Lipid II Is an Intrinsic Component of the Pore Induced by Nisin in Bacterial Membranes*

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Eefjan Breukink†‡§, Hester E. van Heusden‡, Pauline J. Vollmerhaus†, Ewa Swiezewska†, Livia Brunner∗**, Suzanne Walker∗**, Albert J. R. Heck†, and Ben de Kruijff§

The peptidoglycan layers surrounding bacterial membranes are essential for bacterial cell survival and provide an important target for antibiotics. Many antibiotics have mechanisms of action that involve binding to Lipid II, the prenyl chain-linked donor of the peptidoglycan building blocks. One of these antibiotics, the pore-forming peptide nisin uses Lipid II as a receptor molecule to increase its antimicrobial efficacy dramatically. Nisin is the first example of a targeted membrane-permeabilizing peptide antibiotic. However, it was not known whether Lipid II functions only as a receptor to recruit nisin to bacterial membranes, thus increasing its specificity for bacterial cells, or whether it also plays a role in pore formation. We have developed a new method to produce large amounts of Lipid II and variants thereof so that we can address the role of the lipid-linked disaccharide in the activity of nisin. We show here that Lipid II is not only the receptor for nisin but an intrinsic component of the pore formed by nisin, and we present a new model for the pore complex that includes Lipid II.

The cell wall is an essential structure of a bacterium, providing its shape and protecting it from bursting because of the high osmotic pressures of the cytoplasm. This wall is a three-dimensional network built of identical subunits consisting of two amino sugars, N-acetylmuramic acid (GlcNAc) and N-acetylmuramic acid (MurNAc). A pentapeptide, often L-alanyl-glycyl-glycin-glycin-glycin (or L-lysyl-N-alanyl-N-alanine) is attached to the carboxyl group of MurNAc. These subunits are assembled in the cytosol of bacteria using UDP-activated precursors on a special lipid carrier, undecaprenyl phosphate (for a review see Ref. 1). The integral membrane protein MraY and the peripherally membrane-associated MurG that synthesize the precursors Lipid I and II, respectively, are the key enzymes in the last two cytoplasmic steps in the formation of the subunits (Fig. 1). Subsequently, Lipid II is transported across the plasma membrane via an as of yet unknown mechanism. Thereafter, the subunits are polymerized and inserted into the pre-existing cell wall by means of the penicillin-binding proteins (for review see Ref. 2). Numerous antibiotics target the cell wall synthesis, including a diverse group of antibiotics that bind to Lipid II. Perhaps the best known of these antibiotics is vancomycin, the antibiotic of last resort to treat MRSA infections (3). However, there are many others, including the polypeptide nisin, that kill cells by permeabilizing bacterial membranes. Efficient membrane permeabilization by nisin requires an interaction with Lipid II (4, 5). This designates nisin as the first example of a targeted pore-forming peptide antibiotic.

Two recent studies have shed light on the structural requirements within nisin for the interaction with Lipid II. A genetic approach indicated that the N terminus of nisin is involved in the interaction with Lipid II (6), and more recently we could map the binding interface toward specific residues in the N terminus using 15N-labeled nisin (7). It is not clear yet what events lead to pore formation after the initial interaction. Information on later stages in the mode of action of nisin arose from studies using site-specific tryptophan fluorescence experiments. Here it was shown that in the presence of Lipid II, nisin has a stable transmembrane orientation (8). So far, understanding the mode of action of nisin has been focused on nisin itself and not on Lipid II, the receptor it docks on. The role of Lipid II in the mode of action of nisin is largely obscure but is intriguing because tryptophan fluorescence measurements indicated that Lipid II plays additional roles besides acting merely as a receptor for nisin: the presence of Lipid II switches the orientation of nisin from parallel to perpendicular with respect to the membrane surface. Research on Lipid II in general is hampered by the very limited availability of the molecule. To obtain insight into the role of Lipid II in the mode of action of nisin we developed a novel and versatile synthesis route to Lipid II and variants, making use of membrane preparations rich in MraY and MurG supplied with polyisoprenyl phosphates of different chemical composition and length. Using these variants we show that the length of the prenyl chain of Lipid II plays an important role in the activity of nisin, i.e. in maintaining pore stability, but a prenyl chain is not essential. Additionally, by using a Lipid II carrying a pyrene label it could be shown by fluorescence experiments that upon addition of nisin, Lipid II gets recruited into a stable pore structure. Hence, Lipid II is an integral part of the nisin pore.

