The Combination of Electron Capture Dissociation and Fixed Charge Derivatization Increases Sequence Coverage for *O*-Glycosylated and *O*-Phosphorylated Peptides

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Electron capture dissociation (ECD) has become an alternative method to collision-activated dissociation (CAD) to avoid gas-phase cleavage of post-translational modifications carried by side chains from the peptide backbone. Nonetheless, as illustrated herein by the study of *O*-glycosylated and *O*-phosphorylated peptides, the extent of ECD fragmentations may be insufficient to cover the entire peptide sequence and to localize accurately these modifications. The present work demonstrates that the derivatization of peptides at their N-terminus by a phosphonium group improves dramatically and systematically the sequence coverage deduced from the ECD spectrum for both *O*-glycosylated and *O*-phosphorylated peptides compared with their native counterparts. The exclusive presence of N-terminal fragments (*c*-type ions) in the ECD spectra of doubly charged molecular cations simplifies peptide sequence interpretation. Thus, the combination of ECD and fixed charge derivatization appears as an efficient analytical tool for the extensive sequencing of peptides bearing labile groups. (J Am Soc Mass Spectrom 2007, 18, 1405–1413) © 2007 American Society for Mass Spectrometry

The field of proteomics usually aims at differential characterization of the complete pattern of protein expression, for instance between cells in a normal state and those in a pathological state. Protein identification is now routinely achieved on a large scale (tens or hundreds of proteins per day) by combining enzymatic digestion, mass spectrometric analysis of the resulting peptides, and subsequent database search. If few peptides or short peptide sequences are sufficient for protein identifications into databases, a complete determination of the primary structure is nevertheless useful to detect modifications that can be involved in the regulation of protein function. Indeed, the catalytic property, the conformation, the turnover, or the subcellular localization of proteins are largely under the control of various peptide sequence processings named post-translational modifications. These modifications (PTM) involve the proteolytic maturation of the proteins and/or the transfer of chemical groups (phosphorylation, glycosylation, acetylation, sumoylation) on specific amino-acids (serine, threonine, or tyrosine

for *O*-phosphorylations, serine or threonine for *O*-glycosylations, asparagine for *N*-glycosylations). The most obvious strategy for mapping the modifications along the peptide chain involves a comparison between the sequence obtained by mass spectrometric data and the one deduced from the open reading frame coded by the gene sequence. However, a drawback arises from the propensity of groups conjugated to the amino acid side chains to dissociate readily upon collisional activation. For instance, collisionally-activated dissociation (CAD) MS/MS spectra of protonated precursor ions of phosphorylated peptides exhibit intense ions originating from the elimination of metaphosphoric (HPO_3, loss of 80 Da) or orthophosphoric acid (H_3PO_4, loss of 98 Da) [1]. In the case of *O*-N-acetylgalactosamine and *O*-N-acetylglucosamine substituted peptides, singly protonated molecular ions undergo a predominant neutral loss of 203 Da, and doubly charged peptides [2, 3] form an intense fragment ion at *m/z* 204. These favored gas-phase eliminations can help the specific detection of modified peptides by reconstructing ion chromatograms either on specific neutral losses or diagnostic ions. For peptides containing several amino acids potentially modified (serine, threonine), the precise localization of the modification remains a difficult

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task since the fragment ions bearing the modification are often lacking or are of low abundance in the MS/MS spectra.

To overcome the limitations of CAD-MS/MS, numerous strategies have been proposed to localize phosphate, O-GalNAc, and O-GlcNAc moieties [4, 5] that combine β-elimination and mass spectrometric characterization of end products. However, these protocols still suffer from some shortcomings. In particular, both the temperature and duration of the β-elimination reaction have to be carefully adjusted to each sample to avoid nonspecific Ser or Thr dehydration.

Electron capture dissociation (ECD) [6, 7] and, more recently, electron-transfer dissociation (ETD) [8] have emerged as powerful novel dissociation techniques that do not induce fragmentation of labile bonds between conjugated groups and peptides. Basically, the dissociation of the reduced cation species $[M + nH]^{(n-1)+\cdot}$ leads to a series of fragment ions of the c and $z^\cdot$ type. The interest of ECD has now been widely illustrated for the unambiguous assignment of phosphorylation [9], O-glycosylation [6], or γ-carboxylation sites [10], as well as for intact protein sequencing in the so-called top-down approach [11, 12]. It is now established that the electron capture is followed by the formation of long-lived intermediate complexes in which hydrogen rearrangements can take place [13]. These $c/z^\cdot$ hydrogen-bonded complexes can further dissociate to give "classical" $c/z^\cdot$ ions or related species such as z′ or z″ ions if hydrogen atom transfers have taken place before the dissociation [14]. For large proteins, a disruption of the weak intramolecular noncovalent bonds that may prevent the separation of the ECD products is often necessary [15, 16]. This can be done by "activated ion ECD (AI-ECD)" in which the parent ions are collisionally- or IR-heated during electron irradiation. AI-ECD employing laser irradiation following electron capture has also proved to be an efficient way to obtain a substantially higher number of fragments than regular ECD for phosphopeptides [17] or N-glycopeptides [18].

