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Electron Capture Dissociation Mass Spectrometry for Gaseous Protonated Melittin Ions and Its Single Amino Acid Substituted Variants

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Abstract : This study demonstrated the sensitivity of electron capture dissociation mass spectrometry (ECD-MS) to probe subtle conformational changes in gaseous melittin ions induced by the substitution of an amino acid. ECD-MS was performed for triply and quadruply-protonated melittin and its variants obtained by a single amino acid substitution, namely, D-Pro14, Pro14Ala, and Leu13Ala. Although native triply-protonted melittin showed only a few peptide backbone cleavage products, the D-Pro14 and Pro14Ala variants exhibited extensive backbone fragments, suggesting the occurrence of a significant structural or conformational change induced by a single amino acid substitution at Pro14. On the contrary, the substitution at Leu13, namely Leu13Ala (+3), did not cause significant changes in the ECD backbone fragmentation pattern. Thus, the sensitivity of ECD-MS is demonstrated to be good enough to probe the aforementioned conformational change in melittin.

Keywords : Electron Capture Dissociation, Melittin, Amino Acid Substitution, Chiral Inversion, Mass Spectrometry

Introduction

Studies on the structural features of gaseous biomolecules generated by the soft ionization method of electrospray ionization (ESI) have attracted significant attention, especially from the viewpoint of solution-phase structures.[1-6] A variety of mass spectrometry (MS) methods, measurements of collision cross-sections (or ion-mobility),[7-9] H/D exchange,[10,11] and electron capture dissociation (ECD)[3-6,12-14] have been employed in these studies. Among these methods, ECD can be employed to cleave peptide backbones with minimal structural changes induced during ECD tandem MS. Thus, ECD has been used for probing conformational changes of gaseous protein cations.[3,5,6] It can detect structural changes induced by a single D-amino acid substitution in proteins.[4] Adams *et al*. found that ratios of the abundances of z_{18} and z_{19} fragments in the ECD of doubly-protonated "Trp-cage" (20 amino acids) cations significantly changed with the D-substitution of a single amino acid, *i.e*., R_{chiral} = 8.6.[4] Their ECD results for the native form of the doubly-protonated cations favoured the preservation of the solution-phase tertiary structures. Furthermore, since their model system Trp-cage is known to be one of the smallest proteins defined by tertiary structures, the molecular dynamics simulations (MDS) were amenable.[4,15-17] Simulation studies showed that there was a strong correlation between the frequencies of occurrence of neutral H-bonding in MDS results and experimentally observed N-C_α bond cleavage frequencies, thereby supporting the 'neutral H-bonding model' versus the 'charge-solvation model'.[4]

Herein, a 26-mer melittin (GIGAVLKVLTTGLPALISW IKRKRQQ), *venom* peptide from the *Apis mellifera* honey bee, was chosen as a model system for ECD studies. Melittin comprises two amphiphatic helical secondary structure segments separated by a hinge region near the position of proline-14 (Pro14) (see Scheme 1). This peptide shows a striking structural contrast to Trp-cage with defined tertiary structures. Furthermore, owing to its important cytolytic activity, structural information is well documented; for instance, molecular modelling *in vacuo* was performed and D-Pro14 substituted melittin structures in methanol were obtained by nuclear magnetic resonance (NMR) spectroscopy.[18-22] In previous molecular modeling studies, the main structural features of melittin were predicted to be more or less preserved even *in vacuo*.[21,23] The unique cyclic structure of proline forms a kink between the two helical regions.

Herein, ECD was performed for a few melittin variants in which a single amino acid substitution was substituted

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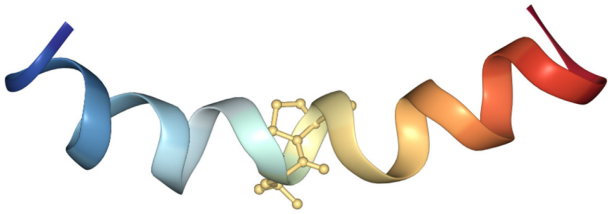

Scheme 1. A Structure of *Venom melittin* from PDB (Protein Data Bank).

for various amino acid positions near Pro14 with an aim to demonstrate the sensitivity of ECD to monitor subtle conformational changes due to a single amino acid substitution.

