Chapter 11

Ion Mobility-Mass Spectrometry to Evaluate the Effects of Protein Modification or Small Molecule Binding on Protein Dynamics

Lauren J. Tomlinson and Claire E. Eyers

Abstract

Ion mobility-mass spectrometry (IM-MS) of intact protein complexes under native conditions is a powerful tool for the analysis of protein complexes and protein–ligand interactions, permitting insight into ligand-induced changes in protein conformation. Here we describe a procedure for analyzing the effects of phosphorylation and/or inhibitor binding on protein kinase conformational flexibility using Protein Kinase A (PKA) as a model system. By calculating the protein collision cross section (CCS) before and after inhibitor binding, and additionally by performing collision-induced unfolding (CIU), we can establish the effects of protein modification or small molecule binding on protein dynamics.

Key words Ion mobility-mass spectrometry, Native mass spectrometry, Collision cross section, Collision-induced unfolding

1 Introduction

Ion mobility-mass spectrometry (IM-MS) of intact protein complexes under native electrospray ionization (ESI) conditions is advancing the structural analysis of proteins, protein complexes, and protein–ligand interactions. The benefits of rapidly obtaining information on protein dynamics and conformational flexibility from small amounts of (partially) purified material means that IM-MS is confidently being implemented alongside more traditional structural techniques such as X-ray crystallography and nuclear magnetic resonance (NMR) to gain insight into the structural alterations associated with protein modification, protein–ligand binding, and protein complex formation [1–5].

Many elegant IM-MS studies are now being published that evaluate the effect of different types of ligands on protein complex formation and protein dynamics, including small molecules [6–13], RNA and DNA oligomers [14, 15], and (proteo)glycans [16, 17].

There are four primary forms of ion mobility that are coupled with MS: drift-tube ion mobility spectrometry (DTIMS), traveling wave ion mobility spectrometry (TWIMS), trapped ion mobility (TIMS), and differential-mobility spectrometry (DMS), a version of which is field-asymmetric ion mobility spectrometry (FAIMS) [18, 19]. DTIMS and TWIMS (and more recently TIMS) are preferentially used in the investigation of protein–ligand interactions, as they can be used to define analyte rotationally average collision cross section (CCS) values permitting direct comparison of conformers, e.g., before and after ligand binding. If required, CCS values can also be correlated with data obtained using software programs, such as MOBCAL [7].

Protein kinases are important drug targets, in large part because of their rate-limiting roles in numerous diseases [20]. Consequently, understanding the structural implications upon binding of small (and large) molecule drugs to these proteins, and the effect of regulatory post-translational modifications, such as phosphorylation, is important. Investigating protein conformational dynamics and the effects on stability is particularly relevant as the field moves toward development of, arguably more specific, "type II" small molecule allosteric inhibitors that bind regions of the protein outside of the relatively conserved ATP-binding site [21, 22].

Here we present a method of utilizing IM-MS to assess the structural effects of phosphorylation and ligand binding on cAMP-dependent protein kinase (PKA) [7, 23]. The described method is readily transferable to the investigation of phosphorylation and ligand-induced changes in other proteins, including other members of the protein kinase superfamily.

2 Materials

Prepare all solutions using HPLC-grade water. All protein solutions should be kept at 4 °C unless otherwise stated.

2.1 Buffer Exchange

1. Ammonium Acetate: 50 mM. Weigh out 0.39 g ammonium acetate and dissolve in 100 mL HPLC-grade water to make 50 mM ammonium acetate buffer. Prechill to 4 °C.

2. HPLC-MS water.

3. Amicon spin filter columns.

4. Bench top centrifuge.

5. Gel loading tips.

2.2 Inhibitor Assay

1. Protein(s) of interest: exemplified here are different forms of the catalytic subunit of PKA (PKAc)—wild-type (WT; hyper-phosphorylated), λ protein phosphatase (λPP)-treated PKAc,

catalytically inactive K72H PKAc, and the non-PKI-binding R133A PKAc variant.

2. Inhibitors: Prepare all stocks of inhibitors (staurosporine (STS), H89 and AT13148) by diluting to 10 mM final concentration in DMSO.

