Comparison of CID Versus ETD Based MS/MS Fragmentation for the Analysis of Protein Ubiquitination

Frank Sobott,^a Stephen J. Watt,^b Julia Smith,^c Mariola J. Edelmann,^d Holger B. Kramer,^d and Benedikt M. Kessler^d

^a Oxford Centre for Gene Function/OXION, Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, United Kingdom

^b Structural Genomics Consortium, University of Oxford, Oxford, United Kingdom

^c Bruker Daltonics, Coventry, United Kingdom

^d Henry Wellcome Building for Molecular Physiology, Department of Clinical Medicine, University of Oxford, Oxford, United Kingdom

Ubiquitination has emerged as one of the major post-translational modifications that decide on protein fate, targeting, and regulation of protein function. Whereas the ubiquitination of proteins can be monitored with classic biochemical methods, the mapping of modified side chains proves to be challenging. More recently, mass spectrometry has been applied to identify ubiquitinated proteins and also their sites of modification. Typically, liquid chromatography tandem mass spectrometry (LC-MS/MS) based approaches, including collision-induced fragmentation (CID), have been successfully used in the past. However, a potential difficulty arises from the unstable nature of this modification, and also that the isopeptide bond linkage between C-terminal glycine and the N(ϵ) lysyl side chain is susceptible to fragmentation under these conditions. Here we investigate the utility of electron-transfer dissociation (ETD)-based fragmentation to detect ubiquitination sites in proteins. Our results indicate that ETD can provide alternative fragmentation patterns that allow detection of gly-gly-modified lysyl side chains, in particular $z+1$ fragment ions derived from triply charged precursor ions. We subsequently applied ETD fragmentation-based analysis and detected novel ubiquitination sites on DNA polymerase B1 that were not easily observed using CID. We conclude that ETD can provide significant alternative fragmentation information that complements CID-derived data to improve the coverage when mapping ubiquitination sites in proteins. (J Am Soc Mass Spectrom 2009, 20, 1652–1659) © 2009 Published by Elsevier Inc. on behalf of American Society for Mass Spectrometry

Covalent attachment of ubiquitin, a small 76 amino acid protein, has been recognized to be critical for protein turnover, targeting, and regulating function [1–3]. Many approaches have been developed to study this post-translational modification on proteins using classic biochemical methods, including the more recently introduced poly-ubiquitin linkage-specific antibodies [4]. Whereas the dynamics of protein ubiquitination can be readily monitored in this way, still little information is available on the topology of ubiquitination, since it remains a challenge to characterize heterogeneous ubiquitin chains and to map the modified sites on protein substrates. A major step towards this was achieved by applying mass spectrometry methods to measure protein ubiquitination (reviewed in [5, 6]). One approach is based on the principle that protein fragments derived from ubiquitinated proteins,

which have been digested with trypsin, contain a glycine-glycine moiety covalently attached to N(ϵ)-lysyl side chains via an isopeptide bond [7]. Analysis by tandem mass spectrometry (LC-MS/MS) using collision-induced dissociation (CID) can often detect this modification and hence be used to map ubiquitination sites that are identified by an additional mass tag of 114.1 Da on lysine residues [7, 8]. In addition, stable isotope derivatives of such orthogonal peptides have been used for the quantitation of protein ubiquitination on specific sites [9, 10].

However, since isopeptide bonds are also sensitive to fragmentation, the detection of this modification using CID may be incomplete and hence more gentle fragmentation methods may provide better coverage and sensitivity of detection. Electron-transfer dissociation (ETD) was recently developed as an alternative fragmentation method to preserve more labile modifications, such as phosphorylation, methylation, acetylation, glycosylation, nitrosylation, and sulfation [11–15]. Whereas CID-based sequencing produces spectra with limited peptide backbone fragmentation, ETD has

Address reprint requests to Dr. F. Sobott and Dr. B. M. Kessler, Department of Clinical Medicine, Henry Wellcome Building for Molecular Physiology, University of Oxford, Roosevelt Drive, Oxford, Oxon OX3 7BN, UK. E-mail: frank.sobott@dpag.ox.ac.uk and E-mail: bmk@ccmp.ox.ac.uk

shown to generate more complete series of ions and thus more extensive sequence information, with the additional advantage of leaving labile post-translational modifications (PTMs) intact [16]. In the following study, we briefly discuss the advantages and disadvantages of the two complementary fragmentation methods, and describe the concept of CID alternating with ETD on a 3D radiofrequency (rf) ion trap.

