Extending the mass limit of collision cross sections of proteins in Orbitrap analyzers through kinetic energy and fragmentation behavior analysis

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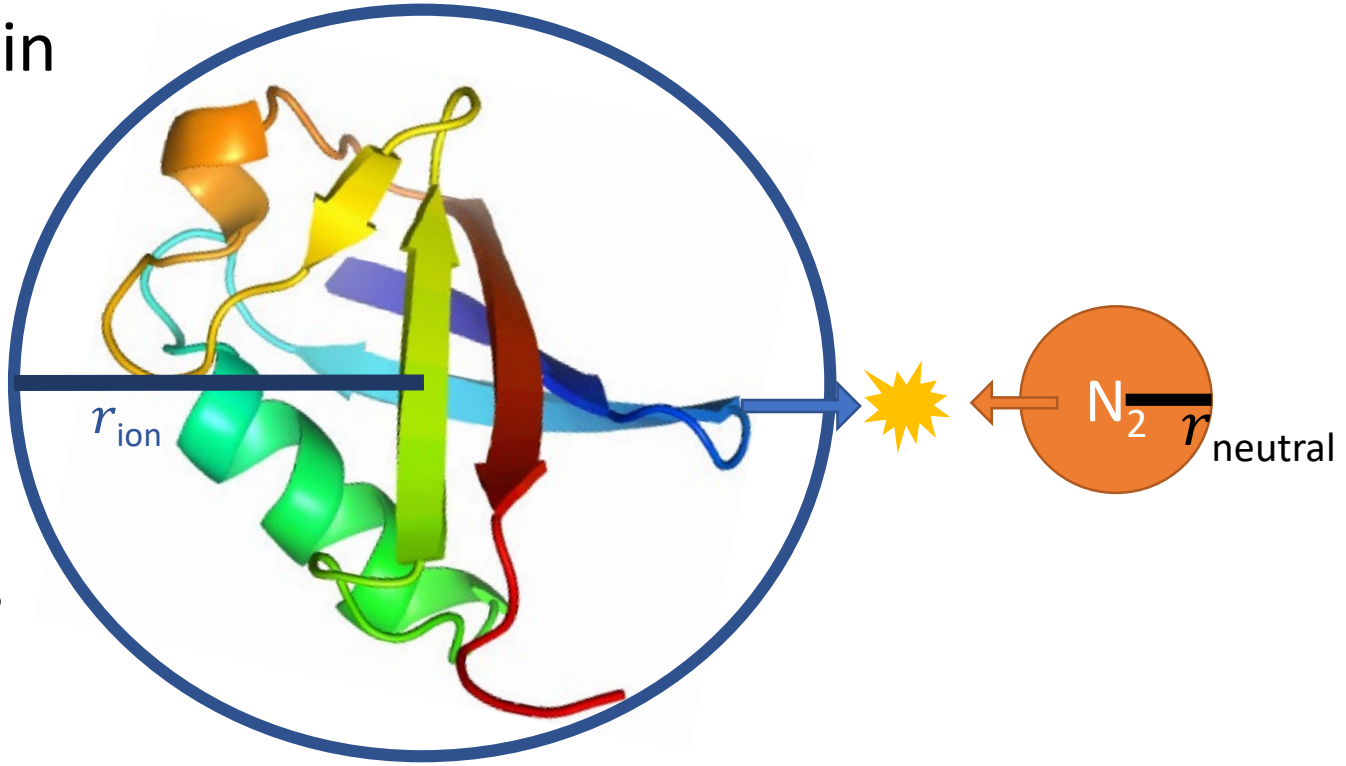
Analyzing Protein Structure in the Gas Phase

- Protein structure plays critical roles in protein function
  - Ligand binding
  - Enzymatic reactions
- Collision cross section (CCS, σ) of a protein may be measured in the gas phase
- Typically measured using ion mobility drift cells



$$\sigma = \pi(r_{ion} + r_{neutral})^2$$

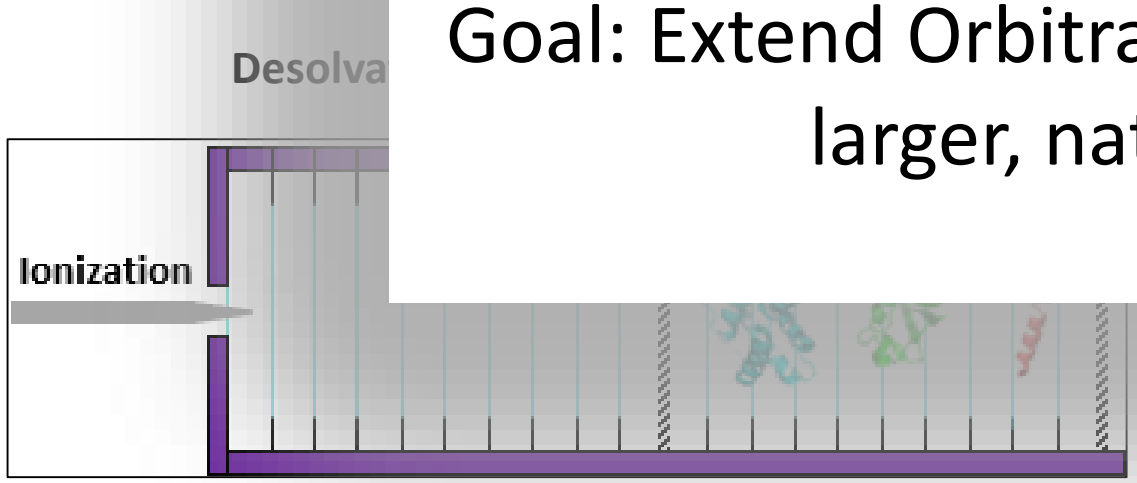
Drift cell vs. Orbitrap CCS Measurements

CCS values are typically measured based on migration times in ion mobility cells

Alternative methods have been developed to measure CCS directly in mass analyzers

- FT electrostatic linear ion trap
- FT-ICR

Goal: Extend Orbitrap measurement of CCSs to larger, native-like proteins



-Dziekonski, E. T.; Johnson, J. T.; Lee, K. W.; McLuckey, S. A. *J. Am. Soc. Mass Spectrom.* **2018**, *29* (2), 242–250.
-Jiang, T.; Chen, Y.; Mao, L.; Marshall, A. G.; Xu, W. *Phys. Chem. Chem. Phys.* **2015**, *18* (2), 713–717.
-Yang, F.; Voelkel, J. E.; Dearden, D. V. *Anal. Chem.* **2012**, *84* (11), 4851–4857.

Measuring CCS in an Orbitrap

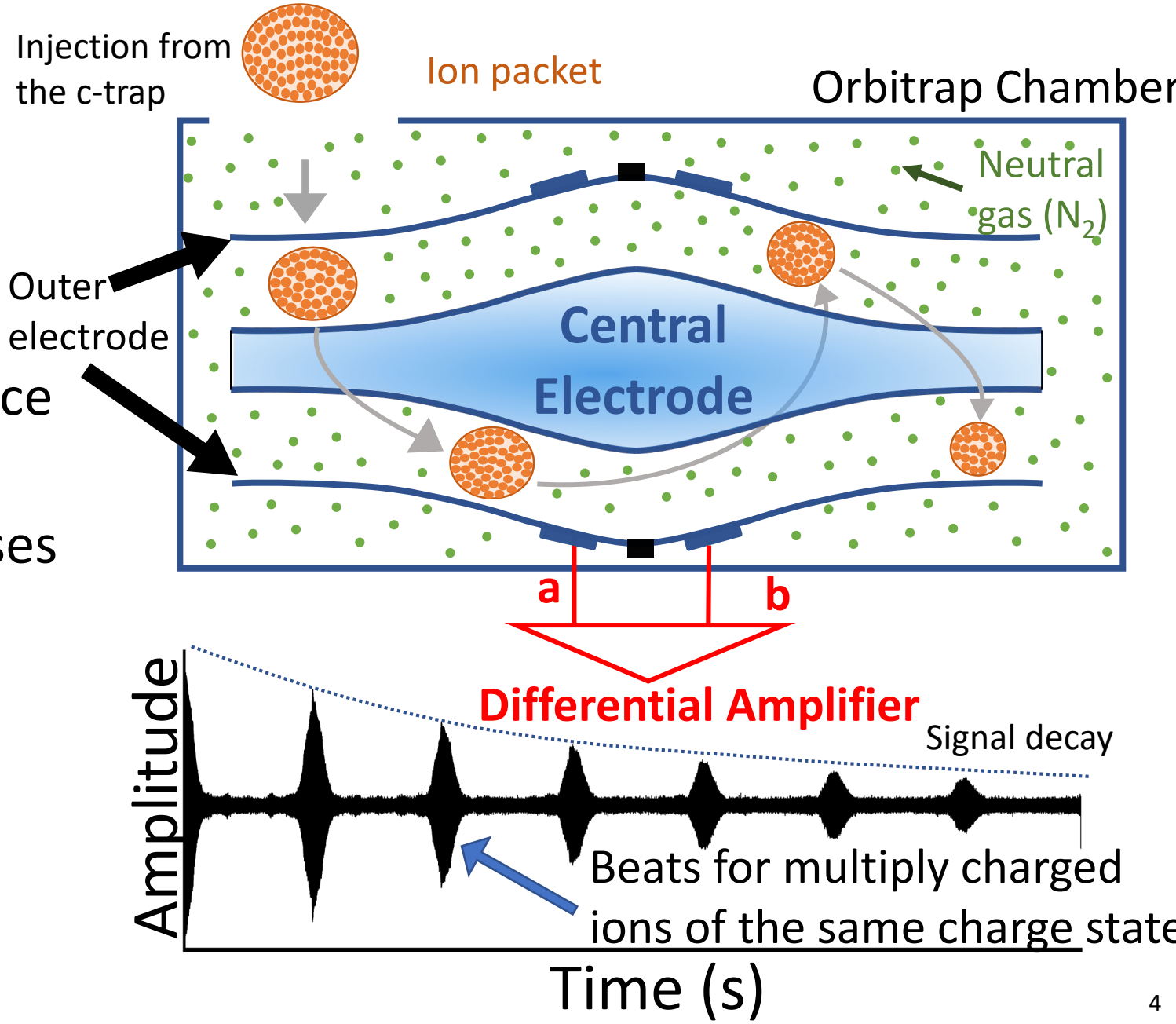
- Ions experience collisions with neutral gas molecules in the Orbitrap MS analyzer

