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The role of the abundant phenylalanines in the mode of action of the antimicrobial peptide clavanin

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Abstract

Clavanin A is a special antimicrobial peptide that acts at the level of the membrane via a pH-dependent mechanism. At neutral pH, clavanin disrupts biological and model membranes in a nonspecific manner, causing efflux of large molecules. At mildly acidic conditions, however, the peptide efficiently kills bacteria by permeabilizing their membrane most likely by interacting with proteins involved in proton translocation [Biochemistry 41 (2002) 7529]. Clavanin A is unusually rich in phenylalanines with 5 out of 23 residues, which suggests that these residues are functionally important. A set of mutants, in which all Phe residues are replaced by either Ile, Leu, Trp, or Tyr was used to investigate the role of these amino acids. The antimicrobial activities of the different peptides both at neutral and low pH show that the presence of phenylalanine is not essential nor optimal, as the Trp, Leu, and Ile mutant are equally or more active than the wild-type component. In general, at neutral pH, the biological activities correlate well with the peptides' ability to interact with membrane lipids. Correspondingly, the permeabilization efficiencies of biological and model membranes of the various derivatives were found to be closely related to their ability to adopt α-helical structures, and follows the order 5L>5W>5I>5Y>wild type. The results suggest an important role for the Phe residues, in providing the peptide in a balanced manner with sufficient hydrophobicity, and therewith membrane affinity, as well as conformational flexibility.

Keywords: Peptide; Antibiotic; Biomembrane; Model membrane; Mechanism; Phenylalanine

1. Introduction

Antimicrobial peptides are widely distributed in nature. Many organisms use these peptides as an innate defense mechanism that protects them against invading microorganisms [2–5]. Generally, these peptides are cationic amphipathic molecules, due to abundant basic and hydrophobic amino acids. These features facilitate interactions of the peptide antibiotics with their primary target, the bacterial membrane [6–8]. The antimicrobial effect of many of these membrane-active peptides is exerted via permeabilization of this target membrane, due to the formation of pore-like structures or other membrane-disrupting modes of organization of the peptides [6,7,9–20]. Despite their similar cationic and amphipathic nature, the primary sequences of the various antimicrobial peptides that have been identified are highly heterogeneous, and some of them are very specific [7,20].

One interesting example in this respect is the class of the clavanins, which were originally isolated from the hemocytes of the marine organism, *Styela clava* [21–23]. The primary structures of the clavanins, A, B, C, D, and E, are highly homologous. They contain 23 amino acids and their C-termini are amidated. The clavanin sequences

Abbreviations: CD, circular dichroism; CF, carboxyfluorescein; cfu, colony forming unit; $DiSC_2(5)$, 3,3′-diethylthiodicarbocyanine iodide; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; ES-MS, electron spray mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; LUV, large unilamellar vesicle; MH, Mueller Hinton; MIC, minimal inhibitory concentration; MRS, Man, Rogasa, Sharpe; OD, optical density; RP-HPLC, reversed phase high performance liquid chromatography; SUV, small unilamellar vesicle; TFA, trifluoroacetic acid; Tris, Tris-(hydroxymethyl)aminomethane.

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distinguish themselves from many other antimicrobial peptides, as they are rich in histidines, glycines, and phenylalanines (Table 1).

Depending on the pH of the environment, clavanin A permeabilizes target membranes via two distinctly different mechanisms [1]. At neutral pH, when the peptide has a slight positive charge, membrane bilayers are destabilized in an aspecific manner driven by hydrophobic interactions with a lack of lipid head group specificity [1,24]. When the histidines are protonated at pH 5.5, clavanin A becomes a much more potent antimicrobial agent than at neutral pH [1,25]. This enhanced activity is accompanied by an increased ability to collapse the membrane potential. Strikingly, lipid bilayers are not efficiently permeabilized at low pH [1], which immediately suggests that at this pH, the peptide uses another cell killing mechanism most likely by interaction with proteins involved in proton translocation [1].

For this more specific mode of action, the histidines and glycines play a major role [1,24]. The latter residues inhibit the formation of α-helical structures in the peptide and thereby disfavour aspecific permeabilization of the bilayer. These properties distinguish the clavanins from most other natural antimicrobial peptides and suggest that they form a special class of membrane active peptides.

Phenylalanine is remarkably abundant in the clavanin A sequence with 5 out of 23 residues. This paper focuses on these aromatic amino acids. Aromatic residues in general appear to play special roles in peptide/protein–membrane interactions because of their interface-seeking properties [24,26]; moreover, the bulky Phe is rather hydrophobic and thereby could contribute significantly to the membrane interaction of the peptide.

