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Peptide Dimethylation: Fragmentation Control via Distancing the Dimethylamino Group

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Abstract

Direct reductive methylation of peptides is a common method for quantitative proteomics. It is an active derivatization technique; with participation of the dimethylamino group, the derivatized peptides preferentially release intense a_1 ions. The advantageous generation of a_1 ions for quantitative proteomic profiling, however, is not desirable for targeted proteomic quantitation using multiple reaction monitoring mass spectrometry; this mass spectrometric method prefers the derivatizing group to stay with the intact peptide ions and multiple fragments as passive mass tags. This work investigated collisional fragmentation of peptides whose amine groups were derivatized with five linear ω -dimethylamino acids, from 2-(dimethylamino)-acetic acid to 6-(dimethylamino)-hexanoic acid. Tandem mass spectra of the derivatized tryptic peptides revealed different preferential breakdown pathways. Together with energy resolved mass spectrometry, it was found that shutting down the active participation of the terminal dimethylamino group in fragmentation of derivatized peptides is possible. However, it took a separation of five methylene groups between the terminal dimethylamino group and the amide formed upon peptide derivatization. For the first time, the gas-phase fragmentation of peptides derivatized with linear ω -dimethylamino acids of systematically increasing alkyl chain lengths is reported.

Introduction

The use of peptide derivatization is a staple of proteomic quantitation.[1] Derivatization-based mass spectrometry (MS) quantitation is characterized by its sample-throughput capabilities allowing multiple samples to be analyzed simultaneously. Different applications are dependent upon fragmentation control of the derivatized peptide. In applications of isobaric mass tagging reagents [1, 2], the derivatized peptides are isobaric, but after fragmentation produce differentiable reporter ions in tandem MS (MS/MS) spectra. Peptide derivatization with these types of reagents are Type I active derivatizations (Scheme 1), classified by the strong signals directly observed from the fragmented derivatization group [3]. One significant pitfall of these reagents is the limited dynamic range, which is caused by all the derivatized peptides sharing common reporter ions; therefore, peptides with similar masses and elution time produce elevated background for the peptides in quantitation [4, 5].

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Reductive methylation has been used as both mass-difference tagging for MS quantitation [6-8] and recently isobaric mass tagging for MS/MS quantitation [9-11]. Dimethylation is a relatively simple reaction only requiring a reducing agent and formaldehyde. This reaction is expeditious without any substantial formation of side products. When stable isotope labels are used in the reducing agent and/or formaldehyde, peptides can be introduced with a designed number of stable isotope labels for MS-based quantitative measurements. This method has been broadly applicable due to its easy implementation [12].

The molecular basis for MS/MS quantitation of peptides with direct dimethylation is the facile collisional cleavage of the first N-terminal amino acid residues. Strong signals for derivatized a_1 ions (stable quaternary amine ions) are characteristic in MS/MS spectra of the derivatized peptides [13-15]. Dimethylation of peptides is considered a Type II active derivatization, where the derivatizing group promotes the cleavage of the first amide bond of peptides (Scheme 1). Collisional fragmentation of directly dimethylated peptides has been studied in detail [14, 15]. When the methyl groups carry differential stable isotope labels, the derivatized peptides can, in principle, be quantified based on the correspondingly labeled a_1 ions. This quantitation using MS/MS measurement could be advantageous. These a_1 ions are peptide-specific and thus lessen the interference from co-eluting peptides experienced for peptides derivatized with isobaric mass tagging reagents. In order to observe such interference, derivatized peptides would need to have similar elution times and masses as well as the same first N-terminal amino acid residue; a rare occurrence. However, authentic quadrupole sampling of dimethylated peptide ions with different numbers of isotope labels as precursors can be problematic. Efforts have been made to develop an isobaric mass tagging capability using peptide dimethylation. One method derivatizes peptides at both N- and C-termini, but with complementary numbers of stable isotope labels. In this method, triplex isobaric peptide termini labeling with dimethylation, peptide precursors are isobaric and fragment ions carry differentiable stable isotope labels and thus the ion intensity of the fragments can be used for quantitation [9].

In line with recent advancements in the utilization of ultra-high resolving MS for proteomics, differences in mass defects of carbon, hydrogen, oxygen, and nitrogen isotopes have been exploited [10, 11, 16-19]. The scope of isobaric mass tagging is thus being expanded, opening exciting new opportunities in proteome quantitation. In principle, all of the currently practiced, derivatization-based quantitative proteomic methods can be adapted to better utilize contemporary mass spectrometers. Two recent reports use peptide dimethylation. Peptides in comparison carry derivatizing groups with different isotopologue labels, i.e., two atoms of ^{13}C versus ^2H . Therefore, during the low-resolution selection of precursor ions, the differentially-labeled peptides are co-selected authentically. However, when MS/MS are recorded with ultra-high resolving power, fragment ions can be baseline-separated for quantitation. Both works use Lys-C for protein digestion so that each resulting peptide carries two derivatizing groups. It is interesting to note that there are more derivatized y ions than b ions available to be used for quantitation [10, 11]; in other words the ϵ -dimethylamino group on the lysine side chain at the peptide C-terminus is more stable than the α -dimethylamino group at the N-terminus.

