How C-Terminal Carboxyamidation Alters the Biological Activity of Peptides from the Venom of the Eumenine Solitary Wasp†

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ABSTRACT: Inflammatory peptides display different types of post-transcriptional modifications, such as C-terminal amidation, that alter their biological activity. Here we describe the structural and molecular dynamics features of the mast cell degranulating peptide, eumenine mastoparan-AF (EMP-AF-NH₂), found in the venom of the solitary wasp, and of its carboxyl-free C-terminal form (EMP-AF-COO−) characterized by a reduced activity. Circular dichroism indicates that both peptides switch from a random coil conformation in water to a helical structure in TFE and SDS micelles. NMR data, in 30% TFE, reveal that the two peptides fold into an α-helix spanning most of their length, while they differ in terms of molecular rigidity. To understand the origins of the conformational flexibility observed in the case of EMP-AF-COO−, a 5 ns MD simulation was carried out for each peptide, in an explicit water/TFE environment. The results show that the two peptides differ in an H-bond between Leu14 NH₂ and the backbone carbonyl of Ile11. The loss of that H-bond in EMP-AF-COO− leads to a significant modification of its structural dynamics. In fact, as evidenced by essential dynamics analysis, while EMP-AF-NH₂ exists mainly as a rigid structure, EMP-AF-COO− presents two helical stretches that fluctuate in some sort of independent fashion. We conclude that the diverse biological activity of the two peptides is not simply due to the reduction of the net positive charge, as generally suggested, but also to a structural perturbation of the amphipathic α-helix that affects their ability to perturb the cell membrane.

The insects of the order Hymenoptera are classified into two groups, based on their life history: social and solitary. The venom of social Hymenoptera evolved for use as a defensive tool to protect the colonies of honeybees and wasps from the attacks of predators. Small peptides are one of the major components of these venoms and include melittin and mast cell degranulating (MCD) peptides in honeybees, while mastoparans and chemotactic peptides are present in social wasps. On the other hand, solitary hymenopteran venoms evolved for use as a tool for paralyzing and/or killing prey. Therefore, the major components of these venoms are neurotoxins, though they also contain MCD peptides. These last ones possess structures closely related to mastoparans from social wasps, presenting amphipathic helical structure and exhibiting similar degranulating activity. These peptides have been characterized both chemically and pharmacologically (1–3). Their potent degranulation, hemolytic, and antimicrobial activity has been ascribed to their ability to interact with the cell membrane surface via the positively charged side of their amphipathic α-helical structure.

Frequently, the biological activity of these peptides is associated with some type of post-transcriptional processing of their precursors, such as C-terminal amidation. This occurs in melittin (4), cercopins (5), dermaseptins (6), PGLa (7), clavanin (8), PR-39 (9), apidaecins (10), dipterocin (11), polyphemusins (12), or penaeidins (13). There are also studies showing an increase in activity following amidation or, alternatively, an activity decrease when the amide is removed (14–16).

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Abbreviations: CD, circular dichroism; DQF-COSY, double-quantum-filtered correlation spectroscopy; ED, essential dynamics; ESI-MS, electrospray ionization mass spectrometry; MCD, mast cell degranulating; MD, molecular dynamics; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser enhancement spectroscopy; rmsd, root-mean-square deviation; rmsf, root-mean-square fluctuation; SDS, sodium dodecyl sulfate; TFE, 2,2,2-trifluoroethanol; TOCSY, total correlation spectroscopy.
Though the precise mechanism of action of these peptides is not known yet, it is believed they target the lipid component of cell membranes, disrupting the membrane structure via either the “barrel stave mechanism” or the “carpet-like mechanism” (17–19).

Recently, a new inflammatory peptide presenting mast cell degranulating activity, eumenine mastoparan-AF (INLLKIAKGIKSL-NH₂, EMP-AF-NH₂), has been isolated from the venom of the solitary wasp Anterhynchium flavormarginatum micado (20–22). The same authors also showed that EMP-AF-NH₂ has a more potent biological activity than its analogue containing a carboxyl-free C-terminus, INLLKIAKGIKSL-COO⁻ (EMP-AF-COO⁻; 20).