To get insight into the role of the phenylalanines in the mode of action, a series of clavanin analogues was prepared in which these residues are replaced either by the aliphatic leucines and isoleucines, or by the other aromatic amino acids tryptophan and tyrosine. Membrane activities were analyzed in monolayer insertion experiments, membrane leakage experiments using model membrane systems, intact bacteria, and mammalian cells, i.e. rabbit erythrocytes, and compared to the ability of the peptides to inhibit the growth of bacteria. The relations between the peptides' structural properties, as evaluated using circular dichroism (CD) spectroscopy, and their membrane activities are established. The results demonstrate that the phenylalanines present in the clavanin sequence do have a profound role in the peptides' membrane and biological activities, which is closely connected with the induced peptide conformation upon interaction with membranes.

Table 1
Clavanin A and derivatives that have the F residues mutated into I, L, W, or Y, and mass spectral analysis data

Peptide sequence	Code	Mass	
		Calculated	Measured
Wild-type clavanin A			
VFQFLGKIIHHVG NFVHGFSHVF-NH_2	5F	2664.43	2664.74 ± 0.52
Mutants			
V**I**Q**I**LGKIIHHVG N**I**VHG**I**SHV**I**-NH_2	5I	2496.04	2496.08 ± 0.21
V**L**Q**L**LGKIIHHVG N**L**VHG**L**SHV**L**-NH_2	5L	2496.04	2496.13 ± 0.19
V**W**Q**W**LGKIIHHVG N**W**VHG**W**SHV**W**-NH_2	5W	2861.31	2861.50 ± 0.07
V**Y**Q**Y**LGKIIHHVG N**Y**VHG**Y**SHV**Y**-NH_2	5Y	2746.13	2746.05 ± 0.16

2. Materials and methods

2.1. Materials

N-α-Fmoc-protected amino acids, coupling reagent, and resin used in peptide synthesis were purchased from Novabiochem (Läufelfingen, Switzerland). Solvents for peptide synthesis and HPLC were obtained from Biosolve (Valkenswaard, The Netherlands). The bacterial strains *Micrococcus flavus* (DSM 1790) and *Lactobacillus sake* (ATCC 15521 = DSM 20017 = CECT 906) were cultured at 30 °C in Mueller Hinton and MRS broth (Oxoid, Unipath Ltd. Basingstoke, Hampshire, England), respectively. The membrane potential-sensitive probe 3,3′-diethylthiodicarbocyanine iodide [$DiSC_2(5)$] was purchased from Molecular Probes Inc. 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). Carboxyfluorescein was purchased from Eastman Kodak Co. (Rochester, NY) and purified as described [27]. All other chemicals used were of analytical or reagent grade.

2.2. Peptide synthesis and purification

Wild-type clavanin A and its mutant peptides (Table 1) were prepared using automated solid phase peptide synthesis as described [24]. The peptides were purified using RP-HPLC on a Waters Symmetry C18 column (Milford, MA), applying a linear gradient of acetonitrile and water containing 0.05% TFA. Analytical RP-HPLC [24] and electron spray mass spectrometry (ES-MS) were performed in order to determine the peptides' purity and molecular masses to confirm their composition.

2.3. Antimicrobial activity measurements

The antimicrobial activities of the clavanin peptides were determined in growth inhibition assays against *M. flavus* (DSM 1790) and *L. sake* (ATCC 15521 = DSM 20017 =

CECT 906). *M. flavus* and *L. sake* cells were grown from an overnight culture to mid-logarithmic phase in Mueller Hinton (MH) and Man, Rogasa, Sharpe (MRS) broth, respectively, diluted to 3.2×10^5 cfu/ml, and incubated with serial dilutions of the peptides in 96-well plates. After 24-h incubation, growth inhibition percentages were calculated from the $OD_{595\ nm}$ values versus that of the growth controls. Subsequently, the minimal inhibitory concentration (MIC) values were derived from the growth inhibition curves.

2.4. Membrane activity on intact M. flavus cells

The fluorescent membrane-potential-sensitive probe, 3,3′-diethylthiodicarbocyanine iodide [$DiSC_2(5)$] [28] was used to determine the effect of the peptides on the membrane integrity of intact *M. flavus* cells. *M. flavus* cells were grown at 30 °C to mid-logarithmic phase, harvested, washed, and resuspended with buffer (100 mM NaCl, 25 mM potassium phosphate buffer containing 0.2% glucose, pH 7) at 4 °C, and stored on ice to immediately start fluorescence measurements.

Therefore, cells were added to a fluorescence cuvette to an OD_{600nm} of 0.03 together with 20 nM $DiSC_2(5)$. The fluorescence emission was monitored at 20 °C using a LS 50B spectrofluorimeter (Perkin-Elmer, Bucks, UK) at 670 nm (excitation of 650 nm) as described before [24]. Full dissipation of the membrane potential was obtained by adding gramicidin D (final concentration 0.06 nM). The membrane potential dissipating activity of the peptides is expressed as,

$$\%\ \text{inhibition} = 100[(F_p - F_0)/(F_g - F_0)]$$

in which F_0 is the stable fluorescence value after addition of the $DiSC_2(5)$ dye, F_p is the fluorescence value 2 min after addition of the peptides, and F_g the fluorescence signal after addition of gramicidin D.

