Research Article

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Comparison of CID, ETD and metastable atom-activated dissociation (MAD) of doubly and triply charged phosphorylated tau peptides

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The fragmentation behavior of the 2+ and 3+ charge states of eleven different phosphorylated tau peptides was studied using collision-induced dissociation (CID), electron transfer dissociation (ETD) and metastable atom-activated dissociation (MAD). The synthetic peptides studied contain up to two known phosphorylation sites on serine or threonine residues, at least two basic residues, and between four and eight potential sites of phosphorylation. CID produced mainly b-/y-type ions with abundant neutral losses of the phosphorylation modification. ETD produced c-/z-type ions in highest abundance but also showed numerous y-type ions at a frequency about 50% that of the z-type ions. The major peaks observed in the ETD spectra correspond to the charge-reduced product ions and small neutral losses from the charge-reduced peaks. ETD of the 2+ charge state of each peptide generally produced fewer backbone cleavages than the 3+ charge state, consistent with previous reports. Regardless of charge state, MAD achieved more extensive backbone cleavage than CID or ETD, while retaining the modification(s) in most cases. In all but one case, unambiguous modification site determination was achieved with MAD. MAD produced 15–20% better sequence coverage than CID and ETD for both the 2+ and 3+ charge states and very different fragmentation products indicating that the mechanism of fragmentation in MAD is unique and complementary to CID and ETD.

Supporting information may be found in the online version of this article.

Keywords: CID; ETD; metastable atom; tau phosphopeptides; peptide sequencing; collision-induced dissociation

INTRODUCTION

Recent advances in mass spectrometry (MS) have given the proteomic field an exceptional new tool to study the effects of post-translational modifications (PTMs). Because of the steady improvements in resolving power, sensitivity and analysis time, tandem MS (MS/MS) has become a preferred method for large-scale phosphorylation identification.[1] Protein phosphorylation is known as one of the most frequent PTMs and is attributed to a wide range of biological functions and regulatory mechanisms.[2] Phosphorylations occur most often on tyrosine, serine and threonine amino acid residues, and the latter two are notably more labile. In the brain, hyperphosphorylation of serine and threonine residues of tau protein in neurofibrillary tangles is considered to be one of the major hallmarks of Alzheimer's disease (AD).[3,4] To date, more than 30 serine and threonine phosphorylation sites have been characterized. However, it is still undetermined which phosphorylated sites are the critical initiation site for the progression of tau into the symptomatic hyperphosphorylated version.[5] The ability to obtain unambiguous modification site determination is crucial for routine biomarker detection and for intricate mapping studies, especially when multiple modifications are present in close proximity.

Identifying tau phosphorylation can be done through direct sequencing methods by utilizing mass spectrometers or Edman degradation techniques or through indirect techniques such as tau phosphorylation-specific antibody interactions.[6] The latter techniques have led to the successful identified of several previously unknown phosphorylation sites and can also be used for AD biomarker detection. However, these techniques are time consuming, require intensive sample preparation and can lead to unambiguous site determination. The majority of tau phosphorylation sites have been identified by MS/MS due to its high sensitivity and selectivity.[7]

The most common MS/MS activation method employed today is collision-induced dissociation (CID). Dissociation occurs through collisions of isolated peptide ions with a neutral bath gas which gives rise to heating and subsequent dissociation. CID provides cleavage of the weaker peptidic bonds such as C–N backbone bonds that lead to the observation of b-/y-type ions. CID also provides internal fragments and neutral losses of water, ammonia, carbon dioxide and labile PTMs. Unfortunately,

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the slow heating nature of CID leads to the significant neutral loss of PTMs, especially phosphate (−80 Da) or phosphoric acid (−98 Da) from phosphorylated peptides.[8,9] Whereas neutral losses can confirm the modification type, such fragments often cannot unambiguously locate the modification site.[8–11] Palumbo and Reid recently demonstrated that during slow heating CID of lower charge-state phosphopeptides, gas-phase rearrangements of the phosphate moiety prior to fragmentation can lead to ambiguous or incorrect residue assignment.[12] The magnitude of this rearrangement problem is thought to be negligible,[13] but the PTM assignments of low charge-state peptides cannot easily be verified by an independent method like electron transfer dissociation (ETD) and electron capture dissociation (ECD).

The introduction of new types of radical-induced dissociation methods has become the preferred way to sequence and identify PTMs. ECD, introduced by McLafferty and co-workers, leads to predominantly N–Cα backbone cleavages while retaining the more labile PTMs.[14] Dissociation occurs through the interaction of free electrons with isolated peptide ions within Fourier transform ion cyclotron resonance mass spectrometer. Recently, developments have enabled ECD to be performed in linear ion traps.[15,16] Similar to ECD, ETD employs radical anions to transfer electrons to peptide cations to produce ECD-like fragmentation and is easier to implement in relatively low-cost RF ion trapping instruments compared to ECD.[17] Compared to CID, ETD and ECD have shown superior analysis of labile PTMs such as phosphorylation,[17,18] glycation/glycosylation,[19–22] and sulfonation,[23,24] among others.