Multiple reaction monitoring (MRM) MS is at the forefront of targeted, quantitative MS due to its high selectivity, sensitivity, and method robustness [20]. Stable isotope labeled references are commonly used to assemble methods for stable isotope dilution (SID) MRM MS. The generation of quantitation reference peptides can be achieved by derivatizing peptides with stable isotope labeled chemicals for mass-difference tagging [21, 22]. Although the quantitation utility of the derivatized reference peptides can be comparable to those produced by metabolic labeling techniques [23], these reagents have yet to be broadly adopted. The limited adoption of the commercial reagents for MRM MS exemplifies a common problem for using active derivatizations of peptides for MRM-based MS quantitation: the derivatizing groups are not efficiently held as intact mass tags for the precursor ions of the derivatized peptides or for their fragments.

MRM-based methods require the detection of strong signals for both precursor ions and fragment ions to obtain low quantitation limits. Furthermore, multiple distinguishable fragment ions are preferred to secure the method's specificity. If the derivatizing group is cleaved from the fragment ions, these fragments are no longer distinguishable among peptides with differential derivatizations. Therefore, MRM-based quantitation can benefit from passive derivatization of peptides (Scheme 1) [3]: during MRM analysis, derivatized peptides preserve the derivatizing groups on the intact peptide precursor ions in the ionization region of the instrument and keep these derivatizing groups on multiple fragment ions during fragmentation of the derivatized peptides in the collision cell. We hypothesize that if the dimethylamino group on derivatized peptides is disengaged from the adjacent amide bond, then a passive derivatization will be achieved for MRM-based peptide analysis. This derivatization will keep the increased gas-phase basicity of dimethylamino peptides, from the tertiary amine of the dimethylamino group, and accordingly the enhanced MS signals [24], but not activate particular peptide bonds in a biased manner. Herein, we present a systematic study of how diversely a dimethylamino group participates in collisional fragmentation of peptides with the increased distance from the first N-terminal amino acid residue. In addition, the change of role for the dimethylamino group from an active participant in fragmentation to a passive mass tagging one is reported.

Experimental

Chemicals

2-aminoacetic acid (>99%), 3-aminopropanoic acid (99%), 4-aminobutanoic acid (>99%), 5-aminopentanoic acid (>97%), 6-aminohexanoic acid (>99%), methanol (>99.9%), diethyl ether (>99%), glacial acetic acid (>99.7%), N,N-diisopropylethylamine (99.5%, DIEA), N-hydroxysuccinimide (98%, NHS), dimethyl sulfoxide (>99.7%, DMSO), anhydrous N,N-dimethylformamide (>99.8%, DMF), 4-(4,6-dimethoxy-1, 3, 5-triazin-2-yl)-4-methylmorpholinium chloride (96.0%, DMTMM), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (98.0%, EDC), and anhydrous dichloromethane (>99.5%, DCM) were purchased from Sigma-Aldrich (St. Louis, MO). Formic acid (88% and 99.5%), trifluoroacetic acid (97%, TFA), acetonitrile (99.9%, ACN), and formaldehyde (37%) were purchased from Fisher (Hanover Park, IL). Concentrated hydrogen chloride acid (36.5% - 38%) was purchased from J.T. Baker (Center Valley, PA). N, N'-diisopropylcarbodiimide

(99%, DIC) was purchased from Alfa Aesar (Ward Hill, MA). The ultrapure water was made from a Direct-Q 3 UV water purification system (EMD Millipore, Billerica, MA). The peptides LSLVPDSEQGEAILPR (95%), LSEPAELTDAVK (99%), YGGFLR (98%), and SVILLGR (99%) were purchased from Peptide 2.0 (Chantilly, VA). The peptide NSILTETLHR (95%) was purchased from Anaspec (Fremont, CA). The isotopic peptide SVIL[L-¹³C₆ ¹⁵N]GR (99%) was purchased from Thermo Fisher (Rockford, IL).

Dimethylamino Acid Synthesis

Each amino acid (8 mmol) was dissolved in formic acid (5 mL, 88%) and formaldehyde (1 mL). The synthesis of 3-(dimethylamino)-propanoic acid (denoted as dim-3), 4-(dimethylamino)-butanoic acid (denoted as dim-4), 5-(dimethylamino)-pentanoic acid (denoted as dim-5), and 6-(dimethylamino)-hexanoic acid (denoted as dim-6) was carried out in a microwave reactor (CEM Discover-S, Matthews, NC), with the following conditions: 100 W, 110 °C, 20 to 120 min. 2-(Dimethylamino)-acetic acid (denoted as dim-2) was refluxed at 100 °C for 1 hour. Concentrated HCl (1 mL) was added after heating. The solvent was dried *in vacuo*. After drying, a white precipitate was obtained and washed with glacial acetic acid (5 mL) three times. The dimethylamino acid was then recrystallized with methanol and diethyl ether. After purification, the reagents were characterized with nuclear magnetic resonance (NMR) spectroscopy (Avance III 400 MHz, Bruker, Billerica, MA) and high-resolution MS (AccuTOF™ DART, JEOL). Detailed syntheses and characterizations can be found in supplemental information (SI) 1-5.

Peptide Derivatization

The dimethylamino acid (1.0 equivalent), NHS (1.2 equivalents), and DIC (1.0 equivalent) were first dissolved in DMSO (10 µL), and then diluted with anhydrous DCM (30 µL). The solution was incubated overnight at room temperature. A solution containing five peptides, NSILTETLHR, LSLVPDSEQGEAILPR, LSEPAELTDAVK, YGGFLR, and SVILLGR dissolved in 10 µL of 10% DIEA/DMF, was added to the activated dimethylamino acid. The solution was incubated overnight at room temperature. The coupling was then quenched with 20% formic acid/H₂O on ice. The solution was first dried with a speed-vac (Savant SC100, Thermo Fisher) then lyophilized (Labconco FreeZone Plus). After drying, the sample was desalted via an empty spin column (Thermo Fisher) packed with hydrophilic-lipophilic-balanced (HLB, Oasis®) reversed-phase sorbent. The sample was then dried and reconstituted with FA/H₂O for liquid chromatography (LC)-MS analysis (Shimadzu HPLC pumps/controller, Kyoto, KYT, Japan and HTC PAL autosampler, Carrboro, NC).

