Characterization of Permeability and Morphological Perturbations Induced by Nisin on Phosphatidylcholine Membranes

Rachida El Jastimi,* Katarina Edwards# and Michel Lafleur*

*Department of Chemistry, Université de Montréal, Montréal, Québec H3C 3J7, Canada, and #Fysikalisk-Kemiska Institutionen, Box 532, S-751 21 Uppsala, Sweden

ABSTRACT Nisin is an antimicrobial peptide used as food preservative. To gain some insights into the hypothesis that its bactericidal activity is due to the perturbation of the lipid fraction of the bacterial plasmic membrane, we have investigated the effect of nisin on model phosphatidylcholine (PC) membranes. We show that nisin affects the PC membrane permeability, and this perturbation is modulated by the lipid composition. Nisin-induced leakage from PC vesicles is inhibited by the presence of cholesterol. This inhibition is associated with the formation of a liquid ordered phase in the presence of cholesterol, which most likely reduces nisin affinity for the membrane. Conversely, phosphatidylglycerol (PG), an anionic lipid, promotes nisin-induced leakage, and this promotion is associated with an increased affinity of the peptide for the bilayer because nisin is a cationic peptide. When the electrostatic interactions are encouraged by the presence of 70 mol% PG in PC, the inhibitory effect of cholesterol is not observed anymore. Nisin drastically modifies the morphology of the dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) multilamellar dispersion without causing a significant change in the gel-to-liquid crystalline phase transition of the lipid. The morphological changes are observed from ^{31}P and ^{2}H NMR and cryo-electron microscopy. From the NMR point of view, the interactions giving rise to a broad signal (quadrupolar interactions and chemical shift anisotropy for ^{2}H NMR and ^{31}P NMR, respectively) are partly averaged out in the presence of nisin. This phenomenon is interpreted by the formation of curved lipid planes that lead to the lipid lateral diffusion occurring in the intermediate motional regime. By cryo-electron microscopy, large amorphous aggregates containing small dense globular particles are observed for samples quenched from 25 and 50°C. Long thread-like structures are also observed in the fluid phase. A structural description of DPPC/nisin complex, consistent with the experimental observation, is proposed. The presence of 30 mol% cholesterol in DPPC completely inhibits the morphological changes induced by nisin. Therefore, it is concluded that nisin can significantly perturb PC bilayers from both the permeability and the structural points of view, and these perturbations are modulated by the lipidic species in the bilayer.

INTRODUCTION

Nisin, a 34-residue peptide, is used as a food preservative because of its antimicrobial activity against several Gram-positive bacteria that might develop in food (Jung, 1991). Since it was proposed that its antibacterial activity takes place at the plasmic membrane level (Ruhr and Sahl, 1985; Kordel and Sahl, 1986), efforts have been devoted to examine the influence of nisin on lipid model membranes to bring some insights into its antibacterial mechanism. Nisin binds to lipid membranes, and its affinity for bilayers is sensitive to the lipid composition: it has been recently established that nisin affinity is increased by negatively charged lipids (Demel et al., 1996; Martin et al., 1996; Breukink et al., 1997; El Jastimi and Lafleur, 1997). This behavior was somehow expected because nisin is a peptide carrying 5 basic residues. In addition, the interaction of nisin with lipids leads to peptide insertion in lipid monolayers and this insertion is promoted by the presence of anionic lipids (Demel et al., 1996; Breukink et al., 1997). Nisin has also been shown to reduce the lateral diffusion of lipids and, again, the decrease is more pronounced for the negatively charged lipids (Kordel et al., 1989; Giffard et al., 1996). Nisin affects the permeability of lipid bilayers, and this modulation is again sensitive to the lipid composition. At this point, there is still some controversy regarding the influence of negatively charged lipids. It was reported that liposomes containing cardiolipin or phosphatidylserine were more susceptible to nisin-induced leakage than were neutral vesicles (Martin et al., 1996; Giffard et al., 1997). Conversely, it was indicated that dioleoyl-*sn*-glycero-3-phosphatidylglycerol (DOPG) vesicles were much more resistant to the action of nisin than were dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC) ones, suggesting a strong inhibitory effect of anionic lipids (Garcìa Garcerá et al., 1993; Driessen et al., 1995). It is now established that nisin-induced leakage does not require the presence of a transmembrane electric potential, even though the presence of such potential increases the perturbations of the membrane permeability caused by nisin (Moll et al., 1997). Recently, it has been shown that nisin affects the organization of lipid bilayers formed by unsaturated phosphatidylethanolamines (PE) by promoting the formation of inverse nonlamellar phases (El Jastimi and Lafleur, 1999).

In this paper, we present results that provide additional details relative to the role played by the lipid composition in nisin-induced leakage, more specifically the influence of cholesterol (chol). It has been shown that the presence of

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Address reprint requests to Michel Lafleur, Department of Chemistry, C.P. 6128, Succ. Centre Ville, Université de Montréal, Montréal, Québec, H3C 3J7, Canada. Tel.: 514-343-5936; Fax: 514-343-7586; E-mail: michel.lafleur@umontreal.ca.

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cholesterol inhibits the morphological changes induced by nisin on unsaturated PE (El Jastimi and Lafleur, 1999) and that this phenomenon may be related to the limited hemolytic action of nisin on red blood cells (Kordel and Sahl, 1986). We examine here whether this inhibition caused by cholesterol may be extrapolated to nisin-induced leakage. The lipid bilayer permeability has been characterized using the fluorophore release technique. In addition, we reveal that nisin drastically affects the structure of dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) vesicles and promotes the formation of a new type of organization. This is, to our knowledge, the first report of morphological perturbations of phosphatidylcholine bilayers induced by nisin. The structural examination has been done using ^{2}H and ^{31}P nuclear magnetic resonance (NMR) and infrared (IR) spectroscopy, and cryo-electron microscopy (EM).

MATERIALS AND METHODS

Nisin, 99% pure, was provided by Aplin and Barrett, Ltd. (Trowbridge, UK). All the phospholipids were obtained from Avanti Polar Lipids (Birmingham, AL), whereas cholesterol, 2-(*N*-morpholino)ethanesulfonic acid (Mes), Triton X-100, and deuterium-depleted water were purchased from Sigma (St. Louis, MO). Calcein was obtained from Molecular Probes (Eugene, OR) and ethylenediaminetetraacetic acid (EDTA) was purchased from Aldrich (Milwaukee, WI).

Fluorescence measurements

In the case of the lipid mixtures, each lipid species was first solubilized in a benzene/MeOH 95/5 (v/v) mixture. Aliquots of these solutions were mixed to provide the desired proportions, and the solution was then freeze dried.

