Chapter 6

Separation and Identification of Peptide Isomers by Free-Radical-Initiated Peptide Sequencing (FRIPS) Combined with Ion Mobility-Mass Spectrometry

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6.1 Abstract

Complex mixtures of peptides with strong sequence homogeneity are often challenging to separate by high performance liquid chromatography. However, ion mobility spectrometry (IMS) provides another dimension of analyte separation that is capable of resolving highly similar peptide structures. This study demonstrates the application of a novel classification device, the radial opposed migration ion and aerosol classifier (ROMIAC), to the separation of peptide isomers. The ROMIAC is coupled to the atmospheric pressure interface of a linear trap quadrupole mass spectrometer, and monitoring distinct dissociation pathways associated with peptide isomers fully resolves overlapping features in the ion mobility data. We find that the addition of the reagent for free-radical-initiated peptide sequencing (FRIPS) to the N-terminus of isomeric peptides improves separation by altering gas-phase peptide structure. The ability of the ROMIAC to operate at atmospheric pressure and serve as a front-end analyzer to continuously transmit ions with a particular mobility facilitates its utilization in a variety of experimental protocols in which additional analyte separation is required.

6.2 Introduction

The field of proteomics has advanced rapidly in the past decade with the development of both novel instrumentation and new experimental techniques.[334,335] The combination of mass spectrometry (MS) methods that simultaneously enable high resolution, mass accuracy, and sensitivity with techniques for improved analyte separation through high performance liquid chromatography (HPLC) or capillary electrophoresis has enabled the analysis of increasingly intricate samples and yielded valuable insight into a vast array of cellular processes.[336-341]. In spite of these improvements, many challenges to the analysis of complex samples remain. For example, many isomeric peptides cannot be separated in standard HPLC columns, precluding sequence identification by HPLC-MS.[342-344] In addition, traditional collision-induced dissociation (CID) experiments often lead to loss of post-translational modifications (PTMs), such as phosphorylation or nitrosylation, hindering the identification of modified amino acid residues.

Several recent developments in the dissociation of gas-phase peptide ions offer improved capabilities for peptide sequencing. The advent of electron capture dissociation (ECD) and electron transfer dissociation (ETD) has greatly enhanced the ability of proteomics experiments to identify PTMs, as these dissociation methods typically cleave selectively at N-C_α bonds and leave side chain modifications intact.[245,249-251,280,345,346] However, these techniques have their own experimental limitations, including inefficient dissociation, especially for low charge-state peptides. Free-radical-initiated peptide sequencing (FRIPS) is an alternative method for the gas-phase sequencing that gives information complementary to that obtained by traditional CID or ECD/ETD experiments.[304] In this

technique, a free radical precursor is attached to the N-terminus of a peptide or protein. When the derivatized peptide is subjected to collisional activation, homolytic bond cleavage adjacent to the free radical precursor generates an acetyl radical at the N-terminus of the peptide, which subsequently initiates dissociation of the peptide by hydrogen atom abstraction from various sites along the peptide backbone. As with other radical-directed dissociation techniques, the product ions formed are highly sensitive to the $C_\beta-H$ bond dissociation energy (BDE) of each amino acid residue, with residues possessing high $C_\beta-H$ BDEs preferentially generating side chain loss and those with low $C_\beta-H$ BDEs leading to backbone dissociation.[248,311]

In the separation of complex mixtures prior to dissociation, ion mobility spectrometry (IMS) adds another useful dimension of separation between HPLC and MS analysis. A wide range of methodologies have been successfully employed to separate peptide and other complex biological mixtures, including drift cells,[347,348] traveling wave ion mobility cells,[349] high-field asymmetric waveform devices (FAIMS),[350] and differential mobility analyzers (DMA).[351,352] These techniques have unique advantages and limitations, but many of them require significant new instrumentation that is costly to build and implement.

