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The EuroBiotech Journal

RESEARCH ARTICLE

Pharmaceutical Biotechnology

The role of C-terminal amidation in the mechanism of action of the antimicrobial peptide aurein 1.2

Mahdi Shahmiri and Adam Mechler*

Abstract

C-terminal amidation is a common feature of wild type membrane disrupting antimicrobial peptides (AMPs). Empirical evidence suggests that this modification increases antimicrobial efficacy. However, the actual role of C-terminal amidation in the molecular mechanism of action of AMPs is not fully understood. Amidation alters two key properties simultaneously: the net charge and helicity of the peptide, both of which are implicated in the mechanism of action. However, the differences between the physicochemical properties of the carboxyl and amide moieties have been disregarded in former studies. In this study we assessed whether the difference in activity is only caused by changes in the helicity and overall charge of a peptide, i.e. whether the chemistry of the terminus is otherwise irrelevant. To do so, the membrane disrupting activity of a modified aurein 1.2 peptide was studied in which a secondary amide was formed with a terminal methyl group, instead of the primary amide as in the wild type peptide. Results of quartz crystal microbalance, dye leakage and circular dichroism experiments show that the activity of the modified peptide is substantially reduced compared to the wild type peptide, in particular that the modified peptide exhibited a much-reduced ability to bind to the membrane. Thus, the primary amide at the C-terminus is required to bind to the membrane, and a secondary amide cannot serve the same purpose. We hypothesize that this difference is related to the hydration state of the terminus. The lack of membrane binding ability of the modified peptide identifies the primary amide moiety at the C terminus as a specific membrane binding motif.

Keywords: antimicrobial peptide, membrane disruption, carpet mechanism, activity

La Trobe Institute for Molecular Science,
La Trobe University, Melbourne VIC 3086
Australia

*Corresponding author: A. Mechler
E-mail: a.mechler@latrobe.edu.au

DOI: 10.2478/ebtj-2020-0004

Introduction

As the occurrence of multidrug resistant strains of pathogens is reaching alarming proportions, there is an increasing urgency to develop novel therapeutic approaches (1). Antimicrobial peptides (AMPs) offer a nature-inspired solution to this problem, in case the human cytotoxicity can be addressed by altering the peptide sequence (2). Most effort on turning AMPs into antibiotics focused on membrane disrupting AMPs (2, 3). These AMPs are 12-50 amino acid long cationic peptides that permeabilize the cytoplasmic membrane by a non-receptor-mediated process, that is, by opening holes in the phospholipid bilayer (4-6). It is a widely-held view that the activity of these peptides is governed by the overall physicochemical properties, such as charge, helicity, amphiphilicity etc. of the peptide (2, 7, 8). Yet, there is mounting evidence that certain residues and moieties play specific roles in the membrane interaction (9, 10). The chemistry of the C-terminus, in particular its naturally occurring amidation, was identified as one of the key factors in membrane disrupting activity (4). This modification greatly enhances the antibacterial activity compared to the same peptide with a free carboxylic acid at the terminus, e.g. in case of dermaseptin s3 the activity increased ~10-fold (11).

As an explanation for this enhanced activity, it was proposed that C-terminal amidation stabilizes α-helical conformation due to the provision of an additional hydrogen

bond (12, 13). Helicity in the presence of lipid membranes correlates well with the activity of cationic membrane disrupting AMPs (14, 15). However, the effect is not limited to α-helical peptides: enhanced activity was also recorded in the case of the β-sheet indolicidin (16). It was also considered that, by eliminating the negative charge of the carboxyl group, C-terminal amidation yields a higher cationic charge compared to identical peptides with a free C-terminus, therefore enhancing peptide binding to negatively charged bacterial membranes (9). Yet it was demonstrated before that charge interaction plays a minor role in the peptide-membrane interaction (10). It was also suggested that C-terminal amidation influences activity by an entirely different, and indirect, pathway by preventing the enzymatic degradation of AMPs (17). This does not explain the activity difference observed *in vitro* (10).

While there is empirical data in support of each of the hypotheses outlined above, designing negative controls had proven problematic as the amidation changes several aspects of the chemistry of the C-terminus simultaneously. The most important of these is a change in the hydration state, caused by eliminating the negative charge of the carboxylate moiety while introducing a stronger dipole moment and two hydrogen bond donors (18). These changes may also affect the interaction of the terminal region with the polar moieties of the lipid headgroups. Thus, while recognizing that there might be individual differences between AMPs, it can be hypothesized that the amidation exerts its effect via polar interactions.

