Electron Transfer Dissociation and Multi-Stage Activation Analysis of Human Kinase Sites of Phosphorylation

Martin Hornshaw[1] and Nick Morrice[2]

[1]Thermo Fisher Scientific, Hemel Hempstead, UK; [2]MRC Protein Phosphorylation Unit, University of Dundee, Dundee, UK

MRC Medical Research Council
Protein Phosphorylation Unit

Abstract

Purpose: Assess the utility of different dissociation techniques for phosphorylation site analysis of human kinases.

Methods: Comparison of neutral loss based technique and multi-stage activation to electron transfer dissociation (ETD) during nano-LC-MS/MS.

Results: The different dissociation techniques were found to be entirely complementary and, rather surprisingly, a number of novel phosphorylation sites were identified on such well known kinases as PKB. ETD data on average identified more sites of phosphorylation and with greater discrimination relative to CID based approaches.

Introduction

The analysis of phosphopeptides on a linear ion trap mass spectrometer has typically been performed by Data Dependent™ MS/MS followed by MS^3 of the putative neutral loss peak if observed in the MS/MS spectrum (neutral loss MS^3). The strength of this approach is the sensitivity in full scan MS^n of linear ion trap technology and the characteristically strong neutral loss peak serving to flag the MS/MS spectrum as potentially belonging to a phosphopeptide. However, two novel techniques have recently been developed that can be applied to the analysis of protein phosphorylation. Here we seek to better understand the utility of multi-stage activation (MSA)[1] and electron transfer dissociation (ETD)[2] applied to the analysis of sites of phosphorylation. Illustrations of the MSA and ETD processes are shown in figure 1.

Materials & Methods

Recombinant human kinases were studied. The proteins were reduced, alkylated and digested. The samples were analyzed by LC-MS^n with nanoflow-reversed phase LC. The multi-stage activation and Data Dependent neutral loss MS^3 experiments were performed on both the Thermo Scientific LTQ Orbitrap™ and LTQ XL™ instruments. ETD experiments were performed exclusively on an LTQ XL. Only data from the LTQ XL is further discussed in this work. The typical workflow in the LTQ XL was to perform alternating CID (MSA) and ETD for the same precursor where precursors were selected in a Data Dependent manner following an enhanced resolution scan to enable charge state determination. In addition, 2+ precursor ions, when ETD was performed, were further activated by supplementary activation, that is, electron transfer dissociation was performed but any unfragmented precursor was then further activated by CID. Database searching was performed with SEQUEST® residing within BioWorks™ 3.3.1 software and with Mascot 2.1 and 2.2 (Matrix Science) against all human entries in Swiss-Prot or against a proprietary phosphoprotein database (MRC).

Results

An example of ETD LC-MS/MS data of a putative phosphopeptide is shown in figure 2. This piece of data is quite illustrative of the efficacy of ETD in the analysis of phosphopeptides. A doubly phosphorylated peptide from SAPK2a searched against the Swiss-Prot database produces a highly confident identification with both Mascot and SEQUEST. Note, no neutral loss was observed, which is typical for ETD MS/MS of phosphopeptides. The difference in Mowse score between the best scoring ID and the second best using Mascot is very significant at 33.4. There are 4 potential sites of phosphorylation on the peptide HTDDEMT*GY*VATR, coloured in blue. The most probable sites of phosphorylation, as determined by ETD, are asterisked. In addition, manual examination of the ETD MS/MS spectrum shows absence of evidence for phosphorylation at 2 potential sites which would be indicated by the presence of ions at *m/z* 336, 340, 1578 and 1582 which are in fact not present in the ETD MS/MS spectrum. Mass differences between c and z ions of 243 and 181 clearly indicate the presence of phosphothreonine and phosphotyrosine at residues 7 and 9, respectively.

For PKB, the ETD data was thoroughly analyzed to see which phosphopeptides were present and what the quality of identification was. A number of novel phosphopeptides were observed with confident identifications (figure 3 and table 1). A very simple attempt was made to quantify the difference between MSA and ETD with respect to characterizing sites of phosphorylation. The best and second best Mowse scores after database searching with Mascot were used to indicate the quality of the identification of the phosphopeptide and the confidence of correct location of the site of phosphorylation.