Experiments

Experiments were performed on a commercial 4.7 T electrospray ionization-Fourier transform mass spectrometer (ESI-FTMS: Ionspec Inc., Lake Forest, CA, USA), equipped with ECD capability. Sample solutions between 20 and 50 μM were prepared in a water:methanol solvent solution (98:2, v/v) buffered at pH ~7.5. Sample solutions were infused directly through a home-pulled fused silica capillary (i.d. = 100 μm) emitter at a flow rate of ~1.0 μL/min, using a syringe pump (Harvard Apparatus 22, Holliston, MA, USA). Mild ESI source conditions were employed, namely the applied capillary voltage was between +1.5 and 2.1 kV while the capillary temperature was held at 120°C.

The generated ions were externally accumulated in a hexapole linear trap for between 300 and 500 ms. The accumulated ions were transferred through rf-only quadrupole guides into a closed cylindrical ion cyclotron resonance (ICR) cell. Ion trapping in the cell was accomplished without the aid of a collision gas. The molecular ions of interest were isolated using single stored waveform inverse Fourier-transform (SWIFT) waveform. An interval of 10 s was given before the application of ECD, and this was performed by exposing the isolated molecular ions to electron irradiation which was generated on the surface of an indirectly heated dispenser cathode with a diameter of 3.4 mm (Heatwave Labs, Watsonville, CA, USA). The electron's kinetic energy was controlled by adjusting the potential difference between the dispenser cathode and the rear trap plate; this was set to between 0.65 and 0.70 eV. The electron beam was applied in the form of a pulse at a length of 1-2 s. Broadband detection of the ions was conducted with 1,024 KW data points and at an ADC rate of 2 MHz in the range between 200 and 2,500 *m/z*. All mass spectra were obtained by aggregating more than 30 scans.

Venom melittin was purchased from Sigma (St. Louis, MO, USA) and used without further purification. Variants of melittin possessing a single amino acid substitution, such as D-Pro14, Pro14Ala, and Leu13Ala, were synthesized (Peptron Inc., Daejeon, Korea) using the Fmoc solid-phase method. Here, Pro14Ala and Leu13Ala represent the melittin variants in which proline-14 and leucine-13 are replaced by alanine, and D-Pro14 refers to the melittin variant in which the L-forms of proline-14 was replaced with the D-form.

Results and Discussion

ECD of melittin cations

A melittin sample solution, which had been prepared in a water:methanol (98:2, v/v) solution buffered at pH ~7.5, was electrosprayed under mild ESI interface conditions. The composition of the sample solution and the mass spectrometry operational parameters were carefully adjusted in order to ensure the favorable formation of the *native-like* melittin conformers. The resulting ESI mass spectrum exhibited a charge distribution in which triply-protonated melittins, $[M+3H]^{3+}$, were most abundant. When more methanol or acetonitrile was added to the ESI solution, the electrospray process was easily facilitated, however, the charge distribution shifted toward higher charge states. This is consistent with the reported literatures.[3,24]

ECD experiments were carried out for triply-protonated (+3) and quadruply-protontated (+4) melittin, respectively, which were isolated by SWIFT. To avoid any possible thermal activation of the melittin ions, there was an interval of 10 s between SWIFT and the electron irradiation event (ECD). The resulting ECD mass spectra are shown in Figure 1. Overall, triply-protonated melittin yielded fewer ECD fragments than quadruply-protonated melittins. For triply-protonated melittin, only a few terminal backbone fragments appeared (Figure 1a). It is also noteworthy that the abundance of singly-reduced species, that is, $[M+3H]^{2+\bullet}$ at *m/z* 1424, was low. To see whether more ECD-friendly conditions induced an increase in the number of ECD fragments for the triply-protonated melittin, an extended electron irradiation, *e.g.*, a 10 s electron gate opening or a higher electron current, was applied. However, the extent of fragmentation in the protein backbone of triply-protonated melittin did not change much (mass spectra not shown). In contrast, ECD of quadruply-protonated melittin exhibited far more extensive backbone *c/z* fragments throughout the entire sequence (see Figure 1b).

