Anal. Chem. **2009,** *81,* 4369–4381

Gas-Phase Intramolecular Phosphate Shift in Phosphotyrosine-Containing Peptide Monoanions

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Phosphotyrosine-containing peptide monoanions $[M - H]^-$ exhibit extensive neutral loss of phosphoric acid (98 Da) upon quadrupole time-of-flight and ion-trap collision-induced dissociation (CID). In contrast, a neutral loss of metaphosphoric acid HPO_3 (80 Da) is negligible from the deprotonated phosphotyrosine peptides. The efficient H_3PO_4 release is unexpected, given the structure of phosphotyrosine. Our study reveals that the abundant $[M - H - 98]^-$ product ions of pTyr-peptides are not a result of consecutive losses of HPO_3 and H_2O but, rather, are induced by an intramolecular interaction of the phosphotyrosine phosphate with deprotonated peptide functions such as hydroxyl, carboxyl, and to a small extent, amide. As a result, an internal phosphotyrosine phosphate shift occurs, and the obtained phosphorylated functionalities undergo elimination of H_3PO_4 to give rise to the $[M - H - 98]^-$ fragments. The mechanism proposed for the phosphoric acid neutral loss is based on extensive CID studies of Ala-substituted model phosphorylated peptides and oxygen-18 labeling. The proposed mechanistic pathway explains the fact that the pTyr phosphate transfer and the subsequent H_3PO_4 neutral loss are not observed for multiply charged anions of pTyr-peptides. Monoanions of pSer-containing peptides undergo the intramolecular phosphate shift as well, although its efficiency is much lower compared to the aromatic phosphorylation sites. These observations facilitate correct identification of pSer-, pThr-, and pTyr-peptides in CID studies. This work demonstrates that the established phosphate-specific neutral loss fragmentation rules of protonated pTyr-peptides cannot be applied to the CID spectra of their $[M - H]^-$ ions.

Reversible phosphorylation on hydroxyl groups of serine, threonine, and tyrosine is a widespread posttranslational modification of proteins that regulates nearly every aspect of cellular life and touches the majority of signaling pathways.[1−3] In eukaryotes, phosphorylation occurs more often on serines and threonines compared to tyrosines, although phosphotyrosine residues play a central role in many signaling pathways of mammalian cells.[4] Abnormal protein phosphorylation is associated with a variety of diseases, including cancer, Alzheimer disease, muscular dystrophy, and others.[5−7] Elucidation of the physiological/pathophysiological role of particular phosphorylation events in living organisms requires the identification of exact sites of phosphorylation in protein sequences as initial step.

In recent years, tandem mass spectrometry (MS/MS) combined with electrospray ionization (ESI) or matrix-assisted laser/desorption/ionization (MALDI) has become the most commonly used method for analysis of protein phosphorylation.[8,9] Thus, in collision-induced dissociation (CID) phosphate-specific fragmentations of phosphopeptides serve as a specific probe for detection and identification of their phosphorylation sites. Following CID, protonated pSer- and pThr-containing peptides undergo elimination of phosphoric acid H_3PO_4 (98 Da), whereas the ionized pTyr-peptides expel, although to a smaller extent, molecules of metaphosphoric acid HPO_3 (80 Da).[10,11] An additional reporter ion of protonated pTyr-peptides is the pTyr immonium ion at m/z 216.043.[12,13] The distinct fragmentation patterns of the two groups of phosphorylated peptides typically allow one to differentiate between the aliphatic and aromatic modification sites of proteins by CID. Sequence-specific fragment ions (b- and y-type product ions) of pSer/pThr-peptides, carrying the phosphorylated residues, also exhibit efficient loss of H_3PO_4. The backbone cleavage product ions of pTyr-containing peptides generally stay intact or exhibit a HPO_3 loss of low abundance. A mechanism of the H_3PO_4 loss of protonated pSer/pThr-peptides obtained upon

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10.1021/ac900244e CCC: $40.75 © 2009 American Chemical Society
Published on Web 04/29/2009

ESI has been studied using stable isotope labeled compounds. CID experiments with C_α-deuterated pSer/pThr-peptides have shown that the fragmentation reaction mainly proceeds via a charge-directed S_N2 mechanism initiated by protonation of the phosphate group.[14] The carbonyl oxygen of the amino acid on the N-terminal side of the pSer/pThr residue performs a nucleophilic attack to the β-carbon of the phosphorylated site, resulting in loss of H_3PO_4 and a cyclic oxazoline-containing product ion. Similar five-ring-containing reaction intermediates and fragments were proposed for a variety of other gas-phase decomposition reactions of ionized peptides.[15−17] A charge-remote β-elimination mechanism, occurring via a six-centered transition state,[11] is also involved in the H_3PO_4 neutral loss of pSer/pThr residues, but its contribution appears to be relatively low.[14] The proposed mechanistic pathways of the H_3PO_4 release explain the absence of the elimination reaction for pTyr residues, since neither the five-ring product ions nor the six-centered transition states can be formed in the case of the aromatic phosphorylation sites.

The strong acidity of the phosphate monoesters[18] induces a reduction of the propensity of phosphorylated peptides to the gas-phase protonation comparing to the unmodified analogues.[19,20] This fact may result in detection problems of low-stoichiometry phosphorylation sites of protonated protein digests, especially in cases when the modified sequences have only one basic amino acid, carry multiple acidic amino acid side chains (aspartic acid (Asp) and glutamic acid (Glu)) or contain several phosphorylated residues. Therefore, examination of protein digests in negative ion mode, as a complement to the analysis of cations, may help to overcome those problems, thus increasing significantly the reliability of mass spectral analysis of protein phosphorylation sites.[21,22] For negative ion CID, three types of phosphopeptide reporter fragmentations have been described: (i) the neutral loss of H_3PO_4 for pSer and pThr but not for pTyr; (ii) formation of low-mass fragments at m/z 97 (= $[H_2PO_4]^-$), m/z 79 (= $[PO_3]^-$), and m/z 63 (= PO_2^-) for pSer, pThr, and pTyr[11,23] and, as a special feature of $[M - nH]^{n-}$ ions of pSer-, pThr-, and pTyr-peptides, (iii) a unique high-mass fragment of the type $[M - nH - 79]^{(n-1)-}$, which is the complementary fragment of the m/z 79 ion.[24]

Besides CID, electron-capture and electron-transfer dissociations (ECD and ETD) are used for analysis of protein phosphorylation. The ECD and ETD methods lead to random N−C_α bond cleavages of peptide backbones producing c- and z-type fragment ions of peptides.[25,26] Both ECD and ETD techniques fragment phosphopeptides with preservation of the phosphate moiety on all fragment ions. At the same time ECD is effectively restricted to Fourier transform ion cyclotron resonance (FTICR) mass spectrometers, and ETD may outperform CID only for highly charged (> +2) precursor ions.[27] Therefore, CID is still the most widely used method for the structural analysis of protein phosphorylation.

The use of MS/MS for the analysis of protein phosphorylation requires clear understanding of the fragmentation patterns of ionized phosphopeptides under the applied conditions. Rearrangement reactions following CID of peptides complicate significantly interpretation of MS/MS data and may lead to difficulties in extracting the correct peptide sequence information. Therefore, a comprehensive knowledge on rearrangement processes occurring during the gas-phase fragmentation of phosphopeptides is indispensable for structural characterization of protein phosphorylation by MS/MS.[28,29] The literature contains several reports on gas-phase rearrangements of phosphorylated peptides. Thus, 98 Da neutral loss of $[M + 2H]^{2+}$ ions of pTyr-peptides produced by ESI upon ion-trap CID was hypothesized to result from a rearrangement reaction involving the aromatic phosphorylation sites.[30] Another study has shown that some of protonated pTyr-peptides produced by MALDI may also undergo, during postsource decay, an efficient neutral loss of 98 Da caused by elimination of phosphoric acid from the ionized phosphopeptides.[31] The H_3PO_4 release was found to be strongly dependent on the presence of aspartic acid and arginine residues in the phosphopeptide sequences. One of the mechanisms proposed for the H_3PO_4 elimination was based on the intramolecular transfer of the pTyr phosphate to the aspartic acid side chain with following cleavage of phosphoric acid from the resulting mixed anhydride. Recently, intramolecular phosphate migration to unmodified hydroxyl-containing amino acid residues was reported for protonated pSer-, pThr-, and pTyr-peptides during ion-trap CID.[32] Although the observed rearrangement reaction was relatively inefficient it occurred during CID of a wide variety of phosphorylated peptides. The propensity of the protonated phosphopeptides for the phosphate transfer was found to be highly dependent on the precursor ion charge state and the mobility of the ionizing proton. It was demonstrated that the rearrangement reaction may lead to wrong assignments of protein phosphorylation sites during ion-trap multistage MS/MS analyses.

