Sulfopeptide Fragmentation in Electron-Capture and Electron-Transfer Dissociation

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Sulfopeptides can be misassigned as phosphopeptides because of the isobaric nature of the sulfo- and the phosphomoieties. Instruments having the ability to measure mass with high accuracy may be employed to distinguish these moieties based on their mass defect (the sulfo-group is 9 mmu lighter than the phosphomoiety). However, the assignment of the exact site(s) of post-translational modification is required to probe biological function. We have reported earlier that peptides with identical sequences containing either O-sulfo- or O-phospho-modifications display different fragmentation behavior (K. F. Medzihradszky et al., *Mol. Cell. Proteom.* **2004,** 3, 429–440). We have also established that O-sulfo moieties are susceptible to side-chain fragmentation during collision-induced dissociation. Our present study provides evidence that neutral SO_3 losses can also occur in electron capture dissociation and electron-transfer dissociation experiments. We also report that such neutral losses may be reduced by fragmenting peptide-alkali metal adducts, such as sodiated or potassiated peptides. (J Am Soc Mass Spectrom 2007, 18, 1617–1624) © 2007 American Society for Mass Spectrometry

Phosphopeptides are the most frequently targeted post-translational modifications in proteomics studies because of their biological significance [1–5]. Sulfopeptides also display an 80 Da mass increase as well as similar chromatographic and mass spectrometric behavior [6]. The sulfopeptides are 9 mmu lighter than their phosphorylated counterparts. They can easily be misidentified as phosphopeptides. However, the most significant difference between phospho- and sulfopeptides is revealed during MS/MS analysis. In CID analyses phosphorylated Tyr residues retain the modification, and even their modified immonium ion is sometimes observed, while phosphorylated Ser and Thr residues undergo β-elimination of H_3PO_4. Thus, the fragments detected in the CID spectra of phosphopeptides either display a +80 Da or a −18 Da mass shift in comparison to the fragments of an unmodified peptide. Under identical conditions, collisional activation of O-sulfopeptides leads to a gas-phase rearrangement reaction that completely eliminates the sulfate from the molecular ion, and the CID fragment spectrum is practically identical to that observed for the unmodified molecule [6]. We have analyzed numerous O-sulfopeptides and have not detected any CID fragments that still contained the sulfate group.

While the different CID behavior proved to be a reliable tool for the differentiation of these two isobaric modifications, the gas-phase elimination of the sulfo-moiety also prevented the assignment of the modification site. Therefore, it seemed essential to evaluate electron-capture dissociation based on reports that most of the labile side chains of peptides and proteins studied remain intact [7]. Electron-transfer dissociation [8, 9] was also employed to analyze synthetic phospho- and sulfopeptides. Here we report our observations on the ECD and ETD fragmentation of sulfopeptides.

Experimental

Materials

Phosphopeptides and sulfopeptides were synthesized as described earlier [6]. Their sequences are as follows: RIEVALsTK; RIEVALpTK; LAGLQDEIGsSLR; LAGLQDEIGpSLR; Ac-LAGLQDEIGsSLR-amide. Caerulein, <QQDsYTGWMDF-amide was purchased from Sigma (St. Louis, MO) "s" indicates that the following amino acid features a sulfate ester on its aliphatic (Ser and Thr) or aromatic (Tyr) hydroxyl, while "p" indicates a phosphate-ester, i.e., phosphorylation.

Mass Spectrometry

Electron-capture dissociation was performed on a hybrid linear ion trap-Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer, LTQ-FT; Thermo, Bremen, Germany) equipped with a 7 T ICR magnet. Phospho- and sulfopeptides at a concentration of 1 to 5 pmol/μL in 0.1% formic acid and 50% acetonitrile were loaded in a static nanospray tip (Proxeon, Odense, Denmark)