For the leakage measurements, solid lipids were hydrated with an Mes (50 mM) buffer (30 mM NaCl, 5 mM EDTA, pH 6) containing 80 mM calcein. After five freeze-and-thaw cycles, the dispersions were extruded through polycarbonate filters with pores of 100-nm diameter, using a LiposoFast from Avestin (Ottawa, ON, Canada). The large unilamellar vesicles (LUV) that were formed were passed on a Sephadex G-50 gel (fine) column to separate the calcein-loaded vesicles from the free calcein. The column had been equilibrated with a buffer (150 mM NaCl, 50 mM Mes, 5 mM EDTA, pH 6) isosmotic with the calcein-containing buffer. In a quartz cuvette, the LUV were diluted to a phospholipid concentration of about 10–20 μM. The residual fluorescence intensity of the encapsulated calcein at high concentration (I_b) was measured. An aliquot of a nisin solution made in the external buffer was added. The leakage could be observed by the increasing fluorescence intensity (I_f) due to the dilution of the calcein in the external milieu. To normalize the release curves, Triton X-100 was added to the cuvette (10 μL of a 0.1 (v/v) % solution) to disrupt completely the vesicles and obtain the fluorescence intensity corresponding to complete release (I_t). The percent of release was then determined by

$$\%\ \text{of release} = \left(\frac{I_f - I_b}{I_t - I_b}\right) \times 100.$$

Figure 1 *A* displays a typical release curve.

These experiments were performed on a SPEX Fluorolog-2 spectrofluorimeter. The excitation and emission wavelengths were 490 and 513 nm, respectively. The bandpath widths of the excitation and the emission monochromators were adjusted to 2.2 and 1.9 nm, respectively. The experiments were done at room temperature and the samples were continuously stirred during the data acquisition.

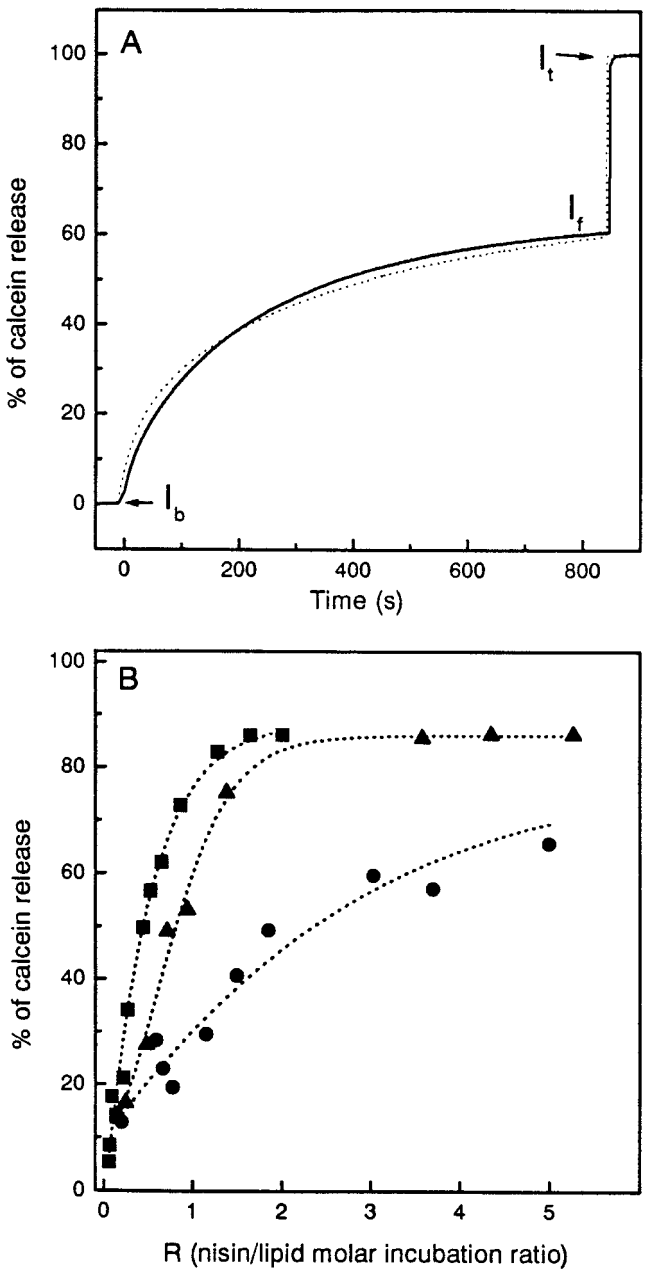

FIGURE 1 (*A*) Calcein release induced by nisin from POPC vesicles at $R = 0.67$ (——) and from POPC/30 mol% chol vesicles at $R = 3.3$ (·····). (*B*) Variation of nisin-induced release as a function of the peptide proportion for POPC (■), POPC/15 mol% chol (▲) and POPC/30 mol% chol (●) vesicles.

Infrared spectroscopy measurements

The sample preparation and the data acquisition were described elsewhere (El Jastimi and Lafleur, 1997). Briefly, nisin in solution in a 50-mM Mes buffer, containing 50 mM NaCl, 2 mM EDTA, pH 5.5, was added to preformed DPPC multilamellar vesicles. The sample was submitted to freeze-and-thaw cycling, from liquid nitrogen temperature to >40°C. For the nisin/DPPC complex, the sample was centrifuged at 3000 × *g* for 1 h to pellet the lipids and the associated nisin, and the spectra were recorded from an aliquot of the pellet. The spectra were obtained by transmission on a BioRad FTS25 spectrometer using a cell formed by 2 CaF_2 windows spaced out by a 5-μm thick Teflon ring mounted in a brass holder whose

temperature was regulated by thermopumps. Each spectrum was obtained from 200 scans with a resolution of 2 cm^{-1}. Before the determination of the peak positions in the C-H stretching region, the contribution of the O-H stretching band of water at 3400 cm^{-1} was eliminated by subtracting a least-square fitted polynomial simulating its edge.

NMR spectroscopy measurements

Solid lipids were hydrated as for the IR spectroscopy samples in a 50-mM Mes buffer containing 150 mM NaCl and 5 mM EDTA, pH 6, made with deuterium-depleted water. The appropriate amount of solid nisin was added to the lipid dispersion, and then, the samples were incubated for 2 h at 50°C. To show that the effects that we report are not specific to the addition of solid nisin to preformed vesicles, an alternative procedure was used for the sample preparation. Appropriate amounts of DPPC and nisin were cosolubilized in a $CHCl_3$:MeOH 1:1 mixture. Most of the organic solvent was removed with a nitrogen gas jet, and the samples were dried completely under vacuum overnight. The samples were then hydrated with the Mes buffer and then incubated at 50°C for 2 h. Similar results were obtained from both sample preparations.

A Bruker DSX-300 spectrometer was used to record the spectra. The ^{31}P NMR spectra were obtained from the free induction decay following a single 70° pulse of 10 μs. 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 ^{2}H NMR spectra were obtained with a quadrupolar echo pulse sequence, with a 90° pulse of 4.0–4.2 μs, an interpulse delay of 54 μs, and a relaxation time of 500 ms. After the second pulse, 8192 points were acquired in quadrature mode, with a dwell time of 5 μs. The number of scans was 20,000. Spin-spin relaxation time, T_{2e}, was obtained from 12 quadrupolar echoes for which the interpulse delay, τ, varied between 54 and 994 μs. T_{2e} was calculated by fitting the decrease of the spectrum area as a function of 2τ with a monoexponential. The sample temperature was controlled by a Bruker VT unit

Cryo-electron microscopy

Samples similar to those used for the NMR spectroscopy were prepared. The two sample preparation procedures were used and both gave similar results. The technique used for the cryo-EM examination of the lipid/nisin samples has been described in detail elsewhere (Bellare et al., 1988). A small drop of the sample dispersion was deposited on a copper grid covered with holey polymer film. Thin sample films, spanning the holes in the polymer film, were prepared by blotting away excess liquid with a filter paper. To avoid artifacts due to temperature changes or evaporation, the blotting procedure was performed at constant temperature (25 or 50°C) and humidity (close to 100%) within a custom-built environmental chamber. The films were then vitrified by quick freezing in liquid ethane and transferred to a Zeiss EM 902 transmission electron microscope for examination. To prevent sample perturbations as well as ice-crystal formation, the specimens were kept below 108 K during both the transfer and the viewing procedures. All observation were made in zero loss bright-field mode and at an accelerating voltage of 80 kV.

