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Nisin promotes the formation of non-lamellar inverted phases in unsaturated phosphatidylethanolamines

Rachida El Jastimi, Michel Lafleur *

Department of Chemistry, C.P. 6128, Succ. Centre Ville, Université de Montréal, Montréal, Qué. H3C 3J7, Canada

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Abstract

Nisin, a peptide used as a food preservative, is shown, by ^{31}P-nuclear magnetic resonance and infrared spectroscopy, to perturb the structure of membranes formed of unsaturated phosphatidylethanolamine (PE) and to induce the formation of inverted non-lamellar phases. In the case of dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE), nisin promotes the formation of inverted hexagonal phase. Similarly, the peptide induces the formation of an isotropic phase, most likely a cubic phase, with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylethanolamine (POPE). It is proposed that the insertion of the peptide in the bilayer shifts the amphiphilic balance by increasing the hydrophobic contribution and is at the origin of the changes in the polymorphic propensities of PE. This is supported by the fact that the presence of cholesterol in the PE bilayer inhibits the power of nisin to perturb the membrane structure, most likely because the peptide insertion is difficult in the fluid ordered phase. This finding provides insight into possible antibacterial mechanisms of nisin.

Keywords: Nisin; Phosphatidylethanolamine; Non-lamellar phase; NMR; Infrared

1. Introduction

Nisin (Fig. 1) is used as food preservative because of its antimicrobial activity against Gram-positive bacteria [1]. It is part of the lantibiotic family because of the presence of lanthionine and methyllanthionine groups. It has been proposed that the site of the antibacterial action of nisin is the plasmic membrane [2]. However, the antimicrobial mechanism has not yet been identified. It has been proposed that nisin can interfere with murein synthesis and the deterioration of the cell wall would be at the origin of its effect [3]. Another hypothesis proposes that nisin interacts with the lipidic components of the membrane as a means to destabilize the membrane. It has been shown, for example, that nisin can induce leakage from lipid vesicles [4,5].

In order to gain insight into the validity of the second hypothesis, we have examined whether nisin has the ability to alter the bilayer structure. In this

Abbreviations: chol, cholesterol; CSA, chemical shift anisotropy; DEPE, dielaidoyl-*sn*-glycero-3-phosphatidylethanolamine; DOPE, dioleoyl-*sn*-glycero-3-phosphatidylethanolamine; EDTA, ethylenediaminetetraacetic acid; H_{II}, inverted hexagonal; MES, 2-[morpholino]ethanesulfonic acid; NMR, nuclear magnetic resonance; PE, phosphatidylethanolamine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylethanolamine; R_i, incubation lipid/peptide molar ratio; T_h, temperature of the lamellar-to-H_{II} phase transition; T_m, temperature of the gel-to-liquid crystalline phase transition

* Corresponding author. Fax: +1 (514) 343-7586;
E-mail: michel.lafleur@umontreal.ca

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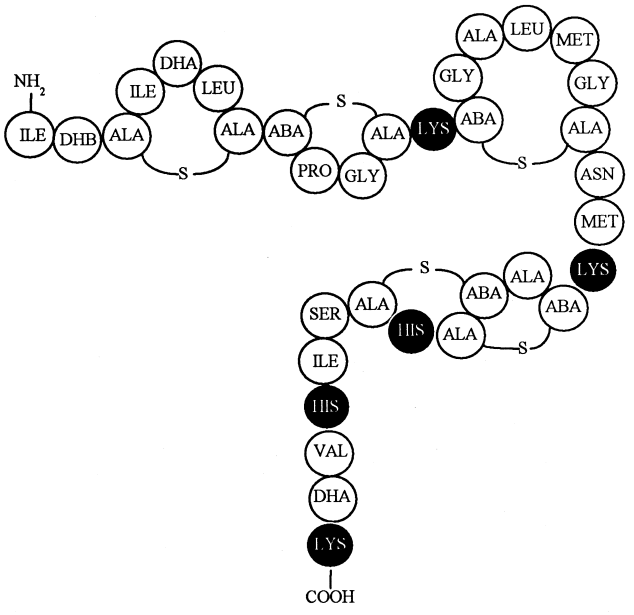

Fig. 1. Primary structure of nisin. The full circles represent basic residues. DHB, dehydrobutyrine; DHA, dehydroalanine; ABA, aminobutyric acid.

