Biochemistry

 Volume 41, Number 24 June 18, 2002

Articles

Clavanin Permeabilizes Target Membranes via Two Distinctly Different pH-Dependent Mechanisms†

Ellen J. M. van Kan,*,‡,§ Rudy A. Demel,§ Eefjan Breukink,§ Arie van der Bent,‡ and Ben de Kruijff§

Department Coating Systems and Active Ingredients, Renewable Resources, Agrotechnological Research Institute, Wageningen University and Research Centre, Bornsesteeg 59, 6708 PD Wageningen, The Netherlands, and Department of Biochemistry of Membranes, Centre for Biomembranes and Lipid Enzymology, Institute of Biomembranes, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands

Received December 14, 2001; Revised Manuscript Received March 15, 2002

ABSTRACT: The pH dependence of the antimicrobial and membrane activity of clavanin A, a peptide antibiotic that is rich in histidines and glycines, was analyzed in growth and membrane leakage experiments. Clavanin A more effectively inhibited the growth of the test organism *Lactobacillus sake* when the pH of the medium was lowered. Whereas the wild-type peptide efficiently released fluorophores from unilamellar vesicles at neutral pH according to a nonspecific permeabilization mechanism, it did not permeabilize model bilayers at low pH. It was therefore suggested that this peptide uses a distinct mode of action under acidic conditions different than that used around neutral pH. However, at low pH, the membrane is still the target for clavanin A, as the peptide collapsed both vital transmembrane proton gradients and ion gradients under these conditions. Clavanin A did not act as a ionophore across phospholipid bilayers, indicating that membrane constituents other than membrane phospholipids are involved in the dissipation of transmembrane ion gradients. Membrane proteins that generate transmembrane ion gradients are suggested to be the targets for clavanin A at low pH. In addition to the histidines, the three glycine residues of clavanin A are shown to play an important role in the specific mode of interaction with these membrane targets. These residues may induce a flexible hydrophobic conformation that allows the peptide to exert different membrane activities. This study demonstrates that clavanin A is a special membrane-active peptide that has access to two markedly distinct pH-dependent modes of actions.

Cationic peptide antibiotics have been identified as important components of the innate defense for many organisms. Clavanin A is supposed to belong to the class of linear cationic amphipathic, α-helical peptides (*1*−*5*). This peptide was originally isolated from the hemocytes of the marine invertebrate *Styela clava* by Lehrer and co-workers (*1*, *6*). Clavanin A is C-terminally amidated and contains 23 amino acids. Its sequence, presented in Table 1, is especially rich in histidines, glycines, and hydrophobic residues such as phenylalanines and valines. Many of the naturally occurring peptide antibiotics are thought to exert their antimicrobial activity via permeabilization of the target membrane (*4*, *5*, *7*−*12*). At neutral pH, the antibacterial effect of clavanin A as tested on *M. flavus* was indeed shown to result from

† This study has been carried out with financial support from the Commission of the European Communities, Agriculture and Fisheries (FAIR) specific RTD program, CT97-3135.

* Corresponding author. Phone: +31 317 475328. Fax: +31 317 475347. E-mail: e.j.m.vankan@ato.wag-ur.nl.

‡ Agrotechnological Research Institute.

§ Utrecht University.

10.1021/bi012162t CCC: $22.00
Published on Web 05/18/2002

Table 1: Clavanin A Gly → Ala Mutants and Their Antimicrobial Activities against *L. sake* (Strain ATCC 15521 = DSM 20017 Cultured in MRS Broth Buffered Using 25 mM Potassium Phosphate Buffer at 30 °C) at pH 6.5, 6.0, and 5.6, Respectively

peptide	sequence	MIC value on *L. sake* (μM)[a]		
		pH 6.5	pH 6.0	pH 5.6
GGG (native)	VFQFL**G**KIIHHV**G**NFVH**G**FSHVF−NH_2	7.8 ± 3.4	1.5 ± 0.0	0.7 ± 0.0
AGG	VFQFL**A**KIIHHV**G**NFVH**G**FSHVF−NH_2	27.2 ± 17.8	2.9 ± 0.0	1.5 ± 0.0
GAG	VFQFL**G**KIIHHV**A**NFVH**G**FSHVF−NH_2	7.8 ± 3.4	1.5 ± 0.0	1.0 ± 0.4
GGA	VFQFL**G**KIIHHV**G**NFVH**A**FSHVF−NH_2	2.9 ± 0.0	1.5 ± 0.0	0.7 ± 0.0
AAG	VFQFL**A**KIIHHV**A**NFVH**G**FSHVF−NH_2	>93	2.9 ± 0.0	1.0 ± 0.4
AGA	VFQFL**A**KIIHHV**G**NFVH**A**FSHVF−NH_2	15.5 ± 6.7	2.9 ± 0.0	1.2 ± 0.4
GAA	VFQFL**G**KIIHHV**A**NFVH**A**FSHVF−NH_2	5.8 ± 0.0	1.5 ± 0.0	0.6 ± 0.2
AAA	VFQFL**A**KIIHHV**A**NFVH**A**FSHVF−NH_2	>46	2.9 ± 0.0	1.4 ± 0.0

[a] MIC values represent the average of three independent antimicrobial assays.

membrane permeabilization. Interactions of clavanin A with membrane systems resulted in the loss of membrane barrier function, observed as rapid dissipation of the transmembrane potential of intact bacterial cells and the release of fluorophores from lipid vesicles. Because the addition of clavanin resulted in the release of components with a molecular mass of up to 10 kDa from large unilamellar vesicles (LUVs),[1] the permeabilization mechanism was assumed to be rather aspecific. Furthermore, the glycines at positions 6 and 13 were found to play an important role in the antimicrobial and membrane activity at neutral pH, presumably in inducing conformational flexibility of the peptide (*13*).

Generally, productive membrane interactions of amphipathic polypeptide antibiotics result from a delicate balance of hydrophobic and electrostatic interactions (*5*, *14*, *15*). Hydrophobic interactions are responsible for the partitioning of nonpolar amino acid side chains into the hydrocarbon core of the membrane. Electrostatic interactions between positively charged amino acid residues and negatively charged phospholipid headgroups increase the peptides' affinity for the membranes (*5*, *10*, *12*, *15*−*20*) and may anchor the peptides in the membrane interface region (*15*, *21*). In combination with the self-assembling properties of the peptides, these interactions might result in the formation of pore-like structures or in other membrane barrier disruptive modes of organization of the peptide (*10*, *16*, *17*, *21*).

The presence of the four histidine residues (with a pK_a ∼ 6) within the clavanin sequence indicates that the net peptide charge is pH-dependent in the physiological range. This could imply that the way in which clavanin interacts with the membrane is also pH-dependent. In agreement with this, it was observed that the peptide did show a higher antimicrobial activity at pH 5.5 than at neutral pH values (*22*, *23*). However, the lipid insertion, as measured using the monolayer technique, proved hardly pH-dependent (*13*), demonstrating that, at both acidic and neutral pH, membrane interaction is primarily driven by hydrophobic forces. This raises the question whether the mode of action of clavanin A at low pH differs from that at neutral pH. To address this issue, in this study, the pH dependence of the antimicrobial and membrane activity of clavanin A was analyzed in growth and membrane leakage experiments using both biological and model membrane systems (*24*−*28*). Next to wild-type clavanin, a series of Gly → Ala mutant peptides (Table 1) was also studied in order to get information on the possible importance of the peptide conformation, as measured by CD, in the pH dependence of its antimicrobial activity. The results demonstrate that, at low pH, clavanin A is a more potent antimicrobial agent than at neutral pH. Interestingly, clavanin A permeabilizes the target membrane via two completely distinct pH-dependent mechanisms.

EXPERIMENTAL PROCEDURES

Materials. Peptide synthesis chemicals, such as *N*-Fmoc protected amino acids, reagents, and resin were purchased from Novabiochem (Läufelfingen, Switzerland). Solvents used for peptide synthesis and HPLC were purchased from Biosolve (Valkenswaard, The Netherlands).

The strain *Lactobacillus sake* (ATCC 15521 = DSM 20017 = CECT 906, MRS medium, at 30 °C) was grown in MRS broth (Oxoid, Unipath Ltd., Basingstoke, Hampshire, U.K.). The phospholipids 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), and 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). The ionophores nigericin and valinomycin (dissolved in ethanol), gramicidin D (dissolved in anhydrous DMSO), and the fluorescent probe calcein were purchased from Sigma Chemical (St. Louis, MO). 5-(and-6)-Carboxyfluorescein diacetate, succinimidyl ester (5(6)-CFDA, SE) (dissolved in anhydrous acetone) and HPTS were both purchased from Molecular Probes Inc. (Eugene, OR). All other chemicals used were of analytical or reagent grade.

Peptide Synthesis and Purification. The clavanin peptides of Table 1 were prepared using automated solid-phase peptide synthesis and purified using RP-HPLC on a Waters Symmetry C18 column (Milford, MA) using previously described methods (*13*). The peptide masses were confirmed using electrospray mass spectrometry.

