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Effect of Chemical Modifications on Peptide Fragmentation Behavior upon Electron Transfer Induced Dissociation

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In proteomics, proteolytic peptides are often chemically modified to improve MS analysis, peptide identification, and/or to enable protein/peptide quantification. It is known that such chemical modifications can alter peptide fragmentation in collision induced dissociation MS/MS. Here, we investigated the fragmentation behavior of such chemically modified peptides in MS/MS using the relatively new activation method electron transfer dissociation (ETD). We generated proteolytic peptides using the proteases Lys-N and trypsin and compared the fragmentation behavior of the unlabeled peptides with that of their chemically modified cognates. We investigated the effect of several commonly used modification reactions, namely, guanidination, dimethylation, imidazolinylation, and nicotinylation (ICPL). Of these guanidination and imidazolinylation specifically target the ε-amino groups of lysine residues in the peptides, whereas dimethylation and nicotinylation modify both N-termini and ε-amino groups of lysine residues. Dimethylation, guanidination, and particularly imidazolinylation of doubly charged Lys-N peptides resulted in a significant increase in peptide sequence coverage, resulting in more reliable peptide identification using ETD. This may be rationalized by the increased basicity and resulting positive charge at the N-termini of these peptides. Nicotinylation of the peptides, on the other hand, severely suppressed backbone fragmentation, hampering the use of this label in ETD based analysis. Doubly charged C-terminal lysine containing tryptic peptides also resulted in an enhanced observation of a single type of fragment ion series when guanidinated or imidazolinylated. These labels would thus facilitate the use of de novo sequencing strategies based on ETD for both arginine and lysine containing tryptic peptides. Since isotopic analogues of the labeling reagents applied in this work are commercially available, one can combine quantitation with improved ETD based peptide sequencing for both Lys-N and trypsin digested samples.

In recent years, mass spectrometry (MS) has become the method of choice for protein identification.[1] Currently, sequencing and identification is often performed with peptides with a molecular weight below 3 kDa. One of the main rationales for reducing a protein to peptides is the superior sequencing of peptides possible by mass spectrometry alongside an advanced armory of MS compatible peptide separation strategies.[2−4]

The protease most often used for digestion of proteins into peptides is trypsin. The tryptic peptides can then be separated by chromatography and subsequently subjected to sequencing by collision induced dissociation (CID) based tandem mass spectrometry (MS/MS). Currently, the most popular strategy to obtain sequence information from the spectra is by employing database search algorithms. These algorithms align experimental data against in silico derived spectra to obtain the most likely candidate sequence.[1,5]

Typically under CID conditions tryptic peptide ions generate a number of different fragment ion series wherein N-terminal (b-ions) and C-terminal (y-ions) fragment ions dominate the tandem mass spectra.[6−8] However, these mixtures of different fragment ion series can create rather complex spectra that contain several layers of potentially redundant information. Reducing spectra to minimal information, ideally originating from a single but complete ion series, will allow easier interpretation either manually or by automated search algorithms. Over the years, a number of strategies have been developed to influence peptide ion fragmentation creating simplified spectra. Under normal MALDI and ESI conditions, peptide fragment ion formation and observation is ultimately linked to protons. The "mobile" proton weakens the peptide bonds, helps to induce bond cleavage, and allows observation of fragment ions.[9−12] Gas phase basicity of the

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(1) Aebersold, R.; Mann, M. *Nature* **2003**, *422*, 198–207.

(2) Sandra, K.; Moshir, M.; D'Hondt, F.; Verleysen, K.; Kas, K.; Sandra, P. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* **2008**, *866*, 48–63.

(3) Tang, J.; Gao, M. X.; Deng, C. H.; Zhang, X. M. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* **2008**, *866*, 123–132.

(4) Cravatt, B. F.; Simon, G. M.; Yates, J. R. *Nature* **2007**, *450*, 991–1000.

(5) Domon, B.; Aebersold, R. *Science* **2006**, *312*, 212–217.

(6) Johnson, R. S.; Martin, S. A.; Biemann, K. *Int. J. Mass Spectrom. Ion Processes* **1988**, *86*, 137–154.

(7) Steen, H.; Mann, M. *Nat. Rev. Mol. Cell Biol.* **2004**, *5*, 699–711.

(8) Roepstorff, P.; Fohlman, J. *Biomed. Mass Spectrom.* **1984**, *11*, 601.

(9) Burlet, O.; Orkiszewski, R. S.; Ballard, K. D.; Gaskell, S. J. *Rapid Commun. Mass Spectrom.* **1992**, *6*, 658–662.

(10) Dongre, A. R.; Jones, J. L.; Somogyi, A.; Wysocki, V. H. *J. Am. Chem. Soc.* **1996**, *118*, 8365–8374.

(11) Tsaprailis, G.; Nair, H.; Somogyi, A.; Wysocki, V. H.; Zhong, W. Q.; Futrell, J. H.; Summerfield, S. G.; Gaskell, S. J. *J. Am. Chem. Soc.* **1999**, *121*, 5142–5154.

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various functional groups present in peptides and their fragments dictate the location of the proton(s) to a major extent. The average residence site of the proton(s) controls which fragment ions are observed. One strategy to create simplified spectra involves modifying functional groups on peptides. Changing the basicity or acidity will affect proton localization and thus the peptide fragmentation. When the protons can be sequestered at a single terminus of the peptide, a single ion series of sequence diagnostic fragment ions may be observed.[13] Most chemical modification strategies prefer MALDI since it generates peptides with only a single proton, allowing proton localization and thus fragmentation to be more easily controlled. Two commonly used methods that simplify spectra by removing the presence of N-terminal fragments are chemically assisted fragmentation (CAF)[14,15] and 4-sulfophenyl isothiocyanate (SPITC).[14] In both these reactions, the N-terminal amino group is modified to a sulfonate derivative which is highly acidic/negatively charged. The sulfonate moiety leads to N-terminal fragments that are either negatively charged or neutral, eliminating their presence in the CID spectrum, which causes the fragmentation spectra to be dominated by C-terminal fragment ions (y-ions). However, the acidic nature of the N-terminus leads to a loss of sensitivity in positive ion mode since peptide ionization is compromised. Other examples of chemical modifications of tryptic peptides to increase ionization efficiency include the derivatization of C-terminal lysines to enhance the basicity/positive charge of the C-terminus. In this case, C-terminal fragments (y-ions) are preferably formed upon CID fragmentation.[16−20] However, the increased basicity of the peptide has also a side effect; the collision energy needs to be increased for efficient fragmentation. The increased collision energy often leads to "overfragmentation" and when insufficient energy is applied low-energy pathways such as cleavage adjacent to aspartates and glutamates dominate.[21,22] The results of the N- and C-terminal labeling methods described above are complementary as they both lead to the increased formation of C-terminal fragment ions, and thus it is not surprising that the two strategies have also been combined.[14,23]