Research has shown that phosphorylation modifications can have a negative effect on the efficiency of dissociating 2+ species with ECD.[25] This phenomenon is attributed to the phosphorylation moiety forming a noncovalent salt bridge with a basic amino acid residue, which is typically not cleaved in ECD. Additionally, ETD studies have shown that fragmentation can be charge-state dependent with a decrease in fragmentation efficiency of the 2+ species leading to few or no backbone cleavages.[26,27] Both of these issues can pose a problem with peptides that do not contain multiple (>2) sites of protonation. The ineffectiveness of ETD and ECD for 2+ peptides is a problem given that a large portion of tryptically digested peptides are doubly charged.[28] McLuckey and co-workers have shown that raising the bath gas temperature during ETD can increase the sequence coverage of 2+ species.[27] Another technique to enhance the effectiveness of ETD of 2+ peptides is to apply supplemental activation in the form of collisional energy to the charge-reduced product ion to increase fragmentation efficiency, a technique known as ETcaD.[29–31]

The complementary nature of the fragment ion types produced by CID and ETD has led many to combine the methods into either a consecutive alternating mode or as an MS^3 mode.[32–34] By utilizing information from both methods, the overall sequence coverage can be increased to more than 90%.[32] Good *et al*. illustrated that CID produces superior peptide identification capabilities for 2+ species, whereas ETD outperforms CID when precursor charge (z) is greater than 2.[30] CID MS/MS spectra of higher charge states are difficult to identify or score because of the multiple charge states of the fragment ions. Automated *de novo* sequencing algorithms built on fusing CID and ETD spectra can make the identification more reliable.[35,36] Similar combinations have been utilized for CID and ECD also increasing peptide identifications compared to CID alone.[37]

Recently, another type of radical-induced dissociation has been introduced that uses metastable atoms as the electron vehicle, or energy source, for dissociating peptide ions.[38–43] We refer to the technique as metastable atom-activated (or induced) dissociation (MAD).[41] Extensive sequence coverage and backbone cleavages with a-, b-, c-, x-, y- and z-type ions, while retaining PTMs, are observed through the interaction of a high kinetic energy beam of helium metastable atoms with isolated peptide ions. Several groups have provided evidence that dissociation occurs through two competing mechanisms, charge reduction (CR) and Penning ionization (PI).[38,39,41,43] The CR mechanism is analogous to the mechanism proposed for ECD and ETD and occurs through an electron transfer from the metastable atom to a site of protonation on the peptide ion.[44–46] The second mechanism, PI, occurs through the detachment of an electron, most likely from an n or π orbital, from the peptide ion by the metastable atom. PI generates a radical peptide ion, a ground-state noble gas atom and a free electron.[38,41,43]

PI has been well characterized for neutral molecules and occurs when the internal energy of the metastable atom is greater than the ionization potential of the neutral.[47,48] Metastable atoms are attracted by dipoles and regions of high electron density such as the lone pairs of electrons on carbonyl oxygen atoms and amide nitrogen atoms.[49–52] PI leads to direct oxidative radical generation unlike the reductive radical generation observed in ETD and ECD. To date, MAD has been applied to multiply charged cations and anions,[38,41,43] 1+ cations,[39–41] disulfide bridges,[43] leucine and isoleucine differentiation,[39,41] proline ring cleavage,[41] phosphopeptide cations,[40,41,43] phosphopeptide anions,[53] sulfopeptides,[53] nitrated/nitroslated peptides,[42] and lipids.[54]

In this work, we investigate and compare the types of fragment ions produced, the sequence coverage and the ability to unambiguously identify the location of PTMs present using CID, ETD and MAD for the 2+ and 3+ charge states of several different tau phosphopeptides. The tau peptides studied contain several potential sites of modification in close proximity, thereby increasing the need to achieve high sequence coverage.

Table 1. A comparison of the percent sequence coverage for CID, ETD and MAD of each tau phosphopeptide

		% sequence coverage					
	Sequence	CID		ETD		MAD	
		2+	*3+*	*2+*	*3+*	*2+*	*3+*
P1a	SRpTPpSLPTPPTREPK	50	64	**36**	71	86	86
P1b	SRTPpSLPTPPTREPK	57	57	**7**	79	100	86
P2a	VAVVRTPPKSPSpSAK	**86**	93	86	93	93	100
P2b	VAVVRpTPPKpSPSSAK	71	71	86	93	93	100
P2c	VAVVRTPPKSPpSSAK	78	78	86	86	100	100
P2d	VAVVRTPPKpSPSSAK	93	86	78	93	78	100
P3a	RSGYSpSPGSPGpTPGSRSR	82	71	59	65	71	76
P3b	RSGYSSPGpSPGpTPGSRSR	47	65	**47**	**65**	65	94
P3c	RSGYSSPGpSPGTPGSRSR	**41**	**41**	71	**65**	**59**	76
P4	TPSLPpTPPTREPK	67		67		92	
P5	TDHGAEIVYKSPVVSGDTpSPR	**85**		**30**		90	
	Average coverage	***67***	***71***	***62***	***73***	***83***	***91***

Bold and underline numbers indicate that a modification site could not be unambiguously identified.

