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Simplifying Fragmentation Patterns of Multiply Charged Peptides by N-Terminal Derivatization and Electron Transfer Collision Activated Dissociation

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N-terminal peptide derivatization strategies used in conjunction with tandem mass spectrometry to yield simplified fragmentation patterns have shown limited success for the *de novo* sequencing of multiply charged peptides, including those predominantly formed in LC−ESI-MS experiments. Significant proton mobilization occurs for multiply charged peptides upon collisional activation, resulting in the formation of both N-terminal and C-terminal product ions rather than an exclusive series of C-terminal ions preferred for *de novo* sequencing algorithms. To circumvent this problem, multiply charged, N-terminally derivatized peptides were subjected to electron transfer reactions with fluoranthene anions to produce singly charged, radical species. Upon subsequent "soft" collision induced dissociation (CID), highly abundant z-type ions were formed nearly exclusively, which yielded simplified fragmentation patterns amenable to *de novo* sequencing methods. Furthermore, the derivatized peptides retained labile phosphoric acid moieties, and the enhanced set of z ions were also observed for peptides not possessing basic C-terminal residues, a type of peptide that poses more challenges to traditional simplification methods based on collision activated dissociation. This improved LC−MS^n strategy was demonstrated for a variety of multiply charged model peptides and a tryptic digest of myoglobin.

Mass spectrometry (MS) has increasingly become the analytical method of choice in the field of proteomics in recent years due to significant advances in instrumentation, sampling techniques, and data interpretation algorithms. Although there have been tremendous inroads, there also remain some unresolved limitations in practical applications. For example in a recent study, 24 proteomics laboratories were given a sample of *Escherichia coli* spiked with 20 proteins and of these participating laboratories, only six correctly identified the 20 proteins.[1] While discouraging, these results were a significant improvement compared to a similar study conducted the previous year. One key problem resulting in protein misidentification is a high false positive rate in computer-guided interpretation of tandem mass spectra. In a typical "bottom-up" approach, proteins are first enzymatically digested, and then tandem MS is performed on the resulting peptides. *De novo* algorithms or cross-correlation scoring methods can then be used to decipher the tandem mass spectra, assign peptide sequences, and integrate the data sets to identify proteins.[2−8] However, often the tandem mass spectra are extremely complex with many redundant product ions that cause errors in spectral interpretation,[9−11] leading to incorrect identification of proteins or unidentified proteins. To enhance the success of *de novo* sequencing methods, measures have been taken to reduce the complexity of product ion spectra. Keough et al. first reported the derivatization of the N-terminus of peptides with sulfonic acid groups, which upon dissociation yield mass spectra with an extensive array of C-terminal ions (e.g., y ions) void of neutralized N-terminal ions (e.g., b ions).[12] This technique greatly improved the accuracy of *de novo* peptide sequencing methods. A number of other studies have described the potential merits of other derivatization procedures for proteomic applications.[10,11,13−34] For example, the N-terminal sulfonation reagent 4-sulfophenyl isothiocyanate (SPITC) in con-

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junction with ESI-MS/MS and MALDI-MS/MS has proved to be useful identifying sites of ubiquitination.[18,19] Our group has used N-terminal sulfonation with infrared multiphoton dissociation (IRMPD) to improve *de novo* sequencing of peptides by eliminating the low mass cutoff inherent to ion traps.[11] Recently, we have synthesized a new N-terminal reagent with a highly IR-absorbing phosphonite group that increased the dissociation efficiencies and sequence information obtained upon IRMPD.[35]

One major drawback to the use of N-terminal derivatization strategies is that the simplification of the fragmentation patterns of peptides is substantially impaired when multiply charged precursor ions are analyzed.[14,15,18,20] For example, when doubly charged SPITC-modified peptides are dissociated, the N-terminal b ions are no longer neutralized due to the presence of the extra mobile proton, and the utility of the derivatization procedure is greatly reduced.[14] This is particularly problematic for LC−ESI-MS applications in which tryptic peptides are typically multiply protonated (i.e., charge states of 2+ and greater).[36] Lee et al. described a method to simplify the fragmentation patterns of these multiply charged ions by using isotopically labeled SPITC, but extra derivatization steps and more elaborate spectral interpretation were needed.[15] Our group has attached UV chromophores to the N-terminus of peptides, and the modified peptides almost exclusively produced y ions upon ultraviolet photodissociation (UVPD), a striking selectivity attributed to the secondary dissociation and rapid annihilation of the UV chromophore-containing b ions.[34] However, because of the slow repetition rate of the laser (10 Hz) and the requirement for multiple laser pulses used in this application, analysis on a chromatographic time scale could not be achieved.

In recent years, other ion activation methods have been developed as alternatives to collision induced dissociation (CID, also known as CAD). Electron-based dissociation techniques such as electron capture dissociation (ECD)[37] in FTICR instruments and electron transfer dissociation (ETD)[38] in ion traps have shown to be particularly promising for characterization of post-translational modifications (PTMs)[38−45] and for increasing peptide sequence coverage.[36,46] Both methods involve the reaction of multiply charged cations with either low-energy electrons (ECD) or radical anions (ETD), producing complementary c- and z-type backbone cleavages that retain labile PTMs such as phosphorylation. For peptides of lower charge, however, these electron-based methods yield charge-reduced radicals from the precursor as the most abundant products with low yields of c and z products.[36,47] Recently, both the Coon and McLuckey groups have implemented supplemental collisional activation (i.e., low-energy CID) of charge-reduced peptide radicals after the electron transfer reaction (ETcaD), thus affording significantly improved peptide sequence coverage.[47−49] Both ETD and ECD are promising new alternatives to CID for sequencing peptides, but the resulting spectra remain rather complex with both N-terminus and C-terminus product ions that can complicate *de novo* interpretation.

