Mechanism of Action of the Peptide Antibiotic Nisin in Liposomes and Cytochrome c Oxidase-Containing Proteoliposomes

F. H. GAO, T. ABEE, AND W. N. KONINGS*

Department of Microbiology, University of Groningen, Kerklaan 30, 9651 NN Haren, The Netherlands

The interaction of the peptide antibiotic nisin with liposomes has been studied. The effect of this interaction was analyzed on the membrane potential (inside negative) and the pH gradient (inside alkaline) in liposomes made from *Escherichia coli* phosphatidylethanolamine and egg phosphatidylcholine (9:1, wt/wt). The membrane potential and pH gradient were generated by artificial ion gradients or by the oxidation of ascorbate, N, N,N',N'-tetramethyl-p-phenylenediamine, and cytochrome c by the beef heart cytochrome c oxidase incorporated in the liposomal membranes. Nisin dissipated the membrane potential and the pH gradient in both types of liposomes and inhibited oxygen consumption by cytochrome c oxidase in proteoliposomes. The dissipation of the proton motive force in proteoliposomes was only to a minor extent due to a decrease of the oxidase activity by nisin. The results in these model systems show that a membrane potential and/or a pH gradient across the membrane enhances the activity of nisin. Nisin incorporates into the membrane and makes the membrane permeable for ions. As a result, both the membrane potential and pH gradient are dissipated. The activity of nisin was found to be influenced by the phospholipid composition of the liposomal membrane.

Nisin is a small gene-encoded antimicrobial peptide produced by *Lactococcus lactis* (1). It belongs to the lantibiotic group and contains dehydro residues (dehydroalanine and dehydrobutyrine) and thioether cross-linkages that are introduced by posttranslational modifications of the amino acids serine, threonine, and cysteine (5, 12). Nisin exhibits antimicrobial activity against a wide spectrum of gram-positive organisms (9, 15, 17). The mechanism of the bacterial action of nisin is still unclear, although it was found as early as 1928 (9) and has been widely used as a preservative in the food and dairy industries (9). Nisin has been shown to effect peptidoglycan synthesis (16) and cytoplasmic membrane permeability properties (17, 18). In addition, nisin was found to cause a rapid efflux of amino acids and Rb^+ from the cytoplasm of a number of gram-positive bacteria such as *Staphylococcus cohnii* and *Bacillus subtilis* (11, 17). As a consequence, the membrane potential dropped dramatically, and it was suggested that nisin dissipates the membrane potential and ionic gradients across the cytoplasmic membrane by affecting its integrity (17). Intact cells of gram-negative bacteria were not affected by nisin. However, cells of *Escherichia coli* became sensitive when the outer membrane was disrupted (11).

Experiments with intact cells and cytoplasmic membrane vesicles of gram-positive bacteria indicated that nisin requires an energized membrane to exert its effect (17). However, this could not be verified with artificial asolectin liposomes (17). Furthermore, no information is available on whether nisin is functionally inserted into the membrane in the presence of a pH gradient (ΔpH) only.

Therefore, we studied in more detail the effect of nisin on the membrane potential (negative inside) and/or the pH gradient (alkaline inside) generated by artificial ion gradients in liposomes made from (i) *E. coli* phosphatidylethanolamine (PE)-egg phosphatidylcholine (PC) (9:1, wt/wt), (ii) soybean PC (asolectin), and (iii) egg PC. In addition, the effect of nisin was investigated on cytochrome c oxidase proteolipo-

somes (COVs) made from *E. coli* PE-egg PC (9:1, wt/wt) in which beef heart cytochrome c oxidase has been incorporated as a H^+ pump. In COVs, a $\Delta\Psi$ and a ΔpH can be formed by the oxidation of the electron donor system ascorbate–N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD)–cytochrome c (3). The results obtained in these model systems show that nisin dissipates both the membrane potential and the pH gradient and that the phospholipid composition of the liposomal membrane affects the activity of nisin.

MATERIALS AND METHODS

Preparation of liposomes. Liposomes were prepared from asolectin, from egg PC, and from a mixture of *E. coli* PE and egg PC (9:1, wt/wt). Appropriate quantities of lipids were dissolved in $CHCl_3$-methanol (9:1, vol/vol), dried under vacuum for 40 min, and then suspended in buffer at a concentration of 20 mg of lipid per ml with a bath sonicator (Sonicon Instruments). Liposomes were obtained by sonication by using a probe sonicator (Soniprep 150; MSE Scientific Instruments, West Sussex, United Kingdom) for 300 s at 4 μm amplitude with intervals of 15 s of sonication and 45 s of rest at 5°C under a constant stream of N_2.

Proton motive force generation in liposomes. Liposomes prepared in 50 mM potassium phosphate (pH 6.0) were diluted 100-fold in 50 mM sodium phosphate (pH 6.0). Upon addition of the K^+ ionophore valinomycin (0.5 μM), a membrane potential ($\Delta\Psi$, interior negative) can be generated. The reaction was performed at 25°C in a Perkin Elmer LS-50 spectrophotofluorometer, and the $\Delta\Psi$ was followed with the fluorescent dye 3,3-dipropylthiacarbocyanine (8 μM) (excitation wavelength, 643 nm; emission wavelength, 666 nm). Liposomes prepared in 20 mM potassium phosphate (pH 6.0) supplemented with 100 mM potassium acetate were diluted 100-fold in 20 mM potassium phosphate (pH 6.0) containing 100 mM potassium–piperazine-N,N'-bis(ethanesulfonate) (K-PIPES) for the generation of a pH gradient (ΔpH, interior alkaline). The reaction was performed at 25°C in a Perkin Elmer LS-50 spectrophotofluo-

---

* Corresponding author.

rometer. The internal pH of the liposomes was determined from the fluorescence of pyranine (excitation wavelength, 450 nm; emission wavelength, 508 nm) entrapped within the liposomes. The entrapment was achieved by adding 100 μM pyranine to the liposomes before sonication. External pyranine was removed by chromatography of the membrane suspension over a Sephadex G-50 column (1 by 6 cm). The ΔpH can be calculated from the difference between the external and internal pHs. A conversion factor Z of 59 at 25°C was used to express ΔpH $(-Z\Delta pH)$ in millivolts.