**EXPERIMENTAL PROCEDURES**

**Materials**

Nisin Z was produced by batch fermentation and purified as previously described (9). 1,2 dioleoyl-sn-glycerol-3-phosphocholine (DOPC)1 was purchased from Avanti Polar Lipids, Inc. UDP-N-acetylmuramic acid (UDP-GlcNAc) was purchased from Sigma. All other chemicals were of analytical grade or better. Long chain polyisoprenes were used.

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1 The abbreviations used are: DOPC, 1,2 dioleoyl-sn-glycerol-3-phosphocholine; E/M, excimer-monomer ratio.
isolated from the appropriate natural source (11) and phosphorylated as described (10). Geranyl-, farnesyl-, and geranylgeranyl phosphates were synthesized from, respectively, geraniol (ICN), farnesol (ICN), and geranylgeraniol (Sigma) as described (11). Right-side-out vesicles of Micrococcus flavus were prepared and isolated as described (12) and stored in liquid nitrogen until use.

Methods

Isolation and Purification and Labeling of UDP-MurNAc-pentapeptide

The isolation and purification of UDP-MurNAc-pentapeptide (lysinyl form) from Staphylococcus simulans was performed as described (15). UDP-MurNAc-pentapeptide was labeled with the lysine residue with the use of pyrenechloride (Molecular Probes); UDP-MurNAc-pentapeptide was dissolved in acetonitrile/100 mM sodium carbonate, pH 9.0 (7:3, v/v), followed by the addition of pyrenechloride (dissolved in N,N-dimethylformamide) up to a 2:1 molar excess. The mixture was incubated for 30 min at room temperature, followed by a C18 HPLC column, and purified using a linear gradient from 50 mM ammonium bicarbonate to 100% methanol.

Synthesis and Purification of Lipid II

Synthesis and Purification of Lipid II-containing Polyprenyl Chains of 4–25 Isoprene Units—M. flavus vesicles (40–80 nmol lipid-P) were incubated together with 100 nmol UDP-GlcNAc, 100 nmol UDP-MurNAc-pentapeptide, 15 nmol polyprenyl phosphate, in 150 μl of buffer containing 100 mM Tris-HCl, pH 8.5, 5 mM MgCl₂, and 1% (w/v) Triton X-100. The suspension was incubated at room temperature for 1 h, followed by extraction of the lipids by 200 μl of 300 mM ammonium bicarbonate (2:3:1). The mixture was then poured into a flask containing 15 ml of cold methanol and concentrated to 1.5 ml. The resulting white suspension was loaded with a step gradient of water/acetonitrile containing 0.1% ammonium bicarbonate (starting from 100% water and decreasing by 5% after each 10 ml). The product eluted in 75–80% water/acetonitrile.

Mass Spectrometry

Electrospray mass spectrometry spectra were recorded with a Micromass Z-spray nanoflow ion source (Micromass Ltd., Manchester, UK). The short chain Lipid II fractions were diluted in 25 mM ammonium acetate (acidified to pH 5 with acetic acid) to a concentration of 20–25 μM, whereas 11-Lipid II was dissolved in ammonium acetate/methanol (50:70, v/v). The capillary and cone voltages were 1.5 kV and 30 V, respectively. The source block temperature was maintained at 80 °C. Spectra were acquired and stored with Micromass MassLynx version 3.4 software.

Fluorescence Experiments

Fluorescence experiments were performed with a SLM-Aminco SPF-500 C fluorometer. All samples (1.2 ml) were continuously stirred in a 10 × 4-mm quartz cuvette and kept at 20 °C using a water bath with continuous circulation.

