**Chapter 6**

**Determination of phosphohistidine stoichiometry in histidine kinases by intact mass spectrometry**

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*Running head: MS-based analysis of pHis stoichiometry*

**Abstract**

Protein histidine phosphorylation has largely remained unexplored due to the challenges of analysing relatively unstable phosphohistidine-containing proteins. We describe a procedure for determining the stoichiometry of histidine phosphorylation on the human histidine kinases NME1 and NME2 by intact mass spectrometry under conditions that retain this acid-labile protein modification. By characterising these two model histidine protein kinases in the absence and presence of a suitable phosphate donor, the stoichiometry of histidine phosphorylation can be determined. The described method can be readily adapted for the analysis of other proteins containing phosphohistidine.

**Key words:** NME1, NME2, histidine phosphorylation, intact mass spectrometry

1. **Introduction**

Protein phosphorylation is an important post-translation modification (PTM) found in eubacteria, archaebacteria, prokaryotes and eukaryotes, typically mediated by target-specific protein kinases [1]. Phosphorylation plays vital roles in signal transduction, mediating the cellular response to external stimuli, and in the regulation of diverse cellular processes including transcription, metabolism, cell cycle progression, apoptosis and differentiation [2, 3].

Histidine (His) phosphorylation has been well studied in prokaryotes, in particular in bacteria due to their crucial involvement in two-component signalling systems (TCS), which comprise a receptor His kinase and an effector protein. Two-component systems were first reported in bacteria in 1980 and their widespread relevance in bacterial signalling systems is now accepted. They are thought to be critically important in the response of cells to environmental factors (sensory responses). However, TCS are not limited to prokaryotes, and can be found in major eukaryotic kingdoms, including plants and fungi [4, 5]. More recently, phosphohistidine (pHis) has been recognised to have roles in vertebrate signalling [6 - 8], and there is growing evidence that this acid-labile PTM may function in a similar cell signalling capacity as described for the much-better studied phosphorylation of serine, threonine and tyrosine. However, pHis analysis is challenging, due to the high free energy of hydrolysis (~12 kcal/mol) of the phosphoramidate (N-P) bond [9], meaning that hydrolysis of the phosphate group occurs rapidly at high temperatures and at low pH [10], conditions often employed for biochemical analysis. The two isomers of phosphohistidine (1-pHis, or 3-pHis) arising due to phosphate group addition to the nitrogen at either the 1 or 3 positions of the histidine imidazole ring, also have a different ΔG° of hydrolysis, with 1-pHis believed to be more labile [11].

NME1 and NME2 are known mammalian histidine kinases and belong to the family of nucleoside diphosphate kinases (NDPKs), a family of proteins encoded by the *nme* (non-metastatic cells) genes. NDPKs are responsible for catalysing the transfer of *γ*-phosphate from nucleoside triphosphate to nucleoside diphosphate. This mechanism of catalytic transfer is associated with the generation of a ‘high-energy’ phosphohistidine intermediate [12, 13].

Mass spectrometry (MS)-based proteomic methods represent a leading technique for protein identification and characterisation of both the sites and stoichiometry of phosphorylation [14-18]. Analysis of intact proteins by MS permits the mass of the phosphorylated protein to be defined, and consequently the average number of phosphorylation sites per molecule to be identified, enhancing peptide-driven analyses (which are discussed in Chapter 15). In general, MS-based analysis is a label-free analytical strategy that overcomes any requirement for radioactive (32/33P) labelling (as presented in Chapters 3 and 4) or for PTM-specific antibodies (as in Chapter 12), and is thus a simple method for evaluating His protein kinase activity.

We present a straightforward MS method to identify phosphorylation-induced mass shifts in two model histidine phosphorylated proteins that can be used to determine relative phosphorylation stoichiometry. To do this, we employ His autophosphorylation of the putative protein histidine kinases NME1 and NME2 as exemplars. This method can be readily adapted to evaluate autophosphorylation of other histidine kinases, or enzyme-mediated histidine phosphorylation of substrate proteins, and for the analysis of proteins containing other unstable phosphoamino acids [19-21].