Reconstitution of cytochrome c oxidase in liposomes. A mixture of 20 mg of dried $E.\ coli$ PE and egg PC (9:1, wt/wt) and 9 mg n-octyl-β-D-glucopyranoside in 1 ml of 50 mM potassium phosphate (pH 6.0) was sonicated until it was clear under a stream of N_2 gas with a probe sonicator. Beef heart cytochrome c oxidase (5.1 nM heme a) was added, and the suspension was then dialyzed at 4°C for 2 h against a 500-fold volume of 50 mM potassium phosphate (pH 6.0). Dialysis was repeated for another 2 h and continued overnight at 4°C.

Proton motive force generation in COVs. COVs in 50 mM potassium phosphate (pH 6.0) were energized by the addition of the electron donor system ascorbate (10 mM, adjusted to pH 6.0 with 10 N KOH), horse heart cytochrome c (10 μM), and TMPD (200 μM) unless indicated otherwise. The $\Delta\Psi$ was measured in the presence of nigericin (10 nM) with 2 μM tetraphenylphosphonium in a thermostated polyvinyl chloride vessel of 2 ml in which a tetraphenylphosphonium ion-selective electrode was inserted as described by Elferink et al. (4). Experiments were performed at 25°C. The magnitude of the $\Delta\Psi$ was calculated with the Nernst equation. A correction for tetraphenylphosphonium binding to liposomal membranes was applied as described by Lolkema et al. (13). An internal volume of 5 μl/mg of lipid was used for COVs (3).

The internal pH in COVs was determined in 50 mM potassium phosphate (pH 6.0) in the presence of the electron donor system and valinomycin (0.5 μM) from the fluorescence of entrapped pyranine (see above).

Beef heart cytochrome c oxidase activity. The activity of beef heart cytochrome c oxidase was measured in an Aminco DW2a double-beam spectrophotometer at 25°C by monitoring the decrease in absorbance of the α peak of horse heart cytochrome c with an absorption coefficient of 19.5 $mM^{-1}\ cm^{-1}$ (reduced-oxidized) at 550 to 540 nm. The reaction mixture (1-ml final volume) contained 50 mM potassium phosphate (pH 6.0), 1% (vol/vol) Triton X-100, and 20 μM reduced cytochrome c. Beef heart cytochrome c oxidase (5.8 nmol of heme) was added to start the reaction. The reaction rate was calculated from the initial slope of cytochrome c oxidation.

Determination of oxygen consumption. Oxygen consumption in COVs was measured in the presence of the electron donor system at 25°C in 50 mM potassium phosphate (pH 6.0) with a Clark-type oxygen electrode.

Protein concentration. Protein was determined by the method of Schaffner and Weissmann (20) with bovine serum albumin as a standard.

Chemicals. Nisin was obtained from Genzyme. Egg PC, $E.\ coli$ PE, and asolectin were obtained from Sigma. Horse heart cytochrome c (type 111) was obtained from Sigma.

RESULTS

Action of nisin in the absence and presence of a membrane potential. Previous experiments indicated that the peptide
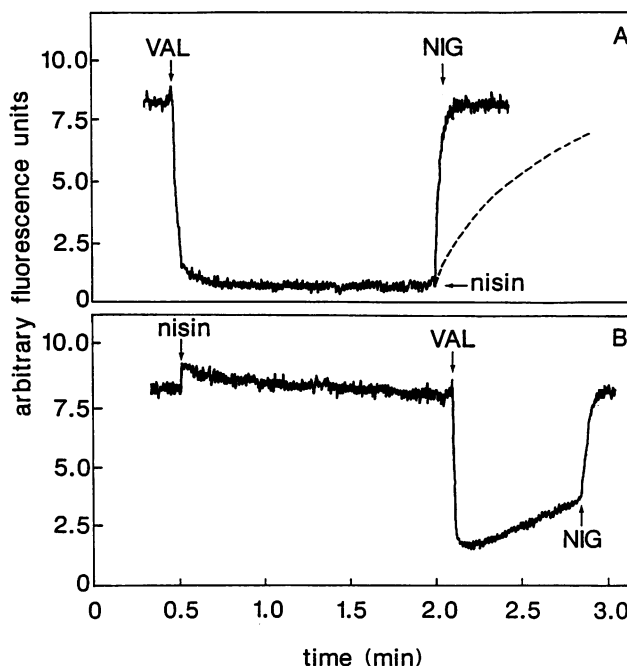

