Fragmentation of Cationized Phosphotyrosine Containing Peptides by Atmospheric Pressure MALDI/Ion Trap Mass Spectrometry

Susanne C. Moyer,* Christopher E. VonSeggern, and Robert J. Cotter

Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, USA

An investigation of phosphate loss from sodium-cationized phosphotyrosine containing peptide ions was conducted using liquid infrared (2.94 μm) atmospheric pressure matrix-assisted laser desorption/ionization (AP MALDI) coupled to an ion trap mass spectrometer (ITMS). Previous experiments in our laboratory explored the fragmentation patterns of protonated phosphotyrosine containing peptides, which experience a loss of 98 Da under CID conditions in the ITMS. This loss of 98 Da is unexpected for phosphotyrosine, given the structure of its side chain. Phosphate loss from phosphotyrosine residues seems to be dependent on the presence of arginine or lysine residues in the peptide sequence. In the absence of a basic residue, the protonated phosphotyrosine peptides do not undergo losses of HPO_3 (Δ 80 Da) nor $\text{HPO}_3 + \text{H}_2\text{O}$ (Δ 98 Da) in their CID spectra. However, sodium cationized phosphotyrosine containing peptides that do not contain arginine or lysine residues within their sequences do undergo losses of HPO_3 (Δ 80 Da) and $\text{HPO}_3 + \text{H}_2\text{O}$ (Δ 98 Da) in their CID spectra. (J Am Soc Mass Spectrom 2003, 14, 581–592) © 2003 American Society for Mass Spectrometry

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Mass spectrometry has become a powerful tool for diagnostic proteomics and is a leading technique for structural determination [1]. The growing recognition that the proteome is not a static entity has placed greater emphasis on identifying post-translational modifications. These modifications are proving to be important regulators in cellular activity and are crucial for a complete understanding of the proteome. Mass spectrometry is the leading analytical technique for the determination of post-translational modifications [2]. As proteomic techniques become more automated, the need for full understanding of potential gas phase fragmentation patterns is vital to rapid identification of structural components [3]. Post-translational modifications prove to be a challenge in the creation of structural databases [2]. Complete understanding of protein function can be gained through careful analysis of post-translational modifications.

Phosphorylation is one of the most common and physiologically important post-translational modifications in proteins and peptides. The ubiquitous nature of phosphorylation in biological systems has necessitated

the development of methods for the identification and characterization of phosphorylation sites. Several groups have studied the gas phase dephosphorylation of phosphorylated peptide and protein ions using electrospray ionization (ESI) or matrix-assisted laser desorption/ionization (MALDI) mass spectrometry [4–17]. Annan and Carr examined phosphopeptide ion fragmentation by postsource decay (PSD) time-of-flight (TOF) mass spectrometry and found that phosphoserine and phosphothreonine containing peptides produced abundant $[\text{MH}-\text{H}_3\text{PO}_4]^+$ ions and less abundant $[\text{MH}-\text{HPO}_3]^+$ ions [5]. Conversely, their PSD data for phosphotyrosine containing peptides showed predominantly $[\text{MH}-\text{HPO}_3]^+$ ions and rarely revealed $[\text{MH}-\text{H}_3\text{PO}_4]^+$ ions. PSD fragmentation patterns revealing losses of either 80 or 98 Da have been used to determine whether an aromatic or aliphatic phosphorylation site is present within a peptide sequence [18].

However, Qin and Chait reported that the MALDI/ITMS CID spectra of protonated phosphotyrosine peptides showed a loss of 98 Da from their molecular ions, corresponding to either a loss of H_3PO_4 or $\text{HPO}_3 + \text{H}_2\text{O}$ [7]. DeGnore and Qin [8] later studied the CID spectra of phosphopeptides by ESI/ITMS. They reported that the loss of phosphate group is charge state dependent and that loss of 98 Da from a phosphotyrosine containing peptide ion was most likely the result of a two-step process involving the loss of HPO_3 followed by the loss of H_2O elsewhere in the peptide.

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Address all reprint requests to R. J. Cotter, Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, 725 N. Wolfe St., B-7 Biophysics Building, Baltimore, Maryland, USA, 21205. E-mail: rcotter@jhmi.edu

*Also of the Department of Chemistry, Johns Hopkins University, Baltimore, MD, USA.