RESULTS

Nisin induces leakage from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC) LUVs. The leakage obtained at an incubation nisin/lipid molar ratio (R) of 0.67 is represented in Fig. 1 *A*. The release of calcein occurs over more than 10 min and reaches 59% after 800 s. The extents of leakage from POPC LUV observed 800 s after the addition of various nisin proportions are reported in Fig. 1 *B*. These results are in quantitative agreement with the carboxyfluorescein release observed from DOPC vesicles (Breukink et al., 1997). As expected, the extent of calcein release increases with increasing amount of nisin and an R of 0.43 is necessary to obtain a 50% release. It appears that it is impossible to empty all the vesicles, and there is always about 15% of calcein that is not released before the addition of Triton X-100, despite high concentrations of nisin (as high as $R = 2$). Such a behavior has already been reported for nisin-induced release from vesicles made from lipids extracted from *Escherichia coli* (Garcìa Garcerá et al., 1993).

Inspired by the inhibiting effect of cholesterol on the action of nisin on unsaturated PE (El Jastimi and Lafleur, 1999), we have examined the effect of cholesterol on nisin-induced leakage from POPC vesicles. Figure 1 *A* shows the release curve from POPC/30 mol% chol vesicles induced by nisin at $R = 3.3$. In these conditions, the rate of leakage is similar to that observed from POPC vesicles. However, about five times more nisin is needed to induce the same extent of leakage from the cholesterol-containing vesicles. This clearly indicates that cholesterol inhibits nisin-induced leakage from POPC vesicles. This inhibition is dependent on the cholesterol proportion in the bilayer. It is shown in Fig. 1 *B* that the inhibition caused by 15 mol% chol in the membrane is much less pronounced than that for a 30 mol% content. Compared to pure POPC vesicles, 1.5 times more nisin is required to cause 50% of calcein release when POPC bilayers contain 15 mol% chol, whereas it is 5.5 times more when the cholesterol content is 30 mol%.

We have also investigated the impact of cholesterol on nisin-induced leakage from negatively charged membranes to examine whether the inhibition observed with POPC was a general behavior. In addition, this set of experiments provides additional information relative to the controversial effect of negatively charged lipids on nisin-induced leakage. Figure 2 *A* shows the calcein release from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylglycerol (POPG) vesicles induced by nisin at $R = 0.14$. First, it is noted that the initial rate of leakage is faster than that observed for POPC vesicles. There is a rapid burst occurring during the first 20 s after the addition of the peptide, followed by a much slower release, practically equivalent to the passive diffusion of calcein across a bilayer. Such a release profile is in agreement with a previous report on carboxyfluorescein release induced by nisin from DOPG vesicles (Breukink et al., 1997). Second, the nisin-induced leakage is more significant in the case of POPG than with POPC. The leakages measured for different nisin contents are presented in Fig. 2 *B*. One nisin per 6 lipids leads to a calcein release of 60% from POPG vesicles, whereas only 15% of release was obtained from POPC vesicles. These results support the promoting effect of negatively charged lipids on nisin-induced lysis proposed previously (Giffard et al. 1997; Breukink et al., 1997). The promotion of the leakage is not linear with the charge density of the bilayer. The releases obtained from POPC/POPG (30/70 and 85/15) mixtures are also reported

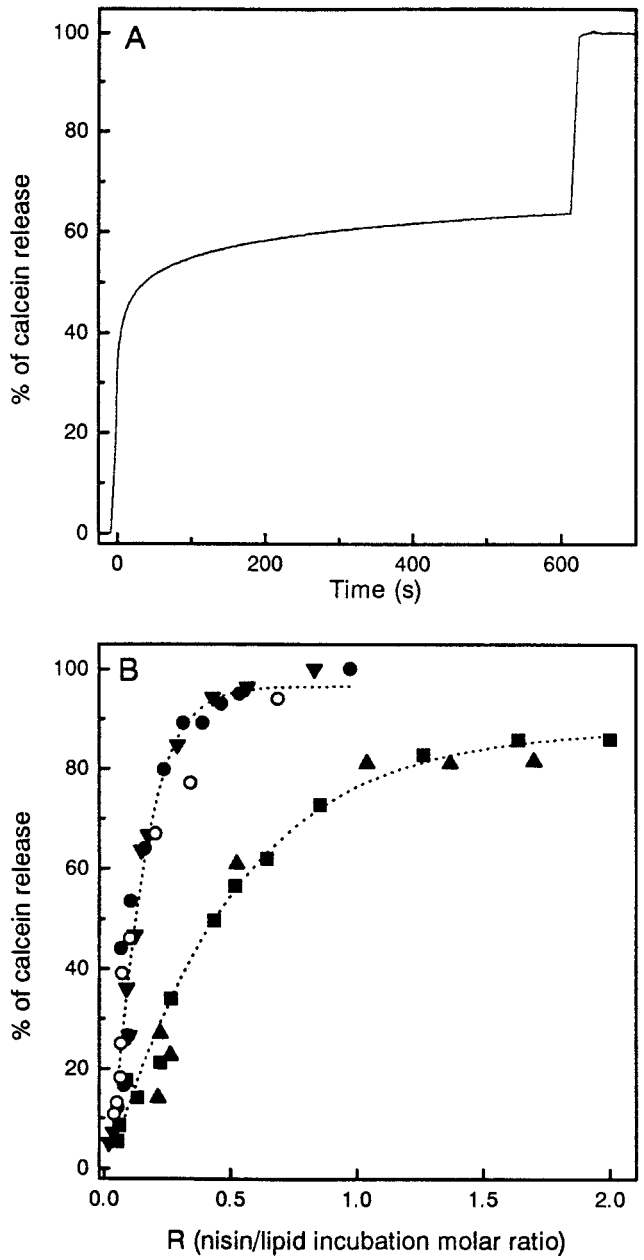

FIGURE 2 (*A*) Calcein release induced by nisin from POPG vesicles at $R = 0.14$. (*B*) Variation of nisin-induced release as a function of the peptide proportion for POPC (■), POPC/POPG (75/15) (▲), POPC/POPG (30/70) (●), and POPG (▼) vesicles. The release obtained from POPC/POPG (30/70) with 30 mol% chol is also presented (○).

in Fig. 2 *B*. The presence of 15 mol% of POPG in a POPC matrix does not significantly enhance the permeability perturbations induced by nisin, and similarly, the presence of 30 mol% POPC in a POPG does not reduce it. This dependence is in good agreement with that recently reported for the nisin-induced release from the DOPC/DOPG system (Breukink et al., 1997). Finally, Fig. 2 *B* also shows the effect of cholesterol on nisin-induced release from vesicles made up from the POPC/POPG 30/70 mixture. In contrast with the observation on pure POPC vesicles, cholesterol does not significantly influence the calcein release obtained in the presence of nisin from these negatively charged vesicles.

