Mechanistic Studies of Lantibiotic-Induced Permeabilization of Phospholipid Vesicles[†]

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ABSTRACT: Nisin is a cationic polycyclic bacteriocin secreted by some lactic acid bacteria. Nisin has previously been shown to permeabilize liposomes. The interaction of nisin was analyzed with liposomes prepared of the zwitterionic phosphatidylcholine (PC) and the anionic phosphatidylglycerol (PG). Nisin induces the release of 6-carboxyfluorescein and other small anionic fluorescent dyes from PC liposomes in a $\Delta\psi$-stimulated manner, and not that of neutral and cationic fluorescent dyes. This activity is blocked in PG liposomes. Nisin, however, efficiently dissipates the $\Delta\psi$ in cytochrome *c* oxidase proteoliposomes reconstituted with PG, with a threshold $\Delta\psi$ requirement of about −100 mV. Nisin associates with the anionic surface of PG liposomes and disturbs the lipid dynamics near the phospholipid polar head group—water interface. Further studies with a novel cationic lantibiotic, epilancin K7, indicate that this molecule penetrates into the hydrophobic carbon region of the lipid bilayer upon the imposition of a $\Delta\psi$. It is concluded that nisin acts as an anion-selective carrier in the absence of anionic phospholipids. *In vivo*, however, this activity is likely to be prevented by electrostatic interactions with anionic lipids of the target membrane. It is suggested that pore formation by cationic (type A) lantibiotics involves the local perturbation of the bilayer structure and a $\Delta\psi$-dependent reorientation of these molecules from a surface-bound into a membrane-inserted configuration.

Among the bacteriocins produced by bacteria, lantibiotics are the most efficient antimicrobial proteins (Klaenhammer, 1993). The paradigm of lantibiotics is nisin. It possesses antimicrobial activity against a broad spectrum of Gram-positive bacteria and has realized commercial application as a food preservative.

The structural gene for nisin codes for the precursor form, comprising a 23-residue leader sequence followed by a 34-residue sequence that corresponds to the mature nisin (Buchman *et al.*, 1988; Dodd *et al.*, 1989; Kaletta & Entian, 1989). Nisin contains several posttranslationally modified amino acid residues, namely α,β-didehydroalanine (Dha), α,β-didehydrobutyrine (Dhb), *meso*-lanthionine and (2*S*,3*S*,6*R*)-3-methyllanthionine; the latter two residues introduce thioether bridges at various locations in the molecule, resulting in a series of cyclic units (Kellner & Jung, 1989). The importance of the posttranslational modifications for biological activity has yet to be established. Another important property of nisin and other lantibiotics belonging to the type A group is that they are cationic. Nisin is soluble in aqueous solution at pH 2, but at high pH values it is inactivated where

it forms oligomers (Liu & Hansen, 1990). It has been suggested that the lanthionines serve to constrain the nisin molecule into a conformation which is required for activity, possibly fixing the molecule in a screw-like or amphiphilic conformation. The dehydroalanines may promote biological activity by reacting with free sulfhydryl groups of the target (Hansen, 1993).

The primary antimicrobial activity of nisin is thought to result from the formation of pores in the target cytoplasmic membrane which leads to a loss of small intracellular molecules and ions and a collapse of the proton motive force (Δp)[1] (Bruno *et al.*, 1992; Gao *et al.*, 1991; Okereke & Montville, 1992; Ruhr & Sahl, 1985; Sahl, 1991). Nisin requires a trans-negative electrical potential $(\Delta\psi)$ for its

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[1] Abbreviations: PC, dioleoylphosphatidylcholine; PG, dioleoylphosphatidylglycerol; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; R$_{18}$, octadecyl rhodamine B; DPC, dodecylphosphocholine; SDS, sodium dodecylsulfate; DPX, *p*-xylenebispyridinium bromide; $\Delta\psi$, transmembrane electrical potential; ΔpH, transmembrane pH gradient; Δp, proton motive force; MQAE, *N*-(ethoxycarbonylmethyl)-6-methoxyquinolium ion; NOESY, nuclear overhauser enhancement spectroscopy; F, fluorescein, free acid; 3, FDA, fluorescein diacetate; CF, 5-(and 6-)carboxyfluorescein, free acid; CFDA-AM, 5-(and 6-)carboxyfluorescein diacetate, acetoxymethyl ester; BCECF, 2′,7′-bis-(2-carboxyethyl)-5-(and 6-)carboxyfluorescein, free acid; BCECF-AM, 2′,7′-bis-(2-carboxyethyl)-5-(and 6-)carboxyfluorescein acetoxymethyl ester; FMG, fluorescein mono-β-D-galactopyranoside; CFDG, 5-(and 6-)carboxyfluorescein-di-β-D-galactopyranoside; FDEX, fluorescein—dextran; *r*, anisotropy; S-13, 5-chloro-3-*tert*-butyl-2′-chloro-4′,4′-nitrosalicylanilide; TPP⁺, tetraphenylphosphonium ion; TMA-DPH, 1-[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene; TMAP-DPH, *N-p*-(6-phenyl(1,3,5-hexatrienyl(phenyl(propyl))))trimethyl-ammonium; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.

action (Sahl *et al.*, 1987; Sahl, 1991; Kordel & Sahl, 1986), but the molecular basis for this has remained ambiguous.

Nisin induces the release of the fluorophore carboxyfluorescein (CF) from liposomes (Kordel *et al.*, 1989) in a Δp-stimulated manner (García Garcerá *et al.*, 1993). The presence of anionic phospholipids in the liposomes strongly inhibited this activity (García Garcerá *et al.*, 1993). In contrast, planar bilayer experiments have demonstrated that there is a strict requirement for a $\Delta\psi$ for pore formation, while anionic phospholipids lower the threshold requirement for $\Delta\psi$ (Benz *et al.*, 1991). To determine the molecular basis for the inhibitory effect of anionic lipids and the apparent discrepancy between the liposomal and planar bilayer experiments, we have analyzed the interaction of nisin and another lantibiotic, epilancin K7, with liposomes in further detail. The latter lantibiotic harbors a unique tyrosine residue (Van de Kamp *et al.*, 1994) that allows a spectroscopical analysis of the interaction of a lantibiotic with the membrane without the need of using an extrinsic probe. The data suggest that nisin and K7 act as anion-selective carriers in liposomes composed of the zwitterionic phosphatidylcholine. Nisin electrostatically interacts with the anionic surface of phosphatidylglycerol liposomes and perturbs the phospholipid dynamics at the surface. K7 inserts in the membrane of PC liposomes upon the imposition of a $\Delta\psi$. The significance of these findings in relation to the *in vivo* action of cationic type-A lantibiotics in general is discussed.