paper, we present a spectroscopic study which reports the perturbations of the lipid lamellar structures by nisin. The investigated lipid systems were formed of unsaturated phosphatidylethanolamine (PE). This choice was motivated by the fact that these lipids already have a propensity to form non-lamellar phases and also because they constitute an abundant class in several bacterial membranes [6]. ^{31}P-Nuclear magnetic resonance (NMR) spectroscopy has been used because it is a powerful tool to characterize the macroorganization of lipid aggregates [7] since the spectra are characteristic of the architecture of the aggregates. Bilayers and inverted hexagonal (H_{II}) phases provide typical profiles, for example. Small structures, such as micelles, bicelles or small unilamellar vesicles, and structures for which the phospholipids diffuse along highly curved surfaces, such as in cubic phases, can lead to the complete averaging of the chemical shift anisotropy and a narrow symmetric line is obtained in the spectrum [7,8]. In addition to phase identification by ^{31}P-NMR, we have characterized the thermotropism of PE/nisin complexes using infrared spectroscopy. Finally, we have investigated the influence of cholesterol on the polymorphic propensities of the lipids in the presence of nisin.

2. Materials and methods

Nisin was obtained from Aplin and Barrett (Trowbridge, UK). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylethanolamine (POPE) and dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE) were purchased from Avanti Polar Lipids (Birmingham, AL, USA). Cholesterol (chol), and 2-(*N*-morpholino)ethanesulfonic acid (MES) were obtained from Sigma (St. Louis, MO, USA). Ethylenediaminetetraacetic acid (EDTA) was bought from Aldrich (Milwaukee, WI, USA).

About 30 mg of lipid was hydrated with 700 μl of 50 mM MES buffer, containing 150 mM NaCl and 5 mM EDTA, pH 6. In the case of the mixtures containing cholesterol, the phospholipid and cholesterol were co-solubilized in an organic solvent (benzene/MeOH 95/5, v/v) and freeze-dried prior to the hydration. The lipid was submitted to five freeze-and-thaw cycles, from liquid nitrogen temperature to a temperature above the temperature of the gel-to-liquid crystalline phase transition (T_m) of the pure phospholipid. Nisin was added to preformed vesicles to mimic its effect on membranes. Because of the considerable lipid concentration required for the IR and NMR techniques and the limited solubility of the peptide, the appropriate amount of solid nisin was added to the preformed vesicles to obtain a defined incubation lipid/peptide molar ratio (R_i). The samples were then incubated for 2 h at a temperature higher than the temperature of the lamellar-to-H_{II} phase transition (T_h) of the lipid. In addition, the samples were submitted again to five freeze-and-thaw cycles, in order to promote the homogeneous distribution of the peptide in the samples.

The ^{31}P-NMR spectra were acquired on a Bruker DSX-300 spectrometer equipped with a 10-mm-coil probe (Morris Instrument), at a frequency of 121.5 MHz. After a single 70° pulse of 10 μs, the free induction decay was recorded. The interactions with the protons were removed by high power decoupling. The relaxation time between consecutive experiments was 5 s and the number of scans was 2000. The temperature was controlled by a Bruker VT unit. The temperature was equilibrated 25 min prior to the data acquisition and the spectra were acquired as a function of increasing temperatures. For the temperature cycling experiments, the details

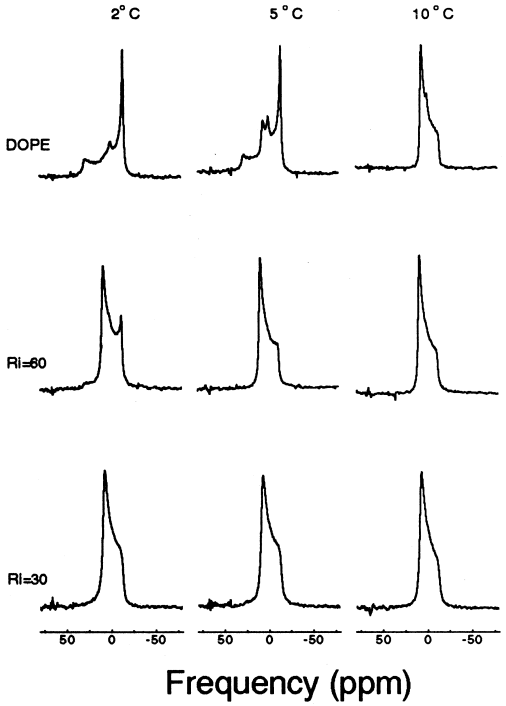

Fig. 2. ^{31}P-NMR spectra of pure DOPE and DOPE/nisin complexes. The temperatures are indicated at the top of each column and the proportion of nisin is displayed on the left of each row.

were: (a 25-min equilibration at the low temperature; acquisition of the spectrum; a 25-min equilibration at the high temperature; acquisition of the spectrum)$_n$.

