

CID and ETD Analysis of Kinase Sites of Phosphorylation with High Mass Accuracy Measurement

¹Martin Hornshaw, ²Kai Scheffler, ³Martin Zeller, ⁴Nick Morrice

¹Thermo Fisher Scientific, Hemel Hempstead, UK, ²Thermo Fisher Scientific, Dreieich, Germany, ³Thermo Fisher Scientific, Bremen, Germany and ⁴MRC Protein Phosphorylation Unit, Dundee, UK

Overview

Purpose: To evaluate ETD and CID (MSA) performance in an LTQ Orbitrap XL ETD™ specifically for the characterization of sites of protein phosphorylation

Methods: Digested human kinases were analyzed by LC-MS using a data dependent or data dependent decision tree approach

Results: Typically ETD produced better MS/MS data in terms of localization of sites of protein phosphorylation

Introduction

Electron transfer dissociation (ETD) and collision induced dissociation (CID) are complementary dissociation techniques. However, CID of phosphopeptides in ion trap mass spectrometers tends to produce MS/MS spectra dominated by intense neutral loss peaks of 98, 49 or 32.7 (1+, 2+ and 3+ respectively) and fairly weak b and y sequence ions. Even though CID MS² data can be improved upon with a subsequent MS³ scan of these intense neutral loss ions, further improvements in our ability to identify phosphopeptides and characterize sites of phosphorylation become increasingly necessary due to the inherent challenges in the analysis of protein phosphorylation, such as their typically low natural abundance. Here we seek to better understand the utility of multi-stage activation (MSA)¹, a rather clever application of CID to produce individual mass spectra that contain both MS² product ions and MS³ product ions from the MS² neutral loss ion and ETD² applied to the analysis of sites of phosphorylation. Illustrations of the MSA and ETD processes are shown below in Figure 1.

FIGURE 1A. Multi-stage activation scan

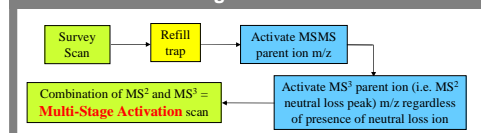
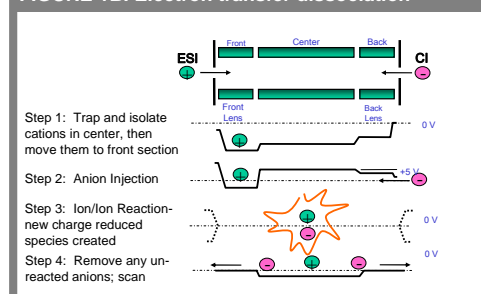


FIGURE 1B. Electron transfer dissociation



Certain observations as to the effect of charge and mass/charge on the quality of CID and ETD spectra have led to the development of an intelligent new data dependent acquisition mode implemented in the LTQ Orbitrap XL ETD termed the Data Dependent Decision Tree (DDDT). Typically, the higher the charge state the better is the fragmentation observed by ETD whereas the opposite is true for CID. Also as mass/charge (*m/z*) increases so ETD efficiency decreases. Thus, certain basic guidelines for acquiring MS/MS data from precursors of a given charge or mass/charge can be arrived at. Example data for ETD at different charge states for the same precursor ion are shown in Figure 2 and a description of the DDDT is given in Figure 3, including the conditions used here.

Methods

Human kinases were reduced, alkylated and enzymatically digested to produce peptides. Samples were analyzed by LC/MS/MS. The digested kinases were either analyzed on an LTQ XL™ instrument enabled with ETD or an LTQ Orbitrap XL ETD instrument. MS/MS data was either acquired as a 'toggle' experiment where every precursor selected by data dependent criteria is subjected to both MSA and ETD or using the DDDT (only on the LTQ Orbitrap XL ETD instrument) such that only one MS/MS spectrum was acquired per precursor based on previously determined charge and mass/charge criteria. A Thermo Scientific Surveyor™ HPLC system equipped with a Micro AS autosampler was interfaced with the nanospray source of the mass spectrometers for online peptide separations using a C₁₈ column. Data analysis was performed using Proteome Discoverer 1.0 software enabled with SEQUEST® and Mascot™ search algorithms.