In this study, doubly protonated peptides derivatized at the N-terminus via reactions with 4-sulfophenyl isothiocyanate (SPITC) or 4-(chlorosulfonyl)phenyl isocyanate (SPC) (Supplemental Figure 1) were converted to charged-reduced radical species after gas-phase electron transfer (ET) reactions with fluoranthene radicals. The resulting singly charged radical species were then subjected to subsequent collisional activation (ETcaD), which produced an enhanced series of z ions void of N-terminal ions. This spectral simplification strategy was performed using a linear ion trap on a liquid chromatographic time scale, and improved *de novo* sequencing was realized for a variety of multiply charged peptides including phosphorylated species.

EXPERIMENTAL SECTION

Materials. Myoglobin and cytochrome c from equine heart and all reagents were purchased from Sigma-Aldrich (St. Louis, MO). The peptides KRPPGFSPFR and ASHLGLAR were obtained from BACHEM (King of Prussia, PA), the phosphorylated peptides KRpTIRR and TRDIYETDYpYRK from AnaSpec (San Jose, CA), and immobilized TPCK-treated trypsin beads from Pierce Biotechnology, Inc. (Rockford, IL). All solvents were purchased from Fisher Scientific (Fairlawn, NJ).

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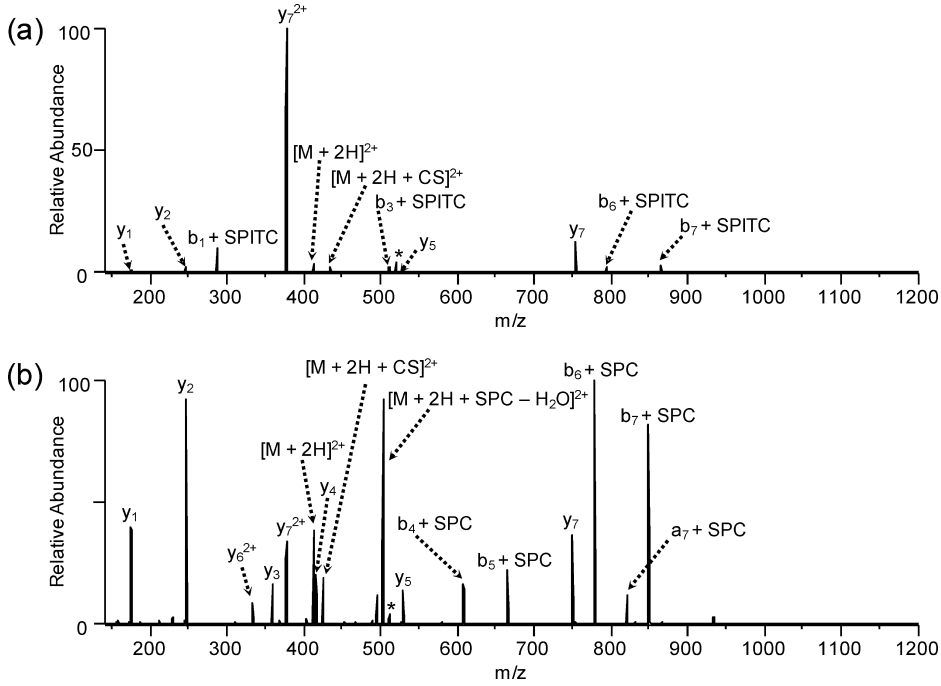

Figure 1. CID mass spectra of the doubly charged peptide ASHLGLAR (a) derivatized with SPITC, 12 mV collision voltage, $q = 0.25$; (b) derivatized with SPC, 14 mV collision voltage, $q = 0.25$. The unmodified peptide is denoted by M and the precursor ion by an asterisk (*). The partial decomposition of the SPITC or SPC moiety resulting in a bonded carbon and sulfur atom attached N-terminally to the peptide is denoted by CS.

Derivatization and Sample Preparation. Before N-terminal derivatization, lysines were converted to homoarginine residues through a guanidination step (except where noted) to ensure modification with 4-sulfophenyl isothiocyanate (SPTIC) exclusively at the N-terminus and not at the ε-amine group of lysines. A stock of 0.05 g of *O*-methylisourea in 50 μL of water was prepared, and 5 μL of this stock solution, 50 μL of 5 N ammonium hydroxide, and approximately 10 nmol of the protein digest or model peptide was combined. This mixture was then incubated for 10 min at 65 °C, after which the sample was desalted via C_{18} spin columns.

The SPITC and 4-(chlorosulfonyl)phenyl isocyanate (SPC) derivatization procedures used in this study were adapted from previous reports.[16,50] Briefly, a stock solution of SPITC was made by dissolving approximately 1 mg of SPTIC in 100 μL of a 20 mM $NaHCO_3$ solution (pH ~9.5). A 20 μL aliquot of this stock was combined with 10 nmol of peptide or protein digest, which was then incubated for approximately 30 min at 55 °C. For SPC-modified peptides, a stock solution was made by dissolving approximately 1 mg of SPC in 100 μL of acetonitrile. A 20 μL aliquot of this stock was combined with 10 nmol of peptide in a 50:50 pyridine/water (v/v) solution, which was then incubated for approximately 30 min at ambient temperature. After incubation, all samples were cleaned up using C_{18} spin columns. For direct infusion experiments, working solutions of 10 μM peptides in 49.5:49.5:1.0 water/methanol/acetic acid (v/v/v) solution were prepared before infusion at a flow rate of 3 μL/min into the mass spectrometer.

For protein samples, 100 μL of immobilized TPCK-treated trypsin beads and 10 μmol of ammonium bicarbonate were used to digest 10 nmol of protein prior to the derivatization procedures. Digestion occurred for 18 h at 37 °C, and the beads were discarded after digestion.

