

Localization of Modified Sites on Peptides and Proteins using High-resolution Differential Ion Mobility Spectrometry

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Overview

- Localization variants of phosphopeptides fully separated by differential ion mobility spectrometry (IMS) or field asymmetric waveform IMS (FAIMS) using planar-gap analyzers and He/N₂ buffer gas
- Variants “co-eluting” in one charge state may be resolved in another, providing multiple separation opportunities. On average, better resolution opportunities for higher charge states
- Baseline separation achieved regardless of the modified residue (S, T, or Y) or number of phosphorylations (1, 2, or 3)
- Resolution uncorrelated with the distance of PTM shift along the backbone, isomers with phospho groups on neighboring sites readily separated

Introduction

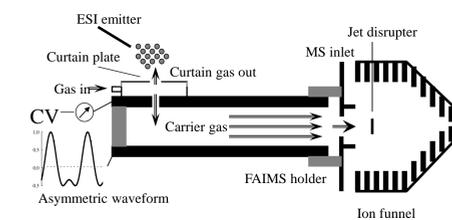
- As analyses of the primary sequences become routine, proteomics is shifting to characterization of post-translational modifications (PTM). Perhaps the most common and important one is phosphorylation, which in eukaryotes occurs at the **Ser (S)**, **Thr (T)**, or **Tyr (Y)** residues
- Most proteins feature multiple sites attaching a given PTM, but hardly any site is always occupied. This creates multiple localization variants, many of which coexist *in vivo* and have differing biological activity
- Identification of variants by collision-induced dissociation (CID) is challenged by the dominant PTM abstraction that yields uninformative fragments and PTM shifts in the excited peptide prior to the dissociation step. Electron capture or transfer dissociation (EC/TD) avoids those problems, but has low sensitivity because of inefficiency and indiscriminate bond cleavage
- Number of unique fragments equals the distance between alternative modified sites along the backbone, and peptides with adjacent sites differ by a single fragment. Hence, variants with nearby sites are hard to distinguish, PTM often assigned to a region instead of a residue
- Peptides featuring three or more attachment sites have variants non-distinguishable by MS/MS (either CID or EC/TD) in principle [1]
- Some variants may be separated by LC, especially the HILIC technique. However, LC is slow and many variants are unresolved or do not elute, especially for multiply modified peptides [2]

Methods

IMS/MS was used to separate and characterize peptide and protein isomers since 1990-s, but until recently not the localization variants of modified peptides. We had shown [3, 4] baseline resolution of all variants of singly or doubly phosphorylated model peptide with three **S** (APLS¹FRGS²LPKS³YVK) using FAIMS

Here, we study peptides from human proteins (tau and Sprouty2) with biomedically important variant phosphorylation, including isoforms with alternative attachment to adjacent sites, **T** and **Y** modification, and triple phosphorylation

We employed a planar FAIMS unit with 1.9 mm gap, coupled to the LTQ ion trap MS (Thermo Fisher) using an ion funnel interface [5]



Scheme of FAIMS unit with MS front end

The residence time in the gap t varied by setting the gas flow rate, here 2 L/min resulting in $t = 0.2$ s

The highest peak voltage (dispersion voltage, DV) was 5.4 kV. For best resolving power R , we used He/N₂ gas with maximum He fraction under the breakdown threshold: 50% for DV = 5.4 kV and 70% for DV = 4 kV. These regimes provide $R \sim 200$ for 3+ and 4+ peptides [6, 7]

Selected tau peptides were synthesized on solid phase and purified to homogeneity by RPLC and CZE, Sprouty2 peptides were obtained from Alta Bioscience (Birmingham, UK). To test the dependence on the solution conditions, some analyses were repeated using infusion solvents with pH = 3 (50/49/1 water/methanol/acetic acid) and pH = 2 (48/48/4 water/methanol/formic acid). Solutions were delivered to the ESI emitter at 0.1 - 0.5 μ L/min, lower values for lower pH

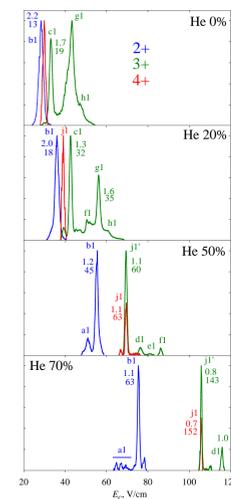
Ions of charge states 2, 3, and 4 were investigated