2.5. Hemolytic activity assay

The hemolytic activity of the peptides was determined using rabbit red blood cells (rRBC). The red blood cells were collected from heparin-treated blood by centrifugation ($2000 \times g$ for 5 min), washed three times, and resuspended in 150 mM NaCl, 10 mM Tris/HCl, pH 7.4 buffer. Erythrocyte suspensions (5×10^8 cells/ml) in physiological buffer were incubated with two-fold serially diluted peptides in 96-well plates for 1 h at 37 °C. After this incubation, the suspensions were centrifuged ($1238 \times g$ for 5 min). The absorbance of the supernatant was measured at 405 nm to detect the release of hemoglobin. Zero hemolysis (blank) and 100% hemolysis were determined from suspensions of rRBC incubated in physiological salt buffer and milliQ water, respectively.

2.6. Monolayer experiments

Monolayer surface pressure measurements were performed using the (platinum) Wilhelmy plate method [29] at room temperature as described before [24]. DOPG/DOPE (1:3) phospholipid monolayers were spread on a subphase of 10 mM potassium phosphate buffer (pH 7). The standard deviations in the monolayer insertion measurements were typically in the order of 5%.

2.7. Dye leakage experiments from lipid vesicles

Carboxyfluorescein (CF)-loaded large unilamellar vesicles (LUVs) were prepared after hydration of dry DOPC films with 15 mM CF solution at pH 7. LUVs were prepared using the extrusion technique according to Hope et al. [30] through 400-nm pore size polycarbonate filters. Nonencapsulated CF was removed by gel filtration on Sephadex G75 columns equilibrated with 150 mM NaCl, 10 mM Tris/HCl buffer (pH 7.0) [24]. The phospholipid content was determined as inorganic phosphate according to Rouser et al. [31].

CF leakage from DOPC LUVs in 150 mM NaCl, 10 mM Tris/HCl buffer (pH 7.0) was monitored by measuring the fluorescence emission at 515 nm (excitation 492 nm) on a SPF 500 C spectrophotometer (SLM instruments Inc., USA) at 20 °C and quantified as reported earlier [24].

2.8. CD measurements

CD spectra were recorded in absence and presence of small unilamellar vesicles (SUVs) on a Jasco-J810 spectropolarimeter in the 180–260 nm range using quartz cuvettes with a path length of 0.1 cm at 20 °C, as previously described [1]. Five scans were averaged and corrected for the contributions of vesicles and solvents. The peptides were dissolved in 10 mM potassium phosphate buffer (pH 7.0) at a concentration of 0.25 mg/ml in the cuvette. After hydration of dry DOPC films with 10 mM potassium phosphate buffer (pH 7.0), SUVs were prepared by sonication using a Branson 250 tip sonicator (Danburry, Connecticut) for 3–5 min with 20-s time intervals and an input power of 40 W until the dispersions were clear. The peptides at a peptide concentration of 0.25 mg/ml were added to DOPC SUVs to a peptide/lipid ratio of 1:25 (mol/mol) and incubated at room temperature for at least 30 min prior to the measurements.

3. Results

3.1. Peptide design and characterization

To study the role of Phe in clavanin, the five aromatic Phe residues (5F) were either replaced with the aliphatic hydrophobic residues isoleucine (5I) or leucine (5L), or

Table 2
Antimicrobial activities against *M. flavus* and *L. sake* of wild-type clavanin A and mutants 5I, 5L, 5W, and 5Y given as MIC values, derived from growth inhibition experiments in MH and MRS broth, respectively

Peptide	Minimal inhibitory concentrations (μM)[a]		
	M. flavus (pH 7.0)	*L. sake* (pH 6.5)	*L. sake* (pH 5.6)
5F	4.9 ± 1.7	6.6 ± 3.7	0.6 ± 0.2
5I	1.6 ± 0.0	2.4 ± 0.9	1.0 ± 0.4
5L	1.0 ± 0.4	3.1 ± 0.0	0.8 ± 0.0
5W	1.4 ± 0.0	22 ± 0.0	0.7 ± 0.0
5Y	11 ± 0.0	>91	11 ± 0.0

[a] The MIC values represent the average values of at least three independent bioactivity measurements.

with the other aromatic residues tryptophan (5W) or tyrosine (5Y) (Table 1). The mass spectral data (Table 1) confirmed the peptides' identity and purity. Their antimicrobial activities were explored in bacterial growth assays.