Recognizing that the biological activity of these cytolytic peptides may be strongly influenced by the conformation they acquire before interacting with the membranes (23, 24), we have decided to study the conformational features of these peptides at that specific stage. To mimic such a unique chemical environment, characterized by a dielectric constant reduced compared to that of bulk water, we have carried out these studies in a H₂O/TFE mixture (24–26). In this work, we focus on the description of the possible structural differences that we believe lead to the diverse biological activity of these two peptides. The NMR-derived solution structures of EMP-AF-NH₂ and EMP-AF-COO⁻ indicate that the removal of the C-terminal NH₂ group induces a localized destabilization of the extended α-helical organization that characterizes the amidated form. To disclose the origin of that structural instability, we carried out 5 ns molecular dynamics (MD) simulations in an explicit water/TFE environment for both peptides. On the basis of ED analysis, a mechanistic model that justifies their structural and functional features is presented.

**MATERIALS AND METHODS**

**Chemicals.** 2,2,2-Trifluoroethanol (TFE) was purchased from Aldrich Inc. Perdeuterated 2,2,2-trifluoroethanol (TFE-d₅) was purchased from Cambridge Isotopes Laboratories. Sodium dodecyl sulfate (SDS) was purchased from Pharmacia Fine Chemicals.

**Peptide Synthesis and Purification.** The peptides were prepared by stepwise manual solid-phase synthesis using N-9-fluorophenylmethoxycarbonyl (Fmoc) chemistry with Novasyn TGS resin (NovaBiochem). Side chain protective groups included tert-butyl for serine and tert-butoxycarbonyl for lysine. Cleavage of the peptide–resin complex was performed by treatment with a trifluoroacetic acid/1,2-ethanediithio/phenol/water mixture (82.5:2.5:5:5 by volume), using 10 mL per gram of complex at room temperature over the course of 2 h. After being filtered to remove the resin, the crude peptides were extracted with ethyl ether and purified using a semipreparative reversed-phase HPLC column (Shiseido C18, 250 mm × 10 mm), under isocratic conditions, with a 40% (v/v) acetonitrile/water mixture containing 0.1% (v/v) trifluoroacetic acid. The homogeneity and correct sequence of the synthetic peptides were assessed by Edman degradation chemistry, analytical HPLC, and ESI-MS analysis.

**Peptide Concentration.** Because of the absence of any aromatic residue, the peptide concentration was determined by quantitative amino acid analysis.

**CD Spectroscopy.** Circular dichroism (CD) experiments were carried out using a Jasco 810 spectropolarimeter (JASCO International Co. Ltd.), coupled to a Peltier Jasco PFD-425S system for temperature control. The peptide samples were dissolved either in a TFE/H₂O solvent mixture, with a variable solvent ratio as indicated in the text, or in the presence of SDS micelles (8 mM SDS, pH 4.5) to yield a final peptide concentration of 50 μM for both EMP-AF-NH₂ and EMP-AF-COO⁻. Spectra were collected from 195 to 250 nm and averaged over four scans at 20 °C, with a quartz cell with a path length of 1 mm. A 0.5 nm step resolution, a 50 nm/min speed, an 8 s response time, and a 1 nm bandwidth were generally used. Following baseline correction, the observed ellipticity θ (mildidegrees) was converted to the molar mean residue ellipticity [θ] (degrees per square centimeter per decimole). The peptide helical content was estimated according to the method of Chen et al. (27).

