Unusual fragmentation of derivatized cysteine-containing peptides

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Abstract

RATIONALE—Modification of cysteines by aminoethylation results in side chains similar to those of lysine. Trypsin cleaves at this modified residue and this labeling method can facilitate the analysis of proteins, specifically antibodies. In this work, the ability to identify peptides containing aminoethylated cysteines is investigated through digestion, covalent labeling, and low-energy ion fragmentation.

METHODS—A prototype antibody was reduced, aminoethylated, and digested with either Lys-N or Glu-C. The resulting peptides were amidinated with SMTA and analyzed by PSD in a MALDI-TOF/TOF mass spectrometer or by CID in an ESI-ion trap/orbitrap mass spectrometer.

RESULTS—PSD and CID fragmentation of peptides with an amidinated aminoethylated cysteine can produce an intense characteristic loss from this modified residue. A neutral loss of 118 Da or charged loss of 119 Da is observed when peptides have low charges. This fragment can form when the cysteine is located in any position in the peptide. The rationalization for this ion is that the amidino group can be initially neutral or protonated and initiates fragmentation.

CONCLUSIONS—The combination of a dual-labeling technique and low-energy fragmentation produces an abundant diagnostic ion for the analysis of cysteine-containing peptides. These 118 and 119 Da losses are observed when protons are sequestered.

INTRODUCTION

Proteins often contain disulfide bonds that help to stabilize their tertiary structure.^[1–2] For example, disulfide bonds connect the heavy and light chains of antibodies and facilitate exposure of complementarity determining regions.^[3] Mass spectrometric analysis of proteins usually includes reduction of disulfide bonds followed by enzymatic digestion.^[4] To prevent reformation of disulfide bonds, cysteine alkylation is commonly performed with reagents such as iodoacetic acid and iodoacetamide.^[5] 2-bromoethylamine is another cysteine reactive molecule that generates a lysine-like residue, called an aminoethylated cysteine.^[6–7] Although it contains a sulfur atom, the side chain of this modified residue is comparable in length to that of a lysine. Although it was reported that trypsin cleaves C-terminal to aminoethylated cysteines^[8–10], Plapp and co-workers found that this occurs less

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readily than at arginines and lysines.^[11–12] The pKa for the side chain amine of an aminoethylated cysteine has been measured to be between 9.2 and 9.8, which is lower than the 10.79 pKa of the lysine ϵ amine.^[13–15] This pKa difference provides an explanation for the reduced enzymatic cleavage at modified cysteines.

Lysine-terminated peptides produced from tryptic digestion of proteins tend to ionize less efficiently by matrix-assisted laser desorption/ionization (MALDI) compared to peptides containing arginine.^[16–17] For this reason, lysine is often guanidinated with O-methylisourea or amidinated with S-methylthioacetimidate (SMTA) to create a more basic group at the side chain amine and improve MALDI signals.^[18–20] Although guanidination modifies lysines, amidination labels both lysines and N-termini. In the fragmentation of guanidinated or amidinated peptides, the amidino group is also able to sequester a proton better than the primary amine in lysine, which results in improved ionization efficiency and sequence coverage.^[21–22] This process of sequestering or mobilizing protons is known to affect peptide fragmentation.^[23–25]

In the present work, a method for identifying cysteine-containing peptides is outlined. A prototype antibody is utilized as a source of these peptides. Cysteines are aminoethylated with 2-bromoethylamine. The ability of the enzyme Lys-N to cleave at aminoethylated cysteines is then investigated. To improve ionization yields, amidination of aminoethylated cysteines is performed. Singly charged peptides containing aminoethylated or amidinated aminoethylated cysteines are produced by MALDI and fragmented by post-source decay (PSD) in a time-of-flight (TOF/TOF) mass spectrometer. In order to investigate the effect of charge state on fragmentation, peptides are also electrosprayed and collisionally fragmented in an ion trap/orbitrap mass spectrometer.

EXPERIMENTAL

Materials

2-bromoethylamine hydrobromide, TRIS hydrochloride, trypsin, α -cyano-4-hydroxycinnamic acid (CHCA), acetylated cysteine (Ac-C), and acetic anhydride were purchased from Sigma (St. Louis, MO, USA). Glu-C was from Promega (Madison, WI, USA). Potassium phosphate was acquired from JT Baker (Phillipsburg, NJ, USA) and ammonium bicarbonate was from Mallinckrodt Chemicals (Phillipsburg, NJ, USA). Concentrated sodium hydroxide was obtained from VWR Analytical (Radnor, PA, USA) and dithiothreitol was purchased from Bio-Rad (Hercules, CA, USA). Genentech (South San Francisco, CA, USA) provided the antibody, rituximab. Fmoc-Arg(Ptf) Wang resin and Fmoc-Cys(Trt)-OH were from Midwest Biotech (Fishers, IN, USA).

