Structure−Function Relations of Variant and Fragment Nisins Studied with Model Membrane Systems†

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ABSTRACT: Nisin, a 34 residue lantibiotic produced by strains of *Lactococcus lactis* subsp. *lactis*, exerts antimicrobial activity against Gram-positive bacteria at the cytoplasmic membrane. The structural aspects of nisin which facilitate membrane interaction and permeabilization have been investigated in planar lipid bilayers and liposomes with proteolytic fragments and site-directed variants. N-Terminal nisin fragments N1−12 and N1−20 had little effect on phospholipid mobility, on macroscopic electrical conductance, or on calcein release from liposomes. By contrast, the I30W nisin A variant induced a time-dependent reduction in lipid mobility, indicative of nisin−membrane surface interactions, as well as a decline in membrane capacitance, rise in conductance, and calcein release from liposomes. In these respects I30W nisin A is similar to native nisin. Charge substitutions were also engineered to generate K12L and H27K nisin A variants, both of which were similar to I30W nisin A with respect to an overall reduction in phospholipid mobility. While the K12L nisin A variant elicited a higher increase in membrane capacitance and electrical conductance than I30W nisin A, the H27K nisin A variant elicited weaker effects. These results point to a substantial role for intramembrane charged residues in controlling ion flow through nisin-doped membranes. Native nisin and variants elicit an enhanced release of calcein from liposomes composed of the negatively-charged phospholipids cardiolipin and phosphatidylserine, compared with phospholipid bearing no net charge, suggesting that an electrostatic attraction encourages the initial nisin−membrane association. The results are discussed in the context of other recently proposed models for nisin action.

Nisin is an antimicrobial peptide that is used in the food industry as a natural preservative because of its ability to inhibit the growth of food spoilage and pathogenic organisms (Delves-Broughton et al., 1996). It is highly modified and belongs to a group of peptides, termed lantibiotics, that undergo extensive posttranslational modification (Sahl et al., 1995). This involves the introduction of intramolecular rings which generate the unusual thioether linkages lanthionine and 3-methyllanthionine, as well as a number of other atypical didehydroamino acids (Jung, 1991).

The target for the antagonistic action of nisin appears to be the cytoplasmic membrane of sensitive bacteria. The nisin-induced bactericidal effect is exerted by disruption of the cytoplasmic membrane, resulting in an efflux of ions, amino acids, and ATP, leading to a collapse of the proton-motive force (pmf)[1] (Ruhr & Sahl, 1985; Kordel & Sahl, 1986; Gao et al., 1991; Bruno et al., 1992; Okereke & Montville, 1992; García Garcerá et al., 1993; Winkowski et al., 1996). This membrane breakdown has been attributed to the formation of channel-like structures that facilitate ion flow in a voltage-dependent manner (Sahl et al., 1987; Benz et al., 1991). However, a recent investigation into the involvement of membrane charge and rigidity suggested that intrinsically unstable pores may be formed as a result of the coinsertion of nisin bound to charged phospholipid head groups following an initial electrostatic interaction at the membrane surface (Driessen et al., 1995).

Although membrane depolarization is generally accepted as the primary mode of action of nisin, little is known about the structural features responsible, and the contribution of individual animo acids is less certain. Investigation into the relationship between the structure of nisin and its function may help to elucidate the underlying principles that govern its mechanism of action. At a crude level this can potentially be achieved through studies on the action of proteolytic fragments of nisin, though more subtle effects can be addressed with site-directed variants.

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[1] Abbreviations: CL, cardiolipin; calcein, 3,6-dihydroxy-2,3-[*N*,*N*′-di(carboxymethyl)aminomethyl]fluoran); DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; FRAP, fluorescence recovery after photobleaching; fPLB, folded planar lipid bilayer; HPLC, high-pressure liquid chromatography; H27K nisin A, nisin variant containing lysine at position 27; I30W nisin A, nisin variant containing tryptophan at position 30; *I*−*V*, current-to-voltage; K12L nisin A, nisin variant containing leucine at position 12; LUV, large unilamellar vesicles; ΔM21 nisin A, nisin variant with methionine deleted at position 21; MIC, minimum inhibitory concentration; N1−12, nisin$_{1-12}$ fragment; N1−20, nisin$_{1-20}$ fragment; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine; pmf, protonmotive force.

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N-Terminal fragments of nisin, generated proteolytically (Dodd et al., 1996a), enable an investigation of the involvement of different regions of the nisin molecule in target membrane associations. The presence of natural lantibiotic variants suggested that it may also be possible to create variant peptides artificially that retain activity. A number of expression systems have been developed for the generation of variants of the lantibiotics nisin, subtilin, Pep5, epidermin, and gallidermin by protein engineering (Dodd et al., 1992, 1995, 1996b; Kuipers et al., 1992, 1995, 1996; Liu & Hansen, 1992; Bierbaum et al., 1994; Ottenwälder et al., 1994). However, the characterization of variant lantibiotics has been minimal and, in the case of nisin, largely restricted to structural confirmations and determination of biological activities (Kuipers et al., 1995; Rollema et al. 1995; Dodd et al., 1996b). In this study, a lactococcal expression system (Dodd et al., 1996b) has been used to generate specifically mutated *nisA* genes that result in the exclusive production of novel nisins including the variant molecules I30W nisin A, K12L nisin A, and H27K nisin A. The amino acids of native nisin selected for modification involve charge alterations, enabling the determination of any role charge may have in sensing voltage within the membrane dielectric, and the introduction of an aromatic acid which has also been exploited for its fluorescent properties (Martin et al., 1996). The interaction of these site-directed nisin variants with artificial model membrane systems is employed here in an attempt to reveal the functional importance of particular residues.

