Structural characterization of free and membrane-bound nisin by infrared spectroscopy

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Abstract

This study reports two new trends about nisin affinity for lipid membranes. First, there is a very strong dependence of nisin binding on the membrane surface charge. As illustrated in this work, the binding of nisin is much greater for phosphatidylglycerol (PG) than for phosphatidylcholine (PC) membranes. This can be rationalized by electrostatic attraction between the positively charged peptide and the negatively charged PG. Second, the affinity of nisin shows a very weak dependence on the lipid phase, the binding to fluid or gel phase membranes being nearly equivalent. Therefore, our results suggest that nisin behaves as an extrinsic peptide. This work also presents the first piece of information relative to the structure of membrane-bound nisin. The Amide I band of the peptide is different for free nisin in water and for membrane-bound nisin. By analyzing this region using self-deconvolution and band fitting, and by comparing with results obtained from nisin dissolved in various H_2O/trifluoroethanol mixtures, it can be inferred that the binding of nisin to phospholipid membranes leads to an increased proportion of β-turns.

Keywords: Nisin; Phospholipid; Infrared spectroscopy; Secondary structure

1. Introduction

Nisin is a positively charged peptide of 34 amino acids. Five thioether bridges, introduced as posttranslational modifications of serine, threonine and cysteine [1] are at the origin of five cycles labelled from A to E, starting from the N-terminal. Because of its antimicrobial activity, nisin is widely used as food preservative for milk products and meats. It is part of the lantibiotic family, since it contains dehydro (dehydroalanine and dehydrobutyrine) residues. It has been shown that the membrane is the primary site for the antibiotic activity of nisin [2]. The amphiphilic properties of the peptide suggest that it can interact

directly with the lipidic portion of the membrane. Actually, it has been recently shown that nisin can induce leaks from vesicles made of natural and synthetic lipids [3–6].

A structural description of the peptide in its active conformation is required in order to get deeper insights into the mechanism of its antimicrobial activity. Because of the large portion of amino acids involved in cycles (about 65%), no uniform secondary structure is expected. However, the covalent restrictions appear to provide some well-defined structural features in the peptide in water. ^{1}H-NMR spectroscopy techniques coupled with computer simulations have suggested the presence of a β-turn in the region DAbu8–Ala11 (cycle B) [7,8]. The ring C appears to be more flexible, and only a helical turn in the region 16–19 has been reported [9]. Finally, nisin structure in aqueous environment includes a β-turn in the Lys22–DAbu23 region [10] and the presence of two consecutive β-turns or an overwound α-helix was proposed for the cycles D and E [8]. The segment 29–34 has been reported to exist under an extended conformation [10].

To understand the mechanism by which the peptide acts as an antimicrobial agent, it is essential to know the structure of nisin when it interacts with membranes. Recent studies have addressed this question. To simulate a membrane environment, trifluoroethanol (TFE) has been used. It was shown that nisin structure is modified in TFE/water mixtures [8,11]. The presence of TFE promotes, as expected, the structural organization of the peptide, and the helical segment observed in water for the region 23–28 extends all the way to the C-terminal in a water/TFE mixture [8]. More recently, a detailed NMR-based study has characterized the structure of nisin bound to micelles made of dodecylsulfate or dodecylphosphocholine [11,12]. This investigation reports structural changes between free nisin in aqueous environment and its micelle-bound form, as discussed below. However, up to now, there is no study providing information about the structure of nisin bound to a phospholipid membrane. This piece of information is essential especially when one considers that the previous studies indicate structural modifications of nisin relative to its environment.

In this paper, we have examined the structure of nisin bound to lipid membranes. In the first part, we

have characterized the binding of the peptide to lipid membranes. Second, the structural characterization of nisin was done in water, TFE and water/TFE mixtures as a preliminary step to investigate the secondary structure from the spectroscopic data. Finally, the structure of membrane-bound nisin was examined by infrared spectroscopy.

2. Materials and methods

Nisin (activity $> 30\,000$ U/mg) was bought from NBS Biologicals (North Mymms, UK). High-pressure liquid chromatography (HPLC) analysis [13] showed that nisin was pure at 88%; the major contaminant was [Ser[33]] nisin. For the infrared spectroscopy, nisin was used without further purification. For the binding experiments, the peptide was purified according to the procedure previously described [13]. All the phospholipids were purchased from Avanti Polar Lipids (Birmingham, USA), and TFE was from Aldrich (Milwaukee, USA).

