

Mapping O-GlcNAc and Phosphorylation Sites in C-MYC and CTD Peptides by Electron Transfer Dissociation Mass Spectrometry

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Overview

Purpose: Demonstrate the utility of Electron Transfer Dissociation (ETD) for mapping of neighboring phosphorylated and O-glycosylation sites in peptides.

Methods: Synthetic peptides bearing different post-translational modifications (PTMs) were analyzed by direct infusion and nESI using a Thermo Scientific LTQ XL mass spectrometer equipped with an ETD option (Thermo Scientific).

Results: Dissociation induced by ETD yields extensive peptide sequence information and in addition preserves labile PTMs. The exact sites of O-linked glycosylation and phosphorylation were unambiguously identified in this work.

Introduction

Modification of Serine/Threonine residues on peptides by phosphorylation or addition of a single O-linked N-acetylglucosamine (O-GlcNAc) plays an important role in cell regulation⁽¹⁾. In many instances, the sites of O-GlcNAcylation or phosphorylation are localized to the same, or neighboring residues on the peptide. Both modifications are extremely dynamic and labile, making them difficult to analyze by traditional mass spectrometry fragmentation techniques such as Collisionally Induced Dissociation (CID). In conventional CID experiments, modifications such as these are often lost prior to fragmentation of the peptide backbone, preventing localization of the site of modification, although the type of modification is often identified. This makes direct identification of sites of O-linked glycosylation almost impossible without employing chemical derivatization techniques. A new fragmentation technique, Electron Transfer Dissociation, or ETD, preserves labile PTMs, enabling both PTM identification and site localization.⁽²⁾ Figure 1 shows the way the ions are precisely controlled within the LTQ XL linear ion trap mass spectrometer for ETD. In this study, we utilize ETD on a linear ion trap mass spectrometer for the detection and localization of neighboring phosphorylated and O-GlcNAcylated sites on peptides.

Methods

Samples

Synthetic peptides bearing 1, 2, 3, 5 or 10 tandem repeats of the RNA Pol II carboxyl terminal domain (CTD), repeat sequence YSPTSPSK (CTD-peptide) or c-Myc

proto-oncogene protein sequence KKFELLPTPPLSPSRR (c-Myc peptide) were synthesized by standard Fmoc chemistry. Synthetic peptides bearing O-linked GlcNAc or phosphate were prepared by incorporating Fmoc-protected serine/threonine-phospho or O-GlcNAc at the desired position, as described by Greis et al.⁽³⁾ Synthetic peptides corresponding to amino acids 1-21 of human histone H3 with different PTMs were obtained from Upstate (Millipore Corporation, Lake Placid, NY).

Mass Spectrometry.

Mass Spectrometer: Thermo Scientific LTQ XL linear ion trap mass spectrometer with ETD and nESI source

Spray Voltage:	1.2 kV
Capillary Temp:	160 °C
Capillary Voltage:	35 V
Tube Lens:	125 V
MS ⁿ Target:	1e4
Mass Range:	50-2000 m/z or 100-4000 m/z
Anion Reagent:	Fluoranthene
Anion Reagent Isolation:	On
Anion Target:	2e5
Max Anion Injection Time:	50 ms
ETD Reaction Time:	50-100 ms

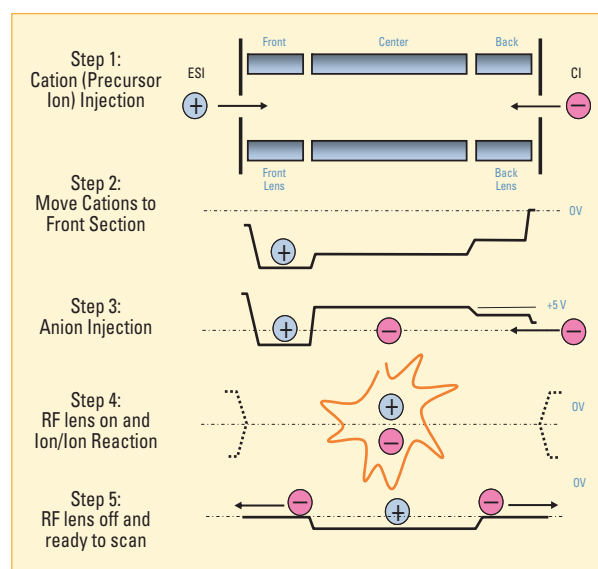


Figure 1: Schematic of the precisely controlled ion-ion reaction within the LTQ XL segmented linear ion trap for Electron Transfer Dissociation.