FIG. 1. Effect of nisin on potassium diffusion potentials ($\Delta\Psi$, interior negative) in $E.\ coli$ PE-egg PC (9:1, wt/wt) liposomes. K^+-loaded liposomes in 50 mM potassium phosphate (pH 6.0) were diluted 100-fold in 50 mM sodium phosphate (pH 6.0), and at the indicated times valinomycin (VAL; 0.5 μM), nigericin (NIG; 0.5 μM), or nisin (0.2 μg/μg of phospholipid) (broken line) was added. The $\Delta\Psi$ was measured with the indicator 3,3-dipropylthiacarbocyanine as described in Materials and Methods.

antibiotic nisin requires a membrane potential for activity (17). We therefore investigated the effect of nisin on ion gradients in liposomes made from $E.\ coli$ PE-egg PC (9:1, wt/wt). K^+-loaded liposomes prepared in 50 mM potassium phosphate (pH 6.0) were diluted 100-fold in 50 mM sodium phosphate (pH 6.0). Upon the addition of the K^+ ionophore valinomycin (0.5 μM), a $\Delta\Psi$ (interior negative) was generated as indicated by the absorbance changes of the $\Delta\Psi$ indicator 3,3-dipropylthiacarbocyanine (Fig. 1A). Subsequent addition of the H^+-K^+ exchanger nigericin (0.2 μM) or nisin (0.2 μg of nisin per μg of lipid) resulted in a dissipation of the $\Delta\Psi$. This indicates that nisin makes the liposomal membranes permeable for ions. No fluorescence change was observed with K^+-loaded liposomes diluted in K^+ buffer upon the addition of valinomycin, nisin, or nigericin (data not shown).

The addition of nisin (0.2 μg/μg of lipid) to K^+-loaded liposomes diluted in Na^+ buffer did not result in the generation of a $\Delta\Psi$ (Fig. 1B). The subsequent addition of valinomycin (0.5 μM) resulted in an immediate generation of a transient $\Delta\Psi$, which was rapidly dissipated by nigericin. This indicates that in the absence of a $\Delta\Psi$ nisin was not able to induce efflux of K^+ from K^+-loaded liposomes, i.e., to make the liposomal membranes leaky. Once a $\Delta\Psi$ is generated, the action of nisin results in an increased ion permeability of the liposomal membrane, and as a consequence the $\Delta\Psi$ dissipates.

Effect of the magnitude of the $\Delta\Psi$ in liposomes on the action of nisin. The influence of nisin on $E.\ coli$ PE-egg PC (9:1, wt/wt) liposomes with artificially imposed K^+ diffusion potentials of different magnitudes was investigated. Different

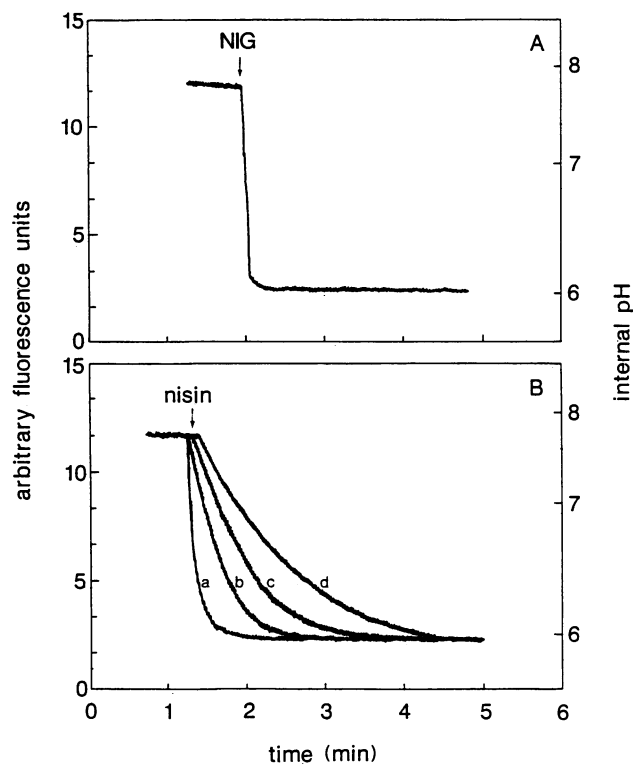FIG. 2. Effect of nisin on the artificial ΔpH (internal alkaline) in liposomes. Liposomes were made from *E. coli* PE-egg PC (9:1, wt/wt). A ΔpH was generated by diluting liposomes in 20 mM potassium phosphate–100 mM potassium acetate (pH 6.0) 200-fold in 20 mM sodium phosphate–100 mM Na-PIPES (pH 6.0). The internal pH was measured with pyranine as described in Materials and Methods. Nigericin (NIG; 0.5 μM) (A) and nisin (B; a, 40 μg; b, 30 μg; c, 20 μg; d, 10 μg) were added as indicated.
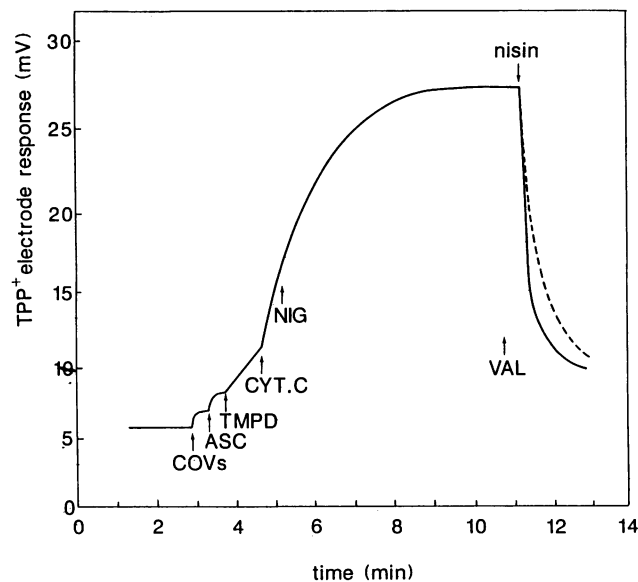