- Collisions lead to loss of coherence of the ion packet

→ The amplitude of beats decreases

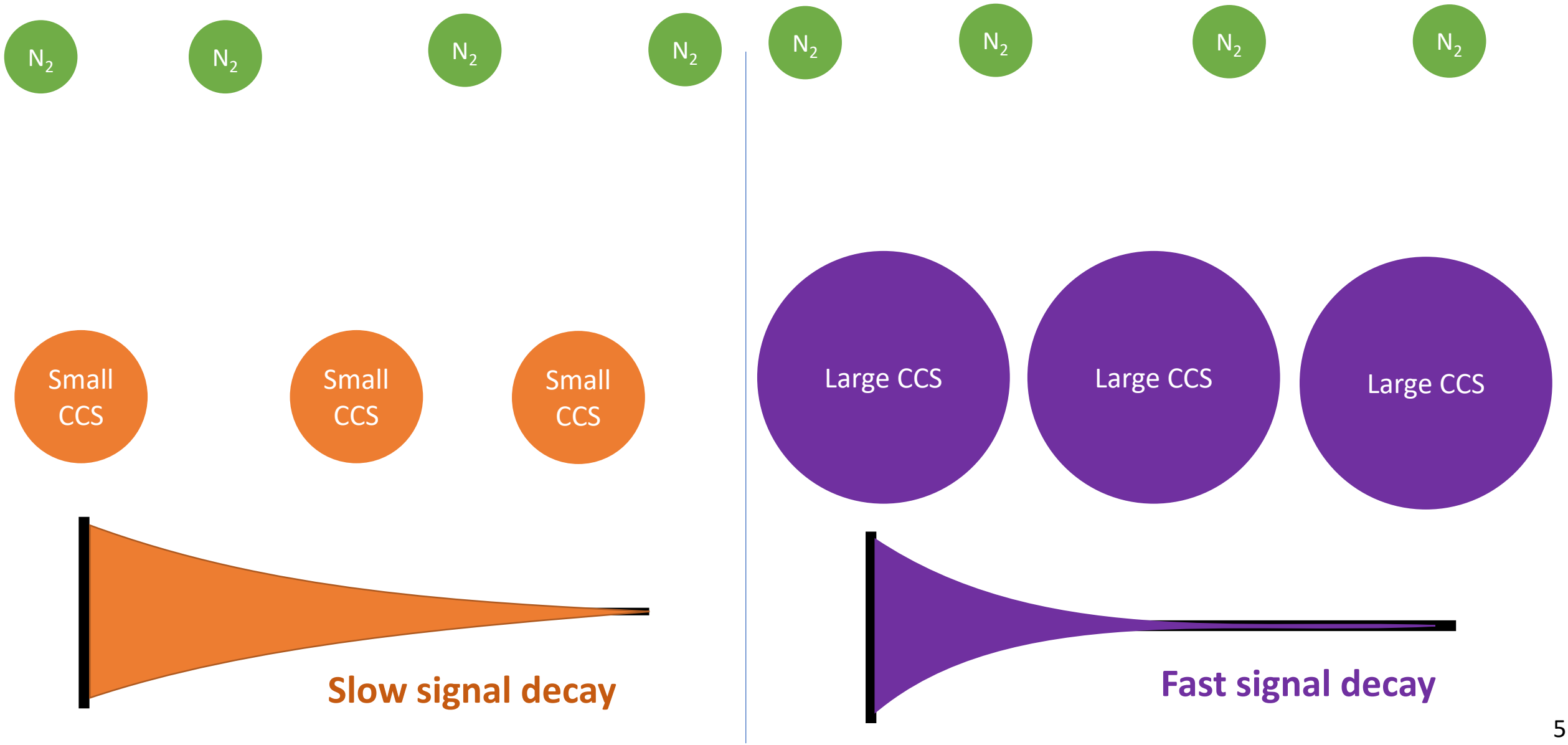
- Rate of decay of beat amplitude

  → CCS



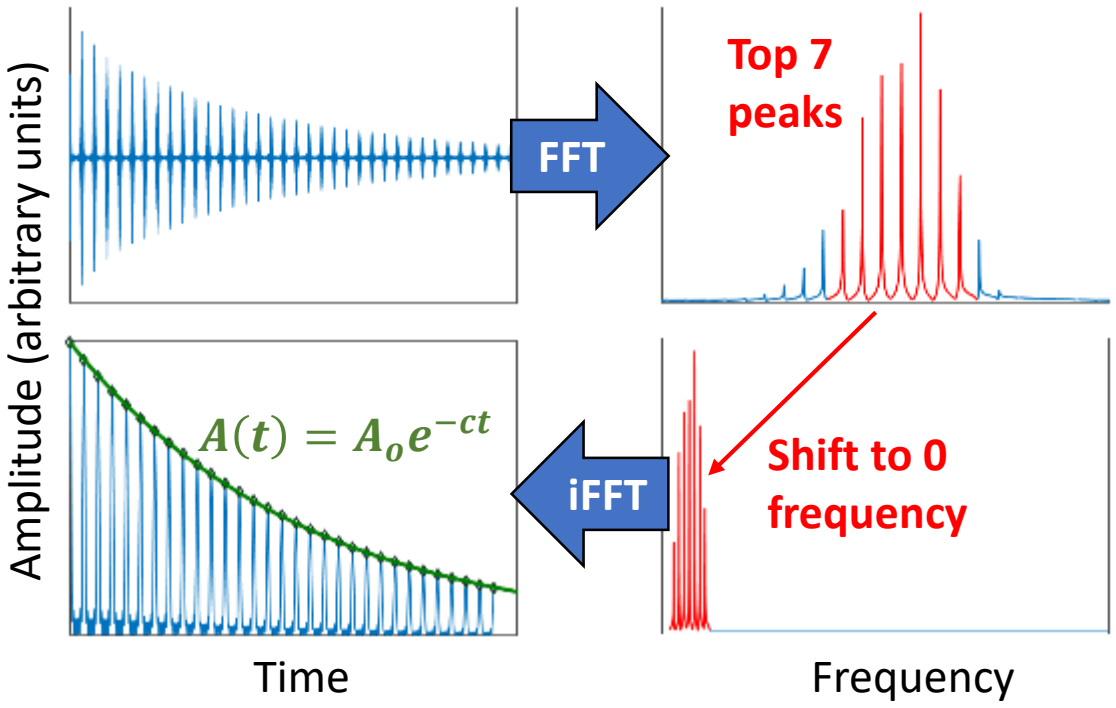
The Orbitrap MS: Ion Decay

Expanded view of a few ions



Orbitrap CCS Method

Find decay rate (c) of analyte ion



Use *c* to calculate CCS

$$\sigma = \frac{c}{f * L * N}$$

$$c = decay\ rate$$

$$\sigma = CCS$$

$$f = frequency\ of\ axial\ ion\ oscillation$$

(inversely proportional to m/z)

$$L = ion\ path\ length\ around\ the\ central\ electrode\ of\ the\ Orbitrap\ for\ one\ oscillation$$

$$N = density\ of\ neutral\ gas\ molecules\ (molecules/m^3)$$

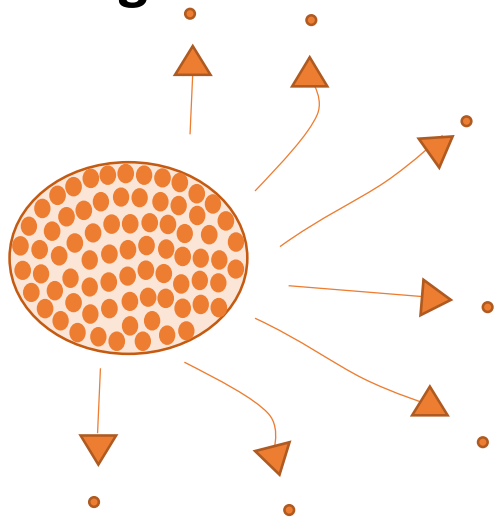
N : determined by calibration using ion of known CCS

Sanders, J. D.; Grinfeld, D.; Aizikov, K.; Makarov, A.; Holden, D. D.; Brodbelt, J. S. Determination of Collision Cross-Sections of Protein Ions in an Orbitrap Mass Analyzer. *Anal. Chem.* **2018**, *90* (9), 5896–5902.