LC-MS and LC-MS/MS Studies

A 10 cm, 1.0 mm I.D. Hypersil Gold HPLC column (Thermo Fisher Scientific, Inc., USA) with C18, 175 Å, and 3 µm resin was used. Solvent A was composed of 98.8% H₂O, 1.0% ACN, and 0.2% FA and solvent B was 98.8% ACN, 1.0% H₂O, and 0.2% FA. The LC gradient was 5% to 35% B for 45 minutes. The column temperature was 60 °C. The hybrid mass spectrometer used was a QSTAR® Elite (AB Sciex, Framingham, MA). Positive electrospray ionization (ESI) was used, with parameters of 5.5 kV for the spray voltage and source temperature of 300 °C. The initial collision energy (CE) and declustering potential

(DP) were obtained for the doubly- and triply-charged peptides from Equations 1 and 2, respectively [25].

$$CE = (0.057 \times m/z) - 4.265 \quad \text{Equation 1}$$

$$CE = (0.031 \times m/z) + 7.082 \quad \text{Equation 2}$$

These equations were empirically adjusted for the non-derivatized peptides (Table S1), and for dim-2-peptides (Table S2). The new equations from the dim-2-peptides were applied to dim-3-through dim-6-peptides.

Energy Resolved (ER)-MS Studies

An AB SCIEX 4000 Qtrap® (AB SCIEX, Framingham, MA) triple quadrupole mass spectrometer with direct syringe infusion of sample solutions was used for the ER-MS studies. The ESI was in positive mode with parameters of 5.5 kV for ion spray and source temperature of 200 °C. Each peptide (500 fmol/μL) was individually infused with a flow rate at 5 μL/min. The DP was optimized before the collision studies were started. The CE was ramped from 5 to 35 V in 0.5 V/s increments. The resolution was set to unit for Q1 and high for Q3. The dwell time was 200 msec. Three replicates were taken for each peptide measured (non-derivatized YGGFLR through dim-6-YGGFLR).

Results and Discussion

Microwave-Assisted Synthesis of Dimethylamino Acids

The established Eschweiler-Clarke reaction [26, 27] was used to dimethylate the primary amines of five amino acids: 2-aminoacetic acid, 3-aminopropanoic acid, 4-aminobutanoic acid, 5-aminopentanoic acid, and 6-aminohexanoic acid. They have increasing numbers of methylene groups, one through five (dim-2 through dim-6, respectively), distancing the amino group from the carboxylic acid. Full conversion of 3-aminopropanoic acid to dim-3 required 8 hours of conventional refluxing. Other amino acids were even less reactive towards amine dimethylation. In an attempt to expedite the dimethylation of 3-aminopropanoic acid to 6-aminohexanoic acid, microwave irradiation was utilized. All of the initial five substrates were converted to the corresponding products within 20 to 120 minutes of irradiation (SI 1-5).

Preparation of Dimethylamino Peptides

Five synthetic peptides were used as models to investigate collisional fragmentation mechanisms of dimethylated peptides: NSILTETLHR, LSLVPDSEQGEAILPR, LSEPAELTDAVK, YGGFLR, and SVILLGR. They possess a wide variety of properties (Table S3). Peptide LSEPAELTDAVK was chosen as a model peptide for lysine-containing peptides, which have two primary amines. Tandem mass spectra for these non-derivatized peptides are shown in Figures S1a-f.

Peptidyl amino groups on the model peptides were derivatized with dimethylamino acids whose carboxylic groups were activated *in situ* (Scheme 2). The activation of carboxylic groups was mediated by DIC and NHS. Solvent played a dominating role in this peptide derivatization. The dimethylamino acids were soluble in aqueous solution. However, derivatization of the peptides using water-soluble mediation reagents, including EDC/NHS and DMTMM, resulted in low and varying yields for the derivatization products. A mixed solvent of DMSO and DCM served well for activating the dimethylamino acids using DIC/NHS. This solvent system was miscible with the mixture of DIEA and DMF, which was used as the solvent for preparing peptide solutions for derivatization.

MS Signal Enhancement of Dimethylamino Peptides

The percentage of chemical conversion (PCC) and signal yield for mass spectrometry (SYMS) were quantitatively measured for peptides dim-2-SVILLGR through dim-6-SVILLGR. After quenching the derivatization reaction, an equal amount of isotopically labelled SVIL[L-¹³C₆¹⁵N]GR was added as the quantitation reference, although GSVIL[L-¹³C₆¹⁵N]GR would be a closer reference. The MS signal for the residual unreacted SVILLGR, compared to that for the isotopic quantitation reference, allowed for calculation of the PCC value for each derivatization. The average PCC for all five derivatizations of SVILLGR was 85% (Equation S1). Further optimization of derivatization reactions was not performed in this work, because these reactions were used to generate sufficient amounts of dimethylated peptides for mechanistic investigations not to be directly applied for proteome quantitation. The SYMS value for a derivatization was calculated based on the MS signal of the derivatized peptide, e.g., dim-2-SVILLGR, and that of the isotopic quantitation reference. The average SYMS for all five derivatized peptides was 170% (Equation S2). This average SYMS was further corrected for the derivatization completeness (or average PCC), giving a corrected SYMS of 200% (Equation S3), for the addition of dimethylamino acids to the N-terminus of SVILLGR.