Here, we describe a new and very efficient phosphate-specific reaction of phosphotyrosine-containing peptide monoanions occurring during quadrupole time-of-flight and ion-trap CID. The gas-phase reaction results in extensive neutral loss of phosphoric

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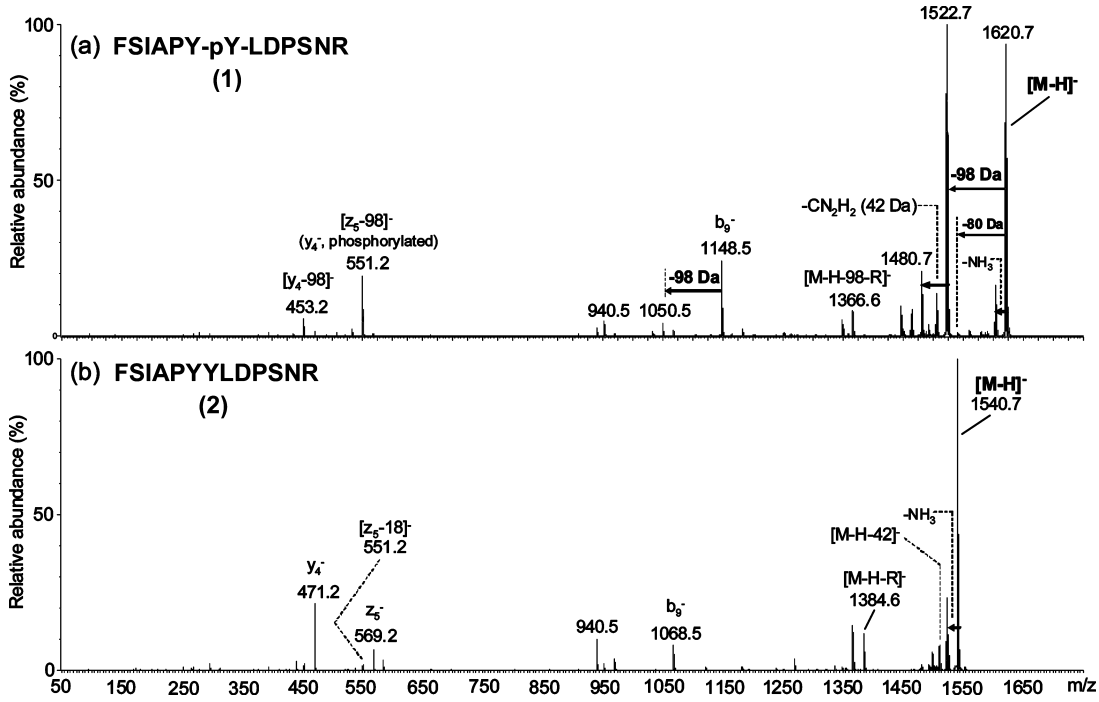

Figure 1. NanoESI-Q-TOF CID spectra of $[M - H]^-$ ions of (a) FSIAPY-pY-LDPSNR **1** (collision energy 62 eV) and (b) FSIAPYYLDPSNR **2** (collision energy 60 eV). The mass spectrum of the deprotonated phosphopeptide **1** exhibits an extensive elimination of 98 Da. A comparison of the pTyr-peptide spectrum with that of its nonphosphorylated analogue **2** indicates that the expelled neutral belongs to phosphoric acid.

acid from the peptide $[M - H]^-$ ions, involving an intramolecular relocation of the phosphotyrosine phosphate upon CID. A detailed mechanism for this unexpected gas-phase fragmentation of deprotonated pTyr-peptides is proposed.

EXPERIMENTAL SECTION

Mass Spectrometry. NanoESI-Q-TOF tandem mass spectra of peptides were acquired on a Q-TOF-2 mass spectrometer (Waters Micromass, Manchester, U.K.) and modified QSTAR Pulsar *i* quadrupole time-of-flight (MDS Analytical Technologies, Concord, Canada) instrument equipped with an automated nanospray chip ion source NanoMate HD (Advion BioSciences, Ithaca, NY). Borosilicate capillaries produced in-house (DKFZ, Heidelberg) using a micropipet puller (type P-87, Sutter Instruments, Novato, CA) and coated with a semitransparent film of gold in a sputter unit type SCD 005 (BAL-TEC, Balzers, Liechtenstein) were used for the Q-TOF-2 experiments. In the Q-TOF CID measurements argon was the target gas in Q-TOF-2, and nitrogen was the collision gas in a modified QSTAR. Ion-trap CID spectra were recorded on a hybrid LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a robotic nanoflow ion source NanoMate HD System (Advion Biosciences, Inc., Ithaca, NY). In the LTQ Orbitrap MS/MS experiments spectra were acquired at the Orbitrap analyzer, the target mass resolution and precursor isolation window were 7500 (at m/z 400) and 1 amu, respectively. Intact peptides were detected in the Orbitrap at 60 000 resolution (at m/z 400). Peptide analytes were dissolved in a 50%/2%/48% (v/v/v) mixture of acetonitrile/formic acid/water to give a concentration of ~10−50 pmol/μL.

Phosphopeptide Synthesis. For solid-phase synthesis of the phosphopeptides the Fmoc strategy[33,34] was employed. A multiple automated synthesizer (Syro II, Multisyntech) was used for the peptide synthesis. Peptide chain assembly was performed by in situ activation of amino acid building blocks by 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate. The Fmoc−Thr(PO(OBzl)OH)−OH, Fmoc−Tyr(PO(OBzl)OH)−OH, and Fmoc−Ser(PO(OBzl)OH)−OH were purchased from Merck Biosciences GmbH.

^{18}O Labeling. The synthetic peptide FAIAPYpYLAPANR was incubated overnight in ^{18}O water (98 atom % ^{18}O, Rotem, Leipzig) and trypsin (Roche) at 37 °C. The reaction resulted in the predominant (over 90%) incorporation of two atoms of ^{18}O into the C-terminal COOH group.

RESULTS AND DISCUSSION

The Phenomenon. The nanospray Q-TOF CID spectrum of the $[M - H]^-$ ion of the pTyr-peptide **1** (FSIAPYpYLDPSNR) exhibits a very intense neutral loss of 98 Da (fragment at m/z 1522.7), see Figure 1a. The $[M - H - 98]^-$ ion is the most prominent product ion in the tandem mass spectrum of **1**. By contrast, the signal of the $[M - H - 80]^-$ fragment ion obtained by an expulsion of HPO_3, which is generally associated with peptide phosphotyrosine residues, is extremely low. Comparison of the CID spectrum of **1** to that of its unmodified analogue **2** (Figure 1b) shows that the efficient 98 Da neutral loss is induced by the presence of the phosphate moiety in the sequence of **1**. Indeed, the CID spectrum of the peptide **2** does not display the corresponding $[M - H - 98]^-$ fragment. The monoisotopic mass of the neutral unit released from the deprotonated phosphopeptide **1** (97.98 Da) implies that the $[M - H - 98]^-$ (m/z 1442.7) ions are generated either by elimination of phosphoric acid or by consecutive losses of HPO_3 (80 Da) and

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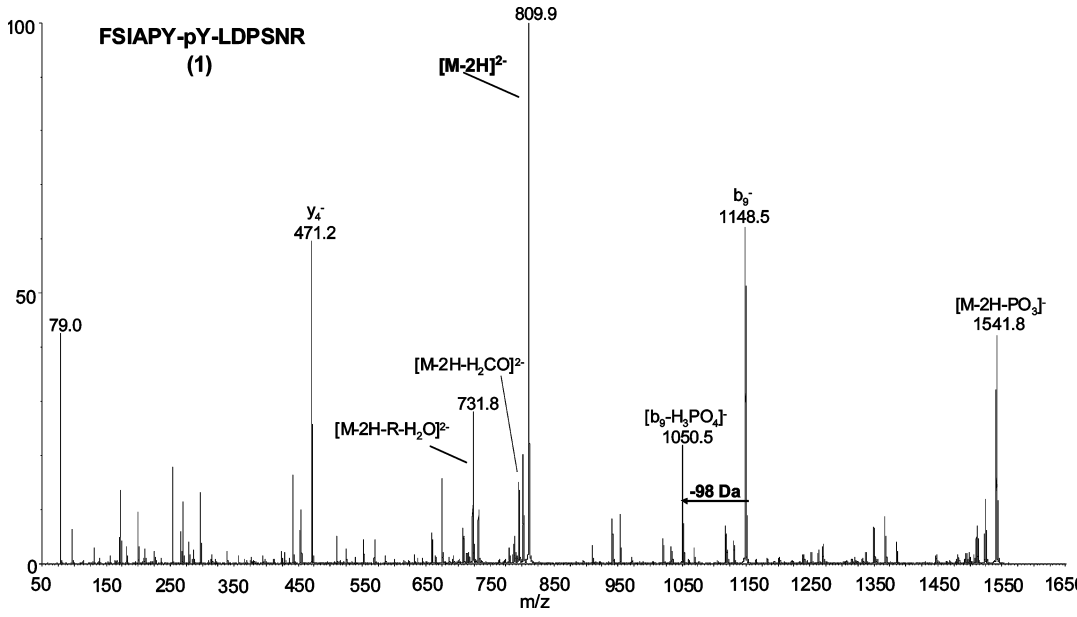

Figure 2. NanoESI-Q-TOF collision-induced dissociation mass spectrum (collision energy 65 eV) of $[M - 2H]^{2-}$ ion of phosphopeptide **1**. The doubly deprotonated pTyr-peptide **1** does not undergo elimination of phosphoric acid.

a H_2O molecule. The two-step process involving a loss of HPO_3 from the pTyr residue of **1** and subsequent expulsion of water elsewhere in the molecule can be excluded on the basis of the very low intensity (RA% 2−3) of the $[M - H - H_2O]^-$ signal in the CID spectrum of the unmodified peptide **2** (Figure 1b). Thus, the experimental data reveal that the singly deprotonated phosphotyrosine-containing peptide **1** undergoes an efficient neutral loss of phosphoric acid upon CID. The observed fragmentation reaction is unexpected, given the structure of the phosphotyrosine side chain.[11,14] Noteworthy, the pTyr-containing backbone fragment b_9^- of the phosphopeptide **1** undergoes the 98 Da neutral loss as well (Figure 1a), despite its lower internal energy in comparison to the peptide molecular ion $[M - H]^-$.

CID Spectrum of $[M - 2H]^{2-}$ Ion of 1 and Positive Ion Mode MS^2 Spectra. In contrast to the fragmentation behavior of the singly charged molecular anion $[M - H]^-$ of **1**, the doubly deprotonated analogue does not undergo elimination of phosphoric acid (Figure 2). The doubly ionized phosphopeptide **1** does not undergo also a neutral loss of 80 Da HPO_3. Prominent signals in the CID spectrum of the $[M - 2H]^{2-}$ ion of **1** belong to PO_3^- (m/z 79), y_4^- (m/z 471.2), b_9^- (m/z 1148.5), $[b_9 - H_3PO_4]^-$ (m/z 1050.5), $[M - 2H - PO_3]^-$ (m/z 1541.8), and $[M - 2H - R]^{2-}$ (m/z 731.8) fragments.