The application of nisin to planar lipid bilayers enables the investigation of its impact on an array of membrane parameters (Giffard et al., 1996). Measurement of the lateral diffusion of phospholipids within the bilayer, using fluorescence recovery after photobleaching (FRAP), can be carried out in parallel with measurements of bilayer capacitance and conductance, facilitating association with the estimated effects on membrane structure and ion permeation properties. Previous work on native nisin has established a pattern of nisin−membrane surface association followed by insertion (Giffard et al., 1996). This work has been extended in the present study to investigate the structure−function relations of biologically active nisin fragments and variants. FRAP has been used to investigate the initial association of variants with the membrane surface and their subsequent penetration into the membrane. Conductance measurements have enabled comparisons of the relative efficacies of different structures in conferring electrical activity in membranes and on the voltage dependence of this interaction. Furthermore, the application of nisin variants to liposomes has enabled an investigation of the interaction of phospholipid composition with peptide structure in generating membrane permeability.

EXPERIMENTAL PROCEDURES

Materials. The following lipids were purchased from Avanti Polar Lipids Inc. (Alabaster, AL): cardiolipin (CL), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (POPS), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE). The fluorescent phospholipid analogue *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE) was purchased from Molecular Probes (Eugene, OR). Nisin (50 000 units/mg) was obtained from Aplin and Barrett (Trowbridge, Wilts, U.K.). Calcein (3,6-dihydroxy-2,3-[*N*,*N*′-bis(carboxymethyl)aminomethyl]fluoran), Triton X-100, hexadecane, hexane, and other chemicals used were of the highest purity available and were purchased from Sigma Chemical Co. (Dorset, U.K.).

Production, Purification, and Characterization of Nisin Variants and Fragments. Nisin variants were generated by site-directed mutagenesis using the gene replacement protocol previously described by Dodd et al. (1996b). Nisin fragments were generated enzymatically by digesting native nisin A with trypsin (N1−12) or α-chymotrypsin (N1−20) (Dodd et al., 1996a). Variant purification was carried out from culture supernatants by hydrophobic interaction chromatography followed by reverse-phase semipreparative HPLC according to Mulders et al. (1991) with the modifications described by Dodd et al. (1996b). Electrospray mass spectrometry carried out in a Fisons Instruments VG Platform (Fisons, Altrincham, U.K.) confirmed the relative molecular masses of purified variants and fragments.

Liposome Production and Calcein Leakage. Calcein was encapsulated in large unilamellar vesicles (LUV) which were prepared from multilamellar vesicles according to Defrise-Quertain et al. (1988) and were diluted in 150 mM NaCl, 0.01 mM EDTA, and 10 mM MES, at pH 6.0, to a lipid concentration of 35 μg/mL. Fluorescence recovery was recorded on a LS-5 fluorescence spectrometer (Perkin-Elmer, Beaconsfield, U.K.) on addition of 4.5 μM of variant peptide to liposomes as described in Martin et al. (1996). Experiments were carried out in triplicate, and standard errors were calculated for each set of values.

"Folded" PLB Formation. fPLB were formed according to the method of Montal and Mueller (1972), using the apparatus adapted by Ladha et al. (1996) and Giffard et al. (1996). In brief, a 25 μm thick Teflon film (Goodfellow, Cambridge, U.K.) containing a small circular hole of 200−400 μm^2 was clamped between the two halves of a Teflon chamber such that the cell chamber was divided by the Teflon septum containing a single hole. Prior to bilayer formation, the area around the hole was precoated with 1% (v/v) hexadecane in hexane. After evaporation of hexane, each compartment was filled with filtered, unbuffered 1 M KCl (pH 5.2−5.6) to a level below the hole. Lipid, 6.5 mM in hexane incorporating NBD-PE at a 1% molar ratio, was layered onto the buffer surface in each compartment, and the hexane was allowed to evaporate. The formation of a bilayer was monitored optically through a microscope via silica windows in the Teflon chamber and electrically via capacitance measurements as the levels of solution in the *trans* compartment, followed by the *cis* compartment, were raised above the hole, forming a bilayer across it.

Membrane potentials were recorded *cis* with respect to *trans* which was held at ground, and nisin was added to the *cis* compartment. Membrane current measurement under voltage clamp conditions used a pair of Ag/AgCl electrodes in direct contact with aqueous solutions and connected to a low-noise operational amplifier (R. A. P. Montgomery, U.K.). The amplified signal was simultaneously displayed on an oscilloscope (Gould, Essex, U.K.) and recorded on a chart recorder or DAT tape (Sony Corp., Japan). For construction of a current−voltage ($I-V$) relationship, the applied voltage was ramped over the range ±140 mV for 45 s in each direction. The membrane RC time constant