2.3 Ion Mobility-Mass Spectrometry

1. Ion mobility-mass spectrometer: Waters G2-Si Synapt.

2. API Calibration (NaCsl) Solution Kit (Waters).

3. TW120–4 Thin-Wall Capillary (4 × 1.2 mm), 10 G.

4. Capillary puller: Sutter P-1000 Puller (*see* **Note 1**).

5. Ceramic capillary cutter.

6. Platinum (Pt) wire (0.125 mm × 5 m), one per capillary, cut to 4 cm lengths.

7. Protein calibrants for CCS determination: β-lactoglobulin A, avidin, transthyretin, concanavalin A, and serum albumin (Sigma-Aldrich) prepared as 5–10 μM in 200 mM ammonium acetate (*see* **Note 2**).

3 Methods

3.1 IM-MS Setup

1. Prepare the capillaries for nano electrospray ionization (nESI) using the capillary puller and the program as shown in Table 1 (*see* **Note 1**).

2. To calibrate the ToF, infuse the Lockspray Flow Control with NaCsl. Start infusing at 20 μL/min, decreasing to 10 μL/min once a strong signal is visible.

3. Calibrate using NaCsl over a m/z range of 500–8000 in resolution mode (*see* **Note 3**).

3.2 Protein Buffer Exchange

1. Prepare the protein solution for IM-MS analysis by buffer exchanging into 50 mM ammonium acetate (*see* **Note 4**) using amicon 0.5 mL spin filter columns (*see* **Note 5**). Dilute the required amount of protein solution (*see* **Note 6**) in ammonium acetate to the capacity of the amicon spin filter (*see*

**Table 1**
Sutter P-1000 Puller settings

Line	Heat	Pull	Vel.	Delay	Pressure	Ramp
1 × 1	489	0	20	120	235	491
2 × 1	489	0	18	120	Delay mode	x
3 × 1	489	0	18	120	Safe heat	x
4 × 1					Jaw temp. 19 °C	

Note **7**). Centrifuge at $14,000 \times g$ for 10 min, in a precooled bench top centrifuge at 4 °C. Discard the flow through. Repeat the dilution and centrifugation step twice (*see* **Note 8**).

2. Following the final buffer exchange spin, invert the filter in to a new low-bind centrifuge tube and spin for 3 min at $14,000 \times g$, 4 °C.

3. Determine the concentration of the final protein solution in ammonium acetate using a NanoDrop spectrophotometer (*see* **Note 9**).

3.3 IM-MS Analysis

1. Cut off ~2 cm from the back end of the capillary using a ceramic capillary cutter (*see* **Note 10**).

2. Place the capillary in the secure clasp holder. Using gel-loading tips, add between 1–3 μL (2–5 μM) of protein sample (either protein CCS calibrant or protein of interest) directly in to the nESI capillary.

3. Spin down the clasp holder containing the capillary for a few seconds in a microfuge to ensure that all of the protein solution has reached the end of the capillary tip.

4. Remove the capillary from the clasp and place the piece of platinum wire inside the capillary (*see* **Note 11**). Affix the metal clasp and the protective rubber insert to the outside of capillary and mount onto the source stage of the Synapt G2-Si instrument.

5. Use the camera function to position the capillary, so that it is in the optimal position (*see* **Note 12**).

6. Increase the nano flow gas to 0.05 bar and the sampling cone to 20 V.

7. Set the instrument to TOF/MS mode and increase the capillary voltage to ~1.1–1.6 kV until a stable signal is observed (*see* **Note 13**).

8. Determine the optimal TriWave settings for the protein of interest by adjusting the Wave Velocity (m/s) and Wave Height (V) (*see* **Note 13**).