FIG. 3. Effect of nisin on the ΔΨ (interior negative) generated in COVs. COVs were made from *E. coli* PE-egg PC (9:1, wt/wt) and beef heart cytochrome *c* oxidase. A ΔΨ was generated in COVs by the oxidation of ascorbate (ASC; 10 mM), TMPD (200 μM), or cytochrome *c* (CYT.C; 10 μM). The ΔΨ was measured with a tetraphenylphosphonium (TPP$^+$) ion-selective electrode as described in Materials and Methods. Nisin (0.15 μg/μg of phospholipid) (broken line), valinomycin (VAL; 0.5 μM), and nigericin (NIG; 0.5 μM) were added as indicated.

values of the ΔΨ (−30 to −120 mV) were obtained by diluting K$^+$-loaded liposomes in the presence of valinomycin with sodium phosphate buffers containing increasing concentrations of K$^+$. A ΔΨ as low as −30 mV could be dissipated by 0.2 μg of nisin per μg of lipid (data not shown).

The rate at which the ΔΨ was dissipated by nisin increased with the magnitude of the ΔΨ. Membrane potentials of −30 and −90 mV were dissipated by 0.1 μg of nisin per μg of lipid within 47 and 9 s, respectively (data not shown).

Effect of nisin on artificial pH gradients in liposomes. Nothing is known about the effect of nisin on ΔpHs. To investigate whether nisin can be activated by a ΔpH in the absence of a ΔΨ, an artificial ΔpH (interior alkaline) was generated by diluting acetate-loaded *E. coli* PE-egg PC (9:1, wt/wt) liposomes into K-PIPES (pH 6.0). The fluorescence change of pyranine, entrapped in these liposomes as a pH indicator, indicated that a ΔpH of 1.8 (interior alkaline) was generated (Fig. 2). The addition of nigericin (0.2 μM) or nisin (0.2 μg/μg of lipid) resulted in an internal acidification of the liposomes as was demonstrated by the decrease in fluorescence intensity of the entrapped pyranine. These results indicate that nisin is able to make the liposomes permeable to protons in the presence of a ΔpH.

The ΔpH was dissipated already by very low concentrations of nisin (0.05 μg of nisin per μg of phospholipid). The rate at which the ΔpH dissipated increased with the concentration of nisin (Fig. 2).

Effects of nisin on the ΔΨ and the ΔpH in COVs. Oxidation of the electron donor system ascorbate-TMPD-cytochrome *c* by COVs resulted in the generation of a ΔΨ of −136 mV (interior negative) (Fig. 3). This ΔΨ was completely dissipated by the K$^+$ ionophore valinomycin (0.5 μM) or nisin (0.4 μg/μg of lipid). As compared with those in liposomes, higher concentrations of nisin were needed to dissipate the ΔΨ generated by cytochrome *c* oxidase activity in COVs (0.05 and 0.4 μg of nisin per μg of lipid, respectively).

The magnitude of the ΔΨ generated in COVs can be varied by using different concentrations of TMPD and cytochrome *c*. Nisin (0.4 μg/μg of phospholipid) was able to dissipate a ΔΨ as low as −25 mV (data not shown).

The effect of nisin on the ΔpH generated in COVs by the oxidation of ascorbate-TMPD-cytochrome *c* was investigated. A ΔpH of 1.1 (−ZΔpH, −65 mV) was generated, which could be dissipated by nigericin (0.5 μM) or by nisin (0.4 μg/μg of lipid), although at a slower rate (Fig. 4). As compared with the effect of nisin on the artificial ΔpH in liposomes, a higher concentration of nisin was needed to dissipate the ΔpH generated in COVs by the cytochrome *c* oxidase activity (0.05 and 0.4 μg of nisin per μg of phospholipid, respectively).

Effect of nisin on the cytochrome *c* oxidase activity and oxygen consumption in COVs. The possibility exists that the dissipation of the ΔΨ and the ΔpH in COVs is due to inhibition of the cytochrome *c* oxidase activity. Therefore the effect of nisin on the oxidation of reduced cytochrome *c* by the solubilized cytochrome *c* oxidase was examined. The activity of the enzyme was inhibited by 48% in the presence of 10 μg of nisin per ml (Fig. 5A). In the presence of the electron donor system, the oxygen consumption in COVs was also inhibited by nisin (Fig. 5B). To investigate whether
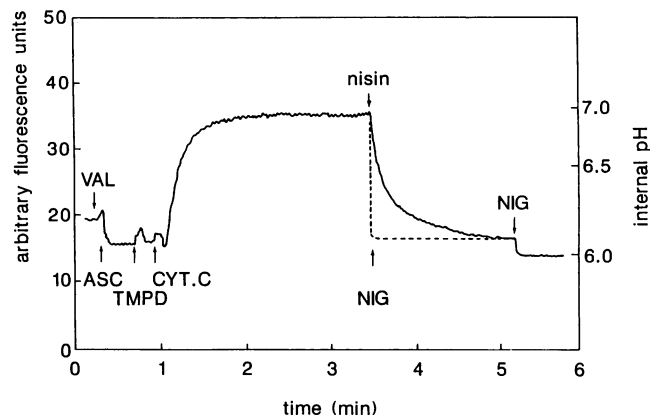
FIG. 4. Effect of nisin on the ΔpH generated in COVs. COVs were made from E. coli PE-egg PC (9:1, wt/wt). In COVs (400 μg of phospholipid), a ΔpH (inside alkaline) was generated, as indicated by the fluorescence change of pyranine, by the oxidation of ascorbate (ASC; 10 mM), TMPD (200 μM), and cytochrome c (CYT.C; 10 μM). Valinomycin (VAL; 0.5 μM), nigericin (NIG; 0.5 μM) (broken line), and nisin (0.25 μg/μg of phospholipid) were added as indicated.

