Lipid II Induces a Transmembrane Orientation of the Pore-Forming Peptide Lantibiotic Nisin†

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ABSTRACT: Nisin is an antimicrobial peptide produced by *Lactococcus lactis* and used as a food preservative in dairy products. The peptide kills Gram-positive bacteria via the permeabilization of the membrane, most probably via pore formation using the cell wall precursor Lipid II as its docking molecule. In this study, site-directed tryptophan spectroscopy was used to determine the topology of nisin in the Lipid II containing membrane, as a start to elucidate the mechanism of targeted pore formation. Three single tryptophan mutants were used, which are viable representatives of the wild-type peptide. The emission spectra of tryptophans located at the N-terminus, the center, and the C-terminus as well as quenching by acrylamide and spin-labeled lipids were investigated using model membrane vesicles composed of DOPC containing 1 mol % Lipid II. Nisin was shown to adopt an orientation where the most probable position of the N-terminus was found to be near the Lipid II headgroup at the bilayer surface, the position of the center of nisin was in the middle of the phospholipid bilayer, and the C-terminus was located near the interface between the headgroups and acyl chain region. These results were used to propose a model for the orientation of nisin in Lipid II containing membranes. Our findings demonstrated that Lipid II changes the overall orientation of nisin in membranes from parallel to perpendicular with respect to the membrane surface. The stable transmembrane orientation of nisin in the presence of Lipid II might allow us to determine the structure of the nisin−Lipid II pores in the lipid bilayer.

Nisin is a well-known member of the lantibiotic family, and it has high antimicrobial activity against a broad range of Gram-positive bacteria. Certain strains of *Lactococcus lactis* produce nisin, and since it has a relatively low toxicity toward humans, the peptide has been employed as a natural food preservative for decades (*1*, *2*).

Nisin consists of 34 amino acids (Figure 1A) and has an overall positive charge. Like other lantibiotics, nisin has some unique structural features (*3*−*6*). During the biosynthesis route, serines and threonines are dehydrated to form dehydroalanine (Dha)[1] and dehydrobutyrine (Dhb). Thereafter, reactions between cysteine side chains and Dha or Dhb residues result in the formation of the intramolecular thioether rings (*3*). These so-called lanthionine rings impose a considerable amount of conformational constraint to the peptide molecule (*7*, *8*). The rings A to C and D to E form two rigid parts in the nisin molecule which are interconnected by a flexible hinge region of three amino acids (Figure 1A) (*8*). Also the C-terminus is rather flexible (*8*−*10*). Due to the presence of the ring structures, hydrophilic and hydrophobic residues are positioned at opposite sides of the molecule (*8*, *11*).

Positive charge and amphipathic characteristics are generally found in peptides which kill bacteria via the permeabilization of their membranes [see reviews (*12*−*15*)]. Extensive studies on the interaction between nisin and model membrane systems revealed that nisin can permeabilize lipid bilayers (*16*−*20*). Special emphasis was placed on the role of anionic phospholipids, which facilitate the binding of nisin and the permeabilization of the membrane [see, for review, (*21*)]. Nisin was proposed to perforate the membrane via the formation of transient pores with lifetimes in the millisecond range (*21*). Although nisin was able to permeabilize the negatively charged model membranes, the wedge-like model proposed for pore formation could not explain the very efficient killing of bacteria at nanomolar concentrations of nisin, whereas micromolar concentrations of nisin were required to induce leakage from model membrane vesicles.

This discrepancy was solved with the discovery that efficient pore formation by nisin is determined by the presence of a specific docking molecule in the bacterial membrane rather than by phospholipid composition (*22*, *23*). Lipid II was found to be the target molecule for pore formation by nisin. When 0.1 mol % or even lower concentrations of the cell wall precursor were incorporated in model membranes, the minimal nisin concentration required to induce leakage from vesicles was changed from micromolar to nanomolar (*22*).

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[1] Abbreviations: 5DOX-PC, 1-palmitoyl-2-stearoyl(5-DOXYL)-*sn*-glycero-3-phosphocholine; 12DOX-PC, 1-palmitoyl-2-stearoyl(12-DOXYL)-*sn*-glycero-3-phosphocholine; 16DOX-PC, 1-palmitoyl-2-stearoyl(16-DOXYL)-*sn*-glycero-3-phosphocholine; CF, carboxyfluorescein; Dha, dehydroalanine; Dhb, dehydrobutyrine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; EPR, electron spin resonance; GlcNAc, *N*-acetylglucosamine; MIC, maximal inhibitory concentration; MurNAc, *N*-acetylmuramic acid; TEMPO-PC, 1,2-dioleoyl-*sn*-glycero-3-TEMPO-phosphocholine.

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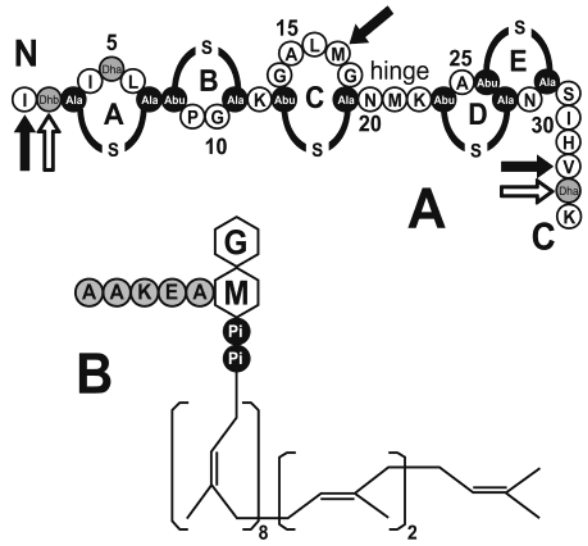

FIGURE 1: Primary structure of nisin Z (A) and a schematic picture of eubacterial Lipid II (B). (A) Posttranslationally modified groups Dha and Dhb are shown in gray circles. Dehydrated residues that have further reacted with cysteine residues to form thioether bonds are shown in black. These characteristic (methyl)lanthionine bonds are presented as −S−. Filled arrows point to the three amino acids which were substituted for tryptophans in the three nisin mutants. Open arrows mark the residues of which posttranslational modification was inhibited due to the presence of tryptophans on positions 1 and 32. (B) *N*-acetylated sugars MurNAc and GlcNAc are shown as hexagons with M and G, respectively. The amino acids of the pentapeptide, which is attached to the MurNAc, are presented as gray circles. The composition of the linear pentapeptide varies among different bacterial genera, but the lysine form is one of the most common found among bacteria (*24*, *25*). The undecaprenyl tail is shown in abbreviated form as well as the pyrophosphate moiety which links the sugar−peptide headgroup and the hydrophobic tail (Pi).

