INTERNAL FRAGMENT ASSIGNMENT CHALLENGES IN TOP-DOWN ELECTRON CAPTURE DISSOCIATION TANDEM MASS SPECTROMETRY

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Introduction

- Top-down tandem mass spectrometry (MS/MS) of intact proteins typically generates sequence-informative fragments from backbone cleavages near the termini.
- This lack of fragmentation in the protein interior is particularly apparent in native top-down MS/MS.
- Improved sequence coverage, critical for reliable annotation of posttranslational modifications (PTMs) and sequence variants, can be obtained from internal fragments generated by multiple backbone cleavage events [1, 2].
- However, internal fragment assignments can be error prone due to isomeric/isobaric fragments from different parts of a protein sequence.
- Electron capture dissociation (ECD) shows superior retention of labile PTMs and has been proposed to allow annotation of structural transitions in collision induced unfolding (CIU) following native MS [3].
- Here, we focus on improved internal fragment annotation in ECD and electron transfer dissociation (ETD) following both native MS and liquid chromatography (LC)/MS via control of the number of electron capture events as well as MS^3 of internal fragment candidates.

Methods

Bovine calmodulin and melittin from honeybee venom were used intact or following trypsin digestion.

LC-MS and LC-ECD MS/MS were performed with an Agilent 1290 HPLC using a Hamilton polystyrene-divinylbenzene PRP-3 or Agilent Poroshell 120 EC-C18 column with an acetonitrile:water/0.1% formic acid solvent system coupled to a 7 T SolariX Q-FT-ICR mass spectrometer (Bruker). The autosampler, column, and drying gas were operated at 10, 40, and 200-250 °C, respectively.

Native ECD MS/MS was performed via a CaptiveSpray nanoelectrospray (nESI) ion source from 50 mM ammonium acetate following calmodulin purification with a Biospin gel filtration column (6 kDa MWCO).

CIU was performed with an ion mobility (IM)-Q-TOF (Agilent 6560c with e-MSion ExD cell) mass spectrometer. ECD-infrared multiphoton dissociation (IRMPD) MS^3 was performed inside the SolariX ICR cell following CHEF isolation. ETD-higher energy collision dissociation (HCD) MS^3 was performed with a Thermo Fisher Orbitrap Fusion Lumos Tribrid instrument.

CIU-ECD of Calmodulin on an Agilent 6560c

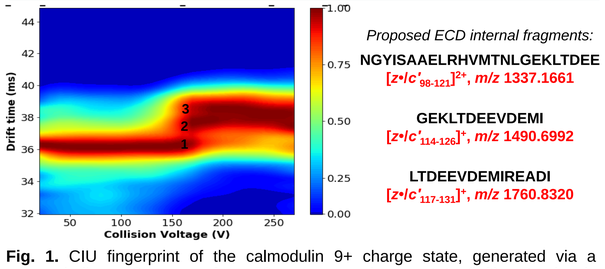

Fig. 1. CIU fingerprint of the calmodulin 9+ charge state, generated via a micronebulizer ESI source from 50 mM ammonium acetate (left). Previously proposed internal fragment candidates (right) showing the highest abundance in CIU feature 2.

Calmodulin LC-Q-FT-ICR MS Tryptic Peptide Mapping Indicates Met1 removal, N-acetylation, and Lys116 Trimethylation

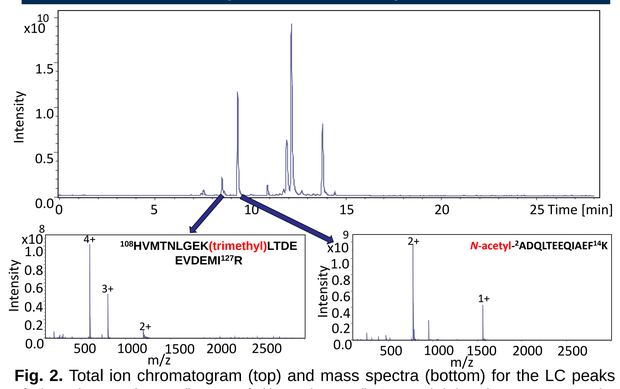

Fig. 2. Total ion chromatogram (top) and mass spectra (bottom) for the LC peaks at elution times of 8.5 (bottom left) and 9.3 (bottom right) minutes, respectively. Observed accurate masses correspond to the trimethylated tryptic peptide [108-127] and the acetylated tryptic peptide [2-14] (lacking Met1), all known calmodulin PTMs. ***Because Lys116 trimethylation was not considered in 2/3 previous ECD internal fragment assignments (Fig. 1), alternative assignments are likely.***