Antimicrobial Activity Measurements. The antimicrobial activities of the peptides were determined in a growth inhibition assay as a function of the pH. Hereto, *L. sake* cells diluted from an overnight culture were incubated with serial dilutions of the peptides in 96-wells plates, essentially as

[1] Abbreviations: CD, circular dichroism; 5(6)-CF, SE, 5-(and-6)-carboxyfluorescein, succinimidyl ester; 5(6)-CFDA, SE, 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester; cfu, colony-forming unit; choline, [2-hydroxyethyl]trimethylammonium; DMSO, dimethyl sulfoxide; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; Fmoc, 9-fluorenylmethoxycarbonyl; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HPTS, 8-hydroxypyrene-1,3,6,-trisulfonic acid; LUV, large unilamellar vesicle; MES, 2-[*N*-morpholino]ethanesulfonic acid; MIC, minimal inhibitory concentration; MRS, Man, Rogasa, Sharpe; SUV, small unilamellar vesicle; RP-HPLC, reversed-phase high-performance liquid chromatography; pH_{in}, intracellular pH; pH_{out}, extracellular pH; TFE, trifluoroethanol; Tris, tris(hydroxymethyl)aminomethane.

described earlier (*13*). The pH in the wells was adjusted to 6.5, 6.0, and 5.6, respectively, by adding potassium phosphate to a final concentration of 25 mM. After 24 h of incubation, the growth inhibition was calculated from the $OD_{595\,nm}$ versus the growth controls to which no peptide was added.

LUVs Preparation and Calcein Leakage Studies. Calcein (MW 622.5) was dissolved in 1 M NaOH, and the pH of the solution was adjusted to the desired pH using 1 M HCl. The fluorescent probe was encapsulated at a self-quenching concentration of 45 mM in LUVs composed of DOPC or DOPG/DOPE 30:70 (molar ratio). LUVs were prepared using the extrusion technique according to Hope et al. (*29*) and 400 nm nucleopore filters. Untrapped calcein was removed by gel filtration on Sephadex G75 columns that were equilibrated with buffer containing 150 mM NaCl and 10 mM Tris or 10 mM MES at corresponding pH. The release experiments were carried out by adding the indicated amounts of peptide to 1.2 mL of buffer containing 16.5 μM calcein-loaded lipid vesicles. The release of the fluorescent dye was detected using emission spectra at 505 nm (excitation 470 nm) on a SPF 500C spectrophotometer (SLM Instruments Inc., Urbana, IL) at 20 °C.

The release of fluorescent dye was calculated according to

$$R_f = 100[(F_t - F_0)/(F_{100} - F_0)]$$

where R_f is the fraction of dye released and F_0, F_t, and F_{100} are the fluorescence intensities at times $t = 0$ s, $t = 120$ s, or after the addition of 10 μL of 10% Triton X-100, respectively.

Measurement of pH_{in} of Intact Bacterial Cells. The intracellular pH was measured by labeling *L. sake* cells with 5(6)-CFDA, SE, as described by Breeuwer et al (*25*). 5(6)-CFDA, SE is a nonpolar molecule that spontaneously penetrates cell membranes and is converted to the anionic pH-sensitive 5-(and-6)-carboxyfluorescein succinimidyl ester (5(6)-CF, SE) by intracellular esterases. Once the probe is internalized, amine-reactive coupling of succinimidyl groups of 5(6)-CF, SE to aliphatic amines of intracellular proteins results in the formation of membrane impermeable pH-sensitive probe conjugates.

L. sake cells from an overnight culture ($OD_{600\,nm} = 0.6$) were washed, resuspended in 50 mM potassium HEPES, 1 mM $MgSO_4$ (pH 7.0) buffer, and incubated for 10 min at 30 °C in the presence of 1 μM 5(6)-CFDA, SE to allow them to take up the probe. After the incubation, the extracellular probe was removed by washing and resuspending the cells in 50 mM potassium phosphate buffer containing 1 mM $MgSO_4$ (pH 7.0). The intracellular nonconjugated 5(6)-CF, SE was extruded from the cells by adding glucose to a final concentration of 10 mM and incubating for 30 min at 30 °C (*25*, *26*, *30*). The cells were washed 3 times and resuspended in 50 mM potassium phosphate and 1 mM $MgSO_4$ (pH 7.0) buffer to remove the extruded probe and were stored on ice prior to use.

Measurements were carried out by diluting the cells to an $OD_{600\,nm}$ of 0.025 in 3 mL of 50 mM potassium phosphate buffer, containing 1 mM $MgSO_4$ (pH 5.8) in a quartz cuvette. The fluorescence emission at 525 nm was monitored at 25 °C using a Perkin-Elmer LS 50B spectrofluorimeter (Bucks, U.K.) at the excitation wavelengths 490 nm (pH-sensitive) and 440 nm (pH-insensitive) by rapidly alternating the monochromator between those wavelengths. The excitation and emission slit widths were 5 and 10 nm, respectively. The ratio of the fluorescence intensity of the encapsulated probe at 490 and 440 nm is a measure of the intracellular pH.

The cells were energized by adding glucose to a concentration of 10 mM. When a stable 490-to-440-nm fluorescence ratio was observed, aliquots of the peptides were added to analyze the effect they have on the intracellular pH. At the end of the measurement, the proton gradient was fully dissipated using nigericin (1 μM). This ionophore eliminates pH gradients across the cell membrane and, therewith, equilibrates the intracellular pH to that of the extracellular medium.

Nigericin (1 μM) and valinomycin (1 μM) were used to calibrate the fluorescence ratio as function of the pH. Therefore, the cells were added to buffers with pH values ranging from 4 to 9. The calibration buffers were prepared from 50 mM glycine, 50 mM citric acid, 50 mM $Na_2HPO_4 \cdot 2H_2O$, and 50 mM KCl buffer, adjusted with HCl or NaOH.

Proton Transport Assay in LUVs. DOPC LUVs containing the pH-sensitive fluorophore pyranine [8-hydroxypyrene-1,3,6,-trisulfonic acid, trisodium salt (HPTS)] were used to probe the proton permeability of the membrane bilayer. Pyranine is highly watersoluble, and its fluorescence emission shows a strong pH-dependence when excited at 450 nm.

Dried DOPC films were hydrated with a 2 mM pyranine solution in 0.2 M NaH_2PO_4/Na_2HPO_4 buffer at pH 7, subjected to 10 freeze−thaw cycles, and subsequently extruded according to Hope et al. (*29*) to prepare pyranine-loaded DOPC LUVs. Untrapped pyranine was removed by gel filtration on a PD-10 column (Pharmacia, Uppsala, Sweden) that was equilibrated with 10 mM MES and 0.2 M Na_2SO_4 buffer of pH 5.5. This gel filtration step could thus be used to exchange buffers and, therewith, generate a pH gradient across the bilayer.

The effect of the clavanin peptides on the proton permeability of the lipid vesicles was monitored by adding aliquots of peptide to 1.2 mL of 10 mM MES and 0.2 M Na_2SO_4 buffer (pH 5.5) containing 38 μM pyranine-loaded DOPC LUVs. The phospholipid concentration was determined using an enzymatic colorimetric method (Phospholipids B test kit) from Wako Chemicals (Neuss, Germany). The fluorescence emission of the pyranine-loaded vesicles was detected at 508 nm (excitation at 450 nm) on a Perkin-Elmer LS 50B spectrofluorimeter (Bucks, U.K.) at 25 °C.

Calibration of the pH-dependent fluorescence signal of the fluorophore was performed by adding pyranine-loaded vesicles to 10 mM MES and 0.2 M Na_2SO_4 buffer with varying pH. The fluorescence emission was measured after destroying the lipid vesicles by Triton X-100 addition to allow the probe to adjust to the pH of the medium.

Potassium Leakage Experiments. Peptide-induced potassium leakage from intact *L. sake* cells was monitored using a potassium selective electrode (G-K15; Philips) and a reference electrode (R44/2-SD/1; Philips) in a thermostated cuvette at 20 °C. From an overnight *L. sake* culture ($OD_{600\,nm} = 0.4$), cells were washed 3 times and resuspended in the potassium- and sodium-free 25 mM MES, 25 mM Tris, 150 mM choline chloride, 10 mM glucose and 5 mM $MgSO_4$ (pH 7) buffer and were stored on ice prior to use. The

measurements were started when cells were suspended to an $OD_{600\,nm}$ of 0.2 in 6 mL of 35 mM MES, 15 mM Tris, 150 mM choline chloride, 1 mM $MgSO_4$, and 10 mM glucose buffer (pH 5.8). Varying peptide concentrations were added while monitoring the K^+ concentration. The total amount of K^+ ions could be determined after the addition of the nonionic detergent *N*,*N*-dimethyldodecylamine-*N*-oxide. This total amount of K^+ present in the cells, the 100% value, was verified after destroying the bacteria by boiling. The potassium ion concentrations were calibrated by adding known aliquots of KCl solution.

