Poster Reprint

ASMS 2024
Poster number WP 470

High-Efficiency Electron Capture Dissociation of Peptides and Proteins After Collision-Induced Unfolding and Ion Mobility

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Introduction

CIU-IM-ECD-MS

Collision induced unfolding (CIU) combined with ion mobility-mass spectrometry (IM-MS) provides valuable information about biomolecule structure.

Combining CIU-IM-MS with a top-down fragmentation technique like electron capture dissociation (ECD) can provide additional information about sequence and modifications for protein structures. For example, the electrons used in ECD can be used as a probe for gas phase structure[1], because exposed regions are more likely to produce detectable fragment ions. Likewise, CIU can also improve the sequence coverage of structures otherwise recalcitrant to ECD.

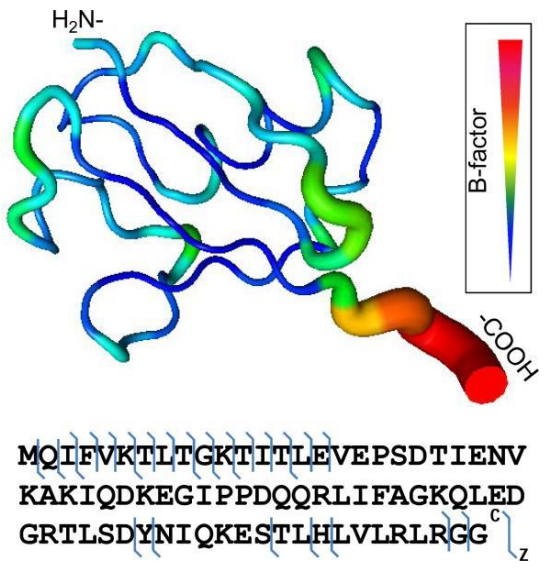


Figure 1. Crystal structure of ubiquitin color-coded by B-factor, a measure of thermal motion. A gentle fragmentation technique like ECD can confirm gas phase structures predicted by B factor. Figure copied from reference 1.

However, it has historically been difficult to perform electron-based fragmentation (ExD) after ion mobility, because most ExD techniques rely on an ion trapping device operating on the same timescale as IM separation, i.e. milliseconds. The "fly-through" design of the "ExD cell" device with microsecond-scale transit times makes it uniquely suited to combining with millisecond-scale IM separation, as long as practical efficiencies can be achieved.

Recent changes to the ExD cell design have led to simplifications to operation and increased ECD efficiency, particularly for lower-charge analytes. The changes include adjusted gas pressure and re-positioning the ExD cell downstream of the collision cell. With these changes, care must be taken to minimize the effects of ExD cell gas pressure & lens tuning on IM resolution. So, in this work we investigate ways to tune the whole system to yield practical ECD efficiency and IM resolution. Furthermore, we leverage the prototype ExD cell to study the relationship between protein collision cross section (CCS) and sequence coverage by ECD.

Experimental

Instrumentation

A 6560C IM-QTOF instrument (Agilent Technologies, Santa Clara, CA) was modified with prototype ECD hardware ("ExD cell", Agilent Technologies, Santa Clara, CA) installed after the collision cell. This modified instrument provides in-source CIU, drift tube ion mobility separation, CID and ECD capabilities. ECD efficiency and IM resolving power were optimized by adjusting gas pressure and ion optics voltages.

The main difference between ECD tunefiles was the delta voltage between lens 4 and the filament bias. Gentle ECD conditions provided more information for native structures compared to aggressive conditions.