Calmodulin Top-Down LC-ECD-MS/MS on the SolariX Q-FT-ICR Instrument

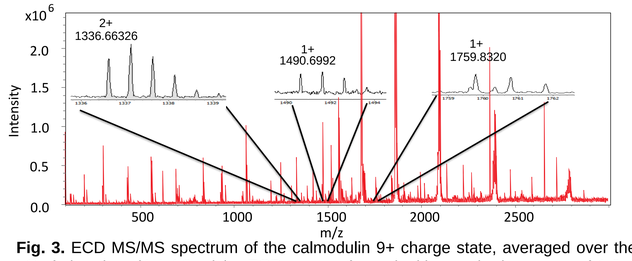

Fig. 3. ECD MS/MS spectrum of the calmodulin 9+ charge state, averaged over the LC elution time (4.4-4.9 min). ECD was performed with a cathode current of 1.6 A and an irradiation time of 100 ms. Similar to previous ECD on the Agilent 6560c following native MS (Fig. 1), fragments at m/z 1336.7, 1490.7 and 1759.8 are observed, suggesting they are not unique to the folded protein. ***Considering the PTMs discussed in Fig. 2, an alternative assignment for the doubly charged fragment is $c'_{23}{}^{2+}$ whereas alternative assignments for the singly charged fragments are y_{12} and z'_{15}.***

Calmodulin Native Top-Down ECD-IRMPD MS^3 on the SolariX Q-FT-ICR Instrument

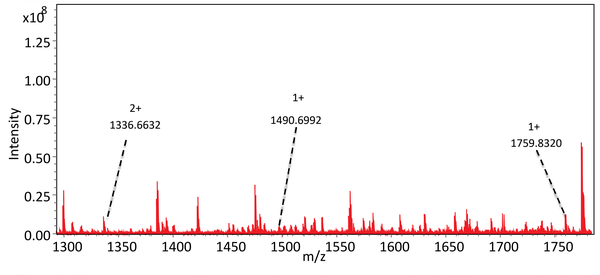

Fig. 4. Because internal fragment formation is more likely following multiple electron capture events (to cleave more than one backbone bond), the 8+• radical species (resulting from a single electron capture), observed following ECD of the 9+ calmodulin charge state (from native MS) was in-cell isolated and subjected to IRMPD. This ECD-IRMPD MS^3 spectrum again showed the fragment ions at 1336.7, 1490.7 and 1759.8, further suggesting they are not internal fragments.

ECD vs. ETD of Unfolded Calmodulin

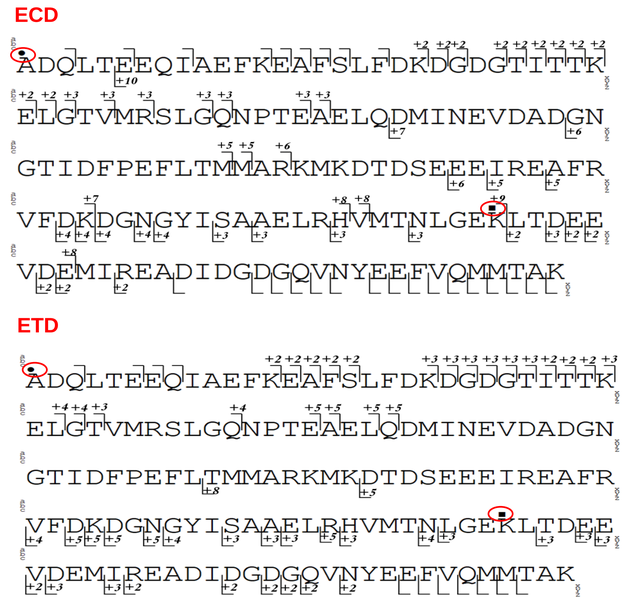

Fig. 5. Fragmentation data from ECD (top) and ETD (bottom) of the calmodulin 16+ charge state. Although the corresponding spectra are not identical, the 1336.7 fragment appears in both cases. Because MS^3 is more efficient on the Oribtrap Fusion Lumos instrument, such experiments were first pursued following ETD.

How do ECD/ETD Fragments Behave in MS^3?

