Published in final edited form as:

J Mass Spectrom. 2018 December ; 53(12): 1178–1188. doi:10.1002/jms.4291.

Electron Transfer Dissociation Mass Spectrometry of Acidic Phosphorylated Peptides Cationized with Trivalent Praseodymium

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Abstract

The lanthanide ion praseodymium, Pr(III), was employed to study metallated ion formation and electron transfer dissociation (ETD) of twenty-seven biological and model highly acidic phosphopeptides. All phosphopeptides investigated form metallated ions by electrospray ionization (ESI) that can be studied by ETD to yield abundant sequence information. The ions formed are $[M + \text{Pr} - \text{H}]^{2+}$, $[M + \text{Pr}]^{3+}$, and $[M + \text{Pr} + \text{H}]^{4+}$. All biological phosphopeptide with a chain length of seven or more residues generate $[M + \text{Pr}]^{3+}$. For biological phosphopeptides, $[M + \text{Pr}]^{3+}$ undergoes more backbone cleavage by ETD than $[M + \text{Pr} - \text{H}]^{2+}$ and, in some cases, full sequence coverage occurs. Acidic model phosphorylated hexa- and octa-peptides, composed of alanine residues and one phosphorylated residue, form exclusively $[M + \text{Pr} - \text{H}]^{2+}$ by ESI. Limited sequence information is obtained by ETD of $[M + \text{Pr} - \text{H}]^{2+}$ with only metallated product ions being generated. For two biological phosphopeptides, $[M + \text{Pr} + \text{H}]^{4+}$ is observed and may be due to the presence of at least one residue with a highly basic side chain that facilitates the addition of an extra proton. For the model phosphopeptides, more sequence coverage occurs when the phosphorylated residue is in the middle of the sequence than at either the N- or C-terminus. ETD of the metallated precursor ions formed by ESI generates exclusively metallated and non-metallated c- and z-ions for the biological phosphopeptides, while metallated c-, z-ions and a few y-ions form for the model phosphopeptides. Most of the product ions contain the phosphorylated residue indicating that the metal ion binds predominantly at the deprotonated phosphate group. The results of this study indicate that ETD is a promising tool for sequencing highly acidic phosphorylated peptides by metal adduction with Pr(III) and, by extension, all non-radioactive lanthanide metal ions.

Keywords

phosphorylated acidic peptides; electron transfer dissociation; phosphorylated residue location; higher charge effect; trivalent lanthanide cations

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Supporting Information

Supporting information may be found in the online version of this article.

Introduction

Post-translational modifications are ubiquitous in nature and allow the functions of peptides or proteins to be altered via covalent interactions.^{1,2} In phosphorylation, a common eukaryotic post-translational modification, phosphate groups reversibly replace hydroxyl groups. About 90% of phosphorylated residues in nature are phosphoserine (pS), while phosphothreonine (pT) and phosphotyrosine (pY) exist in lower abundances collectively contributing to the remaining 10%.³ A few other amino acid residues have been known to undergo phosphorylation although to a much lesser extent than serine, threonine, and tyrosine.⁴ According to the database PhosphoNET, about 80% of human genes code for phosphorylated proteins.⁵

Phosphopeptides can be difficult to analyze. They tend to exist in lower abundances in nature than their non-phosphorylated counterparts.⁶ In addition, the process is reversible and typically has a short lifetime. Mass spectrometry has emerged as an important tool for analysis of phosphopeptides due to its high sensitivity and its ability to locate the site of phosphorylated residues.^{7,8} However, formation of ions from phosphopeptides can be difficult. Low positive mode ionization efficiencies sometimes occur in electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI).^{6,9,10} Highly acidic phosphopeptides such as those employed in the current study often contain one or more acidic aspartic and/or glutamic acid residues. Carboxylic acid and phosphate groups have a high propensity for deprotonation as represented by their pKa's¹¹ and gas-phase acidities (GAs)^{12,13}. The phosphorylated residues are more acidic than aspartic or glutamic acid residues by about 50–90 kJ/mol,^{12,13} so their presence contributes additional acidic character to a phosphopeptide.

Collision-induced dissociation (CID) is commonly employed as an automated tandem mass spectrometry technique (MS/MS) for peptide analysis in the positive ion mode. However, CID may be plagued with loss or migration of the labile phosphate group as a low energy process upon collisional activation, which hinders identification of phosphorylated sites along the peptide backbone.^{6,14,15} Electron-induced dissociation techniques, such as electron capture dissociation (ECD) and electron transfer dissociation (ETD), usually preserve the site of phosphorylation due to more random and nonergodic fragmentation. Several studies on protonated peptides and protein ions have demonstrated the ability of ECD and ETD to provide abundant sequence information as well as the location of phosphorylated sites.^{6,16–24} However, ECD and ETD can be limited by the fact that these processes generally require ions with a charge of 2+ or greater since capture or transfer of an electron to the charged species occurs. Addition of an electron to a singly charged ion (1+) would result in a neutral molecule that cannot be analyzed by mass spectrometry.

Complexation of peptides with metal ions can assist in the formation of multiply charged ions. In an ECD study, Williams and coworkers²⁵ demonstrated that the use of trivalent lanthanide cations can generate multiply charged ions with small peptides by ESI. In addition, previous work by our group^{26,27} demonstrates the use of trivalent lanthanide metal ions to facilitate the formation of multiply charged metallated ions by ESI for non-phosphorylated acidic peptides. In that study, the metallated ions undergo abundant

fragmentation by ETD to yield full sequence information. For phosphopeptides, deprotonation may occur on the phosphate group.^{12,19} Therefore, extra protons may be needed to achieve a positive charge state of 2+ or greater. However, peptide size may restrict addition of three or more protons. A single trivalent lanthanide cation can alleviate the need for several protons by imparting a charge of 3+ that is suitable for ETD and will compensate for the deprotonation of a phosphate group.

