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Chemical derivatization of peptide carboxyl groups for highly efficient electron transfer dissociation

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Abstract

The carboxyl groups of tryptic peptides were derivatized with a tertiary or quaternary amine labeling reagent to generate more highly charged peptide ions that fragment efficiently by electron transfer dissociation (ETD). All peptide carboxyl groups—aspartic and glutamic acid side-chains as well as C-termini—were derivatized with an average reaction efficiency of 99%. This nearly complete labeling avoids making complex peptide mixtures even more complex due to partially-labeled products, and it allows the use of static modifications during database searching. Alkyl tertiary amines were found to be the optimal labeling reagent among the four types tested. Charge states are substantially higher for derivatized peptides: a modified tryptic digest of bovine serum albumin (BSA) generates ~90% of its precursor ions with $z > 2$, compared to less than 40% for the unmodified sample. The increased charge density of modified peptide ions yields highly efficient ETD fragmentation, leading to many additional peptide identifications and higher sequence coverage (e.g. 70% for modified versus only 43% for unmodified BSA). The utility of this labeling strategy was demonstrated on a tryptic digest of ribosomal proteins isolated from yeast cells. Peptide derivatization of this sample produced an increase in the number of identified proteins, a >50% increase in the sequence coverage of these proteins, and a doubling of the number of peptide spectral matches. This carboxyl derivatization strategy greatly improves proteome coverage obtained from ETD-MS/MS of tryptic digests, and we anticipate that it will also enhance identification and localization of post-translational modifications.

Introduction

Mass spectrometry-based proteomics has become a workhorse technique for learning about the proteins that drive biological structure and function. A deeper, richer analysis of the proteins present provides a more comprehensive understanding of the biological system under investigation. Bottom-up proteomics, wherein the peptides from an enzymatic digestion of proteins are analyzed by mass spectrometry, currently provides the most in-depth analysis, but there still exists a need for improvements that identify more peptides. These additional peptides may expand the list of identified proteins, or they may expand the sequence coverage of proteins, thereby revealing other proteoforms [1] (e.g., isoforms from alternative splicing, protein variants arising from genetic variation, and/or proteins with

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various post-translational modifications (PTMs)). Currently, approaches employed by researchers to increase coverage have included the use of longer chromatographic gradients and/or pre-fractionation methods, customized proteomic databases that more closely reflect the sample being analyzed compared to generic databases, and peptide derivatization strategies to improve chromatographic characteristics and electrospray ionization behavior. The latter approach addresses the issue that in many cases, peptides remain unobserved due to their chemical composition (sequence), which can cause poor chromatographic elution, poor ionization, and/or poor fragmentation. We have previously reported a robust and efficient derivitization chemistry for the modification of protein or peptide carboxyl groups with tertiary or quaternary amines [2]. These added amines impart increased charge during positive mode electrospray ionization mass spectrometry. In the present work, we evaluate the utility of this chemistry for increasing performance of electron transfer dissociation (ETD) for peptide fragmentation.

It is well known that ETD is particularly ineffective for the dissociation of peptide dications (z = 2) [3–6]. Consequently, it is common to pair ETD-MS with an alternative enzyme such as endoproteinase LysC instead of trypsin, as LysC gives rise to longer peptides with correspondingly larger charge states. However, LysC still produces more peptides with $z = 2$ (40% of the total) than any other charge state [7], and even though LysC produces more peptides with charge states of $z > 2$, not all such peptides are equivalent in their propensity to yield a quality fragmentation spectrum. We have shown that the percent fragmentation and probability of identification, following ETD, correlates inversely with the residues/charge ratio, which can be approximated by the precursor *m/z* [5]. The reason behind this relationship is as follows. A precursor having a lower residue/charge ratio, or *m/z*, has a higher charge density, which causes increased repulsion between the *c*- and $^{\bullet}$*z-type* fragment ions created by backbone cleavage after electron transfer. When Coulombic repulsion overcomes the non-covalent interactions between the two fragments, they separate and are detected at their respective *m/z* values. If the charge density is too low, the peptide fragments fail to separate after electron-induced bond cleavage, and only the charge-reduced precursor *m/z* value is observed, instead of the desired fragmentation products. This phenomenon is referred to as electron transfer without dissociation, or ETnoD [8, 9].

A few methods have been developed to increase the dissociation of the *c*- and $^{\bullet}$*z-type* fragment ions that are created from ETD of *unmodified* peptides. These approaches typically work by activating the peptide (i.e., increasing its internal energy), either before or after the electron transfer occurs. Some success has been shown with both collisional [10] and IR activation [11] for peptides in low charge states. That said, these tactics would be unnecessary if all precursor ions had charge states greater than 2 and low m/z values.

Researchers have devised several strategies for increasing the charge states of peptides and proteins to improve their analysis by mass spectrometry. One technique involves changing the electrospray conditions with solvent additives [12–14], but a more common strategy is chemical derivatization. The most common derivatization reagents are N-hydroxysuccinimide (NHS) esters due to their compatibility with aqueous solvents, their availability, and the simplicity of the reaction. NHS esters react with amine functional groups (N-termini and lysine side-chains) on peptides and proteins, which has the drawback of eliminating native protonation sites, but this is usually counteracted by designing the modifying reagent with a replacement charging site or a fixed positive charge [15–22]. Others have modified amino groups by alkylation with aldehydes, which preserves the charge sites [2, 22–29]. Another easily-derivatized group is the thiol on the side-chain of cysteine residues [30–34], and two research groups have taken advantage of this to increase the charge states and ETD efficiency of peptides [35, 36]. Reid et al. have converted methionine side-chains into fixed charge groups by alkylation to sulfonium groups [37].

Targeting these sulfur-containing residues is limited by the fact that most peptides do not contain a methionine or cysteine residue. However, carboxyl groups occur nearly universally on all peptides at the C-terminus and also on the side chains of the relatively abundant residues of aspartic and glutamic acid. Furthermore, carboxyl groups are neutral during typical positive-mode ESI-MS, and so attaching basic groups or fixed-positive charges can increase peptide charge states substantially.

A few researchers have derivatized carboxyl groups on peptides to increase charge states during LC-MS [38–42]. All have employed essentially the same derivatization strategy, first developed by Lu and co-workers, namely using the carbodiimide EDC along with HOAt in DMF solvent (EDC: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, HOAt: 1-hydroxy-7-azabenzotriazole, and DMF: dimethylformamide) [38]. Lu attached pyrimidyl piperazine groups to enhance ionization of standard phosphopeptides by MALDI and ESI, and then showed that the increase from +1 to +2 precursors allowed fragmentation by ETD [39]. However, fragmentation was still not particularly efficient for these dication precursors for the reasons described above. Ko and Brodbelt derivatized model peptides, as well as those from a tryptic digest of the small protein cytochrome c, by attaching basic or fixed-charge groups to the peptide carboxyls [42]. They thoroughly analyzed the charge states and ETD fragmentation efficiencies, both of which increased substantially for the modified peptides. Despite efforts to optimize the EDC/HOAt/DMF chemistry, they reported that labeling was incomplete and dependent on the peptide sequence; they estimated the reaction efficiency as ≥70% of the C-terminal carboxyls, but much lower and less consistent for the aspartic acid and glutamic acid side-chain carboxyls.

The completeness of the labeling reaction becomes increasingly important with increasing sample complexity. This is because unmodified and/or partially-modified peptides exist as separate chemical entities with different retention times and masses than the desired fully-modified molecules. Incomplete labeling can easily double or triple the number of species in the mixture, which may be acceptable for simple peptide mixtures but not for proteomics of complex samples where instrument bandwidth already limits the number of peptide identifications. In addition, the database searching aspect of identifying peptides and proteins from complex samples is hindered when it includes too many dynamic modifications, but nearly complete derivatization will allow the use of static (or fixed) modifications during database searching. Thus, for derivatization to be most effective on real-world samples, the labeling reaction efficiency should be very high, and this is more difficult to achieve for the less-reactive carboxyl groups than for amino or thiol groups.

We have developed a derivatization method that yields nearly complete derivatization of all carboxyl groups (Asp, Glu, and C-termini) on small proteins or peptides (Figure 1) [2]. In this 2-step reaction scheme, the native amino groups are first protected by methylating them with formaldehyde, a modification reaction already employed for multiple purposes in proteomics because it leaves the amino groups available as protonation sites [23–27]. The second step is an amidation reaction whereby the peptide carboxylic acids are converted to tertiary or quaternary amine groups. The fact that the relatively abundant amino acid residues of Asp and Glu are modified, in addition to the C-termini, leads to substantially higher charge states after derivatization. In prior work, we employed this chemistry to generate highly charged intact proteins by positive-mode electrospray ionization [28, 43, 44]. Here, we apply it to complex mixtures of peptides, specifically a tryptic digest of a large protein, bovine serum albumin, and a tryptic digest of purified yeast ribosomal proteins. In both cases, the higher charge states afforded by derivatization lead to dramatic improvement in ETD fragmentation and consequently increased protein identifications and sequence coverage.

Experimental

Reagents and Materials

Bovine serum albumin (BSA) and two test peptides, neurotensin and DAENLIDSFQELV, were purchased from Sigma-Aldrich (St. Louis, MO); the other two standard peptides, SDEEEAIVAYTL and EQKLISEEDL, were obtained from Anaspec (Fremont, CA). All reagents, solvents, acids, and buffers were obtained from Sigma-Aldrich unless specified otherwise. (7-Azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyAOP) was purchased from Applied Biosystems (Foster City, CA, USA). The tertiary amines used as labeling reagents were purchased in their free-base forms: 3-(dimethylamino)-1-propylamine (C3-methylTert); 5-(dimethylamino)-amylamine (C5-methylTert, Matrix Scientific, Columbia, SC); N,N-diethyl-1,4-butanediamine (C4-ethylTert, Alfa Aesar, Ward Hill, MA); N,N-diisopropyl-1,5-pentanediamine (C5-isopropylTert, Alfa Aesar); 4-(2-aminoethyl)pyridine (C2-pyridine, Alfa Aesar); 4-(2-aminoethyl)morpholine (C2-morpholine, Aldrich); and 4-(3-aminopropyl)morpholine (C3-morpholine, Alfa Aesar). These amines were converted to their mono- or di-hydrochloride salts by the drop-wise addition of a stoichiometric amount of 1 M HCl in ether while stirring in an ice bath. The ether solvent was removed from the solid products by rotary evaporation, and then acetone was added and subsequently removed by rotary evaporation. (Note: dihydrochloride salts were used for the alkyl tertiary amine reagents, but mono-hydrochloride salts were used for the less basic functional groups, morpholines and pyridine, in order to yield optimal peptide labeling [2].) The quaternary amine, (4-aminobutyl)trimethylammonium dichloride (C4-quat), was synthesized as reported previously [28].