In a previous work, we have confirmed experimentally and with computer simulations that C-terminal amidation is crucial for the activity of aurein 1.2, a small anuran surface active peptide (GLFDIIKKIAESF-NH_2) (10). The short sequence makes this an ideal peptide to study the role of individual moieties in the mechanism of action. Here we report on experiments with a modified peptide where instead of the primary amide of the wild type, a secondary amide is formed at the C-terminus, with a methyl group at the end of the backbone (GLFDIIKKIAESF-NH-CH_3). If the hypotheses assigning key roles to charge and/or helicity, i.e. the hydrogen bonding ability of the C terminus are correct, the activity of this peptide would not be different from the wild type peptide. For comparison, results for the non-amidated peptide (GLFDIIKKIAESF-COOH) are also shown.

Materials and Methods

Materials

3-mercaptopropionic acid (MPA; HPLC grade, >99%) was purchased from Fluka (Switzerland). Chloroform (99.8%, Analytical grade), methanol (>99.9%, Spectrophotometric grade), and ethanol (HPLC/ Spectrophotometric grade) were purchased from Sigma Aldrich (Castle Hill, NSW, Australia). Hydrogen peroxide (H_2O_2) solution (30%), and ammonia solution (28%, ACS reagent) were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Potassium dihydrogen phosphate (KH_2PO_4) and potassium hydrogen phosphate (K_2HPO_4) were purchased from Fluka (Switzerland) at ACS reagent grade; sodium chloride (pro analysis) was purchased from Merck (Darmstadt, Germany). 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), the sodium salt of 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG), and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL, USA). 5(6)-carboxyfluorescein (CF) was purchased from Sigma-Aldrich (USA). Lipids were dissolved in chloroform; for DMPG 3% methanol was added to improve solubility. Desired ratios of lipids: neat DMPC, DMPC/DMPG (4:1), DMPC/cholesterol (9:1), DMPC/DMPG (3:2), were measured into round bottom glass test tubes. The solvent was evaporated under a gentle stream of N_2 and dried overnight. Liposomes were hydrated in the assay buffer (20 mM phosphate buffered saline solution containing 100 mM NaCl at pH 6.9) at 37°C for 30 min, vortexed (~1 min) and briefly sonicated (~30 s) before use.

Quartz Crystal Microbalance

Quartz Crystal Microbalance with Dissipation Monitoring (QCM) measurements were performed with a Q-SENSE E4 system (Q-Sense, Sweden). The sensor crystals used were 5 MHz, AT-cut, polished quartz disks with evaporated gold sensor surface. The changes of resonance frequency (Δf) and energy dissipation (ΔD) upon mass deposition were measured simultaneously at four different eigenmodes (3rd, 5th, 7th and 9th) of the crystal resonance, but only the seventh harmonic of the fundamental frequency of the sensor chip was used for analysis.

Sensor chips were cleaned in the so called base piranha solution: 3:1:1 mixture of 18.2 MΩ deionized water (Ultrapure, Sartorius AG, Germany), hydrogen peroxide and ammonium hydroxide solutions (concentrations as per above) for 20 min at 70 °C. Consecutively the chips were rinsed with deionized water and dried. Chips were modified with 2% (w/w) MPA in propan-2-ol overnight to form a self-assembled monolayer as a support for the biomimetic membrane. Propan-2-ol wash was used to remove excess MPA. During the QCM experiments, first water was injected into the chambers to hydrate the surface of the chips and then assay buffer was introduced, followed by the lipid (in vesicular form) to deposit a membrane. Peptides were injected into the QCM chamber once the formation of a single bilayer membrane: -13-15 Hz frequency and 2.5-3.0×10^{-6} dissipation change was confirmed (19, 20). QCM experiments were repeated at least 3 times for each peptide and at each concentration. Control experiments on MPA coated gold confirmed the absence of peptide binding to the MPA self-assembled monolayer. Hence, all measured structural changes correspond to the interaction between the peptides and the membranes.

QCM Fingerprinting

Plotting the two main signals of QCM: dissipation change against frequency change (f-D curve) for a biomolecular interaction process can be used to provide a mechanistic fin-

gerprint. A detailed description was provided in a previous work (10). QCM fingerprinting was developed and evaluated primarily for the characterization of α-helical AMPs, as interpretation of the trends relies on the geometrical specifics and known membrane disrupting properties of these peptides.

