Mode of Action of the Peptide Antibiotic Nisin and Influence on the Membrane Potential of Whole Cells and on Cytoplasmic and Artificial Membrane Vesicles

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The peptide antibiotic nisin was shown to cause a rapid efflux of amino acids and Rb⁺ from the cytoplasm of gram-positive bacteria (Staphylococcus cohnii 22, Bacillus subtilis W 23, Micrococcus luteus ATCC 4698, and Streptococcus zymogenes 24). It strongly decreased the membrane potential of cells as judged by the distribution of the lipophilic tetraphenylphosphonium cation. Ascorbate-phenazine methosulfate-driven transport of L-proline by cytoplasmic membrane vesicles was blocked after addition of nisin, and accumulated amino acids were released from the vesicles. Soybean phospholipid (asolecin) vesicles were not affected by nisin. The data suggest that the cytoplasmic membrane is the primary target and that membrane disruption accounts for the bactericidal action of nisin.

Nisin is a polypeptide antibiotic produced by different strains of Streptococcus lactis belonging to Lancefield group N (11). It consists of five closely related substances, nisins A through E, which display different antibiotic potencies against gram-positive bacteria (1). Nisin is used mainly in the food and dairy industries (8). Despite its widespread use, its mode of action is not well understood. Reisinger et al. (14) described an effect on murein synthesis. This effect is brought about by complex formation of nisin with the undecaprenyl pyrophosphate-activated intermediates of murein synthesis, resulting in the inhibition of the synthesis of this polymer. In an early paper, Rameiser (13) observed membrane leakiness of nisin-treated clostridia and discussed an effect similar to that of cationic detergents. The structure of nisin was elucidated by Gross and Morell (5), who showed that it contains inner-molecular-ring structures built by the thioether amino acid lanthionine.

The staphylococcin-like peptide Pep-5 is produced by Staphylococcus epidermidis 5 and has been shown to cause the efflux of low-M₉ cytoplasmic compounds (16) and to rapidly abolish the membrane potential of susceptible cells (H.-G. Sahl, J. Bacteriol., in press). The detection of lanthionine in hydrolysates of the staphylococcin-like peptide Pep-5 (17), along with further structural similarities (M, 3,500; isoelectric point, 10.5), suggested a similar mode of action for both.

In this paper we report that addition of nisin to susceptible cells leads to the efflux of accumulated, radioactively labeled markers such as Rb⁺ or amino acids out of the cytoplasm of whole cells and the immediate collapse of the membrane potential. Furthermore, the influence of nisin on cytoplasmic membrane vesicles and artificial phospholipid vesicles was investigated.

MATERIALS AND METHODS

Bacterial strains. Staphylococcus cohnii 22 and Streptococcus zymogenes 24 were isolated in the Institute for Medical Microbiology and Immunology of the University of Bonn, Bonn-Venusberg, Federal Republic of Germany. Bacillus subtilis W 23 was obtained from W. Konings, Groningen, The Netherlands. Micrococcus luteus ATCC 4698 was purchased from the American Type Culture Collection, Rockville, Md.

Growth media. All strains were subcultured weekly on blood agar plates. For the efflux experiments CPY medium containing 0.5% casein hydrolysate (Oxoid Ltd., London, England), 0.5% Bacto-Peptone (Difco Laboratories, Detroit, Mich.), 0.2% yeast extract (Difco), and 5 mM glucose (E. Merck AG, Darmstadt, West Germany) was used. For preparation of membrane vesicles, strains were cultured in tryptone soya broth (Oxoid).

Chemicals. All radiochemicals were obtained from the Radiochemical Center Amersham-Buchler, Brunswick, Federal Republic of Germany. Chloramphenicol, valinomycin, carbonyl cyanide m-chlorophenylhydrazone (CCCP), RNase, DNase, lysozyme, and L-a-phosphatidylycholine were purchased from Sigma, Munich, Federal Republic of Germany; Sephadex G-25 was purchased from Pharmacia, Freiburg, Federal Republic of Germany; Unisolve I was purchased from Zinsser, Frankfurt, Federal Republic of Germany; and nisin was purchased from Koch-Light, Colnbrook, England.