Key Words

- LTQ XL™ Linear Ion Trap Mass Spectrometer
- BioWorks™ 3.3.1
- Glycosylation
- Phosphorylation
- PTM Site Mapping

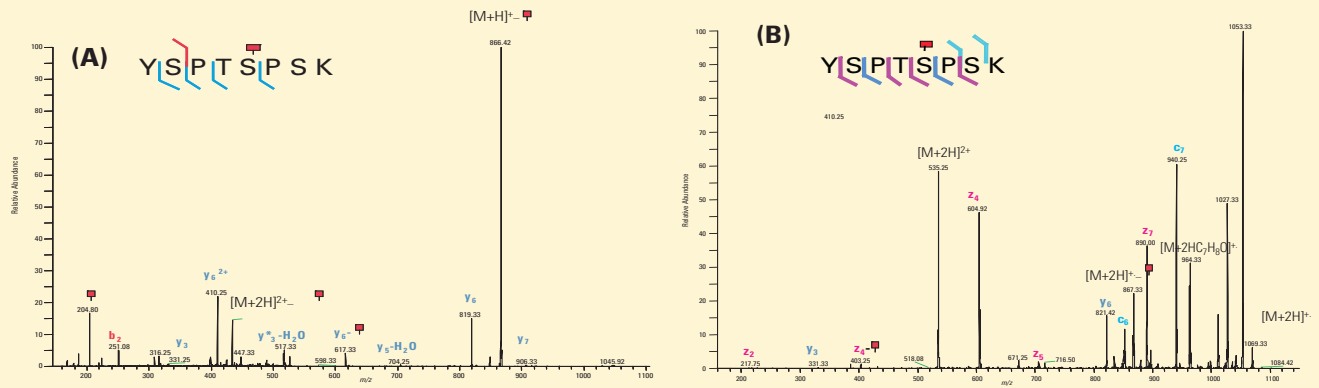


Figure 2: Localization of O-GlcNAc site in YSPTSPSK. (A) CID spectrum of m/z 535.35 ($2+$ precursor). (B) ETD with supplemental activation spectrum of the same peptide precursor.

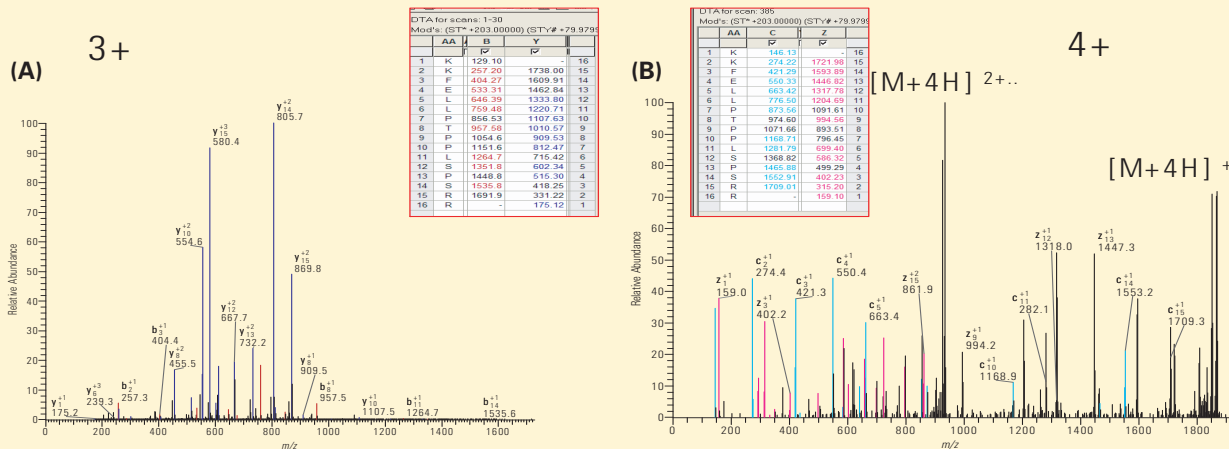


Figure 3: Analysis of the unmodified c-MYC peptide, KKFLELTPPLSPSR. (A) CID spectrum (B) ETD spectrum

Results

Synthetic peptides in this study were chosen because of their biological relevance and complex modified composition. Both motifs participate in protein activity regulation via reversible phosphorylation or O-GlcNacylation.⁽³⁾ Both contain neighboring threonine/serine residues, multiple prolines, and basic residues, and pose a significant challenge for conventional CID. Furthermore, the lysine or arginine residues at carboxyl termini were representative of typical glycopeptides, had they been obtained by digestion with trypsin.