In an alternative approach, phosphate [19] or glycan moieties [20] have been localized by CAD-MS/MS or postsource decay analysis of peptides derivatized at their N-termini with a phosphonium group. It has been shown that when a singly charged cation was selected as an ion precursor, loss of the conjugated moiety could be almost completely suppressed. This feature is likely due to the lack of a mobile proton able to drive a charge-induced fragmentation mechanism.

The present work deals with an evaluation of ECD combined to fixed-charge derivatization in the perspective of a general strategy that would allow concomitantly the localization of modification sites and extensive sequence coverage, ideally from a unique series of fragment ions.

Experimental

Nano-Electrospray Mass Spectrometry

All experiments were carried out with a 7-T APEX III FT-ICR mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) equipped with a 7 tesla actively shielded superconducting magnet, an infinity cell [21], and a nano-electrospray ion source. Nano-electrospray needles were purchased from Proxeon (Odense, Denmark) and filled with 2 μL of the peptide solution (2 pmol/μL in 50% MeOH/water/ 0.2% formic acid). The needles were subsequently opened by breaking the tapered end of the tip under a microscope. A stable spray was observed by applying a voltage of about −700 V between the needle (grounded) and the entrance of the glass capillary used for ion transfer. A source temperature of 50 °C was used for all nano-electrospray experiments. The estimated flow rate was 20–50 nL/min. Ions were stored in the source region in a hexapole guide for 1 s and pulsed into the detection cell through a series of electrostatic lenses. Ions were finally trapped in the cell using either gas-assisted dynamic trapping (Xe pulses, upper pressure around 10^{-7} mbar) with front and back trapping voltages of 3.0 and 3.5 V for trapping, reduced to 0.9 and 0.95 V for detection or static trapping. Mass spectra were acquired from m/z 500 to 3000 with 512 k data points and monoisotopic peaks were automatically labeled using the XMASS 6.1.4 (Bruker Daltonik GmbH, Bremen, Germany) software. For Sustained Off Resonance Irradiation Collision Activated Dissociation (SORI-CAD) [22] experiments, the ion to fragment was isolated by radio frequency (rf) ejection of all unwanted ions using both low-voltage single rf pulses (soft shots) at their resonance frequencies and a chirp excitation covering the region of interest. The fragmentation was performed using an excitation duration of 400 μs (V_{p-p} = 37 dB) at a frequency 500 Hz higher than the cyclotron frequency of the ion of interest. Xenon was used as the collision gas and introduced several times through a pulsed valve to get a pressure in the cell up to 10^{-7} mbar. The excitation/detection of all fragment ions was performed after a 4 s pumping delay. For ECD experiments, a hollow electron emitter was used [23]. The emitter generates a hollow, tubular electron beam and was heated with a 1.9 A current. Isolated multiply charged ions were irradiated during 150 ms with electrons having <1 eV kinetic energy. An external calibration using NaI (2 mg/mL in isopropanol/water) was done daily.

Synthetic Glycopeptides and Phosphopeptides

Glycopeptide syntheses were performed manually using standard Fmoc chemistry protocols on a polystyrene resin functionalized with p-benzyloxybenzyl alcohol and esterified with the C-terminal residue (alanine

Table 1. Primary structure and molecular masses (in Da) before and after TMPP derivatization (+ 573.18841 Da) of all glycopeptides and phosphopeptides studied

Sequence	Peptide designation	Modification	Mr before derivatization	Mr after derivatization
ALGST*T*PPA	**G1**	N-acetylgalactosamine	1219.5820	1792.77041
ALGS*T*TPPA	**G2**	N-acetylgalactosamine	1219.5820	1792.77041
ALGS*TT*PPA	**G3**	N-acetylgalactosamine	1219.5820	1792.77041
ALGS*T*T*PPA	**G4**	N-acetylgalactosamine	1422.6614	1995.84981
GTTPSPVPT*TSTTSAPG	**G5**	N-acetylgalactosamine	1760.8316	2334.02001
KGGGTTSTT*SAPG	**G6**	N-acetylgalactosamine	1323.6154	1896.80381
YLEKRAS*GQAFE	**P1**	Phosphate	1477.6603	2050.84871
YFELILS*PRSKE	**P2**	Phosphate	1560.7589	2133.94731
YPEFPLS*KKK	**P3**	Phosphate	1315.6577	1888.84615
YSDSPS*TST	**P4**	Phosphate	1023.3434	1596.5324

The asterisk (*) indicates the localization of the post-translational modifications.

