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Structural characterization of monomers and oligomers of D-amino acid-containing peptides using T-wave ion mobility mass spectrometry

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Abstract

The D-residues in D-amino acid containing peptides (DAACPs) are proven crucial to biological function of these peptides. Previous ion mobility mass spectrometry (IM-MS) studies revealing oligomerization patterns of amyloid cascade demonstrated conversion from native soluble unstructured assembly to fibril β -sheet oligomers, which has been implicated in amyloid diseases, such as Alzheimer's disease and type 2 diabetes. Although neuropeptides are typically present at very low concentrations in circulation, their local concentrations could be much higher in large dense core vesicles, forming dimers or oligomers. We studied the oligomerization of the protonated peptides and metal adducts of achatin I and dermorphin peptide isomers with IM-MS. Our results suggested that dimerization, oligomerization and metal adduction augment the structural differences between D/L peptide isomers. Dimers and oligomers enhanced the structural differences between D/L peptide isomers in both aqueous and organic solvent system. Furthermore, some oligomer forms were only observed for either D- or L- isomers, indicating the importance of chiral center in oligomerization process. The oligomerization patterns of D/L isomers appear to be similar. Potassium adducts were detected to enlarge the structural differences between D- and L- isomers.

Graphical Abstract

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Keywords

Ion mobility mass spectrometry; IM-MS; D-amino acid containing peptides; DAACPs; native state; organic solvent; monomer; dimer; oligomer; oligomerization pattern; metal adducts; collision cross section; CCS; conformational differences

Introduction

D-amino acid containing peptides (DAACPs) are diastereomers with significant biological functions. Every amino acid (except glycine) can occur in two isomeric forms due to their ability to form stereoisomers around the central α -carbon atom. The DAACP and its corresponding L-form isomer constitute peptide epimers. Before the first DAACP, dermorphin, was isolated in 1981 [1], it was believed that all proteins and peptides were comprised of only L-amino acids. To date, more than 30 endogenous DAACPs have been found in living organisms [2] including crustaceans [3, 4], snails [5–7], spiders [8], frogs [9–11], and even mammals [12, 13]. For example, the spider-venom agatoxins [14], a crustacean hormone [15] and sheep crystallin proteins [16] all contain DAACPs. The D-amino acid residues produced by post-translational isomerization have been proven crucial to the biological activities of DAACPs. For example, the DAACP cardioactive peptide (NdWFamide) significantly enhances the beating amplitude of an *Aplysia* heart at nM level, while the L-isomer has little effect even at μ M level [7, 17]. Achatin I, that contains D-phenylalanine, can excite muscles of the snail, but its L-isomer does not have this function [5]. These observations and reports raise a series of intriguing questions. For example, why are the activities of D/L isomers so different? How much do D/L isomers differ in conformation? Which factors may enhance the differences of these isomers? The first step towards answering these intriguing questions is to develop methods that characterize these structural isomers.

D/L isomer separation is challenging for mass spectrometry (MS) as the isomerization does not change the elemental composition, molecular formula or mass of the peptide. However, the gas phase basicities of the diastereomers can be differentiated when they break apart into fragments [18] and thus can be manifested as different branching ratios among product ions [19]. As a result, MS has become a powerful tool to study DAACPs [20–22]. Fragmentation patterns generated during MS/MS sequencing were used to probe the thermochemical difference between peptide diastereomers during collision-induced dissociation (CID) [20], electron capture dissociation [21] or radical-directed dissociation (RDD) [22]. Although excellent differentiation and quantitation between D/L peptide isomers can be accomplished by these strategies, localization of D-amino acid in peptides is still difficult, as measurement of fragment ion intensities cannot provide accurate positional information of D-amino acids. Our previous study introduced a novel ion mobility mass spectrometry (IM–MS) based strategy enabling site-specific characterization of DAACP isomers to localize D-amino acids [23].