CD Spectroscopy. CD spectra were recorded in absence and presence of small unilamellar vesicles (SUVs) on a Jasco-J810 spectropolarimeter in the range 180−260 nm using quartz cuvettes with a path length of 0.1 cm at 20 °C. Five scans were averaged and corrected for the contributions of vesicles and solvents. The peptides were dissolved in 10 mM potassium phosphate buffer (ranging from pH 5.5 to 7.0) at a concentration of 0.25 mg/mL in the cuvette. DOPC SUVs were prepared by sonication after hydration of dry phospholipid films with 10 mM potassium phosphate buffer (pH 5.5−7.0) to reduce the influence of scattering at low wavelengths. The peptide/lipid ratio was 1:25 (mol/mol) at a peptide concentration of 0.25 mg/mL.

RESULTS

Antimicrobial Activity as Function of the pH. The pH dependency of the antimicrobial activity of clavanin A was tested on the Gram-positive lactic acid bacterium *L. sake*. This organism was selected for its ability to grow well in the pH 5.6−6.5 range used. Figure 1A shows that clavanin effectively inhibits the growth of *L. sake* and that this effect is strongest at pH 5.6, the lowest pH that could be tested properly. To exclude that this is caused by a pH-dependent sensitivity of the microorganism, the effect of gramidicin D, an unrelated neutral pore-forming polypeptide antibiotic, was analyzed. As shown in Figure 1B, the growth inhibition caused by gramicidin was much less pH-dependent than that of clavanin, from which it is concluded that clavanin becomes a much more potent antimicrobial agent at lower pH. Table 1 shows a 10-fold decrease of the MIC value of clavanin A upon lowering the pH of the growth medium from 6.5 to 5.6. The minimal inhibitory concentration (MIC) against *L. sake* of wild-type clavanin A of 7.8 μM at pH 6.5 (Table 1) corresponds well to the value reported for *M. flavus* (*13*), indicating that the peptide inhibits the growth of the bacteria around neutral pH via a similar mechanism.

To obtain insight into the importance of the glycine residues in the pH dependency of the antimicrobial activity of clavanin, the series of Gly → Ala mutants shown in Table 1 were tested. Figure 2 shows the growth inhibition curves, and Table 1 gives the corresponding MIC values. Two effects are immediately clear. First, all mutants show a largely increased growth inhibition activity at lower pH. Second, the differences in growth inhibition observed for the different clavanin mutants decrease strongly at lower pH. The large differences in the abilities to inhibit the growth at pH 6.5 (the MIC values of the clavanin mutants AAA or AAG were at least 6−12 times higher than that of the wild-type peptide) are in a good agreement with the similar differences reported for *M. flavus* at neutral pH and the different abilities of these

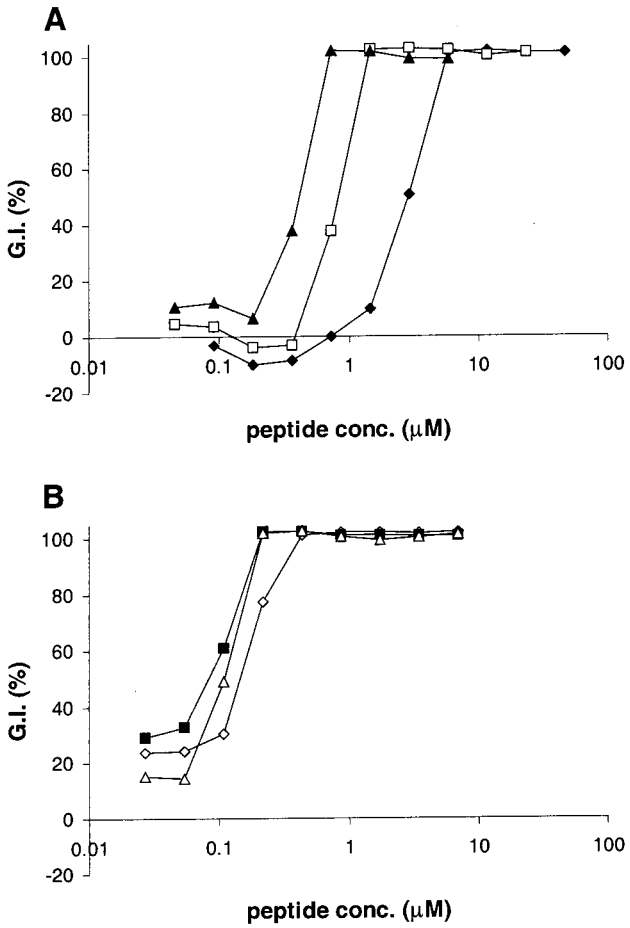

FIGURE 1: (A) Growth inhibition curves of wild-type clavanin A against *L. sake* (strain ATCC 15521 = DSM 20017 cultured in MRS broth buffered using 25 mM potassium phosphate buffer at 30 °C) at (◆) pH 6.5, (□) pH 6.0, and (▲) pH 5.6, respectively. (B) Growth inhibition curves of gramicidin D against *L. sake* at (◇) pH 6.5, (■) pH 6.0, and (△) pH 5.6, respectively.

peptides to insert into lipid monolayers and permeabilize the target membrane, as derived from membrane potential measurements on intact *M. flavus* cells (*13*). The glycines play an important role in the antimicrobial activity at neutral pH but apparently are less crucial at acidic pH values where the histidines become protonated. These results suggest that the antimicrobial activity of clavanin at low pH is distinctly different from that at higher pH values. To get a first indication into the mechanism of the antimicrobial activity at low pH, lipid vesicles were used to study whether these peptide−lipid interactions resulted in membrane destabilization.

Calcein Leakage from DOPC LUVs. The membrane permeabilizing activities were measured as a function of the pH by monitoring the peptide-induced release of the fluorescent dye calcein (*31*) from LUVs. This dye can be used in the pH 5.5−7.5 range. The vesicles were prepared from the zwitterionic phospholipid DOPC, to avoid changes in vesicle charge upon varying the pH. Figure 3A shows the pH-dependent calcein release induced by wild-type clavanin A and mutant AAA. At neutral pH, both peptides induced a similar release of calcein from the LUVs. The amounts of calcein released at pH 7 were comparable with those reported for carboxyfluorescein from DOPC LUVs under the same conditions (*13*).

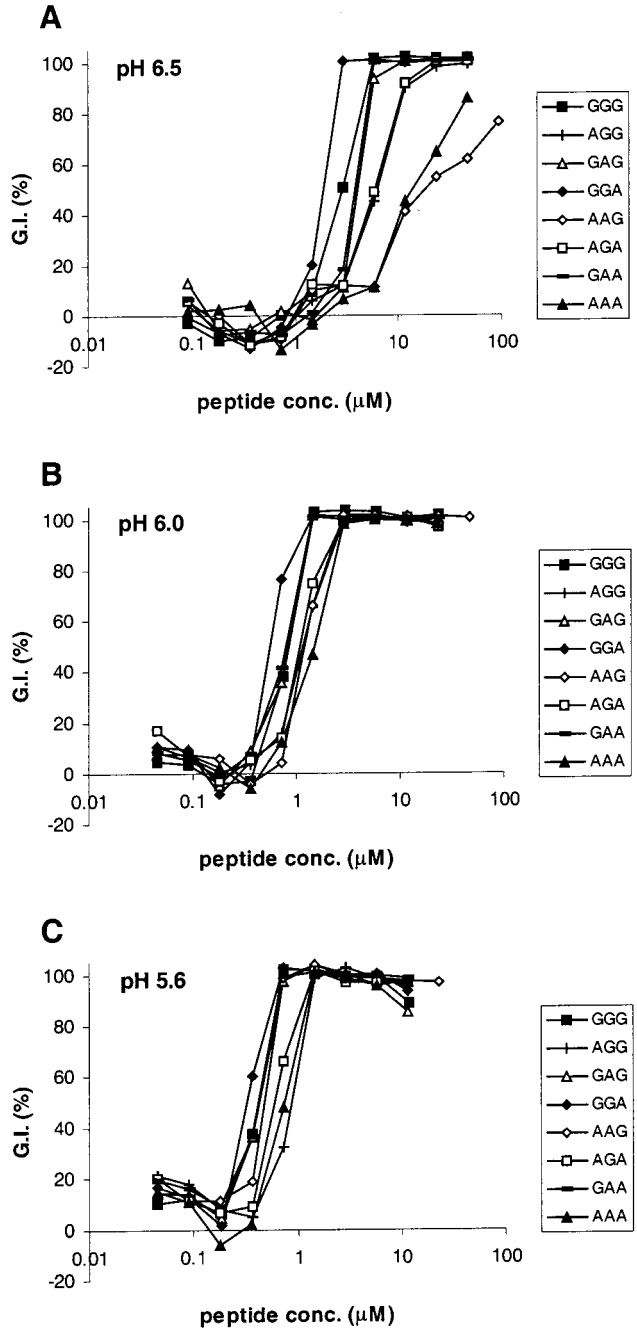

FIGURE 2: Growth inhibition curves of clavanin A Gly → Ala mutants against *L. sake* (Strain ATCC 15521 = DSM 20017, at 30 °C) in MRS broth buffered using 25 mM potassium phosphate buffer at (A) pH 6.5, (B) pH 6.0, and (C) pH 5.6, respectively.