Dye Leakage

Dye release upon peptide interaction was monitored using 5(6)-carboxyfluorescein (CF) loaded vesicles. Liposomes were prepared as described above using a buffer containing 20 mM CF (i.e. close to the lower limit of self-quenching concentration). Excess dye was removed from the medium by dialysis. Leakage of CF from liposomes was detected as an increase in fluorescence intensity, monitored using Spectramax M5 spectrophotometer (Molecular Devices, Silicon Valley, CA, USA) with excitation wavelength of 480 nm while the emission was monitored in a range of 500-530 nm. Measurements were repeated three times.

Circular Dichroism Spectroscopy

CD spectra from 200 to 250 nm were recorded with a Model 420 CD spectrophotometer (AVIV, USA) at 25 °C. 1-mm path length quartz cell was used for all measurements, containing 10 μM peptide and 100 μM lipid in PBS buffer. Each experiment was repeated three times to confirm the trends. Data are shown as mean residue molar ellipticity (deg cm^{-1} $dmol^{-1}$). The percentage of α-helical content was estimated from the molar ellipticity at 222 nm (θ_{222}) using Equation 1 (21).

$$\% \alpha\text{helix} = - ([\theta_{222}] + 2340)/303 \qquad \text{Equation 1}$$

Results

Dye leakage

The simplest test of the membrane disrupting activity is a dye leakage experiment. As shown in **Table 1**, exposing dye loaded neat DMPC liposomes to wild type aurein1.2-NH_2 lead to substantial increase in fluorescence, indicating breach of the membrane integrity as expected for a membrane disrupting peptide. Without C-terminal amidation (aurein1.2-COOH), the peptide exhibited much reduced, but nonzero activity, as seen with QCM before (10). In contrast, the aurein 1.2 sequence with a secondary amide at the C terminus (aurein1.2-NH-CH_3) did not cause any dye release.

Table 1. Dye leakage results with neat DMPC liposomes

Peptides	Fluorescence intensity increase (%)
Aurein 1.2 –NH_2 (wild type)	30
Aurein 1.2 –COOH	1
Aurein 1.2 –NH-CH_3	0

CD spectroscopy

Helicity of the peptide is used as an indirect indicator of membrane binding: most antimicrobial peptides are unstructured in solution but become helical upon binding to the membrane (22). Therefore, conformations of the three aurein 1.2 variants in the presence of different phospholipid mixtures were characterized using CD spectroscopy (**Fig. S1**). Percentage helicity data are shown in **Table 2**. All peptides were largely unstructured in the buffer solution. As expected, the helicity of the wild type peptide increased in the presence of phospholipids, due to membrane binding, and so did the helicity of aurein1.2-COOH that was shown before to bind to membranes but do not disrupt them (10). In contrast, the helicity of aurein1.2-NH-CH_3 did not change appreciably. This is counterintuitive given the helicity-enhancing role of the amide moiety (23). The most likely explanation is that the peptide cannot enter the headgroup zone of the membrane that is believed to facilitate folding.

Fingerprinting analysis

QCM experiments were performed with membrane mixtures that mimic mammalian (zwitterionic) and bacterial (charged) membranes. The primary data, (f-t) and (D-t) sensograms are shown in the supplementary material (**Figs. S2-S5**). For analysis, dissipation change versus frequency change plots (f-D curves) were used. f-D curves show changes in the viscoelastic character of the membrane when it is exposed to the peptides, revealing stages of the molecular mechanism of action (24). **Figure 1** shows the viscoelastic fingerprint analysis of the interactions of aurein1.2-NH-CH_3 with mammalian model membranes. For zwitterionic PC membranes, there is a short [-f, +D] trend that can be explained with weak membrane association. In case of neat DMPC (**Fig. 1** left panels) a second stage (marked with arrow) appears at higher concentrations, revealing a structural change that is likely related to peptide ag-

Table 2. Results of CD measurements

Medium	%Helicity		
	aurein1.2-NH_2	aurein1.2-NH-CH_3	aurein1.2-COOH
PBS	9	4	9
DMPC	25	5	24
DMPC:cholesterol 9:1	20	5	12
DMPC:DMPG 4:1	38	8	32

