

Separation of Isomeric Peptides Using Electrospray Ionization/High-Resolution Ion Mobility Spectrometry

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In this paper, the first examples of baseline separation of isomeric macromolecules by electrospray ionization/ion mobility spectrometry (ESI/IMS) at atmospheric pressure are presented. The behavior of a number of different isomeric peptides in the IMS was investigated using nitrogen as a drift gas. The IMS was coupled to a quadrupole mass spectrometer, which was used for identification and selective detection of the electrosprayed ions. The mobility data were used to determine their average collision cross sections. The gas-phase ions of isomeric peptides were found to have different collision cross sections. In all cases, doubly charged ions exhibited significantly (8–20%) larger collision cross sections than the respective singly charged species. The analysis of mixtures of the isomeric peptides clearly demonstrated the capability of IMS to separate gas-phase peptide ions due to small differences in their conformational structures, which cannot be determined by mass spectrometry. An actual resolving power of 80 was achieved for two doubly charged reversed sequenced pentapeptides. Baseline separation was provided for ions differing by only 2.5% in their measured collision cross sections; partial separation was shown for isomeric ions exhibiting differences as small as 1.1%.

The physiological properties of biologically active compounds are strongly related to their structures. For this reason, structure determination of these high-molecular-weight compounds has been an important issue in analytical chemistry. With the development of electrospray ionization (ESI)^{1,2} and matrix-assisted laser desorption/ionization (MALDI),³ the creation of “intact” gas-phase ions of high-molecular-weight compounds has been enabled. Routinely, mass spectrometry was used to analyze peptides and protein sequences, mostly revealing information about the molecular weight and the primary structure. Moreover, these powerful ionization tools have opened up the field for studies of the conformational structure of biomolecules in the gas phase using mass spectrometry (e.g., refs 4 and 5). Recently, ESI was also found to be a suitable ionization method for ion mobility

spectrometry.^{6,7} Consequently, ion mobility techniques have been extensively developed in the past few years to probe the gas-phase conformation of peptides and proteins.^{8–11} As the ion mobility separation process is complementary to mass spectrometric separations, it can be also integrated into mass spectrometers to obtain multidimensional separations of mixtures and comprehensive information about gas-phase ion structures.^{12–14}

The mobility of an ion is a measure of how rapidly it moves through a buffer (drift) gas under the influence of a weak electric field. For macromolecules, it depends on the charge state of the ion and the “geometry” of the molecule (referred to as average collision cross section) rather than the mass.^{15,16} Knowing the charge state from mass spectrometric measurements, the average collision cross section of an ion can be determined from ion mobility data. These “measured” collision cross sections can be correlated with the gas-phase ion conformation and can be a fairly accurate estimate of molecular size.^{9,17} It has already been shown that ion mobility is able to distinguish between isomeric compounds with identical mass-to-charge ratios (m/z), which cannot be separated from each other using mass spectrometry.^{18,19}

Although interesting information about the conformation of different proteins^{10,17,20,21} and peptides^{8,22} has been reported, one of the major restrictions of ion mobility remained the insufficient resolution at the low buffer gas pressures. Recently, Jarrold and co-workers reported high-resolution IMS with laser desorption/

