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Gas-phase intermolecular phosphate transfer within a phosphohistidine phosphopeptide dimer

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ABSTRACT

The hydrogen bonds and electrostatic interactions that form between the protonated side chain of a basic residue and the negatively charged phosphate of a phosphopeptide can play crucial roles in governing their dissociation pathways under low-energy collision-induced dissociation (CID). Understanding how phosphoramidate (i.e. phosphohistidine, phospholysine and phosphoarginine), rather than phosphomonoester-containing peptides behave during CID is paramount in investigation of these problematic species by tandem mass spectrometry. To this end, a synthetic peptide containing either phosphohistidine (pHis) or phospholysine (pLys) was analyzed by ESI-MS using a Paul-type ion trap (AmaZon, Bruker) and by traveling wave ion mobility-mass spectrometry (Synapt G2-*Si*, Waters). Analysis of the products of low-energy CID demonstrated formation of a doubly 'phosphorylated' product ion arising from intermolecular gas-phase phosphate transfer within a phosphopeptide dimer. The results are explained by the formation of a homodimeric phosphohistidine (pHis) peptide non-covalent complex (NCX), likely stabilized by the electrostatic interaction between the pHis phosphate group and the protonated *C*-terminal lysine residue of the peptide. To the best of our knowledge this is the first report of intermolecular gas-phase phosphate transfer from one phosphopeptide to another, leading to a doubly phosphorylated peptide product ion.

1. Introduction

Mass spectrometry has become an extremely powerful analytical tool for the qualitative and quantitative analysis of protein phosphorylation, being able to provide data as to the position and the extent (or stoichiometry) of the modification [1–3]. Tandem mass spectrometry, wherein precursor ions are induced to dissociate using a variety of activation techniques prior to analysis of the resultant products, is the key step for localization of the modification site. Despite the advantages and availability of electron-driven techniques such as electron transfer and electron capture dissociation (ETD and ECD) [4–8], collision-induced dissociation (CID) remains the most widely used strategy for phosphorylation site mapping.

Serine and threonine phosphorylated peptides usually undergo elimination of phosphoric acid (H_3PO_4) or metaphosphoric acid (HPO_3) [4] under typical low-energy CID conditions. This behavior represents a major challenge, not only to the identification of the phosphorylation site, but also for peptide sequence identification; such predominant loss of the phosphate group limits the formation of sequence specific b and y ions necessary for confident sequence identification and notably for site localization [4]. Under a low proton mobility environment, when the ionizing protons are sequestered to the most basic residues of a tryptic peptide (arginine and lysine), the elimination of (meta) phosphoric acid is thought to be promoted by the occurrence of an intramolecular hydrogen bond between the protonated *C*-terminal lysine or arginine and the oxygens of the phosphate group [9,10]. This interaction lowers the activation energy associated with the proton transfer from the *C*-terminus to the phosphate [10], thus assisting the elimination of H_3PO_4 via intramolecular nucleophilic substitution [9,10]. In

Abbreviations: NCX, non-covalent complex; pArg, phosphoarginine; pHis, phosphohistidine; pLys, phospholysine; TWIMS, travelling wave ion mobility-mass spectrometry.

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an analogous manner to phosphomonoester-containing peptides, peptides with phosphate covalently bound to other amino acids, such as phosphohistidine (pHis), phospholysine (pLys) or phosphoarginine (pArg), are also prone to undergo prevalent neutral loss during CID [11–14]. Additionally, the mass spectrometric (MS) analysis of such phosphopeptides is made even more challenging due to the intrinsic instability of the phosphoramidate bond under the acidic conditions typically required for positive ion mode mass spectrometry [11,15], thus adding yet another hurdle to their identification by MS.

Phosphorylated peptides have been demonstrated to form non-covalent complexes (NCX) due to interaction of the phosphorylated residue with a protonated quaternary amine [16–19]. The guanidinium group of a protonated arginine (PA = 251.2 kcal/mol) [20], and the ε-ammonium group of a protonated lysine (PA = 239.4 kcal/mol) [20], can engage in strong electrostatic interactions with the phosphate of a phosphorylated amino acid residue which holds a partial or net negative charge [16]. Lone pairs of electrons on the oxygens of the *O*-phosphoester can also drive the formation of hydrogen bonds with the protonated side chains of Arg and Lys, thus increasing the overall stability of the complex [9,19,21]. An in-depth description of the strength of the hydrogen bond interaction between phosphorylated residues (Ser and Tyr) and the protonated side chains of Arg and Lys was recently provided by Rapp and co-workers [22,23]. The phosphate-ammonium bond, which plays a major role in biological systems [24], is maximized in the gas phase, a medium where electrostatic interactions are known to be stronger than in solution, due to the considerably lower dielectric constant of the vacuum ($\varepsilon = 1$) as compared to solvents like water ($\varepsilon = 80$). It is therefore expected that any such electrostatic interactions will play an even bigger role in the vacuum, controlling the gas-phase ion chemistry of the species in which it is established.