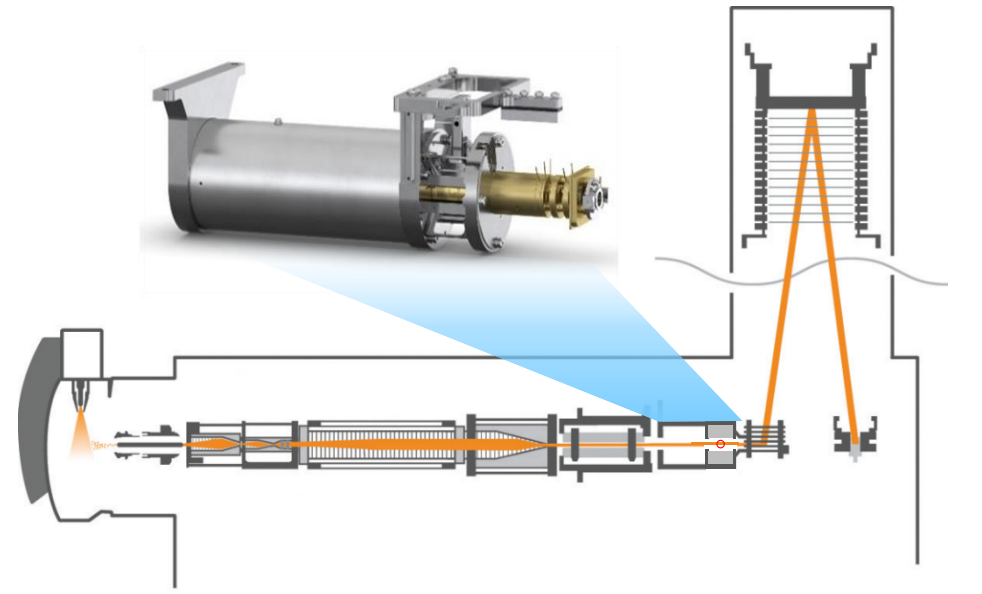


Figure 2. Schematic of the Agilent 6560C IM-QTOF fitted with prototype ExD cell positioned after the collision cell.

Samples

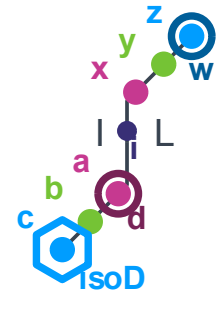
The ExD cell was tuned for ECD using the series of standards shown in Table 1. Samples were introduced using an Agilent nanospray source (G1992A) modified for static spray, or an AJS source (G1958B) with a syringe pump for sample delivery.

Analyte	Formulation	Introduction method
Subtance P	10 µg/mL in 50% methanol + 0.1% FA	Dual AJS + syringe pump
Ubiquitin	5 µM in 100 mM ammonium acetate	Static nanospray
Cytochrome c	5 µM in 100 mM ammonium acetate	Static nanospray
Carbonic anhydrase	5 µM in 100 mM ammonium acetate	Static nanospray
NISTmAb	2.4 mg/mL in 100 mM ammonium acetate	Static nanospray

Table 1 – Samples used & their formulations.

Software

Top-down ECD data were analyzed using IM-MS Browser and ExDViewer (Agilent Technologies, Santa Clara, CA).



Native Cytochrome *C* (12 kDa)