Tau peptides

Tau is a brain protein, its longest splicing form (441 residues) has four regions with variant phosphorylation relevant to Alzheimer's disease [2]. We studied the 226 - 240 sequence VAVVR^{T231}PPK^{S235}PS²³⁷S²³⁸AK that binds Pin1 (a prolyl isomerase enzyme) and microtubules, affecting neuronal function. There are one **T** and three **S**, including two adjacent **S**

Monophosphorylated variants: pT²³¹ (1), pS²³⁵ (2), pS²³⁷ (3), pS²³⁸ (4); 1602.9 Da

Spectra for **1** show usual increase of compensation field (E_C) and resolving power (R) at higher He fractions; peak widths (fwhm) are marked with R underneath

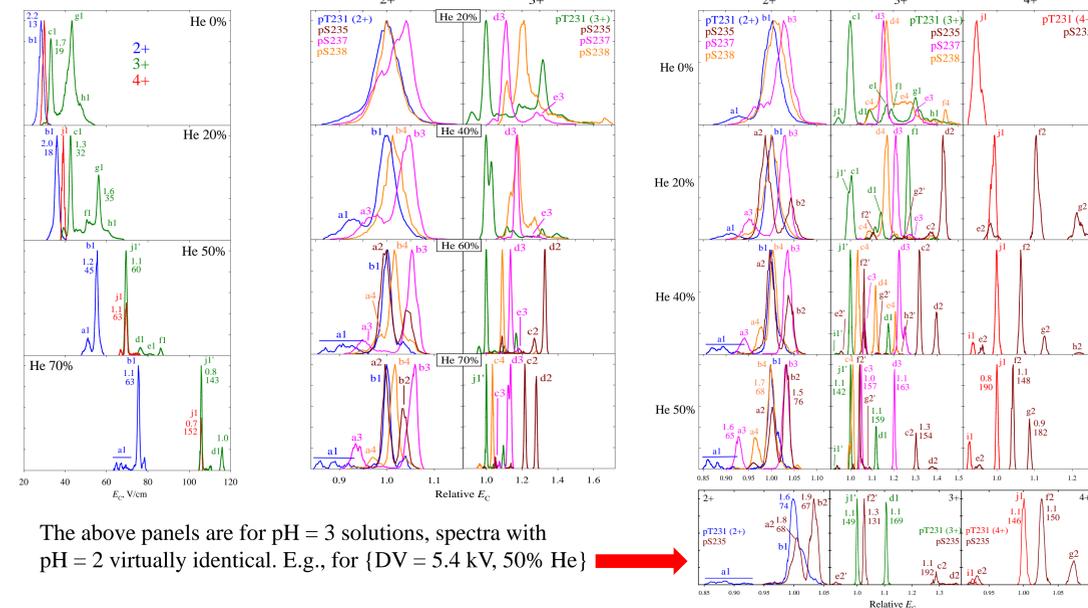


DV = 4 kV

Resolution improves at higher He fractions, all four variants fully resolved at 70% He for 3+ ions

DV = 5.4 kV

Behavior resembles that at DV = 4 kV with He fractions offset by $\sim 30\%$ up, but differences for some variants provide separation flexibility



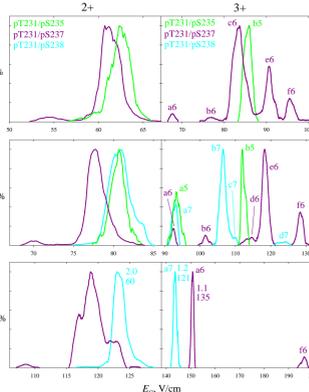
The above panels are for pH = 3 solutions, spectra with pH = 2 virtually identical. E.g., for {DV = 5.4 kV, 50% He}

Bisphosphorylated variants: pT²³¹pS²³⁵ (5), pT²³¹pS²³⁷ (6), pT²³¹pS²³⁸ (7); 1682.8 Da

DV = 5.4 kV

Signal for (5) vanishes above 20% He, perhaps due to peptide unfolding upon stronger field heating at higher He fractions