Recently, we showed that fragmentation behavior of peptide ions cannot only be chemically regulated but also biochemically using the proteolytic enzyme Lys-N which generates peptides that exhibit two basic entities at the N-terminus.[24−27] This concentration of basicity directs the protons/charge to the N-terminus and leads to fragmentation spectra dominated by b-ions in MALDI-CID.[25] We also showed that using ESI-MS in combination with electron transfer dissociation (ETD) for the doubly charged Lys-N peptides that contain solely a single basic residue give rise to simple fragment ion spectra dominated by N-terminal c-ion fragments.[26,27]

At present, little is known about the fragmentation behavior of chemically modified peptides following ETD. Moreover, only a few chemical labeling strategies have been investigated in combination with ETD for quantification (e.g., using the isobaric tags, isobaric tag for relative and absolute quantitation (iTRAQ) and tandem mass tags (TMT)),[28−30] but these reports focus on the fragmentation of the label and not on the changed behavior of the bound peptide. Furthermore, both labels were compromised in their ability to allow quantitation with ETD based tandem mass spectrometry. We set out to investigate the effect of other commonly used chemical labels on the fragmentation behavior of Lys-N and tryptic peptides under ETD conditions. The four commonly used peptide modifications we examined are guanidination,[17−19] dimethylation,[31−33] imidazolinylation (Lys Tag 4H),[20] and nicotinylation (ICPL).[34,35] Since these labels can also be made with differential stable isotopes, they have been previously applied in quantitative proteomics applications, albeit by using CID for fragmentation.

EXPERIMENTAL SECTION

Materials. Bovine serum albumin, iodoacetamide, formaldehyde (37% solution in H_2O), and triethylammonium bicarbonate (1 M solution) were supplied by Sigma-Aldrich (Steinheim, Germany). Ammonia (25% solution in H_2O) and formic acid were obtained from Merck (Darmstadt, Germany). Ammonium bicarbonate, dithiothreitol (DTT), sodium cyanoborohydrate, and *O*-methyl isourea hemisulfate were purchased from Fluka (Buchs, Switzerland). Protease inhibitor cocktail was supplied by Roche Diagnostics (Mannheim, Germany). The water used in these experiments was obtained from a Milli-Q purification system (Millipore, Bedford, MA). Human embryonic kidney (HEK) 293 cells were provided by Dr. Pantelis Hatzis and Dr.

(12) Paizs, B.; Suhai, S. *Mass Spectrom. Rev.* **2005**, *24*, 508–548.
(13) Leitner, A.; Lindner, W. *Proteomics* **2006**, *6*, 5418–5434.
(14) Keough, T.; Lacey, M. P.; Youngquist, R. S. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 2348–2356.
(15) Keough, T.; Youngquist, R. S.; Lacey, M. P. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 7131–7136.
(16) Spengler, B.; Luetzenkirchen, F.; Metzger, S.; Chaurand, P.; Kaufmann, R.; Jeffery, W.; Bartlet-Jones, M.; Pappin, D. J. C. *Int. J. Mass Spectrom.* **1997**, 127–140.
(17) Brancia, F. L.; Oliver, S. G.; Gaskell, S. J. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 2070–2073.
(18) Beardsley, R. L.; Reilly, J. P. *Anal. Chem.* **2002**, *74*, 1884–1890.
(19) Warwood, S.; Mohammed, S.; Cristea, I. M.; Evans, C.; Whetton, A. D.; Gaskell, S. J. *Rapid Commun. Mass Spectrom.* **2006**, *20*, 3245–3256.
(20) Peters, E. C.; Horn, D. M.; Tully, D. C.; Brock, A. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 2387–2392.
(21) Sullivan, A. G.; Brancia, F. L.; Tyldesley, R.; Bateman, R.; Sidhu, K.; Hubbard, S. J.; Oliver, S. G.; Gaskell, S. J. *Int. J. Mass Spectrom.* **2001**, *210*, 665–676.
(22) Qin, J.; Chait, B. T. *Int. J. Mass Spectrom.* **1999**, *191*, 313–320.
(23) Joss, J. L.; Molloy, M. P.; Hinds, L. A.; Deane, E. M. *Int. J. Pept. Res. Therapeutics* **2006**, *12*, 225–235.
(24) Nonaka, T.; Hashimoto, Y.; Takio, K. *J. Biochem.* **1998**, *124*, 157–162.
(25) Boersema, P. J.; Taouatas, N.; Altelaar, A. F. M.; Gouw, J. W.; Ross, P. L.; Pappin, D. J.; Heck, A. J. R.; Mohammed, S. *Mol. Cell. Proteomics* **2009**, *8*, 650–660.
(26) Taouatas, N.; Altelaar, A. F. M.; Drugan, M. M.; Helbig, A. O.; Mohammed, S.; Heck, A. J. R. *Mol. Cell. Proteomics* **2009**, *8*, 190–200.
(27) Taouatas, N.; Drugan, M. M.; Heck, A. J. R.; Mohammed, S. *Nat. Methods* **2008**, *5*, 405–407.
(28) Han, H. L.; Pappin, D. J.; Ross, P. L.; McLuckey, S. A. *J. Proteome Res.* **2008**, *7*, 3643–3648.
(29) Phanstiel, D.; Zhang, Y.; Marto, J. A.; Coon, J. J. *J. Am. Soc. Mass Spectrom.* **2008**, *19*, 1255–1262.
(30) Viner, R. I.; Zhang, T.; Second, T.; Zabrouskov, V. *J. Proteomics*, in press, corrected proof.
(31) Boersema, P. J.; Aye, T. T.; van Veen, T. A. B.; Heck, A. J. R.; Mohammed, S. *Proteomics* **2008**, *8*, 4624–4632.
(32) Hsu, J. L.; Huang, S. Y.; Chow, N. H.; Chen, S. H. *Anal. Chem.* **2003**, *75*, 6843–6852.
(33) Raijmakers, R.; Berkers, C. R.; de Jong, A.; Ovaa, H.; Heck, A. J. R.; Mohammed, S. *Mol. Cell. Proteomics* **2008**, *7*, 1755–1762.
(34) Munchbach, M.; Quadroni, M.; Miotto, G.; James, P. *Anal. Chem.* **2000**, *72*, 4047–4057.
(35) Schmidt, A.; Kellermann, J.; Lottspeich, F. *Proteomics* **2005**, *5*, 4–15.