the dissipation of the ΔΨ and the ΔpH in the presence of increasing concentrations of nisin was completely due to an inhibition of the oxygen consumption, a control experiment was performed in which the oxygen consumption was varied by changing the amount of TMPD. The ΔΨ and the ΔpH in COVs in the absence and presence of increasing concentrations of nisin were plotted as functions of the oxygen consumption (Fig. 6). At the same oxygen consumption rate, the ΔΨ and the ΔpH were always significantly lower in the presence of nisin. These results indicate that the dissipation of the ΔΨ and the ΔpH by nisin in COVs is only to a minor extent due to an inhibition of the cytochrome c oxidase activity.

Effect of the lipid composition on the action of nisin. Liposomes prepared from E. coli PE-egg PC (9:1, wt/wt), asolectin, and egg PC were used to study the effect of the lipid composition on the action of nisin. K^+-loaded liposomes prepared in 50 mM potassium phosphate (pH 6.0) were diluted 100-fold in 50 mM sodium phosphate (pH 6.0). Upon addition of the K^+ ionophore valinomycin (0.5 μM), a membrane potential (ΔΨ, interior negative) was generated in the three types of liposomes (Fig. 7). The ΔΨ was maintained for a prolonged period of time in E. coli PE-egg PC (9:1) liposomes and could be dissipated by nigericin (0.2 μM) or nisin (0.2 μg/μg of lipid) (Fig. 7A). The addition of nisin resulted in hardly any dissipation of the ΔΨ in the egg PC liposomes. In the presence of 0.2 μg of nisin per μg of phospholipid, only 4% of the ΔΨ was dissipated, whereas nigericin (0.5 μM) decreased the ΔΨ by 75% (Fig. 7B).

Asolectin liposomes were leaky, and the ΔΨ dissipated completely within 4 min. The addition of nisin directly after the generation of the ΔΨ resulted in an immediate dissipation of this ΔΨ, which indicates that nisin is active in asolectin liposomes (Fig. 7C). These results show that the action of nisin depends on the phospholipid composition of the liposomal membrane.

DISCUSSION

The results described herein show that nisin can increase the permeability of artificial membranes provided that a ΔΨ
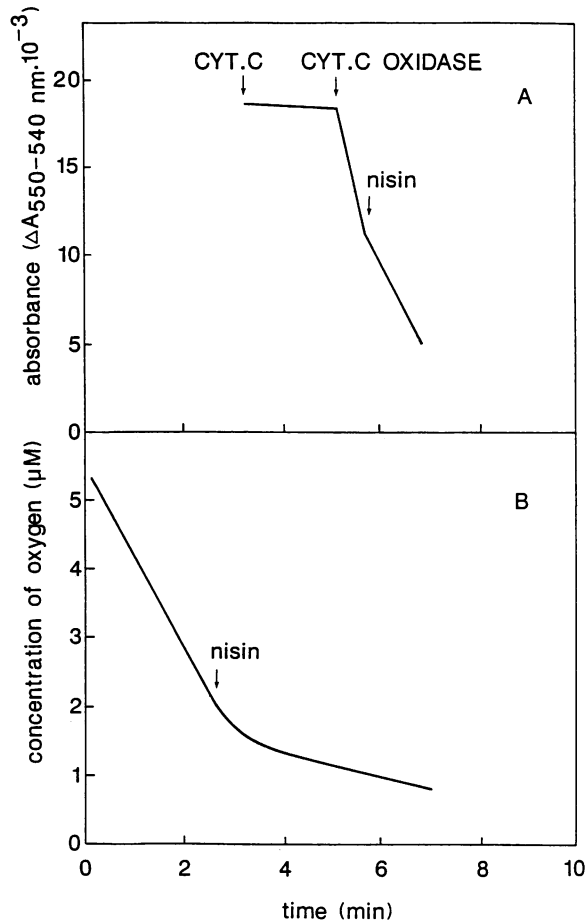

FIG. 5. Effect of nisin on the activity of beef heart cytochrome c oxidase (A) and on oxygen consumption (B) in COVs. Oxidation of reduced cytochrome c (CYT.C) by solubilized cytochrome c oxidase and oxygen consumption in COVs made from E. coli PE-egg PC (9:1, wt/wt) and beef heart cytochrome c oxidase were measured as described in Materials and Methods. At the indicated time, 10 μg of nisin per ml and 0.4 μg of nisin per μg of phospholipid were added in experiments A and B, respectively.

(negative inside) and/or a ΔpH (alkaline inside) is present. In E. coli PE-egg PC (9:1, wt/wt) liposomes, a ΔΨ as low as −30 mV could be dissipated by nisin (0.05 μg/μg of phospholipid). However, the rate at which the ΔΨ is dissipated increases strongly with the magnitude of the ΔΨ. After the addition of nisin, membrane potentials of −30 and −90 mV collapsed completely within 47 and 9 s, respectively.