Although the use of ionization with metals is not yet routine in proteomics research, there is an increasing body of scientific work involving ECD and ETD of metallated peptides.^{25–43} Three studies have focused on metal cationization of phosphopeptides.^{41–43} In a report involving metal oxides, Hakansson and Kweon⁴¹ determined multiple sites of phosphorylation for several peptides using ECD. Mosley and coworkers⁴² employed lanthanide metal (III)-ligand complexes to analyze phosphopeptides, obtaining full sequence coverage by ECD as well as unambiguous location of phosphorylated residues. The identity of the lanthanide metal ion also affected the amount of fragmentation obtained, with easily reduced Eu^{3+} in the Eu(III) -peptide complex providing limited sequence information. In a recent ETD investigation involving zinc(II)-peptide complexes, Asakawa and Osaka⁴³ observed that higher charge states of phosphopeptides lead to more sequence information as well as identification of the phosphorylation site.

Here, the effect of Pr(III) adduction on ETD of acidic model and biological phosphorylated peptides is investigated. In two previous studies,^{26,27} we have observed abundant metallated peptide ion formation by ESI using Pr(III) for non-phosphorylated acidic peptides; these ions yield extensive sequence informative fragmentation by ETD. The current work also probes the effects of the location and identity of phosphorylated residue on ETD.

Experimental

All experiments were performed on the Bruker (Billerica, MA, USA) HCTultra PTM Discovery System high capacity quadrupole ion trap (QIT) mass spectrometer equipped with ESI. The needle was held at ground and a high voltage of -3.5 kV was applied to the capillary entrance, the stainless steel end cap, and the capillary entrance cap. Nitrogen gas served as both the drying gas and the nebulizing gas. Drying gas was heated to 260°C and flowed at $5\text{--}10$ L/min, while the nebulizing gas pressure was optimized between 5 and 10 psi. Metal salt and acidic peptide mixtures at a 10:1 Pr(III) :peptide molar ratio were infused using a KD Scientific (Holliston, MA, USA) syringe pump. The final peptide concentration was 5 μM in a solvent system of acetonitrile:water at a 50:50 volume ratio. All spectra shown are the result of signal averaging 200 scans.

The ETD reagent anion, fluoranthene, was generated in a negative chemical ionization (nCI) source with methane serving as the nCI reagent gas. The anion/cation reaction times were in the range of 180–250 ms and the lower end m/z cutoff was 120. Reagent anion accumulation was set between 8–12 ms and the ion charge control (ICC) between 300,000–400,000 to maximize transfer of the electron. Following the anion/cation reaction, the “smart decomposition” function was activated to further dissociate any charge reduced (ETnoD) ions. Smart decomposition enhances observed ETD product ions by using resonant

excitation to overcome attractive forces (e.g., hydrogen or noncovalent bonding) that may keep peptide fragments together following the electron transfer process. Smart decomposition is described in more detail in previous work.²⁷

Human epidermal growth factor receptor, EGF (988–998), [pSER95, Ala97]-RII, and heat shock protein fragment (hsp) were purchased from Bachem Americas, Inc. (Torrance, CA, USA). The other acidic biological peptides were custom synthesized by Biomatik (Wilmington, Delaware, USA). They were epidermal growth factor receptor (EGF(DL-6)), platelet-derived growth factor beta-receptor hormone (PDGF(pTyrY-8P)), platelet-derived growth factor beta-receptor hormone (PDGF (PTyrL-5P)), and DNA transcription inhibitory peptide and its phosphothreonine and phosphotyrosine analogs. All model peptides were synthesized in our laboratory with an Advanced ChemTech Model 90 peptide synthesizer (Louisville, KY, USA) using standard Fmoc protocol.⁴⁴ Praseodymium(III) nitrate hexahydrate salt was purchased from Alfa Aesar (Ward Hill, MA, USA). Ultrapure Milli-Q 18Ω water was produced with a Barnstead (Dubuque, IA, USA) E-pure system.

Results and Discussion

Spectra are labeled using the established peptide cleavage nomenclature of Roepstorff and Fohlman⁴⁵ as modified by Biemann⁴⁶ to keep track of the number of hydrogens incorporated into product ions formed by ETD. A prime symbol to the left indicates loss of a hydrogen (i.e., $^{\prime}c_n = [c_n - H]^+$) and to the right indicates addition of a hydrogen (i.e., $c_n^{//} = [c_n + 2H]^+$). For easier product ion identification, the following colors are used to label spectra: non-metallated z-ions = red, non-metallated c-ions = blue, metallated z-ions = bright pink, metallated c-ions = green, and metallated y-ions = brown. Precursor ion that did not dissociate is labeled in purple with a large purple diamond arrow head. Neutral loss products and electron transfer without dissociation (ETnoD) ions are labeled in black. The c- and z-ions involve N-Cα bond cleavage, which are typically products in ECD^{47–51} and ETD^{52,53} processes.