Peptide Synthesis

Dipeptide CR was synthesized in-house as previously described.^[26] To generate N-terminally acetylated CR (Ac-CR), acetic anhydride was added to the synthesis cell and allowed to react for 1 hour prior to peptide cleavage from the solid phase resin.

Protein Aminoethylation, Digestion, and Amidination

The disulfide bonds in 60 µg of rituximab were reduced with 5 mM dithiothreitol in water at 37°C for 1 hour. 2-bromoethylamine hydrobromide was prepared for cysteine aminoethylation as previously reported.^[14] 0.1 mL of 2 M 2-bromoethylamine hydrobromide was added to 0.088 mL of 5 M NaOH and incubated for 5 minutes at 55°C. The resulting solution is approximately 1.06 M ethylene imine, which is the cysteine reactive molecule. The aminoethylation reaction with rituximab was performed in a 400 mM TRIS solution and ethylene imine was added to the reduced protein in a ratio of 1500:1. 5 mM Ac-C and Ac-CR were reacted with 100 mM of ethylene imine in a 25 mM of ammonium bicarbonate solution. Reactions proceeded for 2 hours in a sonicator. After protein aminoethylation, reagents and buffer were removed with a 3 kDa spin filter by adding 100 mM ammonium bicarbonate. Lys-N was extracted from mushrooms in-house. Digestions with this enzyme were performed in solutions of 90% ammonium bicarbonate and 10% acetonitrile with 2 µg of Lys-N. Alternatively, 2 µg of Glu-C was employed in 100% sodium phosphate buffer. In either case, rituximab was digested overnight at 37°C.

Amidination of peptides was performed as described in previous work.^[20] A 43.4 g/L SMTA stock solution was prepared in a 250 mM tris(hydroxymethyl)aminomethane or 25 mM ammonium bicarbonate solution. Peptide and SMTA solutions were mixed in equal volumes. The reaction proceeded for 1 hour at room temperature.

Mass Spectrometry

For analysis with an ABI 4800 MALDI-TOF/TOF mass spectrometer (Framingham, MA, USA), peptide mixtures from the digestion of rituximab were injected onto a reversed-phase Eksigent Nano2D liquid chromatograph (Framingham, MA, USA) with a home-packed 150 µm ID C18 column. The eluent was then coupled to an Eksigent Eksport MALDI spotter to separate peptides across 96 sample wells. Following deposition onto a MALDI sample plate, CHCA was added to the dried peptide spots. Singly charged peptide ions were fragmented by PSD. The laser intensity was adjusted for optimal fragmentation and the metastable suppressor was enabled. Spectra were generated by averaging 500-1000 laser pulses.

A Thermo Scientific Orbitrap Fusion Lumos Tribrid mass spectrometer (Waltham, MA, USA) with an ESI source was used to obtain multiply charged peptide ions and fragment them by CID. Peptide mixtures were separated by a reversed-phase Thermo Scientific EASY-nLC 1200 liquid chromatograph with a Thermo Scientific Acclaim PepMap RSLC C18 column. Data dependent ion fragmentation was performed with a 3 Da isolation width, 0.250 activation Q, and 35 eV collision energy.

For analysis of Ac-C and Ac-CR, a Thermo Scientific LTQ ion trap mass spectrometer (Waltham, MA, USA) with an ESI source was used. A mixture consisting of 80% of the reaction solution, 19.9% ACN, and 0.1% FA was directly infused into the mass spectrometer. Ions were fragmented by CID with a 3 Da isolation width, 0.250 activation Q, and a normalized collision energy of 30.