Lipid II provides the cell wall synthesis machinery with subunits for the peptidoglycan polymer (*24*, *25*). It is formed inside the bacterial cell by a two-step assembly of two *N*-acetylated sugars—GlcNAc and MurNAc—to a bactoprenol tail (Figure 1B). The MurNAc sugar also contains a peptide of five amino acids. Lipid II molecules are being transported across the membrane via an unknown mechanism to deliver their sugar−peptide cargo to the extracellular cell wall biosynthesis machinery (*25*).

Direct binding studies showed that the affinity of nisin for membranes was remarkably enhanced by the presence of Lipid II (*26*). Black-lipid membrane studies further showed that pores formed in membranes containing Lipid II had an increased size and lifetime, suggesting a direct involvement of the lipid in pore formation [Wiedemann, I., Benz, R., and Sahl, H. G. (2002) (manuscript in preparation)]. Therefore, nisin was found to be the first peptide antibiotic known to use a specific target in the membrane for very efficient permeabilization of membranes (*22*).

In this study, site-directed tryptophan spectroscopy (*27*) was applied to determine the topology of nisin in the Lipid II containing membrane in order to gain more insight into the membrane-permeabilizing action of nisin (*28*). Changes in emission spectra together with quenching from the lipid and solvent phase were used to propose a model for the orientation adopted by nisin upon the interaction with Lipid II in the membrane.

MATERIALS AND METHODS

Materials. Wild-type nisin Z and tryptophan variants I1W/Dhb2T (1W), M17W (17W), and V32W/Dha33S (32W) were produced and purified as described (*29*). Nisin stock solutions with concentrations between 0.25 and 0.50 mM in 0.05% acetic acid were stored at −20 °C. Protein concentrations were determined using a bicinchoninic acid protein assay (Pierce Chemical Corp.), with bovine serum albumin as a standard. Phospholipids 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG), and spin-labeled (SL) lipids 1,2-dioleoyl-*sn*-glycero-3-TEMPO-phosphocholine (TEMPO-PC), 1-palmitoyl-2-stearoyl(5-DOXYL)-*sn*-glycero-3-phosphocholine (5DOX-PC), 1-palmitoyl-2-stearoyl(12-DOXYL)-*sn*-glycero-3-phosphocholine (12DOX-PC), and 1-palmitoyl-2-stearoyl(16-DOXYL)-*sn*-glycero-3-phosphocholine (16DOX-PC) were obtained from Avanti Polar lipids Inc. Carboxyfluorescein (CF) was obtained from Kodak and purified as described (*30*). Lipid II was obtained via a synthesis and purification procedure which was developed in our group [Breukink, E., Vollmerhaus, P. J., Swiezewska, E., Heck, A. J. R., and de Kruijff, B. (2002) (submitted for publication)]. All other chemicals were of analytical grade or better.

Vesicles. Large unilamellar vesicles of varying composition were prepared in 50 mM MES, pH 6.0, 100 mM K_2SO_4 (K^+-buffer) by extrusion techniques (*31*) using filters with a 0.2 μm pore size. For CF-leakage experiments, vesicles were prepared in K^+-buffer containing 50 mM carboxyfluorescein and treated as described (*17*).

The Lipid II content was chosen to be 1 mol % of the total lipid amount to minimize membrane-perturbing effects in combination with low vesicle-related scattering in the fluorescence spectra (*32*). Parallel control experiments were performed with similarly charged membranes using DOPC vesicles with 3% DOPG, since the net negative charge of the Lipid II molecule is 3.

Electron spin resonance (ESR) spectra were recorded on a Bruker ESP 300E to check for the relative abundance of unpaired electrons in samples containing equal amounts of the four different SL-lipids (*33*). Only small differences were found between the varying SL-lipids. Therefore, concentrations were adjusted only slightly to ensure that within the vesicles containing the four different SL-lipids, equal amounts of paramagnetic quenching groups were present. Phospholipid and Lipid II concentrations were based on inorganic phosphate determination after destruction of the lipids with perchloric acid (*34*).

Carboxyfluorescein-Leakage. The CF-leakage assay was performed to test the pore-forming activities of nisins 1W, 17W, and 32W in comparison to wild-type nisin, using model membranes containing 0.5 mol % Lipid II (*22*). CF-leakage as well as all tryptophan fluorescence experiments were performed with the same SLM-Aminco SPF-500 C fluorometer; all samples were continuously stirred in a 10 × 4 mm quartz cuvette and kept at 20 °C using a water bath with continuous circulation. For the CF-leakage experiments, different nisin concentrations were used (10−100 nM), and measurements were performed at least 3 times. The activity of nisin variants in the presence of Lipid II was calculated as described (*26*).

Fluorescence Measurements. The effect of Lipid II containing membranes on the fluorescence characteristics of the tryptophan variants of nisin was assessed with spectral recordings between 300 and 400 nm (bandwidth 5 nm) as well as single-wavelength recordings at 350 nm (various bandwidths) (*28*). Fluorescence recordings of 1.0 μM 1W, 17W, and 32W in K^+-buffer were performed with an excitation wavelength of 280 nm (bandwidth 5 nm), in the absence and presence of DOPC vesicles containing 1% Lipid II (or 3% DOPG). Fluorescence spectra and single-wavelength recordings were corrected for by blank subtraction and dilution effects. Additionally, the absorbance of each sample was measured after the fluorescence experiment, at the wavelength of excitation and emission on a Perkin-Elmer UV/Vis Lambda 18 spectrophotometer. These results were used to calculate the inner filter effect as described (*35*).