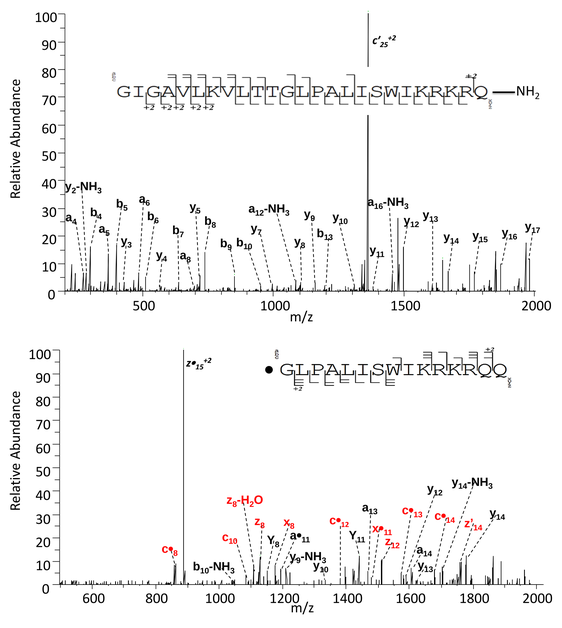

Fig. 6. Prior to attempting MS^3 to settle ambiguous ECD/ETD MS^2 fragment assignments, we sought to establish the CID/HCD behavior of ECD/ETD product ions. As expected, an even-electron *c'*-type ion from melittin ETD showed typical *a*/*b*/*y*-type fragments upon HCD-MS^3 (top). By contrast, a radical *z*•-type ion from melittin ETD showed both even-electron and radical-driven dissociation (bottom).

ETD-HCD MS^3 of the 1336.7 Fragment Ion

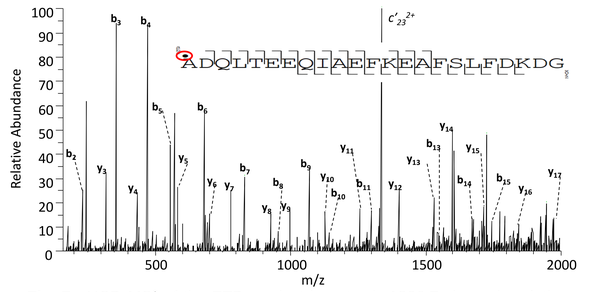

Fig. 7. HCD MS^3 of the ETD product ion at m/z 1336.7 showed a plethora of fragments matching the $c'_{23}{}^{2+}$ assignment, including N-terminal acetylation (indicated by "•") and Met1 loss, i.e., this fragment is not an internal one. The observed even-electron type fragmentation is further evidence against an internal fragment assignment, which would include a radical site.

ETD-HCD MS^3 of a 1314.6 Fragment Ion

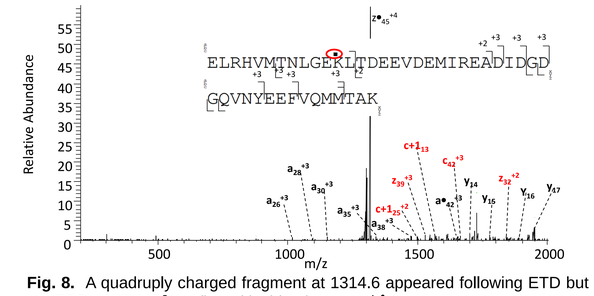

Fig. 8. A quadruply charged fragment at 1314.6 appeared following ETD but not ECD. HCD MS^3 confirmed its identity as $z_{45}{}^{4+\bullet}$.

Conclusions

- A combination of bottom-up LC-MS, top-down LC-ECD, and ETD confirmed N-terminal methionine loss + acetylation and Lys116 trimethylation as PTMs in calmodulin.
- Three ECD/ETD fragment ions (m/z 1336.7^{+2}, 1490.7, and 1759.8), previously assigned as internal fragments in native top-down ECD, were also found in LC-ECD-MS/MS, suggesting they are not unique to native conditions.
- These fragments also appeared in ECD-IRMPD MS^3 of the 8+• calmodulin radical species, generated via a single electron capture event, further suggesting they are not internal fragments.
- Even-electron *c*-type ions show typical fragmentation behavior in MS^3 whereas radical z-type ions show a mixture of even-electron and radical-driven dissociation.

Acknowledgments

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References

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