EXPERIMENTAL PROCEDURES

Materials. Purified nisin was purchased from NBS Biologicals (North Mymms, Hatfield, Herts, U.K.) or obtained from Aplin & Barrett and stored at −20 °C in acetic acid solution at pH 3.0 at 10 mg/mL. Gramicidin D was purchased from Sigma. Both were used without further purification. Epilancin K7 was purified from *Staphylococcus epidermidis* strain K7 (Pulverer & Jeljaszewicz, 1976) as described (Van de Kamp *et al.*, 1994). *p*-Xylenebispyridinium bromide (DPX), anti-fluorescein rabbit IgG, highly purified carboxyfluorescein (CF), and unless indicated all other fluorescent dyes were purchased from Molecular Probes, Inc. (Eugene, OR). Dioleoylphosphatidylcholine (PC) and -glycerol (PG), *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE), and 1-palmitoyl-2-(*n*-doxylstearoyl)-*sn*-glycero-3-phosphatidylcholine (n = 5, 10, or 16) were obtained from Avanti Polar Lipids Inc. (Birmingham, AL). Porcine liver esterase (EC 3.1.1.1) and *Escherichia coli* β-galactosidase (EC 3.2.1.23) were obtained from Sigma Chem. Co. (St. Louis, MO). Beefheart cytochrome *c* oxidase was isolated as described (Driessen & Konings, 1993).

Formation of Liposomes and Entrapment of Dyes. Large, unilamellar liposomes were formed by extrusion from multilamellar liposomes. Dioleoylphosphatidylcholine (PC) or -glycerol (PG) dissolved in CHCl₃/MeOH (9/1, v/v) were thoroughly dried under vacuum for 1 h. Where indicated, the dissolved lipids were supplemented with NBD-PE (2 mol %), R₁₈ (4 mol %), or doxyl PCs (20 mol %). The dry lipid film was suspended at 20 mg/mL in 50 mM potassium 4-morpholineethanesulfonate (KMes), pH 6.0, and 75 mM K₂SO₄ and supplemented with a fluorescent dye at the concentration as indicated. The lipids were rehydrated and dispersed by ultrasonic irradiation using a bath sonicator (Sonicor, Sonicor Instruments, New York) and were sub-

jected to three to five cycles of freezing in liquid nitrogen and thawing in a water bath of 25 °C to form multilamellar liposomes. These were sized by extrusion through 400-, 200-, and 100-nm polycarbonate filters (Avestin Inc., Ottawa, ON, Canada) using a small volume extrusion apparatus (LiposoFast, Avestin) (MacDonald *et al.*, 1991). Nonencapsulated dye was removed from the liposomes by rapid gel filtration using a P-10 spin column (Pharmacia) in 50 mM KMes, pH 6.0, and 75 mM K₂SO₄.

Determination of the Efflux of Fluorescein Derivatives. Release of carboxyfluorescein (CF) was determined as described (Goessens *et al.*, 1988). Liposomes were loaded with 100 mM CF in 10 mM KMes, pH 6.0, 5 mM K₂SO₄, and 1 mM EDTA. External CF was removed by gelfiltration using 10 mM KMes, K₂SO₄, and 1 mM EDTA. The release of fluorescein thiocyanate dextran (molecular mass of 3 kDa) and of other fluorescein derivatives was determined by quenching the fluorescence by externally added anti-fluorescein rabbit IgG. Release of the MQAE was monitored in the presence of the quencher DPX at a concentration of 20 mM. Release of nonfluorescent esters or sugar derivatives was monitored through the use of an excess of externally added esterase or β-galactosidase at 1−10 μg/mL to release the fluorescein. Dyes at 10 μM concentration were entrapped into the liposomal lumen by freeze/thaw extrusion as described. External dye was removed by the use of a P10 spin column. In the case of the fluorescein−dextran, nonencapsulated dye was removed from the liposomes with a Sephadex G-100 column as described. Liposomes were used at a final concentration of 50−80 μg/mL and nisin was added to a concentration of 10 μg/mg of phospholipid, unless indicated otherwise. The release of the indicator dyes was determined in the presence and absence of an imposed $\Delta\psi$ (see below). Excitation and emission wavelengths used were (Ex/Em): MQAE, 350 nm/460 nm; F, FDA, FMG, CFDG, and FDEX, 490 nm/514 nm; CF and CFDA-AM, 492 nm/514 nm; and BCECF and BCECF-AM, 482 nm/530 nm. Maximal (100%) fluorescence was obtained by addition of Triton X-100 (0.2% by vol.) to the liposome suspension. The initial rate of dye release was calculated from the tangent of the efflux curve at the point of nisin addition, and corrected for nonspecific dye efflux. All fluorescence measurements were performed with a SLM/AMINCO 4800C fluorescence spectrometer at 25 °C using a thermostatically controlled, magnetically stirred cell-holder.

Imposition of a Transmembrane Electrical Potential. To determine the effect of a transmembrane electrical potential, $\Delta\psi$, inside negative, on the rate of CF release, K⁺-loaded liposomes were diluted 200-fold into a solution containing 10 mM NaMes, pH 6.0, and 75 mM Na₂SO₄ in the presence of the K⁺ ionophore valinomycin (2 nmol/mg of lipid). For the imposition of a $\Delta\psi$, inside positive, Na⁺-loaded liposomes were diluted into a solution containing 10 mM KMes, pH 6.0, and 75 mM K₂SO₄. Generation of a $\Delta\psi$ was followed with the fluorescent indicators 3,3′-diethylthiadicarbocyanine iodine [DiSC₂(5); $\Delta\psi$, inside negative] (Ex/Em, 649 nm/671 nm) (Driessen & Konings, 1993) and bis(3-propyl-5-oxoisooxazol-4-yl)pentamethine oxonol (Oxonol VI; $\Delta\psi$, inside positive) (Ex/Em, 599 nm/634 nm) (Apell & Bersch, 1987).

Formation of Cytochrome c Oxidase Proteoliposomes and Measurement of Transmembrane Electrical Potential. Beefheart cytochrome *c* oxidase was reconstituted into liposomes as described (Driessen & Konings, 1993). After dialysis,

liposomes were frozen into liquid nitrogen, thawed, and extruded through 100-nm polycarbonate filters as described above. Proteoliposomes were energized with the electron-donor cytochrome c/TMPD/ascorbate in the presence of the K^+/H^+ antiporter nigericin (10 nM) to prevent the generation of a ΔpH. The magnitude of $\Delta\psi$ was titrated with the uncoupler 5-chloro-3-*tert*-butyl-2'-chloro-4',4'-nitrosalicylanilide (S-13). $\Delta\psi$ was calculated from the transmembrane distribution of tetraphenylphosphonium ion as measured with an ion-selective electrode (Driessen & Konings, 1993; Lolkema et al., 1982).

Stopped-Flow Fluorescence Spectroscopy. The rapid kinetic reaction of nisin binding to liposomes was analyzed with the MilliFlow stopped-flow reactor of SLM/AMINCO (Urbana, IL). The two reactants, i.e., solutions of liposomes and nisin or epilancin K7, contained in the drive syringes whose pistons were simultaneously driven by a gas-operated plunger, were forced through a mixing chamber into an fluorescence observation cell (6.3 μL) with quartz windows. Both drive syringes, the mixing chamber, and the observation cell were maintained at 25 °C. Variable ratio mixing was achieved by changing the Hamilton Gastight syringes. Drive pistons were operated at the pressure recommended by the manufacturer for the respective syringe volume. The death-time of the mixing device was 0.8 ms. Changes in fluorescence were recorded with a resolution of 1 ms (NBD) or averaged over 10 ms time intervals (Tyr). NBD data were fitted to a single-exponential rise to determine the initial rate of association. The excitation and emission wavelengths used were 290 nm/340 nm for Tyr and 460 nm/534 nm for NBD. Slit widths were set at 4 nm.

