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RESEARCH ARTICLE

Enhanced Electron Transfer Dissociation of Peptides Modified at C-terminus with Fixed Charges

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Abstract

The impact of the conversion of carboxylates in peptides to basic or fixed charge sites on the outcome of electron transfer dissociation (ETD) is evaluated with respect to ETD efficiency and the number of diagnostic sequence ions. Four reagents, including benzylamine (BA), 1-benzylpiperazine (BZP), carboxymethyl trimethylammonium chloride hydrazide (GT), and (2-aminoethyl)trimethylammonium chloride hydrochloride (AETMA), were used for the carboxylate derivatization, with the first two replacing the acidic carboxylate groups with basic functionalities and the latter two introducing fixed charge sites. The ETD efficiencies and Xcorr scores were compared for both nonderivatized and derivatized tryptic and Glu-C peptides from cytochrome *c*. Derivatization of the carboxylate increases the average charge states, the number of fragment ions, and the dissociation efficiencies of peptides, especially for the fixed charge reagent, AETMA.

Key words: Electron transfer dissociation, C-terminus, Fixed charge site, Peptide

Introduction

Tandem mass spectrometry has been used with great success to elucidate the sequences of biomolecules such as nucleic acids [1], peptides [2–5], and oligosaccharides [6, 7]. Both the well-explored dynamics and mechanisms of collision induced dissociation (CID) [8–10] and extensively developed computer algorithms that have facilitated spectral interpretation [11, 12] have cemented the landmark status of CID for tandem mass spectrometry. Although collision-induced dissociation (CID) remains the most popular method for ion activation, newer alternatives, such as photon-based activation methods, including infrared multiphoton dissociation (IRMPD) [13–18], and ultraviolet photodissociation (UVPD) [19–24], as well as electron-based methods, such as electron capture dissociation (ECD) [25, 26] and electron transfer dissociation (ETD) [27–29] have emerged as promising strategies that complement CID or even outperform CID for certain types of applications, especially in the context of proteomics.

Both ECD and ETD have shown superior capabilities for the characterization of post-translational modifications (PTMs) of peptides compared to CID [25, 27]. Whereas collisional activation produces *b/y* types of fragment ions, electron-based activation promotes cleavages of N–C_α bonds that result in *c*- and *z*-type ions with retention of post-translational modifications on the amino acid side chains, unlike CID. One of the main drawbacks of ECD and ETD arises from their significant dependence on the charge state of the selected precursor ion. Precursor ions in low charge states tend to undergo charge reduction, often preferentially relative to production of the informative *c*- and *z*-type ions. In order to alleviate this handicap, several methods have been explored. For example, McLafferty et al. developed activated ion ECD, a method combining electron capture and collisional activation, to increase sequence coverage for

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top-down proteomics applications [30]. Both the Coon [27] and McLuckey groups [31, 32] have developed methods that combine electron activation with subsequent collisional activation of the charge-reduced ions (in a tandem process sometimes abbreviated as ETcaD), resulting in improved sequence coverage for peptides in low charge states. Our group also reported that ETcaD of N-terminal derivatized peptides afforded simplified MS/MS spectra containing predominantly C-terminal *z*-type ions, leading to successful de novo sequencing of peptides [33]. In addition, the use of supplemental IR activation prior to ECD or during ETD significantly increased the sequence coverage of peptides in low charge states upon disruption of intramolecular interactions that otherwise prevented disassembly of the constituent fragment ions [34, 35].

An alternative method to improve ECD or ETD efficiencies is to increase the charge states of the targeted precursor ions prior to activation. Charge states of ions can be manipulated by covalent attachment of fixed charge sites, or incorporation of very basic functionalities that enhance protonation, or by altering the electrospray conditions to enhance the production of ions in higher charge states. Williams and coworkers [36–38] were among the first to use a solution additive such as m-nitrobenzyl alcohol (m-NBA) to promote production of more highly charged peptides. The Jensen group [39] demonstrated that this method proved a natural strategy for enhancing the formation of more highly charged ions prior to electron-based activation, offering increased sequence coverage upon ETD of supercharged tryptic peptides. Derivatization strategies also can be employed to generate ions in higher charge states. For example, Smith et al. attached fixed charge quaternary ammonium groups to the carboxylic acid groups of proteins, resulting in charge states that shifted by up to five charges for lysozyme [40, 41]. O'Connor et al. labeled the lysine side chains of peptides with 2,4,6-trimethylpyridinium groups, and the labeled peptides exhibited fewer backbone cleavages and more prominent side-chain cleavages upon ECD [42]. Chamot-Rooke *et al.* derivatized the N-termini of peptides with phosphonium groups to improve the sequence coverage obtained upon ECD of O-glycosylated and O-phosphorylated peptides [43]. The Chait group pursued the derivatization of cysteines with *N,N*-dimethyl-2-chloro-ethylamine (DML), resulting in incorporation of very basic amine groups and ultimately yielding better sequence coverage upon subsequent ETD [44]. After conversion of the N-terminal and lysine side-chain amine groups of disulfide-linked peptides to fixed charge quaternary amines, McLuckey et al. reported that disulfide bond cleavages were enhanced upon electron-transfer ion-ion reactions [45]. Yang and coworkers increased the charge states of phosphopeptides by derivatizing their carboxy termini with 1-(2-pyrimidyl) piperazine, thus creating doubly charged ions that were suitable for sequencing by ETD [46]. Recently, our group reported greater ETD efficiencies and improved sequence coverage of peptides upon derivatization of cysteine residues by using (3-acrylamidopropyl)trimethyl ammonium chloride (APTA), a reagent that incorporates a fixed charge tag into the peptides [47]. Reid et al. used an amine-selective fixed charge sulfonium ion reagent to increase the ionization efficiencies and the charge states of phosphopeptides, thus improving sequence coverage upon ETD [48]. In a series of studies, the Turecek group reported the use of fixed charge reagents with tunable electronic properties that resulted in a broader array of *c*-type fragment ions and with no deleterious impact on *z*-type fragment ions upon ETD of peptides [49–51].