**NMR Spectroscopy.** The samples for NMR measurements were prepared by dissolving the peptides in a TFE-d₅/H₂O mixture (30:70, v/v) at pH 4.5 to yield a concentration of 1.0 mM for both EMP-AF-NH₂ and EMP-AF-COO⁻. 1H NMR experiments were carried out on a Varian Inova 500AS spectrometer (Varian, Inc.) operating at 499.730 MHz for the 1H frequency and at 20 °C. The proton chemical shifts were referenced to 4,4-dimethyl-4-silapentane-1-sulfonate (DSS, 0.00 ppm). To assign the peptide resonance peaks, standard methods were used, including double-quantum-filtered (DQF)-COSY (28), total correlation spectroscopy (TOCSY) using a DIPSI spin-lock sequence and a field strength of 10 kHz (29), and nuclear Overhauser enhancement spectroscopy (NOESY) (30, 31) experiments. NOESY spectra with mixing times of 100, 200, and 300 ms were recorded to check for spin diffusion. All two-dimensional experiments were carried out in the phase-sensitive mode using the States method (32). The spectral width was typically 6000 Hz, and 512 τ₁ increments with 32 transients of 1024 complex points for each free induction decay were recorded. In the case of the DQF-COSY experiments, 1024 τ₁ increments were collected each with 32 transients of 4096 complex points. Water suppression was achieved by low-power continuous wave irradiation during the relaxation delay or WET pulse technique.

Data were processed on a Silicon Graphics Octane 2 workstation using FELIX NMR 2000 (Accelrys Inc.) and nmrPIPE/nmrVIEW (33, 34). Prior to Fourier transformation, the time domain data were zero-filled in both dimensions to yield a 4K × 2K data matrix. When necessary, a fifth-order polynomial baseline correction was applied after transformation and phasing. The DQF-COSY experiment was processed to a 16K × 2K data matrix to obtain 0.36 Hz/point of digital resolution for coupling constant measurements.

To obtain interproton distance constraints, the cross-peak volumes of the 300 ms NOESY spectra were measured and calibrated with respect to the cross-peak volume of geminal β-protons, corresponding to an interproton distance of 1.8 Å. The obtained NOE values were classified as strong, medium, and weak, corresponding to upper bound distances of 2.8, 3.5, and 4.0 Å, respectively. The lower bound was taken to be the sum of the van der Waals radii (1.8 Å) for the interacting protons.
Molecular Modeling. The NMR-derived solution structures of EMP-AF-NH$_2$ and EMP-AF-COO$^-$ were computed using the simulated annealing methods in the DYANA (35) refine module. The modeling protocol was based on the methods implemented by Pristovšek et al. (36). Each round of refinement started with 20 random conformers, and the 10 models with the lowest target function were used to analyze constraint violations and assign additional NOE constraints to be included in the subsequent calculation. This process was repeated until all NOEs had been inserted and all violations eliminated. In the final round of refinement, a total of 100 structures were calculated, and the 40 conformers with the lowest target function (0.41 ± 0.02 Å$^2$ for EMP-AF-NH$_2$ and 0.29 ± 0.01 Å$^2$ for EMP-AF-COO$^-$) were considered for analysis. After simulated annealing had been carried out, these 40 structures, with no distance violation larger than 0.2 Å and no dihedral angle violation greater than 5°, were energy minimized with a full consistent valence force field (37, 38) by steepest descents and conjugated gradients using several thousand interactions until the maximum derivative was less than 0.001 kcal/Å. All calculations were carried out on an Octane2 workstation (Silicon Graphics Inc.) using the DISCOVER (Accelrys Inc.) software package, together with INSIGHTII as the graphic interface. The quality of the final structures was analyzed using PROCHECK-NMR (39) and CNS (40).

MD Simulations. The MD calculations and trajectory analysis were performed using the GROMACS suite of programs (41) running on a Compaq AlphaServer ES40 multiprocessor. Molecular visualization procedures were carried out using the INSIGHTII graphical environment on an Octane2 workstation.

(1) System Setup. The initial structures were immersed in a triclinic box filled with a mixture of simple point charge (SPC) water molecules (42) and TFE model (43) molecules, corresponding to an approximately 30:70 (v/v) TFE/H$_2$O mixture. The resulting systems were composed by 67 TFE and 800 H$_2$O molecules for EMP-AF-NH$_2$ and 64 TFE and 804 H$_2$O molecules for EMP-AF-COO$^-$. Each box size was adjusted to ensure a minimum distance of 0.8 nm between the peptide and the periodic box edges. The N-terminus was treated as charged ammonium, and in the case of the carboxyl-free peptide, the C-terminus was treated as a carboxylate group. The net charges of the peptides (+4 for EMP-AF-NH$_2$ and +3 for EMP-AF-COO$^-$) were neutralized with a corresponding number of Cl$^-$ counterions.