9. Once a good signal is established, and TriWave settings optimized, ensure the 'Add Drift Time' function button is ticked and acquire data over the required *m/z* range (*see* **Note 14**). *See* Fig. 1 for native MS and IM-MS data for the exemplar hyperphosphorylated PKAc, before and after treatment with λ protein phosphatase (PKA λPP), as well as two protein variants: K72H PKAc which is catalytically inactive and therefore not phosphorylated, and R133A PKAc which is unable to bind PKI (the inhibitor of PKA).
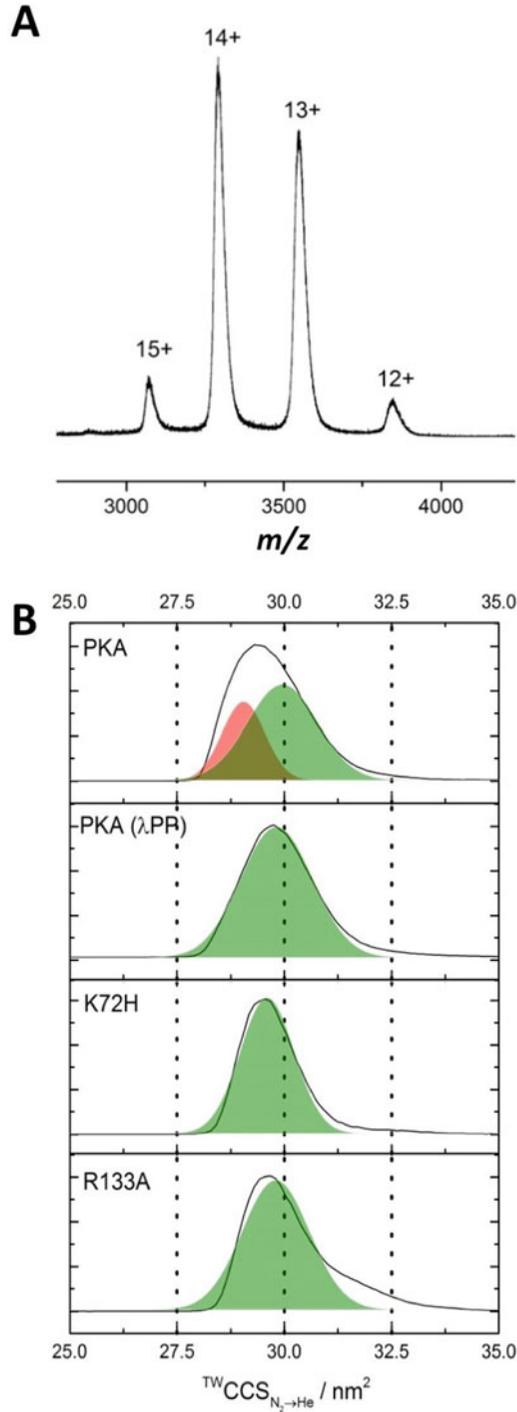
Fig. 1 Structural analysis of variants of the PKA catalytic subunit (PKAc) by IM-MS. (**a**) ESI mass spectrum of PKAc obtained under native conditions; charge states are indicated. (**b**) $^{TW}CCS_{N_2 \rightarrow He}$ for the $[M+13H]^{13+}$ form of untreated WT PKAc (PKA), PKAc following treatment with $Mn^{2+} - \lambda$ protein phosphatase (PKA

3.4 Calibration of the TWIMS for CCS Determination

1. To determine CCS values for the protein of interest, the drift time through the instrument must first be calibrated under the optimal MS acquisition parameters used for the protein. Calibrate the drift time using the CCS Calibration setting on the Intellistart program. Acquire IM-MS data as described in Subheading 3.3.

2. Open the data file in the MassLynx software using the 2: TOFMS (500:8000) ES+ function.

3. Extract the peak of interest from Total Ion Chromatogram (TIC) to open spectrum.

4. Right click across the peak in the spectrum view to open another chromatogram window.

5. In the chromatogram window, click edit and copy the chromatogram list. Paste these values (drift times and the corresponding intensities) in to Microsoft Excel or a similar program of choice.

6. Refer to the Collision Cross Section (CCS) database (https://depts.washington.edu/bushlab/ccsdatabase/) provided by the Bush Lab [24] to determine the native-like ion value for CCS $(He)/nm^2$ of the chosen CCS calibrants.

7. Calculate the reduced cross section Ω' of the protein calibrants using Eq. 1:

$$\Omega' = \Omega \frac{\sqrt{\mathsf{u}}}{q} \tag{1}$$

where μ is the reduced mass and q is the charge state.

8. Plot (Ω') versus (tD') and fit a straight line using Eq. 2 to convert the drift time scale to a CCS (He) scale:

$$\Omega' = \Omega \frac{q}{\sqrt{u}} tDr^{A°} exp(C) \tag{2}$$

where A is the determined slope of the fit and C is the intercept.

