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Review

The lantibiotic nisin, a special case or not?

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Abstract

Nisin is a 34-residue-long peptide belonging to the group A lantibiotics with antimicrobial activity against Gram-positive bacteria. The presence of dehydrated residues and lanthionine rings (thioether bonds) in nisin, imposing structural restrains on the peptide, make it an interesting case for studying the mode of action. In addition, the relatively high activity (nM range) of nisin against Gram-positive bacteria indicates that nisin may be a special case in the large family of pore-forming peptides antibiotics. In this review, we attempted to dissect the mode of action of nisin concentrating on studies that used model membranes or biological membranes. The picture that emerges suggests that in model membrane systems, composed of only phospholipids, nisin behaves similar to the antimicrobial peptide magainin, albeit with an activity that is much lower as compared to its activity towards biological membranes. This difference can be contributed to a missing factor which nisin needs for its high activity. Novel results have identified the factor as Lipid II, a precursor in the bacterial cell wall synthesis. The special high affinity interaction of nisin with Lipid II resulting in high activity and the active role of Lipid II in the pore-formation process make nisin a special case.

Keywords: Antibiotic; Lipid II; Pore-forming; Membrane; Peptide

Contents

Abbreviations: DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphoserine; MIC, minimal inhibitory concentration; MurNAc, *N*-acetylmuramic acid; GlcNAc, *N*-acetylglucosamine; UDP, uridinediphosphate

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1. Introduction

Of all the antimicrobial peptides known, only a very few of them are actually allowed to be used either as a preservative in the food industry or as an antibiotic in health care. The 34-residue-long peptide nisin is one of these few, and has been used as a food preservative for a long time. It is part of the family of lantibiotics (lanthionine-containing antibiotics), which are modified peptides, and are divided into two groups, the elongated type A group and the globular type B group (for reviews see [1–3]). Of the latter group, it is believed that they predominantly inhibit enzyme reactions. Nisin is part of the type A group. The peptide shares similar characteristics with other antimicrobial peptides. It is overall positively charged (+4) and its structure possesses amphipathic properties. However, some structural properties make nisin rather special. Nisin is post-translationally modified such that serine and threonine residues are dehydrated to become dehydroalanine and dehydrobutyrine. Subsequently, five of the dehydrated residues are coupled to upstream cysteines, thus forming the thioether bonds that produce the characteristic lanthionine rings (Fig. 1). Two naturally occurring nisin variants that have similar activities, nisin A and nisin Z, have been found [4,5]. Nisin A differs from nisin Z in a single amino acid residue at position 27, being a histidine in nisin A and an asparagine in nisin Z [4]. The studies described in this review were done either with nisin A or Z. Since there is no difference in activities between the two peptides, this review will not discriminate between the two peptides and mainly use the term nisin. The thioether bonds give nisin two rigid ring systems, one N-terminally and one C-terminally located. A hinge region (residues 20–22), that often is found in antimicrobial peptides, separates the ring systems. Due to the ring structures, the nisin molecule is maintained in a screw-like conformation that possesses amphipathic characteristics in two ways: the N-terminal half of nisin is more hydrophobic than the C-terminal half; and the hydrophobic residues are located at the opposite side of the hydrophilic residues throughout the screw-like structure of the nisin molecule. Therefore, in contrast to the peptides described in this issue that adopt most of their (amphipathic, α-helical) structure only upon interaction with membranes, nisin already possesses a fair amount of structure in the aqueous phase. Only slight variations in the conformations of the N-terminus and the C-terminal five residues are observed upon binding to membrane-mimicking micelles [6,7].

Nisin kills its target by pore formation in the target membrane. Over the past 10 years, much insight into the mechanism of pore formation has been achieved (see [1,3,8]). This review will focus on the interactions of nisin with model and biological membranes, and the attempts that have been made to elucidate its mode of action. It will be divided into different sections that deal with the interaction of nisin with model and biological membranes. The last part of the review will focus on a recent discovery of great importance for understanding the mechanism of action.

2. The interaction of nisin with biological membranes

Nisin is predominantly active against Gram-positive bacteria. Only in conjunction with chemically induced damage of the outer membrane can it also act against Gram-negative bacteria, such as *Escherichia coli* or *Salmonella* species. Initially, the antimicrobial activity of nisin was thought to be caused by reacting with sulfhydryl groups of enzymes via the dehydro residues [9] or by inhibition of cell wall synthesis [10,11]. However, experiments with intact bac-

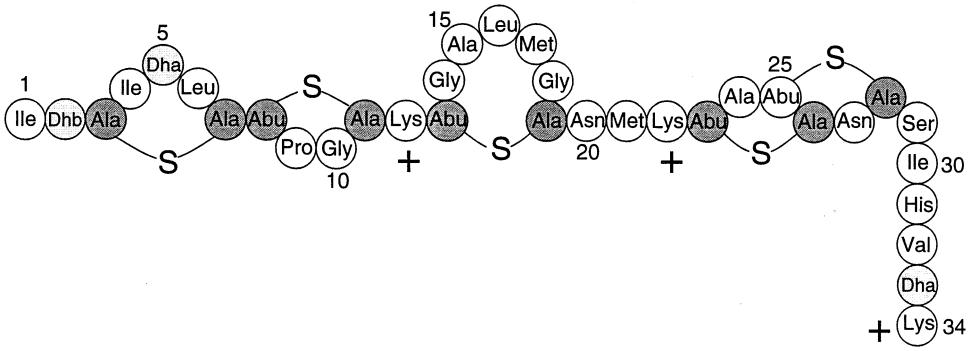

Fig. 1. The primary structure of nisin Z. The typical residues found in lanthionins are dark gray for lanthionine residues and light gray for dehydrated residues. Dha, dehydroalanine; Dhb, dehydrobutyrine, Ala–S–Ala, lanthionine; Abu–S–Ala, β-methyllanthionine.

terial cells and isolated plasma membrane vesicles have shown that treatment of nisin resulted in rapid efflux of small cytoplasmic compounds, e.g. amino acids, ATP, or pre-accumulated rubidium [12,13]. It is therefore now generally accepted that the bacterial plasma membrane is the target for nisin, and that nisin kills the cells by pore formation. The pore formation causes collapse of vital ion gradients and concomitant dissipation of the proton motive force of the bacteria, resulting in cell death.