Titration experiments using individual samples were performed to investigate the concentration dependency of the spectral changes observed in the fluorescence spectra of nisins 1W, 17W, and 32W. Fluorescence intensities were measured at 350 nm and averaged over recording times of 20 s. Intensities as well as complete spectra were measured before and after addition of Lipid II containing membranes. The Lipid II:nisin ratios used for the individual samples varied from 0.1 to 2:1, and samples were measured at least in duplicate.

Acrylamide Quenching. Insertion of the peptide nisin into the bilayer containing Lipid II was investigated with the water-soluble quencher acrylamide (*36*). From the excitation wavelengths tested between 280 and 295 nm, 290 nm was found to be the most suitable for these experiments. Fluorescence was recorded at 350 nm and averaged over measuring times of approximately 20 s. Aliquots from an aqueous 3.0 M acrylamide solution were added stepwise, and an equilibration time of 60 s was taken to allow homogeneous distribution of the quencher. The acrylamide titration of nisin samples with Lipid II containing membranes was started 5 min after the addition of the membranes. Titrations were performed at least in duplicate, and data were analyzed according to the Stern−Volmer equation (*36*):

$$F_0/F = 1 + K_{sv} \times [Q] \quad (1)$$

where F_0 is fluorescence in the absence and F fluorescence in the presence of increasing quencher concentrations [Q]. The Stern−Volmer constant K_{sv} is the product of the bimolecular quenching rate k_q and the fluorescence lifetime τ_0:

$$K_{sv} = k_q \times \tau_0 \quad (2)$$

Both the Stern−Volmer constant and the bimolecular quenching rate can be used as measure for the accessibility of the tryptophan to acrylamide.

Spin-Labeled Lipid Quenching. The position of the tryptophans at the N-terminus, center, and C-terminus of nisin in the Lipid II containing membranes was assessed by considering the quenching effect of four different SL-phospholipids on the fluorescence of 1W, 17W, and 32W. Single-wavelength recordings at 350 nm as well as spectral recordings were performed in the absence and presence of DOPC vesicles containing 1% Lipid II and an additional amount of 10%, 25%, or 50% of one of the spin-labeled lipids (TEMPO-PC, 5DOX-PC, 12DOX-PC, or 16DOX-PC). The quenching efficiency Q_{eff} of each SL-lipid was calculated via

$$Q_{eff} = (1 - F_h/F_0) \times 100 \quad (3)$$

in which F_h is the fluorescence in the presence of quencher at depth h and F_0 the fluorescence in the absence of quencher. If tryptophans become inserted deeply into symmetrically labeled bilayers, fluorescence will most likely become quenched by spin-labels residing in both leaflets; therefore, the following equation was used (*37*):

$$\ln [F_h/F_0] = S/(\sigma\sqrt{2\pi}) \times \exp\left\{-\frac{1}{2}[(h - h_m)/\sigma]^2\right\} + S/(\sigma\sqrt{2\pi}) \times \exp\left\{-\frac{1}{2}[(h + h_m)/\sigma]^2\right\} \quad (4)$$

where h is the distance between the bilayer center and the quenching moiety, S is the area under the curve, and σ is the dispersion coefficient. The Gaussian-based eq 4 was fitted to the (h, ln [F_h/F_0]) plot to obtain the value for h_m, which reflects the most probable distance between the fluorophore and the bilayer center.

RESULTS

Site-directed tryptophan spectroscopy was applied to gain insight into the Lipid II induced topology of membrane bound nisin, since this technique had successfully been used before to determine the orientation of nisin in model membranes lacking Lipid II (*28*). Tryptophan emission spectra were recorded, and the insertion into the less accessible parts of the membrane was assessed with acrylamide quenching. Quenching of nisin variants with different spin-labeled lipids revealed the position where the tryptophans became inserted into the Lipid II containing membranes.

Characterization of the Tryptophan Mutants. The tryptophan variants 1W, 17W, and 32W used in this study were previously shown to have only slightly lower antimicrobial activities compared to wild-type nisin (*28*). To test if the tryptophan variants exhibit comparable membrane-permeabilizing abilities as the wild type, the pore formation activity in the presence of Lipid II was tested using a carboxyfluorescein-leakage assay (*22*, *26*). The pore-forming activities of 100 nM nisin were calculated from the leakage measured 30 s after addition to the CF-loaded vesicles. Leakage values relative to wild-type nisin (100%) were 82 (±2)%, 90 (±1)%, and 90 (±6)%, for 1W, 17W, and 32W, respectively. Based on these results and the previously found minimal inhibitory concentrations (MIC values) (*28*), the tryptophan mutants of nisin were considered as valid representatives of the wild type for studying the nisin−Lipid II interaction in model membranes.

Emission Spectra. To get a first insight into the effects on the fluorescence properties of 1W, 17W, and 32W, emission spectra were recorded in the absence and presence of Lipid II containing membranes. The nisin to Lipid II ratio was chosen as 1:1, which corresponds to the nisin−Lipid II binding stoichiometry (*26*). Spectra and single-wavelength measurements were performed after an incubation time of 5