Similarly to the $[M - 2H]^{2-}$ ions, positively charged molecules of the pTyr-peptide **1** do not expel phosphoric acid upon CID (Figure 3). The tandem mass spectra of the $[M + H]^+$, $[M + 2H]^{2+}$, and $[M + 3H]^{3+}$ ions of **1** do not show products of phosphoric acid elimination neither from the intact ionized peptide nor from its fragment ions. The CID spectrum of the $[M + H]^+$ ion has a small signal at m/z lower than that of the precursor ion by 98 Da (Figure 3c). However, the presence of a low-abundant m/z 1542.7 $[M + H - HPO_3]^+$ fragment allows one to assume that the observed peak is a result of water elimination from the HPO_3 neutral loss product ion of the peptide. The observed dephosphorylation pattern of the $[M + H]^+$ ion of **1** is typical for protonated pTyr-peptides upon CID.[11,35] The 80 Da neutral loss fragments are generally more abundant for singly charged protonated pTyr-peptides in comparison to their doubly and multiply charged analogues.

The suppression of the 98 Da neutral loss during CID of the positively charged peptide **1** reveals that the highly efficient H_3PO_4 elimination of its $[M - H]^-$ ions is induced by the negative charge. Furthermore, the absence of the H_3PO_4 release during fragmentation of the doubly deprotonated peptide molecules $[M - 2H]^{2-}$ clearly indicates that a proton abstraction at the phosphate moiety leads to the suppression of the H_3PO_4 elimination as well. Indeed, since pK_{a1} value of the phosphate monoester is the lowest one in comparison to the other two acidic positions of the peptide **1** (pTyr, ca. 0.9 pK_{a1}; D, side-chain pK_a 3.9; R, C-terminal pK_a 1.8),[18,36] a big portion of the doubly deprotonated peptide ions are expected to carry one of the negative charges on the pTyr phosphate moiety, turning the probability for the occurrence of a neutral phosphate group into negligible. In contrast, a significant portion of the activated $[M - H]^-$ ions of **1** may have a structure with the noncharged phosphate. The phenomenon of the extensive proton transfers within activated deprotonated peptides prior to CID fragmentation is well-known.[37] The observed correlation between the number of the negative charges of the ionized phosphopeptide **1** and the efficiency of its H_3PO_4 neutral loss enables one to assume that the 98 Da elimination is induced by an intramolecular interaction of the phosphotyrosine phosphate group with a negatively charged functionality of the modified peptide. An electrostatic repulsion of the two negative charges of the ionized pTyr phosphate and an interacting moiety is probably the reason for the absence of the H_3PO_4 elimination from the $[M - 2H]^{2-}$ ions of **1**. Thus, the uncharged state of the phosphotyrosine side chain is a

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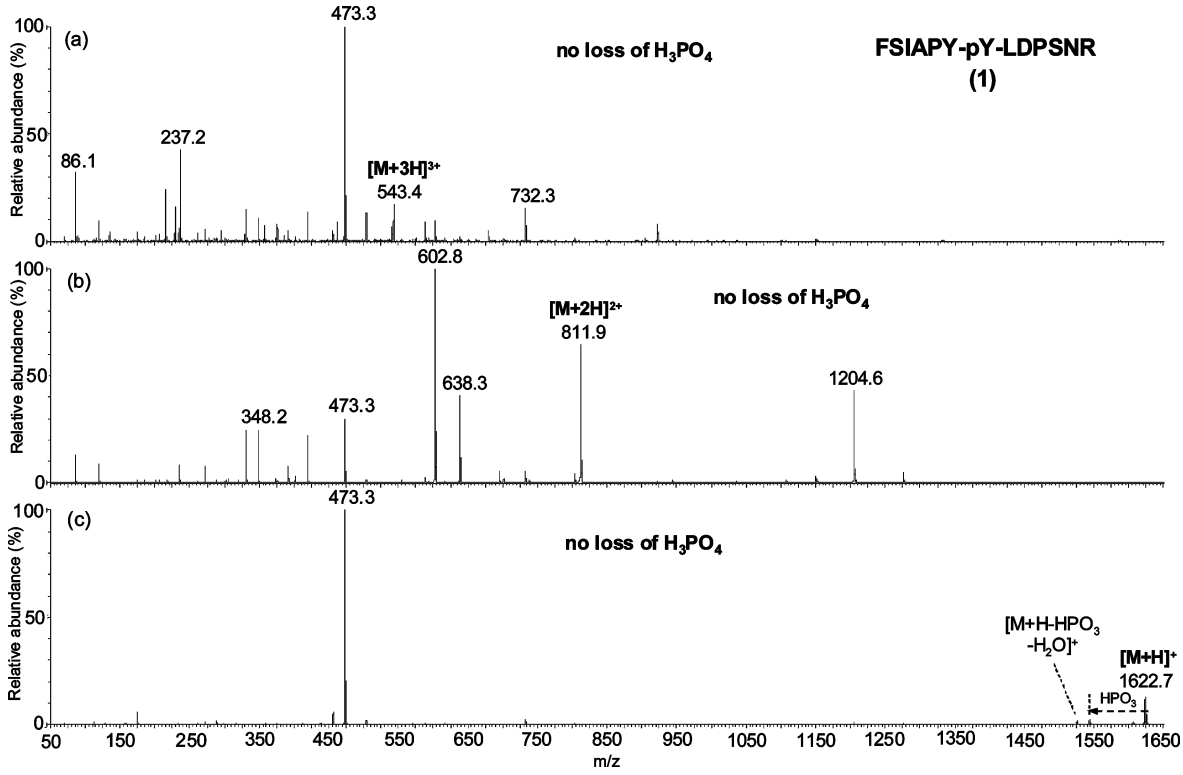


Figure 3. NanoESI-Q-TOF collision-induced dissociation mass spectra of positively charged phosphopeptide **1**: (a) $[M + 3H]^{3+}$ ions, collision energies 45−75 eV; (b) $[M + 2H]^{2+}$ ions, collision energies 50−70 eV; (c) $[M + H]^{+}$ ions, collision energies 65−80 eV. Average MS/MS spectra at a range of collision energies are shown for each charge state. No elimination of phosphoric acid is observed from the protonated molecules of the pTyr-peptide **1**.

prerequisite for the dephosphorylation reaction of the $[M - H]^-$ ions of the pTyr-peptide **1**.

Negative Ion MS/MS Spectra of Isomeric pTyr-Peptides. To verify an involvement of the supposed intramolecular interaction in the elimination of phosphoric acid from the $[M - H]^-$ ions of **1**, the negative ion mode CID behavior of phosphopeptides IYQIQS-pY-R **3** and pY-IYQIQSR **4** has been examined. The peptides **3** and **4** are positional isomers that differ by the location of the phosphotyrosine residue in the peptide sequence. A comparison of the H_3PO_4 neutral loss fragment relative abundances reveals that the elimination reaction is significantly more efficient for the peptide **4**, containing the N-terminal pTyr residue, than for the sequence **3**, in which phosphotyrosine is the second amino acid from C-terminus (Figure 4). These data strongly support the assumption that the unusually intense elimination of phosphoric acid from $[M - H]^-$ ions of pTyr-peptide **1** is not a local event at the modified amino acid but is, rather, induced by an intramolecular interaction between the phosphorylated side chain and a negatively charged peptide function. The difference in the intensities of the H_3PO_4 neutral loss ion signals in the CID spectra of **3** and **4** indicates that the relative location of the reacting centers in the phosphopeptide **4** is more favorable than that of the peptide **3**.

Identification of Functional Groups Involved in the H_3PO_4 Elimination. To examine what functions of the pTyr-peptide $[M - H]^-$ ions undergo an intramolecular interaction with phosphotyrosine phosphate during the H_3PO_4 loss reaction, the CID behavior of model phosphopeptides **5**−**11** has been investigated. The sequences **5**−**11** are variants of peptide **1**, which eliminates phosphoric acid very efficiently, and differ from the original peptide by the number of free carboxyl and hydroxyl moieties. Since a probability of COOH- and OH-containing peptide groups to be ionized in the negative ion ESI-MS(-MS/MS) is much higher than that of the other functionalities,[36] the chance that they initiate the gas-phase pTyr phosphate transfer is very high. In the sequences **5**−**11**, the number of the carboxyl and hydroxyl proton-donor functions has been gradually decreased either via exchange of the Ser and Asp residues of peptide **1** for alanine (peptides **6**−**10**), or through its C-terminal derivatization (peptides **6**−**9**, **11**), or by replacing Tyr-6 of **1** by phenylalanine (peptide **5**). Negative ion CID data of $[M - H]^-$ ions of the peptides **5**−**11** are presented in Figure 5 and Table 1.

FSIAP**F**-pY-LDPSNR **5**
FA**I**APY-pY-L**A**PA**N**R-**NH$_2$** **6**
FA**I**APY-pY-L**A**P**S**NR-**NH$_2$** **7**
F**S**IAPY-pY-L**A**PA**N**R-**NH$_2$** **8**
FA**I**APY-pY-L**D**PA**N**R-**NH$_2$** **9**
FA**I**APY-pY-L**A**PA**N**R **10**
FSIAPY-pY-LDPSNR-**NH$_2$** **11**

The results of the negative ion mode CID measurements reveal that removal of the side-chain Tyr-6 hydroxyl of the peptide **1** to give rise to the sequence **5** does not affect the efficiency of the H_3PO_4 loss reaction (Figures 1a and 5a, Table 1). Indeed, in the CID spectra of **1** and **5** the most abundant product ions are the $[M - H - 98]^-$, b_9^- (*m*/*z* 1132.5), and $[z_5 - 98]^-$ (*m*/*z* 551.2) ions, which are of similar relative abundances for both peptides.