3. The interaction of nisin with model membrane systems

Insight into the molecular aspects of pore formation by nisin has been obtained by using model systems. The first group to show that nisin can permeabilize model membranes composed of only phospholipids was the group of Sahl [14]. They showed that nisin at a peptide/lipid molar ratio above 1:20 could permeabilize membranes composed of the zwitterionic phospholipid DOPC. The positively charged nature of nisin would suggest that it should interact preferably with anionic lipids. Indeed, several studies indicated that nisin binds efficiently to membranes containing anionic lipids [14–18]. Surprisingly, in early studies, no leakage could be detected from membranes containing negatively charged lipids [14,17,19]. The anionic lipids even seemed to inhibit dye leakage from vesicles. Studies that are more recent gave a very different picture. Efficient dye leakage could only be detected in the presence of anionic lipids [15,18,20,21]. In addition, the activity against isolated bacterial membrane vesicles was strongly dependent on the presence of anionic lipids [15]. The reason for this discrepancy remains a mystery.

The mechanism leading to pore formation by nisin can be divided into different steps. Obviously, the first step is binding to the target membrane, most likely followed by insertion into the lipid phase of the membrane. This insertion step will finally lead to pore formation. We will discuss the interaction of nisin with model membranes further according to these steps.

3.1. Nisin binding

The basic character of nisin suggests that it preferably interacts with negatively charged membranes. Indeed, every group that has studied the binding of nisin to model membranes has shown that nisin binds preferably to membranes containing anionic lipids. The binding of nisin has been studied with varying methods. Most studies used indirect methods to measure nisin binding [14,17,18,22,23]. Two groups used direct methods based on centrifugation of lipid vesicles to separate membrane-bound peptides from free [15,16]. Only one study systematically varied the anionic lipid content of the membranes [15]. It was shown that beyond an anionic lipid content of 40%, the amount of bound nisin increased dramatically (Fig. 2). This suggests that nisin needs a relatively large amount of anionic lipids for efficient binding. In general, Gram-positive bacteria have relative higher concentrations of anionic lipid in their plasma membrane as compared to Gram-

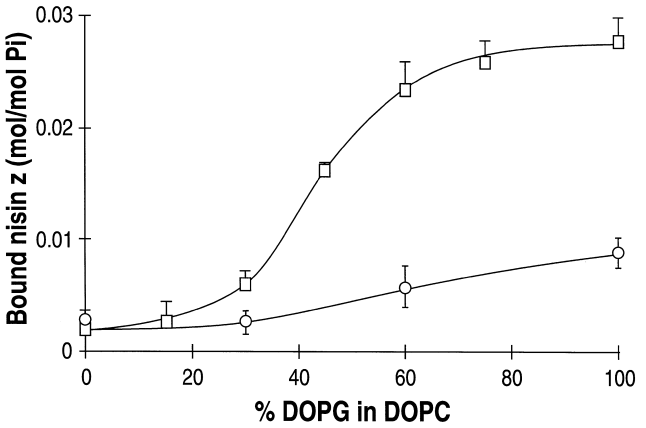

Fig. 2. Binding of nisin Z (□) and [Glu-32]-nisin Z (○) to lipid vesicles with different DOPG/DOPC ratios at a constant nisin: phospholipid molar ratio of 1:25. Data were redrawn from [15].

negative species [24]. Thus, this may partly explain the higher activity of nisin towards Gram-positive bacteria [15].

The binding isotherms of nisin displayed a biphasic character, suggesting a cooperative interaction [15]. Recent isothermal titration calorimetry (ITC) experiments showed that binding of nisin to DOPC/DOPG (3:1) membranes at low concentrations is largely determined by the surface charge (E. Breukink, J. Seelig and B. de Kruijff, unpublished observations). The intrinsic binding constant of nisin was about a factor of 10 lower than that of magainin [25]. This is in accordance with the relative activity of the two peptides in dye-leakage assays using model membranes of different composition (see Section 4).

Very useful information about the structural elements in nisin which are responsible for membrane binding came from studies using nisin variants derived by mutagenesis or chemical modification [15,18,23] or fragments of nisin [18]. Changes in the C-terminus had severe effects on the nisin binding. Introducing a negatively charged residue in the C-terminus on position 32 of nisin almost completely abolished the anionic lipid-dependent binding of nisin (Fig. 2) [15]. The apparent binding constant for this variant was also dramatically lowered as compared to wild-type nisin in ITC experiments (E. Breukink, J. Seelig and B. de Kruijff, unpublished observations). Fragments of the N-terminus of nisin (N1–12 and N1–20) displayed hardly any affinity for membranes as compared to full-length nisin [18]. In contrast to the large effects on nisin binding by changes in, or complete removal of the C-terminus, changes in the N-terminus had only minor effects. Even the loss of a positive charge by replacement of lysine-12 by a leucine residue did not effect the initial interaction of nisin with the membrane [18]. A major change that resulted in opening of the first lanthionine ring and concomitant loss of activity also hardly affected the binding of this variant [23]. Together, these results show that the C-terminus plays an important role in the binding of nisin to the target membrane by mediating the initial electrostatic interaction of nisin with the membrane. The results described above would suggest that one could increase the activity of nisin simply by increasing the positive charge of the C-terminus. However, this is not the case, since increasing the positive charge at the C-terminus by replacing valine at position 32 with lysine did not increase antibacterial activity [26], although the affinity of this mutant for membranes containing anionic lipids was increased compared to wild-type nisin (E. Breukink, J. Seelig and B. de Kruijff, unpublished observations). Moreover, adding more positive charges to the C-terminus even appeared to be detrimental for the activity of nisin [27] (see Section 3.3).