Preparation of Ribosomal Protein Sample

Purification of yeast ribosomes was adapted from published reports [45, 46]. Briefly, the YIT613 strain of Saccharomyces cerevisiae (a gift from Dr. Toshifumi Inada of Nagoya University) was grown, and then yeast cells were harvested at an OD_{600} of 0.8. Ribosomes were isolated using an ANTI-FLAG M2 Affinity Gel column (Bio-Rad, Hercules, CA) and eluted using FLAG peptide (synthesized at the Biotechnology Center at the University of Wisconsin). Ribosomes were subsequently concentrated by ultracentrifugation, and then ribosomal proteins were isolated from ribosomal RNA using a 66% acetic acid wash and acetone precipitation.

Protein Digestion and Peptide Derivatization

The BSA and ribosomal proteins were dissolved in 8M urea/25mM NH_4HCO_3, reduced with DL-dithiothreitol (DTT), alkylated with iodoacetamide (IAA), diluted 10-fold with 25mM NH_4HCO_3 and then digested overnight at 37 °C with porcine trypsin (Promega, Madison, WI) at an enzyme:protein ratio of 1:50. The digested samples were purified using C18 solid-phase extraction columns (1cc/50mg SepPak, Waters, Milford, MA).

Standard peptides or those from tryptic digestion of protein samples were derivatized first at their amino groups and subsequently at their carboxyl groups, as reported in our previous Methods paper [2]. Peptides were dissolved, at a concentration of 2 mg/mL, in 50:50 methanol:H_2O containing 40 mM formaldehyde (HCHO), 60 mM borane-pyridine complex and 50 mM 4-methylmorpholine (NMM), which maintains the appropriate basic pH for the reaction. After reacting at room temperature for 1–2 hours, the mixture was evaporated to dryness in a vacuum centrifuge. The resulting dimethylated peptides were dissolved in dimethyl sulfoxide (DMSO) at 1.5 mg/mL. The mono- or di-hydrochloride salt of the desired amine labeling reagent and 4-methylmorpholine (used to buffer the pH) were dissolved in a minimal amount of water (e.g. 30 μmol of amine salt and 2.6 μL of NMM in

5–8 μL of water for a 100 μg batch of peptides in 67 μL of DMSO). The peptide/DMSO solution was added to the aqueous amine/NMM solution and mixed to yield final concentrations of 450 mM amine and 350 mM NMM. Solid PyAOP, which activates the carboxyl groups, was added to a concentration of 60 mM, and the reaction was allowed to proceed for 2 hours at room temperature before quenching by adding aqueous 0.1% formic acid such that the DMSO was diluted to 5% (v/v). The quenched reaction was extracted twice with chloroform to remove reaction by-products, and the aqueous layer was dried in a vacuum centrifuge, followed by re-suspending the solid products in 0.1% TFA and desalting on a C18 solid-phase extraction column.

While all of the results reported in this paper made use of the reaction conditions above, certain precious samples may require the use of lower peptide concentrations. We performed one experiment with the test peptide neurotensin at 40-fold lower concentration and found that derivatization under these conditions was just as complete (>99%, data not shown). Note that only the peptide concentration was lower; it is important to maintain high concentrations of all other reagents.

Mass Spectrometry and Data Analysis

Unlabeled and labeled samples of the individual test peptides were analyzed by direct infusion into the electrospray interface of a Bruker micrOTOF mass spectrometer. The mass spectra were analyzed manually to find the peak intensities for all charge states of the completely derivatized product and any underivatized or partiallyderivatized products. These values were used to calculate the efficiency of the labeling reactions and the average charge states for the test peptides, as reported in Table 1. A similar data analysis was performed for the BSA tryptic peptides listed in Table 1, but those mass spectra were acquired by LC-MS on a different instrument (see next paragraph). More specifically, the average charge state values were calculated by manually analyzing full scan MS1 spectra for the BSA tryptic peptides listed in Table 1. The labeling reaction efficiencies for these peptides were calculated using LC-MS peak areas from the extracted ion chromatograms obtained at the theoretical masses of the fully-, partially-, and un-derivatized peptide products.

The unlabeled and labeled tryptic digests of BSA were injected into a capillary HPLC-ESI-MS/MS system consisting of a Waters nanoAcquity HPLC (Milford, MA) connected to an electrospray ionization (ESI) ion-trap/orbitrap mass spectrometer (LTQ Orbitrap Velos, Thermo Scientific, San Jose, CA). The BSA digest samples (0.9 pmol in 9 μL of 0.1% formic acid, 1.5% acetonitrile) were loaded for 12 min at 1.5 μL/min onto a trapping column prepared by packing 5 cm of 5 μm-diameter 300Å-pore C18 beads (Western Analytical Products, Murrieta, CA) into a 100 × 365 μm fused silica capillary having a frit at one end. The analytical column (100 × 365 μm) was packed with 10 cm of the same C18 beads, but instead of packing against a frit, the capillary tip was pulled to ~1 μm with a P-2000 laser puller (Sutter Instruments, Novato, CA). Peptides were eluted at 0.2 μL/min in an aqueous mobile phase containing 0.1 % formic acid with a gradient of increasing acetonitrile (1.5–35% in the first 60 min, increasing to 70% in the next 6 min). A full-mass scan (150–2000 *m/z*) was performed in the orbitrap at a resolution of 60,000. The six most intense peaks with $z > 1$ from the full scan were selected for fragmentation by ETD, employing 3.5×10^5 fluoranthene reagent ions and 5×10^4 precursor ions with a reaction time of 50 ms. Higher charge states generally require shorter ETD reaction times, but the outcome of experiments in this paper were not particularly sensitive to reaction time—35, 50, and 70 ms were tested, and 50 ms was nearly optimal for both labeled and unlabeled peptides. The isolation width for the precursor ions was 2.0 *m/z*, and the product ions from fragmentation were analyzed in the orbitrap detector at a resolution of 7500. Dynamic exclusion was enabled with a repeat count of two over 20 s and an exclusion window of 60 s.

The unlabeled and labeled tryptic digests of ribosomal proteins were analyzed in the same manner as the BSA digests with the following differences. The amount injected was approximately 0.06 μg of ribosomal protein digest, as estimated by a BCA assay (Pierce, Rockford, IL). During the sample-loading step (30 min at 0.5 μL/min, 2% acetonitrile, 0.1% formic acid), effluent from the trapping column was sent through the 15 cm analytical column (3 μm MAGIC aqC18 beads, Bruker-Michrom, Auburn, CA) and analyzed by MS. Gradient elution employed a flow rate of 0.3 μL/min in 0.1% formic acid with increasing acetonitrile (2–8% in the first minute, up to 30% over the next 120 min, and increasing to 70% over the next 10 min). The ten most intense peaks with $z > 2$ were fragmented by ETD (4×10^5 reagent ions, 1×10^5 precursor ions, reaction time 50 ms) using an isolation width of 2.5 *m/z*. Dynamic exclusion used a repeat count of three over 30 s and an exclusion time of 120 s. Note that $z = 2$ precursors were excluded for this more complex ribosomal sample to maximize time spent on $z > 2$ precursors, which have a higher probability of generating a peptide identification from an ETD spectrum; whereas $z = 2$ precursors were included for the relatively less complex BSA digest sample because there is sufficient time in that case to analyze all peptide precursors.

The mass spectra from the tryptic digest samples were searched against the appropriate organism protein databases from Uniprot (*Bos taurus* for BSA, downloaded 10/26/2009; *S. cerevisiae* for yeast ribosomes, downloaded 5/5/2011) using the SEQUEST algorithm of Proteome Discoverer (Thermo Scientific). Mono-isotopic masses were used for both precursor and fragment ions with tolerances of 15 ppm and 0.06 Da, respectively (except that the fragment tolerance was 0.1 Da for the ribosomal samples). The searches allowed for up to two missed trypsin cleavages. The results were filtered using a 1% peptide false discovery rate (FDR). Variable methionine oxidation (+15.995 Da) and static carbamidomethylation of cysteines (+57.021 Da) were used in all searches. All labeled samples were searched with static dimethylation of the N-termini and lysines (+28.031 Da). Also, the following *static* modifications were employed at the C-termini and all aspartic acid and glutamic acid residues for data acquired from the correspondingly labeled samples: C3-methylTert (+84.105 Da), C5-methylTert (+112.136 Da), C4-ethylTert (+126.152 Da), C5-isopropylTert (+168.323 Da), C4-quat (+112.136 Da), C2-pyridine (+104.074 Da), C2-morpholine (+112.100 Da), and C3-morpholine (+126.116 Da).

Results and Discussion

Labeling Efficiency

The peptide derivatization reactions employed in this work proceed nearly to completion, thereby minimizing any increase in sample complexity that would result from multiple incomplete reaction products for each peptide. The dimethylation of primary amines (Lys and N-termini) with formaldehyde and a reducing agent has been reported by us and others to be essentially complete [2, 23, 27, 28]. Note that dimethylation is a necessary first step because it prevents the native peptide amino groups from reacting with the peptide carboxyl groups that are activated by PyAOP during the second step. The second reaction, amidation of peptide carboxyls (Asp, Glu, and C-termini), is a more difficult reaction to drive to completion, but excellent labeling efficiencies were achieved with the reaction conditions reported in the Experimental Section.