Biological peptides

The sequences of the acidic biological phosphopeptides studied are shown in Table 1. These peptides contain a variety of amino acid residues and include one or more aspartic (D) or glutamic acid (E) residues and one phosphoserine (pS), phosphothreonine (pT), or phosphotyrosine (pY) residue. Phosphorylated residues are written in bold and italicized font. Peptide chain lengths vary from 5 to 19 residues.

The most abundant metallated ions formed by ESI are $[M + Pr]^{3+}$ and $[M + Pr - H]^{2+}$, as shown in Table 1. Heat shock protein fragment (hsp) and [pSER95, Ala97]-RII also generate $[M + Pr + H]^{4+}$, with protonation undoubtedly occurring due to the presence of a highly basic arginine residue (gas-phase basicity of arginine (GB) = 1006.6 kJ/mol⁵⁴) that can accept a proton. (These are also the only two peptides in the study to form multiply protonated ions.) The other peptides in this study do not contain an arginine residue. In addition, the greater lengths of the chains for these two peptides (14 and 19 amino acid residues) may allow the peptide to accept both a trivalent lanthanide metal ion and a proton.

Conversely, the highly acidic nature of pS in hsp still causes some deprotonation to form $[M + Pr - H]^{2+}$, albeit in lesser abundance than $[M + Pr]^{3+}$ and $[M + Pr + H]^{4+}$.

The hendecapeptide EGF(988–998) forms $[M + Pr - H]^{2+}$ and $[M + Pr]^{3+}$. A proline residue, which is the fifth most basic amino acid residue⁵⁵ (GB of proline = 886.0 kJ/mol^{54,56}), may accept a proton^{26,57} and facilitate the formation of $[M + Pr + H]^{4+}$. This is in addition to the normal protonation sites; i.e., the N-terminus and the side chains of arginine, lysine, and histidine. In the prior study,²⁶ the twelve residue EGF(985–996) with a proline formed the 4+ ion. However, EGF(988–998) with a proline, a shorter chain (eleven residues), and a highly acidic pY only forms $[M + Pr]^{3+}$. The acidic nature of the phosphorylated residue and possibly the shorter peptide chain length in EGF(988–998) may hinder the formation of $[M + Pr + H]^{4+}$.

The highly acidic octapeptide, DNA transcription inhibitory peptide, and its analogs form $[M + Pr - H]^{2+}$ and $[M + Pr]^{3+}$. These three peptides have the sequence EDDXDEEN, where X is varied from pS in the main peptide to pT or pY in the analogs. The type of phosphorylated residue had minimal, if any, effect on the ions formed by ESI. This is consistent with the fact that the GAs of the three phosphorylated amino acid amides are very similar and highly acidic.¹² (Refer to Supplemental Table S1 for GAs of the phosphorylated amino acid amides. Amide groups at the C-terminus are reflective of an amino acid residue incorporated into a peptide.) Seven of the eight residues in the peptides are acidic, which supports the formation of $[M + Pr - H]^{2+}$. More uncertain is the formation of abundant $[M + Pr]^{3+}$ despite the highly acidic nature of all three peptides. In our previous work,²⁶ a heptapeptide with six acidic residues only formed the deprotonated species, $[M + Pr - H]^{2+}$. The increase in chain length from seven to eight residues may contribute to the formation of $[M + Pr]^{3+}$.

The peptides with less than seven amino acid residues, PDGF beta-receptor (pTyrL-5P) and human epidermal growth factor receptor (DL-6), generate only $[M + Pr - H]^{2+}$. Pentapeptide pTyrL-5P contains pY, which is the most acidic of the three phosphorylated residues (by ~30 kJ/mol¹²) and undergoes facile deprotonation. Lack of formation of 3+ and 4+ metallated ions for the hexapeptide DL-6 is consistent with the fact that the peptide contains four acidic sites (including a pY residue and the C-terminal carboxyl group) that will easily deprotonate. In addition, the small size of pTyrL-5P may limit the ability of the peptide to coordinate at multiple sites to both a trivalent lanthanide metal ion and a proton.

Each multiply charged ion generated by ESI was further investigated by ETD. Figure 1 shows the ETD spectra of $[M + Pr + H]^{4+}$ for hsp and [pSER95, Ala97]-RII. As with our previous studies,^{26,27} the 4+ ion yields the most extensive sequence coverage. The spectra contain high signal-to-noise ratios (S/N) allowing for easy identification of product ions. A mix of complementary non-metallated and metallated c- and z-ions form with $c_n^{/+}$ and $[c_n + Pr]^+$ as the c-series and $z_n^{/+}$ and $[z_n + Pr]^+$ as the z-series. All metallated product ions contain the phosphorylated residue, suggesting metal interaction with the phosphate group. (In metal-based enrichment strategies, the phosphate group acts as a Lewis base and readily binds to a metal carrying a positive charge.⁵⁸) Most ETD product ions are singly charged although a few form with a charge of 2+. Metallated c- and z- ions lack one hydrogen

relative to the direct backbone cleavage site. Cooper and coworkers¹⁸ also observed $^{1/c_n} = [c_n - H]^+$ in an ECD study of phosphorylated peptides containing arginine and pS. The location of pS in hsp could be definitively identified at residue 7 as shown in Figure 1(A). This contrasts CID where loss or migration of the phosphate group complicates spectra and can prevent unambiguous identification of phosphorylation sites.^{6,14,15} Definitive localization of phosphorylated residues is a major advantage of analyzing such peptides by ETD rather than by CID.