Assumptions for Orbitrap CCS Measurements

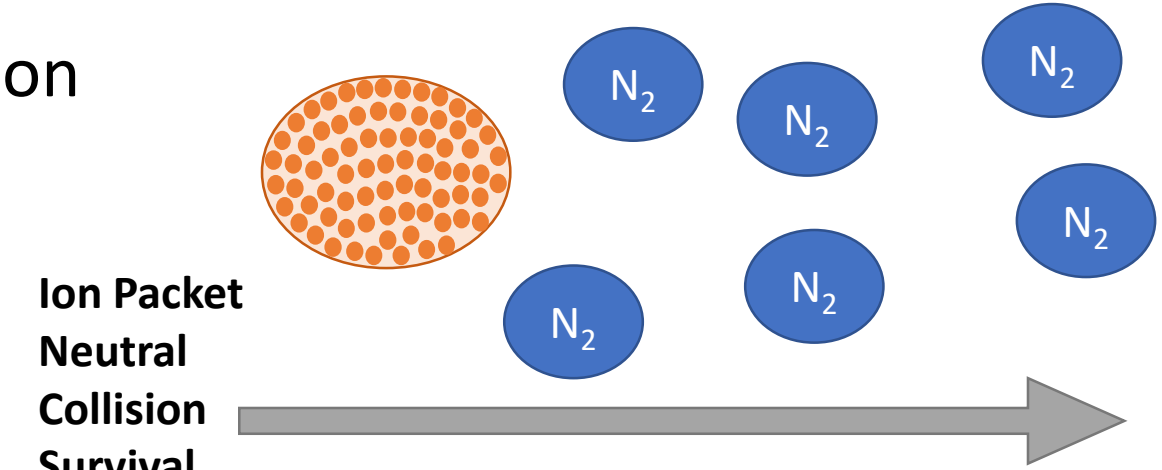
There are two mechanisms of decay: dephasing and collisions

- The decay must be dominated by the collisions rather than dephasing. Dephasing occurs at too much or too low space charging
  - Too much space charging, or too little space charging (lack of self bunching) results in dephasing and a higher than expected decay rate (over-estimation of CCS)



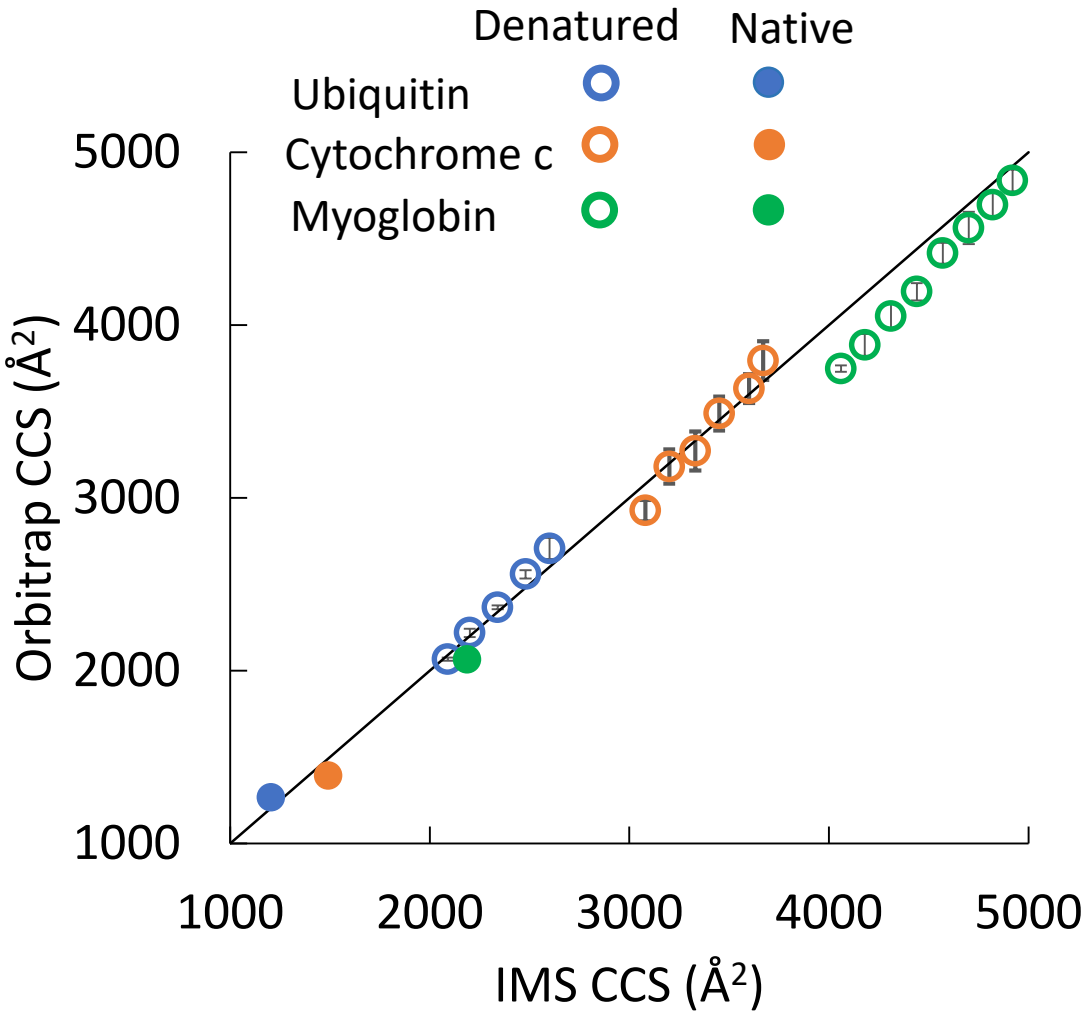
Dephased Ion Packet

- Every collision results in removal of an ion from the ion packet
  - Insufficient energy of collision results in lower than expected decay rate (under-estimation of CCS)



Previous Orbitrap CCS Results

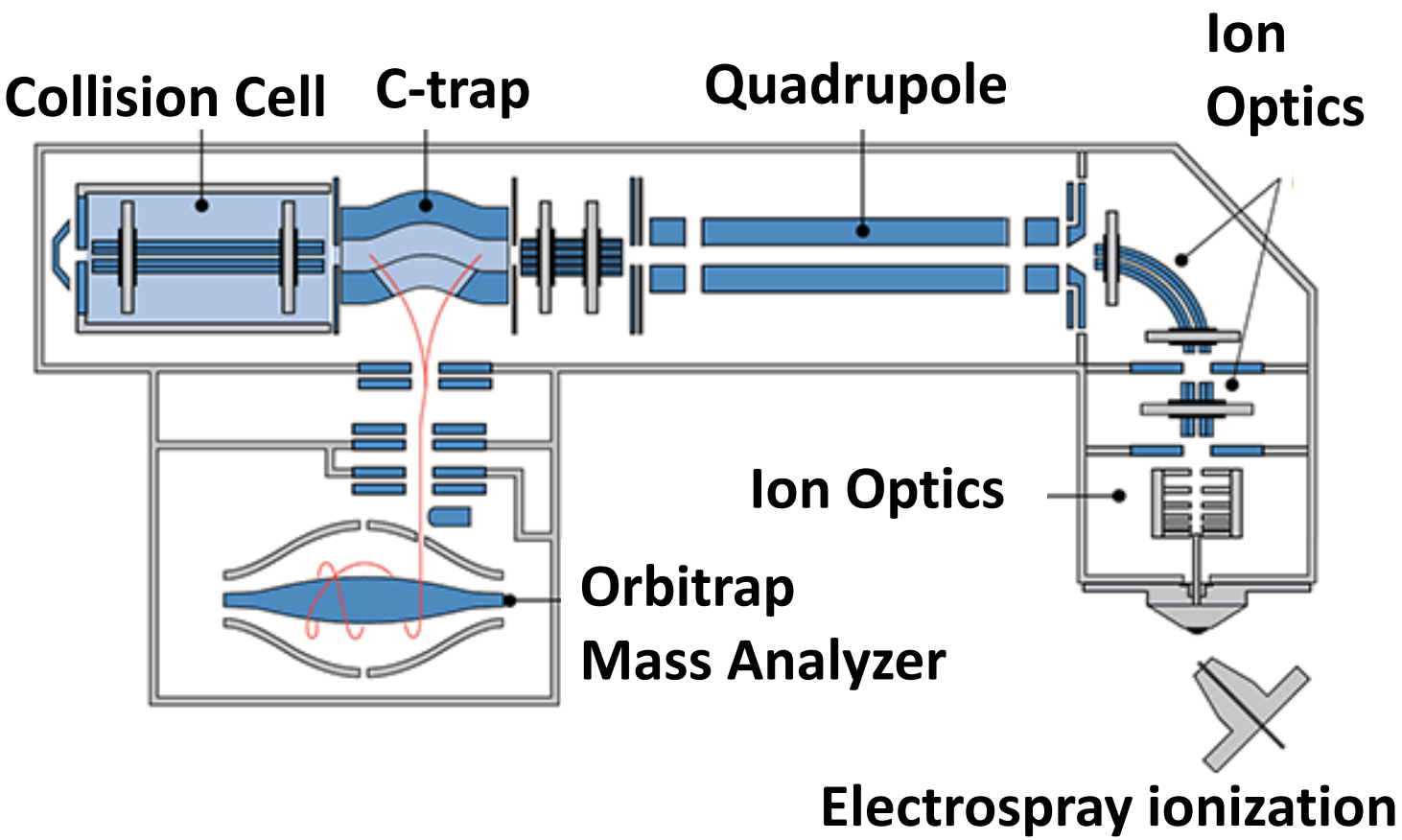
- Previous work focused on smaller proteins, up to 16 kDa.
- All previous data was collected on an Elite Orbitrap mass spectrometer.
- The Orbitrap CCS method showed good agreement with CCS values from ion mobility.



Bush, M. F.; Hall, Z.; Giles, K.; Hoyes, J.; Robinson, C. V.; Ruotolo, B. T. *Anal. Chem.* **2010**, *82* (22), 9557–9565.

Orbitrap CCS Measurements using a modified QE High Field Orbitrap

- 960,000 resolution
  - 2 second transients
- Expansion of CCS methods to native proteins
  - Proteins: 20-50 kDa
  - Monomers and multimers
  - Aqueous solutions
  - Lower charge states