**2 Materials**

Prepare all solutions and buffers using HPLC grade water and acetonitrile where applicable. Store all reagents at room temperature. Nucleotides can be stored frozen (-20°C) prior to use. Protein solutions should be maintained on ice unless otherwise stated. For longer term storage, proteins should be kept at -20 °C in a glycerol-containing buffer of suitable pH.

***2.1 Phosphorylation Assay***

1. Histidine kinases: NME1 and NME2. Enzymes should be ~0.1 – 0.3 mg/mL in 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM DTT, 10% (v/v) glycerol (*see* **Notes 1 and 2**).
2. Low bind micro-centrifuge tubes.
3. HPLC water.
4. 1 M Tris-HCl (pH 8.0): Weigh out 121 g Tris Base. Add to a graduated cylinder with ~800 mL of water. Mix and adjust to a pH of 8.0 with HCl (*see* **Note 3**). Make up to 1 L with water.
5. 1 M NaCl: Weigh out 58 g of NaCl. Add to a graduated cylinder and make up to 1 L with water.
6. Nucleotides (10 mM): Make up a stock of adenosine triphosphate (ATP) in water and adjusted to pH 8.0 with NaOH (*see* **Note 4**).
7. Assay buffer: 50 mM Tris-HCl (pH 8.0), 100 mM NaCl. Add 500 μL of 1 M NaCl and 1 mL of 1 M Tris-HCl (pH 8.0) to 8.5 mL of water. Check that the pH is still at 8.0 (*see* **Note 5**).
8. Assay buffer with nucleotides: 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 μM nucleotide. Add 500 μL of 1 M NaCl, 1 mL of 1 M Tris-HCl (pH 8.0) and 10 μL of the appropriate phosphate donor (*see* **Note 4**) to a low-bind micro-centrifuge tube. Make up to 10 mL with water. Check that the pH is still at 8.0 (*see* **Note 5**).

***2.2 Liquid chromatography-Mass spectrometry (LC-MS)***

1. HPLC Water
2. Buffer A: 0.1% (v/v) formic acid in HPLC grade water: Add 1 mL of formic acid to 1 L HPLC-grade water in a clean glass bottle.
3. Buffer B: 0.1% (v/v) formic acid in HPLC grade acetonitrile. Add 1 mL of formic acid to 1 L acetonitrile in a clean glass bottle.
4. TFA (0.1 % v/v): Add 0.5 mL of TFA to 500 mL of HPLC grade water in a clean glass Duran® bottle.
5. (Glu1)-Fibrinopeptide B calibrant (GluFib): 100 fmol/µL in 0.1% formic acid.
6. Myoglobin: 500 fmol/μL in buffer A.
7. MS sample vials (*see* **Note 6**).
8. LC-MS instrument: We use a Waters Synapt G2-S*i* in-line with a Nano Acquity UPLC system (*see* **Note 7**).
9. MassPREP Micro Desalting Column(Waters).
10. MassLynx data analysis software (*see* **Note 8**).

**3 Methods**

* 1. ***MS calibration***
1. To calibrate the time-of-flight (ToF) mass analyser, infuse the Lockspray Flow Control with GluFib. Start at 20 μL/min and decrease to 10 μL/min once a strong signal is observed at *m/z* 785.8.
2. Calibrate the ToF using the Intellistart program, acquiring data for GluFib over a *m/z* range of 200 – 4000, in resolution mode.
3. Repeat if necessary to ensure a mass accuracy of 1 ppm or below (*see* **Note 9**).
	1. ***LC-MS set up***
4. Wash the UPLC column with 50% buffer A/50% buffer B for 1 minute at a flow rate of 8 mL/min.
5. Programme the LC-MS gradient as shown in table 1.
6. Define the MS settings as listed in table 2.
7. To evaluate instrument configuration, inject 0.5 μL of 500 fmol/μL of myoglobin into the sample loop and acquire mass spectra over the period of reverse-phase chromatographic separation (*see* **Note 10**).

***3.3 Autophosphorylation assay***

1. Label two low-bind tubes for each enzyme being analysed, one for the assay, and the other for the negative control.
2. To each tube add 200 ng of recombinant enzyme (*see* **Note 11)**. To the assay tube, make up to 20 µL with assay buffer containing the appropriate nucleotide/phosphate donor. To the negative control tube, make up to 20 µL with assay buffer (no phosphate donor) (*see* **Notes 12 and 13**).
3. Incubate all vials at room temperature for 5 min (or longer as required) and place back on ice (*see* **Note 14**).

