Observation of Phosphorylation Site-Specific Dissociation of Singly Protonated Phosphopeptides

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In ultraviolet photodissociation of phosphopeptide ions with a basic residue (arginine, lysine, or histidine) at the N-terminus, intense $a_n - 97$ peaks were observed. These ions were formed by cleavage at phosphorylated residues only. For multiply phosphorylated peptides, this site-specific cleavage occurred at every phosphorylated residue. H/D exchange studies showed that $a_n - 97$ was formed by H_3PO_4 loss from $a_n + 1$ radical cations. The site-specificity of phosphate loss observed here is in contrast to the nonspecific phosphate loss from b_n and y_n reported previously. Characteristics of the reaction and its potential utility for phosphopeptide analysis are discussed. (J Am Soc Mass Spectrom 2010, 21, 53–59) © 2010 American Society for Mass Spectrometry

Tandem mass spectrometry [1–3] is widely used for structural and mechanistic studies of protonated peptides generated by matrix-assisted laser desorption/ionization (MALDI) [4] and electrospray ionization (ESI) [5]. The internal energy acquired by a precursor ion at the time of its formation can be the driving force for its dissociation such as in post-source decay (PSD) [6]. Dissociation can be further enhanced by using ion activation techniques such as collisionally activated dissociation (CAD) [3, 7], ultraviolet photodissociation (UV-PD) [8, 9], electron capture dissociation (ECD) [10], and electron-transfer dissociation (ETD) [11].

Tandem mass spectra for singly protonated peptides—to be called peptide ions—are dominated by b and y type product ions (see references [12] and [13] for fragment ion notation) and their consecutive dissociation products. Notable exceptions are the spectra for peptide ions with arginine recorded at high internal energy such as in high-energy CAD and UV-PD. Here, new series of sequence ions, viz. (a_n, d_n) or (x_n, v_n, w_n) for peptide ions with arginine at the N- or C-termini, respectively, become dominant. Biemann [13] suggested that dissociation at high internal energy is initiated by C_α -CO homolytic cleavage, generating $a_n + 1$ or $x_n + 1$ radical cations when arginine is at the N- or C-terminus, respectively. $a_n + 1$ is thought to further dissociate to a_n and d_n , and $x_n + 1$ to x_n , v_n , and w_n .

Phosphorylation of serine (S), threonine (T), and tyrosine (Y) residues in proteins is the key mode of signal transduction and amplification for eukaryotes [14, 15]. Even though ECD and ETD are regarded as the most powerful mass spectrometric techniques for phos-

phoprotein and phosphopeptide analysis, other techniques such as PSD and CAD are also used [16–19]. One of the main problems with the latter techniques is that intact sequence ions such as b_n and y_n are often missing or appear weakly for S/T-phosphorylated peptide ions. Instead, dephosphorylated sequence ions such as $b_n - H_3PO_4$ and $y_n - H_3PO_4$ appear prominently [20].

Recently, we studied dissociation of phosphopeptide ions using photodissociation multi-stage (MS^n) time-of-flight (TOF) mass spectrometry [21]. Through reaction intermediate monitoring with PD- MS^3 , dephosphorylated sequence ions were found to be formed by consecutive reactions mainly via the corresponding intact sequence ions, e.g., $MH^+ \rightarrow b_n \rightarrow [b_n - H_3PO_4]^+$. The second step of such a reaction, viz. dephosphorylation, was found to occur regardless of the position of the phosphorylated residue. Hence, even though the appearance of $[b_n - H_3PO_4]^+$ is an indication that b_n is a phosphorylated sequence ion, it alone is not useful in pinpointing the position of the phosphorylated residue.

In PD- MS^2 spectra for phosphopeptide ions with a basic residue at the N-terminus, we observed some prominent peaks assignable to $a_n - 97$. Investigation for a variety of phosphopeptides utilizing H/D exchange has found that these peaks are formed by H_3PO_4 loss from $a_n + 1$, which, in turn, is formed by C_α -CO cleavage of peptide ions. More importantly, $a_n - 97$ has been found to form only when the phosphate group is at the C-terminal of $a_n + 1$, a case of site-specific reaction. The results are reported in this article.

Experimental

A homebuilt MALDI-PD tandem TOF was used to record PSD and PD spectra of phosphopeptide ions.