Results

As can be observed in Figure 2, the data quality improves in ETD for the same peptide as charge increases or *m/z* decreases. The opposite was true for CID. The best sequence coverage (data not shown) and Mowse score was obtained for the 4+ ETD data. In addition, the difference in Mowse score between the best and second best identifications was greatest for ETD overall for the three charge states observed. All 3 ETD spectra produced highly confident identifications whereas this was only true for the 2+ CID data with an intermediate quality identification obtained for the 3+ CID data and none at all for the 4+ CID data.

FIGURE 2. Effect of charge state on ETD and CID data

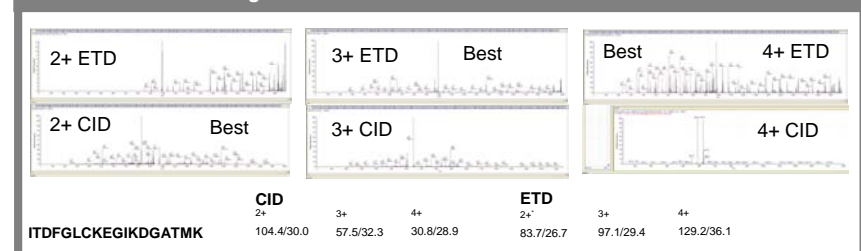


FIGURE 3. (A) Example data dependent decision tree cartoon. (B) Actual DDDT conditions used in these experiments.

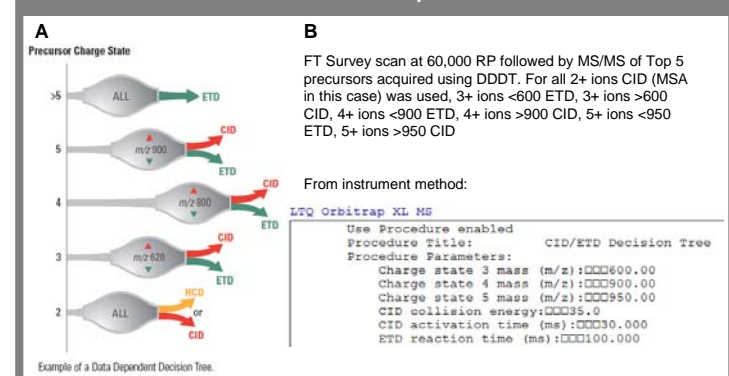


TABLE 1. CID and ETD give similar sequence coverage and quality of identification by database searching with Mascot (Mowse score)

Kinase	Dissociation Technique			
	MSA		ETD	
	Sequence Coverage (%)	Av Mowse Score	Sequence Coverage (%)	Av Mowse Score
MAPKAP-K2	98	58.1	93	57.9
PKB	86	63.6	93	70.8
MST2	77	52.9	89	58.7
PLK1	81	57.9	89	53.7

Table 1 shows that MS/MS data from both CID (in this case MSA) and ETD is approximately equivalent in quality in terms of sequence coverage obtained and Mowse score following database searching with Mascot. Thus, ETD and CID are equivalent dissociation techniques for peptide identification.

Table 2 shows that ETD produces more discrimination of potential sites of phosphorylation than MSA. In the experiment below and on other human kinases (data not shown) acquired with

the toggle approach ETD consistently gives a higher difference in Mowse score and XCorr (data not shown) for the highest and second highest ranking identifications. In addition, more sites of phosphorylation are observed using ETD compared with MSA. ETD seems the most likely to identify sites of phosphorylation.

TABLE 2. Phosphopeptides Identified by ETD and MSA for MST2.

CID and ETD IDs are MOWSE scores of best and second best peptide identifications. The Difference between 1st and 2nd Scores and the total number of phosphopeptides identified is used as an indication of the ability to identify sites of phosphorylation.