***3.4 Intact mass spectrometry analysis***

1. Immediately after the *in vitro* protein kinase assay, transfer each reaction mixture to a clean MS sample vial.
2. Place the vials in the auto sampler (*see* **Note 15**) and analyse the samples using the LC-MS parameters as defined in section 3.2.

***3.4 Data analysis***

Data analysis should be performed using instrument-specific software. In our case, using the Synapt G2-*Si* (Waters), all data analysis was done using MassLynx software.

1. Extract the chromatographic peak of interest from the Total Ion Chromatogram (TIC)
2. Combine the mass spectra for the protein over the chromatographic elution window and view the averaged mass spectrum.
3. Process the MS data using the MaxEnt 1 function to generate a deconvoluted (zero charge state) mass spectrum. Adjust the mass range according to the size of the protein being analysed.
4. Amend the MaxEnt 1 deconvolution settings according to the experimental parameters. We typically use: 0.5 Da/channel resolution; Uniform Gaussian distribution with a width at half height of 0.500 Da; Minimum intensity ratios for left and right at 33% (*see* **Note 16**).
5. The deconvoluted spectrum will open in a new window. Compare the generated MaxEnt 1 mock data with the originator mass spectrum to evaluate how well the deconvolution has worked (*see* **Note 17**).
6. Compare the MS data from the control reaction (with no phosphate donor) with the phosphate donor-containing kinase assay reaction mixture. Examples of the MS data generated for NME1 and NME2 following *in vitro* autophosphorylation in the absence and presence of ATP are presented in Figures 1 and 2.
7. Calculate the stoichiometry of histidine phosphorylation by comparing the relative heights of the phosphorylated and non-phosphorylated protein in the reaction mixture containing the phosphate donor.
8. To validate that the change in mass is due to heat-labile histidine phosphorylation, heat the samples at 80 °C for 10 min and repeat the LC-MS analysis as outlined in section 3.4.
9. **Notes**
10. Histidine kinases can be made by recombinant protein expression and purification (*see* Chapters 2 and 5), or purchased.
11. A buffer suitable to maintain the activity and stability of the protein should be used. We find that these proteins are generally stable in the buffer used for the final purification step (*see* Chapter 5): 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM DTT, 10% (v/v) glycerol.
12. Make sure that the Tris-base is dissolved completely in the water prior to adjusting the pH. 1 M HCl can be used initially, although a lower concentration of HCl should be used for final pH adjustment.
13. Phosphate donor type and concentration may need to be adapted according to the specific requirements of the recombinant enzyme. For example, PGAM uses DPG as a phosphate donor instead of ATP.
14. The pH can be checked using indicator paper. If the pH requires minor adjustment at this point, use dilute concentrations of HCl or NaOH as appropriate.
15. We use TruView™ LCMS sample vials which minimise sample loss.
16. Any LC-MS instrumentation can be used that is capable of mass resolution of 20,000 or greater.
17. Data analysis software used will be dependent on the make of LC-MS instrumentation.
18. If the mass accuracy is greater than 1 ppm during ToF calibration, calibration should be repeated.
19. To minimise the time the sample is kept on ice after *in vitro* phosphorylation, the mass spectrometer should be calibrated and the LC-MS system set up and evaluated prior to the start of the enzyme assay.
20. Ensure the protein is kept on ice prior to starting the assay.
21. Some proteins may have residual phosphorylation prior to the start of the assay. It is essential to evaluate a control sample of the enzyme in the absence of a phosphate donor to determine the change in phosphorylation stoichiometry arising as a result of the assay.
22. This assay configuration assumes a single time point for analysis. If undertaking a time-course is of interest, increase the relative volume of the assay and components, and remove 20 µL to a clean Eppendorf tube kept on ice.
23. The intact protein MS analysis should be performed as soon as possible after the end of the *in vitro* assay.
24. Set the autosampler to remain at 4°C throughout the experiment.
25. It is often useful to generate a course-grained survey spectrum using a resolution of 10-25 Da/channel to define the major protein components in the mixture and allow the mass range to be suitably adjusted.
26. The channel resolution settings can be further adjusted if required to between ~0.25 - 1 Da to gain a zero charge mass spectrum of high signal to noise.