The folding of peptide monomers and oligomers, including conformational changes and oligomerization patterns, has been investigated by IM-MS [24–27]. In neurodegenerative diseases, amyloid cascades transform the native unstructured peptide into β -sheet oligomers forming insoluble plaques [28–30]. The Bowers group studied this conformational conversion with IM-MS. Their study revealed that the unstructured soluble peptide assemblies and insoluble amyloid plaque followed different oligomerization patterns, as shown by different distribution trends of collision cross-section as a function of aggregation state [24]. Although neuropeptides and neurotransmitters are typically present at very low concentrations throughout the nervous system, their concentrations could be much higher in neuronal organelle. For example, the concentration of a neuropeptide in a large dense-core vesicle is 3–10 mM [31], the concentration of acetylcholine (ACh) in synaptic vesicles is ~260 mM [32], the vesicular concentration of catecholamine in chromaffin cells is 190–300 mM [33] and that of dopamine in midbrain neurons is ~300 mM [34]. This paradox prompted an interesting question whether concentrated DAACPs could also form oligomers, and if so, what kind of biomedical consequences the DAACP oligomerization might have. Elucidating the oligomerization pattern and structural changes is important for understanding the activities of DAACPs. Therefore, we set out to study the conformational differences of monomer, dimers, and oligomers, using traveling wave IM-MS.

Peptide oligomers that form different assemblies in solution have been characterized [35–37]. The growth of the oligomers were monitored with IM-MS by measuring collision cross section (CCS). The correlation function between CCS (Y) and oligomer size (n) is different depending on the assembly types [24]. For example, in spatially isotropic self-assembly, the relation is $Y = Y_{\text{mon}} * n^{2/3}$, where Y_{mon} is the monomer CCS. While in fibrillary self-assembly, $Y = a * n + k$, where a and k are constants. Thus, the types of self-assembly could be distinguished via the correlation function between CCS and oligomer size, referred to as oligomerization pattern here after. In this study, we investigated the oligomerization patterns and conformational changes induced by metal binding and other factors. According to our study, dimerization, oligomerization and metal adduct formation augment the structural differences of D/L peptide isomers and thus improving the separation and resolution of these important epimers in IM-MS.

Methods

Chemicals and Biological Samples

Methanol was obtained from Sigma-Aldrich (St. Louis, MO). Optima grade water, acetonitrile (ACN), acetic acid (HAc) and ammonium acetate (certified ACS) were purchased from Fisher Scientific (Pittsburgh, PA). Peptide standards were purchased from American Peptide Company. [D-Ala]-dermorphin ($Y_{(D/L)}AFGYPS$), [D-Phe]-achatin-I ($G_{(D/L)}FAD$) and their all-L forms were synthesized in Biotechnology Center, University of Wisconsin Madison.

CCS Calibration and Measurement

The CCS calibration strategy is similar to that described by Bush et al. [38], but we used a drift-time/CCS power relationship instead of a quadratic relationship [39]. Since T-Wave IM-MS utilizes non-uniform, dynamic electric field to push ions through a buffer gas [40], the Mason-Schamp equation was empirically modified as equation (1). The variable Ω is the CCS, z is the ion charge state, e is the fundamental charge, K_b is Boltzmann's constant, T is temperature, m is the mass of the analyte, M is the mass of the drift gas, P is the pressure, N is the buffer gas number density, and t_D is the ion's drift time or arrival time. The constants A and B result from the non-uniformity of the electric field and must be determined by calibration. The CCS can be normalized by reduced mass and charge state (Ω'), and all other variables can be collapsed into a single term (A') in equation (2). A' and B will be determined by using polyalanine as calibration standard.

$$\Omega = \frac{ze}{16} \left[\frac{18\pi}{K_b T} \left(\frac{1}{m} + \frac{1}{M} \right) \right]^{1/2} \frac{760}{P} \frac{T}{273.15} \frac{1}{N} A t_D^B \quad (1)$$

$$\Omega' = A' t_D^B \quad (2)$$

Polyalanine was suspended in 50% ACN, 1% HAc at 0.1 mg mL^{-1} , and acquired under six different T-Wave IM wave velocity (WV)/wave height (WH) ratios ($\text{m s}^{-1} \text{ V}^{-1}$): 500/30, 500/35, 600/35, 600/40, 700/35, and 800/40. WH refers to the magnitude of the sequential voltage pulses that make up the traveling wave, and WV refers to the propagation speed of those pulses. Instrument parameters were optimized to minimize ion heating during transmission. These parameters include the spray voltage (nano ESI capillary voltage), cone voltage, extraction cone voltage (orthogonal skimmer cone), trap bias (energy for injection into helium cell), and helium cell DC (energy for injection into IM cell) set to 2.0 kV, 30 V, 4 V, 35 V, and 15 V, respectively. The source temperature was set to 70°C . Data processing was conducted using Waters MassLynx 4.1 and DriftScope 2.1. The measured drift time profiles were calibrated into absolute CCS with a home-built software package.