Conventional MS/MS methods, which use vibrational activation for peptide fragmentation, such as CID, suffer from competing dissociation pathways that can lead to product ion spectra that are complex and sometimes difficult to interpret. In CID, activation of the peptide ions proceeds via multiple collisions with the inert background gas, such as helium. Due to a rapid vibrational redistribution (equilibration) of the internal energy, this process becomes equivalent to an internal “heating” of the precursor ions. When the internal energy reaches the fragmentation threshold, the weakest bonds are cleaved preferentially, no matter if they are present in the peptide backbone or in the side chains, such as PTMs. CID typically generates y - and b -type fragment ions [17], but not equally along the backbone. For instance, cleavage near the amino acids E, D, and P is preferred, and abundant internal fragmentation and loss of PTMs occur frequently.

In contrast, methods that take advantage of the energy released in the exothermic capture (ca. 6 eV) of a thermal electron, such as electron capture dissociation (ECD) first developed by McLafferty et al. on Fourier transform-ion cyclotron resonance (FT-ICR) instrumentation [18], achieve nonergodic fragmentation without prior internal redistribution of energy. This gas-phase radical process leads to a rapid bond cleavage mainly at the sites where the originally formed radical was trapped [19], thereby promoting random N–C α bond breaks that yield c - and z -type ion fragments and leave side-chains intact.

Since electrons cannot easily be trapped alongside peptide ions in rf traps, an analogous method called ETD, which uses a radical anion as the electron-transfer species, has been developed by Hunt et al. on linear ion traps [12]. Recently, this approach has been implemented on commercial instrumentation for the first time, namely the widely used 3D (nonlinear) quadrupole ion trap [20]. In this setup, reagent anions are formed from a neutral compound (fluoranthene) in a separate, gated negative chemical ionization (nCI) source and transferred into the 3D trap, where they are trapped alongside peptide cations for a defined amount of time (Figure 1). When the electron-transfer reaction is complete, product ions are swept out of the cell and detected in a conventional way. This enables rapid cycling between alternate CID and ETD fragmentation on the same set of precursor ions in a data-dependent manner while peptides are eluting from an LC column.

In this study, we applied CID- and ETD-based fragmentation using LC-MS/MS to map ubiquitination sites in proteins, and compared the two fragmentation meth-

Figure 1. Scheme of the CID-ETD ion trap configuration. The design of the high capacity ion trap tandem mass spectrometer accommodates a gated negative chemical ionization (nCI) source that allows conventional collision induced dissociation (CID) and electron-transfer dissociation (ETD) in the same 3D ion trap setup. ETD reagent anions are formed from fluoranthene vapor via collisions with thermal electrons in a methane atmosphere and transferred into the trap, where they are trapped alongside peptide cations for a defined amount of time.

ods for their ability to generate product ions allowing increased confident assignment of this relevant PTM in biological samples.

Experimental

Reagents and Sample Preparation

Trypsin based proteolysis of 100 μ g di-ubiquitin (Biomol, Plymouth Meeting, PA, USA) was carried out essentially as described [21]. In brief, proteins were denatured in 100 mM ammonium bicarbonate solution pH 7.8 (Sigma, St. Louis, MO, USA) for 40 min at 90 °C, followed by reduction with DTT (Sigma) and alkylation with iodoacetamide (Sigma). The solution was diluted 5-fold with 100 mM ammonium bicarbonate pH 7.8 buffer. Trypsin (Promega, Southampton, Hampshire, UK) at a final concentration of 20 ng/ μ L was added, followed by incubation for 18 h at 37 °C. Formic acid was used to acidify the solution and the samples were kept at –20 °C until analysis. Preparation of DNA polymerase β -1 was performed as described [22], and gel bands corresponding to ubiquitinated material were in-gel digested with trypsin as reported previously [23], with the exception that chloroacetamide instead of iodoacetamide was used for the alkylation step.

Intact Mass Analysis

The mass of intact, undigested di-ubiquitin was confirmed by LC-MS, using an Agilent LC/MSD TOF system with reversed-phase HPLC coupled to electrospray ionization and an orthogonal time-of-flight mass analyzer (Agilent, Santa Clara, CA, USA). Proteins were desalted before mass spectrometry by rapid elution off a C3 column with a 6 min gradient of 5%–95% acetonitrile in water with 0.1% formic acid.