The enhanced MS signal for the derivatized peptides can be attributed to the increased gas-phase basicity, compared to the underivatized SVILLGR. These derivatizations convert a primary N-terminal amino group to a tertiary amine which is more basic and results in more favorable protonation in the gas phase. Signal enhancement of 130% to 240% was also reported for peptides with dimethylated lysine side chains, compared to their non-dimethylated counterparts [24]. The increased basicity of peptides carrying the dimethylamino group is likely also attributing to the charge-state shift from a mixture of singly- and doubly-charged ions for SVILLGR and YGGFLR to the mostly doubly charged for their derivatized counterparts, and predominantly doubly-charged LSLVPDSEQGEAILPR to a doubly and triply-charged mixture (Figure S7a-c). The coalescence to doubly-charged SVILLGR from the singly- and doubly-charged mixture could also attribute to the increase in SYMS.

Fragmentation Dependence of Dimethylamino Peptides on Alkyl Chain Length

Tandem MS was performed on a QqTOF mass spectrometer for all of the derivatized peptides. The derivatized YGGFLR peptides are used as the exemplary peptides for discussion, with all others in the SI (Figures S1-6). The CE was empirically adjusted using

Equations 1 and 2 to ensure proper fragment ion production. The dim-2-peptides were used for the empirical adjustment, and the CE was increased by 1.1 to 1.5 times, compared to the suggested values from the equations (Table S3). The CE was adjusted until an approximate <10% precursor ion was observed after collision-induced dissociation (CID). Equations 1 and 2 were then corrected accordingly for other dimethylamino peptides. As shown in Scheme 3, fragment ions are denoted with typical nomenclature except for the following: (1) b and a ions carrying the derivatizing group are denoted as *b and *a respectively, (2) b and a ions carrying a cyclization product of the derivatizing group are denoted as ^cb and ^ca respectively, (3) ions from the bond cleavage between the α and β carbon from the dimethylamino group is denoted as f₁, (4) b and a ions carrying an acetyl group (the complementary products from the α - β cleavage) are denoted as ^ab and ^aa, respectively, and (5) the ions from the amide bond cleavage between the derivatizing group and the original N-terminus of the peptide is denoted as f₂, and the corresponding production of the singly-charged ion as the original peptide is denoted as °M. A general trend of the increased coverage of b and derivatized b ions was observed for the derivatized peptides compared to their underivatized counterparts (Figures S1-6) [6].

Dim-2 derivatization results in peptides with the same chemical structure as peptides with direct reductive dimethylation; collisional fragmentation mechanism for the directly methylated peptides has been studied in detail (Scheme 4a) [14, 15]. For instance, dim-2-YGGFLR (MS/MS spectrum shown in Figure 1) is the same as dimethylated GYGGFLR. Dimethylated peptides are known to produce enhanced signals for *a₁ ions [13-15]. In the nomenclature system used in this paper, the f₁ ion was observed for dim-2-YGGFLR (or *a₁ for dimethylated GYGGFLR) at *m/z* 58.06 (Figure 1; Scheme 4). Generation of f₂ ions (or *b₁ for dimethylated GYGGFLR) at *m/z* 86.20 can be accounted for by the oxozolium mechanism [28] or the aziridin-2-one pathway (Scheme 4b) which further produces the f₁ ion [29]. Generation of f₂ ions suggests the favorable protonation of the dimethylamino group and a facile intramolecular proton transfer to activate the adjacent amide group in the derivatized peptides (Scheme 4b). In other words, there is an active participation of the derivatizing group in the peptide fragmentation.

Dim-3 derivatization extends the distance by one additional methylene group between the dimethylamino group and the first amide group on the derivatized peptides, which is formed upon the attachment of a dimethylamino propionic acid. Two intense ions at *m/z* 58.06 (f₁) and *m/z* 754.36 [MH-f₁]⁺ were observed for dim-3-YGGFLR, together with significant signals for acetyl b ions (^ab₁, ^ab₂, ^ab₃, and ^ab₄), shown in Figure 2. The sum of *m/z* 58.06 and *m/z* 754.36 made up the mass balance for dim-3-YGGFLR, suggesting the existence of a labile bond. Generation of these 2 ions can be readily explained by the McLafferty-type rearrangement (Scheme 5) [30]. It should be noted that although the ion structure for f₁ ions for dim-2-peptides and dim-3-peptides are the same, mechanisms for the ion generation are different (Schemes 4 and 5). In general, the f₁ ion was observed for all dim-3 peptides (Figure 2 and Figure S3). On the other hand, not all of the corresponding [MH-f₁]⁺ ions were observed (Figure S3), which could be due to further breakage of the primary [MH-f₁]⁺ fragment ions under experimental conditions. According to the categorization of peptide

derivatization shown in Scheme 1, dim-3 reaction would be classified as Type I active derivatization, where there is preferential cleavage within the derivatizing group.