The IR spectra were acquired on a Bio-Rad FTS25 spectrometer equipped with a cell composed of two CaF_2 windows spaced out with a 5-μm-thick Teflon ring, inserted in a thermoregulated brass holder. The number of scans was 100 and the resolution was 2 cm^{-1}.

3. Results

Fig. 2 displays ^{31}P-NMR spectra of pure DOPE and DOPE/nisin complexes as a function of temperature. For the pure lipid, the spectrum at 2°C shows a profile typical of the fluid lamellar phase, with a chemical shift anisotropy (CSA) of 43 ppm [7,9]. Upon heating, the formation of the H_{II} phase is observed by the apparition of a component characteristic of this type of organization. This component is typical of a system which experiences axially symmetric motion. However, it is inverted relative to a lamellar phase spectrum and its CSA is reduced by a factor of about 2, as a consequence of the lipid diffusion around the H_{II} cylinders [7,10]. At 10°C, the spectrum is essentially representative of the H_{II} phase and its CSA is 21 ppm. It is possible to follow quantitatively the lamellar-to-H_{II} phase transition by determining the relative area of each spectral component. Fig. 3 shows that pure DOPE undergoes a transition from a lamellar-to-H_{II} phase between 3 and 8°C, with a mid-point at 6°C; this temperature of the lamellar-to-H_{II} phase transition (T_h) is in agreement with the literature [11]. In the presence of nisin, the promotion of the H_{II} phase is observed, even at an R_i of 60. Actually, at 2°C, nisin added at an R_i of 30 destabilizes completely the lamellar structure to induce the formation of a H_{II} phase (see Figs. 2 and 3). The thermally induced lamellar-to-H_{II} transition becomes less cooperative and is shifted towards lower temperatures for the complexes with nisin. At an R_i of 10, the DOPE/nisin complexes exist only in the H_{II} phase over the whole temperature range investigated.

The effect of nisin on POPE has also been investigated. The thermotropism is first characterized by

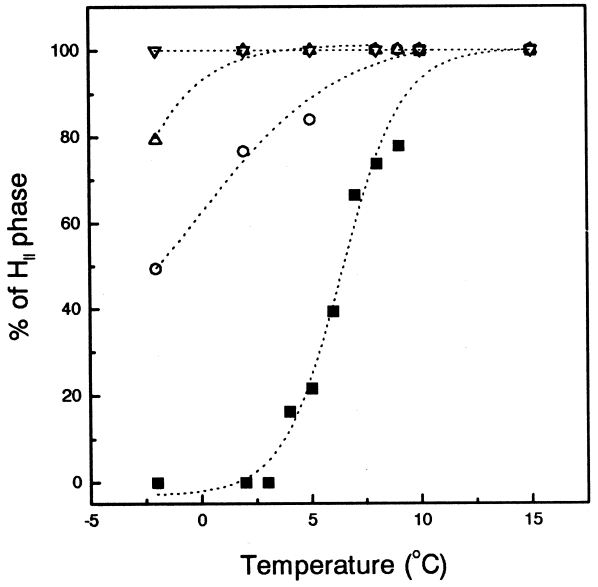

Fig. 3. Progression of the H_{II} phase content in DOPE and DOPE/nisin complexes, as a function of temperature. (■), pure DOPE, and DOPE/nisin complexes with an R_i of (○) 60, (△) 30, and (▽) 10.

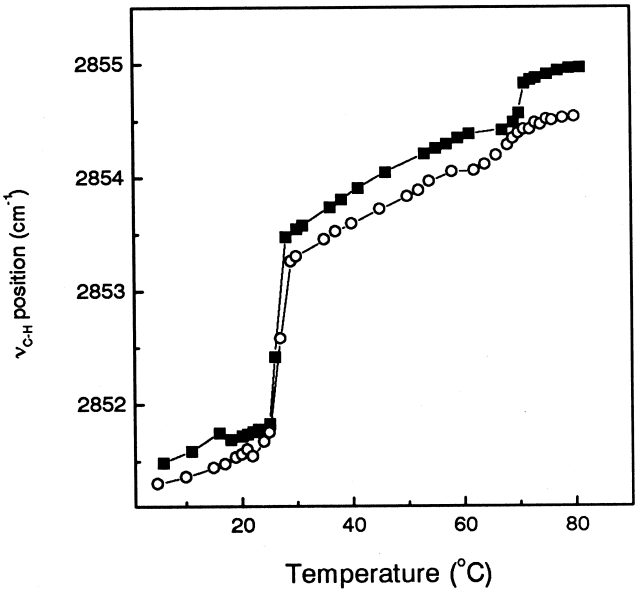

Fig. 4. Thermotropism of pure POPE (■) and POPE/nisin complex (○) with an R_i of 10, as probed by the position of the ν_{C-H} band.