Recently, Metzger and Hoffman [12] proposed two intriguing mechanisms to explain the loss of H_3PO_4 from phosphotyrosine containing peptide ions under MALDI PSD conditions. The first mechanism involves the transfer of HPO_3 to an aspartic acid side chain, followed by a cleavage of H_3PO_4 and subsequent formation of a succinimide. The second mechanism entails protonation of the phosphate group of phosphotyrosine by an arginine residue followed by the loss of H_3PO_4 resulting in a phenyl cation on the tyrosine residue. Later work by Moyer et al. also reported that the CID fragmentation of protonated phosphotyrosine containing peptides resulted in loss of 98 Da, but suggested that this occurs only when there is an arginine or lysine residue present in the peptide sequence [19]. Their results would suggest that the loss of 98 Da from phosphotyrosine containing peptides could be the result of either charge remote or charge directed fragmentation processes, rather than a rearrangement.

The AP-MALDI/ITMS configuration has proven to be useful in obtaining structural information for peptides and protein digests [19–23] as well as for the identification and characterization of posttranslational modifications [23]. Recent work in our laboratory has demonstrated the utility of infrared AP-MALDI/ITMS using an Er:YAG laser (2.94 μm) for the analysis of carbohydrates [24] and posttranslationally modified peptides [25]. The infrared AP-MALDI/ITMS instrument is capable of utilizing liquid matrices, such as glycerol. The glycerol matrix produces protonated, sodiated and potassiated ions as well as sodium and potassium salts.

In the present work, liquid infrared (2.94 μm) AP MALDI was utilized to evaluate the effect of alkali metal cation attachment on the gas phase dephosphorylation patterns of phosphotyrosine containing peptides under collision induced dissociation (CID) conditions. While previous work suggests that phosphate loss from phosphotyrosine residues is dependent on the presence of arginine or lysine residues in the peptide sequence, we now show similar CID fragmentation behavior of sodium and potassium cationized phosphotyrosine-containing peptides.

Experimental Section

Peptides

The peptide SVL(pY)TAVQPNE was purchased from AnaSpec (San Jose, CA). All other peptides were synthesized at the Peptide Synthesis Core Facility (Johns Hopkins University School of Medicine, Baltimore, MD). All peptides were dissolved in 18 M Ω water to a concentration of 100 μM .

Liquid AP-MALDI Matrix

Glycerol was obtained from Sigma (St. Louis, MO) and used without further purification.

Liquid Infrared AP-MALDI/ITMS

Mass spectra were obtained on a LCQ quadrupole ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) equipped with a modified commercial atmospheric pressure matrix-assisted laser desorption/ionization (AP-MALDI) source (Mass Technologies, Burtonsville, MD). The source is equipped with a Bioscope UV+ laser (Bioptric Lasersysteme, Berlin, Germany) that has both a Nd:YAG laser at 355 nm and an Er:YAG laser at 2.94 μm . The laser was focused onto the MALDI target with a sapphire lens ($F = 150$ mm). For this work, the Er:YAG laser was utilized. The Er:YAG laser generated approximately 30 ns pulses at 5 Hz with approximately 400 μJ /pulse. The laser was run asynchronously with the trapping cycle and spectra were acquired at 300 ms per scan. A potential of 2.5 kV was applied between the sample target and the inlet capillary. The transfer capillary temperature was set to 200 $^\circ\text{C}$. Samples were prepared by mixing approximately 1 μL of glycerol with 1 μL of analyte solution on the AP-MALDI target plate. The resulting mixture was then analyzed in liquid form by infrared AP MALDI/ITMS.