3.2. Nisin insertion and orientation

After binding of nisin to the membrane, the amphiphilic properties of the peptide allow it to insert into the lipid phase of the membrane. This insertion step has been studied using the monolayer technique [15,28], and quenching by depth probes of the fluorescence of tryptophan variants of nisin [20,29]. The monolayer studies showed that the presence of anionic phospholipids was essential for efficient insertion of nisin in the lipid phase of the membrane. Nisin variants with either extensions at the N-terminus, or with minor changes in the first ring, displayed severely reduced abilities to insert into lipid monolayers. In contrast, changes in the C-terminus hardly affected the ability of nisin to insert into the lipid phase of the membrane [15,27]. These results indicate that primarily the N-terminal part of nisin inserts into the lipid phase of the membrane. Calculation of the molecular hydrophobicity potential [30] of nisin shows that the N-terminus of the peptide is the

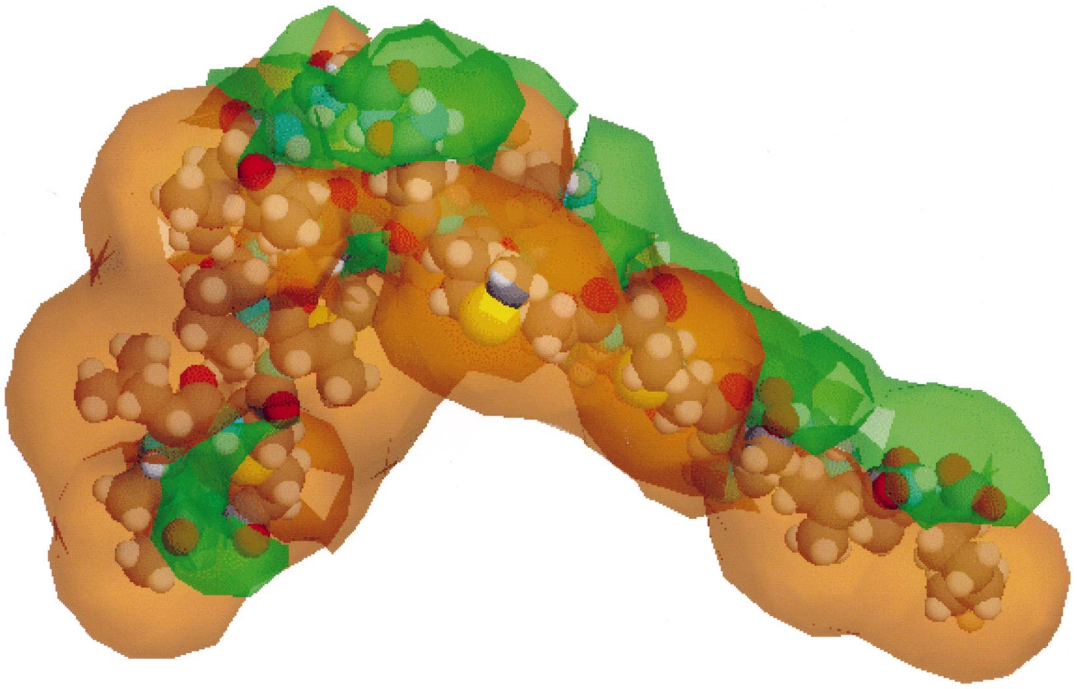

Fig. 3. Molecular hydrophobicity potential (MHP) around nisin which is represented in CPK. Green envelopes represent hydrophilic potentials and orange/brown envelopes, the hydrophobic ones. Figure adapted from Lins et al. [31].

most hydrophobic (Fig. 3), suggesting that hydrophobic interactions are predominantly responsible for insertion of the N-terminus of nisin into the lipid phase of the membrane [31].

These conclusions were supported by experiments using variants containing unique tryptophan residues at three positions (1, 17 and 32) in the molecule. By measuring the tryptophan fluorescence quenching by spin-labeled lipids, the position of the three tryptophan residues in the membrane was determined. It was shown that the tryptophan at the N-terminus had the deepest location in the membrane, while the C-terminal tryptophan was located close to the membrane surface. Moreover, it became clear that an increase in the amount of anionic lipids resulted in an increased depth of insertion of the nisin molecule. With the information on the depth of the tryptophan residues and the NMR structure of nisin [6], it became possible to estimate the orientation of nisin in the membrane (Fig. 4) [29]. As can be seen from this figure, the N-terminus is more deeply inserted than the C-terminus, while the peptide has an overall parallel orientation towards the membrane surface. However, from experiments using one variant of nisin possessing a tryptophan at position 30, it was concluded that the C-terminus inserted deeper into the membrane as compared to the N-terminus [20]. This is not consistent with the picture presented in Fig. 4 and therefore we propose another interpretation of these results. When a tryptophan residue is placed at position 30 in the structure of nisin as shown in Fig. 4, it indeed possesses a deep location in the bilayer, in agreement with the results of Martin et al. [20]. Thus, this residue is not deeply inserted into the bilayer due to deep insertion of the complete C-terminus, but merely possesses a relative deeper location in a nisin molecule, which maintains its overall parallel orientation to the bilayer. This shows the importance of using multiple tryptophans for these kind of experiments. Interestingly, with a totally independent approach using molecular dynamics calculations of nisin structures in a simulated bilayer interface, an orientation was predicted similar to that presented in Fig. 4 [31]. In conclusion, the stable orientation of nisin in the membrane is parallel with respect to the membrane surface.

3.3. Pore formation by nisin

Pore formation by nisin is most likely a coopera-

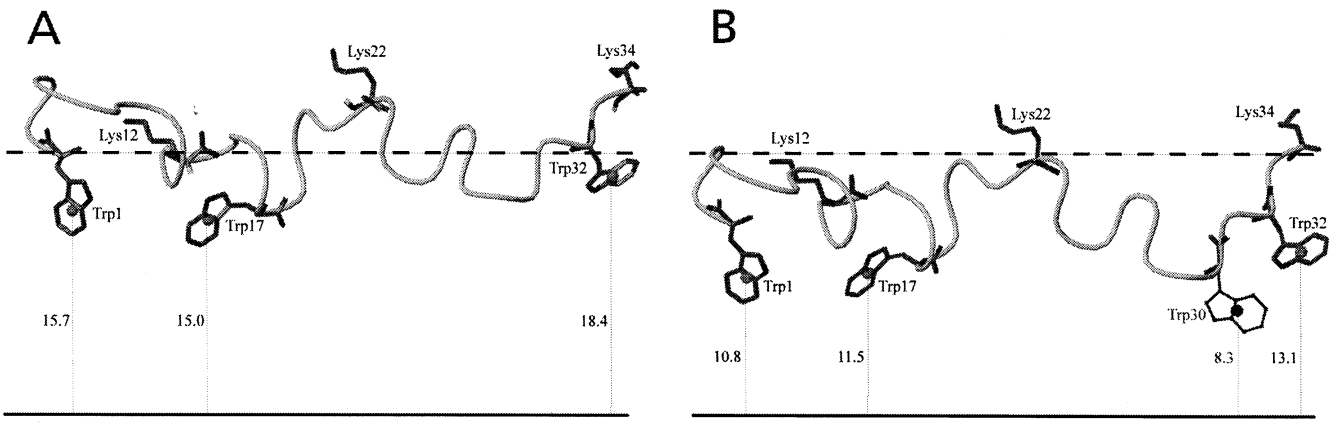

Fig. 4. Modelling of the orientation of nisin in the membrane. Shown are backbone tracings of the nisin structure in the presence of 50% PG (A) and 90% PG (B), showing only the tryptophan and lysine residues. A and B are views along the peptide axis. The larger dots in the tryptophan rings represent the centers of gravity of the tryptophan side chains which were used to position the tryptophans at their respective distance from the bilayer center (solid line). The dashed line represents the lipid–water interface. The tryptophan at position 30 in (B) was placed in the peptide structure after orientation of the peptide according to the depth profile of the other tryptophan residues, see text for further details. Figure adapted from [29].