or glycine). Briefly, the protected amino-acids and the glycosylated building blocks [Fmoc-Ser(α-GalNAc{OAc}$_3$)-OH or Fmoc-Thr(α-GalNAc{OAc}$_3$)-OH] (3 equivalents) were incorporated in the peptide chain using 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU)/1-hydroxybenzotriazole (HOBT)/di-isopropylethylamine (DIEA) as the coupling reagent. Fmoc protection was removed with 20% piperidine in DMF. The products were cleaved from the resin with aqueous trifluoroacetic acid (95%). Deacetylation of the sugar residue was achieved with a catalytic amount of sodium methoxide in methanol at pH 11. The glycopeptides were purified by reverse phase high-performance liquid chromatography using a Perkin-Elmer pump system with a UV detector at 230 nm. The column was a Waters Delta Pak C18 (15 μ, 300Å, 7.8 × 300 mm) and the gradient was performed with water (0.1% TFA)/acetonitrile (from 0% to 30%) over 20 min. The compounds were charac-

terized by amino acid analysis (AAA) and electrospray mass spectrometry (ESMS).

N-Terminus Modified Acetylphosphonium Derivatives

To a solution of a peptide (20–100 pmol) in 2 μL acetonitrile/water (2/8) (vol/vol) was added 2 μL of 50 mM Tris-HCl buffer (pH 8.2) followed by 1 μL of 1 mM (N-succinimidyloxycarbonylmethyl)tris(2,4,6-trimethoxyphenyl)phosphonium bromide (Sigma-Aldrich, Saint-Quentin Fallavier, France). The mixture was vortexed for 30 s and then sonicated in a water bath for 30 min (final bath temperature <30 °C). The resulting mixture was evaporated (centrifugal evaporator) to dryness and the residue redissolved in 50% MeOH/water/0.2% formic acid. The derivatives were stored at −20 °C and further analyzed directly by nano-electrospray.

Table 2. c and z ions cleavages observed for ECD experiments performed on doubly charged native and phosphonium derivatized peptides (except P3 for which triply charged ions were selected)

Peptide	ECD fragment ions on the native peptide	ECD fragment ions on the phosphonium derivatized peptides
G1	A L G S T*T* P P A	TMPP-A L G S T*T* P P A
G2	A L G S*T* T P P A	TMPP-A L G S*T* T P P A
G3	A L G S*T T* P P A	TMPP-A L G S T T* P P A
G4	A L G S*T*T* P P A	TMPP-A L G S*T*T* P P A
G5	G T P S P V P T*T S T T S A P G	TMPP-G T P S P V P T*T S T T S A P G
G6	K G G G T T S T T S A P G	TMPP-K G G G T T S T*S A P G
P1	Y L E K R A S*G Q A F E	TMPP-Y L E K R A S*G Q A F E
P2	Y F E L I L S* P R S K E	TMPP-Y F E L I L S* P R S K E
P3	Y P E F P L S* P K K K	TMPP-Y P E F P L S* P K K K
P4	Y S D S P S*T S T	TMPP-Y S D S P S*T S T

The asterisk (*) indicates the localization of the post-translational modifications in the peptide sequence.
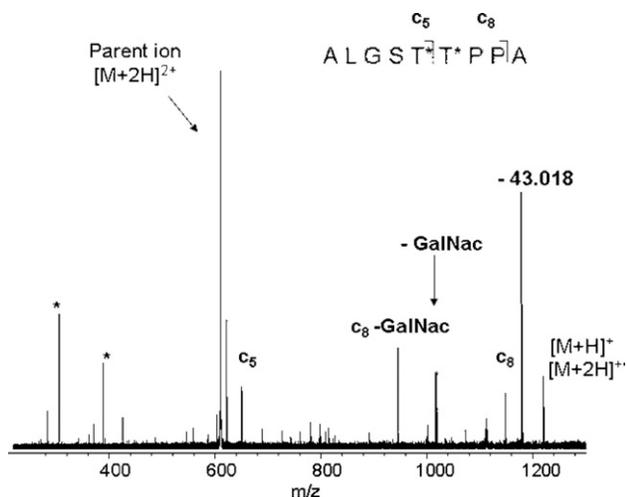
Figure 1. ECD of 2^+ (m/z 610.7983) of the glycosylated peptide **G1** (two GalNAc groups).

Results and Discussion

As expected, all protonated glycopeptides and phosphopeptides under study (Table 1) undergo an intense loss of the PTM (data not shown) when subjected to SORI-CAD. The lability of these PTM groups is well known and is induced by a preferential transfer of a mobile proton to the sugar-peptide glycosidic bond or to the phosphoester bond.

ECD Analysis of Native Peptides

ECD results for all peptides under study are gathered in the Table 2. For all glycopeptides and for phosphopeptides **P1**, **P2**, and **P4**, the doubly charged ions were isolated and irradiated with electrons. For the phosphopeptide **P3**, the triply charged species was much more intense and was chosen for ECD experiments.

Native Glycopeptides

Although ECD is often very useful for glycosylation site localization, for all glycopeptides under consideration, ECD experiments on the most intense doubly charged ions yielded an incomplete c ions series and nearly no z ion. The ECD spectrum of the (2^+) ion of diglycosylated peptide **G1** is shown in Figure 1. Clearly the glycosylation site cannot be assigned on the basis of this spectrum since one GalNAc could be either on Ser3 or on Thr4.