the shift of the ν_{C-H} band in the IR spectra (Fig. 4). The position of this band is sensitive to the conformational order of the lipid acyl chains and, as a consequence, is a useful probe for lipid transitions [12,13]. For the pure lipid, at temperatures below 25°C, the ν_{C-H} frequency is 2851.5 cm^{-1}, a value characteristic of a gel phase lipid. The gel-to-liquid crystalline phase transition is observed at 25°C by the abrupt shift of the band to 2854 cm^{-1}, a value typical of fluid bilayers. This T_m is in agreement with the literature [14,15]. A second transition is observed at 70°C, corresponding to the lamellar-to-hexagonal phase transition [14,15]. The amplitude of this transition is much smaller than that of the gel-to-liquid crystalline phase transition and this observation is related to the limited conformational disorder introduced during the second transition [13]. The presence of nisin at an R_i of 10 does not influence the gel-to-liquid crystalline phase transition. Conversely, the peptide induces a decrease in the cooperativity of the lamellar-to-non lamellar phase transition and shifts it slightly towards lower temperatures. It was also observed in the IR spectra of the POPE/nisin complexes that the amide I band of nisin showed a similar profile to those observed for nisin bound to phosphatidylcholine and phosphatidylglycerol bilayers [16]: the maximum of the band envelope was at 1661 cm^{-1} and there was a component at 1638 cm^{-1} (data not shown). This indicates that a considerable proportion of nisin interacts with the PE membranes and this interaction leads to a change in the secondary structure of the peptide, likely an increase in the β-turn content, as proposed previously [16].

In order to further characterize the transition, ^{31}P-NMR has been used (Fig. 5). For pure POPE, the

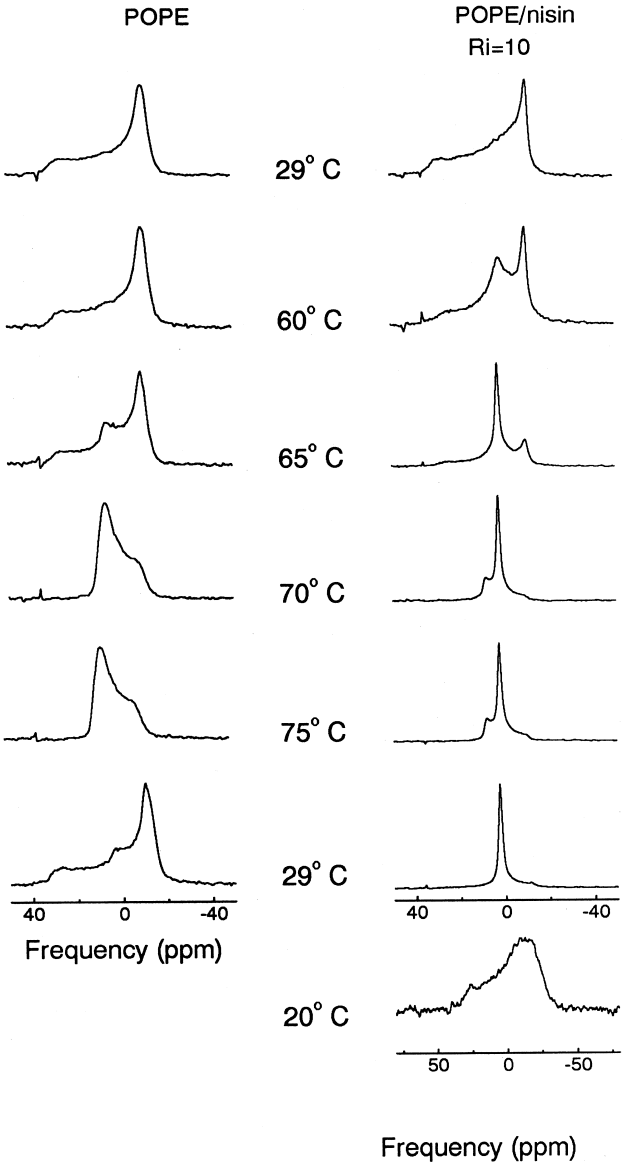

Fig. 5. ^{31}P-NMR spectra of pure POPE and POPE/nisin complex with an R_i of 10. The spectra were recorded from the top to the bottom. It is noted that the scale of the spectrum recorded at 20°C is different.