Interestingly, full sequence coverage was not obtained by ETD of $[M + Pr + H]^{4+}$ for [pSER95, Ala97]-R11, as shown in Figure 1(B). Cleavages are absent from the C-terminal side of arginine (R^9) to the N-terminal side of the phosphoserine (pS^{11}). The absence of cleavages along this portion of the peptide suggests the formation of a salt-bridge between the side chain of the highly basic R and the highly acidic pS. In a study by Cooper and Creese,¹⁹ it was postulated that salt bridges between the deprotonated pS and protonated arginine and lysine residues hindered fragmentation. Salt bridges between phosphorylated and basic amino acid residues occur in nature and are involved in biological functions such as stabilization of protein secondary structures and activation of proteins.^{59–61} However, the formation of $[^{1/c_{15}} + Pr]^+$ facilitates location of the phosphate group at pS^{11} .

Electron transfer dissociation of $[M + Pr]^{3+}$ can also provide full sequence information. For EGF(988–998) shown in Figure 2 and DNA transcription inhibitory peptide and analogs shown in Figure 3, most of the c- and z-ions are singly charged, although a few are doubly charged. For EGF(988–998), these c- and z-ions correspond to cleavage between all residues except N-terminal to proline and result in full sequence coverage. The side chain of proline forms a ring incorporating the backbone, which prevents dissociation of the peptide into two separate products following N-C α bond cleavage.^{6,18,19,62–64} Full sequence coverage results in unambiguous identification of the location of the phosphorylated residues in the middle of the sequence for DNA transcription inhibitory peptide and its analogs. All metallated product ions contain the phosphorylated residue.

For the other biological peptides, ETD of $[M + Pr]^{3+}$ does not generate complete sequence information. In Figure 4(A), only 71% sequence coverage occurs for hsp. Lack of cleavage between adjacent residues from the C-terminal side of pS^7 to the N-terminal side of V^{10} limits the ability to locate phosphorylation sites. For the octapeptide pTyr Y-8P in Figure 4(B), formation of non-metallated and metallated c- and z-ions results in 70% sequence coverage. In Figure 4(C), [pSER95, Ala97]-R11 with 19 residues only 61% of the peptide sequence is deciphered.

A few metallated product ions form that do not incorporate the phosphorylated residue. For pTyr Y-8P in Figure 4(B), $[^{1/z_n} + Pr]^+$, $n = 6$ and 7 , contain an acidic aspartic acid residue as well as a C-terminal carboxyl group; i.e., two acidic sites. The Pr(III) can coordinate with non-phosphorylated acidic residues, such as the C-terminal carboxyl groups or side chains of aspartic or glutamic acid. ETD of these structures may form product ions lacking the phosphorylated residue. In our previous work,²⁶ as well as a study by Williams and coworkers²⁵ with non-phosphorylated acidic peptides, metallated ions form when at least two acidic sites are incorporated into the product. No other peptide in the study forms

metallated product ions without the phosphate group. The location of the phosphorylated residue at the N-terminus in pTyr Y-8P may provide greater conformational flexibility in allowing the seven residues to coordinate with the Pr(III). Metal adduction consistently occurs for peptides with at least seven residues as observed in our previous studies.^{26,27} In the other phosphopeptide with an N-terminal residue, pTyrL-5P, the phosphorylated residue may be necessary to allow the peptide to coordinate with Pr(III). For pTyr Y-8P, metallated z-ions also form in low abundance compared to the other peptides. This may indicate that the more abundant product ions are generated from a structure containing the phosphate group complexed to Pr(III), causing electron-driven processes to occur in this region. The least amount of sequence coverage occurs from ETD of $[M + Pr - H]^{2+}$. In Supplemental Figures S1-S8, ETD spectra of $[M + Pr - H]^{2+}$ for pTyrL-5P, EGF(DL-6), pTyr Y-8P, EGF(988–998), hsp, and DNA transcription inhibitory peptide and its analogs contain primarily singly charged metallated c- and z-ions. Sequence coverage is: PDGF(pTyrL-5P) = 66%, EGF(DL-6) = 60%, PDGF(pTyrY-8P) = 33%, EGF(988–998) = 78%, hsp = 23%, and DNA transcription inhibitory peptide and analogs = 60%. The site of phosphorylation could not be identified for any of these peptides due to lack of cleavage on both sides of the modified residue. Similar decreased product ion formation for ETD of $[M + Pr - H]^{2+}$ was found in our previous work^{26,27} for non-phosphorylated acidic peptides as well as in a phosphorylated study by Asakawa and Osaka.⁴³

Model peptides

Eighteen model peptides were investigated to determine the effect of structural features on metallated ion formation by ESI and on ETD of acidic phosphopeptides adducted to Pr(III). Factors considered include the type of phosphorylated amino acid residue and location of the residue within the sequence. The hexapeptides have the general sequences pXAAAAA (pXA5), AApXAAA (A2pXA3), and AAAApXA (A4pXA), while the octapeptides are pXAAAAAA (pXA7), AAApXAAAA (A3pXA4), and AAAAAApXA (A6pXA), where A is alanine and pX is pS, pT, or pY. In Fmoc synthesis,⁴⁴ the peptide is built from the C-terminus on an insoluble polystyrene bead called a Wang resin. No phosphorylated amino acid Wang resins are commercially available; therefore, peptides of the sequence AAAAAAApX could not be synthesized. However, such peptides would be of limited utility to study here because the tryptic enzymes that are commonly used in bottom-up proteomics research do not generate peptides with C-terminal phosphorylated residues.^{65,66} Table 2 shows the metallated precursor ions formed by ESI and the ETD product ions for all of the model peptides.

