# Fragmentation of Amidinated Peptide Ions

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The collision-induced dissociation characteristics of amidinated and unmodified tryptic peptides are compared using an ion trap mass spectrometer with both electrospray ionization and matrix-assisted laser/desorption ionization (MALDI). Several fragmentation pathways in a number of tryptic peptides of various precursor charge states are found to be enhanced. The additional information conveyed by the observed fragment ions should facilitate protein identifications.   (J Am Soc Mass Spectrom 2004, 15, 158–167) © 2004 American Society for Mass Spectrometry

Over the past decade the combination of emerging methods in biological mass spectrometry and separations science has enabled the accurate, rapid, and sensitive characterization of the protein complements of complex biological mixtures [1–3]. One of the most common techniques used to identify proteins involves the gas phase fragmentation of one or more of their enzymatic or chemical digestion products, followed by matching of MS/MS data to theoretical sequences in a database [4, 5]. This general approach has been applied with both electrospray (ESI) and matrix-assisted laser/desorption ionization (MALDI) using a number of different ion activation methods [6–10] and mass analyzers [11–13]. For many years low-energy collision induced dissociation (CID) has been the activation method of choice [2]. However, techniques such as high-energy CID [6], surface induced dissociation (SID) [7], photo dissociation (PD) [8], electron capture dissociation (ECD) [9], and infrared multi-photon dissociation (IRMPD) [10] have also proven useful. Since these methods employ different activation mechanisms, they often provide complementary sequence specific information. Regardless of the technique employed, the interpretation of tandem mass spectra without automated methods is not feasible in a proteomic experiment entailing the analysis of thousands of unknowns. There are usually several different observed ion types and accurately predicting relative fragmentation efficiencies is not currently possible. Furthermore, peptides rarely yield a contiguous series of ions that would facilitate de novo sequencing. Because of the complex nature of peptide fragmentation, a number of computer database searching algorithms have been developed to facilitate the interpretation of tandem mass spectra [4, 5]. With such tools spectra can be rapidly compared with the predicted fragment ion masses of candidate sequences found in a database. Candidates are typically defined as peptides whose calculated masses are isobaric with a given precursor ion. One of the criteria used in matching peptide sequences is the similarity of predicted and experimentally acquired mass spectra. In the SEQUEST algorithm, for example, each acquired spectrum is cross-correlated with those predicted for candidate sequences and the result is quantified with a score (Xcorr). However, this score alone is not sufficient to determine if a match is unambiguous. It is possible for multiple candidate sequences to be given a high score, thus increasing the uncertainty of a protein assignment. This difficulty is exacerbated by large databases and less accurate mass analyzers since these factors increase the number of candidate peptides, thus leading to more false positive matches.

It has long been recognized that mass spectrometric peptide sequencing techniques would be improved if the relative fragmentation efficiencies of the cleavage sites in a peptide could be predicted. Along with the calculated masses of fragment ions, current algorithms use only rudimentary knowledge of fragmentation efficiencies when generating hypothetical MS/MS spectra. Thus, the fragmentation of peptide ions has been extensively researched throughout the development of biological mass spectrometry [14]. Often, the goals of these studies include the identification of residues that enhance specific ion fragmentation pathways. For example, numerous researchers have described the highly efficient fragmentation of backbone amide bonds on the N-terminal side of proline residues [15–19]. Likewise, others have elucidated dissociation pathways that are promoted by acidic residues [20, 21]. The research groups of Gaskell and Wysocki have examined this phenomenon by analyzing the dissociation of acidic residue-containing peptides as a function of the relative number of ionizing protons and arginine residues [21]. They found that formation of b- and y-type fragment ions from cleavage on the C-terminal side of acidic residues was enhanced when the number of ionizing

protons did not exceed the number of highly basic, charge-sequestering arginine residues. They also found that chemical derivatizations that add a basic group or a fixed charge induce a similar effect [22, 23]. Based on these findings, they proposed that the acidic hydrogen of aspartic acid, rather than a mobile proton, promotes backbone cleavages via a seven-membered ring intermediate. Recently, Tabb et al. performed a broader investigation of the fragmentation of electrosprayed tryptic peptides in a quadrupole ion trap [24]. A number of trends were identified in this study. The propensity with which a-, b-, and y-ions are formed was defined as a function of fragment ion size. Also, they elucidated the tendencies of specific amino acid residues to promote the formation of certain types of fragments. Knowledge of these trends should enable mass spectra to be predicted from candidate sequences more accurately and thus improve database searching algorithms.