When the calcein leakage from LUVs was measured at lower pH values (Figure 3A), the permeabilization efficiencies of the two peptides GGG and AAA markedly diverged. Wild-type clavanin A was unable to release the fluorophore from DOPC vesicles at pH 5.5. Under these low pH conditions, however, this peptide actually displayed higher antimicrobial activities against *L. sake* than at neutral pH. These observations again indicate that, at low pH, the wild-type peptide acts via a different mechanism than at neutral pH, where it was suggested to induce rather aspecific membrane permeabilization (*13*). Under acidic conditions, the membrane integrity to molecules of at least the size of calcein (MW 622.5 Da) seems to be retained.

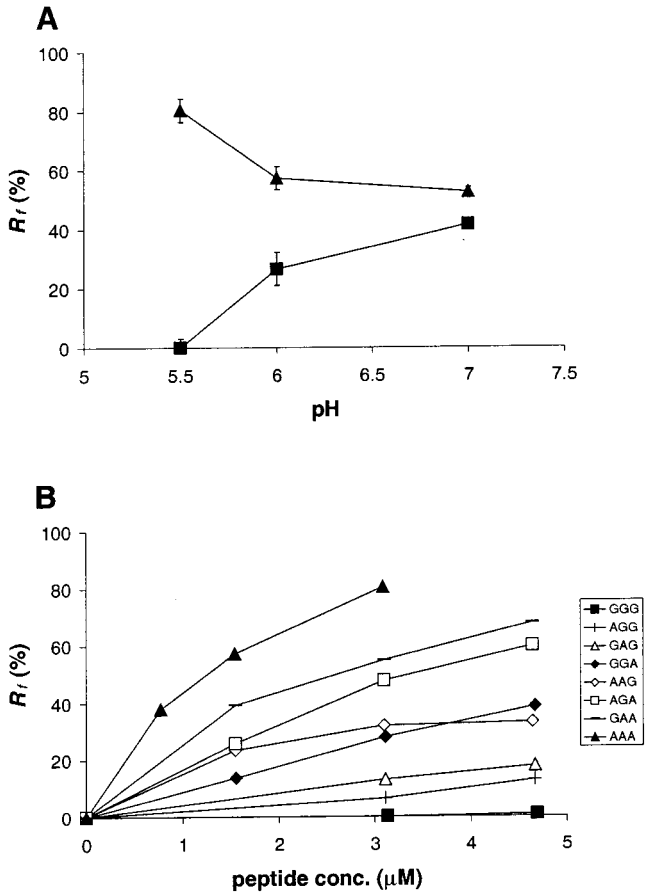

FIGURE 3: (A) Release of the fluorescent probe calcein from DOPC LUVs at pH 5.5, 6.0, and 7.0, respectively, measured 2 min after the addition of wild-type clavanin A (■) and derivative AAA (▲) at a concentration of 3 μM. (B) Concentration-dependent release of the fluorescent probe calcein from DOPC LUVs at pH 5.5, measured 2 min after the addition of the clavanin peptides.

In contrast to wild-type clavanin, its mutant AAA more effectively releases calcein from DOPC LUVs at pH 5.5 than at neutral pH (Figure 3A). This suggests an important role of the glycines in the controlling the mode of action by which the peptide induces membrane destabilization at low pH.

This concept was verified by analyzing the calcein release induced by the full range of Gly → Ala mutant peptides at pH 5.5 (Figure 3B). At all concentrations tested, the largest differences were observed between wild-type clavanin A and mutant AAA. The other examined clavanin derivatives display intermediate bilayer destabilizing properties. The extent of bilayer destabilization was roughly proportional to the number of Gly → Ala substitutions with some position specific effects. The differences in permeabilizing efficiency of the various clavanin derivatives at low pH were in contrast with their comparable MIC values against *L. sake* under acidic conditions. This indicates that, depending on the presence of the glycines, the peptides may use an alternative mode of action than aspecific membrane permeabilization to exert their antimicrobial effect.

To exclude that the discrepancy between the calcein release and biological activities of particularly wild-type clavanin results from the use of DOPC as model lipid, vesicles were prepared of a DOPG/DOPE (30:70) mixture which mimics a bacterial phospholipid composition. Table 2 shows the calcein release percentages from these DOPG/DOPE (30:70) LUVs at both acidic and neutral pH. At low pH, the

Table 2: Release of the Fluorescent Probe Calcein (as Percentage of the Total Amount of Encapsulated Calcein) from DOPG/DOPE 30:70 LUVs at Acidic and Neutral pH, Measured 2 min after the Addition of Clavanin A and Its Gly → Ala Mutants to a Final Peptide Concentration of 3 μM

	calcein release (%)[a] DOPG:DOPE 30:70 LUVs	
peptide	pH 5.5	pH 7.4
GGG	25 ± 8	55 ± 3
AGG	42 ± 6	68 ± 14
GAG	34 ± 8	61 ± 5
GGA	67 ± 18	72 ± 14
AAG	63 ± 2	69 ± 6
AGA	63 ± 10	63 ± 9
GAA	68 ± 4	70 ± 15
AAA	79 ± 3	65 ± 4

[a] Average release percentages and standard deviations were calculated from three independent measurements.

calcein release from these negatively charged vesicles shows a general trend that is similar to that observed for PC vesicles (i.e., wild-type clavanin is least effective, the AAA mutant is the most potent, and the other Gly → Ala mutants show intermediate behavior). However, for this membrane composition, the addition of the wild-type peptide does result in a small but significant leakage of calcein at pH 5.5.

From the discrepancy between the ability to permeabilize a lipid bilayer to calcein and the antimicrobial activity of clavanin A at low pH, the question arises whether the membrane is still the target. Therefore, it is analyzed if the peptide is able to permeabilize the membrane of intact *L. sake* cells under acidic conditions.

Impact on the L. sake Proton Gradient. The pH-sensitive fluorescent dyes, such as carboxyfluorescein or derivatives thereof, have previously been used to measure the intracellular pH (pH_{in}) in bacteria and eukaryotic cells (*26*, *27*). In this study, *L. sake* cells were loaded with the cell-permeant dye 5(6)-CFDA, SE to study the effects of the antimicrobial clavanin peptides on the pH_{in}. Once the probe has been internalized, it is well-retained within the cells due to the formation of membrane impermeable pH-sensitive probe conjugates (*25*, *26*).

The ratios between the pH-sensitive and pH-insensitive fluorescence emission signals at 525 nm of 5(6)-CFDA, SE-loaded cells that were obtained after excitation at 490 and 450 nm, respectively, were monitored to indicate the intracellular pH. The 5(6)-CFDA, SE-loaded *L. sake* cells were suspended in 50 mM potassium phosphate and 1 mM $MgSO_4$ buffer of pH 5.8 and energized by the addition of 10 mM glucose, as indicated by the first arrow in Figure 4A. After a constant fluorescence ratio was observed, varying concentrations of clavanin A (second arrow) were added, resulting in a decreased signal when the peptide was able to disturb the proton gradient across the membrane. The ionophore nigericin (1 μM, third arrow) was finally added to fully dissipate the transmembrane ion gradients. The fluorescence 490-to-450 nm ratios were calibrated by measuring suspended 5(6)-CFDA, SE-loaded cells in buffers of various pH, after addition of both the ionophores valinomycin and nigericin to equilibrate the pH_{in} and known pH_{out}. The pH_{in} of the cells during the measurements was subsequently derived from the sigmoidal calibration curve shown in Figure 4B (inset). The pH_{in} of energized *L. sake* cells was thus found

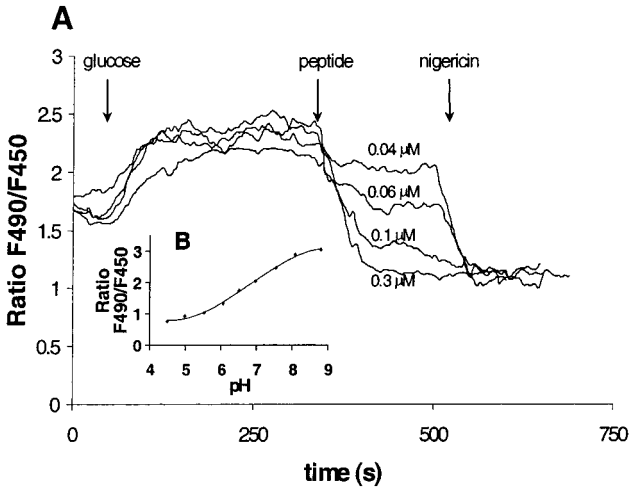

FIGURE 4: (A) Fluorescence emission ratios at 525 nm of 5(6)-CFDA, SE conjugated *L. sake* cells ($OD_{600\ nm}$ in cuvette = 0.025) in 50 mM potassium phosphate and 1 mM $MgSO_4$ buffer (pH 5.8) after excitation at 490 and 450 nm as an indication for the intracellular pH. Typical results are shown upon addition of varying concentrations of clavanin A after a constant signal was observed. The H^+ gradient was fully dissipated using 1 μM nigericin. (B, inset) Calibration of the ratio of fluorescence emission at 525 nm on 5(6)-CFDA, SE conjugated *L. sake* cells ($OD_{600\ nm}$ in cuvette = 0.025) in 50 mM glycine, 50 mM citric acid, 50 mM $Na_2HPO_4 \cdot 2H_2O$, and 50 mM KCl buffer, adjusted with HCl or NaOH (calibration buffer) after excitation at 490 and 450 nm using 1 μM valinomycin and 1 μM nigericin to equilibrate the pH_{out} and pH_{in}.