Ubiquitination Analysis by LC and Tandem MS

For the analysis of digested protein material, liquid chromatography was performed using an Ultimate 3000 nano-HPLC system (Dionex, Sunnyvale, CA, USA) comprising a WPS-3000 well-plate micro auto sampler, a FLM-3000 flow manager and column compartment, a UVD-3000 UV detector, an LPG-3600 dual-gradient micro-pump, and an SRD-3600 solvent rack controlled by Hystar (Bruker Daltonics, Billerica, MA, USA) and DCMS link 2.0 software. Samples were concentrated on a trapping column Dionex (Sunnyvale, CA, USA), 300 μm i.d., 0.1 cm) at a flow rate of 30 $\mu\text{L}/\text{min}$. For the separation with a C18 Pepmap column (75 μm i.d., 15 cm, Dionex), a flow rate of 300 nL/min was used as generated by a cap-flow splitter cartridge (1/1000). Peptides were eluted by the application of a 30 min linear gradient: solvent A (95% H_2O , 5% acetonitrile, 0.1% formic acid), 0%–45% Solvent B (95% acetonitrile, 5% water, 0.1% formic acid). LC was interfaced directly with a 3D high capacity ion trap mass spectrometer (HCTultra; Bruker Daltonics) utilizing 10 μm i.d. distal coated SilicaTips (New Objective, Woburn, MA, USA) and nano-ESI mode. SPS parameter settings on the ion trap were tuned for a target mass of 850 m/z , compound stability 100% and a smart ICC target of 200,000.

MS/MS analysis was initiated on a contact closure signal triggered by HyStar software. Up to four precursor ions were selected per cycle with active exclusion (0.5 min) for both CID and ETD, excluding singly charged ions. CID fragmentation was achieved using helium gas and a 30%–200% collision energy sweep with amplitude 1.0 (ions are ejected from the trap as soon as they fragment). For electron-transfer dissociation experiments we used a prototype nCI source as described elsewhere [20], which was tuned to maximize output of ETD reagent ion (202 m/z), with the following parameters: reagent ion ICC 600,000, ionization energy 70 eV, emission current 2.6 μA , reactant remove cut-off 210 m/z , without supplemental activation. A typical MS/MS cycle consists of (1) full-range MS spectrum and precursor ion selection, (2) precursor ion accumulation (ca. 10 ms) and CID MS/MS spectrum, (3) precursor ion accumulation followed by ETD ion accumulation (ca. 5 ms) followed by ETD reaction time (50–100 ms; variable) and recording of ETD MS/MS spectrum. Steps (2) and (3) are repeated automatically for each precursor in the cycle.

Data Processing and Analysis

Raw LC-MS/MS data were processed and Mascot compatible files created using DataAnalysis 3.4 software (Bruker Daltonics) with the following parameters: compounds (autoMS) threshold 10,000, number of compounds 500, retention time windows 0.5 min for C18 (30 min gradient).

Two searches were performed in parallel in each case for CID and ETD, using Mascot software (version 2.2)

[24] and the SwissProt database (release 54.0, 07/2007, number of entries 276,256) with the following parameters: 2+ and 3+ ions, peptide tolerance 1.2 Da, $^{13}\text{C} = 1$, fragment tolerance 0.6 Da, missed cleavages: 2, instrument type: ESI-TRAP (for CID) and ETD-TRAP, respectively, for the two separate datasets. In cases where ubiquitination was examined, the gly-gly tag on lysine residues (+114.1 Da) was included as a variable modification as described [10]. The interpretation and presentation of MS/MS data were performed according to published guidelines [25]. Individual MS/MS spectra for peptides with a Mascot Mowse score lower than 40 were inspected manually and only included if a series of at least four continuous fragment ions were observed.

Results and Discussion

The aim of the present study was to compare CID and ETD based MS/MS fragmentation for mapping ubiquitinated lysine side chains in substrate proteins. In the first experiment, we used a lys 48 -linked di-ubiquitin preparation as a model substrate [26]. LC-TOF analysis of the intact, undigested protein material confirmed the sequence mass ($M_{r,\text{calc.}} = 17,201.7$ Da, observed $M_r = 17,201.5$ Da) and revealed >98% purity of the synthetic dimer (Figure 2).