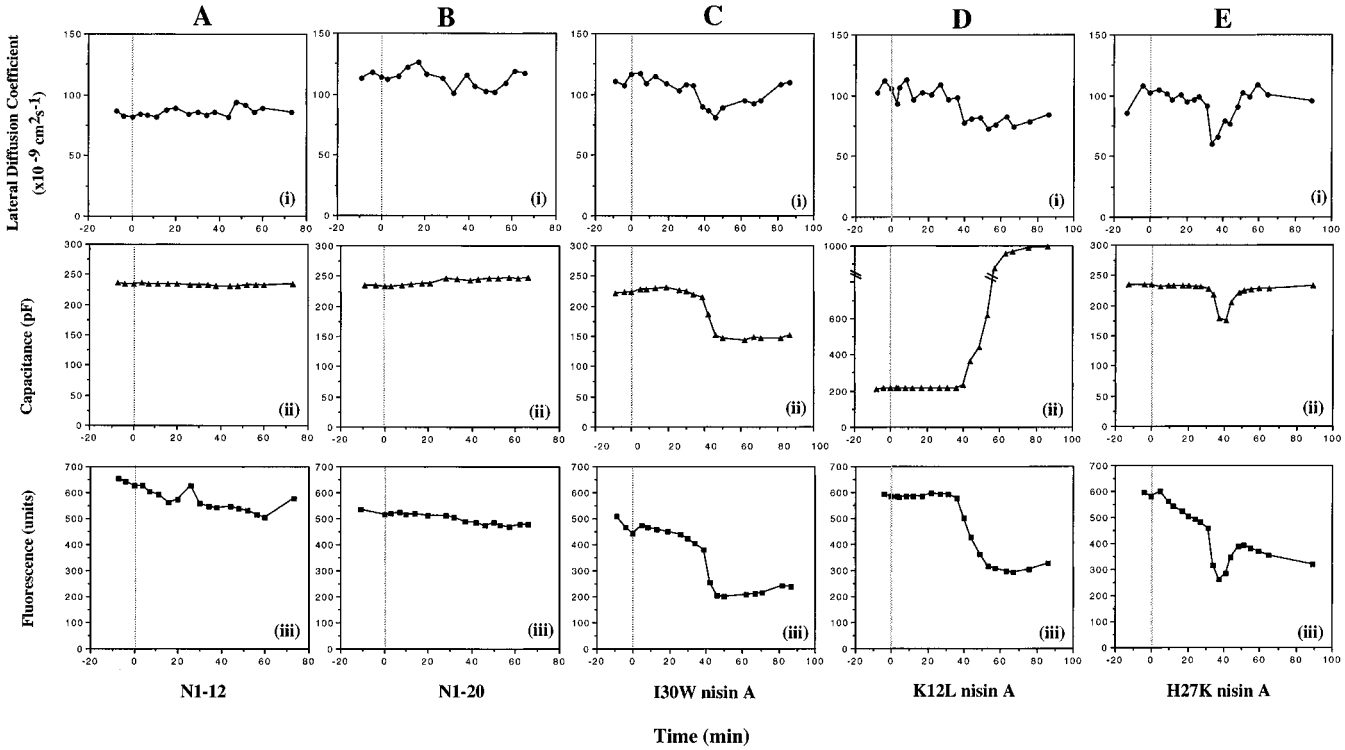


FIGURE 1: Time-dependent effect of the addition of 10 μM nisin fragments or variants on FRAP parameters and capacitance. (A) N1−12, (B) N1−20, (C) I30W nisin A. (D) K12L nisin A or (E) H27K nisin A were added to a 4:1 DOPC:CL (molar ratio) bilayer containing NBD-PE at a 0.01 molar ratio, formed in the presence of unbuffered 1 M KCl, pH 5.2. Bilayer areas were in the range 280−350 μm^2. Lateral diffusion and fluorescence values were derived from an average of 10 FRAP curves, errors were below 5%. Capacitance was measured immediately after each set of FRAP data were collected. Each variant data set is representative of three determinations. The grey line marks time 0 at which peptide was added.

was of the order of 2 s, and therefore the measured $I-V$ relationship approximated its steady state.

Fluorescence Recovery after Photobleaching (FRAP). FRAP was used to measure the lateral diffusion of the fluorescent probe NBD-PE in fPLB as described by Ladha et al. (1994, 1996) and Giffard et al. (1996). A conventional FRAP setup based on an upright Nikon Optiphor microscope (Nikon, Surrey, U.K.) was modified to enable measurements of phospholipid mobility in PLB. On formation of a bilayer with an acceptable capacitance, a laser spot [diameter ($1/e^2$) 3.3 μm] was focused on the center of the bilayer. The spot size was small enough for the bilayer to act as a "near infinite" reservoir. At least five FRAP measurements were recorded prior to the addition of nisin. Variant and fragment nisins were added to a final concentration of 10 μM. Measurements were then taken every 5−10 min for the duration of the experiment. FRAP data were analyzed using nonlinear least-squares fitting to an expression which defines the time dependence of fluorescence recovery observed with a circular beam of Gaussian cross-sectional intensity (Yguerabide et al., 1982).

Apparent Membrane Thickness Estimation. The apparent membrane thickness was estimated from membrane capacitance as described in Giffard et al. (1996).

RESULTS

Effect of Variants on fPLB As Monitored by FRAP. The interaction of nisin fragments and variants with fPLB monitored by FRAP is presented in Figure 1. Each data set is representative of three separate measurements of events recorded on the addition of each nisin fragment or variant to a 4:1 DOPC:CL bilayer. For the nisin variants, the lateral diffusion coefficient, capacitance, and fluorescence tended to decrease with time, but this was not so for the fragments. An average of the principal effect on each recorded membrane parameter is provided in Table 1 as a percentage of its initial value prior to addition of nisin. Controls in the absence of nisin revealed no consistent or significant change in the lateral diffusion coefficient, capacitance, or fluorescence for periods in excess of 80 min.

Table 1: Time-Dependent Effect of 10 μM Nisin Fragments and Variants on a 4:1 DOPC:CL (Molar Ratio) Bilayer Containing NBD-PE at a 0.01 Molar Ratio Monitored by FRAP

	% control level[a]			
nisin variant	lateral diffusion coefficient	capacitance	fluorescence	app increase in membrane thickness (nm)
N1−12	99 ± 2[b]	98 ± 2	92 ± 0	0.1 ± 0
N1−20	93 ± 2	101 ± 1	91 ± 2	
I30W nisin A	68 ± 1	71 ± 4	58 ± 4	6.6 ± 1.6
K12L nisin A	69 ± 3	leak	49 ± 3	
H27K nisin A	59 ± 3	84 ± 2	74 ± 4	3.7 ± 0.8

[a] Data have been normalized to values at $T = 0$. Typical absolute values for controls were of the order of 100×10^{-9} cm^2 s^{-1} for the diffusion coefficient, 220 pF for capacitance, and 600 units for fluorescence. [b] The values shown are maximal effects recorded between 35 and 60 min after addition of nisin, and are given as the mean ± SE for between 3 and 4 PLB.