Table 1 lists the results from amidating several different peptides with a selection of different amines. For example, the test peptide neurotensin (pyroELYENKPRRPYIL) gave ≥98.7% reaction efficiency for each of the eight different amine labels. Note that this reaction efficiency is calculated as the percentage of carboxyl groups on the peptides that were successfully derivatized. One may also wish to know the percentage of peptides that were completely modified. For example, a peptide with two, three, or four carboxyl groups

would yield 97.6%, 96.4% or 95.3% of completely modified peptides, respectively—calculated from $(0.988)^n$ where n is the number of carboxyls and 98.8% is the average reaction efficiency from all of the peptides listed in Table 1.

The high reaction efficiency per carboxyl group appears to be independent of the number of carboxyl sites, their position within the peptide, or the complexity of the peptide sample. Excellent efficiencies were obtained for all eight peptides shown in Table 1, regardless of the number of carboxyls (ranging from two to five) and even when the carboxyl-containing residues were clustered within the peptide (e.g. S***deee***AIVAYT***l***). Table 1 also lists results from amidation reactions performed on a more complex sample mixture, a tryptic digest of bovine serum albumin (BSA). The reaction efficiencies for the BSA peptides were mostly >98%, demonstrating that the amidation reaction proceeds nearly to completion even for complex peptide mixtures. These very high reaction efficiencies are important for proteomics of real-world samples because they minimize the increase in sample complexity and also allow the use of static modifications during database searching.

Charge States and Signal Intensity

The peptide charge states observed for positive-mode electrospray ionization increase substantially upon derivatizing the peptides at their carboxylic acid sites with tertiary or quaternary amines. An example is shown in Figure 2 for the peptide DAFLGSFLYEYSR from a tryptic digest of BSA. The ESI-MS spectrum for this peptide in its unmodified form shows only the +2 charge state, whereas the C4-quat amidated version of this peptide yields predominantly +4 ions, some +5 ions, and a small number of +3 ions. The weighted average charge state for this peptide increased from 2.0 to 4.4 upon modification at its three carboxyls: aspartic acid (D), glutamic acid (E), and Cterminus. The spectrum for the C5-methylTert amidated peptide is similar to the C4-quat one, but it has a slightly smaller +5 peak and thus a weighted average charge state of 4.3. Table 1 lists the weighted average charge states for numerous other modified peptide examples, and in all cases a substantial increase in charge state is observed.

Another potential benefit of modifying peptides with tertiary or quaternary amines is improved signal intensity. The spectra in Figure 2 provide an example of this effect because the y-axis intensity value increased by more than an order of magnitude after peptide modification. Note that we have observed an intensity increase upon modification for many, but not all, peptides. The additional signal may arise from a few possible factors. One likely factor is an improvement in ionization efficiency during ESI due to the chemical modifications of the peptide. Another is that the mass-to-charge ratio is lower for the more highly charged modified peptides, and the mass spectrometer transfer and detection efficiencies may be greater in this *m/z* range. A third factor that likely plays a role for some peptides is a decrease in peptide hydrophobicity as a result of attaching additional positive charge sites. We have shown in other work that alkylating peptides with butyl or hexyl groups *increases* their hydrophobicity, making certain peptides more detectable by LC-MS [29]. In the current work, the *decrease* in hydrophobicity is demonstrated by the earlier elution times during reverse-phase LC for modified peptides (e.g. DAFLGSFLYEYSR elutes at 44.5 min for the C4-quat modified peptide and 47.0 min for the C5-methylTert modified peptide, as compared to 60.3 min for the unmodified form). This unmodified peptide is near the high end of the suitable hydrophobicity range; its hydrophobicity index is 50.6 in SSRCalc (http://hs2.proteome.ca/SSRCalc/SSRCalcX.html) [47]. Appending charged amino groups makes it less hydrophobic and increases its signal intensity (it is not known to what extent this is due to improved chromatographic elution and to what extent it changes the ionization efficiency). It is possible that some peptides having already low relative hydrophobicities will be rendered too hydrophilic by this type of chemical

modification, although the high sequence coverages observed below for BSA indicate this is not a major problem.

The magnitude of the charge state increase depends upon a number of factors. As might be expected, the predominant factor is the number of modification sites, *i.e.* more modifications lead to larger increases in charge state. The examples from Table 1 show average increases of 1.4, 2.2, 2.8, and >3.0 for peptides with 2, 3, 4, and 5 C4-quat modifications, respectively. Note that the charge state increase is never as large as the number of modifications even when appending the fixed-charge C4-quat group. This result is mostly due to Coulombic repulsion from the additional charges decreasing protonation of basic sites on the peptide, as has been discussed in detail for peptide and protein ionization [44, 48]. Other secondary factors influencing the observed charge state increase may include the number or density of native basic sites and their relative positions to each other and to the modification sites; an in-depth analysis of these factors is beyond the scope of this paper. Another variable affecting the charge state increase is the type of functional group attached. The fixed-charge C4-quat group always shows the largest increase for a given peptide, but somewhat surprisingly, the aliphatic tertiary amines usually produce an equivalent increase—except for the cases involving five modified residues. These aliphatic tertiary amines (C3-methylTert, C5-methylTert, C4-ethylTert, and C5-isopropylTert) have a high gas-phase basicity, which makes them very likely to be protonated during ESI-MS. The morpholine and pyridine functional groups were chosen for their somewhat lower gas-phase basicities, and consequently they do produce a smaller increase in charge state than the aliphatic tertiary and quaternary amines.

Figure 3 shows the distribution of charge states obtained for the various unmodified and modified tryptic peptides from a BSA digest. These data were obtained by a comprehensive analysis of the peptide precursor charge states ($z \geq 2$) from the LC-MS runs. Nearly 60% of the unmodified tryptic peptides appear in the +2 charge state, which is undesirable for ETD fragmentation. Dimethylation of the amine groups on the peptides, the first part of our two-step labeling method, produces little change in charge states. However, amidation of the carboxyls with amine labels leads to additional basic sites on the peptides and consequently a substantial shift to higher charge states. Furthermore, the magnitude of the charge state increase follows the expected trend of the pKa, or more accurately, the gas-phase basicity (GB) of the amine label. The pyridine group has the lowest pKa and GB, and so those peptides appear predominantly in the +3 charge state. The morpholines have the next lowest GB and thus usually produce +3 and +4 peptides. The alkyl tertiary amines have quite high pKas and GBs leading to a predominance of +4 peptides. Finally, since the quaternary amine is a fixed positive charge, it gives the highest charge states, although they are not much higher than those obtained using the alkyl tertiary amines. This charge state plot clearly demonstrates that the low charge states of unmodified tryptic peptides are converted to significantly higher charge states through labeling the peptides at their carboxylic acid sites. The next section illustrates how the higher charge states obtained from modified peptides improve ETD fragmentation.

ETD Fragmentation

The MS/MS spectra in Figure 4 demonstrate a dramatic increase in ETD fragmentation for tertiary and quaternary amine modified peptides compared to the unmodified form. These ETD spectra for DAFLGSFLYEYSR were acquired using precursor ions from the most abundant charge state, +2 for the unmodified peptide and +4 for each of the modified peptides (refer to Figure 2 for the full scan MS spectra). The ETD spectrum in Figure 4 for the unmodified peptide illustrates the typical lack of fragment ions for +2 precursor ions (i.e. only 3 of the 24 possible *c*- and $^{\bullet}$*z-type* products are observed). In contrast, the +4 precursors

for the modified peptides yield all 24 possible *c*- and •*z-type* fragments, and the *m/z* peaks corresponding to each fragment have substantial intensity.

The higher charge state precursors of the modified peptides are able to produce fragments in somewhat higher charge states. In the examples in Figure 4, the +4 precursors yield numerous +1 fragment ions (in fact all 24 possible ones for the C5-methylTert case), but also +2 fragments are observed for c_{10}–c_{12} and $^{\bullet}z_4$–$^{\bullet}z_{12}$ ions. The reason for the appearance of only these particular +2 fragments relates to the position of the tagged glutamic acid residue. The $c_{10–12}$ ions include this charge-tagged residue and have observable +2 peaks, as opposed to the $c_{1–9}$ ions, which do not have this additional tertiary/quaternary amine and consequently only produce +1 peaks. The same argument explains the charge states observed for the •*z-type* products. Even more highly charged parent ions, such as +5 precursors, produce additional +2 fragments and some +3 fragments for these modified tryptic peptides (data not shown). While the appearance of fragments in multiple charge states does lead to more complicated MS/MS spectra, the increased fragmentation from these charge-tagged peptides is very helpful for peptide identification. Furthermore, if spectral complexity becomes a limiting factor for confident identification by database searching, then one could follow ETD with a proton transfer reaction to charge reduce all fragments to +1 ions [49].

Few peaks other than the *c*- and •*z-type* products are observed in these ETD spectra of modified peptides. There are the usual peaks due to left over precursor ions and charge-reduced precursors (shown in green in Figure 4). The remaining peaks are all shown in black, and these correspond to neutral loss fragments of the charge-reduced precursors, which is a common occurrence during electron transfer dissociation. These modified peptides tend to give neutral losses of 15 and 17 Da, easily attributable to loss of a methyl group or ammonia, but also 44, 45, 58, and 59 Da [8, 50, 51]. Looking at the structures of the labels, one could easily imagine losing di- or tri-methylamine (45 or 59 Da), and the 44 and 58 Da losses could result from hydrogen atom transfer during the rearrangement of the radical that leads to the neutral loss. Notice that the peak intensities for the precursor and charge-reduced precursors are similar to those of the *c*-and •*z-type* fragment ions, indicating that nearly all of the peptide ions undergo fragmentation. The fragmentation efficiency is calculated by summing all *c*- and •*z-type* product ion intensities and then dividing by the sum of all fragment and precursor ion intensities in the ETD spectrum [42]. The ETD efficiencies for the DAFLGSFLYEYSR peptide in its unmodified, C4Quat-modified, and C5-methylTert-modified forms are 2%, 80%, and 90%, respectively. Clearly, the high charge density (or low residue/charge ratio) of the modified peptides leads to very efficient fragmentation by ETD. (One could reasonably expect a similar enhancement of fragmentation efficiency during electron capture dissociation (ECD) [52, 53]).