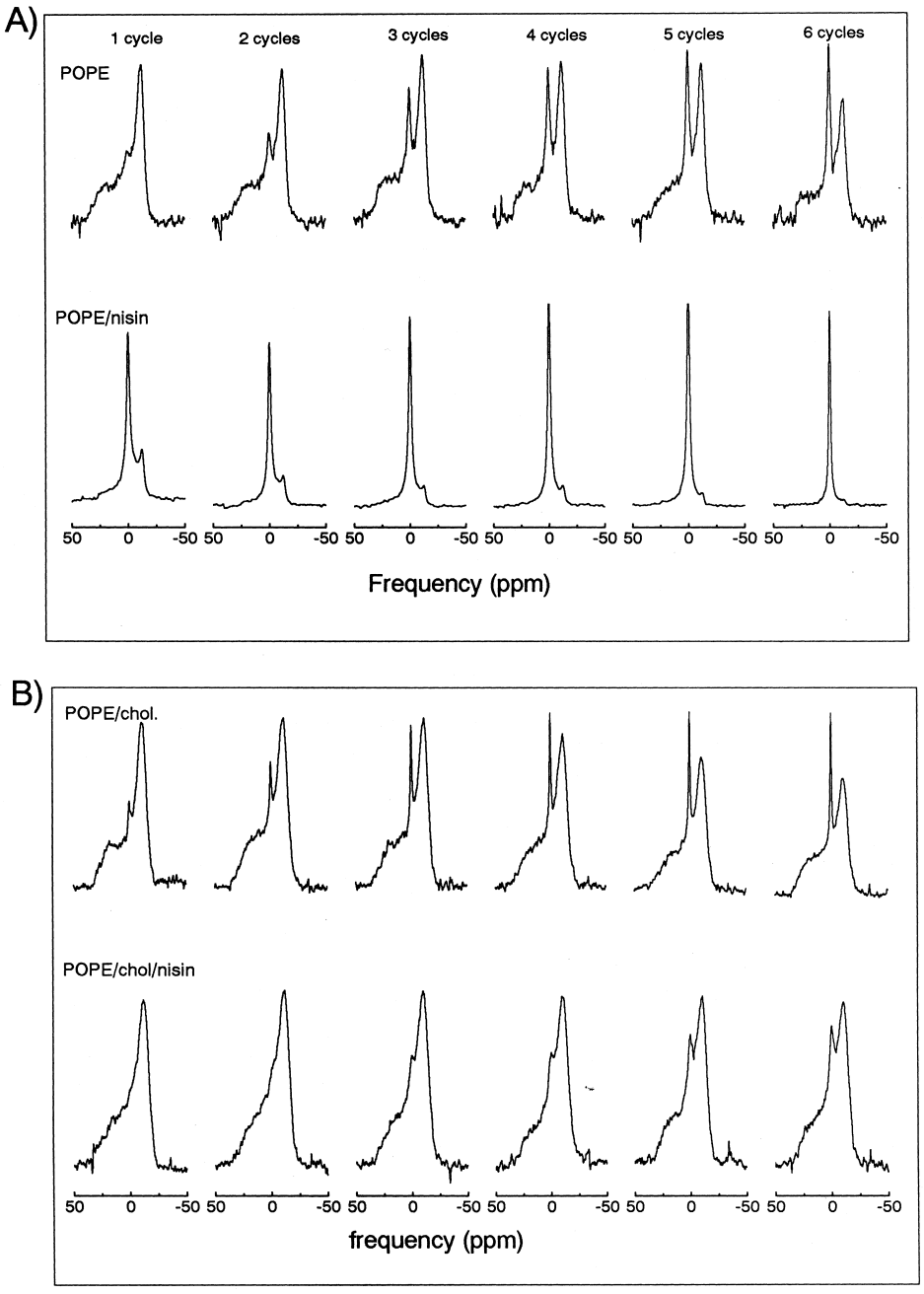

Fig. 6. ^{31}P-NMR spectra recorded: (A) at 65°C for pure POPE and POPE/nisin complex with an R_i of 10; and (B) at 50°C for POPE/30 mol% chol mixture and POPE/30 mol% chol/nisin complex at an R_i of 10. The samples with and without cholesterol were submitted to temperature cycles between 50 and 65°C, and 65 and 75°C, respectively.