Carboxyfluorescein-loaded DOPC vesicles containing 0.1 mol % of Lipid II with varying chain composition were obtained as described (6). The pore-forming activity of 100 nm nisin toward 25 μM carboxyfluorescein-loaded vesicles containing 0.1 mol % Lipid II with varying membrane anchors was determined as described (5). Pore stability was tested by preincubation of 125 nm nisin with 250 μM "empty" DOPC vesicles containing 0.1 mol % Lipid II with varying chains at room temperature in a total volume of 200 μl for 2 min. Subsequently, 100 μl of this mix was added to the cuvette containing carboxyfluorescein-labeled vesicles carrying 0.1 mol % 11-Lipid II, such that the final concentrations were 10 nm nisin, 20 μM empty variant-Lipid II-containing vesicles and 10 μM 11-Lipid II-containing carboxyfluorescein-loaded vesicles.

Pyrene excimer formation was followed with spectral recordings between 360 and 550 nm (λex 350 nm, bandwidth 5 nm) using DOPC vesicles containing 0.1–20 mol % pyrene-labeled Lipid II. The effect of nisin on pyrene-labeled Lipid II was measured using 50 μM DOPC vesicles containing 0.5 mol % pyrene-labeled Lipid II. To prevent influence of quenching effects of nisin on the monomer fluorescence of pyrene on the excimer/monomer ratios, these were calculated using the monomer fluorescence of the initial sample before nisin addition.

RESULTS

Synthesis of Lipid II and Variants—To study lipid II interacting with nisin it is essential to have sufficient amounts of this molecule and variants thereof. So far, access to lipid II and variants was limited to groups having considerable synthetic expertise (14, 16, 17). We reasoned that it should be possible to produce Lipid II if membrane preparations carrying sufficient MraY and MurG activity were provided with the appropriate UDP-activated amino sugars and undecaprenyl phosphate in the presence of a suitable detergent. Indeed, total conversion of undecaprenyl phosphate (11-P) to Lipid II could be observed in the presence of sufficient amounts of substrates and enzyme activity (Fig. 2, compare lanes 1–3). In the absence of UDP-GlcNAc, Lipid I production was evident from a product running in lane 4, whereas addition of only UDP-GlcNAc to the reaction mixture did not result in a detectable conversion of 11-P (lane 5). Also, fluorescently labeled Lipid II variants were produced if labeled UDP-MurNAc-pentapeptide were used (lanes 6–7). From these reaction mixtures Lipid I and II purification is relatively straightforward, and production of 50–100-mg quantities can be easily achieved with purities.
around 98% (not shown). This approach can be applied similarly to various polyisoprenyl phosphate substrates (Table I) with the exception of the short chain polyprenyls, geranyl and farnesyl phosphate. Both isopentenyl- and dimethylallylphosphate were tested. Both geranylgeranyl and phytyl phosphate are substrates.

![Fig. 2. Bypassing the bacterial membrane limits for 11-P. Lipid II synthesis was performed as described under “Methods” using bacterial membranes of M. flavus with no external addition of 11-P (lane 1) and with addition of 10 nmol 11-P (lane 2). Lanes 1–6, spots were stained by iodine vapor. Lane 7, UV detection of pyrene-labeled Lipid II. a, pyrene-labeled UDP-MurNAc-pentapeptide was used in this case.](image)

**Table 1.** Substrate specificity of the Lipid II-synthesizing enzymes for polyisoprenoid phosphates

| n-prenyl¹ | Substrate² | Solubility in water |
|-----------|------------|---------------------|
| 1-P       | No²        | +                   |
| 2-P       | Yes        | +                   |
| 3-P       | Yes        | +                   |
| 4-P       | Yes        | +                   |
| 7-P       | Yes        | –                   |
| 8-P       | Yes        | –                   |
| 11-P or PP| Yes        | –                   |
| 12-PP     | Yes        | –                   |
| 15-PP     | Yes        | –                   |
| 18-PP     | Yes        | –                   |
| 21-PP     | Yes        | –                   |
| 25-PP     | Yes        | –                   |
| Dol-8-P   | Yes        | –                   |
| Dol-18-PP | Yes        | –                   |
| Alkyl-C12-P | Yes      | –                   |

¹ n denotes the number of isoprenoid units that compose the polyprenyl chain.
² Both isopentenyl- and dimethylallylphosphate were tested.
³ Both geranylgeranyl and phytyl phosphate are substrates.
⁴ Synthesis included ATP to allow conversion into 11-P.