A common, neutral loss of dimethylamine was observed for doubly-charged ions of dim-4-, dim-5-, and dim-6-YGGFLR, producing corresponding doubly-charged fragments (Scheme 6). Dim-4 derivatization produced a very strong fragment ion at m/z 390.75 (cyc^{2+} , Figure 3). Dim-5-YGGFLR gave a doubly-charged ion at m/z 397.73 (cyc^{2+}) as the most intense fragment (Figure 4). These 2 ions are proposed as YGGFLR carrying an imido lactone at the N-terminus (Scheme 6). A complement of b and a ions carrying the imido lactone, i.e., $^{\text{c}}\text{b}$ and $^{\text{c}}\text{a}$ ions, were observed for dim-4- and dim-5-YGGFLR. Formation of these ions can be explained by nucleophilic substitution of the amide oxygen to the α -methylene carbon of the dimethylamino group (Scheme 6). Similar mechanisms were reported in a fragmentation study of lysylglycine [31] and used for explaining the charge mobilization of peptides derivatized with a quaternary amine [32] and loss of dimethylamine from N^{e} -dimethyllysine [33]. Dim-6-YGGFLR produced a similar cyclization product at m/z 404.72 (cyc^{2+} , Figure 5), but at a much reduced intensity and without observable complementary $^{\text{c}}\text{a}$ and $^{\text{c}}\text{b}$ ions. The intensity differences could be related to the stability of imido lactone rings.

Doubly-charged dim-6-YGGFLR, compared to dim-4- and dim-5-YGGFLR, produced full series of $^{\text{a}}$ and $^{\text{b}}$ ions carrying the derivatization group, together with y ions (Figures 3-5). This suggests that in the dim-6-peptide the preferential cleavage via the mechanism in Scheme 5 becomes much less competitive. In other words, the dim-6 derivatization is passive (Scheme 1). Similar results were also observed for other dim-6 derivatized peptides (Figure S6). It is also interesting to note that the intensity for the residual doubly-charged precursor was high (Figure 5), using the collision energy obtained from the same calculation as those for other derivatized peptides (Table S2), dim-2-YGGFLR through dim-5-YGGFLR.

Double derivatization of LSEPAELTDAVK was obtained at both the N-terminus and the lysine side chain. For example, dim-4-LSEPAELTDAVK (Figure S4d) produced fragments that corresponded to the cyclization occurring at both sites. The first cyclization product (m/z 727.45) was 80% relative intensity and the second (m/z 704.90) was 30% relative intensity. In comparison, one cyclization product (m/z 741.43) was observed for dim-5-LSEPAELTDAVK at 15% relative intensity (Figure S5e), which reports the bond cleavage at the N-terminus.

Another Preferential Cleavage Pathway of Dim-4-, Dim-5-, and Dim-6-Peptides

Two ions related to cleavage of the derivatizing group from the doubly-charged dim-4-YGGFLR precursor were observed; one dominantly at m/z 114.10 (f_2) and the other strong ion at m/z 712.38 ($^{\text{c}}\text{M}$, Figure 3). Dim-5-YGGFLR and dim-6-YGGFLR, cleaving at the same amide bond, also produced ion pairs at m/z 128.15 and 712.38 and at m/z 142.12 and 712.34, respectively. However, the relative ion intensities for the corresponding f_2 ions, although strong, were not dominantly high for dim-5- and dim-6-YGGFLR (Figures 4 and 5). The favorable cleavage of the amide bond can be accounted for by the mechanism proposed in Scheme 7. The intensity differences among these ions are on the same order of the facility for intramolecular proton transfer, requiring the formation of a pseudo ring of 7,

8, or 9 atoms, with the increasing entropy penalty. Although dim-2- and dim-3-peptides have a more facile intramolecular proton transfer through a pseudo 5- or 6-membered ring (Schemes 4b and 5), the nucleophilic attack of the deprotonated dimethylamine to the carbon of protonated amide would experience high ring constraints; the generation of similar f_2 ions would require the formation of a ring with three or four atoms following the mechanism in Scheme 7. Protonated aziridin-2-ones, however, are viable intermediates for a_1 ions of peptides upon collisional dissociation (Scheme 4b) [29].

ER-MS to Categorize Peptides into Active and Passive Derivatization Groups

ER-MS is an excellent tool to evaluate the energy requirements for the generation of fragment ions and elucidation of fragmentation patterns and mechanisms [28]. ER-MS was performed for YGGFLR and dim-2- through dim-6-YGGFLR. The CE profiles, in the laboratory frame, for the breakdown of the derivatized peptides were recorded on a triple quadrupole instrument. After normalization to the initial precursor ion intensity, triplicate measurements were combined as a single data set for sigmoidal dose-response fitting to produce the survival curve (Figure 6), giving a CE value at 50% (CE^{50}) of residual precursor ions. Fitting plots for all of the six YGGFLR peptides are shown in Figure S8 and the formulae and the goodness of fitting are reported in Table S4. CE^{50} values for these peptides were plotted against m/z values of doubly-charged precursor ions (Figure 7). Dim-2-, dim-3-, dim-4-, and dim-5-YGGFLR correlated linearly with a slope of 0.153 and adjusted R^2 of 0.9994. Together with the fact that different pathways operate to preferentially produce f_1 and/or f_2 ions and their complementary fragment ions (Schemes 4-7), this linear correlation suggests that these different mechanisms result in minimal differential effects on CE required for the breakage of doubly-charged precursor ions.