(i) Effect of N1−12 and N1−20 on Bilayer Physical Parameters. A comparison of the effects of fragments N1−12 and N1−20 is presented in Figure 1A,B and also in Table 1. Minimal effects on bilayer parameters were observed in response to either fragment.

(ii) Effect of Site-Directed Variants on Bilayer Physical Parameters. The interactions of site-directed variants on

fPLB are shown in Figure 1C,D,E (see also Table 1), and the major effect on lateral diffusion, capacitance, and fluorescence displays corresponding time dependence. The effects of I30W nisin A on membrane parameters (Figure 1C, Table 1) were comparable with those of native nisin (Giffard et al., 1996), with the exception of a delay in capacitance recovery. Hence, I30W nisin A has been taken as representative of native nisin interactions. The lateral diffusion coefficient reduced to 68% of control levels in the presence of I30W nisin A [Figure 1C(i)] with hints of a biphasic reduction in the lateral diffusion coefficient. Capacitance decreased to 71% of the control level [Figure 1C-(ii)]. The concurrent fluorescence decrease to 58% of control values remained low though the slight subsequent increase in values detected suggests that fluorescence may increase again [Figure 1C(iii)].

The effects of K12L nisin A on bilayer physical parameters differed greatly from those of I30W nisin A (Figure 1D, Table 1). The lateral diffusion coefficient fluctuated to a greater degree than has been observed with other peptides [Figure 1D(i)]. The overall decrease was to 69% of control levels though no steep decline in the lateral diffusion coefficient was observed. The effect of K12L nisin A on bilayer capacitance was vastly different from that of the other variants [Figure 1D(ii)]. A very large increase was observed that started coordinately with the effect on NBD-PE fluorescence. An increase in capacitance on this scale is indicative of a leak (i.e., a possible partial destabilization of the membrane). The reduction of NBD-PE fluorescence to 49% of its initial level appeared to be maintained for an extended time in comparison with I30W nisin A.

H27K nisin A displayed a decrease in lateral diffusion coefficient slightly greater than I30W nisin A [Figure 1E(i), Table 1]. A small initial decrease was followed by a second reduction in the lateral diffusion coefficient to 59% of control values which was observed at around 30 min, earlier than the equivalent reduction with I30W nisin A. Bilayer capacitance was less affected, with a reduction to 84% of the control level occurring as the decrease in lateral diffusion reached its maximum effect [Figure 1E(ii)]. Capacitance levels returned quickly to original values. A sharp decrease in fluorescence to 74% of initial control values transpired at the same time as the impact on capacitance [Figure 1E(iii)]. Fluorescence values rose after their sharp fall, but did not recover completely.

Effect of Fragments and Variants on the Macroscopic Conductance of fPLB. Nisin variants and fragments were added to a fPLB composed of 4:1 DOPC:CL across which a voltage ramp was imposed from 0 to 140 mV in positive and negative directions. Representative macroscopic $I-V$ relationships (from at least three individual experiments) are shown in Figure 2 for each nisin variant and fragment.

Addition of N1−12 led to reduced conductance in comparison with I30W nisin A that appeared to be polarity-independent (note the difference in ordinate scaling in Figure 2A). Thus, a short N-terminal stretch of amino acids was still capable of inducing membrane conductance in a voltage-dependent manner, although to a considerably lower degree and at high clamping voltages. The influence of N1−20 was greater, inducing conductance to I30W nisin A levels at high *cis*-positive potentials (Figure 2B). This $I-V$ relationship retained slight hysteresis.

I30W nisin A induced a conductance pattern that resembled the $I-V$ relationship of native nisin (Giffard et al., 1996), exhibiting a rectifying current with large hysteresis at *cis*-negative potentials (Figure 2C). These conducting units appeared slow to respond to the change to a positive-going voltage. At *cis*-positive potentials, there is possibly a region of apparent negative conductance. The effects of the other nisin variants were more surprising. K12L nisin A induced the greatest difference from I30W nisin A in macroscopic conductance (Figure 2D). Vast current flow was detected in both positive and negative sections of the voltage ramp that reached saturation levels above ±50 mV. The influence of H27K nisin A (Figure 2E) was also substantially different to the response of I30W nisin A. An almost symmetrical $I-V$ relationship was observed with slight hysteresis at both polarities. Current only reached I30W nisin A levels at high clamping potentials (> ±130 mV).

Effect of Variants on the Single Channel Conductance in fPLB. The lack of reproducibility observed with native nisin at the level of individual conductance events precluded a thorough investigation of single channel behavior with nisin variants. However, while recording the membrane conductance evoked by 10 μM I30W nisin A, single conductance events were observed, and recordings were attempted with each variant and fragment. Sample current traces are displayed in Figure 3 (variant K12L nisin A is not shown as current levels saturated at well below −100 mV). Traces from native nisin, I30W nisin A, H27K nisin A, N1−12, and N1−20 are shown at different amplification levels. I30W nisin A current traces appeared similar to native nisin when scaling is taken into account, which correlates with the similarities in macroscopic $I-V$ relationships of these two molecules. The burst-like trace of H27K nisin A suggests the formation of an unstable membrane-conducting conformation. The small openings of N1−12 were similarly spikey, and openings in the N1−20 current trace were even more difficult to distinguish.

Variant- and Fragment-Induced Liposome Permeabilization. Nisin variants and fragments evoked permeabilization of liposomal membranes. This has been monitored as an increase in fluorescence due to leakage from vesicles of calcein entrapped at self-quenching levels, and is presented in Figure 4 as a percentage of the total fluorescence recovery on addition of Triton X-100. The calcein leakage induced by nisin variants and fragments varied considerably with different phospholipid compositions.

(i) Effect of Nisin Fragments on Liposomal Membranes. Strikingly, but not surprisingly in the context of $I-V$ relationships, the ability of nisin fragments to permeabilize liposomal membranes was low in comparison with native nisin, and this was regardless of phospholipid composition (Figure 4). The release of calcein was analogous for each fragment with all lipid compositions except DOPC. The fluorescence recovery induced by both fragments appeared to be reduced by an increasing percentage of PS such that no release was observed from 100% PS vesicles. Similarly, the incorporation of CL reduced the effect of both fragments in comparison with native nisin-induced leakage.