Efflux of radioactively labeled compounds. Strains were cultured in CPY medium at 37°C to an absorbance of ca. 1 at 600 nm. Centrifuged cells were resuspended 1:2.5 in fresh medium containing 0.2% Bacto-Peptone, 0.8% glucose, 0.4% yeast extract, 2 mM potassium phosphate (pH 7), and 100 μg of chloramphenicol per ml. After 10 min of preincubation, radioactively labeled compounds (l-[³H]glutamate, 39 Ci mmol⁻¹; l-[¹⁴C]lysine, 334 mCi mmol⁻¹) were added (final concentration, 1 μCi ml⁻¹), and the culture was immediately divided into two parts; one was treated with nisin (final concentration, 10 μg ml⁻¹) and the other was run as a control. After 30 min of incubation, the untreated control was further subdivided; one part received nisin (10 μg ml⁻¹) so that we could follow its effect on pre-accumulated amino acids. Samples were filtered through cellulose nitrate filters (pore size, 0.45 μm; Millipore Corp., Bedford, Mass.) and washed twice with 5 ml of 200 mM potassium phosphate buffer (pH 7) containing 100 μM unlabeled glutamate or

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lysine. Filters were dried and transferred to counting vials filled with scintillation fluid (Unisolve I). The radioactivity was measured with a liquid scintillation counter. For efflux experiments with $^{86}$Rb$^+$, the same basic procedure was carried out with some modifications. The CPM medium contained 10 mM sodium phosphate buffer (pH 7) and 10 µM potassium phosphate. $^{86}$Rb$^+$ (0.5 µCi ml$^{-1}$; 1.6 µCi mg$^{-1}$) was added to the main culture. Centrifuged cells were resuspended in 10 mM sodium phosphate buffer (pH 7). The washing buffer contained 100 mM sodium phosphate buffer (pH 7) and 200 mM potassium chloride.

Efflux experiments with membrane vesicles were performed at 25°C in a final volume of 1 ml. Membrane vesicles were diluted 1:50 in 50 mM potassium phosphate buffer (pH 7) containing 5 mM MgSO$_4$. L-$^{14}$Cproline (339 mCi mmol$^{-1}$) was added (final concentration, 1 µCi ml$^{-1}$), and the suspension was preincubated for 4 min.

The active transport reaction was started by the addition of the artificial electron donor system ascorbate-phenazine methosulfate (Asc/PMS) (20 mM and 100 µM, respectively). After 10 min, nisin was added (10 µg ml$^{-1}$). Aliquots (80 µl) were quickly filtered through cellulose nitrate filters (pore size, 0.22 µm; Millipore) and washed once with 5 ml of 100 mM lithium chloride (pH 7) containing 5 mM unlabeled proline. The filters were dried and counted as described above. Membrane vesicles of _M. luteus_ transported amino acids only after brief sonication. This phenomenon was not further investigated.

**Preparation of membrane vesicles.** For vesicle preparation _Staphylococcus cohnii_ 22, _B. subtilis_ W 23, and _M. luteus_ ATCC 4698 were grown at 37°C with vigorous aeration in tryptone soya broth (Oxoid) to an absorbance of ca. 1 at 600 nm. At 30 min before the cells were harvested by centrifugation, chloramphenicol (100 µg ml$^{-1}$) was added. Membrane vesicles were prepared essentially as described by Bisschop and Konings (2), with the following modifications. Cells were suspended in 50 mM potassium phosphate buffer (pH 7) containing 5 mM MgSO$_4$ at 30°C at a concentration of 4 g (wet weight) per liter. Lysozyme, DNase, and RNase were added to final concentrations of 100, 2, and 2 µg ml$^{-1}$, respectively. The membrane vesicles were resuspended in 50 mM potassium phosphate buffer (pH 7) containing 5 mM MgSO$_4$ and diluted to a concentration of 2 to 3 mg of membrane protein ml$^{-1}$. Portions (500 µl) in Eppendorf plastic tubes were rapidly frozen and stored in liquid nitrogen. For efflux experiments, membrane vesicles were quickly thawed in a water bath at 40°C and subsequently put on ice. Protein was determined by the method of Lowry et al. (9).