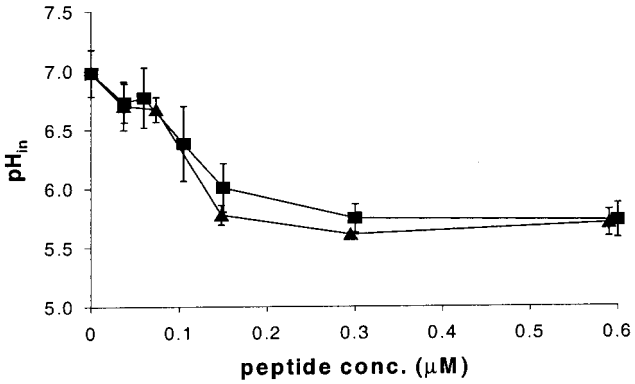

FIGURE 5: Effect on the intracellular pH of 5(6)-CFDA, SE conjugated *L. sake* cells after the addition of wild-type clavanin A (■) and mutant AAA (▲) in 50 mM potassium phosphate, 1 mM $MgSO_4$ buffer of pH 5.8 derived from the fluorescence emission ratio after excitation at 490 and 450 nm as described in the Experimental Procedures section. Data points and error bars represent average values and standard deviations of three independent measurements, respectively.

to be around 7.0 and was lowered as result of adding the wild-type peptide in a concentration-dependent manner (Figure 4A), to fully adjust to the external pH of 5.8 after addition of nigericin.

Both wild-type clavanin A (GGG) and mutant AAA collapse the transmembrane proton gradient of 5(6)-CFDA, SE-loaded cells at submicromolar concentrations, as shown in Figure 5. At peptide concentrations as low as 200 nM, this proton gradient is fully disturbed. Addition of the wild-type peptide or AAA to intact bacteria is shown to have an essentially identical effect on the proton permeability of the membranes. Their mode of action, however, may differ significantly. Analogue AAA is expected to allow protons to cross the membrane, as it efficiently released fluorophores

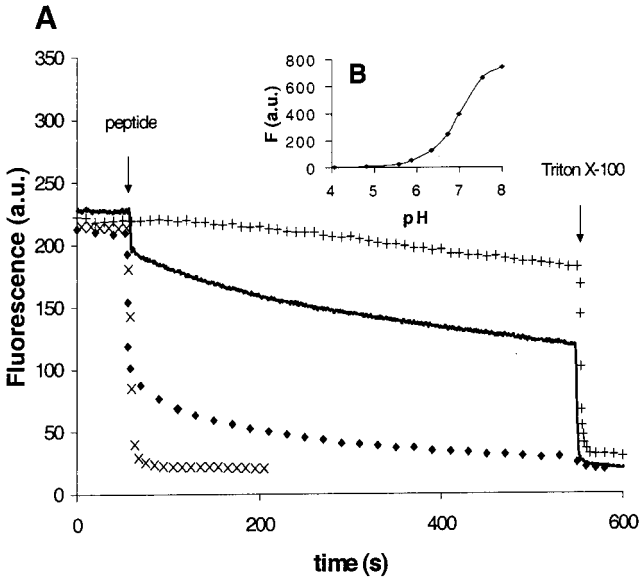

FIGURE 6: (A) Pyranine fluorescence emission at 508 nm (excitation wavelength 450 nm) of HPTS-loaded DOPC LUVs in 10 mM MES and 0.2 M Na_2SO_4 buffer (pH 5.5) to monitor the effect of wild-type clavanin A (solid line, P/L = 0.3), derivative AAA (◆, P/L = 0.3), and nigericin (×, P/L = 0.03) on the membrane permeability to protons, derived from changes in the intravesicular pH (no peptide added (+)). (B, inset) Calibration curve of the HPTS fluorescence emission at 508 nm (excitation at 450 nm) in DOPC LUVs suspended in 10 mM MES and 0.2 M Na_2SO_4 buffer adjusted with NaOH to the indicated pH. The fluorescent signal of pyranine adapted the pH of the external buffer after adding Triton X-100 to the DOPC LUVs.

from model membranes. GGG does not do the latter and, hence, may use a more specific mechanism in which transmembrane proton transport is facilitated. To determine whether the antimicrobial effect of wild-type clavanin A results from a specific protonophore activity at the membrane level, proton transport was measured using lipid vesicles.

Proton Transport into LUVs. Lipid vesicles containing the pH-sensitive probe pyranine (HPTS) were used to evaluate the proton flux across membrane bilayers. Pyranine was encapsulated in DOPC LUVs in a manner similar to calcein. For the measurement of transmembrane proton transport, generation of a pH gradient across the phospholipid bilayer is required. This pH gradient has to be stable during the course of the measurements (2−3 h), which could be established using 0.2 M NaH_2PO_4/Na_2HPO_4 buffer (pH 7.0) inside the vesicles and 10 mM MES and 0.2 M Na_2SO_4 buffer (pH 5.5) on the outside. Buffers containing, for instance, potassium or chloride ions cannot be used as membranes are more permeable to these ions than to sodium ions or larger anions. Also, the presence of small (organic) buffer compounds is undesirable as they can act as ionophores.

The fluorescence emission at 508 nm of pyranine-loaded DOPC LUVs after excitation at 450 nm was monitored in the absence and presence of the peptides (Figure 6A). Addition of a high concentration (11 μM) of wild-type clavanin A (corresponding to a P/L ratio of 0.3) lowered the fluorescence of the dye slightly. This reduced signal, however, does not correspond with a dramatic decrease of the pH_{in}. Two minutes after addition of the peptide, the pH_{in} decreased from 6.7 to only 6.5 (Table 3), as derived from the calibration curve of the HPTS emission (Figure 6B). This

Table 3: Effect of Wild-Type Clavanin A and Mutant AAA on the pH_{in} of Pyranine-Loaded DOPC LUVs, Determined 2 min after Addition of the Peptides Using the HPTS Fluorescence Calibration Curve to Derive the pH Value

	intravesicular pH derived from HPTS fluorescence	
P/L	GGG	AAA
0	6.7	6.7
0.1	6.6	6.2
0.2	6.5	6.1
0.3	6.5	5.9

observation markedly contrasts the efficient proton transport induced by wild-type clavanin A in *L. sake* cells (compare Figure 4). The pH_{in} of the pyranine-containing vesicles was found to be nearly constant during the measurements when no peptide was added, varying only slightly from pH 6.7 to 6.6 in 10 min. When nigericin, an effective protonophore, was added, the HPTS signal instantaneously dropped to attain the pH of the external medium of 5.5 (Figure 6A).

Addition of mutant AAA lead to a more rapid and more extensive decrease in the pyranine fluorescence compared to the wild-type peptide, representing a pH_{in} shift to 5.9 at the highest peptide−lipid ratio of 0.3 (Figure 6A and Table 3). This decrease in HPTS signal observed after adding AAA is expected in view of the ability of this mutant peptide to cause the release of calcein at acidic pH (Figure 3A). Lowering of the fluorescence might originate from the release of the fluorophore pyranine (MW 524 Da) to the external medium (pH 5.5), proton influx into the vesicles, or a combination of both effects.

These results strongly suggest that, at low pH, clavanin exerts its antimicrobial activity by permeabilizing the target membrane to protons via another mechanism than that at neutral pH. To test whether membrane permeabilization is restricted to protons, the effect of the peptide on the barrier function toward K^+ ions was analyzed.

Potassium Leakage from L. sake. The membrane permeability for potassium ions after addition of the clavanin peptides GGG and AAA to intact *L. sake* cells was monitored using a potassium selective electrode (*24*). The K^+ ion efflux from cells can thus be measured with high sensitivity. Figure 7A represents the concentration-dependent K^+ efflux relative to total intracellular K^+, observed 2 min after addition of the peptides GGG and AAA. Addition of wild-type peptide GGG results in a moderate release of K^+ ions from the cells. Only after 15 min at the highest GGG concentration tested nearly complete dissipation of the K^+ ion gradient from the cells is obtained (Figure 7B). Comparing these results to those observed for the induced proton permeability suggests that the potassium gradient is less and perhaps only secondarily affected by the peptide at low pH. Peptide AAA, on the other hand, reaches maximal release of these cations after 2 min. The K^+ ions are rapidly and efficiently released from the bacterial cells when derivative AAA is added, thus indicating instantaneous membrane permeation consistent with a general membrane destabilizing activity of this mutant peptide.