Results and Discussion

In the present study, the low energy gas phase CID fragmentations of phosphotyrosine containing peptides resulting from sodium and potassium attachment were studied. In previous work, it was demonstrated that protonated phosphotyrosine containing peptides would undergo a loss of 98 Da, corresponding to a loss of $\text{HPO}_3 + \text{H}_2\text{O}$, if the peptide sequence also contained an arginine or lysine residue [19]. If the peptide contained only one arginine residue in its sequence, losses of both 80 and 98 Da, corresponding to losses of HPO_3 and $\text{HPO}_3 + \text{H}_2\text{O}$, respectively, were evident in the CID spectra. However, peptide sequences with a lysine residue or two or more arginine residues would display only the loss of 98 Da. Conversely, protonated phosphotyrosine containing peptides that did not possess a basic arginine or lysine residue did not undergo losses of 80 or 98 Da in their CID spectra. This was even the case in peptides that contained several acidic residues (aspartic or glutamic acids) in their sequence. This finding is in contrast with the mechanism postulated by Metzger and Hoffman [12], where a loss of H_3PO_4 (98 Da) from a phosphotyrosine containing peptide proceeds through transfer of HPO_3 from the phosphotyrosine side chain to an aspartic acid side chain followed by elimination of H_3PO_4 from the aspartic acid side chain and the resulting succinimide formation. However, other data presented by Metzger and Hoffman displayed compelling evidence that the loss of 98 Da from a phosphotyrosine containing peptide was the result of a loss of HPO_3 from the phosphotyrosine residue and H_2O from elsewhere in the

Figure 1. (a) MS^2 of the $[M+H]^+$ ion of SVL(pY)TAVQPNE at m/z 1300. No loss of phosphate is observed in this spectrum, (b) MS^3 of the b_8 ion of SVL(pY)TAVQPNE at m/z 942. No loss of phosphate is observed in this spectrum, (c) MS_2 of the $[M+Na]^+$ ion of SVL(pY)TAVQPNE at m/z 1322. The asterisks (*) indicate a sodiated ion. Losses of 80 and 98 Da from the sodium cationized parent ion, corresponding to losses of HPO_3 and HPO_3+H_2O , respectively, are observed

peptide. In that work, they deuterated the phenyl ring of a phosphotyrosine residue. A loss of 99 Da in the fragmentation spectrum could indicate that H_2DPO_4 was eliminated from the phosphotyrosine side chain, whereas a loss of 98 Da would signify losses of HPO_3 from the phosphotyrosine side chain and H_2O from another location within the peptide; their PSD data revealed a loss of 98 Da.

Our previous findings suggest that arginine and lysine residues may play a role in the elimination of HPO_3 from a phosphotyrosine residue via a charge remote or charge induced fragmentation process [19]. Charge-remote fragmentation is described as a class of gas-phase decompositions that occur at a location that is physically remote from the charge site [26–29]. Charged-induced fragmentations occur when a direct interaction between the charge site location and the site of fragmentation initiates fragmentation at that site [26]. According to Gross [30], charge-remote fragmentation

usually occurs under high energy CID conditions, however it is possible for these types of reactions to occur under low energy CID or metastable decay processes.

Several researchers have noted losses of HPO_3 from protonated phosphotyrosine peptides in low energy CID or PSD spectra [5,7–9,12,14]. However these peptides all contained an arginine or lysine in their sequences. Fragmentation of a protonated peptide proceeds differently when a peptide sequence is devoid of arginine or lysine residues. In previous work [19], we showed that the protonated peptides, AALIEDA E(pY)AAAG, AAAAADAA(pY)AAAA and SV L(pY)TAVQPNE, as well as the tryptic digest fragment, V(pY)IHPF, did not lose HPO_3 in their CID fragmentation spectra. Figures 1a and 1b show the MS/MS and the MS^3 spectra of the protonated peptide, SV L(pY)TAVQPNE. HPO_3 is not eliminated from the protonated peptide ion under low-energy CID conditions. When the $[M+Na]^+$ ion at m/z 1322 (Figure 1c)

Figure 2. (a) MS² of the [M+Na]⁺ ion of AAAAADAA(pY)AAAA at *m/z* 1180. The asterisks (*) indicate a sodiated ion. (b) MS³ of the sodiated *y*₇ ion of AAAAADAA(pY)AAAA at *m/z* 710. The asterisks (*) indicate a sodiated ion.

and the [M+K]⁺ ion at *m/z* 1338 (data not shown) of SVL(pY)TAVQPNE are analyzed by CID, fragment ions corresponding to losses of 80 and 98 Da (HPO₃ and HPO₃ + H₂O, respectively) are observed.