Limited protein backbone dissociations in the ECD of low charge state proteins can be generally understood by looking at tertiary structures. These are prominent in the lower charge states, and can hold two ECD peptide backbone fragments together through a variety of interactions, including London interactions, dipole-dipole, ion-dipole, and more importantly, hydrogen bonds. As a

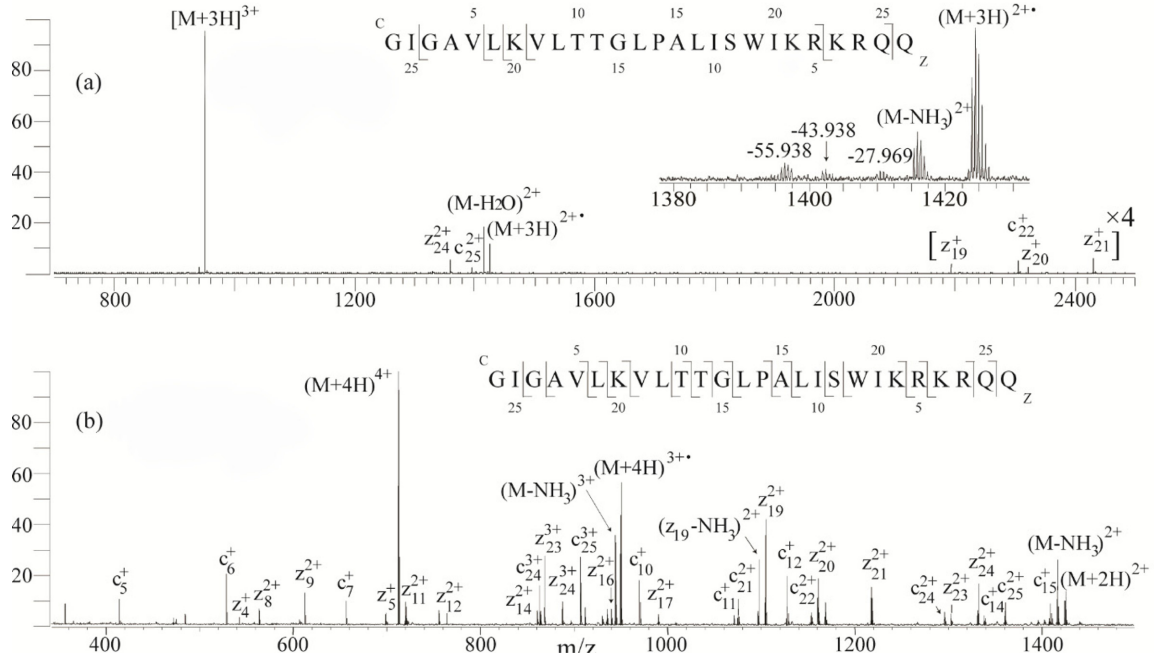

Figure 1. ECD mass spectra for the (a) triply-protonated (+3) and (b) quadruply-protontated (+4) mellittin.

result, fewer backbone fragments could be observed. However, a short sequence of melittin (26 amino-acid residues) is not likely to allow for the presence of many tertiary structures. Previous molecular modelling studies for melittin predicted that the extensive α-helical structure in the solution was preserved *in vacuo* and that tertiary structures were not prevalent under these conditions.[21,23] The lack of tertiary structures in triply-protonated melittin is also evident by the abundance of the singly-reduced species, $[M+3H]^{2+\bullet}$ (see Figure 1a). In general, it is known that the singly-reduced species represents two ECD-induced complementary fragment pairs that are held together through the variety of interactions ubiquitous in tertiary structures.[3,14] On the other hand, the low electron capture efficiency of triply-protonated melittin might be associated with limited ECD protein backbone dissociations. The electron capture efficiency was previously reported to be proportional to the triple of the charge, *i.e.*, z^3.[25] However, other melittin variant cations of similar size and in the same charge state exhibit more enhanced ECD fragmentation (*vide infra*).