31P NMR Spectroscopy. Sample preparation was as described by Van Echteld et al. (1982). PC and PG were dissolved in $CHCl_3$ at 20 mg of lipid/mL. The lipid solution (1 mL) was mixed with 0.5 mL of a solution containing 9 mg of nisin dissolved in MeOH, or 4 mg of gramicidin D dissolved in $CHCl_3$/ MeOH (1/1, v/v). The polypeptide to lipid ratio was about 1:10. Solvent was evaporated under a stream of nitrogen, and samples were further dried under vacuum for 3 h. Lipid mixtures were allowed to swell overnight in 0.5 mL of 50 mM potassium acetate, pH 5.0, with 10% D_2O and were subsequently vortexed in the presence of glass beads.

$31P$ NMR spectra were recorded at 161.97 MHz on a Bruker AM400 spectrometer interfaced to an Aspect 3000 computer. The temperature was 298 K. Spectra were proton decoupled by composite pulse decoupling. The spectral width was 200 ppm, the number of datapoints was 8192, the number of scans was 2048, and the relaxation delay was 1 s. The free induction decay was multiplied by an exponential function with a line broadening of 50 Hz. Spectra were referenced to external 85% H_3PO_4 diluted with 10% D_2O.

Time-Resolved Fluorescence Spectroscopy. Excited-state lifetimes of NBD and TMA-DPH were determined by the phase delay and demodulation technique at 6-, 18-, and 30-MHz modulation frequency as described. Excitation of NBD was at 460 nm with a bandwidth of 0.5 nm, and emission was measured at 534 nm with a bandwidth of 4 nm. Excitation of TMA-DPH was at 355 nm with a bandwidth of 1 nm, and emission was measured at 450 nm with a bandwidth of 4 nm. Lifetimes were referenced against a dilute solution of 1,4-bis(4-methyl-5-phenyloxazol-2-yl)-benzene in ethanol ($\tau = 1.45$ ns) or a glycogen scatter reference ($\tau = 0$ ns). The phase (τ_ϕ) and modulation (τ_m) lifetimes as determined at the different frequencies were fitted in a two-component heterogeneity analysis to yield the individual lifetimes using the software package supplied by SLM Inc. The χ_R^2 values for these fits were between 20 and 40.

Other Fluorescence Spectroscopy Determinations. For lipid redistribution measurements, PC and PG liposomes were prepared with 4 mol % of the cationic membrane probe octadecyl rhodamine B (R_{18}) (Hoekstra et al., 1984). R_{18}-labeled liposomes were mixed with an equal amount of nonlabeled liposomes (final concentration 250 μM), and lipid transfer was initiated by the addition of nisin. Fluorescence measurements were performed as described using excitation and emission wavelengths of 556 and 577 nm, respectively. The terbium/DPA fusion assay was performed as described (Wilschut & Papahadjopoulos, 1979).

Steady-state tyrosine fluorescence emission spectra of epilancin K7 were recorded with an excitation wavelength of 290 nm, and the emission levels were referenced against the intensity of the excitation beam. Slit widths were set at 1 nm, and spectra were corrected for the raman fluorescence contribution of water and the linearity of the photomultipliers using the correction-set provided by the manufacturer.

TMA(P)-DPH fluorescence polarization measurements were performed as described (In't Veld et al., 1991) using Glan−Thompson polarizers in the excitation (355 nm) and emission (450 nm) channels. TMAP-DPH is a TMA-DPH analogue with a three-carbon spacer that allows a deeper penetration of the DPH reporter group into the hydrophobic core of the lipid bilayer as compared to TMA-DPH. The tetramethylammonium group anchors the molecule at the lipid surface in a position perpendicular to the acyl chains of the phospholipids. Liposomes (40 $\mu g/mL$) were preincubated with 0.5 μM TMAP-DPH (added from a DMSO stock solution) for 1 h in the dark at 25 °C. The polarization (P) was calculated using

$$P = \left[\frac{R_{vert}}{R_{horz}} - 1\right]\left[\frac{R_{vert}}{R_{horz}} + 1\right]^{-1} \tag{1}$$

where R_{vert} is the ratio of the corrected parallel and perpendicular signals with the excitation polarizer placed in the vertical orientation, and R_{horiz} is the ratio of parallel and perpendicular signals with the excitation polarizer placed in the horizontal orientation. Anisotropy (r) values were derived from the polarization using

$$r = \frac{2P}{3 - P} \tag{2}$$

Miscellaneous Procedures. The concentration of the liposome preparations was determined by phosphate analysis using malachite green (Driessen et al., 1991) after destruction of dried lipid with 70% $HClO_4$. Light scattering was detected at a 90° angle using a beam of 400 nm. The size of the liposomes was determined by photon correlation spectroscopy using a Nicomp submicron particle sizer (Avestin Inc., Ottawa, ON, Canada) (Elferink et al., 1994).

RESULTS

Nisin Acts as an Anion Carrier in Phosphatidylcholine Liposomes. Addition of the lantibiotic nisin (Figure 1) (García Garcerá et al., 1993) to PC liposomes causes the
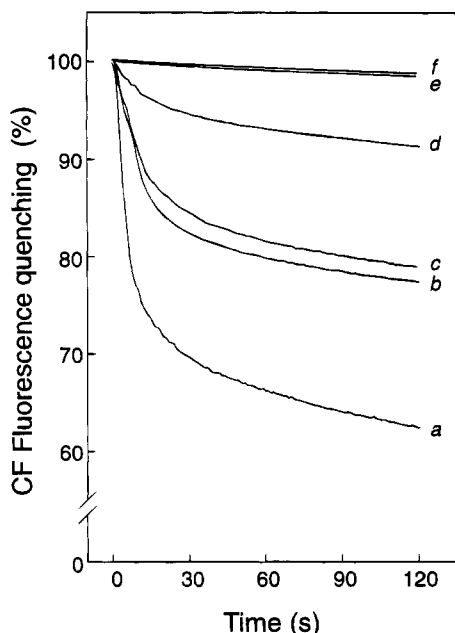
FIGURE 1: Effect of $\Delta\psi$ on the efflux kinetics of nisin-induced carboxyfluorescein efflux from PC and PG liposomes. Potassium-loaded PC (*a* and *b*) or PG (*e* and *f*) liposomes were diluted into a buffer containing sodium (*a* and *e*; $\Delta\psi$, inside negative) or potassium (*b* and *f*; no $\Delta\psi$) ions. Sodium-loaded PC liposomes were diluted into a buffer containing sodium (*c*; no $\Delta\psi$) or potassium (*d*; $\Delta\psi$, inside positive). At zero time, 10 μg of nisin per mg of phospholipid was added. In the absence of nisin, traces identical to *f* were obtained for both the PC and PG liposomes in sodium or potassium-containing buffer.