In the present study, peptides are derivatized at their carboxylate groups via attachment of basic amine groups or quaternary amines with fixed charges. Derivatization of the C- termini of peptides as well as amino acid side chains is recognized as a versatile tool for manipulating peptide properties like hydrophobicity, ionizability, charge sites, and chromatographic elution behavior. Although amine derivatization has been the most widely used method, carboxylate derivatization also offers a compelling target for enhancing charge states because these sites are typically among the most acidic in peptides and, thus, most susceptible to deprotonation. Xu et al. reported a highly effective carboxylate derivatization method based on an 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) coupling method in which a very basic pyrimidyl piperazine moiety was attached [52]. In the present work, carboxylate derivatization via a similar EDC coupling strategy is used to enhance the charge states of peptides and their resulting ETD efficiencies by using four reagents: benzylamine (BA), 1-benzylpiperazine (BZP), carboxymethyl trimethylammonium chloride hydrazide (also known as Girard's reagent T, GT), and (2-aminoethyl)trimethylammonium chloride hydrochloride (AETMA), with the first two converting the carboxylates to basic amine groups and the latter two incorporating fixed charge sites. The reagents are shown in Figure 1. The resulting derivatized peptides yield higher charge states upon ESI compared with the nonderivatized peptides and, as shown in this report, the resulting peptides undergo more effective ETD.

Experimental

Materials and Reagents

All reagents were purchased from Sigma Aldrich (St. Louis, MO, USA) except the GT reagent, which was obtained from TCI America (Boston, MA, USA). All peptides were obtained from BACHEM (King of Prussia, PA, USA), including YGGFLK, RPPGFSPFR (bradykinin), DRVYIHPFHL (angiotensin I), RQpSVELHSPQLPR (aquaporin II), ASHLGLAR, and pyrLYENKPRRPYIL (neurotensin). Ac-AAADKAAAR was synthesized at the Protein Microanalysis Facility at the University of Texas at Austin. Cytochrome *c* was purchased from Sigma Aldrich (St. Louis, MO, USA). The C_{18} resin was obtained from

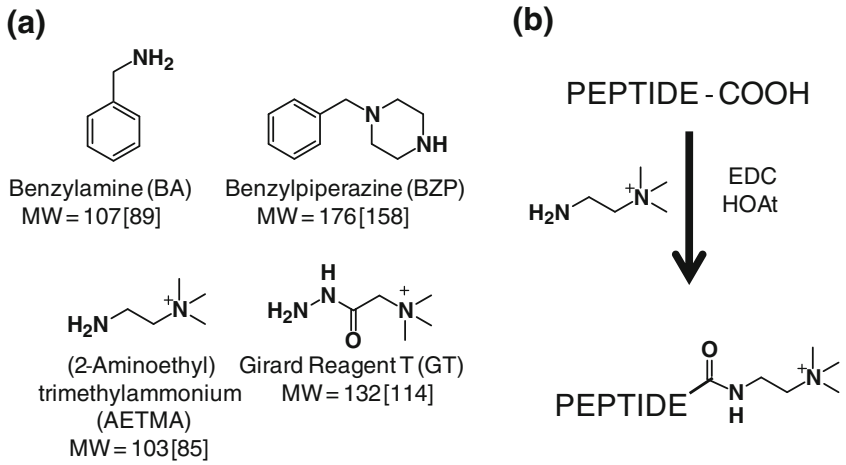

Figure 1. **(a)** Structure of the reagents used for derivatization in this study. In parentheses next to the compound name is the abbreviation and in brackets in the nominal mass addition. **(b)** Scheme showing the expected carboxylate derivatization route

Supelco (Bellefonte, PA, USA) and used for peptide purification. All solvents were purchased from Fisher Scientific (Fair Lawn, NJ, USA) and used without further purification.