(2) Simulation Parameters. The calculations were performed using the GROMACS all-hydrogen force field. The systems were simulated using periodic boundary conditions with a cutoff radius of 0.8 nm for short-range interactions and updating the neighbor pair list every 10 steps. Long-range electrostatic interactions were treated with the Particle Mesh Ewald method (44, 45). The LINCS algorithm (46) was used to constrain all bond lengths and SETTLE (47) to constrain water geometries. A time step of 2 fs was chosen for integrating the equations of motion. The simulations were performed at a constant temperature (293 K) and pressure (1 bar).

(3) Simulation Protocol. A canonical α-helix conformation was used as a starting model for both EMP-AF-NH$_2$ and EMP-AF-COO$^-$ peptides. The solvated peptides were submitted to an initial relaxation of solvent and ions through 500 steps of the steepest descents algorithm while keeping the peptide backbone coordinates fixed. After the constraints had been removed, the systems were submitted to another short minimization step and the molecular dynamics simulations proceeded without any restraint for 5 ns. The atomic positions were recorded every 1.0 ps throughout each trajectory.

ED Analysis. ED was used to obtain a quantitative characterization of the dynamic properties of each system (48). The covariance matrices describing the positional fluctuations of the Ca atoms were diagonalized, resulting in sets of eigenvectors and corresponding eigenvalues. The eigenvectors indicate directions in a 3N-dimensional space (where N is the number of atoms) where there are concerted fluctuations of the atoms. The eigenvalues are equivalent to the total mean square displacement of the atoms along their corresponding eigenvectors. Concerted intramolecular motions within the subspace can be studied by projecting the trajectory onto the individual eigenvectors.

RESULTS

CD Spectroscopy. As shown in panels a and b of Figure 1, the two peptides present a random conformation in water, while they acquire a helical conformation at a TFE concentration higher than 15%. The insets of panels a and b of Figure 1 show that the increase in the helical content, as judged by the intensity of the 222 nm band (27), reaches its maximum at ~30% TFE and can be estimated to be 62% for EMP-AF-NH$_2$ and 29% for EMP-AF-COO$^-$. Figure 1c shows the CD spectra of both peptides in the presence of 8 mM SDS. Also in this case, the peptides acquire an α-helical configuration. However, comparing the CD spectra of the peptides in 40% TFE with those in the presence of 8 mM SDS, we observe that, in the case of EMP-AF-NH$_2$, in SDS micelles there is a net increase in the optical activity that, combined with the shift of the negative band from 206 to 208 nm, suggests that the peptide acquires a higher α-helical content (83%). As for EMP-AF-COO$^-$, on the other hand, not many differences are observed. In fact, the calculation of the helical content gives a value of 31%.

NMR Spectroscopy. Sequential resonance assignments of EMP-AF-NH$_2$ and EMP-AF-COO$^-$ in a 30:70 (v/v) TFE/H$_2$O mixture has been performed on the basis of the combined use of two-dimensional TOCSY and NOESY experiments (49).

The proton chemical shifts of the two peptides together with the $J_{\text{N-H}}$ coupling constants, estimated from the antiphase cross-peak in the DQF-COSY experiment, have been deposited in the BioMagResBank (BMRB) database as entries 5883 (EMP-AF-NH$_2$) and 5916 (EMP-AF-COO$^-$).