Sequence Coverage

A major benefit of amidating peptides for improved ETD is that numerous additional peptides are confidently identified during database searching of LC-MS/MS data from complex mixtures of peptides. The additional identified peptides could lead to more identified proteins and/or to an increase in the sequence coverage of proteins already identified by other peptides. To illustrate an increase in sequence coverage upon amidation, Figure 5 plots the sequence coverage of BSA obtained for various labels. The initial value of 43% coverage for unmodified BSA is reasonable for ETD-only fragmentation of a tryptic digest. (Note that higher coverage is obtained from HCD (59%) and CID (67%), data not shown, and this is due to the synergy between tryptic peptides and collisional dissociation methods [5, 54]. Also note that collisional dissociation of the derivatized peptide samples yields lower sequence coverage than for the unmodified peptides, data not shown, and this result is not surprising given the high charge state and low *m/z* values of these precursors

[5]). Figure 5 shows that dimethylation of the peptide amino groups leads to a small improvement in the ETD sequence coverage, over the 43% for unmodified peptides. Subsequent amidation with tertiary or quaternary amines causes an additional large increase, and the ETD sequence coverage reaches about 70% for the alkyl tertiary amines.

The differences in sequence coverage obtained from the various amidation labels are not explained simply by the change they cause to the precursor charge states. As discussed above, the increase in charge state follows the gas-phase basicity of the appended functional groups: pyridine < morpholine < alkyl tertiary amine < quaternary amine. The morpholine tags yield higher sequence coverage than the unmodified or dimethylated peptides but less than the alkyl tertiary amines, as expected. The pyridine tag, however, produces only 7% coverage, far below the unmodified sample itself, despite the fact that labeling with pyridine does increase the charge states (Figure 3). We speculate that the pyridine functional group sequesters the transferred electron and produces uninformative side-chain fragmentation products rather than backbone cleavages, as has been observed for ETD and ECD of various peptides having heterocyclic aromatic groups [8, 55–57]. Interestingly, Turecek has found that a pyridinium group, attached to a peptide's N-terminus, served as a good functional group for improving ETD [58, 59]. While similar in structure, the pyridinium differs from the pyridine employed here in that the nitrogen atom resides on the opposite side of the aromatic ring and it is quaternary, rather than tertiary. Presumably, these differences in tag structure are responsible for the discordant results.

Also somewhat surprisingly, the C4-quat label is not quite as effective as the alkyl tertiary amines at increasing sequence coverage, even though its fixed charge generates the most highly charged peptides. We have considered three possible explanations for this behavior. First, the alkyl quaternary ammonium group may not be as efficient as the tertiary amino group for generating backbone cleavage products after electron transfer. McLuckey and co-workers have explored this in-depth and found that the trimethyl ammonium group yields a lower percentage of electron transfer products than a lysine amino group, which they attribute to a smaller recombination energy for the quaternary amine and its inability to transfer a H-atom to the peptide backbone [8]. Second, we have noticed that the chromatographic peaks of C4-quat labeled peptides are broader (data not shown), and these wider, shorter peaks are detrimental in terms of decreasing chromatographic separation and mass spectrometric sensitivity. This peak-width problem is likely inconsequential for the BSA digest sample, but it could be an issue with more complex mixtures such as whole-cell lysates. Finally, the quaternary ammonium groups impart the most hydrophilicity to the peptides, which may cause some peptides to be lost during the chromatographic trapping step. This third hypothesis was supported by the results from one LC-MS run without a trapping step; it was found that in the absence of such losses during trapping, the C4-quat labeled sample produced 70% sequence coverage for BSA, comparable to that obtained with the alkyl tertiary amines.

The enhancement of ETD sequence coverage from amidating tryptic peptides is even more dramatic if one excludes missed cleavages by trypsin. Database searches not allowing missed cleavages yielded only 25% sequence coverage for unmodified BSA but 54% for the C5-isopropylTert sample. The reason for this larger effect (more than doubling the sequence coverage) is that missed cleavages for the unmodified BSA allow more peptides to surpass the +2 charge that is particularly problematic for ETD. In fact, many studies utilizing ETD fragmentation employ LysC rather than trypsin to generate longer, more highly charged peptides. While advantageous because it minimizes the quantity of +2 peptides, LysC does not have a significant effect on the residues/charge ratio that strongly affects whether dissociation happens after electron transfer [5]. Amidation of peptide carboxyl groups, however, does lower the residues/charge ratio leading to higher dissociation efficiency,

while also allowing the use of trypsin, which is the least expensive, most robust, and easiest to use enzyme for digestion of proteomic samples.

Ribosomal Proteins

We applied the labeling strategy to a real-world sample of much greater complexity, namely a tryptic digest of purified yeast ribosomal proteins. Yeast ribosomes are thought to contain about 80 different proteins, and the Uniprot database that we used contained 127 protein entries from the 40S and 60S subunits due to the inclusion of numerous closely related protein isoforms. We identified 86 and 92 ribosomal proteins from individual LC-ETD-MS runs of the unmodified and the C3-methylTert amidated samples, respectively. These analyses resulted in greater than 90% of the peptide spectral matches (PSMs) arising from 40S and 60S ribosomal proteins. Consequently, we chose to summarize the data for only these ribosomal proteins, rather than also including the data from the other proteins that had much lower abundance and were not the focus of the sample preparation.

As shown in Figure 6, labeling the tryptic peptides of these ribosomal proteins leads to an increase in the number of identified proteins, peptides and PSMs. Only a small increase in the number of identified proteins was obtained after C3-methylTert labeling, but that is not surprising given the purified nature of this sample and the fact that nearly all of the expected proteins were already observed in the unmodified run. However, the amidation labeling did lead to a substantial improvement in the number of identified peptides, a 58% increase from 389 to 613, as well as more than doubling the number of PSMs. The additional observed peptides led to a >50% increase in sequence coverage of ribosomal proteins, which is comparable to the 63% increase (70% versus 43%) observed for the BSA digest.

The additional sequence coverage afforded by labeling samples at their carboxylic acid sites with tertiary/quaternary amines will likely be beneficial for identifying PTMs and pinpointing their locations. We are currently applying this labeling approach to help comprehensively analyze PTM changes to ribosomal proteins from yeast grown under different conditions. Maximizing sequence coverage in proteomics requires a multipronged approach that we believe should include ETD of amidated peptides, but also other types of fragmentation, other enzymes besides trypsin, and pre-fractionation or multi-dimensional chromatography.

Conclusions

The peptide derivatization strategy reported in this paper appends tertiary or quaternary amines to the peptide carboxyl groups present at the C-terminus and in aspartic and glutamic acid residues. The amidation reaction employed is not unlike carbodiimide chemistry utilized by others, but it produces nearly complete labeling of all peptide carboxyls. The labeled peptides tend to have higher signal intensities than their unlabeled counterparts, presumably due to improved chromatographic elution and/or ionization efficiency. The most important outcome of the derivatization, however, is the substantial increase in charge states for the labeled peptides and the corresponding enhancement of ETD fragmentation efficiency. The increased number of fragment ions having high signal intensity leads to additional peptide identifications during database searching of the mass spectra, and consequently more protein identifications and higher sequence coverages. Confident identification of more unique peptides will enable discovery of more protein variation in biological samples. The derivatization method is robust enough to be applied to complex proteomic samples, as demonstrated by the analysis of yeast ribosomes.

The optimal labeling reagents for amidation of tryptic peptides are those containing an alkyl tertiary amine. This functional group produced the best ETD-MS results, although alkyl

quaternary amines and morpholines were only slightly poorer, and that likely has more to do with their impact on chromatography than their effect on electron transfer dissociation per se. The four alkyl tertiary amines all performed similarly for the BSA digest sample, and thus we chose not to expend the time and money testing each of them on the more complex sample of yeast ribosomal proteins. The C3-methylTert reagent that we did employ on the ribosome sample yielded excellent results. In future work, one may wish to choose a particular alkyl tertiary amine based on whether to impart more or less hydrophilicity (e.g., C3-methylTert versus C5-isopropylTert).

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References

1. Smith LM, Kelleher NL. Proteoform: a single term describing protein complexity. Nat. Methods. 2013; 10:186–197. [PubMed: 23443629]
2. Krusemark CJ, Frey BL, Smith LM, Belshaw PJ. Complete chemical modification of amine and acid functional groups of peptides and small proteins. Methods Mol. Biol. 2011; 753:77–91. [PubMed: 21604117]
3. Syka JE, Coon JJ, Schroeder MJ, Shabanowitz J, Hunt DF. Peptide and protein sequence analysis by electron transfer dissociation mass spectrometry. Proc. Natl. Acad. Sci. U.S.A. 2004; 101:9528–9533. [PubMed: 15210983]
4. Pitteri SJ, Chrisman PA, Hogan JM, McLuckey SA. Electron transfer ion/ion reactions in a three-dimensional quadrupole ion trap: reactions of doubly and triply protonated peptides with SO2*. Anal. Chem. 2005; 77:1831–1849. [PubMed: 15762593]
5. Good DM, Wirtala M, McAlister GC, Coon JJ. Performance characteristics of electron transfer dissociation mass spectrometry. Mol. Cell. Proteomics. 2007; 6:1942–1951. [PubMed: 17673454]
6. Wiesner J, Premsler T, Sickmann A. Application of electron transfer dissociation (ETD) for the analysis of posttranslational modifications. Proteomics. 2008; 8:4466–4483. [PubMed: 18972526]
7. Swaney DL, McAlister GC, Coon JJ. Decision tree-driven tandem mass spectrometry for shotgun proteomics. Nat. Methods. 2008; 5:959–964. [PubMed: 18931669]
8. Xia Y, Gunawardena HP, Erickson DE, McLuckey SA. Effects of cation charge-site identity and position on electron-transfer dissociation of polypeptide cations. J. Am. Chem. Soc. 2007; 129:12232–12243. [PubMed: 17880074]
9. Xia Y, Han H, McLuckey SA. Activation of intact electron-transfer products of polypeptides and proteins in cation transmission mode ion/ion reactions. Anal. Chem. 2008; 80:1111–1127. [PubMed: 18198896]
10. Madsen JA, Brodbelt JS. Simplifying Fragmentation Patterns of Multiply Charged Peptides by N-Terminal Derivatization and Electron Transfer Collision Activated Dissociation. Anal. Chem. 2009; 81:3645–3653. [PubMed: 19326898]
11. Ledvina AR, Beauchene NA, McAlister GC, Syka JEP, Schwartz JC, Griep-Raming J, Westphall MS, Coon JJ. Activated-Ion Electron Transfer Dissociation Improves the Ability of Electron Transfer Dissociation to Identify Peptides in a Complex Mixture. Anal. Chem. 2010; 82:10068–10074. [PubMed: 21062032]
12. Iavarone AT, Jurchen JC, Williams ER. Supercharged protein and peptide ions formed by electrospray ionization. Anal. Chem. 2001; 73:1455–1460. [PubMed: 11321294]
13. Lomeli SH, Yin S, Loo RRO, Loo JA. Increasing: Charge While Preserving Noncovalent Protein Complexes for ESI-MS. J. Am. Soc. Mass Spectrom. 2009; 20:593–596. [PubMed: 19101165]
14. Kjeldsen F, Giessing AMB, Ingrell CR, Jensen ON. Peptide sequencing and characterization of post-translational modifications by enhanced ion-charging and liquid chromatography electron-

transfer dissociation tandem mass spectrometry. Anal. Chem. 2007; 79:9243–9252. [PubMed: 18020370]