The underivatized YGGFLR showed positive deviation of 1.81 volts from the linear correlation (Figure 7). This deviation signifies the peptide as a mechanistic indicator, reporting that there is no special bond in the peptide which is particularly labile to gas-phase collision. The same is true for the positive deviation of 2.59 volts for dim-6-YGGFLR (Figure 7). Although dim-4- to dim-6-YGGFLR share a common fragmentation pathway releasing the derivatizing groups as N,N-dimethyl lactams (Scheme 7), the increased distance between the dimethylamino group and the amide bond formed upon the peptide derivatization leads to a discrete increase in the stability of intact precursors from dim-5- to dim-6-YGGFLR, shown as the positive deviation (Figure 7). This agrees with the observation that the ϵ -dimethylamino group on the lysine side chain is a passive mass tag (Figure S2e), stable to collisional fragmentation in the gas phase [10, 11].

Two Routes for Selective Activation of Dimethylamino Peptides

There are two general mechanisms for a derivatizing group actively participating in preferential fragmentations around the N-termini of derivatized peptides. Both start from the preferential protonation of the dimethylamino group. This proton then either: (1) transfers to the adjacent amide group acting as an acid catalyst to facilitate fragmentation reactions (the mobile-proton regime) as in Schemes 4b, 5, and 7, or (2) directly polarizes the α -methylene carbon of the protonated dimethylamino group for the nucleophilic substitution by the adjacent amide oxygen (the charge-directed regime) as in Scheme 6. Proton migration from

basic amino acids, including histidine, lysine, and arginine, has been shown to promote preferential neighboring cleavages [34-36]. The first general mechanism also operates in the preferential fragmentation of peptides derivatized with isothiocyanates [37, 38]. The importance of the intramolecular proton transfer is evident in this study. When the intramolecular proton transfer becomes less favorable for dim-6-YGGFLR, the preferential activation of the adjacent amide bond diminishes and so do the subsequent fragmentation products. In comparison, proton transfer from non-methylated ϵ -amine at the peptide N-terminus faces competing paths due to the complex intramolecular solvation of the protonated amine group [39]; therefore activation of the first amide group via acid catalysis confronts similar competitions.

For the second general mechanism, although activation of the α -methylene carbon of the protonated dimethylamino group stays the same more or less, the direct nucleophilic substitution by the amide oxygen becomes less entropically favorable with the distancing of the protonated dimethylamino group from the neighboring amide. Succinctly, a quantized change happens from dim-5-YGGFLR to dim-6-YGGFLR or from active derivatization to passive derivatization (Scheme 1).