In addition of characterizing the effect of nisin on bilayer permeability, we have examined the influence of the peptide on the polymorphism of phosphatidylcholine aggregates. First, we have characterized the thermotropism of DPPC/nisin complex, at $R = 0.2$, using IR spectroscopy. Figure 3 shows the gel-to-liquid crystalline phase transition as reported by the shift of the methylene symmetric C-H stretching ($\nu_{C\text{-}H}s$) band. This vibrational mode is a suitable probe for this transition because its position is sensitive to the conformational order of the lipid acyl chains (Cameron et al., 1980; Mantsch and McElhaney, 1991). The temperature of the gel-to-liquid crystalline phase transition of pure DPPC is clearly observed at 41°C by the abrupt shift of the band by about 2 cm^{-1}. The gel-to-liquid crystalline phase transition is not considerably affected by the presence of nisin, the most notable effect being a decrease in the cooperativity. This result suggests that DPPC remains in a bilayer form with nisin. It has been shown previously that the gel-to-liquid crystalline phase transition of POPE is also not very sensitive to the presence of nisin (El Jastimi and Lafleur, 1999). In the IR spectra, the profile of the Amide I band is affected by the presence of DPPC (El Jastimi and Lafleur, 1997); the maximum of the band is shifted from 1653 to 1660 cm^{-1} upon binding. This shift is also observed for the complexes prepared by the addition of solid nisin, indicating that a considerable proportion of the peptide interacts with the lipids and this association leads to a change of its secondary structure as previously reported (El Jastimi and Lafleur, 1997).

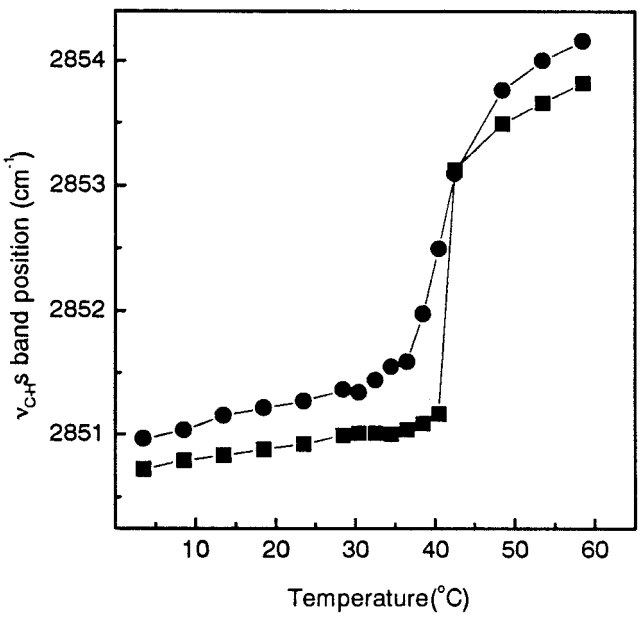

FIGURE 3 Thermotropism of DPPC (■) and DPPC/nisin at $R = 0.2$ (●), as probed by the $\nu_{C\text{-}H}s$ band position.

The morphology of DPPC/nisin complexes has been also examined by ^{31}P and ^{2}H NMR spectroscopy. Figure 4 presents the ^{31}P NMR spectra obtained for DPPC in the presence and the absence of nisin. For pure DPPC, the spectra are typical of multilamellar vesicles, and the gel-to-liquid crystalline phase transition is observed by the decrease of the chemical shift anisotropy (CSA), going from 51 ppm at 26°C, typical of a gel phase, to 42 ppm at 45 and 50°C, indicative of a fluid lamellar phase (Seelig, 1978). The spectrum of the DPPC/nisin complex, at $R = 0.2$, recorded at 26°C, is typical of an axially symmetric system similar to that obtained from phospholipid bilayers. At 45°C, there is the disappearance of the spectrum typical of MLV and a broad line slightly asymmetric is obtained. Its width at half-height is 13 ppm. It becomes narrower with increasing temperature to reach a width of 9 ppm at 50°C (it was 15 ppm when the sample was prepared from the lipid/nisin cosolubilized in organic solution). This change is completely reversible and the bilayer spectrum is reobtained when the sample is cooled back to 26°C (data not shown). Identical results were obtained with the samples made using the chain-perdeuterated DPPC that were used for the ^{2}H NMR experiments (see below). Upon the dilution of the DPPC/nisin complexes, there is the appearance of a broad component with a profile similar to a powder pattern with a CSA in the range of that measured for a bilayer (data not shown). These results

