**Ion mobility-mass spectrometry to evaluate the effects of protein modification or small molecule binding on protein dynamics**

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**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 analysing 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 ionisation (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 three primary forms of ion mobility that are coupled with MS: drift-tube ion mobility spectrometry (DTIMS), travelling-wave ion mobility spec­trometry (TWIMS), and differential-mobility spectrometry (DMS) also known as field-asymmetric ion mobility spectrom­etry (FAIMS) [18]. DTIMS and TWIMS 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 [19]. 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 towards 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 [20, 21].

Here we present a method of utilising IM-MS to assess the structural effects of phosphorylation and ligand binding on cAMP-dependent protein kinase (PKA) [7, 22]. The described method is readily transferable to the investigation of phosphorylation and ligand-induced changed 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. Pre-chill 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; hyperphosphorylated); 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
1. API Calibration (NaCsl) Solution Kit (Waters)
2. TW120-4 Thin-Wall Capillary (4 x 1.2 mm), 10G
3. Capillary puller: Sutter P-1000 Puller (*see* **Note 1**)
4. Ceramic capillary cutter
5. Platinum (Pt) wire (0.125 mm x 5 m), cut to 4 cm lengths.
6. 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 set up***

1. Prepare the capillaries for nano electrospray ionisation (nESI) using the capillary puller and the programme 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* **Note 7**). Centrifuge at 14,000 *g* for 10 min, in a pre-cooled 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 minutes at 14,000 *g*, 4°C.
3. Determine the concentration of the final protein solution in ammonium acetate using 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) controls (*see* **Note 13**).
9. Once a signal is established, and TriWave settings optimised, ensure the ‘Add Drift Time’ function button is ticked and acquire data over the required *m/z* range (*see* **Note 14**).See Figure 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).

***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 programme. Acquire IM-MS data as described in section 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 [23] to determine the Native Like Ion value for CCS (He)/nm2 of the chosen CCS calibrants.
7. Calculate the reduced cross section Ω' of the protein calibrants using equation [1]:

*Equation 1*:

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

1. Plot (Ω') versus (tD’) and fit a straight line using equation [2] to convert the drift time scale to a CCS (He) scale:

*Equation 2*:

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

1. Plot the converted nonlinear scale to against the corresponding intensity values.
2. Using OriginPro9 (or similar), plot the CCS values calculated in step 9 (x-axis) against the intensity values for each conformer (y-axis).
3. 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 ~10:1. 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 section 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 step 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 section 3.4.
2. Isolate this single charge state in the quadrupole for further investigation.
3. Increase the CID trap energy gradually from ~ 20 V to 41 V, acquiring IM-MS data (as detailed in section 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-Colour (Fig. 3).

**4 Notes**

1. The programme in Table 1 should be used as a starting guide and adapted to achieve the optimal capillary shape. See manufacturer’s instructions for 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 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 recommended 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 the molecular weight cut off 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 NanoDrop for protein concentration determination as this requires very little material. If a NanoDrop spectrophotometer is not available, determine protein centration 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 could 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 optimised.
13. To determine optimal spectra, adjust the wave height and velocity settings until the peak observed in the drift time spectra is at the centre of the x axis.
14. We typically acquire over a TOF MS Range of 500 – 8000 *m/z* for ~10 min, suing 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. Ion mobility–mass spectrometry as a tool to investigate protein–ligand interactions. Anal Bioanal Chem (2017) 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. Current Opinion in Chemical Biology. 42: 167-176. doi: 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. doi: 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. Current opinion in chemical biology. 42: 25-33. doi: 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. Current Opinion in Chemical Biology. 17 (5): 809-817. doi: 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. Biochemical Journal. 473 (19): 3159-3175.
8. Wongkongkathep, P., Han, JY., Choi, TS., Yin, S., Kim, HI., Loo, JA.. (2018). J Am Soc Mass. 29(9): 1870-1880. doi: 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 Science. 28 (3): 598-608. doi: 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. doi: 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. doi: 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. Nature Chemical Biology. 12: 94–101. doi: 10.1038/nchembio.1988.
13. Hozefa, A., Bate, C., Williams, A., Virdee, J., Jeggo, R., Spanswick, D.,Scopes, D.I.C., 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. doi: 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. Journal of The American Society for Mass Spectrometry. 1-11.
15. Lixa, C., Mujo, A., Q. de Magalhães, MT., Almeida, FCL., Lima, LMTR., Pinheir, AS. (2018). Oligomeric transition and dynamics of RNA binding by the HuR RRM1 domain in solution. Journal of Bimolecular NMR. 72 (3,4): 179-192. doi: 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. doi: 10.1021/acs.analchem.8b04673.
17. Zhao, Y., Yang, JY., Thieker, DF., Xu, Y., Zong, C., Boons, GJ., Liu, J., Woods, RJ., Moremen, KW., Amste, IJ. doi: 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. Nature Chemistry. 6 (4): 281-294. doi: 10.1038/nchem.1889.
19. Khajehali, E., Malone, DT., Glass, M., Sexton, PM., Christopoulos, A., Leach, K. (2015). Biased Agonism and Biased Allosteric Modulation at the CB1 Cannabinoid Receptor. Molecular Pharmacology. 1-59. doi: https://doi.org/10.1124/mol.
20. 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. doi: 10.2174/0929867323666161006113418.
21. 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 Research. 78(1): 1.-16. doi: 10.1158/0008-5472.CAN-17-2291.
22. Masterson, L.R., Mascioni, A., Traaseth, N.J., Taylor, S.S., Veglia, G. (2008). Allosteric cooperativity in protein kinase A. Proc. Natl Acad. Sci. USA. 105: 506–511.
23. Bush, M. (2019). Collision Cross Section Database. Bush Lab. <https://depts.washington.edu/bushlab/ccsdatabase/>.

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

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| ***Line*** | ***Heat*** | ***Pull*** | ***Vel.*** | ***Delay*** | ***Pressure*** | ***Ramp*** |
| 1 x 1 | 489 | 0 | 20 | 120 | 235 | 491 |
| 2 x 1  | 489 | 0 | 18 | 120 | Delay mode XXSafe Heat Jaw Temp 19 °C  |
| 3 x 1  | 489 | 0 | 18 | 120 |
| 4 x 1 |  |  |  |  |

**Figure Legends**

**Figure 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 - TWCCSN2→He for the [M+13H]13+ form of untreated WT PKAc (PKA), PKAc following treatment with Mn2+-λ protein phosphatase (PKA λPP), and the K72H and R133A variants. The hyperphosphorylated PKAc had a TWCCSN2→He value of 29.4 nm2. TWCCSN2→He value increased by 1.5% following treatment with λPP. Half-height width of the CCS distribution reduced from 2.2 nm2 (28.4–30.6 nm2) to 1.8 nm2 (28.8–30.6 nm2) 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.

**Figure 2: Small-molecule inhibitor binding to PKAc.** A - Native ESI mass spectra. B - TWCCSN2→He values in the presence of DMSO vehicle or with 10-fold 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.

**Figure 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.