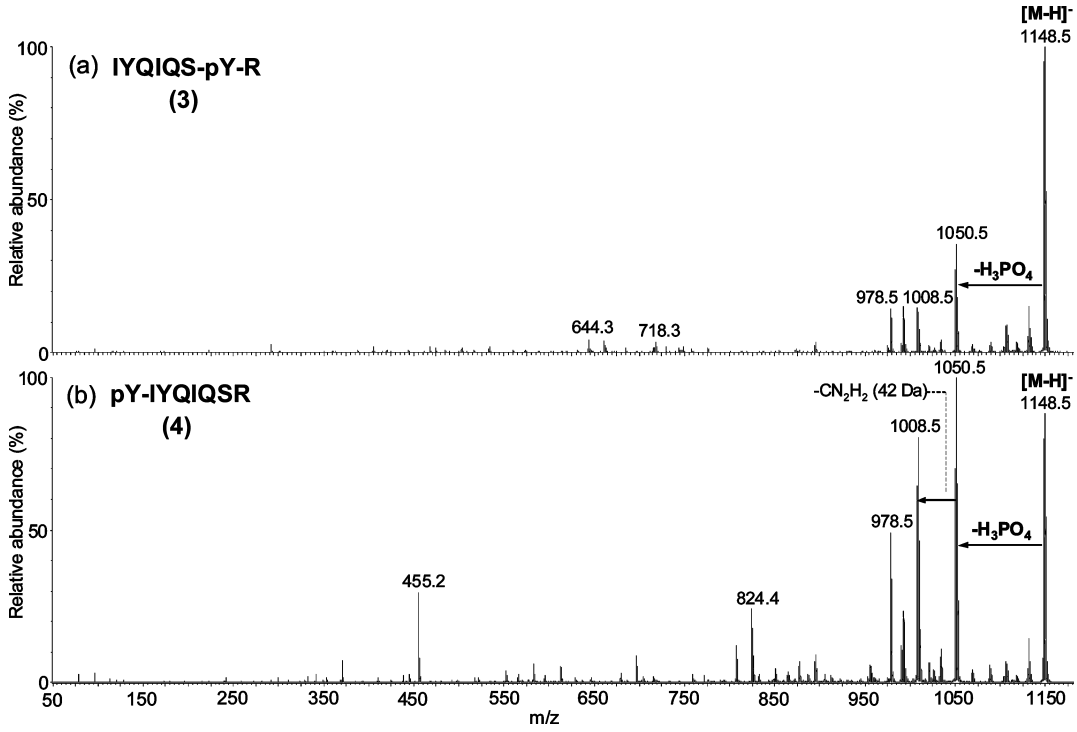


Figure 4. NanoESI-Q-TOF collision-induced dissociation spectra (collision energy 52 eV) of $[M - H]^-$ ions of (a) IYQIQS-pY-R **2** and (b) pY-IYQIQSR **3**. The mass spectra of the isomeric phosphopeptides exhibit different extents of elimination of phosphoric acid. The data support the hypothesis of the involvement of an intramolecular interaction in H_3PO_4 neutral loss reaction of the deprotonated pTyr-peptides.

These data indicate that the Tyr-6 residue of peptide **1** does not facilitate the observed H_3PO_4 elimination from the adjacent pTyr group.

In contrast to the fragmentation behavior of the peptide **5**, the negative ion CID data of **6** show a significant suppression of the $[M - H - 98]^-$ product ion formation. Thus, whereas the signal of the H_3PO_4 neutral loss fragment is the most dominant in the CID spectrum of the $[M - H]^-$ ion of **1**, the relative abundance of the $[M - H - 98]^-$ product ion of **6** reaches only 9% (Figures 1a and 5b, Table 1). These data clearly demonstrate that the set of the carboxyl and hydroxyl functions of **1**, replaced in the sequence of **6**, indeed includes moiety(ies) that is(are) involved in the phosphoric acid neutral loss reaction. The minor $[M - H - 98]^-$ ion observed in the CID spectrum of **6** might be a result from both an interaction of pTyr phosphate with the deprotonated side-chain/terminal peptide amides and possible sequential loss of water from the minor HPO_3 neutral loss peptide fragment. Although the acidity of amide groups (side-chain pK_a of N is ca. 13)[36] is significantly lower compared to carboxy groups, an abstraction of amide protons occurs during negative ion CID of peptides.[37] It is reasonable to assume that the contribution of the consecutive losses of HPO_3 and H_2O to the 98 Da elimination of **6** is insignificant, because a signal of H_2O neutral loss of the peptide **2** is very low (Figure 1b). In comparison to peptide **6**, peptide **2** contains two extra hydroxyl and two extra carboxyl groups. Therefore, its extent of H_2O elimination should be considerably more pronounced compared to that of peptide **6**. Since the structure that is expected to be obtained following an internal phosphorylation of the ionized amide groups is not anticipated to undergo a facial elimination of phosphoric acid (in contrast to the products of the phosphate transfer to the deprotonated OH and COOH groups), the significant decrease of the $[M - H - 98]^-$ ion signal in the CID spectrum of **6** is reasonable.

It should be noted that an absence of the $[M - H - 80]^-$ fragment in the CID spectrum of **6** provides an additional support to the statement that the loss of 98 Da from $[M - H]^-$ represents a loss of H_3PO_4 and not a combined loss of HPO_3 and water. If the $[M - H - 98]^-$ fragment of **1** would be produced by the consecutive losses of HPO_3 and H_2O, the CID spectrum of **6** should definitely show the $[M - H - 80]^-$ signal, because H_2O elimination from a peptide without carboxyl and hydroxyl functions is not favored. In addition, the CID data of **6** indicate that peptide backbone fragments do not lose a water molecule as well.

The CID spectra of the peptides **7** and **8** reveal that a sequential reinsertion of the two serine residues of the peptide **1** into the sequence **6** leads to an increase of the $[M - H - 98]^-$ ion signal in comparison to that of the peptide **6** (Figure 5, parts c and d, Table 1). These results enable us to conclude that the presence of Ser-2 and Ser-11 in the sequences of the peptides **7** and **8**, respectively, accelerates the phosphoric acid neutral loss of the deprotonated pTyr-peptides. Since the elimination reaction is conditioned by deprotonation of a peptide function different from the phosphate moiety, it is reasonable to assume that the H_3PO_4 neutral losses of the peptides **7** and **8** are induced by an interaction of pTyr side chain with the deprotonated serine side-chain alkoxyls to give rise to the intramolecular phosphate transfer, as shown in Scheme 1. Apparently, the observed $[M - H - 98]^-$ fragments of **7** and **8** are the products of H_3PO_4 neutral loss from the phosphoserine residues formed in the gas phase instead of an elimination occurring at the originally phosphorylated tyrosine residue. It is highly probable that the conformation adopted by the $[M - H]^-$ ion of **8** in the gas

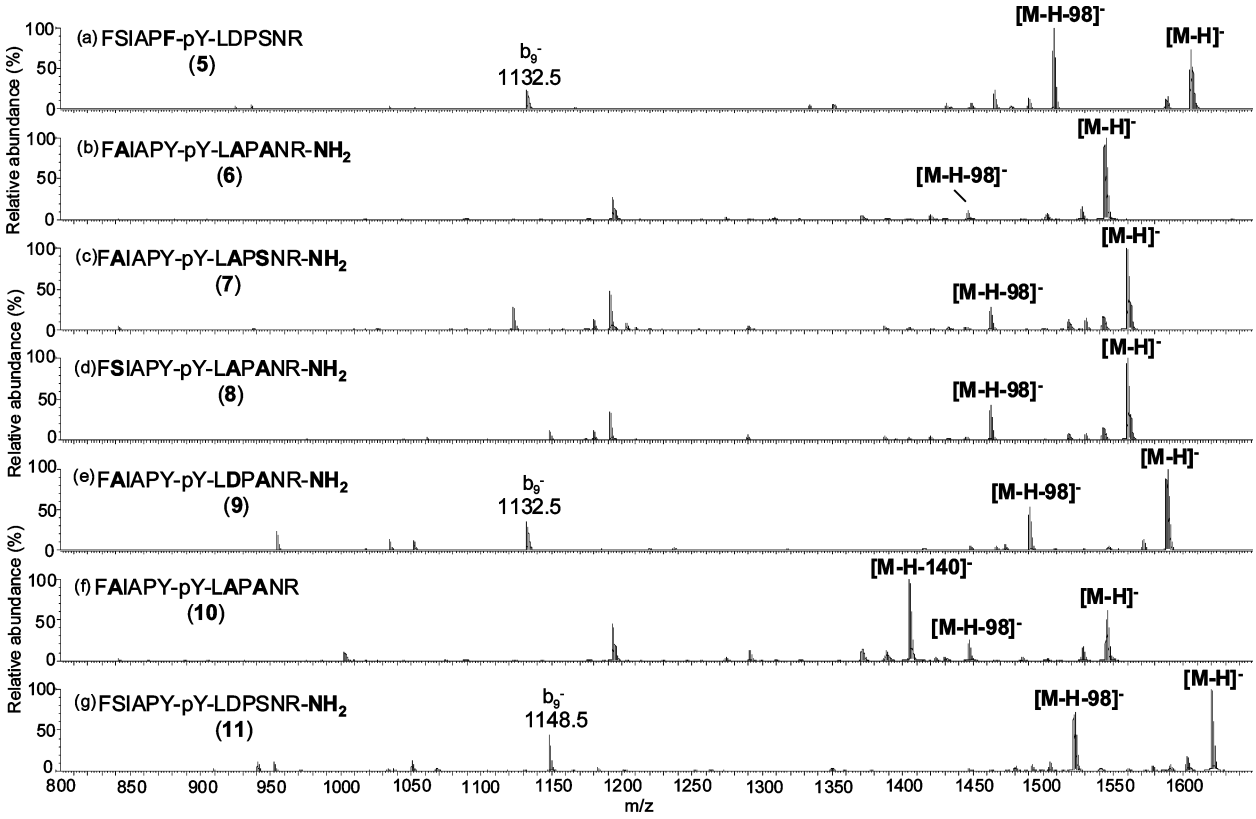


Figure 5. NanoESI-Q-TOF partial negative ion CID spectra of $[M - H]^-$ ions of the model peptides **5**−**11**: (a) **5**, collision energy 62 eV; (b) **6**, collision energy 60 eV; (c) **7**, collision energy 61 eV; (d) **8**, collision energy 61 eV; (e) **9**, collision energy 63 eV; (f) **10**, collision energy 60 eV; (g) **11**, collision energy 65 eV. The CID data indicate a clear correlation between a number of carboxyl and alkoxyl moieties of pTyr-peptides and the efficiency of H_3PO_4 elimination from their $[M - H]^-$ ions.