(ii) Effect of Site-Directed Variants on Liposomal Membranes. The effect of site-directed variants on the permeability of liposomal suspensions was more substantial than that of the nisin fragments (Figure 4). The percentage of

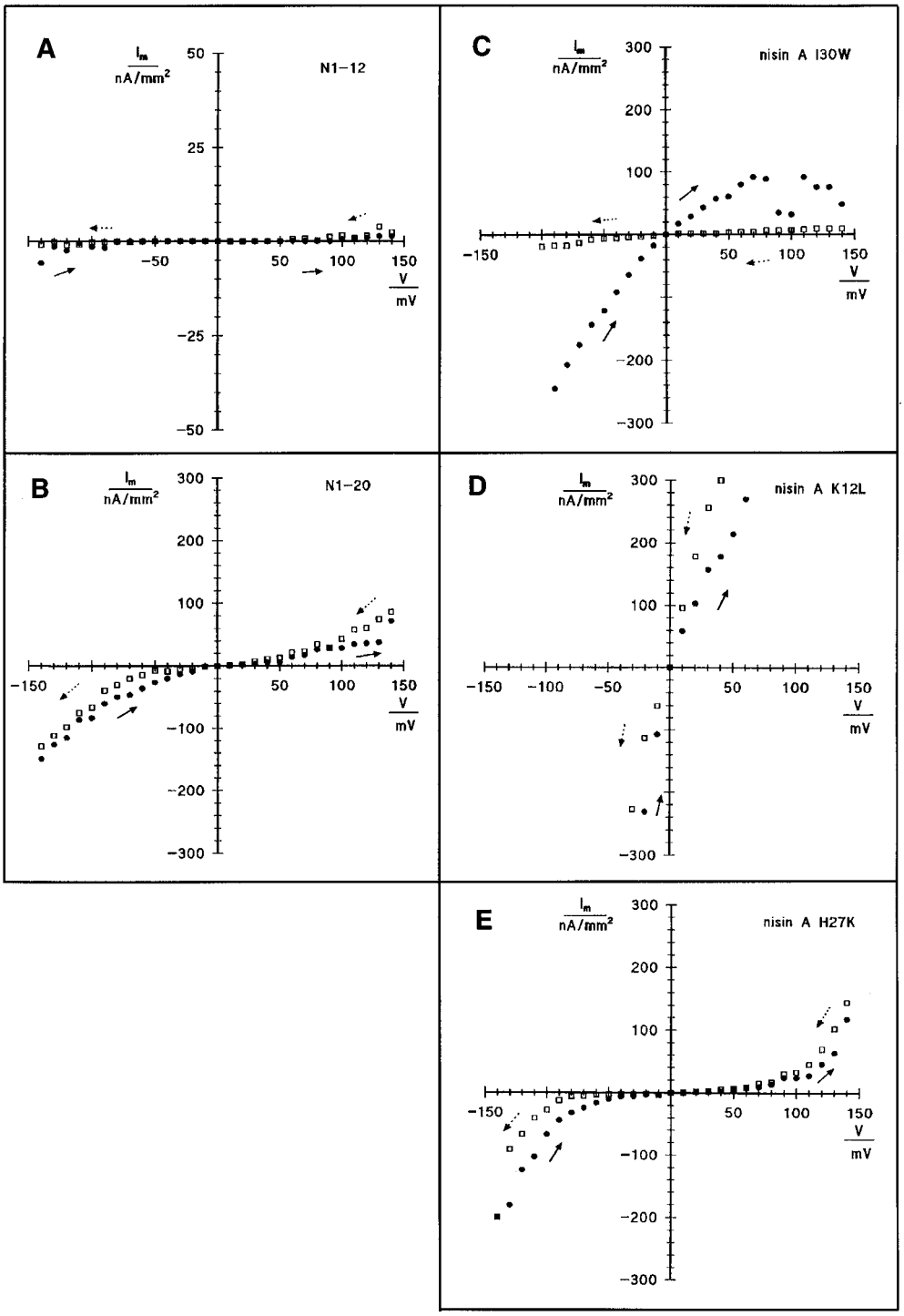


FIGURE 2: $I-V$ relationships of nisin fragments and variants in 4:1 DOPC:CL fPLB. 10 μM variant or fragment was added to a bilayer formed from 4:1 DOPC:CL (molar ratio) in symmetrical 1 M KCl, pH 5.6. A voltage ramp of $\pm$140 mV was applied, and the membrane current passed was recorded. A representative $I-V$ relationship for each variant and fragment is shown. (Note the change in ordinate scale in panel B)

calcein released varied with each variant, and with modified phospholipid charge ratios, but remained below that of native nisin (with the exception of K12L nisin A). Sufficient I30W nisin A was available only to compare the effect of increasing the phospholipid charge:mole ratio on variant activity (Figure 4). Although I30W nisin A-induced calcein release was less efficacious than native nisin in the presence of CL, considerable activity was retained. The variant K12L nisin A showed its strongest reduction in activity compared to native nisin in the presence of CL. The introduction of PS failed to induce K12L nisin A activity to native nisin levels; nevertheless, the release of calcein was still comparable. In fact, K12L nisin A was the only variant to show calcein leakage that increased over and above that observed with native nisin (Figure 4). This enhanced disruption was greatest with DOPC liposomes, but was also observed with 1:1 DOPC:POPE vesicles. The effect of the variant H27K nisin A was most comparable to native nisin with liposomes composed of DOPC (Figure 4). The introduction of anionic phospholipid hindered the activity of H27K nisin A in relation to