All eighteen model phosphopeptides form only $[M + Pr - H]^{2+}$ by ESI. These peptides produce only singly protonated ions, $[M + H]^+$, in low intensity in the absence of Pr(III). None form $[M + Pr]^{3+}$ or $[M + Pr + H]^{4+}$, indicating that the facile deprotonation of the phosphorylated residue is dominant even in the presence of metallation. In addition, this shows that an N-terminal basic site alone is not sufficient to allow the peptide ions to form in the 3+ or higher charge state. Like the model hexapeptides, biological EGF(DL- 6) contains six residues and forms only $[M + Pr - H]^{2+}$. Also, in our previous work with non-phosphorylated acidic peptides,²⁶ hexapeptides only form $[M + Pr - H]^{2+}$. The fact that the

model octapeptides do not form $[M + Pr]^{3+}$ is somewhat unexpected because the octapeptide DNA transcription inhibitory peptide and its analogs yield abundant $[M + Pr]^{3+}$.

The $[M + Pr - H]^{2+}$ ions were subjected to ETD for all model peptides. The result was singly charged metallated product ions, which always contain the phosphorylated residue. The ETD spectra of the model pS peptides are shown in Figure 5, while spectra for pT and pY peptides are in Supplemental Figures S9-S20.

Structural effects such as position of phosphorylated residue can affect dissociation of peptides.^{18,61,67-69} When the phosphorylated residue is at the N-terminus in model hexapeptides, ETD of $[M + Pr - H]^{2+}$ results in cleavage at only two backbone sites producing $[c_n + Pr]^+$, $n = 4$ and 5 , as shown in Figure 5(A) and Supplemental Figures S9 and S10. For the pXA7 octapeptides shown in Figure 5(D) and Supplemental Figures S11 and S12, cleavage occurs at three sites. Formation of only c-ions suggests that the metal ion is binding at the phosphorylated side chain on the N-terminal residue. Generation of only N-terminal product ions also indicates a stronger interaction of the phosphate group with Pr(III) than occurs with a carboxylic acid group. Aspartic or glutamic acid residues at the N-terminus do not restrict formation of C-terminal ions in non-phosphorylated acidic peptides adducted to Pr(III).²⁶

The most extensive sequence coverage occurs when the phosphorylated residue is in the middle of the peptide sequence. Three to four backbone cleavages were found for the hexapeptides and six for the octapeptides. (See Figures 5 (B) and (E) and Supplemental Figures S13-S16.) Lack of cleavage adjacent to the phosphorylated residue makes determination of the phosphorylated site ambiguous; however, the location can be narrowed down to two residues in the middle of the sequence.

Limited sequence information is obtained from the ETD spectra of $[M + Pr - H]^{2+}$ when the phosphorylated residue is located penultimate to the C-terminus. In Figure 5(C) and Supplemental Figures S17 and S18 for A4pXA, only $[c_5 + Pr]^+$ and $[z_5 + Pr]^+$ form. Octapeptides (A6pXA) yield singly charged metallated c-, z-, and y- ions corresponding to three or four cleavage sites, as found in Figure 5(F) and Supplemental Figures S19 and S20. Location of the exact site of phosphorylation is impossible with such limited fragmentation.

The three octapeptides with C-terminal penultimate phosphorylated residues, A6pXA, form y-ions, which involve $(O\equiv C)-N$ cleavage and were not observed for the other peptides in this study. A6pTA (Supplemental Figure S19) forms metallated y- and c-ions, while A6pSA (Figure 5(F)) and A6pYA (Supplemental Figure S20) produce z-ions with one c- and one y-ion each, all of which are metallated. Formation of y-ions in electron-based techniques such as ECD and ETD has been observed from $[M + 2H]^{2+}$ for peptides with alkyl side chains.⁷⁰⁻⁷³ The strings of six alanine residues in A6pXA undoubtedly cause the y-ion formation, which is proposed to involve a CID-like mechanism. The y-ions here lack three hydrogens, $^{111}y_n = [y_n - 3H]$, which is probably due to deprotonation at the phosphorylated residue.

Dissociation trends and structural effects

The type of phosphorylated residue affects the extent of fragmentation by ECD and ETD.^{19,74} In general, peptides containing pS undergo less backbone cleavage than peptides with pT or pY. For example, A2pSA3 cleaves at one less site than A2pTA3 and A2pYA3 (Figure 5(B) and Supplemental Figures S13 and S14). In addition, A6pSA has one less backbone cleavage than A6pTA or A6pYA (Figure 5F and Supplemental Figures S19 and S20). Cooper and Creese¹⁹ also observed that pS hampered backbone dissociation for protonated peptide ions. These researchers postulated that this was mostly likely due to formation of salt bridges between the deprotonated pS and highly basic side chain of arginine or lysine. (They did not study peptides containing pT and pY.) Steric hindrance from the bulkier side chains of pT and pY may limit their ability to form salt bridges.