Derivatization and Sample Preparation

For the derivatization of carboxylic acids in peptides, a procedure from a recent report [52] was used after slight modification. Before the EDC coupling, the basic amines such as side chains of arginine, lysine, and histidine, were protected with di-tert-butyl dicarbonate. The solutions of amine-containing reagents (~0.05 M) in diethylformamide (DMF, 10 μL), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 10 mg/mL, 10 μL) in DMF, and coupling reagent 1-hydroxy-7-azabenzotriazole (HOAt, 10 mg/mL, 10 μL) in DMF were sequentially added to a solution of peptides (~1 mM) in water (5 μL). After several seconds of vortexing, a 10 % trifluoroacetic acid solution was added and allowed to react for 1 h. The addition of 10 % TFA solution removes the t-Boc protecting group from all basic amines. The samples were dried using a vacuum centrifuge in order to terminate the reaction. The dried peptides were purified using a home-made spin column packed with Supelco C_{18} resin. For the experiments involving tryptic digestion of three proteins, trypsin (1.0 mg/mL in 1 mM HCl) was added to ~100 μg of protein (1/50 trypsin/protein w/w) in 100 mM NH_4HCO_3. The digestion solution was incubated for 16 h at 37 °C, then desalted using the home-made C18 column. The desalted digests were derivatized with AETMA in the same manner as described above for the peptides. Glu-C digestion of the proteins was performed in the same manner as tryptic digestion except Glu-C protease was diluted with pure water while trypsin was diluted in 1 mM HCl. In addition, Glu-C digestion buffer was 1× PBS buffer instead of ammonium bicarbonate.

Mass Spectrometry

A Thermo Fisher LTQ XL mass spectrometer (San Jose, CA, USA) with ETD capabilities was used for all experiments. The purified peptide samples were diluted to ~10 μM in 70/30 water/acetonitrile (vol/vol) and infused at 3 μL/min with a PHD 2000 syringe pump. The ETD experiments were performed by injecting 1×10^6 fluoranthene reagent anions into the trap, and the ETD reaction time was fixed at 100 ms. The precursor ion population for ETD was set to 1×10^4. The ETD MS/MS data of nonderivatized and AETMA-derivatized digests of cytochrome *c* were applied to SEQUEST through the Thermo Fisher Scientific Proteome Discoverer 1.0 software using the following parameters: 2 maximum miscleavages with trypsin and Glu-C, ±1.5 Da precursor mass tolerance, ±0.8 fragment mass tolerance, and signal: noise ratio cutoff of 3. Sequences of each protein were used for searching the spectral data obtained for the nonderivatized and AETMA-derivatized tryptic and Glu-C digests. The AETMA-derivatization of the C-termini was set as a dynamic modification. The reported Xcorr scores are a measurement of the overlap between the experimental MS/MS spectra and the theoretical MS/MS spectra generated based on known protein sequences. For each Xcorr score, a higher confidence is proportional to a higher score.

HPLC-MS

The purified nonderivatized and AETMA-derivatized tryptic and Glu-C peptides were separated by using a Dionex UltiMate 3000 nano RSLC system (Sunnyvale, CA, USA). An Acclaim PepMap RSLC C18 (75 μm×15 cm, 2 μm particle size) column (Santa Clara, CA, USA) was used. A pre-concentration column [Dionex Acclaim PepMap100 C18; Santa Clara, CA, USA (75 μm×2 cm, 3 μm particle size)] was used. The mobile phases were 0.1 % formic acid in water (Eluent A) and 0.1 % formic acid in acetonitrile

(Eluent B) applied in a gradient mode. The gradient applied was: 0–5 min, 5 % eluent B; 5–60 min, linear increase to 50 % eluent B at 0.3 μL/min. Data-dependent analyses were performed for all LC-MS/ETD runs by first acquiring an ESI mass spectrum (*m/z* 400–2000), then six ETD analyses were performed on the six most abundant ions in the ESI mass spectrum. The injection concentration and volume were 1 μM and 1 μL, respectively.

Result and Discussion

The purpose of this study was aimed at enhancing the ETD fragmentation efficiencies of peptides after conversion of the carboxylate groups to amides or to amides bearing quaternary amines. This derivatization procedure served the two-fold purpose of replacing the most acidic groups [C-terminus, glutamic acid (E) and aspartic acid side chains (D)] with more basic groups or ones with fixed charges, thus increasing the charge states of the peptides, as well as adding a readily trackable mass tag to facilitate assignment of fragment ions. A series of four carboxylate derivatization reagents were used (see Figure 1a), and all could be readily coupled to peptides via the simple one-step procedure outlined in Figure 1b. For the present study, the efficiencies of the reactions and changes in the charge states of the peptides were evaluated, along with an assessment of the ETD mass spectra with respect to the numbers of diagnostic sequence ions produced and the overall dissociation efficiencies compared with that obtained for the nonderivatized peptides.