Table 1: Nisin-Induced Release of Fluorophores by PC Liposomes

| compound | mass (dalton) | charge | initial permeation rate (%/s) | | |
| | | | −nisin | +nisin | |
			±$\Delta\psi$	−$\Delta\psi$	+$\Delta\psi$
MQAE	326	+	0.06	0.08	0.08
F	332	−	0.17	6.4	16.2
FDA	416	0	2.1	2.1	2.1
CF	376	−	0.03	5.7	14.8
CFDA-AM	532	0	1.9	1.9	1.9
BCECF	520	−	0.02	1.2	2.9
BCECF-AM	821	0	1.5	1.5	1.5
FMG	494	−	0.08	0.11	0.19
CFDG	701	−	0.02	0.02	0.02
FDEX	~3000	−	<0.01	<0.01	<0.01

release of CF. Imposition of a transmembrane electrical potential, $\Delta\psi$, inside negative, accelerates CF release from PC liposomes (traces a and b). Imposition of a $\Delta\psi$ of opposite polarity, i.e., inside positive, limits the release of CF (traces c and d). Liposomes composed of the anionic PG retain the CF after the addition of nisin (i.e., up to 100 μg/mg of phospholipid) both in the absence or presence of a $\Delta\psi$ (traces e and f).

The characteristics of the nisin-induced dye release from PC liposomes were further studied by testing a series of dyes that differed in size and polarity. Rapid release was only observed with fluorescein and carboxyfluorescein (Table 1). Much slower release was observed with 2',7'-bis(2-carboxy-ethyl)-5-(and 6-)carboxyfluorescein (BCECF), while CF derivatives containing one or two galactopyranoside residues (FMG and CFDG) were almost not released. In each of these cases, except for CFDG, the release was further stimulated by the imposition of a $\Delta\psi$, inside negative. Nisin had no effect at all on the release of anionic fluorescein dextran with

Table 2: Effect of Nisin on $\Delta\psi$ in Cytochrome *c* Oxidase Proteoliposomes[a]

| lipid | S-13 (nM) | $\Delta\psi$ (mV)[b] | |
		−nisin	+nisin
PG	0	−147	−5
	0.1	−107	−17
	0.2	−89	−78
	0.3	−78	−72
	0.4	−43	−37
PC	0	−128	−102
	0.1	−88	−82
	0.2	−69	−55

[a] Nisin was added at 10 μg/mg of phospholipid. [b] Nigericin was added at a final concentration of 10 nM.

a molecular mass of 3000 or higher. Each of the fluorescein derivatives tested carried net negative charge. By reacting the free carboxyl groups with an ester, the net charge can be neutralized, resulting in a nonfluorescent molecule. Influx of these dyes can be measured conveniently through the use of liposome-entrapped esterase that converts the ester into a fluorescent molecule. The esters showed an increased passive permeability (Table 1). Nisin was, however, completely ineffective in accelerating influx any further, even though the anionic fluorescein derivatives permeate the liposomal membrane at much higher rates in the presence of nisin. The cationic fluorescent indicator *N*-(ethoxycar-bonylmethyl)-6-methoxyquinolium ion (MQAE) was not released by nisin. Under conditions that nisin elicits CF release from PC liposomes, little effect was observed on the $\Delta\psi$ when measured with the fluorophores $DiSC_2(5)$ (inside negative) and oxonol VI (inside positive) (not shown; see also Table 2). These results suggest that nisin induces the release of nonbulky anionic fluorescent indicators from liposomes in a $\Delta\psi$-stimulated manner, possibly as an anion carrier. This activity is specific for PC liposomes and not observed with PG liposomes.

Nisin Dissipates the $\Delta\psi$ in Cytochrome c Oxidase Proteoliposomes Composed of Phosphatidylglycerol. Nisin has previously been shown to dissipate the $\Delta\psi$ in cytochrome *c* oxidase proteoliposomes prepared from *E. coli* phospholipids (Gao *et al.*, 1991). Cytochrome *c* oxidase was reconstituted into PG and PC liposomes and supplied with the electron-donor system cytochrome *c*/TMPD/ascorbate. The iono-phore nigericin was added to assure that $\Delta\psi$ was the only component of the Δp. The generation of a $\Delta\psi$, inside negative, was followed through the use of a TPP^+-selective electrode that monitors the external concentration of this lipophilic cation. Both liposome preparations maintained a high $\Delta\psi$ (Table 2). In the presence of nisin (Table 2), $\Delta\psi$ was almost completely dissipated in the PG liposomes whereas addition of nisin had only a small effect on the steady-state $\Delta\psi$ in PC liposomes. To determine if nisin requires a threshold $\Delta\psi$ for activity, the steady-state $\Delta\psi$ prior to the addition of nisin was progressively lowered through the use of small quantities of the uncoupler S-13. When the $\Delta\psi$ was below −100 mV, nisin appeared to be hardly effective in PG liposomes (Table 2). Lowering of the $\Delta\psi$ in PC liposomes by S-13 had no further effect on the inability of nisin to dissipate the $\Delta\psi$ in these liposomes. These data confirm previous black lipid membrane studies (Sahl *et al.*, 1987) and demonstrate that nisin requires a threshold $\Delta\psi$ for pore formation in PG liposomes, while it is nearly ineffective in dissipating $\Delta\psi$ in PC liposomes.
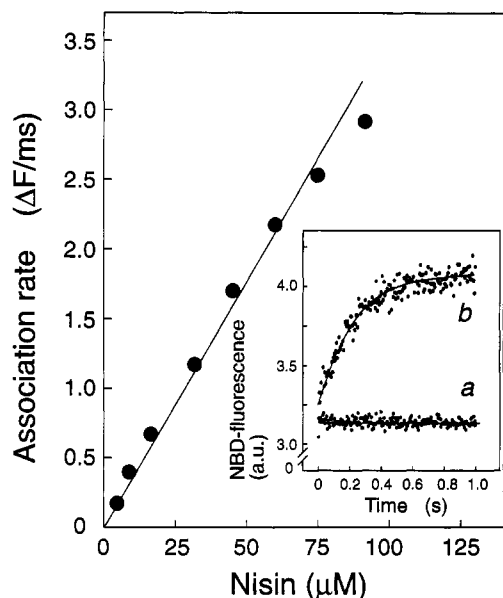
FIGURE 2: Binding of nisin to PG liposomes. Nisin and PG liposomes containing 2 mol % NBD-PE were rapidly mixed with a stopped flow device as described in Experimental Procedures. The final concentration of the PG liposomes was 235 μM. Association rate was calculated from best fits to a single-exponential rise function. (Inset) Kinetics of the NBD fluorescence increase upon mixing of PC (*a*) or PG (*b*) liposomes labeled with 2 mol % NBD-PE with nisin at a concentration of 50 μM.

