Poster Reprint

ASMS 2024
Poster number TP 150

Top-down Characterization of Intact Proteins via On-line Charge-stripping and Electron Capture Dissociation

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Introduction

Top-down electron capture dissociation (ECD) can be more efficient for denatured protein samples. This is due to larger electron capture cross sections for higher charge state ions with unfolded protein structures. However, proteins under denaturing conditions also typically have a wide charge state envelope, resulting in lower signal intensities for a given charge state. To overcome this problem, we have introduced a chemical charge stripping method to lower the number of charge states with a hope to maintain the unfolded protein structure. Charge stripping occurs via micro-droplet reactions in the electrospray ionization chamber. Concentration of the charge state envelope into fewer peaks of higher intensity provided for improved ECD fragmentation and sequence coverage. Mechanistic insights were obtained by application of ion mobility (IM) mass spectrometry.

Experimental

Sample Preparation

Ubiquitin (bovine), Cytochrome C, Myoglobin, and Carbonic anhydrase were purchased from Sigma Aldrich (St. Louis, MO). NIST mAb was purchased from NIST (Gaithersburg, MD). Denatured solutions of 1-5 uM protein concentration were prepared in 15% acetonitrile (ACN) in water with 0.1 % formic acid (FA); native solutions were in 100 uM ammonium acetate. Intact mAbs were buffer exchanged with corresponding buffers, using Amicon Ultra 0.5 mL 10 kDa centrifugal molecular weight cutoff filters (Sigma-Aldrich, St. Louis, MO, USA). Working solutions of intact mAbs were prepared at 1 mg/mL either in 100 mM ammonium acetate (native solution) or in 50% ACN/water with 0.1% FA before introducing into the mass spectrometer.

Data Analysis

Data processing was performed with IM MS Browser and ExDViewer softwares (Agilent Technologies, Inc., USA). ExDViewer allowed determination of protein sequence coverage and evaluation of the ECD efficiency based on ion intensities for all isotopic peaks. ECD/IM experiments were controlled and tuned with imsRealTime software (Agilent Technologies, Inc., USA). Autotune procedure of ExDControl software (Agilent Technologies, Inc., USA) was used for tuning ExD cell in both MS1 and MS2 modes.

Experimental

Instrumental Analysis

ECD experiments were performed using both 6560 (Figure 1) and 6545XT AdvanceBio LC/Q-TOF (Agilent Technologies, USA) mass spectrometers modified to enable ECD by installation of a second generation ExD cell (Agilent Technologies, USA). The ExD cell consists of seven electrostatic lens elements, two ring magnets, and an electron emitting filament. All voltages are supplied by a separate power supply regulated by ExD Control software (Agilent Technologies, USA). A dilute solution of charge stripping reagent (dimethylaminopropylamine in water, 1:10 v/v) was infused through the reference nebulizer of the Dual Agilent Jet Stream (AJS) electrospray ionization ion source at flow rates from 0.01 to 0.2 µL/min, depending on the desired charge stripping level. Protein samples were infused through the main nebulizer of Dual AJS source at a flow rate of 5-10 µL/min. Proteins of different sizes from ubiquitin to monoclonal antibodies (mAbs) were studied with this approach. To find softer conditions for ion spraying, the Agilent static nanospray ion source was also used to introduce the proteins under study.

Experiments on collisional activation prior to ECD were conducted by applying a voltage difference between capillary exit and the fragmentor (in collision induced unfolding (CIU) experiments) or by applying a collision voltage in collision cell in the same way as in collision induced dissociation (CID) experiments.