Mass Spectrometry and Liquid Chromatography. Mass spectrometric analysis was performed on a Thermo Fisher (San Jose, CA) LTQ XL two-dimensional linear ion trap mass spectrometer equipped with an ETD unit. Fluoranthene anions were introduced as the electron-transfer reagent for ETD experiments with reaction times of 100 ms. Standard parameters (*q*-value equal to 0.25 and activation times of 30 ms) were used for all CID experiments. ETcaD was carried out using ET reaction times of 100 ms, followed by isolation of the charge-reduced radical, and a 30 ms CID period. For all MS^n experiments, a product ion was considered to be detected in an ETD, CID, or ETcaD mass spectrum if the ion peak had a signal/noise ratio equal to or greater than 3.

A Hitachi L-7000 (Hitachi Ltd.) analytical HPLC system was used for liquid chromatographic separations. Samples containing 10 μM digested protein were injected (10 μL) onto a Symmetry300 reversed-phase C_{18} column (Waters, Milford, MA) (2.1 mm × 50 mm, 3.5 μm packing) with a matched guard column (2.1 mm × 10 mm, 3.5 μm packing). Eluents consisted of 0.2% formic acid in water (A) and 0.2% formic acid in acetonitrile (B). Gradient elution was performed as follows: 95% (A) for 2 min followed by a linear gradient to 40% (A) over 60 min at a flow rate of 0.300 mL/min. Data-dependent aquisition was used for all LC−MS^n analysis with the first scan event being a full mass spectrum at a m/z range of 400 − 2000 and subsequent events consisting of ETD, CID, and ETcaD of the one or two most abundant peaks. A normalized collision energy of 35% (61−142 mV) was used for all LC−CID-MS analyses.

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RESULTS AND DISCUSSION

N-Terminally Derivatized Multiply Charged Peptides. Two commonly used commercial reagents (e.g., SPITC and SPC) were used for N-terminal derivatization reactions of the peptides, resulting in a mass addition of 215 Da for SPITC and 199 Da for SPC. The structures of both reagents, SPITC and SPC, are shown in Figure 1 in the Supporting Information. As noted previously, the chlorine atom in SPC is hydrolyzed and replaced with a hydroxyl group in aqueous solution.[16] The sulfonic acid group present in both reagents introduces a negative charge at the N-terminus, thus reducing the overall charge of the peptides and simplifying the tandem mass spectra by effectively neutralizing N-terminal product ions (e.g., b, a, and c ions). This simplification of the product ion spectra of singly charged sulfonate-derivatized peptides has been documented previously and has proven to be especially beneficial for *de novo* sequencing strategies.[10−12,26] Upon CID, an enhanced set of C-terminal ions (y ions) are produced, and redundant N-terminal products (b, a ions) are formed as neutral species and are not detected. However, when more than one positive charge resides on the derivatized peptide, the N-terminal products formed upon CID may retain a charge, thus nulling the benefit of the derivatization process for spectral simplification. This increased spectral convolution is illustrated in Figure 1 for the doubly charged derivatized peptide ASHLGLAR in which N- and C-terminal product ions of multiple charge states are detected. Upon ESI, the N-terminal derivatized peptide ASHLGLAR was predominantly observed in the 2+ charge state (data not shown). CID of the doubly charged ions yielded complex spectra containing both y and redundant a and b ions (with these N-terminal ions retaining the original SPITC- or SPC-modification) (see parts a and b of Figure 1). Moreover, product ions in multiple charge states are observed, as is the case for the y_7 ion, which further complicates spectral interpretation. This loss in spectral simplification for multiply charged peptides is the primary reason that N-terminal sulfonation reactions have mainly been employed for MALDI-MS, not ESI-MS, applications.

An alternative approach, as described in the present study, relies on using ETD (via ETcaD) rather than CID for characterization of the N-terminal derivatized peptides. In this case, doubly charged SPITC- or SPC-modified peptides were subjected to electron transfer reactions, producing low amounts of sequence ions and predominantly charge-reduced peptide radical cations (singly charged) as illustrated in Figure 2 in the Supporting Information. Subsequently, the resulting charged-reduced ions were subjected to collisional activation, a net process termed ETcaD, which yielded a simple set of C-terminal (z ions) without any redundant N-terminal ions (c, b, or a ions) (see Figure 2). The ETcaD spectrum of doubly charged SPITC-modified ASHLGLAR is illustrated in Figure 2a, displaying a complete series of z-type ions and no N-terminal ions. Some z product ions were observed as radicals and others as even electron ions, a result which is discussed in more detail later. Also, a product ion attributed to the loss of both the SPITC moiety and ammonia (net loss of 232 Da) was observed in all ETcaD spectra; this characteristic loss can be used as a marker for every SPITC-modified peptide. It is a spectral feature that is especially convenient when the derivatization of peptide mixtures might be incomplete, and one needs a facile means to screen the species of interest. As also seen in Figure 2a, product ions attributed to partial cleavage of the SPITC moiety are observed in the m/z range of 850−1040. These product ions are thought to stem from the low-energy, radical-driven dissociation processes of ETcaD since many of these ions were not observed upon CID of the singly charged, even-electron peptides. Regardless, these ions do not cause substantial spectral congestion as they do not overlap with the z ion series, and these were formed in low abundance.

ETD of SPITC- or SPC-derivatized peptides with more than two charges resulted in significant abundances of both c- and z-ions and increased sequence coverage than obtained upon ETD of the doubly charged SPITC- or SPC-derivatized peptides, which is analogous to the ETD behavior of unmodified peptides (i.e., more efficient formation of both c- and z- ions with increasingly higher precursor charge state). However, significant abundances of nondissociated charge-reduced peptides were also observed. Because the ETD spectra for the SPITC- and SPC-modified peptides in the higher charge states display both the N-terminal and C-terminal sequence ions, the resulting spectra contain redundant ions that are less amenable to *de novo* sequencing.