**References**

1. Loomis, W. F., Shaulsky, G. & Wang, N. (1997). Histidine kinases in signal transduction pathways of eukaryotes. *J. Cell Sci.* 110**:** 1141–1145.
2. Manning, G., Whyte, D. B., Martinez, R., Hunter, T. & Sudarsanam, S. (2002). The Protein Kinase Complement of the Human Genome. *Science.* 298 (5600): 1912.
3. Cohen, P. (2002). The origins of protein phosphorylation. *Nature cell biology*. 4(5):. 127-30.

# Adam, K. & Hunter, T. (2018). Histidine kinases and the missing phosphoproteome from prokaryotes to eukaryotes. *Pathology in Focus.* 98: 233-247.

# Kee, J.M. and T.W. Muir. (2012). Chasing phosphohistidine, an elusive sibling in the phosphoamino acid family. *ACS chemical biology*. 7 (1): 44-51.

# Klumpp, S. & Krieglstein, J. (2009). Reversible Phosphorylation of Histidine Residues in Proteins from Vertebrates. *Science Signalling.* 2 (61): 1-4.

1. Fuhs, S.R., et al. (2015). Monoclonal 1- and 3-Phosphohistidine Antibodies: New Tools to Study Histidine Phosphorylation*.* *Cell*. 162(1): 198-210.
2. Srivastava, S., et al. (2016). Histidine phosphorylation relieves copper inhibition in the mammalian potassium channel KCa3.1. *eLife*. 5.
3. Puttick, J., Baker, E., & Delbaere, L. (2008). Histidine phosphorylation in biological systems. [*Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics*](https://www.sciencedirect.com/science/journal/15709639)*.* 1784 (1): 100-105.
4. Attwood, P. V. (2013). P/N bond protein phosphatases. *Biochimica et Biophysica Acta*. 1834: 470-478.
5. Attwood, P. V. & Muimo, R. (2018). The actions of NME1/NDPK-A and NME2/NDPK-B as protein kinases. *Laboratory Investigation*. 98: 283-290.
6. Attwood, P.V. and Wieland, T. (2015). Nucleoside diphosphate kinase as protein histidine kinase. *Naunyn-Schmiedeberg's archives of pharmacology*. 388(2): 153-60.
7. Wu, Z., Tiambeng, T. N., CaI, W., Chen, B., Lin, Z., Zachery R. Gregorich, Z. R. & Ge, Y. (2018). Impact of Phosphorylation on the Mass Spectrometry Quantification of Intact Phosphoproteins. *Anal Chem*. 90 (8): 4935-4939.
8. Haydon, C.E., Eyers, P. A., Aveline-Wolf, L.D., Resing, K.A., Maller, J.L. (2003). Identification of novel phosphorylation sites on Xenopus laevis Aurora A and analysis of phosphopeptide enrichment by immobilized metal-affinity chromatography. *Mol Cell Proteomics*. 2 (10): 1055-67.
9. Schweppe, R.E., Haydon, C.E., Lewis, T.S., Resing, K.A., Ahn, N.G. (2003).The characterization of protein post-translational modifications by mass spectrometry. *Acc Chem Res.* 36 (6): 453-6.

# Johnson, H., Eyers, C.E, Eyers, P.A, Beynon, R.J., Gaskell, S.J. (2009). Rigorous determination of the stoichiometry of protein phosphorylation using mass spectrometry. *J Am Soc Mass Spectrom*. 20 (12): 2211-20.

# Byrne, D.P., Vonderach, M., Ferries, S., Brownridge, P.J., Eyers, C.E., Eyers, P.A. (2016). cAMP-dependent protein kinase (PKA) complexes probed by complementary differential scanning fluorimetry and ion mobility-mass spectrometry. *Biochem J.* 473 (19): 3159-75.