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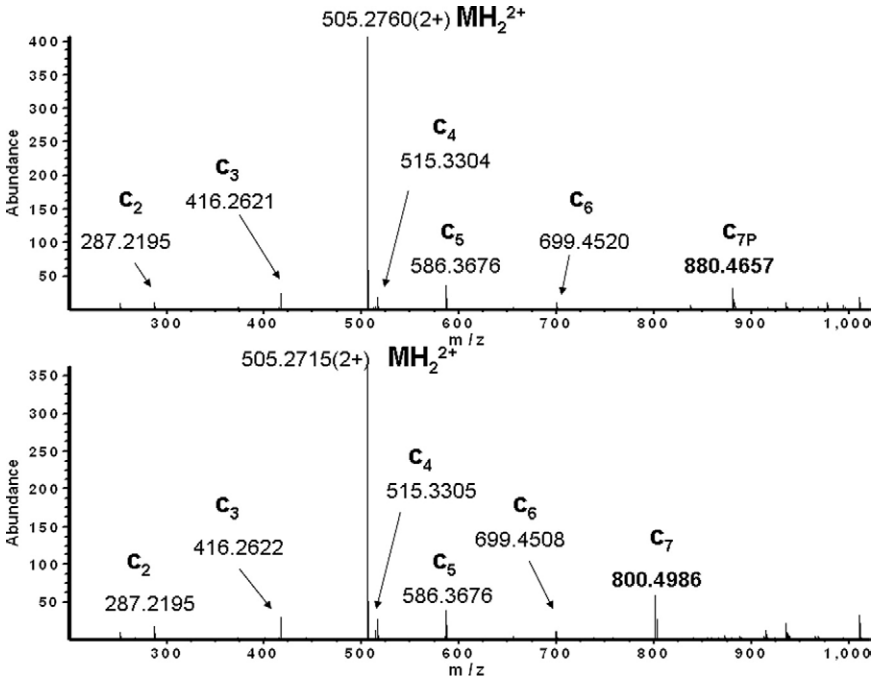


Figure 1. Comparison of the ECD spectra of phosphorylated (upper panel) and sulfated (lower panel) synthetic peptide, RIEVALTK. In both peptides Thr-7 is modified. While fragment $\mathbf{c}_7$ of the phosphopeptide features the modification, a gas-phase rearrangement reaction resulted in complete sulfate loss in the sulfopeptide fragment. (Supplementary Table S1.)

for ionization. Electron beam time and electron energy were set at 300 ms and 5 eV, respectively. Xcalibur 2.0 SR2 (Thermo, Bremen, Germany) was used for both data acquisition and data processing.

Some experiments were performed (Figure 1) in the National High Magnetic Field Laboratory, Tallahassee, FL on a homebuilt 9.4 Tesla passively shielded ESI-Q-FT-ICR mass spectrometer [10] controlled by a modular ICR data acquisition system (MIDAS) [11]. A Nanomate chip system (Advion BioSciences Inc., Ithaca, NY) consisting of a 10 × 10 grid of reproducibly formed 10 μm i.d. spray nozzles with low nanoliter per minute flow rates was used for sample introduction. The instrument configuration and operating conditions for ECD on this instrument have been previously described [12]. Ion transients of individual scans were collected and 100 transients were summed before apodization, fast Fourier transform, and conversion to a mass spectrum with MIDAS software [11].

Electron-transfer dissociation was performed on a linear ion trap (LTQ) equipped with ETD, at Thermo, San Jose, CA. Phospho- and sulfopeptides were infused into the instrument with a flow rate of 200 nL/min. Acquisition conditions: fluoranthene radical anion target: 2.5e5; activation time 100 ms. The ETD data of the terminally blocked sulfopeptide were acquired at a 5× higher fluoranthene anion target value.

Results

A series of synthetic phospho- and sulfopeptides were subjected to ECD analysis on different Fourier transform ion cyclotron resonance (FT-ICR) instruments (see the Experimental section), (Figures 1, 2, 3, and 4, and Tables S1–S7 in the Supplementary Material section, which can be found in the electronic version of this article). ECD spectra acquired on the different FT-ICR instruments featured similar fragmentation patterns eliminating the possibility of instrumental artifacts. The fragment ions were detected mostly above the mass of the precursor *m/z* values. For the phosphopeptides studied (Figure 1, upper panel, Suppl. Table S1; Figure 2, middle panel, Suppl. Table S2), the side-chain moieties were retained in the fragment ions. However, sulfopeptides with identical sequences did not yield any fragments that retained the modification (Figure 1, lower panel, Suppl. Table S1; Figure 3, middle panel, Suppl. Table S4; Figure 4, Suppl. Table S6). This phenomenon was observed for all sulfated residues, i.e., for Tyr-, Thr-, and Ser-modified peptides as shown. Interestingly, ECD spectra of all peptides studied contained a high number of "unusual" fragment ions, namely, $\mathbf{z} + \mathbf{1}$ and $\mathbf{c} - 1^{\bullet}$ fragments, as well as the products of side-chain fragmentations from the molecular ion or from the major sequence ions. Some of these findings, such as **w** ion formation and side-chain losses have been previously reported [13–15]. The formation of $\mathbf{z} + 1$ and $\mathbf{c} - 1^{\bullet}$ ions is illustrated in Scheme **1**. This mechanism has been proposed by Bakken et al. [16].