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Product ions appearing in PD spectrum (Figure 1b) are rather similar to those in PSD. $[M + H - H_2O]^+$ in this spectrum appears as a negative peak because its intensity in the laser-on spectrum is weaker than in the laser-off spectrum (PSD). This can occur when $[M + H - H_2O]^+$ undergoes PD itself; PSD product ions move with the same velocity as their precursor ion. We have mentioned earlier that C_α -CO homolytic cleavage to $a_n + 1$ and its further dissociation to a_n and d_n are characteristic high-energy channels for peptide ions with arginine at the N-terminus. In agreement with this spectral correlation, $a_n + 1$ ($n = 1, 2$) and d_{na} ($n = 2, 3$) are present in PD spectrum. Here, d_{na} represents d_n formed by OX ($X = H$ or $PO(OH)_2$) loss from $a_n + 1$. Even though a_n ($n = 1-3$) ions also appear in the spectrum, it is not certain whether each a_n is formed via

Figure 1. (a) PSD and (b) 193 nm PD spectra of [RTpTST + H]⁺ formed by DHB-MALDI. Identities of product ions consistent with H/D exchange results are marked.

$a_n + 1$ because a product ion with the same chemical composition, but probably with a different structure, can also be formed by CO loss from b_n [27]. Let us denote these two different a_n ions, those from $a_n + 1$ and b_n , as a_n^h and a_n^l , respectively. Generation of a_n^h from $a_n + 1$ is shown in Scheme 1. a_n^h and a_n^l can be distinguished by H/D exchange because the hydrogen lost in the formation of the latter is exchangeable while that of the former is not [25]. Splitting of the a_2 peak in PD into a_2^l and a_2^h upon H/D exchange is shown in Figure 2a. Even though a_2^h is weaker than a_2^l in this case, strong intensity of $a_2 + 1$ suggests that the high-energy dissociation channel is competing effectively with the low-energy one.

There was one strong peak in PD spectrum that could not be easily assigned, viz. the peak at m/z 314.2. After checking various possible chemical compositions with the mass 314.2 Da, we concluded that it was probably $a_3 - 97$. If the ion is formed via loss of 97—the most likely candidate for 97 is $\text{NH}_3 + \text{HPO}_3$ —from a_3 , there are two possibilities for $a_3 - 97$, namely $a_3^l - \text{NH}_3 - \text{HPO}_3$ and $a_3^h - \text{NH}_3 - \text{HPO}_3$. They will be called Structures 1 and 2, respectively. Alternatively, $a_3 - 97$ may actually be $a_3 + 1 - 98$, viz. $a_3 + 1 - \text{H}_3\text{PO}_4$ (or, might be $a_3 + 1 - \text{HPO}_3 - \text{H}_2\text{O}$). In our attempt to distinguish the three cases by H/D exchange, we realized that upon H/D exchange, $a_3 + 1$ might lose either D_3PO_4 or HD_2PO_4 —two hydrogens in the phosphate group, $\text{OPO}(\text{OH})_2$, are exchangeable. Even though we do not know the structures of the product ions formed by D_3PO_4 or HD_2PO_4 losses, they will be called Structures 3 and 4, respectively. Upon H/D exchange, the m/z ratios of the structures 1, 2, 3, and 4 become 321.2, 322.2, 323.3, and 324.3, respectively, and hence can be distinguished. The $a_3 - 97$ region in 193 nm PD spectrum of $[\text{RTpTST} + \text{H}]^+$ after H/D exchange is shown in Figure 2b. Clearly $a_3 + 1 - \text{D}_3\text{PO}_4$, i.e., Structure 3, is the main contributor to the original $a_3 - 97$ peak, even though other structures also contribute.

It is to be emphasized that $a_4 - 97$ does not appear in PD spectrum ($a_1 - 97$ and $a_2 - 97$ cannot be formed because neither $a_1 + 1$ nor $a_2 + 1$ contains a phosphate group). This suggests that $a_n - 97$ is generated only from $a_n + 1$ formed by $\text{C}_\alpha\text{-CO}$ cleavage at the C-terminal of the phosphorylated residue (for brevity, this will be called $\text{C}_\alpha\text{-CO}$ cleavage at the phosphorylated residue). It is to be mentioned that $a_3 - 97$ appears in PSD spectrum (Figure 1a) also, even though as an extremely weak peak. Finally, it is to be mentioned that

Figure 2. (a) a_2 and (b) $a_3 - 97$ spectral regions of 193 nm PD spectrum of $[\text{RTpTST} + \text{H}]^+$ after H/D exchange. The extent of H/D exchange for the precursor ion estimated from its isotopic pattern in MALDI spectrum was 98.5%.

the trend found for $[\text{RTpTST} + \text{H}]^+$ was also observed for other peptide ions with arginine at the N-terminus investigated in this work, viz. $[\text{RAPSPVA} + \text{H}]^+$ and $[\text{RRAPSPA} + \text{H}]^+$ (PD spectra in the Supplemental Data, which can be found in the electronic version of this article).