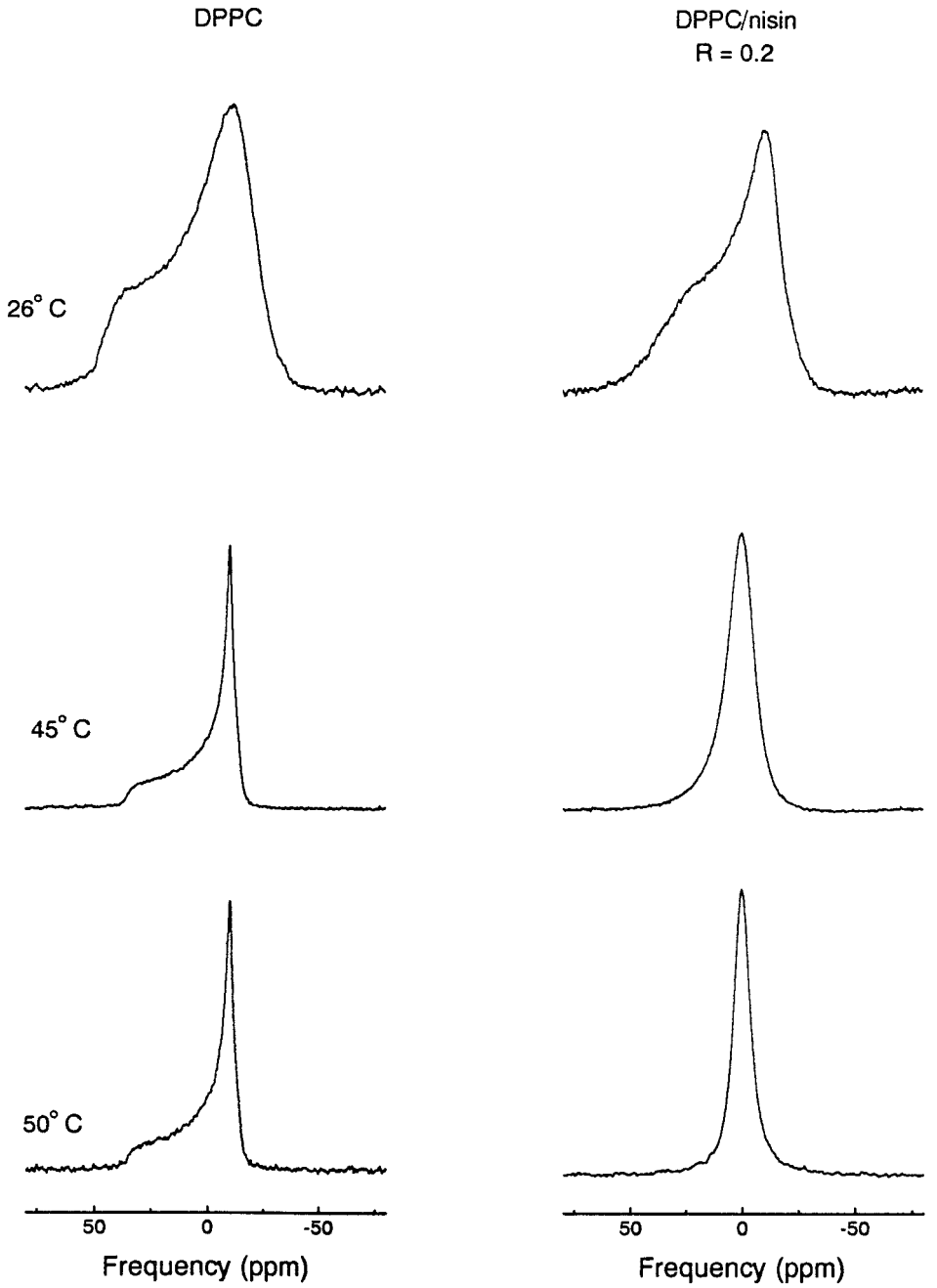

FIGURE 4 ^{31}P NMR spectra of pure DPPC and DPPC/nisin complex at $R = 0.2$. The acquisition temperatures are indicated on the left of the figure.

indicate that the dilution of the sample shifts the equilibrium away from the complex giving rise to the isotropic line, and bilayer-type structures are reformed.

The behavior of the DPPC/nisin complexes was also examined by ^{2}H NMR spectroscopy, using DPPC bearing perdeuterated palmitoyl chains (DPPC-d_{62}). The gel-to-liquid crystalline phase transition of DPPC-d_{62} considerably modifies its spectrum (Fig. 5). In the gel phase (26°C), the spectrum of DPPC-d_{62} shows a broad and featureless signal accompanied by a central doublet associated with the terminal methyls (Davis, 1979). In the fluid phase (50°C), the spectrum is a superposition of several partly resolved powder patterns typical of axial symmetry motions (Davis, 1979). The quadrupolar splitting of the outermost doublet, a signal associated with the methylene groups near the polar head group, is about 20 kHz (measured for the lipids whose long axis is perpendicular to the external magnetic field). In the gel phase, nisin has no considerable effect on the spectrum, in agreement with the results obtained by ^{31}P NMR. There is a progressive decrease of the splitting of the signal associated with the terminal methyls and the apparition of a small isotropic component. However, the relative area of the latter represents always less than 5% in the case of DPPC-d_{62}/nisin complex at $R = 0.067$. In the fluid phase, the spectrum of DPPC-d_{62}/nisin complex ($R = 0.067$) shows three features. First, the component typical of the bilayer signal has a similar width as that measured for the pure phospholipid. Second, there is a broadening of the peaks as illustrated by the figure insert. Finally, there is the apparition of an isotropic line. At $R = 0.2$, the loss of resolution is enhanced. Actually, no distinct signal associated with the methylene groups along the acyl chains can be resolved (see the insert). However, a broad component, remnant of the lipid bilayer signal, remains observable.

The degradation of the spectral resolution can be due to a variation of the spin-spin relaxation time, T_{2e} (Bayerl and Bloom, 1990; Fenske and Cullis, 1993). We have measured T_{2e} at 50°C for DPPC-d_{62}, with ($R = 0.067$) and without nisin. For the pure lipid, T_{2e} is 370 $\pm$ 6 μs, in agreement with values previously reported for fluid-phase phosphatidylcholine bilayers (Bienvenue et al., 1982; Dico et al., 1997). T_{2e} decreases to 220 $\pm$ 23 μs for the complex with nisin. A similar reduction of T_{2e} of the phospholipid chains accompanied with a limited influence on the orientational chain order was also observed in the presence of rhodopsin (Bienvenue et al., 1982) and the pulmonary protein SP-B (Dico et al., 1997); a degradation of the spectral resolution could also be observed in these cases.

NMR spectroscopy is a molecular technique that provides indirect indications relative to the macrostructure of the lipid aggregates. To get information on another length scale, we have examined the nisin/DPPC complexes ($R = 0.2$) by

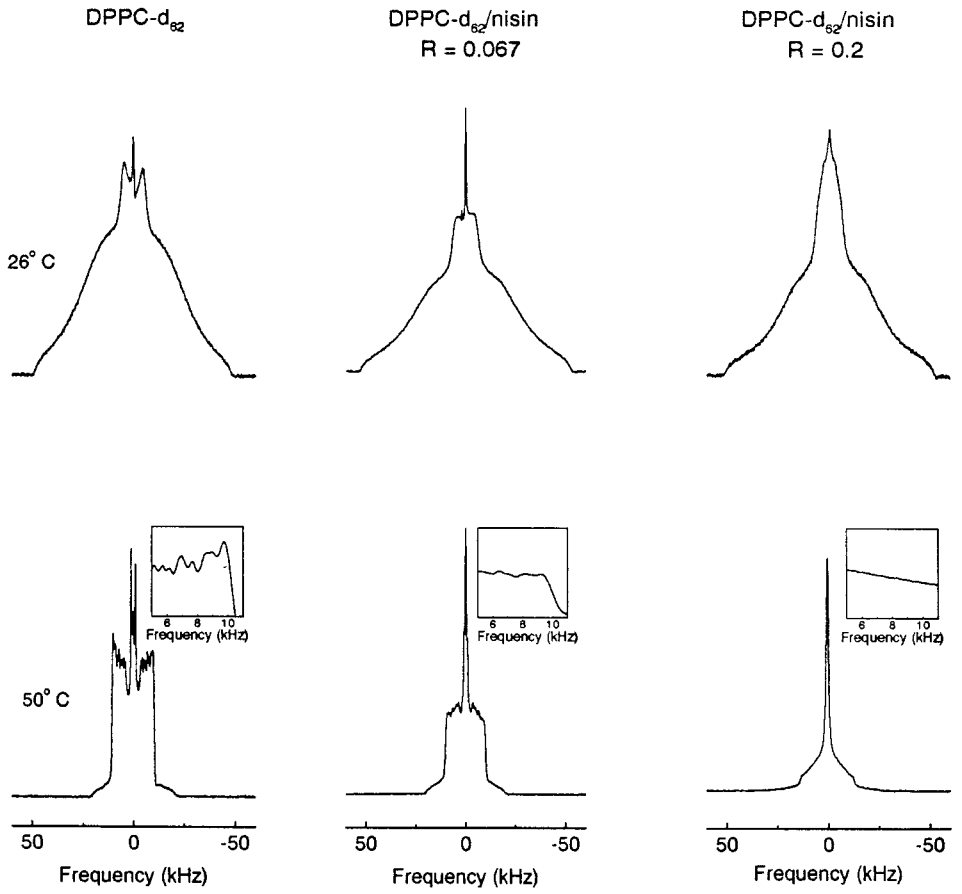

FIGURE 5 ^{2}H NMR spectra of pure DPPC-d_{62} and DPPC-d_{62}/nisin complex at $R = 0.067$ and 0.2. The acquisition temperatures are indicated on the left of the figure. The inserts represent a zoom of the region between 5 and 11 kHz of the spectra obtained from the fluid phase.