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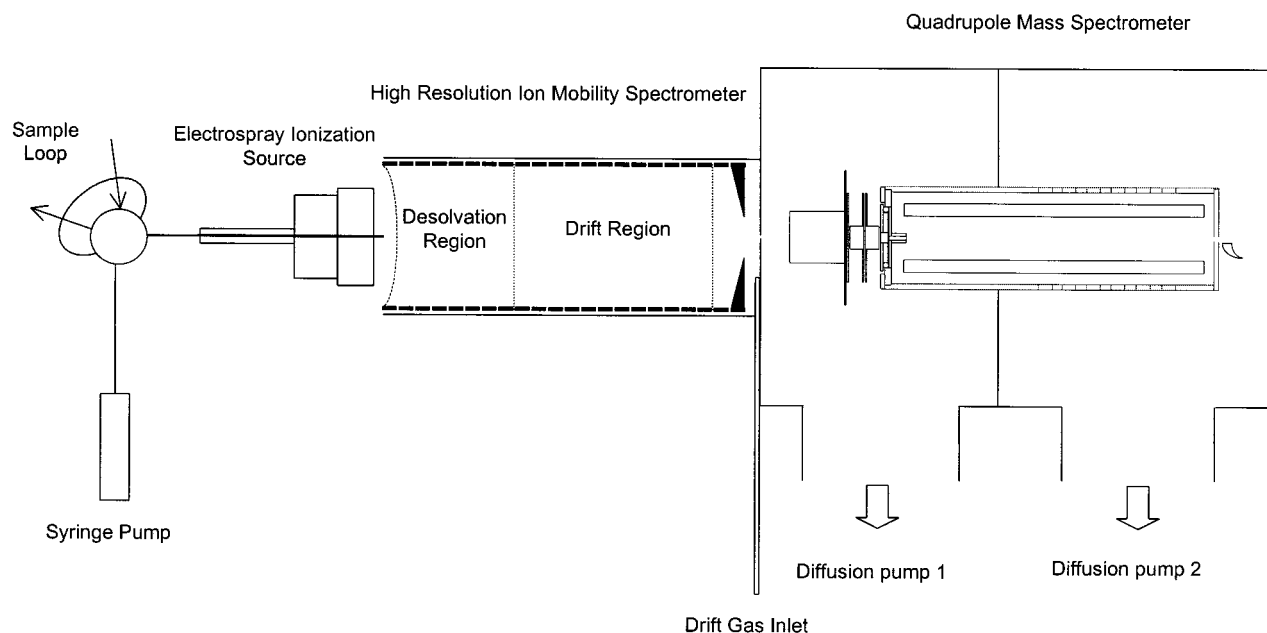


Figure 1. Schematic diagram of instrumental setup (not drawn to scale) including (a) electrospray ionization source, (b) ion mobility spectrometer, and (c) quadrupole mass spectrometer

Table 1. Ion Mobility Measurements of Gas-Phase Peptide Ions

peptide sequence	MW	ion	m/z	K_0 (cm ² /(V s))	Ω (Å ²)
Ser-Asp-Gly-Arg-Gly	490.5	[SG + H] ⁺	491.5	0.850	185.0
		[SG + 2H] ²⁺	246.3	1.412	222.7
Gly-Arg-Gly-Asp-Ser		[GS + H] ⁺	491.5	0.850	185.0
		[GS + H] ²⁺	246.3	1.485	211.7
Sar-Arg-Gly-Asp-Ser-Pro	601.6	[SP + H] ⁺	602.6	0.743	210.5
		[SP + 2H] ²⁺	301.8	1.370	228.4
Gly-Arg-Gly-Asp-Thr-Pro		[GP + H] ⁺	602.6	0.738	212.0
		[GP + 2H] ²⁺	301.8	1.338	233.8
Tyr-Ala-Gly-Phe-Leu	569.7	[TL + H] ⁺	570.7	0.743	210.8
Tyr-D-Ala-Gly-Phe-Leu		[TdAL + H] ⁺	570.7	0.738	212.2
Tyr-D-Ala-Gly-Phe-D-Leu	771.9	[TdAdL + H] ⁺	570.7	0.735	213.1
Leu-Arg-Arg-Ala-Ser-Leu-Gly		[LG + 2H] ²⁺	387.0	1.105	281.8
Leu-Arg-Arg-Ala-Ser-Val-Ala		[LA + 2H] ²⁺	387.0	1.111	280.2