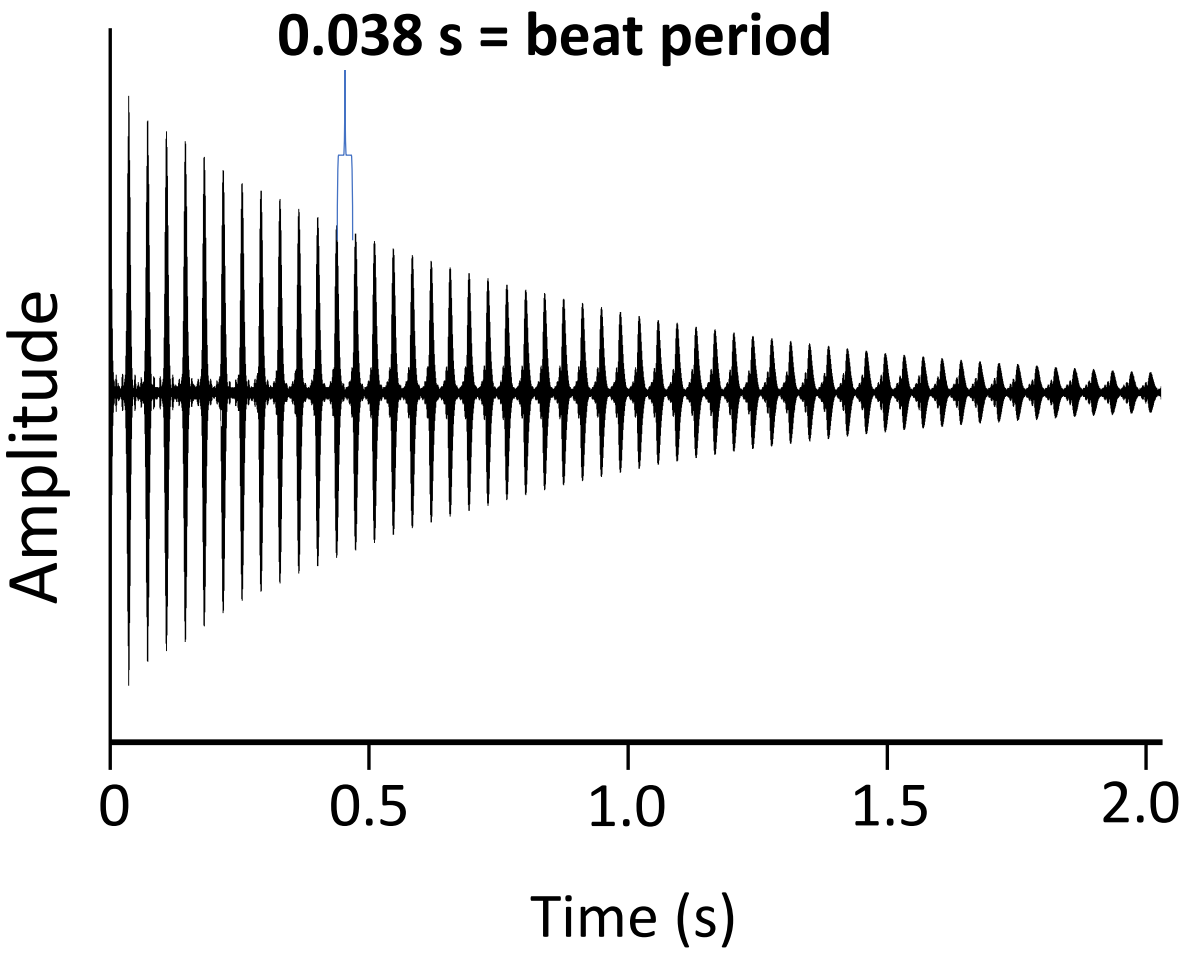
Mass Limit of Orbitrap CCS Measurements

- Theoretical mass limit imposed by number of beats in the transient
  - Number of beats is dependent on mass and charge
  - Need at least 3 beats for decay fitting equation

- Small proteins in high charge states have many beats

$$beat\ period = coefficient * \frac{mass^{3/2}}{charge^{1/2}}$$

Example:
Ubiquitin 9+ (8.5 kDa)

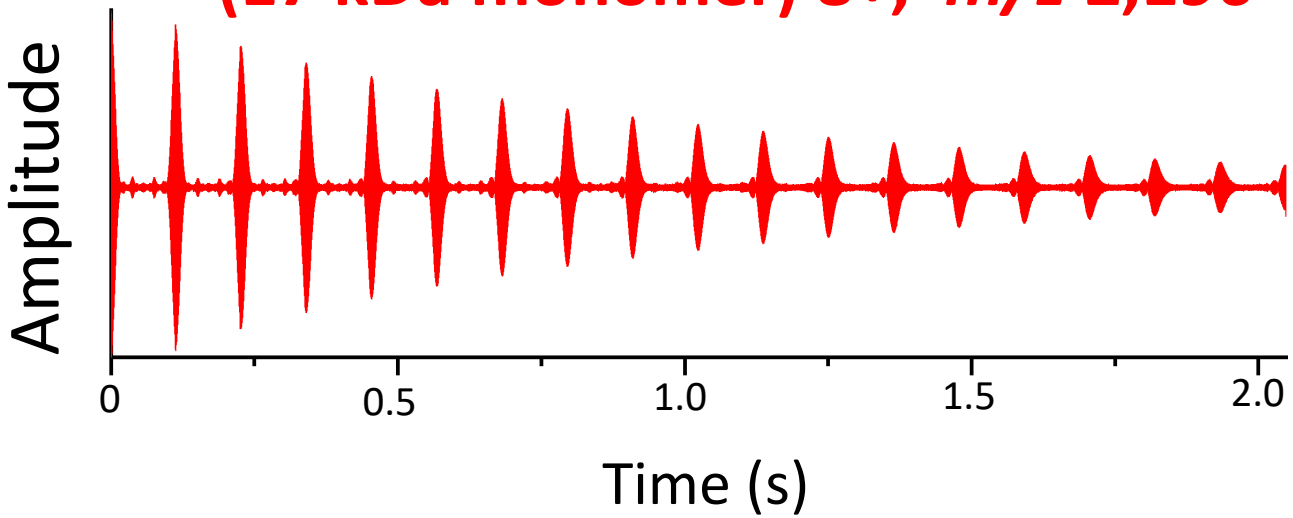


Makarov, A.; Denisov, E. Dynamics of Ions of Intact Proteins in the Orbitrap Mass Analyzer. *J. Am. Soc. Mass Spectrom.* **2009**, *20* (8), 1486–1495.

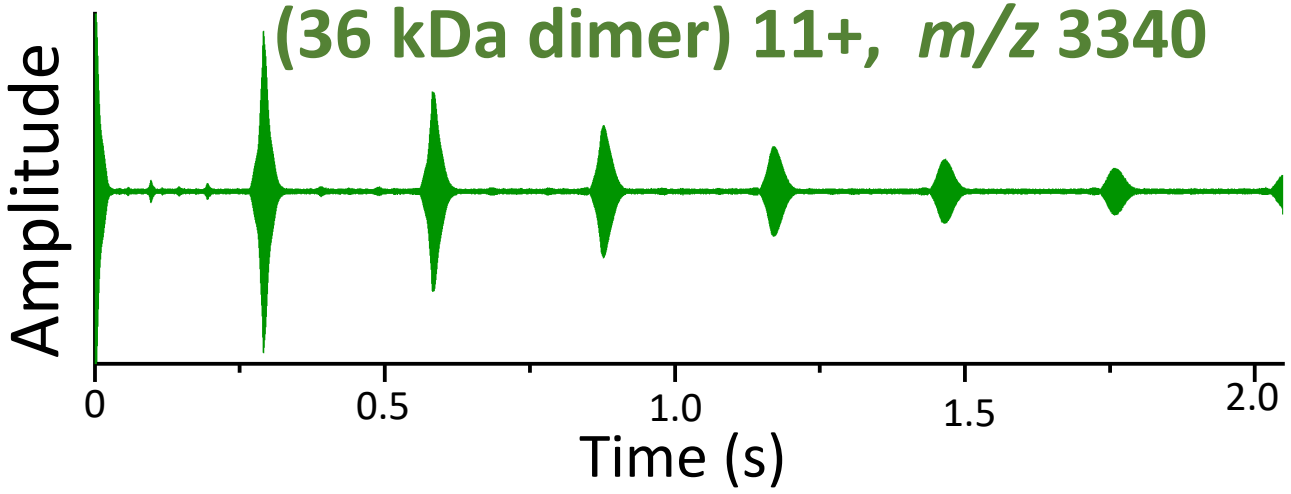
Mass Limit of Orbitrap CCS Measurements

- Our focus: larger proteins, lower charge states
  - Fewer beats per transient
- Max transient time 2 seconds
- Upper mass limit of ~60 kDa to obtain three beats in a transient