The samples were introduced for analyses using metal coated glass nanospray tips (static nanospray). Such tips generally produce some sodium- and/or potassium adduct formation, and this was indeed detected for each sample (Figures 2 and 3, top panels).

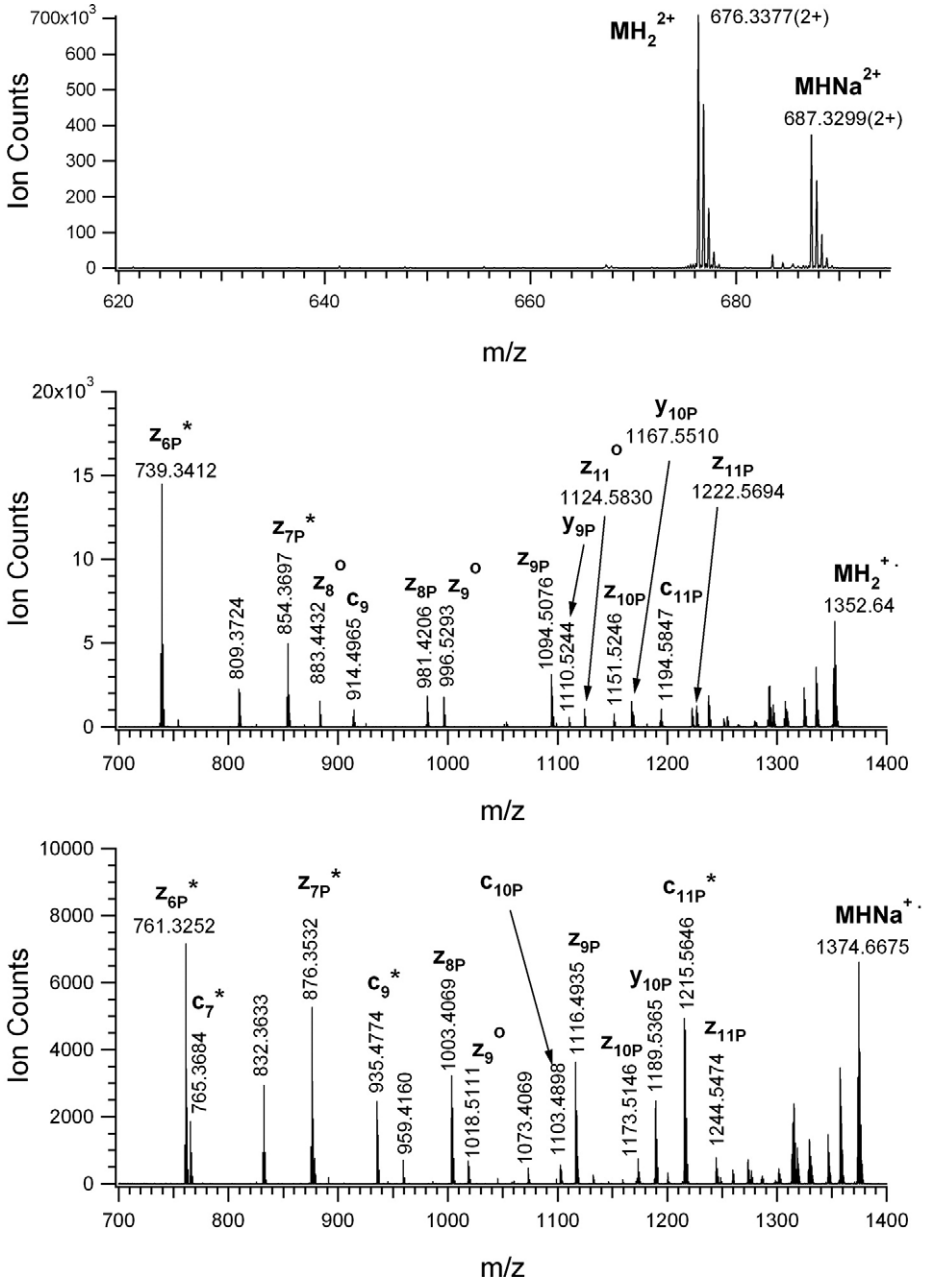


Figure 2. The upper panel shows the ESIMS spectrum of synthetic phosphopeptide, LAGLQDEIGpSLR. No detectable β-elimination occurs under normal MS acquisition conditions. The middle panel shows the ECD spectrum generated from the doubly protonated ion as the precursor (Suppl. Table S2). The lower panel shows the ECD spectrum of the doubly charged protonated and sodium-adduct ion (Suppl. Table S3). Asterisk labels indicate $\mathbf{z} + 1$ or $\mathbf{c} - 1^{\bullet}$ fragments, "p" indicates the fragment contains the modification, while the ^{O} label indicates the elimination of H_3PO_4. Each fragment retained the Na.

When these alkali metal ion adducts were selected as precursors, side-chain fragmentation was virtually eliminated from the ECD spectra of phosphopeptides and sulfopeptides (Figures 2 and 3, bottom panels, Suppl. Tables S3 and S5, respectively). Interestingly, all fragments observed retained the metal ion, not just the highly acidic residue-containing fragments as might be expected. Potassium adducts and doubly sodiated ions also displayed similar fragmentation patterns (data not shown).

Some of the phospho- and sulfopeptide samples were also subjected to electron-transfer dissociation analyses (Figures 5 and 6). While the phosphopeptide fragmentation was similar (Figure 5, lower panel), the sulfopeptide fragmentation was different from that observed in ECD (Figure 3 versus Figure 5, upper