9. Plot the converted nonlinear scale against the corresponding intensity values.

Fig. 1 (continued) PP), and the K72H and R133A variants. The hyperphosphorylated PKAc had a $^{TW}CCS_{N_2 \to He}$ value of 29.4 nm^2. $^{TW}CCS_{N_2 \to He}$ value increased by 1.5% following treatment with λPP. Half-height width of the CCS distribution reduced from 2.2 nm^2 (28.4–30.6 nm^2) to 1.8 nm^2 (28.8–30.6 nm^2) with addition of λPP. Two overlapping conformations of PKA are indicated in red and green. (Reproduced from Byrne et al. 2016 with permission from the Biochemical Journal)

10. Using OriginPro9 (or similar), plot the CCS values calculated in **step 9** (*x*-axis) against the intensity values for each conformer (*y*-axis).

11. Normalize the inputted data by clicking on Analysis–Mathematics–Normalize Columns–Normalize to [0,1] and plot the best fit line. This can then be used to determine the CCS value for all similar analytes according to their measured drift times under the same conditions (Fig. 1).

### 3.5 Evaluating the Effect of Inhibitor Addition on Protein Conformation

1. Mix the protein solution (in ammonium acetate) with the required concentration of inhibitor, to achieve a molar ratio ~1:10 protein:inhibitor. Leave the mixture at room temperature for ~10 min to facilitate binding prior to IM-MS analysis.

2. Prepare a capillary and collect IM-MS data as described in Subheading 3.4.

3. Calculate the CCS value of the different charge states of the ligand-bound protein (Fig. 2) according to the procedure outlined in Subheading 3.5 (*see* **Note 15**).

3.6 Collision-Induced Unfolding

1. Determine the lowest charge state of suitable intensity for CIU analysis following IM-MS data acquisition of the protein as described in Subheading 3.4.

2. Isolate this single charge state in the quadrupole for further investigation.

3. Increase the CID trap energy gradually from ~20 to 41 V, acquiring IM-MS data (as detailed in Subheading 3.4) in two-volt intervals.

4. Repeat **steps 2–4** for each observed charge state.

3.7 CIU Data Analysis

1. Open the CIU data files in the MassLynx software using the 2: TOFMS (500:8000) ES+ function.

2. Extract the peaks of interest from Total Ion Chromatogram (TIC) to open the spectra.

3. Right click across the peak in the spectrum view to open another chromatogram window.

4. In the chromatogram window, click edit and copy the chromatogram list. Paste these values (drift times and the corresponding intensities) in to Microsoft Excel or similar program of choice.