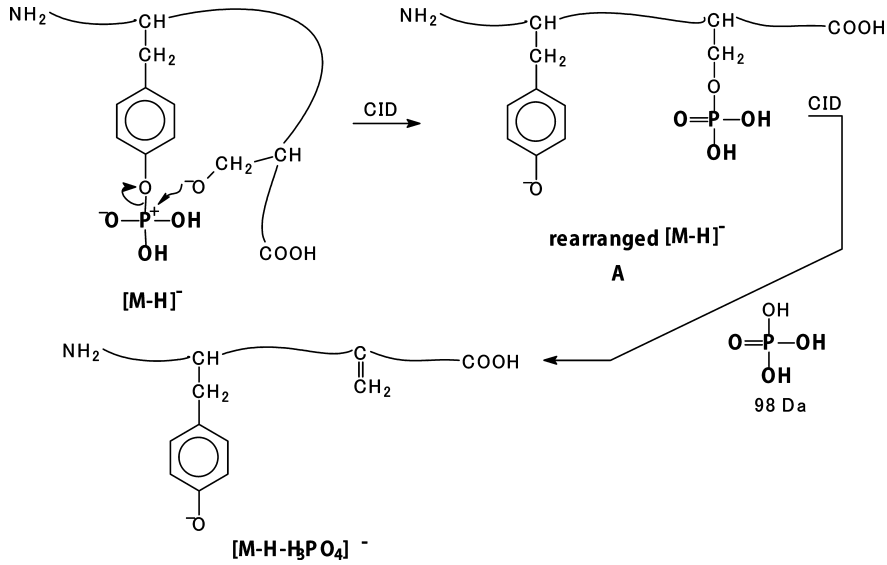
Table 1. Partial Q-TOF CID Mass Spectra of $[M - H]^-$ Ions of Phosphopeptides 5−11 and 15−23[a,b]

	relative abundance, %		
peptide	$[M - H]^-$	$[M - H - 98]^-$	$[M - H - 140]^-$
FSIAP**F**-pY-LDPSNR **5**[c]	64	100	21
FA**I**APY-pY-LA**PA**NR-**NH$_2$** **6**[d]	100	9	<0.1
FA**I**APY-pY-LA**PS**NR-**NH$_2$** **7**[e]	100	26	2
FS**I**APY-pY-LA**PA**NR-**NH$_2$** **8**[e]	100	41	4
FA**I**APY-pY-L**DPA**NR-**NH$_2$** **9**[f]	100	50	5
FA**I**APY-pY-LA**PA**NR **10**[d]	53	19	100
FSIAPY-pY-LDPSNR-**NH$_2$** **11**[g]	100	62	5
GSTAENAE-pY-LR **15**[h]	100	88	50
DQQDFFPK-pY **16**[i]	100	42	<0.1
DQQD-pY-FFPK **17**[i]	73	100	<0.1
ISLDNPDQ-pY **18**[j]	100	90	<0.1
ISLDNPDQ-pY-NH$_2$ **19**[j]	100	90	<0.1
HYQP-pY-APPR **20**[k]	93	62	100
HYQP-pY-APPR-NH$_2$ **21**[k]	100	16	6
D-pY-MGWMDF-NH$_2$ **22**[d]	86	68	<0.1
N-pY-pY-GWMDF-NH$_2$ **23**[l]	100	45	<0.1

[a] The molecular ions are obtained by negative ion nanoESI. [b] The Q-TOF CID spectra of **5**−**11** and **15**−**21** were recorded on Q-TOF-2; the data for **22** and **23** were obtained using modified QSTAR Pulsar *i* Q-TOF. [c] Collision energy 62 eV. [d] Collision energy 60 eV. [e] Collision energy 61 eV. [f] Collision energy 63 eV. [g] Collision energy 65 eV. [h] Collision energy 57 eV. [i] Collision energy 51 eV. [j] Collision energy 49 eV. [k] Collision energy 54 eV. [l] Collision energy 70 eV.

phase leads to more efficient interaction of the phosphorylated residue with the ionized serine, relatively to the sequence **7** (relative abundances of the $[M - H - 98]^-$ ion of **8** and **7** are 41% and 26%, respectively). In addition, the bulky Pro residue which is on N-terminus of Ser-11 of **7** may play a role in the relative suppression of the H_3PO_4 elimination of **7** in comparison to efficiency of the neutral loss from the peptide **8**. In the negative ion mode, the H_3PO_4 neutral loss of phosphoserine occurs via a "charge-remote" β-elimination which leads to a dehydroalanine structure formation at the decomposing peptide position.[11] Both charge-directed E2 elimination and S_N2 neighboring group participation mechanisms,[14] requiring a preliminary intramolecular protonation of the phosphotyrosine phosphate, seem to be much less probable than the β-elimination.

Scheme 1. Proposed Mechanistic Pathway for H_3PO_4 Elimination of $[M - H]^-$ Ions of pTyr-Peptides Involving an Intramolecular Interaction with Ser Side Chains[a]



[a] Following collision-induced activation, the phosphopeptide $[M - H]^-$ ions represent a mixture of isomeric structures with a different location of the negative charge. Some of these structures carry a neutral pTyr phosphate group, which can be attacked by a negatively charged function. The outlined reaction is induced by the interaction of the neutral pTyr phosphate group with the deprotonated alkoxyl moieties to give rise to the intramolecular migration of the pTyr phosphate to a Ser side chain. The rearranged peptide $[M - H]^-$ ions **A** undergo H_3PO_4 elimination by the "charge-remote" β-elimination mechanism.

In order to examine if the secondary alkoxy moiety of threonine may induce the pTyr H_3PO_4 elimination as well, the $[M - H]^-$ ion of the Thr-containing peptide **12** (FAIAPY-pY-LAPTNR-NH_2) was subjected to CID. The CID data obtained (not shown) were compared to those of peptide **7**. The sequences of peptides **7** and **12** differ exclusively at position 11, which carries Ser (**7**) or Thr (**12**). The tandem mass spectrum of **12** displayed product ions which were analogous to those generated upon CID from peptide **7**, including the $[M - H - 98]^-$ fragment ion. The observed fragmentation behavior of peptide **12** indicated that deprotonated Thr residues of pTyr-peptides might be involved in the pTyr phosphate transfer as well. At the same time, the intensity of the neutral loss observed in the CID spectrum of **12** was lower than that of peptide **7** (RA% 20 vs 30), which can be attributed to a steric hindrance by the Thr β-methyl group.

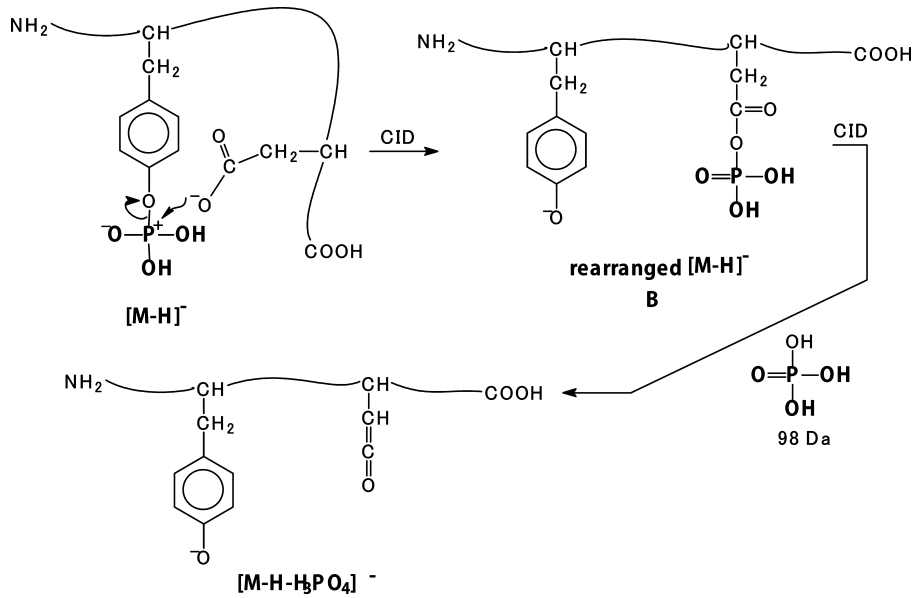
The negative ion CID spectrum of model peptide **9** with Asp-6 exhibits an increased efficiency of H_3PO_4 elimination compared to that of peptide **6** with Ala-6 (see Figure 5, parts e and b, Table 1). The ratio RA% $[M - H]^-$/RA% $[M - H - 98]^-$ in the CID spectrum of **9** is even higher than those of the peptides **7** and **8** (Table 1), whereas the intensities of their other fragment ions are very similar. Since the sequence **9** differs from that of **6** by the presence of Asp-9 instead of Ala-9, the obtained results clearly indicate that the aspartic acid residue is involved in the neutral loss of H_3PO_4 from the deprotonated peptide. It is reasonable to assume that the 98 Da loss of **9** is initiated by an intramolecular attack of the negatively charged Asp side-chain carboxyl at the pTyr phosphate group, as outlined in Scheme 2. The resulting mixed anhydride **B** can undergo a facile elimination of phosphoric acid to give rise to the ketene-containing neutral loss $[M - H - 98]^-$ fragment of the peptide. A possibility of the formation of a stable succinimide ring as a result of the elimination of phosphoric acid by the phosphorylated aspartic acid residues has been examined as well. To this end, the CID spectrum of the $[M - H]^-$ ion of **9** (FAIAPY-pY-LDPANR-NH2, Figure 5e) has been compared to that of the peptide **13** (FAIAPY-pY-LEPANR-NH_2). Since glutamic acid has two side-chain methylene groups instead of one in the case of Asp residue, the succinimide ring cannot be formed by a nucleophilic attack of C-terminal to Glu amide nitrogen at the carbonyl of phosphorylated Glu side chain. The CID spectrum of the Glu-containing peptide **13** (data not shown) exhibited a very abundant $[M - H - 98]^-$ fragment, similar to the sequence **9**. Therefore, succinimide ring formation was excluded from the mechanism presented in Scheme 2.

A further support to the involvement of an Asp side chain in the phosphate shift in peptide **9** is provided by the relative abundances of the b_9^- ions of phosphorylated and unmodified peptides **1** and **2** (m/z 1148.5 and 1068.5, respectively, see Figure 1). The b_9^- fragments are generated by an amide bond cleavage C-terminally to the Asp residue following an abstraction of its C_α-proton.[37] Since phosphorylation of the Asp side chain increases the acidity of the Asp $C_\alpha H$ group[38] the more efficient formation of the b_9^- fragment observed in the CID spectrum of the phosphopeptide **1** supports the hypothesis of the pTyr phosphate shift to the side chain of Asp.