ETD of Model Peptides

First, a series of model peptides, both the ones without and with aspartic acid and glutamic acid residues, were derivatized using the four different reagents. Upon modification of the carboxylates, the peptides undergo mass shifts of 89 Da for benzylamine (BA), 158 Da for benzylpiperazine (BZP), 85 Da for (2-aminoethyl)trimethyl ammonium (AETMA), and 114 Da for carboxymethyl trimethylammonium chloride hydrazide (Girard's reagent T, GT), for each modification site. In order to estimate the derivatization efficiencies, two peptides, ASHLGLAR and RQpSVELHSPQSLPR, were subjected to the derivatization procedure using the AETMA reagent, then the resulting mixture was analyzed by LC-MS. The reconstructed total ion chromatograms are illustrated in Supplemental Figure 1. The efficiencies of the derivatization reaction were estimated to be approximately 70 % or greater based on the peak areas of the derivatized and nonderivatized peptides. Extensive reaction optimization did not lead to significant further improvements in derivatization efficiencies (not beyond 70 % for some of the peptides), a factor that should be considered when adopting the strategy for more complex biological samples. As discussed below, the drawback of the non-quantitative derivatization efficiency may be offset by the improved ETD dissociation efficiencies.

The impact of the derivatization reactions on the charge states of the peptides was monitored by calculating the average charge state and maximum charge state after each of the four reactions relative to the charge states of the nonderivatized peptides. The results are shown in bar-graph form in Supplemental Figure 2 for eight representative peptides in which the average charge state is plotted, with the maximum charge state shown on the top of each bar. For the most part, each derivatization reaction increases the maximum charge state by one charge, an outcome that is consistent with conversion of the acidic C-terminus to either a more basic group that can be protonated upon ESI (for BZP or BA) or a fixed positive charge site (for AETMA and GT). The increase in average charge state is most dramatic for the AETMA- and GT-derivatized peptides, with an average increase in charge state of one. Coupling the peptide with BA did not result in a significant increase in charge state, presumably due to the modest basicity of the BA moiety. Derivatization with BZP, which like BA does not attach a fixed charge but incorporates a very basic tertiary amine moiety, resulted in a net shift in charge state on par with the two reagents that attached fixed charge quaternary amines.

The series of nonderivatized and derivatized peptides were subjected to ETD, and the spectral results were evaluated in terms of the number of diagnostic fragment ions (generally *c* and *z* ions) and the dissociation efficiencies. Examples of the ETD mass spectra obtained for ASHLGLAR (2+) and its AETMA-derivatized counterpart (3+) are shown in Figure 2. The fragment ions that retain the AETMA modification are labeled accordingly in Figure 2. In addition to the formation of *c* and *z* ions, charge reduction also occurs as a prominent process for both the nonderivatized and derivatized peptides. As exemplified by the data in Figure 2a, the nonderivatized peptides generally yielded fewer fragment ions upon ETD and lower sequence coverage. The ETD spectra of the AETMA-derivatized peptides typically displayed an entire series of *z*-type fragments and nearly all of the *c*-type ions (Figure 2b). The ETD spectral quality obtained for the other peptides is similar, and the results for the total number of *c*-type and *z*-type fragment ions are summarized in bar graph form in Figure 3 and the overall ETD efficiencies are shown in Figure 4.

As presented in Figure 3, the derivatized peptides generally produced a greater number of diagnostic fragment ions than the nonderivatized peptides. For example, nonderivatized pyr-LYENKPRRPYIL (3+) produced 17 *c/z* fragment ions; however, the corresponding AETMA-derivatized and GT-derivatized peptides yielded 29 and 24 informative fragment ions, respectively. The derivatization process tended to increase both the number of *c*- and *z*-type fragment ions, although the number of *z*-type ions increased more dramatically than the number of *c*-type ions. The specific distribution of the number of *c* and *z* ions for three peptides is summarized in Table 1. This enhancement of *z*-type ions is rationalized by the fact that the added fixed charge is located at the C-terminus, thus favoring the formation of charged *z*-type ions. The difference in preferential formation of *c*- and *z*-type ions is less