5. Copy data for all the acquired voltage settings in to OriginPro9.

6. Normalize the inputted data by clicking on Analysis–Mathematics–Normalize Columns–Normalize to [0,1].

7. Copy all and add to matrix.

8. Create a CIU plot by going to Plot-Contour-Color (Fig. 3).
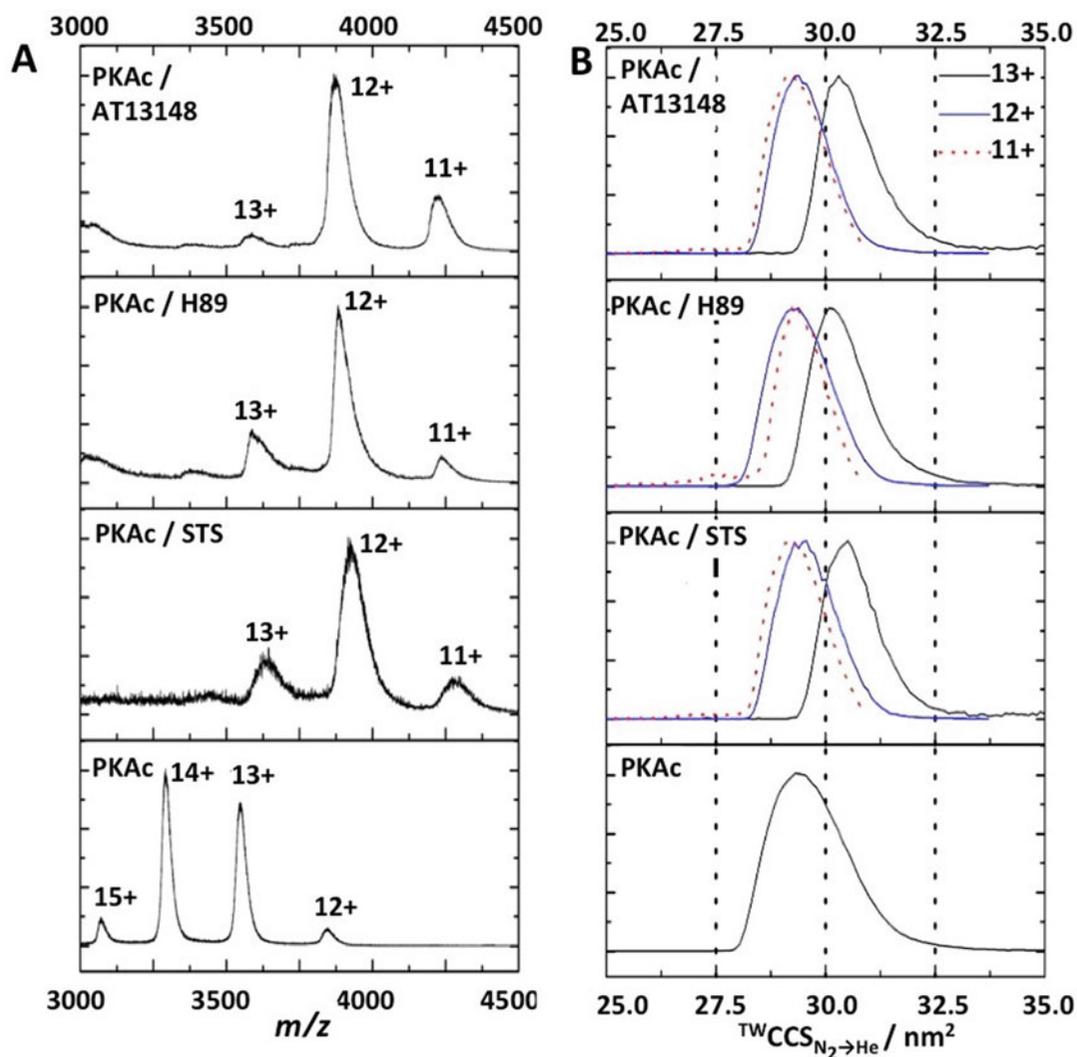
Fig. 2 Small-molecule inhibitor binding to PKAc. (**a**) Native ESI mass spectra. (**b**) $^{TW}CCS_{N_2 \rightarrow He}$ values in the presence of DMSO vehicle or with tenfold molar excess of staurosporine (STS), H89, or AT13148. CCS distributions are presented for $[M+11H]^{11+}$ (red dotted line), $[M+12H]^{12+}$ (blue line), and $[M+13H]^{13+}$ (black line). (Reproduced from Byrne et al. 2016 with permission from the Biochemical Journal)

4   Notes

1. The program in Table 1 should be used as a starting guide and adapted to achieve the optimal capillary shape. *See* manufacturer's instructions for the use of other puller models. The fine end of the capillary tip should be ~0.4 cm in length.

