Chapter 6

Determination of Phosphohistidine Stoichiometry in Histidine Kinases by Intact Mass Spectrometry

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Abstract

Protein histidine phosphorylation has largely remained unexplored due to the challenges of analyzing 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 characterizing 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-translational 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 signaling 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 signaling 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

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kingdoms, including plants and fungi [4, 5]. More recently, phosphohistidine (pHis) has been recognized to have roles in vertebrate signaling [6–8], and there is growing evidence that this acid-labile PTM may function in a similar cell signaling 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 loss 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 on the nitrogen at either the one or three 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* (nonmetastatic cells) genes. NDPKs are responsible for catalyzing 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 characterization 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 ($^{32/33}$P) 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 [19–22].

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 microcentrifuge 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 adjust 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 microcentrifuge 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 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

3.1 MS Calibration

1. To calibrate the time-of-flight (ToF) mass analyzer, 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**).

3.2 LC-MS Setup

1. Wash the UPLC column with 50% buffer A/50% buffer B for 1 min at a flow rate of 8 mL/min.
2. Program the LC-MS gradient as shown in Table 1.
3. Define the MS settings as listed in Table 2.
4. 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 Auto-phosphorylation Assay

1. Label two low-bind tubes for each enzyme being analyzed, 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 analyze the samples using the LC-MS parameters as defined in Subheading 3.2.

Table 1
LC/MS gradient

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

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

Table 2
MS instrument settings

Source	Capillary (kV) Sampling cone Source offset	3.0 40 80
Temperature (°C)	Source Desolvation	100 250
Gas flow settings	Cone gas (L/h) Desolvation gas (L/h) Nebulizer (bar)	50 300 6.0

Acquisition parameters for intact protein analysis using the Synapt G2-S*i* (Waters)

3.5 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 analyzed.
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 Figs. 1 and 2.
7. Calculate the stoichiometry of histidine phosphorylation by comparing the relative heights of the phosphorylated and

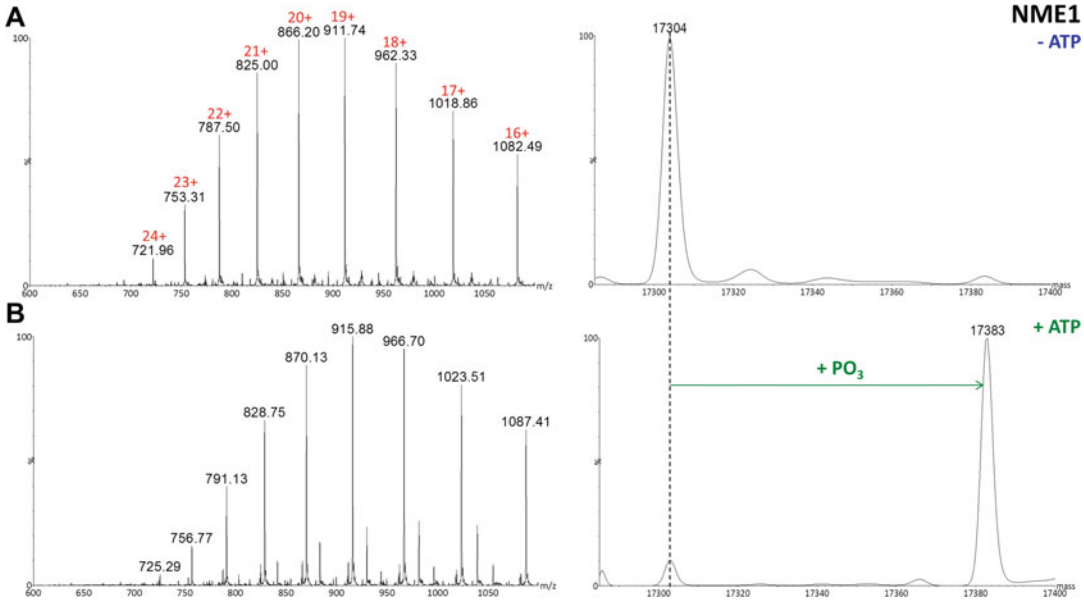

Fig. 1 Intact mass analysis of NME1 in the absence and presence of ATP. In vitro kinase assays was carried out for 5 min at room temperature with 200 ng recombinant protein and 10 μM ATP. The reaction mixtures were then analyzed by LC-MS using a Waters G2-S*i* Synapt. MS data were deconvoluted using 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