Tokameh Mahmoudi from The Netherlands Institute for Developmental Biology, (Utrecht, The Netherlands). Sep-Pak Vac tC18 1 cm^3 cardriges were obtained from Waters Corporation (Milford, MA). Nicotine *N*-hydroxy succinimide ester (Nic-NHS ester) in DMSO was a gift from Dr. Keidel, Max-Plank-Institute for Biochemistry (Martinsried, Germany). 2-Methoxy-4,5-dihydro-1*H*-imidazole (Lys Tag 4H) was a gift from Agilent Technologies. Metalloendopeptidase Lys-N (*Grifola Frondosa*) was obtained from Seikagaku Corporation (Tokyo, Japan).

Cell Lysing and Digestion of Proteins. A protein extract of a HEK293 cell lysate was prepared as described previously.[26] The protein concentration was estimated by a Bradford assay. HEK293 proteins and bovine serum albumin (BSA) were reduced and carbamidomethylated using DTT and iodoacetamide. Subsequently, the proteins were digested with Lys-N or trypsin at 37 °C overnight.

Strong Cation Exchange (SCX) Chromatography. The HEK293 peptide solution was desalted prior to SCX chromatography using a C_{18} Sep-Pak cartridge (Waters Corporation, Milford, MA), dried in a vacuum centrifuge, and resuspended in 10% formic acid. SCX was performed on a SCX system as described previously.[26] Collected fractions were desalted and dried in a vacuum centrifuge.

Labeling of Peptides. For guanidination a 5 M solution of *O*-methyl isourea hemisulfate in water was prepared immediately before usage. Peptides (1−10 μg) were resuspended in 30 μL of 1 M Na_2CO_3 and mixed with 6 μL of 5 M *O*-methyl isourea hemisulfate. A pH of 10.5−11 was estimated by the use of indicator strips. The solution was incubated for 30 min at 65 °C. The reaction was terminated by adding 14 μL of 50% formic acid.

For imidazolinylation, a 2 M solution of 2-methoxy-4,5-dihydro-1*H*-imidazole in water was prepared immediately before usage. Peptides (1−10 μg) were resuspended in 10 μL of 1 M Na_2CO_3 and mixed with 45 μL of 2 M 2-methoxy-4,5-dihydro-1*H*-imidazole. A pH of 11−12 was estimated by the use of indicator strips. The solution was incubated for 3 h at 55 °C. The reaction was terminated by addition of 5 μL of 50% formic acid.

For nicotinylation, 25 μL of a 33 mg/mL nicotine *N*-hydroxy succinimide ester in DMSO solution was added to 1−10 μg of peptides resuspended in 5 μL of 50% MeCN. This mixture was left at room temperature for 2 h. Additional 10 μL of nicotine *N*-hydroxy succinimide ester solution was added after 15, 30, and 45 min.

For dimethylation, 1 μg of peptides was dissolved in 100 μL of triethylammonium bicarbonate. Formaldehyde (4 μL of a 4% solution) and 4 μL of 0.6 M cyanoborohydrate solution were added to the peptides, and the solution was left shaking for 1 h at room temperature. The reaction was terminated by addition of 1% ammonia solution.

NanoLC−ESI-CID/ETD-MS/MS. The peptides were subjected to NanoLC−MS/MS analysis with CID and ETD or ETcaD for doubly charged peptides. The NanoLC−MS/MS consists of a HCT ultra PTM Discovery system (Bruker Daltonics) equipped with an Agilent 1200 system HPLC. The columns were made in-house. The material used for the precolumn is Aqua C18 (Phenomenex, Torrance, CA). Reprosil-pur C18 3 μm (Dr. Maisch, Ammerbuch-Entringen, Germany) was used for the 25 cm analytical capillary column with 50 μm inner diameter. Mobile-phase buffer A consisted of 0.1 M acetic acid in water, and mobile-phase buffer B consisted of 0.1 M acetic acid in 80% acetonitrile.

Protein Identification. Raw MS data were converted to peak lists using Data Analysis software (Bruker Daltonics, version 4.0) Spectra were searched against the IPI (International Protein Index) Human database version 3.36 (69 012 sequences; 29 002 682 residues) or a homemade BSA database (8 sequences; 2973 residues) using Mascot software version 2.2.0 (www.matrixscience.com), with Lys-N or trypsin cleavage specificity. The database search was made with the following parameters set to consider a peptide tolerance of ±0.5 Da, a fragment tolerance of ±1.2 Da, allowing 1 missed cleavage, carbamidomethyl (C) as fixed modification, oxidation (M), and the appropriate chemical labeling as a variable modification. Tandem mass spectra assigned with a Mascot score ≥ 25 (p-value ≤0.05) were accepted.

Statistical Analysis. The sequence coverage for each peptide from the BSA digests was calculated by dividing the observed number of ions for a particular fragment ion series by the maximum theoretical number of observable ions. The differences in sequence coverage between labeled and unlabeled peptides were determined, and the average and the standard deviation of these differences were calculated.

The number of ions for the individual fragment ion series of the peptides from the HEK 293T cell lysate digests were calculated and exported from Mascot. Also, for the HEK 293T cell lysate the differences in the number of ions between labeled peptides and their unlabeled cognates were determined and the average and standard deviation of the differences were calculated. p-values were calculated based on the number of compared peptides and the corresponding average and standard deviation using a two sample one-sided t test. In all cases, differences were considered to be significant at p-values < 0.05.

RESULTS AND DISCUSSION

Electron transfer dissociation of doubly charged peptides leads to the generation of an entire population of product species that are singly charged.[36] The generation of this singly charged population has been exploited by our group through the use of Lys-N in order to predominantly produce a single type of fragment ion series in ETD. The underlying principle for creating these simplified spectra is the manipulation of basicity where Lys-N creates peptides with all amino groups placed at the N-terminus. We observed that creating such peptides lead to preferentially N-terminal fragment ions. To carry out a more in-depth analysis of the effect of basicity on peptide fragmentation we modulated the basicity of the amino groups. A number of labeling strategies exist within proteomics for modifying amino groups.[13,17,20,31,32,34,35,37] For specific derivatization of the ε-amine of lysine, we utilized the two reagents: *O*-methyl isourea[17] and 2-methoxy-4,5-dihydro-1*H*-imidazole[20] (Figure 1). The latter reagent creates a moiety with a higher basicity than the original ε-amino and the guanidino group produced by *O*-methyl isourea. Additionally, two further labeling strategies were characterized for derivatization of all

(36) Syka, J. E. P.; Coon, J. J.; Schroeder, M. J.; Shabanowitz, J.; Hunt, D. F. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 9528–9533.
(37) Gevaert, K.; Impens, F.; Ghesquiere, B.; Van Damme, P.; Lambrechts, A.; Vandekerckhove, J. *Proteomics* **2008**, *8*, 4873–4885.