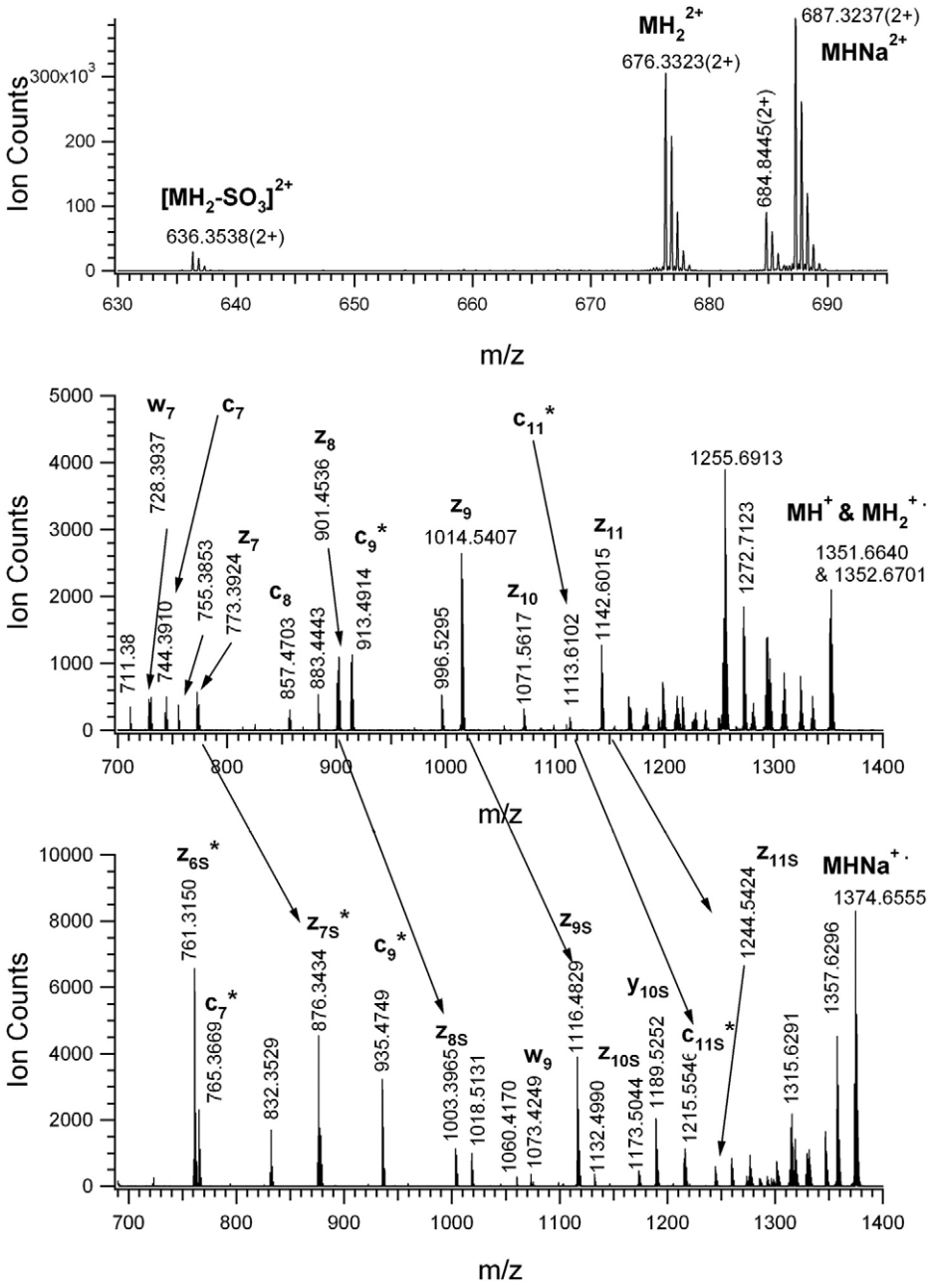


Figure 3. The upper panel shows the ESIMS spectrum of synthetic sulfopeptide, LAGLQDEIGsSLR. Some sulfate loss occurs under normal MS acquisition conditions. The middle panel shows the ECD spectrum generated from the doubly protonated ion as the precursor (Suppl. Table S4). The lower panel shows the ECD spectrum of the doubly charged protonated and sodium-adduct ion (Suppl. Table S5). Asterisk labels indicate $\mathbf{z} + 1$ or $\mathbf{c} - 1^{\bullet}$ fragments, "s" indicates the fragment contains the modification. Each fragment retained the Na.

panel; Figure 6 versus Suppl. Table S7). For example, the sulfopeptide with free N- and C-termini produced a spectrum analogous to the corresponding CID spectrum (Figure 5, upper panel). However, in this case a few fragments did retain the sulfo-group. The same peptide sequence with blocked N- and C-termini yielded ECD-like fragmentation with the modifying moiety still attached to most fragments (Figure 6).

Discussion

As pointed out by Zubarev and coworkers [17], the recombination energy of a free electron and a multiply charged ion is between 4 to 7 eV, depending on its charge state. Three to 4 eV is required to break the N–C bond to produce **c** and $\mathbf{z}^{\bullet}$ ions [17]. The majority of the remaining deposited energy (up to 4 eV) is dissipated as vibrational energy in the product ions (and product