tive process, which is preceded and/or accompanied by aggregation of peptides in the membrane. In several studies, evidence for aggregation of nisin in the membrane was found. The shape of the binding isotherms of nisin in the presence of anionic lipid-containing membranes were indicative of a process where the peptides aggregate once inside the membrane [15]. The fluorescence of the tryptophan variants of nisin displayed self-quenching most likely caused by aggregation of the peptides [29]. Results from planar lipid bilayer studies also suggested that aggregation of nisin monomers occurred in the membrane [22].

The actual pore formation by nisin was studied with dye-leakage assays that showed a strong dependency of the membrane permeabilizing activity of nisin on the presence of negatively charged lipids [15,18,20]. It seems that the anionic lipids promote the nisin activity independent of the headgroup type, since DOPS [18], cardiolipin [18,20] as well as DOPG [15], all promoted the nisin activity in leakage assays using calcein- [18,20] or carboxyfluorescein-based [15] leakage assays. The nisin-induced leakage of carboxyfluorescein from mixed DOPG/DOPC vesicles, displayed a similar dependency on the amount of DOPG present as was observed for the binding (compare Figs. 2 and 5). This suggests that the amount of bound nisin greatly influences the amount of leakage that occurs.

Only limited information about the pore state of nisin is available. The size of the nisin pore, estimated from black-lipid membrane experiments, was 1 nm [32]. From the preferential release of the negatively charged carboxyfluorescein over the positively charged potassium ion, it was proposed that nisin is anion-selective [15]. It was suggested that this is due to the alignment of positively charged residues in the nisin pore. Support for this comes from studies with a nisin variant in which lysine-12 was replaced by a leucine residue. The increased conductance and the decreased dependence on the polarity of the applied potential of pores produced by this variant caused the authors to suggest that the lysine at position 12 controls the flow of ions through the bilayer [18].

The fluorescence experiments discussed above indicated that nisin had an overall stable orientation, parallel with respect to the membrane surface. This seems to contradict the existence of a nisin pore, in which the nisin monomers possess a transmembrane orientation. Thus, it seems plausible to suggest that nisin permeabilizes the membrane due to local disturbance of the bilayer. Evidence against this suggestion came from recent experiments which showed that nisin-induced leakage is paralleled by translocation of its C-terminus across the membrane [27]. Hence, nisin must have obtained a transmembrane orientation at some point in time causing leakage of ions. To reconcile these two results, one has to

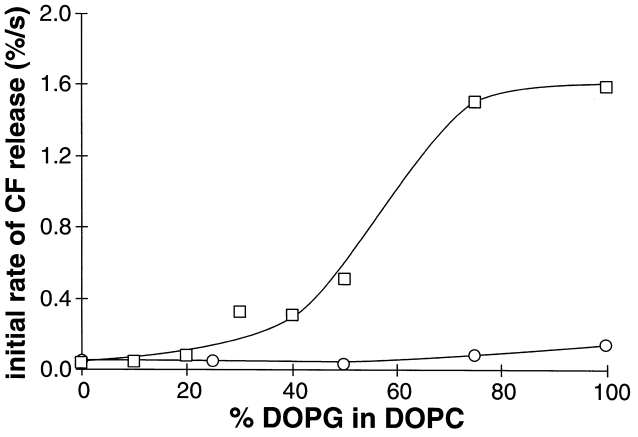

Fig. 5. The initial rate of nisin induced carboxyfluorescein leakage from vesicles with different DOPC/DOPG ratios. Nisin Z (□), and [Glu-32]-nisin Z (○). Data redrawn from [15].

assume that the nisin pore is of transient nature. This is in accordance with the short pore-lifetime of nisin in black-lipid membrane experiments, which is in the millisecond range [33].

A transient pore, together with translocation of the C-terminus suggests that nisin has the same mode of action (at least in phospholipid model membrane systems) as magainin, an antimicrobial peptide of animal origin. For this peptide, it was shown that it also forms transient pores and upon relaxation of the pore the peptide translocates in total to the inner leaflet of the membrane [34,35]. For nisin, it was only shown that the C-terminus translocates across the membrane upon pore formation. However, due to the relatively high concentration of peptides on the outer leaflet, it is unlikely that the peptides flip back to this leaflet upon relaxation of the pore. Rather, the peptides translocate completely relieving the stress on the outer leaflet.

It was suggested that phospholipids are active members of the pores formed by magainin [36]. Magainin pores are formed by both peptides and lipids, and resemble a torus, in which the outer monolayer of the membrane folds back to the inner monolayer. It could be calculated that the lipids in the pore possess an overall positive curvature [37]. It is possible that nisin possesses a similar pore structure. However, direct experimental evidence is lacking. Moreover, recent experiments using ^{31}P-NMR magic angle spinning (MAS) of phospholipid bilayers and wideline ^{2}H-NMR demonstrated that all the lipid molecules in the system were in a bilayer phase [38]. Interestingly, this study also demonstrated that nisin can selectively recruit the negatively charged lipid PG, which causes the lipid environment of nisin to be enriched in PG as compared to the overall lipid composition. This suggests that the negatively charged lipids in the membrane play a more active role than just providing binding sites.

3.4. The membrane potential

Nisin was shown to be less active against de-energized cells [12]. For instance, nisin could totally dissipate the membrane potential of *S. cohnii* cells which were fully energized, while it could only partially dissipate the membrane potential of starved cells. In addition, the activity of nisin against intact cells of *S. simulans* seemed to depend on the magnitude of the membrane potential [33]. It was therefore proposed that nisin needs an energized membrane for its activity. From experiments with isolated membrane vesicles similar conclusions were drawn [12,39].