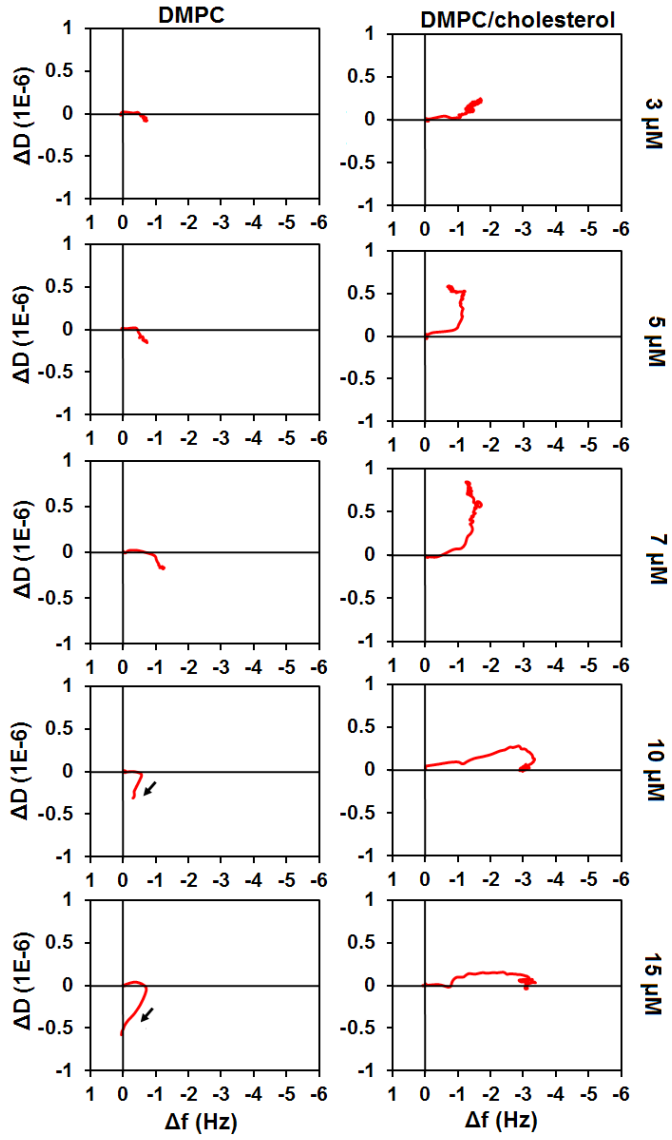

Figure 1. ΔD vs Δf plots of aurein1.2-NH-CH_3 interaction with DMPC and DMPC:cholesterol (9:1) membranes at different concentrations. The effect is shown for the seventh harmonic of the fundamental frequency of the sensor chip.