In conclusion, the very potent antimicrobial activity of wild-type clavanin at low pH most likely is the result of a specific change in the membrane permeability to small

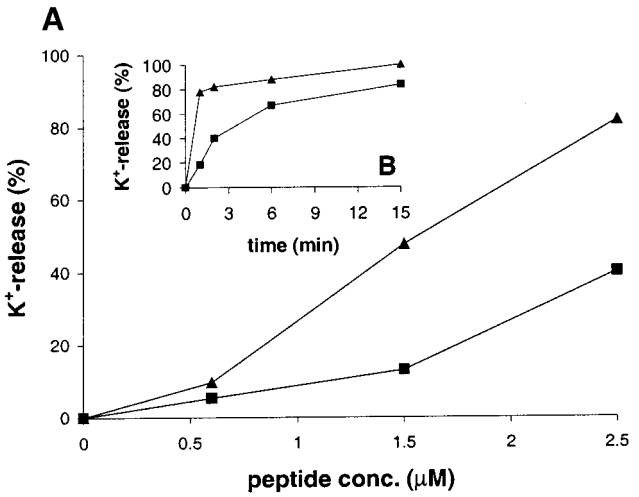

FIGURE 7: (A) Potassium release percentages from intact *L. sake* cells in potassium-free buffer (pH 5.8) measured using a potassium selective electrode 2 min after the addition of wild-type clavanin A (■) and mutant AAA (▲). (B, inset) Time-dependent potassium release from intact *L. sake* cells in potassium-free buffer (pH 5.8) monitored using a potassium selective electrode after addition of 2.5 μM wild-type clavanin A (■) and mutant AAA (▲).

cations. The glycines must be essential for this effect because their replacement by alanines causes a markedly different aspecific membrane permeabilizing activity at low pH. Given that glycines have distinct conformational properties, the mode of action at low pH of GGG and AAA might be related to different (bio)active conformations. To investigate this possibility, the pH dependency of the secondary structure of the clavanins was investigated using CD.

CD Spectroscopy in Aqueous Solution. CD spectra of wild-type clavanin A were measured in 10 mM potassium phosphate buffer as a function of the pH and in the structure-promoting solvent TFE (Figure 8A). In TFE, clavanin A displayed a typical α-helix spectrum (Figure 8A), with characteristic minima at 208 and 222 nm. In aqueous solution at pH 5.5, the wild-type peptide displayed a spectrum typical of a random coil structure. The loss of entropy and repulsive forces between the positively charged histidines, lysine, and the N-terminal end of the peptide at this pH may prevent its folding into a well-defined secondary structure, such as an α helix, in aqueous solution.

When the pH of the aqueous buffer was raised, the shape of the spectra changed. However, at these higher pH values, the wild-type peptide did not adopt specific secondary structures. At pH 7.0, the CD signal intensity did not decrease, indicating that the peptide was well-dissolved and nonaggregated. As it is hard to retrieve reliable results from spectra that do not show a well-known secondary structure pattern, the spectra were not quantitatively analyzed. Also, the mainly unordered conformations of clavanin A in buffer solutions of varying pH did not show a two-state equilibrium, as no isodichroic point was observed for its spectra. Its folding behavior in aqueous solution is, therefore, suggested to be complex and pH-dependent.

CD Spectroscopy in the Presence of SUVs. Upon interaction with membranes, the peptide may fold into a bioactive conformation. To test this possibility, wild-type clavanin A was added to DOPC SUVs (P/L = 1:25) in 10 mM potassium phosphate buffer. At pH 7, the intensity of the CD spectrum of the wild-type peptide in the presence of DOPC SUVs

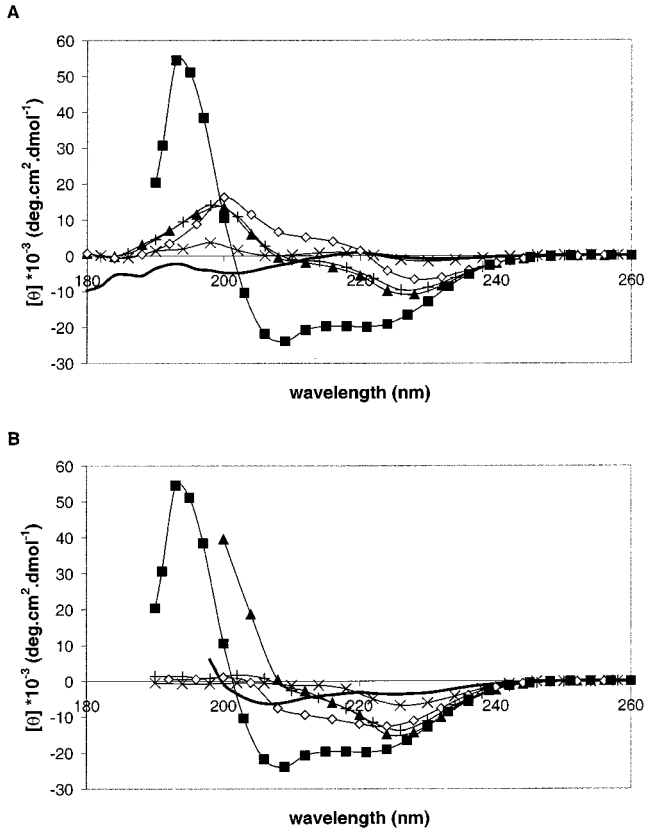

FIGURE 8: (A) CD spectra of wild-type clavanin A (94 μM) both in 10 mM potassium phosphate buffer as function of the pH ((—) pH 5.5, (×) pH 5.75, (◇) pH 6.0, (+) pH 6.5, and (▲) pH 7.0) and in TFE (■). (B) CD spectra of wild-type clavanin A (94 μM) in the presence of DOPC SUVs (peptide/lipid = 1:25) in 10 mM potassium phosphate buffer as function of the pH ((—) pH 5.5, (×) pH 5.75, (◇) pH 6.0, (+) pH 6.5, and (▲) pH 7.0) and in TFE (■).

(Figure 8B) was increased compared to its spectrum in aqueous solution (Figure 8A). This demonstrates an interaction between the peptide and the vesicles, in agreement with the efficient insertion of clavanin A into phospholipid monolayers (*13*). The absence of an isodichroic point showed the folding behavior of clavanin A upon interaction with lipid vesicles to be complex. Its conformation varies when the pH is changed from 5.5 to 7.0 (Figure 8B).

The CD spectra determined in the presence of DOPC SUVs for both wild-type clavanin A and mutant AAA at pH 5.5 and pH 7.0 are compared in Figure 9. Both under acidic and neutral pH conditions, the degree of α helicity of derivative AAA upon membrane interaction was higher than that of the wild-type compound, consistent with the higher helical propensity of alanine over glycine. Analysis of the spectra via commonly used deconvolution methods did not give reliable results in that the calculated curve did not fit the experimental data well. As a semiquantitative alternative, the helix content of similar peptides was compared by calculating the ratio of the molar ellipticity values of the minimum at 222 nm and the intensity of the minimum between 195 and 210 nm (*32*). For a highly helical peptide, this parameter will approach 1, and in a random structure, this will be close to 0. This parameter for mutant AAA (1.3 and 0.8 at pH 7 and 5.5, respectively) is closer to 1 than that of the wild-type peptide (2.1 and 0.6 at pH 7 and 5.5, respectively). This suggests that peptide AAA is more helical

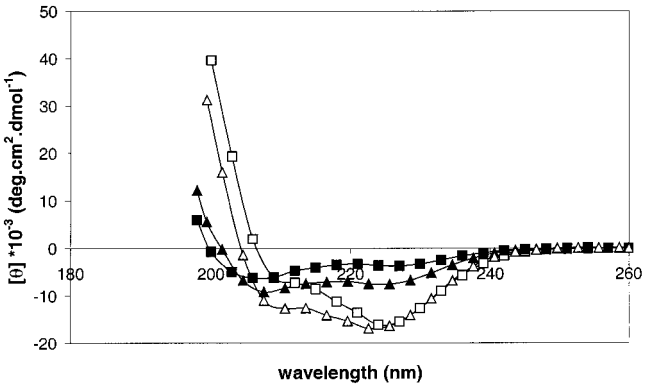

FIGURE 9: CD spectra of wild-type clavanin A (squares) and mutant AAA (triangles) in the presence of DOPC SUVs (peptide/lipid = 1:25, peptide concentrated = 94 μM) in 10 mM potassium phosphate buffer at pH 5.5 (closed symbols) and pH 7.0 (open symbols).

in the presence of vesicles than GGG, both at neutral and acidic pH conditions.

DISCUSSION

In this study, the pH dependence of the antimicrobial and membrane activity of the peptide antibiotic clavanin A was investigated. Various experiments on intact cells and model membrane systems revealed that clavanin A exerts its antimicrobial activity by permeabilizing the target membrane via two distinctly different pH-dependent mechanisms. The histidine and glycine residues within the clavanin sequence play an important role in these mechanisms.