**Prenyl Chain Length Dependence of the Pore-forming Activity of Nisin**—The availability of Lipid II variants with different composition and/or length of the polyprenyl chain allowed us to study the importance of the unusual bactoprenol membrane anchor of Lipid II for nisin-mediated pore formation. As shown in Fig. 4, Lipid II with a wild-type undecaprenyl (11-LII) chain resulted in nisin activities that were close to optimal. Lipid II variants that contained a longer prenyl chain were tolerated and resulted in only a minor drop in nisin activity. With shorter prenyl chains a different picture was observed. First, a slight increase in leakage could be observed for the nisin activity in the presence of 7-Lipid II. Then, upon further shortening the prenyl chain to 4 isoprene units, a severe drop in the nisin activity could be observed. For 3-Lipid II-containing vesicles, the nisin activity dropped even further to the level obtained for the control vesicles containing no Lipid II. Evidently, short prenyl chains are tolerated much less by nisin.

In addition, nisin displayed similar activity against a Lipid II variant in which the α-prenyl unit is saturated (dolichol-type polysoprenyl chain) as compared with a polyprenyl chain of similar length. Thus, nisin does not discriminate at the level of the α-prenyl unit. Interestingly, nisin activity could even be observed if a Lipid II variant was used carrying a C20-alkyl chain anchor, which has a length comparable with 5 isoprene units. The amount of leakage fell in between the values of 4- and 7-Lipid II. These results indicate that for optimal nisin activity, the Lipid II headgroup should be well anchored in the membrane by a hydrophobic tail of which the chemical composition is not of great importance.

These lower activities of nisin with the chain length variants of Lipid II could be caused by an inhibition of the receptor function of Lipid II. However, this is highly unlikely for two reasons. First, the Lipid II headgroup is identical for all length variants; second, the results with the C20-alkyl-Lipid II suggest no specific interaction of nisin with the prenyl chain. Thus, it is more likely that later stages in the mode of action of nisin are affected, like the pore stability or structure. To test whether
pore stability is affected, we preincubated nisin with DOPC vesicles containing 0.1 mol % of variant-Lipid II but lacking carboxyfluorescein. The stoichiometry of the nisin-Lipid II interaction was previously determined to be 1:1 (5, 6); therefore, to ensure complete binding of nisin the nisin/Lipid II ratio was 1:2 during preincubation. Subsequently, the mixture was tested for (residual) nisin activity by adding it to DOPC vesicles containing both 11-Lipid II and carboxyfluorescein (Fig. 5). For control purposes, nisin was first preincubated with DOPC vesicles lacking Lipid II. In this case, a maximum amount of leakage (about 60%) is obtained as expected, considering the low affinity of nisin for DOPC membranes (22, 23). Hardly any activity of nisin was detected if nisin was preincubated with Lipid II variants with prenyl chains of 7 isoprene units or longer, indicating that pores formed by nisin with these variants are stable. In contrast, preincubation of nisin with Lipid II variants with shorter anchors did result in significant nisin activity, and the nisin activity showed inverse dependence on the anchor length. Thus, the interaction of nisin with short chain Lipid II variants leads to the formation of unstable pores.

Further support for the formation of unstable pores by nisin in the presence of 4-Lipid II came from analysis of the leakage kinetics, which showed that an amount of nisin was continuously released from the complex, with 4-Lipid II resulting in leakage rates of the carboxyfluorescein from the 11-Lipid II-containing vesicles that were linear in time as opposed to leakage rates that leveled off when a fixed amount of nisin was added to the 11-Lipid II-containing vesicles (not shown). Our conclusion that the length rather than the nature of the hydrophobic chain determines pore stability is supported by the nisin activities obtained after preincubation with the long chain dolichol-type Lipid II and the short chain C20-alkyl Lipid II-containing vesicles (compare Figs. 5 and 4).

**FIG. 3.** Mass spectra of Lipid II variants. A, mass spectrum of 11-LII. The inset is a zoomed-in portion containing the doubly protonated ion of 11-LII and adducts of ammonia and sodium. The 11-LII sample was dissolved in 30/70 H2O/MeOH. B, mass spectrum of 3-LII. The inset is a zoomed-in portion of the spectrum containing the protonated ion of 3-LII. See “Results” for details.