Direct Infusion

IM-MS was performed with the nanospray source of the Synapt G2 HDMS (Waters Corporation, Milford, MA, USA) in positive ionization mode with nitrogen as IM-MS buffer gas. The average pressures (mbar) of the instrument backing, source, trap, and helium cell were 2.73, 1.22×10^{-3} , 2.66×10^{-2} , and 1.46×10^{-3} , respectively. For experiments conducted with aqueous buffer, analytes were dissolved in 500 mM ammonium acetate buffer at concentrations of 1 mg/ml (~ 1.3 mM). For experiments conducted in organic solvent, analytes were dissolved in 50:50 water/methanol solution and sample concentration was 1 mg/ml (~ 1.3 mM). The samples were directly infused into a Synapt G2 HDMS mass spectrometer equipped with a nano-ESI ion source at a rate of 0.05 $\mu\text{L}/\text{min}$ (Waters, Milford, MA, USA). The ion of interest was isolated by the quadrupole. The CCS distributions were converted from extracted drift time distributions with a window of 0.001 Th.

Results and Discussion

1. Dimerization enhances structural difference in both aqueous and 50% methanol/water solvents

For the D/L peptide pairs we studied, dimers presented larger conformational difference compared to monomers. The drift time for monomer and dimer of achatin and dermorphin isomers were recorded in IM-MS under six different WH/WV settings. To obtain decent IM-MS signals, we acquired data for 30–60 minutes for each set. Due to a limited sample amount the IM-MS profile of achatin I in aqueous condition was not recorded; the data was only acquired in 50% methanol/water. The profiles of dermorphin were recorded in both solvents. In both aqueous and 50% methanol/water solution, the monomers of those pairs did not differ much in mobility, but dimers exhibited much bigger differences (Fig. 1). We further converted the drift time into absolute collision cross section using polyalanine as standard calibrant. The relative CCS difference, which is the CCS difference between D-form and L-form divided by CCS of L-form, was calculated. In organic solvent, the difference increased from 0.57% for the achatin monomer to 2.91% for the achatin dimer. For dermorphin, the difference was null for the monomer in both aqueous and 50% methanol/water solvents, and increased to 1.2% and 1.1 % for the dimer respectively.

Although the structures of diastereomers are very different around the chiral center and in most cases could be easily separated by chiral or even normal reversed-phase HPLC, the chirality did not largely affect the collision cross section or the mobility of the molecules as was measured in IM-MS. The reason might be for short peptides (e.g., 4–7 amino acid peptide) their secondary structures were not significantly affected by isomerization, especially when measured in the gas phase. The relatively low resolution of IM-MS, or T-Wave IM-MS more specifically, could further contribute to the small differences observed. IM-MS separates gas phase ions based on their differential mobility through a buffer gas. IM-MS gains advantages on rapid separation speed (on the millisecond timescale vs. HPLC typically on the scale of seconds), and low detection limit (attomole amounts, which is a 10-fold improvement compared to HPLC) [41]. However, T-Wave IM-MS does suffer from low resolving power ($R < 100 \Omega / \Delta\Omega$), meaning that an ion with Ω of 1,000 $\text{\AA}^2$ can theoretically be well separated from an ion with $\pm 10 \text{\AA}^2$ difference in Ω [41]. As a result, although the

local conformation around the chiral center of the DAACP monomers differs largely, the conformational difference we observed in IM-MS could be quite small. A benchmark study performed by the Ruotolo's group showed the relative standard deviation values in CCS, as a function of Weight Height, for alcohol dehydrogenase (ADH) tetramers demonstrating a small amount of variation, ranging from 0.1% to 0.3% [42]. The relative CCS differences of the dimers observed in our study, being 1% – 2.9%, were 10–30 times larger than the typical systematic variation, suggesting that the conformational differences between the D/L dimers measured in IM-MS study are rather significant. Although the measured peptides may have different conformations in organic or native solution, the overall trend in both native (aqueous) and denatured conditions suggested that dimerization enhanced conformational differences between D/L peptide isomers.

2. Oligomers were detected for dermorphin isomers in aqueous and 50% methanol/water solvents with unique conformers to D- or L- isomers being observed

Our IM-MS data suggested that peptide oligomerization was observed for D/L dermorphin in both aqueous and 50% methanol/water solvents. We tested our hypothesis that D/L dermorphin can form oligomers at 1 mg/ml (~ 1.3 mM) in aqueous and 50% methanol/water solvents. High order oligomers were detected in both D- and L-forms and in both solvents. We annotated each ion with its number of subunits and charge state, shown in Fig. 2. Fewer number of different oligomer peaks were detected in aqueous solution as compared to 50% methanol/water solvent, while more salt adducts were detected in aqueous solution. The reason could be that the ionization efficiency of the peptide oligomers in aqueous samples was poor and thus not all the oligomers were observed in IM-MS or much more non-specific binding occurred in organic solvent and thus more oligomers were observed.