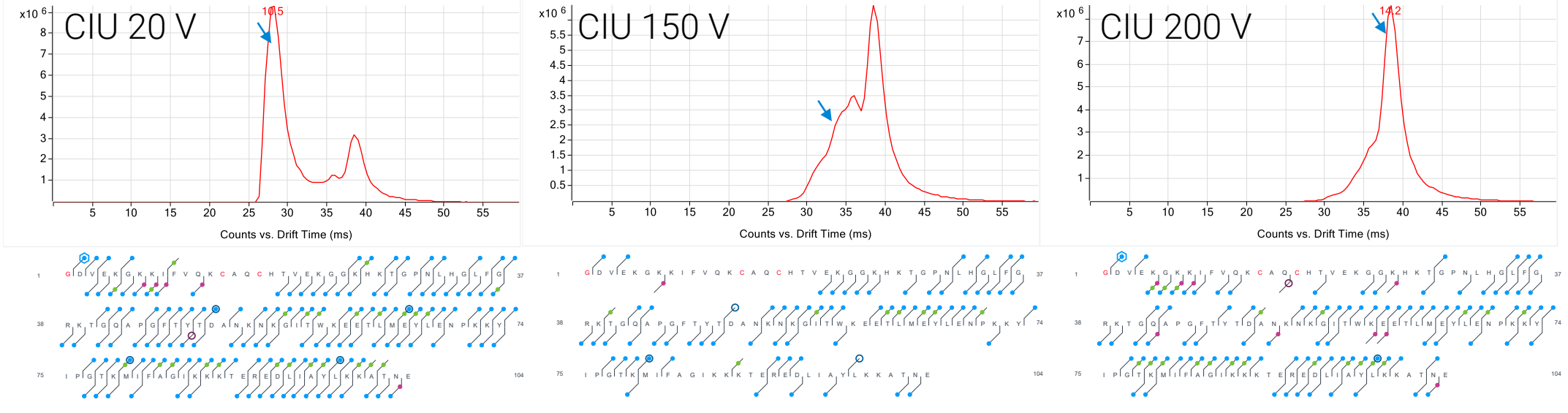
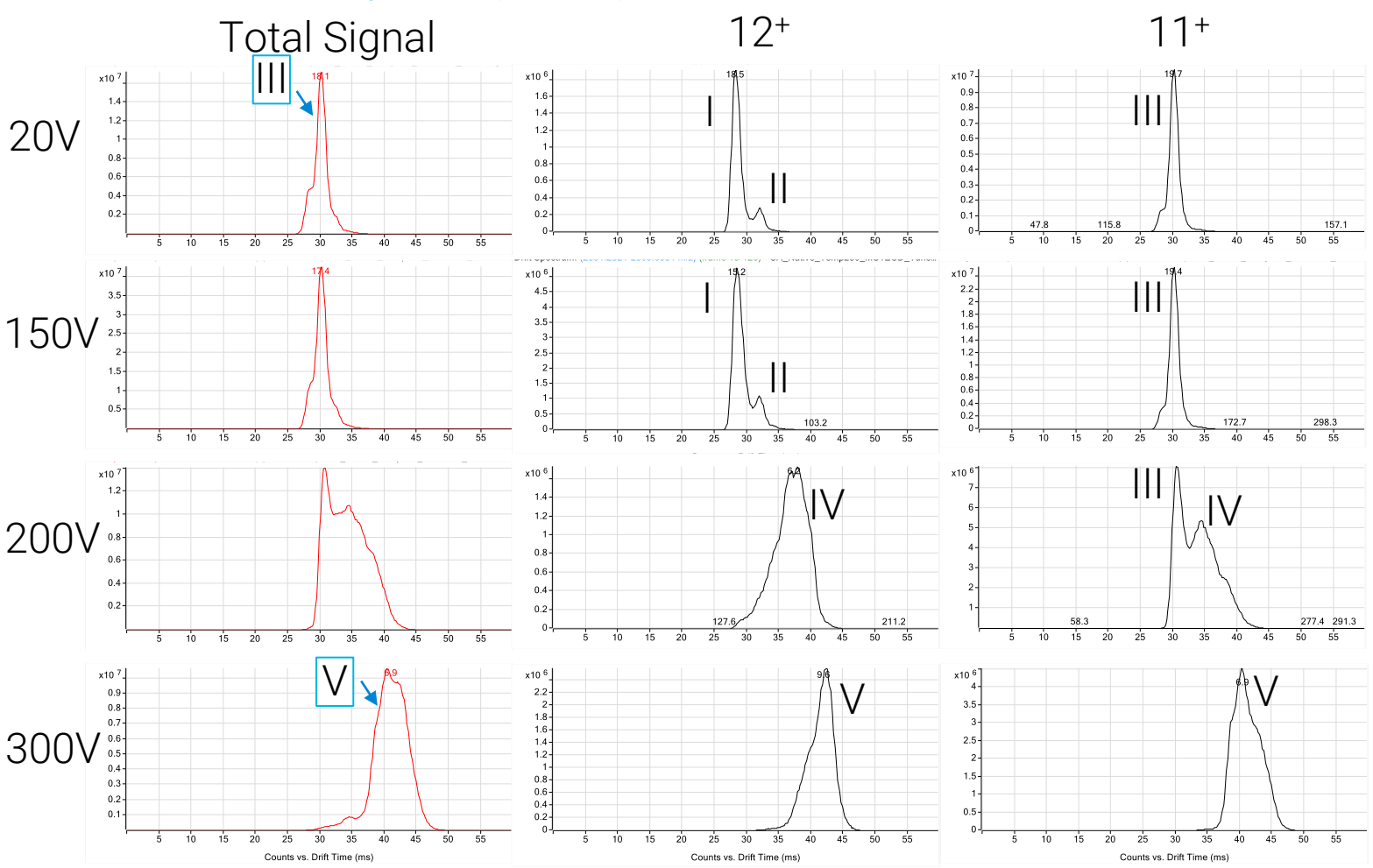