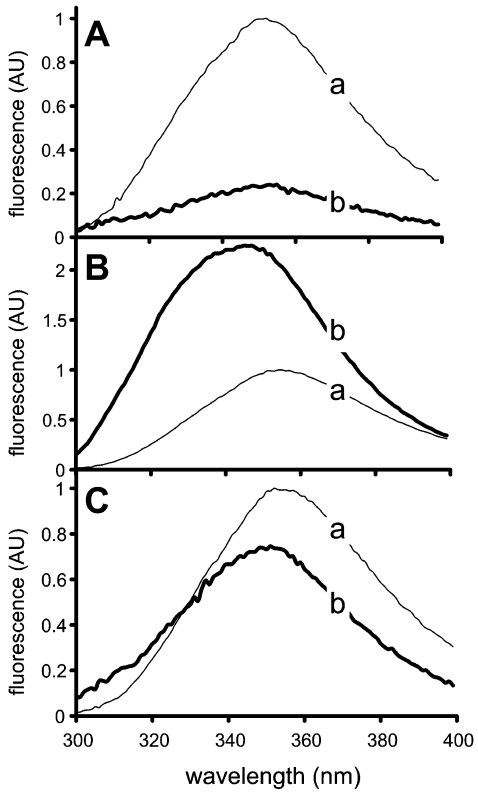

FIGURE 2: Fluorescence emission spectra of nisin tryptophan variants in the absence and presence of Lipid II containing membranes. Spectra were recorded for 1.0 μM 1W (A), 17W (B), and 32W (C) in K^+-buffer in the absence (tracings a) and in the presence (tracings b) of model membranes, composed of 100 μM Lipid−Pi DOPC containing 1 mol % Lipid II. An excitation wavelength of 280 nm was used, and optimal signal-to-noise ratios were obtained by adjusting the fluorometer settings for each tryptophan variant individually. The intensity at the emission maximum in buffer was set to 1 for 1W, 17W, and 32W separately, to facilitate comparison of the effects. Spectra were corrected for dilution effects, blank subtraction, and the inner filter effect as described under Materials and Methods.

Table 1: Emission Maxima (λ_{max} in nm) and Intensity Changes of 1 μM Nisin Solutions Occurring upon Addition of Phospholipid Vesicles

	buffer	100 μM DOPC; 1 mol % Lipid II		100 μM DOPG[b]	
	λ_{max}	λ_{max}	Δintensity[a]	λ_{max}	Δintensity
1W	352	352	0.2	340	1.1
17W	355	344	2	348	1.3
32W	355	350	0.9	348	1.1

[a] Relative intensity changes expressed as the ratio of fluorescence at λ_{max} in the presence of bilayers ($F_{\lambda,PL}$) to fluorescence at λ_{max} from nisin in buffer ($F_{\lambda,0}$). [b] $\Delta\lambda_{max}$ and Δintensity values were taken from (*28*).

min. The spectral changes were followed for 60 min and were found to be rapid and completed within the first 5 min (data not shown).

Tracings *a* in Figure 2A−C represent the emission spectra of 1W, 17W, and 32W in buffer. The spectra had a nearly similar shape, and visual inspection of the spectra revealed that the emission maximum of 1W was at a slightly lower wavelength than the maxima of 17W and 32W (see Table 1).

Tracings *b* in Figure 2A−C show the fluorescence of the three tryptophan variants in the presence of DOPC vesicles containing 1 mol % Lipid II in the membrane. Addition of Lipid II containing membranes to 1W caused a large reduction of the fluorescence intensity, but no shift of the emission maximum. A completely different picture was obtained for the fluorescence of 17W. The addition of Lipid II containing membranes caused a blue shift and a concomitant intensity increase. Intermediate effects were observed for 32W fluorescence.

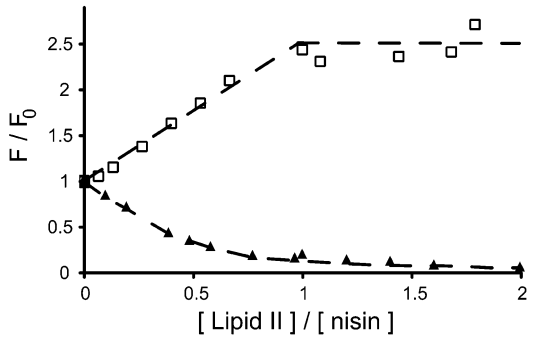

FIGURE 3: Nisin:Lipid II ratio-dependent change of 1W and 17W tryptophan fluorescence. Different amounts of model membrane vesicles of DOPC containing 1% Lipid II were added to 1.0 μM nisin samples. Single-wavelength recordings were performed at 350 nm using 280 nm as the excitation wavelength. Fluorescence intensities before (F_0) and after addition of Lipid II containing vesicles (F) were used to calculate F/F_0 values for 1W (▲) and 17W (□), which were plotted against the Lipid II:nisin molar ratio.

The spectral changes were only caused by the interaction between nisin and Lipid II in the membrane because control incubations with model membranes consisting of 3 mol % DOPG (to achieve similar charge density as with Lipid II) in DOPC did not cause any significant spectral change compared to nisin in buffer (data not shown).

Table 1 shows a summary of the effects of Lipid II containing vesicles on intensity and the emission maximum, and compared them to previous data in the presence of DOPG vesicles (*28*). The effects on the fluorescence spectra of 1W, 17W, and 32W were more divergent in Lipid II containing membranes than for membranes containing anionic lipids, especially at the N-terminus of nisin. This indicates that in the presence of Lipid II the mode of insertion of nisin is altered.

Quantification of Spectral Effects. To investigate how the fluorescence of 1W, 17W, and 32W quantitatively depended on Lipid II, the emission of 1.0 μM solutions of the tryptophan variants was recorded at 350 nm before and after addition of varying amounts of Lipid II containing membranes.

Increasing the concentration of Lipid II containing membranes caused a gradual and nearly proportional decrease of 1W fluorescence at 350 nm, until the effect saturated around the molar ratio of 1:1 nisin to Lipid II (Figure 3). The increase of 17W fluorescence was nearly proportional to the increase of Lipid II concentration, until the Lipid II amount had reached the same concentration as that of nisin. After this point had been reached, the fluorescence remained constant.

Single-wavelength recordings could not be used accurately to characterize the change in 32W fluorescence, since changes were small and of complex behavior. However, above a nisin to Lipid II ratio of 1:1, addition of more Lipid

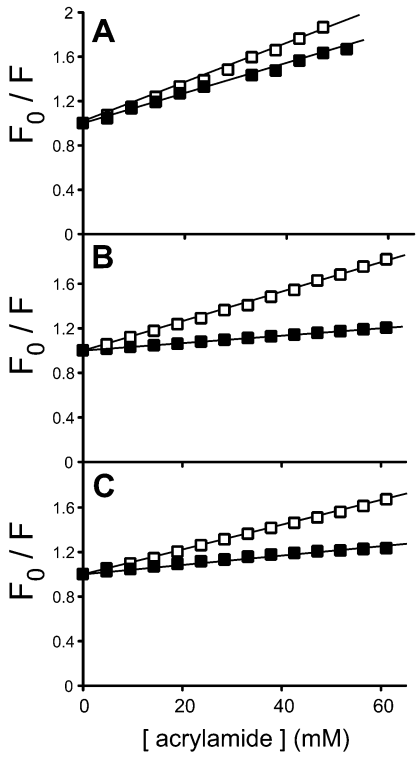

FIGURE 4: Acrylamide quenching of nisin tryptophan variants. Acrylamide quenching of tryptophan fluorescence measured for samples containing 1.0 μM 1W (A), 17W (B), or 32W (C) in the absence (□) and presence of model membrane vesicles of 100 μM Lipid−Pi DOPC with 1 mol % Lipid II (■). Single-wavelength recordings were performed at 350 nm using an excitation wavelength of 290 nm. Fluorescence measured in the absence (F_0) and presence (F) of the quencher acrylamide was used to construct these plots and to derive the Stern−Volmer constant K_{sv} (*36*).