Formation of z_{n-1} versus y_{n-1} Product Ions. It has previously been shown that y_{n-1} is the most abundant product formed upon CID of SPITC-modified peptides, both singly and doubly charged.[14,15] This phenomenon is illustrated in Figure 1a in which both the singly and doubly charged y_7 ions dominate the CID mass spectrum obtained for doubly protonated SPITC-ASHLGLAR. This process is promoted by nucleophilic attack of the sulfur atom of the SPITC moiety on the carbonyl oxygen atom of the adjacent amino acid, resulting in an Edman-type degradation.[14,32] Derivatization of peptides with SPC, which is identical to SPITC except for substitution of an oxygen atom for the sulfur, alleviates the domination of the y_{n-1} ion due to the reduced nucleophilicity of the oxygen.[16] This leads to a more even distribution of y ions that is preferable for *de novo* sequencing (Figure 1b); however, both y and b series were produced upon CID of the doubly protonated, SPC-derivatized peptide which led to numerous redundant ions and a cluttered spectrum. These CID patterns are characteristic of other doubly charged, SPITC- and SPC-derivatized peptides: dominant formation of the y_{n-1} ion for the former and more importantly cluttered spectra for both; thus, neither was ideal for sequencing peptides in mixtures using an LC−ESI-MS^n approach.

In contrast, ETcaD of the SPITC-modified peptides using low supplemental collision voltages yielded a broad series of z ions and did not result in enhanced formation of the y_{n-1} (or corresponding z_{n-1}) product ions, a result that was surprising given the lability of the peptide bond that normally is cleaved to form the y_{n-1} ion upon CID (see Figure 2a with comparison to Figure 1a). However, ETcaD using a higher collision voltage produced a more abundant y_{n-1} ion, in addition to the series of z ions (Figure 2b). The y_{n-1} ion is easily identifiable because it is 16 Da greater in mass than the z_{n-1} ion, but its presence can largely be eliminated by using lower energy ETcaD conditions as demonstrated in Figure 2a. To optimize the formation of the preferred z-type ions over the low energy y_{n-1} product, the collision voltage and q-value were varied systemati-

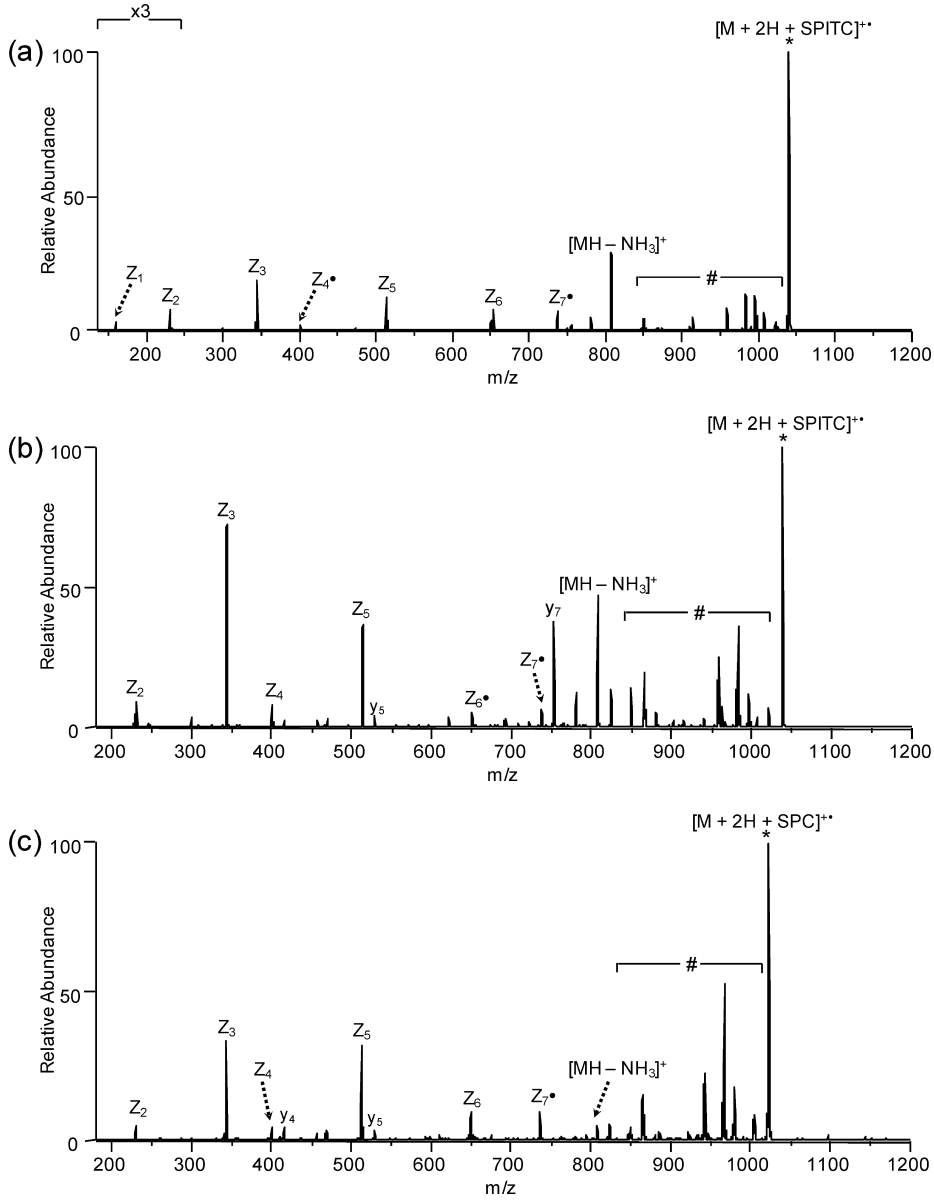

Figure 2. ETcaD mass spectra of the doubly charged peptide ASHLGLAR (a) derivatized with SPITC, 24 mV subsequent collision voltage, $q = 0.12$; (b) derivatized with SPITC, 42 mV subsequent collision voltage, $q = 0.16$; (c) derivatized with SPC, 42 mV subsequent collision voltage, $q = 0.16$. Product ions due to partial decomposition of the SPITC or SPC moiety are denoted by #. The unmodified peptide is denoted by M, and the charge-reduced radical precursor ion is denoted by *.