Nisin Associates with the Surface of Phosphatidylglycerol Liposomes. To analyze binding of nisin to the membrane, an assay was developed that monitors the fluorescence of a nitrobenzoxadiazole group attached to the polar head group of phosphatidylethanolamine. NBD-PE can be used to follow bilayer-to-hexagonal phase transitions since these transitions cause an increase in the NBD fluorescence intensity due to the transfer of the NBD group from a polar to an apolar environment (Allen *et al.*, 1990). The addition of nisin to PG liposomes containing 2 mol % of NBD-PE caused a large increase in fluorescence intensity, while no effect was observed with PC liposomes (inset in Figure 2). Bilayer-to-hexagonal phase transitions are usually accompanied with liposome disruption and release of the aqueous contents. Since PG liposomes are not permeabilized by nisin, it seems unlikely that the change in NBD fluorescence intensity is due to hexagonal phase formation (see also at the ^{31}P NMR experiments). Alternatively, we propose that the binding of nisin to the phospholipid surface protects the NBD group from the polar solvent water, thereby causing an increase in NBD-fluorescence intensity. Nearly all (>95%) of the fluorescent signal in the absence of nisin had the very short lifetime (τ_o) of 1.1 ns characteristic of NBD in an aqueous environment. In the presence of nisin, however, there was a significant increase in the number of NBD molecules with a long τ_o value, i.e., 5.9 ns (about 27% with 100 μM nisin). These results suggest that a major fraction of the NBD molecules is shielded from water by nisin. Since the fluorescence changes occurred within a second after the addition of nisin, the kinetics of binding of nisin to PG membranes was further analyzed by stopped-flow fluorescence spectroscopy. At a fixed liposome concentration, the rate of association increased as a linear function of the nisin concentration (Figure 2). From the linear portion of this plot a pseudo-first-order rate constant for association (k_{ass}) was calculated, yielding a value of 3.3 $\times$ 10^8 %$\cdot$s$^{-1}\cdot$M^{-1}. The association rate was not affected by
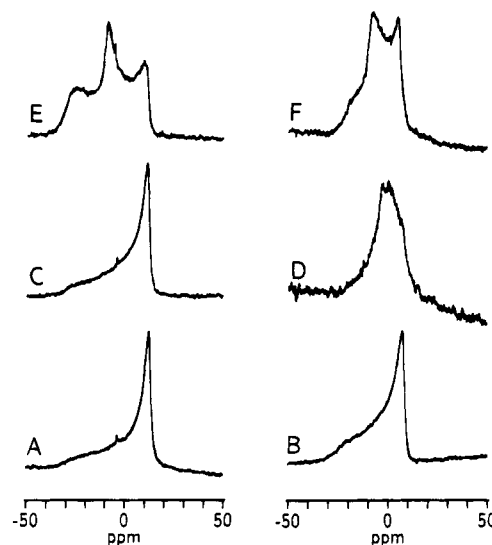

FIGURE 3: ^{31}P NMR of PG and PC liposomes in the presence of nisin and gramicidin. ^{31}P NMR spectra of aqueous dispersions of (A) PC, (B) PG, (C) nisin and PC, (D) nisin and PG, (E) gramicidin D and PC, and (F) gramicidin D and PG. The polypeptide to lipid ratio was about 1:10.

the simultaneous imposition of a $\Delta\psi$, inside negative (not shown). Dissociation of nisin from the membrane was analyzed by following the fluorescence changes after the addition of a 10-fold excess of nonlabeled PG liposomes to a solution containing nisin prebound to NBD-labeled PG liposomes. Only a slow decrease in NBD fluorescence was noted with a rate of less than 1%$\cdot$s^{-1} (not shown). The calculated dissociation constant, $K_d = k_{dis}/k_{ass}$, is therefore approximately 3 nM. The low value for the K_d implies that the interaction between nisin and PG liposomes is extremely tight. Even in the presence of a $\Delta\psi$, we observed no change in NBD fluorescence when PC liposomes were used, suggesting that nisin interacts in a different manner with PC and PG liposomes.

Nisin Perturbs the Bilayer Structure of Phosphatidylglycerol Liposomes. ^{31}P NMR measurements were performed to determine the hydration state and dynamics of the phospholipid head groups of PC and PG in the presence of nisin. The spectrum of a mixture of nisin and PG (Figure 3D) showed a peak 7 ppm low field from the peak in the spectrum of PG (Figure 3B). There was no change in the typical bilayer spectrum of PC liposomes (Figure 3A) in the presence of nisin (Figure 3C). As reported before (van Echteld *et al.*, 1982), gramicidin induced a significant change of the ^{31}P spectrum both with PC and PG liposomes (Figure 3E,F), typical for a bilayer-to-hexagonal phase transition. These results are indicative of a perturbation of the bilayer structure of PG by nisin. From the shape of the signal, it appears that nisin dehydrates the phospholipid headgroups of PG, and not of PC liposomes. The reduced signal-to-noise ratio of the ^{31}P NMR spectrum (Figure 3D) suggests that nisin immobilizes some of the PG. These data are complementary to the NBD-PE fluorescence measurements.

Nisin Mediated Lipid Transfer between Phosphatidylglycerol Liposomes. Light-scattering experiments indicated that the addition of nisin causes an extensive aggregation of the PG liposomes (not shown). We did not analyze these turbidity changes any further but used instead the anionic fluorescent membrane probe octadecyl rhodamine B (R_{18}) to monitor changes in the lipid distribution among liposomes
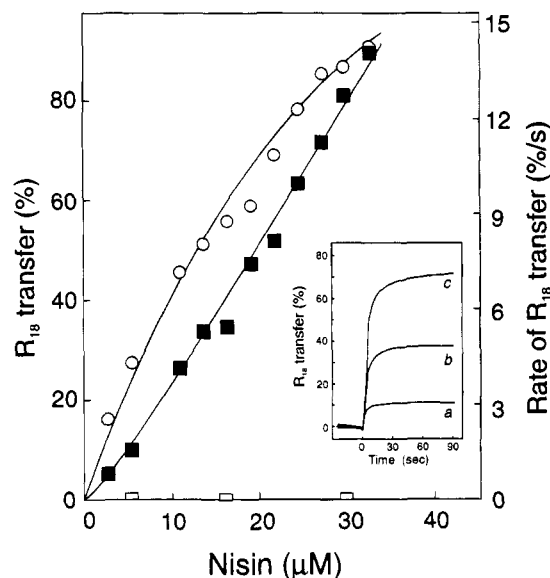
FIGURE 4: Nisin-induced lipid transfer. Rate (■) and extent (○) of the nisin induced lipid transfer between R_{18}-labeled and unlabeled PG liposomes was calculated from the increase in R_{18} fluorescence upon the addition of nisin at the concentration indicated. (□) Lipid transfer between PC liposomes. The final concentration of the PG liposomes was 250 μM. (Inset) Kinetics of R_{18} fluorescence increase with 5 (*a*), 15 (*b*), and 30 (*c*) μM nisin.
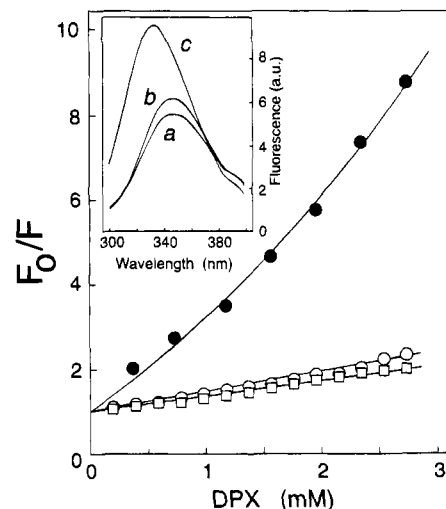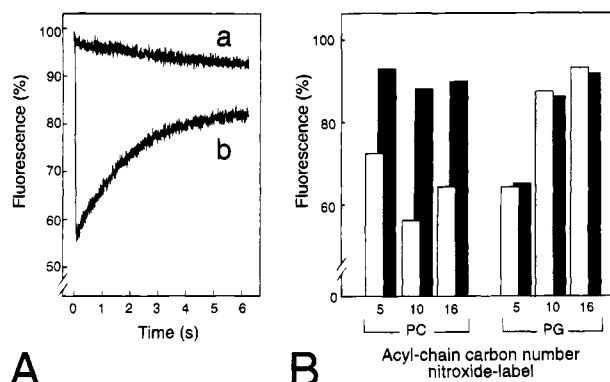