For the structural investigation of free nisin, the peptide was dissolved in the various solvents to have a final concentration of nisin of 10 (w/v)%. The sample was then put in a closed cell described previously [14]. Briefly, the cell was made of two CaF_2 windows with a Teflon spacer of 5 μm. This cell was mounted in a brass holder whose temperature was computer-controlled using thermopumps. The spectra of nisin were recorded at 25°C.

Lipid–nisin complexes were prepared by adding nisin to preformed lipid multilamellar vesicles. First, the lipid was hydrated with a buffer containing 50 mM 2-[morpholino]ethanesulfonic acid (Mes), 50 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA) (pH 5.5), and was vortexed above the temperature of the gel-to-liquid crystalline phase transition (T_m) to ensure complete hydration. Nisin in solution in the same buffer was then added to obtain an incubation lipid/nisin molar ratio (R_i) of 5. The sample was freeze-and-thawed five times, from liquid nitrogen temperature to a temperature above T_m. An aliquot of the sample was transferred between two CaF_2 windows spaced out by a 5-μm-thick Teflon ring. In the case of the DPPC/nisin complex, the sample was centrifuged at $3000 \times g$ for 1 h and an aliquot of the pelleted complex was used to record the spectrum.

This method ensured that the absorption is due to bound nisin. The cell was mounted in the brass holder described above. The spectra were recorded at 25°C.

The infrared spectra were recorded on an FTS-25 Bio-Rad spectrometer equipped with a water-cooled globar source and a medium-band mercury-cadmium-telluride detector. For each spectrum, 200 scans at a resolution of 2 cm^{-1} were coadded, and Fourier transformed using a triangular apodization function.

The spectra in the region of the Amide bands (1720–1580 cm^{-1}) were corrected for the solvent contribution by subtracting the spectrum of the solvent. For water, the subtraction factor was determined using the association band at 2125 cm^{-1} and the subtraction was satisfactory when the region between 1750–2650 cm^{-1}, where there is no absorption, was flat [14]. For TFE, solvent bands at 1454, 1414 and 1372 cm^{-1} were used as internal standards. For H$_2$O/TFE mixtures, both contributions were corrected individually using the same internal standards. The small overlap of the Amide II band with the Amide I band was subtracted by simulating the side of the Amide II band with a polynomial of fourth order. For the bound nisin, the carbonyl stretching bands of the lipids interfere with the Amide I band of the peptide. Nisin introduces a perturbation at the membrane interface and causes changes in the lipid carbonyl band shape. This prevented us from correcting for this contribution using the lipid spectrum. The contribution of the lipid was corrected by simulating the carbonyl band with two bands between 1680–1785 cm^{-1} and by subtracting these fitted bands in the region of the Amide I band. The 1750–1580 cm^{-1} region of the corrected spectra was Fourier self-deconvolved to identify the number of components in the Amide I band. Subsequently, the Amide I band was curve-fitted. These mathematical treatments were done using GRAMS software (Galactic Industry, Salem, USA).

To determine the proportion of bound nisin in our lipid/nisin samples, a simple binding test was done. Different amounts of a phospholipid dispersion prepared in the Mes buffer was added to a 100-μl aliquot of nisin dissolved in the Mes buffer. Buffer was then added to have a nisin concentration of 0.5 mg/ml in all the samples. The samples were freeze-and-thawed five times, and then centrifuged for 1 h at 3000 $\times$ g, at 7°C, to isolate the lipid–nisin complexes from the free nisin. Nisin concentration in the supernatant was measured by Lowry protein assay [15], using bovine serum albumin as a standard. Phospholipid concentration in the supernatant was also determined, using the Fiske–SubbaRow method [16]. A control experiment showed that all the nisin remained in the supernatant after centrifugation in the absence of lipid. No lipids were detected in the supernatant after centrifugation in the presence or the absence of nisin.