FIGURE 1a. Illustration of Multi-Stage Activation Scan

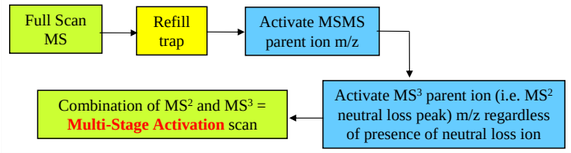

FIGURE 1b. Illustration of Electron Transfer Dissociation Process

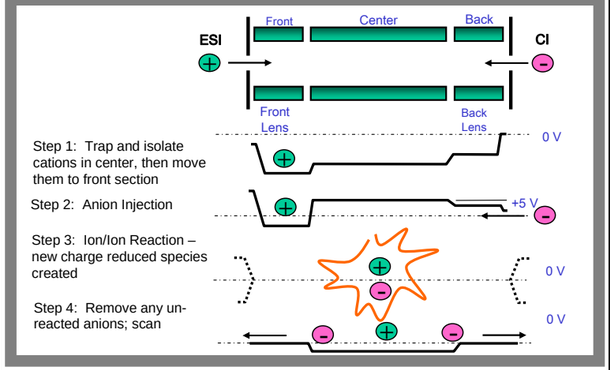

FIGURE 2. Example of Identification of Sites of Phosphorylation using ETD. Database searching was performed with Mascot and SEQUEST in BioWorks. Difference in MOWSE score for best and second best ID is significant. In addition, manual examination of the data revealed absence of evidence for phosphorylation at other potential sites.

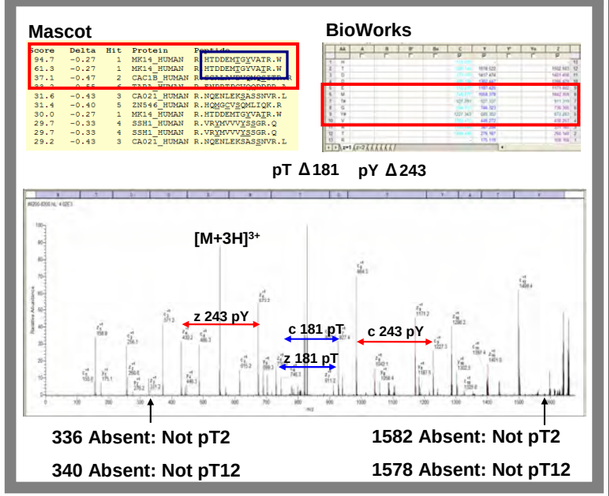

FIGURE 3. Sequence coverage of PKB analyzed by ETD. A number of novel phosphopeptides were observed (in blue).

1 MSYYHHHHHH DYDIPTTENL YFQGAMGSMD FRSGSPSDNS GAEEMEVSLA
51 KPKHRVTMNE FEYLKLLGKG TFGKVILVKE KATGRYYAMK ILKKEVIVAK
101 DEVAHTLTEN RVLQNSRHPF LTALKYSFQT HDRLCFVMEY ANGGELFFHL
151 SRERVFSEDR ARFYGAEIVS ALDYLHSEKN VVYRDLKLEN LMLDKDGHIK
201 ITDFGLCKEG IKDGATMKTF CGTPEYLAPE VLEDNDYGRA VDWWGLGVVM
251 YEMMCGRLPF YNQDHEKLFE LILMEEIRFP RTLGPEAKSL LSGLLKKDPK
301 QRLGGGSEDA KEIMQHRFFA GIVWQHVYEK KLSPPFKPQV TSETDTRYFD
351 EEFTAQMITI TPPDQDDSME CVDSERRPHF PQFDYSASGT A

ETD: 93% sequence coverage (shown in red)

Black indicates that the peptide has been previously observed while blue indicates that this has not been previously observed. Red letters indicate putative sites of phosphorylation

SGSPSDNSGAEEMEVSLAKPK Observed as un-, mono-, di- and tri-phosporylated

HRVTMNEFEYLKLLGK HRVTMNEFEYLK HRVTMNEFEYLKLLGKGTFGK

ILKKEVIVAKDEVAHTLTENR KEVIVAKDEVAHTLTENR EVIVAKDEVAHTLTENR

ITDFGLCKEGIKDGATMKTFCGTPEYLAPEVLEDNDYGR
EGIKDGATMKTFCGTPEYLAPEVLEDNDYGR Observed as mono- and diphosphorylated

TLGPEAKSLLSGLLK TLGPEAKSLLSGLLKKDPK

KLSPPFKPQVTSETDTR

TABLE 1. Phosphopeptides Identified by ETD and MSA for PKB.