Figure 2a is the MS/MS spectrum of the [M+Na]⁺ ion of AAAAADAA(pY)AAAA at *m/z* 1180. The two predominant fragment ions in this spectrum are the [M+Na-98]⁺ ion at *m/z* 1082 and the sodiated *y*₇ ion at

Table 1. CID Fragmentations of C-fragmentations of C-terminally amidated phosphotyrosine peptides

	AAA(pY)AAA-NH ₂	AAAYAAA-NH ₂
MS ² of [M + H] ⁺	ΔNH_3 , $\Delta(\text{NH}_3 + \text{H}_2\text{O})$, x_6 , b_6 , a_6 or x_6 , y_5 or c_5 , b_5 , y_4 or c_4 , b_4 or A(pY)AA or AA(pY)A, A(pY)A or AA(pY) or (pY)AA	ΔNH_3 , $\Delta(\text{NH}_3 + \text{H}_2\text{O})$, ΔCONH_2 , x_6 , x_6^- , NH_3 , b_6 , y_5 , b_5 , y_4
MS ³ of [M + H] ⁺	$\Delta\text{H}_2\text{O}$, x_6 , x_6^- , NH_3 , b_6 , x_5^- , NH_3 , b_5 or AA(pY)AA, x_4^- , NH_3 , A(pY)AA or AA(pY)A, {A(pY)AA or AA(pY)A}-30, A(pY)A or AA(pY) or (pY)AA, {A(pY)A or AA(pY) or (pY)AA}-30, A(pY) or (pY)A, x_3 , x_3^- , NH_3 , b_3 {A(pY) or (pY)A}-30	$\Delta\text{H}_2\text{O}$, x_6 , x_6^- , NH_3 , b_6 , b_6^- , H_2O , a_6^- , NH_3 , b_5 , a_5 , a_5^- , NH_3 , y_4 , b_4 , a_4 , AAY or AYA or YAA, x_3 , x_3^- , NH_3 , b_3
MS ² of [M + Na] ⁺	ΔNH_3 , $\Delta\text{H}_2\text{O}$, $\Delta(\text{NH}_3 + \text{H}_2\text{O})$, x_6 , $\Delta\mathbf{80}$, y_6 , b_6 , $\Delta\mathbf{98}$, x_5 or a_6 , x_6^- , $\mathbf{80}$, y_5 or c_5 , b_5 , a_5 or x_4 , y_4 or c_4 , b_4 or A(pY)AA or AA(pY)A, a_5^- -80 or x_4^- -80, a_5^- -98 or x_4^- -98	ΔNH_3 , $\Delta\text{H}_2\text{O}$, $\Delta(\text{NH}_3 + \text{H}_2\text{O})$, ΔCONH_2 , x_6 , y_6 , b_6 , a_6 , y_5 , b_5 , a_5 , y_4 , b_4 , a_4
MS ² of [M + K] ⁺	$\Delta\text{H}_2\text{O}$, $\Delta(\text{NH}_3 + \text{H}_2\text{O})$, x_6 , y_6 , $\Delta\mathbf{80}$, b_6 , $\Delta\mathbf{98}$, a_6 or x_5 , c_5 or y_5 , b_5 , a_5 or x_4 , $\mathbf{x_6-80}$, c_4 or y_4 , b_4 , a_4	$\Delta\text{H}_2\text{O}$, $\Delta(\text{NH}_3 + \text{H}_2\text{O})$, ΔCONH_2 , b_6 , y_5 , b_5 , a_6 , y_4 , b_4 , c_4
MS ² of [M - H + 2Na] ⁺	$\Delta\text{H}_2\text{O}$, $\Delta\mathbf{NaPO_3}$, $\Delta(\mathbf{NaPO_4} + \text{H}_2\text{O})$, y_4 or c_4 , a_5 or x_4 , b_4	not available

m/z 710. The sodiated y_7 ion is a result of cleavage C-terminal to the aspartic acid residue. Although a sodiated b_6 ion is present at m/z 493, it is of relatively low intensity, suggesting that cleavage at this site favors rearrangement to form the sodiated y_7 over the sodiated b_6 ion. Beauchamp and coworkers showed that sodiated peptides undergo cleavage C-terminal to an aspartic acid residue [31]. They proposed a zwitterionic peptide that forms a salt-bridge between the carboxylate of the aspartic acid and the sodium ion, leaving a “mobile proton” to transfer within the peptide, facilitating charge-induced fragmentation under CID conditions. The mobile proton model, developed by Wysocki and coworkers [32–35], describes charge-induced fragmentations of protonated peptides that result from rapid intramolecular proton transfers occurring upon collisional activation of the molecule.