3. Results

Fig. 1 shows the binding of nisin to phosphatidylcholine, a zwitterionic lipid, in the upper panels, and to phosphatidylglycerol, a negatively charged lipid, in the lower panels. Panels B and D of Fig. 1 show the binding of nisin to gel phase lipids (the T_m of both 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylglycerol (DPPG) is 41°C [17]) whereas panels A and C show nisin binding to lipids in the liquid crystalline phase (the T_m of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC) and 1-palmi-
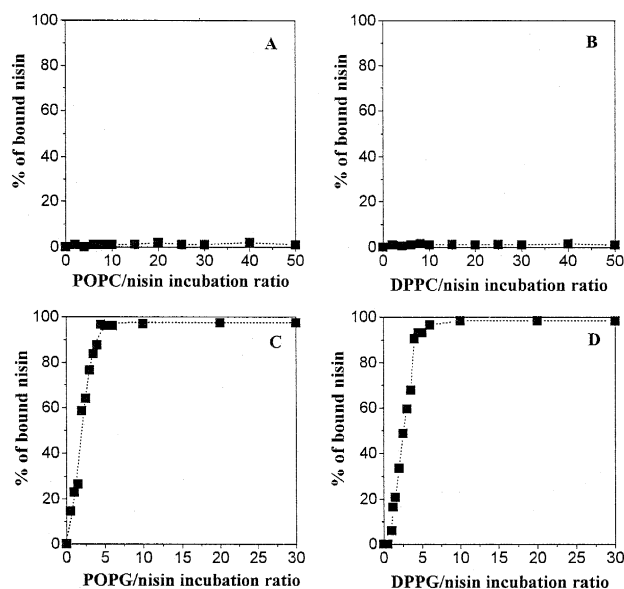

Fig. 1. Binding curves of nisin to (A) POPC, (B) DPPC, (C) POPG, and (D) DPPG.

toyl-2-oleoyl-*sn*-glycero-3-phosphatidylglycerol (POPG) is −2°C [17] and +1°C [18], respectively). Two trends are inferred from these results. First, the affinity of the peptide is greatly dependent on the lipid charge. For the zwitterionic lipids, there is no significant binding of nisin to the vesicles, even for lipid/nisin molar incubation ratio (R_i) as high as 50. Conversely, almost all the nisin is bound to PG vesicles at a R_i of 5. It is therefore clear that electrostatic interactions increase drastically the affinity of the peptide for the membranes. Second, nisin binding is almost independent of the lipid phase. The affinity curves of nisin for DPPC and for POPC show that there is no significant binding of the peptide in these conditions. The binding curves obtained with saturated and unsaturated PG both show that almost all the nisin is bound when the R_i reaches a value of about 5. When these two curves are compared closely, the affinity seems to be slightly higher for POPG than for DPPG, the curves being slightly shifted toward lower R_i for the unsaturated lipid.

Prior to the analysis of the spectra of bound nisin, we have investigated the structure of nisin in various solvents. The Amide I region of the IR spectra of nisin in water, TFE and a 50:50 water/TFE mixture is shown in Fig. 2; the spectra, the deconvolved and the simulated results are displayed. The Amide I band appears to include four components which are at about 1673 ± 3, 1656 ± 2, 1636 ± 3, and 1612 ± 4 cm^{-1} [1]. These components can be clearly identified on the deconvolved spectra. It is also possible to simulate the band using four components and the positions obtained from the simulations are always close (within 3 cm^{-1}) to the frequency measured on the deconvolved spectra. In the band simulation, the position of the low frequency component has been restricted to be between 1600 and 1615 cm^{-1}. A component in that region was identified in the deconvolved and the simulated spectra of nisin in solution with a proportion of TFE higher than 75%. This component, even though still detectable in the deconvolved spectra for nisin in solvent with lo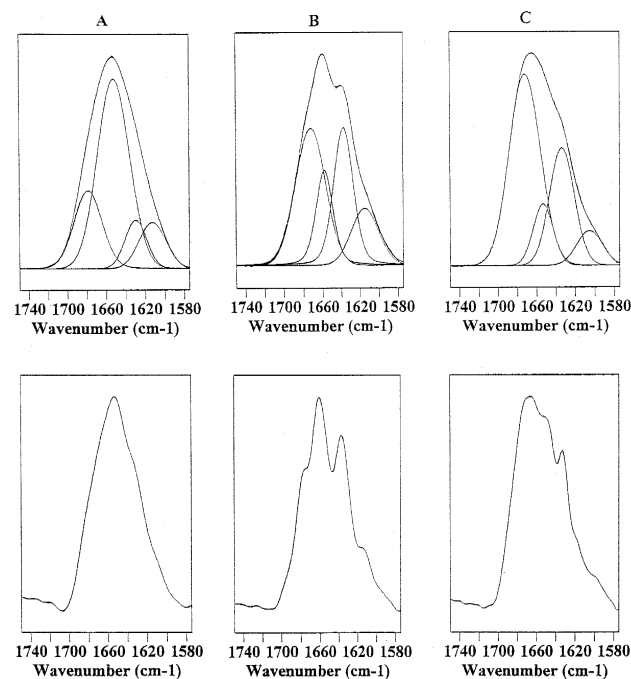wer proportion of TFE, was not always found in the mathematical solution of the band simulation. Consequently, we imposed a positional limitation for this component. The restriction improved drastically the reproducibility of the band decomposition solutions for independent samples in identical conditions. The results indicate that the secondary structure of nisin is sensitive to the solvent. This is shown in a straightforward manner by the shift of the maximum of the overall Amide I band from 1653 cm^{-1} in water to 1663 cm^{-1} in TFE. As seen on the decomposed spectra, this shift is concomitant with changes in the relative intensity of the components, especially for the bands at 1673 and 1656 cm^{-1}. The frequencies of the components, as determined on the deconvolved and decomposed spectra, do not appear to be sensitive to the solvent whereas their relative proportions are. A series of H$_2$O/TFE mixtures was made, and the Amide I band of nisin dissolved in these solvents was analyzed by curve fitting; the results are summarized in Fig. 3. First, to establish without doubt that the Amide I band of nisin is sensitive to the solvent, Fig.