15. Thompson A, Schafer J, Kuhn K, Kienle S, Schwarz J, Schmidt G, Neumann T, Johnstone R, Mohammed AK, Hamon C. Tandem mass tags: a novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS. Anal. Chem. 2003; 75:1895–1904. [PubMed: 12713048]
16. Ross PL, Huang YN, Marchese JN, Williamson B, Parker K, Hattan S, Khainovski N, Pillai S, Dey S, Daniels S, Purkayastha S, Juhasz P, Martin S, Bartlet-Jones M, He F, Jacobson A, Pappin DJ. Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. Mol. Cell. Proteomics. 2004; 3:1154–1169. [PubMed: 15385600]
17. Mirzaei H, Regnier F. Enhancing electrospray ionization efficiency of peptides by derivatization. Anal. Chem. 2006; 78:4175–4183. [PubMed: 16771548]
18. Chamot-Rooke J, van der Rest G, Dalleu A, Bay S, Lemoine J. The combination of electron capture dissociation and fixed charge derivatization increases sequence coverage for O-glycosylated and O-phosphorylated peptides. J. Am. Soc. Mass Spectrom. 2007; 18:1405–1413. [PubMed: 17560119]
19. Xiang F, Ye H, Chen RB, Fu Q, Li LJ. N,N-Dimethyl Leucines as Novel Isobaric Tandem Mass Tags for Quantitative Proteomics and Peptidomics. Anal. Chem. 2010; 82:2817–2825. [PubMed: 20218596]
20. Lu YL, Zhou X, Stemmer PM, Reid GE. Sulfonium Ion Derivatization, Isobaric Stable Isotope Labeling and Data Dependent CID- and ETD-MS/MS for Enhanced Phosphopeptide Quantitation, Identification and Phosphorylation Site Characterization. J. Am. Soc. Mass Spectrom. 2012; 23:577–593. [PubMed: 21952753]
21. Wuhr M, Haas W, McAlister GC, Peshkin L, Rad R, Kirschner MW, Gygi SP. Accurate multiplexed proteomics at the MS2 level using the complement reporter ion cluster. Anal. Chem. 2012; 84:9214–9221. [PubMed: 23098179]
22. Hennrich ML, Boersema PJ, van den Toorn H, Mischerikow N, Heck AJ, Mohammed S. Effect of chemical modifications on peptide fragmentation behavior upon electron transfer induced dissociation. Anal. Chem. 2009; 81:7814–7822. [PubMed: 19689115]
23. Hsu JL, Huang SY, Chow NH, Chen SH. Stable-isotope dimethyl labeling for quantitative proteomics. Anal. Chem. 2003; 75:6843–6852. [PubMed: 14670044]
24. Hsu J-L, Huang S-Y, Shiea J-T, Huang W-Y, Chen S-H. Beyond Quantitative Proteomics: Signal Enhancement of the a1 Ion as a Mass Tag for Peptide Sequencing Using Dimethyl Labeling. J. Proteome Res. 2005; 4:101–108. [PubMed: 15707364]
25. Fu Q, Li L. De Novo Sequencing of Neuropeptides Using Reductive Isotopic Methylation and Investigation of ESI QTOF MS/MS Fragmentation Pattern of Neuropeptides with NTerminal Dimethylation. Anal. Chem. 2005; 77:7783–7795. [PubMed: 16316189]
26. Melanson JE, Avery SL, Pinto DM. High-coverage quantitative proteomics using amine-specific isotopic labeling. Proteomics. 2006; 6:4466–4474. [PubMed: 16897685]
27. Boersema PJ, Raijmakers R, Lemeer S, Mohammed S, Heck AJR. Multiplex peptide stable isotope dimethyl labeling for quantitative proteomics. Nat. Protocols. 2009; 4:484–494.
28. Krusemark CJ, Ferguson JT, Wenger CD, Kelleher NL, Belshaw PJ. Global amine and acid functional group modification of proteins. Anal. Chem. 2008; 80:713–720. [PubMed: 18184016]
29. Kulevich SE, Frey BL, Kreitinger G, Smith LM. Alkylating Tryptic Peptides to Enhance Electrospray Ionization Mass Spectrometry Analysis. Anal. Chem. 2010; 82:10135–10142. [PubMed: 21114270]
30. Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. Nat. Biotechnol. 1999; 17:994–999. [PubMed: 10504701]
31. Ren DY, Julka S, Inerowicz HD, Regnier FE. Enrichment of cysteine-containing peptides from tryptic digests using a quaternary amine tag. Anal. Chem. 2004; 76:4522–4530. [PubMed: 15283597]
32. Yi EC, Li XJ, Cooke K, Lee H, Raught B, Page A, Aneliunas V, Hieter P, Goodlett DR, Aebersold R. Increased quantitative proteome coverage with (13)C/(12)C-based, acid-cleavable isotope-

coded affinity tag reagent and modified data acquisition scheme. Proteomics. 2005; 5:380–387. [PubMed: 15648049]

33. Williams DK Jr, Meadows CW, Bori ID, Hawkridge AM, Comins DL, Muddiman DC. Synthesis, characterization, and application of iodoacetamide derivatives utilized for the ALiPHAT strategy. J. Am. Chem. Soc. 2008; 130:2122–2133. [PubMed: 18220392]
34. Wang J, Zhang J, Arbogast B, Maier CS. Tandem Mass Spectrometric Characterization of Thiol Peptides Modified by the Chemoselective Cationic Sulfhydryl Reagent (4-Iodobutyl)Triphenylphosphonium- J. Am. Soc. Mass Spectrom. 2011; 22:1771–1783. [PubMed: 21952891]
35. Ueberheide BM, Fenyo D, Alewood PF, Chait BT. Rapid sensitive analysis of cysteine rich peptide venom components. Proc. Natl. Acad. Sci. U.S.A. 2009; 106:6910–6915. [PubMed: 19380747]
36. Vasicek L, Brodbelt JS. Enhanced electron transfer dissociation through fixed charge derivatization of cysteines. Anal. Chem. 2009; 81:7876–7884. [PubMed: 19722535]
37. Reid GE, Roberts KD, Simpson RJ, O'Hair RA. Selective identification and quantitative analysis of methionine containing peptides by charge derivatization and tandem mass spectrometry. J. Am. Soc. Mass Spectrom. 2005; 16:1131–1135. [PubMed: 15923125]
38. Xu YW, Zhang LJ, Lu HJ, Yang PY. Mass Spectrometry Analysis of Phosphopeptides after Peptide Carboxy Group Derivatization. Anal. Chem. 2008; 80:8324–8328. [PubMed: 18821779]
39. Zhang LJ, Xu YW, Lu HJ, Yang PY. Carboxy group derivatization for enhanced electron-transfer dissociation mass spectrometric analysis of phosphopeptides. Proteomics. 2009; 9:4093–4097. [PubMed: 19658101]
40. Qiao XQ, Sun LL, Chen LF, Zhou YA, Yang KG, Liang Z, Zhang LH, Zhang YK. Piperazines for peptide carboxyl group derivatization: effect of derivatization reagents and properties of peptides on signal enhancement in matrix-assisted laser desorption/ionization mass spectrometry. Rapid Commun. Mass Spectrom. 2011; 25:639–646. [PubMed: 21290451]
41. Zhang JM, Al-Eryani R, Ball HL. Mass Spectrometry Analysis of 2-Nitrophenylhydrazine Carboxy Derivatized Peptides. J. Am. Soc. Mass Spectrom. 2011; 22:1958–1967. [PubMed: 21952763]
42. Ko BJ, Brodbelt JS. Enhanced Electron Transfer Dissociation of Peptides Modified at Cterminus with Fixed Charges. J. Am. Soc. Mass Spectrom. 2012; 23:1991–2000. [PubMed: 22895859]
43. Frey BL, Krusemark CJ, Ledvina AR, Coon JJ, Belshaw PJ, Smith LM. Ion-ion reactions with fixed-charge modified proteins to produce ions in a single, very high charge state. Int. J. Mass Spectrom. 2008; 276:136–143. [PubMed: 19802328]
44. Krusemark CJ, Frey BL, Belshaw PJ, Smith LM. Modifying the Charge State Distribution of Proteins in Electrospray Ionization Mass Spectrometry by Chemical Derivatization. J. Am. Soc. Mass Spectrom. 2009; 20:1617–1625. [PubMed: 19481956]
45. Inada T, Winstall E, Tarun SZ, Yates JR, Schieltz D, Sachs AB. One-step affinity purification of the yeast ribosome and its, associated proteins and mRNAs. RNA. 2002; 8:948–958. [PubMed: 12166649]
46. Simons SP, McLellan TJ, Aeed PA, Zaniewski RP, Desbonnet CR, Wondrack LM, Marr ES, Subashi TA, Dougherty TJ, Xu ZY, Wang IK, LeMotte PK, Maguire BA. Purification of the large ribosomal subunit via its association with the small subunit. Anal. Biochem. 2009; 395:77–85. [PubMed: 19646947]
47. Krokhin OV, Spicer V. Peptide Retention Standards and Hydrophobicity Indexes in Reversed-Phase High-Performance Liquid Chromatography of Peptides. Anal. Chem. 2009; 81:9522–9530. [PubMed: 19848410]
48. Schnier PD, Gross DS, Williams ER. On the Maximum Charge-State and Proton-Transfer Reactivity of Peptide and Protein Ions Formed by Electrospray-Ionization. J. Am. Soc. Mass Spectrom. 1995; 6:1086–1097. [PubMed: 24214055]
49. Coon JJ, Ueberheide B, Syka JEP, Dryhurst DD, Ausio J, Shabanowitz J, Hunt DF. Protein identification using sequential ion/ion reactions and tandem mass spectrometry. Proc. Natl. Acad. Sci. U.S.A. 2005; 102:9463–9468. [PubMed: 15983376]