Another possible explanation for the low ECD efficiency for triply-protonated melittins is the presence of certain structural features that do not allow for substantial ECD fragmentations. Furthermore, the addition of an extra proton to triply-protonated melittin may cause substantial structural changes for quadruply-protonated melittin, so that it enhanced ECD fragmentation of the protein backbone.

Distribution of protons in the melittin cations

Ionic and neutral hydrogen bonds play important roles in maintaining secondary and tertiary structures in gaseous molecules.[4] Ionic hydrogen bonds, particularly those that incorporate a proton (H^+), are stronger by 8 to 30 kcal/mol than their neutral counterparts.[26] Therefore, they are critical in governing the conformations that proteins will adopt, particularly in the gas phase.[4] Due to this, extensive knowledge about the relevant protonation sites is important for understanding the related ECD spectra and possible correlation found in gaseous protein structures. Indeed, placing protons at different sequence positions was previously shown to promote different structural characteristics in ion-mobility experiments and the associated MDS.[27]

The locations of protons on melittin cations can be inferred from the gas-phase basicity of the free amino acids (Arg > His > Lys ~ N-terminal amino group) and from the charge states of the fragments obtained via ECD.[3] Melittin contains two arginine (R22 and R24) and three lysine (K7, K21, and K23) residues in the sequence. However, it should be noted that when inferring protonation sites based on the charge states of the associated ECD fragments, a long-range proton (or hydrogen atom) transfer, as a precursor step for ECD, is not considered. That is, ECD is presumed to occur only in the neighboring residues of the protonated amino acid residue in the direction of either the N- or C-terminal. For melittin molecules that have

extended solution-phase conformations with a substantial number of α-helical secondary structures, ignoring the long-range proton transfer appears to be reasonable.

In the ECD mass spectrum for quadruply-protontated (+4) melittins, a series of *z*-type dications, namely z_{11}^{2+}, z_{12}^{2+}, z_{14}^{2+}, z_{16}^{2+}, z_{17}^{2+}, z_{19}^{2+}, z_{20}^{2+}, z_{21}^{2+}, z_{23}^{2+}, and z_{24}^{2+}, were observed. The presence of this series of dications, particularly z_{11}^{2+} and z_{12}^{2+}, indicates that two protons residing on the C-terminal region, most likely in the C-terminal KRKR (21-24) region. The other two protons are thought to be located at the N-terminus and in the central regions, respectively. Since a proton initiates peptide backbone dissociation from its neighbors by combining with an electron, the presence of N-terminal *z*-type dications, such as z_{21}^{2+}, z_{23}^{2+}, and z_{24}^{2+}, suggests that one proton likely resides at the N-terminus and the other proton is located in the roughly central region of melittin (presumably at K7). This proton distribution pattern is also in agreement with the charge state distribution of *c*-type fragments.

A series of *c*-type singly protonated fragments, *i.e.*, $c^{+}_{5\text{–}7,\ 10\text{–}12,\ 14,\ 15}$, are likely to be produced via electron capture at K7; that is, there are two protons residing on the N-terminal amino group and K7, respectively. In addition, doubly-protonated *c*-type fragments, such as $c^{2+}_{21,\ 22,\ 24,\ 25}$, and triply-protonated fragments, such as c^{3+}_{24} and c^{3+}_{25}, also support the aforementioned proton distribution pattern observed for quadruply-protontated (+4) melittin.

For triply-protonated (+3) melittin, the scarcity of fragment ions makes it difficult to infer the location of the protonation sites. Nevertheless, analysis of the charge states of the fragments could still be made. This indicates that there are two protons residing on the N-terminus and in the central region (K7), and another proton is in the KRKR region. This proton distribution pattern is supported by the presence of z_{19}^{+}, z_{20}^{+}, and z_{21}^{+} monocations and z_{24}^{2+} dication.