Figure 3. CIU settings of 20, 150, or 200 V were used to unfold the gas-phase structure of 7^+ native cytochrome c, resulting in 2-3 distinct structures with drift times of 28, 33, and 38 ms, respectively (blue arrows). ECD sequence coverage was similar for all three structures, with lack of coverage around the covalent heme modification site. The slightly lower sequence coverage for the structure with drift time 33 ms may be due to lower precursor intensity, resulting in fragment ion peaks with insufficient signal-to-noise ratios for the restrictive matching settings used.

Native Carbonic Anhydrase (29 kDa)



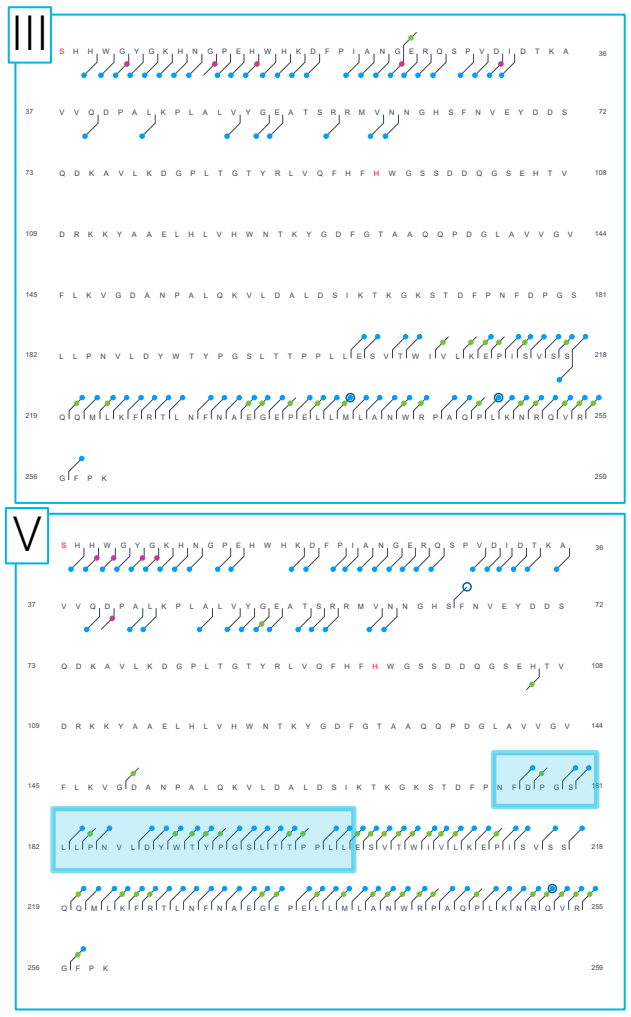


Figure 4. CIU settings of 20, 150, 200, or 300V were used to unfold the gas-phase structures of 11^+ and 12^+ native zinc-binding carbonic anhydrase. For the 12^+ precursor, there were two distinct structures (I & II) at 20V CIU, with an additional structure (IV) revealed at >200V CIU. The 11^+ precursor had a structure with slightly greater drift time (III) and did not yield a more unfolded structure (IV) until >200V CIU. A possible fifth structure (V) was observed at high CIU voltages for both charge states. Sequence coverage by ECD was greater for the unfolded structure (V) compared to the compact structure (III), particularly around residues 180-210. This suggests that this region near the C-terminus becomes more exposed with unfolding. Some key residues in this region, for example T199, are important for stabilizing the zinc atom. There is also a hydrophobic pocket in this region that is important for the native structure of CA.