ionization²³ and electrospray ionization,²⁴ but for the latter, no actual separation of distinct ions has yet been published. We have recently shown the high-resolution capability of atmospheric pressure ion mobility spectrometry.¹¹ Resolutions of 57–78 for singly to triply charged ions of a heptapeptide (fragment 1–7 of dynorphin A) and 216 for the (M + 11H)¹¹⁺ ion of cytochrome c have been attained, which were close to the maximum diffusion-limited resolution for these ions. With this resolving power, it should be possible to separate ions due to even small differences in their conformation and the respective average collision cross sections. Initial results, which clearly demonstrated the capability of our IMS instrument to separate isomeric peptides, have been first presented at the 6th International Workshop on Ion Mobility Spectrometry.²⁵ In this paper, we show the actual separation of a number of different isomeric peptide pairs and discuss the results with respect to the gas-phase conformations of the respective ions.

EXPERIMENTAL SECTION

Instrumentation. All experiments were carried out in a high-resolution ion mobility spectrometer coupled to a quadrupole mass spectrometer, which is shown in Figure 1 and has been described in detail earlier.¹¹ As an ionization source, a specially designed water/air-cooled electrospray was used in order to prevent solvent evaporation in the electrospray needle. A detailed description of this device can be found in ref 7.

The electrosprayed ions were introduced through a target screen as solvated droplets into the desolvation region of the ion mobility spectrometer, where the solvent evaporated before they entered the drift region of the spectrometer. The desolvation region (7.2 cm) and the drift region (13 cm) were separated from each other by an ion gate constructed from a Bradbury–Nelson design¹⁵ using parallel wires of Alloy 46 (California Fine Wire Co., Grover Beach, CA 93483) with a 0.64 mm spacing. It was “closed”

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by applying 75 V across adjacent wires, creating an electric field of 1000 V/cm orthogonal to the drift field. Positive ions approaching the gate were then collected on the negative wires and could not pass. To "open" the gate, all the wires were referenced to the drift field voltage. Ions were pulsed into the drift region by opening the ion gate for 0.2 ms at a frequency of 20 Hz. In the drift tube, the ions migrated under the influence of a weak electric field through a counterflow of preheated nitrogen drift gas. As the drift tube was operated at ambient air pressure, higher voltages could be dropped across the drift region enhancing the resolution without significantly increasing the ion energy.

A 40 μm pinhole served as the barrier between the atmospheric region of the ion mobility spectrometer and the vacuum region of the mass spectrometer. The mass spectrometer used for these studies was a C50-Q (ABB Extrel, Pittsburgh, PA) consisting of a set of atmospheric pressure ionization (API) ion lenses and a quadrupole mass filter with rods that were 20 cm in length and 0.95 cm i.d. After passing through the quadrupole mass filter, ions were detected with an electron multiplier (ABB Extrel, model 051-72) equipped with ion-counting capability. The vacuum chamber of the mass spectrometer was maintained at 2.2×10^{-4} Torr in the ion optical region and 1.5×10^{-5} Torr in the electron multiplier region.

The output signal of the mass spectrometer was connected directly to a Keithley 427 amplifier (Keithley Instruments, Cleveland, OH) and was amplified by a gain of 10^9 before entering the data acquisition system. Three different operational modes were utilized to collect data in these experiments. First, non-mobility-selected mass spectra were obtained by opening the ion gate of the IMS and scanning the mass spectrometer to mass-identify all ions in the ion plasma. Second, the mass analyzer was used as an ion detector for the IMS to record non-mass-selected ion mobility spectra. Last, the single ion-monitoring mode of the mass spectrometer was used to monitor only ions with a selected mass-to-charge ratio.

General Operating Conditions. The potentials applied to the electrospray needle and the target screen were 9500 and 5500 V, respectively, producing a potential difference of 4000 V. The potential on the target screen also provided the initial potential for the drift tube, creating a drift field of 279 V/cm throughout the desolvation and the ion drift region. The total drift voltage across the drift region was 3625 V. The nitrogen drift gas was maintained at a temperature of 250 °C and a flow rate of 800 mL/min. The drift tube was operated at ambient air pressure, which was measured for each individual experiment.