Figure 2b is the MS³ spectrum of the sodiated y_7 ion of AAAADAA(pY)AAA at m/z 710. This ion was selected for fragmentation to determine whether or not a loss of 98 Da would be observed in a peptide fragment that did not contain an acidic residue. The major fragment ions observed in this spectrum correspond to the loss of H_2O at m/z 692, the loss of HPO_3 at m/z 630 and the loss of $\text{HPO}_3 + \text{H}_2\text{O}$ (98 Da) at m/z 612. In this case, it is likely that H_2O is eliminated from the C-terminal carboxy-group via a retro-Koch type of reaction as described by Harrison et al. [36, 37].

The peptides, AAAYAAA-NH₂ and AAA(pY)AAA-NH₂ were studied in order to evaluate the differences in fragmentation between non-phosphorylated and phosphorylated protonated and metal-cationized peptide ions. The C-termini of these peptides were amidated in order to prevent direct peptide dehydration from the C-terminus. A summary of the CID fragmentations of these peptides is presented in Table 1. Figure 3a is the MS/MS spectrum of the [M+H]⁺ ion of AAAYAAANH₂ at m/z 607. This spectrum demonstrates that the protonated peptide ion does not undergo extensive backbone fragmentation. In addition, the fragmentation spectrum does

not reveal a direct loss of H_2O from the peptide. The major fragment ion at m/z 590 is a result of a loss of NH_3 from the peptide ion.

Figure 3b is the MS/MS spectrum of the [M+Na]⁺ ion at m/z 629. The degree of backbone fragmentation of this ion is increased over that of the protonated ion. In addition, the most intense fragment peak, located at m/z 611, suggests that dehydration of this molecule occurs as a result of loss of oxygen from the peptide backbone. Water loss from the tyrosine residue is unlikely, due to the resonance stabilization of the phenolic oxygen. These findings are in agreement with those of Gaskell et al., describing dehydration of peptide ions under CID conditions [38], where loss of water from the peptide backbone is common, while dehydration from a tyrosine side chain is unlikely. Reid and coworkers [39] later went on to describe the mechanism of water loss from the peptide backbone to occur via a retro-Ritter reaction, where protonation of the carbonyl oxygen on an amide followed by proton transfer from the amide nitrogen to the protonated carbonyl resulted in the subsequent loss of water and the formation of a nitrilium ion. The presence of the sodiated fragment ions, y_4 at m/z 416, b_4 at m/z 399 and a_4 at m/z 371, as well as the presence of low intensity sodiated fragment ions c_3/y_3 at m/z 253 and b_3 at m/z 237 suggests that the sodium resides on the molecule directly C-terminal or N-terminal to the tyrosine residue.

Similar fragmentation patterns are evident in the MS/MS spectrum of the [M+K]⁺ shown in Figure 3c. Either the peptide is preferentially cationized at a position close to the tyrosine residue during ionization, or a process of rearrangements occur during CID that promote backbone fragmentation until the cation reaches a position near the tyrosine residue. Tandem mass spectrometry of alkali metal cationized peptides has been described extensively by Gross [40, 41], Adams [42,43], Russell [44,45], and Tang [46]. Russell [45] suggested that the sodium ion interacted with the amino terminus or the amide nitrogen in a small tyrosine containing peptide. Gross [40, 41] and Tang

Figure 3. (a) MS² of the [M+H]⁺ ion of AAAYAAA-NH₂ at *m/z* 607. (b) MS² of the [M+Na]⁺ ion of AAAYAAA-NH₂ at *m/z* 629. The asterisks (*) indicate a sodiated ion. (c) MS² of the [M+K]⁺ ion of AAAYAAA-NH₂ at *m/z* 645. The asterisks (*) indicate a potassiated ion.