In conjunction with the experiments with intact bacteria, nisin activity was dependent on the applied potential in model membrane experiments [17,19,32,33,40]. These experiments showed that a threshold potential was needed before any nisin activity could be measured [33]. The threshold potential was dependent on the amount of anionic lipids present [32]. Opposing membrane potentials inhibited the nisin activity [17,33].

Later studies also showed an effect of the presence of a membrane potential on nisin activity towards model membrane vesicles [15]. However, in the presence of high amounts of anionic lipids, nisin could efficiently induce dye leakage from vesicles in the absence of a membrane potential.

The action of the membrane potential is most likely in the initial stages of the pore formation. It probably acts via an electrophoretic action on the charges of nisin to pull it into the membrane, resulting eventually in a transmembrane orientation. In view of the above results on the insertion of the N-terminus of nisin, the membrane potential probably acts on the N-terminal positive charges, i.e. the N-terminus and lysine at position 12. In effect, the membrane potential lowers the energy barrier for pore formation. Support for this suggestion came

from studies using a nisin variant in which the lysine at position 12 was replaced by a leucine (K12L) [18]. This variant displayed no dependency on a threshold potential, which is most likely caused by the replacement of the positive charged residue in the N-terminus by a hydrophobic residue facilitating the insertion of nisin.

3.5. *Model of the pore formation by nisin in lipid bilayers*

A general model can be build up which describes the mechanism of action of nisin as deduced from model membrane experiments. Nisin first binds with its C-terminus via electrostatic interactions with the anionic lipids (Fig. 6, step I). This is then followed by dipping of the N-terminus into the lipid phase of the membrane, and the peptide adopts an overall parallel orientation with respect to the membrane surface (step II). Upon insertion into the membrane, nisin may locally disturb the phospholipids in such a way that it induces a positive interface curvature, especially in the presence of PG. In addition, nisin recruits negatively charged lipids, thus creating a locally higher concentration of these lipids in its surrounding area.

Previously, it has been proposed that nisin follows the barrel-stave model of pore formation [13,41]. In this model, peptides first adopt a transmembrane orientation before they aggregate to form water filled pores. As was correctly stated by Sahl [13], this would imply exposure of charged groups in the lipid phase of the bilayer, which is energetically highly unfavorable. Therefore, it is more likely that several (probably 4–6) peptides adopt a transmembrane orientation in a cooperative manner, probably involving pre-aggregation (although this remains to be resolved) of the peptides at the surface of the bilayer. Taking into consideration the deeper insertion of the N-terminus of nisin, it is tempting to speculate that initiation of pore formation results in translocation of the N-terminus to the inner leaflet of the membrane. Thus, nisin forms pores in which the N-termini are located on the *trans* side with respect to the side of addition (step III). Whether or not phospholipids are active members of the pore remains to be determined. The so-formed pore is not stable, and will fall apart (as can be concluded from the life

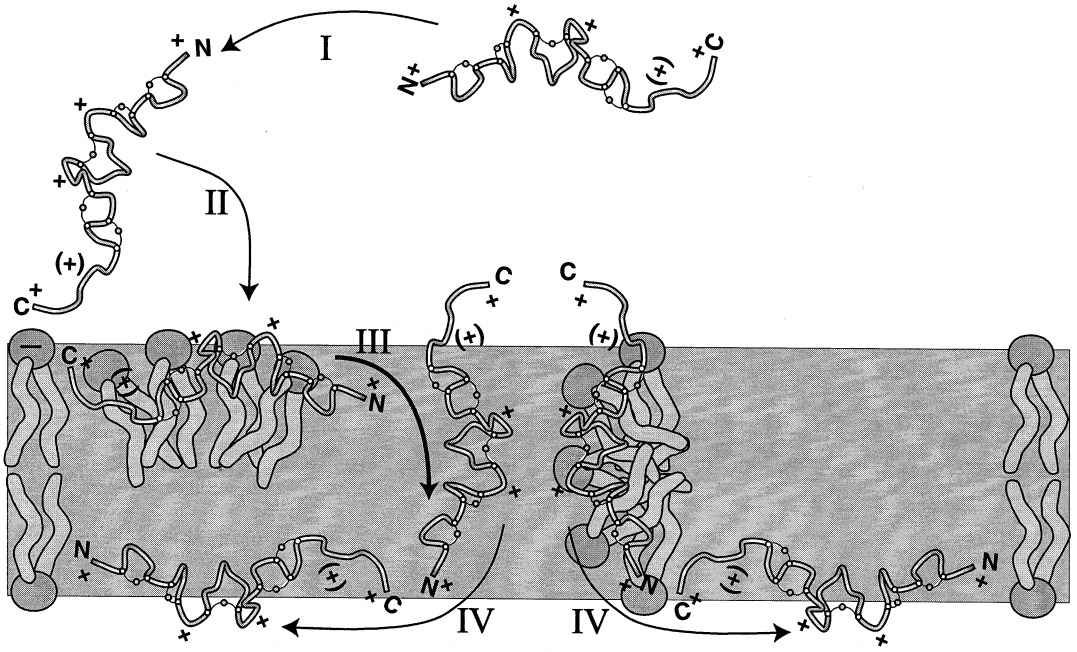

Fig. 6. Model for the mode of action of nisin. Binding of nisin via its C-terminus (step I), insertion of nisin into the membrane (step II), pore formation by nisin (step IV) in which the right part represents the situation when phospholipids are active members of the pore, and translocation of the whole peptide (step IV).

time described above). The relatively high peptide concentration on the outer leaflet of the bilayer will drive the relaxation of the pore towards the inner leaflet of the bilayer. In effect, the whole peptide might translocate during pore relaxation (step IV).

4. What makes nisin a special case?

The above-described model for the mechanism of action of nisin resembles the model for the mode of action proposed for the antimicrobial peptide magainin [36]. The similar characteristics of nisin as compared to magainin and related peptides, i.e. the positive charge and amphiphilic properties, probably account for these similar modes of action. When the activity of nisin is compared to the activity of magainin towards model membrane systems in dye-leakage experiments, the latter is even more active (Fig. 7A). This is actually in correspondence to the higher intrinsic binding constant of magainin as mentioned above. This raises the question of what the advantages are of the special structural elements that nisin possesses? This becomes evident when the activities of the two peptides against Gram-positive bacteria are compared. In MIC-value determinations, nisin is generally a factor 100–1000 more active than magainin against, e.g. micrococci, streptococci and staphylococci. Moreover, when the ability of the two peptides to dissipate the membrane potential of intact *M. flavus* bacteria was tested, nisin also displayed a factor of 1000 higher activity (Fig. 7B, Breukink et al. unpublished observations). These activities of nisin are in the nM range, while its activity in the model membrane systems described above are in the μM range. Apparently, the nisin activity towards intact bacteria is two to three orders of magnitude higher, which is not accounted for by an interaction solely with the phospholipids of the target membrane. Thus, there must be a factor present in the target membrane which somehow dramatically increases the nisin activity.