Higher charge states can generate an increased number of product ions and improved sequence coverage with better location of phosphorylated residues.^{75–77} This trend is seen for several peptides in the current study. Backbone cleavage occurs on both sides of the pS^8 in ETD of $[M + Pr + H]^{4+}$ for hsp (Figure 1(A)) and allows unambiguous identification of the site of phosphorylation. The location of pS could not be determined by ETD of the $[M + Pr]^{3+}$ (Figure 4(A)) or $[M + Pr - H]^{2+}$ (Supplemental Figure S5). Another example is EGF(988–998) where the exact location of pY at residue 5 can be conclusively determined by ETD of the $[M + Pr]^{3+}$ (Figure 2) but not for $[M + Pr - H]^{2+}$ (Supplemental Figure S4). In an ECD study by Asakawa and Osaka⁴³ involving phosphopeptides complexed with divalent zinc, precursor ions with higher charge states generate more product ions and provide increased sequence coverage when compared to precursor ions with lower charges.

Overall efficiency of fragmentation varies among the peptides. For example, in Figure 1 showing ETD mass spectra for hsp, almost all of $[M + Pr + H]^{4+}$ dissociates. However, for DNA transcription inhibitory peptide and its analogs, the precursor ion $[M + Pr]^{3+}$ is the most intense ion in their ETD spectra (Figure 3) indicating a low dissociation efficiency. Nonetheless, full sequence coverage is obtained. Peptide sequence coverage and ETD efficiency of $[M + Pr - H]^{2+}$ for the model hexa- and octapeptides is low with the undissociated precursor ion being the base peak in most spectra. The only exception is for octapeptide A3pSA4 where the non-dissociated precursor is about half the intensity of the base peak, which is the reduction product $[M + Pr - H]^+$ and is not sequence informative.

Neutral losses were present in ETD spectra of the acidic phosphopeptides. Many peptides exhibit a loss of 59 Da corresponding to $C_2H_3O_2$ from the side chains of aspartic and glutamic residues. This 59 Da loss is also observed in previous ETD and ECD studies involving non-phosphorylated acidic peptides^{26,27,78} Neutral losses from the phosphate group, such as H_3PO_4 and HPO_3 , are common in CID^{17,79–84} and sometimes in ECD of protonated peptides.⁸⁵ However, no such ions were observed in this study of metallated peptides; this is useful for locating phosphate groups.

Conclusions

The utilization of trivalent metal cationization for the analysis of highly acidic phosphopeptides by ETD has been demonstrated. Upon addition of Pr(III), all peptides in

the study form multiply charged ions by ESI that can be studied further by ETD. In contrast, in the absence of Pr(III) only the two peptides containing highly basic arginine residues form multiply protonated ions. ETD of $[M + Pr - H]^{2+}$ provides the least amount of sequence coverage and location of the phosphorylated residue is not possible. In contrast, location of the site of phosphorylation and full sequence coverage is often possible with both $[M + Pr]^{3+}$ and $[M + Pr + H]^{4+}$. Even highly acidic biological phosphopeptides with eight residues can be fully sequenced by ETD of $[M + Pr]^{3+}$. While $[M + Pr + H]^{4+}$ yields the most sequence informative fragmentation, generation of the protonated and metallated ion requires the presence of a basic amino acid residue in the peptide chain. In the current ETD study, only Pr(III) was employed, but our previous work²⁷ indicates that any of the trivalent lanthanide metal cations (except promethium and europium) would be suitable for the analysis of acidic phosphopeptides.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors gratefully acknowledge support from the National Institutes of Health (1R15GM109401A).

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Figure 1.

ETD mass spectra of $[M + Pr + H]^{4+}$ for (A) heat shock protein fragment (hsp) and (B) [pSER95, Ala97]-RII. The following colors are used to label the ions: non-metallated z-ions = red, non-metallated c-ions = blue, metallated z-ions = bright pink, and metallated c-ions = green. Undissociated precursor ion is labeled in purple with a large purple diamond arrow head. Neutral loss products and electron transfer without dissociation (ETnoD) ions are labeled in black. A prime symbol to the left indicates loss of a hydrogen (i.e., ${}^{\prime}c_n = [c_n - H]^+$) and to the right indicates the addition of a hydrogen relative to the cleavage site (i.e., $c_n^{\prime\prime} = [c_n + 2H]^+$).

ETD mass spectrum of $[M + \text{Pr}]^{3+}$ for EGF(988–998). Refer to the Figure 1 caption for an explanation of the color codes and prime symbols.

Figure 3. ETD mass spectrum of $[M + \text{Pr}]^{3+}$ for (A) EDDpSDEEN, (B) EDDpTDEEN, and (C) EDDpYDEEN. Refer to the Figure 1 caption for an explanation of the color codes and prime symbols.

Figure 5.

ETD mass spectra of $[M + \text{Pr} - \text{H}]^{2+}$ for (A) pSAAAAA (pSA5), (B) AApSAAA (A2pSA3), (C) AAAApSA (A4pSA), (D) pSAAAAAAA (pSA7), (E) AAApSAAAA (A3pSA4), and (F) AAAAAApSA (A6pSA). Refer to the Figure 1 caption for an explanation of the color codes and prime symbols.

Table 1.

ESI ion formation and corresponding ETD product ions for acidic phosphorylated biological peptides cationized by Pr(III).