Over the years a number of chemical derivatizations have been employed to facilitate the interpretation of tandem mass spectra and to probe dissociation pathways of peptides [21–23, 25–27]. For these purposes researchers have employed derivatizations that add acidic [21, 25], fixed-charge [22], or basic [23, 26] moieties. Often the goal of labeling a peptide is to better predict the types of fragments that are produced and use this knowledge to reliably derive sequence specific information. One theme that is common in this type of research is the development of de novo sequencing methods. Lacey and coworkers labeled peptide N-termini with sulfonic acid derivatives to facilitate this task [25]. They demonstrated that this yielded contiguous ladders of y-type ions and eliminated other ion types in both CID and post-source decay (PSD) experiments. Due to the selective formation of a single ion-type, the identity and location of residues could be deduced from the mass differentials between adjacent peaks in MS/MS spectra. Alternatively, other derivatizations have provided specific, but less complete sequence information. Gaskell and co-workers converted peptide N-termini to phenylthiocarbamoyl (PTC) derivatives [27]. This modification, long utilized in Edman degradation, induces selective dissociation of N-terminal peptide bonds in gas phase fragmentation experiments, enabling the facile identification of N-terminal residues. However, because PTC labeled peptides usually did not yield other sequence ions, the utility of this derivatization to protein identifications via tandem mass spectrometry is limited. Nevertheless, the identity of N-terminal residues helps to constrain database searches in peptide mass mapping experiments [28]. In other work focused on controlling peptide fragmentation, Gross and coworkers investigated alkali metal cationized peptides and found that cleavage of C-terminal residues was generally promoted [29]. This phenomenon involved metal cations binding to C-termini, leading to a seven-membered ring intermediate and subsequent cleavage of the C-terminal residue.
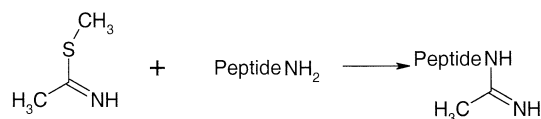


**Scheme 1**. Acetamidination of peptides.

The present study focuses on the effects induced by amidine labels in low energy CID of tryptic peptides. This form of labeling has previously been employed as an elucidative probe of protein function and, very recently, as a tag in comparative proteomics [30, 31]. In the labeling reaction (Scheme **1**) the free amino groups of peptide N-termini and lysine side chains are selectively modified, leading to mass shifts of 41 Da per reactive site.

We have investigated the effect of this label on CID spectra of peptides, and propose an intermediate structure that explains some of our observations. Finally, we consider the impact of using the N-terminal residue of peptides as a database searching constraint. The utilization of this constraint reduces the number of candidates extracted from a sequence database by approximately an order of magnitude and will improve database-matching techniques by reducing the occurrence of false-positive assignments.

## Experimental

### Materials

The proteins cytochrome *c* (horse), hemoglobin (human), serum albumin (bovine), carbonic anhydrase II (bovine), pyruvate kinase (rabbit), and TPCK-treated trypsin (bovine) were obtained from Sigma (St. Louis, MO). $\alpha$-cyano-4-hydroxycinnamic acid (CHCA) and tris-(hydroxymethyl)aminomethane were also purchased from Sigma. Anhydrous diethyl ether, thioacetamide, and ammonium bicarbonate were supplied by Fisher (Fair Lawn, NJ). Iodomethane, formic acid, and 2,5-dihydroxybenzoic acid (2,5-DHB) were purchased from Aldrich (Milwaukee, WI). Acetonitrile and trifluoroacetic acid (TFA) were obtained from EM Science (Gibbstown, NJ).

### Labeling Tryptic Peptides

*S*-methyl thioacetimidate was synthesized as previously described by Thumm et al. [30] and Beardsley and Reilly [31]. Tryptic digests were prepared by combining model proteins with TPCK-treated trypsin (1:100 protein to trypsin molar ratio) in 25 mM ammonium bicarbonate and incubating this mixture for 12 h at a temperature of 37°C. These mixtures were acetamidinated by mixing equal volumes of a digest aliquot and a 43.4 g/L solution of *S*-methylthioacetimidate that was dissolved in 250 mM tris-(hydroxymethyl)aminomethane. These reactions were incubated at ambient temperature for 1 h prior to addition of TFA to a concentration

of 2.0% (vol/vol). The synthesis of *S*-methyl thio-acetimidate and peptide labeling reactions were performed in a fume hood.