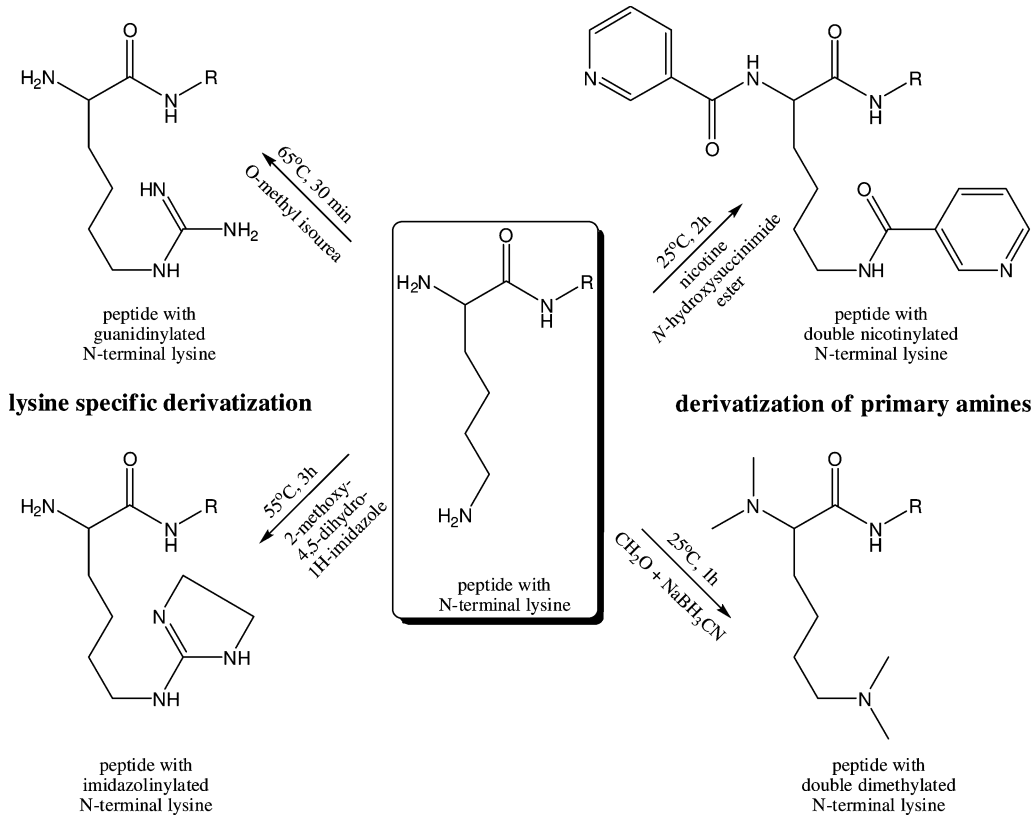

Figure 1. Overview illustrating the lysine (guanidination and imidazolinylation) and amino group (nicotinylation and dimethylation) specific peptide derivatization chemistries.

primary amines, nicotine *N*-hydroxy succinimide ester[34,35] and formaldehyde in the presence of cyanoborohydrate[31,32,38,39] (Figure 1). For both of these reagents the local basicity of the modified residues is also enhanced upon derivatization.

In order to provide a platform for the labeling comparison, a test bed of peptides was generated by digesting bovine serum albumin (BSA) with either trypsin or Lys-N. In the case of Lys-N, the lysine residue would be present at the N-terminus and thus the labels would exclusively modify this region of the peptide. In the cases of guanidination and imidazolinylation, a single label will be placed on the lysine. Dimethylation and nicotinylation will modify both amino groups and thus two labels will be placed at the N-terminus (Figure 1). Tryptic peptides will possess an α-amino group at the N-terminus and potentially an ε-amino group (lysine) at the C-terminus. Guanidination and imidazolinylation will label only a subset of tryptic peptides exclusively at the C-terminal lysine. Dimethylation and nicotinylation will label all tryptic peptides on the N-terminus, and those containing a C-terminal lysine will also be labeled at the C-terminus.

Proteolytic peptides of BSA were generated by trypsin or Lys-N, and aliquots were labeled using the four different reagents. The labeled peptides and their unlabeled counterparts were analyzed by CID and ETD on a three-dimensional quadrupole ion trap. Furthermore, doubly charged peptide ions were subjected to automated supplemental collisional activation (ETcaD) in ETD experiments.[40] As previously reported and mentioned above, ETD spectra of unlabeled doubly charged Lys-N peptides containing one basic amino acid are dominated by c-type fragment ions.[27] The intensities in this series of fragment ions are greatest in the high mass range and decrease with mass. Typical examples of such spectra are shown in Figure 2. The peptide modification reactions chosen for this study (guanidination, dimethylation, nicotinylation, and imidazolinylation) are all known to increase basicity of either or both α- and ε-amino groups present in a peptide. Consequently, the gas phase basicity driven effect of observing solely c-type fragments is still present. Nicotinylated peptides showed nonsequence informative ETcaD fragmentation, which will be discussed in detail below. The main difference between the spectra of unmodified and guanidinated, dimethylated, and imidazolinylated Lys-N peptides was found in the relative intensities for the c-ions observed. All three labels, albeit to different extents, reduced the generally observed signal drop from high to low mass for the c-ion series. Strikingly, additional low mass c-ions could now be observed in the majority of spectra of guanidinated, dimethylated, and imidazolinylated peptides when compared to their unlabeled cognate. The appearance of low mass c-ions was most pronounced for imidazolinylated peptides relative to guanidinated and dimethylated, correlating well with the gas phase basicity of these derivatives (Figure 2). Performing a manual fragment-ion count based on the spectra annotated by scaffold for the doubly charged peptides from the Lys-N digest of BSA and their labeled counterparts revealed that the sequence cover-

(38) Lemeer, S.; Pinkse, M. W. H.; Mohammed, S.; van Breukelen, B.; den Hertog, J.; Slijper, M.; Heck, A. J. R. *J. Proteome Res.* **2008**, *7*, 1555–1564.
(39) Boersema, P. J.; Raijmakers, R.; Lemeer, S.; Mohammed, S.; Heck, A. J. R. *Nat. Protoc.* **2009**, *4*, 484–494.
(40) Swaney, D. L.; McAlister, G. C.; Wirtala, M.; Schwartz, J. C.; Syka, J. E. P.; Coon, J. J. *Anal. Chem.* **2007**, *79*, 477–485.