An additional evidence for the intramolecular pTyr phosphate shift upon negative ion CID of **1** is a significant difference in the intensities of the m/z 453.2, m/z 471.2, m/z 551.2, and m/z 569.2 ion signals in the CID spectra of the peptides **1** and **2** (Figure 1). An interpretation of the origin of the observed product ions reveals that all of these signals belong to backbone fragments of **1** and **2** containing either four or five C-terminal amino acids of the peptides. The m/z 569.2 and m/z 551.2 signals in the CID spectrum of **2** represent the z_5 and $z_5 - H_2O$ ions, respectively.

(38) Edelson-Averbukh, M.; Pipkorn, R.; Lehmann, W. D. *Anal. Chem.* **2007**, *79*, 3476–3486.

Scheme 2. Proposed Mechanistic Pathway for H_3PO_4 Elimination of $[M - H]^-$ Ions of pTyr-Peptides via an Intramolecular Phosphate Shift to an Asp Side Chain[a]



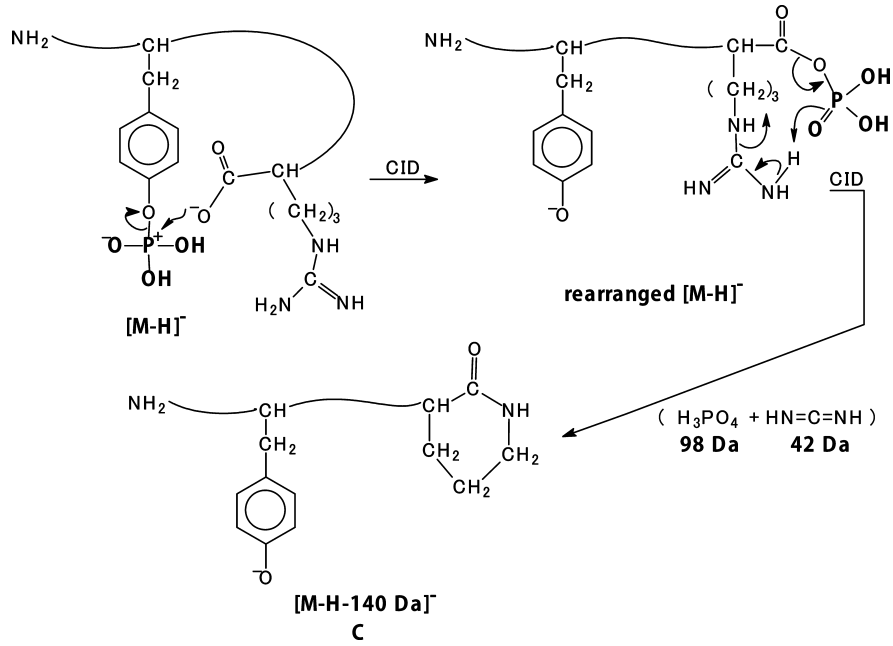
[a] The H_3PO_4 neutral loss from $[M - H]^-$ ions of pTyr-peptides may involve an intramolecular shift of the pTyr phosphate to the deprotonated carboxyl of an Asp side chain. The resulting mixed anhydride **B** can undergo a facile elimination of H_3PO_4 to give rise to the ketene-containing structure of the $[M - H - 98]^-$ fragment.

The z-type fragments are produced by Asp N–C_α bond cleavage following either Asp C_β-proton abstraction[37] or an intramolecular interaction of Asp CH group with a negatively charged peptide function, in analogy with Ser/Thr cleavages.[38] In contrast to the peptide **2**, the CID spectrum of **1** exhibits a relatively abundant *m/z* 551.2 ion and a strongly suppressed *m/z* 569.2 ion signal. This observation indicates that in the case of the phosphopeptide **1**, the *m/z* 551.2 fragment is not produced by H_2O neutral loss of the peptide *m/z* 569.2 z_5^- fragment, but it is rather a result of H_3PO_4 elimination from a phosphate-containing z_5^- fragment. Indeed, a shift of the pTyr phosphate to the side chain of aspartic acid should increase the efficiency of the Asp N–C_α bond cleavage[38] decreasing a stability of the modified species. If the phosphate is shifted to the other moieties of the z-type fragment (Ser side chain or perhaps also C-terminal COOH), the neutral loss reaction of the phosphate-containing z_5^- fragment should be efficient as well. Thus, the increased efficiency of the *m/z* 551.2 product ion formation of **1** accompanied by the strong suppression of the fragment ion at *m/z* 569.2 strongly supports the hypothesis of the intramolecular gas-phase migration of the pTyr phosphate group of **1**. Noteworthy, it is also possible that a part of the *m/z* 551.2 ion signal of **1** corresponds to the phosphorylated y_4^- fragment of the peptide. Indeed, the relative abundance of the *m/z* 471.2 y_4^- product ion of the unmodified peptide **2** is quite significant, whereas the corresponding signal in the CID spectrum of **1** is strongly suppressed (see Figure 1). The mass spectrum of **1** shows, instead, the *m/z* 453.2 ion signal produced by elimination of H_3PO_4 from the phosphorylated y_4^- peptide ion. Since a neutral loss of H_2O from the y_4^- ion of **2** is inefficient (Figure 1b) it is reasonable to assume that a very minor part of the *m/z* 453.2 ion signal of **1** is produced by water elimination from unmodified y_4^- peptide fragment. Thus, a significant suppression of the *m/z* 569.2 z_5^- and *m/z* 471.2 y_4^- ion signals in the CID spectrum of phosphopeptide **1**, together with the very low extent of H_2O elimination from the y_4^- fragment of the peptide **2**, clearly indicates that the majority of z_5^- and y_4^- fragments of pTyr-peptide **1** carry the phosphate group not at its original location.

In order to analyze if in analogy to the Asp and Glu carboxyl groups the C-terminal COOH group may also initiate a phosphate shift, the CID behavior of the model peptide **10**, FAIAPY-pY-LAPANR, has been investigated. The sequence of peptide **10** is designed on the basis of that of **1**, while it does not carry any of the amino acid residues which have been shown above to be efficiently involved in the intramolecular pTyr phosphate shift (Ser-2, Asp-9, and Ser-11). Instead, the C-terminus of the pTyr-peptide **10** is free. The CID spectrum of **10** (Figure 5f) shows that the $[M - H]^-$ ion of the phosphorylated peptide undergoes the phosphoric acid neutral loss, and the extent of this reaction is more significant than that of **6** (Figure 5b). These results reveal that the ionized C-terminus of pTyr-peptides may initiate the intramolecular gas-phase migration of pTyr phosphate as well. In contrast to the CID behavior of the pTyr-peptides **5**, **7**–**9**, the $[M - H - 98]^-$ fragment ion in the CID spectrum of **10** (Figure 5f) is not the most abundant neutral loss product ion. The most intense signal in the CID spectrum of **10** arises from a fragment produced by a neutral loss of 140 Da from the ionized pTyr-peptide. The extremely abundant $[M - H - 140]^-$ ion of **10** can be attributed to consecutive neutral losses of phosphoric acid and carbodiimide (MW 42 Da HN=C=NH) from the peptide molecular ion. The 42 Da elimination is a characteristic reaction of Arg-containing peptides in the negative ion CID.[38,39] This reaction proceeds via a deprotonation of the guanidino NH_2 of Arg and typically produces fragment ions of relative abundances below 50%. In the case of the pTyr-peptide **10**, the intramolecular phosphate shift to its C-terminus is expected to facilitate

(39) Waugh, R. J.; Bowie, J. H.; Gross, M. L. *Aust. J. Chem.* **1993**, *46*, 693–702.

Scheme 3. Proposed Mechanistic Pathway for Neutral Loss of 140 Da from $[M - H]^-$ Ions of pTyr-Peptides Carrying C-Terminal Arginine[a]



[a] The 140 Da elimination takes place following an intramolecular transfer of pTyr phosphate to the deprotonated C-terminus of pTyr-peptides and abstraction of the NH_2 guinidino proton of the terminal arginine by the phosphate moiety.

significantly the 42 Da neutral loss (Figure 5, part b vs part f), because of a possible proton transfer from the guanidino to the phosphate group, which leads to an expulsion of both carbodiimide and phosphoric acid, as shown in Scheme 3. The neutral loss product ion is expected to have a stable structure of a six-membered cyclic amide (see C in Scheme 3). The increased abundance of the $[M - H - 98]^-$ fragment in the CID spectrum of **10** (Figure 5f), in comparison to that of **6**, reveals that the phosphate group transferred to the C-terminus of **10** may abstract a different proton from the guanidino moiety resulting in the expulsion of H_3PO_4 only. A decrease of the $[M - H - 140]^-$ ion signal observed in the CID spectrum of another peptide, FSIAPY-pY-LDPSNR-NH_2 **11** (Figure 5g), relative to that of **1**, supports the assumed involvement of the pTyr phosphate migration to the ionized C-terminus of **1**. Indeed, amidation of the C-terminal carboxyl moiety in the sequence of **11** should reduce significantly an ionization efficiency of the peptide terminus, suppressing a migration of the pTyr phosphate to the C-terminus.