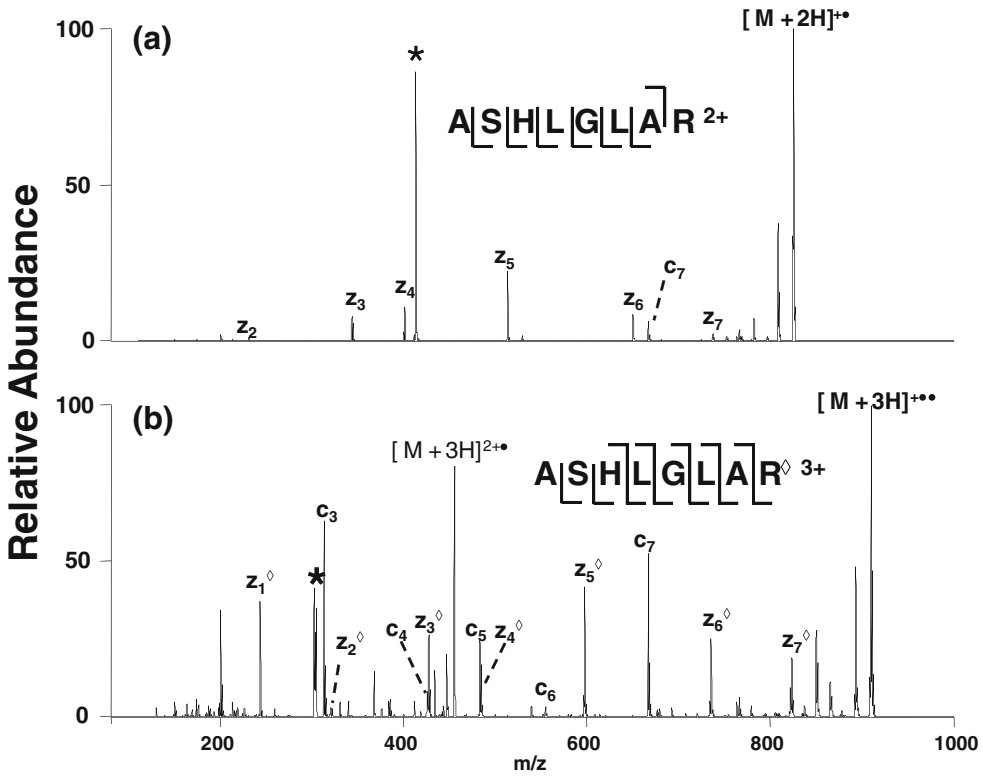

Figure 2. ETD mass spectra for ASHLGLAR **(a)** doubly protonated nonderivatized and **(b)** triply protonated AETMA-derivatized. Those fragment ions that retain the AETMA-modification are denoted by superscript ◊. The selected precursor ions are indicated by*

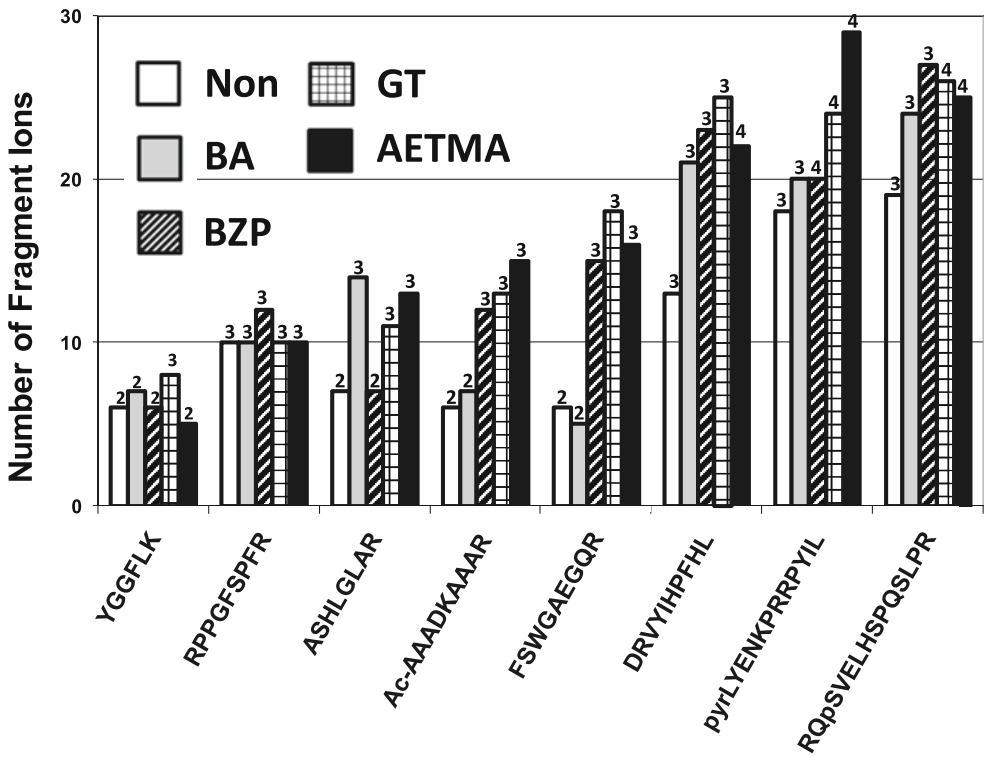

Figure 3. Total number of c and z fragment ions produced by ETD for the model peptides, either nonderivatized or derivatized with BA, BZP, GT, or AETMA. The number on top of each bar represents the maximum charge state which was the one used for ETD