The solvent used for the electrosprayed samples was a mixture of methanol/water (1/1, v/v) acidified with 1% acetic acid. The solvent flow through the needle was controlled at 5 $\mu\text{L}/\text{min}$. The cooling gas flow for the electrospray source was 630 mL/min, and the needle temperature was held at 40 °C. For sample injection, a 70 μL external sample loop connected by a six-port valve (C6W, Valco Instruments, Houston, TX) was filled and then switched into the electrospray solvent flow.

Calculations. All of the reduced mobility constant (K_0) and collision cross section (Ω) data reported in this study were deduced from measured drift times (t_d). The standard deviation of repetitive drift time measurements was always below 0.5%. The reduced mobility constants were calculated according to the

following equation:

$$K_0 = \frac{L^2}{V t_d} \left(\frac{273.15}{T} \right) \left(\frac{P}{760} \right)$$

where L is the length of the drift tube (in cm), V is the drift voltage, T is the temperature of the drift gas (in kelvin), and P is the pressure in the drift region (in Torr).

The average ion-neutral collision cross section Ω was calculated from a simple rearrangement of the Mason-Schamp equation,¹⁵

$$\Omega = \frac{3ze}{16N_0} \left(\frac{2\pi}{\mu kT} \right)^{1/2} \frac{V t_d}{L^2} \left(\frac{T}{273.15} \right) \left(\frac{760}{P} \right)$$

where e is the charge of a single proton, z is the number of charges on the ion, N_0 is the number density of the drift gas at standard conditions (1 atm, 273.15 K), μ [$=mM/(m+M)$] is the reduced mass of an ion (m) and the drift gas (M), and k is Boltzmann's constant.

Chemicals. The following isomeric peptides purchased from Sigma (St. Louis, MO) were used as test compounds in this study: (1) Gly-Arg-Gly-Asp-Ser (GS), Ser-Asp-Gly-Arg-Gly (SG); (2) Sar-Arg-Gly-Asp-Ser-Pro (SP) (sarcosine (Sar) = *N*-methylglycine), Gly-Arg-Gly-Asp-Thr-Pro (GP); (3) Tyr-Ala-Gly-Phe-Leu-([Ala²]leucine enkephalin, TL), Tyr-D-Ala-Gly-Phe-Leu (TD-AL), Tyr-D-Ala-Gly-Phe-D-Leu ([D-Ala², D-Leu⁵]leucine enkephalin, TD-AD-L); (4) Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide, LG), Leu-Arg-Arg-Ala-Ser-Val-Ala (Val⁶, Ala⁷-Kemptide, LA)

For injection into the IMS via the electrospray unit, standard solutions (0.1 mM) were prepared in methanol/water containing 1% of acetic acid. All solvents used were reagent grade.

RESULTS AND DISCUSSION

For all peptides investigated, singly and/or doubly protonated ions have been observed in our ESI/IMS experiments. For each peptide ion, the reduced mobility constant K_0 and the average collision cross section Ω were calculated. Table 1 summarizes these results along with the peptide sequence, the molecular weight, and the observed mass-to-charge ratio of the respective ions. It must be pointed out that the "measured" collision cross sections are significantly different when different drift gases are used. Thus, the cross section data given here are only used in a relative manner and cannot be directly compared with data from studies using drift gases other than nitrogen.

Figure 2 shows the ion mobility spectrum of a mixture of the reversed sequenced peptides SG and GS recording only the doubly charged ion species in the single ion-monitoring mode at a mass-to-charge ratio of 246. Although the only difference between these two peptides was the reversed amino acid sequence, the ion mobility process provided baseline separation for the two isomeric ions. The ion mobility resolving power exhibited in this spectrum was calculated to be 80, which in chromatographic terms is equivalent to 36 000 theoretical plates. The separation of the two ions was due to a different molecular size measurable as the collision cross section. The measurements indicated a 5% difference between the two doubly charged isomeric ions with cross sections of 222.7 Å² for [SG + 2H]²⁺ and 211.7 Å²