cally during the CID activation period. Figure 3 illustrates the resulting trend for formation of the z_{n-1} versus y_{n-1} ions for doubly protonated SPITC-modified ASHLGLAR. As seen in Figure 3, the z_{n-1} dominates at lower energy ETcaD conditions and the y_{n-1} ion dominates at higher energy ETcaD conditions (i.e., higher q-value and/or greater CID voltage). These experiments parallel Coon's initial ETcaD work where more b and y ions (instead of c and z) were seen at higher q-values and greater collision energies.[47] The absence of any y product ions using lower energy ETcaD conditions suggests that the radically driven dissociation pathway that leads to z-type ions could be a lower energy process compared to the process that leads to the formation of the y_{n-1} ion.

Another way to suppress the formation of the y_{n-1} ion is to use SPC as the derivatization reagent. Figure 2c shows the ETcaD mass spectrum of doubly charged SPC-modified ASHLGLAR, and no y_{n-1} ion was formed using any ETcaD conditions; however, some low abundance y ions (e.g., y_4 and y_5) were detected at high q-values and CID energies. Because the SPC derivatization procedure involves the use of a less desirable 50% pyridine solution and reaction yields were often low, SPITC was used as the N-terminal derivatizing reagent for the remainder of the study. The SPITC reaction procedure consistently produced the highest yields of modified peptides. For example, guanidination and SPITC derivatization of the tryptic digest of myoglobin (described in more detail in subsequent sections) resulted in 100% conversion of unmodified peptides to modified species.

LC−MS^n Analysis of SPITC-Derivatized Tryptic Peptides. To evaluate the analytical utility of the ETcaD method for protein characterization, myoglobin was selected as a model protein for tryptic digestion and SPITC derivatization. Under LC−MS conditions, most of the resulting SPITC-modified tryptic peptides were observed in the 2+ charge state upon ESI. Thus,

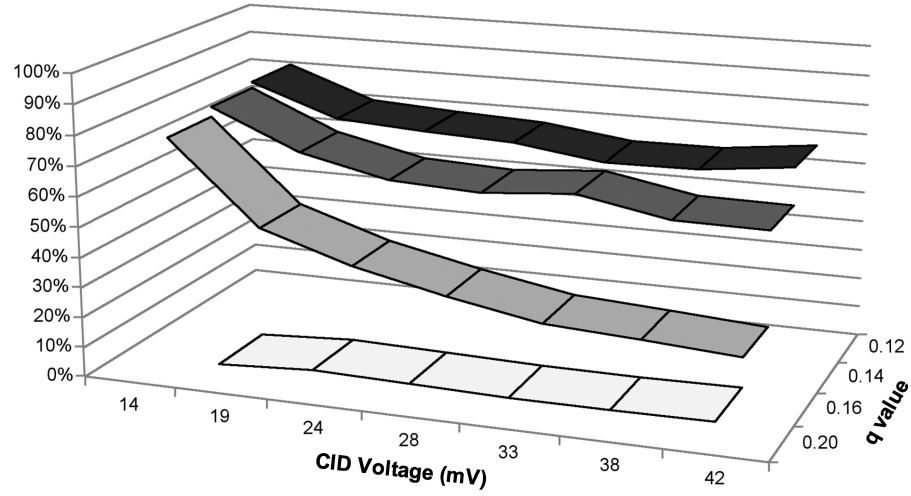

Figure 3. Percent abundance of z_{n-1} versus y_{n-1} (i.e., $z_{n-1}/(z_{n-1} + y_{n-1}) \times 100$) in ETcaD spectra as a function of CID voltage and *q*-value for doubly charged SPITC-ASHLGLAR.

for data-dependent LC−MS experiments, the doubly charged peptides were typically the ones subjected to ion activation. ETcaD, CID, and ETD spectra were collected in a data-dependent manner for the tryptic peptides in several charge states for separate LC runs of SPITC-derivatized and nonderivatized peptides. The peak areas of the product ions for all C-terminal ions and all N-terminal ions were summed separately and converted to percentages. The results are displayed in Figure 4 to allow ready comparison of the preference for formation of C-terminal (*z* ions) versus N-terminal product ions and other redundant ions such as ones in multiple charge states and redundant C-terminal species. The extreme C-terminal product ion selectivity for the ETcaD spectra obtained for the doubly charged SPITC-peptides is readily apparent, as demonstrated by the percentages of C-terminal ions ranging from 95.3% to 100.0% with an average of 97.1% in Figure 4. In comparison, CID of the doubly charged SPITC-modified peptides produced more complicated spectra containing both N-terminal and C-terminal ions. Three of the peptides (LFTGHPETLEK, FDKFK, and HLKTEAEMK) appeared to show high C-terminal ion selectivity upon CID, but this was due to production of very dominant y_{n-1} ions and not due to production of a comprehensive series of y ions as would be needed for effective *de novo* sequencing. Because of the limited m/z range of the ion trap (up to 2000 m/z), larger tryptic peptides such as YLEFISDAIIHVLHSK were unable to be analyzed by the ETcaD method after N-terminal derivatization. Although ETD alone and ETD with supplemental CID of unmodified peptides has been shown to increase the level of sequence coverage and also to promote retention of labile modifications in the product ions,[36,38,47] these electron transfer based methods yield spectra that are often much more complicated than traditional CID spectra because highly abundant c-, z-, and a-type ions are present as well as low abundance y ions. Thus, the SPITC derivatization procedure in conjunction with ETcaD, not ETD alone, is a key factor that leads to the preferential formation of C-terminal ions. Both ETcaD and CID can be set up as successive scan events during a single data-dependent LC−MS run; therefore, these methods can be used in a complementary rather than competitive mode to increase the overall sequence coverage. In the present study, the average sequence coverage obtained was 86% for all N-terminally derivatized peptides analyzed by ETcaD (shown in Figure 4) with two major factors that limited the sequence coverage: (1) the low mass cutoff (LMCO) of the ion trap instrument prohibited detection of the lowest m/z product ions and (2) miscleavages from proline residues inherent to ETD. The high sequence coverage obtained was comparable to previous reports of 89% coverage of unmodified peptides by ETcaD.[47] Also, the increased spectral simplification afforded by ETcaD of the SPITC-modified peptides could better unveil miscleavages stemming from the presence of proline residues based on characteristics gaps in the observed series of z ions. In summary, the combination of ETcaD with SPITC-derivatization affords the best outcome of high sequence coverage and most simplified spectra containing nearly exclusively z ions.