[46] proposed that metal cation binding occurs at the carbonyl oxygens of the C-terminus and the adjoining amino acid residue; upon collisional activation, rearrangement of the alkali metal cationized peptide and subsequent expulsion of the C-terminal residue ensues. Furthermore, Gross [41] described metal cationized peptide fragments where the cation was stabilized by aromatic sidechains within the peptide sequence.

Figure 4a is the MS/MS spectrum of the [M+H]⁺ ion of AAA(pY)AAA-NH₂ and Figure 4b is the MS³ spectrum of the [M+H-NH₃]⁺ fragment ion. Although several peaks, corresponding to backbone fragments are present, there is no evidence of loss of HPO₃ from the phosphotyrosine residue, thus accentuating the inherent stability of this particular site under low energy CID conditions. Scheme 1a summarizes the major fragmentations that occur in the CID spectra of the [M+H]⁺ and [M+H-NH₃]⁺ ions of AAA-(pY)AAANH₂.

However, when the MS/MS spectrum of the [M+Na]⁺ ion of AAA(pY)AAA-NH₂ was acquired

(Figure 5a), losses of H₂O (*m/z* 691), HPO₃ (*m/z* 629) and HPO₃ + H₂O (*m/z* 611) were observed. There are no major differences in the types of backbone fragmentation observed for the sodiated ions of the non-phosphorylated and the phosphorylated peptides. But, the presence of sodiated (*a*₅/*x*₄—80) and (*a*₅/*x*₄—98) ions supports the hypothesis that the sodium ion initially orients itself or rearranges to a position near the phosphotyrosine residue. Scheme 1b summarizes the major fragmentations present in the MS/MS spectrum of the [M+Na]⁺ ion of AAA(pY)AAA-NH₂.

Figure 5b is the MS/MS spectrum of the [M-H+2Na]⁺ ion of AAA(pY)AAA-NH₂ at *m/z* 731. As seen in this spectrum, major fragmentations associated with losses of H₂O (*m/z* 713), NaPO₃ (*m/z* 629) and H₂O + NaPO₃ (*m/z* 611) are generated. From this data, it can be surmised that the second sodium is located on the phosphotyrosine side chain in the form of a sodium salt with a deprotonated phosphate oxygen. In addition, backbone fragmentation of this ion essentially does not occur. There are a few very minor singly charged, doubly sodiated ions: *b*₄ at *m/z* 501, *y*₄/*c*₄ at *m/z* 518 and

Figure 4. (a) MS² of the [M+H]⁺ ion of AAA(pY)AAA-NH₂ at m/z 687. (b) MS³ of the [M+H]⁺ ion of AAA(pY)AAA-NH₂ at m/z 670.

a_4/x_4 at m/z 544. These fragmentations are presented in Scheme 1c.

Figure 5c is the MS/MS spectrum of the [M+K]⁺ ion of AAA(pY)AAA-NH₂ at m/z 725. Fragment ions in this

spectrum with the greatest intensity correspond to the loss of H₂O at m/z 707 and to the potassiumated (x_6-80) ion at m/z 600. The presence of the minor potassiumated fragmentation ions of (b_4-98) at m/z 397, (pY) at m/z 300

Scheme 1. (a) CID fragments of the [M+H]⁺ ion of AAA(pY)AAA-NH₂. (b) CID fragments of the [M+Na]⁺ ion of AAA(pY)AAA-NH₂. Enhancement of backbone fragmentation, as well as loss of HPO₃ from the phosphotyrosine side chain is evident. (c) CID fragments of the [M-H+2Na]⁺ ion of AAA(pY)AAA-NH₂. Addition of the second sodium causes a reduction in backbone fragmentation. Loss of 102 Da, corresponding to a loss of NaPO₃, identifies the location of the second sodium to be on the phosphate group of the phosphotyrosine side chain.

Figure 5. (a) MS² of the [M+Na]⁺ ion of AAA(pY)AAA-NH₂ at *m/z* 709. The asterisks (*) indicate a sodiated ion. (b) MS² of the [M-H+2Na]⁺ ion of AAA(pY)AAA-NH₂ at *m/z* 731. The asterisks (*) indicates a sodiated ion and a double asterisks (**) indicates a singly charged doubly sodiated ion. (c) MS² of the [M+K]⁺ ion of AAA(pY)AAA-NH₂ at *m/z* 725. The asterisks (*) indicate a potassiated ion.

and (*b*₃—H₂O) at *m/z* 234, suggests the initial presence or the rearrangement of the potassium to a position near the phosphotyrosine residue.