2. In order to determine CCS values, it is an absolute requirement that the drift time through the instrument be calibrated under the same conditions as used for the analyte protein (and ligand
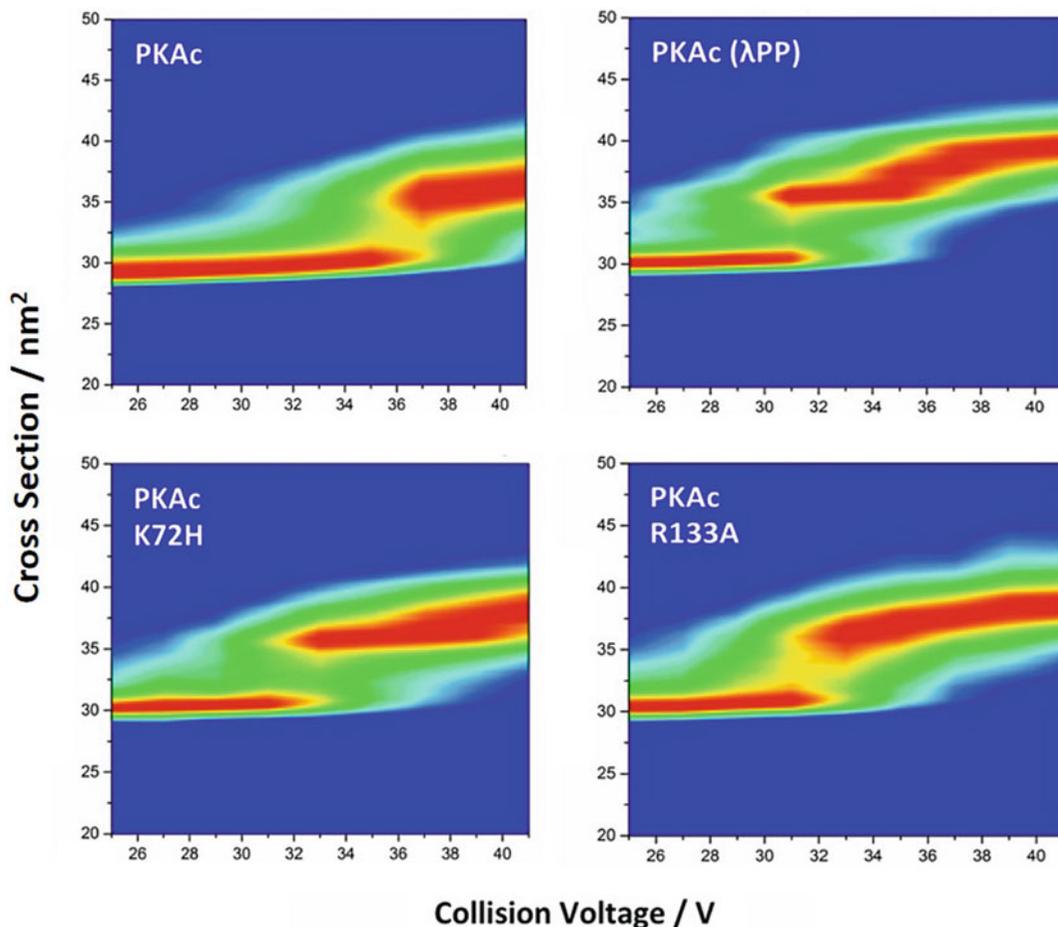
Fig. 3 Collision-induced unfolding of PKAc variants. CIU profiles of PKAc wild-type (WT), λPP-treated, K72H and R133A. PKAc is more stable and requires higher collision energy to begin unfolding at ~36 V. λPP-treated PKA starts to unfold at ~31 V. Both PKAc variants are less stable than PKAc WT and begin unfolding at ~32 V. (Reproduced from Byrne et al. 2016 with permission from the Biochemical Journal)

complex). It is ideal to use protein calibrants that are of a similar mass to the one under investigation. We typically use β-lactoglobulin A, avidin, transthyretin, concanavalin A, and serum albumin. It should be noted that determination of CCS values are not an absolute requirement to evaluate ligand-induced protein conformational changes, but they are required to undertake cross-comparison studies using different techniques, or for analyses undertaken on different instruments, or under different conditions.

3. The ToF should be calibrated to achieve an acceptable tolerance of 1 ppm or below. If the mass accuracy is greater than 1 ppm, calibration should be repeated.

4. While we recommend 50 mM ammonium acetate as a starting concentration, this should be evaluated for your protein of interest, with ranges of 25–250 mM ammonium acetate typically being employed.

5. Use spin filter columns with a molecular weight cutoff appropriate to the protein of interest.

6. The amount of protein subjected to buffer exchange will be dependent on the starting concentration and the relative stability of the protein during buffer exchange. The final concentration of protein required for analysis is ~1 mg/mL or greater; this should be taken into consideration when deciding on the amount of protein required for this step.

7. Amicon spin filters should be pre-equilibrated with the required concentration of ammonium acetate prior to use.

8. Ammonium acetate buffer should be kept cold on ice throughout the buffer exchange step.

9. We recommend using a NanoDrop for protein concentration determination as this requires very little material. If a Nano-Drop spectrophotometer is not available, determine protein concentration using a standard Bradford assay or similar.