**FIG. 4.** Activity of nisin toward vesicles containing Lipid II variants with different chain lengths and composition. Nisin (100 nM) was added to carboxyfluorescein-loaded DOPC vesicles (25 μM lipid-P) containing 0.1 mol % of n-Lipid II. The amount of leakage was determined 1 min after the addition of nisin. The bars represent the average of two experiments. The numbers denote the amount of prenyl units present in the prenyl chain. Dol-LII is a Lipid II variant with a dolichol chain of 18–20 prenyl units. C20-Lipid II is a Lipid II variant containing an alkyl chain 20 C atoms long. Control represents the amount of leakage that is obtained from DOPC vesicles in the absence of Lipid II.

- **chain length vs. CF-leakage (%)**
  - 3-LII
  - 4-LII
  - C6-11-LII
  - 7-LII
  - 11-LII
  - 16-LII
  - 18-LII
  - 21-LII
  - Dol-LII
  - Control

- **m/z vs. m/z**
  - [Headgroup + H]^+”
  - [β-LE + H]^+”
  - [β-LE + 6H]^+”
  - [β-LE + 11H]^+”
  - [β-LE + 16H]^+”
  - [β-LE + 21H]^+”
  - [β-LE + 26H]^+”
  - [β-LE + 31H]^+”
  - [β-LE + 36H]^+”
  - [β-LE + 41H]^+”
  - [β-LE + 46H]^+”
Assembly of a Pore Complex

Fig. 5. Assay for the stability of the nisin-Lipid II complex. Nisin was preincubated in the presence of empty DOPC vesicles containing 2-fold excess of n-Lipid II and subsequently added to carboxyfluorescein-loaded DOPC vesicles containing 0.1 mol % 11-Lipid II. Final concentrations were 10 nM nisin, 10 μM carboxyfluorescein-vesicles containing 0.1 mol % 11-Lipid II, and 20 μM empty vesicles containing 0.1 mol % n-Lipid II. The numbers denote the amount of prenyl units present in the prenyl chain. Dol-LII is a Lipid II variant with a dolichol chain of 18 prenyl units present in the prenyl chain. C20-Lipid II is a Lipid II variant containing an alkyl chain 20 C atoms long. The control represents the amount of leakage that is obtained from 10 nM nisin after preincubation with DOPC vesicles in the absence of Lipid II.

The effect of prenyl chain length on pore stability strongly suggests that Lipid II plays additional roles in the mode of action of nisin besides its receptor function. Therefore, we hypothesized that Lipid II may actually be part of the pore complex. 

Lipid II Is an Integral Part of the Pore Formed by Nisin—To test whether Lipid II is part of the pore complex, we chose to label it with pyrene. Pyrene monomers exhibit characteristic fluorescence emission maxima at ~378, 398, and 417 nm. In addition, pyrene can display a unique fluorescence peak at longer wavelengths (~490 nm), which occurs only when two pyrene rings reside within 10 Å of each other and form an excited state dimer, which is called an excimer. Because of this property, pyrene has been extensively used to address protein conformational alterations and spatial proximity issues (24, 25) as well as to study the behavior of lipids in membranes (for review see Refs. 26 and 27).

To test whether this approach is also applicable to pyrene-labeled Lipid II we first examined the fluorescence characteristics of pyrene-Lipid II in DOPC membranes (Fig. 6). Hardly any excimer fluorescence was observable at concentrations below 2 mol % Lipid II. Above this concentration, the monomer fluorescence peaks started to decrease upon increasing Lipid II concentration; concomitantly, excimer fluorescence centered at 490 nm appeared. Because the nisin activity was only marginally affected by the presence of the pyrene moiety on Lipid II (not shown), pyrene-Lipid II was considered to be a suitable probe to test whether nisin and Lipid II form an oligomeric complex.