50. Cooper HJ, Hudgins RR, Hakansson K, Marshall AG. Characterization of amino acid side chain losses in electron capture dissociation. J. Am. Soc. Mass Spectrom. 2002; 13:241–249. [PubMed: 11908804]
51. Rumachik NG, McAlister GC, Russell JD, Bailey DJ, Wenger CD, Coon JJ. Characterizing Peptide Neutral Losses Induced by Negative Electron-Transfer Dissociation (NETD). J. Am. Soc. Mass Spectrom. 2012; 23:718–727. [PubMed: 22290482]
52. Iavarone AT, Paech K, Williams ER. Effects of charge state and cationizing agent on the electron capture dissociation of a peptide. Anal. Chem. 2004; 76:2231–2238. [PubMed: 15080732]
53. Jones AW, Cooper HJ. Dissociation techniques in mass spectrometry-based proteomics. Analyst. 2011; 136:3419–3429. [PubMed: 21698312]
54. Huang YY, Triscari JM, Tseng GC, Pasa-Tolic L, Lipton MS, Smith RD, Wysocki VH. Statistical characterization of the charge state and residue dependence of low-energy CID peptide dissociation patterns. Anal. Chem. 2005; 77:5800–5813. [PubMed: 16159109]
55. Jones JW, Sasaki T, Goodlett DR, Turecek F. Electron capture in spin-trap capped peptides. An experimental example of ergodic dissociation in peptide cation-radicals. J. Am. Soc. Mass Spectrom. 2007; 18:432–444. [PubMed: 17112737]
56. Li XJ, Cournoyer JJ, Lin C, O'Connor PB. The Effect of Fixed Charge Modifications on Electron Capture Dissociation. J. Am. Soc. Mass Spectrom. 2008; 19:1514–1526. [PubMed: 18657441]
57. Chung TW, Turecek F. Amplified histidine effect in electron-transfer dissociation of histidine-rich peptides from histatin 5. Int. J. Mass Spectrom. 2011; 306:99–107.
58. Chung TW, Turecek F. Selecting fixed-charge groups for electron-based peptide dissociations - A computational study of pyridinium tags. Int. J. Mass Spectrom. 2008; 276:127–135.
59. Chung TW, Moss CL, Zimnicka M, Johnson RS, Moritz RL, Turecek F. Electron-Capture and -Transfer Dissociation of Peptides Tagged with Tunable Fixed-Charge Groups: Structures and Dissociation Energetics. J. Am. Soc. Mass Spectrom. 2011; 22:13–30. [PubMed: 21472540]

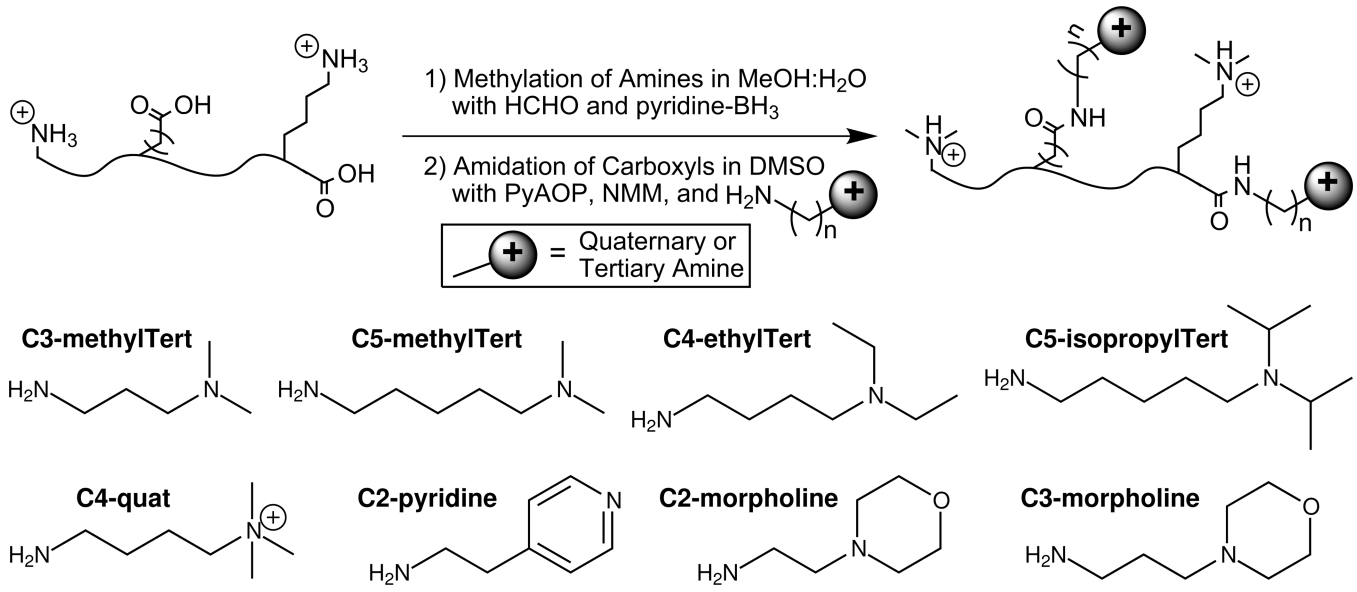

Figure 1.

Labeling strategy to increase peptide charge states. First, the native primary amine groups in the peptides are dimethylated to prevent them from reacting in the second step. Then, the carboxylic acid groups are amidated with a labeling reagent having a primary amine on one end and a tertiary or quaternary amine on the other end. The structures and abbreviated names are shown for the eight labeling reagents employed in this work. (All chemical reagent abbreviations are defined in the Experimental section)

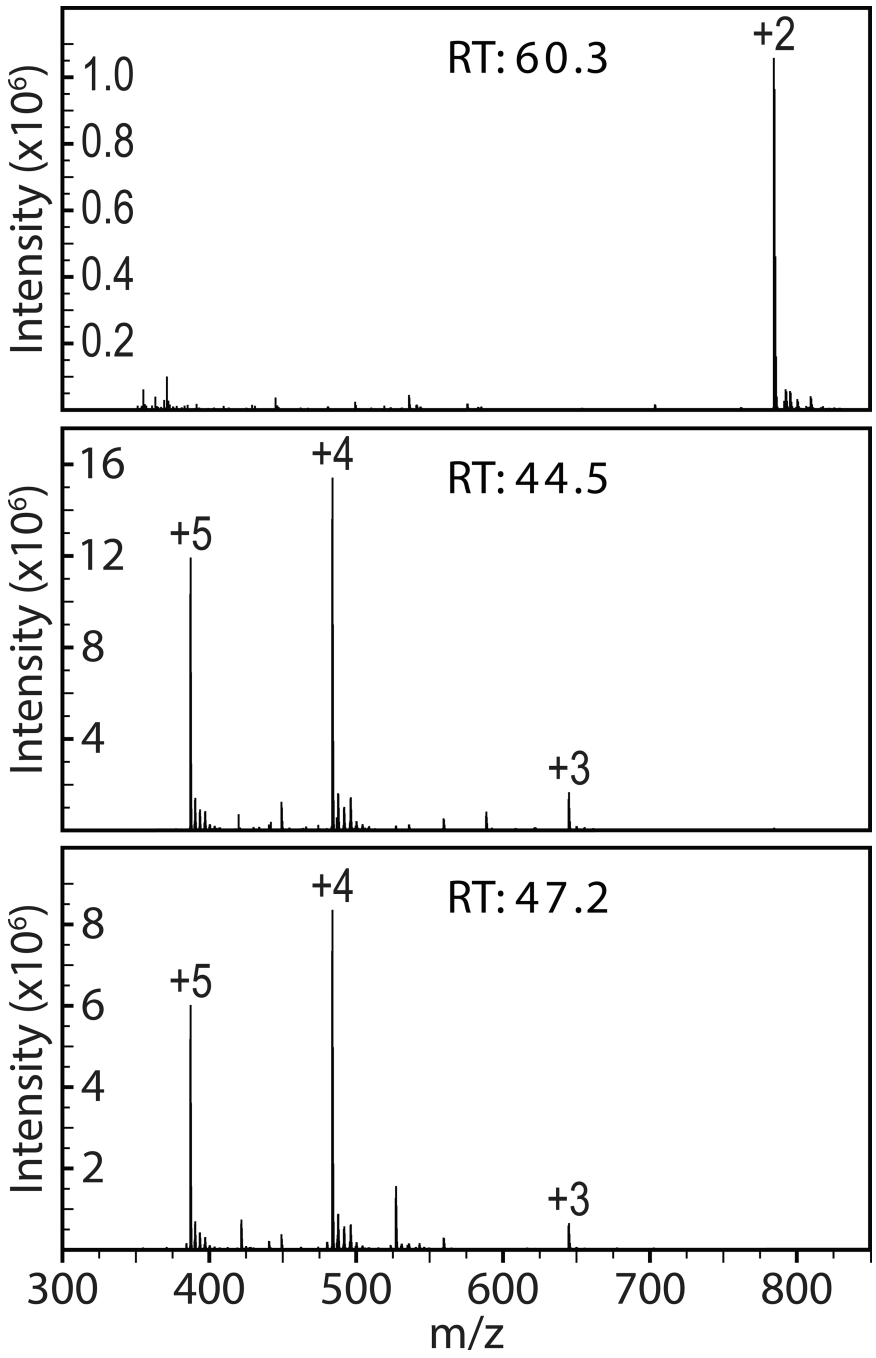

Figure 2.