Transient of Myoglobin (17 kDa monomer) 8+, *m/z* 2,196

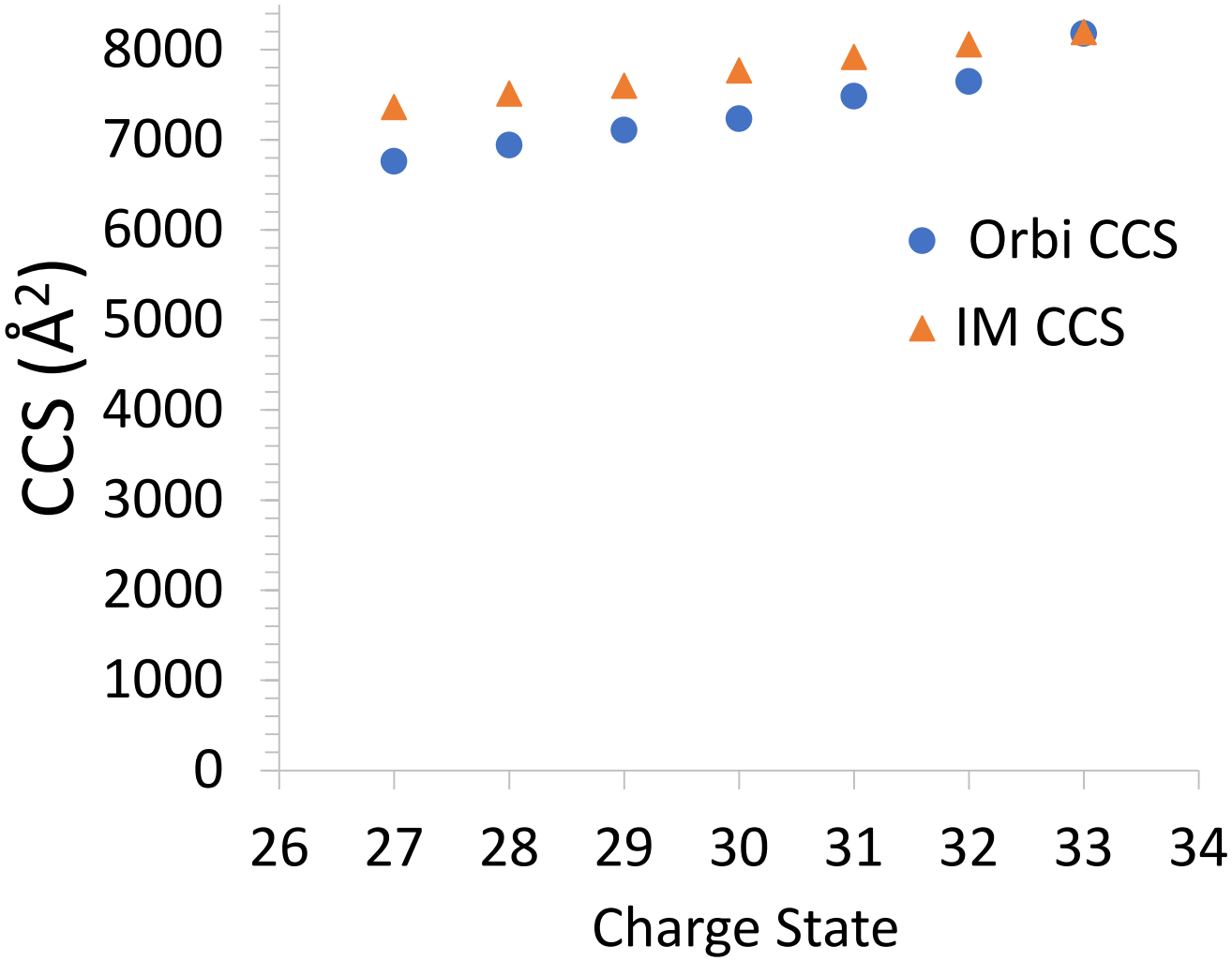


Transient of Beta Lactoglobulin (36 kDa dimer) 11+, *m/z* 3340



Orbitrap CCS Measurements of Larger Proteins in High Charge States

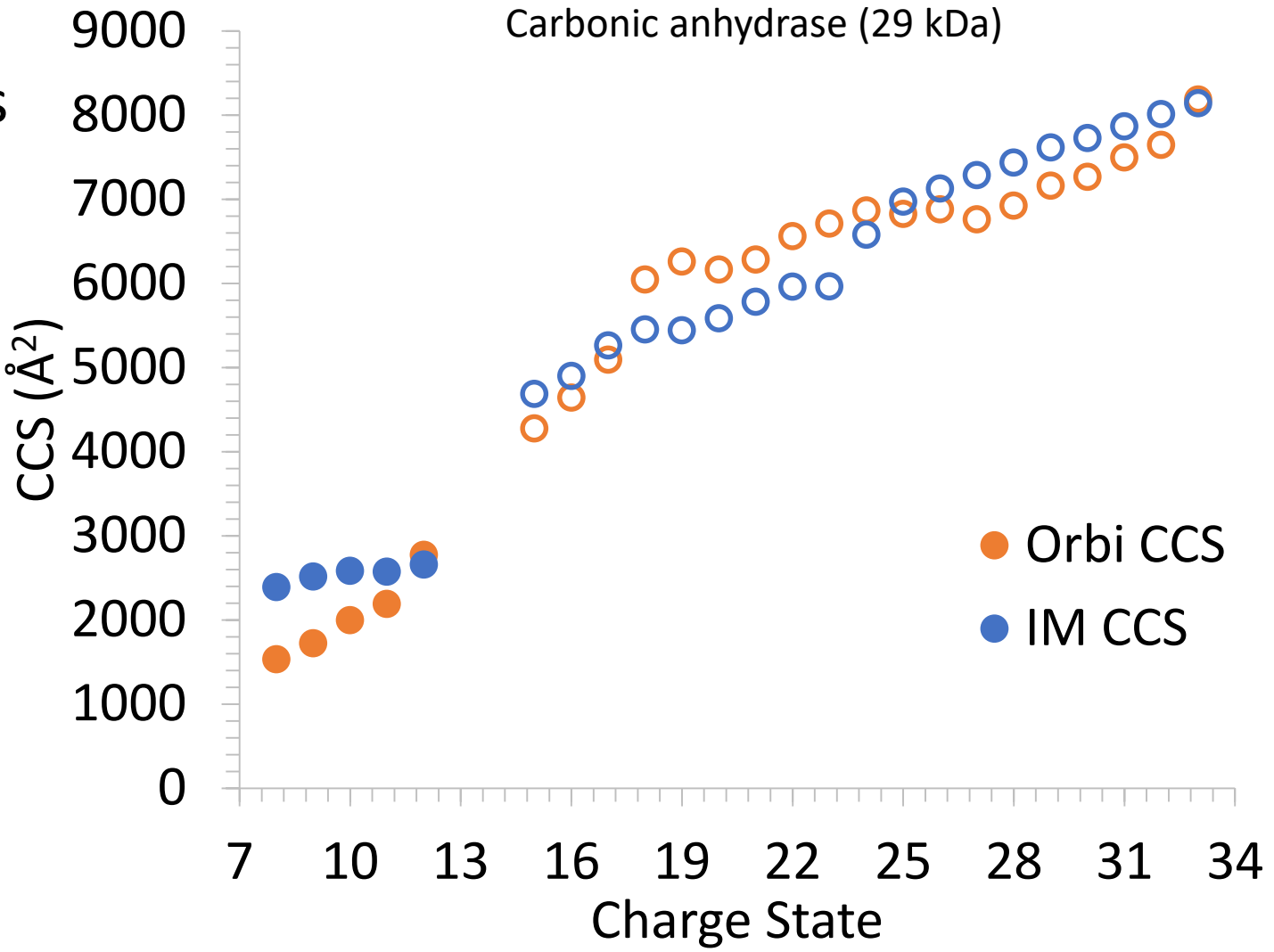
- Our previous study focused on small (<16 kDa) proteins
- New work: Orbitrap CCSs of larger proteins in high charge states show good agreement with IM CCSs
- Example: Denatured carbonic anhydrase (29 kDa) sprayed from denaturing solutions



Orbitrap CCS Measurements of Larger Proteins in Lower Charge States

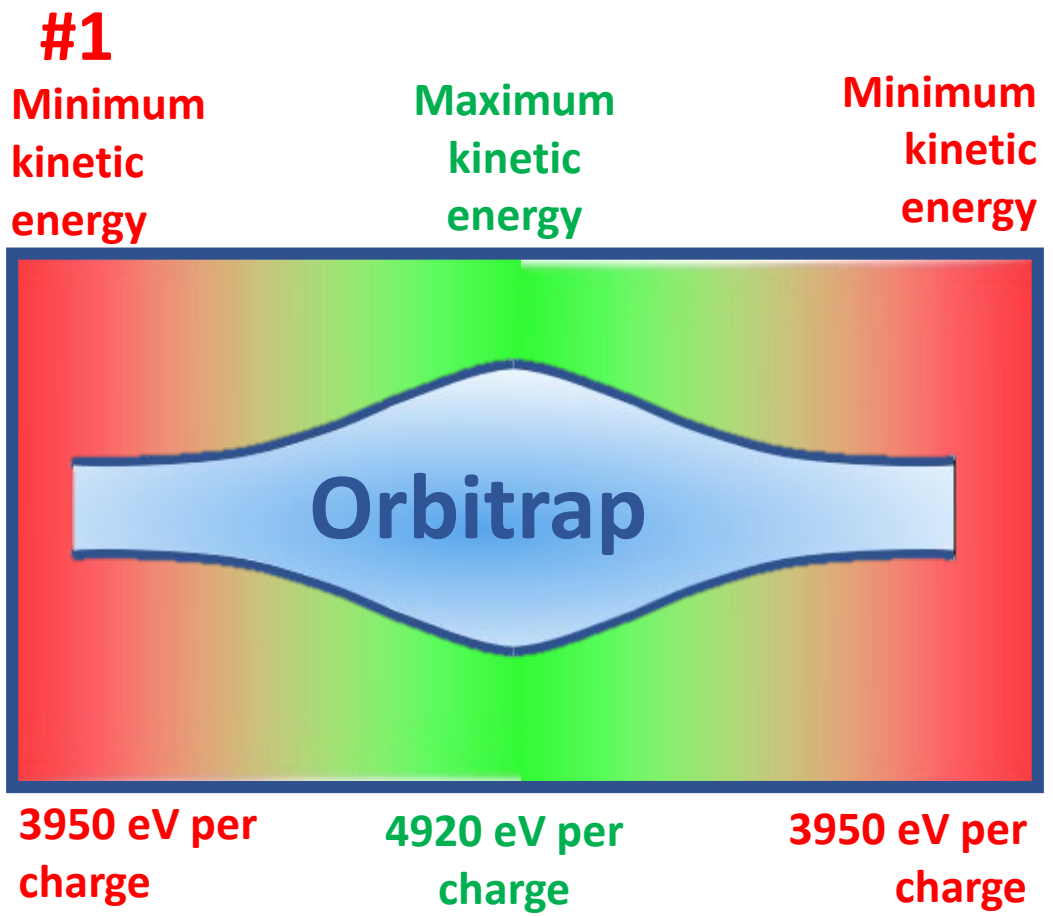
- Orbitrap CCSs of large proteins in low charge states show increasing divergence from IM CCSs.

- Why are the CCS values underestimated for lower charge states of carbonic anhydrase?