Phosphorylated Peptides. One of the biggest advantages of using electron-based dissociation techniques such as ETD is that labile modifications of amino acids generally remain intact, a factor that greatly aids in identifying PTMs.[38−45] However, loss of phosphoric acid is still a substantial dissociation pathway for phosphopeptides examined by ETcaD in a linear ion trap.[47] To circumvent this shortcoming, we evaluated the use of ETcaD for analysis of the N-terminal sulfonated phosphorylated peptide KRpTIRR. The ETcaD spectra for the SPITC-derivatized peptides (i.e., ET of the 2+ and 3+ charge states, then collisional activation of the resulting singly charged radicals; see parts a and b of Figure 5, respectively) displayed an enhanced series of z ions with minimal loss of phosphoric acid. Conversely, ETcaD of doubly charged unmodified KRpTIRR (Figure 5c), CID of singly charged SPITC-modified KRpTIRR (Figure 5d), and CID of doubly charged SPITC-modified KRpTIRR (Figure 5e) all showed abundant losses of phosphoric acid and lower sequence coverage.[51] A possible explanation for the minimized loss of phosphoric acid for SPITC-derivatized peptides in comparison to unmodified peptides upon

(51) When KRpTIRR was guanidinated before N-terminal derivatization, the already extremely basic peptide became even more basic with the conversion of the lysine to a homoarginine residue. This significantly reduced the abundance of the doubly charged ion, making it difficult to analyze; therefore, KRpTIRR was not guanidinated to ensure comparison of the ETcaD method with CID. This was the only lysine-containing peptide not guanidinated in this study.

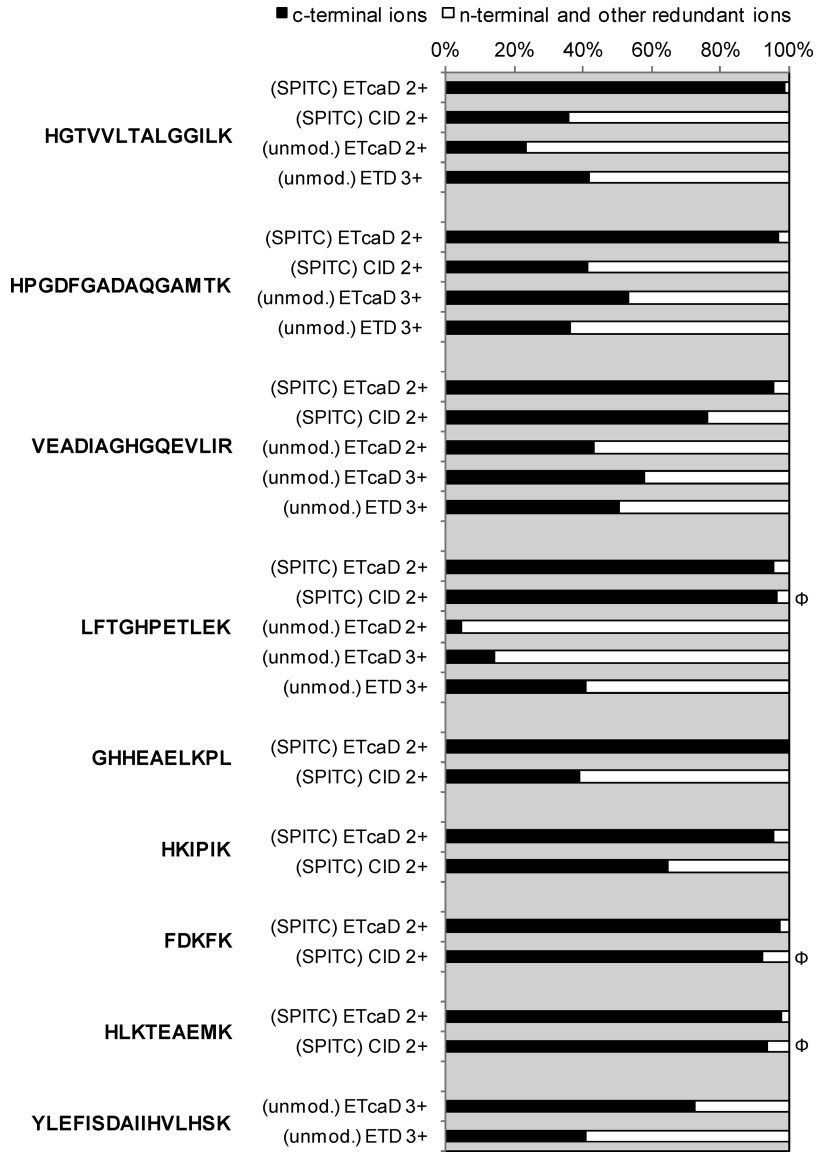

Figure 4. Comparison of ETcaD, CID, and ETD results for multiply charged tryptic peptides from myoglobin obtained by LC−MS^n. SPITC-derivitized peptides are denoted by (SPITC), and unmodified peptides by (unmod.). The peak areas of the product ions for all C-terminal ions and all N-terminal ions were summed separately and converted to percentages. All lysines are guanidinated for SPITC modified peptides. Φ denotes peptides with high apparent C-terminal ion selectivity upon CID but which was actually due to production of very dominant y_{n-1} ions and not due to production of a comprehensive series of y ions as would be needed for effective *de novo* sequencing.