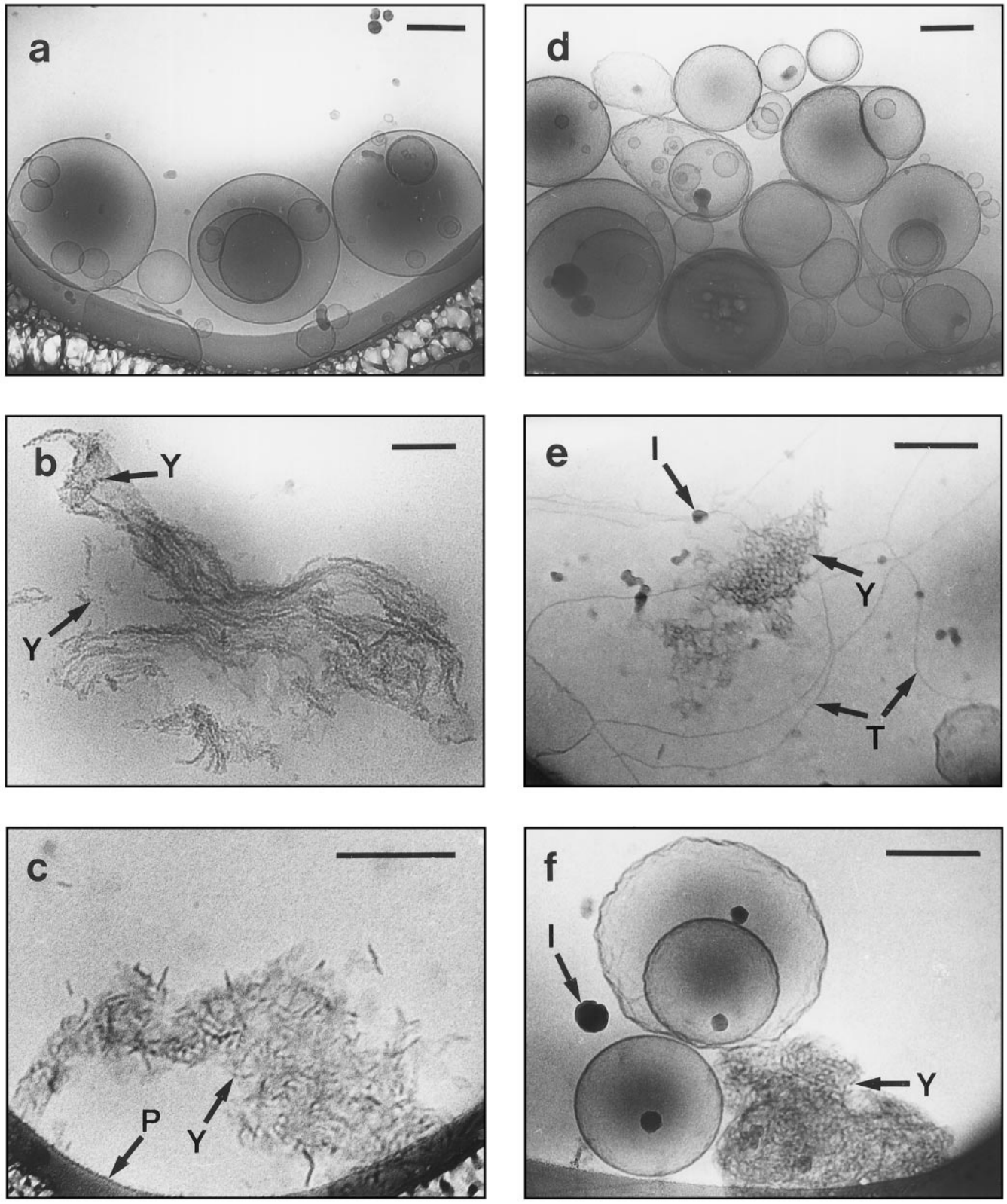

FIGURE 6 Micrographs obtained from (*a* and *d*) FATMLV of pure DPPC and (*b*, *c*, *e*, and *f*) DPPC/nisin samples ($R = 0.2$). The micrographs *a–c* were obtained from samples frozen from the gel phase (25°C), whereas *d–f* were obtained from the fluid phase (50°C). Y indicates some dense globular particles, T indicates thread-like aggregates, P indicates the edge of the polymer support, and I indicates ice crystals deposited on the surface of the sample after vitrification. The bar length corresponds to 200 nm.

cryo-EM (Fig. 6). The micrographs of pure DPPC vesicles are displayed for samples frozen from the gel (Fig. 6 *a*) and the fluid phase (Fig. 6 *d*). The observed structures are similar to those obtained by freeze-fracture EM on frozen and thawed MLV (FATMLV) (Mayer et al., 1985). In the presence of nisin, no typical MLV could be observed when the sample is frozen from the gel phase (25°C) (Fig. 6, *b* and *c*). Instead, crystalline-like aggregates that appear to contain a large number of small globular dense particles (as seen as dark dots) completely dominate the sample. The dense particles seem to align, one relative to the others, and form filaments of about 5–10 nm of diameter. When the DPPC/nisin sample is frozen from the fluid phase (50°C), three types of structures can be detected (Fig. 6, *e* and *f*). First, there is a very polydispersed population of vesicles. Second, diffuse amorphous structures of various shape and size are commonly observed. Within these aggregates, the small dense particles are also observed. It is likely that these aggregates are the fluid form of those observed when the sample is quenched from the gel phase. Third, one can observe long entangled and connected, thread-like aggregates similar to those formed by phosphatidylcholine in the presence of sodium decyl sulfate (Silvander et al., 1996).

Finally, the effect of cholesterol on the morphological changes induced by nisin on DPPC was examined by ^{2}H and ^{31}P NMR. Figure 7 presents the spectra obtained from DPPC-d_{62}/30 mol% chol mixture with ($R = 0.2$) and without nisin. The spectra of the pure lipid are typical of the liquid-ordered phase formed by this mixture (Vist and Davis, 1990). They show that, at both temperatures, the lipid molecules experience axially symmetric motions and have their acyl chains relatively ordered, the largest splitting at 26°C being 61 kHz. The addition of nisin does not significantly influence the spectra. The only small difference is the apparition of a small isotropic line, which contributes less than 5% of the total signal. ^{31}P NMR spectra of DPPC/30 mol% chol mixture at $R = 0.2$ were also recorded at 26, 45, and 50°C, and the presence of peptide does not modify the NMR signal in these cholesterol-containing samples (data not shown).