A single amino acid substitution

In aqueous native melittin, proline-14 forms a kinked region between two α-helical segments due to its cyclic structure. Its unique structural role in melittin has brought substantial attention to structural and functional-activity assessments of the melittin variants that have been modified at the proline-14 residue. These include the variants with chiral inversion at the proline-14 such as D-Pro14.[28] It has been shown that D-Pro14 has noticeable differences in its backbone conformation when compared with its L-Pro14 counterpart, particularly, in the hydrogen bonding network around the kinked region 12-17.[28] As another example, ^{1}H NMR analysis has shown that the NH of leucine-16 in aqueous native melittin was hydrogen bonded with glycine-12 in a bond that straddled the kink, whereas leucine-16 was involved in a hydrogen bond with leucine-13 as part of the β-turn encompassing residues 13-16 for D-Pro14 in methanol.[28] D-proline-14 substitution also triggered significant biological consequences as it was found to exhibit no haemolytic or cytotoxic activity.[57]

In the present study, a similar approach to single amino-acid substitution was employed in order to assess the structural role of proline-14 in gaseous melittin cations. For this, D-Pro14, Pro14Ala, and Leu13Ala were synthesized, and ECD spectra were acquired for triply- and quadruply-protonated variants. In the Pro14Ala and Leu13Ala variants, proline-14 and leucine-13 were substituted for alanine, respectively.

ECDs for triply-protonated melittin variants were found to be sensitive enough for monitoring the conformational differences between the native molecule and the variants. As shown in Figure 2(a) and (b), the ECD spectra for D-Pro14 and Pro14Ala variants in the triply-protonated state showed significantly enhanced backbone fragmentations in comparison to the ECD spectrum for triply-protonated native melittin. In terms of the number of cleaved backbone interresidue bonds, D-Pro14 and Pro14Ala yielded a total of twenty-three and twenty backbone bond cleavages, respectively, whereas native melittin had seven (see Figure 1a). Although D-Pro14 and Pro14Ala had very similar numbers of backbone interresidue bond cleavages, there were noticeable differences in the relative abundance of individual ECD products and the locations of the backbone cleavages. For example, the interresidue bond between valine-18 and leucine-9 in Pro14Ala was cleaved, showing c_8^{+} fragments, whereas cleavage at this location was absent in D-Pro14. Conversely, c_{17}^{+} and z_9^{+} fragments were absent in the ECD spectrum for Pro14Ala but appeared in the ECD spectrum for D-Pro14. It was also found that the L→D chiral inversion at proline-14 led to more prominent cleavages around proline-14 than the Pro14 → Ala14 substitution, which was evident in the appearance of peaks c_{14}^{+}, c_{15}^{+}, z_{11}^{+}, and z_{12}^{+} in the ECD spectrum for D-Pro14 (Figure 2a).

In addition, proline-14 substitution induced more drastic conformational changes than leucine-13 substitution did. When leucine-13 was replaced with alanine, ECD cleavage occurred just as sparsely as it did in the native one, but the cleavage pattern was only slightly different. For example, in the ECD spectrum of triply-protonated native melittin cations, a few fragment peaks appeared below the *m/z* values of the singly reduced peak, $[M+3H]^{2+\bullet}$, whereas no fragment peak appeared for triply-protonated Leu13Ala with the exception of a peak associated with the loss of a water molecule (see Figure 2c).

For quadruply-protonated cations, the ECD spectra for all the native and variants melittins showed extensive backbone fragmentation, but the ECD fragmentation patterns were noticeably different from each other (ECD spectra not shown). However, the variations were not as large as those associated with the triply-protonated cations, indicating that, for probing conformational changes, ECD