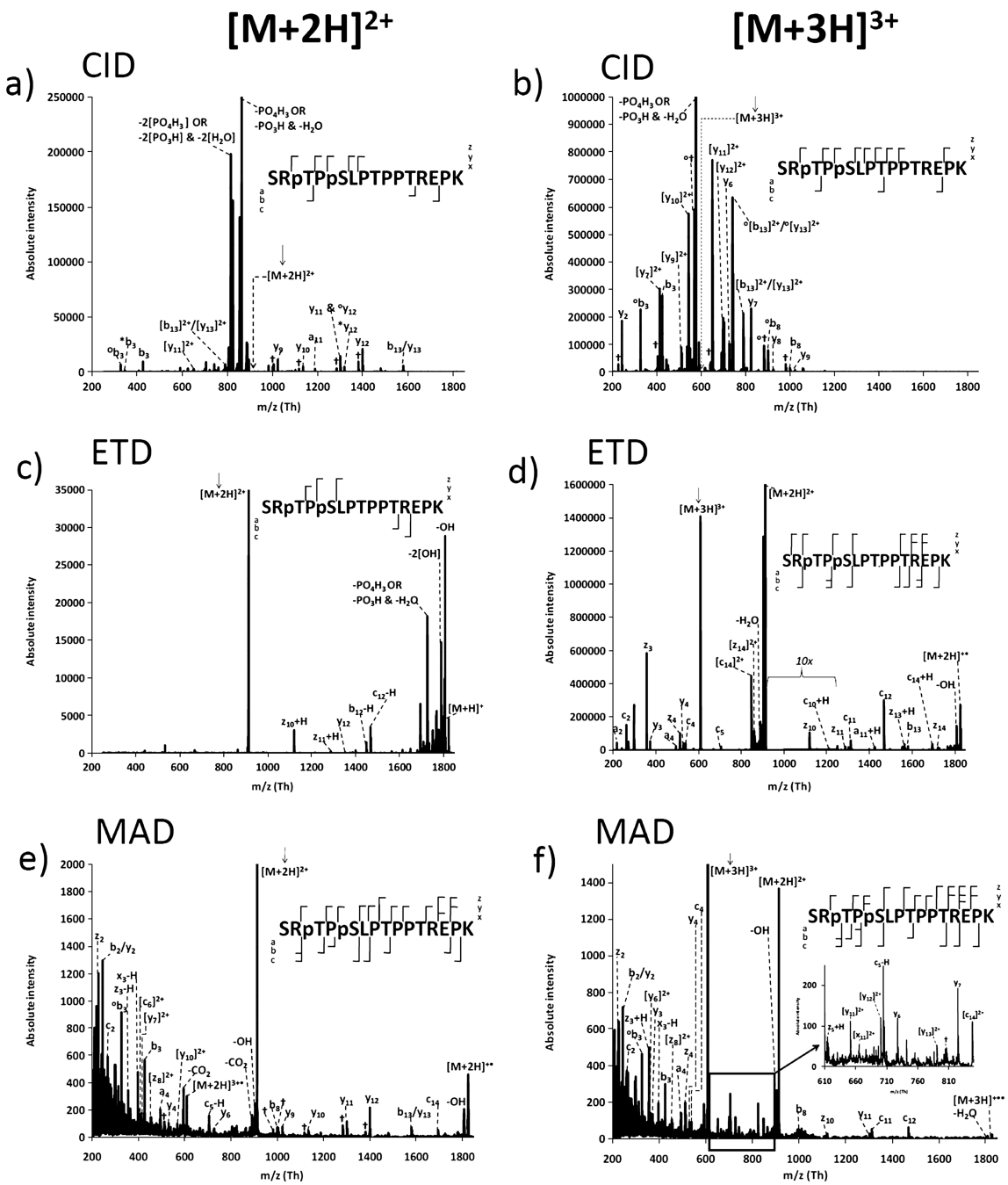

Figure 1. Comparison of the dissociation of the di-phosphorylated tau peptide P1a SRpTPpSLPTPPTREPK (tau210-224) generated of the 2+ charge state through (a) CID, (c) ETD, (e) MAD and of the 3+ charge state through (b) CID, (d) ETD and (f) MAD. The precursor is indicated by an arrow. Neutral losses are indicated by the following: water (†), phosphorylation modification corresponding to 98 Da (°) and to 80 Da (*). Fragments that do not retain the modification are omitted from the peptides sequence inserts.

EXPERIMENTAL

Preparation of peptides

Phosphopeptides were synthesized on a Syro 2000 multiple peptide synthesizer (MultiSyn Tech GmbH) according to the procedure detailed by Singer *et al.*[4] Subsequent RP-HPLC purification and sequence confirmation by matrix-assisted laser desorption/ionization time-of-flight MS was performed (4700 proteomics analyzer, Applied Biosystems GmbH, Darmstadt, Germany). All peptides were reconstituted to a 1:1 ratio of methanol (Sigma-Aldrich, HPLC grade, St. Louis, Mo) and water with 1% acetic acid (Sigma-Aldrich). Peptide concentrations were 5 μM for CID and ETD experiments and between 10 and 20 μM for MAD experiments.