Full scan MS spectra for the peptide DAFLGSFLYEYSR from LC-MS runs of unmodified (top), C4-quat modified (middle), and C5-methylTert modified (bottom) samples of a BSA tryptic digest. The retention times (RT) for these spectra are listed, and the charge states of the peaks are labeled. Note that the small peaks in these spectra are not due to side products of the derivatization reactions. Rather, there are peptide oxidation peaks (multiples of +16 Da) present in the unmodified spectrum as well as the modified ones, and other miscellaneous peaks are due to other peptides that happen to partially co-elute with this DAFLGSFLYEYSR peptide

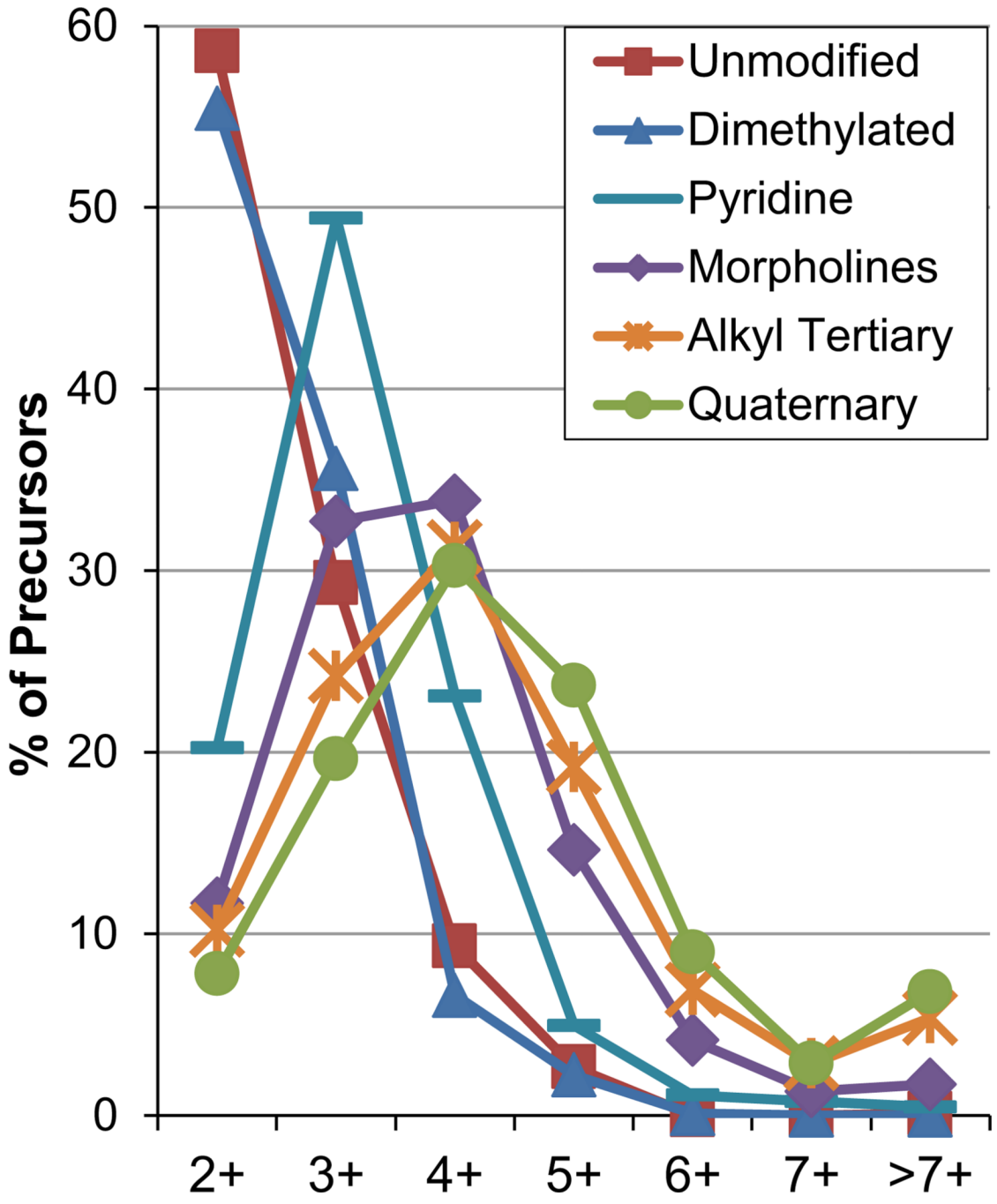

Figure 3.
Charge state distributions of peptide precursors from unmodified and modified tryptic digests of BSA. The data include precursor mass values >250 *m/z* from a retention time window of 22–53 min and exclude +1 ions; these cut-offs were chosen to minimize the number of non-peptide precursors. The four aliphatic tertiary amine labels were averaged and plotted together because they offered very similar charge state distributions; the same was true for the two morpholine labels

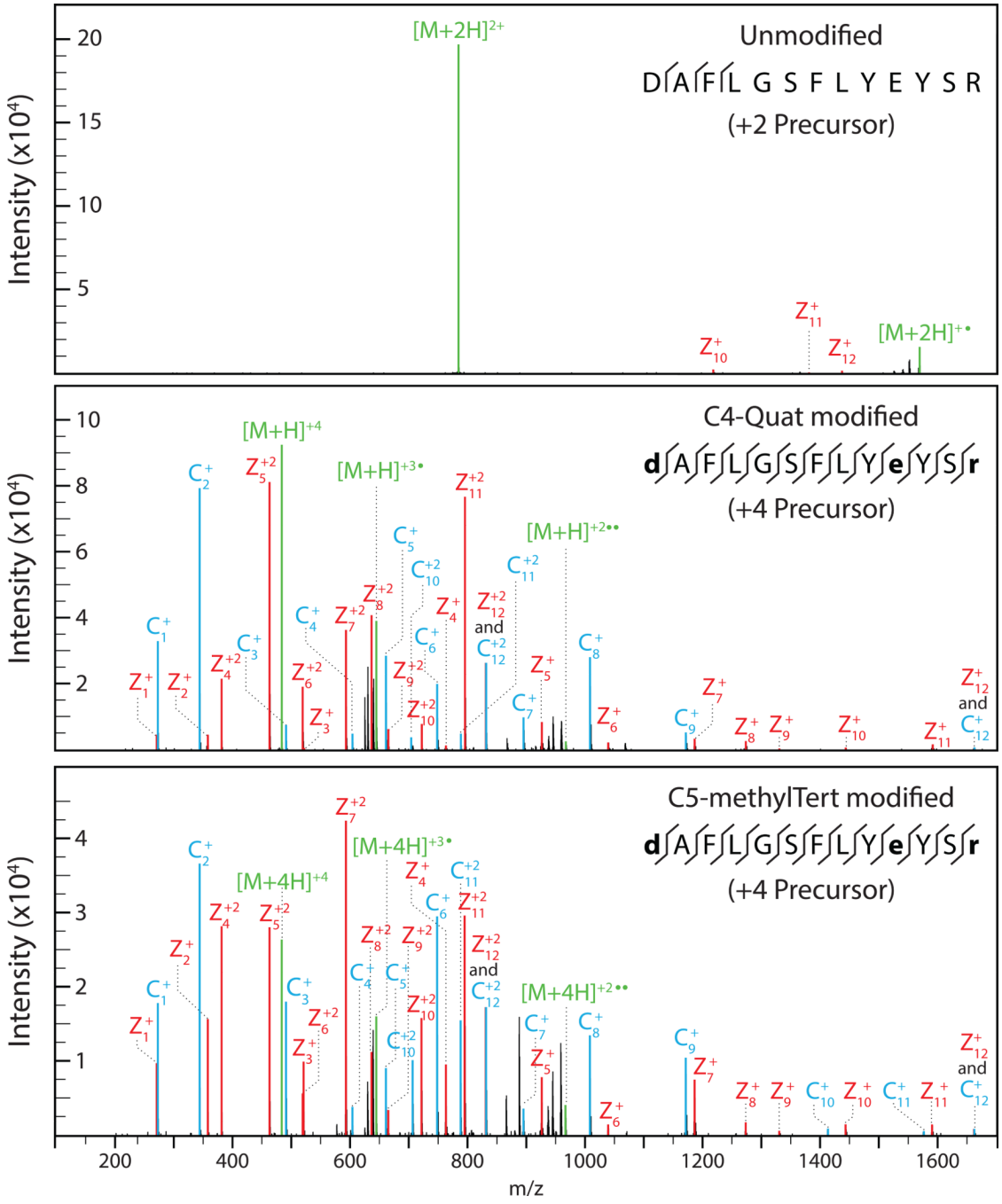

Figure 4.