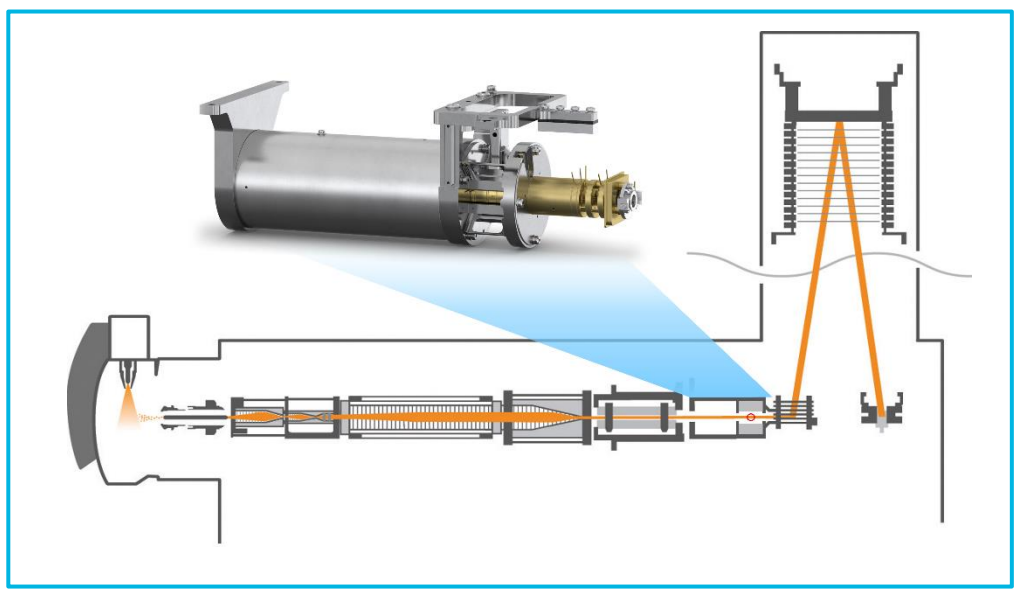

Figure 1. Schematic of Agilent 6560 IM Q-ToF mass spectrometer with ECD cell shown in insert.

In-spray charge reduction in Dual AJS source using dimethylaminopropylamine.

In the present work, intact proteins in denaturing solution, were fragmented using ECD. All proteins were subjected to proton stripping reactions with the charge stripping reagent, dimethylaminopropylamine, prior to ECD. Proton stripping level was controlled by ramping the flow rate of reagent solution and could be stopped at the level with required ECD efficiency. For denatured ubiquitin, for example, the charge levels down to 4+, 3+ or 2+ precursors were produced (Figure 2).