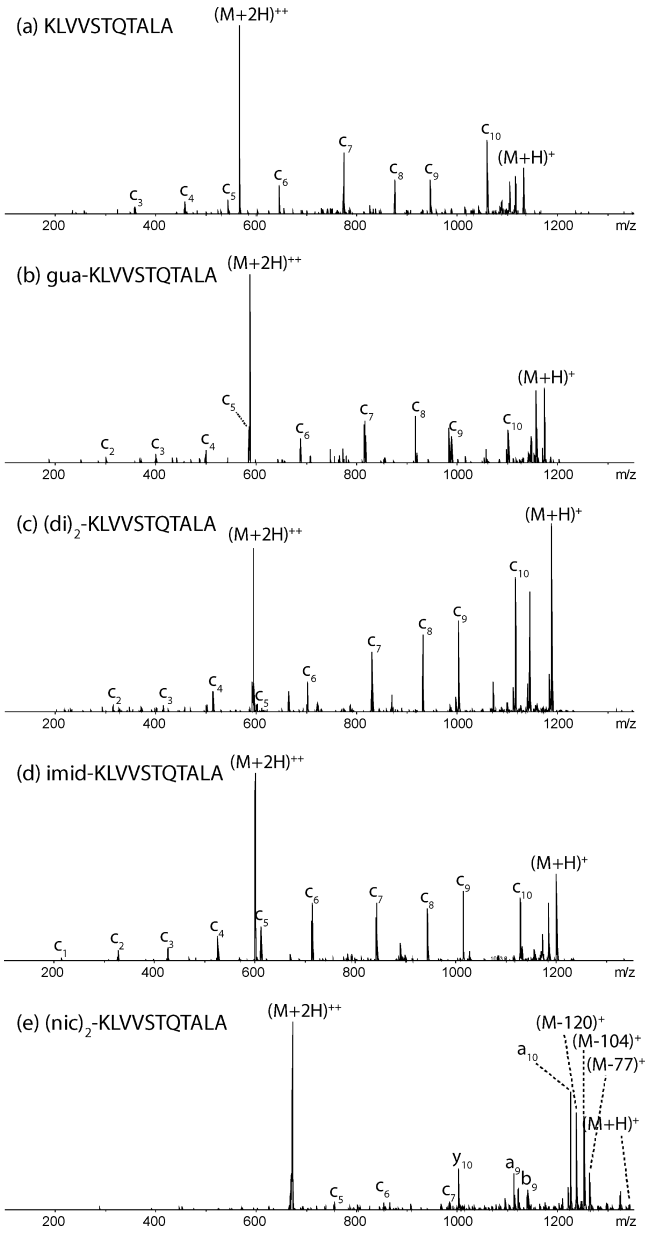

Figure 2. Comparison of ETcaD fragmentation spectra of the doubly charged Lys-N peptide KLVVSTQTALA derived from bovine serum albumin (BSA): (a) unmodified, (b) guanidinated (gua), (c) double dimethylated (di), (d) imidazolinylated (imid), and (e) double nicotinylated (nic).

age of the guanidinated and imidazolinylated peptides is significantly greater in the modified than in the unlabeled peptides (Figure 3). On the basis of a two sample one-sided *t* test, the probabilities for the observed increase in c-ions generated by guanidination and imidazolinylation were $p < 0.02$ and $p < 0.001$, respectively (Supplemental Table 1 in the Supporting Information).

Next we also evaluated the effect of the different labels on the ETD induced fragmentation of tryptic peptides (Figure 4). As expected, doubly charged tryptic peptides with a single lysine residue resulted in MS/MS spectra containing a mixture of c- and z-ions. The observed distribution of c- and z-ions can be rationalized by the location of the basic moieties at each of the two termini of these peptides. ETD induced fragmentation of peptides containing a C-terminal arginine resulted in dominant z-ion series reflecting the higher basicity of this residue versus the primary amine present at the N-terminus (see the Supporting Information).[27,41−43] The ETD spectra of the guanidinated or imidazolinylated tryptic peptides became generally more dominated by z-ions. This may be rationalized as *O*-methyl isourea or 2-methoxy-4,5-dihydro-1*H*-imidazole only labels the ε-amino group of lysines, increasing the basicity at the C-terminus of lysine containing tryptic peptides. The reduction in signal observed with fragment ion mass for the z-ion series of the labeled peptides is abated with respect to unmodified peptides (Figure 4) in line with the trends observed for Lys-N peptides. The relative intensities of c-ions against z-ions are lower compared to their unlabeled cognates providing further evidence that basicity is directing product ion detection in ETD spectra of doubly charged ions. In contrast to the other two labels, dimethylation of tryptic peptides showed no significant difference compared to unmodified peptides (Supplemental Table 1 in the Supporting Information). Dimethylation modifies both α- and ε-amino groups, which in the case of lysine containing tryptic peptides, will mean that both the N- and C-terminus are derivatized. Performing a manual fragment ion-count for tryptic BSA peptides with a C-terminal lysine revealed that the guanidinated and imidazolinylated peptides had a statistically significantly greater peptide sequence coverage based on only z-ions compared to the unlabeled counterpart (Figure 5), each with *p*-values < 0.03 (Supplemental Table 1 in the Supporting Information). Strikingly, the imidazolinylation of lysine containing tryptic peptides generated ETD spectra containing extensive z-ion series (i.e., few c-ions) that may facilitate de novo sequencing strategies based on ETD (Figure 5 and Supplemental Table 1 in the Supporting Information).

In order to test our findings in a proteomics environment, we progressed to a more complex sample which in our case was created by the use of a human cell lysate. We evaluated both trypsin and Lys-N with dimethylation, guanidination, or imidazolinylation. Proteolytic peptides were, initially, separated by low pH strong cation exchange (SCX) chromatography.[26] At pH 2.7, all carboxylic acid residues will be neutral and charge will be dictated mostly by protonated basic moieties.[44−46] Under such conditions, SCX enables separation of peptides on the basis of the number of basic residues present. Fractions containing peptides with a single basic amino acid or two basic amino acids were selected and chemically modified. Appearance of fragment ions for the labeled peptides were compared against their unlabeled cognates using a two sample one-sided *t* test to calculate the probability for the difference in c- and z-ion populations. Pleasingly, the trends observed mirrored those reported above for the BSA peptides. The number of c-ions detected in the guanidinated and imidazolinylated doubly charged Lys-N peptides were significantly greater compared to their unlabeled cognates with a corresponding *p*-value < 0.0001 (Table 1). In this larger data set we could also confirm that more c-ions are observed by

(41) Kalli, A.; Hakansson, K. *J. Proteome Res.* **2008**, *7*, 2834–2844.
(42) Cooper, H. J. *J. Am. Soc. Mass Spectrom.* **2005**, *16*, 1932–1940.
(43) Mohammed, S.; Lorenzen, K.; Kerkhoven, R.; van Breukelen, B.; Vannini, A.; Cramer, P.; Heck, A. J. R. *Anal. Chem.* **2008**, *80*, 3584–3592.
(44) Ballif, B. A.; Villen, J.; Beausoleil, S. A.; Schwartz, D.; Gygi, S. P. *Mol. Cell. Proteomics* **2004**, *3*, 1093–1101.
(45) Beausoleil, S. A.; Jedrychowski, M.; Schwartz, D.; Elias, J. E.; Villen, J.; Li, J. X.; Cohn, M. A.; Cantley, L. C.; Gygi, S. P. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 12130–12135.
(46) Pinkse, M. W. H.; Mohammed, S.; Gouw, L. W.; van Breukelen, B.; Vos, H. R.; Heck, A. J. R. *J. Proteome Res.* **2008**, *7*, 687–697.