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

Peptide Sequence	CID (MSA) 1st and 2nd IDs		ETD 1st and 2nd IDs	
	1st	2nd	1st	2nd
SGSPSDNSGAEEMEVSLAKPK	107	13	122	11
SGSPSDNSGAEEMEVSLAKPK+p	126	120	107	107
SGSPSDNSGAEEMEVSLAKPK+2p	101	94	108	108
SGSPSDNSGAEEMEVSLAKPK+3p	74	63	45	45
HRVTMNEFEYLK	65	22	85	13
HRVTMNEFEYLK+p	68	31	91	6
HRVTMNEFEYLKLLGK	74	25	119	25
HRVTMNEFEYLKLLGK+p	66	32	101	28
HRVTMNEFEYLKLLGKGTFGK	76	15	98	12
HRVTMNEFEYLKLLGKGTFGK+p	ND	ND	70	23
EVIVAKDEVAHTLTENR	121	13	98	22
EVIVAKDEVAHTLTENR+p	94	90	79	67
KEVIVAKDEVAHTLTENR	142	19	99	28
KEVIVAKDEVAHTLTENR+p	54	47	81	57
ILKKEVIVAKDEVAHTLTENR+p	70	67	81	62
EGIKDGATMKTFCGTPEYLAPEVLEDNDYGR+p	82	82	102	76
EGIKDGATMKTFCGTPEYLAPEVLEDNDYGR+2p	71	55	82	56
ITDFGLCKEGIKDGATMKTFCGTPEYLAPEVLEDNDYGR+p	76	75	67	41
ITDFGLCKEGIKDGATMKTFCGTPEYLAPEVLEDNDYGR+2p	67	65	56	49
TFCGTPEYLAPEVLEDNDYGR+p	87	85	27	27
TLGPEAKSLLSGLLK+p	76	50	93	49
TLGPEAKSLLSGLLKKDPK+p	39	34	87	58
KLSPPFKPQVTSETDTR+p	24	17	54	28
RPHFPQFDYSASGTA+p	68	63	27	24

Summary: 1st-2nd/1st and 1st-2nd CID 0.16 and 10.2 (n=17) ETD 0.305 and 24.8 (n=18)

Typically, if more than one potential site of phosphorylation exists, then the best and second best scores after database searching will be for the same peptide sequence but with different potential sites of phosphorylation. ETD data, on average, was better able to discriminate between potential sites of phosphorylation than was MSA data (table 1 for PKB and table 2 for a second example). In addition, more phosphopeptides were detected with ETD than with MSA. Lastly, a number of novel phosphorylation sites were observed (see figure 3 for the PKB example).

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) 1st and 2nd ID		ETD 1st and 2nd ID	
	1st	2nd	1st	2nd
IAYSKDFETLKVDFLSKLPEMLK+p	56	33	93	41
DFETLKVDFLSKLPEMLK+p	10	9 (3rd ID correct)*	65	20
LKKLSEDSLTKQPEEVFDVLEK+p	57	55	26	24
LKKLSEDSLTKQPEEVFDVLEK+2p	53	51	55	47
KLSEDSLTKQPEEVFDVLEK+p	78	45	71	69
KLSEDSLTKQPEEVFDVLEK+2p	nd	nd	24	24
LSEDSLTKQPEEVFDVLEK+p	69	65	65	49
LRNKTLIEDEIATILK+p	nd	nd	55	14
NKTLIEDEIATILK+p	74	17	48	11
LADFGVAGQLTDTMAK+p	80	62	111	80
LADFGVAGQLTDTMAKR+p	100	78	66	47
ATATQLLQHPFIK+p	62	59	56	46
AKRHEEQQRELEEEEENSDEDELDSHTMVK+2p	nd	nd	43	24
RHEEQQRELEEEEENSDEDELDSHTMVKTSVESVGTMR+2p	nd	nd	42	25
RHEEQQRELEEEEENSDEDELDSHTMVK+p	nd	nd	119	51
RHEEQQRELEEEEENSDEDELDSHTMVK+2p	nd	nd	111	87
HEEQQRELEEEEENSDEDELDSHTMVK+p	nd	nd	73	42
HEEQQRELEEEEENSDEDELDSHTMVK+2p	nd	nd	57	41
NKSHENCNQNMHEPFPMSK+p	53	13	63	10
VPQDGDFDFLKNLSLEELQMR+p	104	29	86	21
NLSLEELQMR+p	30	18	51	13
ALDPMMEREIEELRQRYTAK+p	17	15 (6th ID correct)*	45	43
QRYTAKRQPILDAMDAK+p	23	19 (2nd 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)

Conclusions

Of importance to the study of protein phosphorylation is the nature of the peptide fragmentation induced by electron transfer dissociation. Primarily c and z type product ions are generated *without neutral loss*. Thus, for example, phosphothreonine can be detected in an ETD MS/MS spectrum by the observation of pairs of peaks differing in mass by 181. This leads to enhanced interpretability of ETD MS/MS spectra relative to CID. *Work here demonstrates that ETD is better able to discern the location of sites of phosphorylation than is CID (MSA). Additionally, more phosphopeptides were confidently identified with ETD than with CID (MSA)*.

Future work will involve investigating the effect of high mass accuracy and resolution measurements of ETD MS/MS spectra in an orbital trap on confidence in assignment of sites of phosphorylation.

References

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

Part of Thermo Fisher Scientific

Thermo SCIENTIFIC