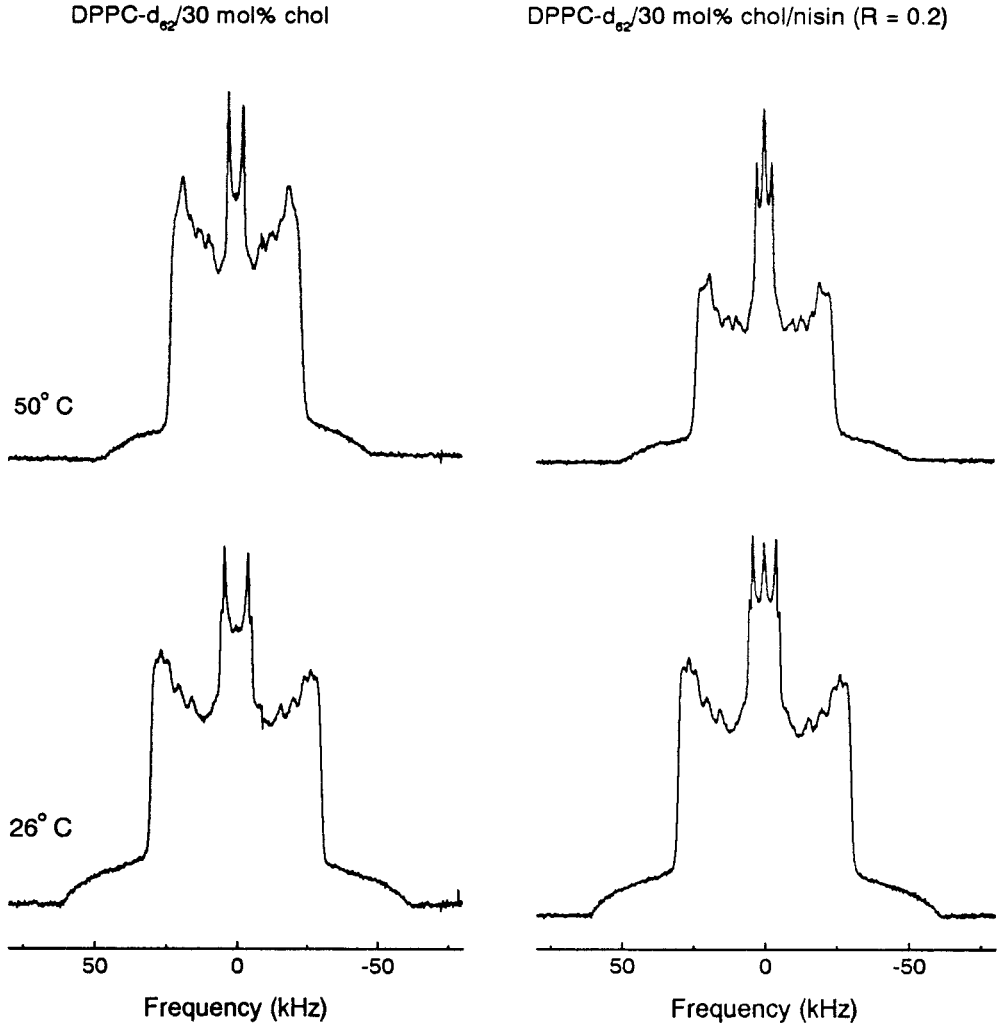

FIGURE 7 ^{2}H NMR spectra of pure DPPC-d_{62}/30 mol% chol mixture and DPPC-d_{62}/30 mol% chol in the presence of nisin at $R = 0.2$. The acquisition temperatures are indicated on the left of the figure.

DISCUSSION

The results presented here provide additional information relative to the dependence of the membrane permeability perturbations induced by nisin on the lipid composition. First, this study reveals that nisin-induced leakage from POPC vesicles can be inhibited by the presence of cholesterol in the membrane. The most likely rationale for this inhibition is the formation of the liquid ordered phase when cholesterol is present in phosphatidylcholine bilayers (Vist and Davis, 1990; Thewalt and Bloom, 1992). Because of the more ordered and tighter lipid chain packing in this phase, the affinity of nisin for the membrane is probably decreased and, as a consequence, its lytic power is inhibited. This inhibitory effect is much more pronounced for a cholesterol content of 30 mol% than 15 mol%. At 30 mol%, the lipids exclusively form a liquid-ordered phase, and this is likely associated to the bilayer resistance to the action of nisin. Such resistance is probably a common feature because it has been already reported that cholesterol reduces the lytic activity of several other peptides including magainin (Matsuzaki et al. 1995), melittin (Benachir et al., 1997), and the synthetic peptide GALA (Nicol et al., 1996).

Second, it is shown that the presence of negatively charged lipids in the bilayer promotes the ability of nisin to induce leaks, in agreement with previous works (Breukink et al., 1997, Martin et al., 1996). The ability to promote the leakage is not linear with the anionic lipid content: the promotion being observed between 15 and 70 mol% of POPG in POPC, in agreement with a previous study on DOPC/DOPG systems (Breukink et al., 1997). This dependence on negative charge density of the bilayer surface correlates nicely with the peptide insertion quantified as surface pressure increase on monolayers; the increase of the nisin penetration was also observed when the phosphatidylglycerol proportion varies from 20 to 70% in a dioleoyl-*sn*-glycero-3-phosphatidylethanolamine matrix (Demel et al., 1996). Nisin has a greater affinity for the negatively charged bilayers than for the zwitterionic ones (Breukink et al., 1997; El Jastimi and Lafleur, 1997). Therefore, the increased affinity, related to attractive electrostatic interactions between the negatively charged lipids and the positively charged residues of the peptide, is most likely associated with the promoting effect of anionic lipids on nisin-induced leakage. The promotion of leakage by negatively charged lipids has also been observed for other cationic peptides including tachyplesin I (Matsuzaki et al., 1991), and magainin (Matsuzaki et al., 1995). These peptides have in common a very weak affinity for zwitterionic bilayers, and their interactions with membranes are dominated by electrostatic interactions. This behavior contrasts with the case of melittin (Dufourcq and Faucon, 1977; Beschiaschvili and Seelig, 1990), and the amphipathic model peptide KLAL (Dathe et al., 1996) which display a considerable affinity for zwitterionic membranes. For example, the concentration of melittin or KLAL required to induce 50% of leakage from POPC vesicles is 200 times less than that of nisin (Benachir and Lafleur, 1995; Dathe et al., 1996). Despite the fact that negatively charged lipids increase the affinity of these peptides for the membranes (Dufourcq and Faucon, 1977; Beschiaschvili and Seelig, 1990; Dathe et al., 1996), these lipids inhibit the peptide-induced leakage (Ohki et al., 1994; Benachir and Lafleur, 1995; Dathe et al., 1996). The inhibition of the perturbing effect of the peptides caused by the negatively charged lipids has been rationalized by the strong anchoring of the peptide at the membrane interface preventing its relocation in the membrane that leads to the formation of defects responsible for the release of the entrapped material. The impact of negatively charged lipids on the leakage induced by peptides does not show a general trend and peptides activated and peptides inhibited by anionic lipids can be distinguished. The category in which a peptide would fall appears to be representative of the balance between the electrostatic and hydrophobic interactions. Interestingly, the bilayer affinity of KLAL can be reduced by replacing two residues by their D-amino acid isomers; this change being associated with a reduction of the hydrophobic domain of the peptide (Dathe et al., 1996). In the case of this mutant, the lysis of POPC vesicles was much reduced compared to KLAL but was stimulated by the presence of anionic lipids. This finding reinforces the proposed association between the impact of negatively charged lipids on peptide-induced release and the nature of the peptide/bilayer interactions.

