RESEARCH ARTICLE

Discrimination Between Peptide O-Sulfo- and O-Phosphotyrosine Residues by Negative Ion Mode Electrospray Tandem Mass Spectrometry

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Abstract

Unambiguous differentiation between isobaric sulfated and phosphorylated tyrosine residues (sTyr and pTyr) of proteins by mass spectrometry is challenging, even using high resolution mass spectrometers. Here we show that upon negative ion mode collision-induced dissociation (CID), pTyr- and sTyr-containing peptides exhibit entirely different modification-specific fragmentation patterns leading to a rapid discrimination between the isobaric covalent modifications using the tandem mass spectral data. This study reveals that the ratio between the relative abundances of $[M-H-80]^-$ and $[M-H-98]^-$ fragment ions in ion-trap CID and higher energy collision dissociation (HCD) spectra of singly deprotonated +80 Da Tyr-peptides can be used as a reliable indication of the Tyr modification group nature. For multiply deprotonated +80 Da Tyr-peptides, CID spectra of sTyr- and pTyr-containing sequences can be readily distinguished based on the presence/absence of the $[M-nH-79]^{(n-1)-}$ and $[M-nH-79-NL]^{(n-1)-}$ ($n=2, 3$) fragment ions (NL=neutral loss).

Key words: Sulfotyrosine, Phosphotyrosine, Negative ion CID, Electrospray

Introduction

Tyrosine-O-sulfation is a common post-translational modification of proteins entailing covalent attachment of sulfate to the tyrosine side chain. It occurs mostly on secreted and trans-membrane spanning proteins. Many classes of proteins contain sulfotyrosine, including G protein-coupled receptors, adhesion molecules, coagulation actors hormones, and extracellular matrix proteins, which are implicated in a variety of pathophysiological processes [1, 2]. Tyrosine sulfation is involved in modulation of

protein–protein interactions, regulation of proteolytic processing, and modification of secretion rates [3, 4]. As many as 1% of all tyrosine residues of the total protein in an organism can be sulfated, making it the most common post-translational modification of the tyrosine side chain hydroxyl [5].

Elucidation of the physiological/pathophysiological role of particular sulfation events in living organisms requires the identification of exact sites of sulfation in protein sequences. Classic chemical sequencing by Edman degradation and amino acid analysis following acid hydrolysis fails to detect this modification because of the rapid decomposition of the modified residues at low pH and upon high temperature conditions [6]. In the recent decades, mass spectrometry has been established as a powerful method for analysis of a wide

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variety of protein post-translational modifications (PTMs) including the labile ones. However, currently no methods for unambiguous identification of this covalent modification by mass spectrometry are available [7]. Sulfotyrosine identification by mass spectrometry is hampered by its instability in the gas phase. Upon CID, peptide cations comprising tyrosine-O-sulfated residues undergo a facile elimination of sulfur trioxide SO_3 (80 Da) to produce unmodified tyrosine even at very low collision energies [8, 9]. The sulfotyrosine degradation partly occurs upon electron capture and electron transfer dissociation conditions (ECD and ETD respectively) [10, 11] or even under the usually non-fragmenting conditions of soft-ionizing electrospray ionization (ESI) [12–14].

Negative ion mode mass spectrometry can facilitate significantly the characterization of protein sulfation sites relatively to analysis of protonated species. First, this approach can provide a more realistic insight into degree of protein sulfation. It has been demonstrated that the sulfotyrosine degradation is more pronounced in the case of protonated peptides in comparison to their deprotonated counterparts [15]. A decisive role of the proton in the sTyr dissociation has been shown by increased stability of sTyr-peptide $[\text{Na}]^+$ adducts relatively to their protonated analogues [16]. Another advantage of the negative polarity for MS analysis of sulfotyrosine residues over the positive ion mode is its ability to ionize the modified peptides via proton abstraction. It is well known that peptides carrying strongly acidic moieties such as sulfate and phosphate and those rich in amino acids with acidic side chains (Asp, Glu) or containing multiple acidic modification sites are ionized more efficiently in the negative ion mode [17–19]. Protonation efficiency of peptides that may contain multiple basic residues is determined by the ratio between the number of proton-donor and proton-acceptor functions [20]. Noteworthy, the sTyr sulfate group is not only more acidic than the tyrosine phosphate ester, but sulfotyrosine residues in proteins are also typically surrounded by several acidic amino acids [21–23]. In addition to the higher stability and the enhanced ionization efficiency of sTyr residues in the negative ion mode, the extreme lability of sulfotyrosine at low pH does not favor a prolonged exposure of the sulfated samples to acidic liquid chromatography buffers [24]. The tyrosine-O-sulfate hydrolysis can be minimized by use of higher pH solvents (with $\text{pH} > 4$) [4], which are optimal for negative ion mode mass spectrometric analysis.

Another major challenge in analysis of protein sulfation by mass spectrometry is distinguishing the sulfated side chains from the isobaric phosphorylated residues. Phosphate and sulfate moieties have the same nominal mass (80 Da) while their monoisotopic masses differ by 9.4 mDa (sulfate addition=79.9568; phosphate addition=79.9663). Although high resolution and high mass accuracy instruments could, in principle, distinguish the corresponding masses, unequivocal assignments of the two PTMs would be error-prone because of interfering

chemical noise and ion statistics limitations [25]. A comparison of liquid chromatography retention times of identified +80 Da Tyr-peptides of protein digests with those of synthetic sulfated/phosphorylated analogues may help to resolve uncertainty regarding the nature of the tyrosine modifying group [9, 26, 27]. However, this approach produces ambiguous data in cases when sulfated and phosphorylated peptide forms co-elute or have similar retention times and it cannot be applied to a large-scale protein analysis.