RESULTS AND DISCUSSION

Aminoethylated Peptides from Lys-N Digest

The disulfide bonds in rituximab were reduced and the cysteines were aminoethylated as shown in Scheme 1a. Aminoethylated rituximab was digested with Lys-N, which cleaves N-terminal to lysines. After LC separation, peptides were analyzed by PSD and CID. For example, C(+43)EVTHQGLSSPVT is a peptide with a 43 Da aminoethyl label on an N-terminal cysteine. The PSD spectrum for this singly charged peptide is displayed in Figure 1a. Abundant b-type ions that contain the aminoethylated cysteine are identified, while y-type ions that do not contain the modification also appear. In order to compare the effect of charge state on fragmentation, this peptide was also electrosprayed and then collisionally activated. The spectra for the doubly and triply charged peptides are in Figures 1b and 1c. Similar to the PSD spectrum, abundant b-type ions that contain the aminoethylated cysteine are observed, although some of these are multiply charged. Although it is not obvious why the b_{12}^{2+} ion is such a dominant feature in these two spectra, it is possibly due to a salt-bridge or zwitterion involving threonine.^[25, 27] Several other peptides that also have N-terminal aminoethylated cysteines were obtained from the Lys-N digestion, and are listed in Table S1. Although rituximab contains 16 cysteines, Lys-N peptides are not expected at all sites because of the locations of other lysines. Note that two examples contained missed cleavages, one of which includes the protein's C-terminal cysteine. Nevertheless, these results indicate that Lys-N is capable of cleaving on the N-terminal side of aminoethylated cysteine.

Amidinated Aminoethylated Peptides

To improve the ionizability of peptides and compare the effects of chemical labeling on fragmentation, rituximab peptides obtained from Lys-N digestion were amidinated with SMTA as shown in Scheme 1b, LC separated, and analyzed using both PSD and CID. Interestingly, guanidination, a similar amine modification method, does not occur at aminoethylated cysteines, most likely due to their lower pKa.^[13–15] Nevertheless, after amidination of C(+43)EVTHQGLSSPVT, the singly charged (+41)C(+84)EVTHQGLSSPVT was identified. In this notation, the +41 indicates an amidinated N-terminal amine and the +84 denotes an amidinated aminoethylated cysteine. The PSD mass spectrum for this amidinated aminoethylated peptide in Figure 2a contains only two intense peaks that correspond to loss of 118 Da from the precursor mass and an additional loss of ammonia. The only other fragments present are very low intensity b_1 and y_{12} ions. These low intensity ions result from cleavage of the peptide bonds between the C and E residues as facilitated by the amidino group at the N-terminus.^[23] The CID spectrum of the doubly charged peptide appears in Figure 2b. Formation of the 1364.7 Da $[MH-118]^+$ product ion from the 741.9 Da doubly charged precursor requires loss of a singly charged 119 Da fragment. Additionally, several low intensity b-118 ions also appear. The CID spectrum for the triply charged peptide shown in Figure 2c contains a very intense b_{12}^{2+} ion that did not lose 118 Da and a few other low abundance ions, but the dominant M-118 ions from Figures 2a and 2b are absent. Other examples with an N-terminal amidinated aminoethylated cysteine are shown in Figures S1 and S2. In both cases, abundant M-118 ions are formed from activation of the +2 charged peptide but not from the +3 charged

peptide. Comparison of Figures 1 and 2 demonstrates that amidination drastically affects the fragmentation of this peptide when it is in a low charge state.

Aminoethylated rituximab was also digested with Glu-C to obtain peptides with a modified cysteine at locations other than their N-terminus. For example, (+41)SPVTK(+41)SFN*RGEC(+84) contains a missed cleavage site and an amidinated aminoethylated cysteine that is at the C-terminus of the rituximab light chain. The N* corresponds to a deamidated asparagine. The PSD spectrum for the singly charged peptide is in Figure 3a and CID spectra for the +2, +3, and +4 charged peptides are in Figures 3b, c, and d. Intense 118 Da losses were observed for the +1 and +2 charged peptides. However, +3 and +4 charged peptides did not exhibit this characteristic loss and instead intense b- and y-type ions were produced. (+41)YK(+41)C(+84)K(+41)VSNK(+41)ALPAPIE is another peptide obtained from Glu-C digestion that contains an amidinated aminoethylated cysteine that is not at either terminus. PSD of the +1 charged peptide (Figure 4a) leads to an abundant [MH-118]⁺ fragment, while CID of the +3 charged peptide (Figure 4b) forms [M+2H-118]²⁺ and [M+3H-118]³⁺. The abundant [M+2H-118]²⁺ fragment indicates loss of 119 Da, similar to Figure 2b. The +4 (Figure 4c) and +5 (Figure 4d) charged peptides did not produce this loss and primarily formed intense higher charged b-type ions, such as b₁₄³⁺ and b₁₄⁴⁺, respectively.