The opposed migration aerosol classifier (OMAC) is a new instrument that operates with equipotential inlet and outlet flows, reducing loss of ions and particles due to electric field distortions.[353] The OMAC employs parallel porous or screen electrodes and can readily be integrated with the atmospheric pressure interface of a mass spectrometer. A gas cross-flow enters and exits the classification region through porous electrodes within the classification region, exerting a drag force on translating ions or particles. Mobility

classification is accomplished by balancing this drag force with the force from an electric field applied antiparallel to the gas flow. The use of antiparallel drag and electric forces increases the distance that particles or ions must diffuse to degrade instrument resolution, improving instrument performance. A radial geometry further decreases diffusional losses and enhances resolution by elimination of electric field deformities from proximate dielectric walls. We refer to the instrument as a radial opposed migration ion and aerosol classifier (ROMIAC).

The ability of the ROMIAC to classify ions with high transfer efficiency makes it well-suited for the analysis of biological samples, especially peptides, which possess mobility diameters on the order of a single nanometer. This study combines this novel front-end classifier with peptide derivatization by the FRIPS reagent to achieve improved peptide separation prior to sequence analysis by mass spectrometry. Specifically, the ROMIAC is utilized to separate several isomeric peptide samples as a demonstration of the ability of this instrument to improve analysis of peptide samples with significant sequence homogeneity. The addition of the FRIPS reagent to the peptides is found to enhance peptide isomer separation through alterations in gas-phase peptide structure.

6.3 Methods

6.3.1 Materials

High-purity methanol was obtained from J. T. Baker Avantor (Center Valley, PA), and high-purity water as well as ACS grade glacial acetic were purchased from EMD Millipore (Billerica, MA). The model peptides AARAAATAA, AATAAARAA, AARAAHAMA, and AARAAMAHA, were obtained from Biomer Technologies (Pleasanton, CA) and used without further purification.

6.3.2 Synthesis of TEMPO-based FRIPS Reagent

The (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO)-based FRIPS reagent recently developed by Sohn and co-workers, based upon the procedure outlined by Lee and co-workers,[305] was synthesized and employed for free radical generation.[94,306,321] An overview of the synthetic procedure is found in Chapter 5, and full synthetic details have been detailed in a previous publication.[321]

6.3.3 Sample Preparation

Solutions of the underivatized peptides were prepared from a 1 mg/mL stock solution in water, which was then diluted in 49% methanol, 49% water, and 2% acetic acid (v/v) for a 50 μM solution. To derivatize the model peptides, approximately 1 mg of a peptide was dissolved in 1 mL of a 50/50 (v/v) mixture of acetonitrile/water, vortexed for 3 min, sonicated for 15 min, and centrifuged at 4500 rpm for 5 min. A reaction mixture of 50 μL of peptide supernatant, 10 μL of a 10 μg/μL solution of FRIPS reagent in acetonitrile in a 100 mM triethyl ammonium bicarbonate buffer (pH 8.5) was prepared. The reaction was allowed to proceed for 2 hr and then quenched by addition of 2 μL of formic acid. The

solvent was removed with use of a rotary evaporator, and the sample was resuspended in 10 µL of 0.1% trifluoroacetic acid and purified using a C_{18} ZipTip (Millipore, Billerica, MA) according to manufacturer protocol. The eluted sample was increased to a final volume of 500 µL in 49% methanol, 49% water, and 2% acetic acid (v/v).

6.3.4 Experimental Setup

The experimental setup consists of an electrospray ion source (ESI), the ROMIAC, and a Thermo LTQ-MS (Thermo Fisher, Waltham, MA). Full details of the experimental apparatus and calibration procedure can be found elsewhere in work by Mui and co-workers.[95] Nitrogen gas enters the ESI chamber perpendicular to the spray needle and conveys ions to the ROMIAC. Nitrogen is also utilized as the cross-flow gas through the ROMIAC, regulated with a proportioning solenoid valve and exhausted through a vacuum pump with the flow rate kept constant by a critical orifice at the inlet to the vacuum pump. All experiments were run at cross-flow gas rate of 34.3 L min^{-1} and a carrier gas flow rate of 1.70 L min^{-1}, resulting in a theoretical resolution (R_{nd}) of 20.2. Gas flow was controlled by a custom LabView interface utilizing a proportional-integral-differential (PID) algorithm with feedback from differential pressure transducers. Analytes were at atmospheric pressure for the entire journey from the ESI spray needle to the LTQ-MS inlet, the duration of which is estimated to be on the order of tens to hundreds of milliseconds.