Mass spec analysis by CID and ETD of the synthetic peptide corresponding to the C-terminal domain of RNA polymerase II-CTD peptide is presented in Figure 2. CID analysis, as expected, displayed a prominent neutral loss of the O-linked glycan but did not provide adequate information to identify the exact site of modification, as seen in Figure 2A. Further fragmentation using MS³ on the neutral loss peak would not have been helpful due to complete loss of the modification. In the previous study⁽³⁾ of the same peptide by conventional CID mass spectrometry, the exact site was only identified after alkaline-induced beta-elimination, which converts serine and threonine residues to alanine and 2-aminobutyric acid, respectively. We were able to specify the exact site of O-glycosylation as Serine 5 out of four potential sites

based on an almost complete series of z ions, while the complementary c ions were not observed. Using supplemental activation (resonance CID) with a reaction time of 100 msec provided maximum peptide backbone fragmentation with minimal or no loss of the glycan (Figure 2B).

The c-Myc peptide corresponds to a sequence in an important regulatory domain (family of proto-oncogene proteins), and undergoes constant *in vivo* phosphorylation/O-glycosylation.⁽⁴⁾ Potential sites for phosphorylation/O-glycosylation are Threonine 8 and Serine 12 and 14. The c-Myc peptide is highly basic and generates several charged species via electrospray ionization: namely 2+, 3+, and 4+, the higher charge states species are more favorable for ETD analysis than CID.

Unmodified, monophosphorylated and mono-O-glycosylated cMyc-peptides of concentration 250 fmol/uL were infused in 50% AcN/50% 0.1% formic acid (v/v) and analyzed using both CID and ETD. The results are displayed in Figures 3–5. The 3+ c-Myc peptide species was selected for CID fragmentation and the 4+ species was selected for ETD. With CID, we were unable to obtain a complete b/y ion series even for the unmodified peptide (Figure 3A), nor localize sites of modification (Figure 4). The existence of multiple prolines and basic residues prevented the generation of a full ion series, and significant neutral loss fragments were observed for both phosphate