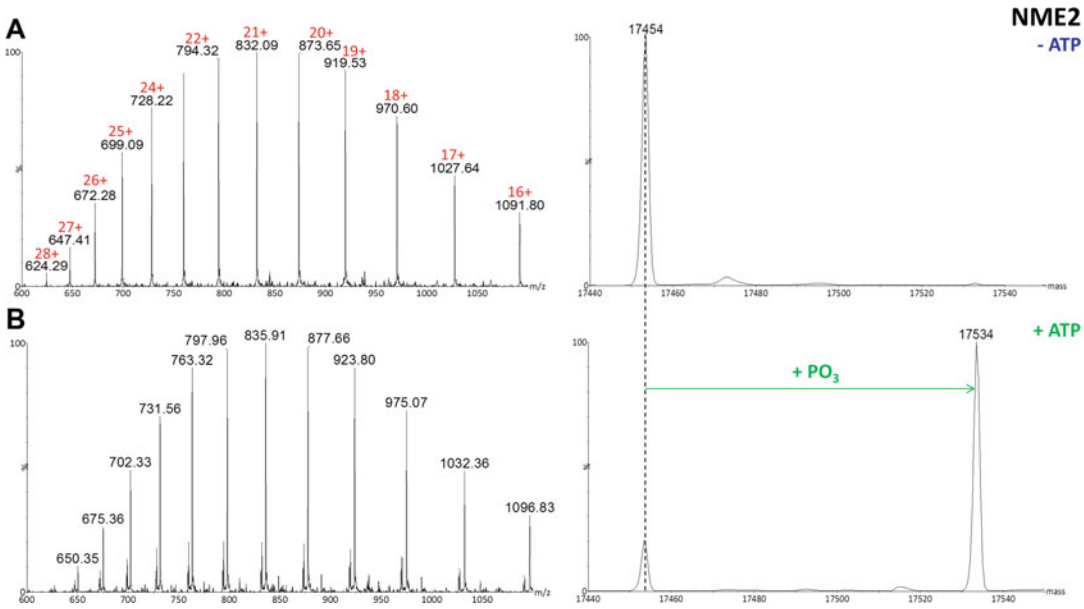

Fig. 2 Intact mass analysis of NME2 in the absence and presence of ATP. In vitro kinase assays was carried out for 5 min at room temperature with 200 ng recombinant protein and 10 μM ATP. The reaction mixtures were then analyzed by LC-MS using a Waters G2-S*i* Synapt. MS data were deconvoluted using 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 nonphosphorylated 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

nonphosphorylated 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 Subheading 3.4.

4 Notes

1. Histidine kinases can be made by recombinant protein expression and purification (*see* Chapters 2 and 5), or purchased.
2. 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.
3. 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.

4. 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.
5. 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.
6. We use TruView™ LCMS sample vials which minimize sample loss.
7. Any LC-MS instrumentation can be used that is capable of mass resolution of 20,000 or greater.
8. Data analysis software used will be dependent on the make of LC-MS instrumentation.
9. If the mass accuracy is greater than 1 ppm during ToF calibration, calibration should be repeated.
10. To minimize 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.
11. Ensure the protein is kept on ice prior to starting the assay.
12. 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.
13. 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.
14. The intact protein MS analysis should be performed as soon as possible after the end of the in vitro assay.
15. Set the autosampler to remain at 4 °C throughout the experiment.
16. It is often useful to generate a coarse-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.
17. The channel resolution settings can be further adjusted if required to between ~0.25 and 1 Da to gain a zero charge mass spectrum of high signal to noise.

References

1. Loomis WF, Shaulsky G, Wang N (1997) Histidine kinases in signal transduction pathways of eukaryotes. J Cell Sci 110:1141–1145
2. Manning G, Whyte DB, 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. Nat Cell Biol 4(5):127–130
4. Adam K, Hunter T (2018) Histidine kinases and the missing phosphoproteome from prokaryotes to eukaryotes. Pathol Focus 98:233–247
5. Kee JM, Muir TW (2012) Chasing phosphohistidine, an elusive sibling in the phosphoamino acid family. ACS Chem Biol 7(1):44–51
6. Klumpp S, Krieglstein J (2009) Reversible phosphorylation of histidine residues in proteins from vertebrates. Sci Signal 2(61):1–4
7. Fuhs SR et al (2015) Monoclonal 1- and 3-phosphohistidine antibodies: new tools to study histidine phosphorylation. Cell 162 (1):198–210
8. Srivastava S et al (2016) Histidine phosphorylation relieves copper inhibition in the mammalian potassium channel KCa3.1. eLife 5:pii: e16093
9. Puttick J, Baker E, Delbaere L (2008) Histidine phosphorylation in biological systems. Biochim Biophys Acta 1784(1):100–105
10. Attwood PV (2013) P/N bond protein phosphatases. Biochim Biophys Acta 1834:470–478
11. Attwood PV, Muimo R (2018) The actions of NME1/NDPK-A and NME2/NDPK-B as protein kinases. Lab Investig 98:283–290
12. Attwood PV, Wieland T (2015) Nucleoside diphosphate kinase as protein histidine kinase. Naunyn Schmiedeberg's Arch Pharmacol 388 (2):153–160
13. Wu Z, Tiambeng TN, CaI W, Chen B, Lin Z, Zachery R, Gregorich ZR, Ge Y (2018) Impact of phosphorylation on the mass spectrometry quantification of intact phosphoproteins. Anal Chem 90(8):4935–4939
14. Haydon CE, Eyers PA, Aveline-Wolf LD, Resing KA, Maller JL (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):1055–1067
15. Schweppe RE, Haydon CE, Lewis TS, Resing KA, Ahn NG (2003) The characterization of protein post-translational modifications by mass spectrometry. Acc Chem Res 36 (6):453–456
16. Johnson H, Eyers CE, Eyers PA, Beynon RJ, Gaskell SJ (2009) Rigorous determination of the stoichiometry of protein phosphorylation using mass spectrometry. J Am Soc Mass Spectrom 20(12):2211–2220
17. 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
18. Ferries S, Perkins S, Brownridge PJ, Campbell A, Eyers PA, Jones AR, Eyers CE (2017) Evaluation of parameters for confident phosphorylation site localization using an orbitrap fusion tribrid mass spectrometer. J Proteome Res 16(9):3448–3459
19. Attwood PV, Besant PG, Piggott MJ (2011) Focus on phosphoaspartate and phosphoglutamate. Amino Acids 40(4):1035–1051
20. Besant PG, Attwood PV, Piggott MJ (2009) Focus on phosphoarginine and phospholysine. Curr Protein Pept Sci 10(6):536–550
21. 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. https://doi.org/10.1101/202820
22. Hardman G, Perkins S, Brownridge PJ, Clarke CJ, Byrne DP, Campbell AE, Anton Kalyuzhnyy A, Myall A, Eyers PA, Jones AR, Eyers CE (2019) Strong anion exchange-mediated phosphoproteomics reveals extensive human non-canonical phosphorylation. The EMBO Journal. https://doi.org/10.15252/embj.2018100847