The origin of the novel 118 Da neutral or 119 Da ion fragment losses was further investigated. An accurate mass of the [MH-118]⁺ ion in Figure 2b measured with an orbitrap is 1364.6788 Da and the mass of the precursor MH⁺ (where M is (+41)C(+84)EVTHQGLSSPVT) ion derived from measurement of the doubly charged [M+2H]²⁺ ion is 1482.7367 Da. Their difference is 118.0579 Da, which is consistent with the calculated mass of HS-CH₂-CH₂-NH-CN₂-CH₃ to within 0.0014 Da. This neutral loss corresponds to cleavage between the β-carbon and sulfur of the amidinated aminoethylated cysteine. This interpretation was supported by further fragmentation of certain ions. Collisional activation of the [MH-118]⁺ product ion from (+41)C(+84)EVTHQGLSSPVT leads to the spectrum in Figure 5a. This spectrum displays an abundant loss of ammonia but provides little information about the location of the original 118 Da loss. However, isolation and collisional excitation of the [MH-118-NH₃]⁺ ion produces the MS³ spectrum in Figure 5b. Abundant b- and a-type ions now appear, all of which are calculable based on N-terminal losses of 118 Da and ammonia from protonated (+41)C(+84)EVTHQGLSSPVT. This is consistent with the 118 Da loss arising from the amidinated aminoethylated cysteine. Figure 5c contains the MS³ spectrum obtained by activating the b₁₂²⁺ product ion from triply charged (+41)C(+84)EVTHQGLSSPVT. Intense ions result from the loss of 118 Da. Note that while fragmentation of the *triply* charged peptide in Figure 2c did not produce these M-118 ions, activation of *doubly* charged fragment ions containing the intact amidinated aminoethylated cysteine can generate this characteristic loss, similar to the doubly charged peptide in Figure 2b. Evidently, peptide charge state directly impacts fragmentation patterns; charges need to be sequestered in order to observe M-118 ions.^[24, 28–29]

As shown in Figure 1, the 118 Da loss resulting from cleavage of the C_β-S bond does not occur when cysteines are only aminoethylated. To demonstrate this, the amino acid cysteine with an acetylation group on the N-terminus was aminoethylated and collisionally activated.

The resulting spectrum, in Figure S3a, displays a very low intensity peak 1 that corresponds to cleavage of the C β -S bond along with two other ions that result from side chain fragmentation. After amidinating the aminoethylated acetylated cysteine, the amidino group on the cysteine side chain should be the only particularly basic site in this amino acid, and therefore it should be protonated. Upon activation of the singly charged ion, loss of 118 Da (peak 2 in Figure S3b) is not a dominant process but peak 1, at 119 Da, corresponding to protonated HS-CH $_2$ -CH $_2$ -NH-CN H -CH $_3$ is more prominent. Analysis of the labeled dipeptide Ac-C(+84)R is further enlightening, since in this case arginine is the most basic site in the molecule. When the singly charged ion is fragmented, (Figure S3c), loss of neutral 118 Da is the most abundant product.

The above results indicate that cleavage of the C β -S bond can be a dominant process when a peptide contains an amidinated aminoethylated cysteine, but it depends on the charge state. In general, the number of charges on a peptide must be less than the number of basic sites (sum of arginines, histidines, and amidination labels) in order to abundantly produce the 118 Da loss. In other words, this fragmentation pathway is favored when charges are sequestered, analogous to the fragmentation of peptides C-terminal to aspartic acid residues.^[24] If charges are mobile, they can move to the N-terminal areas of residues to initiate fragmentation.^[27] The 119 Da ion in Figure S3b, did not appear in other spectra presented here due to the low mass cutoff of the mass spectrometer. However, examples in Figures 2b, 4b, S1a, and S2a suggest that a 119 Da ion is probably formed because there is a charge state difference between the precursor and the M-118 product ion. Therefore, based on the observation of both 118 and 119 Da fragments, HS-CH $_2$ -CH $_2$ -NH-CN H -CH $_3$ can be lost as a neutral or as an ion, though the former seems to be a more preferred process. The observation of the 119 Da ion is likely affected by the size of the peptide and the number of basic sites.

The signature fragmentation of oxidized methionine and phosphopeptides may provide some valuable mechanistic insights. In oxidized methionine, the S=O group in the side chain has been shown to abstract a proton from the β -carbon in situations when charges are sequestered.^[30–32] Upon rearrangement of electrons, HSO-CH $_3$ is released leaving a double bond between the β - and γ -carbons. Likewise, phosphopeptides can proceed through a charge-remote β -elimination, in which an oxygen from the phosphorylation group abstracts a proton from the α -carbon and rearranges electrons to release H $_3$ PO $_4$.^[33–35] In the present case, the amidino group may act in an analogous manner by abstracting a proton from the α -carbon to initiate the fragmentation. The abstracted hydrogen rearranges to HS-CH $_2$ -CH $_2$ -NH-CN H -CH $_3$ which is released. This mechanism is illustrated in Scheme 2. If the amidino group is initially protonated, a resonance structure can still enable the amine to abstract a hydrogen atom from the α -carbon, which leaves as a charged 119 Da ion.