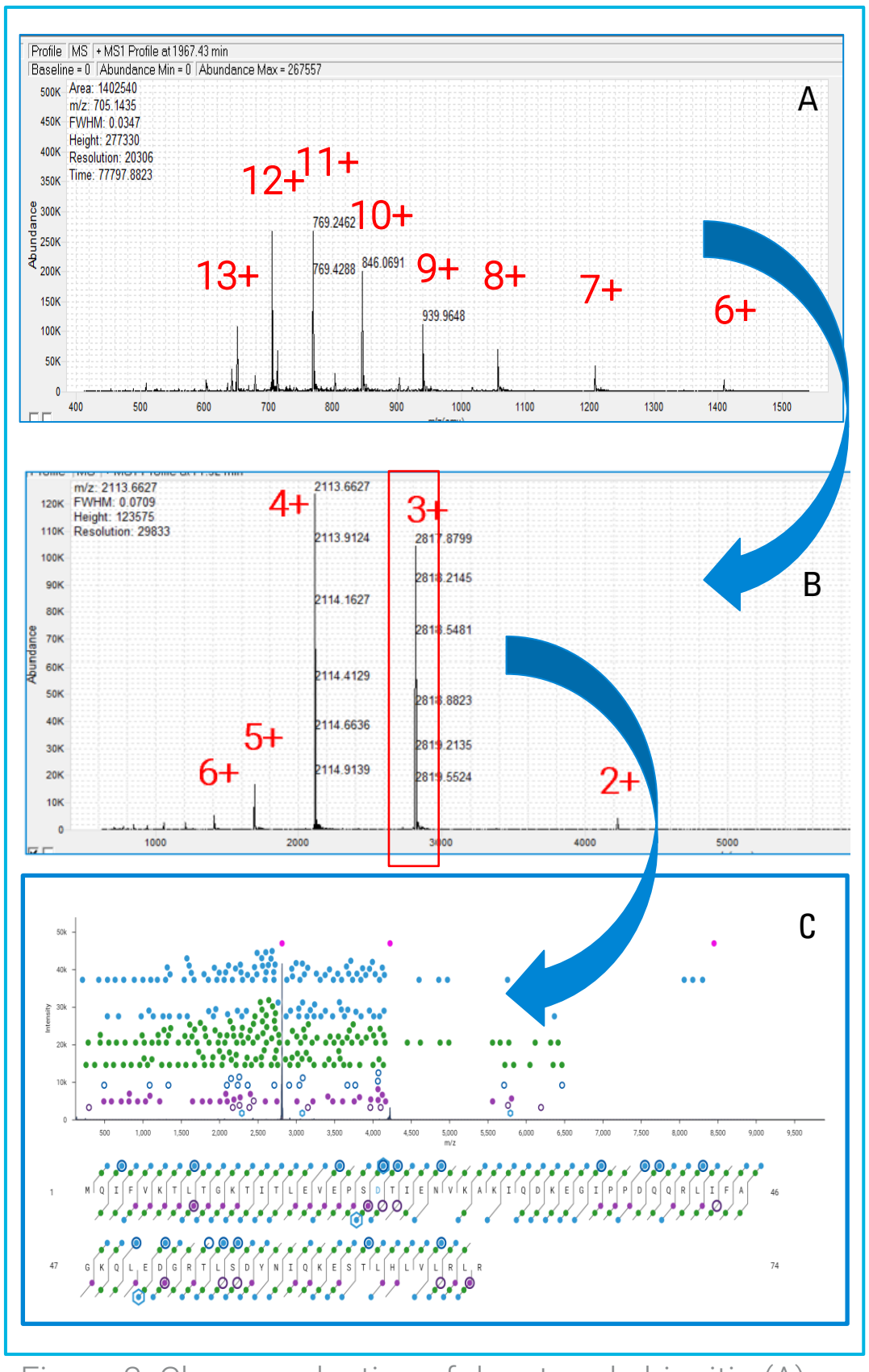

Figure 2. Charge reduction of denatured ubiquitin (A) to the most prominent 4+ and 3+ charge states (B) and product-ion ECD spectrum with complementary 100 V CID energy for 3+ precursor (C) showing 100% sequence coverage.

ECD spectra of ubiquitin different structures.

Structural differences between protein ions, inferred from the IM results, were correlated with the differences observed in their ECD mass spectra. Presented below are data for 6+ ubiquitin, but comprehensive data were obtained for other ubiquitin charge states as well as for other proteins mentioned in the Experimental. ECD spectra of native ubiquitin correlated well with X-ray crystallography data (see insert in Figure 3(A) with ubiquitin B-factor taken from Reference 1).