$[\text{RGpTpTIT} + \text{H}]^+$ and $[\text{RLpTpSTpSSF} + \text{H}]^+$

One hundred ninety-three nm PD spectra of these peptide ions are shown in Figure 3. The peaks at m/z 270.1 and 451.0 in PD spectrum for $[\text{RGpTpTIT} + \text{H}]^+$ (Figure 3a) are due to $a_3 + 1 - \text{H}_3\text{PO}_4$ and $a_4 + 1 - \text{H}_3\text{PO}_4$, respectively, as confirmed through H/D exchange. It is to be noted that $a_5 - 97$, that cannot be formed by the site-specific reaction, is absent in the spectrum. Similarly, the peaks at m/z 326.2, 493.1, and 761.0 in PD spectrum for $[\text{RLpTpSTpSSF} + \text{H}]^+$ (Figure

Scheme 1. Formation of a_n^h from $a_n + 1$.

Figure 3. (a) 193 nm PD spectrum for $[\text{RGpTpTIT} + \text{H}]^+$, (b) 193 nm, and (c) 266 nm PD spectra for $[\text{RLpTpSTpSSF} + \text{H}]^+$.

3b) are due to $a_3 + 1 - \text{H}_3\text{PO}_4$, $a_4 + 1 - \text{H}_3\text{PO}_4$, and $a_6 + 1 - \text{H}_3\text{PO}_4$, respectively. Here again, $a_5 - 97$ and $a_7 - 97$ that can not be formed by the site-specific reaction are absent. We also recorded 266 nm PD spectrum for $[\text{RLpTpSTpSSF} + \text{H}]^+$ (Figure 3c). $a_n - 97$ ($n = 3, 4$, and 6) peaks appear in this spectrum also, even though with weaker intensities than in 193 nm PD, indicating that the mechanism for their formation is unaffected by the excited-state accessed by photoabsorption (amide backbone excitation at 193 nm versus aromatic side-chain excitation at 266 nm).

$[\text{KApSpSSG} + \text{H}]^+$, $[\text{HTpTTTK} + \text{H}]^+$, and $[\text{HTpTTTR} + \text{H}]^+$

Lysine and histidine are weaker bases than arginine. For unphosphorylated peptide ions with lysine or histidine, but without arginine, it is well known that $\text{C}_\alpha\text{-CO}$ cleavages do not effectively compete with b/y channels. Figure 4a shows 193 nm PD spectrum for $[\text{KApSpSSG} + \text{H}]^+$. Some a_n ions appear prominently in this spectrum. A H/D exchange experiment showed that these ions were formed mostly via b_n , not via $a_n +$

Figure 4. 193 nm PD spectra for (a) [KApSpSSG + H]⁺, (b) [HTpTTTK + H]⁺, and (c) [HTpTTTR + H]⁺.

1, in agreement with the spectral correlation mentioned above. Similar trend was observed in PD of a phosphopeptide ion with histidine at the N-terminus, [HTpTTT + H]⁺ (spectrum shown in the Supplemental Data). In PD spectra for phosphopeptide ions with lysine or histidine at the N-terminus, however, $a_n - 97$ ions formed by cleavage at phosphorylated residues

appeared prominently, such as $a_3 - 97$ and $a_4 - 97$ in Figure 4a. H/D exchange experiments identified these as $a_n + 1 - H_3PO_4$. On the other hand, most of the peaks ($a_n + 1$, a_n^h , and $a_n - 97$) traceable to C_α -CO cleavage at unphosphorylated residue appear in Figure 4a. That is, C_α -CO cleavages at phosphorylated residues effectively compete with b/y channels, while

those at unphosphorylated residues do not. $a_n - 97$ peaks appear prominently even when lysine and/or histidine residues are present at both ends of a phosphopeptide ion, as demonstrated for [HTpTTTK + H]⁺ in Figure 4b. Namely, it seems that $a_n - 97$ can be formed abundantly whenever there is a basic residue to the N-terminal side of a phosphorylated residue.

Observations presented so far suggest that one role of the basic residue at the N-terminus is to retain the additional (ionizing) proton such that the N-terminal side product in C_α-CO cleavage appears as the charged species, viz. $a_n + 1$. As a test, we recorded 193 nm PD spectrum (Figure 4c) for [HTpTTTR + H]⁺. Here again, $a_3 - 97$ appears at m/z 295.0. However, its intensity is very weak, in agreement with the fact that arginine is far more basic than histidine and hence can better retain the additional proton. For the same reason, v_n , x_n , y_n and dephosphorylated sequence ions x_n^* and y_n^* dominate the spectrum. The same trend was observed for other phosphorylated peptide ions with arginine at the C-terminus, [VAApSIR + H]⁺ and [VAATpSR + H]⁺, studied in a previous work [21]. v_n and x_n are presumably formed via $x_n + 1$, which is the product of C_α-CO cleavage with charge retention at the C-terminus. Unlike phosphopeptide ions with a basic residue at the N-terminus, however, the site-specific dephosphorylation of $x_n + 1$ —viz. formation of $x_n - 97$ —was not observed in these cases.