Instrumentation and method

CID and ETD experiments were performed on a Thermo LTQ Orbitrap XL ETD equipped with a nano-ESI source (Thermo Fisher Scientific GmbH, Bremen, Germany). MAD experiments were

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performed on a modified Bruker EsquireLC (Bruker Daltonics, Bremen, Germany) with an Ion Tech FAB gun (P50 PSU, Teddington, UK) as the metastable atom source. The FAB gun was positioned approximately 2 cm above the ring electrode. The ring electrode contained a 2 mm hole to allow metastable atoms to enter the trap directly above the center of the trap. The deflection electrodes consisted of two 4–40 stainless steel nuts at the end of two flat-ended screws positioned 1 cm below the exit orifice of the FAB gun to prevent ions and free electrons from entering the trap. The metastable atom beam was pulsed on during the part of the MS duty cycle reserved for collisional activation. Detailed instrumentation modification and metastable atom beam pulse production has been described previously.[41]

CID and ETD

All CID and ETD experiments were performed on the Thermo LTQ-Obritrap. The 2+ and 3+ charge state of the phosphopeptides were generated by nano-ESI and directly injected at a flow rate of 0.4–0.6 mL/h. An isolation window of *m/z* 1 was used to isolate all precursor ions and the low mass cut off (LMCO) value was dependent on the precursor mass. The collisional activation energy was set to 35 mV for all CID experiments, whereas the ETD activation time varied between 100 and 250 ms depending on precursor charge state and peptide. The CID MS/MS spectra were acquired in intervals between 25 and 50 s consisting of 7–12 averaged scans. ETD MS/MS spectra consisted of 5–9 scans and were acquired between 25 and 50 s intervals.

MAD

All MAD experiments were performed on the Bruker 3D ion trap. The 2+ and 3+ charge states of the phosphopeptides were generated by ESI at a flow rate of 0.2 mL/h. The isolation window was varied between *m/z* 1.0 and 2.0 based on the lability of the peptide and charge state. A low mass trapping voltage was set to 200 *m/z* to minimize background ions from the PI of residue pump oils. The vacuum pressure in the MS chamber outside the trap with He bath gas leaking out of the trap was 1.8 mbar. An additional 1.0 mbar of pressure from the FAB gun was added to produce the He metastable atom beam. The FAB gun anode was powered at 6 keV, and the metastable atom beam was activated for 100–250 ms, depending on the charge state and fragmentation behavior of each peptide. The MAD MS/MS spectra were acquired in 2 min intervals which consisted of 60–70 scans with 5 averages per scans. All fragment ion identifications and assignments on the Bruker instrument were manually determined based on predicted fragmentation patterns and were within ±0.8 *m/z* of the expected product ions. Peaks were only assigned if the intensities were at least three times the signal-to-noise ratio. The 2+ and 3+ ions were identified according to the expected *m/z* value and the presence of an isotope envelope peak at *m/z* +0.5 and *m/z* +0.3, respectively.

RESULTS AND DISCUSSION

Details of the peptides used in this study are listed in Table 1. A total of eleven isoforms of five different peptides are studied, with one or two known phosphorylation sites on either a threonine or serine residue. The peptides contain between 4 and 8 potential sites of phosphorylation, which makes unambiguous PTM localization a challenge. Additionally, the peptides contain between 2–5 proline residues, which are known to hinder both CID and ETD backbone cleavage or direct cleavage to the N-terminal side.[55–57] Throughout the text, peptides will be referred to by their given number (P1–P5). Phosphopeptides had a precursor ion mass increase of 80 Da relative to the unmodified peptides, confirming the addition of $-HPO_3$ group. Peptide sequence inserts in all figures omit those fragment ions that have undergone a neutral loss of the PTM modification. It is important to note that there is a positive correlation between the abundance of isolated precursor ions available for MAD or ETD and the quality of the resulting tandem mass spectra. In the Bruker EsquireLC ion trap instrument, we are severely limited by space charge effects in the 3D ion trap and are only able to reach a maximum of 10^5 – 10^6 AU signal intensity for the isolated precursor ions. The LTQ Orbitrap is able to store many more precursor ions in the 2D ion trap and thus has a significant starting advantage. In addition, the high resolution of the Orbitrap enables greater confidence in assigning product ions than the Bruker 3D ion trap. We acknowledge that many of the peptides in this study provided superior ETD spectra when the 4+ precursor ions of the tau peptides were used in the LTQ instrument. However, the benefit of high charge states is well known for ETD[30] and is beyond the scope of the current article. Higher charge states are also harder to obtain for peptides with few basic residues.

Effect of precursor charge state

To study the effects of charge state, the percent sequence coverage was compared for each peptide via CID, ETD and MAD. Percent sequence coverage is defined in the typical way as the percent of the backbone bonds that are cleaved at least once. For this reason, efficiencies cannot exceed 100%. Clearly, 100% coverage of one ion type (e.g. all b ions or all z ions) is more useful for *de novo* sequencing, but no attempt is made here to report the percent coverage of each