3.2. Antimicrobial activities

The activities of the clavanin derivatives on the gram-positive bacteria *M. flavus* and *L. sake* were determined as described before [1,24]. The MIC values of wild-type clavanin A and its mutants 5I, 5L, 5W, and 5Y against these two test organisms are depicted in Table 2. Clavanin A effectively inhibited the growth of both these test organisms around neutral pH, displaying MIC values around 5 and 7 μM for *M. flavus* and *L. sake*, respectively, in agreement with our previous data [1,24].

Peptide mutants, 5L, 5I, and 5W, fully inhibited *M. flavus* growth at concentrations ranging from 1.0 to 1.6 μM, and therewith proved more potent than the wild-type peptide. This result demonstrates that the Phe residues are not essential for the antimicrobial activity. The tyrosine mutant 5Y appeared less active at neutral pH than the native peptide. Comparable results were obtained against *L. sake*. The peptides 5I and 5L were more potent than native clavanin A around neutral pH. Mutant 5W appeared less potent against this lactic acid bacterium.

The lactic acid bacterium *L. sake* was selected for its ability to grow well at mildly acidic pH. Previously, wild-type clavanin A was shown to become more potent at low pH conditions, which is accompanied by a different mode of action [1]. Upon lowering the pH of the growth medium from 6.5 to 5.6, the MIC value of wild-type clavanin A against *L. sake* showed about a 10-fold decrease. Also, the clavanin mutants displayed increased activities when the pH of the medium was lowered to 5.6. Except for mutant 5Y, the differences between the various peptides are much less pronounced at low pH, which suggests that there is no special role for Phe in the low-pH mechanism. Therefore, we concentrated at neutral pH conditions to further analyze the effect of the Phe residues on the mode of action of clavanin.

3.3. Membrane permeabilization of intact bacterial cells

The membrane potential-sensitive carbocyanine dye $DiSC_2(5)$ was used to determine the effect of the clavanins on the membrane integrity of intact *M. flavus* cells at neutral pH, using the protocols described earlier [24]. This probe accumulates in the membrane interior of energized cells and, therewith, its fluorescence signal is strongly quenched, as visible in Fig. 1A (first arrow). After a stable signal is observed, wild-type clavanin A and derivative 5W (second arrow) were added. The typical tracings show that mutant 5W much more effectively disturbs the membrane integrity than the wild-type peptide, as indicated by an increase in fluorescence due to a collapse of the ion gradients that generate the membrane potential. The membrane-potential-dissipating activities of these membrane-active peptides can be determined in relation to the effect of gramicidin D (third arrow), which fully collapses the membrane potential.

All clavanin peptides tested against energized *M. flavus* cells demonstrated efficient permeabilization of the bacterial

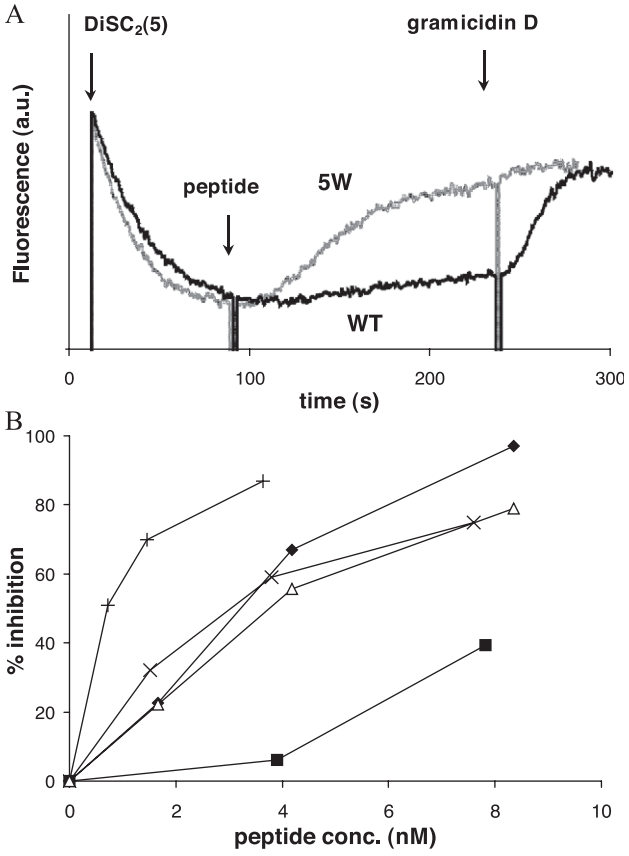

Fig. 1. (A) Fluorescence of the membrane-potential-sensitive probe $DiSC_2(5)$ to determine the effect of wild-type clavanin A and derivative 5W (peptide concentration 4 nM) on the membrane potential of intact *M. flavus* cells. Fluorescence measurements were performed as described in Materials and methods. (B) Effect of clavanin A (■) and its mutants 5I (△), 5L (◆), 5W (+), and 5Y (×) on the membrane potential of intact *M. flavus* cells (OD_{600} = 0.03) measured using the membrane-potential-sensitive dye $DiSC_2(5)$ at pH 7.0.

membranes (Fig. 1B) with the smallest effect observed for wild-type clavanin A. The four derivatives proved highly effective in dissipating the membrane potential. Their improved membrane permeabilizing activities do not point to a specific role for Phe.