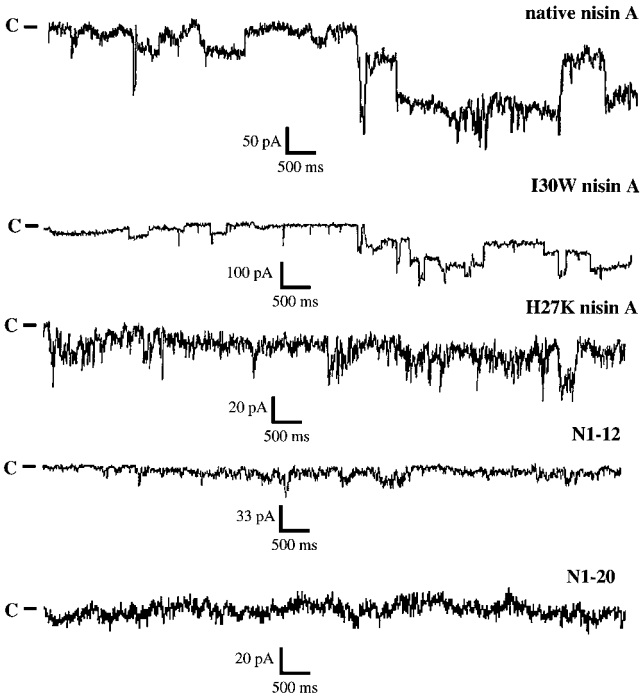


FIGURE 3: fPLB current traces on addition of 10 μM native nisin, fragment or variant. 10 μM of each variant was added to a fPLB composed of 4:1 DOPC:CL (molar ratio) in 1 M KCl, pH 5.6. The membrane voltage was clamped at −100 mV. K12L nisin A did not produce identifiable activity. Each experiment was recorded at a different gain, hence the difference in scale presented here.

native nisin. However, when CL supplied the negative charge at a 1:1 molar ratio with DOPC, the release of calcein induced by H27K nisin A increased.

DISCUSSION

The molecular mechanisms of the primary mode of action of nisin have been investigated with variants and fragments of nisin using artificial membrane systems. A measure of *in vivo* activity can be estimated from the minimum inhibitory concentrations (MICs) of the nisin fragments and variants. The high MICs of N1−12 and N1−20 [several thousandfold greater than native nisin (Chan et al., 1996; Giffard, 1996)] reflect the reduction in membrane interactions observed here. However, the similarities between MICs determined for native nisin and the variants used in this study (Dodd et al., 1996b) belie the differences that have become apparent in the interaction of each variant with artificial membranes. These differences have been observed at each stage of membrane interaction, ie. association, insertion, and permeabilization, and here an attempt has been made to try to clarify this in terms of peptide function.

Membrane Association. It has been demonstrated that in the presence of native nisin there is an initial decrease in the lateral diffusion coefficient and fluorescence (Giffard et al. 1996). These features have been proposed to signify an association of nisin with phospholipid at the surface of a fPLB and were not obvious with either of the nisin fragments N1−12 and N1−20, or with K12L nisin A. These molecules have altered charge distributions, suggesting that removal of positive charge from the N- or C-terminal region of nisin affected the initial peptide−membrane interaction. The conservative exchange of substituting a nonpolar isoleucine with a nonpolar tryptophan in the C-terminal region of nisin (I30W nisin A) did not appear to modify membrane surface associations. Similarly, the initial interaction of H27K nisin A with the bilayer surface resembled that of native nisin. The proposed electrostatic interactions between the cationic charges of nisin and anionic CL (as used in these artificial bilayers) thus appear to have importance in this initial step toward membrane disruption.

Membrane Insertion. A second phase was observed in the interaction of native nisin with fPLB which resulted in a concomitant decrease in the lateral diffusion coefficient, fluorescence, and capacitance (Giffard et al. 1996). This is thought to signify further association of nisin with the membrane, leading to penetration of the hydrophobic core. In equivalent experiments involving nisin variants, it was found that the introduction of a C-terminal tryptophan (I30W nisin A) appeared to prolong peptide insertion into the membrane despite the limited difference in initial interaction. Possibly the structural impact imposed on the membrane by I30W nisin A is not easily relieved. The introduction of a tryptophan at position 1 in nisin Z has been shown to reduce peptide insertion into monolayers composed of DOPG, implicating penetration of the N-terminal part of nisin Z into the lipid phase. However, at position 17, tryptophan had less of an effect on monolayer penetration (Demel et al., 1996). Quenching experiments with aqueous and membrane-restricted quenchers (iodide and spin-labeled lipids, respectively) established that the C-terminally located tryptophan in I30W nisin A was positioned around 0.4 nm from the center of liposomal bilayers containing CL (Martin et al., 1996). This implied that the C-terminal domain of nisin inserts into the hydrophobic core. Flexibility between the N- and C-terminal domains may be a prerequisite for peptide insertion into the hydrophobic core. Deletion of both the asparagine and methionine residues at positions 20 and 21 in nisin A reduced antimicrobial activity to low levels and decreased the penetration of monolayers (Demel et al., 1996) and may be a reason for the lack of biological activity detected with similar variant ΔM21 nisin A (Giffard, 1996).

The removal of the C-terminal domain of nisin had a dramatic effect at this stage in nisin−membrane interactions. Coincidental reductions in FRAP parameters were not observed with N1−12 and N1−20, implying that membrane fluidity was less affected. These fragments of nisin may have insufficient length to slow down phospholipid diffusion, to interact with one another, or to insert into the hydrophobic core. The reduced association with the membrane surface that occurs when the C-terminus is missing may lead to the observed decrease in membrane penetration. It is also speculated that an inability of nisin fragments to form oligomers, or aggregates of sufficient dimensions, may hinder penetration of the lipid bilayer.