An alternative and frequently more reliable approach to discrimination between sulfated and phosphorylated protein residues by mass spectrometry is through the use of the differences in tandem mass spectral data (MS/MS) of the covalently modified peptides. In positive ion CID, both sulfo- and phosphotyrosine-containing peptides undergo elimination of 80 Da (SO_3 and HPO_3 for sTyr and pTyr, respectively) although the desulfation reaction is typically significantly more pronounced [12, 28, 29], which is often used for predicting the nature of the tyrosine modification [7]. However, the elimination reactions of both protonated sTyr- and pTyr-containing peptides are strongly affected by the length of the modified peptides, their charge states and the amino acid compositions hampering an unambiguous identification of the Tyr modifying group nature [30]. Thus, for example, multiple modification sites, basic residues, and longer chains of sulfopeptides induce an increase in stability of the sulfated sequences suppressing their modification-specific decomposition [7, 8]. Protonated pTyr peptides give rise upon CID to a characteristic m/z 216.043 immonium ion [31]. However, similarly to the loss of 80 Da neutrals, the degree of the immonium ion formation varies considerably with phosphopeptide amino acid sequence [32]. Furthermore, multiple fragments observed in the low mass regions of the peptide positive ion mode CID spectra frequently interfere with pTyr immonium ion signal [31] and is usually not detectable in ion-trap CID spectra because of the low mass cut-off [33]. In the negative ion mode, we recently demonstrated that pTyr peptide monoanions undergo an extensive elimination of phosphoric acid (H_3PO_4) [34], while the multiply charged peptide species produce phosphate-induced $[\text{M}-\text{nH}-79]^{(n-1)-}$ fragment ions [35]. For sulfated peptides, an expulsion of SO_3 has been occasionally observed for singly deprotonated molecules [15]. There is no information available on the gas-phase chemistry of multiply charged sulfopeptide anions upon tandem mass spectrometry. Multiply deprotonated sulfopeptides, $[\text{M}-\text{nH}]^{n-}$ ($n > 1$), are not only very abundant species in the negative ion mode nanoelectrospray spectra of sulfopeptides but are frequently the only ions that can be used for tandem mass spectrometry analysis.

In this work, we investigate whether negative ion mode CID modification-specific patterns of phospho- and sulfotyrosine-containing peptides can be used for a reliable discrimination between these isobaric protein tyrosine modifications by mass spectrometry. In this study, comparative analysis of CID chemistry of pairs of deprotonated

sTyr- and pTyr peptides differing by the Tyr modifying group exclusively has been performed. We formulate here clear rules for discrimination between these two types of protein tyrosine modifications by negative ion ion-trap CID and HCD.

Materials and Methods

Mass Spectrometry

NanoESI-MS and MS/MS spectra of peptides have been acquired on an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a robotic nanoflow ion source NanoMate HD System (Advion Biosciences, Inc., Ithaca, NY, USA). Two tandem mass spectrometry techniques have been used: CID in a linear ion trap (IT-CID) and CID in an external octapole collision cell (HCD) of the LTQ Orbitrap instrument. The CID experiments were carried out by mass selecting the desired ions with a 1.5–5 m/z units window and subjecting them to the following typical conditions: normalized collision energy (NCE) between 10% and 30% for LTQ CID and 30–85 eV collision energy (CE) for the HCD measurements. NCEs used for the IT-CID spectra experiments have been ramped till maximal ion-trap fragmentation of the precursor ions was observed. Activation parameter q was set to 0.25 and activation time of 30 ms was applied. All Orbitrap MS/MS spectra were acquired with target mass resolution of 7500 (at m/z 400). Intact peptide ions were detected in the Orbitrap at 60,000 resolution (at m/z 400). Peptide analytes were dissolved in a 50%/0.5%/49.5% (vol/vol/vol) mixture of methanol/formic acid/water to a concentration of ~10–50 pmol/ μ L. Sequences of natural sulfopeptides **1a–6a** and their synthetic phosphorylated analogues **1b–6b** are listed in Table 1. The sulfotyrosine-containing peptides were purchased from Bachem AG and Sigma-Aldrich. The phosphorylated analogues of sTyr-containing peptides were produced by solid-phase synthesis.

Phosphopeptide Synthesis

For solid-phase synthesis of the phosphopeptides the Fmoc strategy [36, 37] was employed. A multiple automated

synthesizer (Syro II; Multisynthetech) 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.

Results and Discussion

Ion Trap CID Modification-Induced Neutral Loss Reactions of sTyr- and pTyr Peptide [M – H][–] Ions

Nanoelectrospray LTQ-Orbitrap CID spectra of singly deprotonated sulfated peptides **1a–6a** exhibit highly intense signals of product ions formed by elimination of 79.9568 Da (SO_3) from the peptide molecular ions $[M - H]^-$ (Table 2, Figure 1). The expulsion of SO_3 from the negatively charged sulfopeptides **1a–6a** can occur either through a simple sulfur–oxygen bond cleavage of the deprotonated sTyr side chain (route A, Scheme 1) or via a rearrangement of the sulfotyrosine neutral form accompanied by intramolecular proton transfer (route B, Scheme 1). Noteworthy, the $[M - H]^-$ ion of the sulfopeptide **6a** undergoes an extremely efficient neutral loss of H_2O causing a partial reduction of the peptide $[M - \text{H} - 80]^-$ ion relatively to the other peptides (Table 2). The unusually facile expulsion of water from **6a** is induced by N-terminal location of Glu, the side chain of which can rapidly interact with the N-terminal peptide amine to form a stable cyclic amide structure [38].