3.4. Hemolytic activities

Rabbit erythrocytes were used as a convenient model system for mammalian cells from which the release of hemoglobin is a measure for membrane permeabilization. In Fig. 2, the hemolytic activities of clavanin A and its mutants 5I, 5L, 5W, and 5Y are compared. Wild-type clavanin A proved nonlytic against the erythrocytes at peptide concentrations up to 400 μM. For the peptide mutants, the hemolytic activities increased in the order of 5Y < 5I < 5W < 5L (Fig. 2). These results demonstrate a difference of several orders of magnitude in membrane activity of the peptide against bacterial and mammalian cells. Secondly, the peptides' membrane permeabilizing properties follow largely the order that was observed for *M. flavus*.

3.5. Monolayer insertion

To gain insight into the importance of the Phe residues in the clavanin A sequence for interaction with membrane lipids, peptide–lipid interactions were analyzed by the monolayer technique. For this approach, the DOPG/DOPE (1:3) system was selected. This lipid composition shows a very similar interaction with clavanins as zwitterionic lipids [24]; however, it resembles more the lipid composition of bacterial membranes. Peptide insertion into phospholipid monolayers was monitored after its injection to a 0.7 μM concentration underneath the monolayer surface at varying initial surface pressures (π_i) ranging from 15 to 35 mN/m at pH 7. At this concentration, wild-type clavanin A induces a maximal surface pressure increase ($\Delta\pi$) [24].

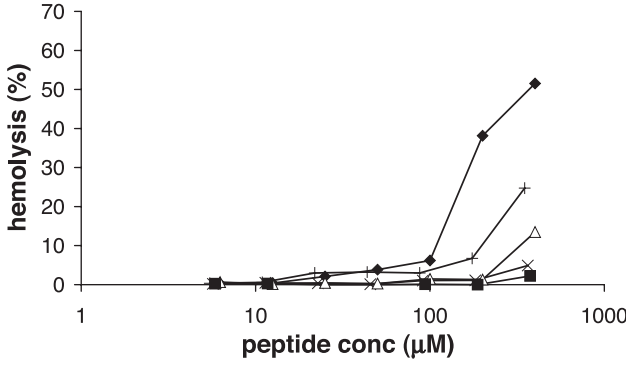

Fig. 2. Hemolytic activities of wild-type clavanin A (■) and mutants 5I (△), 5L (◆), 5W (+), and 5Y (×) on rabbit erythrocytes under physiological conditions in 150 mM NaCl, 10 mM Tris/HCl buffer at pH 7.4.

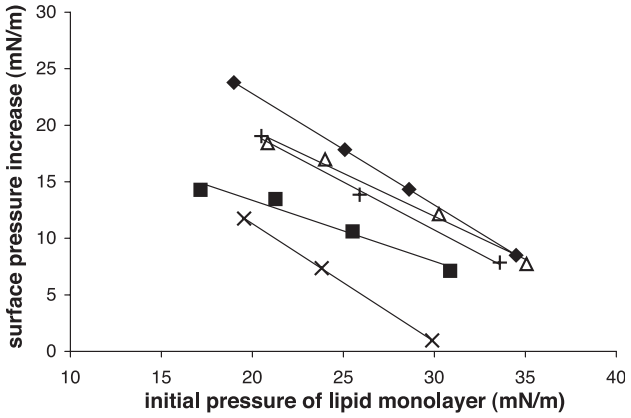

Fig. 3. Penetration of wild-type clavanin A (■), mutant 5I (△), mutant 5L (◆), mutant 5W (+), and mutant 5Y (×) (0.7 μM) into DOPG/DOPE (1:3) lipid monolayers spread at 10 mM potassium phosphate buffer, pH 7.0. The increases in surface pressure ($\Delta\pi$, *y*-axis) after adding peptide stock solutions (1 mg/ml) underneath the monolayers to yield a peptide concentration of approximately 0.7 μM are plotted versus the initial monolayer surface pressure (π_i, *x*-axis).

The maximal surface pressure increases induced by wild-type clavanin and its four derivatives derived from typical monolayer insertion curves (data not shown) are plotted as a function of the initial surface pressure in Fig. 3. The five peptides all efficiently inserted into the monolayers at low initial surface pressures as witnessed from the increased surface pressure. With a maximal surface pressure increase of 10 mN/m at an initial surface pressure of 20 mN/m, peptide mutant 5Y displayed the lowest extent of insertion between the phospholipids. Its ability to penetrate into the monolayer rapidly decreased upon raising the initial monolayer pressure. Extrapolation of the monolayer insertion data yields the exclusion pressure, defined as the pressure at which the peptide cannot penetrate into the monolayer. For peptide 5Y, this value is at 31 mN/m. Wild-type clavanin and its mutants, 5I, 5W, and 5L, show more pronounced lipid interactions. Their exclusion pressures lie in the 42–46 mN/m range, which is well above the physiological range of 30–35 mN/m [29], suggesting that these peptides are able to insert in biological membranes. Among these peptides, wild-type clavanin showed the least strong interaction. In order to investigate whether these peptide–lipid interactions can lead to destabilization of the membranes, the ability of the peptides to permeabilize phospholipid vesicles was analyzed.