FIGURE 5: Quenching of K7 tyrosine fluorescence by water-soluble DPX. Stern—Volmer plots of the quenching of tyrosine fluorescence of epilancin K7 by DPX. Nonbound K7 (□), and K7 bound to PG (●) and PC (○) liposomes. The concentration of epilancin K7 was 80 μg/mL. Liposomes were present at 310 μM. Excitation and emission wavelengths were set at 290 and 340 nm, respectively. (Inset) Aromatic fluorescence spectra of soluble epilancin K7 (*a*) and epilancin K7 associated with PC (*b*) and PG (*c*) liposomes. Spectra were corrected for the raman contribution of water.

(Hoekstra *et al.*, 1984). The fluorescence of this probe is quenched at high concentrations, and, upon lipid transfer to nonlabeled liposomes, the self-quenching is relieved. PG liposomes were mixed in a one-to-one ratio with PG liposomes containing 4 mol % R_{18}. A rapid redistribution of R_{18} among the PG liposomes was observed upon the addition of nisin (inset, Figure 4), while PC liposomes were completely ineffective (Figure 4, □). The kinetics of this event are rather atypical. After a short, initial burst of lipid transfer, a plateau level was reached that remained stable. With increasing nisin concentration, both the rate (■) and extent (○) of the transfer increased. Lipid redistribution occurs at an about 200-fold slower rate than binding of nisin to the liposomes. This phenomenon may be due to membrane fusion or may reflect true lipid transfer between aggregated liposomes. On the basis of the CF release studies, this fusion event has to be exceptionally nonleaky. Fusion can be followed as the mixing of aqueous contents using the terbium (Tb^{3+})/dipicolinic acid (DPA) assay (Wilschut & Papahadjopoulos, 1979). Fusion of DPA and Tb^{3+}-containing liposomes results in the formation of a Tb^{3+}—DPA complex that is highly fluorescent. This assay, however, did not reveal any significant level of nisin-dependent fusion whereas Ca^{2+}-induced fusion was readily observed (not shown). We, therefore, conclude that the nisin-dependent aggregation of PG liposomes allows a rapid lipid transfer without occurrence of bilayer fusion. In addition, fluorescence polarization experiments with TMA-DPH indicate that under these conditions, i.e., from 0 to 0.15 mol of nisin to PG, the anisotropy (*r*) increased from 0.191 to 0.269. A far less dramatic increase in the *r* value (from 0.162 to 0.169) was detected with TMAP-DPH that probes the membrane viscosity deeper into the hydrophobic core of the membrane. In contrast, hardly any change was observed in the TMA-DPH and TMAP-DPH fluorescence *r* value with PC liposomes, i.e., 0.159—0.161 and 0.189—0.194, respectively. The fluorescence lifetime (τ_o) of TMA-DPH in PG and PC liposomes increased from 0.55 to 1.1 ns and 0.47 to

0.53 ns, respectively. These data suggest that nisin restricts the fluidity of PG membranes near to the interface between the aqueous phase and the lipid head groups.

Membrane Binding and Insertion of Epilancin K7, a Lantibiotic with a Unique Tyrosine Residue. Epilancin K7, a novel lantibiotic, contains a unique tyrosine residue in the middle of the molecule (Van de Kamp *et al.*, 1994). Like nisin, epilancin K7 provokes an $\Delta\psi$-dependent release of CF from PC liposomes, whereas it is ineffective with PG liposomes (not shown). It tightly associates with PG liposomes with a K_d of 1.5 nM (i.e., k_{ass} of 4.8 × 10^8 %·s^{-1}·M^{-1} and a k_{diss} of 0.7%·s^{-1}), and dissipates the $\Delta\psi$ in cytochrome *c* oxidase proteoliposomes composed of PG and not of PC (not shown). These data suggest that epilancin K7 roughly interacts with membranes in a manner similar to that found for nisin. The Tyr fluorescence of epilancin K7 allows a more detailed spectroscopical analysis of the interaction of lantibiotics with membranes. The Tyr fluorescence emission maximum of epilancin K7 is exceptionally high, i.e., 345 nm instead of 305 nm, and is comparable to tryptophan. Excited state ionization, facilitated by nearby proton acceptors, can result in the ionization of the Tyr even at a neutral pH (Lakowicz, 1986; Szabo *et al.*, 1978). This phenomenon can give rise to a red-shifted emission spectrum of a tyrosinate complex (Lakowicz, 1986; Szabo *et al.*, 1978). Addition of increasing amounts of PG liposomes to epilancin K7 in solution results in an increased Tyr fluorescence with a large blue-shift of the emission maximum of about 18 nm (inset, Figure 5). Less dramatic changes were observed when PC liposomes were used. The changes in the fluorescence characteristics of the Tyr residue of epilancin K7 upon binding to PG are indicative for a lowering of the degree of ionization of the Tyr.

The Tyr fluorescence of epilancin K7 bound to PG liposomes is efficiently quenched by the membrane-impermeable cationic quencher DPX with a Stern—Volmer constant of 0.43 mM^{-1} (Figure 5). Much higher concentrations of DPX are needed to quench the Tyr fluorescence of free and PC liposomes-associated epilancin K7, i.e., *K* of

FIGURE 6: Quenching of K7 tyrosine fluorescence by PC analogues harboring doxyl moieties at a different position in the acyl chain. (A) Time course of the quenching of tyrosine fluorescence of epilancin K7 by 1-palmitoyl-2-stearoyl-(10-doxyl)-*sn*-glycero-3-phosphatidylcholine incorporated at 20 mol % into PC liposomes. Curves represent the tyrosine fluorescence in the absence (*a*) and presence (*b*) of an imposed $\Delta\psi$, inside negative. (B) Quenching of the tyrosine of epilancin K7 by doxyl moieties present at the 5-, 10-, or 16-position of the acyl chain of PC and PG. Open and filled areas represent the minimal tyrosine fluorescence levels of epilancin K7 in the presence and absence of a $\Delta\psi$. Peak values were calculated from the transient changes in Tyr fluorescence in the absence and presence of an acyl-chain bound doxyl label. Further experimental details were as given in the legend to Figure 5.