These results add further evidence to support the model proposed by Ruhr and Sahl (17) for the primary bactericidal action of nisin. Macroscopic conductivity measurements with planar lipid bilayers (black lipid membranes) indicated that nisin and the closely related lantibiotic Pep5 produced by Staphylococcus epidermidis have to be regarded as membrane-depolarizing agents that act in a voltage-dependent fashion. The investigators observed that the electrical potential must be negative inside versus outside when nisin or Pep5 is added to the outside and that the electrical potential over the black lipid membranes formed from dioleoyl phosphatidylcholine-phosphatidylserine (4:1 molar ratio in n-decane) must be ca. −100 mV (10, 19). This potential differs markedly from the magnitude of the ΔΨ (−30 mV)

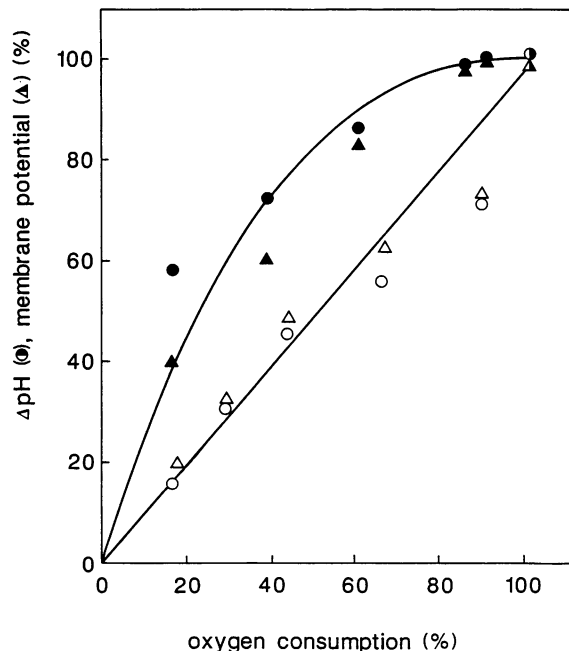FIG. 6. ΔpH (○, ●) and ΔΨ (△, ▲) generation in COVs in the absence (●, ▲) and presence (○, △) of nisin, plotted as a function of the oxidation rate. COVs were energized in the presence of nisin by the addition of ascorbate (20 mM), cytochrome c (20 μM), and TMPD (200 μM). Increasing concentrations of nisin (0.05, 0.1, 0.2, 0.3, and 0.4 μg/μg of phospholipid) inhibited oxygen consumption. In the absence of nisin, as a control experiment, the oxygen consumption was varied by different concentrations of TMPD. The ΔΨ and the ΔpH were determined as described in Materials and Methods. The 100% values for ΔΨ and $-Z$ΔpH were -136 and -65 mV, respectively.

that was found in this study to be dissipated by nisin in *E. coli* PE-egg PC (9:1, wt/wt) liposomes. An explanation for this difference in threshold potentials, necessary for nisin activity, could possibly be found in the different compositions of the membranes used in our experiments and the black lipid membrane experiments (19).

The results of experiments (Fig. 7) in which asolectin and egg PC were used to study the effect of the lipid composition on the action of nisin are consistent with this suggestion. The ΔΨ generated by valinomycin-mediated K^+ diffusion potentials in *E. coli* PE-egg PC (9:1, wt/wt) and asolectin liposomes was dissipated by nisin. In asolectin liposomes only a transient membrane potential could be generated because of the higher ionpermeability of these liposomes. This explains why Ruhr and Sahl did not observe an effect of nisin in asolectin liposomes loaded with ^{14}C-labeled L-glutamate (17). In their experiments, nisin was added approximately 3 to 4 min after a ΔΨ was generated by the addition of valinomycin to K^+-loaded liposomes. Most likely the ΔΨ was already dissipated in this period of time, no functional insertion of nisin occurred, and therefore no efflux of ^{14}C-labeled glutamate was observed.

In contrast, the ΔΨ in egg PC liposomes was maintained for a long period of time and was not dissipated by nisin (Fig. 7). The activity of nisin is clearly affected by the phospholipid composition of the liposomal membrane. In addition, these experiments with artificial membranes show that there is no need for an integral membrane component that serves as a receptor for nisin.
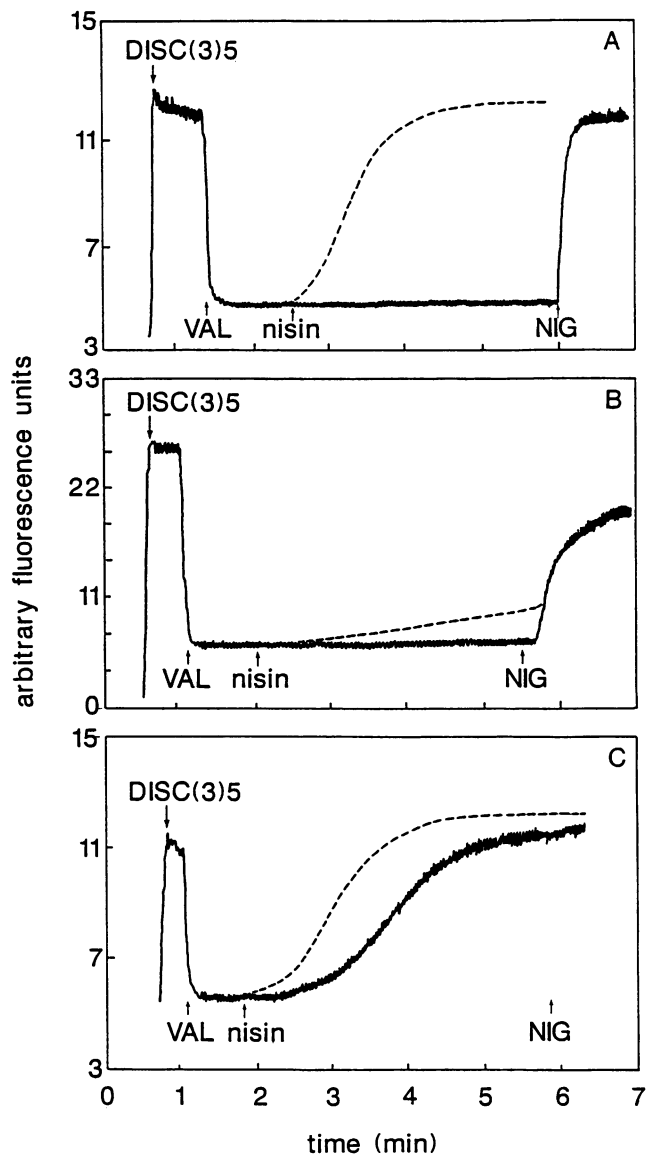