Around neutral pH, wild-type clavanin A effectively inhibits the growth of the Gram-positive lactic acid bacterium *L. sake*. Interactions of clavanin A with membrane phospholipids result in permeabilization of the target membrane, as visible from the dissipation of the transmembrane potential of intact bacteria and the release of fluorescent probes from model bilayers (*13*). This loss of membrane barrier function was shown to result from a nonspecific permeabilization mechanism (*13*), such as, for instance, the carpet model described by Shai and co-workers (*10*, *33*) in which the antimicrobial peptides permeabilize the membrane in a detergent-like manner.

The present study demonstrates that clavanin A becomes a more potent antimicrobial agent against *L. sake* when the pH of the medium in the growth experiments is lowered. Enhanced antibacterial and antifungal activities for clavanin A at these mildly acidic conditions have also been described by Lehrer and co-workers (*22*). This enhanced activity was suggested to result from an increased affinity of clavanin for the anionic bacterial membrane after protonation of its histidine residues which have a p$K_a \sim 6$. However, wild-type clavanin A inserts with comparable efficiency in both neutral and negatively charged phospholipid monolayers. Moreover, this insertion is essentially pH-independent, demonstrating that, at both acidic and neutral pH, clavanin interacts with membrane lipids and that these interactions are primarily driven by hydrophobic forces (*13*).

Despite its high affinity for membrane phospholipids at low pH (*13*), the present data show that wild-type clavanin A is unable to induce the release of calcein from DOPC vesicles and only causes moderate release from mixed DOPG/DOPE model membranes. Apparently, clavanin A uses a distinctly different mechanism under acidic conditions to inhibit bacterial growth than the nonspecific membrane permeabilization that takes place at neutral pH.

However, the membrane is still the target for clavanin A under acidic conditions. This can be concluded from the observation that the wild-type peptide both collapses the membrane barrier function to protons and potassium ions and inhibits growth of *L. sake* cells at submicromolar concentrations. The loss of a vital transmembrane proton gradient will strongly interfere with cell metabolism (*34*, *35*). Whether the potassium efflux from the bacteria is a primary effect or the indirect result from the loss of the proton gradient could not be determined.

The aromatic imidazole ring system of histidine has a pK_a of about 6, making this residue well-equipped to be de- and reprotonated within the physiological pH range. Nevertheless, despite its numerous histidines, clavanin A proved unable to transport protons across model bilayers. The proton permeability of intact bacterial cells induced by clavanin A at low pH, therefore, is assumed to involve membrane components other than membrane phospholipids.

The question arises whether not only the histidines but also the abundant glycine residues play a role in the mode of action that is displayed at low pH. The role for the glycines was established using a series of Gly → Ala mutant peptides. Under acidic conditions, the Gly → Ala derivatives are as potent as the wild-type peptide against the test organism *L. sake*. However, the membrane permeabilizing action of these peptides becomes more and more aspecific when the number of Gly → Ala mutations is increased. Hence, their activity is similar to that of wild-type clavanin A at neutral pH (e.g., derivative AAA very effectively releases calcein from lipid vesicles at acidic pH and destroys the barrier function of both biological and model membranes to small cations). The glycines within the clavanin sequence, therefore, appear to be essential for the ion-selective membrane permeabilization mechanism at low pH. From the essentially identical antimicrobial activities of the various Gly → Ala derivatives at low pH, the selective and the nonspecific permeation mechanisms are assumed to both take place, where the relative contribution of the two to the antimicrobial activity depends on the specific mutant.

Summarizing, clavanin A permeabilizes the target membrane via two distinct pH-dependent mechanisms. At neutral pH, wild-type clavanin A, as many naturally occurring cationic antimicrobial peptides, acts via a nonspecific membrane permeabilizing mechanism to induce cell death (*5*, *7*, *9*, *10*). The carpet model, as described by Shai and co-workers (*10*, *33*), in which the amphipathic peptides cover the membrane in a carpet-like organization, represents such a mode of action. The peptides insert into the membrane, anchoring their hydrophilic residues in the membrane interface region via electrostatic interactions and penetrating their apolar side chains more deeply into the hydrocarbon core of the membrane. Once a threshold concentration is reached, the membrane collapses (Figure 10A).

The results clearly demonstrate that, despite its good interactions with membrane lipids when the histidine residues are protonated, wild-type clavanin A is unable to induce the loss of barrier function of phospholipid bilayers. Apparently, it uses more specific interactions to permeabilize

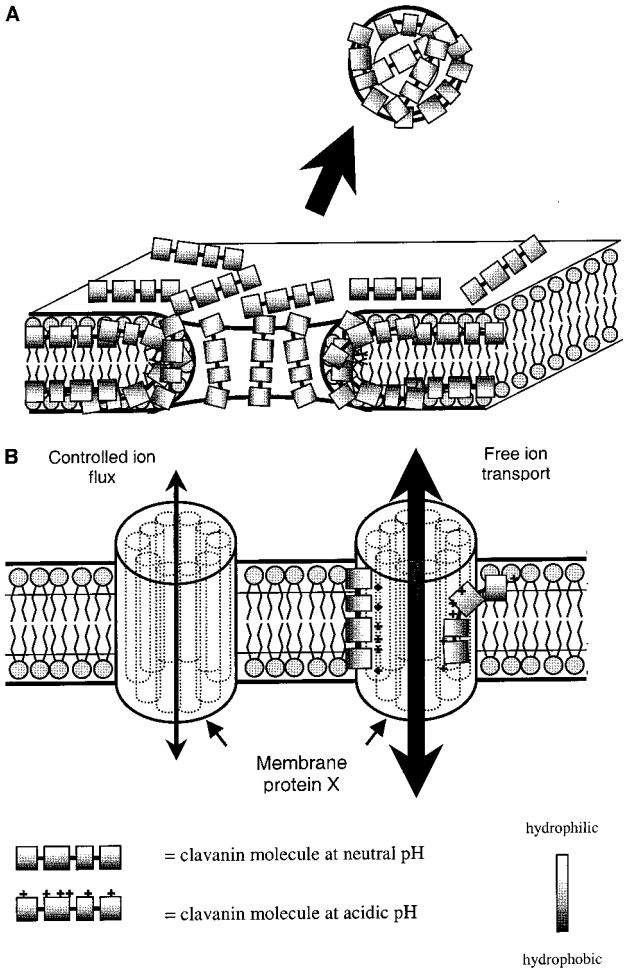

FIGURE 10: (A) Nonspecific membrane permeabilization mechanism of clavanin A at neutral pH, as based on the carpet model proposed by Shai and co-workers (*10*, *33*). (B) Putative model of specific membrane permeabilization mechanism of clavanin A at low pH, in which the peptide is assumed to interfere with the function of regulatory membrane proteins as target molecules by binding to their transmembrane segment.

biological membranes. In this respect, membrane protein complexes that are involved in generating transmembrane proton or ion gradients, such as ATP synthases or proton pumps (*36*, *37*), are suggested to be more likely target molecules of clavanin A than membrane carbohydrates or lipid molecules. A putative model for the specific mode of interaction of clavanin A with membrane proteins is visualized in Figure 10B. The basis of the model is the assumption that binding of wild-type clavanin A to these proteins interferes with their function. The peptides' highly hydrophobic character argues for interactions with transmembrane protein segments.

The protonated histidines (*38*) as well as the glycines, which have a tendency to "break" helices, promote the peptides' flexibility and prevent the formation of amphipathic helical structures, as shown by CD measurements. The glycines may act as hinges between the various peptide segments, thereby allowing for optimal interaction with the membrane target. On the other hand, it is observed that they prevent nonspecific phospholipid bilayer destabilization at low pH. Mutations of glycines into helix-stabilizing alanines result in an increased α-helical structure, as measured using CD both at neutral and acidic pH, which makes these mutant peptides less flexible. Certain conformational flexibility seems essential for the peptides' effective insertion into phospholipid monolayers (*13*) and their interference with potential target molecules in the specific model. In contrast to native clavanin A, the more helical, amphipathic derivative AAA displays reduced insertion efficiency into monolayers. Perhaps surprising at first sight, this peptide retains its ability to nonspecifically permeabilize lipid bilayers upon interaction with target membranes at both neutral and low pH, according to the model proposed in Figure 10A. The relative few AAA molecules that do insert at neutral pH may form clusters that are capable of permeabilizing the membrane. Vice versa, the high insertion of wild-type clavanin into monolayers at low pH does not necessarily imply that the peptide organizes in permeabilizing structures. The apparent discrepancies between the permeabilizing efficiency of model membranes and intact cells are likely to be due to one or more of the numerous differences between these systems.

In view of the specific mechanism of clavanin A at low pH, the behavior of another class of histidine-rich peptides, the histatins, becomes relevant. These histatins show broad-spectrum antifungal and antibacterial activities (*39*), are nonlytic to lipid membranes (*40*, *41*), and bind to specific sites on the cell membrane (*42*, *43*). Nonlytic efflux of ATP from target cells is observed when adding one member of this peptide class, histatin 5, inducing cell death (*43*, *44*). Apparently, this histatin 5 targets to the energized mitochondrion by dissipating its transmembrane potential, after it has been internalized (*41*, *45*). Other peptide antibiotics, such as pyrrhocoricin (*46*) and efrapeptin (*47*), have been reported to interfere with ATP synthesis or hydrolysis, due to specific interaction with target molecules.