Conclusion

Reagents for quantitative MS are essential tools in proteomic technologies. Full utilization of contemporary MS advancement requires a clear appreciation of chemical principles governing the gas-phase fragmentation of derivatized peptides [40]. In certain applications, *active* cleavage of the derivatizing group is preferred to produce quantitative reporter ions and simple spectra of sequence ions for concurrent peptide identification; common examples are proteomic peptides derivatized with tandem mass tagging reagents. In other applications like MRM MS, it is advantageous to have the derivatizing group associated with multiple fragment ions. This requires a derivatizing group staying *passive* as a ubiquitous mass tag during fragmentation processes of derivatized peptides [3]. Understanding the underlying principles of gas-phase chemistry of derivatized peptides, as revealed by the investigational reagents in this study, can guide rational design of novel reagents for quantitative MS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Yao X. Derivatization Or Not: A Choice in Quantitative Proteomics. *Anal Chem.* 2011; 83:4427–4439. [PubMed: 21495688]
2. 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, Bartlett-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]
3. Shi Y, Bajrami B, Yao X. Passive and Active Fragment Ion Mass Defect Labeling: Distinct Proteomics Potential of Iodine-Based Reagents. *Anal Chem*. 2009; 81:6438–6448. [PubMed: 19572545]
 4. Ow SY, Salim M, Noirel J, Evans C, Rehman I, Wright PC. iTRAQ Underestimation in Simple and Complex Mixtures: “The Good, the Bad and the Ugly”. *J Proteome Res*. 2009; 8:5347–5355. [PubMed: 19754192]
 5. Karp NA, Huber W, Sadowski PG, Charles PD, Hester SV, Lilley KS. Addressing Accuracy and Precision Issues in iTRAQ Quantitation. *Mol Cell Proteomics*. 2010; 9:1885–1897. [PubMed: 20382981]
 6. Hsu JL, Huang SY, Chow NH, Chen SH. Stable-Isotope Dimethyl Labeling for Quantitative Proteomics. *Anal Chem*. 2003; 75:6843–6852. [PubMed: 14670044]
 7. Boersema PJ, Raijmakers R, Lemeer S, Mohammed S, Heck AJ. Multiplex Peptide Stable Isotope Dimethyl Labeling for Quantitative Proteomics. *Nat Protoc*. 2009; 4:484–494. [PubMed: 19300442]
 8. Wu Y, Wang F, Liu Z, Qin H, Song C, Huang J, Bian Y, Wei X, Dong J, Zou H. Five-Plex Isotope Dimethyl Labeling for Quantitative Proteomics. *Chem Commun (Camb)*. 2014; 50:1708–1710. [PubMed: 24394284]
 9. Koehler CJ, Arntzen MO, de Souza GA, Thiede B. An Approach for Triplex-Isobaric Peptide Termini Labeling (Triplex-IPTL). *Anal Chem*. 2013; 85:2478–2485. [PubMed: 23316706]
 10. Bamberger C, Pankow S, Park SK, Yates JR. Interference-Free Proteome Quantification with MS/MS-Based Isobaric Isotopologue Detection. *J Proteome Res*. 2014; 13:1494–1051. [PubMed: 24417624]
 11. Zhou Y, Shan Y, Wu Q, Zhang S, Zhang L, Zhang Y. Mass Defect-Based Pseudo-Isobaric Dimethyl Labeling for Proteome Quantification. *Anal Chem*. 2013; 85:10658–10663. [PubMed: 24180428]
 12. Kovanich D, Cappadona S, Raijmakers R, Mohammed S, Scholten A, Heck AJ. Applications of Stable Isotope Dimethyl Labeling in Quantitative Proteomics. *Anal Bioanal Chem*. 2012; 404:991–1009. [PubMed: 22644145]
 13. Hsu JL, Huang SY, Shiea JT, Huang WY, Chen SH. 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]
 14. 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 N-Terminal Dimethylation. *Anal Chem*. 2005; 77:7783–7795. [PubMed: 16316189]
 15. Locke SJ, Leslie AD, Melanson JE, Pinto DM. Deviation from the Mobile Proton Model in Amino-Modified Peptides: Implications for Multiple Reaction Monitoring Analysis of Peptides. *Rapid Commun Mass Spectrom*. 2006; 20:1525–1530. [PubMed: 16642461]
 16. Rose CM, Merrill AE, Bailey DJ, Hebert AS, Westphall MS, Coon JJ. Neutron Encoded Labeling for Peptide Identification. *Anal Chem*. 2013; 85:5129–5137. [PubMed: 23638792]
 17. McAlister GC, Huttlin EL, Haas W, Ting L, Jedrychowski MP, Rogers JC, Kuhn K, Pike I, Grothe RA, Blethrow JD, Gygi SP. Increasing the Multiplexing Capacity of TMTs using Reporter Ion Isotopologues with Isobaric Masses. *Anal Chem*. 2012; 84:7469–7478. [PubMed: 22880955]
 18. Werner T, Becher I, Sweetman G, Doce C, Savitski MM, Bantscheff M. High-Resolution Enabled TMT 8-Plexing. *Anal Chem*. 2012; 84:7188–7194. [PubMed: 22881393]
 19. Hebert AS, Merrill AE, Stefely JA, Bailey DJ, Wenger CD, Westphall MS, Pagliarini DJ, Coon JJ. Amine-Reactive Neutron-Encoded Labels for Highly Plexed Proteomic Quantitation. *Mol Cell Proteomics*. 2013; 12:3360–3369. [PubMed: 23882030]
 20. Abbatiello SE, Mani DR, Schilling B, Maclean B, Zimmerman LJ, Feng X, Cusack MP, Sedransk N, Hall SC, Addona T, Allen S, Dodder NG, Ghosh M, Held JM, Hedrick V, Inerowicz HD, Jackson A, Keshishian H, Kim JW, Lyssand JS, Riley CP, Rudnick P, Sadowski P, Shaddock K, Smith D, Tomazela D, Wahlander A, Waldemarson S, Whitwell CA, You J, Zhang S, Kinsinger CR, Mesri M, Rodriguez H, Borchers CH, Buck C, Fisher SJ, Gibson BW, Liebler D, Maccoss M, Neubert TA, Paulovich A, Regnier F, Skates SJ, Tempst P, Wang M, Carr SA. Design,

Implementation and Multisite Evaluation of a System Suitability Protocol for the Quantitative Assessment of Instrument Performance in Liquid Chromatography-Multiple Reaction Monitoring-MS (LC-MRM-MS). *Mol Cell Proteomics*. 2013; 12:2623–2639. [PubMed: 23689285]