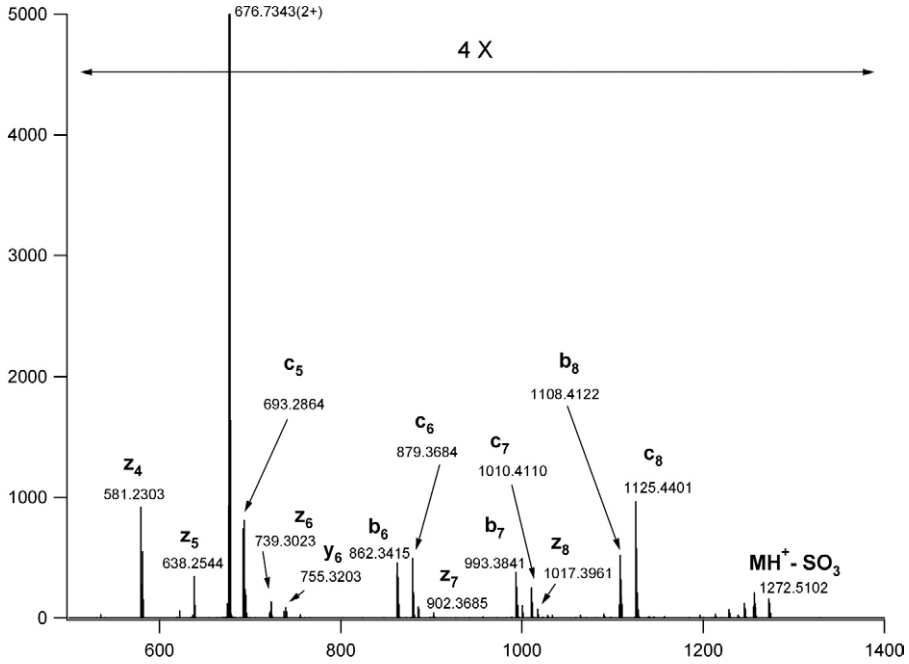
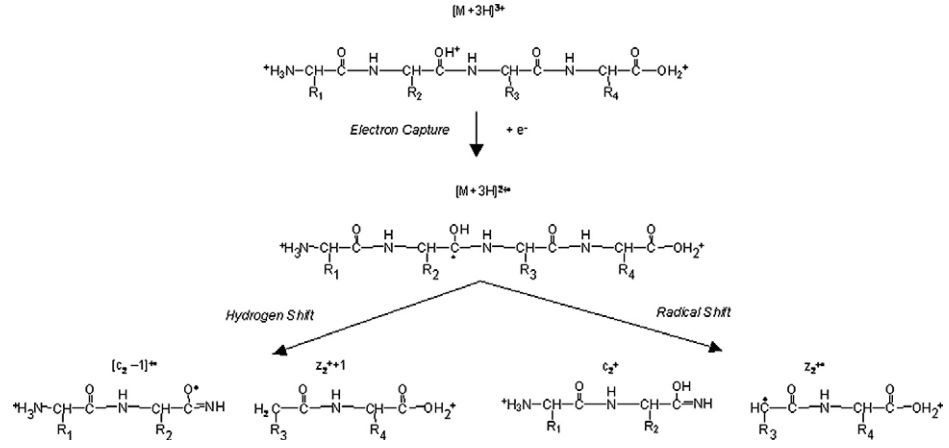


Figure 4. ECD spectrum of caerulein: <QQDsYTGWMDF-amide (Suppl. Table S6). No sulfated fragment was detected in this experiment.

neutrals). Although in phosphopeptides, the energy can be quickly distributed throughout many bonds of the product species without further fragmentation, this quantity of energy may be just sufficient to cause SO_3, a very stable neutral, to be cleaved from a sulfopeptide.

In ETD analysis, electrons are delivered to peptide cations via a molecular carrier such as fluoranthene, whose electron affinity is 0.63 eV [18]. The energy for excitation of the product ions is the total electron energy minus the EA value. In the case of sulfopeptides, the reduction may not be sufficient to prevent the liable neutral loss from the product ions as we have observed. In addition, collision activation of peptide cations with the electron carrier (fluoranthene radical anion) also may play some role in the fragmentation process. Although Mikesh et al. [19] observed retention of sulfate group on ETD fragments of triply protonated peptide GRLGsSRAGR, the preservation of SO_3 group may be the result of (1) the presence of three arginine residues, causing this particular peptide to be heavily charged and (2) the location of a positively charged arginine residue adjacent to the sulfated residue that may stabilize the negatively charged $-SO_3$ group. In general, however, sulfopeptides are more labile than their phospho-analogs and thus we are conducting further experiments on the ECD/ETD of metallized



Scheme 1. Electron capture dissociation processes. Since electron capture requires reduction of at least one charge and retention of at least one charge for fragment ions to be observed, a triply charged peptide is used to illustrate the process. The electron captured species can undergo either a radical shift towards the C-terminus to form **c** and $\mathbf{z}^{\bullet}$ ions or a hydrogen shift to give $\mathbf{c} - 1^{\bullet}$ and $\mathbf{z} + 1$ ions.