Figure 2 displays the plot of the α-proton chemical shifts index (CSI; 50) for the two peptides. In the case of EMP-AF-NH$_2$, the negative values observed from residue Leu3 to Leu14, together with the $J_{\text{N-H}}$ coupling constant of <6.0 Hz for almost all residues, suggest the existence of a helical segment extending over that region. As for EMP-AF-COO$^-$, the data suggest the presence of an α-helical conformation in the Leu3–Lys8 region. However, the smaller α-proton $\Delta\delta$ values coupled with the slightly higher $J_{\text{N-H}}$ indicate the existence of a less stable helical conformation.
The pattern of inter-residue NOEs for the two peptides is reported in Figure 3, and it shows a good correlation with the CSI values. In fact, the medium-range $d_{RR}(i, i + 3)$, $d_{RN}(i, i + 3)$, and $d_{RN}(i, i + 4)$ NOEs together with the intense $d_{NN}(i, i + 1)$ indicate an $\alpha$-helical fold spanning residues 3–14 for EMP-AF-NH$_2$. In the case of EMP-AF-COO$^-$, though overall the NOEs are consistent with a helical conformation extending over the same region, the smaller number or absence of $d_{RR}(i, i + 3)$, $d_{RN}(i, i + 3)$, and $d_{RN}(i, i + 4)$ from residue 9 to 14 confirms the presence of a less stable helical conformation in the C-terminus.

Molecular Modeling. A total of 180 distance constraints, of which 36 are medium-range, for EMP-AF-NH$_2$ and 210 distance constraints, including 24 medium-range, for EMP-AF-COO$^-$ were used for structure calculation using DYANA (35).

Figure 4a is the superposition of the 20 minimum energy structures of EMP-AF-NH$_2$. The models have been superimposed in the region of residues 3–14 and show that the peptide folds into a very well-defined amphipathic $\alpha$-helix. A quite different situation is encountered in the case of EMP-AF-COO$^-$, where the analysis of the NMR-derived structures reveals the existence of various conformational families. Figure 4b shows that a total of 35 minimum energy structures present a well-defined helical stretch spanning residues 3–8. On the other hand, an analysis of the peptide C-terminus allows identification of four main structural families where residues 9–14 give rise to discrete helical conformations. Overall, the peptide appears to be characterized by a significant degree of molecular plasticity that results in a clear structural fluctuation.

The structural statistics are summarized in Table 1, and they show no significant violation of the distance restraints, indicating a good correspondence between the experimental NOEs and the calculated models. PROCHECK-NMR (39) has been used to evaluate the quality of the energy-minimized structures. As can be seen in Table 1, the backbone rmsd for all residues is 0.26 ± 0.13 Å for EMP-AF-NH$_2$ and
The root-mean-square fluctuation (rmsf) of the backbone atoms of the two peptides (Figure 5) was calculated over the last 2 ns of the MD simulation, where an equilibrium state was reached. The results clearly show that, in agreement with the experimental results, while the N-terminal region of the peptides exhibits comparable behavior, there is an increased level of fluctuation of the backbone atomic positions in the C-terminal region of EMP-AF-COO\(^-\) after residue 9.

To disclose the specific intramolecular interactions responsible for these diverse conformational dynamics, we analyzed the behavior of the H-bonds involved in the stabilization of the \(\alpha\)-helix secondary structure. Figure 6 shows the result of this analysis for the two peptides. As for EMP-AF-COO\(^-\) (Figure 6a, top panel), the repulsive interaction between Leu14 COO\(^-\) and Ile11 CO turns out to affect the stability of the Ile10 CO–Leu14 NH H-bond that is part of the \(\alpha\)-helix H-bond network (Figure 6a, bottom panel). Interestingly, this H-bond reaches an equilibrium state after 2 ns, oscillating at \(\sim 4\) Å, thus suggesting it must be unstable. Differently, in the case of EMP-AF-NH\(_2\) (Figure 6b), the distance between the C-terminus amide NH\(_2\) group (Leu14 NH\(_2\)) and the backbone carbonyl of Ile11 (Ile11 CO) (Figure 6b, top panel) and the distance between the backbone carbonyl of Ile10 (Ile10 CO) and the backbone amide of Leu14 (Leu14 NH) (Figure 6b, bottom panel) do not vary throughout the MD simulation, thus being compatible with the formation of stable H-bonds.