3.6. CF leakage from lipid vesicles

The ability of the peptides to induce membrane leakage was monitored using carboxyfluorescein (CF)-loaded large unilamellar DOPC vesicles at neutral pH. Typical fluorescence tracings are depicted in the insert of Fig. 4, which shows that the release of CF induced by clavanin mutant 5L is much more efficient that that induced by the wild-type molecule.

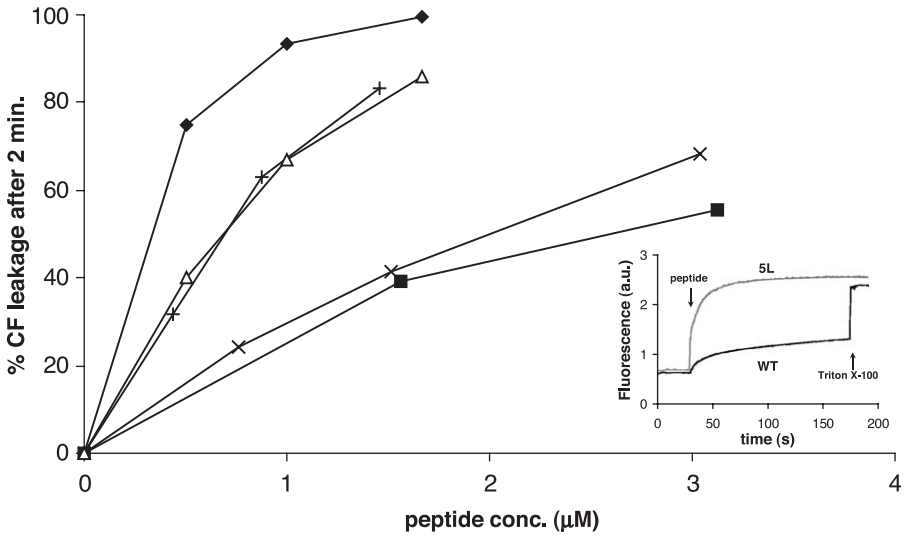

Fig. 4. Release of the fluorescent probe carboxyfluorescein from DOPC LUVs in 10 mM Tris/HCl, 150 mM NaCl buffer (pH 7.0), measured 2 min after the addition of wild-type clavanin A (■), mutant 5I (△), mutant 5L (◆), mutant 5W (+), and mutant 5Y (×). Insert: Typical CF leakage from DOPC LUVs in 10 mM Tris/HCl, 150 mM NaCl buffer (pH 7.0) after addition (first arrow) of wild-type clavanin A and derivative 5L (peptide conc. 1.5 μM). Full release of CF was obtained after addition of Triton X-100 (second arrow). The relative levels of CF release after 2 min are depicted at the *y*-axis.

Fig. 4 summarizes the data for all mutants at different peptide concentrations as leakage after 2 min. All peptides were able to induce CF release from DOPC vesicles, but wild-type clavanin A and mutant 5Y least efficiently permeabilized these model membranes consistent with the monolayer insertion data.

To see whether the differences in membrane permeabilizing activities between the peptides result from distinctions in conformational behaviour, the secondary structures of the derivatives were investigated using CD spectroscopy.

3.7. Conformational studies

The CD spectra of wild-type clavanin A and the four peptide derivatives, measured in 10 mM potassium phosphate buffer (pH 7.0), are shown in Fig. 5. The five peptides display distinctly different CD spectra, demonstrating that the folding behaviour of the peptides in aqueous solution highly depends on the nature of the substitution of Phe by other amino acids. The wild-type clavanin A spectrum in aqueous solution did not represent a strictly defined secondary structure pattern, as described previously [1]. Peptide mutant 5L, under these conditions, showed a CD spectrum typical of an α-helix conformation with characteristic double minima at 208 and 222 nm [32,33]. The 5L spectrum resembles the spectrum of wild-type clavanin A in TFE; however, its intensity is somewhat lower than that of clavanin A in TFE [1,24]. Peptide mutant 5I, on the other hand, displayed a typical β-sheet spectrum with one single minimum around 216 nm [32]. The tyrosine mutant 5Y did not fold into a well-defined α-helix or β-sheet structure, but