C-Terminal ^{18}O Labeling of FAIAPY-pY-LAPANR 10. In order to provide a direct support for the phenomenon of the intramolecular pTyr phosphate shift during CID of pTyr-peptide $[M - H]^-$ ions, ^{18}O labeling of C-terminal carboxyl of the model phosphopeptide **10** has been performed. It is reasonable to assume that if the hypothesis of the gas-phase intramolecular pTyr phosphate migration is correct, the labeled $[M - H]^-$ ions of **10**, containing two C-terminal ^{18}O atoms, will eliminate upon CID 142 Da, instead of 140 Da observed for the unlabeled analogue. Indeed, according to Scheme 3, one of the oxygen atoms of phosphoric acid eliminated from the rearranged C-terminally phosphorylated $[M - H]^-$ ions of pTyr-peptides stems from the C-terminal COOH group. Thus, the deprotonated $^{18}O_2$ molecules of peptide **10** are anticipated to lose HN=C=NH (42 Da) and $H_3P^{16}O_3{}^{18}O$ (100 Da) to give rise to the corresponding $[M - H - 142]^-$ product ions. A partial nanoESI product ion spectrum of the $[M - H]^-$ ion of the $[^{18}O_2]$-labeled peptide **10** at m/z 1548.8 is presented in Figure 6. The negative ion mode tandem mass spectrum demonstrates that, as expected, the $[^{18}O_2]$-labeled peptide undergoes neutral loss of 142 Da, indicating loss of $[^{18}O_1]$-labeled phosphoric acid (100 Da) and carbodiimide. The signal of the resulting product ion appears at m/z 1406.7. The experimental data clearly indicate an intramolecular pTyr phosphate transfer to the C-terminus of the $[M - H]^-$ ion of **10** during CID. Noteworthy, the CID spectrum of the labeled peptide **10** provides an additional support to an involvement of the deprotonated Asn side-chain amide in the intramolecular phosphate transfer of pTyr-peptide monoanions, as proposed above on the basis of the CID data of the model peptide **6** (Figure 5b). Indeed, the tandem mass spectrum at Figure 6 exhibits, in addition to the neutral loss of $H_3PO_3{}^{18}O$ (signal at m/z 1448.7), a comparable elimination of unlabeled phosphoric acid (signal at m/z 1450.7). It is reasonable to assume that the observed H_3PO_4 neutral loss is a result of an intramolecular interaction of pTyr phosphate with the deprotonated Asn side chain. As already discussed for the peptide **6**, contribution of consecutive losses of HPO_3 and unlabeled H_2O to the m/z 1450.7 signal of the $^{18}O_2$ molecules of **10** seems to be negligible. Besides, it should be noted that the minor signals observed at 1 and 2 Da below those of the annotated peaks in Figure 6 arise from the decompositions of $^{18}O_1$ molecules of **10**, whose molecular ion isotopic envelope coincides partly with that of the $^{18}O_2$ peptide.

pTyr/pSer Exchange of 10. To clarify the factors that cause the observed intramolecular phosphotyrosine phosphate migration during CID of the negatively charged pTyr-peptides, the CID spectrum of $[M - H]^-$ ion of peptide **14** (FAIAPY-pS-LAPANR) has been acquired. The model peptide **14** is a phosphoserine-containing analogue of **10** carrying pSer instead of pTyr at position 7. Since phosphoric acid can be released from pSer side chains directly, it is expected that the pTyr/pSer exchange in the sequence of **10** will reduce the efficiency of the 140 Da

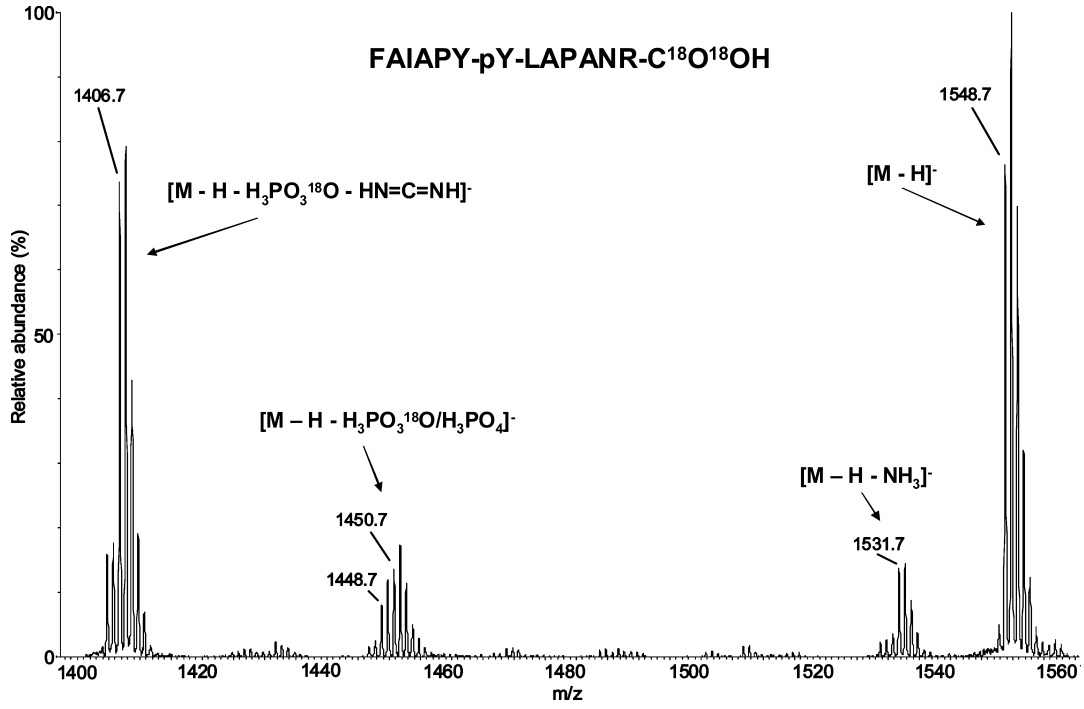


Figure 6. Partial negative ion nanoESI-Q-TOF CID spectrum (collision energy 60 eV) of $[M - H]^-$ ion of C-terminally $[^{18}O_2]$-labeled peptide **10** (annotated *m*/*z* values refer to monoisotopic ions). The 142 Da neutral loss from the labeled peptide provides a direct confirmation of a migration of the peptide pTyr phosphate to the C-terminal COOH during negative ion CID.

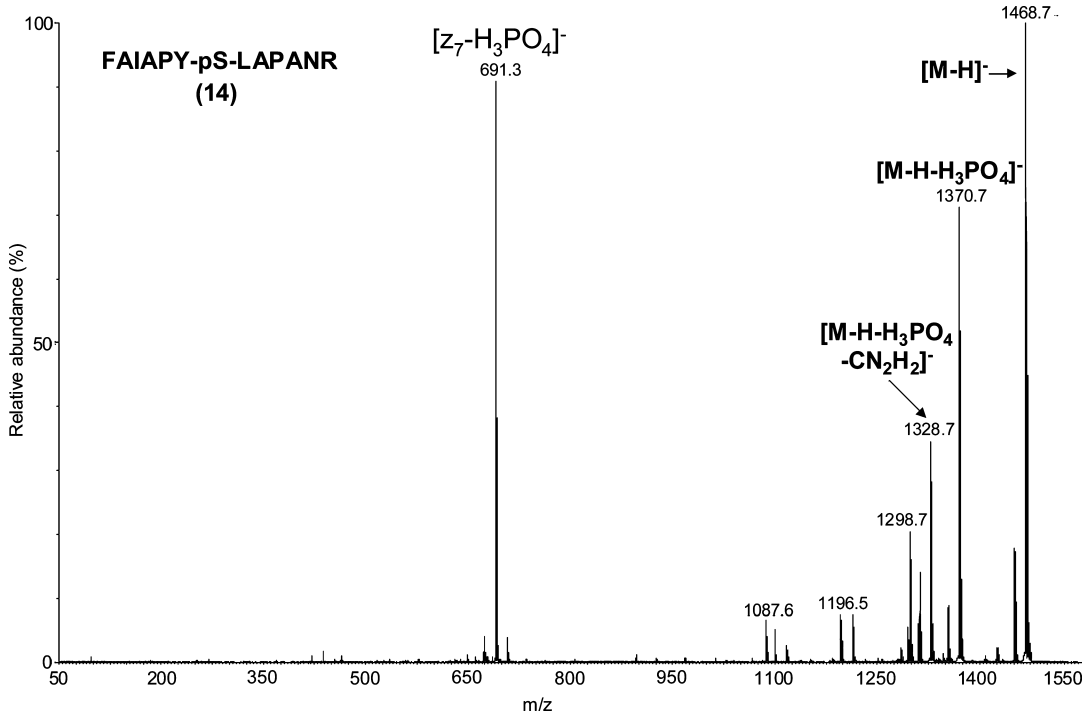


Figure 7. NanoESI-Q-TOF CID spectrum of the $[M - H]^-$ ion of phosphopeptide **14** (collision energy 55 eV). The mass spectrum shows a significant decrease in the efficiency of the 140 Da neutral loss of the peptide $[M - H]^-$ ions compared to the pTyr analogue **10**.

elimination from the corresponding $[M - H]^-$ ions. The tandem mass spectrum of the $[M - H]^-$ ion of **14** is presented in Figure 7. As expected, the CID data reveal a substantial decrease of the relative abundance of the $[M - H - 140]^-$ fragment ion, compared to the spectrum in Figure 5f. The most intensive signal of neutral loss product ions of the ionized pSer-peptide corresponds to the $[M - H - 98]^-$ fragment. The experimental data allow one to conclude that the efficient intramolecular migration of pTyr phosphate during CID of pTyr-peptide monoanions $[M - H]^-$ is a result of a high kinetic stability of phosphotyrosine residues. The higher kinetic stability of pTyr residues, relatively to that of pSer side chains, is a result of both the bigger strength of the phenoxy C–O bond (compared to that of alkoxy) and the lack of possibility