Ion mobility spectra were obtained by stepping through a range of voltages and monitoring the LTQ-MS signal. The LTQ-MS was scanned from *m/z* 50 to 2000 and averaged over three microscans of 10 ms maximum duration. The instrument was operated with a capillary temperature of 50° C, a capillary voltage of 0 V, and a tube lens voltage of

88 V. For model peptide collision-induced dissociation (CID) experiments, the singly-protonated parent ion was isolated with an isolation width of 3 m/z (window of m/z in the trap during isolation prior to collisional activation) and a normalized collision energy of 10%. The experimental setup was calibrated using tetraalkylammonium halide salts as mobility standards for instrument calibration (IC) and also using biomolecules with known cross sections for mobility calibration (MC).

6.4 Results and Discussion

6.4.1 CID and FRIPS of Peptide Isomers

Two sets of peptide isomers, AARAAATAA/AATAAARAA and AARAAHAMA/AARAAMAHA, were analyzed in this study. To identify diagnostic product ions for each species, CID was performed on the singly-protonated ion of each of the peptides, as shown in Figure 6.1 with the product ions unique to each isomer highlighted. The CID spectra of untagged peptides are dominated by b- and y-type ions generated via proton-catalyzed dissociation of the peptide backbone,[247] whereas addition of the FRIPS reagent results in free-radical-initiated dissociation that is highly selective for specific amino acids in the peptide sequence.[304,311] As shown in Figure 6.2a, CID of peptides derivatized with the FRIPS reagent generates an acetyl radical at the N-terminus of the peptide by homolytic cleavage of the C–O bond, followed by hydrogen atom abstraction and dissociation. The product ions utilized to differentiate the model peptide isomers are formed according to the processes shown in Figure 6.2b-d. For threonine-containing peptides, the observed $[a_6+H]^{\bullet}$ and z_7 ions are generated by hydrogen atom abstraction from C_β of the threonine side chain, followed by N–C_α bond cleavage and

subsequent loss of isocyanic acid from the N-terminal product ion (Figure 6.2b). The z_7-H ion may further decompose to yield the y_6 ion. The c_6 ion is formed by a similar process in which hydrogen atom transfer to the N-terminal carbonyl oxygen occurs in concert with N–C_α bond cleavage (Figure 6.2c).[321] For isomeric peptides containing histidine, unique product ions are formed by abstraction of hydrogen from C_β of histidine followed by cleavage of the C_α–C bond (Figure 6.2d).[311]