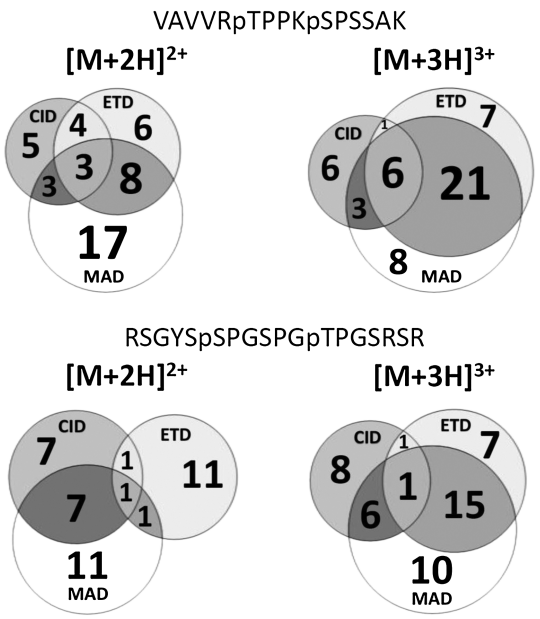

Figure 2. Venn diagrams comparing the number of product ions types produced for CID, ETD and MAD of both the 2+ and 3+ charge states of the di-phosphorylated Tau peptides P2b VAVVRpTPPKpSPSSAK (tau226-240) and P3a RSGYSpSPGSPGpTPGSRSR (tau194-210).

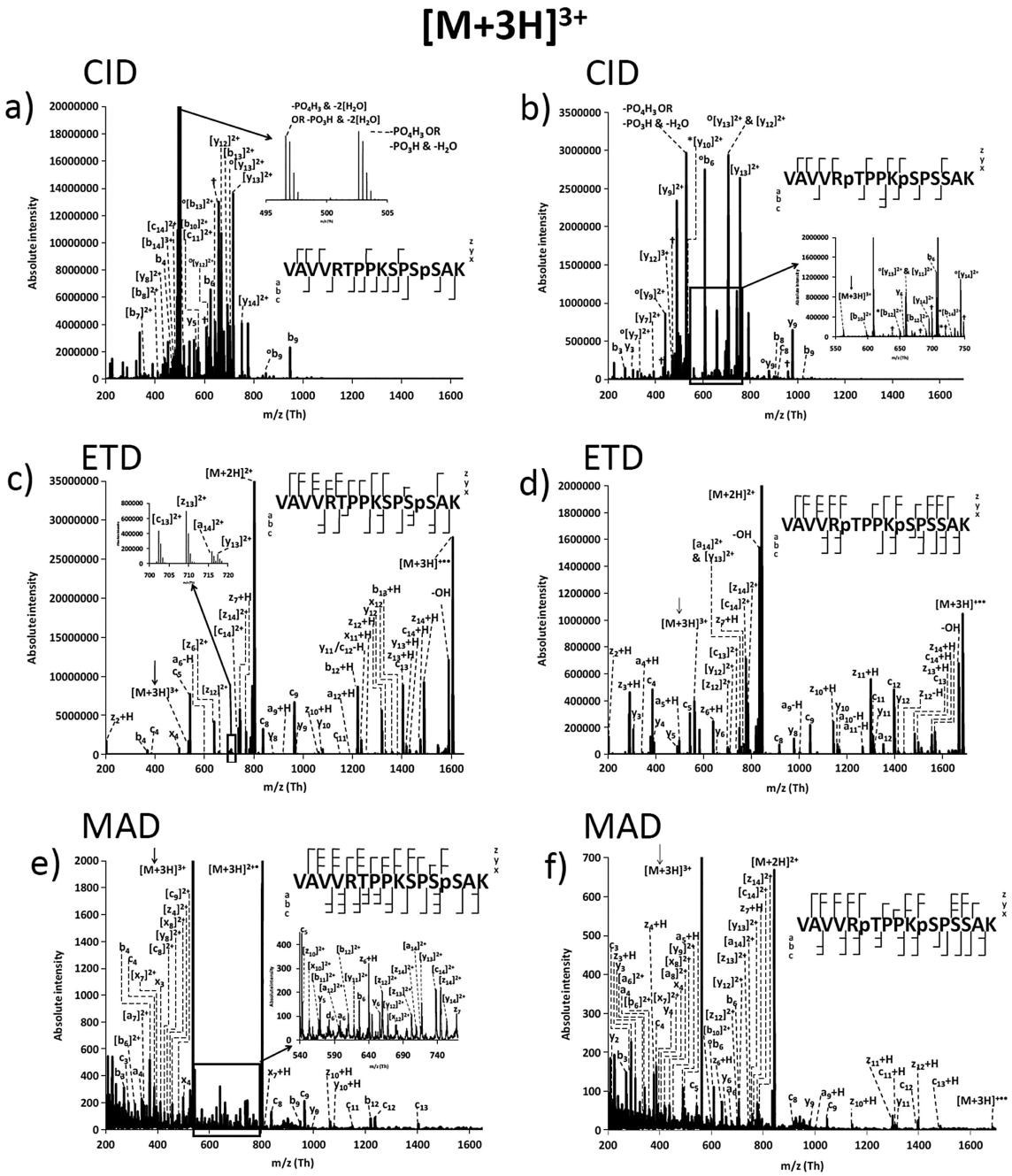

Figure 3. Comparison of the dissociation of the 3+ charge state of the mono-phosphorylated tau peptide P2a VAVVRTPPKSPSpSAK (tau226-240) through (a) CID, (c) ETD, (e) MAD and of the di-phosphorylated tau peptide P2b VAVVRpTPPKpSPSSAK (tau226-240) through (b) CID, (d) ETD (f) and MAD. The precursor is indicated by an arrow. Neutral losses are indicated by the following: water (†), phosphorylation modification corresponding to 98 Da (^{o}) and to 80 Da (*). Fragments that do not retain the modification are omitted from the peptides sequence inserts.