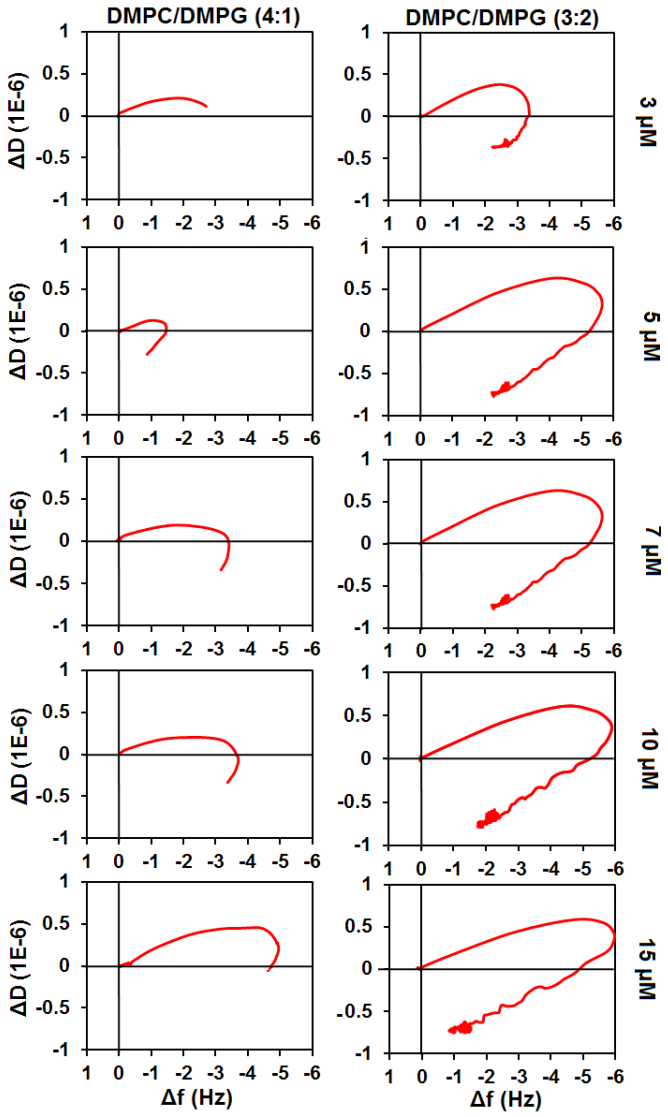

Figure 2. ΔD vs Δf plots of aurein1.2-NH-CH_3 interaction with DMPC/DMPG (4:1) and DMPC/DMPG (3:2) at different concentrations. The effect is shown for the seventh harmonic of the fundamental frequency of the sensor chip.

gregation and/or partial membrane penetration. Addition of 10 % cholesterol eliminated this second stage (**Fig. 1** right panels), instead the amount of binding increased somewhat at higher concentrations.

In the case of charged membranes (**Fig. 2**) the amount of initial binding is higher, ~ -4 Hz for (4:1) and ~ -6 Hz for (3:2), suggesting that charge might play a role in the association; in case of the higher charged lipid content there are signs of some membrane disruption, although it is still not full disintegration as in case of the wild type peptide.

The comparison of ΔD vs Δf plots for aurein1.2 (wild type) and aurein1.2–CH_3 exposed to different membranes is shown in **Figure 3** (neutral membranes) and **Figure 4** (charged membranes). Here the scale is adjusted to accommodate the trendlines of the wt peptide; the traces shown are taken from (10). As it can be seen, only negligible activity was observed for the aurein1.2-NH-CH_3 compared to the wild type peptide, indicating the importance of C-terminal amidation for the peptide activity.

Discussion

The three different termini are shown in **Figure 5** for comparison. As our previous results showed, both aurein1.2-NH_2 and aurein1.2-COOH could bind to all membranes, in spite of the different net charge (0 vs. +1) (10). In contrast, the terminal CH_3 group reduced the amount of membrane binding substantially, even though also providing +1 charge as in case of the primary amide. This is further evidence that membrane *binding* is not charge driven. Dye leakage experiments also confirmed the lack of any membrane disrupting activity of aurein1.2-NH-CH_3; as the primary and secondary amides at the C terminus yield equivalent net charge for the peptide, the difference in overall *activity* cannot be related to charge effects either.