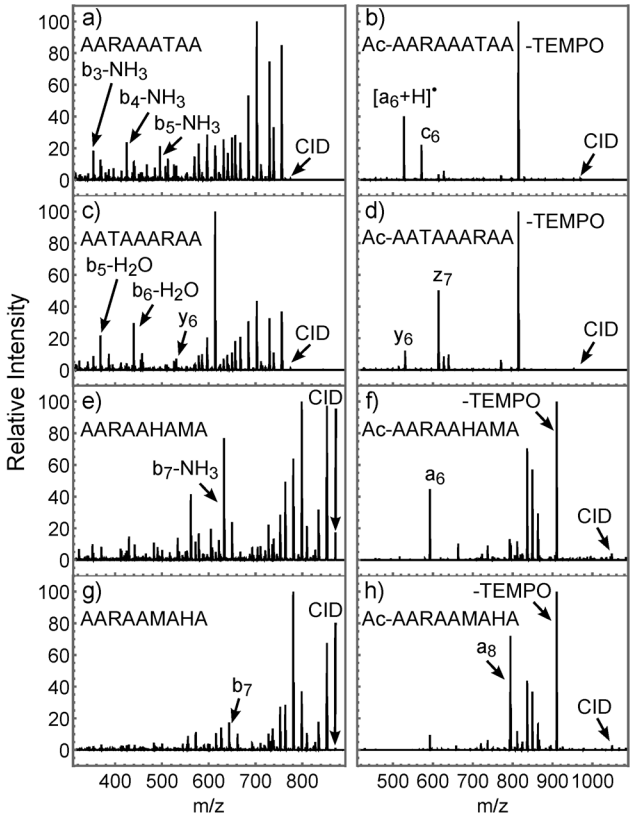

Figure 6.1. CID and FRIPS spectra of peptide isomers. Shown in a-d are the MS^2 spectra of AARAAATAA and AATAAARAA, both underivatized (a, c) and tagged with the FRIPS reagent (b, d). Shown in e-h are the MS^2 spectra of AARAAHAMA and AARAAMAHA, both underivatized (e, g) and tagged with the FRIPS reagent (f, h). Labeled product ions are specific to each isomer and are used for identification during ion mobility separation. Product ions in the FRIPS spectra are referenced to the *m/z* of the peptide with an acetyl radical at the N-terminus.

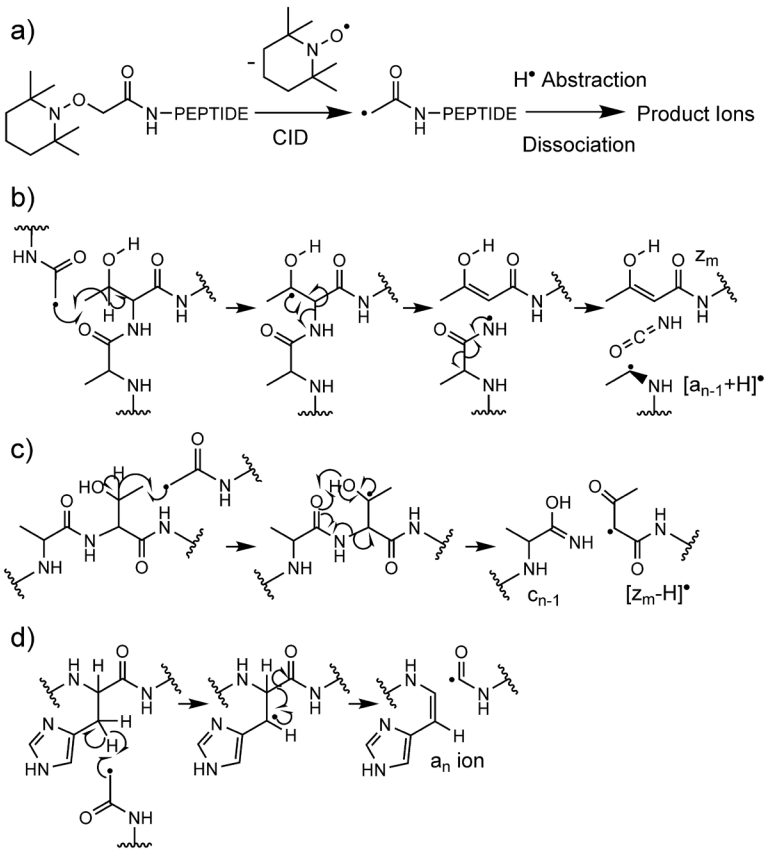

Figure 6.2. Free radical dissociation processes in peptides derivatized with the FRIPS reagent. The FRIPS methodology employed here is shown schematically in (a). The TEMPO-based FRIPS reagent is coupled to the N-terminus of the peptide, and subsequent collisional activation leads to loss of the TEMPO moiety, generating an acetyl radical. This acetyl radical then abstracts a hydrogen atom from diverse sites along the peptide, leading to dissociation of the backbone or neutral loss of amino acid side chains. Unique product ions are proposed to occur at threonine residues via the mechanisms shown in (b) and (c), resulting in (b) $[a_6+H]^•$ and z_7 ion and (c) c_6 ion formation. Backbone dissociation at histidine residues occurs by the mechanism illustrated in (d), leading to a_6 and a_8 ion generation.