To examine whether the extensive 80 Da neutral loss of singly deprotonated sTyr-peptides **1a–6a** can be used to distinguish the sulfated sequences from the corresponding phosphotyrosine-containing analogues, negative ion mode IT-CID Orbitrap spectra of $[M - H]^-$ ions of **1b–6b** have been analyzed. A comparison of the experimental data obtained for the sulfated and phosphorylated peptide pairs **1–6** reveals a considerable difference in fragmentation patterns of the singly deprotonated sTyr- and pTyr peptides. Indeed, while 80 Da neutral loss reaction is the major modification-induced decomposition pathway of the sulfated peptides **1a–6a**, the RAs of the corresponding 80 Da (HPO_3) elimination product ions of **1b–6b** do not exceed 2% (Table 2, Figures 1 and 2). In contrast, 98 Da neutral loss products of the phosphopeptides **1b–6b** (formed by elimination of H_3PO_4 for pTyr sequences) [34] are significantly more abundant in comparison to the sulfated analogues **1a–6a**. The $[M - \text{H} - 98]^-$ fragments of sulfopeptides can be generated upon CID by secondary fragmentation of the ionized peptides via sequential losses of SO_3 and H_2O from the ionized molecules. MS^3 experiments performed for the $[M - \text{H} - 80]^-$ ions of **2a**, **4a–6a** (data not shown) are either dominated by or display H_2O neutral loss $[M - \text{H} - 80 - \text{H}_2\text{O}]^-$ ions exclusively. These results are in a full agreement with the observed secondary fragmentation of the sulfopeptide $[M - H]^-$ ions during IT-CID to produce the $[M - \text{H} - 98]^-$

Table 1. Sequences and Molecular Weights of sTyr- and pTyr Peptide Pairs

Peptide ^a	MW ^b Da
N-sY-sY-GWMDF-NH ₂ 1a	1253.3463
D-sY-MGWMDF-NH ₂ 2a	1142.3507
sY-GGFL 3a	635.2261
RD-sY-TGW-Nle-DF-NH ₂ 4a	1250.5026
GDFFEEIPEE-sY-LQ 5a	1547.5973
EQFDD-sY-GHMRF-NH ₂ 6a	1522.5605
N-pY-pY-GWMDF-NH ₂ 1b	1253.3653
D-pY-MGWMDF-NH ₂ 2b	1142.3603
pY-GGFL 3b	635.2356
RD-pY-TGW-Nle-DF-NH ₂ 4b	1250.5121
GDFFEEIPEE-pY-LQ 5b	1547.6068
EQFDD-pY-GHMRF-NH ₂ 6b	1522.5700

^asY=sulfotyrosine; pY=phosphotyrosine; Nle=norleucine;

^bMonoisotopic molecular weight (in Daltons)

Table 2. Relative Abundances (RA%) of Modification-Induced Neutral Loss Product Ions in Ion-Trap Orbitrap CID^a Spectra of Singly Deprotonated Sulfated and Phosphorylated Peptides 1–6

Sulfopeptide	[M-H-80 (SO ₃)] [−]	[M-H-98 (SO ₃ +H ₂ O)] [−]	Phospho-peptide	[M-H-80 (HPO ₃)] [−]	[M-H-98 (H ₃ PO ₄)] [−]
1a	100	<0.1	1b	2	100
2a	100	41	2b	<0.1	100
3a	100	0.3	3b	<0.1	14 ^b
4a	100	47	4b	1	36 ^c
5a	100	24	5b	<0.1	100
6a	56 ^c	36	6b	<0.1	85 ^c

^aNCE 20%.^bThe main fragment of the CID spectrum of **3b** is represented by a decomposition product of the phosphopeptide [M-H-98][−] ion (unmodified *m/z* 293 c₃[−] peptide ion). The deprotonated phosphopeptide **3b** eliminates H₃PO₄ so efficiently that the resulting [M-H-98][−] ion undergoes the extensive fragmentation in spite of the resonant nature of the ion trap decompositions.^cThe most intense signal in the CID spectrum belongs to the peptide [M-H-H₂O][−] product ion

fragments. The direct elimination of 98 Da in the form of H₂SO₄ from sTyr residues of deprotonated sTyr peptides does not seem to be a viable option for formation of the sTyr peptide [M-H-98][−] ions, since the resultant product would possess a highly reactive benzyne moiety. Indeed, CID data of the [M-H-2×80][−], [M-H-80][−], and [M-H-80-H₂O][−] ions of **1a**, **3a**, and **2a**, **4a–6a**, respectively, clearly indicate the presence of unmodified Tyr residues at the positions of the former sulfated peptide residues. The observed distinctive IT-CID decomposition behavior of the sulfo- and phospho-tyrosine-peptide pairs clearly demonstrates that the intensive signals of the [M-H-80][−] ion in the tandem mass spectra of

the deprotonated sulfated peptides **1a–6a** are directly related to the presence of the sulfated tyrosine residues and are not induced by decomposition of any other peptide function. Noteworthy, the similar extents of the [M-H-98][−] ion formation of **2a/5a** and those of [M-H-H₃PO₄-H₂O][−] fragments of **2b** and **5b** (Figures 1 and 2) further support the stepwise loss of 98 Da during CID of the deprotonated sulfated peptides. Noteworthy, ion-trap Orbitrap CID data of [M-H][−] ions of unmodified analogues of the peptides **1**, **3**, and **5** (N-Y-Y-GWMDF-NH₂, Y-GGFL and GDFEEIPEE-Y-LQ) have been also analyzed (data not shown) to confirm that the [M-H-80][−] and the [M-H-98][−] fragment ions of the

Figure 1. Ion-trap Orbitrap CID spectra (NCE 20 %) of [M-H][−] ions of sulfated peptides (a) **1a**, (b) **2a**, (c) **3a**, and (d) **5a**

Scheme 1. SO₃ elimination routes of sulfotyrosine-containing peptides in the negative ion mode

sulfo- and phosphotyrosine-peptides **1–6** are indeed the modification-specific product ions of these peptides. The tandem mass spectra of the deprotonated unmodified analogues display neither 80 nor 98 Da neutral loss products for these precursor ions.