II containing membranes did not further influence the fluorescence characteristics of 32W (data not shown).

Acrylamide Quenching. To test if the tryptophans of 1W, 17W, and 32W became inserted into Lipid II containing membranes, accessibility to the aqueous quencher acrylamide was measured in the presence and absence of the membranes containing Lipid II. Membranes containing Lipid II were added to obtain samples in which equimolar amounts of nisin and Lipid II were present. In buffer, the increase of the acrylamide concentration caused an efficient quenching of the fluorescence of 1W, 17W, and 32W, as indicated by the linear increase in the F_0/F ratio (open symbols, Figure 4). This is in accordance with the expected accessibility of the tryptophan residues of peptides dissolved in buffer (*28*, *38*). In the presence of Lipid II containing vesicles, quenching is considerably reduced in which the largest effects were observed or 17W (Figure 4B) and 32W (Figure 4C). The minor reduction in quenching observed for 1W is in accordance with a location of this residue in an aqueous environment accessible to acrylamide. Control experiments using 3% DOPG containing vesicles (data not shown) revealed that the presence of Lipid II in the zwitterionic phospholipid matrix is required to induce the reduction of the quenching of tryptophan fluorescence from 1W, 17W, and 32W.

The curves in Figure 4 were used to calculate the Stern−Volmer constants (K_{sv}) (Table 2). In the presence of Lipid II containing membranes, the K_{sv} values were reduced for

Table 2: Stern−Volmer Quenching Constants (K_{sv} in M^{-1}) and Bimolecular Quenching Rates[a] (k_q in M^{-1} s^{-1}) for 1.0 μM Nisin in Buffer and in the Presence of 100 μM Lipid−Pi DOPC Vesicles Containing 1 mol % Lipid II

		buffer	Lipid II LUVETs	DOPG LUVETs[b]
1W	K_{sv} (M^{-1})	17.6	13	4.8
	k_q (M^{-1} s^{-1})	18	57	
17W	K_{sv} (M^{-1})	13	3.3	3.7
	k_q (M^{-1} s^{-1})	13	1	
32W	K_{sv} (M^{-1})	10.8	4.2	3.8
	k_q (M^{-1} s^{-1})	11	5	

[a] See text for details on the way the bimolecular quenching rates are obtained. [b] K_{sv} data obtained from (*28*).

1W, 17W, and 32W. The largest reduction of accessibility was found for 17W, whereas the tryptophans of 1W remained rather accessible according to their K_{sv}. Alternatively, the bimolecular quenching rate (k_q) can be used as a measure for the accessibility of fluorophores. If fluorescence lifetime values are not available, their relative change can be approximated by the relative change in intensities (*52*). For illustrative purposes only, we have assumed that the lifetime for each of the three mutants in buffer equals 1 ns and projected the lifetimes of membrane-bound peptides in accordance with changes of the intensities given in Table 1. Overall, the same picture emerges, as compared to the K_{sv} values. However, the k_q value for 1W suggests that this tryptophan becomes even more water-exposed in the presence of Lipid II than in buffer, indicating a structural change of the N-terminus upon binding of Lipid II.

Data presented in Tables 1 and 2 showed that the spectral characteristics and accessibility of 1W, 17W, and 32W tryptophans were affected differently by anionic and Lipid II containing membranes (*28*). Thus, the way in which nisin is inserted in the lipid bilayer is apparently changed due to the presence of Lipid II.

Spin-Labeled Lipid Quenching. To obtain more exact information on the position of different nisin parts in the Lipid II containing membrane, the effect of quenchers in the membrane was determined. The quenching moiety consisted of a covalently linked nitroxide group with an unpaired electron (spin-label), positioned either at the headgroup or attached to the hydrocarbon chain at C-atom number 5, 12, or 16. The fluorescence of the three nisin mutants was measured in the presence of Lipid II enriched membranes containing each of the varying spin-labeled lipids.

None of the spin-labeled lipids quenched the fluorescence of 1W (data not shown). The relation between the position of the quencher and the efficiency of quenching is shown in Figure 5 for 17W and 32W. Comparable quenching profiles were obtained for membranes containing 10% or 50% spin-labeled lipids (data not shown). The presence of spin-labeled lipids in vesicles containing 3% DOPG in DOPC did not affect the 17W and 32W fluorescence.

Tryptophan fluorescence intensities of 17W and 32W were most effectively reduced by 16DOX-PC and 5DOX-PC, respectively, i.e., the deepest quencher and the quencher near the interface. The results obtained for 17W and 32W, with vesicles containing 25% spin-labeled lipids (Figure 5), were mathematically converted via the distribution analysis method (*37*, *39*) to values corresponding to the most probable

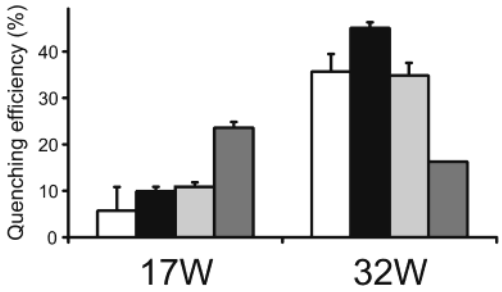

FIGURE 5: Spin-labeled lipid quenching of nisin tryptophan variants. Quenching of 17W and 32W fluorescence in the presence of 25% spin-labeled lipids. In 100 μM Lipid−Pi DOPC vesicles containing 1 mol % Lipid II, either TEMPO-PC (white bar), 5DOX-PC (black bar), 12DOX-PC (light gray bar), or 16DOX-PC (dark gray bar) was present. Single-wavelength recordings were done at 350 nm using an excitation wavelength of 280 nm. The quenching efficiencies were calculated from nisin fluorescence in the presence of Lipid II containing membranes with and without spin-labeled lipids ($n = 3$).