Indications about the identity of the unknown factor came from earlier studies which suggested that nisin inhibited the cell wall synthesis [11]. In agreement with this suggestion, it was later found that nisin binds to Lipid II, a membrane-bound precursor in peptidoglycan synthesis [10]. However, inhibition

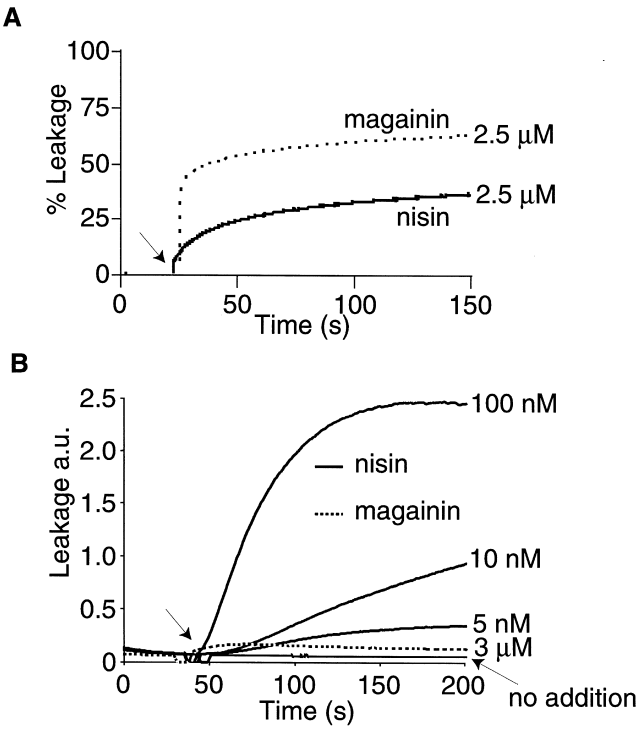

Fig. 7. (A) Activity of nisin and magainin towards model membranes composed of a lipid extract from *M. flavus*. The peptide activity was measured by monitoring the leakage of carboxyfluorescein from vesicles made from a lipid extract of *M. flavus* by measuring the increase in fluorescence due to dilution of the dye from self-quenching concentrations as described [15]. The concentration of both nisin and magainin was 2.5 μM. The arrowheads mark the time point of peptide addition. (B) Activity of nisin and magainin towards intact *M. flavus* cells. The peptide activity was measured by monitoring their effect on the membrane potential with the fluorescent membrane-potential-sensitive probe 3,3′-diethylthiodicarbocyanine iodide [DiS-C_2(5)] [50]. Cells were grown until midlog phase, harvested, and washed once with 250 mM sucrose, 5 mM $MgSO_4$, 10 mM potassium-phosphate pH 7.0 and resuspended in the same buffer. Cells were added to the fluorescence cuvette at a cell density of $OD_{600} = 0.075$ together with DiS-C_2(5) at 1 μM.

of cell wall synthesis by nisin, like the formation of pores in model systems, was only detected at concentrations well above the MIC-values of the respective strains. Therefore, it was suggested that Lipid II might somehow participate in pore formation by nisin [12].

Lipid II is composed of a membrane anchor of 11 polyisoprene residues (undecaprenyl) to which, via a pyrophosphate, the basic building block of the cell wall, MurNAc(pentapeptide)-GlcNAc, is attached (Fig. 8). Lipid II is synthesized on the cytosolic

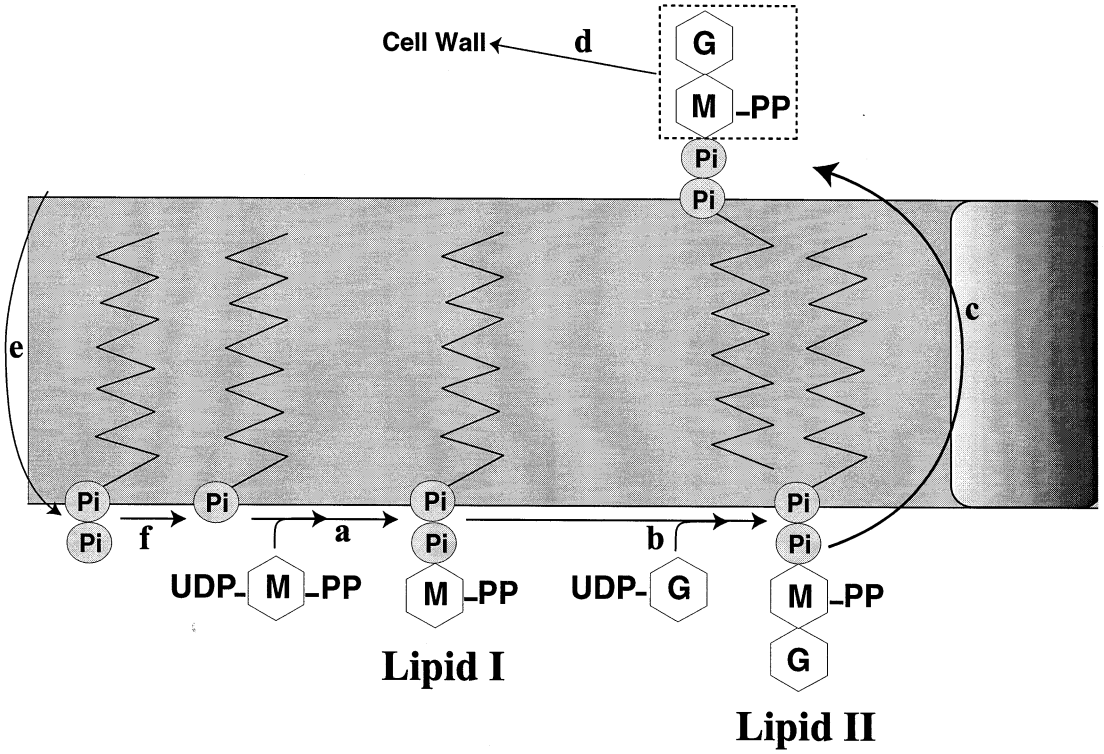

Fig. 8. Model for the central role of Lipid II in the cell wall biosynthesis. See text for details.