A similar fragmentation pattern was obtained for all glycopeptides lacking a basic amino acid (**G1**–**G5**). For peptides lacking amino acids of high gas-phase basicity (arginine, lysine, histidine), such as peptides **G1**–**G5**, the preferential formation of c ions (versus z ions) upon ECD is due to their higher stability [6, 24]. Moreover, it has been shown that this kind of peptide has often a nonclassical behavior upon ECD, a common feature being the formation of b ions. It was demonstrated by

Cooper [25] and confirmed very recently by Haselmann and Schmidt [26] that the formation of these ions (which are true N-terminal b-type ions) can be explained by typical MS/MS pathways (low-energy threshold backbone dissociation) from the even-electron charged-reduced cation. These b ions were not detected in the ECD spectra of all glycopeptides under study. Conversely, an important fragmentation observed for peptides **G1**–**G4** (and to a lesser extent **G5** and **G6**) is a loss of GalNAc (loss of 203.079 Da from [MH]$^+$ ions). This fragmentation (which is the major one in SORI-CAD experiments) can be explained like the formation of b ions, i.e., via the mobile proton pathway from the [MH]$^+$ ions formed by hydrogen loss. Another explanation could be a vibrational excitation of the precursor ions due to collisions with the trapping gas still residing in the ICR cell. This is unlikely since experiments realized with static trapping gave the same results. Moreover, a vibrational excitation of selected ions followed by ECD would have led to AI-ECD experiments and numerous backbone cleavages. As mentioned above, ECD spectra obtained for all glycopeptides under study exhibited only a few fragmentations, which excludes the possibility of important vibrational excitation.

Ions corresponding to either the loss of 43.018 Da (C_2H_3O) from the radical cationic species or 42.016 Da (C_2H_2O) from the even electron species [M + H]$^+$ are observed in all spectra. This fragmentation has been discussed in details by Mormann et al. [27] who proposed a mechanism based on the attack of a hot hydrogen atom at the nitrogen of the N-acetyl group of one glycan leading to an N-centered hypervalent radical, which could subsequently fragment by loss of an acetyl radical (43.0178 Da). Nevertheless, it should be noted that the attack of the hydrogen atom should be more likely at the carbonyl oxygen which has a higher hydrogen affinity than the amide nitrogen and therefore this mechanism seems unlikely. Other mechanisms involving, as the first step, either a direct electron capture by one amide OCN π^* orbital from the peptide backbone followed by a facile cleavage of the N-C$_\alpha$ bond (superbase mechanism) [28–30] or through a bond electron-transfer from one of the Rydberg orbital of the hypervalent species formed by the attachment of the electron to a positively charged site [31, 32] may play a role here. The elimination of neutral ketene as a secondary fragmentation of the reduced species can be excluded since SORI-CAD of the [M + H]$^+$ species (selected before the electron capture) does not give any loss of 42 Da. Careful analysis of the experimental data on this set of glycopeptides does not lead to any indication on which mechanism is involved in this process.

Another fragmentation observed for glycopeptides **G1**–**G4** is a loss of the GalNAc moiety from c_8. This fragmentation was first thought to be a water loss from a z ion, but an internal calibration of all ECD spectra definitely rejected this hypothesis. Such glycan loss has been observed mainly from (z+1H$^.$) type fragment ions
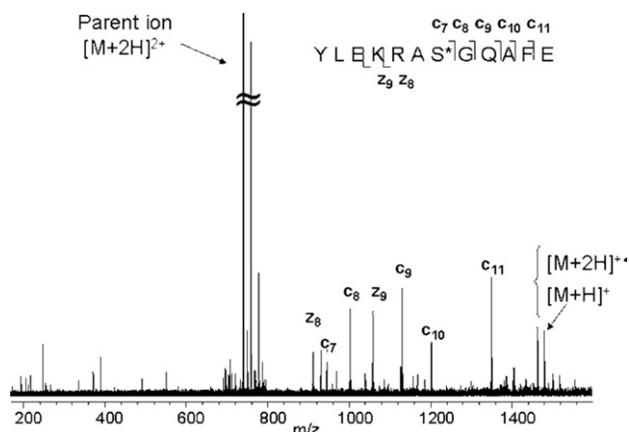
Figure 2. ECD of 2^+ (m/z 739.8374) of the phosphorylated peptide **P1**.

but also from c ions [6, 28]. The formation of this (c_8-GalNAc) intense ion can be explained by two different mechanisms. The first one is a direct loss of GalNac (low-energy pathway) from energetic c_8 ions or the formation of c_8-GalNAc is consecutive to the direct loss of GalNac from $[M + 2H]^{+\cdot}$.

For the glycopeptide **G5**, which contains one GalNac and no basic amino acid, half of the sequence (C-terminal moiety) can be retrieved in ECD experiments based on the presence of c ions.