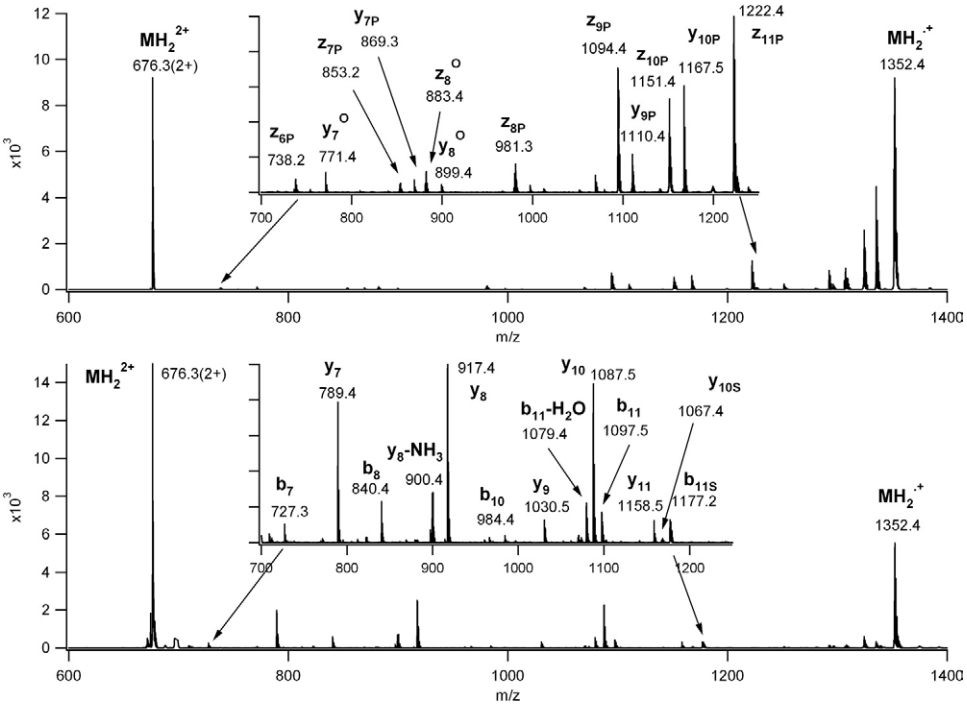


Figure 5. ETD spectrum of LAGLQDEIGpSLR (lower panel) and LAGLQDEIGsSLR (upper panel) acquired on the linear ion trap (LTQ) under identical acquisition conditions. The insets show the *m/z* 700–1250 range magnified. Letters "p" and "s" in the fragment assignments indicate the presence of the modification,^{O} indicates gas-phase elimination of H_3PO_4.

peptide precursor ions as a general method for determination of the sulfo-group location.

The presence of any nonvolatile salt, even at low concentrations, is usually avoided by mass spectrometrists because it may hamper the ionization of peptides, lower the detection sensitivity by dispersing the signal, or interfere with the chromatography, or simply clog the capillaries. However, there are numerous examples in the literature when metal-ion adducts have been used to facilitate sample detection or the elucidation of structure. In most cases, such successes were associated with otherwise readily fragmented com-