Kinetic Energy of Ions in the Orbitrap MS

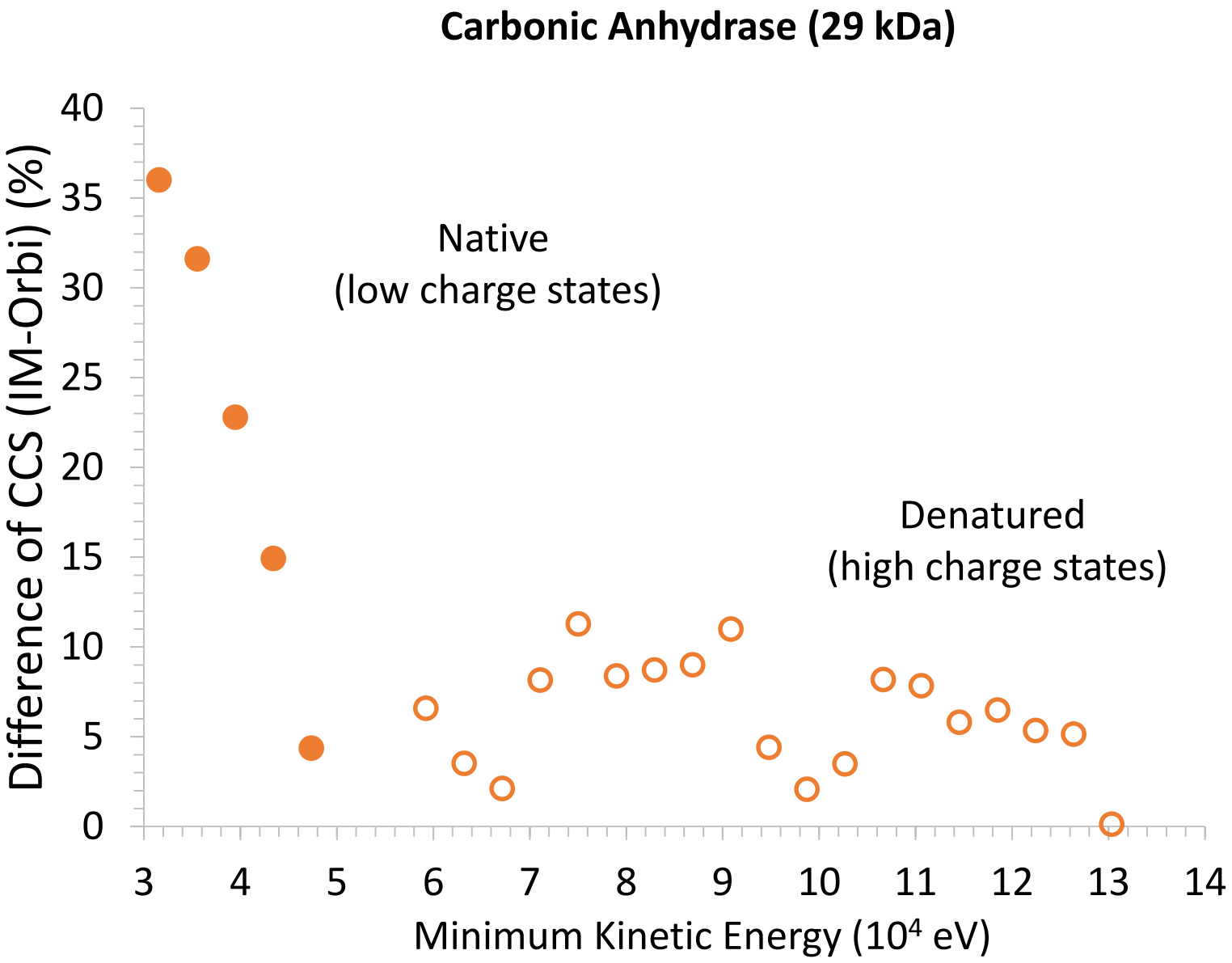
- Single ion charge detection studies have shown that large ions (100 kDa to MDa) survive in the Orbitrap for indefinitely.
  - These ions have high m/z, low frequency, and low kinetic energies
- For ions <20 kDa, differing kinetic energies lead to mixed ion survival/decay
  - Ion survival may happen in minimum kinetic energy regions
  - Ion decay may happen in maximum kinetic energy region
- Compare Orbitrap CCS error to minimum kinetic energy for each ion since ions are more likely to survive collisions at their minimum kinetic energy thus deviating from ideal Orbitrap CCS experimental conditions



Kinetic energy of ions in the Orbitrap MS is only dependent on charge owing to energy imparted during ion injection

Wörner, T. P.; Aizikov, K.; Snijder, J.; Fort, K. L.; Makarov, A. A.; Heck, A. J. R. Frequency Chasing of Individual Megadalton Ions in an Orbitrap Analyzer Improves Precision of Analysis in Single Molecule Mass Spectrometry. bioRxiv 2021, 2021.06.15.448530.

Impact of Kinetic Energy on Orbitrap CCS Measurements

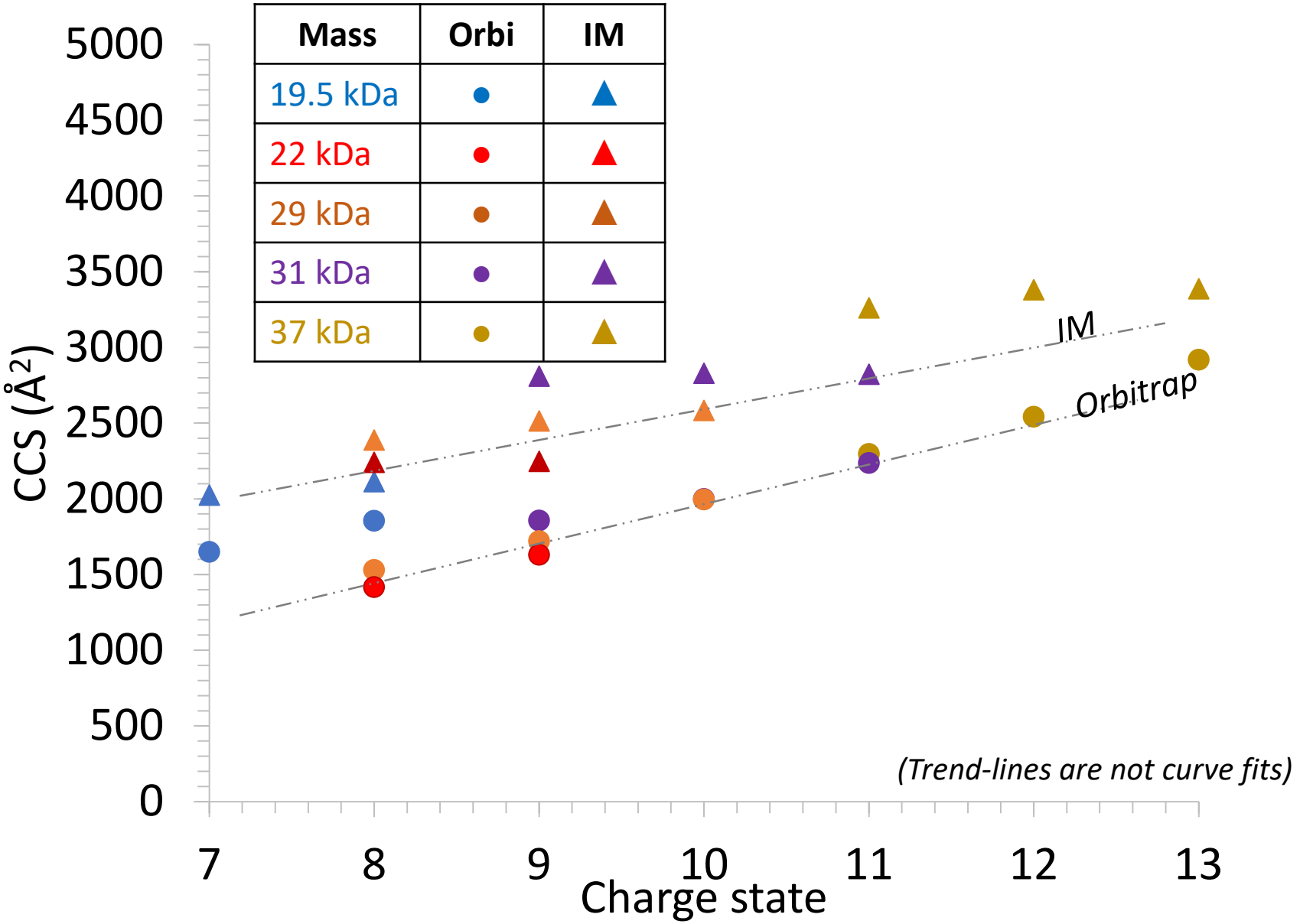


- <u>High charge states:</u> Ion kinetic energy decreases with decreasing charge, but still meets the minimum threshold so that each collision will result in removal of the ion from the ion packet

- <u>Low charge states:</u> Low kinetic energies allow ions to survive some collisions, resulting in underestimation of CCS.

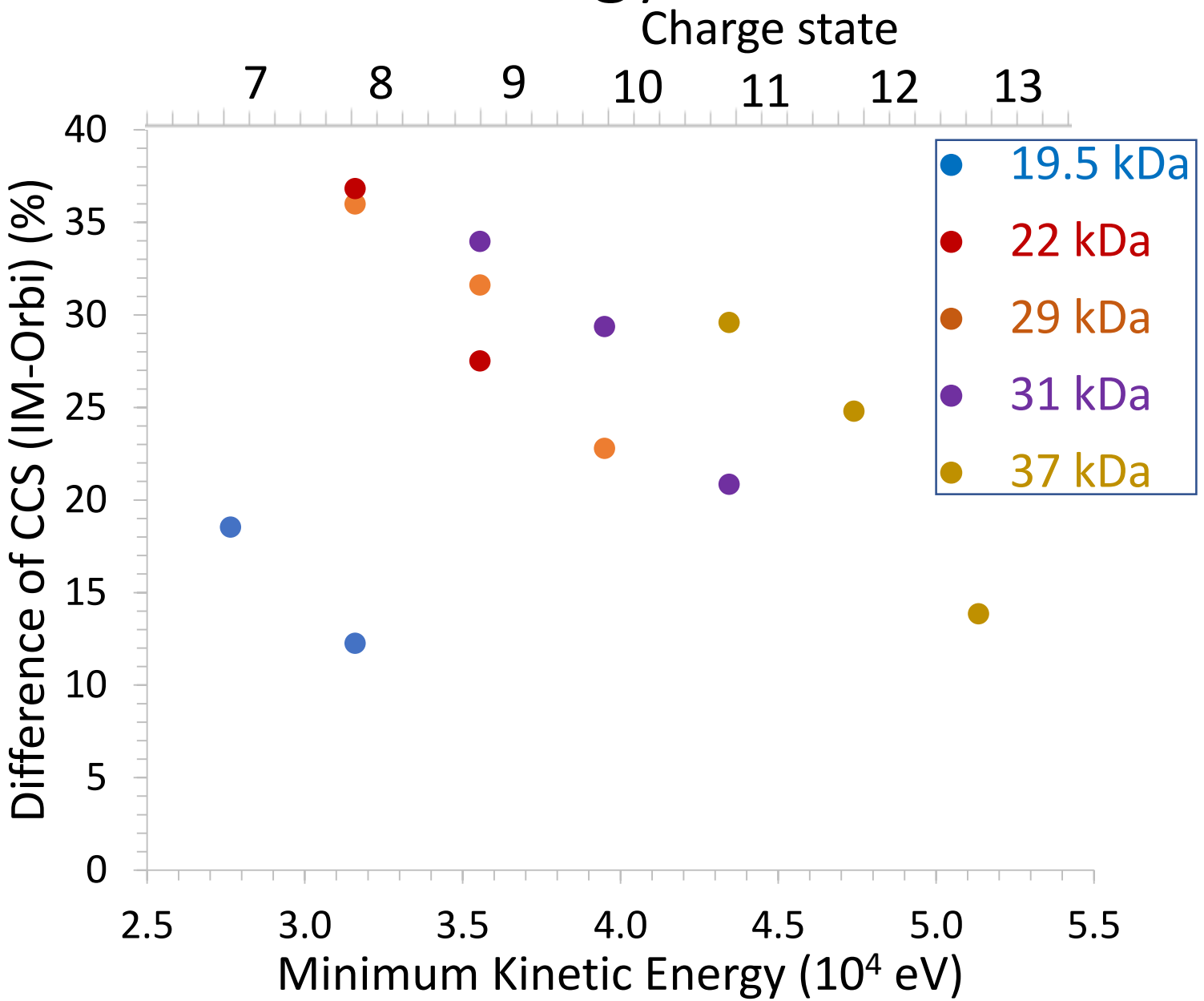
Orbitrap CCS Results for High Mass Proteins