spectra recorded below 65°C are typical of the fluid lamellar phase, with a CSA of 41 ppm. For higher temperatures, the spectrum shows a profile typical of the H_{II} phase. This transition is in agreement with the thermotropism described here by IR spectroscopy as well as with the literature [14,15]. A lamellar phase spectrum is obtained when the sample is cooled back to 29°C, indicating that the lamellar-to-hexagonal phase transition is readily reversible. When nisin is added in an R_i of 10, no significant changes are observed in the spectrum at 29°C. At 60°C, the spectrum reveals that a fraction of the

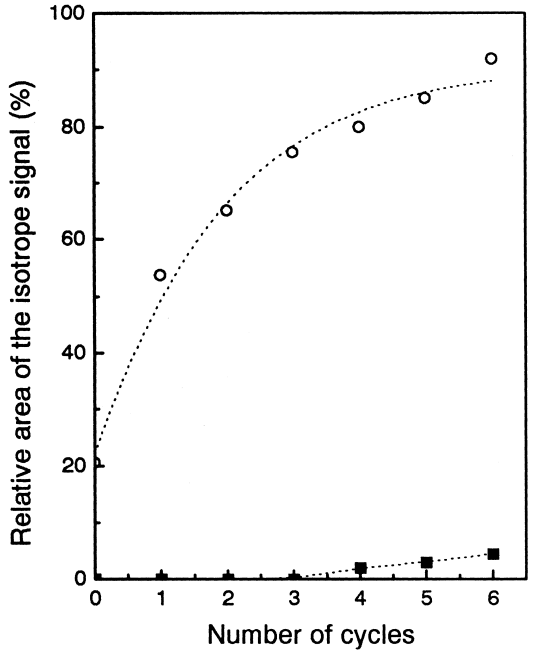

Fig. 7. Evolution of the relative area of the narrow line at 65°C as a function of the number of temperature cycles between 65 and 75°C. ■, Pure POPE; and ○, POPE/nisin complex with an R_i of 10.

phospholipids experiences isotropic motion as indicated by the apparition of a narrow line. This contribution increases with temperature to reach a relative area of 46% at 65°C. At 70 and 75°C, the remaining lamellar structure is transformed into a H_{II} phase which coexists with the isotropic structure. When the sample is cooled down to 29°C, the relative fraction of the component associated with isotropic motions becomes greater. When the temperature is decreased to 20°C, i.e. in the gel phase of the pure phospholipid, there is the disappearance of the narrow line which is replaced by a spectrum typical of a gel-phase lipid, with a CSA of about 50 ppm [7].

Previous studies have shown that some unsaturated phosphatidylethanolamines (e.g. dielaidoyl-*sn*-glycero-3-phosphatidylethanolamine, DEPE, and DOPE) form cubic phases when the samples experience several temperature cycling below and above T_h [17–19]. In order to define the influence of nisin on the formation of the cubic phase, the samples were submitted to a series of six cycles between 60 and 75°C. Fig. 6A shows the spectra obtained at 60°C during this experiment. It is observed that the relative area of the signal associated with isotropic motions increases with the number of cycles for the pure POPE. This behavior is similar to that previously observed for DEPE and DOPE [17,18]. In these cases, the narrow line was attributed to the formation of a cubic phase as confirmed by X-ray diffraction [18,19]. The same cycles were imposed on the POPE/nisin complex. After the first cycle, the narrow signal already represents a considerable proportion of the spectrum, about 20%. Its amplitude increases with the number of cycles as presented in a quantitative way on Fig. 7. After six cycles, the lipid gives rise almost exclusively to the narrow line in the presence of nisin, whereas the line represents less than 10% of the pure POPE spectrum. The component associated with isotropic motions is observed as long as the sample temperature is maintained above the T_m of pure POPE and the system is *reset* to a bilayer structure if the sample is cooled below T_m.