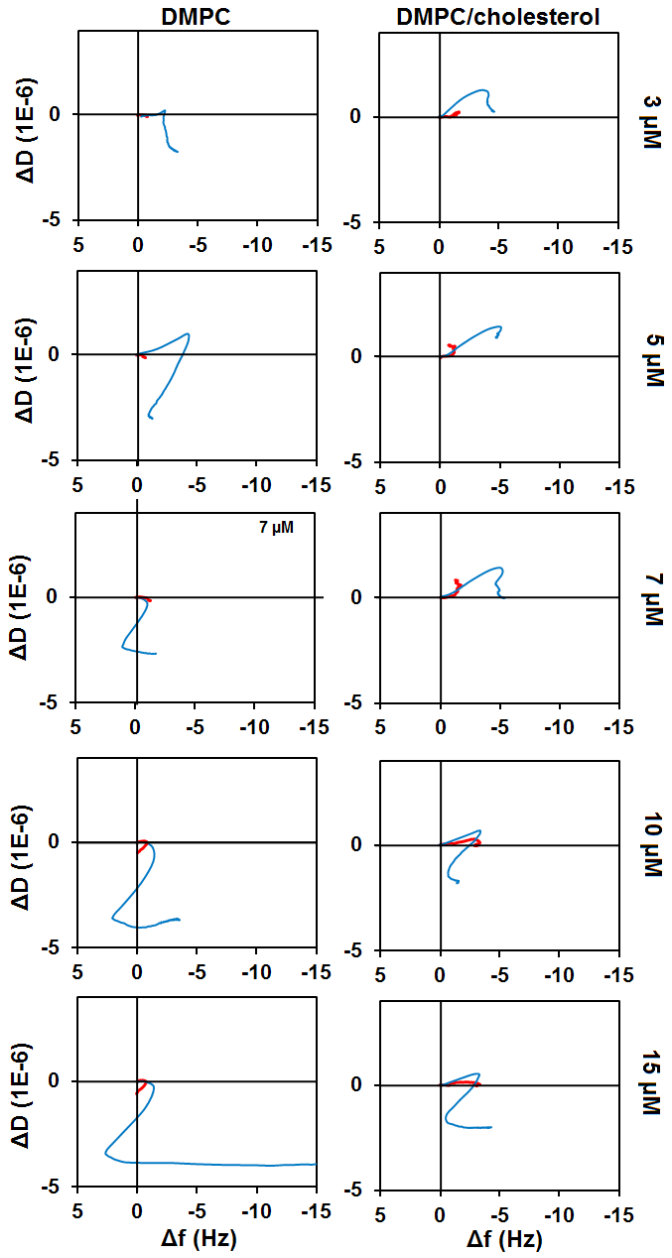

Figure 3. Comparison of the viscoelastic fingerprints of aurein1.2-NH-CH_3 (red line) and aurein1.2-NH_2 (blue line). The effect is shown for the seventh harmonic of the fundamental frequency of the sensor chip.

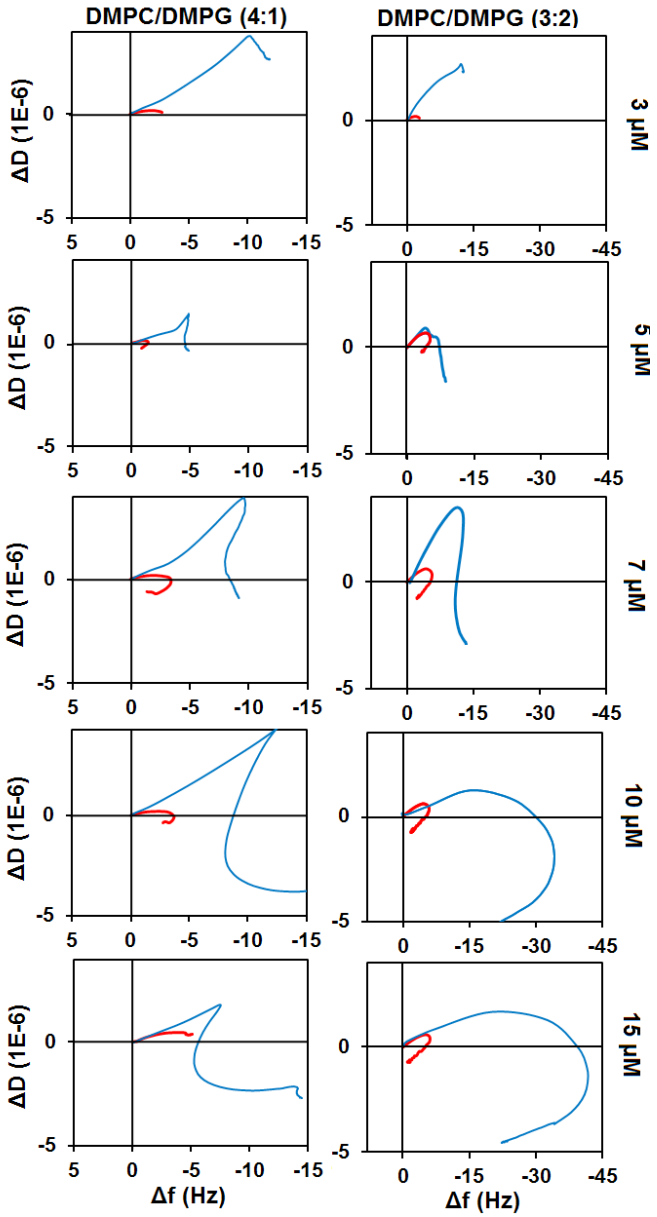

Figure 4. Comparison of the viscoelastic fingerprints of aurein1.2-NH-CH_3 (red line) and aurein1.2-NH_2 (blue line). The effect is shown for the seventh harmonic of the fundamental frequency of the sensor chip.