- Other proteins show similar trend of increasing Orbitrap CCS error with decreasing charge state
- Disagreement between CCS values measured by IM versus Orbitrap depends on both charge state and mass



Variation of CCS Error with Kinetic Energy and Mass

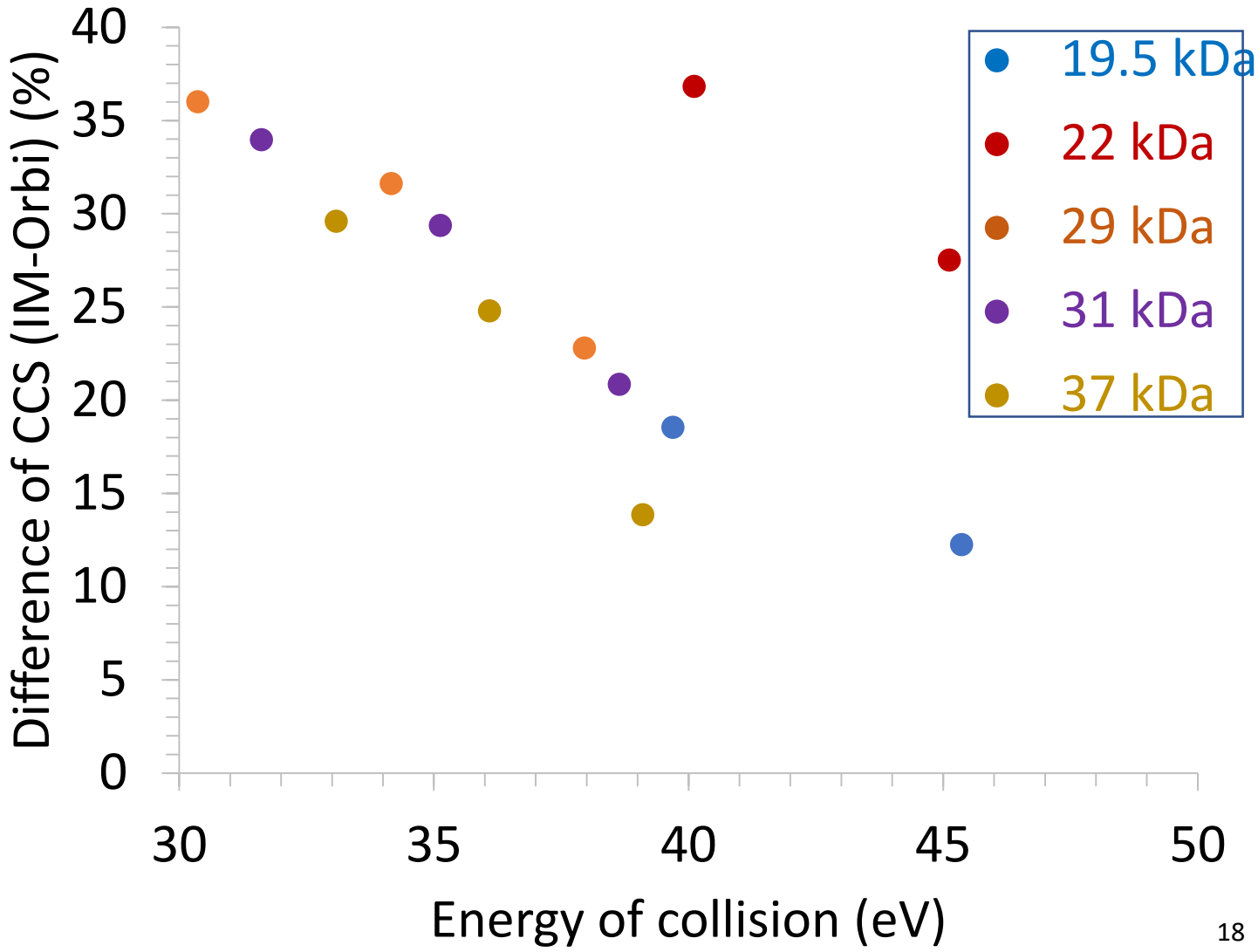
- Decreasing kinetic energy, increasing mass leads to increase in CCS error
- Both protein mass and ion kinetic energy correlate with the magnitude of CCS error
- Further examination of the effect of protein mass is warranted



Variation of CCS Error with Energy of Collision

$$E_{collision} = \frac{Mass_{neutral}}{Mass_{neutral} + Mass_{ion}} Kinetic\ Energy_{ion}$$

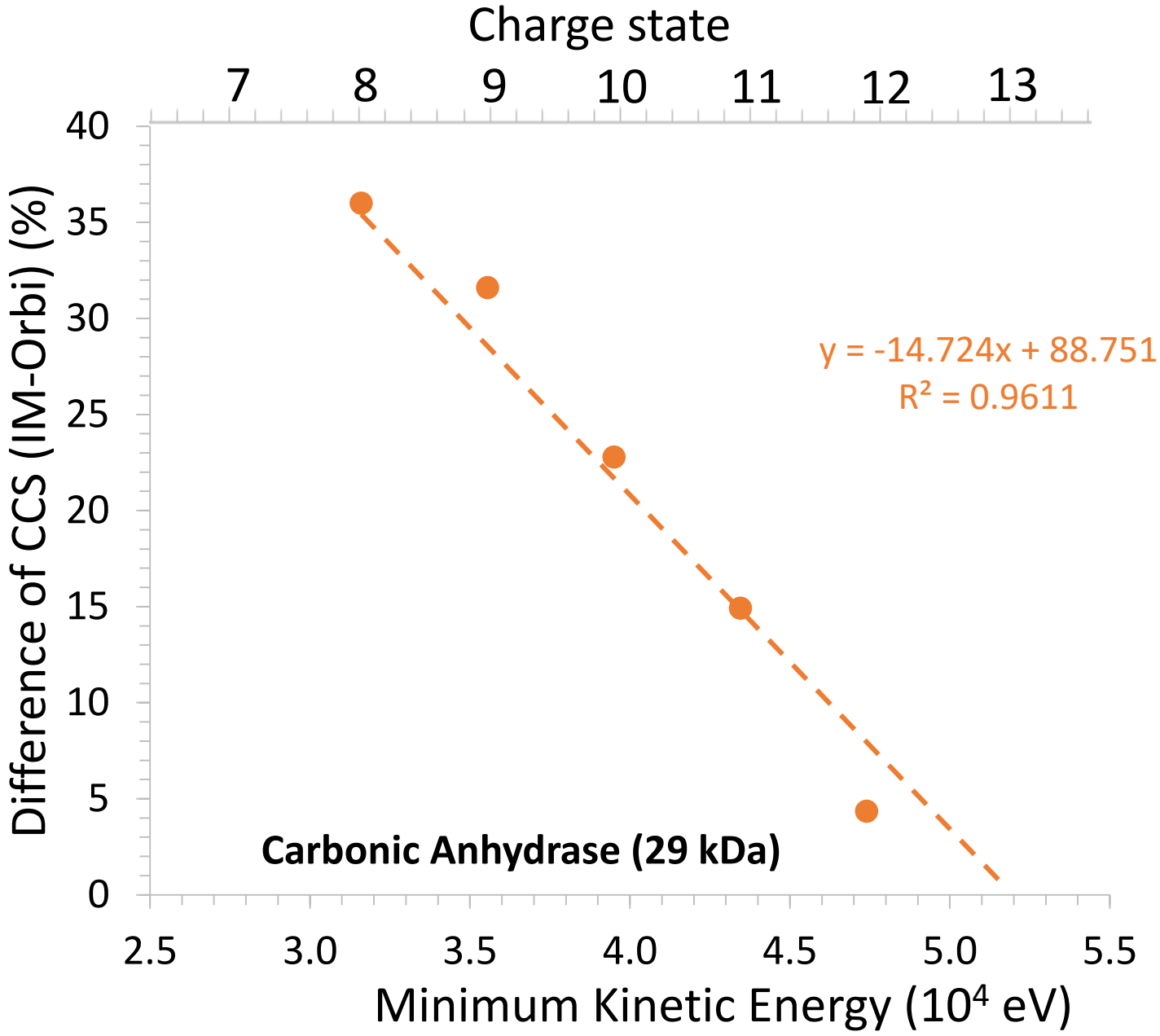
- Energy of collision also considers ion mass
- Energy of collision is a determining factor on loss of coherence of ion packet
- Agreement in the relationship between energy of collision and CCS error for most data points