Table 3: Most Probable Distances (Å) of the Tryptophan Residues to the Center of the DOPC Bilayer Containing 1% Lipid II (Calculated with Distribution Analysis)[a]

	Lipid II	DOPG/DOPC (9:1)[b]
1W	−	9.9 ± 0.4
17W	0.0 ± 0.5[c]	11.5 ± 0.2
32W	13.4 ± 0.3[c]	13.1 ± 0.2

[a] As described (*37*). [b] Data from (*28*). [c] The values of the dispersion parameter (sigma) obtained by fitting the quenching profile with eq 4 were found to equal 4.7 and 7.0 Å for 17W and 32W, respectively.

insertion depths (Table 3) [for positions in the DOPC bilayer, see ref (*40*)]. These values implicate that the tryptophan of 32W can be found near the interface of the hydrocarbon tails and the headgroups, whereas the tryptophan of 17W is positioned at the center of the bilayer. Compared to anionic membranes, Lipid II causes the 17W tryptophan to shift to a 12 Å deeper position in the membrane.

DISCUSSION

In this study we have performed site-directed tryptophan fluorescence to determine the orientation of nisin in membranes containing Lipid II. The tryptophan emission spectra obtained for 1W, 17W, and 32W as well as the accessibility measurements with aqueous quenchers and spin-labeled lipid quenching were used to propose a model for the orientation of nisin in membranes containing Lipid II.

Nisins 1W, 17W, and 32W showed comparable fluorescence characteristics in buffer, with the fluorescence maxima centered around 355 nm. This is indicative for tryptophan residues located in a hydrophilic environment (*35*). However, fluorescence intensities varied significantly between 1W, 17W, and 32W, as was previously found and ascribed to differences in the surrounding amino acids of the tryptophans (*28*). Especially the proximity of the thioether sulfur atom of the lanthionine bridge of ring A may be responsible for the reduction of the fluorescence quantum yield of 1W (*41*).

The presence of Lipid II containing membranes altered the fluorescence characteristics of the tryptophan variants in remarkably different ways. The fluorescence of 32W and especially 17W was substantially blue-shifted, whereas the 1W fluorescence showed no blue shift at all. Recording of complete spectra has revealed that in the presence of increasing quencher concentrations (acrylamide as well as spin-labeled lipids) the shape of the 1W, 17W, and 32W spectra remained the same. As a consequence, the Stern−Volmer plots are linear, which is indicative for quenching of a homogeneous population of fluorophores. Apparently, all tryptophans of 1W, 17W, and 32W all become located in different environments. Below, the determination of the most probable positions of the N-terminus, the center, and the C-terminus of nisin in the Lipid II containing membrane will first be discussed individually.

N-terminus. The fluorescence of the N-terminal mutant was very effectively quenched by the presence of Lipid II containing membranes. Three possible causes of the reduction of tryptophan fluorescence can be considered; i.e., (1) intermolecular self-quenching of tryptophan (*35*), (2) intramolecular quenching (*41*, *42*), and (3) quenching by external groups in the membrane (*42*). The first possibility may occur within aggregates of nisin whereby the tryptophans of the individual nisin molecules are in close proximity to each other. From the absence of relief of self-quenching upon dilution of the 1W pool with wild-type nisin (several ratios were tested), this possibility was ruled out (not shown). Although the second option cannot be excluded, we favor the third possibility. From the absence of the blue shift and the accessibility toward the aqueous quencher acrylamide, it can be expected that the 1W tryptophan resides in the hydrophilic headgroup region where charged moieties might be in the close proximity of the tryptophan, and cause fluorescence quenching. Therefore, the most likely candidate quencher is the Lipid II pyrophosphate group. Quenching abilities of single phosphate groups originating from phospholipids have been reported before (*38*, *43*). That pyrophosphate groups may even be more potent quenchers as compared to single phosphates was suggested by control experiments using solutions of sodium phosphate and pyrophosphate which showed that the latter was a better quencher of tryptophan in solution (data not shown). Moreover, the recently discovered importance of the N-terminus of nisin in the recognition and binding of Lipid II supports the localization of 1W near the pyrophosphate (*26*, *44*). Additionally, NMR studies showed that the pyrophosphate of Lipid II becomes immobilized in the presence of nisin, due to docking of nisin with its protonated N-terminal amine group onto the negatively charged pyrophosphate of Lipid II [Bonev, B. B., and Breukink, E. (2002) (manuscript in preparation)].

Center. Completely different data were obtained for nisin 17W. The blue shift of 12 nm, accompanied by an intensity increase found for 17W fluorescence, suggested that the tryptophan is inserted into the hydrophobic part of the Lipid II containing membrane. Indeed, the acrylamide experiments demonstrated that the 17W tryptophan was inserted into a less accessible location compared to 32W and especially to 1W (Table 2). The depth determination using spin-labeled lipid quenching measurements confirmed these results and revealed that the 17W tryptophan becomes inserted at the deepest position in the Lipid II containing membrane, placing it in the middle of the bilayer (Table 3). However, the emission maximum of 17W in the presence of Lipid II containing membranes is much longer than that of a typical W residue embedded in the hydrocarbon core (*51*). Furthermore, the residue still remains accessible to the water-soluble

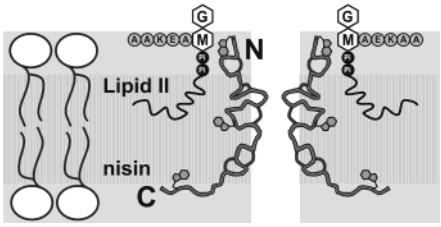

FIGURE 6: Model for the orientation of nisin in phospholipid bilayers containing Lipid II. This figure shows a cross section of the membrane containing the putative nisin−Lipid II pore complex in the membrane. The perpendicular nisin orientation was based on the most probable positions of tryptophans in 1W, 17W, and 32W, which are depicted as tryptophanyl side chains of the nisin molecule (see also Discussion). Lipid II is also included (see Figure 1). The structure of the C55 polyprenyl chain is simplified for illustrative reasons, since the conformation in a membrane-like environment is unclear (*32*).

quencher acrylamide. This indicates that the residue is located close to the pore wall.