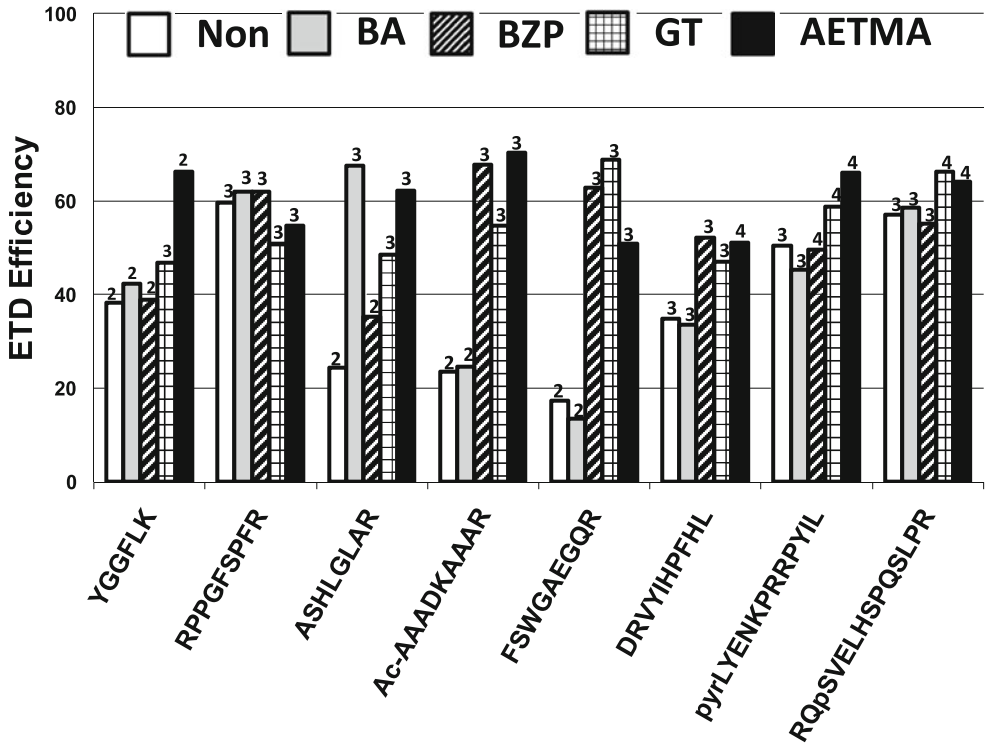

Figure 4. Overall ETD efficiencies for the model peptides, either nonderivatized or derivatized with BA, BZP, GT, or AETMA. The number on top of each bar represents the charge state of the peptide selected for ETD

dramatic for those peptides that also have a very basic arginine residue closer to the N-terminus for which a proton may be more readily sequestered on the arginine.

The peptides that contained amino acids with acidic side chains (D, E) had the potential to undergo multiple derivatization reactions. However, the abundances of these multi-derivatized products were too low to allow extensive analysis or make them feasible for use in proteomics applications. As noted in Table 2, the number of fragments that incorporate modifications at D/E residues is lower than the number of fragments which specifically incorporate the modification at the C-terminus. For example, although the peptide RQpSEVELHSPQSLPR has two acidic side chains in the peptide sequence, no fragments from glutamic acid derivatization were detected upon ETD of the singly derivatized peptide (i.e., none of the *c* ions contained the AETMA mass shift). Thus, neither multi-derivatized products nor ones which were modified at the acidic side chains were considered further due to their low abundance.

The ETD efficiencies of the peptides are displayed in Figure 4. The ETD efficiencies were calculated according to the following formula:

$$\text{ETD Efficiency} = \frac{\Sigma_i F_i}{P + \Sigma_i F_i}$$

where P is the abundance of the surviving precursor ion plus all charge reduction products, and F_i is the abundance of each *c*- and *z*-type fragment ion. The results in Figure 4 confirm the benefits of the carboxylate derivatization

Table 1. Number of c/z Ions for Each Model Peptide

	m/z	# c ions	# z ions
RPPGFSPFR			
Nonderivatized (+3)	353	5	5
BA (+3)	383	5	5
BZP (+3)	407	5	5
GT (+3)	392	5	4
AETMA (+3)	382	5	5
ASHLGLAR			
Nonderivatized (+2)	412	1	6
BA (+3)	305	5	7
BZP (+2)	491	0	6
GT (+3)	313	4	6
AETMA (+3)	303	5	7
DRVYIHPFHL			
Nonderivatized (+3)	433	7	6
BA (+3)	463	10	10
BZP (+3)	485	11	11
GT (+3)	469	10	13
AETMA (+4)	346	9	13

Table 2. Number of c/z Fragments Containing the AETMA Modification Specifically Located at the C-terminus or at D/E Side Chains

AETMA Derivatization	C-terminal	D/E
Ac-AAADKAAAR	16	1
FSWGAEGQR	20	2
DRVYIHPFHL	15	7
pyrLYENKPRRPYIL	22	9
RQpSEVELHSPQSLPR	25	0

strategy. Although the ETD efficiencies of the BA- and BZP-derivatized peptides increase by approximately 6 %–14 % on average compared with the nonderivatized peptides, the ETD efficiencies of the AETMA- and GT-derivatized peptides increase on average by 17 % to 22 %. As a specific example of the more pronounced improvements upon AETMA-derivatization, the ETD efficiency of AETMA-FSWGAEGQR was 51 %, which was more than double the dissociation efficiency of the nonderivatized peptide. Some of the peptides showed more dramatic changes in ETD efficiencies than others in Figure 4 (for example, note the relatively unimpressive change in ETD efficiencies for RQpSVELHSPQSLPR), but overall the best carboxylate reagent was AETMA in terms of giving consistently good derivatization and ETD efficiencies, as well as total number of diagnostic fragment ions produced.