Oligomers of either D- or L- form assume certain conformations that were absent from the other form, indicating the importance of chiral center for oligomerization. As is shown in the representative IM-MS drift time distributions of dermorphin oligomers (Fig. 3), many D/L dermorphin oligomers share similar conformations while different conformations are also present in oligomers such as decamer at +4 charge state and docosamer at +6 charge state. We also studied the dynamic conversion of oligomer conformation by adjusting the ratio of D/L isomers among 100% D-dermorphin, 50%/50% D- and L-dermorphin, and 100% L-dermorphin. As shown in Fig. 4, all conformers, which were unique to D- or L- isomers, were present in the 50/50 mixture. For example, for the 10/4 oligomer a larger percentage of the molecules existed as the more extended form in pure D form while in pure L form the majority adopted the more compact conformation and the 50/50 mixture contained both conformations.

3. Oligomerization pattern

Collision cross section (CCS) reflects the size and shape differences of different ions, and correlation between CCS and number of subunit in the molecules provides information on oligomerization type of the oligomers. We measured the mobility of D/L dermorphin at six different WH/WV settings and converted the drift time to absolute CCS using polyalanine as calibration standard. The average CCS under six settings was reported as the representative CCS value of the dermorphin oligomers. The oligomerization pattern was determined by

calculating the CCS distribution vs. the number of oligomer subunits. The oligomerization patterns of D/L dermorphin are likely similar in organic solvent (Fig. 5A). The distribution could be fitted into an exponential function with the form of $Y = A * X^B$, where Y is the CCS value, X represents the number of subunits, and A and B are constants. The fitted equation for D-dermorphin is $Y = 212.66 * X^{0.6311}$, while the equation for L-dermorphin is $Y = 216.07 * X^{0.6193}$. Since constants A and B are close in both D/L forms, we assume that the oligomerization patterns of D/L dermorphin are likely to be very similar. Their fitted equations are close to spatially isotropic self-assembly model and it is very likely the D/L dermorphin oligomers undergo isotropic assembly. Although chirality affected oligomer conformations, it did not trigger alternative self-assembly patterns in aqueous or 50% methanol/water based on the results in the current study.

Most of the observed oligomers formed by L-form dermorphin are present in a more extended conformation compared to their D-form counterparts. For all the L-form oligomers, we compared the conformations with those of D-form counterparts by calculating the CCS difference between L- and D- form oligomers under six different sets of wave height/wave velocity measurements. The results were shown in Fig. 5B, and the CCS difference was increased from null for the monomer to $1 \sim 4 \text{ \AA}^2$ for different oligomers. L-form oligomers exhibited larger CCS suggesting that the L-form oligomers have more extended conformation in comparison to the D-forms. It might be very possible that the trend was solvent specific. For the limited IM-MS oligomer peaks detected in aqueous experiments, the D-forms exhibited slightly more extended conformation compared to L-form oligomers, however the differences are relatively modest. Overall, our observation supported that the chiral center played an important role in forming oligomers and ion mobility was capable to detect those small changes.

Other techniques such as native gel analyses, high-resolution atomic force microscopy, electron microscopy and molecular structural modeling could be combined with IM-MS to verify peptide oligomerization *in vitro*. Applying native gel analyses of full-length tau and deletion constructs, the Bowers' group demonstrated that the N-terminal region produced multiple bands, which was consistent with oligomerization [43]. High resolution atomic force microscopy was used to directly image populations of small oligomers and observe features that can be attributed to oligomers with different number of subunits [44]. By combining electron cryomicroscopy, 3D reconstruction, and integrative structural modeling methods, Matthias Schmidt and co-workers determined the molecular architecture of a fibril formed by A β (1–42). Their model revealed that the individual layers of the A β fibril were formed by peptide dimers with face-to-face packing [45]. To confirm the oligomer formation of DAACPs and their oligomerization pattern in large dense core vesicles, we are going to employ electron microscopy, high resolution atomic force microscopy, and molecular simulations to further validate our hypothesis proposed by the IM-MS study. To date, we have tested several other DAACPs, including D-/L-GFFD and D-YRFG, demonstrating potential applicability of the methods to other L/D pairs. Additional DAACP isomers will be investigated to reveal general trends of DAACP oligomerization.