side of the plasma membrane via addition of first the UDP-activated amino sugar MurNAc(pentapeptide) to undecaprenylphosphate (step a). This product is called Lipid I. Then the second UDP-activated amino sugar GlcNAc is attached, resulting in the complete Lipid II molecule (step b). Subsequently Lipid II somehow flips to the exterior side of the plasma membrane (step c), a process that is proposed to involve a specific membrane transporter. The two amino sugars, including the pentapeptide, are then coupled to the cell wall (step d). The remaining undecaprenylpyrophosphate is transported back to the cytosolic side of the membrane (step e) where it is dephosphorylated to undecaprenylphosphate (step f), and the cycle can start again. It should be emphasized that several steps in this cycle require energy input. For instance, the additions of amino acids to the UDP-MurNAc to form the pentapeptide are ATP-dependent steps [42,43]. In addition, the translocation of Lipid II across the membrane most likely requires energy input, since related transport mechanisms also using the undecaprenol carrier were highly dependent on the proton motive force and a high energy phosphate pool [44,45].

Only recently, it could be shown that Lipid II is indeed an important factor in the pore formation by nisin [46]. When the Lipid II biosynthesis was blocked by ramoplanin, which inhibits step b in Fig. 8 [47], the nisin sensitivity of intact cells decreased. Moreover, nisin-induced carboxyfluorescein leakage from multilamellar liposomes was dramatically increased upon incorporation of purified Lipid II. In the presence of Lipid II, the activity of nisin in dye-leakage assays is in the nM range, the same concentration range as the MIC values (E. Breukink et al. unpublished observations). Thus, Lipid II is the high affinity target for nisin. Upon interaction with Lipid II, nisin forms pores in the membrane resulting in cell death. This nM activity of nisin was achieved in the absence of a membrane potential. Bearing in mind that the Lipid II cycle requires energy input, it seems logical that any disturbance in the energy status of the bacterium, e.g. by dissipating the membrane potential, disturbs the Lipid II cycle. Thus, the lower sensitivity of several bacteria to nisin due to dissipation of the membrane potential (see Section 3.4 is most likely not the result of a decreased direct effect of the reduced membrane potential on nisin.

Rather, a reduction in the Lipid II pool causes the reduced nisin sensitivity of the cells.

4.1. The Lipid II–nisin interaction

Several experiments gave the first insights into the structural requirements for the interaction of nisin with Lipid II. Undecaprenol and undecaprenylphosphate had no effect on the nisin activity [46]. Since nisin binds also to Lipid I [10,46], this suggests that the first sugar moiety of Lipid II is important for the interaction with nisin. Interestingly, nisin displayed activity against certain *Methanobacterium* species [48]. These bacteria contain a cell wall polymer named 'pseudomurein', which resembles the more common murein of eubacteria in some features. Namely, it is also build up of two aminosugars, one of which contains a pentapeptide. The amino sugars are also transported via Lipid II. However, the first sugar of this Lipid II is now GlcNAc not containing a pentapeptide, while the second sugar is *N*-acetyltalosaminuronic acid, to which a pentapeptide is attached. The differences between the pentapeptides are quite large, which suggest that this is not the binding site for nisin. However, the sugar backbones of the first amino sugar in both Lipid II species are quite similar in structure. Therefore, it is tempting to speculate that this is the recognition site of nisin. The pyrophosphate and/or the isoprene moiety are probably also important for the interaction, since recognition of the sugar alone would mean that the whole cell wall would act as a massive sink for nisin.

Several experiments suggested that the N-terminus of nisin is important for the interaction of nisin with Lipid II. From comparison of the nisin N-terminus with other lantibiotics that did or did not interact with Lipid II, it was suggested that the first two rings are important for the binding to Lipid II [46]. Support for this conclusion came from experiments using nisin variants with modifications in the N-terminus (I. Wiedemann and H.-G. Sahl, unpublished observations). An interesting observation in this regard also arose from experiments using nisin fragments [49]. Specifically, an N-terminal nisin fragment (residues 1–12) antagonized the activity of intact nisin towards *L. lactis* cells. It is tempting to speculate that this inhibition was caused by binding of the N-terminal fragment to Lipid II. Interestingly, the N-terminal nisin fragment was not able to antagonize the activity of subtilin against *L. lactis* cells. The N-terminus of subtilin resembles the nisin N-terminus, but has some variations [13]. This suggests that these variations could have increased the affinity of subtilin for Lipid II with respect to the nisin affinity for Lipid II. These results show that the special structural elements of nisin, the ring structures, are important for the interaction with Lipid II.

5. Concluding remarks

Nisin is indeed a special case, as it uses a combination of a high affinity interaction with the membrane anchored Lipid II and pore formation to kill bacterial cells. It is the combination of these properties that make nisin such an effective molecule.

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References

[1] H.G. Sahl, in: J. Marsh (Ed.), Antimicrobial Peptides, John Wiley and Sons, Chichester, 1994, pp. 27–42.
[2] R. Jack, G. Bierbaum, C. Heidrich, H.G. Sahl, Bioessays 17 (1995) 793–802.
[3] G. Jung, in: G. Jung, H.-G. Sahl (Eds.), Nisin and Novel Lantibiotics, ESCOM Science Publishers, Leiden, 1991, pp. 1–31.
[4] J.W. Mulders, I.J. Boerrigter, H.S. Rollema, R.J. Siezen, W. de Vos, Eur. J. Biochem. 201 (1991) 581–584.