Finally, the glycopeptide **G6**, which contains one GalNAc and one lysine as the N-terminal amino acid exhibits a rather common behavior upon ECD with an acceptable sequence coverage due to the exclusive presence of c ions. Note that among these c ion series, c_3–c_7 isotopic patterns indicate a mixture of c and c-H$^\cdot$ ions.

Taken together, these results show that two major factors influence the quality of the ECD spectra in term of sequence coverage. First, the peptide sequence and more particularly the presence of a basic amino acid such as arginine, lysine, or histidine influences the fragmentation pattern observed upon ECD. Indeed, we confirm in this study that peptides that do not contain any basic amino acids yield fragmentations due mainly to the mobile proton pathway (loss of the PTM in our case), as already proposed by Cooper [25] and Haselmann and Schmidt [26]. Second, the proximity of the glycans probably induces a steric hindrance which can preclude a hydrogen migration to the peptidic bond (necessary for the "Cornell" mechanism but not for the "superbase" one).

Native Phosphopeptides

The results are far better than for the glycopeptides since for all phosphopeptides studied the sequence coverage is sufficient to localize the phosphorylation site. Note that the phosphopeptides **P1-P3**, which give the highest sequence coverages, contain at least two basic amino acids. Nevertheless, all fragment ions are of low abundance and no complete c or z ion series can be

obtained. The ECD spectrum of phosphopeptide **P1**, which does not contain any proline residue and should thus give a whole sequence coverage, is depicted in Figure 2.

For this sample, the analysis of c and z ions clearly indicates that the remaining proton remains on the most basic residue (arginine). The same conclusion can be drawn for **P2**. Neither loss of phosphate nor b ions is observed. For **P4**, which does not contain any basic amino acids, an abundant loss of one and two molecules of water is observed from $[M + H]^+$ ions and also from c_7 and c_8 ions. The formation of these dehydrated c ions can be rationalized by the formation of intact c ions that lose consecutively one or two molecules of water. An alternative mechanism would be the formation of $[MH_2^{+\cdot} - H_2O]$ ions, which could yield dehydrated c ions. The absence of $[MH_2^{+\cdot}]$ and $[MH_2^{+\cdot} - H_2O]$ ions in the ECD spectrum does not favor this latter mechanism. Note that the sequence of **P4** is rich in hydroxyl functions, which explain the facile loss of water molecules.

These results on both glycopeptides and phosphopeptides show that the localization of labile PTM by ECD can sometimes be difficult. We show in this study that this is especially the case for peptides lacking basic amino acids and it is highlighted when several modifications are close to each other in the sequence. For that reason, the peptides displaying these features were chosen as good candidates to develop ECD after fixed-charge derivatization.

ECD Analysis of Charge Derivatized (TMPP) Peptides

It has been shown, some years ago, that the addition of a fixed charge group to the N-terminus of a peptide can create important changes in its fragmentation patterns [33]. For example, the use of TMPP-acetic acid N-hydroxysuccinimide ester (TMPP-AcOSu) allows the formation of peptides containing a highly stabilized phosphonium cation on their N-terminus. This charge derivatization produces better fragmentation and simpler spectra during collision activated dissociation experiments since an exclusive and often complete series of N-terminal fragment ions is obtained [34]. This derivatization procedure has also been applied successfully to tryptic digests [35]. In the case of MALDI experiments, where no additional proton is available that precludes the "mobile proton" model, this can be explained by charge-remote fragmentation [36, 37]. Very recently, coupling of TMPP derivatization with C-terminal digestion was shown to improve peptide de novo sequencing by MALDI MS [38].

The same derivatization procedure has been applied to phosphorylated peptides further analyzed in an ion trap using electrospray as the ionization source [14]. The authors showed that some a and b ions obtained in the MS/MS experiments still contain the phosphate
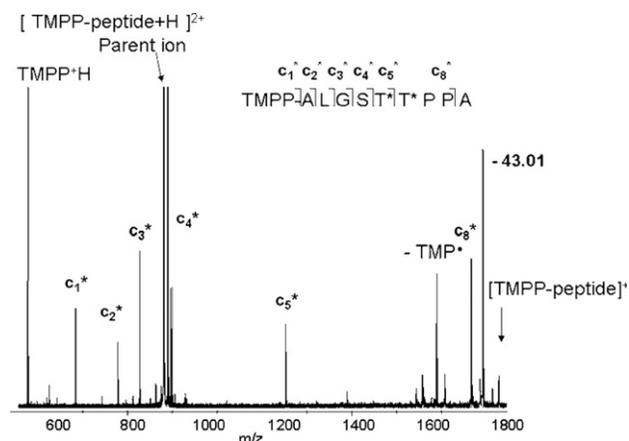
Figure 3. ECD of 2^+ (*m/z* 896.8888) of glycosylated peptide **G1** derivatized with TMPP.

group (especially when low collision energies where used), which is not the case for nonderivatized species. Similar results were obtained more recently for glycopeptides by Czeszak et al.[20] who showed that the MALDI-PSD spectra of *O*-glycopeptides derivatized with a phosphonium allowed unambiguous assignment of the GalNAc moieties in the sequence by providing informative *a* and *b* ions. The main limitation of this method for sequencing glycosylated or phosphorylated peptides by SORI-CAD is that the fragments allowing the localization of the modifications in the peptides are generally of low abundance.