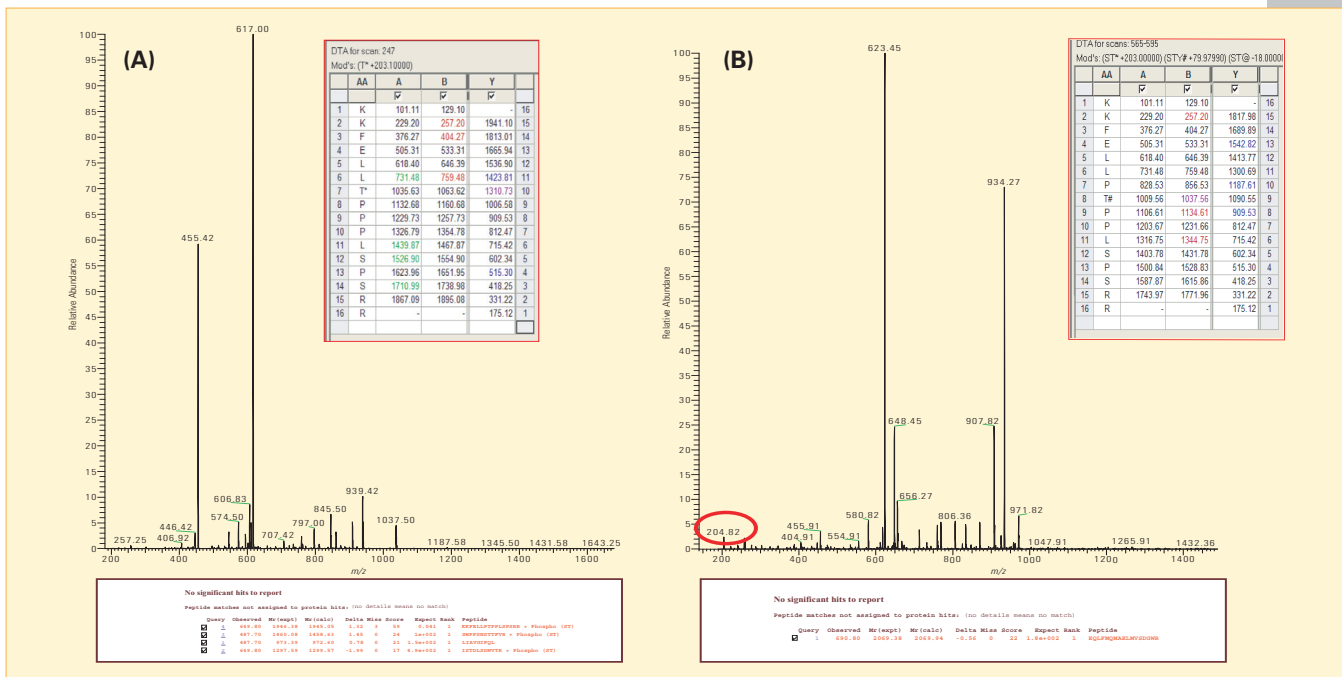


Figure 4: CID spectra of mono phospho (A) and glyco (B) c-MYC peptide (3+ charge) KKFELLPT*PPLSPSRR. Inserts show Mascot results from a no enzyme search against Swiss Prot database with no positive ID

and glyco groups (Figure 4). The use of Neutral loss initiated MS³ did confidently identify the site of phosphorylation but not glycosylation.

Figure 5 shows that ETD generated a near complete set of *c/z* ions for the 4+ species without loss of phosphoric acid or O-GlcNAc, enabling unambiguous identification of the PTM site for c-Myc peptide isoforms at Threonine 8 (corresponding to Threonine 58 in the human form of the c-Myc protein). High confidence results for ETD data were obtained from the database search by using both search engines: BioWorks 3.3.1 (SEQUEST®) and Mascot™ (Figure 5, inserts). Note: *c*₆, *c*₈, *c*₉, *c*₁₀, *z*₄, *z*₇, *z*₈ and *z*₁₀ fragment ions were not observed due to the ring structure of proline. The fragment ions most important for PTM site identification are circled.

Histones contain multiple basic residues and various PTMs *in vivo*, which complicates their analysis by CID.⁽²⁾ Figure 6 shows the results of ETD analysis of different charge states of a synthetic peptide corresponding to the N-terminus of human H3 histone (1-21 + GGK-biotin). This peptide has two sites of modification: Serine 10, which is phosphorylated and Lysine 14, which is acetylated. Because of the abundant number of basic residues in the sequence of this peptide, various charge states are generated, and precursors at *m/z* 712 (4+), *m/z* 570 (5+) and *m/z* 475 (6+) were analyzed. The ETD spectrum of the 5+ charge precursor provided the best results in terms of protein ID and PTM site localization, and produced a near complete series of predominantly singly or doubly

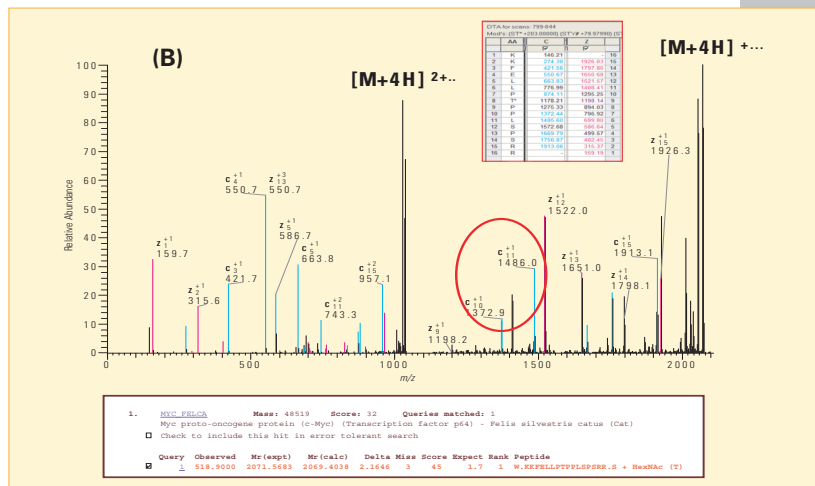
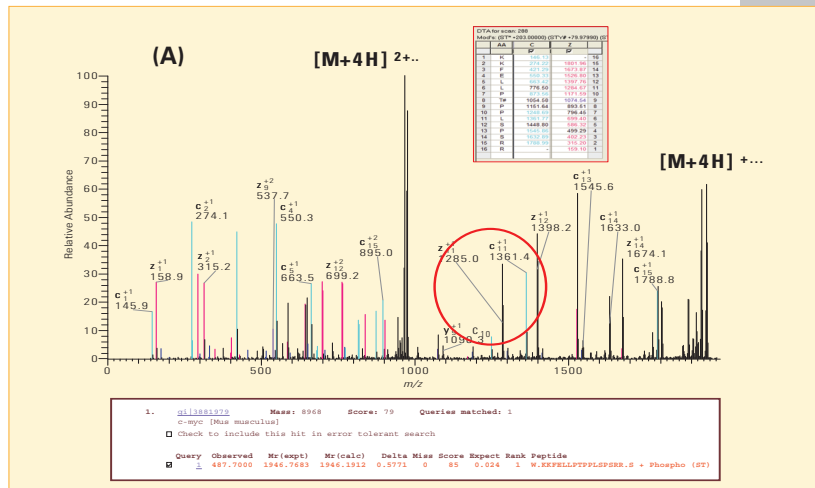