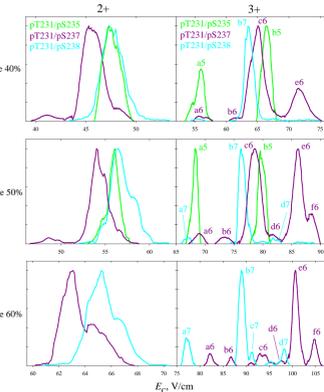
All variants, including **6** and **7** with adjacent alternative sites, resolved at 20% He for 3+ ions



DV = 4 kV

Signal for (5) now vanishes above 50% He, supporting the notion of heat-induced unfolding

All variants resolved at 50 - 60% He, for 3+ ions



Results

Sprouty peptides

Sprouty2, a regulator of the receptor tyrosine kinase signaling, has at least 15 phosphorylated sites: 11 **S**, three **T**, and one **Y** [8].

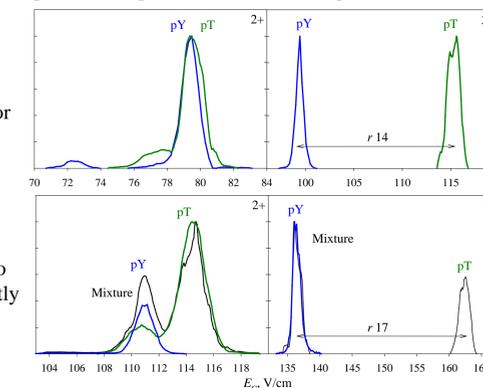
This is in line with the relative abundances in human proteome: 84% **S**, 14% **T**, and 2% **Y** [3]. Separation of previously unexplored modification patterns were evaluated using two tryptic sequences

51 - 64 sequence: NTNEY⁵⁵T⁵⁶EGPTVVPR (1655.7 Da)

Allows evaluating FAIMS separation for variants involving rare **Y** phosphorylation, in particular alternative modification of adjacent **Y** and **T**. As expected, separation improves with increasing He fraction

DV = 4 kV, 70% He

Variants separated for 3+, but not 2+ ions



DV = 5.4 kV, 40% He

Some separation also for 2+ ions, apparently due to thermal unfolding

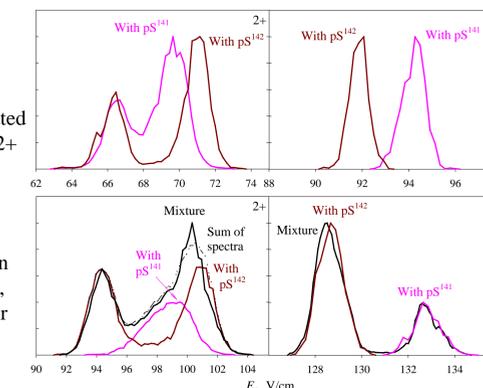
Resolution of 17: the highest seen in FAIMS for localization variants

135 - 151 sequence: LLGS¹³⁸S¹³⁹FS¹⁴¹S¹⁴²GPVADGIIR (1754.9 Da)

With four **S** (including two pairs of adjacent **S**), permits variant triple modification. We looked at pS¹³⁸pS¹³⁹pS¹⁴¹ and pS¹³⁸pS¹³⁹pS¹⁴²

DV = 4 kV, 70% He

Variants fully separated for 3+ ions, but not 2+



DV = 5.4 kV, 40% He

Slight resolution gain in both charge states, validation by data for the mixture plotted

Sprouty2 peptides show excellent separation of variants with adjacent alternatively modified sites or triple phosphorylation

Conclusions

- High-resolution FAIMS is broadly able to separate localization variants of phosphopeptides, including those with one, two, or three phospho groups on Ser, Thr, or Tyr
- Separation uncorrelated with the distance of PTM shift along the backbone, variants with adjacent alternative attachment sites (that challenge MS/MS approaches most) readily resolved
- Effective variant separation is due to substantial orthogonality between FAIMS and MS dimensions: CVs for isomeric peptides differ by up to 1.5 times (vs. $\sim 10\%$ for mobility in conventional IMS). Achieved resolution up to 17 for variant pairs and peak capacity up to ~ 60 in one charge state
- Variant separations in different charge states mutually independent; the total peak capacity exceeds 100. Multiplicity of charge states must be considered when comparing peak capacity with separations in solution
- Absolute CVs accurate enough to identify variants based on “CV tags”, without MS/MS. Spectra essentially unchanged at least over some range of solution pH, which should enable identifications over an LC mobile phase gradient

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