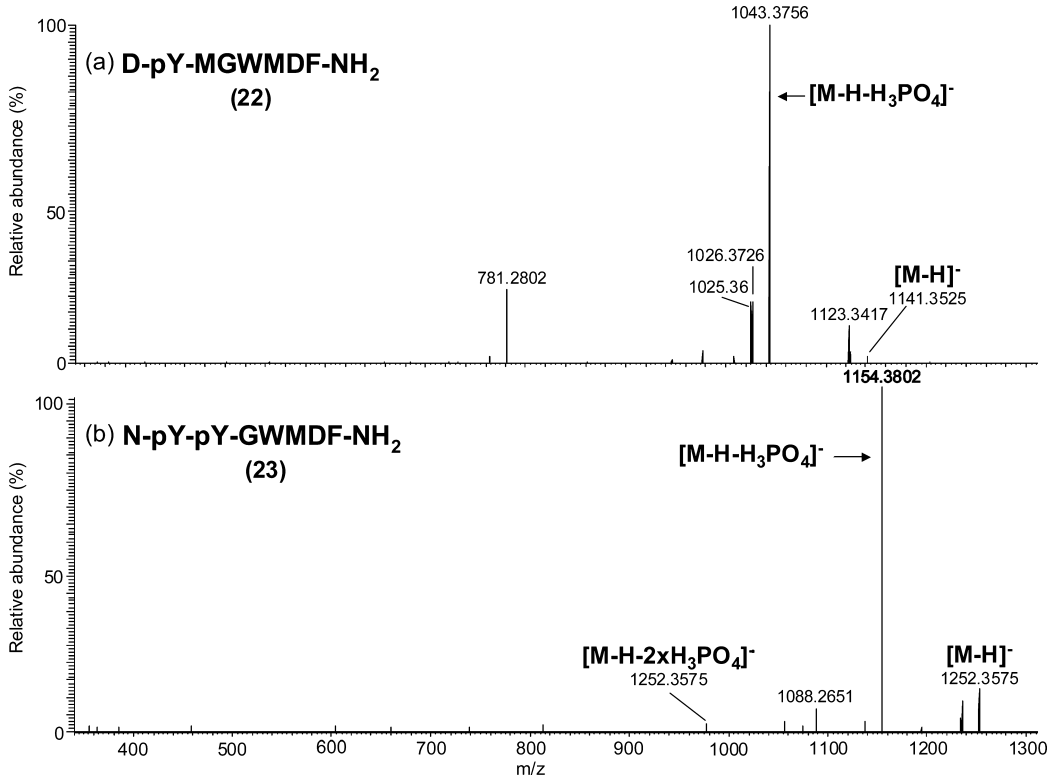


Figure 8. NanoESI-LTQ Orbitrap CID spectra of $[M - H]^-$ ions of phosphopeptides (a) **22** (normalized collision energy 17%) and (b) **23** (normalized collision energy 18%). The phosphate-induced fragmentation behavior of $[M - H]^-$ ions of pTyr-peptides in ion-trap CID is very similar to that observed upon Q-TOF CID.

to undergo a facial charge-remote H_3PO_4 β-elimination process. Thus, in the case of the aromatic phosphorylation sites, there is no competition of the H_3PO_4 release involving the intramolecular phosphate shift with an efficient phosphorylation by alternative mechanistic pathways. It is interesting to point out that the higher RA% of the $[M - H - 140]^-$ fragment ion in the CID spectrum of **14** (Figure 7) relatively to that of the $[M - H - 42]^-$ ion of the unmodified peptide **2** (Figure 1b) suggests that a small fraction of the pSer-containing deprotonated molecules of **14** undergo an intramolecular migration of the phosphate to the peptide C-terminus, in spite of the fact that phosphoserine may eliminate phosphoric acid via the facile β-elimination reaction.

Generality of H_3PO_4 Neutral Loss Phenomenon during CID of pTyr-Peptide $[M - H]^-$ Ions. To estimate the generality of the observed pTyr phosphate transfer during CID of singly deprotonated pTyr-peptides, nanoESI-Q-TOF tandem mass spectra of $[M - H]^-$ ions of a group of synthetic phosphopeptides **15**–**23** have been examined. To test whether the gas-phase rearrangement of the negatively charged phosphopeptides is limited to CID of the modified peptides in the linear collision cell, some of the pTyr-peptides were subjected to nanoESI-ion-trap CID as well. The CID data of **15**–**23** are presented in Table 1 and Figure 8.

The nanoESI-Q-TOF CID spectra of all phosphopeptides **15**–**23** show a neutral loss of H_3PO_4 from their $[M - H]^-$ ions (Table 1). These data suggest that a release of phosphoric acid is a characteristic feature of CID of $[M - H]^-$ ions of phosphotyrosine-containing peptides. Noteworthy, none of the negatively charged pTyr-peptides undergoes elimination of metaphosphoric acid HPO_3, which is the widely accepted typical neutral loss of pTyr-peptides upon CID. Besides, the CID mass spectra reveal that the efficiency of $[M - H - 98]^-$ fragment ion formation is very similar for peptides **18** and **19**, in spite of the fact that the C-terminus of **19** is amidated. These data indicate that the deprotonated C-terminal COOH group of pTyr-peptides in which the modified residue is located on the C-terminus is not involved in the H_3PO_4 elimination reaction. The experimental results can be explained by the rigidity of the pTyr phenyl ring which probably prevents an intramolecular interaction between the C-terminal pTyr phosphate and the peptide C-terminus. In the case of the peptide sequences **18** and **19**, there are two aspartic acid residues which may interact with the C-terminal phosphotyrosine inducing the efficient loss of H_3PO_4 upon CID (see Table 1). An additional support for the observation that the C-terminal carboxy group of pTyr-peptides with pTyr at the C-terminus does not participate in the internal phosphate shift is a higher intensity of the H_3PO_4 neutral loss fragment ion signal in the CID spectrum of the peptide **17** compared to that of **16**. Note, the N-terminal location of pTyr residues does not preclude the intramolecular phosphate migration during CID of the phosphopeptide monoanions (Figure 4b). In contrast to the peptide pair **18** and **19**, the Q-TOF CID spectra of **20** and **21** show a significant difference in the abundances of their neutral loss product ions. Indeed, whereas the tandem mass spectrum of **20** is dominated by the $[M - H - 98]^-$ and $[M - H - 140]^-$ ion signals, the corresponding fragment ions of the amidated analogue **21** are of low abundance. Furthermore, the peak of the 98 Da neutral loss fragment of **21** is more intensive compared to the $[M - H -$

140]$^-$ ion, but for the nonamidated sequence **20** the 140 Da elimination reaction is the most efficient fragmentation pathway. The suppressed formation of the [M − H − 98]$^-$ fragments from peptide **21** can be attributed to the fact that this peptide does not carry any side residue that may induce an efficient pTyr phosphate relocation with subsequent elimination of phosphoric acid. The observed differences in the fragmentation patterns of **20** and **21** correlate very well with those observed in the CID mass spectra of the peptides **6** and **10** (Figure 5, parts b and f). These data clearly demonstrate that sequences of tryptic pTyr-peptides carrying a C-terminal arginine can be rapidly recognized by negative ion CID using their [M − H − 140]$^-$ fragment as a reporter ion.

Similarly to the Q-TOF CID dephosphorylation pattern, the ion-trap CID spectra of [M − H]$^-$ ions of pTyr-peptides exhibit abundant signals of fragments produced by elimination of phosphoric acid (Figure 8). The 98 Da neutral loss reaction is the most efficient fragmentation pathway for both deprotonated phosphopeptides **22** and **23** measured in the ion-trap CID. Furthermore, the signals of the [M − H − 98]$^-$ fragment ions of **22** and **23** are more dominant in their ion-trap CID spectra compared to their Q-TOF tandem mass spectra. These observations are in accordance with expectations because first, primary fragment ions mostly stay intact in the ion-trap CID, and second, longer times of ion activation in the ion trap increase the probability of rearrangement reactions. In addition, an LTQ Orbitrap CID spectrum of phosphopeptide **24** (RD-pY-TGW-Nle-DF-NH_2) was acquired (data not shown). Similarly to the ion-trap CID behavior of **22** and **23**, the spectrum exhibits intensive signal of H_3PO_4 neutral loss product. Nevertheless, the relative abundance of the peptide neutral loss fragment [M − H − 98]$^-$ was lower than those of **22** and **23** (RA% 50 at normalized collision energy of 18%). The dominant fragment of the [M − H]$^-$ ion of **24** upon ion-trap CID is produced by elimination of H_2O from the ionized peptide.

CONCLUSIONS

This work reveals that, in contrast to what is expected based on the structure of the phosphotyrosine side chain, singly deprotonated pTyr-peptides tend to lose upon CID a molecule of phosphoric acid (98 Da) instead of HPO_3 (80 Da). The [M − H − 98]$^-$ fragment ions are generated both during quadrupole time-of-flight and ion-trap CID. Our study has demonstrated that the efficient H_3PO_4 elimination from [M − H]$^-$ ions of the modified peptides occurs following an intramolecular relocation of the pTyr phosphate group upon CID. Besides the phosphoaromatic sites, it was observed that phosphoserine peptide side chains may undergo the gas-phase phosphate shift as well, although the rearrangement in this case is much less efficient.

The H_3PO_4 neutral loss reaction of the ionized pTyr-peptides is a characteristic feature of the phosphopeptide fragmentation upon CID, and it can be used for detection of the modified sequences by MS/MS. Importantly, the CID spectra of phosphopeptide [M − H]$^-$ ions cannot be used to distinguish between the aromatic and aliphatic protein phosphorylation sites (pTyr and pSer/pThr, respectively) because of the similar dephosphorylation patterns. At the same time, the negative ion CID spectra of peptides with C-terminal arginine, in which the signal of [M − H − 140 Da]$^-$ product ion is higher than that of the 98 Da neutral loss fragment, can be considered as an indication for the original location of the phosphate on the aromatic side chain. The discovered phenomenon of H_3PO_4 elimination from pTyr-peptide monoanions helps to prevent wrong identifications of phosphorylation sites in peptides containing both hydroxyalkyl (Ser/Thr) and hydroxybenzyl (Tyr) side chains by the negative ion CID studies.

This study provides an additional aspect to phosphate-specific chemistry of phosphorylated peptides in the gas phase. The discovered shift of the pTyr phosphate group to deprotonated hydroxyl-containing peptide functions presents an example of intramolecular gas-phase base-catalyzed transesterification reaction. The mechanism of the pTyr phosphate shift of the deprotonated gaseous pTyr-peptides is analogous to that of in vivo dephosphorylation of phosphotyrosine residues by protein tyrosine phosphatases.[40] Finally, since there are multiple examples for the analogy between reactions of ions in gaseous and condensed phases, a possibility of an occurrence of the described phosphate shifts in solutions of peptides and proteins has to be completely excluded prior to application of experimental procedures to characterization of protein phosphorylation.

ACKNOWLEDGMENT

M.E.-A. acknowledges the financial support of the German Cancer Research Center (DKFZ), where a part of the work was done, via a guest scientist scholarship. M.E.-A. acknowledges the support of the German Research Foundation (DFG) through the Grant for a Temporary Position as a Principal Investigator (Eigene Stelle).

Received for review February 2, 2009. Accepted April 7, 2009.

AC900244E

(40) Tonks, N. K. *Nat. Rev. Mol. Cell Biol.* **2006**, *7*, 833–846.