Analysis of the negative ion mode ion-trap CID data of the singly charged sulfo- and phosphotyrosine-containing

peptides **1a–6a** and **1b–6b** clearly indicate that the different nominal masses of the sTyr- and pTyr-specific peptide fragments allow a rapid recognition of the tyrosine modifying group nature. Thus, the CID spectra of pTyr-containing peptides are characterized by highly suppressed [M-H-80]⁻ ions and abundant [M-H-98]⁻ product ions. In contrast, sTyr peptide precursor ions exhibit abundant [M-H-80]⁻ fragments and significantly more suppressed [M-H-98]⁻ ions. The data listed in Table 2 indicate that sulfopeptides with N-terminal aspartic/glutamic acids (**2a** and **6a**) and those in which Asp/Glu side chains are adjacent to nucleophilic amine/carbonyl-containing side residues (**4a** and **5a**) undergo an enhanced release of water to produce the [M-H-98]⁻ fragments. The sulfopeptide IT-CID mass spectra indicate that even in case of peptide **6a**, the sequence of which is extremely prone to the H₂O expulsion because of the possibility of a resulting terminal five-membered cyclic amide formation, the RA of the diagnostic peptide [M-H-80]⁻ fragment exceeds significantly the [M-H-98]⁻ ion signal (Table 2).

General guidelines for using IT-CID spectra of [M – H]⁻ ions of +80 Da peptides to distinguish between sTyr- and pTyr-containing sequences are presented in Scheme 2. Thus, if RA% of [M-H-98]⁻ fragment in CID spectrum of a +80 Da peptide is considerably (by at least 20-fold) higher than that of the [M-H-80]⁻ ion then it is most likely that the

Figure 2. Ion-trap Orbitrap CID spectra (NCE 20%) of [M – H]⁻ ions of pTyr peptides (a) **1b**, (b) **2b**, (c) **3b**, and (d) **5b**

Scheme 2. Discrimination between sTyr- and pTyr-containing peptides using IT-CID data of the $[M - H]^-$ ions

precursor ion belongs to a pTyr-containing peptide. In contrast, if RA of the peptide $[M-H-80]^-$ fragment exceeds that of the 98 Da neutral loss ion there is a strong indication for the occurrence of a sulfotyrosine-containing peptide. Energy-resolved CID experiments performed for the peptide pairs **1–6** reveal that ion-trap CID analysis at NCE of 20% can be effectively used to differentiate between the isobaric Tyr modifications of singly deprotonated sTyr- and pTyr peptides within the mass range of 600–1600 Da. It is important to point out that the modification-specific patterns outlined in Scheme 2 do not enable differentiation between aromatic (Tyr) and aliphatic (Ser, Thr) modified residues of +80 Da modified peptides in which there are more than one hydroxyl-containing residue. Indeed, the $[M - H]^-$ ions of pSer- and pThr peptides also undergo efficient H_3PO_4 (98 Da) neutral loss upon IT-CID [29] while peptides with rarely occurring sulfoserine and sulfothreonine residues [27] are expected to eliminate SO_3 neutrals similarly to their sTyr analogues.

Interestingly, the ion-trap CID data of the peptide pairs **1–6** indicate that $[M - H]^-$ ion of the peptide pY-GGFL **3b** is significantly more stable in comparison to the deprotonated molecules of other phosphopeptides **1b**, **2b**, **4b–6b** (e.g., Figure 2). Similarly, the sulfated analogue **3a** exhibits an increased stability of the $[M - H]^-$ ion in comparison to the other analyzed sulfated peptides (e.g., Figure 1). Indeed, the molecular ion of the pentapeptide **3b** only decomposes completely at NCE of 35% while $[M - H]^-$ ions of the long phosphorylated sequences **1b**, **2b**, **4b–6b** disappear from their tandem mass spectra at NCE of 20–22%. The observed stability of the deprotonated modified enkephalin is probably caused by the N-terminal location of the modified Tyr residues, which favors their stabilization via internal hydrogen bonding with the peptide N-terminal amine. The hydrogen bond formation is expected to reduce the $[M - H]^-$ ion entropy to a significantly smaller extent for peptides with N-terminally located modified residue relatively to these with internal modified side chains, facilitating the formation of the stabilized molecular ions. A partial negative charge on the pTyr phosphate group as well as reduction of the sulfate proton lability caused

by the H bridging are also expected to contribute to the suppression of the modified side chain decomposition of the peptides **3a** and **3b**. Since the sequence **3** does not contain amino acids causing efficient fragmentations other than these occurring on the modified Tyr residues, the singly deprotonated peptides display enhanced stability upon IT-CID. The observed phenomenon correlates very well with the known solution chemistry of sTyr-peptides. It has been shown that peptide basic residues, in particular Arg side chains, increase acid-stability of sTyr-peptides by forming salt bridges with sulfoester moieties [4, 8]. The similar effect of Arg residues has been previously observed in gaseous sTyr-peptide ions [8].