Fig. 2. Amide I band of nisin dissolved in (A) H$_2$O, (B) H$_2$O/TFE 50:50, and (C) TFE. The upper panels show the experimental and the simulated spectra, as well as the four components obtained from the band fitting. The lower panels show the deconvolved spectra. The spectra were recorded at 25°C.

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[1] The averages and the standard deviations were calculated from the values obtained for the decomposed spectra recorded in ten different water/TFE mixtures.

3A shows that the maximum of the band is progressively shifted from 1653 to 1663 cm^{-1} when the proportion of TFE is increased in the mixture. The origin of this shift towards higher frequency can be associated to the increase of the intensity of the band at 1673 cm^{-1} relative to that at 1656 cm^{-1} as seen in Fig. 3B. The component at 1656 cm^{-1} dominates the spectrum recorded in water whereas it represents less than 10% of the band when nisin is dissolved in TFE. In parallel, the contribution of the component at 1673 cm^{-1} increases from about 20% in water to 50% in TFE. The variations in the relative intensity of these bands are observed mainly between 0% and 50% of TFE in water. When the proportion of TFE is greater than 50%, the relative contribution of these two components remains fairly constant. The relative area of the two other components, at 1636 and 1612 cm^{-1}, is not strongly affected by the variation of the solvent, their respective contributions remaining around 20% and 10%.

In addition, we have recorded the spectrum of nisin dissolved in D$_2$O (data not shown). The spectrum was similar to that obtained in H$_2$O and showed also four components after deconvolution. The decomposition with four components led to bands at 1671 cm^{-1}, 1649 cm^{-1}, 1625 cm^{-1}, and 1593 cm^{-1} with respective areas of 25%, 49%, 20% and 6%.

Fig. 4 shows the Amide I band of nisin in the presence of DPPC or DPPG vesicles. The maximum of the Amide I band envelope is at about 1660 cm^{-1}.

Fig. 4. Amide I band of nisin bound to (A) DPPC and (B) DPPG, in a lipid/nisin incubation molar ratio of 5, at 25°C

This value is close to that observed for nisin dissolved in the H$_2$O/TFE 50:50 mixture. Actually, the overall shape of the Amide I band of nisin in the presence of lipids is similar to the spectrum recorded in the H$_2$O/TFE 50:50 mixture. The deconvolved spectra also show the four components observed for free nisin, and the band fitting results are again close to those obtained with nisin dissolved in the H$_2$O/TFE 50:50 mixture; the relative areas of the bands at 1669, 1657, 1638, and 1615 cm^{-1} are 55%, 15%, 22%, and 8%, respectively, for DPPC-bound nisin and 46%, 6%, 36%, and 12%, respectively, for DPPG-bound nisin.