To map the modified site, lys 48 -linked di-ubiquitin was digested with trypsin and subsequently subjected to LC-MS/MS analysis using an rf ion trap mass spectrometer with CID and ETD capabilities (see the Experimental section). The tryptic peptide LIFAGKQLEDGR ($M_{r,\text{calc.}} =$

Figure 2. LC-ESI-TOF MS of the intact di-ubiquitin conjugate protein. Di-ubiquitin containing lys 48 -linked isopeptide bonds generated by enzymatic conjugation of a lys 48 cys and a Ub-asp 77 variant was analyzed by LC-ESI-TOF MS. Mass reconstruction of the $[M + H]^+$ mass was performed using Mass Hunter software (Agilent); observed mass: 17,201.5 Da, theoretical mass: 17,201.7 Da.

1346.5 Da) containing a glycine-glycine tag on lysine 48 (+114.1 Da) was detected after 15.4–15.6 min as $[M + 2H]^{2+}$ and $[M + 3H]^{3+}$ precursor ions when analyzed by LC-MS (Figure 3a and b). Both were in turn selected as precursor ions and subjected to CID and ETD based MS/MS (Figure 4).

Using CID, an almost complete y-ion series was observed for the 2+ precursor ion covering the gly-gly modified lys⁴⁸ side-chain (11 fragment ions total, Figure 4a top panel). However, CID-based fragmentation of the 3+ peptide ion did not reveal any complete series of critical fragment ions that would allow the mapping of the modified side chain (11 fragment ions total). The product ion spectrum was dominated by the doubly charged y₁₀²⁺ fragment ion, and all larger y-ions were doubly charged thus making the MS/MS spectrum appear complex below m/z 800, thereby complicating the peak assignment. No significant amount of dissociation of the diglycyl side chain seems to occur for the y₇-y₁₁ fragments, which would lead to species such as y₇-K(G), m/z 902.4, or y₇-K, m/z 845.3, for either 2+ or 3+ precursor ions.

In contrast, LC-MS/MS runs using ETD of the doubly charged precursor resulted in very incomplete fragmentation, predominantly leading to the charge-reduced 1+ molecular peptide ion $[M + H]^+$ at m/z 1460.6, which is formally a product of hydrogen abstraction from a $[M + 2H + e]^{•+}$ radical intermediate (or a direct proton transfer from the $[M + 2H]^{2+}$ cation onto the reagent anion). We found that the energy released by electron-transfer to a doubly charged pep-

tide is not sufficient to cause efficient fragmentation of the peptide backbone, producing only a few large C-terminal fragments. It should be noted here that we use the Biemann nomenclature [27], where the C-terminal free radical ion, which differs from a y-ion by 16 Da, is defined as $z + 1$ (also called z^*), while the 1 Da heavier species formed by addition of a H[•] radical as a byproduct of the ETD reaction is referred to as $z + 2$ (sometimes confusingly called $z + 1$). ETD of 2+ precursors also leaves only one charge between two corresponding fragments, with half the fragments being neutral and therefore invisible in the mass spectra. ETD of the triply charged precursor, on the other hand, yields many more fragments of the modified LIFAGKQLEDGR peptide (15 fragment ions, Figure 4b bottom panel), presumably due to the higher amount of Coulombic energy released upon electron-transfer. In this case, we observed a complete $z + 1$ ion series (apart from the $(z + 1)_1$ ion, which is outside the m/z range, below m/z 200), as well as $z + 2$ and c-ions. Only singly charged peptide fragments are observed, as expected from the ETD mechanism where one charge is lost due to electron-transfer to the 3+ peptide ion, leaving two charges for each fragment pair [12].

It is a common feature in such ETD spectra that the general efficiency of fragmentation is reduced compared with CID, with abundant precursor $[M + 3H]^{3+}$ as well as charge-reduced, unfragmented $[M + 2H]^{2+}$ and $[M + H]^+$ peptide ions surviving in the MS/MS spectra. We observe this irrespective of a systematic variation of ETD parameters, such as the reagent to

Figure 3. LC-MS analysis of tryptic peptides derived from di-ubiquitin. Lys⁴⁸-linked di-ubiquitin was digested in solution with trypsin and subjected to LC-MS/MS analysis using a CID-ETD 3D high capacity ion trap tandem mass spectrometer. (a) Total ion chromatogram (top trace) and total MS(*n*) ion intensity (bottom trace). Arrows indicate retention time of the precursor ion of the tryptic LIFAGKQLEDGR peptide containing the gly-gly modified lys residue. (b) MS spectrum at 15.4–15.5 min showing the gly-gly modified peptide LIFAGKQLEDGR precursor ions $[M + 2H]^{2+}$, 730.8 Da and $[M + 3H]^{3+}$, 487.5 Da.