**Preparation of L-α-phosphatidylcholine liposomes.** To prepare "asolecint" vesicles, L-α-phosphatidylcholine from soybeans (type 1V; Sigma) was used without further purification. Asolecin (80 mg) was homogenized in 2 ml of buffer (10 mM Tris-hydrochloride [pH 7], 1 mM CaCl$_2$, 100 mM KCl) by sonication in a bath-type sonicator (Bransonic 12). After the addition of L-$^{14}$Cglutamic acid (39 Ci mmol$^{-1}$; final concentration, 2 µCi ml$^{-1}$), sonication was continued until the suspension became opalescent (10 to 20 min). Liposomes were frozen subsequently in liquid nitrogen and thawed quickly in a water bath at 40°C. The turbid mixture was briefly sonicated to clarity and chromatographed on a Sephadex G-25 column (1 by 12 cm; flow rate, ca. 1 ml min$^{-1}$) with 10 mM Tris-hydrochloride (pH 7), 100 mM KCl, and 1 mM CaCl$_2$ at room temperature to remove untrapped radioactivity. Fractions containing the liposomes were collected and used for experiments in a 100-fold dilution. To impose a K$^+$ diffusion potential of 120 mV (negative inside), 100 mM choline chloride was added to the dilution buffer instead of 100 mM KCl. The potential was then generated by means of 4 µM valinomycin.

**Estimation of the membrane potential.** Cells were grown in 100 ml of tryptone soya broth at 37°C with vigorous aeration to an absorbance of ca. 0.8 to 1 at 600 nm. Cells were centrifuged, washed twice, and suspended in 10 ml of 10 mM potassium phosphate buffer (pH 7)–1 mM MgSO$_4$. The suspension was either kept at 37°C in a shaking water bath or starved to reach immediately. The experiments were performed at 22°C. To monitor the membrane potential, 0.2 µCi of [14C]tetraphenylphosphonium (TPP$^+$; 50.6 mCi mmol$^{-1}$) was added. For tests with energized cells, the energization was achieved by the addition of 20 mM glucose. Nisin was added at a concentration of 10 µg ml$^{-1}$. Samples (100 µl) were filtered on cellulose nitrate filters (pore size, 0.45 µm; Schleicher & Schuell) and washed twice with 5 ml of buffer. Dried filters were counted as described above.

Controls were treated with 50 µM CCCP. Counts were corrected for unspecified binding of TPP$^+$ by subtracting the radioactivity of 10% butanol-treated aliquots. For calculation of the membrane potential ($\Delta\psi$), TPP$^+$ concentrations were applied to the Nernst equation ($\Delta\psi = (2.3 \times R \times T/F) \times \log (TPP^+_{in}/TPP^+_{out})$). The internal volume of bacterial cells was determined by means of the silicon oil centrifugation technique described by Harris and van Dam (6), using

![FIG. 1. Accumulation of L-$^{14}$Cglutamic acid and L-$^{14}$Clysine into chloramphenicol-treated cells (○) and efflux of the radioactive amino acids after nisin addition (10 µg ml$^{-1}$) (C); (●) uptake of the radioactive markers by nisin pretreated cells. Aliquots (1 ml) of the culture were filtered over cellulose nitrate filters and washed twice with 5 ml of buffer, and the filters were counted for radioactivity.](http://aac.asm.org/)
RESULTS

Influence of nisin on amino acid and \(^{86}\text{Rb}^+\) accumulation in whole cells. To demonstrate the influence of nisin on active transport, we examined the uptake of different radioactively labeled amino acids into several randomly selected chloramphenicol-treated gram-positive bacteria. Cells pretreated with nisin were unable to carry out active transport of amino acids, in contrast to the untreated control. The addition of nisin to cells which had accumulated the amino acid led to a rapid efflux within less than 2 min (Fig. 1). Similar effects were obtained for the cation \(^{86}\text{Rb}^+\), which was tested as a K\(^+\) analog. In this case the efflux was completed within 1 min after nisin addition (Fig. 2). The facts that similar results were obtained for all strains and that the efflux velocity for small ions such as Rb\(^+\) is as rapid as for larger positively and negatively charged amino acids indicate that there is no selectivity for charge and size of the efflux mediated by nisin. This is in contrast to channel-forming colicins (7) and suggests that a more generalized membrane perturbation is brought about by nisin, rather than a channel with finite size.