4. Potassium adducts of dermorphin isomers

For some of the oligomers, potassium adduct was detected to enhance the conformational differences between oligomers formed by D/L dermorphin isomers. Potassium adducts were detected in both aqueous and 50% methanol/water solvents. However, in aqueous solution most of the adducts dissociated in the drift tube during IM-MS measurements, and we were not able to measure the CCS. For some oligomers, e.g. M+K, 3M+H+K, L- and D- forms showed obvious difference, while some other forms, e.g. 2M+H+K and 4M+H+K, little differences were observed (Fig. 6). We further tested the conformational dynamic conversion by changing the ratio of D/L dermorphin among 100% L- form, 50/50 D- and L-forms, and 100% D-form. The 50/50 mixture contained conformations from both D- and L-forms.

Conclusions

We studied dimerization, oligomerization and metalation of the D/L peptide isomers using IM-MS. Dimers, oligomers and metal adducts were detected in both aqueous and 50% methanol/water solutions. The relative CCS difference of the peptide isomers increased when monomers were converted into dimers, indicating that dimerization enhanced the structural difference of peptide isomers in both aqueous and 50% methanol/water solutions. In both solvent systems, oligomers were detected. The IM-MS results showed some conformers found only for D- or L- isomers, suggesting the importance of chiral center in oligomerization process. The CCS vs. aggregation state distribution of the dermorphin isomers exhibited similar trend while oligomerization enlarged the CCS difference from null for the monomer to $1 \sim 4 \text{ \AA}^2$ for different oligomers. Potassium adducts augmented the structural differences between D/L isomers and the dynamic conversion of oligomer conformations showed 50/50 mixture presented unique conformations in either D- or L-forms. In summary, we demonstrate that IM-MS can be utilized to probe the structural differences of D/L peptide isomers at dimer, oligomer and metalation. These differences may provide helpful molecular clues to address the intriguing question why D/L isomers differ significantly in biological activity. Further biochemical investigation and in-depth characterization of structure-function relationship of DAACPs in monomer and oligomer conditions will be needed to gain a clear mechanistic understanding of the process.

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Figure 1.

Dimerization enhanced structural differences of peptide isomers. A) Achatin I ($G_{(D/L)}$ FAD, MW 408.41) in 50% methanol/water, the relative CCS difference increased from 0.57% for the monomer to 2.91% for the dimer. B) Dermorphin ($Y_{(D/L)}$ AFGYPS, MW 802.89) in 50% methanol/water, the relative CCS difference increased from null for the monomer to 1.2 % for the dimer. C) Dermorphin in aqueous solution, the relative CCS difference increased from null for the monomer to 1.1% for the dimer.

Figure 2.

Oligomers of dermorphin isomers were detected in both 50% methanol/water for D-dermorphin (A) and L-dermorphin (B) and aqueous solution for D-dermorphin (C) and L-dermorphin (D). Each ion was annotated with its number of subunits and charge state. For example, 2/1 means a dimer at +1 charge state.

Figure 3. Drift time profiles of the dermorphin oligomers in aqueous buffer with some conformers that were unique to D- or L- isomers.

Figure 4.
Dynamic conversion of dermorphin oligomer conformations in 50% methanol/water.

Figure 5.

(A) Oligomerization pattern of dermorphin isomers in 50% methanol/water as shown in the collision cross section vs. aggregation state distribution. The oligomerization patterns of D/L dermorphin isomers appear to be similar. (B) The collision cross section difference between L- and D-dermorphin oligomers in 50% methanol/water, calculated as the CCS of L-isomers subtract the ones of the corresponding D-isomers. For monomer and oligomer with seven subunits, there was one single measurement and no error bar was obtained for these two data points.

Figure 6.

The drift time profiles and their dynamic conversion of dermorphin oligomer conformations of potassium adducts in 50% methanol/water. Monomer and trimer adducts showed obvious difference between D- and L- forms, while the dimer and tetramer adducts did not. The conformational dynamic conversion studies with the ratio of D/L dermorphin changed among 100% L- form, 50/50 D- and L-forms, and 100% D-form showed that the 50/50 mixture preserved conformations from both D- and L-forms. For 100% D-form dermorphin M+K peak, there were two peaks, indicating the presence of at least two conformations.