This study demonstrates that clavanin A is a special membrane-active peptide that has access to two distinct modes of actions, depending on the pH of the environment. Whereas a putative model for the unique mode of action at low pH is presented, the exact nature of the interactions of clavanin A with specific constituents of biological membranes at low pH remains to be resolved. In addition, it remains to be answered if this duality has a physiological relevance to *Styela clava*, the organism that produces the peptide. Possibly, there is a role in protecting *Styela clava* against its own toxin. Clavanin peptides are present in hemocytes of this marine organism, generally within distinctive cytoplasmic granules (*22*, *48*). Although specific information for *Styela clava* is lacking, there are indications that the hemocytes of tunicates such as *Styela clava* contain specific cell compartments with an internal pH of 5.0 (*22*). When the tunicate stores its clavanins in specialized, low pH compartments that lack the specific membrane target, the host is protected against its own membrane-active peptides.

ACKNOWLEDGMENT

The authors thank Dr. Annemieke Ultee and Dr. Roy Moezelaar for the useful suggestions related to the intracellular pH measurements.

REFERENCES

1. Lee, I. H., Zhao, C., Cho, Y., Harwig, S. S. L., Cooper, E. L., and Lehrer, R. I. (1997) *FEBS Lett.*, 158−162.
2. Hancock, R. E. W., and Lehrer, R. (1998) *TIBTECH 16*, 82−88.

3. Epand, R. M., and Vogel, H. J. (1999) *Biochim. Biophys. Acta 1462*, 11−28.
4. Van't Hof, W., Veerman, E. C. I., Helmerhorst, E., and Van Nieuw Amerongen, A. (2001) *Biol. Chem. 382*, 597−619.
5. Tossi, A., Sandri, L., and Giangaspero, A. (2000) *Biopolymers 55*, 4−30.
6. Zhao, C., Liaw, L., Lee, I. H., and Lehrer, R. I. (1997) *FEBS Lett. 410*, 490−492.
7. Matsuzaki, K. (1999) *Biochim. Biophys. Acta 1462*, 1−10.
8. Boman, H. G. (1991) *Cell 65*, 205−207.
9. Nicolas, P., and Mor, A. (1995) *Annu. Rev. Microbiol. 49*, 277−304.
10. Oren, Z., and Shai, Y. (1999) *Biopolymers 47*, 451−463.
11. Wieprecht, T., Dathe, M., Epand, R. M., Beyermann, M., Krause, E., Maloy, W. L., MacDonald, D. L., and Bienert, M. (1997) *Biochemistry 36*, 12869−12880.
12. Sitaram, N., and Nagaraj, R. (1999) *Biochim. Biophys. Acta 1462*, 29−54.
13. Van Kan, E. J. M., Van der Bent, A., Demel, R. A., and De Kruijff, B. (2001) *Biochemistry 40*, 6398−6405.
14. Dathe, M., Wieprecht, T., Nikolenko, H., Handel, L., Maloy, W. L., MacDonald, D. L., Beyermann, M., and Bienert, M. (1997) *FEBS Lett. 403*, 208−212.
15. Dathe, M., and Wieprecht, T. (1999) *Biochim. Biophys. Acta 1462*, 71−87.
16. Hong, J., Oren, Z., and Shai, Y. (1999) *Biochemistry 38*, 16963−16973.
17. Matsuzaki, K., Nakamura, A., Murase, O., Sugishita, K., Fujii, N., and Miyajima, K. (1997) *Biochemistry 36*, 2104−2111.
18. Dathe, M., Nikolenko, H., Meyer, J., Beyermann, M., and Bienert, M. (2001) *FEBS Lett. 501*, 146−150.
19. Wang, W., Smith, D. K., and Chen, H. M. (1999) *Biochim. Biophys. Acta 1473*, 418−430.
20. Wiese, A., Munstermann, M., Gutsmann, T., Lindner, B., Kawahara, K., Zahringer, U., and Seydel, U. (1998) *J. Membr. Biol. 162*, 127−138.
21. Dathe, M., Schümann, M., Wieprecht, T., Winkler, A., Beyermann, M., Krause, E., Matsuzaki, K., Murase, O., and Bienert, M. (1996) *Biochemistry 35*, 12612−12622.
22. Lee, I. H., Cho, Y., and Lehrer, R. I. (1997) *Infect. Immun. 65*, 2898−2903.
23. Taylor, S. W., Grey Craig, A., Fischer, W. H., Park, M., and Lehrer, R. I. (2000) *J. Biol. Chem. 275*, 38417−38426.
24. Breukink, E., Van Kraaij, C., Demel, R. A., Siezen, R. J., Kuipers, O. P., and De Kruijff, B. (1997) *Biochemistry 36*, 6968−6976.
25. Breeuwer, P., Drocourt, J.-L., Rombouts, F. M., and Abee, T. (1996) *Appl. Environ. Microbiol. 62*, 178−183.
26. Glaasker, E., Konings, W. N., and Poolman, B. (1996) *Mol. Membr. Biol. 13*, 173−181.
27. Boyer, M. J., and Hedley, D. W. (1994) *Methods in Cell Biology*, pp 135−148, Academic Press Inc., New York.
28. Silberstein, A., Mirzabekov, T., French Anderson, W., and Rozenberg, Y. (1999) *Biochim. Biophys. Acta 1461*, 103−112.
29. Hope, M. J., Bally, M. B., Webb, G., and Cullis, P. R. (1985) *Biochim. Biophys. Acta 812*, 55−62.
30. Molenaar, D., Abee, T., and Konings, W. N. (1991) *Biochim. Biophys. Acta 1115*, 75−83.
31. Allen, T. M. (1984) in *Liposome technology* (Gregoriadis, G., Ed.), pp 177−182, CRC Press, Boca Raton, FL.
32. Bruch, M. D., Dhingra, M. M., and Gierasch, L. M. (1991) *Proteins: Struct., Funct., Genet. 10*, 130−139.
33. Shai, Y. (1999) *Biochim. Biophys. Acta 1462*, 55−72.
34. Cox, S. D., Mann, C. M., Markham, J. L., Gustafson, J. E., Warmington, J. R., and Wyllie, S. G. (2001) *Molecules 6*, 87−91.
35. Cox, S. D., Mann, C. M., Markham, J. L., Bell, H. C., Gustafson, J. E., Warmington, J. R., and Wyllie, S. G. (2000) *J. Appl. Microbiol. 88*, 170−175.
36. Elston, T., Wang, H., and Oster, G. (1998) *Nature 391*, 510−513.
37. Lanyi, J. K., and Pohorille, A. (2001) *Trends Biotechnol. 19*, 140−144.
38. Armstrong, K. M., and Baldwin, R. L. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 11337−11340.
39. Helmerhorst, E. J., Van't Hof, W., Veerman, E. C. I., Simoons-Smit, I., and Van Nieuw Amerongen, A. (1997) *Biochem. J. 326*, 39−45.
40. Helmerhorst, E. J., Van't Hof, W., Breeuwer, P., Veerman, E. C. I., Abee, T., Troxler, R. F., Van Nieuw Amerongen, A., and Oppenheim, F. G. (2001) *J. Biol. Chem. 276*, 5643−5649.
41. Gyurko, C., Lendenmann, U., Troxler, R. F., and Oppenheim, F. G. (2000) *Antimicrob. Agents Chemother. 44*, 348−354.
42. Edgerton, M., Koshlukova, S. E., Lo, T. E., Chrzan, B. G., Straubinger, R. M., and Raj, P. A. (1998) *J. Biol. Chem. 273*, 20438−20447.
43. Koshlukova, S. E., Lloyd, T. L., Araujo, M. W. B., and Edgerton, M. (1999) *J. Biol. Chem. 274*, 18872−18879.
44. Koshlukova, S. E., Araujo, M. W. B., Baev, D., and Edgerton, M. (2000) *Infect. Immun. 68*, 6848−6856.
45. Helmerhorst, E. J., Breeuwer, P., Van't Hof, W., Walgreen-Weterings, E., Oomen, L. C. J. M., Veerman, E. C. I., Van Nieuw Amerongen, A., and Abee, T. (1999) *J. Biol. Chem. 274*, 7286−7291.
46. Kragol, G., Lovas, S., Varadi, G., Condie, B. A., Hoffmann, R., and Otvos, L. (2001) *Biochemistry 40*, 3016−3026.
47. Abrahams, J. P., Buchanan, S. K., Van Raaij, M. J., Fearnley, I. M., Leslie, A. G. W., and Walker, J. E. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 9420−9424.
48. Lehrer, R. I., Lee, I. H., Menzel, L., Waring, A., and Zhao, C. (2001) *Adv. Exp. Med. Biol. 484*, 71−76.

BI012162T