4. Discussion

First of all, we have investigated the affinity of nisin for lipid vesicles. Two conclusions are inferred from the binding experiments. First, the affinity of nisin is much greater for negatively charged than for zwitterionic lipids. This can be easily rationalized on the basis of electrostatic interactions because nisin has a net positive charge of $+5$ and likely interacts
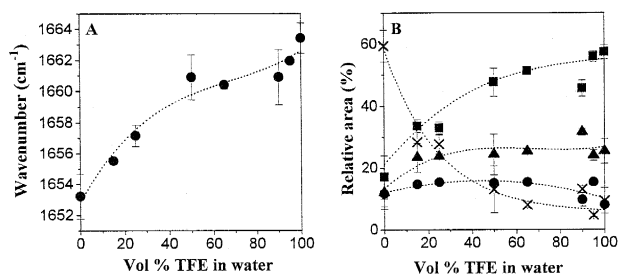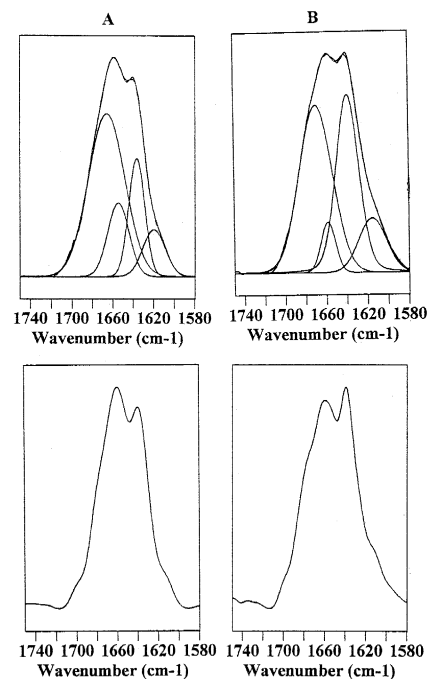

Fig. 3. Effect of the solvent of the Amide I band of nisin. (A) Position of the overall band and (B) variation of the relative area of the four components of the Amide I band determined from band fitting, as a function of the proportion of TFE in H$_2$O/TFE mixtures; ($\blacksquare$) 1673 cm^{-1} component, ($\times$) 1656 cm^{-1} component, ($\blacktriangle$) 1636 cm^{-1} component, and ($\bullet$) 1612 cm^{-1} component. The error bars represent the standard deviation on three measurements; when the bar is not apparent, it means that the deviation is small relative to the symbol.

with the negatively charged interface of the membrane. Our results are consistent with the binding kinetics study which indicates that nisin binding to negatively 1,2-dioleoyl-*sn*-glycero-3-phosphatidylglycerol (DOPG) vesicles was found much faster than the binding to zwitterionic 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC) vesicles [5]. The drastic change in affinity reported here may also be partly at the origin of the enhanced influence of nisin on membrane fluidity when the bilayer contains negatively charged phosphatidylserine [19]. Increased binding of positively charged peptides to negatively charged bilayers has been already reported for several systems including melittin [20,21], cardiotoxin II [22], and polymyxin B [23]. Second, the affinity of nisin for vesicles is not strongly dependent on the lipid phase, the binding curves being similar for gel-phase and liquid crystalline-phase lipids. This result suggests that nisin interacts mainly at the membrane interface level and the contact between the peptide and the hydrophobic core of the bilayer is limited. When a peptide has a significant hydrophobicity and inserts in the membrane, the binding is more dependent on the lipid phase. For example, melittin shows an affinity much higher for fluid than for gel-phase bilayers [20] and it is interpreted as a facilitated insertion of the peptide in the membrane when the lipid chains are disordered. The results presented here suggest that nisin behaves as an extrinsic peptide.