Figure 5: Single scan ETD spectra of mono phospho (A) and glyco (B) c-MYC peptide (charge 4+) KKFELLPT*PPLSPSRR acquired in high mass range. Inserts show MASCOT results from a no enzyme search against Swiss Prot database resulting in a confident ID

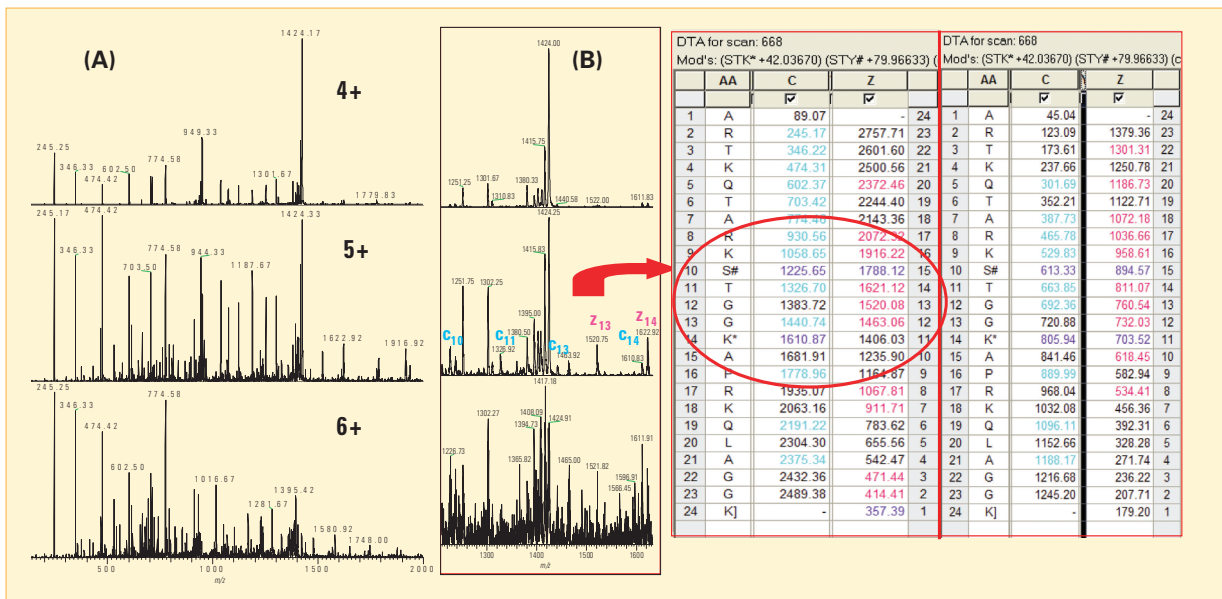


Figure 6: PTM analysis of the synthetic analog of a human histone H3 peptide (1-21 GGG-biotin) by ETD: ARTKQTARKpSTGGackAPRKQLAG ETD spectra of the precursor at different charge states: m/z 50-2000(A); m/z 1200-1650(B), matched ions for charge state of 5+(C)

charged c and z ions. The low charge state fragment ions make spectral interpretation easy and allow for confident protein identification and PTM mapping (Figure 6B&C). ETD fragmentation of the 4+ precursor didn't generate enough information to pinpoint the exact phosphorylation site as Serine 10 (Figure 6B, top panel). Though the 6+ charge spectrum produced the highest number of ions, many of the product ions were highly charged, which complicates data analysis (Figure 6, bottom panel). Singly charged product ions most important for PTM site identification are shown in Figure 6B and circled.

Conclusions

Electron transfer dissociation demonstrated excellent capabilities for mapping neighboring phosphorylated and O-GlcNacylated sites in peptides without chemical derivatization, regardless of charge state.

The exact site of O-glycosylation in the CTD-O-GlcNAc peptide was determined to be Serine 5 using ETD on the 2+ species with supplemental activation.

High confidence results for ETD data were obtained from the database search by using both BioWorks 3.3.1 (SEQUEST) and Mascot.

Successful ETD fragmentation and sequencing of larger peptides (~25 amino acids) can be performed by selecting optimal charge state species.

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