6.4.2 Separation of Peptide Isomers

Following the identification of diagnostic product ions for each isomer, a mixture of isomeric peptides was separated by the ROMIAC and sequenced by CID within the ion trap, both with and without attachment of the FRIPS reagent. For untagged AARAAATAA

and AATAAARAA, the peak signals from the three diagnostic product ions of each isomer (Figure 6.1a,c) appear at the same voltage (considering errors), precluding resolution of the isomers (Figure 6.3a,b). However, the addition of the FRIPS reagent enhances peptide isomer separation by ~7 V or ~14 $Å^2$ (Figure 6.3c,d), allowing for isomer identification by CID. A similar but less dramatic separation enhancement occurs for the AARAAHAMA and AARAAMAHA isomers; unique product ions from the untagged peptides (Figure 6.1e,g) are separated by ~1 V (Figure 6e,f), but tagging of these peptides with the FRIPS reagent increases separation in the ion mobility spectrum by ~3 V or ~4 $Å^2$ (Figure 6.3g,h). The difference in separation enhancement between the two pairs of model peptides upon addition of the FRIPS reagent is not surprising, since the structural similarity is much greater between AARAAHAMA and AARAAMAHA than between AARAAATAAA and AATAAARAA. In the former pair of isomers, the location of the likely protonation site (Arg) is not altered, and the His and Met residues change position only slightly. It is evident in both cases, however, that the addition of the FRIPS reagent to the N-terminus improves separation. The reason for this improvement is not entirely clear but is likely due to disruption of interactions between the altered N-terminus and other sites on the peptide backbone. In addition, the radical-driven dissociation of the TEMPO-tagged peptides generates a more distinct spectrum than CID, although the overall sequence coverage is diminished.