Since the addition of a fixed charge, such as a phosphonium group, seemed to substantially modify the behavior of peptides subjected to collision-activated dissociation, we aimed to evaluate the changes induced by such derivatization on ECD fragmentation. Ions selected in this series of experiments were mostly doubly charged, which meant that the charge carriers were a phosphonium group and a proton. For one peptide, the triply charged species (which was the most intense in the spectrum) was subjected to ECD. In that case, the charge carriers were one phosphonium and two protons.

Such ECD experiments on peptides cationized with other charge carriers than protons have previously been realized, but never with a phosphonium species. Williams et al. reported some ECD experiments on mono- or dimetalated peptides using lithium and cesium [39]. They showed in their study that ECD of $[M + 2Li]^{2+}$ and $[M + 2Cs]^{2+}$ ions produced mono- and dimetalated analogues of the fragment ions formed from $[M + 2H]^{2+}$ with the proportion of dimetalated to monometalated fragment ions increasing with fragment ion mass. For $[M + Li + Cs]^{2+}$ species, the abundance of cesiated fragment ion exceeded that of lithiated in the ECD spectra by 10:1 and all *c* and *z* ions formed from ECD of the $[M + Li + H]^{2+}$ were lithiated. These results were explained by a preferred neutralization of the cation of highest recombination energy. The authors evaluated the recombination energy of protonated, lithiated, and cesiated glycine to be 116, 85,

and 72 kcal/mol, respectively. Zubarev et al. performed ECD on angiotensin II using Zn^{2+} as charge carriers and on polyglycols (PG) cationized with two sodium or two ammonium ions. For all experiments, fewer fragment ions are observed compared with protonated species [40]. Marshall et al. showed that ECD of simultaneously protonated and cationized oligodeoxynucleotide dications yielded more product ions than that generated from the doubly protonated species [41]. More recently, divalent alkaline-earth metal ions were used as charge carriers for ECD of several model peptides as well as Substance P [42]. Again, information complementary to the ECD of protonated peptides was obtained. The authors also pointed out that doubly charged molecular ions could be generated by simply doping a small amount of alkaline-earth metal salt into the analyte solution whereas doubly protonated system were sometimes difficult to attain. Similar experiments on sulfated peptides lacking basic amino acid revealed the analytical potential of this approach [43].
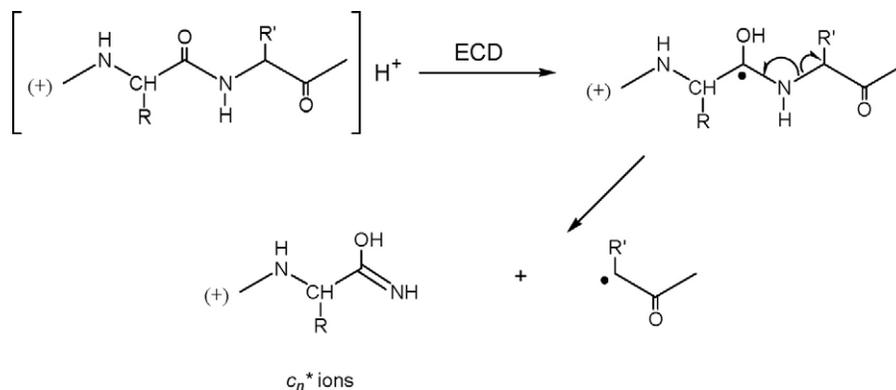
TMPP-Glycopeptides

ECD experiments on glycopeptides **G1**–**G6** gave informative spectra containing different kind of fragment ions. The ECD spectrum obtained for the glycopeptide **G1** shown in Figure 3 is representative of the results obtained for the glycopeptides. Note that Tables 2 and 3, respectively, gather the bond cleavages observed for all peptides subjected to ECD and (TMPP derivatization + ECD) and the sequence coverage obtained for all these ECD experiments.

The most interesting ions observed are c^* ions. These *c*-type ions are marked with an asterisk to indicate that the charge carrier is a phosphonium and not a proton. Their probable structure is depicted in Scheme **1**. Note that no *z* ions are observed, indicating that the charge always remains on the N-terminus (on the phosphonium group).