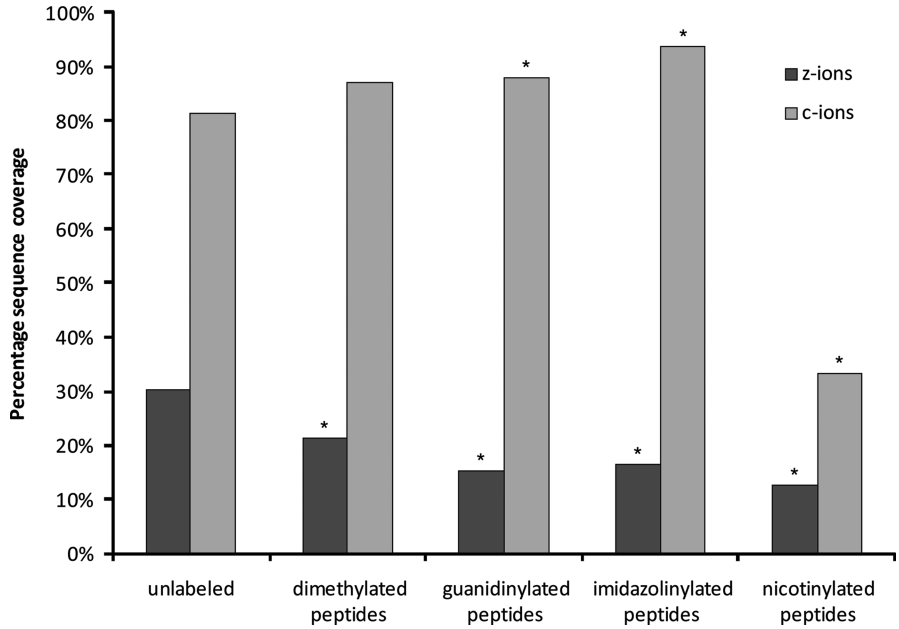

Figure 3. Average percentage of peptide sequence coverage based on c- and z-ions found in spectra of peptides resulting from a Lys-N digest of BSA and their labeled counterparts. All these BSA peptides ($n = 8$) contain a single lysine residue at the N-terminus. An asterisk indicates significant ($p < 0.05$) increase in c-ions and decrease in z-ions, respectively, between labeled peptides and their unlabeled cognates.

double dimethylated doubly charged Lys-N peptides compared to their unlabeled cognates with a corresponding p-value < 0.05. In agreement with the results obtained for the peptides originating from the BSA digest, in the cell lysate derived data we also observed significantly more z- and less c-ions for C-terminal guanidinated and imidazolinylated tryptic peptides compared to their unlabeled cognates (p-values < 0.0001), whereas no significant changes in c- or z-ion amounts could be observed for tryptic peptides dimethylated at both termini. All p-values for all combinations of protease and chemical labels are summarized in Table 1.

Peptides containing more than one basic amino acid are predominantly triply or higher charged in ESI, and these triply charged peptides, in contrast to doubly charged peptides, produce spectra with almost complete complementary c- and z-ion series upon ETD. In some cases, these peptides can generate doubly charged precursors. In the data sets from the cell lysate, we could observe the effect of labeling on doubly charged peptides containing a histidine or an arginine. A "typical" example with labeled analogues is shown in Figure 6. Unlabeled doubly charged Lys-N or tryptic peptides with one histidine/arginine residue produce both c- and z-ions. Exact populations of different fragment ion series are dependent on the location of the histidine/arginine residue. Compared to the unlabeled cognate, guanidination and imidazolinylation of these peptides reduce the signal drop from high mass to low mass for the c-ion series with Lys-N peptides (Figure 6) and analogues for the z-ion series with C-terminal labeled tryptic peptides. On average, more than 1.5 additional c-ions and z-ions can be observed for guanidinated or imidazolinylated Lys-N and tryptic peptides, respectively, in comparison to their unlabeled cognate (Supplemental Table 1 in the Supporting Information). It follows that the sequence coverage obtainable with a single ion series is increased upon chemical labeling. Additionally, the relative intensities of dominant z-ions compared to the c-ions present are decreased in guanidinated and imidazolinylated Lys-N peptides and vice versa the number of c-ions is decreased for the guanidinated and imidazolinylated tryptic peptides (the corresponding p-values are summarized in Supplemental Table 1 in the Supporting Information).

As mentioned above, the majority of the nicotinylated peptides, when subjected to ETcaD, did not yield spectra revealing significant fragmentation (Figure 7). In our experiments, CID and ETD spectra were recorded for each precursor ion and thus characterization of ETD spectra was possible via CID spectra (Figure 7). A characteristic pattern was observed in several spectra of doubly nicotinylated peptides consisting of fragments with 77, 104, and 120 Da lower than the peptide mass. We hypothesize that these fragments are generated by the loss of pyridine-, dehydropyridinecarboxaldehyde-, and nicotinamide-species. Apart from these losses, b- and y-ions are also observed in spectra of nicotinylated Lys-N peptides. The b- and y-ions are most likely generated by the supplemental collisional activation. Nicotinylated peptides generated by both trypsin and Lys-N exhibit these same tendencies in ETD (Figures 2 and 4). In line with these findings, Li et al. observed decreased backbone and enhanced side chain cleavage upon labeling peptides with 2,4,6,-trimethylpyridine (TMP) in ECD.[47] Moreover, Belyayev et al. found decreasing backbone and enhanced side chain fragmentation upon labeling peptides with coumarin derivatives.[48] Both groups reasoned that their tags provide sites where a radical can be resonance stabilized and therefore might function as a radical trap. Further evidence is provided by Jones et al. who used a tag that contains a pyridinyl group and obtained similar results to those described here.[49] Additionally, the imidazole moiety of histidine has also been shown to have a subtle negative impact on dissociation after electron

(47) Li, X. J.; Cournoyer, J. J.; Lin, C.; O'Connor, P. B. *J. Am. Soc. Mass Spectrom.* **2008**, *19*, 1514–1526.
(48) Belyayev, M. A.; Cournoyer, J. J.; Lin, C.; O'Connor, P. B. *J. Am. Soc. Mass Spectrom.* **2006**, *17*, 1428–1436.
(49) Jones, J. W.; Sasaki, T.; Goodlett, D. R.; Turecek, F. *J. Am. Soc. Mass Spectrom.* **2007**, *18*, 432–444.