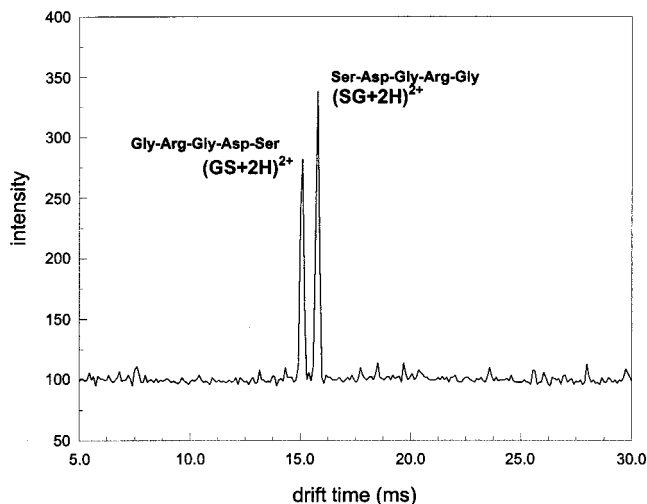
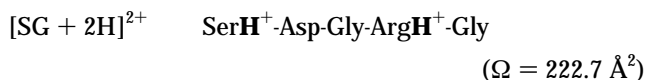


Figure 2. Ion mobility spectrum of a mixture of two isomeric pentapeptides with inverse amino acid sequence. The spectrum was obtained with nitrogen as a drift gas at 250 °C and at atmospheric pressure. The mass spectrometer was operated in the single ion-monitoring mode at a mass-to-charge ratio of 246 showing only the doubly charged gas-phase ions of the peptides.

for $[GS + 2H]^{2+}$, respectively. In contrast, a separation of the singly charged ions of these peptides was not possible. For both the $[SG + H]^+$ and the $[GS + H]^+$ ions, the same collision cross section of 185.0 Å² was determined (see Table 1).

In the positive ion mode, the mechanism of electrospray ionization of the peptides is the attachment of one or two protons to produce gas-phase cations. Presumably, these protons attach to the peptides in the liquid phase as the solvent evaporates. Thus, the maximum charge state of a peptide ion and the most likely position of the charges at a given pH of the solution are defined by the number of basic sites and their respective basicities. For the doubly charged ions of the inverse pentapeptides SG and GS, it can reasonably be predicted that the charges are located at the N-terminal residue and the basic arginine group as follows:



In the case of $[SG + 2H]^{2+}$, an elongated conformation, where the charges are separated from each other and stabilized by neighboring carbonyl sites,²⁶ should be favored, because such a "stretched out" conformation minimizes Coulombic repulsion of the two charges.¹⁰ In contrast, $[GS + 2H]^{2+}$ has two positive charges close to each other providing a much higher Coulombic repulsion energy. As movement of the two charge sites is limited by covalent bonding, the ion has to be stabilized by carbonyl sites on the backbone of the sequence. To do so, the backbone needs to pull around the charge sites folding the peptide in a more compact gas-phase conformation and thus exhibiting a smaller