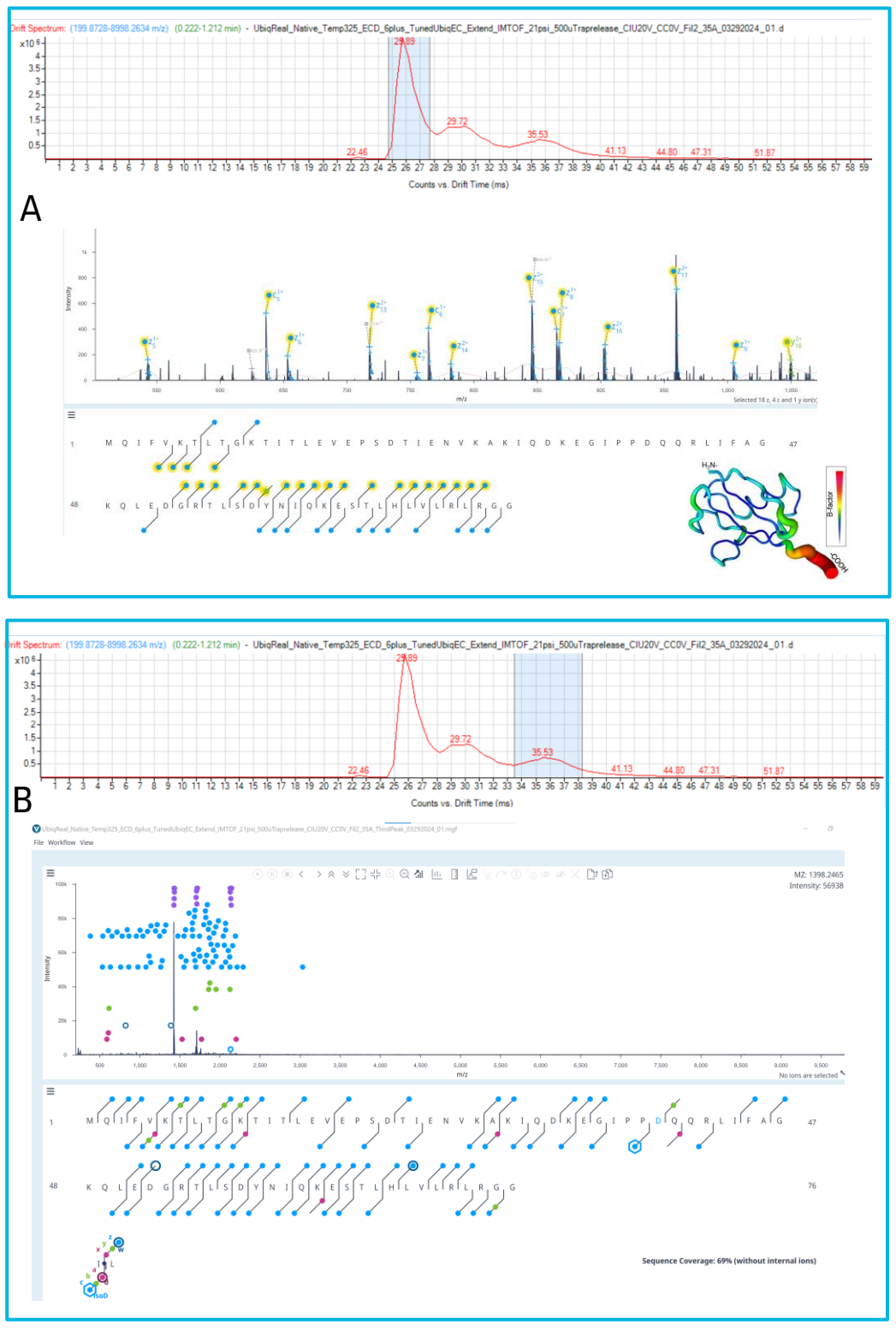

Figure 3. Drift time spectrum (top panel), ECD spectrum (mid panel) and sequence coverage map determined with the ExDViewer software (bottom panel) for 6+ precursor of ubiquitin with native structure (A) and partially unfolded gas-phase structure (B). ECD spectrum for the intermediate gas-phase structure (drift peak between 28-32 msec) was also recorded but it is not included here.

Results and Discussion

Ion mobility separation of protein charge states with different structures generated from native solutions and denatured solution with charge stripping.

The structural changes that result from the charge-stripping reaction sprayed from denatured solutions and similar charge states from native solutions were examined with IM mass spectrometry. These folding/unfolding processes were found to be protein-size dependent. Structural differences between these ions, inferred from the IM results, were correlated with the differences observed in their ECD mass spectra.