2.3 and 2.1 mM^{-1}, respectively. Quenching of the Tyr fluorescence was studied further with 1-palmitoyl-2-(*n*-doxylstearoyl)-*sn*-glycero-3-phosphatidylcholine derivatives bearing a spin-label in positions 5, 10, or 16. Efficient quenching was observed only when the doxyl group was present at the 5-position (Figure 6B). With PC liposomes, only a lower level of quenching was observed. $\Delta\psi$, inside negative (less than -100 mV), had little effect on the Tyr fluorescence (not shown) and the extent of quenching (Figure 6) when epilancin K7 was bound to PG liposomes. With the PC liposomes, a transient, $\Delta\psi$-dependent, quenching of the Tyr fluorescence was observed with doxyl labels at the 5, 10, or 16 position (Figure 6A, B). This process was followed by a slower relaxation to the base level (Figure 6A). These results suggest that $\Delta\psi$ promotes the transient, deep penetration of the Tyr residue of epilancin K7 into PC membranes.

DISCUSSION

Lantibiotics of the Type A group such as nisin are thought to mediate their bacteria-killing effect by permeabilizing the target cell membrane for small solutes and ions, thereby interfering with energy transduction and the maintenance of intracellular pools of metabolites (Bruno *et al.*, 1992; Gao *et al.*, 1991; García Garcerá *et al.*, 1993; Kordel & Sahl, 1986; Okereke & Montville, 1992; Ruhr & Sahl, 1985; Sahl, 1991). Previous *in vitro* studies have shown that nisin induces efflux of the fluorescent dye carboxyfluorescein from liposomes composed of the zwitterionic phosphatidylcholine (PC), whereas it is ineffective with the negatively charged phosphatidylglycerol (PG) (García Garcerá *et al.*, 1993). We now show that nisin conducts transmembrane anion movements in PC liposomes and that this activity is specific for small anionic solutes. These anion movements are stimulated by a $\Delta\psi$, inside negative, and suppressed by a $\Delta\psi$, inside positive. Studies with epilancin K7, a lantibiotic with a unique tyrosine residue, suggest that $\Delta\psi$ may stimulate the anion flux by stabilizing the membrane-inserted state of the

molecule. The nisin-induced anion flux is slow and does not result in a major dissipation of the $\Delta\psi$ (Table 2). Nisin, however, seems to interact differently with PG liposomes. It seems to interact with the surface of these liposomes, possibly in an electrostatic fashion. The nature of this interaction presumably prevents nisin from transporting anionic fluorophores in PG liposomes. The surface-bound nisin perturbs the lipid bilayer structure without affecting the global permeability of the PG membrane. Pore formation in PG liposomes appears to require a $\Delta\psi$ of at least -100 mV.

What lessons can be learned from this *in vitro* study, and what does it mean for the *in vivo* action? It seems unlikely that the observed anion selectivity of nisin in PC liposomes explains the specificity of nisin *in vivo*. *In vivo*, nisin forms pores in the target cytoplasmic membrane with a low specificity (Sahl, 1991). This process strictly requires $\Delta\psi$, in contrast to the anion flux observed with PC liposomes. From the kinetics of amino acid release by lactic acid bacteria (B. González, A. J. M. Driessen, M. Soares, and W. N. Konings, unpublished results) and other Gram-positive bacteria (Bruno *et al.*, 1992; Okereke & Montville, 1992; Ruhr & Sahl, 1985; Sahl, 1985, 1991), it is evident that nisin has a more potent effect on the intracellular amino acid pools than dissipation of the Δp alone. Target membranes in general contain large amounts of anionic phospholipids, and these would antagonize the nisin-dependent anion fluxes observed *in vitro*. Pore formation by nisin in PG liposomes in the presence of a high $\Delta\psi$ may, therefore, closer mimic the *in vivo* mechanism. In the dye-release assays, however, it has not been possible to generate a $\Delta\psi$ of the magnitude needed to allow pore formation. Imposed ion-diffusion gradients are limited by the dilution factor and secondary ion fluxes, while the gradients are only short-lived. In a previous study of Gao *et al.* (1991), nisin dissipated the $\Delta\psi$ and ΔpH in liposomes composed of *E. coli* lipids apparently without the need for a threshold level. Nisin was not active with liposomes composed of egg PC. The effects on $\Delta\psi$ were only observed at a nisin concentration 15–50-fold higher than used in the current study. The exuberant ability of nisin to bind acidic phospholipids may, even in the absence of a $\Delta\psi$, destabilize the *E. coli* liposomes by binding the PG and cardiolipin that stabilize the phosphatidylethanolamine [about 70 mol % (Driessen *et al.*, 1988)] in a bilayer structure. In this respect, the $\Delta\psi$ in cytochrome *c* oxidase proteoliposomes composed of a mixture of dioleoylphosphatidylethanolamine and -glycerol (3/1, mol/mol) is dissipated by nisin concentrations as low as 1 μg/mg of phospholipid (A. J. M. Driessen, unpublished results).

In the absence of stable interactions with anionic phospholipids, the cationic nisin may act as an anion carrier and function according to the following mechanism. When added from the outside, nisin binds to the lipid surface and transverses across the membrane in a $\Delta\psi$ (inside negative) stimulated manner. It then binds the anion (such as carboxyfluorescein) on the inside and subsequently traverses across the membrane as a binary complex with the anion. On the outer surface, the anion is released and nisin recycles to bind another anion on the inside. The anions should not be too bulky and/or hydrophilic. The computed log *P* [the log of the octanol–water partition coefficient (Viswanadhan *et al.*, 1989)] values for the charged-neutralized (i.e., protonated) CF and F are positive, i.e., 0.07 and 0.38, respectively. These compounds are thus able to partition

into a hydrophobic phase when charge neutralized. In principle this can be realized when the anions interact with the cationic nisin in an electrostatic manner. This may explain the observed anion selectivity of nisin in PC liposomes. Penetration of nisin and epilancin K7 into the membrane is likely to be rate-limiting, and for K7 the data suggest that this step is promoted by $\Delta\psi$ (Figure 6).

An increase in the negative surface charge of the membrane due to the inclusion of anionic phospholipids inhibits the formation of the anion-selective pore (García Garcerá et al., 1993). Nisin may remain bound as a partially neutralized complex at the lipid surface or interface. The "sites" on nisin that bind anions in PC liposomes may be occupied by the anionic lipids and would thus not be available to bind other negatively charged groups. NMR data indicate that nisin in solution adopts a rather flexible structure with poor overall resolution, but with an amphiphilic character (Van de Ven et al., 1991). Nisin binds to micelles of zwitterionic dodecylphosphocholine (DPC) or of anionic sodiumdode-cylsulphate (SDS) (Van den Hooven et al., 1993) in approximately similar conformations with the charged side exposed to the aqueous phase (H. W. van den Hooven, unpublished results).