10. Extreme care should be taken when cutting the capillary to ensure a clean cut, and that no glass enters the capillary which may cause the capillary to block. Both ends of the capillary should be checked under a microscope prior to use.

11. Ensure the platinum wire goes to the tip of the capillary without pushing through the tip.

12. The optimal position of the nESI capillary with respect to the instrument orifice is instrument dependent. Using the camera as a guide, the position of the capillary should be adjusted to ~1 cm away from the orifice such that signal is optimized.

13. To determine optimal spectra, adjust the wave height and velocity settings until the peak observed in the drift time spectra is at the center of the x-axis.

14. We typically acquire over a TOF MS range of 500–8000 m/z for ~10 min, using a scan time of 5 s.

15. Evaluation of the effects of ligand binding on protein conformation requires comparison of the CCS values for the same protein charge states ± ligand.

References

1. Göth M, Pagel K (2017) Ion mobility–mass spectrometry as a tool to investigate protein–ligand interactions. Anal Bioanal Chem 409:4305. https://doi.org/10.1007/s00216-017-0384-9

2. Eyers CE, Vonderach M, Ferries S, Jeacock K, Eyers PA (2018) Understanding protein–drug interactions using ion mobility–mass spectrometry. Curr Opin Chem Biol 42:167–176.

https://doi.org/10.1016/j.cbpa.2017.12.013

3. Eschweiler J, Kerr R, Rabuck-Gibbons J, Ruotolo BT (2017) Sizing up protein–ligand complexes: the rise of structural mass spectrometry approaches in the pharmaceutical sciences. Annu Rev Anal Chem 10:25–44. https://doi.org/10.1146/annurev-anchem-061516-045414

4. Ben-Nissan G, Sharon M (2017) The application of ion-mobility mass spectrometry for structure/function investigation of protein complexes. Curr Opin Chem Biol 42:25–33. https://doi.org/10.1016/j.cbpa.2017.10.026

5. Shuai N, Rabuck JN, Ruotolo BT (2013) Ion mobility-mass spectrometry of intact protein-ligand complexes for pharmaceutical drug discovery and development. Curr Opin Chem Biol 17(5):809–817. https://doi.org/10.1016/j.cbpa.2013.06.019

6. Nshanian M, Lantz C, Wongkongkathep P et al (2019) Native top-down mass spectrometry and ion mobility spectrometry of the interaction of tau protein with a molecular tweezer. J Am Soc Mass Spectrom 30(1):16–23. https://doi.org/10.1007/s13361-018-2027-6

7. Byrne DP, Vonderach M, Ferries S, Brownridge PJ, Eyers CE, Eyers PA (2016) cAMP-dependent protein kinase (PKA) complexes probed by complementary differential scanning Fluorimetry and ion-mobility mass spectrometry. Biochem J 473(19):3159–3175

8. Wongkongkathep P, Han JY, Choi TS, Yin S, Kim HI, Loo JA (2018) Native top-down mass spectrometry and ion mobility MS for characterizing the cobalt and manganese metal binding of α-synuclein protein. J Am Soc Mass Spectrom 29(9):1870–1880. https://doi.org/10.1007/s13361-018-2002-2

9. Yuwei T, Lippens J, Netirojjanakul C, Campuzano IDG, Ruotolo B (2018) Quantitative collision-induced unfolding differentiates model antibody–drug conjugates. Protein Sci 28(3):598–608. https://doi.org/10.1002/pro.3560

10. Rabuck-Gibbons JN, Lodge JM, Mapp AK, Ruotolo BT (2019) Collision-induced unfolding reveals unique fingerprints for remote protein interaction sites in the KIX regulation domain. J Am Soc Mass Spectrom 30(1):94–102. https://doi.org/10.1007/s13361-018-2043-6

11. Rabuck JN, Suk-Joon H, Ko KS, Fox CC, Soellner MB, Ruotolo BT (2013) Activation state-selective kinase inhibitor assay based on ion mobility-mass spectrometry. Anal Chem 85