Discussion

We have suggested that retaining the additional proton in the N-terminal product of C_α-CO cleavage is an important role of the basic residue at the N-terminus in this site-specific reaction. For phosphopeptide ions without any basic residue, the additional proton would be mobile and may have a finite chance to be at the N-terminal side of a phosphorylated residue. Then, site-specific formation of $a_n - 97$ might occur in such cases also, just as for [HTpTTTK + H]⁺. To check such a possibility, we re-examined PD spectra for phosphopeptide ions without a basic residue ([ASSpSG + H]⁺, [GApSSSG + H]⁺, and [TTPVpTT + H]⁺) collected as a part of our previous study [21]. Indeed, site-specific $a_n - 97$ peaks were found in such spectra also, but only as extremely weak peaks. This indicates that the basic residue at the N-terminus is doing more than just retaining the additional proton. That is, it may not be a spectator but an active participant in C_α-CO cleavage, somehow making the reaction more efficient. For example, the additional proton attached to the basic residue might interact with the reaction center and make the C_α-CO bond more labile.

It has been mentioned that C_α-CO cleavage channels do not effectively compete with b/y channels in high-energy dissociation of unphosphorylated peptide ions with lysine or histidine but without arginine. Even though the same trend was found to hold for phosphopeptide ions in this work, there was one notable

exception, viz. efficient C_α-CO cleavage at the phosphorylated residue. To summarize, two conditions must be met for the specific C_α-CO cleavage, i.e., phosphorylation of the residue where the cleavage occurs and presence of a basic residue at the N-terminus (or toward the N-terminal side). It seems that the critical energy for C_α-CO cleavage gets smaller when these two conditions are met, even though how these conditions act is unclear.

We have also mentioned that the site-specific dephosphorylation of $x_n + 1$ —viz. formation of $x_n - 97$ —does not occur for phosphopeptide ions with arginine at the C-terminus even though $x_n + 1$ is the dominant ionic product of C_α-CO cleavage in such cases. Let us consider $a_n + 1$ phosphorylated at the C-terminal (–pC_α•) and $x_n + 1$ phosphorylated at the N-terminal (•CO–NH–pC_α–), each formed by homolytic C_α-CO cleavage. Let us suppose that dephosphorylations of these radical cations are radical directed. Then, more efficient dephosphorylation of $a_n + 1$ than $x_n + 1$ may be attributed to the fact that the radical site in the former ion is closer to the phosphate substituent than in the latter. Or, prominence of $a_n - 97$ together with absence of $x_n - 97$ may be an indication that dephosphorylation of $a_n + 1$ occurs by radical site initiation. We have found in the H/D exchange study that the hydrogen atom transferred to the phosphate group (OPO(OH)₂) is exchangeable. However, we do not know where this hydrogen is coming from and how it participates in the radical directed reaction, if it does.

Recently, Diedrich and Julian [28] reported site-specific radical directed dissociation of peptide ions at phosphorylated residue. Instead of studying intact phosphopeptides, C_β-OPO(OH)₂ bond was first cleaved, i.e., the phosphate group was eliminated, and then replaced by C_β-S bond by addition of 2-naphthalenethiol. 266 nm photolysis of the derivatized peptide ions cleaved the C_β-S bond and generated radical cations with naphthalenesulfide loss together with radical site-specific a_n and d_n ions. Even if the loss of H₃PO₄ from $a_n + 1$ observed in the present work is radical directed, its mechanism would be different from the above because the radical positions are different, C_α in the present case versus C_β in Diedrich and Julian's case.

Conclusion

Site-specific dissociation at phosphorylated residue found in the present work can be useful for the analysis of phosphopeptides, especially for pinpointing phosphorylated residues in multiply phosphorylated peptides with multiple serine/threonine residues. The fact that this reaction occurs for intact phosphopeptide ions can be particularly useful in this regard. Propensity of a phosphate substituent to make the adjacent C_α-CO bond more labile may also find interesting applications for the structural study of proteins.

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Appendix A Supplementary Material

Supplementary material associated with this article may be found in the online version at [doi:10.1016/j.jasms.2009.09.003](https://doi.org/10.1016/j.jasms.2009.09.003).

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