Peptide name	Number of residues	Number of acidic residues ^a	Sequence ^{b,c}	Metallated ion formed by ESI	c _n -ions	z _n - ions
PDGF beta-receptor (pTyrL-5P)	5	2	<i>pY</i> ¹ VPML ⁵	[M + Pr – H] ²⁺	[c _n + Pr] ⁺ , n = 3, 4	- ^d
Human epidermal growth factor receptor (DL-6)	6	4	D ¹ ANE ⁴ <i>pY</i> ⁵ L ⁶	[M + Pr – H] ²⁺	[c _n + Pr] ⁺ , n = 5	[z _n + Pr] ⁺ , n = 4, 5
PDGF beta-receptor (pTyr Y-8P)	8	3	<i>pY</i> ¹ MAPYD ⁶ NY ⁸	[M + Pr – H] ²⁺	[c _n + Pr] ⁺ , n = 6, 7	-
				[M + Pr] ³⁺	[c _n + Pr] ⁺ , n = 5–7	z _n ^{+/+} , n = 2 [z _n + Pr] ⁺ , n = 6, 7
DNA transcription inhibitory peptide	8	8	E ¹ D ² D ³ <i>pS</i> ⁴ D ⁵ E ⁶ E ⁷ N ⁸	[M + Pr – H] ²⁺	[c _n + Pr] ⁺ , n = 6, 7	[z _n + Pr] ⁺ , n = 6, 7
				[M + Pr] ³⁺	c _n ^{+/+} , n = 1–3 [c _n + Pr] ⁺ , n = 5–7 [c _n + Pr] ²⁺ , n = 7	z _n ^{+/+} , n = 2–4 [z _n + Pr] ⁺ , n = 6, 7
DNA transcription inhibitory peptide (pT) analog	8	8	E ¹ D ² D ³ <i>pT</i> ⁴ D ⁵ E ⁶ E ⁷ N ⁸	[M + Pr – H] ²⁺	[c _n + Pr] ⁺ , n = 6, 7 [c _n + Pr] ²⁺ , n = 7	[z _n + Pr] ⁺ , n = 6, 7
				[M + Pr] ³⁺	c _n ^{+/+} , n = 1–4 [c _n + Pr] ⁺ , n = 6, 7 [c _n + Pr] ²⁺ , n = 7	z _n ^{+/+} , n = 2–4 [z _n + Pr] ⁺ , n = 6, 7
DNA transcription inhibitory peptide (pY) analog	8	8	E ¹ D ² D ³ <i>pY</i> ⁴ D ⁵ E ⁶ E ⁷ N ⁸	[M + Pr – H] ²⁺	[c _n + Pr] ⁺ , n = 6, 7	[z _n + Pr] ⁺ , n = 6, 7
				[M + Pr] ³⁺	c _n ^{+/+} , n = 1–3 [c _n + Pr] ⁺ , n = 6, 7	z _n ^{+/+} , n = 2–4 [z _n + Pr] ⁺ , n = 6, 7
Human epidermal growth factor receptor, EGF(988–998)	11	5	D ¹ AD ³ E ⁴ <i>pY</i> ⁵ LIPQQG ¹¹	[M + Pr – H] ²⁺	[c _n + Pr] ⁺ , n = 6	z _n ^{+/+} , n = 5

Peptide name	Number of residues	Number of acidic residues ^a	Sequence ^{b,c}	Metallated ion formed by ESI	c _n -ions	z _n -ions
						[¹ / _{z_n}] ⁺ Pr] ⁺ , n = 7–10
				[M + Pr] ³⁺	c _n ^{/+} , n = 2–4 [¹ / _{c_n} + Pr] ⁺ , n = 5, 6, 8–10 [¹ / _{c_n} + Pr] ²⁺ , n = 10	z _n ^{/+} , n = 2, 3, 5, 6 [¹ / _{z_n} + Pr] ⁺ , n = 7–10 [¹ / _{z_n} + Pr] ²⁺ , n = 9, 10
Heat shock protein fragment (hsp)	14	3	CLNRQLpS ⁷ SGVSE ¹² IR ¹⁴	[M + Pr – H] ²⁺	[¹ / _{c_n} + Pr] ⁺ , n = 11– 13	[¹ / _{z_n} + Pr] ⁺ , n = 11– 13
				[M + Pr] ³⁺	c _n ^{/+} , n = 4–6 [¹ / _{c_n} + Pr] ⁺ , n = 13 [¹ / _{c_n} + Pr] ²⁺ , n = 10, 13	z _n ^{/+} , n = 1–4 [¹ / _{z_n} + Pr] ⁺ , n = 8–13 [¹ / _{z_n} + Pr] ²⁺ , n = 8, 9, 11
				[M + Pr + H] ⁴⁺	c _n ^{/+} , n = 2–6 [¹ / _{c_n} + Pr] ⁺ , n = 7–13	z _n ^{/+} , n = 1–7 [¹ / _{z_n} + Pr] ⁺ , n = 7–13 [¹ / _{z_n} + Pr] ²⁺ , n = 11, 12
RII Phosphopeptide, [pSer ⁹⁵ , Ala ⁹⁷]-cAMP-dependent protein kinase regulatory subunit type II (81–99), [pSER95, Ala97]-RII	19	5	D ¹ LD ³ VPIPGRFD ¹¹ RRVpS ¹⁵ VAAE ¹⁹	[M + Pr] ³⁺	[¹ / _{c_n} + Pr] ⁺ , n = 11, 15–18 [¹ / _{c_n} + Pr] ²⁺ , n = 15–18	z _n ^{/+} , n = 8 [¹ / _{z_n} + Pr] ⁺ , n = 9, 10, 14, 17, 18 [¹ / _{z_n} + Pr] ²⁺ , n = 16
				[M + Pr + H] ⁴⁺	c _n ^{/+} , n = 2, 3, 5, 7–9, 11 [¹ / _{c_n} + Pr] ⁺ , n = 10– 12, 15–18 [¹ / _{c_n} + Pr] ²⁺ , n = 11, 16–18	z _n ^{/+} , n = 3, 4, 8 [¹ / _{z_n} + Pr] ⁺ , n = 9–12, 14, 16– 18

^a Acidic residues refer to the number of aspartic acid (D), glutamic acid (E), phosphorylated amino acid (pS, pT, pY), and C-terminal residues in the peptide sequence. If an aspartic acid or glutamic acid residue is located at the C-terminus, then it is counted as only one acidic residue.