Another possible mechanism would involve the neighboring oxygen in the amide abstracting the hydrogen from the α -carbon to initiate cleavage of the C β -S bond, resulting in an oxazoline structure. This is similar to the fragmentation of phosphopeptides, that proceeds through an S $_N$ 2 pathway.^[36]

These tentative mechanisms require further investigation.

CONCLUSIONS

Aminoethylation of cysteines creates a cleavable site for Lys-N that may facilitate analysis of proteins that contain disulfide bonds. Amidination of the primary amine in the side chain of an aminoethylated cysteine increases its basicity. If a peptide contains an amidinated aminoethylated cysteine and its charges are sequestered, its fragmentation produces an abundant neutral 118 Da or charged 119 Da loss corresponding to cleavage of the C β -S bond in this modified residue. This signature ion can enable recognition of cysteine-containing peptides. Collisional activation of fragments formed from the combined loss of 118 Da + NH₃ can provide more information about the peptide. Mobile protons inhibit this characteristic loss, as evidenced by the fragmentation of peptides in higher charge states. Likewise, activation of low charge state product ions that contain the intact amidinated aminoethylated cysteine can also produce this 118 Da loss. The fragmentation spectra of Ac-C(+84), Ac-C(+84)R, and larger amidinated aminoethylated peptide ions indicate that this signature loss is observed when protons are sequestered.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(a) PSD mass spectrum in a MALDI-TOF/TOF mass spectrometer of the singly charged C(+43)EVTHQGLSSPVT peptide ion (1400.6 Da). CID mass spectra in a ESI-ion trap-orbitrap mass spectrometer of the (b) doubly (700.8 Da) and (c) triply charged (467.6 Da) peptide ions. * indicates ammonia loss and ‡ denotes water loss.

Figure 2.

(a) PSD mass spectrum in a MALDI-TOF/TOF mass spectrometer of the singly charged (+41)C(+84)EVTHQGLSSPVT peptide ion (1482.7 Da). CID mass spectra in a ESI-ion trap-orbitrap mass spectrometer of the (b) doubly (741.9 Da) and (c) triply charged (494.9 Da) peptide ions. * indicates ammonia loss and ‡ denotes water loss.

Figure 3.

(a) PSD mass spectrum in a MALDI-TOF/TOF mass spectrometer of the singly charged (+41)SPVTK(+41)SFN*RGEC(+84) peptide ion (1491.8 Da). CID mass spectra in a ESI-ion trap-orbitrap mass spectrometer of the (b) +2 (746.4 Da), (c) +3 (497.9 Da), and (d) +4 charged (373.7 Da) peptide ions. * indicates ammonia loss and † denotes water loss.

Figure 4.

(a) PSD mass spectrum in a MALDI-TOF/TOF mass spectrometer of the singly charged (+41)YK(+41)C(+84)K(+41)VS NK(+41)ALPAPIE peptide ion (1909.2 Da). CID mass spectra in a ESI-ion trap-orbitrap mass spectrometer of the (b) +3 (637.0 Da), (c) +4 (478.0 Da), and (d) +5 charged (382.6 Da) from peptide ions. * indicates ammonia loss and ‡ denotes water loss.

Figure 5.

CID MS³ spectra in a ESI-ion trap-orbitrap mass spectrometer of the fragments (a) [MH-118]⁺ (1364.7 Da) and (b) [MH-118-NH₃]⁺ (1347.7 Da) from doubly charged (+41)C(+84)EVTHQGLSSPVT. The loss of 118 Da and ammonia are from the amidinated aminoethylated cysteine and the amidine label at the N-terminus, respectively. The MS³ spectrum for (c) b₁₂²⁺ (682.3 Da) is from triply charged (+41)C(+84)EVTHQGLSSPVT. * indicates ammonia loss and † denotes water loss.

Scheme 1.

(a) Cysteine aminoethylation with 2-bromoethylamine and (b) amidation of the primary amines with SMTA.

Scheme 2.

Proposed mechanism for the fragmentation of $\text{Ac-C}(+84)\text{R}$. RH^+ is protonated arginine.