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Electron capture dissociation mass spectrometry of phosphopeptides: Arginine and phosphoserine

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ABSTRACT

We have previously shown that the presence of phosphorylation can inhibit detection of electron capture dissociation (ECD) fragments of doubly charged peptide ions. The presence of non-covalent interactions, in the form of salt-bridges or ionic hydrogen bonds, prevents the separation of fragments following backbone cleavage. Here, we show the electron capture dissociation mass spectrometry of a suite of model peptides designed to investigate the relationship between phosphoserine and arginine position, namely AApSA$_n$RA$_m$KA (n = 0–6, m = 6–0), the presence of lysine residues (AApSAAKAARAKA) and AAApSARAAAAKAAAK, and the presence of proline A(A/P)ApSARAAA(A/P)KAAAK. The latter are analogous to the peptides studied previously. The results show that the presence of phosphoserine and basic amino acid residues alone does not inhibit ECD fragmentation, even when the number of basic amino acid residues is greater than the precursor charge state. Neither did the presence of proline in the peptide sequence suppress ECD backbone cleavage. Nevertheless, the presence and relative position of the phosphorylated residue do alter the observed backbone fragmentation abundance. In addition, the presence of phosphorylation appears to inhibit cleavage within the arginine side-chain regardless of the relative position of the arginine residue. The results suggest that ECD fragmentation behaviour is dependent on the three-dimensional structure of a peptide rather than its sequence.

1. Introduction

Electron capture dissociation is a tandem mass spectrometry technique which has proved highly useful in the analysis of protein post-translational modifications (PTMs) [1]. Capture of electrons by multiply charged peptide ions results in cleavage of N-Cα bonds in the peptide backbone producing c and $z^{\bullet}$ fragment ions. Importantly for the analysis of PTMs, the backbone fragments tend to retain the modifications [2–6].

Phosphorylation of proteins is one of the most important post-translational modifications and is responsible for numerous cellular processes. Phosphorylation and dephosphorylation are key events in signal transduction, controlling processes including gene expression, cell growth and proliferation. Structural consequences of phosphorylation are variable and dependent of the position of the phosphorylated residue and interactions with neighbouring residues [7–10]. Computational analyses have suggested that a significant proportion of phosphorylation sites are stabilized via electrostatic interactions with amino acid side chains [11].

Many studies have successfully applied ECD mass spectrometry to the characterization of phosphorylation in peptides and proteins [12–16]. Other work has focused on the effect of phosphorylation on ECD behaviour [17–19]. Turecek and co-workers considered the ECD of doubly-protonated serine phosphorylated pentapeptides containing a single basic amino acid residue (arginine) [20]. The major dissociation channel observed was loss of a hydrogen atom plus phosphoric acid from the charge-reduced species. This unusual fragmentation was attributed to dipole-guided electron capture at the guanidinium side-chain. Previous work in our laboratory revealed that the presence of phosphorylation can have an inhibitory effect on the generation of ECD fragments from doubly-charged peptide ions [17]. Peptides based on the sequence APLSFRGSLPKYSYVK, in which the serine residues were variably phosphorylated, were subjected to ECD and no fragments were detected between the phosphoserine and the arginine residue and/or the lysine residue at position 11. It was concluded that non-covalent interactions between the pSer and Arg and/or Lys were preventing the separation of any fragments formed. More recently, we have used a combination of travelling wave ion

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mobility mass spectrometry and molecular dynamics simulations to probe the structures of those peptides [18]. The results suggest that for the peptide phosphorylated at Ser4 a salt-bridge structure exists, whereas for the peptide phosphorylated at Ser12 ionic hydrogen bonds predominate.

Here, we present the ECD mass spectrometry of doubly charged ions from a suite of model phosphopeptides, see Table 1. The aim was to investigate the relationship between phosphoserine and basic amino acid residues. Pep-01 to Pep-08 were designed to test the effect of the position of the arginine residue in relation to the phosphoserine residue on fragment ion abundance; Pep-09 and Pep-10 were designed to examine the effect of an additional basic residue (lysine); Pep-11 to Pep-18 are analogues of the phosphopeptides studied in our earlier work [17,18] in which all residues with the exception of phoshoserine, proline, lysine and arginine are replaced with alanine. The results suggest that the presence of phosphoserine and basic amino acid residue alone are insufficient to inhibit detection of ECD fragments, even when the number of basic amino acid residues exceeds the charge state of the precursor ion. The inclusion of proline did not result in reduced fragmentation of the phosphopeptides (other than the expected absence of fragments directly N-terminal to the proline residue). The relative abundance of the $z^{\bullet}$ fragment directly C-terminal to the serine is reduced upon phosphorylation, mirrored by a concomitant increase in abundance of the N-terminal $z^{\bullet}$ fragment, when arginine is positioned $i \leq +2$. No general trend in abundance of fragments directly N-terminal or C-terminal to arginine and the position of the arginine residue was apparent. For the 12-mer peptides, loss of phosphoric acid was observed in conjunction with loss of ammonia, with the exception of Pep-10 where loss of H_3PO_4 from the charge-reduced radical ion and the charge-reduced hydrogen deficient species was observed. For Pep-12, no loss of phosphoric acid was observed. Loss of CH_5N_3 from the arginine side chain was reduced as a result of phosphorylation but was independent of the position of the arginine relative to the phosphoserine.