FIG. 7. Effect of the lipid composition on the action of nisin in liposomes. Liposomes were made from *E. coli* PE-egg PC (9:1, wt/wt) (A), egg PC (B), and asolectin (C) in 20 mM potassium phosphate–100 mM potassium acetate (pH 6.0). A ΔΨ was generated by diluting liposomes (200 μg of phospholipid) 200-fold in 20 mM sodium phosphate–100 mM sodium acetate (pH 6.0). At the indicated positions, valinomycin (VAL; 0.5 μM), nigericin (NIG; 0.5 μM), and nisin (0.2 μg/μg of phospholipid) (broken line) were added. DISC(3)5, 3,3-dipropylthiacarbocyanine.

No information was available about the effect of nisin on the ΔpH. We therefore studied the effect of nisin on pH gradients generated by acetate diffusion potentials in *E. coli* PE-egg PC (9:1, wt/wt) liposomes. Strikingly, nisin appeared to be very effective in dissipating a ΔpH of 1.8 ($-Z$ΔpH, -108 mV) in the absence of a ΔΨ (Fig. 2). Dissipation of the ΔpH was already observed in the presence of 0.05 μg of nisin per μg of phospholipid. The rate of ΔpH dissipation increased with higher nisin concentrations. In fact, dissipation of the ΔΨ and ΔpH in liposomes by nisin displayed the same concentration dependence, which suggests that the two

components of the proton motive force are equally effective in promoting insertion and pore formation of nisin in the membrane.

The pH gradient-dependent pore-forming activity of nisin differs clearly from the effect of the pH gradient on channel-forming toxins in animals. The heavy chains of diphtheria, tetanus, botulinum, and anthrax toxins form voltage-regulated, pH-dependent channels in planar phospholipid bilayer membranes (2, 5, 6). These toxins show a marked stimulation of channel-forming activity upon decreasing the pH in the compartment to which the toxin is added. The increase in channel activity was however predominantly a reflection of an increased rate of binding of the toxin to the bilayer (2).

It was previously assumed that the requirement for a transnegative potential for activity of nisin was due to the cationic nature of this peptide. The lantibiotics nisin and Pep5 contain three and eight positively charged residues, respectively (7, 10). Kordel et al. (10) performed additional experiments with modified Pep5 in which the lysine residues were succinylated. Surprisingly, the overall negative charge of the modified peptide did not result in a requirement for an electrical potential (positive inside), and a current-voltage characteristic similar to that of unmodified Pep5 was observed (10). This observation, combined with our results showing that nisin is also effectively inserted in the liposomal membrane in the presence of a ΔpH (alkaline inside) only, indicates that the insertion mechanism of these cationic peptides remains to be elucidated.

The antibiotic action of nisin toward the gram-positive bacteria *L. lactis* subsp. *cremoris* and *Clostridium* species has long since been recognized and has provided the basis for the introduction of nisin as a food preservative (9). A large number of bioenergetic studies concerning proton motive force generation and the regulation of the cytoplasmic pH have been performed with lactic acid bacteria and other fermentative bacteria (8, 14, 21). During the growth of these bacteria, the pH of the medium decreases due to the production of acids. In *L. lactis* subsp. *cremoris*, at an external pH of 6.0 in the presence of a fermentable sugar, the proton motive force is composed of a small $\Delta\Psi$ (approximately -40 mV) and a large pH gradient ($-Z\Delta pH$, -80 mV) (14). In sugar-fermenting clostridia the $\Delta\Psi$ component is reported to be very low and is not noticeably affected by membrane-permeabilizing agents (8, 21). However, in the initial growth phase the cells maintain a large pH gradient ($-Z\Delta pH$, -60 mV), which eventually dissipates due to the effect of the produced weak acids (8, 21). The effective inhibition of growth of these bacteria by nisin can now be explained by our observation that nisin also functionally inserts in the membrane in the presence of a pH gradient (alkaline inside).

The effect of nisin on the $\Delta\Psi$ and ΔpH was also investigated in proteoliposomes (COVs) made from *E. coli* PE-egg PC (9:1, wt/wt) in which beef heart cytochrome *c* oxidase has been incorporated as a H^+ pump. Higher concentrations of nisin were needed in COVs, as compared with those needed in liposomes, to dissipate the $\Delta\Psi$ and the ΔpH (0.4 and 0.05 μg of nisin per μg of phospholipid, respectively). This is obviously due to the high H^+-pump capacity of the cytochrome *c* oxidase, which can, to a certain extent, compensate for the leak introduced by nisin.