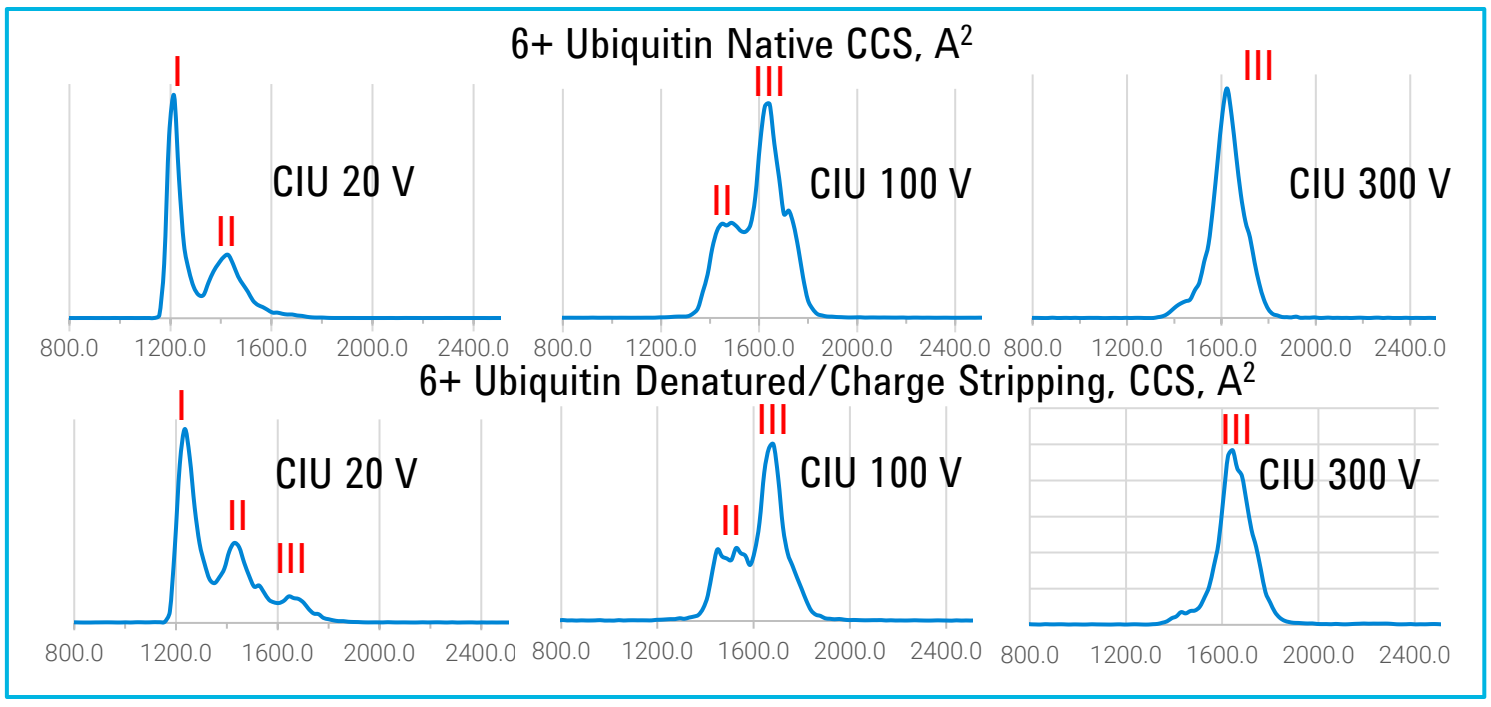

Figure 4. Ubiquitin 6+ precursor from native solution (top panels) and from denatured solutions with charge stripping (bottom panels) recorded with different CIU energies. At low CIU energy, native solution showed two compact structures – native (I) and gas-phase (II), as proved with ECD spectra (Fig. 3). Denatured solutions with charge stripping produced another 6+ structure (III) that is the most elongated 6+ structure. With increasing CIU energy, native structure disappears, whereas structure III becomes dominant structure,