IT-CID Modification-Specific Fragmentation Patterns of $[M - nH]^{n-}$ ($n > 1$) Ions of pTyr- and sTyr Peptides

Negative ion mode nanoESI mass spectra of the analyzed sTyr-/pTyr peptides **1a**, **2a**, **4a–6a**, **1b**, **2b**, and **4b–6b** display doubly charged peptide ions $[M - 2H]^{2-}$ at higher intensities than the corresponding singly charged $[M - H]^-$ species. The MS spectra of **5a** and **5b** also exhibit $[M - 3H]^{3-}$ ions with RA of 8% and 5%, respectively. To study modification-specific patterns of the multiply charged molecules of the modified peptides **1–6** IT-CID spectra of the $[M - 2H]^{2-}$ and $[M - 3H]^{3-}$ ions have been analyzed. The experimental data (Table 3) revealed a very prominent difference in the dissociation patterns of the multiply deprotonated sTyr- and pTyr peptides, especially in m/z areas above the peptide precursor ions. Indeed, the mass spectra of the phosphorylated sequences **1b–6b** are characterized by presence of a series of high mass fragments $[M - nH-79]^{(n-1)-}$ and $[M - nH-79-NL]^{(n-1)-}$ ($n > 1$), the signals of which do not appear in the CID spectra of the sulfated analogues (**1a–6a**). These results demonstrate that multiply deprotonated sulfotyrosine-containing peptides do not undergo dissociation of sTyr residues to produce two anionic species, similarly to the phosphorylated analogues. The high mass regions of IT Orbitrap CID spectra of sulfopeptide $[M - 2H]^{2-}$ and $[M - 3H]^{3-}$ ions are free of signals, which allows a rapid differentiation of the sulfated sequences from pTyr-containing peptides (Table 3, Figure 3). The ion trap CID data clearly demonstrate that the $[M - nH-79]^{(n-1)-}$ and $[M - nH-79-NL]^{(n-1)-}$ ($n > 1$) fragment ions of multiply deprotonated pTyr peptides can be reliably used to distinguish between protein sulfation and phosphorylation sites, even using a lower resolution mass analyzer (Scheme 3). The high mass fragments of multiply deprotonated phosphopeptides are produced by phosphate anion PO_3^- cleavage from doubly ionized phosphotyrosine side chains of the peptides [35]. The $[M - nH-79-NL]^{(n-1)-}$ fragments are formed by elimination of neutral species from the phosphate-induced product ions, the identity of which is determined by a phosphopeptide primary structure.

Table 3. Relative Abundances (RA%)^a of Modification-specific Fragments of Multiply Deprotonated Modified Peptides 1a-6a and 1b-6b in Ion-trap CID Mass Spectra

Peptide	Relative abundance, RA%
1a ^b	$[M - 2H]^{2-}$: $[M-2H-SO_3-2xH_2O]^{2-}$ (1.4%), $[M-2H-SO_3-H_2O]^{2-}$ (6.7%), $[M-2H-SO_3]^{2-}$ (100%)
1b ^b	$[M - 2H]^{2-}$: $[M-2H-H_3PO_4]^{2-}$ (1%), $[M-2H-PO_3-H_2O]^{2-}$ (19.4%), $[M-2H-PO_3]^{2-}$ (100%)
2a ^{b,c}	$[M - 2H]^{2-}$: $[M-2H-SO_3]^{2-}$ (<0.1%)
2b ^{b,c}	$[M - 2H]^{2-}$: $[M-2H-PO_3-H_2O-NH_3]^{-}$ (1.4%), $[M-2H-PO_3-H_2O]^{-}$ (10.9%), $[M-2H-PO_3]^{-}$ (25.62%)
3a ^{c,d}	$[M - 2H]^{2-}$: m/z 214.0174 (4%) $[M-2H-L-SO_3]^{-}$ (1%)
3b ^d	$[M - 2H]^{2-}$: $[M-2H-PO_3]^{-}$ (100%)
4a ^{b,c}	$[M - 2H]^{2-}$: $[M-2H-SO_3]^{2-}$ (2%)
4b ^{b,c}	$[M - 2H]^{2-}$: $[M-2H-HPO_3]^{2-}$ (2%), $[M-2H-PO_3-44-H_2O]^{-}$ (2%), $[M-2H-PO_3-44]^{-}$ (4.7%), $[M-2H-PO_3]^{-}$ (15%)
5a ^g	$[M - 2H]^{2-b}$: $[M-2H-H_2O-SO_3]^{2-}$ (61%), $[M-2H-SO_3]^{2-}$ (61%) $[M - 3H]^{3-c}$: $[M-3H-SO_3]^{3-}$ (2%), $[M-3H-H_2O-SO_3]^{3-}$ (6%)
5b	$[M - 2H]^{2-b}$: $[M-2H-H_3PO_4-H_2O]^{2-}$ (53.7%), $[M-2H-H_3PO_4]^{2-}$ (100%), $[M-2H-PO_3-2xH_2O]^{-}$ (2.6%), $[M-2H-PO_3-H_2O]^{-}$ (9%), $[M-2H-PO_3]^{-}$ (8%) $[M - 3H]^{3-d}$: $[M-3H-PO_3-H_2O]^{2-}$ (3.5%), $[M-3H-PO_3-H_2O]^{2-}$ (100%), $[M-3H-PO_3]^{2-}$ (97.3%)
6a ^{b,c}	$[M - 2H]^{2-b}$: $[M-2H-H_2O-SO_3]^{2-}$ (4.1%), $[M-2H-SO_3]^{2-}$ (11%)
6b ^b	$[M - 2H]^{2-b}$: $[M-2H-H_3PO_4-H_2O]^{2-}$ (3.5%), $[M-2H-H_3PO_4]^{2-}$ (100%), $[M-2H-PO_3-2xH_2O]^{-}$ (3.5%), $[M-2H-PO_3-H_2O]^{-}$ (8%), $[M-2H-PO_3]^{-}$ (1%)

^aRA of the fragment ions are given in brackets.^bNCE 20%.^cThe base peak of the spectrum belongs to the peptide $[M-2H-H_2O]^{2-}$ fragment.^dNCE 18%.^eNCE 15%.^fThe base peak of the spectrum belongs to the peptide b_4^{-} fragment.^gThe base peaks of the peptide CID spectra belong to $[M-2H-H_2O]^{2-}$ and $[M-3H-H_2O]^{3-}$ fragments, respectively