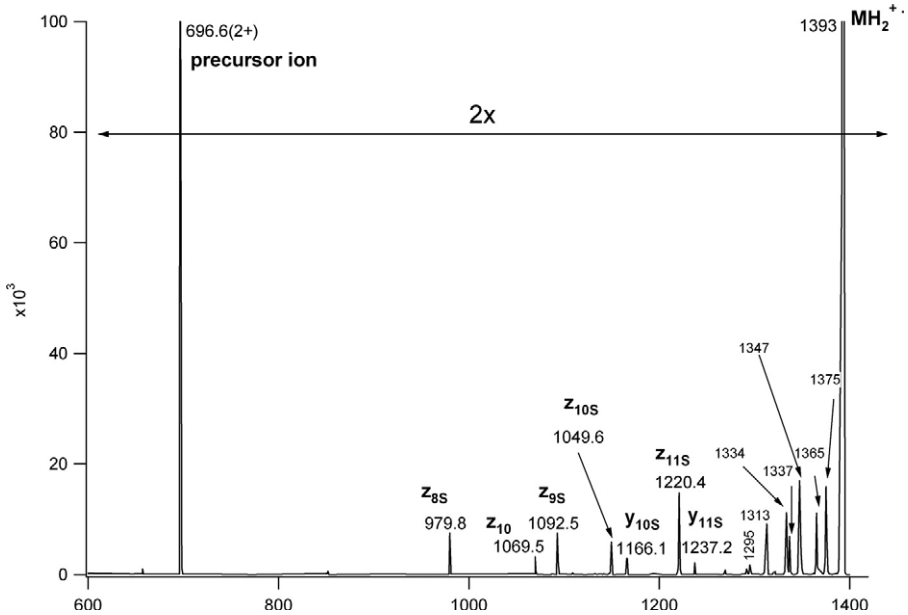


Figure 6. ETD spectrum of synthetic AcLAGLQDEIGsSLR-amide; "s" indicates the sulfate still attached to the fragments. The ECD fragments observed for this peptide are listed in Suppl. Table S7.

pounds, such as different classes of carbohydrates and nucleotides. For example, Na-adduct formation of N-linked glycopeptides was shown beneficial in CID analysis [20]. Li-adducts of glycerophosphocholine lipids provided information on the fatty acids that is lacking in the CID spectra of the protonated species [21]. In the analysis of oligodeoxynucleotide ions, the replacement of the phosphate protons with metal ions leads to stabilization of the molecular ion and, thus, dramatic changes in the fragmentation pattern [22]. In addition, CID analysis of lithiated fatty acids showed that the metal adduct formation protected the carboxylic acid and eliminated the CO_2 loss from the fragmentation [23]. Similar to these findings, we found that replacing the sulfate proton with a sodium ion stabilized the labile modification and prevented the loss of SO_3. As indicated by Zubarev et al. [17], the recombination energy (RE) of the charge carrier atom provides a thermodynamic measurement of electron capture efficiency. The RE is calculated from the ionization potential of the atom, its cation affinity, and its neutral atom affinity towards singly charge-reduced peptides. Although it is difficult to precisely calculate the affinities, Williams and coworker [24] provided the estimated recombination energy of the charge carrying hydrogen, lithium, and cesium atoms. The RE for sodium is likely between 72 and 85 kcal/mol, the values corresponding to cesium and lithium, respectively. Since an electron is 200 times more likely to recombine with H^+ compared to Li^+ and is 10 times more likely to neutralize Li^+ than Cs^+, we can estimate that neutralization for Na^+ is several hundred times less probable than for H^+. Our observation of sodiated ECD fragments is consistent with the above argument, since the sodium cation is more likely to localize near the negatively charged sulfomoiety. Neutralization is more likely to occur at a distance from the sulfated residue and, therefore, there is a smaller chance for energy deposition on the residue to promote additional loss of the sulfate group. It is well known that alkali metal cations stabilize acidic groups by formation of a "salt bridge" in the gas phase [25]. Unlike in CID experiments, in ECD/ETD analyses in the absence of additional internal energy, the "salt bridge" is likely to survive. Metal ion adducts of peptides have been studied earlier by ECD [26].åThe observations reported in that study—most fragments retaining the metal ion as well as enhanced $\mathbf{c} - 1^\bullet$ and $\mathbf{z} + 1^\bullet$ ion formations are in good agreement with our findings. However, abundant $\mathbf{c} - 1^\bullet$ and $\mathbf{z} + 1$ fragments are frequently observed in ECD spectra of multiply protonated peptides. Further studies may elucidate which structural features promote the "hydrogen shift" versus the "radical shift" (Scheme **1**).

Conclusions

Sulfopeptides are more susceptible to neutral losses even under ECD conditions than phosphopeptides of identical amino acid sequences. ETD appears to be a gentler activation technique than ECD. However, SO_3 is still eliminated from most fragments. Na^+ adduct formation with the concomitant elimination of the sulfate proton prevents SO_3 losses and permits assignment of the modification site. Since sodium adducts are a common occurrence in MS experiments, one may not need to add sodium to the sample to carry out such an experiment.

Acknowledgments

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