In addition, beef heart cytochrome *c* oxidase activity appeared to be inhibited by nisin. With a concentration of 0.4 μg of nisin per μg of phospholipid, the oxygen consumption in COVs was decreased by 80%. Inhibition of oxygen

consumption by nisin has been observed in the gram-positive bacterium *S. cohnii* (11). This suggests that nisin, besides forming pores, might have additional effects on electron transfer chain components.

The results discussed above show that these (proteo)liposomes provide a powerful model system for studying the mechanism of insertion and the mode of action of nisin or modified nisin molecules.

REFERENCES

1. **Berridge, N. J., G. G. F. Newton, and E. P. Abraham.** 1952. Purification and nature of nisin. Biochem. J. **52:**529–535.
2. **Blaustein, R. O., T. M. Koehler, R. J. Collier, and A. Finkelstein.** 1989. Anthrax toxin: channel-forming activity of protective antigen in planar phospholipid bilayers. Proc. Natl. Acad. Sci. USA **86:**2209–2213.
3. **Driessen, A. J. M., W. de Vrij, and W. N. Konings.** 1986. Functional incorporation of beefheart cytochrome *c* oxidase into membranes of *Streptococcus cremoris*. Eur. J. Biochem. **154:**617–624.
4. **Elferink, M. G. L., K. J. Hellingwerf, and W. N. Konings.** 1987. The relation between electron transfer, proton motive force and energy consuming processes in cells of *Rhodobacter sphaeroides*. Biochim. Biophys. Acta **730:**379–386.
5. **Hoch, D. H., M. Romera-Mira, B. E. Ehrlich, A. Finkelstein, B. R. Dasgupta, and L. L. Simpson.** 1985. Channels formed by botulinum, tetanus, diphteria toxin in planar lipid bilayers: relevance to translocation of proteins across membranes. Proc. Natl. Acad. Sci. USA **82:**1692–1696.
6. **Kagan, B. L., A. Finkelstein, and M. Colombini.** 1981. Diphtheria toxin fragments form large pores in phospholipid bilayer membranes. Proc. Natl. Acad. Sci. USA **78:**4950–4954.
7. **Kaletta, C., and K. D. Entian.** 1989. Nisin, a peptide antibiotic: cloning and sequencing of the *nisA* gene and posttranslational processing of its peptide product. J. Bacteriol. **171:**1597–1601.
8. **Kashket, E. R.** 1987. Bioenergetics of lactic acid bacteria: cytoplasmic pH and osmotolerance. FEMS Microbiol. Rev. **46:**233–244.
9. **Klaenhammer, T. R.** 1988. Bacteriocins of lactic acid bacteria. Biochemie **70:**337–349.
10. **Kordel, M., R. Benz, and H. G. Sahl.** 1988. Mode of action of the staphylococcinlike peptide Pep5: voltage-dependent depolarization of bacterial and artificial membranes. J. Bacteriol. **170:**84–88.
11. **Kordel, M., and H. G. Sahl.** 1986. Susceptibility of bacterial, eukaryotic and artificial membranes to the disruptive action of the cationic peptides pep 5 and nisin. FEMS Microbiol. Lett. **34:**139–144.
12. **Liu, W., and J. Norman Hansen.** 1990. Some chemical and physical properties of nisin, a small protein antibiotic produced by *Lactococcus lactis*. Appl. Environ. Microbiol. **56:**2551–2558.
13. **Lolkema, J. S., K. J. Hellingwerf, and W. N. Konings.** 1982. The effect of probe binding on the quantitative determination of the proton motive force in bacteria. Biochim. Biophys. Acta **681:**85–94.
14. **Poolman, B., K. J. Hellingwerf, and W. N. Konings.** 1987. Regulation of the glutamate/glutamine transport system by intracellular pH in *Streptococcus lactis*. J. Bacteriol. **167:**2272–2276.
15. **Ramseier, H. R.** 1960. Die Wirkung von Nisin auf *Clostridum butylicum*. Arch. Mikrobiol. **37:**57–94.
16. **Reisinger, P., H. Seidel, H. Tsesche, and W. P. Hammes.** 1980. The effect of nisin on murein synthesis. Arch. Microbiol. **127:**187–193.
17. **Ruhr, E., and H. G. Sahl.** 1985. Mode of action of the peptide antibiotic nisin and influence on the membrane potential of whole cells and on cytoplasmic and artificial membrane vesicles. Antimicrob. Agents Chemother. **27:**841–845.

18. **Sahl, H. G.** 1985. Bactericidal cationic peptides involved in bacterial antagonism and host defence. Microbiol. Sci. **2:**212–217.

19. **Sahl, H. G., M. Kordel, and R. Benz.** 1987. Voltage-dependent depolarization of bacterial membranes and artificial lipid bilayers by the peptide antibiotic nisin. Arch. Microbiol. **149:**120–124.

20. **Schaffner, W., and C. Weissmann.** 1973. A rapid, sensitive and specific method for the determination of protein in dilute solution. Anal. Biochem. **56:**502–514.

21. **Terracciano, J. S., and E. R. Kashket.** 1986. Intracellular conditions required for initiation of solvent production by *Clostridium acetobutylicum.* Appl. Environ. Microbiol. **52:**86–91.