The formation of the isotropic phase in the presence of nisin was also examined with the POPE/30 mol% chol system. The thermotropism of the cholesterol-containing system has been determined by IR (Fig. 8). For POPE/30 mol% chol mixture, the gel-to-liquid crystalline phase transition is abolished because the matrix exists in a liquid ordered phase as recently reported [15]. In addition, it is observed that cholesterol favors the formation of the H_{II} phase as

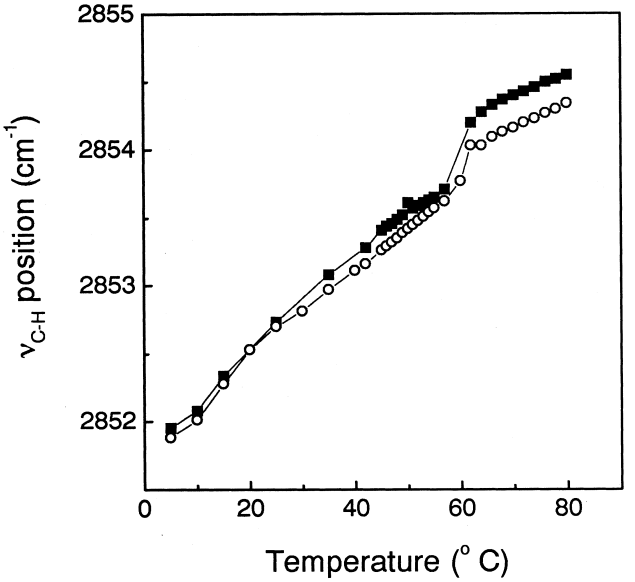

Fig. 8. Thermotropism of: (■) POPE/30 mol% chol mixture; and (○) POPE/30 mol% chol/nisin complex with an R_i of 10, as probed by the position of the ν_{C-H} band.

previously reported [14,15]. This is illustrated by the shift of the lamellar-to-hexagonal phase transition which occurs at 70°C for pure POPE, whereas it is observed at 58°C for POPE/30 mol% chol mixture. The presence of nisin appears to have very little effect on the thermotropism of this lipid mixture. Conversely to the observation on POPE/nisin complexes, the lamellar-to-hexagonal phase transition is not significantly affected by nisin when the lipid matrix contains cholesterol. The behavior of these systems has also been characterized by ^{31}P-NMR (Fig. 6B). The T_h of POPE/30 mol% chol mixture is 58°C. Therefore, the sample temperature was cycled between 50 and 65°C and the spectra were recorded at 50°C. Similarly to the observation on pure POPE, there is the appearance of a narrow signal in the spectrum of POPE/30 mol% chol mixture, and its proportion increases with the number of cycles. After six cycles, the relative area of the narrow line corresponds to less than 10% as in the case of POPE. So, despite its significant influence on the H_{II} phase formation, cholesterol has very little effect on the tendencies of POPE to form cubic phases. The presence of nisin, at an R_i of 10, does not considerably alter this behavior; after six cycles, about 3% of the lipids exist in the isotropic phase. Therefore, it can be concluded that the presence of cholesterol in the lipid matrix inhibits the power of nisin to induce the formation of an isotropic phase.

4. Discussion

The results presented here reveal that nisin promotes the formation of non-lamellar inverted phases in PE matrices. First, it is shown that nisin favors the H_{II} phase with DOPE and this promotion has been observed for relatively low peptide proportion (R_i of 60). Second, nisin promotes the formation of an isotropic phase with POPE, as shown by the considerable increase of the area of the narrow line in the ^{31}P-NMR spectra. Several lipid structures can lead to a component associated with isotropic motions, including micelles, bicelles, small vesicles and cubic phases [7,8]. The POPE/nisin complexes give rise to a narrow line, the relative intensity of which increases with cycling the sample temperature around the T_h and which disappears when the sample is cooled down below the T_m of the pure phospholipid. These features have been observed for other PE systems and they were associated with the cubic phase based on X-ray diffraction results [17–19]. Because of the similarities between the systems, it is most likely that the observed component associated with isotropic motions is attributed to a cubic phase.