The exchange of a histidine for a lysine in the C-terminal domain led to an earlier insertion that appeared to be of a more transient nature than that of native nisin, but the greatest effect on the mechanism of membrane interaction was observed with the replacement of lysine with leucine in the N-terminal domain. The initial interaction of K12L nisin A with fPLB bilayers was followed by a change in capacitance and fluorescence that was rapid and substantial in comparison with that observed for native nisin. This dramatic increase in capacitance implies that the barrier to ion flow provided by the membrane could be more easily breached by insertion of this variant.

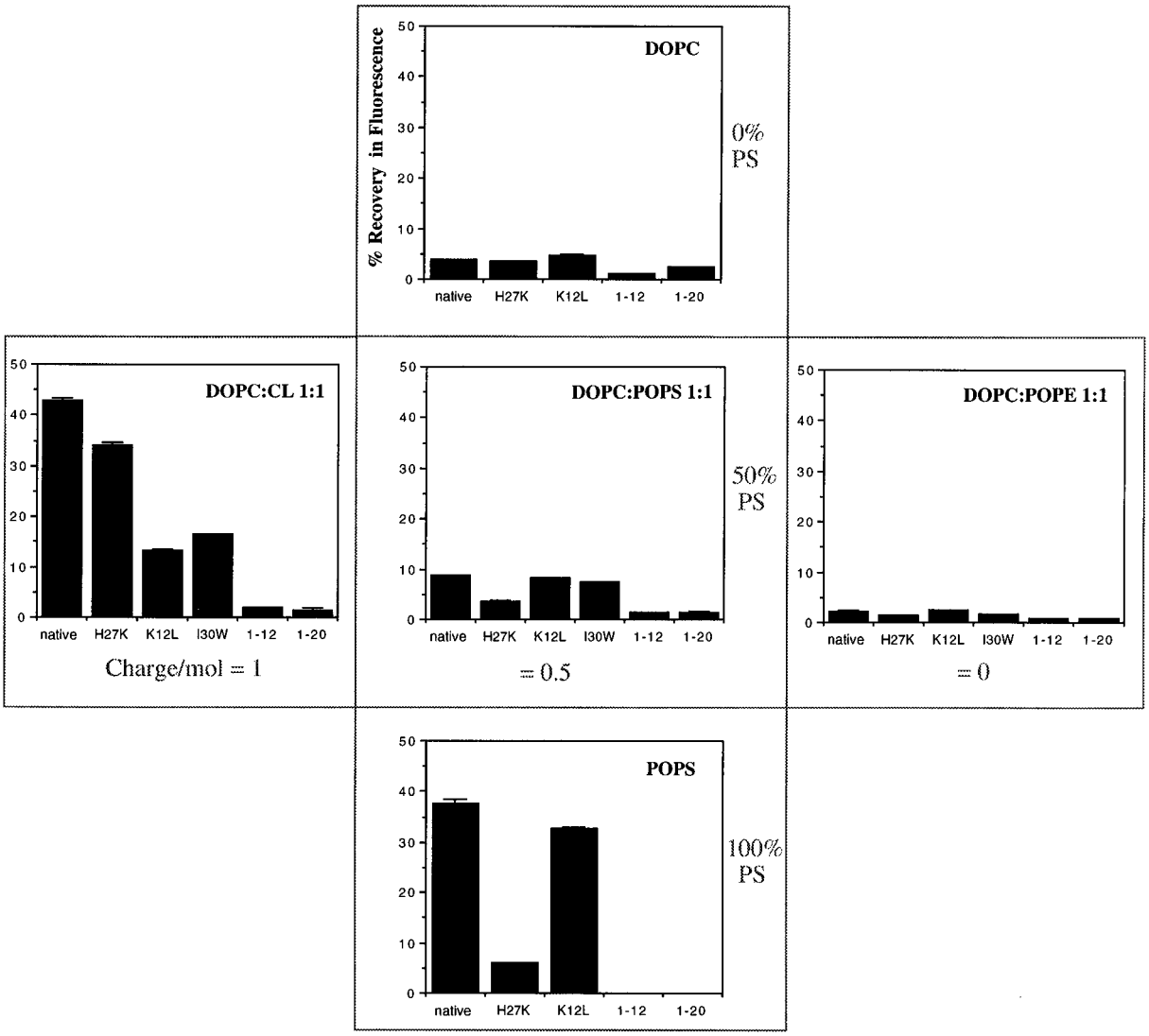


FIGURE 4: Calcein release from liposomes of varying phospholipid compositions on addition of native nisin, fragment or variant. Fluorescence recovery from liposomes was monitored on addition of 4.5 μM peptide in relation to the total fluorescence released by 0.3% (w/v) Triton X-100. The lipid concentration was 35 μg/mL in 150 mM NaCl, 0.01 mM EDTA, and 10 mM MES at pH 6.0. Osmolarity was maintained at 290 mOsM. Standard errors have been added for each set of conditions.

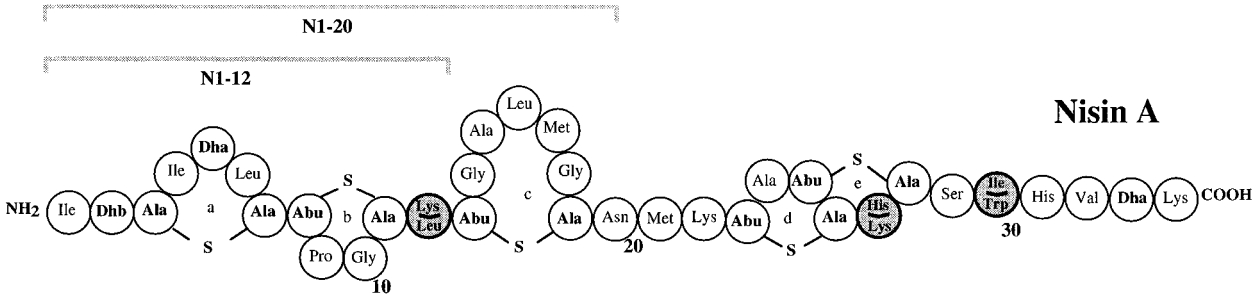
Membrane Permeabilization. The variation in the permeabilizing effects of fPLB by variants and fragments was exposed by observing their $I-V$ relationships. Native nisin has been shown to exhibit rectifying properties in planar bilayers, and responds to voltage ramps in a hysteretic $I-V$ relationship (Giffard et al., 1996). Fragments of nisin were less capable of effective permeabilization of fPLB, and removal of the C-terminal domain reduced the dependence of conductance on polarity and diminished rectification. The extremely low current induced by N1−12 in comparison with N1−20 suggests that the presence of ring C (Figure 5) may enhance the diameter of a conducting structure in the membrane. Liposomal permeabilization by nisin fragments was similarly reduced in comparison with native nisin and appeared to be unaffected by lipid head group charge.