The gas-phase fragmentation of HPO₃ (80 Da) and HPO₃ + H₂O (98 Da) from of sodium and potassium cationized phosphotyrosine peptides is similar to that observed in the CID spectra of protonated phosphotyrosine peptides containing arginine or lysine residues. The data suggests that this is a loss of HPO₃ from the phosphotyrosine residue and H₂O from the peptide. It is likely that the CID fragmentations associated with backbone cleavage and dehydration of the peptide ions are the result of charge-induced fragmentations initiated by a "mobile proton". Backbone cleavages in protonated peptides typically result from protonation at the site of cleavage [34]. Loss of water from the peptide is likely to occur via a retro-Ritter reaction, as described by Reid et al. [39] or a retro-Koch reaction described by Harrison and

coworkers [36,37] and by Hunt et al. [47]. These reactions require protonation of either a carbonyl oxygen or a C-terminal carboxylic acid, with dehydration occurring when a mobile proton is transferred to the site of the protonated oxygen [39].

We propose that loss of HPO₃ from the alkali metal cationized phosphotyrosine containing peptide is due to the close proximity of the metal cation to the phosphotyrosine residue. Collisional activation of the phosphotyrosine containing peptide in the presence of a charge, in this case sodium or potassium, favors fragmentation between the phenolic oxygen and the phosphorous by proton transfer from a phosphate oxygen to the phenolic oxygen and subsequent elimination of the neutral HPO₃. Schemes 2a and 2b present two possible coordinations of a sodium ion with a phosphotyrosine containing peptide. The fragment ion spectrum of alkali metal cationized peptide, AAAYAAA-NH₂, showed a major loss of H₂O from

Scheme 2. (a) Possible orientation of a sodium ion on a phosphotyrosine containing peptide. Interaction of the sodium ion with a carbonyl oxygen is stabilized by the aromatic ring of the phosphotyrosine side chain. (b) Possible orientation of a sodium ion on a phosphotyrosine containing peptide. Interaction of the sodium ion with a carbonyl oxygen and an amide nitrogen with stabilization by the aromatic ring of the phosphotyrosine side chain. The interaction with both the carbonyl oxygen and the amide nitrogen could result in a proton residing on the peptide backbone.

the peptide ion. This would suggest that the losses of 80 and 98 Da observed in the alkali metal cationized peptide, AAA(pY)AAA-NH₂, would be the result of loss of HPO₃ and HPO₃ + H₂O, respectively, rather than the concerted loss of H₃PO₄ (98 Da) from the phosphotyrosine residue.

Conclusions

Sodium and potassium cationized phosphotyrosine peptides undergo fragmentation in their CID spectra

similar to that observed in the CID spectra of protonated phosphotyrosine peptides containing arginine or lysine residues. In many cases the loss of 98 Da is observed. This type of fragmentation pattern proved to be confusing as it corresponds to a loss of H₃PO₄, a fragmentation typically associated with dephosphorylation of an aliphatic side chain (serine and threonine). The data suggests that loss of 98 Da from aromatic phosphorylation is actually a loss of HPO₃ from the phosphotyrosine residue and H₂O from the peptide. It is likely that the fragmentations associated with backbone cleavage and dehydration of the peptide ions are the result of charge-induced fragmentations initiated by a "mobile proton." Loss of water from the peptide is likely to occur via a retro-Ritter reaction, as described by Reid et al. [39] or a retro-Koch reaction described by Harrison and coworkers [36,37] and by Hunt et al. [47]. We propose that loss of HPO₃ from the phosphotyrosine residue is the result of the proximity of the alkali metal ion to the phosphotyrosine residue, where the presence of a charge, in this case a sodium or potassium ion, favors CID fragmentation between the phenolic oxygen and the phosphorous. This fragmentation likely occurs due to an intramolecular proton transfer from an acidic hydrogen located on a phosphate oxygen to the phenolic oxygen, resulting in subsequent elimination of the neutral HPO₃.

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