The promotion of non-lamellar phase by nisin indicates that the association of the peptide with the bilayer modifies the amphiphilic balance. This change could be due to the penetration of the peptide in the bilayer. Nisin has a large hydrophobic section; actually, the segment 1–19 is exclusively hydrophobic if ^{12}Lys is excluded. The insertion of this part of the peptide in the bilayer would be thermodynamically favored. The insertion could be such that the ammonium of ^{12}Lys is in contact with the aqueous environment because of the stretching of its butyl chain; this 'snorkelling effect' has already been reported for other amphipathic peptides [20]. The insertion of nisin in the bilayer would lead to an increase of the hydrophobic contribution and, as a consequence, would favor non-lamellar inverted phases. This hypothesis is supported by fluorescence results providing information relative to the position of nisin in the membrane [21]. It was shown, using (W30)nisin, a nisin analog for which ^{30}Ile residue was replaced by a Trp, that the Trp is located near the center of bilayers formed with an equimolar mixture of egg-PE/egg-PC. Moreover, a recent study has shown that nisin inserts into DOPE monolayers, as displayed by an increase of surface pressure [22]. These findings reinforce the proposed penetration of the peptide in the lipid matrix, which would lead to a change in the curling propensities. Such a model has been suggested as a more general rationale for the modulation of lipid polymorphism by peptides [23]. It was the basis to explain the influence of melittin, a toxin extracted from bee venom, and cardiotoxin, a component of snake venom, on lipid polymorphism. At least two other antimicrobial peptides, gramicidin S [24] and alamethicin [19] also promote the formation of inverted non-lamellar phases in PE matrices. The promotion of non-lamellar phases by non-polar molecules, such as alkanes [25,26], squalene [27], and fluarizin, a drug used in cardiovascular pharmacology [28], has also been associated to their location in the hydrophobic core of the membrane.

It is also possible that nisin remains at the surface and causes a dehydration of the polar head group. Such a decrease of the effective area of the interfacial groups would also lead to the formation of non-lamellar inverted phases. Dehydration has been shown to promote non-lamellar structures [25,29]. However, this mechanism is less likely since the carbonyl stretching region of the infrared spectra does not indicate a significant change of the proportion of lipid ester carbonyl participating to hydrogen bonds for POPE and DOPE bilayers in the presence and the absence of nisin (data not shown).

The present study also shows that the presence of cholesterol in the membrane protects the POPE assemblies from the formation of nisin-induced isotropic phase. As previously reported, cholesterol promotes the formation of H_{II} phase in POPE [14,15], but here, it is shown that it also prevents nisin from altering this behavior. It has been shown that 30 mol% chol in a POPE matrix leads to the formation of a liquid ordered phase [15], as previously observed for PC/chol systems [30,31]. It has been suggested in the case of mastoporan [32] and melittin [33], that the liquid ordered phase limits the peptide insertion in the bilayer because of its tight lipid chain packing. An increase in the lipid chain order was observed in both the lamellar and H_{II} phase when cholesterol was present in POPE matrix [15]. Such a tighter lipid packing may limit the insertion of nisin in the lipid assemblies and, as a consequence, be at the origin of the inhibition of the promotion of the isotropic phase by nisin.

To our knowledge, this paper reports the first results showing that nisin promotes the formation of non-lamellar inverted phases. The phenomenon provides some insight into the possible antimicrobial mechanism of the peptide since the destabilization of the lamellar structure leads to the disruption of the membrane integrity. The formation of inverted non-lamellar phases by nisin can be associated with an increase of the spontaneous radius of curvature of the lipid plane [26,34]. This local change of curvature propensity may introduce a stress that leads to the perturbation of the membrane integrity. Interestingly, the promotion of non-lamellar inverted phases was also observed for gramicidin S [24] and alamethicin [19], two antibiotic peptides, suggesting that a change at the polymorphic propensities of the membrane may be a key factor in the antibiotic mechanism of some peptides. The proposed role of these polymorphic changes in the antibacterial mechanism is also consistent with the significant proportion of PE in the membranes of several Gram-positive bacteria [6]. Moreover, it has been shown that other lantibiotics, such as duramycin and cinnamycin, have specific interactions with PE species [35–37]. For example, it has been shown that duramycin leads to the aggregation of vesicles made of unsaturated PE [35]. Interestingly, the authors also reported the absence of PE in a duramycin-resistant strain of *Bacillus subtilis*. The change in curvature properties induced by nisin does not appear, however, to be related in a straightforward manner to the leakage of the vesicular content induced by this peptide since it has been shown that the presence of PE does not influence considerably nisin-induced leakage [4,38]. It should be added that nisin has a much greater effect on the transitions involving a change in lipid layer curvature than on the gel-to-liquid crystalline phase transition which involves mainly chain disordering, as already observed with apolar molecules including squalene [27] and fluarizin [28]. Therefore, the role of the non-bilayer forming lipids of the bacterial membranes in the response to nisin should be examined carefully. Finally, the protective effect of cholesterol may be at the origin of the resistance of erythrocytes to the effects of nisin previously reported [39].

Acknowledgements

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