The strong dipole and consistently higher hydrogen-bonding ability of the amide, and the resulting increased helicity is also cited as a source of increased activity of C-amidated peptides. There is no significant difference between the hydrogen bonding ability of primary and secondary amides, thus aurein1.2-NH-CH_3 should exhibit comparable helicity to the wild type peptide, which it does not do. It is believed that α-helicity of this highly amphipathic peptide is the result of a templating process, between the lipid headgroup and tail zone, where the hydrophobic residues align to the membrane core and the hydrophilic residues are exposed to water (25). From the fact that aurein1.2-NH-CH_3 is not becoming α-helical at all in the presence of membranes one can speculate that the peptide is not entering even the headgroup zone of the membrane, the weak binding seen in QCM is likely caused by loose surface association and/or a local change of buffer viscosity due to the presence of the peptides (26).

Thus, the presence of the small methyl group inhibits activity while in all other physicochemical characters the wild type peptide and aurein1.2-NH-CH_3 are identical. Hence, the two main reasons cited for increased activity of the C-terminal amidated peptides are ruled out. Inhibition of membrane binding may be caused by a feature of the chemistry of the C-terminus. The main difference between the wild type aurein 1.2 and aurein1.2-NH-CH_3 is the hydration state of the terminus: one fewer hydrogen-bond donor and the methyl moiety restricts water

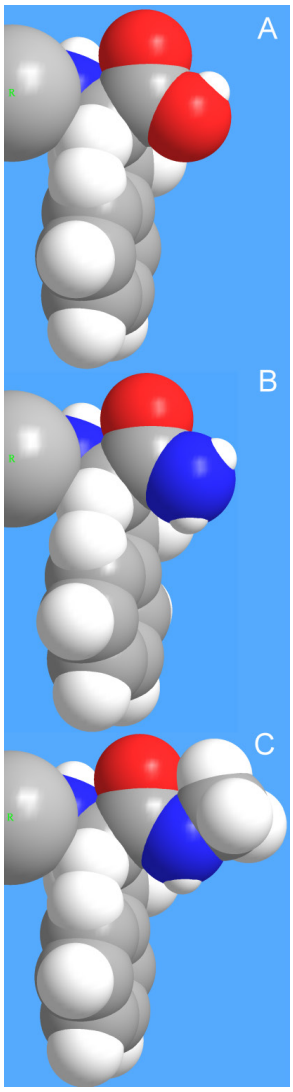

Figure 5. Comparison of the space filling models of the C-terminal region. The last residue (F13) is shown in full; the rest of the peptide is represented with an R group (large sphere in the figure). A) unmodified carboxyl terminus; B) primary amide (wild type) terminus; C) secondary amide with methyl terminus.

access to the amide. Thus, the observed difference in activity is likely related to the hydration of the C terminus of the peptide.

It is challenging to extrapolate the molecular mechanism of action identified in model membranes to the expected activity, or the lack thereof, against bacteria. Changing physicochemical characteristics of an AMP can interfere with its ability to reach the membrane where it can exert its primary, membrane disrupting mode of action. It is known that wild type aurein 1.2 exhibits moderate activity against both Gram positive and Gram negative bacteria with MIC values in the 50-100 µg/ml concentration range (27) thus the membrane disrupting mechanism appears to proceed irrespective of the membrane/cell wall structure. This suggests that the peptide can easily diffuse through to the bacterial membrane surface. The modification discussed here would inhibit interaction with the membrane itself; therefore it is expected that the effect of the modification would be the same in both Gram positive and Gram negative bacteria, that is, lack of activity. Hence it is feasible to assume that the antimicrobial action of aurein1.2 is also dependent on the presence of a primary amide at the C-terminus.

Conclusions

It was shown by using quartz crystal microbalance, dye leakage and circular dichroism experiments that the activity-regulating effect of the C-terminal amidation of aurein1.2 peptide is not related to charge or helical stability of the peptide. A secondary amide cannot serve the same purpose as the primary amide, in particular, the modified peptide exhibited a much-reduced ability to bind to the membrane. Thus, the primary amide at the C-terminus is required to bind to the membrane. We hypothesize that this difference is related to the hydration state of the terminus in the presence of the methyl moiety. We have demonstrated before that in the mechanism of aurein1.2 action phenylalanine residues act as membrane anchors; the results presented here suggest that membrane anchoring is the second step of membrane attachment, whereas the first step involves the primary amide at the C-terminus.

Conflict of Interest

The authors declare that they have no conflicts of interest.

Ethical Compliance

This article does not contain any studies involving human participants or animals performed by any of the authors.

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