### Fragmentation of Multiply Protonated Peptides

Both unmodified and acetamidinated tryptic peptides were analyzed in LC-MS/MS experiments. Samples were injected onto a 1 mm i.d. C-18 reversed phase column (Grace Vydac, Hesperia, CA) and eluted with a linear gradient of organic modifier. The gradient was delivered at a flow rate of 50 $\mu$L/min ranging from 95% Solvent A, 5% Solvent B (A = 0.1% formic acid in water and B = 0.1% formic acid in acetonitrile) to 60% A, 40% B over 60 min. The effluent was split such that 90% of the flow was directed to waste while 10% was delivered to the ESI source. An ion trap mass spectrometer (LCQ-Deca XP Plus, Thermo Finnigan, San Jose, CA) was used for all experiments involving electrospray. MS/MS experiments were performed using a data dependent precursor ion selection strategy. Therefore, the most abundant ion in a full MS scan was selected for CID in the subsequent scan event. Full MS scans were acquired using automatic gain control (AGC) and an *m/z* range of 400–1700. Once isolated, precursor ions were activated by applying a narrowband ($\pm$1 u) resonant RF excitation waveform for 30 ms. The activation energy was normalized by adjusting the amplitude of the resonance excitation RF voltage to compensate for the *m/z*-dependent fragmentation of precursor ions. This voltage is directly proportional to precursor *m/z* and the available range of voltages is established by setting an arbitrarily defined "normalized collision energy" value [32]. In all experiments involving multiply charged peptides the normalized collision energy was set to a value of 35%. Also, an activation Q of 0.25 was applied in these studies.

### Analysis of Singly Protonated Peptides

MALDI mass spectrometry was employed in the study of singly charged peptides using both ion trap (Thermo Finnigan LCQ Deca XP Plus with a Mass Tech atmospheric pressure source) and time of flight (Bruker Reflex III, Bremen, Germany) mass analyzers. MALDI spots were prepared in these experiments using CHCA matrix. This compound was dissolved in a solvent composed of 50% acetonitrile (vol/vol) and 0.1% TFA (vol/vol) in water to a concentration of 10 g/L. Peptide samples were combined with the matrix solution in a 1:9 volumetric ratio and 1 $\mu$L of this mixture was deposited onto a probe. Ion trap mass spectra were acquired using both MS and MS/MS modes, but with a modification to the method used in ESI experiments. Full MS spectra of tryptic digests were acquired over an *m/z* range of 315–2000 without using automatic gain control. Instead, the ion injection time was maintained at 300 ms. This injection time is much higher than that typically employed in electrospray analyses with AGC,

and was chosen to be compatible with the low repetition rate (10 Hz) of the AP/MALDI ion source. The CID of these peptides was performed using a normalized collision energy of 50%, an activation Q of 0.25, an activation time of 30 ms and, unless otherwise noted, wideband activation. The latter enabled excitation of ions having masses up to 20 u less than the precursor. This allowed us to further break down large fragment ions that were abundantly generated by the loss of $NH_3$ from precursor ions. A normalized collision energy of 50%, rather than the 35% employed in the electrospray experiments, helped to compensate for the loss of sensitivity for product ions that is common when wideband activation is used.

Reflectron MALDI-TOF mass spectra of an acetamidinated tryptic digest of hemoglobin were acquired using either 2,5-DHB or CHCA as the matrices. MALDI spots were prepared with 2,5-DHB by mixing 1 $\mu$L of matrix solution with 0.5 $\mu$L of the labeled hemoglobin digest on probe. 2,5-DHB was dissolved to a concentration of 40 g/L in 20% acetonitrile (vol/vol) and 0.1% TFA (vol/vol) in water to make the matrix solution. MALDI spots were prepared with CHCA as above except only 0.7 $\mu$L of the matrix/analyte solution was deposited on probe. In all MALDI spot preparations the acetamidinated digest mixtures were used without any purification prior to mixing with matrix solutions.