individual ion type. This information can be gleaned from the fragmentation pattern inserts in the various figures. Table 1 and Fig. 1 compare the fragment ions generated from CID, ETD and MAD of the 2+ (left column) and 3+ (right column) charge states of the di-phosphorylated peptide SRpTPpSLPTPPTREPK (P1a). For the 2+ precursor, the percent sequence coverage achieved for CID, ETD and MAD was 50%, 36% and 86%, respectively. For the 3+ precursor, the sequence coverage increases for CID and ETD to 64% and 71%, respectively, whereas the coverage for MAD remains at 86%. Combining the ETD 2+ and 3+ charge-state information increases the sequencing efficiency to 79%. Combining the MAD 2+ and 3+ provides 93% coverage. No additional information is obtained by combining the CID information.

Table 1 and Venn diagrams in Fig. 2 illustrate the number of fragment ion types generated for each of the three dissociation methods for the 2+ and 3+ species of the di-phosphorylated peptide P2b and P3a. CID of P2b and P3a produced similar product ions regardless of charge state. ETD and MAD both produced more product ion types for

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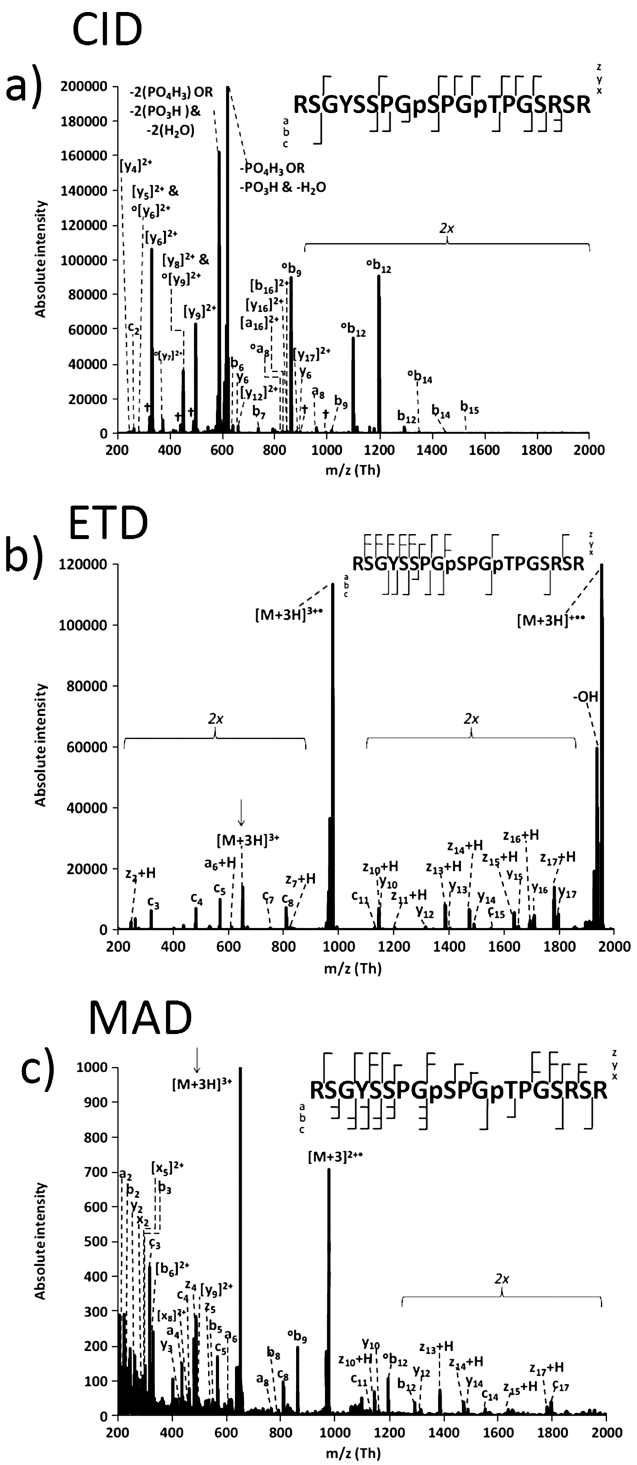

Figure 4. Comparison of the dissociation of the 3+ charge state of the di-phosphoryalted tau peptide P3b RSGYSSPGpSPGpTPGSRSR (tau194-210) through (a) CID, (b) ETD, (c) MAD. The precursor is indicated by an arrow. Neutral losses are indicated by the following: water (†), phosphorylation modification corresponding to 98 Da (°) and to 80 Da (*). Fragments that do not retain the modification are omitted from the peptides sequence inserts.