C-terminus. The 32W tryptophan shows intermediate effects for all techniques used. First, the emission was blue-shifted, but less than observed for fluorescence of 17W. Second, the 32W tryptophan became less accessible to the quenching by acrylamide, and the effect was larger than for 1W but less than for 17W. Also according to the spin-labeled lipid quenching results, the most probable position of the tryptophan at the C-terminus is an intermediate one, not completely buried in the hydrophobic region of the membrane, but near the phospholipid headgroups.

Orientation of Nisin in Lipid II Containing Membranes. The shallow location of the N-terminus of nisin in the presence of Lipid II is completely opposite to the deepest location it had in the presence of only high amounts of anionic phospholipids (*28*). A similar large shift was observed for the location of the tryptophan at position 17. Only the C-terminal 32W was located at a similar position in the presence of Lipid II as compared to membranes containing only high amounts of anionic lipids. The most probable orientation of nisin that would place the tryptophans in these locations in the bilayer in the presence of Lipid II is a transmembrane orientation (Figure 6). This implies that the presence of Lipid II shifts the orientation of nisin with respect to the membrane surface from a parallel [anionic lipids; see (*28*)] to a perpendicular orientation. However, a transmembrane orientation for a single nisin molecule would be energetically very unfavorable because it would not only expose charged residues (i.e., lysine 12 and 22) to the hydrophobic interior of the bilayer, but also expose the (unfolded) backbone. Therefore, oligomerization of nisin and Lipid II is essential to overcome the unfavorable thermodynamics of burying the peptide backbone into the hydrocarbon core of the bilayer. Thus, the nisin molecule must be present in a pore-like structure in which the hydrophilic residues, including the positively charged lysine and histidine side-chains, are pointed inward, forming a water-filled channel through the phospholipid bilayer. From the size of the pore (2 nm) calculated from the results of black-lipid membrane experiments and the size of the nisin molecule [20 × 50 Å (*45*)], it can be estimated that about 4−6 nisin monomers constitute a single pore. Figure 6 shows a cross section of this pore, presenting only two monomers of nisin.

The model is supported by the observation that the pores formed by nisin in black-lipid membranes in the presence of Lipid II have a lifetime of seconds compared to milliseconds in the absence of the cell wall precursor (*46*). This indicates that the pore structure of nisin in the presence of Lipid II is significantly different from the pore structure in its absence.

In the absence of Lipid II, nisin follows a mode of action similar to other pore-forming antibacterial peptides, like magainin (*47*), which is best described by the Shai-Matsuzaki-Huang model (*12*). These peptides form transient wormhole-like pores in which the phospholipids can freely move between the peptide monomers (*47*, *48*). Several results we have obtained in this study suggest that the pores formed by nisin in the presence of Lipid II do not have this wormhole-like formation. Our observation that the fluorescence of 1W could not be quenched by spin-labeled lipids including one with a quenching moiety at the headgroup, but was accessible to acrylamide, suggests that the pore structure of nisin in the presence of Lipid II is closed laterally. Additionally, the quenching efficiency of the spin-labeled lipids (*49*) for 17W was low in comparison to the efficiency found for these quenchers in the absence of Lipid II (*28*). Such a low accessibility for lipidic quenchers would not be expected, especially for such a deeply embedded residue, if the phospholipids were free to diffuse along the peptide monomers (*43*, *50*). Although this still remains speculation at this point, Lipid II probably forms a tight seal between the nisin molecules, acting like the mortar between the bricks of a wall. The observations that the N-terminus docks on the pyrophosphate and the 1:1 stoichiometry of the nisin−Lipid II interaction (*26*) [Bonev, B. B., and Breukink, E. (2002) (manuscript in preparation)] support the model of a tight nisin−Lipid II oligomeric pore complex.

This stability of the nisin−Lipid II pore complex offers ample opportunity for the elucidation of the structure of a pore formed by an antibiotic peptide up to atomic resolution. Future research will be directed toward this challenge.

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REFERENCES

1. . Delves-Broughton, J., Blackburn, P., Evans, R. J., and Hugenholtz, J. (1996) *Antonie Van Leeuwenhoek 69*, 193−202.
2. *2.* Hurst, A. (1981) *Adv. Appl. Microbiol. 27*, 85−123.
3. Jack, R. W., and Jung, G. (2000) *Curr. Opin. Chem. Biol. 4*, 310−317.
4. Sahl, H. G., Jack, R. W., and Bierbaum, G. (1995) *Eur. J Biochem. 230*, 827−853.
5. Kupke, T., and Gotz, F. (1996) *Antonie Van Leeuwenhoek 69*, 139−150.
6. Sahl, H. G., and Bierbaum, G. (1998) *Annu. Rev. Microbiol. 52*, 41−79.
7. Lian, L. Y., Chan, W. C., Morley, S. D., Roberts, G. C., Bycroft, B. W., and Jackson, D. (1992) *Biochem. J. 283*, 413−420.
8. van De Ven, F. J., and Jung, G. (1996) *Antonie Van Leeuwenhoek 69*, 99−107.
9. Van de Ven, F. J., Van den Hooven, H. W., Konings, R. N., and Hilbers, C. W. (1991) *Eur. J. Biochem. 202*, 1181−1188.