Linear Regression to Model Ion Energy Thresholds

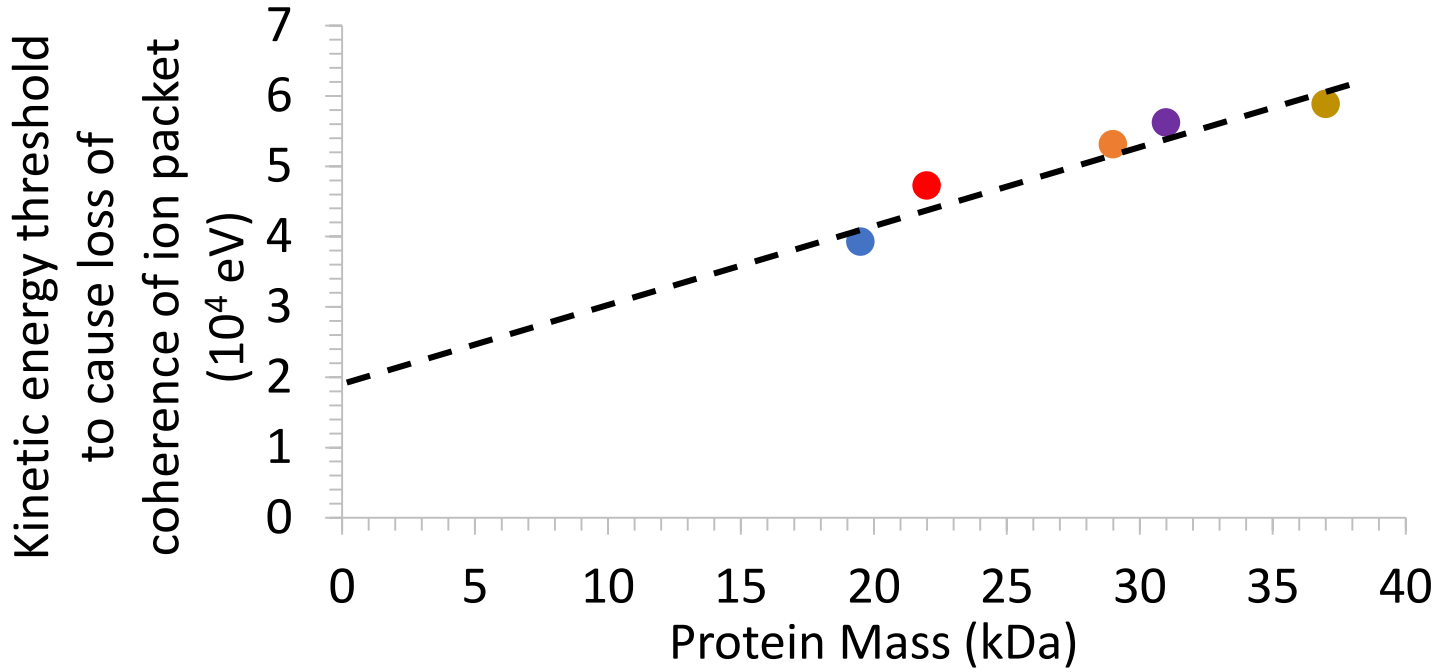
- Perform linear regression to estimate the kinetic energy threshold at which the protein is removed from the ion packet upon experiencing a single collision (x-intercept)

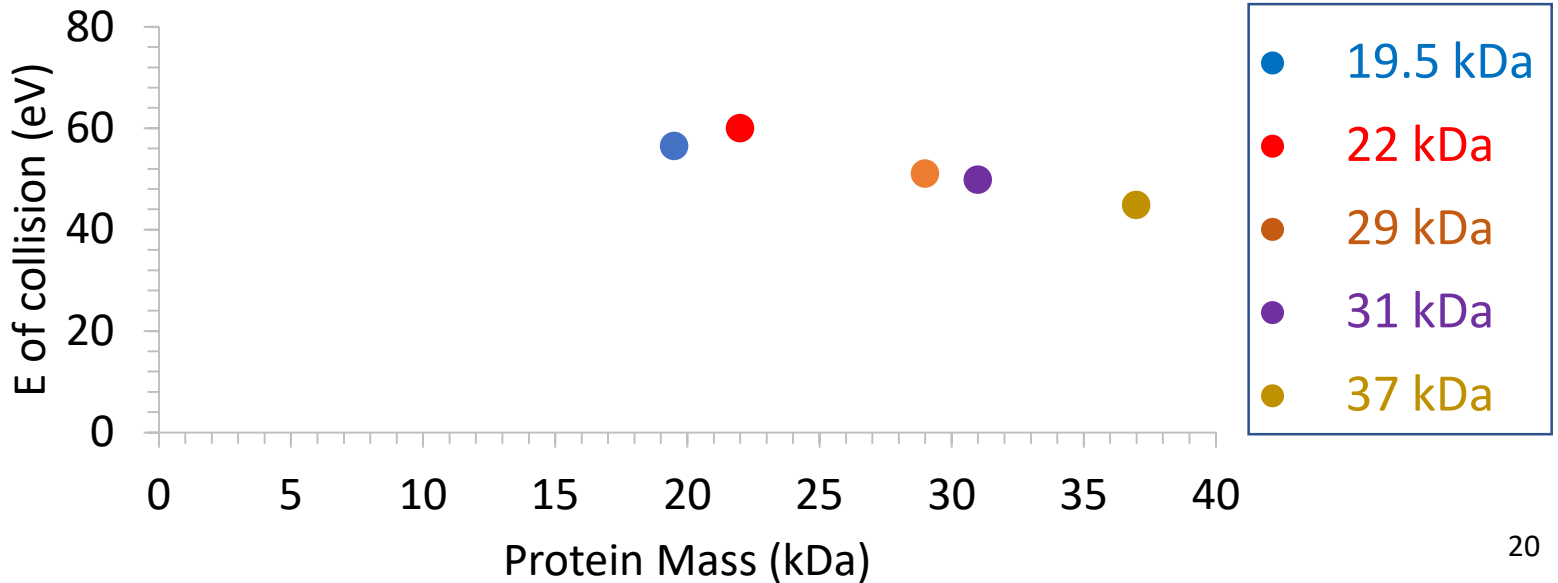
- Perform same linear regression for energy of collision



Energy Thresholds

- Kinetic energy threshold for loss of coherence with ion packet correlates with protein mass.
- Energy of collision threshold shows agreement among proteins
- Both protein mass and ion kinetic energy influence prolonged ion survival in the Orbitrap.



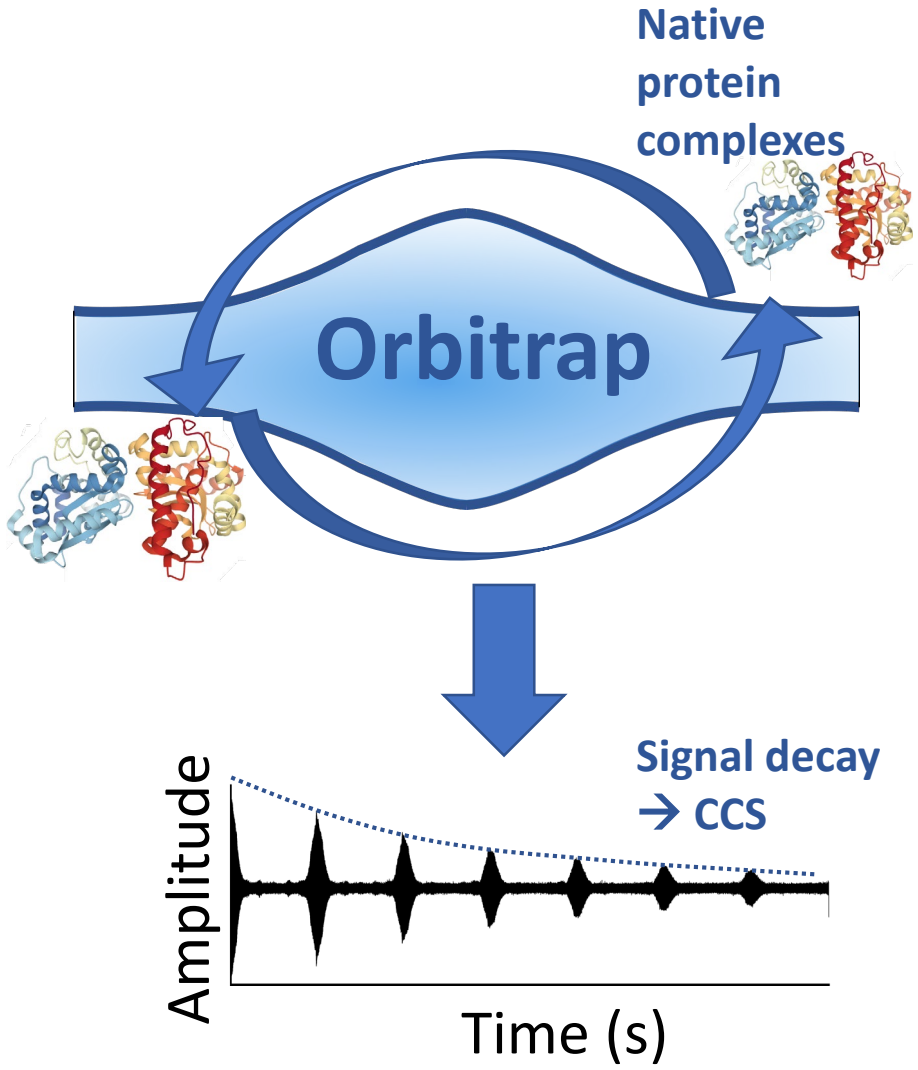


Conclusions

- Number of signal beats and signal decay must be considered for measurement of CCSs of larger proteins in low charge states.

- Orbitrap CCS measurements of 20-50 kDa proteins are accurate for high charge states but underestimate collision cross sections of low charge states owing to prolonged ion survival:
  - lower kinetic energies
  - higher protein mass

- Future work will investigate the structural stability and binding energies of proteins/protein complexes in the Orbitrap mass spectrometer.



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- Dr. Kyle Fort