The presented results show that, in contrast to the observation on POPC vesicles, the inhibitory effect of cholesterol on nisin-induced leakage is clearly much limited for negatively charged bilayers. It has been shown, from binding experiments, that the affinity of nisin to lipid bilayers is essentially dictated by electrostatic interactions and that the bilayer order, even drastically modified by the gel-to-liquid crystalline phase transition, has a minor effect (El Jastimi and Lafleur, 1997). In the case of vesicles containing both cholesterol and POPG, it appears that the changes of the hydrophobic core order induced by the presence of cholesterol has very little effect when electrostatic interactions are promoted by the presence of POPG. This phenomenon may be associated with the easier penetration of nisin in DOPG monolayers than in DOPC (Demel et al., 1996).

In addition to the permeability, nisin also affects the morphology of DPPC bilayers. In the fluid phase, nisin drastically decreases the CSA of the ^{31}P NMR signal of the phospholipids, and the obtained spectra do not show a powder pattern but a line centered at the isotropic chemical shift. In contrast, the ^{2}H NMR spectra still show a signal with a width typical of the bilayer but with a considerable line broadening. To effectively reduce the CSA in ^{31}P NMR and quadrupolar interactions in ^{2}H NMR, lipid motions that change the molecule orientation relative to the external magnetic field have to be fast relative to the NMR time scale (τ_{NMR}), which is associated with the inverse of the spectral width of the signal (Fenske and Cullis, 1993). Because the signal width is significantly different for ^{31}P and ^{2}H NMR spectra, the associated τ_{NMR} are also different. In the case of ^{31}P NMR, the spectral width, at 121 MHz, is $\sim$6 kHz, leading to a τ_{NMR} of about 150 μs. In the case of

^{2}H NMR, the signal is ~120 kHz wide, leading to a shorter τ_{NMR} of about 8 μs. Because of these different time scales, a motion can be effectively more rapid in ^{31}P NMR than in ^{2}H NMR. This phenomenon has already been observed for LUV with a diameter of about 120 nm (Fenske and Cullis, 1993). Experimentally, a broad isotropic line is observed in the ^{31}P NMR spectrum of such LUV, whereas there is only a partial averaging in their ^{2}H NMR spectrum, causing a loss of resolution but leaving a broad and almost featureless component (Fenske and Cullis, 1993). For these vesicles, the correlation times associated with the lipid diffusion along the curved lipid plane and the tumbling of the vesicles fall in the intermediate motional regime (Burnell et al., 1980; Fenske and Cullis, 1993). With the same rationale, lipid diffusion along a curved surface with a correlation time in the intermediate regime can be at the origin of the NMR results obtained with the DPPC/nisin complexes. The formation of unilamellar vesicles with small radius would be consistent with the NMR results, but can be ruled out from the cryo-EM pictures. The pictures obtained from the sample quenched from 50°C indicate the formation of large amorphous aggregates and thread-like structures that are likely at the origin of the NMR changes; the polydispersed vesicles also observed are probably related to the remnants of powder patterns observed in the ^{31}P NMR spectra, particularly for diluted samples. In addition, in the presence of nisin, the sample does not become translucent and it is possible to pellet the complex by centrifugation, ruling out the formation of small vesicles. Therefore, it is proposed that the curved lipid planes are present in the large amorphous aggregates. The hypothesis of curved planes is reinforced by the decrease of T_{2e} observed in the presence of nisin relative to that of the pure phospholipid; lipid diffusion along a curved surface is a motion contributing to the spin-spin relaxation, as illustrated by the T_{2e} decrease observed with LUV radius decrease (Bayerl and Bloom, 1990; Fenske and Cullis, 1993). Indeed, other factors can be at the origin of the modifications of the NMR spectra, but the formation of highly curved lipid planes appears to be the most consistent with the combined results from cryo-EM and NMR.

A gel-to-liquid crystalline phase transition is observed for the DPPC/nisin complexes. It is clearly indicated by the disordering of the chains at 40°C, as illustrated by the shift of the $\nu_{C\text{-}H}$s band in IR spectroscopy. The phase transition is also detected by the changes in the ^{2}H as well as the ^{31}P NMR spectra. Finally, the aspect of the aggregates as pictured by cryo-EM undergoes significant changes between 25 and 50°C. The fact that such a transition is still observed strongly suggests that DPPC molecules still form bilayers in the aggregates with nisin. The formation of a gel phase bilayer below 40°C is supported by all the spectroscopic data (powder pattern with axial symmetry in ^{31}P NMR, spectra with a significant asymmetry parameter in ^{2}H NMR, $\nu_{C\text{-}H}$s band position typical of the gel phase).

The molecular organization of DPPC/nisin complexes should provide lipid planes with a small radius of curvature inferred from the NMR results, a lipid bilayer that would undergo a lipid phase transition, and should lead to large amorphous/long thread-like aggregates observed by cryo-EM. By cryo-EM, it was also observed that the aggregates contain several dense globular particles that are proposed to be nisin aggregates. Therefore, the amorphous particles are proposed to contain aggregated nisin particles in the presence of curved lipid bilayers. The complex appears also to exist under a thread-like micellar form. The details of these structures remain to be defined but the present results clearly establish that the antimicrobial peptide alters the vesicular lipid structures.

At this point, the driving force of such a reorganization is not clearly identified. The insertion of nisin in the hydrophobic core of the DPPC bilayers may be at the origin of the morphological changes. The results presented here suggest a disordering of the gel phase, which may be associated with the penetration of the peptide in the bilayer. The frequency of the $\nu_{C\text{-}H}$s band in the IR spectra is slightly higher for the nisin/DPPC complex than for the pure lipid, suggesting an increased conformational disordering (Cameron et al., 1980; Mantsch and McElhaney, 1991). A decrease of the quadrupolar splitting of the terminal methyl in the presence of nisin is observed in the ^{2}H NMR spectra recorded at 26°C, indicating increased motions at the center of the bilayer. Nisin insertion has been also reported in monolayers formed of DOPC (Demel et al., 1996). In addition, a study on a nisin mutant for which ^{30}Ile was replaced by a Trp indicated that the fluorescent residue was preferentially located near the center of the neutral bilayers made of a phosphatidylcholine/phosphatidylethanolamine mixture (Martin et al., 1996). Finally, it was reported recently that nisin induces the formation of structures involving curved lipid planes (inverted hexagonal and cubic phases) with unsaturated PE (El Jastimi and Lafleur, 1999) and this morphological change may be also related to the shift of the amphiphilic balance caused by the penetration of the peptide in the lipid assemblies. The disruption of the vesicular structure leads unquestionably to a drastic alteration of the membrane integrity and should be examined as a possible contribution of the antibacterial mechanism of nisin.

Finally, it is shown that the reorganization induced by nisin is prevented by the presence of cholesterol in the bilayer. It appears that the sterol inhibits the two perturbations of the phosphatidylcholine membranes induced by nisin investigated in this paper, namely the changes in permeability and the morphological perturbations. The changes induced by nisin on POPE macrostructure were also inhibited by the presence of cholesterol (El Jastimi and Lafleur, 1999). It appears that membranes existing in the liquid ordered phase, induced by the presence of high cholesterol concentration, are more resistant to the attack of nisin. This phenomenon may be at the origin of the lack of hemolytic power of nisin previously reported (Kordel and Sahl, 1986) because red blood cell membranes contain a significant proportion of cholesterol. However, it should be added that cholesterol effect is not observed when the membrane contains 70% of anionic POPG. This phenome-

non reinforces the inference that nisin–lipid interactions are dominated by electrostatic interactions and the contribution of the hydrophobic interactions plays a secondary role.

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