Peptide	CID (MSA) ID		ETD ID	
	1st	2nd	1st	2nd
IAYSKDFETLKVDFLSKLPMLK+p	56	33	93	41
DFETLKVDFLSKLPMLK+p	10	9 (3 rd ID correct)*	65	20
LKLSLSESLTKQPEEVFDVLEK+p	57	55	26	24
LKLSLSESLTKQPEEVFDVLEK+2p	53	51	55	47
KLSESLTKQPEEVFDVLEK+p	78	45	71	69
KLSESLTKQPEEVFDVLEK+2p	nd	nd	24	24
LSESLTKQPEEVFDVLEK+p	69	65	65	49
LRNKTLEIEIATILK+p	nd	nd	55	14
NKTLEIEIATILK+p	74	17	48	11
LADFGVAGQLTDTMAK+p	80	62	111	80
LADFGVAGQLTDTMAKR+p	100	78	66	47
ATATQLLQHPFIK+p	62	59	56	46
AKRHEEQRELEEEENSDEDELDSHTMVK+2p	nd	nd	43	24
RHEEQRELEEEENSDEDELDSHTMVKTSVESVGTMR+2p	nd	nd	42	25
RHEEQRELEEEENSDEDELDSHTMVK+p	nd	nd	119	51
RHEEQRELEEEENSDEDELDSHTMVK+2p	nd	nd	111	87
HEEQRELEEEENSDEDELDSHTMVK+p	nd	nd	73	42
HEEQRELEEEENSDEDELDSHTMVK+2p	nd	nd	57	41
NKSHENCNQNMHEPFPMSK+p	53	13	63	10
VPQDGFDFLKNLSLEELQMR+p	104	29	86	21
NLSLEELQMR+p	30	18	51	13
ALDPMMERIEELRQRYTAK+p	17	15 (6 th ID correct)*	45	43
QRYTAKRQPILDAMDAK+p	23	19 (2 nd ID correct)*	70	57
YTAKRQPILDAMDAK+p	nd	nd	63	61

SUMMARY: 1st-2nd/1st and 1st-2nd CID 0.27 and 19.4 (n=15) ETD 0.37 and 25 (n=24)

Figure 4 shows an example of a DDDT data set. A survey scan was followed by MS/MS of the 5 most intense ions. Choice of dissociation is based upon predefined criteria (Fig. 3). In the example below 3 CID scans have taken place because 3 of the top 5 precursors were doubly charged ions. The other two precursors were a 3+ precursor with a *m/z* of <600 and a 4+ precursor with an *m/z* < 900 and thus ETD was performed as the dissociation technique most likely to result in good data.

FIGURE 4. Example data dependent decision tree MS/MS data

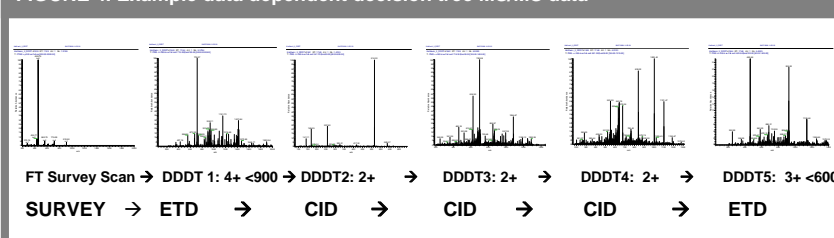


FIGURE 5. Comparison of 3+ ETD data from RSK1 kinase. (A) Sequence, (B) ETD measured in linear ion trap and (C) ETD measured in LTQ Orbitrap



The LTQ Orbitrap XL ETD has the ability to detect ions in either the LTQ or in the Orbitrap. Characteristics of detection in the LTQ are speed and sensitivity whereas detection in the Orbitrap results in higher resolution and mass accuracy. Figure 5 shows ETD data measured in either (B) the LTQ or (C) the orbitrap. For example, in Figure 5C the c¹₁₀ ion measured in the Orbitrap with external calibration gives a measured mass of 1077.4363 whereas measured in the LTQ (fig. 5B) the mass is recorded at 1077.2888. The theoretical mass of the c¹₁₀ ion is 1077.4361. Other ions measured in the Orbitrap achieve a similar mass accuracy. However, scan speed is higher in the LTQ and for these samples better sequence coverage was obtained when MS/MS spectra were measured in the LTQ (data not shown).

Conclusions

- ETD data is better able on average to locate sites of phosphorylation than MSA
- ETD and CID are equivalent in identifying peptides by database searching
- DDDT is a more efficient mode of data acquisition than a 'normal' toggle experiment since each precursor is selected for only one MS/MS event
- The most efficient mode of data acquisition is to measure survey scans in the LTQ Orbitrap while measuring MS/MS scans in parallel in the LTQ XL

References

Schroeder et al., Anal. Chem. (2004) 76, 3590-3598. Syka et al., PNAS USA (2004) 101, 9528-9533

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