ETcaD is that the loss of SPITC or SPTIC + ammonia moieties are even lower energy pathways in comparison to the loss of phosphoric acid, rendering the latter pathway less competitive for the SPITC-derivatized peptides.

The peptide TRDIYETDYpYRK was also N-terminally derivatized with SPITC and subjected to ETcaD analysis. Like the peptide KRpTIRR, SPITC-TRDIYETDYpYRK showed very high sequence coverage upon ETcaD with little neutral loss of phosphoric acid, which is illustrated for the triply charged species in Figure 3 in the Supporting Information. These results illustrate that the combination of N-terminal derivatization and ETcaD can simultaneously simplify spectra and aid identification of PTMs.

Benefits of Enhanced z Ions over Enhanced y Ions. Although ETcaD of multiply charged N-terminally sulfonated peptides and CID of singly charged N-terminally sulfonated peptides are complementary methods for data-dependent LC−MS analyses, there are significant advantages of the former method. One obvious advantage is that the more highly charged, larger peptides provide greater protein sequence coverage, and their fragmentation patterns can only be successfully simplified by the ETcaD technique. Also as previously described, the z ions produced during ETcaD retain labile PTMs. In contrast, CID of the even-electron, singly charged SPITC-derivatized peptides produced fragment ions which did not retain PTMs and offered lower sequence coverage for phosphopeptides. Furthermore, a highly basic residue (e.g., arginine or homoarginine) is needed at the C-terminus to enhance the y ions for CID analysis of SPITC-modified peptides.[14] Without these highly basic residues, the proton is not sequestered on the C-terminus (i.e., it is more mobile), resulting in a more diverse array of both C-terminus and N-terminus ions and lower sequence coverage. However, a highly basic arginine or homoarginine is not needed at the C-terminus when using ETcaD due to the different radical dissociation mechanism inherent to electron-based methods, yet the resulting