LC-MS/ETD of Trypsin and Glu-C Digests of Cytochrome c

The results from the derivatized model peptides summarized above revealed that attachment of fixed charge moieties (i.e., AETMA) via carboxylate derivatization generally led to production of more ETD fragments. Thus, this method was applied for the ETD analysis of tryptic and Glu-C peptides from cytochrome *c*. For this phase of the study, the best-performing reagent, AETMA, was used for the carboxylic acid derivatization after enzymatic digestion of the protein. Two proteases (trypsin and Glu-C) were used to create mixtures of peptides, with trypsin selected because of its general popularity for proteomics applications and Glu-C chosen because Glu-C digestion results in production of peptides possessing acidic residues (D or E) at the C-terminal. This means that the AETMA-moiety will more likely be located at the C-terminus, and the C-terminal but not N-terminal fragment ions will contain AETMA upon MS/MS. Examples of the ETD mass spectra obtained from cytochrome *c* for two representative peptides are illustrated in Figure 5 and Supplemental Figure 3, in which only six and seven c- and z-type ions are detected for the underivatized peptide (2+). In contrast, derivatization with AETMA results in production of triply charged ions, and the resulting ETD mass spectra display nearly all possible c- and z-type fragments. Derivatization with AETMA also generally decreases the retention times of the peptides. Although this is not necessarily a significant shortcoming for routine protein identification, it means that some of the peptides may elute in the earlier part of the gradient (more aqueous), thus reducing their ionization efficiencies.

The ETD efficiencies for several peptides from cytochrome *c* are summarized in Figure 6 for the nonderivatized and analogous AETMA-derivatized peptides. Although many of

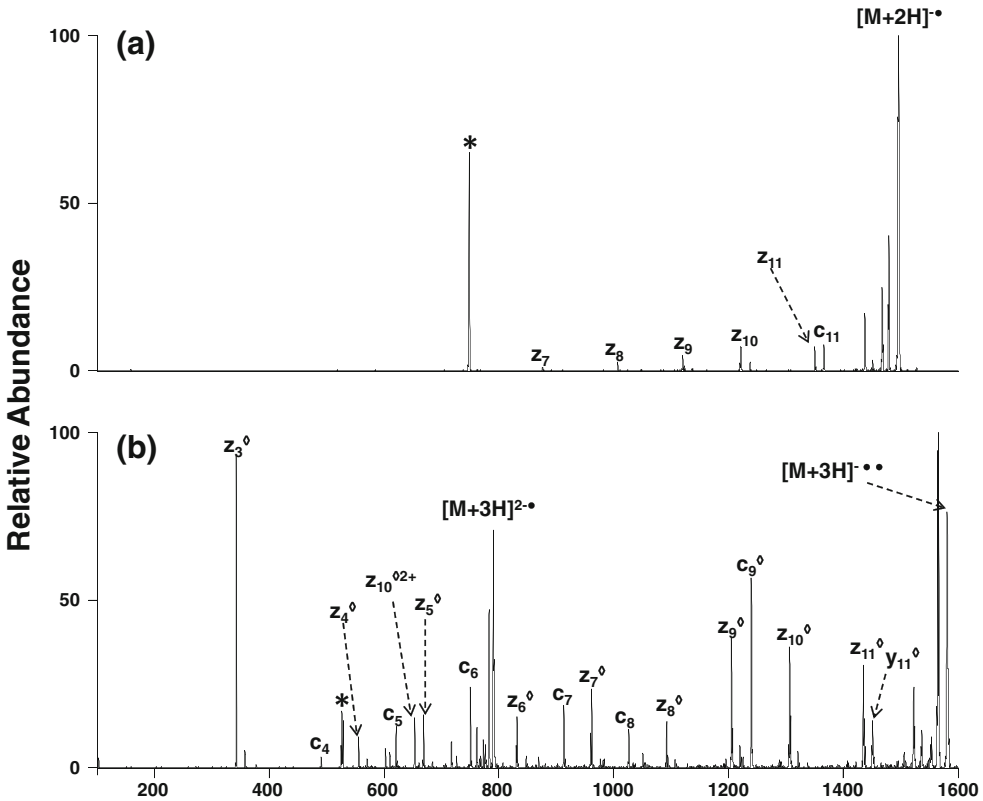

Figure 5. ETD mass spectra for the EETLMEYLENPK peptide from tryptic digestion of cytochrome *c* **(a)** doubly protonated nonderivatized and **(b)** triply-charged AETMA-derivatized. AETMA-derivatization is denoted by superscript of ◊. The selected precursor ions are indicated by *

