. A., Siezen, R. J., de Kruijff, B., and Kuipers, O. P. (1998) The orientation of nisin in membranes, *Biochemistry 37*, 8153−8162.
14. Breukink, E., Van Heusden, H. E., Vollmerhaus, P. J., Swiezewska, E., Brunner, L., Walker, S., Heck, A. J., and De Kruijff, B. (2003) Lipid II is an intrinsic component of the pore induced by nisin in bacterial membranes, *J. Biol. Chem. 26*, 26.
15. Kuipers, O. P., Rollema, H. S., Yap, W. M., Boot, H. J., Siezen, R. J., and de Vos, W. M. (1992) Engineering dehydrated amino acid residues in the antimicrobial peptide nisin, *J. Biol. Chem. 267*, 24340−24346.
16. Rouser, G., Fkeischer, S., and Yamamoto, A. (1970) Two-dimensional then layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots, *Lipids 5*, 494−496.
17. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) Measurement of protein using bicinchoninic acid, *Anal. Biochem. 150*, 76−85.
18. Hope, M., Bally, M. B., Webb, G., and Cullis, P. R. (1985) Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume, and ability to maintain a membrane potential, *Biochim. Biophys. Acta 812*, 55−65.
19. Lakowicz, J. R. (1999) in *Principles of Fluorescence Spectroscopy* (Lakowicz, J. R., Ed.) pp 445−486, Kluwer Academic/Plenum Publishers, New York.
20. Ochoa de Alda, J. A., Llama, M. J., and Serra, J. L. (1995) Effective detergent/chlorophyll ratio and detergent concentration in the aqueous phase during solubilization of *Phormidium laminosum* membranes, *Biochim. Biophys. Acta 1240*, 209−215.
21. Woody, R. W. (1995) Circular dichroism, *Methods Enzymol. 246*, 34−71.
22. Sahoo, D., Narayanaswami, V., Kay, C. M., and Ryan, R. O. (2000) Pyrene excimer fluorescence: A spatially sensitive probe to monitor lipid-induced helical rearrangement of apolipophorin III, *Biochemistry 39*, 6594−6601.
23. Lehrer, S. S. (1995) Pyrene excimer fluorescence as a probe of protein conformational change, *Subcell. Biochem. 24*, 115−132.
24. Zhao, M., Zen, K. C., Hubbell, W. L., and Kaback, H. R. (1999) Proximity between Glu126 and Arg144 in the lactose permease of *Escherichia coli*, *Biochemistry 38*, 7407−7412.
25. Zhao, H., Mattila, J. P., Holopainen, J. M., and Kinnunen, P. K. (2001) Comparison of the membrane association of two antimicrobial peptides, magainin 2 and indolicidin, *Biophys. J. 81*, 2979−2991.
26. Lehrer, S. S. (1997) Intramolecular pyrene excimer fluorescence: A probe of proximity and protein conformational change, *Methods Enzymol. 278*, 286−295.
27. Kol, M. A., de Kroon, A. I., Rijkers, D. T., Killian, J. A., and de Kruijff, B. (2001) Membrane-spanning peptides induce phospholipid flop: A model for phospholipid translocation across the inner membrane of *E. coli*, *Biochemistry 40*, 10500−10506.
28. Kol, M. A., van Dalen, A., de Kroon, A. I., and de Kruijff, B. (2003) Translocation of phospholipids is facilitated by a subset of membrane-spanning proteins of the bacterial cytoplasmic membrane, *J. Biol. Chem. 278*, 24586−24593.
29. Dykes, G. A., Hancock, R. E., and Hastings, J. W. (1998) Structural variations in nisin associated with different membrane mimicking and pH environments, *Biochem. Biophys. Res. Commun. 247*, 723−727.
30. Wiedemann, I., Benz, R., and Sahl, H. (2004) Lipid II-mediated pore formation by the peptide antibioitic nisin−A black lipid membrane study, *J. Bacteriol.* in press.
31. Matsuzaki, K., Murase, O., Fujii, N., and Miyajima, K. (1995) Translocation of a channel-forming antimicrobial peptide, magainin 2, across lipid bilayers by forming a pore, *Biochemistry 34*, 6521−6526.
32. He, K., Ludtke, S. J., Huang, H. W., and Worcester, D. L. (1995) Antimicrobial peptide pores in membranes detected by neutron in-plane scattering, *Biochemistry 34*, 15614−15618.

BI049476B