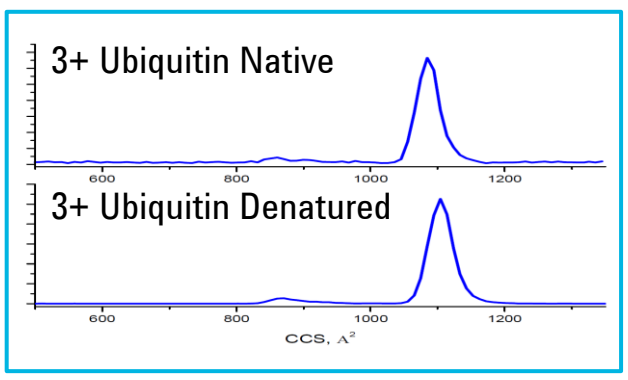

Figure 5. Ubiquitin 3+ precursors were produced only with charge stripping. The structures were found identical for both solutions. No structure changes were observed up to CIU 350 V, proving structure for 3+ is compact and stable.

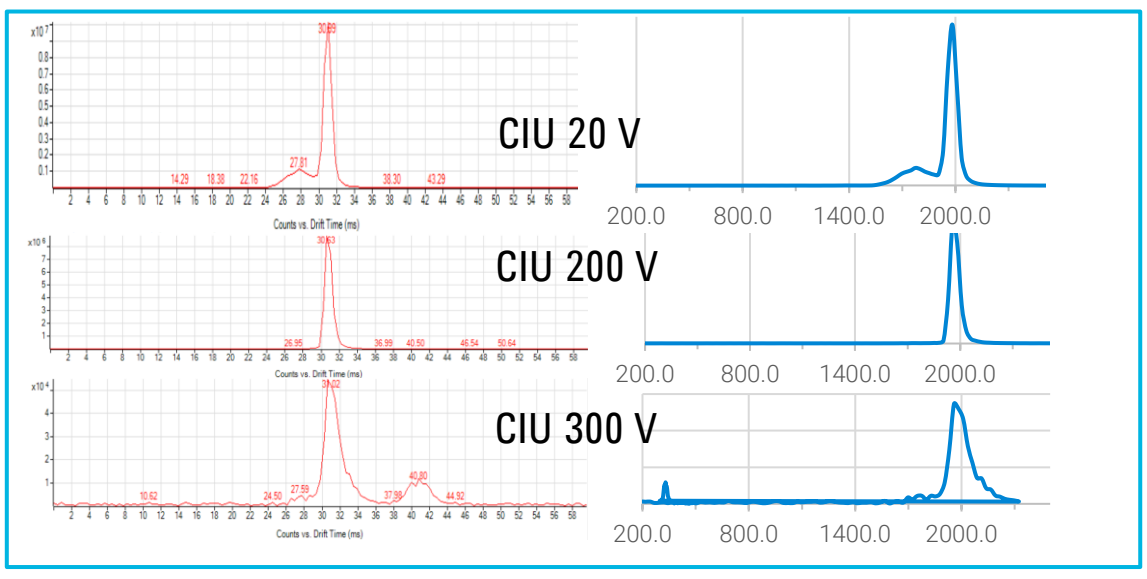

Figure 6. Ubiquitin 8+ drift time (left panels) and CCS spectra (right panels). The most compact structure (28 ms) disappears at 200 V and larger CIU energies. A new feature (at 41 ms in the drift spectrum) appears at CIU 300 V; this drift peak was not from 8+ precursor, however, but from a singly-charged fragment ion with the same m/z as 8+ precursor; considering its charge state, it appears as a narrow peak at lower CCS (right panel). Similar fragmentation (not elongation) with increasing CIU energy was typical for other higher than 8+ ubiquitin precursors.

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Conclusions

- ECD with complementary collisional activation applied for charge stripped proteins resulted in complete sequence coverage for ubiquitin triply-charged state.
- Combined application of ECD and IM proved survival of native structures for proteins as small as ubiquitin at mass spectrometric conditions. It was shown that ECD spectra for native structures matched well with liquid (NMR) and X-ray structures of studied proteins.
- Charge stripping by microdroplet reaction of higher charge state denatured proteins resulted in production of unfolded structures of lower protein charge states with high ECD efficiency.

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.

DE67510535

Published in USA, May 31,2024

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