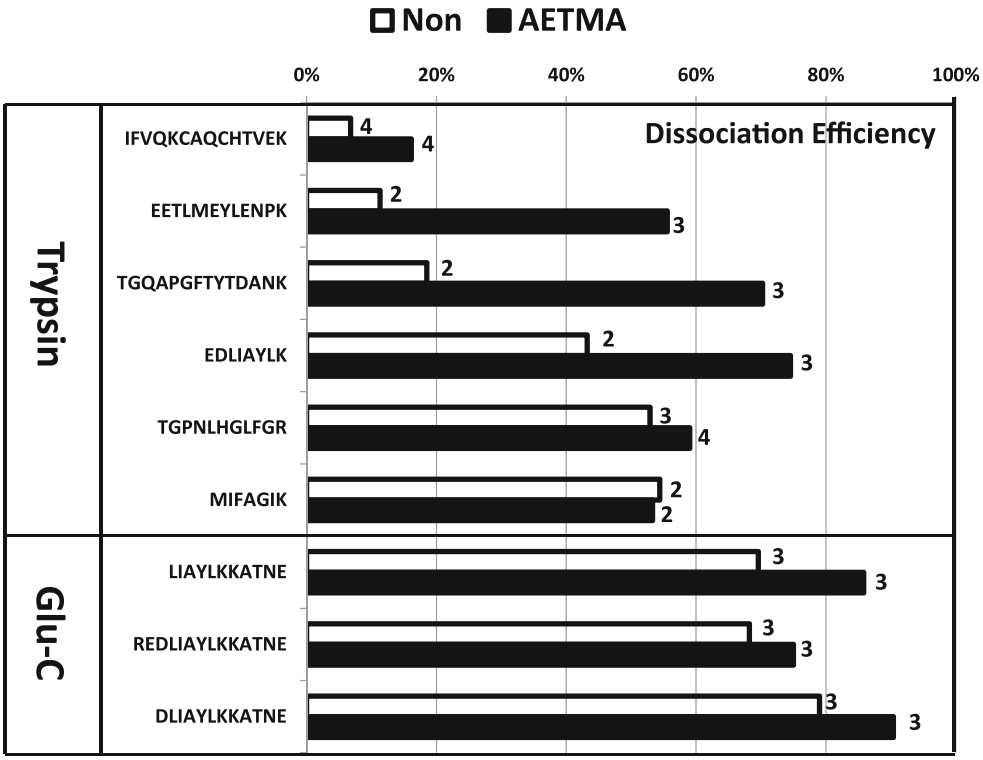

Figure 6. Overall ETD efficiencies for peptides from cytochrome *c* protein after trypsin or Glu-C digestion; either nonderivatized or derivatized with AETMA. The number next to each bar represents the charge state of the peptide selected for ETD

the peptides have two or three potential derivatization sites (E/D or C-termini), only the C-termini derivatized peptides were included in the summary in Figure 6. SEQUEST search was performed with the inclusion of dynamic C-terminal and D/E derivatization. In every case, the derivatized peptides generated higher ETD efficiencies than the nonderivatized tryptic peptides. For example, the ETD efficiency for TGQAPGFTYTDANK was 20 % for the nonderivatized peptide, but the ETD efficiency was greater than 70 % for AETMA-derivatized peptide. The numbers of ETD fragment ions from the tryptic peptides are tabulated in Supplemental Figure 4. Several peptides (e.g., EETLMEYELNPK, TGQAPGFTYDANK, EDLIAYLK, and DLIAYLKKATNE) showed significant increases in the total number of *c*- and *z*-fragment ions after AETMA-derivatization. For example, AETMA-TGQAPGFTYTDANK produced 19 more *c/z* ions upon ETD than the nonderivatized peptide.

The LCMS/ETD data for the cytochrome *c* digests were analyzed by using Proteome Discoverer in order to investigate the impact of derivatization on the Xcorr scores in which higher confidence is associated with higher scores. Table 3 summarizes the results obtained using SEQUEST with triplicate runs. The maximum Xcorr and total Xcorr scores increased for the AETMA-derivatized tryptic and GlucC digests relative to the scores obtained for the corresponding nonderivatized digests. The increase of the Xcorr scores indicates higher confidence in the protein and peptide identification, and thus the AETMA-derivatization procedure yields more informative MS/MS spectra than that of nonderivatized peptides. Application to biological samples is underway, and this will allow a more extensive assessment of the peptide derivatization efficiencies in complex mixtures (impact on sensitivity) relative to the gain in ETD efficiencies (impact on peptide identification).

Table 3. SEQUEST Analysis of LC-MS/ETD Results for Nonderivatized and AETMA-derivatized Peptides from Tryptic and Glu-C Digest of Cytochrome *c*

	Cytochrome *c*			
	Trypsin		Glu-C	
	Nonderivatized	AETMA	Nonderivatized	AETMA
# of peptide	26	49	7	13
Σ peptide Xcorr	40±8	71±10	10±4	20±3
Max peptide Xcorr	6.1	6.9	4.9	6.6

Conclusions

We have demonstrated that the carboxylate derivatization of peptides represents a viable modification strategy for improving ETD efficiencies, and the derivatization and subsequent purification procedures should be amenable for routine protein identification utilizing commercially-available reagents and instrumentation. The derivatization reactions utilizing the BA, BZP, GT, and AETMA reagents demonstrated high reaction efficiencies, estimated at 50 % or greater, thus affording ample ion abundances for subsequent ETD experiments as well as increasing the average peptide charge states by one. Additionally, the carboxylate-derivatized peptides exhibited higher ETD efficiencies relative to underivatized peptides. In particular, the GT and AETMA reagents, both which incorporate fixed charges, generated the highest ETD efficiencies along with greater numbers of diagnostic fragment ions in comparison to the BA and BZP reagents or the underivatized peptides. For the peptides produced from cytochrome *c*, derivatization with the AETMA reagent resulted in improved ETD efficiencies and greater *c* and *z* ion populations compared with the underivatized peptides.

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