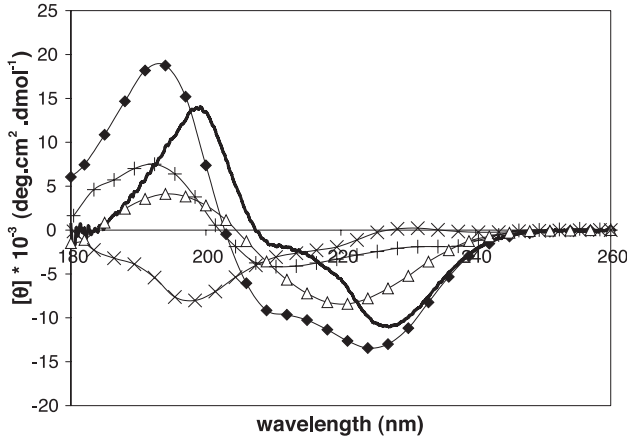

Fig. 5. CD spectra of wild-type clavanin A (—), mutant 5I (△), mutant 5L (◆), mutant 5W (+), and mutant 5Y (×) (peptide concentration = 0.25 mg/ml) measured in 10 mM potassium phosphate buffer, pH 7.0.

Table 3
Ellipticity values, retention behaviour during analytical RP-HPLC, and calculated peptide hydrophobicities of wild-type clavanin A and derivatives 5I, 5L, 5W, or 5Y

Peptide	$-\theta_{222\ nm} \times 10^{-3}$ [a]		Retention time (min)[b]	H[c]
	Aq.	DOPC		
5F	7.9	13	34.1	0.15
5I	8.3	14	32.5	0.17
5L	13	23	36.4	0.13
5W	2.9	18	34.0	0.10
5Y	1.2	10	23.4	0.02

[a] Mean residue ellipticity (deg cm^2 $dmol^{-1}$) of the peptides (0.25 mg/ml) in 10 mM potassium phosphate buffer in the absence and presence of DOPC SUVs (P/L = 1:25) at 222 nm at 20 °C.

[b] The retention times were determined during analytical RP-HPLC on a Symmetry C18 column using a linear gradient of acetonitrile and water containing 0.05% TFA.

[c] Peptide hydrophobicity (H) was calculated using the numerical hydrophobicities of the individual amino acid residues of the "consensus" hydrophobicity scale according to Eisenberg et al. [43].

displayed a random coil spectrum with an intense negative band at 198 nm [33]. Finally, the tryptophan mutant 5W did not show a CD spectrum corresponding to a defined secondary structure. Interpretation of this CD spectrum was complicated due to the intrinsic contributions of these tryptophans to the CD signal [33,34]. The ellipticities at 222 nm of the clavanin derivatives in buffer (pH 7) are depicted in Table 3 as an estimate of the α-helix degree of the various peptides.

The differentiation in the CD spectra observed at neutral pH is lost in 10 mM potassium phosphate buffer at pH 5.5, at which the histidine residues become protonated. Under these conditions, all peptides show random coil spectra (data not shown) similar to that observed for the tyrosine mutant 5Y at pH 7.

When antimicrobial peptides have the possibility to interact with membranes, the formation of secondary structures is usually promoted. To investigate this for the various clavanin mutants, the peptides were added to SUVs prepared from DOPC (at a P/L molar ratio of 1:25), in 10 mM potassium phosphate buffer (pH 7.0). Information on the secondary structure of the various peptides in presence of these phospholipid vesicles was obtained from the CD spectra shown in Fig. 6.

In the presence of DOPC SUVs, the shape of the CD spectrum of wild-type clavanin A is not affected but the intensity is increased compared to its spectrum in aqueous solution (Fig. 5), demonstrating the interactions of the peptide with the vesicles.

In clear contrast, the interaction between all four mutant peptides and the DOPC SUVs results in the promotion of α-helix structures as demonstrated by the characteristic spectral line shapes (Fig. 6). The spectrum of peptide mutant 5L is essentially identical to the wild-type clavanin A spectrum in the α-helix inducing solvent TFE [1,24]. The intensity of the CD signal varies for the various peptide mutants, suggesting varying degrees of α-helicity. The helicity in the membrane-bound state, as estimated from the molar ellipticity measured at 222 nm, followed the order of 5L>5W>5I>5Y (Table 3). Interestingly, these helicities in presence of lipid vesicles correlate well with the retention times of the clavanins in RP-HPLC (Table 3), which suggests that the peptides adopt similar conformations upon interaction with and comparably bind to both the stationary phase in RP-HPLC and lipid vesicles.