Upon addition of increasing nisin concentrations to DOPC vesicles containing a low amount of Lipid II (0.5%), excimer fluorescence appeared (Fig. 7) that coincided with a decrease in the monomer fluorescence. This immediately demonstrates that nisin brings Lipid II molecules together, most likely by recruiting them into a pore structure. The relationship between the excimer/monomer ratio (E/M) and the nisin concentration is depicted in the insert. The increase in the E/M ratio levels off around 0.5 μM nisin to a final value of 0.06. This final E/M ratio was independent of the initial pyrene-Lipid II concentration in the vesicles (tested for 0.1–1.0% Lipid II, not shown), which is indicative of a fixed distance between Lipid II molecules within the oligomeric structure and also indicates that this oligomeric structure is uniform with respect to the number of Lipid II molecules it contains.

DISCUSSION

In this study we have determined the fate of Lipid II upon interaction with the pore-forming peptide nisin and the role of the prenyl chain of Lipid II in this interaction. This was made possible because of a new method we developed to produce Lipid II (and Lipid I) in large amounts and, if desired, with varying chain lengths and/or fluorescent-labeled. The chain variants of Lipid II showed that for optimal nisin activity a minimal chain length is required to obtain a stable pore complex. Insufficient anchoring of the complex actually results in the release of nisin molecules (Fig. 5). This indicates that the stability of the nisin-Lipid II interaction is dominated by the stability of the pore complex. Obviously, the prenyl chain of Lipid II is important for the activity of nisin, but it is not essential because a C20-alkyl chain could substitute the prenyl chain. From this information it can be concluded that there is no specific interaction of nisin with the lipid anchor of Lipid II. Consequently, the interaction takes place specifically at the pyrophosphate-MurNAc(pentapeptide)-GlcNAc head-
group, which is mediated by the N terminus of nisin (6, 7).

These effects of the prenyl chain on late stages of the mode of action of nisin strongly suggested that Lipid II has additional roles besides that of a receptor merely catalyzing nisin insertion. With pyrene-labeled Lipid II we could show that nisin brings Lipid II molecules into close proximity. Because these experiments were performed under conditions in which nisin forms pores, we can conclude that Lipid II is recruited by nisin and is an actual constituent of the pore complex. In addition, the distance between two Lipid II molecules within the complex could be calculated to a first approximation from the final E/M ratio of the pyrene fluorescence of the complex. With help of the insert in Fig. 6, this ratio can be correlated to the E/M ratio of pyrene-Lipid II at a 10% concentration in a DOPC bilayer. Thus, the distance of two pyrene-labeled Lipid II molecules in the pore complex is equal to the average distance between these molecules when present at 10 mol % in a DOPC bilayer. If it is assumed that Lipid II was ideally mixed with the DOPC molecules and that the fluorescence characteristics of nisin-bound pyrene-Lipid II are comparable with that of the free molecule, the distance between two Lipid II molecules in the pore complex is estimated to be 18 Å (for these calculations, the surface area of Lipid II was estimated from molecular dynamics simulations to be 200 Å).²

Non-targeted antimicrobial peptides, like magainin and dermaseptin among others, are proposed not to possess a defined pore structure but rather can be present in multiple aggregational states (for review see Ref. 28). Our observation that the final E/M ratio of pyrene fluorescence was independent of the initial concentration of Lipid II in these vesicles (not shown) indicates that nisin-Lipid II pore complexes have a uniform structure composed of identical numbers of nisin and Lipid II molecules. This information, taking also into account the dimensions of the nisin-Lipid II pore that was determined to be 2 nm³ tempted us to propose a model for the pore complex. The dimensions of nisin in extended conformation are 2.2 × 2.7 × 4.2 nm. Thus, the most likely possible stoichiometry is a pore formed by five to eight nisin molecules and an identical number of Lipid II. Because there seems to be no restriction in length of the anchor, Lipid II most likely is situated at the outer boundaries of the pore complex. This also satisfies the 18 Å distance between two Lipid II molecules. This tight pore configuration does not allow phospholipid molecules to partition between two nisin molecules as was proposed before (8).

In conclusion, our method for Lipid II synthesis has allowed us to unravel the role of Lipid II in the mode of action of nisin. This synthesis method is very easy and will be invaluable for facilitating studies on the interaction of Lipid II with proteins and other antibiotics and for research on bacterial cell wall synthesis.

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