Other modification-induced fragmentation reactions of multiply charged peptides 1–6 observed upon negative ion IT-CID are elimination of SO_3 and H_3PO_4 for sulfated and

phosphorylated sequences, respectively. Interestingly, in contrast to the fragmentation patterns of the peptide $[M-H]^{-}$ ions, the neutral losses of the multiply deprotonated

Figure 3. Ion-trap Orbitrap CID spectra (NCE 20%) of $[M - 2H]^{2-}$ ions of sTyr-/pTyr peptide pairs (a) 1a, (b) 1b, (c) 5a, and (d) 5b

Scheme 3. Discrimination between sTyr- and pTyr-containing peptides using IT-CID data of the $[M - nH]^{n-}$ ($n=2, 3$) ions

peptides are efficient only for sequences **1** and **5** that are characterized by highly acidic nature of their primary structures (Tables 1 and 3). Indeed, peptides **1a** and **1b** carry two highly acidic modified residues while the sequences **5a** and **5b** contain a considerable proportion of Asp/Glu amino acids (about 50% of the peptide functions). The observed strong dependence of the SO_3 elimination efficiency of the deprotonated sTyr-peptides **1a–6a** on the precursor ion charge state and the peptide amino acid sequences provides further insight into the desulfation mechanism of sTyr-peptides in the negative ion mode. Thus, the SO_3 loss suppression for $[M - 2H]^{2-}$ ions of **2a–4a**, **6a**, relatively to their singly charged analogues, combined with the highly efficient expulsion of SO_3 from both singly and multiply charged molecules of **1a** and **5a**, are a clear evidence for dominance of sTyr side chain rearrangement (route B of Scheme 1) over the simple bond cleavage (route A of Scheme 1). Indeed, suppose that the SO_3 expulsion does not involve proton migration to a sulfotyrosine phenyl oxygen (route A of Scheme 1), but is instead initiated by sulfate proton abstraction. In such a case, the efficiency of the elimination reaction would grow with an increase of the deprotonated peptide charge state, diminishing for sequences with a higher proportion of proton-donor functions. The experimental data presented in Tables 2 and 3 clearly demonstrate that the desulfation trend of deprotonated sulfotyrosine-containing peptides upon ion-trap CID is the opposite one. The observed suppression of SO_3 elimination during CID of the multiply deprotonated sulfopeptides **2a–4a**, **6a** relative to their singly charged analogues can be explained by the reduced number of peptide precursor ions carrying uncharged sulfate moieties in the case of multiply deprotonated species. Although the catalyzing proton can, in principle, originate in peptide functions other than the sulfate group, the strong reduction of the $[M-2H-SO_3]^{2-}$ fragment in the mass spectra of **2a–4a** indicates that energy requirements of such interaction makes the expulsion of SO_3 less favorable in comparison to alternative peptide decomposition routes. The high intensities of the SO_3 neutral loss

fragments in the IT-CID spectra of $[M - 2H]^{2-}$ ions of **1a** and **5a** (and the moderate extent of desulfation for **6a**) reveals a facilitating effect of the peptide acidic functionalities on the sulfate-induced reaction. The smaller the number of the peptide labile protons available for the catalysis of SO_3 loss from the $[M - nH]^{n-}$ ($n>1$) ions, the higher the number of anionic reactive centers driving the modified sequences to fragmentation modes competing with the sTyr desulfation. Thus, six COOH groups of **5a** can not only undergo deprotonation instead of sTyr modifying group but they can also serve as proton donors accelerating the sTyr decomposition. Noteworthy, the decreased extent of the neutral loss reaction for the triply charged molecules of **5a** (Table 3), relatively to the molecular dianion, clearly supports this hypothesis.

Similarly to the desulfation behavior of $[M - 2H]^{2-}$ ions of sulfopeptides, elimination of H_3PO_4 from doubly deprotonated pTyr peptides is strongly sequence-dependent. Indeed, while the $[M-2H-H_3PO_4]^{2-}$ fragments give rise to the base signals in IT-CID spectra of phosphopeptides **5b** and **6b** the neutral loss ions are absent or strongly suppressed in the spectra of other phosphorylated sequences (Table 3). Recently, we demonstrated that the elimination of H_3PO_4 from deprotonated pTyr peptides is induced by an intramolecular attack of the uncharged pTyr phosphate group by anionic functions of the modified peptides [34]. Elimination of H_3PO_4 from multiply deprotonated pTyr peptides has not been previously observed during CID of phosphorylated peptides. The experimental data of **1b–6b** (Table 3) demonstrate for the first time that the phosphate relocation of phosphotyrosine-containing peptides can also take place during negative ion CID of their multiply charged ions of peptides in which pTyr residues are located in vicinity of D/E side chains. Indeed, the acidic side chains can efficiently compete with pTyr for the negative charge of the ionized peptides, increasing the probability for phosphotyrosine to exist in its neutral form. Noteworthy, the peptides **5b** and **6b** carry the motifs -EEpY- and -DDpY-, respectively, in addition to the other acidic functions. A suppression of the H_3PO_4 loss from $[M - 2H]^{2-}$ ions of **1b** further underlines the significant effect of the peptide sequence on the efficiency of the modification-induced reaction. Thus, although the pTyr residue of **1b** is adjacent to another modified side chain, the $[M - 2H]^{2-}$ ion of the peptide does not expel H_3PO_4 efficiently, probably because of the internal solvation of one of the phosphate groups with adjacent Asn-1 of the peptide. In summary, the strong peptide sequence dependence observed for both SO_3 and H_3PO_4 elimination reaction efficiencies of multiply deprotonated sTyr- and pTyr-containing peptides, respectively, (Table 3) clearly indicates that in contrast to the behavior of the $[M - H]^-$ ions, the neutral loss fragmentation reactions of the peptide $[M - nH]^{n-}$ ($n>1$) ions upon CID cannot be used for a reliable recognition of the tyrosine modifying groups.