Further evidence for specific electrostatic interactions between sites on the lantibiotic and phospholipids emerges from the study of epilancin K7. This novel lantibiotic, which shows many of the functional characteristics as found for nisin, has a unique Tyr residue at position 15 (Van de Kamp et al., 1994) which was used here as an intrinsic probe to monitor the interaction of K7 with phospholipid vesicles. The fluorescence emission spectrum of free K7 indicates the presence of an excited state tyrosinate (see inset of Figure 5) (Szabo et al., 1978). The observation of this tyrosinate is probably due to electrostatic stabilization of the ionized state. NMR data suggest that a lysine residue at position 14, next to Tyr15, is involved in this stabilization. 2D ^{1}H NOESY of K7 in aqueous solution shows that the side chains of Tyr15 and Lys14 are very close in space (M. van de Kamp, unpublished results), resulting in significant ring-current shift contributions to the Lys14 side-chain proton chemical shifts (Van de Kamp et al., 1994). A nearby Lys14 $N^\zeta H_3^+$ will stabilize the excited-state Tyr $O^{\eta-}$. This interaction also explains the observation that the (ground-state) pK_a of Tyr15 in K7 in solution (9.2 ± 0.2) (M. van de Kamp, unpublished results) is smaller than the pK_a of a free Tyr (10.2 ± 0.2) (Bundi & Wüthrich, 1979). The intrinsic Tyr fluorescence of epilancin K7 changes when K7 interacts with anionic PG liposomes but not when it interacts with zwitterionic PC liposomes. Binding of K7 to the anionic phospholipid surface results in a major blue-shift of the Tyr emission (see inset of Figure 5), which is indicative of a weaker excited-state ionization of Tyr15. Similarly, the Tyr pK_a is affected differently by association of K7 with zwitterionic DPC or anionic SDS micelles (K7—DPC, $pK_a = 10.6 \pm 0.3$; K7—SDS, $pK_a \geq 12.0$) (M. van de Kamp, unpublished results). The relatively large effects with the PG liposomes and the SDS micelles can be explained by the substitution of the interaction of the negatively charged phosphate (PG) or sulfate (SDS) with the Lys14 $N^\zeta H_3^+$ for the Lys14 $N^\zeta H_3^+$—$O^{\eta-}$ Tyr15 interaction. Moreover, the abundant presence of anionic head group will destabilize the ionized state of Tyr15. In the case of zwitterionic PC liposomes and DPC micelles, the electrostatic effect on the Lys14 $N^\zeta H_3^+$—$O^{\eta-}$ Tyr15 interaction and on the Tyr ioniza-

tion will be smaller, in line with the relatively small effects on the Tyr fluorescence and the Tyr pK_a. The inferred presence of the Lys14 $N^\zeta H_3^+$—$O^{\eta-}$ Tyr15 interaction in free and PC liposome associated K7 but not in PG liposome associated K7 corresponds with the observation that DPX quenches the Tyr fluorescence much better for PG-bound K7 than for free and PC-bound K7 (Figure 6).

Several models have been proposed to explain the pore-forming ability of nisin. The "insertion model" assumes that the molecules are initially bound at the lipid surface, and subsequently, in the presence of a $\Delta\psi$, they may flip into a membrane-spanning orientation. The membrane-inserted molecules may form a cluster around a central pore as in the "barrel-stave model" (Ojcius & Young, 1991). Alternatively, a "wedge model" can be envisioned where the amphiphilic molecules adhere to the surface of the membrane causing destabilization of the bilayer structure thereby promoting pore formation, such as proposed for annexins (Demange et al., 1994). Our data suggest that the binding of nisin to anionic phospholipids indeed results in a local disorder of the phospholipid head groups. This process is, however, not directly responsible for pore formation as there is no general loss of the permeability barrier or integrity of the membrane. Pore formation requires the presence of a high $\Delta\psi$. Nisin has a strong effect on the lipid dynamics [membrane fluidity (see also Kordel et al. (1989)), dehydration of the lipid surface, nonbilayer intermediate structures, and stimulation of lipid transfer between liposomes, i.e., bilayer—bilayer contacts] when incubated with liposomes at high concentrations. The PG surface-bound form of nisin presumably represents a true intermediate in the pore formation process. $\Delta\psi$ may change the orientation of nisin in the membrane relative to the plane of the membrane, either forming a wedge or a true membrane span, and possibly carrying the anionic lipids along to form a water-filled pore (see Figure 7).

Nisin forms rectifying (i.e., work only in one direction) channels in highly polarized (>100 mV) planar bilayers (Benz et al., 1991; Sahl et al., 1987). These channels are instable, and single-channel conductances are highly variable. The lifetime of these channels is in the order of milliseconds to seconds, and the fluctuations in amplitude and lifetime increase in a steep fashion with rising voltage. The "apparent" threshold decreases both with the amount of nisin and the amount of negatively charged phospholipids, while only the trans-negative potential induces membrane conductance. Sidedness seems not to be a general property of lantibiotics, and so far is only observed for nisin (Sahl et al., 1987) and Pep5 (Kordel et al., 1988; Schüller et al., 1989). These molecules may insert and function in an oriented manner. Gallidermin and epidermin form nonrectifying channels with higher stability (Benz et al., 1991). Their membrane insertion is less $\Delta\psi$-dependent as compared to nisin. $\Delta\psi$ likely promotes pore formation by favoring or stabilizing the membrane-inserted (wedged) state of the lantibiotic. $\Delta\psi$ would thus not only increase the frequency of insertion but also extend the lifetime of the inserted state.

In conclusion, nisin acts as an anion carrier in PC liposomes. This action may be pertinent to the mode of action of nisin in vivo, where it forms nonselective pores in membranes with a broad lipid composition. It is suggested that nonselective pores arise from the interaction of nisin with anionic phospholipids instead of with anionic solutes. In the presence of a $\Delta\psi$, the surface-bound nisin may plunge
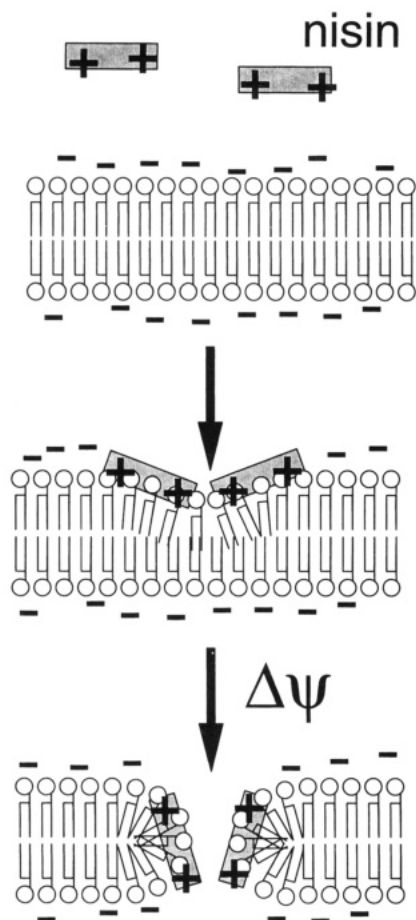
FIGURE 7: Model for the action of nisin in PG liposomes. See Discussion.

into the membrane and further disorder the bilayer structure. The locally disturbed bilayer may form an integral part of the nonspecific, water-filled nisin-dependent pore.

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