These c^* ions are of high abundance in all spectra and

Table 3. Sequence coverages for peptides G1-G6 and P1-P4 obtained for ECD experiments carried out on the native and phosphonium derivatized peptides. The sequence coverage is calculated as the ratio between the number of cleaved peptide bonds and the number of peptidic bonds in the peptide

Peptide	ECD sequence coverage obtained on native peptide (%)	ECD sequence coverage obtained on phosphonium derivatized peptide (%)
G1	25	75
G2	37.5	75
G3	37.5	75
G4	37.5	75
G5	50	75
G6	75	91.5
P1	63.5	82
P2	72.5	82
P3	45.5	63.5
P4	50	87.5

Scheme 1. Proposed structure for the c^* ions formed from ECD of (2^+) TMPP derivatives.

may be accompanied by ($c^* - H^.$) ions. The analysis of Table 3 indicates that TMPP derivatization allows a better sequence coverage in all cases. For instance, the sequence coverage of the native glycopeptide **G1** is 25% before derivatization while of 75% after phosphonium derivatization i.e., all the peptide bonds except the Xxx–Pro linkage have been cleaved.

The exclusive presence of these c^* ions clarifies the spectrum since it avoids the overlap of c and z isotopic patterns that sometimes occurs and complicates the interpretation. No loss of GalNAc was observed. For the glycopeptide **G1**, all c^* ions are observed with a high abundance except c_6^* and c_7^*, both corresponding to the cleavage of an Xxx–Pro bond. These results show that the presence of a fixed charge on the glycopeptides has a positive influence on the results, on sequence coverage as well as in the quality of the data obtained. Indeed, the additional mass brought by the TMPP allows the smaller c ions (c_1, c_2) that are rarely observed in ECD spectra to be detected easily. The presence of these ions can be of prime importance for structural purpose. In the same vein, the mass range chosen for all MS/MS spectra starts at m/z 500 instead of m/z 100, which, for a given number of data points (512 k for all our experiments) increases resolution and mass accuracy.

The analysis of all ECD spectra also indicates that competitive fragmentations take place in the phosphonium moiety. These fragmentations are probably initiated by the capture of the electron by the phosphonium group (Scheme **2**) to give a hypervalent phosphorus radical. The stability of hypervalent nitrogen radicals has been demonstrated some years ago by Turecek and Syrstad [44] and theoretical calculations are underway for phosphorus species.

This unstable species can lose a trimethoxyphenyl radical ($-TMP^.$ in the spectrum) by homolytic cleavage of the P–C bond to give a stable phosphine still bound to the protonated peptide. Another important fragmentation corresponds to the formation of the stable protonated trimethoxyphenyl phosphine at m/z 533. This latter elimination can be rationalized by a proton transfer from the charged peptide to the phosphine moiety to give an ion-molecule complex, which easily dissociates

to give the protonated phosphine. In that case, the TMPP moiety acts as a good leaving group. An attachment of a hydrogen radical to the phosphonium group followed by the cleavage of the phosphorus–methylene bond cannot be excluded.

Finally, no loss of GalNAc is observed in the spectra but the elimination of 43.01 Da is still present in high abundance.

These results demonstrate that the addition of a phosphonium group completely modifies the reactivity
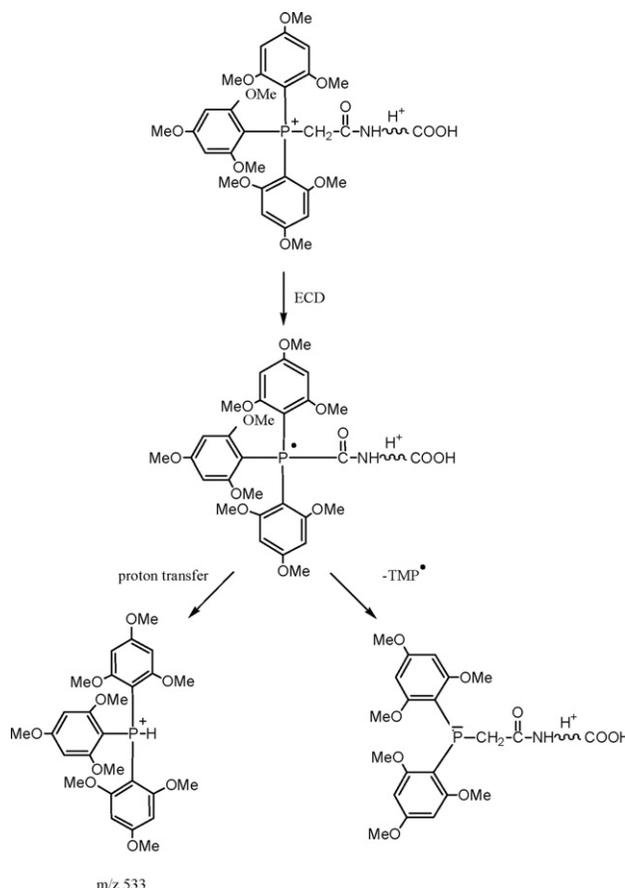
Scheme 2. Proposed structures for the fragment ions related to the TMPP moiety.