ETD-MS/MS spectra for the peptide DAFLGSFLYEYSR from LC-MS runs of unmodified (top), C4-quat modified (middle), and C5-methylTert modified (bottom) samples of a BSA tryptic digest. The *c*- and •*z-type* fragment peaks are blue and red, respectively; precursor and charge-reduced precursor peaks are green; all other peaks are black. Lower case bold letters are used in the sequence to indicate the modified residues

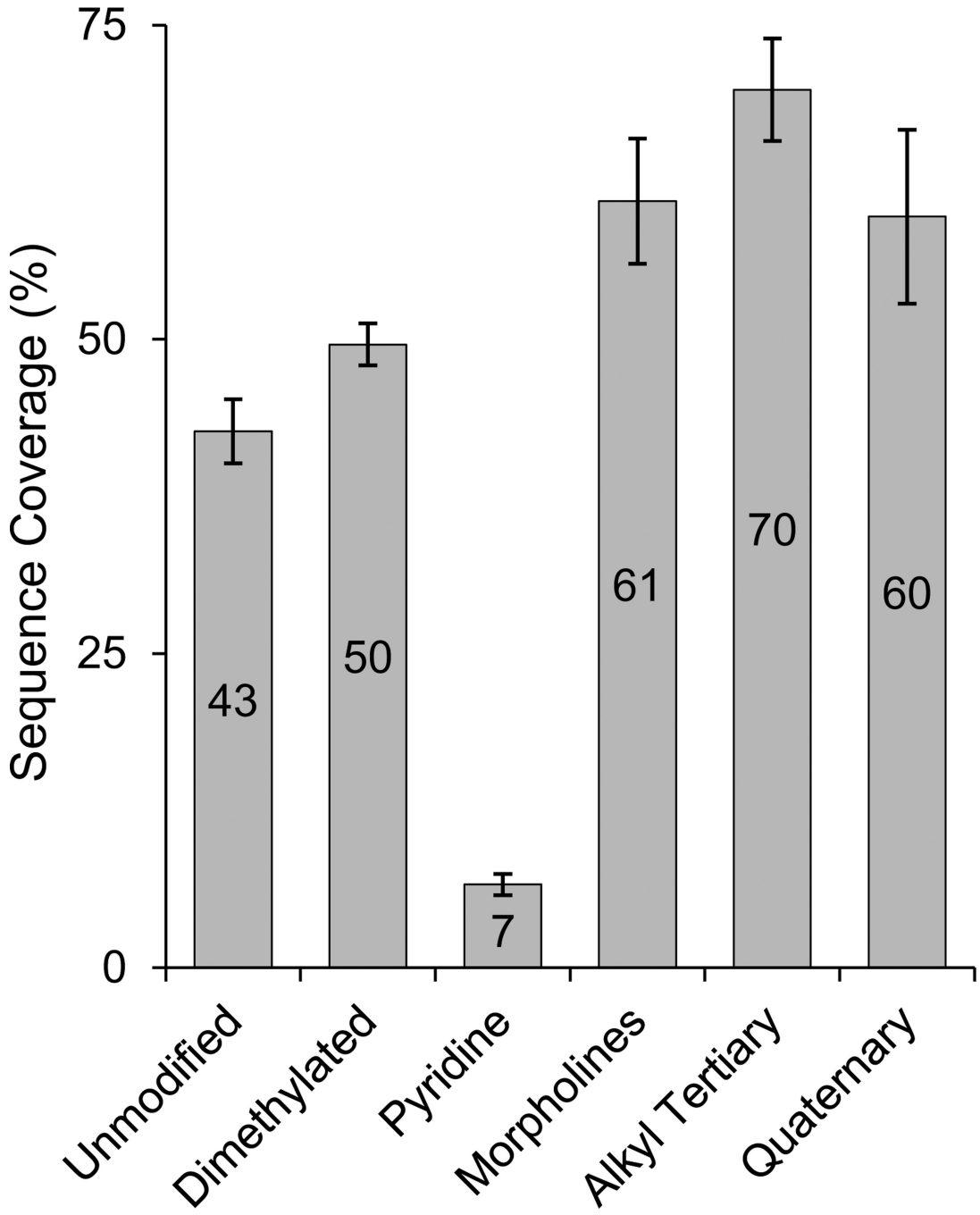

Figure 5.
Sequence coverage obtained by ETD for unmodified and various modified tryptic digests of BSA. As in Figure 3, the alkyl tertiary amine (and morpholine) results were averaged and plotted together. Values are the average of at least 3 LC-MS/MS runs, and the error bars represent +/− 1 standard deviation

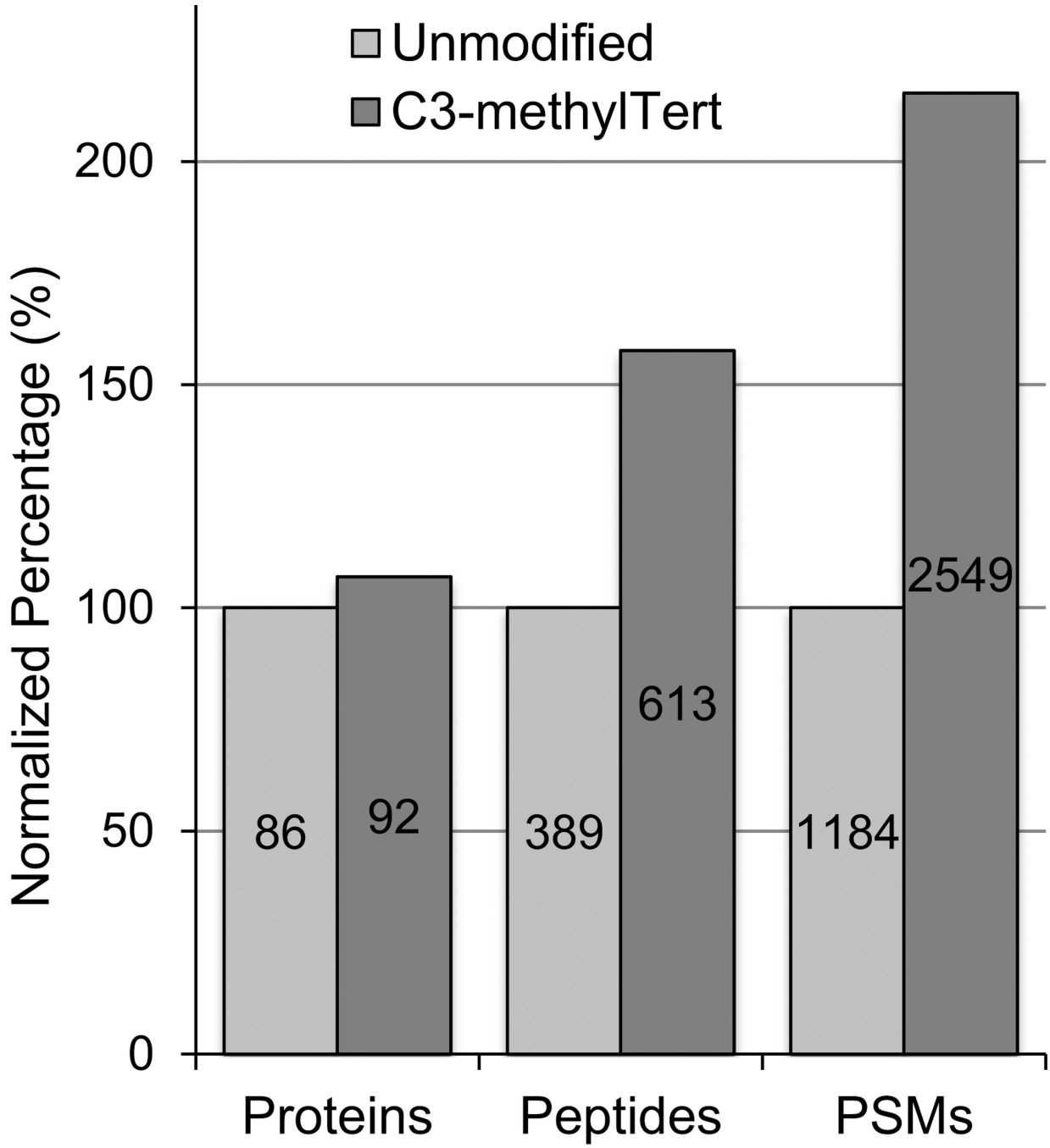

Figure 6.
ETD-MS results for unmodified and C3-methylTert modified tryptic digests of yeast ribosomal proteins

Table 1

Efficiency of the labeling reaction and charge state increases observed for several example peptides

Lower case bold letters are used in the sequence to indicate the residues with amidated carboxyl groups. (Note that all N-terminal and lysine-side-chain amino groups are dimethylated, but those modifications are not highlighted in the sequences).

	# COOH	Modification	Rxn Eff.	Average Charge State		
Test peptides			(%)	Before	After	Increase
pyroELY***e***NKPRRPYI***l***	2	C2-pyridine	98.8	2.9	3.8	0.9
"	2	C2-morpholine	99.2	"	3.9	1.0
"	2	C3-morpholine	99.8	"	4.0	1.1
"	2	C3-methylTert	98.7	"	4.3	1.4
"	2	C5-methylTert	98.8	"	4.3	1.4
"	2	C4-ethylTert	99.3	"	4.2	1.3
"	2	C5-isopropylTert	99.6	"	4.3	1.4
"	2	C4-quat	98.8	"	4.3	1.4
dA***e***NLI***d***SFQ***e***l***v***	5	C3-methylTert	99.4	< 2[a]	4.8	> 2.8
"	"	C4-quat	98.2	"	5.1	> 3.1
S***deee***AIVAYT***l***	5	C3-methylTert	99.5	< 2[a]	4.6	> 2.6
"	"	C4-quat	99.6	"	5.0	> 3.0
eQKLI***Seedl***	5	C4-quat	97.7	< 2[a]	5.0	> 3.0
Example BSA tryptic peptides[b]						
dAFLGSFLY***e***YS***r***	3	C5-methylTert	99.4	2.0	4.3	2.3
"	"	C4-quat	99.0	"	4.4	2.4
ddPHACYSTVF***dk***	4	C5-methylTert	98.5	2.7	5.5	2.8
"	"	C4-quat	98.2	"	5.5	2.8
SLHTLFG***de***LC***k***	3	C5-methylTert	96.7	2.7	4.6	1.9
"	"	C4-quat	98.5	"	4.6	1.9
LVN***e***LT***e***FA***k***	3	C5-methylTert	97.7	2.0	4.3	2.3
"	"	C4-quat	98.7	"	4.3	2.3

[a] For these three peptides, the peaks in the mass spectra showed predominantly +2 ions, no +3 ions, and only very minor levels of +1 ions; however, the instrument was not tuned to give good intensity for the high m/z +1 ions. Thus, the average charge states are less than 2, but a more precise value is not known.

[b] Four random example peptides, rather than all observed peptides for the BSA digest.