10. van den Hooven, H. W., Doeland, C. C. M., van de Kamp, M., Konings, R. N. H., Hilbers, C. W., and van de Ven, F. J. M. (1996) *Eur. J. Biochem. 235*, 382−393.
11. Lins, L., Ducarme, P., Breukink, E., and Brasseur, R. (1999) *Biochim. Biophys. Acta 1420*, 111−120.
12. Zasloff, M. (2002) *Nature 415*, 389−395.
13. Hancock, R. E., and Chapple, D. S. (1999) *Antimicrob. Agents Chemother. 43*, 1317−1323.
14. Bechinger, B. (1997) *J. Membr. Biol. 156*, 197−211.
15. Shai, Y. (1999) *Biochim. Biophys. Acta 1462*, 55−70.
16. Kordel, M., Schuller, F., and Sahl, H. G. (1989) *FEBS Lett. 244*, 99−102.
17. Breukink, E., van Kraaij, C., Demel, R. A., Siezen, R. J., Kuipers, O. P., and de Kruijff, B. (1997) *Biochemistry 36*, 6968−6976.
18. el-Jastimi, R., and Lafleur, M. (1997) *Biochim. Biophys. Acta 1324*, 151−158.
19. Driessen, A. J., van den Hooven, H. W., Kuiper, W., van de Kamp, M., Sahl, H. G., Konings, R. N., and Konings, W. N. (1995) *Biochemistry 34*, 1606−1614.
20. Giffard, C. J., Dodd, H. M., Horn, N., Ladha, S., Mackie, A. R., Parr, A., Gasson, M. J., and Sanders, D. (1997) *Biochemistry 36*, 3802−3810.
21. Breukink, E., and de Kruijff, B. (1999) *Biochim. Biophys. Acta 1462*, 223−234.
22. Breukink, E., Wiedemann, I., van Kraaij, C., Kuipers, O. P., Sahl, H., and de Kruijff, B. (1999) *Science 286*, 2361−2364.
23. Brotz, H., Josten, M., Wiedemann, I., Schneider, U., Gotz, F., Bierbaum, G., and Sahl, H. G. (1998) *Mol. Microbiol. 30*, 317−327.
24. van Heijenoort, J. (1994) in *Bacterial Cell Wall* (Hakenbeck, R., Ed.) pp 39−72, Elsevier, Amsterdam.
25. van Heijenoort, J. (2001) *Glycobiology 11*, 25R−36R.
26. Wiedemann, I., Breukink, E., van Kraaij, C., Kuipers, O. P., Bierbaum, G., de Kruijff, B., and Sahl, H. G. (2001) *J. Biol. Chem. 276*, 1772−1779.
27. Soulages, J. L., and Arrese, E. L. (2000) *Biochemistry 39*, 10574−10580.
28. Breukink, E., van Kraaij, C., van Dalen, A., Demel, R. A., Siezen, R. J., de Kruijff, B., and Kuipers, O. P. (1998) *Biochemistry 37*, 8153−8162.
29. Kuipers, O. P., Bierbaum, G., Ottenwalder, B., Dodd, H. M., Horn, N., Metzger, J., Kupke, T., Gnau, V., Bongers, R., van den Bogaard, P., Kosters, H., Rollema, H. S., de Vos, W. M., Siezen, R. J., Jung, G., Gotz, F., Sahl, H. G., and Gasson, M. J. (1996) *Antonie Van Leeuwenhoek 69*, 161−169.
30. Ralston, E., Hjelmeland, L. M., Klausner, R. D., Weinstein, J. N., and Blumenthal, R. (1981) *Biochim. Biophys. Acta 649*, 133−137.
31. Hope, M., Bally, M. B., Webb, G., and Cullis, P. R. (1985) *Biochim. Biophys. Acta 812*, 55−65.
32. de Ropp, J. S., and Troy, F. A. (1985) *J. Biol. Chem. 260*, 15669−15674.
33. Abrams, F. S., and London, E. (1993) *Biochemistry 32*, 10826−10831.
34. Rouser, G., Fkeischer, S., and Yamamoto, A. (1970) *Lipids 5*, 494−496.
35. Lakowicz, J. R. (1999) in *Principles of fluorescence spectroscopy* (Lakowicz, J. R., Ed.) pp 445−486, Kluwer Academic/Plenum Publishers, New York.
36. Eftink, M. R., and Ghiron, C. A. (1976) *Biochemistry 15*, 672−680.
37. Ladokhin, A. S. (1999) *Biophys. J. 76*, 946−955.
38. de Kroon, A. I. P. M., Soekarjo, M. W., de Gier, J., and de Kruijff, B. (1990) *Biochemistry 29*, 8229−8240.
39. Ladokhin, A. S. (1997) *Methods Enzymol. 278*, 462−473.
40. Chattopadhyay, A., and London, E. (1987) *Biochemistry 26*, 39−45.
41. Cowgill, R. W. (1970) *Biochim. Biophys. Acta 207*, 556−559.
42. Steiner, R. F., and Kirby, E. P. (1969) *J. Phys. Chem. 73*, 4130−4135.
43. Yeager, M. D., and Feigenson, G. W. (1990) *Biochemistry 29*, 4380−4392.
44. Hsu, S.-T., Breukink, E., de Kruijff, B., Kaptein, R., Bonvin, A. M. J. J., and van Nuland, N. A. (2002) *Biochemistry 41*, 7670−7676.
45. Goodman, M., Palmer, D. E., Mierke, D., Ro, S., Nunami, K., Wakamiya, T., Fukase, K., Horimoto, S., Kitazawa, M., Fujita, H., Kubo, A., and Shiba, T. (1991) in *Nisin and novel lantibiotics* (Jung, G., and ÊSahl, H.-G., Eds.) pp 59−75, ESCOM Science Publishers, Leiden.
46. Sahl, H. G., Kordel, M., and Benz, R. (1987) *Arch. Microbiol. 149*, 120−124.
47. Matsuzaki, K., Sugishita, K., and Miyajima, K. (1999) *FEBS Lett. 449*, 221−224.
48. Ludtke, S. J., He, K., Heller, W. T., Harroun, T. A., Yang, L., and Huang, H. W. (1996) *Biochemistry 35*, 13723−13728.
49. Kachel, K., Asuncion-Punzalan, E., and London, E. (1995) *Biochemistry 34*, 15475−15479.
50. Ladokhin, A. S., and Holloway, P. W. (1995) *Biophys. J. 69*, 506−517.
51. Wimley, W. C., and White, S. H. (2000) *Biochemistry 39*, 4432−4442.
52. Ladokhin, A. S. (1999) *Anal. Biochem. 276*, 65−71.

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