Several studies have reported on the strength of the phosphate-guanidinium non-covalent bond network [16–19]. In one of the earliest reports, Jackson et al. [17] investigated the dissociation patterns of the phosphate-arginine non-covalent bond using a Ser phosphorylated peptide and an Arg-rich basic peptide as components of the NCX. When subjected to CID, two major dissociation channels were observed: disruption of the NCX with concomitant separation of the two peptides, and the formation of a new ionic product corresponding to a species which was 80 Da larger than the basic peptide. This suggested that CID resulted in cleavage between the serine oxygen and the phosphorous atom, releasing HPO_3 which was still engaged in electrostatic interaction with the guanidinium moiety in the Arg-rich peptide, highlighting the remarkable strength of this interaction.

Evidence of phosphate relocation during phosphopeptide CID, resulting in sequence scrambling and the limited formation of non-native phosphorylated peptides have been reported in both positive [21,25–27], and negative [28,29] ion mode. However, to our knowledge there is no evidence to date of intermolecular phosphate transfer to a previously phosphorylated peptide ion, resulting in the formation of a doubly 'phosphorylated' CID product. Unlike Ser, Thr and Tyr which form phosphomonoesters with a single phosphate group, His can be diphosphorylated on the 1- and 3-positions of the imidazole ring [30]. The high hydrolysis energy of the phosphoramidate bond also means that pHis/pLys/pArg residues are more likely to participate in phosphate group transfer than pSer/pThr/pTyr, a necessity of the function of pHis in two-component signaling systems [31]. Here we investigate the behavior of these more reactive pHis/pLys peptides during CID.

Using the synthetic phosphorylated peptide p(FVIAFILHLVK), containing either pHis or pLys, we demonstrate that electrospray ionization (ESI) results in a pHis homodimer which, following low-energy CID in a Paul-type ion trap, generates a product ion 80 Da bigger than the precursor ion, indicative of phosphate transfer between the two components of the dimer. MS^3 experiments on the product of the phosphate transfer confirmed its identity as a doubly 'phosphorylated' peptide. Although the exact nature of the bond between the additional phosphate moiety and the original singly phosphorylated peptide has not yet been ascertained, interaction with the *N*-terminus can be excluded. That a phosphate moiety can remain bound to a positively charged Arg residue upon CID of the corresponding NCX has already been reported [14–17]; here we present the first evidence to demonstrate that such phosphotransfer can occur to an already phosphorylated peptide ion.

2. Materials and methods

2.1. Peptide phosphorylation

Peptide FVIAFILHLVK (98% purity) was synthesized (Genescript) and provided as dry powder. Potassium phosphoramidate ($KNH_2PO_3H_2$; KPA) was synthesized from phosphoryl chloride and ammonia as described previously [32]. Phosphorylation was performed in water at pH 8 by adding 25 equivalents of KPA to 1 equivalent of peptide. The reaction was left to proceed overnight at room temperature, after which the solution was diluted to a final concentration of 1 pmol/μL in CH_3CN:H_2O 50:50 (v/v) and used for MS analysis.

2.2. ESI-QIT MS/MS

Phosphorylated peptide solutions were diluted in CH_3CN:H_2O 1:1 (v/v) to 1 pmol/μL and directly infused into an AmaZon ion trap (Bruker) through an electrospray source, at a flow rate of 1 μL/min. Source and octopole ion guide settings were adjusted to minimize in-source dissociation. In particular, the desolvating voltage in the ESI source and the acceleration voltages in the ion funnel were adjusted to final values of 110 and 80 V (with the standard acquisition parameters being 140 and 100 V respectively). Lower values of these two potentials resulted in a reduced ion transmission. Full scan ESI-mass spectra were acquired in the 150–2000 m/z range. CID product ion mass spectra were obtained using He as the collision gas. The MS/MS fragmentation amplitude was set at 1.20 V, and ramped from 30 to 300% of the set value.

2.3. Traveling wave ion mobility mass spectrometry

Phosphorylated peptide solutions were diluted in CH_3CN:H_2O 1:1 (v/v) to 1 pmol/μL and directly infused into a Synapt G2-*Si* HDMS instrument (Waters) through a nanospray source, at a flow rate of 0.5 μL/min. The capillary, cone voltage and source temperature were typically set to 2.7 kV, 40 V and 80 °C respectively. The IM traveling wave speed was set to 630 m/s and the wave height set at its maximum 40 V. The nitrogen drift gas flow was set at 90 mL/min for all experiments. Phosphopeptide CID was induced in the transfer cell using argon collision gas at collision energy (CE) of 30 V. For analysis of the dimer, the capillary voltage was set at 1.95 kV, while the wave speed was reduced to 311 m/s. Mass spectra were processed using MassLynx V4.1 and mobilograms using DriftScope v2.1 (both Waters, UK).

3. Results and discussion

3.1. Synthesis and characterization of p[FVIAFILHLVK]

The products of the reaction of the non-phosphorylated synthetic peptide FVIAFILHLVK with potassium phosphoramidate ($KNH_2PO_3H_2$; KPA) was assessed by ESI-MS/MS. The full scan ESI

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