Figure 4. CID versus ETD MS/MS spectra of the lys⁴⁸(gly-gly) containing peptide LIFAGKQLEDGR. (a) Collision-induced dissociation (CID) based MS/MS spectra of the $[M+2H]^{2+}$ (top panel) and $[M+3H]^{3+}$ (lower panel) precursor ions. Matched b- and y-ions are indicated in the spectra as well as the peptide sequence. The lys (gly-gly) modification (K(GG)) was mapped based on the mass difference between the y6 and y7 ions in the spectrum derived from the doubly charged precursor ion. (b) Electron-transfer dissociation (ETD) based MS/MS spectra of the $[M+2H]^{2+}$ (top panel) and $[M+3H]^{3+}$ (lower panel) precursor ions. Matched z+1, z+2, and c-fragment ions are indicated in the spectra and the peptide sequence. The lys(gly-gly) modification (K(GG)) was mapped based on the mass difference between the (z+1)6 and (z+1)7 ions in the spectrum derived from the triply charged precursor ion.

peptide ion ratio in the trap (as controlled, e.g., by the reagent anion accumulation time) or parameters which govern the ion confinement, such as the shape of the trapping field or the ETD reaction time (not shown here). The appearance of an incomplete reaction is most likely due to the incomplete overlap of the two ion clouds of reagent anions and peptide cations in the 3D trap, which leads to a variable amount of interaction between the two different species and thus also to

heterogeneous reaction products. Nevertheless, the signal-to-noise ratio of the ETD spectra appears much improved over CID, and the number and intensity of unassigned species ("noise peaks", i.e., ions which are not part of the main b/y or c/z fragment ion series) is typically much lower. The presence of unreacted precursor ions in the ETD spectra has the advantage that it confirms precursor ion mass and charge state within the same spectrum.

Taken together, these features of ETD may contribute to an increase in confidence of assignment of fragment ions. Based on our results, we also conclude that CID- and ET-based fragmentation can be complementary in obtaining information about ubiquitinated side chains that are present in protein substrates.

To test this hypothesis, we examined the ubiquitination pattern of DNA polymerase β (DNA Pol β , Figure 5a) using both fragmentation methods in LC-MS/MS. The N-terminal domain (10 kDa) of DNA polymerase β was previously shown to be ubiquitinated by the E3 ubiquitin ligase CHIP in vitro and in living cells [22]. In vitro ubiquitinated recombinant DNA Pol β was separated by SDS-PAGE and the corresponding gel band digested with trypsin. We performed LC-MS/MS of this digest with data-dependent precursor ion selection (see the Experimental section) and alternating cycles of CID and ETD fragmentation of the same precursor. Using Mascot, ubiquitination was detected with both methods in the triply charged m/z 459.6 peptide (73–83 tryptic peptide from DNA Pol β , $M_{r,calc.} = 1376.6$ Da). No corresponding doubly charged peptide was observed in LC-MS.

Based on CID fragmentation, we found peptides covering 30% of the DNA Pol β sequence (highlighted in Figure 5a) and an incomplete MS/MS spectrum for the modified peptide IDEFLATGKLR that contains the

gly-gly modified lys⁸¹ side chain (Figure 5b). From the same LC-MS/MS run we obtained an independent ETD dataset, which gave sequence coverage of 27% (Figure 5a), but, more importantly, yielded a higher number of significant peptide fragments which allow confident assignment of the ubiquitin modification at lys⁸¹.

This side-by-side analysis of alternating CID and ETD fragmentation highlights some common features that we usually observe in ion trap LC-MS/MS. First, the same peptides tend to be identified by both CID and ETD, with the sequence coverage generally slightly lower in ETD data due to the absence of shorter peptides, which form predominantly doubly charged ions in electrospray ionization. We suspect that other tryptic peptides, which are absent in both datasets, are lost before entry into the mass spectrometer, either on surfaces, such as sample vials or capillary walls, or during chromatographic separation or in the electrospray process, presumably because they are either too hydrophobic or too hydrophilic, or too short or too long. Based on this and many similar observations, we believe that the main limiting factor towards increased sequence coverage lies with processes upstream of the mass spectrometric analysis.