To understand in more detail the dynamic features of the two peptides, we carried out an ED analysis of the trajectories produced during those last 2 ns. Figure 7a shows the plot of the first 10 eigenvectors that represent the peptides’ main degrees of freedom (52, 53). Since the first three values account for more than 60% of the peptides’ conformational fluctuations, we analyzed these three eigenvectors to obtain information about the existence of concerted displacements of some residues during the MD simulation. With respect to the eigenvalues, we did not observe a significant difference between the two peptides in their overall molecular dynamics (Figure 7a). However, the calculation of the per residue displacements along the first eigenvector of each peptide (Figure 7b) provides evidence there are some differences in their molecular dynamics. In fact, in the case of EMP-AF-NH\(_2\), the principal concerted fluctuation involves mainly the first five residues, while for EMP-AF-COO\(^-\), the main concerted fluctuation comprises residues in both the N- and C-termini. Overall, the ED analysis indicates that the two peptides experience concerted motions encompassing diverse regions of their primary sequence, in agreement with the existence of distinct conformational families, as evidenced by the NMR-derived structures (Figure 4). In addition, the finding that the N- and C-terminal regions of EMP-AF-COO\(^-\) do not present any significant correlation of their motions explains why, for this peptide, it is not possible to identify a family of NMR-derived structures where the \(\alpha\)-helical backbone (residues 3–14) can be superimposed as in the case of EMP-AF-NH\(_2\) (Figure 4).
DISCUSSION

Most of the inflammatory peptides presenting mast cell degranulating activity share common properties. In fact, they are generally composed of fewer than 60 amino acids, their net charge is positive, they are hydrophobic, and/or they present amphipathic α-helical structures (20). In addition, in most cases they are membrane active.

Recently, Konno and co-workers (20) isolated from the venom of the solitary wasp the EMP-AF-NH₂ peptide together with its C-terminal carboxyl-free form, both characterized by mast cell degranulating activity. They found that EMP-AF-NH₂ had a 50% higher degranulating activity in rat peritoneal mast cells than EMP-AF-COO⁻, at 10⁻⁵ M. Even more interestingly, they found that EMP-AF-COO⁻ turned out to lose completely its hemolytic effect on human erythrocytes, exhibited instead by EMP-AF-NH₂ at 4.5 × 10⁻⁵ M (20).

Though it may occur that amidated and nonamidated forms do not differ substantially in their biological activity (54, 55), in a number of cases, it has been shown that the activity of the carboxyl-free form is significantly impaired (7, 56). Similarly, it has been reported that synthetic C-terminally...
amidated analogues of peptides, whose native forms are not amidated, exhibit increased biological activity (14–16, 57). Thus, the reduced inflammatory and antimicrobial activity of nonamidated peptides is generally ascribed to a reduced net positive charge or to a reduced helical structure.

Recently, however, Shalev et al. (59), while studying the consequences of carboxy amidation on dermaseptin S3, found that the enhanced bacterial growth inhibition potency exhibited by the amidated form had to be associated with a more rigid and extended $\alpha$-helical structure. One point they could not explain, though, was why amidation produces such a significant conformational change throughout most of the peptide sequence. These observations prompted us to investigate in more detail the origin of the differences in the cytolytic activity of EMP-AF-NH$_2$ and EMP-AF-COO$^-$.

Addition of negative charges at the negative pole of the $\alpha$-helix macrodipole is expected to destabilize the helical structure (15). In fact, certainly the CD experiments indicate a lower helicity for the C-terminal carboxyl-free peptide, both in TFE and in the presence of SDS micelles (Figure 1a,b).

As has been suggested by others (17, 23, 24), we do believe that a crucial step that affects the efficiency with which the peptides perturb and permeabilize the membranes is associated with the conformational changes that take place just before the effective interaction with the membrane surface. Therefore, we have chosen to study the structure of the peptides in an $\text{H}_2\text{O}/\text{TFE}$ mixture that is known to mimic that environment (23, 24).

The NMR-derived solution structures of EMP-AF-NH$_2$ and EMP-AF-COO$^-$ (Figure 4) show that while both peptides possess an amphipathic helical structure, only EMP-AF-NH$_2$.
The behavior of EMP-AF-NH$_2$ and EMP-AF-COO$^-$ involves intramolecular interactions responsible for the diverse behavior common to the membrane-interacting lantibiotics. It has been inferred that this might be a structural feature switching from the membrane-bound to the unbound state, as structural changes have been observed for nisin while solvent-exposed location. Relying on the fact that similar flipping of amide protons from a solvent-protected to a solvent-exposed location, molecular dynamics simulation, in vacuum, for the lantibiotic membrane active peptides, by using extensive restrained molecular dynamics simulation, has provided novel information, at the atomic level, about the mechanism responsible for the conformational features and functional properties observed for the two peptides.