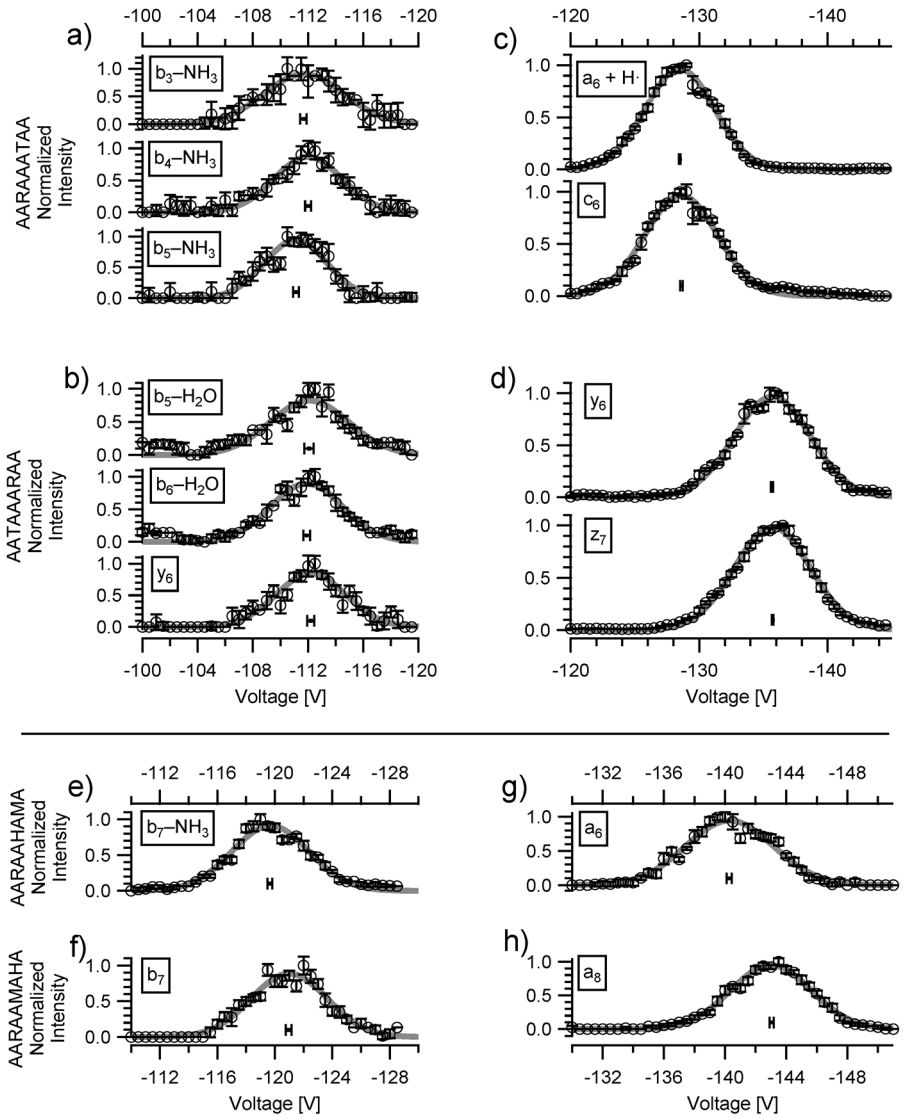

Figure 6.3. Separation of peptide isomers derivatized with the FRIPS reagent utilizing the ROMIAC. Shown are mass-resolved, normalized product ion signals as a function of applied voltage. Data point error bars indicate 1 standard deviation of the normalized signal. Gray line: Gaussian-fitted function to the signal; dot marker: centroid of fit, with 95% confidence interval error bars. (a) Non-TEMPO-tagged AARAAATAA CID fragments. (b) Non-TEMPO-tagged AATAAARAA CID fragments. (c) TEMPO-tagged AARAAATAA CID fragments. (d) TEMPO-tagged AATAAARAA CID fragments. (e) Non-TEMPO-tagged AARAAHAMA CID fragment. (f) Non-TEMPO-tagged AARAAMAHA CID fragment. (g) TEMPO-tagged AARAAHAMA CID fragment. (h) TEMPO-tagged AARAAMAHA CID fragment.

6.5 Conclusions

This study demonstrates the ability of IM-MS experiments using a ROMIAC to effectively separate peptides with similar sequences. Specifically, model peptide isomer separation was successfully conducted with untagged AARAAHAMA/AARAAMAHA using ROMIAC-IMS, and separation was demonstrably enhanced by derivatization with the FRIPS reagent, allowing the separation of AARAAATAA/AATAAARAA isomers. Although the modest resolution of ~20 achieved in these experiments was readily able to separate peptide isomers, greater resolution may be achieved in future work by increasing the cross flow rate.

The ROMIAC is desirable for further IMS applications, as it suffers fewer diffusional losses of ions than the conventional DMA and readily achieves resolution sufficient for separation of peptides. Additionally, the ROMIAC provides continuous transmission of ions, allowing for targeted monitoring of specific analytes. The ROMIAC can be easily interfaced to any mass spectrometer with an atmospheric pressure inlet. Improvements to the ROMIAC-MS interface should greatly enhance ion transfer efficiency and hence sensitivity. Given the ability to identify isomeric peptides shown in this study, the ROMIAC may be utilized in future experiments as a prefilter to reject abundant ion components and enhance the MS capability for detection of low abundance ions. For example, it could be used for real-time investigations of low abundance human serum proteins, especially those known to be markers for disease, by eliminating abundant background proteins and thereby improving the dynamic range of MS to detect the desired proteins.