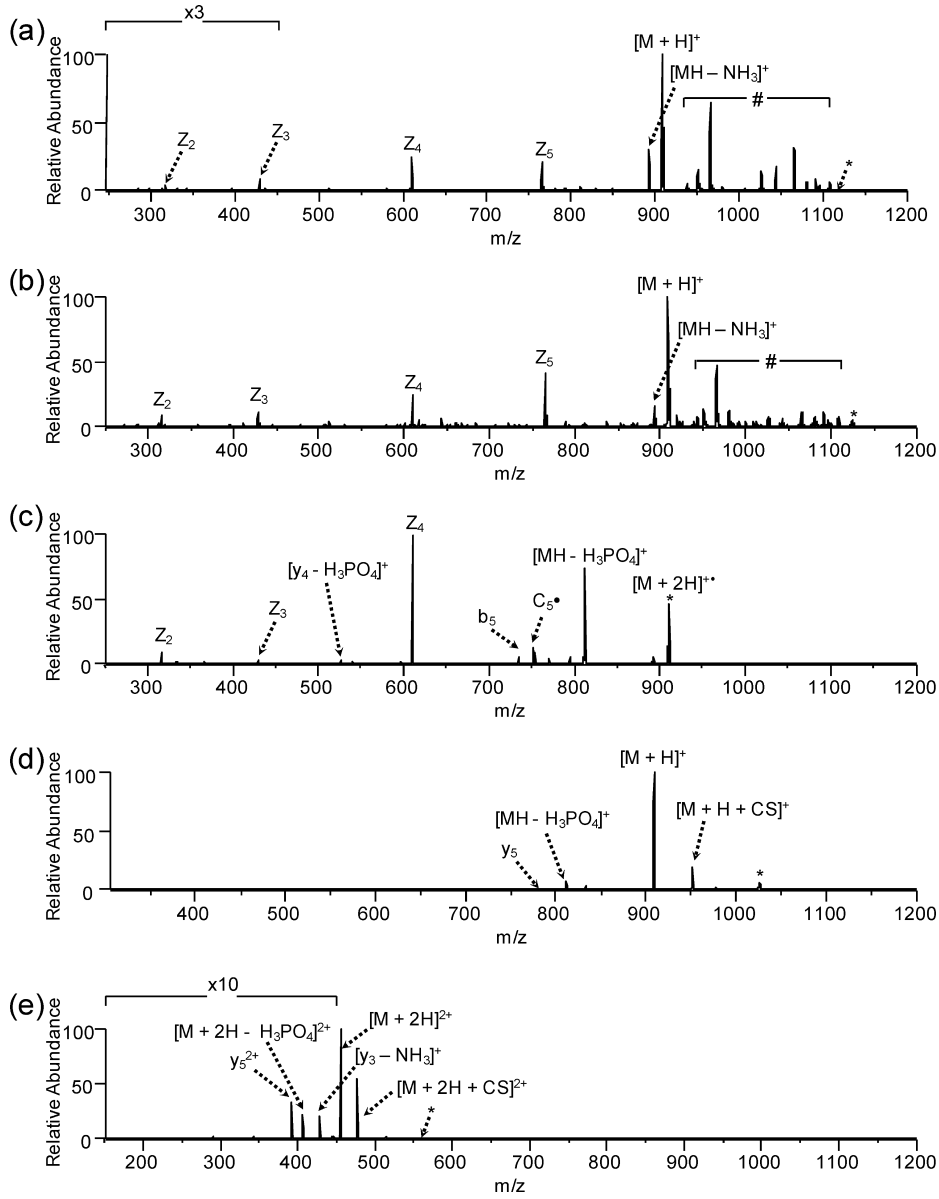

Figure 5. ETcaD of the phosphorylated peptide KRpTIRR (a) derivatized with SPITC (doubly charged), 25 mV subsequent collision voltage, $q = 0.20$; (b) derivatized with SPITC (triply charged), 30 mV subsequent collision voltage, $q = 0.20$; (c) unmodified (doubly charged), 21 mV subsequent collision voltage, $q = 0.20$; and CID of KRpTIRR (d) derivatized with SPITC (singly charged), 45 mV collision voltage, $q = 0.25$; (e) derivatized with SPITC (doubly charged), 14 mV collision voltage, $q = 0.25$. Product ions due to cleavage of a portion of the SPITC moiety are denoted by #, and precursor ions for CID spectra are denoted by *. The unmodified peptide is denoted by M.

spectra still offer high sequence coverage and yield a single dominant series of product ions. This advantage has already been seen for the peptide GHHEAELKPL in Figure 4. Therefore, the combination of ETcaD and N-terminal derivatization could potentially be used for spectral simplification of a variety of peptide mixtures created from proteins using enzymes that do not necessarily cleave after lysines and arginines.

Formation of Even Electron versus Radical z Ions. Since both even electron and radical z product ions are formed by ETcaD, it was relevant to assess the formation preference of these two types of z ions. These two types can generally be distinguished from each other as both the even electron and radical ion peaks are usually present at each z ion position and differ by one mass unit. However, the ability to predict the preference for formation of the radical or even electron z ions would enhance the success of *de novo* sequencing algorithms since some amino acids differ in mass by only 1 amu. To gain insight into the z ion distributions, area counts of all even electron and radical z ions were tabulated for each z ion (z_1, z_2, $z_3 \ldots z_{14}$) from 15 different peptides corresponding to a total of 23 different spectra, which included different charge states and two different CID q-values and collision voltages. Both SPITC-modified model peptides and SPITC-modified peptides from tryptic digests of myoglobin and cytochrome c were included in this facet of the study. The percentages of even electron z ions relative to the total amount of even electron and radical z ions are summarized in Figure 6 for each z_n product. As illustrated in Figure 6, there is a notable increase in the formation of even electron z ions as the length of the z ion decreases. The identity of specific amino acids adjacent to the cleaved amide bond had little or no impact on the extent of even electron z ion formation. However, further investigation with large data sets may be warranted. There was also no trend in the

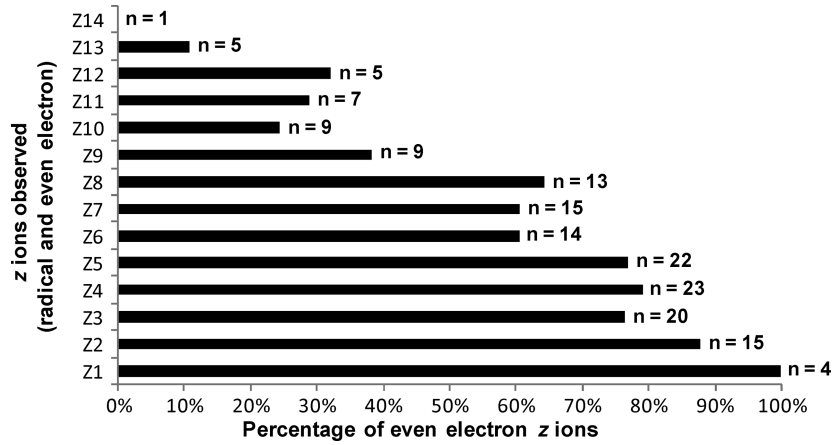

Figure 6. Percentage of even electron versus radical z ions as a function of z ion length for 15 different peptide precursor ions and a total of 23 different spectra. n = the total number of spectra that had observable z ions at each specified sequence position.

formation of one type of z ion when using lower or higher energy ETcaD conditions.

The proposed mechanism for the formation of even electron z species from z radical species has been described previously, and it involves the formation of a hydrogen bond between the z radical and a c even electron species (either in an intramolecular or intermolecular process).[52] The z radical strips a hydrogen atom from the c ion, forming an even electron z ion and a radical c product.[52] This pathway is consistent with the trend seen in Figure 6: the longer z ions may have greater opportunity to fold and form stabilizing intramolecular hydrogen bonds, thus suppressing hydrogen bond formation with c ions and thus reducing even electron ion formation. For the case of SPITC-derivatized peptides, the c products expected upon separation of the z and c species are neutralized instead of remaining positively charged.

CONCLUSIONS

ETcaD of multiply charged, N-terminally sulfonated peptides results in significant spectral simplification via almost exclusive formation of z-type ions. This method offers advantages not only compared to CID of N-terminally derivatized species but also compared to electron transfer based methods (i.e., ETD, ETcaD) of unmodified peptides. The enhanced series of z ions was shown to retain labile phosphoric acid moieties and also allow efficient sequencing of a broad range of peptides, including proline-rich ones and nontryptic species. High sequence coverage was attained for all peptides with the largest limiting factor being the low mass cutoff inherent to the ion trap, which sometimes limited the detection of the lowest m/z ions. With the use of a photodissociation method, such as IRMPD instead of CID during the subsequent activation step, it is anticipated that the LMCO problem could be alleviated.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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