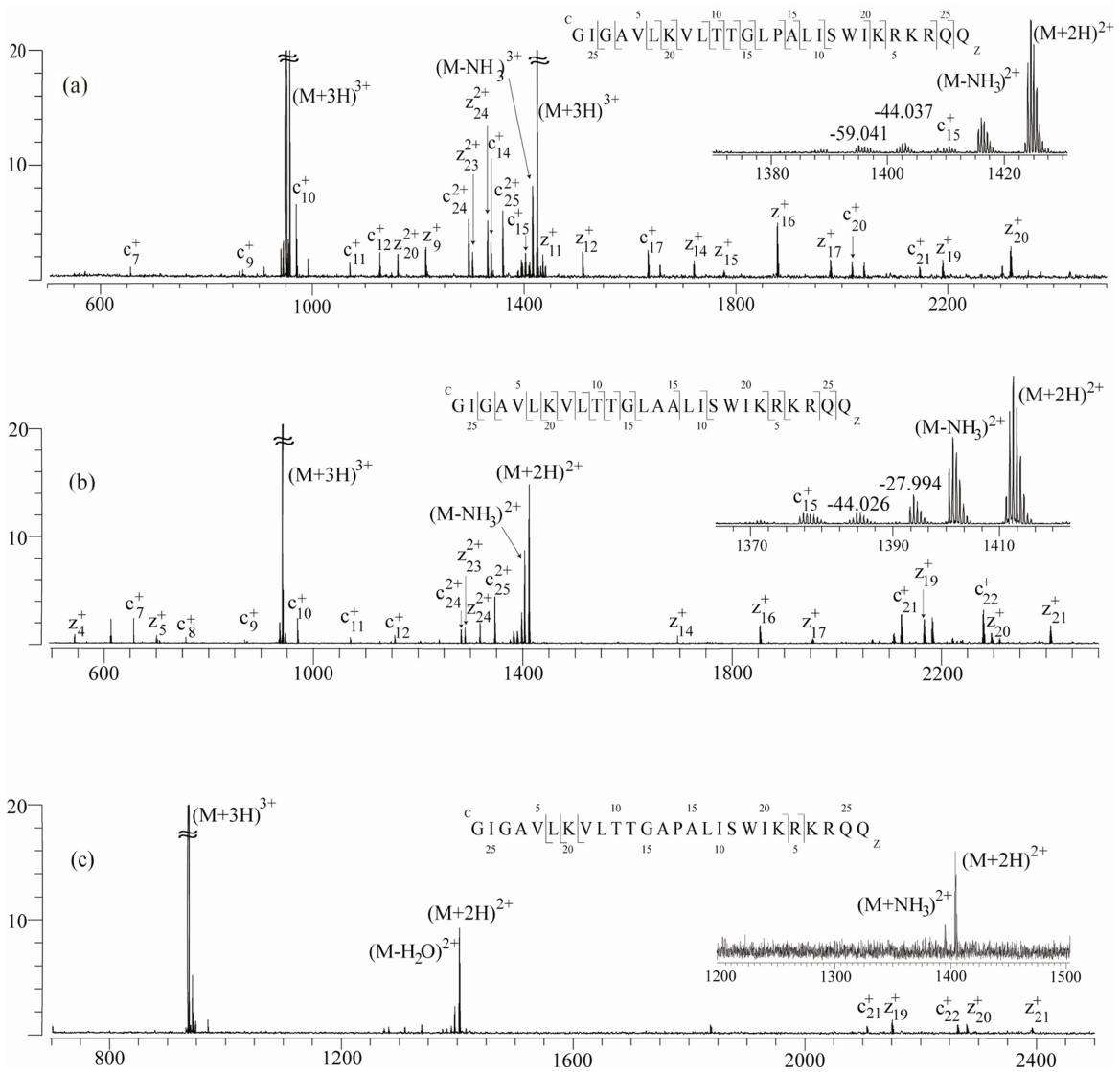

Figure 2. ECD mass spectra for (a) D-Pro14, (b) Pro14Ala and (c) Leu13Ala mellitin variants cations in the triply-protonated (+3) state.

of proteins in high charge states was not as effective as that in low charge states. This is presumably due to the collapse of tertiary conformations by Coulombic repulsions.

To summarize, the substitution of proline-14 was found to cause significant changes in the ECD fragmentation pattern. This finding suggests that proline-14 plays an important role in determining the gas-phase structures.

Conclusions

Herein, it was demonstrated that ECD was a sensitive tool for the investigation of subtle conformational changes of melittin which had been induced by substitution of an amino acid. Collaborative MD simulations are expected to provide useful and detailed information about the structural changes induced by substitution of a single amino acid in the gas-phase melittin ions.

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