of the glycopeptides under study. Two major factors may explain this feature. First, it has been shown that fragmentations observed in ECD of peptides strongly depend on the gas-phase structure of the precursor ions (hydrogen bonding, salt-bridge). Changing the free N-terminus for a TMPP moiety may induce an important modification of the gas-phase structure of the peptide. Second, the energetic of the electron capture is completely modified. In the native peptide, the system contains two protons, which are probably on the most basic sites of the peptide whereas the system after derivatization contains one proton and one phosphonium on the N-terminus. The presence of regular ECD fragments (c^* ions) and fragment ions from the TMPP moiety clearly indicates that the recombination of the electron with the phosphonium group can compete with its recombination with the charged peptide. The improvement in sequence coverage with the c^* ions obtained for all derivatized glycopeptides, regardless of their sequence, is probably an indication of a mechanism that does not involve directly the initial protonation site. Indeed, if the initial protonation site led to the fragmentation, the opposite trend (increased fragmentation with an increased number of protons) should be expected. Thus, these results are probably a good indication of the prevalence of a mechanism involving an electron recombination in the π^* carbonyl orbitals of the polypeptide backbone rather than an alternative mechanism.

TMPP-Phosphopeptides

Similar results are obtained with phosphonium derivatized phosphopeptides since ECD spectra of all doubly charged TMPP-phosphopeptides (**P1**, **P2**, **P4**) contain nearly complete c^* ions series (Table 2). **P3**, for which the triply charged ion was selected for fragmentation, gives both c^* and regular z ions. These z ions are due to the presence of basic residues on the C-terminus of the peptide (KKK). For example, the ECD spectrum obtained for the phosphopeptide **P1** is depicted in Figure 4.
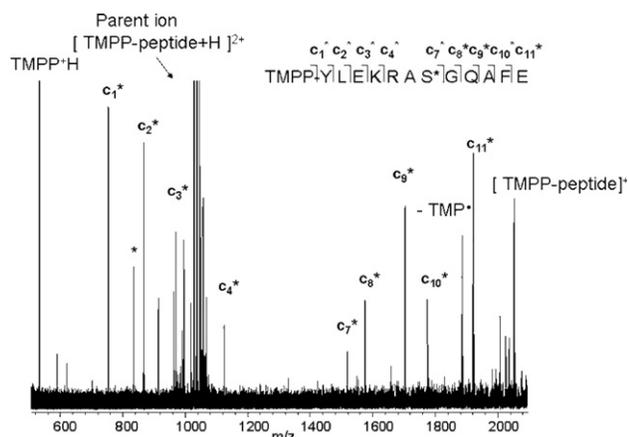

Figure 4. ECD of 2^+ (m/z 1025.928) of phosphorylated peptide **P1** derivatized with TMPP.

For this peptide, we observe abundant c_1^*–c_4^* and c_7^*–c_{11}^* series allowing an easy assignment of the phosphate in the sequence. The absence of c_5^* and c_6^{**} ions may be explained by the formation of a salt bridge between the negatively charged phosphate and the guanidinium moiety of the arginine. Indeed, the ECD spectrum of the doubly charged ion formed from TMPP-YLEKRASGQAFE (same sequence but no phosphate) contains c_5^* and c_6^*. This result indicates that the phosphate group plays an important role in the gas-phase structure of the peptide and in the lack of reactivity observed in some instances. We propose that in the case of the phosphorylated peptide, the c/z complex formed upon ECD does not disrupt because of a strong bonding between a positively charged arginine and a negatively charged phosphate.

The most important increase of sequence coverage can be observed for the phosphopeptide **P4**, which does not contain any basic amino acids. Moreover, the abundant losses of water observed for the native peptide are completely absent here.

As observed for TMPP-glycopeptides, ECD spectra of all TMPP-phosphopeptides exhibit a loss of a TMP radical and formation of an intense TMPP-H$^+$ ion at m/z 533. No loss of phosphate is observed.

Conclusions

Our results clearly show that ECD combined with fixed charge derivatization is a powerful and new analytical tool to increase the sequence coverage of peptides bearing one or several labile post-translational modifications. A full sequence coverage is obtained for nearly all glycopeptides and phosphopeptides studied, considering that the Xxx–Pro bond is rarely cleaved in ECD experiments. No loss of the modification is observed. In all cases, intense c_1 and c_2 ions, which very often lack in ECD spectra, can be retrieved in the spectra. The presence of these ions can be of prime importance for structural purpose. The derivatization is fast, inexpensive, and quantitative, and could thus be applied to complex mixtures such as tryptic digests. Moreover, the quality of the ECD spectra of (2^+) ions and the exclusive presence of c^* fragments for these species can help for de novo sequencing. The difference in reactivity between protonated native peptides and their phosphonium derivatives may arise from the probable modification of the peptide structure and from the difference in energetic due to the presence of the phosphonium in the system. The presence of classical and numerous ECD fragments (c^* ions) and also of fragment ions originating from the TMPP moiety clearly indicate that the recombination of the electron with the phosphonium group can compete with its recombination with the π^* carbonyl orbitals of the polypeptidic chain.

Acknowledgments

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