Modification-Specific Reactions of Deprotonated sTyr- and pTyr Peptides Upon HCD

HCD spectra of deprotonated modified peptides **1–6** have been examined in order to test whether the prominent differences observed in ion-trap CID fragmentation patterns of sTyr- and pTyr sequences are preserved during CID in a quadrupole collision cell. The HCD data of the modified peptides reveal that sulfation- and phosphorylation-specific fragmentation patterns of the singly deprotonated sTyr- and pTyr peptides are identical to those observed upon ion trap CID (Figures 1, 2, and 4, Tables 2 and 4). Although, as expected, the HCD mass spectra exhibit more intense signals of the peptide backbone fragments, sulfated and phosphorylated Tyr residues can be distinguished using the mass spectral data. The HCD spectra of all the examined sulfated sequences display higher signals of $[M-H-80]^-$ ions in comparison to the corresponding $[M-H-98]^-$ fragments while the tandem mass spectra of the phosphorylated peptides reveal the opposite trend (Table 4). Energy-resolved study of the HCD fragmentations of the modified peptides **1–5** (CE was ramped from 30 to 70–80 eV in increments of 5 eV) found CE of 50 eV suitable for differentiation between sulfo- and phosphotyrosine residues of the singly deprotonated analyzed peptides with molecular weights between 600 and 1600 Da. The collision energies that differed from the optimized 50 eV turned

out to be either insufficient for extensive modification-specific fragmentation of the peptides or were too high to preserve the sulfate/phosphate-induced signals throughout HCD process. The HCD spectral data presented in Table 4 clearly demonstrate that in addition to IT CID, this type of fragmentation can be effectively used for distinguishing sTyr and pTyr residues of peptide monoanions.

HCD mass spectral data of the $[M-2H]^{2-}$ and $[M-3H]^{3-}$ ions of peptides **1–6** and **5a/5b**, respectively (data not shown), displayed modification-specific fragmentations analogous to those observed in their IT-CID spectra (Table 3). Similarly to the ion-trap CID behavior, doubly and triply deprotonated phosphopeptides **1b–6b** exhibit upon HCD the $[M-nH-79]^{(n-1)-}$ and $[M-nH-79-NL]^{(n-1)-}$ ($n > 1$) product ions, that are not formed by the sulfated analogues. A collision energy of 50 eV has been found suitable for discrimination between the HCD spectra of all of the examined sulfated and phosphorylated peptides using the high mass $[M-nH-79]^{(n-1)-}$ and $[M-nH-79-NL]^{(n-1)-}$ ($n = 2, 3$) phosphopeptide signature ions. The HCD spectra of pTyr peptides are rapidly recognized based on the presence of the high mass marker ions, which are absent in the tandem mass spectra of the corresponding sulfated analogues. Noteworthy, HCD spectrum of $[M-2H]^{2-}$ ion of sulfated Leu-enkephalin **3a** exhibited deprotonated sulfotyrosine immonium ion at m/z 214.0174 $\text{NH}=\text{CHCH}_2\text{C}_6\text{H}_4\text{OSO}_3^-$ (RA% of 8.5–30 at collision energies of 65–90 eV, respectively), the analogue of which was

Figure 4. HCD (CE 50 eV) of $[M-H]^-$ ions of sTyr-/pTyr peptide pairs (a) **2a**, (b) **2b**, (c) **5a**, and (d) **5b**

Table 4. Relative Abundances (RA%)^a of [M-H-80]⁻ and [M-H-98]⁻ Fragment Ions in HCD Spectra of Singly Deprotonated Sulfo- and Phosphopeptides^b

Sulfopeptide	[M-H-80 (SO ₃) ⁻]	[M-H-98 (SO ₃ +H ₂ O)] ⁻	Phospho-peptide	[M-H-80 (HPO ₃) ⁻]	[M-H-98 (H ₃ PO ₄) ⁻]
1a	10	<0.1	1b	14	48
2a	100	26	2b	3.5	32
3a	100	<0.1	3b	1	6
4a	100	76	4b	2	27
5a ^c	100	17	5b	4	100

^aCE 50 eV.^bRA of [M - H]⁻ ions of **6a** and **6b** were in the negative ion mode ESI-MS spectra too low in order to allow HCD analysis

not observed in the mass spectra of any of the phosphorylated peptides. A low abundant m/z 214 ion signal appears also in the corresponding IT-CID spectrum of **3a** (Table 3). The deprotonated sTyr immonium ion is not generated neither for the singly deprotonated molecules of **3a** (which is consistent with the higher lability of the sulfate within sTyr-peptide monoanions, Tables 2 versus 3) nor from the other multiply charged sulfated peptides (**1a**, **2a**, **4a–6a**). These data indicate that a formation of sulfotyrosine deprotonated immonium ion is favored by N-terminal location of the peptide modified residue (Scheme 4).

Noteworthy, HCD spectra of [M - nH]ⁿ⁻ ($n=1, 2, 3$) ions of sulfopeptides **1a–6a** do not display bisulfate ion HSO₄⁻ (m/z 97) or fragments with mass-to-charge ratios below m/z 97 at any of the collision energies in the range 30–80 eV. These data indicate that deprotonated sTyr-peptides do not undergo dissociation of the Ar-O-modification bond, contrary to the [M - nH]ⁿ⁻ ($n=1, 2, 3$) ions of phosphopeptides **1b–6b**. The phosphorylated peptides exhibit m/z 97 H₂PO₄⁻ and m/z 79 PO₃⁻ fragments at RA varying from 0.5 to 70% upon HCD. The absence of sulfate-induced low mass product ions (m/z 80 SO₃⁻ and m/z 97 HSO₄⁻) in HCD spectra of sulfopeptides **1a–6a** indicates that the m/z 79 and 97 inorganic phosphate ions of pTyr peptides can be used for distinguishing pTyr- and sTyr peptide side chains, in

addition to the [M-H-H₃PO₄]⁻ and [M-nH-79] ($n=2, 3$) reporter ions. Apparently, application of the inorganic phosphate anions to detection of pTyr peptides is hampered in ion trap CID experiments by the low mass cut-off of the IT-CID spectra.