[5] G.W. Buchman, S. Banerjee, J.N. Hansen, J. Biol. Chem. 263 (1988) 16260–16266.
[6] H.W. van den Hooven, C.C.M. Doeland, M. van de Kamp, R.N.H. Konings, C.W. Hilbers, F.J.M. van de Ven, Eur. J. Biochem. 235 (1996) 382–393.
[7] H.W. van den Hooven, C.A.E.M. Spronk, M. Vandekamp, R.N.H. Konings, C.W. Hilbers, F.J.M. van de Ven, Eur. J. Biochem. 235 (1996) 394–403.
[8] R.W. Jack, J.R. Tagg, B. Ray, Microbiol. Rev. 59 (1995) 171–200.
[9] E. Gross, J.L. Morell, J. Am. Chem. Soc. 93 (1971) 4634–4635.
[10] P. Reisinger, H. Seidel, H. Tschesche, W.P. Hammes, Arch. Microbiol. 127 (1980) 187–193.
[11] P.E. Linnett, J.L. Strominger, Antimicrob. Agents Chemother. 4 (1973) 231–236.
[12] E. Ruhr, H.G. Sahl, Antimicrob. Agents Chemother. 27 (1985) 841–845.
[13] H.-G. Sahl, in: G. Jung, H.-G. Sahl (Eds.), Nisin and Novel Lantibiotics, ESCOM Science Publishers, Leiden, 1991, pp. 347–358.
[14] M. Kordel, F. Schueller, H.G. Sahl, FEBS Lett. 244 (1989) 99–102.
[15] E. Breukink, C. van Kraaij, R.A. Demel, R.J. Siezen, O.P. Kuipers, B. de Kruijff, Biochemistry 36 (1997) 6968–6976.
[16] R. Eljastimi, M. Lafleur, Biochim. Biophys. Acta 1324 (1997) 151–158.
[17] A.J.M. Driessen, H.W. van den Hooven, W. Kuiper, M. Vandekamp, H.G. Sahl, R.N.H. Konings, W.N. Konings, Biochemistry 34 (1995) 1606–1614.
[18] C.J. Giffard, H.M. Dodd, N. Horn, S. Ladha, A.R. Mackie, A. Parr, M.J. Gasson, D. Sanders, Biochemistry 36 (1997) 3802–3810.
[19] M.J. Garcera, M.G. Elferink, A.J. Driessen, W.N. Konings, Eur. J. Biochem. 212 (1993) 417–422.
[20] I. Martin, J.M. Ruysschaert, D. Sanders, C.J. Giffard, Eur. J. Biochem. 239 (1996) 156–164.
[21] K. Winkowski, R.D. Ludescher, T.J. Montville, Appl. Environ. Microbiol. 62 (1996) 323–327.
[22] C.J. Giffard, S. Ladha, A.R. Mackie, D.C. Clark, D. Sanders, J. Membr. Biol. 151 (1996) 293–300.
[23] G.N. Moll, J. Clark, W.C. Chan, B.W. Bycroft, G.C.K. Roberts, W.N. Konings, A.J.M. Driessen, J. Bacteriol. 179 (1997) 135–140.
[24] W.M. O'Leary, S.G. Wilkinson, in: C. Ratledge, S.G. Wilkinson (Eds.), Microbial Lipids, Vol. 1, Academic Press, New York, 1988, pp. 117–201.
[25] M.R. Wenk, J. Seelig, Biochemistry 37 (1998) 3909–3916.
[26] C. van Kraaij, E. Breukink, H.S. Rollema, R.J. Siezen, R.A. Demel, B. de Kruijff, O.P. Kuipers, Eur. J. Biochem. 247 (1997) 114–120.
[27] C. van Kraaij, E. Breukink, M.A. Noordermeer, R.A. Demel, R.J. Siezen, O.P. Kuipers, B. de Kruijff, Biochemistry 37 (1998) 16033–16040.
[28] R.A. Demel, T. Peelen, R.J. Siezen, B. de Kruijff, O.P. Kuipers, Eur. J. Biochem. 235 (1996) 267–274.
[29] E. Breukink, C. vanKraaij, A. vanDalen, R.A. Demel, R.J. Siezen, B. deKruijff, O.P. Kuipers, Biochemistry 37 (1998) 8153–8162.
[30] R. Brasseur, J. Biol. Chem. 266 (1991) 16120–16127.
[31] L. Lins, P. Ducarme, E. Breukink, R. Brasseur, Biochim. Biophys. Acta 1420 (1999) 111–120.
[32] R. Benz, G. Jung, H.-G. Sahl, in: G. Jung, H.-G. Sahl (Eds.), Nisin and Novel Lantibiotics, ESCOM Science Publishers, Leiden, 1991, pp. 359–372.
[33] H.G. Sahl, M. Kordel, R. Benz, Arch. Microbiol. 149 (1987) 120–124.
[34] K. Matsuzaki, A. Nakamura, O. Murase, K. Sugishita, N. Fujii, K. Miyajima, Biochemistry 36 (1997) 2104–2111.
[35] K. Matsuzaki, O. Murase, N. Fujii, K. Miyajima, Biochemistry 34 (1995) 6521–6526.
[36] K. Matsuzaki, Biochim. Biophys. Acta 1376 (1998) 391–400.
[37] K. Matsuzaki, K. Sugishita, N. Ishibe, M. Ueha, S. Nakata, K. Miyajima, R.M. Epand, Biochemistry 37 (1998) 11856–11863.
[38] B.B. Bonev, W.C. Chan, B.W. Bycroft, G.C.K. Roberts, A. Watts, submitted for publication.
[39] M. Kordel, H.G. Sahl, FEMS Microbiol. Lett. 34 (1986) 139–144.
[40] F.H. Gao, T. Abee, W.N. Konings, Appl. Environ. Microbiol. 57 (1991) 2164–2170.
[41] T. Abee, FEMS Microbiol. Lett. 129 (1995) 1–9.
[42] M. El Sherbeini, W.M. Geissler, J. Pittman, X. Yuan, K.K. Wong, D.L. Pompliano, Gene 210 (1998) 117–125.
[43] J.J. Emanuele Jr., H. Jin, B.L. Jacobson, C.Y. Chang, H.M. Einspahr, J.J. Villafranca, Protein Sci. 5 (1996) 2566–2574.
[44] P.A. Marino, K.E. Phan, M.J. Osborn, J. Biol. Chem. 260 (1985) 14965–14970.
[45] B.C. McGrath, M.J. Osborn, J. Bacteriol. 173 (1991) 3134–3137.
[46] H. Brotz, M. Josten, I. Wiedemann, U. Schneider, F. Gotz, G. Bierbaum, H.G. Sahl, Mol. Microbiol. 30 (1998) 317–327.
[47] E.A. Somner, P.E. Reynolds, Antimicrob. Agents Chemother. 34 (1990) 413–419.
[48] W.P. Hammes, J. Winter, O. Kandler, Arch. Microbiol. 123 (1979) 275–279.
[49] W.C. Chan, M. Leyland, J. Clark, H.M. Dodd, L.Y. Lian, M.J. Gasson, B.W. Bycroft, G.C.K. Roberts, FEBS Lett. 390 (1996) 129–132.
[50] P.J. Sims, A.S. Waggoner, C.H. Wang, J.R. Hoffmann, Biochemistry 13 (1974) 3315–3330.