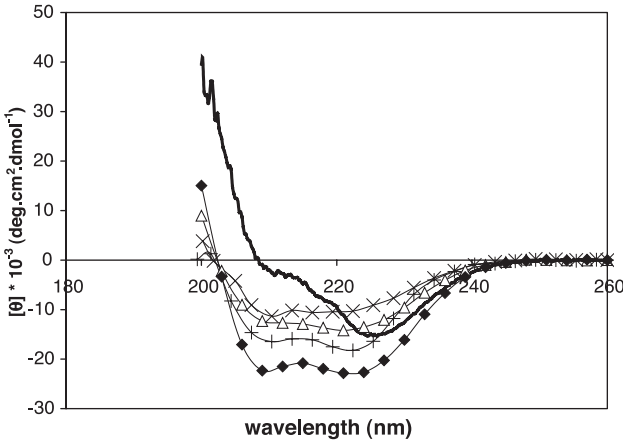

Fig. 6. CD spectra of wild-type clavanin A (—), mutant 5I (△), mutant 5L (◆), mutant 5W (+), and mutant 5Y (×) (peptide concentration = 0.25 mg/ml) measured in presence of DOPC SUVs (peptide/lipid molar ratio = 1:25) in 10 mM potassium phosphate buffer, pH 7.0.

4. Discussion

Clavanin derivatives were synthesized to investigate whether the phenylalanines have a specific role in the mode of action of the native peptide. As mutations of these Phe residues lead to large differences in the peptides' biological effects and membrane activities, it can be concluded that these amino acids are important for the mechanism of clavanin A.

Clavanin has a dual activity. It very efficiently kills bacteria at low pH by permeabilizing their membrane most likely by interacting with proteins involved in proton translocation [1]. The results clearly demonstrate that the phenylalanines do not play a special role in this low pH mechanism, as all mutants except 5Y showed comparable increased activities at pH 5.6. The low activity of derivative 5Y most likely results from its reduced affinity for membrane lipids due to its low hydrophobicity (see Table 3). At low pH, the histidines are (partially) protonated. We postulated that these residues are actively engaged in the increased proton permeability across the bacterial cytoplasmic membrane [1].

Apparently, the phenylalanines can be substituted by either tryptophans or hydrophobic leucines or isoleucines without loss of activity. This suggests that one role of Phe in the wild-type sequence may be to provide sufficient hydrophobicity, and therewith, membrane affinity.

Also, at neutral pH, clavanin is a potent antimicrobial agent; however, with a reduced activity, it most interestingly uses a different mode of action. The peptide disrupts biological membranes and lipid bilayers in a nonspecific way, causing efflux of large molecules and cell death. Interestingly, the abundant Phe residues appear to be non-optimal for this aspecific mechanism. For replacement of Phe by either tryptophan, isoleucine and leucine causes an increased membrane activity and cell killing at neutral pH. This suggests that peptide hydrophobicity plays a determining role in the nonspecific neutral pH mechanism. This suggestion is further supported by the behaviour of mutant 5Y that displays the lowest activity among the mutant peptides.

In general, the antimicrobial activity of the clavanin peptides and their ability to interact with membrane lipids are well correlated. Yet, small deviations were observed that demonstrate the more subtle character of the clavanin–membrane interaction. In this respect, apart from the overall

peptide hydrophobicity, peptide properties, such as the positioning at the membrane surface, anchoring capacity in the membrane interface region [26], bulkiness of the side chain groups, and/or folding in the membrane-bound state, are expected to affect the mode of (inter)action. The present study provides further insight into the mechanism by which clavanin kills bacteria at neutral pH values.

The CD studies reveal a striking correlation between the peptides' ability to adopt α-helical structures and the efficiency by which they permeabilize the membrane. This connection extends even to membranes of eukaryotic cells. Although the peptides display a strongly reduced ability to permeabilize the red blood cell membrane, still the same order of efficiency is observed among the various clavanin mutants. This behaviour is consistent with the classical behaviour of the peptide antibiotics that permeabilize bacterial membranes in an aspecific manner.

Increased helicity coupled to amphipathicity provides the peptide with the ability to permeabilize the membrane via a general effect on the membrane lipids [10,35–40]. In that respect, clavanins resemble peptides like melittin and magainin. An important difference between clavanin and peptides like melittin and magainin is the much less basic character of clavanin and the observed much reduced role of electrostatics in the membrane activity of the peptide.

The reduced effect of clavanin on red blood cells is in full line with this more generalized mechanism. The presence of large amounts of sphingolipids and cholesterol in the plasma membrane of eukaryotic cells rigidifies and thickens the plasma membrane [41,42], and thereby inhibits the membrane permeabilizing effect of the peptide.

Finally, we would like to return to the roles of the abundant phenylalanines in the clavanin sequence for its antimicrobial effect. Our CD studies suggest that within the context of the clavanin sequence, these residues together with the glycines provide the peptide with substantial conformational flexibility and introduce sufficient hydrophobicity for its interaction with membrane systems. The structural flexibility appears to be crucial for the specific mechanism. Additionally, it might provide a way by which the producing organism protects itself against the potential harmful membrane effects of the peptide.

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