Results and Discussion

Native NIST Monoclonal Antibody (150 kDa)

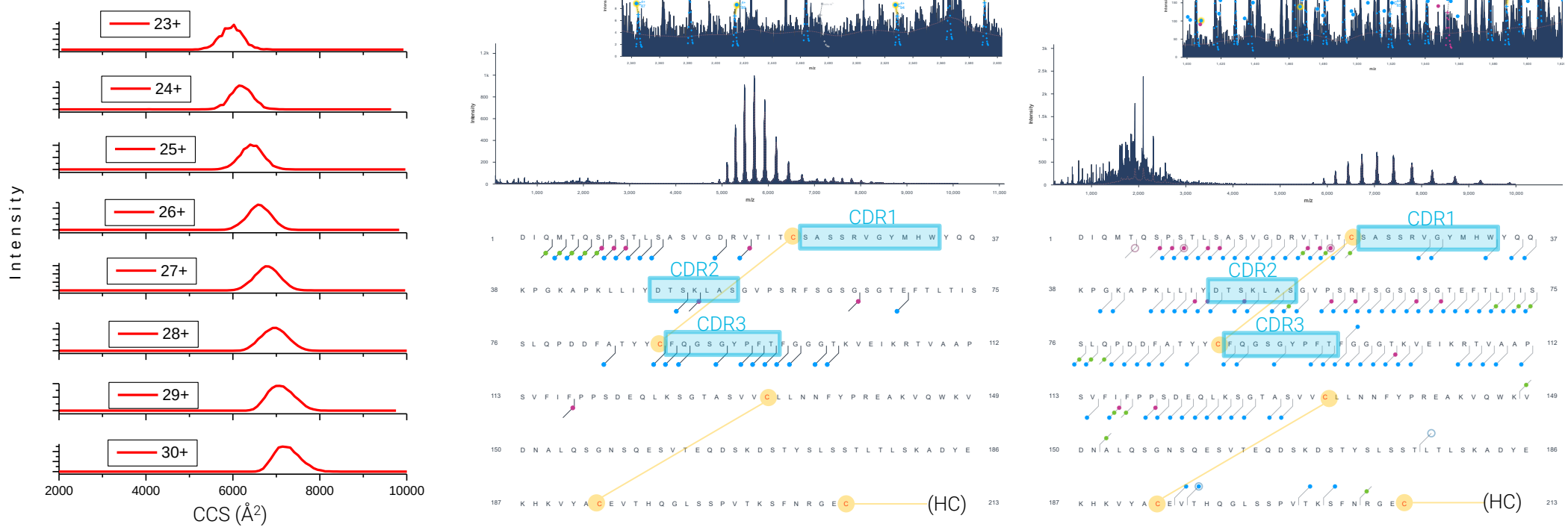


Figure 5. Charge states 22-30⁺ were observed for intact native NISTmAb. Higher charge states tended to have greater CCS. The quadrupole was used to transmit only *m/z* > 4000, ECD was performed on multiple charge states, and the results were matched to the NISTmAb light chain (LC) sequence. With gentle ECD conditions, only the N-terminus and CDR3 region of the LC yielded detectable ECD fragment ions, consistent with the presence of intra- and inter-subunit disulfide bonds. With more aggressive ECD conditions (experiments performed on a 6545XT), the C23-C87 disulfide was broken and greater coverage of the LC was obtained. Inset spectra: close-up of CDR3 fragments.