On the other hand, the major advantage of ETD is that it often results in better peptide fragmentation data, allowing more confident assignment of sequence tags as

Figure 5. Mapping lys⁸¹-linked ubiquitin in DNA polymerase B using CID and ETD based MS/MS. Recombinant mono-ubiquitinated DNA polymerase B was prepared as described [22], separated by SDS-PAGE, and the corresponding gel band subjected to in-gel trypsin digestion and analysis by LC-MS/MS. (a) Sequence coverage obtained by analysis using CID- (boxed) or ETD- based (underlined) MS/MS fragmentation. The asterisk indicates the ubiquitination site. (b) CID MS/MS spectrum. (c) ETD MS/MS spectrum.

well as mapping of post-translational modifications. Since ETD-based fragmentation also produces a second independent, orthogonal dataset to CID data, the combination of both methods within the timeframe of a single LC run increases the confidence of sequencing and PTM mapping of LC-separated peptides significantly.

Conclusions

ETD is a recently introduced method in tandem mass spectrometry, which was shown to provide more comprehensive coverage of peptide sequences and post-translational modifications. In particular, this approach has the advantage of preserving unstable side-chain modifications during fragmentation [11, 28]. Several different instrumental approaches have been developed to utilize electrons for fragmentation, including ECD in FT-ICR mass spectrometers [29], ECD in radio-frequency linear ion trap mass spectrometers [30], and electron-transfer ion/ion reactions in quadrupole/linear ion trap mass spectrometers [31], hybrid quadrupole-hexapole FT-ICRs [32], and hybrid linear ion trap-Orbitrap mass spectrometers [33]. More recently, ETD has also been implemented on quadrupole time-of-flight mass spectrometers [34, 35].

Our approach utilizes a device in which electrons are transferred from singly charged fluoranthene radical anions to multiply protonated peptides in an rf quadrupole ion trap (3D high capacity trap), and induce fragmentation of the peptide backbone along pathways that are analogous to those observed in ECD [20]. In this rapid process, side-chain modifications remain largely unaffected by the dissociation event, thus preserving post-translational modifications and, in many cases, also noncovalent interactions. In the case of protein ubiquitination, the most successful approach has been based on proteolytic digestion with trypsin and the detection of the gly-gly mass tag either using conventional ion trap [7] or high mass accuracy mass spectrometers [36]. However, this approach may suffer from a number of drawbacks. For instance, a potentially false positive interpretation of this modification was suggested to occur by using iodoacetamide (IAA) as alkylating reagent, since two IAA moieties give rise to an identical mass tag as two glycyl residues [37]. However, iodoacetamide is predominantly thiol-reactive and under normal experimental conditions does not readily react with primary amines if samples are not heated during alkylation steps (unpublished observations). In any case, the reported use of the milder alkylating agent chloroacetamide avoids any possible ambiguities. In addition, gly-gly modifications on lysyl side chains are also subjected to fragmentation in a similar manner as peptide backbone amides, and hence may be missed under conventional fragmentation methods. Here we observed that the gly-gly tag largely remained intact in both CID and ETD spectra. We also obtained better sequence coverage with highly charged precursor ion peptides (3+) in ETD compared with the corresponding CID spectra, which usually yielded better fragmenta-

tion from precursor ions with lower m/z ratios (2+) (Figure 4). In this context, it is worthwhile pointing out that the fragment ion yield in ETD can be significantly enhanced in some cases by using supplemental activation [38], an approach that we will study systematically in a forthcoming publication.

Since both fragmentation methods provide information from differentially charged precursor ions, the results are complementary and hence extend the discovery rate of modified side chains. Currently available search engines such as Mascot [24], Phenyx [39], OMSSA [40], or Modiro [41] all have properties to evaluate ETD spectra, and the latest version of Mascot allows for searching of combined ETD and CID datasets. It will also be interesting to determine whether ETD can be applied to alternative approaches for the detection of ubiquitination, such as using a Glu-C digest and detection of a 12 mer peptide fragment [42, 43]. This approach would be valuable to distinguish modification by ubiquitin-like proteins (Ubls) versus conventional ubiquitination based on different C-terminal sequences. Also, proteolytic digests using Lys-N, which yield almost complete c-ion series in ETD, could be well suited for this purpose [44]. Finally, multiple-reaction-monitoring (MRM) [45] and “middle-down” mass spectrometry [46] are now also applied to the detection of protein ubiquitination, both of which could potentially benefit from being combined with ETD.

Protein ubiquitination remains a challenging PTM for proteomic analysis and is potentially more widespread than originally thought, as it is generally observed on lysyl side chains, but was also found to be present on protein N-termini [47], cysteine [48], and perhaps other amino acid residues. We believe that proteomic approaches, which integrate CID with ETD fragmentation and utilize combined dataset for sequence assignment, will pave the way to better identification and sequence coverage of proteins as well as more confident and complete assignments of post-translational modifications.

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