(15):6995–7002. https://doi.org/10.1021/ac4012655

12. Saunders C, Young LM, Mahood RA, Jackson MP, Revill CH, Foster RJ, Smith AD, Ashcroft AE, Brockwell DJ, Radford SE (2016) An in vivo platform for identifying inhibitors of protein aggregation. Nat Chem Biol 12:94–101. https://doi.org/10.1038/nchembio.1988

13. Hozefa A, Bate C, Williams A, Virdee J, Jeggo R, Spanswick D, Scopes DIC, Treherne JM, Mazzitelli S, Chawner R, Eyers CE, Doig AJ (2012) The N-methylated peptide SEN304 powerfully inhibits Aβ(1–42) toxicity by perturbing oligomer formation. Biochemistry 51 (42):8338–8352. https://doi.org/10.1021/bi300415v

14. Vonderach M, Byrne DP, Barran PE, Eyers PA, Eyers CE (2018) DNA binding and phosphorylation regulate the core structure of the NF-κB p50 transcription factor. J Am Soc Mass Spectrom 30(1):128–138

15. Lixa C, Mujo A, de Magalhães MTQ, Almeida FCL, Lima LMTR, Pinheir AS (2018) Oligomeric transition and dynamics of RNA binding by the HuR RRM1 domain in solution. J Bimol NMR 72(3–4):179–192. https://doi.org/10.1007/s10858-018-0217-y

16. Wang Y, Park H, Lin H, Kitova EN, Klassen JS (2019) Multipronged ESI–MS approach for studying glycan-binding protein interactions with glycoproteins. Anal Chem 91 (3):2140–2147. https://doi.org/10.1021/acs.analchem.8b04673

17. Zhao Y, Yang JY, Thieker DF, Xu Y, Zong C, Boons GJ, Liu J, Woods RJ, Moremen KW, Amster IJ (2018) A Traveling Wave Ion Mobility Spectrometry (TWIMS) study of the Robo1-heparan sulfate interaction. J Am Soc Mass Spectrom. https://doi.org/10.1007/s13361-018-1903-4

18. Lanucara F, Holman SW, Gray CJ, Eyers CE (2014) The power of ion mobility-mass spectrometry for structural characterization and the study of conformational dynamics. Nat Chem 6(4):281–294. https://doi.org/10.1038/nchem.1889

19. Gabelica V, Shvartsburg AA, Afonso C, Barran P, Benesch JLP, Bleiholder C, Bowers MT, Bilbao A, Bush MF, Larry Campbell J, Campuzano IDG, Causon T, Clowers BH, Creaser CS, De Pauw E, Far J, Fernandez-Lima F, Fjeldsted JC, Giles K, Groessl M, Hogan CJ, Hann S, Kim HI, Kurulugama RT, May JC, McLean JA, Pagel K, Richardson K, Ridgeway ME, Rosu F, Sobott F, Thalassinos K, Valentine SJ, Wyttenbach T (2017) Recommendations for reporting ion mobility Mass

Spectrometry measurements. Mass Spectrom Rev 38(3):291–320

20. Khajehali E, Malone DT, Glass M, Sexton PM, Christopoulos A, Leach K (2015) Biased agonism and biased allosteric modulation at the CB1 cannabinoid receptor. Mol Pharmacol 88 (2):368–379. https://doi.org/10.1124/mol

21. Verkhivker GM (2017) Leveraging structural diversity and allosteric regulatory mechanisms of protein kinases in the discovery of small molecule inhibitors. Curr Med Chem 24 (42):4838–4872. https://doi.org/10.2174/0929867323666161006113418

22. Wilson LJ, Linley A, Hammond DE, Hood FE, Coulson JM, MacEwan DJ, Ross SJ, Slupsky JR, Smith PD, Eyers PA, Prior IA (2017) New perspectives, opportunities, and challenges in exploring the human protein kinome. Cancer Res 78(1):1–16. https://doi.org/10.1158/0008-5472.CAN-17-2291

23. Masterson LR, Mascioni A, Traaseth NJ, Taylor SS, Veglia G (2008) Allosteric cooperativity in protein kinase A. Proc Natl Acad Sci U S A 105:506–511

24. Bush M (2019) Collision cross section database. Bush Lab. https://depts.washington.edu/bushlab/ccsdatabase/