Substance P (1.4 kDa)

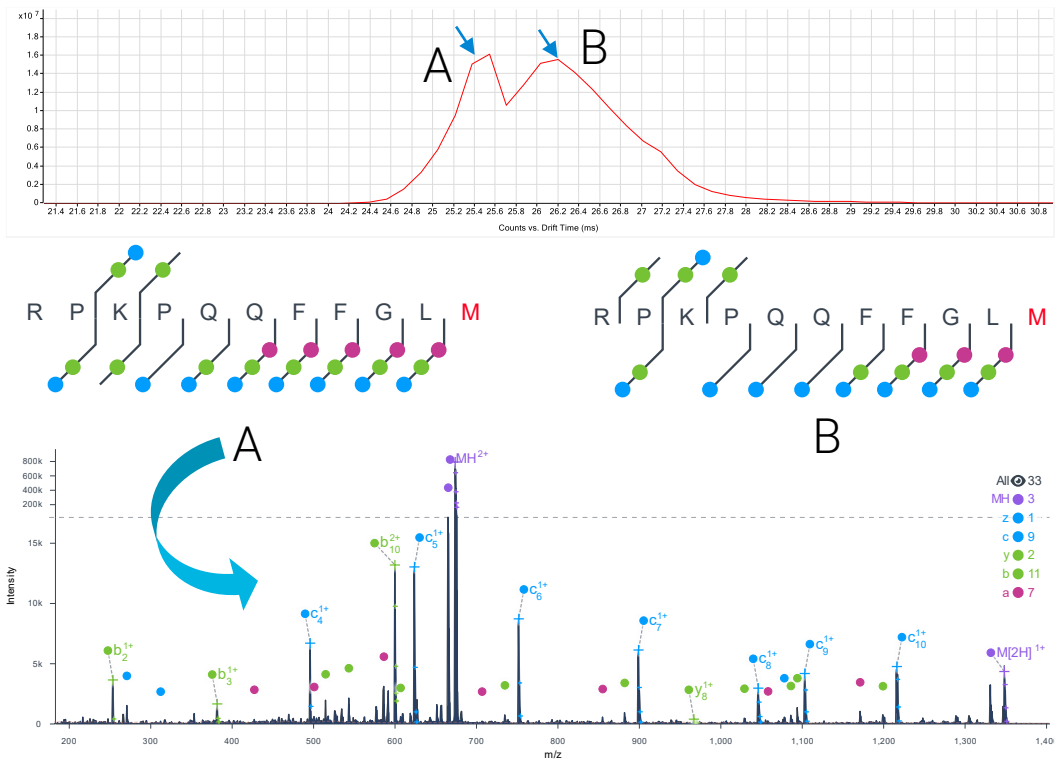


Figure 6. The peptide Substance P was infused and separated using IM. Two structures were observed in the drift spectrum, but could not be baseline separated. ECD spectra for the 2⁺ precursor were very similar for both structures. Most ECD fragment ions had signal-to-noise ratios >100, and ECD efficiency was approximately 5-10%. This relatively high efficiency for peptides overcomes historical constraints for ECD and extends the utility of the technique for applications such as differentiating aspartate/isoaspartate and leucine/isoleucine at the peptide level in an IM-MS/MS experiment.

https://www.agilent.com/en/promotions/asms

Conclusions

- A prototype ECD cell with increased gas pressure yielded efficient ECD with minimal loss of IM resolution. ExD cell tunings which yielded the most efficient ECD were unsuitable for IM experiments, so a compromise was struck primarily via tuning middle ExD cell voltages.
- Combining CIU-IM with ECD allows the direct linkage of higher-order structure information back to primary structure
- CIU effectively unfolds gas-phase protein structures, sometimes enhancing sequence coverage by ECD
- High efficiency ECD with IM is applicable even for 2⁺ peptides

References

[1]Zhang et al. Native electrospray ionization and electron-capture dissociation for comparison of protein structure in solution and the gas phase. Int J Mass Spectrom, 2013, 354-355.

DE58331904

Published in USA, May 31,2024

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