The binding of nisin induces a change in the secondary structure of the peptide as inferred from the Amide I band. In order to provide a structural analysis, we have examined the Amide I band in H_2O/TFE mixtures and compared the structural description obtained with the literature. Our results show that, despite the cycles that should provide some rigidity to the peptide, the secondary structure of nisin is sensitive to the environment. The Amide I band is made up of four components as assessed from both the deconvolved spectra and the reasonable fits obtained by band simulations. The band at 1656 cm^{-1}, which is the main component when nisin is dissolved in water, is representative of α-helix and/or unordered conformation [24–26]; these two structures give rise to Amide I components which are difficult to resolved in a spectrum recorded in H_2O. More insights are provided by the results obtained in D_2O. The main component of the Amide I is shifted to

1649 cm^{-1}, and this frequency is associated with unordered conformation [24]. Therefore, the band at 1656 cm^{-1} observed in H_2O is attributed to unordered segments. The band at about 1673 cm^{-1} has been associated to β-turn [24,27]. The band at 1636 cm^{-1} is generally attributed to β-sheet [24,28]. This is rather surprising, because β-sheet structures have never been reported for nisin in aqueous solution. More recently, a study has suggested that β-turns can also give rise to a band in the region between 1630–1640 cm^{-1} [29]; this component observed in nisin may therefore be associated to β-turns. The contribution at 1612 cm^{-1} is probably not due to the Amide I vibration but to side chain vibrations of the histidine residues and the antisymmetric mode of the terminal carboxylic group [30]. This attribution is also supported by the fact that the relative contribution of this component is more or less insensitive to the solvent. The Amide I band indicates that nisin adopts mainly unordered and β-turn structures. These results are in agreement with the literature. NMR studies and simulations of nisin in water have suggested the presence of β-turns in the region 8–11 in the cycle B [7,8], in the 22–23 residues area [10], and in the cycles D and E [8]. A helical turn has also been proposed for the 16–19 residues [9]. The addition of TFE to water appears to promote the formation of β-turns in nisin as deduced from the change in relative intensity of the 1673 cm^{-1} component compared to that at 1656 cm^{-1}. This is consistent with the ability of TFE to induce regular structures such as β-turns in peptides [29,31]. This phenomenon has been associated with the dielectric constant of TFE ($\epsilon = 27$) which is lower than that of water and closer to that of the protein interior [31]. The structural variations induced by TFE in water are more pronounced between 0% and 50% TFE. Similarly, it has been reported that TFE induces changes in nisin structure as characterized by NMR, and these changes were more important between 0% and 70% TFE in water [11]. To conclude this section, the analysis of the Amide I band suggests that nisin structure in water is mainly disordered and β-turns and that the proportion of these components is sensitive to the solvent polarity, TFE promoting the formation of β-turns at the expense of the disordered component.

The Amide I band of nisin is also affected by the binding of the peptide to membranes. The changes

caused by the binding are very similar to those brought by TFE. Actually, the maximum of the over-all band, the positions and the relative intensities of the components are close to those observed for the spectrum of nisin in solution in the H_2O/TFE 50:50 mixture. So it is suggested that membrane-bound nisin contains a higher proportion of β-turns than the free form in water, as inferred from the increased contribution of the band at 1673 cm^{-1}. A previous investigation has also reported a change in the secondary structure of nisin upon its binding to an amphipathic interface (a micelle in that case) [11,12]. The increased β-turn content that we observed is in agreement with the structural changes observed upon nisin binding to a micelle. The segment $Met_{21}-Ala_{24}$ was shown to form a β-turn in the bound state, a structural feature that was not observed in water [12]. Other conformational changes were reported in ring A. Despite the fact that our results do not allow us to identify the amino acids involved in the conformational reorganization, the structural changes upon binding can be clearly highlighted by infrared spectroscopy and they are similar to those observed when TFE is added to water. A similar conclusion was inferred from NMR and circular dichroism results [11] for which the structure of micelle-bound nisin was found similar to that obtained in water/TFE mixtures but different than that obtained for free nisin in water. Despite the drastic difference in affinity reported above, the nature of the membrane interface does not appear to be critical for the secondary structure of bound nisin because the Amide I band of the peptide bound to PC and to PG vesicles is very similar. This is consistent with the studies using micelles that report no significant difference between the structure of nisin bound to zwitterionic or anionic micelles [11,12]. Despite the small apparent affinity constant of nisin for DPPC, the bound form is observable because the high lipid concentration necessary for the infrared study shifts the equilibrium predominantly toward the lipid-associated peptide side, and the complexes can be isolated by centrifugation.

This study provides the first results about the structure of membrane-bound nisin. Our results show that the association with the membrane interface does affect the conformation of the peptide, the binding promoting the formation of β-turns. These results also validate the hypothesis that H_2O/TFE mixtures and micelles are appropriate systems to mimic phospholipid membranes in conformational studies of bound nisin.

Acknowledgements

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