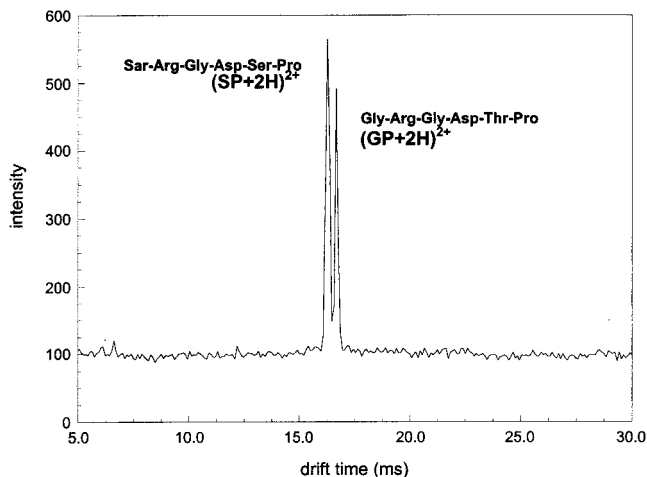


Figure 3. Ion mobility spectrum of a mixture of two isomeric hexapeptides different by the N-terminal amino acid and the fourth amino acid. The spectrum was obtained with nitrogen as a drift gas at 250 °C and at atmospheric pressure. The mass spectrometer was operated in the single ion-monitoring mode at a mass-to-charge ratio of 302 showing only the doubly charged gas-phase ions of the peptides.

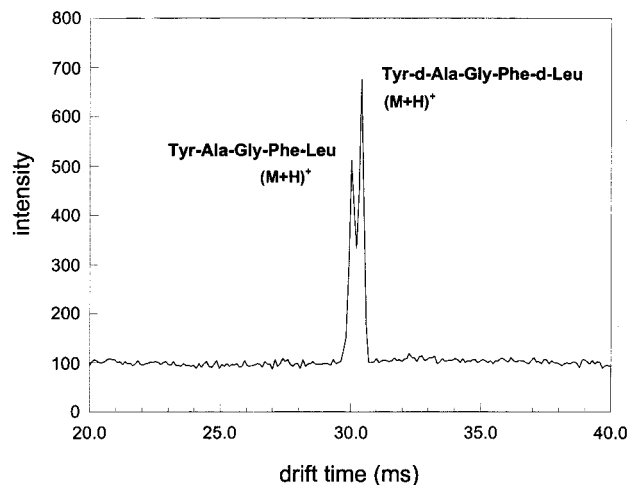


Figure 4. Ion mobility spectrum of a mixture of singly charged [Ala²]-leucine enkephalin (TL) and its D-form stereoisomer, [D-Ala²,D-Leu⁵]-leucine enkephalin (TD-Ad-L). The spectrum was obtained with nitrogen as a drift gas at 250 °C and at atmospheric pressure. The mass spectrometer was operated in the single ion-monitoring mode at a mass-to-charge ratio of 571 showing only the singly charged gas-phase ions of the peptides.

collision cross section compared to the "stretched out" isomer ion. This example shows that the location of the charges in the molecule can affect the gas-phase conformation and enable separation of isomeric peptides in the gas phase.

For the singly charged ions $[SG + H]^+$ and $[GS + H]^+$, the same collision cross section in the gas phase was determined, which was smaller than for the doubly charged ions of the same peptides (Table 1). In the absence of Coulombic repulsion, these ions exhibit a more compact ("folded") gas-phase conformation than the "stretched-out" doubly charged ones. This may be due

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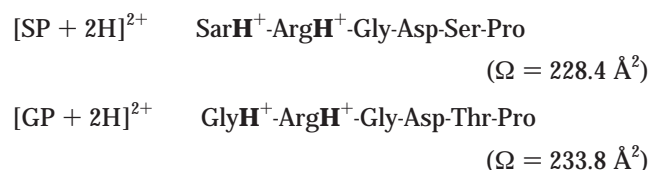
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to stabilization of the charge (most likely located on the arginine residue) by backbone carbonyl groups forming a charge center.⁸ Another possible explanation would be a salt bridge structure as recently proposed for singly charged bradykinin.²⁷

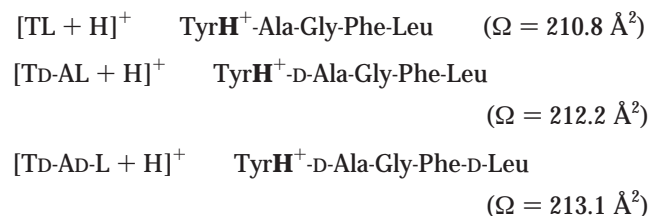
Similar results were observed for another isomeric peptide pair, Sar-Arg-Gly-Asp-Ser-Pro and Gly-Arg-Gly-Asp-Thr-Pro. The difference between these two hexapeptides is that a methyl group located on the amino group in sarcosine is moved to serine, converting the sarcosine to glycine and the serine to threonine. The collision cross sections of the singly charged ions were again significantly smaller than for the doubly charged ones. This was presumably due to folding of the peptide around the charge located on the strongly basic arginine group. The difference of 1.5 Å² (0.7%) in the collision cross sections determined for the two singly charged ions cannot be considered as significant and is thus not further discussed.