# Ferries, S., Perkins, S., Brownridge, P.J., Campbell, A., Eyers, P.A., Jones, A.R., Eyers, C.E. (2017). Evaluation of Parameters for Confident Phosphorylation Site Localization Using an Orbitrap Fusion Tribrid Mass Spectrometer. *J Proteome Res*. 16 (9): 3448-3459.

# Attwood PV, Besant PG, Piggott MJ. (2011). Focus on phosphoaspartate and phosphoglutamate. *Amino Acids.* 40(4):1035-51.

# Besant PG, Attwood PV, Piggott MJ. (2009). Focus on phosphoarginine and phospholysine. *Curr Protein Pept Sci.*10(6):536-50.

# Hardman G, Perkins S, Ruan Z, Kannan N, Brownridge P, Byrne DP, Eyers PA, Jones AR, Eyers CE. (2017) Extensive non-canonical phosphorylation in human cells revealed using strong-anion exchange-mediated phosphoproteomics. bioRxiv 202820; doi: https://doi.org/10.1101/202820

**Table 1. LC/MS gradient**. Details of the LC-MS gradient used for intact protein separation by C4 reverse-phase chromatography. Flow rate and buffer composition (% A and % B) are listed.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Time (min)** | **Flow (μL/min)** | **Buffer A (%)** | **Buffer B (%)** | **Curve** |
| Initial | 25 | 95 | 5 | 6 |
| 0.10 | 40 | 95 | 5 | 6 |
| 5.10 | 40 | 95 | 5 | 6 |
| 5.20 | 25 | 95 | 5 | 6 |
| 6.00 | 25 | 95 | 5 | 6 |
| 6.10 | 25 | 95 | 5 | 6 |
| 7.60 | 25 | 10 | 90 | 6 |
| 7.90 | 25 | 95 | 5 | 6 |
| 8.60 | 25 | 10 | 90 | 6 |
| 8.90 | 25 | 95 | 5 | 6 |
| 9.60 | 25 | 10 | 90 | 6 |
| 11.60 | 25 | 10 | 90 | 6 |
| 11.70 | 25 | 95 | 5 | 6 |

**Table 2. MS instrument settings.** Acquisition parameters for intact protein analysis using the Synapt G2-S*i* (Waters).

|  |  |  |
| --- | --- | --- |
| Source | Capillary (kV) | 3.0 |
| Sampling Cone | 40 |
| Source Offset | 80 |
| Temperature (°C) | Source | 100 |
| Desolvation | 250 |
| Gas Flows  | Cone Gas (L/h) | 50 |
| Desolvation Gas (L/h) | 300 |
| Nebuliser (Bar) | 6.0 |

**Figure legends**

**Figure 1. Intact mass analysis of NME1 in the absence and presence of ATP.** *In vitro* kinase assays was carried out for 5 minutes at room temperature with 200 ng recombinant protein and 10 µM ATP. The reaction mixtures were then analysed by LC-MS using a Waters G2-S*i* Synapt. MS data were deconvoluted using MaxEnt 1 within MassLynx. **A)** *Left* Combined mass spectra for NME1 (no ATP) over the period of LC elution. Numbers in red indicate the observed charge states; *Right* zero charge state mass spectrum for NME1. **B)** *Left* Combined mass spectra for NME1 following incubation with ATP; *Right* zero charge state mass spectrum for phosphorylated NME1. A difference in mass due to phosphorylation (compare panels on the right) can be clearly observed and a stoichiometry of 90% phosphorylation can be observed.

**Figure 2. Intact mass analysis of NME2 in the absence and presence of ATP.** *In vitro* kinase assays was carried out for 5 minutes at room temperature with 200 ng recombinant protein and 10 µM ATP. The reaction mixtures were then analysed by LC-MS using a Waters G2-S*i* Synapt. MS data were deconvoluted using MaxEnt 1 within MassLynx. **A)** *Left* Combined mass spectra for NME2 (no ATP) over the period of LC elution. Numbers in red indicate the observed charge states; *Right* zero charge state mass spectrum for non-phosphorylated NME2. **B)** *Left* Combined mass spectra for NME2 following incubation with ATP; *Right* zero charge state mass spectrum for phosphorylated NME2. A difference in mass due to phosphorylation (compare panels on the right) can be clearly observed and a stoichiometry of 80% phosphorylation can be observed.