2. Experimental methods

2.1. Materials

Model peptides were custom synthesized by GenicBio (Shanghai, China) and used without further purification. Solvents: Ammonium acetate (50 mM) (Sigma-Aldrich St. Louis, Missouri, USA) in water (LC-MS grade, Fisher-Scientific Leicestershire, UK). Methanol (LC-MS grade), water (LC-MS grade), and formic acid (LC-MS grade) were purchased from Fisher-Scientific (Leicestershire, UK).

2.2. Model peptides

1 mg of peptides Pep-01 to Pep-05 was dissolved in 1 ml solution of methanol/water/formic acid (39.9:60:0.1). The peptides Pep-06 to Pep-18 were dissolved in 1:1 methanol/water to a concentration of ~1 mg/ml. Stock solutions were diluted in methanol, water, and formic acid (49.5:49.5:1) to a final concentration of ~1 μM. In addition, 1 mg of the crude peptides was dissolved in 1 ml of 50 mM ammonium acetate (pH = 7.5) and further diluted in the same solution to a final concentration of ~1 μM.

2.3. Mass spectrometry

Samples were ionized via nanospray ionization (nESI) in positive ion mode using an Advion Biosciences Triversa electrospray source (Advion Biosciences, Ithaca, NY, USA), at a flow of ~200 nL/min. Pep-01 to Pep-12 were analyzed with 7 Tesla LTQ FT Ultra (Thermo Fisher Scientific, Bremen, Germany) and Pep-13 to Pep-18 were analyzed with a 7 Tesla solariX-XR (Bruker Daltonics, Bremen, Germany). ECD in the LTQ-FT Ultra was performed by isolating the precursor ion of interest in the linear ion trap (automatic gain control (AGC) target 5×10^5 charges, maximum fill time 2 s, isolation width 5 Th). ECD used thermal electrons from an indirectly heated dispenser cathode [21] for 420 ms at "standard" cathode potential of −3.34 V. The precursor ions in the solariX-XR instrument were isolated in the quadrupole and ECD used thermal electrons at a current of 1.50 A, with a cathode bias of 0.6 V and lens potential of 10 V. Pulse length was varied according to each precursor ion between 0.5 and 0.9 s.

Ion mobility experiments of Pep-05 Pep-06, and Pep-07 were performed in a SYNAPT G-2 HDMS mass spectrometer (Waters Corp., Milford, MA, USA) equipped with Triversa electrospray source and a travelling-wave ion mobility cell (TW-IMS) maintained at 3 mbar of nitrogen. TW-IMS was operated at a wave velocity of 350 m/s and wave amplitude of 18.5, 19, and 19.5 V. Collision cross sections (CCS) calibration was performed following the procedure described by Ruotolo et al. [22] and the CCS of the calibration standards were obtained from the reported values by Counteman and Clemmer [23]. Standards for calibration included tryptic digest peptides of BSA, cytochrome *c* and myoglobin.

Data analysis was performed with Xcalibur software 3.0 (Thermo Fisher Scientific), Data Analysis 4.2 software (Bruker Daltonics), and with in-house software developed at the National University of Colombia. Analyses of Pep-01 to Pep-12 (LTQ FT Ultra) were performed in triplicate. Results from triplicate analyses were used to calculate the standard deviation of the normalized relative intensity (I_i) of each fragment ion (Eq. (1) in supplementary material). *p* values were calculated to determine the significance between fragment ion relative intensities using the Student's *t*-test ($n = 3$) at 95% significance level.

3. Results and discussion

3.1. ECD fragmentation patterns

The doubly charged species of the peptides presented in Table 1 were isolated and fragmented by ECD generating low abundance *c* and $z^{\bullet}$ fragment ions [24]. In addition, ECD resulted in $c^{\bullet}$ ($c^{\bullet} = c - H$), and *z* fragment ions ($z = z^{\bullet} + H$) [25]. The fragmentation patterns observed following ECD of the doubly charged peptide ions are summarized in Fig. 1. The ECD mass spectra are shown in Fig. 2 and Supplemental Figures S1–S12. Full fragment ion assignments are given in Supplementary Tables S1–S18. The results show complete sequence coverage for the peptides Pep-01 to Pep-08 regardless of the presence of phosphorylation or site of the basic arginine residue. Similar results were obtained for Pep-09 and Pep-10. Some inhibition of fragmentation was observed in the central region of Pep-12 when compared with its unmodified counterpart (Pep-11). These results are in contrast to our earlier work, in which inhibition of ECD fragmentation in the region between the phosphate group and the basic residues was observed in doubly charged serine phosphorylated peptides with fifteen amino acid residues (e.g. APLpSFRGSLPKSYVK) [17,18]. In that work, we concluded that non-covalent interactions (either salt-bridges or ionic hydrogen bonds) between the phosphate group and the basic amino acid side chains were preventing the separation of any ECD fragments. The results for Pep-01 to Pep-08 suggest that the presence of both phosphate and basic amino acid residue alone is insufficient to result in inhibition of fragmentation, nor does increasing the number of basic amino acid residues over the charge state, as in Pep-10, result in inhibition of fragmentation. Pep-12 (AAA**pS**A**R**AAAA**K**AAA**K**), which is an analogue of APLpSFRGSLPKSYVK, with the same location of the arginine, lysine and phosphoserine residues, does show