21. DeSouza LV, Taylor AM, Li W, Minkoff MS, Romaschin AD, Colgan TJ, Siu KW. Multiple Reaction Monitoring of mTRAQ-Labeled Peptides Enables Absolute Quantification of Endogenous Levels of a Potential Cancer Marker in Cancerous and Normal Endometrial Tissues. *J Proteome Res*. 2008; 7:3525–3534. [PubMed: 18630974]
22. DeSouza LV, Krakovska O, Darfler MM, Krizman DB, Romaschin AD, Colgan TJ, Siu KW. mTRAQ-Based Quantification of Potential Endometrial Carcinoma Biomarkers from Archived Formalin-Fixed Paraffin-Embedded Tissues. *Proteomics*. 2010; 10:3108–3116. [PubMed: 20661955]
23. Oppermann FS, Klammer M, Bobe C, Cox J, Schaab C, Tebbe A, Daub H. Comparison of SILAC and mTRAQ Quantification for Phosphoproteomics on a Quadrupole Orbitrap Mass Spectrometer. *J Proteome Res*. 2013; 12:4089–4100. [PubMed: 23898821]
24. Gropengiesser J, Varadarajan BT, Stephanowitz H, Krause E. The Relative Influence of Phosphorylation and Methylation on Responsiveness of Peptides to MALDI and ESI Mass Spectrometry. *J Mass Spectrom*. 2009; 44:821–831. [PubMed: 19301359]
25. Maclean B, Tomazela DM, Abbatiello SE, Zhang S, Whiteaker JR, Paulovich AG, Carr SA, Maccoss MJ. Effect of Collision Energy Optimization on the Measurement of Peptides by Selected Reaction Monitoring (SRM) Mass Spectrometry. *Anal Chem*. 2010; 82:10116–10124. [PubMed: 21090646]
26. Eschweiler W. Replacement of Hydrogen Atoms by the Methyl Group by Formaldehyde, Bound at Nitrogen. *BER*. 1905; 38:880–882.
27. Clarke HT, Gillespie HB, Weisshaus SZ. Action of Formaldehyde on Amines and Amino Acids. *J Am Chem Soc*. 1933; 55:4571–4587.
28. Harrison AG. To B Or Not to B: The Ongoing Saga of Peptide B Ions. *Mass Spectrom Rev*. 2009; 28:640–654. [PubMed: 19338048]
29. Harrison AG, Csizmadia IG, Tang TH, Tu YP. Reaction Competition in the Fragmentation of Protonated Dipeptides. *J Mass Spectrom*. 2000; 35:683–688. [PubMed: 10862119]
30. McLafferty FW. Mass Spectrometric Analysis: Molecular Rearrangements. *Analytical Chemistry*. 1959; 31:82–87.
31. Csonka IP, Paizs B, Lendvay G, Suhai S. Proton Mobility and Main Fragmentation Pathways of Protonated Lysylglycine. *Rapid Commun Mass Spectrom*. 2001; 15:1457–1472. [PubMed: 11507760]
32. He Y, Reilly JP. Does a Charge Tag really Provide a Fixed Charge? *Angew Chem Int Ed Engl*. 2008; 47:2463–2465. [PubMed: 18300222]
33. Yalcin T, Harrison AG. Ion Chemistry of Protonated Lysine Derivatives. *J Mass Spectrom*. 1996; 31:1237–1243. [PubMed: 8946732]
34. Farrugia JM, Taverner T, O'Hair RAJ. Side-Chain Involvement in the Fragmentation Reactions of the Protonated Methyl Esters of Histidine and its Peptides. *Int J Mass Spectrom*. 2001; 209:99–112.
35. Tsapralis G, Nair H, Zhong W, Kuppannan K, Futrell JH, Wysocki VH. A Mechanistic Investigation of the Enhanced Cleavage at Histidine in the Gas-Phase Dissociation of Protonated Peptides. *Anal Chem*. 2004; 76:2083–2094. [PubMed: 15053674]
36. Hiserodt RD, Brown SM, Swijter DF, Hawkins N, Mussinan CJ. A Study of b1+H2O and B1-Ions in the Product Ion Spectra of Dipeptides Containing N-Terminal Basic Amino Acid Residues. *J Am Soc Mass Spectrom*. 2007; 18:1414–1422. [PubMed: 17543534]
37. van der Rest G, He F, Emmett MR, Marshall AG, Gaskell SJ. Gas-Phase Cleavage of PTC-Derivatized Electrosprayed Tryptic Peptides in an FT-ICR Trapped-Ion Cell: Mass-Based Protein Identification without Liquid Chromatographic Separation. *J Am Soc Mass Spectrom*. 2001; 12:288–295. [PubMed: 11281604]
38. Diego PA, Bajrami B, Jiang H, Shi Y, Gascon JA, Yao X. Site-Preferential Dissociation of Peptides with Active Chemical Modification for Improving Fragment Ion Detection. *Anal Chem*. 2010; 82:23–27. [PubMed: 19904916]

39. Laskin J, Yang Z, Song T, Lam C, Chu IK. Effect of the Basic Residue on the Energetics, Dynamics, and Mechanisms of Gas-Phase Fragmentation of Protonated Peptides. *J Am Chem Soc.* 2010; 132:16006–16016. [PubMed: 20977217]
40. Barlow CK, O'Hair RAJ. Gas-Phase Peptide Fragmentation: How Understanding the Fundamentals Provides a Springboard to Developing New Chemistry and Novel Proteomic Tools. *J Mass Spectrom.* 2008; 43:1301–1319. [PubMed: 18819114]

Figure 1.
MS/MS Spectrum of Dim-2-YGGFLR

Figure 2.
MS/MS Spectrum of Dim-3-YGGFLR

Figure 3.
MS/MS Spectrum of Dim-4-YGGFLR

Figure 4.
MS/MS Spectrum of Dim-5-YGGFLR

Figure 5.
MS/MS Spectrum of Dim-6-YGGFLR

Figure 6.

Survival Curve of Dim-6-YGGFLR. The precursor was selected in Q1 and Q3 with the CE being ramped from 5 to 35 volts in 0.5 V/s increments. Three replicates were obtained for each YGGFLR peptide, and the data was normalized to the max intensity. After sigmoidal dose-response fitting the CE⁵⁰ was obtained.

Figure 7.
 CE^{50} and m/z Correlation for Dimethylamino YGGFLR Peptides

Scheme 1.
Categorization of Peptidyl Chemical Derivatizations

Scheme 2.

General Reaction for Derivatizing Peptidyl Amines with Dimethylated Amino Acids

n = 1 for dim-2, 2 for dim-3, 3 for dim-4, 4 for dim-5, 5 for dim-6

Scheme 3.

Nomenclature of Fragment Ions as Represented by Dim-3-YGGFLR

Scheme 4.
Mechanism of f_1 Production from Dim-2 Derivatized Peptides

Scheme 5.
Mechanism of f_1 Production from Dim-3 Derivatized Peptides

Scheme 6.

Imido Lactone Formation at the N-terminus of Dim-4-, Dim-5-, and Dim-6-YGGFLR

n = 1 for dim-4, 2 for dim-5 and 3 for dim-6

Scheme 7.

N,N-Dimethyl Lactam Ion Loss from Dim-4-, Dim-5-, and Dim-6-YGGFLR

n = 1 for dim-4, 2 for dim-5, and 3 for dim-6