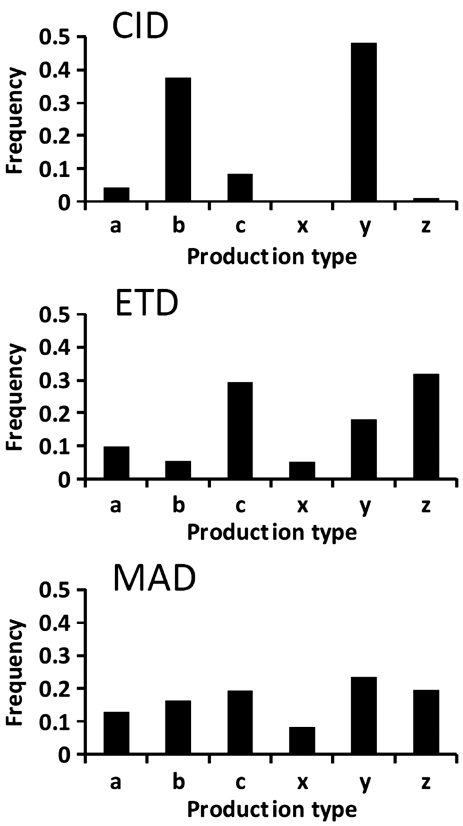

Figure 5. Charts plotting the total frequency of occurrence of the product ion types for CID, ETD and MAD during this study.

the 3+ precursor compared to the 2+ precursor, producing 14 and 12 more product ions for ETD and MAD, respectively. When comparing the average percent coverage for each dissociation method for the eleven isoforms, ETD and MAD both provided 1–3 more backbone cleavages with the 3+ precursor compared to the 2+ precursor. The amount of sequence information generated with CID was roughly the same for either charge state at ~70% sequence coverage. Compared to CID and ETD, MAD achieved 15–20% better sequence coverage for either charge state.

McLuckey and co-workers proposed several reasons for the ETD fragmentation behavior of the 2+ versus 3+ species.[26] One reason for better efficiency with 3+ precursors is that the electron transfer process is simply more exothermic. In CR mode, MAD is equally affected by this phenomenon. However, MAD still achieves ~20% better sequence coverage than ETD for the same charge states. The benefit of MAD over ETD could well reside in the presence of a second fragmentation mechanism such as PI. As with ETD, the electron transfer mechanism in MAD probably leads to the ~8% sequence coverage improvement between the 2+ and 3+ charge states in the phosphopeptides studied. The 20% increase of sequence coverage between ETD and MAD might be attributable to an additional mechanism such as PI, which occurs through electron detachment, not electron transfer.

In PI, the reaction exothermicity is the difference between the potential energy of the metastable atom (~20 eV for He^M) and the ionization energy of biomolecular ion. The exothermicity of electron detachment of polypeptides using electron detachment dissociation (EDD) is approximately 5 eV.[58] Because the ionization potential of the polypeptides is probably in the region of 11–16 eV,[59] we estimate the reaction exothermicity of PI using helium to be in the region of 6–10 eV. Evidence for the PI mechanism can be seen clearly in the Figs. 1e and 5c, where the unfragmented $[M + nH]^{(n+1)+\cdot}$ ions are readily observed.

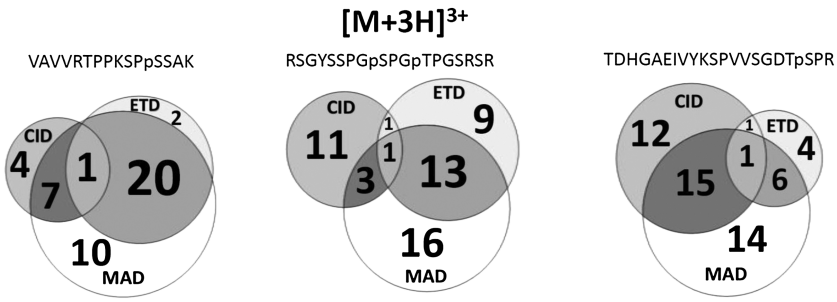

Figure 6. Venn diagrams comparing the number of product ions types produced for CID, ETD and MAD of 3+ charge states of the three tau peptides (a) p2a VAVVRTPPKSPpSSAK (tau226-240), (b) P3b RSGYSSPGpSPGpTPGSRSR (tau194-210) and (c) P5 TDHGAEIVYKSPVVSGDTpSPR (tau386-406).

Fragmentation behavior of phosphorylated tau peptides

The major dissociation channels consistently observed in CID are the neutral losses of 98 Da from the precursor and product ions and are indicative of a phosphoric acid loss ($-H_3PO_4$) or the combination of a HPO_3 (80 Da) and water (18 Da). In ETD and MAD, the most abundant product is usually the charge-reduced species, without fragmentation (i.e. ETnoD). ETD typically generated two intense charge-reduced species, the $[M+3H]^{2+\cdot}$ and $[M+3H]^{+\cdot}$ ions. Compared to ETD, MAD spectra show less intense charge-reduced species. In ETD, many neutral losses are observed from the two charge-reduced species. These neutral losses include $-H^{\cdot}$, –OH, –CO, $-[OH+NH_3]$, $-[CO_2+H]$, and $-[H_3PO_4/HPO_3+H_2O]$ (Supplemental Fig. S1). In contrast to ETD, MAD shows only three neutral losses from the charge-reduced $[M+3H]^{2+\cdot}$ product ion: $-H^{\cdot}$, –OH and $-[H_3PO_4/HPO_3+H_2O]$ (Supplemental Fig. S1). Furthermore, ETD was unique in producing neutral losses of $-[CH_5N_3]$ and $-[C_4HN_{10}]$, which correspond to neutral losses from the side chain of arginine from peptides P1a and P3b.[60]

For CID of peptide P1a, SRpTPpSLPTPPTREPK, in Fig. 1, CID and MAD provided enough cleavages to unambiguously assign the position of the modifications. CID provided quite poor sequence coverage, presumably due to the abundance of proline residues that direct cleavage to their N-terminal side, and because of the three basic residues that decrease the mobility of charging protons. ETD of the 2+ charge state did not provide cleavages between the first three residues, thereby resulting in ambiguous identification between Ser-1 and Thr-3.