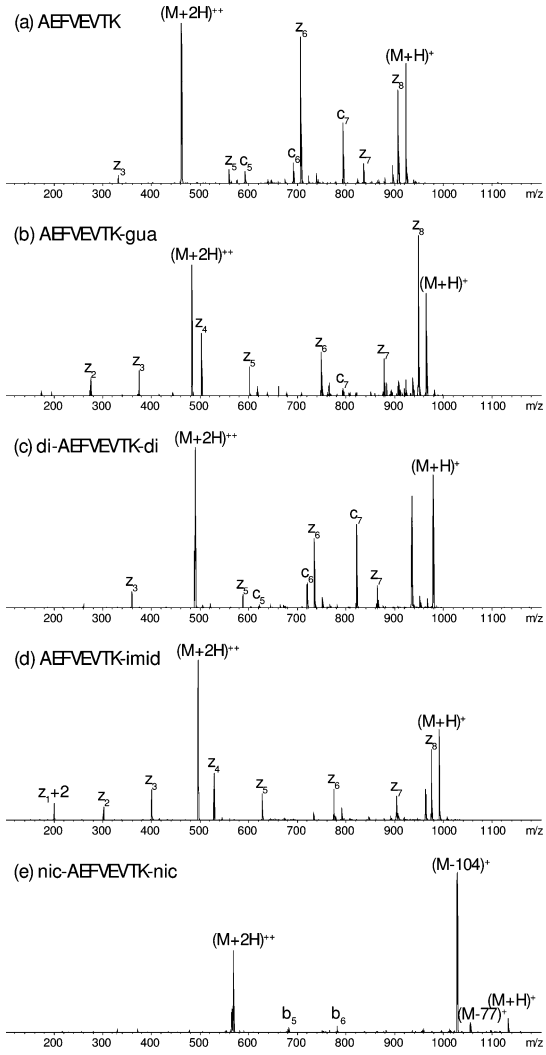

Figure 4. Comparison of ETcaD fragmentation spectra of the doubly charged tryptic peptide AEFVEVTK derived from BSA: (a) unmodified, (b) C-terminal guanidinated (gua), (c) N- and C-terminal dimethylated (di), (d) C-terminal imidazolinylated (imid), and (e) N- and C-terminal nicotinylated (nic).

capture.[50] Nicotinic acid is a member of the heterocyclic aromatic family along with the above-mentioned reagents. We hypothesize that the nicotinic tag is functioning as a radical trap leading to the observed suppression of backbone fragmentation in ETD.

Quantification of proteins is becoming an increasingly important aspect of proteomics. It is most often carried out by comparison of differential metabolic or chemical isotope labeled samples.[37] The quantitative labels used at present have almost exclusively been used on CID instrumentation. To our knowledge only ETD experiments with iTRAQ[28,29] and TMT[30] have been performed up to now. These tandem mass spectrometric based quantitation strategies were both compromised when used with ETD. The labels we investigated in this work and their isotopes are routinely used for differential isotopic labeling in CID.[19,20,31,32,35] In ETD we observed the guanidination, N-dimethylation, and imidazolinylation to be well suited particularly in combination with Lys-N peptides. Furthermore, all labels except nicotinylation improve the level of peptide sequence information obtainable by

(50) Xia, Y.; Gunawardena, H. P.; Erickson, D. E.; McLuckey, S. A. *J. Am. Chem. Soc.* **2007**, *129*, 12232–12243.

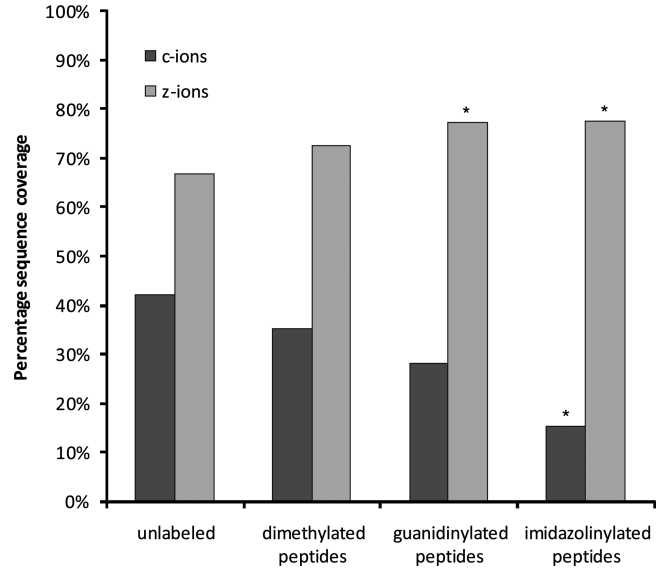

Figure 5. Average percentage of peptide sequence coverage based on c- and z-ions found in spectra of peptides resulting from a tryptic digest of BSA and their labeled counterparts. All peptides ($n = 6$) contain a single lysine at the C-terminus. An asterisk indicates significant ($p < 0.05$) decrease in c-ions and increase in z-ions, respectively, between labeled peptides and their unlabeled cognates.

Table 1. Statistical Significance of the Observed Trends in the ETD Fragment Ion Spectra of Lys-N and Tryptic Labeled and Unlabeled Peptides[a]

KX_n-peptides[b] (Lys-N)			
HEK 293T cell lysate			
	increased number of annotated c-ions	decreased number of annotated z-ions	number of labeled peptides compared against unlabeled peptides
guanidination	$p < 0.000\ 01$	$p < 0.0001$	206
imidazolinylation	$p < 0.000\ 01$	$p < 0.001$	108
dimethylation	$p < 0.05$	$p < 0.001$	186

X_nK-peptides[c] (tryptic)			
HEK 293T cell lysate			
	decreased number of annotated c-ions	increased number of annotated z-ions	number of labeled peptides compared against unlabeled peptides
guanidination	$p < 0.000\ 01$	$p < 0.000\ 01$	127
imidazolinylation	$p < 0.000\ 01$	$p < 0.000\ 01$	75
dimethylation	$p = 0.182\ 338$	$p = 0.397\ 96$	187

[a] *p*-values derived from a *t*-test based on the number of c- and *z*-ions observed for labeled versus unlabeled Lys-N or tryptic peptides (HEK 293T cell lysate). *P*-values smaller than 0.05 indicate a significant increase or decrease of ions for the labeled peptides compared to their unlabeled cognates. [b] Peptides with a N-terminal lysine where X is any amino acid besides H/K/R. [c] Peptides with a C-terminal lysine where X is any amino acid besides H/K/R.

ETD based tandem mass spectrometry where imidazolinylation demonstrated the largest improvement. In fact, labeling in a quantification experiment can potentially also improve the de novo sequencing aspect of the experiment.