^{*b*} Bold font is used to indicate acidic residues. A number immediately following an acidic residue indicates its position in the peptide sequence.

^{*c*} Bold italicized font is used to indicate phosphorylated amino acid residues. A number immediately following a phosphorylated residue indicates its position in the peptide sequence.

^{*d*} - means that no ions were observed.

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Table 2.

ESI ion formation and corresponding ETD product ions for phosphorylated model peptides cationized by Pr(III).

Peptide sequence	Number of residues	Number of acidic residues ^{a,b}	Metallated ion formed by ESI	c _n -ions	z _n -ions	y _n -ions
<i>pS</i> ^I AAAAA ⁶ (pSA5)	6	2	[M + Pr – H] ²⁺	[c _n + Pr] ⁺ , n = 4, 5	- ^c	
<i>pT</i> ^I AAAAA ⁶ (pTA5)	6	2	[M + Pr – H] ²⁺	[c _n + Pr] ⁺ , n = 4, 5	-	
<i>pY</i> ^I AAAAA ⁶ (pYA5)	6	2	[M + Pr – H] ²⁺	[c _n + Pr] ⁺ , n = 4, 5	-	
AA <i>pS</i> ³ AAA ⁶ (A2pSA3)	6	2	[M + Pr – H] ²⁺	[c _n + Pr] ⁺ , n = 4, 5	[z _n + Pr] ⁺ , n = 5	
AA <i>pT</i> ³ AAA ⁶ (A2pTA3)	6	2	[M + Pr – H] ²⁺	[c _n + Pr] ⁺ , n = 4, 5	[z _n + Pr] ⁺ , n = 5	
AA <i>pY</i> ³ AAA ⁶ (A2pYA3)	6	2	[M + Pr – H] ²⁺	[c _n + Pr] ⁺ , n = 4, 5	[z _n + Pr] ⁺ , n = 5	
AAAA <i>pS</i> ⁵ A ⁶ (A4pSA)	6	2	[M + Pr – H] ²⁺	[c _n + Pr] ⁺ , n = 5	[z _n + Pr] ⁺ , n = 5	
AAAA <i>pT</i> ⁵ A ⁶ (A4pTA)	6	2	[M + Pr – H] ²⁺	[c _n + Pr] ⁺ , n = 5	[z _n + Pr] ⁺ , n = 5	
AAAA <i>pY</i> ⁵ A ⁶ (A4pYA)	6	2	[M + Pr – H] ²⁺	[c _n + Pr] ⁺ , n = 5	[z _n + Pr] ⁺ , n = 5	
<i>pS</i> ^I AAAAAAA ⁸ (pSA7)	8	2	[M + Pr – H] ²⁺	[c _n + Pr] ⁺ , n = 5–7	-	
<i>pT</i> ^I AAAAAAA ⁸ (pTA7)	8	2	[M + Pr – H] ²⁺	[c _n + Pr] ⁺ , n = 5–7	-	
<i>pY</i> ^I AAAA ⁴ AAAA ⁸ (pYA7)	8	2	[M + Pr – H] ²⁺	[c _n + Pr] ⁺ , n = 5–7	-	
AA <i>pS</i> ⁴ AAAA ⁸ (A3pSA4)	8	2	[M + Pr – H] ²⁺	[c _n + Pr] ⁺ , n = 5–7	[z _n + Pr] ⁺ , n = 5–7	
AA <i>pT</i> ⁴ AAAA ⁸ (A3pTA4)	8	2	[M + Pr – H] ²⁺	[c _n + Pr] ⁺ , n = 5–7	[z _n + Pr] ⁺ , n = 5–7	
AA <i>pY</i> ⁴ AAAA ⁸ (A3pYA4)	8	2	[M + Pr – H] ²⁺	[c _n + Pr] ⁺ , n = 5–7	[z _n + Pr] ⁺ , n = 5–7	
AAAAAA <i>pS</i> ⁷ A ⁸ (A6pSA)	8	2	[M + Pr – H] ²⁺	[c _n + Pr] ⁺ , n = 7	[z _n + Pr] ⁺ , n = 5–7	[y _n + Pr] ⁺ , n = 6
AAAAAA <i>pT</i> ⁷ A ⁸ (A6pTA)	8	2	[M + Pr – H] ²⁺	[c _n + Pr] ⁺ , n = 7		[y _n + Pr] ⁺ , n = 5–7
AAAAAA <i>pY</i> ⁷ A ⁸ (A6pYA)	8	2	[M + Pr – H] ²⁺	[c _n + Pr] ⁺ , n = 7	[z _n + Pr] ⁺ , n = 5–7	[y _n + Pr] ⁺ , n = 6

^a Bold font is used to indicate acidic residues. A number immediately following an acidic residue indicates its position in the peptide sequence.

^b Bold italicized font is used to indicate phosphorylated amino acid residues. A number immediately following a phosphorylated residue indicates its position in the peptide sequence.

^c - means that no ions were observed