Interestingly, in contrast to the negative ion ion-trap CID, HCD spectrum of the [M - H]⁻ ion of the doubly sulfated peptide **1a**, enables a rapid recognition of the presence of two sulfotyrosine residues in the modified peptide sequence. Indeed, HCD spectrum of the singly deprotonated peptide species exhibits an extensive loss of two molecules of SO₃ originated in the two sTyr residues to give rise to the corresponding [M-H-160]⁻ fragment ion as a base peak (Figure 5a). It is important to point out that the [M - 2H]²⁻ ions of **1a** do not undergo a complete desulfation upon HCD exhibiting a desulfation pattern consistent with the known ‘ladder’ fragmentation behavior of multiply sulfated peptides in the negative ion mode [27, 39] (Figure 5b and Table 3). The striking difference observed upon HCD in the extents of the [M-H-2xSO₃]⁻ and [M-2H-2xSO₃]²⁻ ion formation of **1a** reveals a clear correlation between the efficiency of sTyr-peptide desulfation in the negative ion mode and the probability of the sulfotyrosine deprotonation. Indeed, it is reasonable to assume that the suppressed desulfation of the [M - 2H]²⁻ ion of **1a** upon HCD is a direct consequence of

Scheme 4. Formation of m/z 214 deprotonated sulfotyrosine immonium ion of [M - 2H]²⁻ ion of **3a** upon HCD

Figure 5. HCD spectra (CE 50 eV) of (a) $[M-H]^-$ and (b) $[M-2H]^{2-}$ ion of **1a**

the fact that the modified peptide N-sY-sY-GWMDF-NH₂ has only one acidic function (Asp-7) in addition to the sTyr residues. As a result, big majority of the peptide $[M-2H-SO_3]^{2-}$ fragment ions are expected to carry their second sulfotyrosine monoester moiety in its ionized form which suppresses their dissociation. In contrast, in case of the singly charged peptide fragment $[M-H-SO_3]^-$, the negative charge can be efficiently shared upon HCD conditions between both the second sTyr and the peptide Asp-7 side chains to allow the release of SO₃ neutrals from the uncharged sulfotyrosine residues. The observed HCD behavior of the $[M-H]^-$ ion of **1a** is, to the best of our knowledge, the first example of the complete desulfation process of deprotonated multiply sulfated peptides in the gas phase.

Conclusions

In this work, we have presented a new approach to mass spectrometry-based discrimination between isobaric sulfated and phosphorylated protein tyrosine residues, using different fragmentation routes of deprotonated sTyr- and pTyr peptides in the gas phase. The method is applicable to both

ion-trap CID and HCD data and does not impose any requirements on the charge states of the modified peptides obtained during negative ion nanoESI. The negative ion mode recognition of sTyr- and pTyr peptides does not require preliminary knowledge of peptide sequences and, thus, can be effectively applied to detection of the covalent modifications on tyrosine residues of peptides from unknown proteins.

The results of this study demonstrate for the first time that IT-CID and HCD tandem mass spectra of $[M-H]^-$ ions of phosphotyrosine-containing peptides can be rapidly distinguished from their sulfated counterparts on the basis of the combination of their highly abundant $[M-H-98]^-$ product ions and the strongly suppressed $[M-H-80]^-$ fragments. Indeed, the tandem mass spectra of the sulfated analogues display an opposite trend revealing that the value of ratio $RA\%[M-H-80]^-/RA\%[M-H-98]^-$ is in excess of one even for sulfopeptides undergoing an extremely efficient dehydration in the gas-phase. The mass spectral data of multiply deprotonated sTyr- and pTyr peptides can be also unambiguously differentiated from each other based on the absence or presence of the cluster of high mass fragment ions of type $[M-nH-79]^{(n-1)-}$ and $[M-nH-79-NL]^{(n-1)-}$ ($n=2, 3$) for sTyr-

and pTyr peptides, respectively. This work reveals that relative efficiencies of SO_3 and H_3PO_4 elimination reactions of multiply deprotonated +80 Da Tyr-peptides are strongly dependent on the peptide amino acid compositions and should not be used for determination of the tyrosine modifying group nature in the negative ion mode.

The results of this study provide experimental evidence for the catalysing role of tyrosine-O-sulfate protonation during SO_3 release from deprotonated sTyr-peptides upon CID. In addition, it demonstrates that despite of the less selective character of peptide fragmentations upon HCD, this tandem mass spectrometry technique allows a clear recognition of sulfated and phosphorylated peptide tyrosine residues in the negative polarity. Furthermore, the HCD spectra of $[\text{M} - \text{H}]^-$ ions of multiply sulfated peptides reveal the advantage of this approach over the ion-trap CID for a rapid determination of a number of peptide sulfated Tyr residues.

It is important to point out that the distinctive negative ion mode fragmentation signatures of sTyr- and pTyr-peptides described in this work can be employed for the analysis of the tyrosine modifications even using MS equipment of medium and low resolution. The reported tandem mass spectral data can be used as a benchmark against which low and higher energy CID spectra of unknown +80 Da Tyr-modified peptides can be compared with high level of confidence.

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