The doubly charged ions, however, were significantly different in their collision cross sections by 2.3%. Figure 3 shows the IMS separation of the two doubly charged ions [SP + 2H]²⁺ and [GP + 2H]²⁺. A near-baseline separation of these two ions could be achieved by our ion mobility spectrometer, indicating that the resolving power of this instrument allows baseline separation for compounds with cross section differences as low as 2.5%.



These data suggest that the doubly charged ion of GP exhibits a more elongated conformation in the gas phase than the one of SP. The reason for this measured difference in the gas-phase ion conformation might be the different gas-phase basicities of the N-terminal groups, which are 888.7 kJ/mol for sarcosine and 852.2 kJ/mol for glycine.²⁸ Possibly, the ions undergo different intramolecular interactions in order to overcome the strong Coulombic repulsion between the two charge sites. In GP, for example, the proton on the N-terminal end could be shifted to the carbonyl group on the backbone causing a different gas-phase conformation. It has already been shown that the energy difference between those kinds of structures is not significant due to the low basicity of glycine.²⁶

The three investigated stereoisomeric pentapeptides form another set of interesting data. They have only one basic site in the peptide, but differ by the substitution of D- for L-amino acids. As expected, only singly charged ions were produced for these isomers:



Substituting one or two amino acids in the peptide with the respective D-amino acids led to a small, but measurable increase in collision cross section. As can be seen from Figure 4, our IMS instrument was able to partly separate TL from the stereoisomer with two D-amino acids (Td-AD-L), although baseline separation was not attained. The charge located on the N-terminal end group can be stabilized through interaction with the carbonyl group on the peptide backbone. Possibly, the interaction of the charge with the carbonyl groups was more difficult with D-amino acids and the molecule became twisted, exhibiting larger collision cross sections. In this context, it has to be pointed out that in order to propose definitive gas-phase ion conformations a more detailed study has to be carried out. In this work, we only focused on the IMS instrumentation as an analytical tool of resolving isomeric peptide ions in the gas-phase.

Finally, kemptide and Val⁶,Ala⁷-kemptide were analyzed and their doubly charged ions [LG + 2H]²⁺ and [LA + 2H]²⁺ were monitored in the single ion-monitoring mode. However, they could not be separated from one another by the IMS. Although a small, reproducible difference in their drift times could be established with repetitive measurements, the resolution of the instrument was not sufficient to separate the ions. Thus, the measured collision cross sections of the two isomeric peptide ions showed only a very small difference (0.5%), which cannot be regarded as significant (see Table 1). This is not surprising, as there is only a small difference in the sequence of the peptides (shift of one methyl group from leucine to glycine at the C-terminal end), which does not involve the basic sites, where the charges are most probably located. Further improvement of the resolving power of the instrument is necessary to enable a significant distinction of these two peptide ions with IMS.

CONCLUSION

A number of isomeric peptides were electrosprayed into an ion mobility spectrometer and efficiently separated on the basis of their gas-phase ion mobility differences. With the same ionic mass and charge state, the measured mobility differences of these peptide isomers were caused by their collision cross section differences, which were directly related to the structural conformation of the ions in the gas phase. The achieved resolving power for doubly charged peptide ions was sufficient to baseline separate isomers with collision cross section differences as low as 2.5%. With this improved resolving power, IMS is repositioned among current analytical separation methods as a fast and highly efficient separation technique complementary to mass spectrometric methods.

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