C-terminal lysine containing tryptic peptides when guanidinated or imidazolinylated exhibited simplified spectra that contained

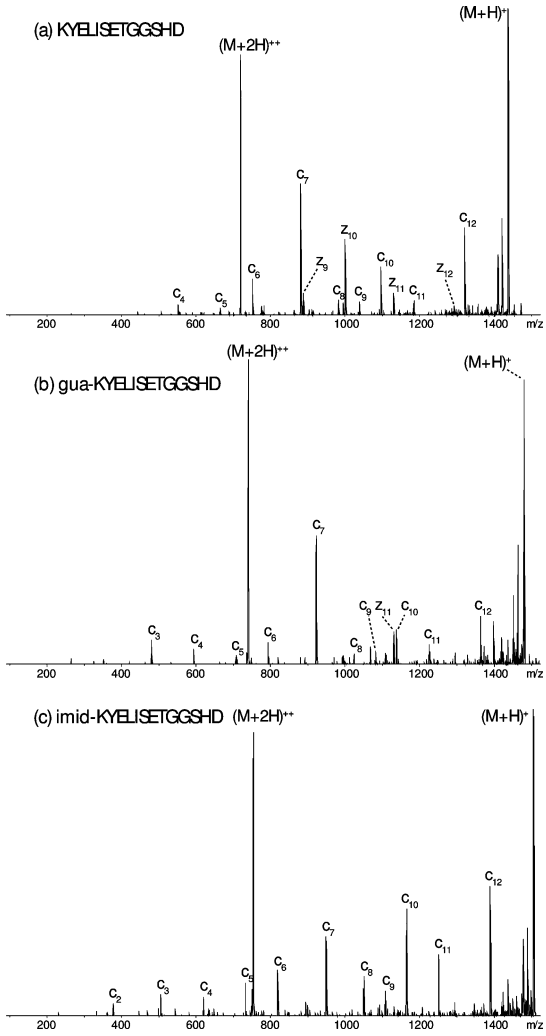

Figure 6. Comparison of ETcaD fragmentation spectra of the histidine containing doubly charged peptide KYELISETGGSHD identified from a Lys-N digested HEK293T cellular lysate: (a) unmodified, (b) guanidinated (gua), and (c) imidazolinylated (imid). Note that almost a full peptide sequence coverage is obtained on the basis of c-ions for the imidazolinated peptide, whereby the z-ions observed for the unlabeled peptide are suppressed.

nearly exclusively z-ions when no other basic residue was present. Both these reagents would allow tryptic pools of peptides when combined with low-pH SCX and ETD to create a de novo sequencing proteomic experiment similar to that previously proposed for Lys-N.[26,27] Doubly charged arginine containing tryptic peptides already exhibited a strong z-ion series, and here we show that chemical labeling enables similar signatures for lysine containing peptides. Unlike peptides generated by Lys-N, tryptic peptides require chemical modification in order to allow the generation of simplified spectra. However, trypsin creates, potentially, a larger pool of peptides for de novo sequencing. The fourth modification we investigated, nicotinylation, is also used for quantification in CID.[34,35] In ETD, nicotinylation of Lys-N and tryptic peptides hampered peptide identification since it suppresses peptide backbone fragmentation. Thus nicotinylation is inappropriate for any strategy that will involve ETD based sequence readouts.

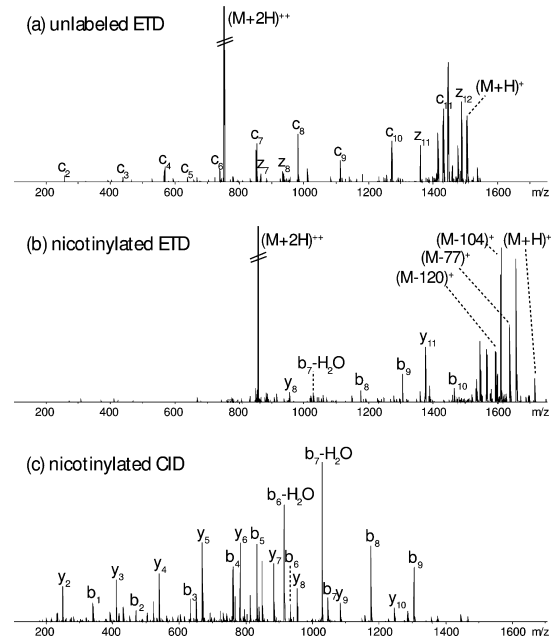

Figure 7. Tandem mass spectra of the Lys-N peptide KEYEATLEEccA derived from BSA. Cysteines of the peptide are carbamidomethylated. (a) ETcaD spectrum of the unlabeled peptide. (b) ETcaD spectra of the nicotinylated peptide. (c) CID spectrum of the nicotinylated peptide.

CONCLUSIONS

In recent years it has become apparent that ETD is a very useful and complementary activation method to fragment peptides in mass spectrometry. Here, we investigated the fragmentation behavior in ETD of chemically modified doubly charged peptides. Such chemical modification is performed quite often in proteomics to improve MS analysis, peptide identification, and/or to enable protein/peptide quantification. We used four different generally available labels, all targeting N-terminal amino groups and/or the ε-amino group of lysine. We found that guanidinated, dimethylated, and imidazolinylated peptides generated by Lys-N resulted upon ETD in even more extensive simple sequence ladders, potentially further facilitating de novo sequencing of such peptides. Guanidination and imidazolinylation of lysine terminated tryptic peptides also showed similar simplified spectra to those achieved by Lys-N peptides and arginine terminated tryptic peptides. In general the most striking positive effects were observed for imidazolinylated peptides. Modification of peptides by nicotinylation suppressed peptide backbone fragmentation in ETD, greatly reducing the number of successful peptide identifications. As all the chemical modifications that were studied in this work are amenable for a quantification strategy based on the incorporation of stable isotopes they except for nicotinylation can provide additional advantages to proteomic comparative experiments based on ETD sequencing.

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SUPPORTING INFORMATION AVAILABLE

Supplementary data set containing one excel file (Supplemental Table 1). This material is available free of charge via the Internet at http://pubs.acs.org (Two scaffold files including most of the data used in this study are available free of charge at https://proteomecommons.org/tranche/data-downloader.jsp?fileName=1dJj54qjNLW6%2FJg%2FSh9ryNFqf%2F10sAXkUqd0eiRkpH%2FwrpNSPsvvKIp9OBpzvgoJwuSTl8GUjdsft5fWfTnqgIMTmUoAAAAAAAAHSw%3D%3D; the passphrase is effpepchem1. The software to view the files (Scaffold viewer) is available free of charge at http://www.proteomesoftware.com/Proteome_software_prod_Scaffold_download-main.html. The first scaffold file contains the results of the tryptic and Lys-N digestion of BSA, and the second file contains the results of the Lys-N digestion of the cell lysate).

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