Effect on the membrane potential of whole cells. The efflux of ions like K\(^+\) should result in a dramatic effect on the membrane potential of bacteria treated with nisin. This was tested by monitoring the membrane potential with the lipophilic cation TPP\(^+\) (Fig. 3). It is shown that nisin leads to a rapid decrease of \(\Delta\psi\). Under favorable conditions, i.e., when cells are fully energized, the TPP\(^+\) concentration dropped even under the counts obtained for the butanol-treated controls, yielding a positive value for \(\Delta\psi\). With cells which are de-energized in incubation in buffer at 37°C for 60 min, the effect is less dramatic. This correlates with the observation that stationary-phase cells are less susceptible to the killing action of Pep-5 than are log-phase cells (15) and indicates a need for a membrane potential for nisin action. An analogous situation is reported for channel-forming colicins, which are known to need a membrane potential for insertion into phospholipid bilayers (7). The results are also in accordance with those obtained for Pep-5 (Sahl, in press) and agree with the observation that ATP and K\(^+\) efflux from

FIG. 2. Efflux of \(^{86}\text{Rb}^+\) (○) from Staphylococcus cohnii 22 and B. subtilis W 23 after addition of nisin (10 \(\mu\)g ml\(^{-1}\)) to untreated controls. Aliquots (800 \(\mu\)l) of the culture were filtered over cellulose nitrate filters and washed twice with 5 ml of buffer, and the filters were counted for radioactivity.

FIG. 3. Influence of nisin on the membrane potential of Staphylococcus cohnii 22. (○) Energized cells; (●) starved cells. The experiments were performed at 22°C. The membrane potential was calculated as described in the text.

Pep-5-treated bacteria is severalfold faster from energized than from de-energized cells (16).

Influence of nisin on cytoplasmic membrane vesicles. The results described above strongly implicate the cytoplasmic membrane as the primary target of nisin action. Therefore, cytoplasmic membrane vesicles were prepared from nisin-sensitive gram-positive bacteria. The uptake of L-[\(^{14}\text{C}\)]proline into cytoplasmic membrane vesicles was initiated by energization with Asc/PMS. Nisin inhibited Asc/PMS-driven uptake of L-[\(^{14}\text{C}\)]proline by cytoplasmic membrane vesicles (Fig. 4). The efflux of radioactively labeled substrates from

FIG. 4. Accumulation (●) of L-[\(^{14}\text{C}\)]proline by cytoplasmic membrane vesicles of Staphylococcus cohnii 22, B. subtilis W 23, and W. luteus ATCC 4698 and efflux of the amino acid (○) after addition of nisin (10 \(\mu\)g ml\(^{-1}\)). Membrane vesicles were prepared as described in the text and diluted 1:50 with dilution buffer. After addition of L-[\(^{14}\text{C}\)]proline and incubation for 4 min, the transport of proline was started with Asc/PMS. Aliquots (80 \(\mu\)l) were filtered over cellulose nitrate filters and washed twice with buffer, and filters were counted for radioactivity.

[^3H]H\(_2\)O and [\(^{14}\text{C}\)]ribose. Protein determination of whole cells was performed by the method of Stickland (19).
vesicles can be compared with the efflux obtained with whole cells.

The need for an energized membrane for proper interaction of nisin with the cytoplasmic membrane can also be deduced from Fig. 5, which shows the influence of nisin and CCCP pretreatment of vesicles. In each case the vesicles were incubated with L-[3H]glutamate and nisin or CCCP, respectively, and the experiment was started with energization of the vesicles with Asc/PMS. CCCP-treated vesicles were unable to transport L-glutamate because the protonophore prevents the generation of a potential. In contrast, nisin-treated vesicles accumulated the amino acid on energization until a certain potential was reached. During this short period nisin is obviously not effective. When the potential was high enough for a proper interaction of nisin with the cytoplasmic membrane, a net uptake of proline was no longer detectable and the accumulated amino acid started to efflux from the internal space of the vesicles. However, when vesicles were preincubated with nisin and Asc/PMS and the experiment was started with L-glutamate, no uptake was observed from the beginning (data not shown).