To identify the pattern through which the ideal $\alpha$-helix is transformed along the MD simulation, we analyzed the hydrogen bond distances that define the $\alpha$-helix conformation at the C-terminus. As shown in Figure 6b, in the EMP-AF-NH$_2$ peptide, the presence of the Ile11 CO–Leu14 NH$_2$ H-bond stabilizes the C-terminal region, while in EMP-AF-COO$^-$, the fact that the H-bond is substituted by the onset of a charge repulsion destabilizes the Ile10 CO–Leu14 NH$_2$ H-bond. Such a destabilization, however, does not produce an unfolding of the helix in that region. Instead, it provides the peptide with some new degrees of conformational freedom as revealed by the NMR-derived solution structures of EMP-AF-COO$^-$ (Figure 4b,c). Recognizing that the biological activity of peptides and proteins depends not only on the presence of specific secondary structure elements but also on their concerted motion, we have used ED analysis to disclose these functionally relevant dynamic features. As a result, EMP-AF-NH$_2$ can be depicted like a rigid amphipathic helix ideal for interacting efficiently with the membrane surface, as has been found for other peptides (65, 66). Interestingly, as for mastoparans B and X in a TFE/H$_2$O mixture (67, 68) or in a membrane-bound state (69), only the first two N-terminal residues experience a certain degree of flexibility, a structural feature considered relevant for the peptide biological activity (68). As for EMP-AF-COO$^-$, instead, the peptide structure consists of two helical segments joined at the Gly9 residue and fluctuating independently. This molecular motion generates a perturbation of the peptide hydrophobic and polar face that is expected to hamper its ability to efficiently interact with the membrane surface.

**CONCLUSIONS**

The enhanced potency of amidated inflammatory peptides is often attributed to their increased positive charge (70–73). It has been postulated that positive charges promote interactions with the predominantly negatively charged membranes and facilitate the interaction of the peptides with the cell membrane (74), leading to its permeabilization.

In the case of EMP-AF-COO$^-$, we have shown that, besides the expected reduction of its positive charge, the presence of the free carboxyl group at C-terminus introduces a local molecular instability that is able to partly destabilize not only the hydrogen bond pattern of that stretch of helix but the peptide as a whole. In fact, the regular amphipathic helix that in EMP-AF-NH$_2$ extends over the membrane surface, as has been found for other peptides (65, 66). Interestingly, as for mastoparans B and X in a TFE/H$_2$O mixture (67, 68) or in a membrane-bound state (69), only the first two N-terminal residues experience a certain degree of flexibility, a structural feature considered relevant for the peptide biological activity (68). As for EMP-AF-COO$^-$, instead, the peptide structure consists of two helical segments joined at the Gly9 residue and fluctuating independently. This molecular motion generates a perturbation of the peptide hydrophobic and polar face that is expected to hamper its ability to efficiently interact with the membrane surface.

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The enhanced potency of amidated inflammatory peptides is often attributed to their increased positive charge (70–73). It has been postulated that positive charges promote interactions with the predominantly negatively charged membranes and facilitate the interaction of the peptides with the cell membrane (74), leading to its permeabilization.

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entire peptide is disrupted in EMP-AF-COO by the onset of an independent fluctuation of the N- and C-terminal regions. Such structural perturbation, by amplifying its difficulty in interacting with the membrane surface, is expected to enhance the reduction of the peptide biological activity.

The fact that the CD data indicate an increase in helicity for EMP-AF-NH$_2$ on going from TFE to SDS micelles, and no differences for EMP-AF-COO, strongly supports this view.

We believe this mechanistic model may help in the understanding of the relationship between the structure of inflammatory peptides and their target membranes and can be useful in devising new biologically active peptides.

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REFERENCES