The 3+ charge state of two isoforms of peptide P2 are shown in Fig. 3. P2a is mono-phosphorylated at position Ser-13 and P2b is di-phosphorylated at positions Thr-6 and Ser-10. All three methods, with the exception of CID of the 3+ form of P2a, provide high sequence coverage and allow unambiguous PTM localization for both charge states for all four isoforms studied. Informative spectra were observed for all three methods for P4, TPSLPpTPPTREPK, as illustrated in Supplemental Fig. S2. MAD achieved the highest percent sequence coverage of the three techniques. However, all three methods were able to cleave between the four potential sites of phosphorylation and were able to unambiguously identify the location of the modification to the Thr-6 residue.

Figure 4 shows the dissociation of peptide P3b, which contains two phosphorylation sites at positions Ser-9 and Thr-12. MAD of P3b achieved 94% sequence coverage and cleaved between all amino acids with the exception of the Pro-7 and Gly-8 residues. ETD and CID produced less sequence coverage, but only ETD was unable to identify the phosphorylation site. Similar results are also observed for ETD of the 2+ charge state of P3b, with no cleavage between Thr-12 and Ser-17 residues. Additional spectra on P5 are included in the supplemental material.

Figure 5 summarizes the frequency of occurrence of all the product ion types generated from this study of the tau phosphopeptides. CID produces mainly b-/y-type ions with few a-, b- and z-type ions. ETD produced mainly c-/z-type ions, and more specifically in the form of c-· and z′-type ions. In ETD, y-type ions are more common than a- or b-type ions, but all three are observed in much lower intensities than the c-/z-type ions. ETD is also known to be ineffective at proline residues and was unable to produce c-/z-ions on the N-terminal side of proline. In several cases, ETD produced a- and y-type ions. For example, in Fig. 3c, the peptide P2a produced the y_8 and y_9 ions. These observations of a-, b- and y-type ions in the ETD spectrum are consistent with several other ETD/ECD studies.[61–63] MAD produces similar frequencies for all product ion types with the exception of x-type ions, which were slightly less common. For multiply charged cations, the main difference between ETD and MAD is that MAD can induce dissociation through PI. In theory, PI can directly generate radicals anywhere along the peptide backbone where there is a polar group or lone pair of electrons, such as carbonyl oxygen atoms or amide nitrogen atoms, so the fragmentation pattern is expected to be less dependent on sites of protonation.[41]

Venn diagrams in Fig. 2 and Fig. 6 illustrate the complementary nature of CID, ETD and MAD. CID and ETD typically share 1–7 (<10%) of the fragment ion types and are therefore more complementary. MAD generates abundant b-, c-, y- and z-type ions and therefore has more in common with both CID and ETD. In general, MAD produces in one spectrum what CID and ETD must produce in two spectra. MAD therefore provides spectra that might be highly beneficial for data mining.[35,36]

CONCLUSION

On average, CID produced sequence coverages of ~70% for the 2+ and 3+ charge states and failed to unambiguously identify the modification site ~20% of the time. Additionally, abundant neutral losses of the phosphorylation modification and water were observed which significantly complicated the spectra. ETD generated a variety of a-, b-, c-, y- and z-type ions, but the c-/z-ions observed were in greatest abundance. The sequence coverage varied between 7% and 93% and ETD failed to unambiguously identify the location of the PTM approximately 30% of the time. MAD generated the most backbone cleavages, between 83% and 91% sequence coverage for the 2+ and 3+ charge states, respectively, and only failed to unambiguously identify the modification site on one occasion (approximately 5% of the time).

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CID and ETD are routine methods for sequencing peptides and analyzing phosphorylation modifications. However, our data has shown that MAD offers an alternative method to complement CID and ETD. MAD produces higher sequence coverage than either CID or ETD for this specific small class of peptides and can unambiguously locate the site of multiple modifications. Whereas true *de novo* sequencing would be very difficult or impossible to achieve with MAD because of the significant abundance of low mass background ions, multiple techniques are required to retrieve a *de novo* sequence for an unknown peptide/protein. Typically, one uses CID followed by ETD or ECD, or whatever else is available in one's laboratory. Thus, partial sequences could come from any/all techniques, even if the phosphorylation site is lost in CID. MAD can clearly help elucidate partially known sequences, especially those with labile PTMs. Most researchers who sequence peptides *de novo* will always be happy to get a third, fourth or fifth fragmentation technique to close a gap or to confirm an uncertain sequence. MAD is certainly capable to act as a complementary technique to serve this need.

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Supporting Information

Supporting information may be found in the online version of this article.

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