**Effect of nisin on artificial vesicles.** To further characterize the effect of nisin on phospholipid bilayers, its influence on artificial membrane vesicles was studied. Asolectin liposomes were prepared in the presence of L-[14C]glutamic acid and tested for the ability to retain the radioactivity on nisin treatment. No efflux of the trapped labeled amino acid from the liposomes could be observed, whether or not a valinomycin-induced K⁺ diffusion potential was generated (Fig. 6). The radioactive marker could only be liberated by a detergent.

It cannot be decided whether this result reflects the in vivo situation, e.g., the inability of nisin to impair nonbacterial membranes, or is simply due to an inappropriate test system. This point needs further clarification.

**DISCUSSION**

The data presented here strongly suggest that the primary target of nisin activity is the cytoplasmic membrane. This must be concluded from the observation that one of the earliest events after nisin addition is the rapid, nonspecific efflux of different substrates like amino acids and cations from sensitive cells and their membrane vesicles. Furthermore, we could demonstrate an immediate collapse of the membrane potential on addition of nisin to whole cells.

Although we did not examine the effect of nisin on ΔpH separately, we can assume that it is affected similarly by the rapid and nonspecific efflux of low-M̄ compounds. Therefore, nisin-treated cells must be deprived of both components of the proton motive force, and it seems reasonable to argue that this leads to a total cessation of any biosynthesis, including murein synthesis. Cessation of biosynthesis has already been shown for the staphylococcin-like peptide Pep-5 (15). Reisinger et al. (14) described murein synthesis as a target of nisin activity. They showed that nisin inhibited the murein synthesis in *Gaffkyia homari* by forming a complex with the undecaprenyl pyrophospho- N-acetylmuramylpeptide, thus trapping murein precursors in the cytoplasmic membrane. However, the evaluation of our experiments strongly suggests that this has to be regarded as a secondary effect on nisin action. The complete membrane depolarization occurs within 1 min, whereas maximum complex formation and subsequent inhibition of cell wall biosynthesis take longer and were achieved with ca. 10-fold higher concentrations than were used in our studies. Furthermore, the cytoplasmic membrane was proposed as a target of cationic antibiotics as early as 1953 by Newton (12) and in 1960 by Ramseier (13), who demonstrated an increase of the absorbance at 260 nm in supernatants of nisin-treated clostridia. The observation indeed suggested an efflux of low-M̄ compounds as was shown in this study. In general, the results obtained with nisin are strikingly similar to those for the staphylococcin-like peptide Pep-5 (15, 16; Sahl, in press), which is consistent with the structural similarities between these compounds (17). Pep-5 and colicin V (21), which is of similar size, are not able to influence asolectin vesicles either.

The inability of these peptides to influence soybean phospholipid vesicles could point to a need for an integral membrane component which could serve as a mediator for...
nisin binding to membranes and which is lacking in nonbacterial membrane extracts. In this respect, the murein precursors could facilitate nisin interaction with the cytoplasmic membrane resulting in the membrane disintegration demonstrated by our results.

The cytoplasmic membrane as a primary target is also known for a group of colicins (E1, A, Ia, and K) disrupting the membrane potential by forming an ion-permeable channel of definite pore size (7). They have been shown to require a potential for membrane insertion and channel formation (see reference 7 for a review). Our data suggest that nisin also needs a threshold potential for interaction with membranes (Fig. 3 and 5).

The strong basic nature of nisin and Pep-5 and their similar molecular weights (about 3,500) are features which are shared by several other bactericidal peptides; among others, staphylococcal 8-lysin (4), melittin from bee venom (20), basic peptides from saliva (10) and from neutrophil granulocytes (18), and lysozyme from platelets (3). Their structural similarities could point to a general principle underlying their bactericidal action. This is supported by the observation that for some of these peptides an induction of autolysis was reported; for nisin and Pep-5 this was also observed and is elsewhere reported in detail (G. Bierbaum and H.-G. Sahl, in press).

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LITERATURE CITED