The removal of a positive charge from the N-terminal domain in variant K12L nisin A also reduced polarity dependence but led to an immense increase in conductance. This is suggestive of a leak effect, implying that the lysine at position 12 has an important role in controlling the flow of ions through bilayers doped with nisin. In this scenario, lysine-12 could have a role in gating (i.e., regulating) the flow of charge through a nisin pore, and its exchange for a leucine appears to remove any restriction previously imposed on the flow of ions across the membrane. An enhanced release of calcein by K12L nisin A from liposomes with little or no anionic lipid, in comparison to native nisin, is consistent with the increase in macroscopic bilayer conductance for this variant.

The loss of polarity dependence by the replacement of a histidine with lysine at position 27 was surprising. Bilayer measurements were carried out in solution at pH 5.6, which is below the histidine pK_a of 6.5 in free solution. However, the pK_a of histidine in a membrane microenvironment may be vastly removed from that in free solution, and hence the change in charge distribution of H27K nisin A within the membrane environment remains unclear. If charge is unaffected, the variation in conductance may have been mediated by a conformational change in the C-terminal region.

Liposomal permeabilization induced by I30W nisin A and H27K nisin A was enhanced by the presence of CL which hindered K12L nisin A activity. The packing structure of lipids with various head groups will vary between liposomes and fPLB and hence affect the peptide-induced modulation of lipid chemistry and dynamics (Beschiaschvili & Seelig, 1990). Thus, variation in fragment and variant activities

a)



b)

	N1-12	N1-20	K12L	H27K	I30W
Association	- - - -	- - - -	-	0	0
Insertion	- - - -	- - - -	++++	-	0
Permeabilisation	- - - -	- -	++++	-	0

FIGURE 5: Interaction of nisin fragments and variants used in this study with model membrane systems. (a) Structure of the nisin molecule showing regions present in the nisin fragments (lines labeled N1−12 and N1−20) and location of amino acid substitutions in the engineered nisin variants (shaded circles). (b) Summary of the altered membrane interactions due to peptide modification. Values have been assigned relative to that of native nisin: 0 = similar effect to native nisin; + = enhanced effect; − = reduced effect. The unusual residues dehydroalanine, dehydrobutyrine, and α-aminobutyric acid are represented as Dha, Dhb, and Abu, respectively.

observed with differing lipid types and molar charge ratios suggests that prediction of structural variant behavior will be difficult without further investigations and comparisons.

The only other study to investigate the effect of charge on lantibiotic−bilayer interaction involved Pep5 (Kordel *et al.*, 1988). Succinylation of lysine residues was used to introduce an overall negative charge to the peptide. In this case permeabilization of artificial membranes was not inhibited, despite virtual inactivity against intact cells. However, a large increase in the applied *cis*-positive transmembrane potential was required to induce peptide-mediated conductance. Thus, alterations in peptide charge can be detected in artificial systems that are more difficult to observe *in vivo*.

Proposed Model. A model for the interaction of nisin with membranes is proposed on the basis of the present data in which nisin initially interacts with the surface of the membrane hindering the diffusion of phospholipid. This association is affected by peptide charge and size, suggesting the involvement of electrostatic interactions between nisin and phospholipid head groups with probable disturbance of membrane structure. Nisin molecules may form aggregates on the surface prior to, and possibly in parallel with, partial insertion into the hydrophobic core of the bilayer. At this stage, the interaction of the small fragments of nisin would be hindered. However, assembly of the structurally altered variants would create greater, varied disruption of phospholipid organization. This may lead to the formation of structures within the bilayer capable of inducing conductance in response to transmembrane potentials, which is largely consistent the barrel stave model proposed as an explanation for the activity of nisin (Sahl, 1991). An amalgamation of amphipathic monomers with hydrophilic faces forming the center of a barrel-like pore would create an aqueous channel through which ion flow could be regulated. The enhanced membrane conductance observed with K12L nisin A suggests that lysine located in the N-terminal domain may be involved in this role. Fluctuations in membrane conductance could be due to further aggregate insertion and also the concomitant random disruption of membrane integrity proposed by Driessen et al. (1995).

CONCLUSIONS

Significant insights into the nisin−membrane interaction can be obtained through the combined measurement of the effects of nisin on PLB conductivity and capacitance. The variant and fragment−membrane interactions in comparison with those of native nisin have been summarized in Figure 5 and, though consistent with the different stages of interaction observed with native nisin, have revealed differences that become apparent at each stage of nisin−membrane interaction. The use of artificial model systems has enabled the variations in antagonistic effects to be scrutinized to a greater depth than has been possible with intact cells as they are uncomplicated by bacterial homeostatic systems or other modifying proteins. This reductionist approach may be applicable to the study of other bacteriocins leading to the design of improved food-grade antimicrobials. The introduction of a series of alternative amino acids at one position could generate further useful information, and comparison of the effects of such a series of mutations might reduce speculation and enable better assessment of each structural effect. Further study of nisin−membrane interactions should encompass a comparison of lipid structure and dynamics, as well as membrane permeability.

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