## Results and Discussion

### Charge State Distribution Shifts

Having demonstrated that MALDI ion yields of amidine-labeled peptides exceeded those of their unmodified counterparts [31], we hypothesized that the increased protonation of amidinated peptides resulted from charge delocalization on the resonance-stabilized groups. To further investigate this phenomenon, we have compared the charge state distributions of electrospray ionization mass spectra of acetamidinated and unmodified tryptic peptides. Mass spectra of these peptides were acquired between MS/MS scans during an LC-MS analysis as described in the experimental section. In all, 26 unmodified and acetamidinated peptides were compared. The peptides used in this study were derived from the tryptic digests of several model proteins including hemoglobin, cytochrome *c*, carbonic anhydrase, and serum albumin. In an attempt to gauge the relative propensity for acetamidinated and unmodified peptides to form multiply charged ions the charge state distributions of these peptides were compared by calculating an average charge state for each case. These values were determined by weighting the contributions of particular charge states based on their *relative* intensities. Therefore these comparisons do not reflect the total ion yields of each peptide. Figure 1 summarizes data for the 26 pairs of peptides. In this plot the molecular weights of the unmodified peptides are provided on the abscissa while the average charge states are displayed on the ordinate. The darker and lighter
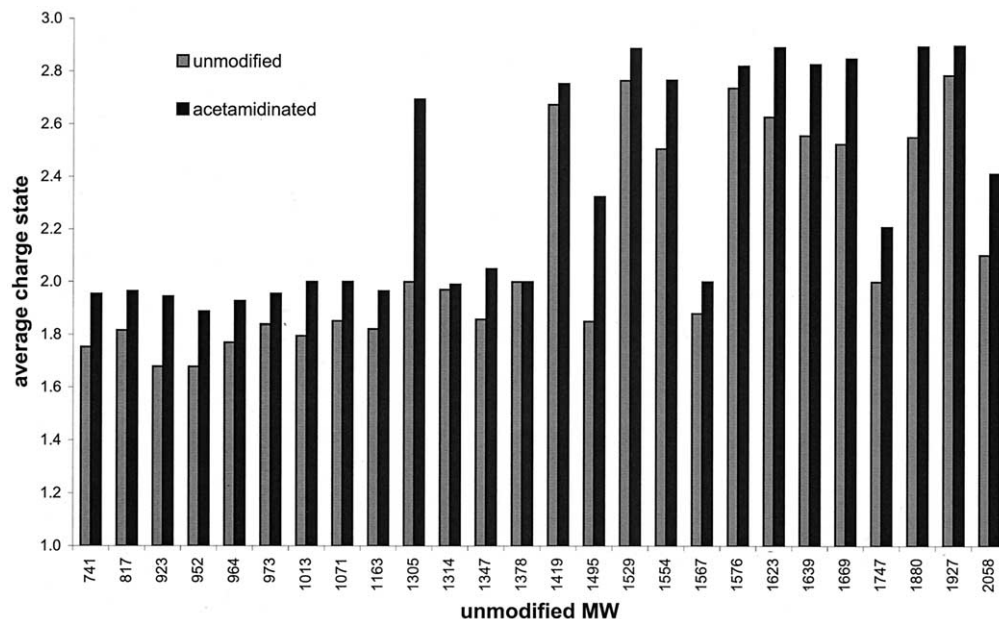
**Figure 1.** Comparison of average charge states of tryptic peptides before and after acetamidination.

bars represent acetamidinated and unmodified peptides, respectively. Not surprisingly, amidination increased the average charge state value in almost every case. In the MW 1378 case, the acetamidinated and unmodified peptides only appear as $[M + 2H]^{2+}$ ions so the average charge states are equal. We have considered the possibility that the different eluting conditions of amidinated and unmodified peptides could play a role in the observed charge state distributions since previous studies have indicated that higher concentrations of acetonitrile generally lead to increased average charge states in ESI [33] and amidinated peptides elute at approximately 1% (vol/vol) higher acetonitrile in reversed phase LC. To probe this issue, we analyzed these peptides from identical electrospray conditions. Labeled and unlabeled peptides from a tryptic digest of hemoglobin were purified by reversed phase LC, collected into the same solution and simultaneously electrosprayed by direct infusion. The results of this analysis were in excellent agreement with the data from the original LC experiments. We thereby concluded that the amidine labels rather than the solvent composition led to the observed increased protonation of peptides.

### Fragmentation of Electrosprayed Amidine Labeled Peptides

The effect of amidination on the fragmentation of electrosprayed peptides was considered next. For this purpose, both unmodified and acetamidinated tryptic peptides of several proteins (pyruvate kinase, hemoglobin, carbonic anhydrase II, and serum albumin) were analyzed in LC-MS/MS experiments. Only the precursor ions that were at least doubly charged were consid-

ered. In total, the tandem mass spectra of 29 peptides were observed for which a direct comparison between the unmodified and acetamidinated forms could be made. Furthermore, MS/MS spectra of a total of 51 acetamidinated and 41 unmodified peptides from these digests were acquired. The fact that not every peptide was paired was most often the result of multiple components co-eluting. Therefore, an MS/MS spectrum could not be acquired in some cases because there was not enough time to perform CID on every component. This problem was exacerbated in our experiments since to attain more reliable results we repeated the MS/MS scans of a precursor ion five times before it was placed on an exclusion list and other ions could be analyzed. It would have been possible to obtain more labeled and unmodified pairs for direct comparison by repeating the analysis of these samples and focusing on unpaired peptides by placing their precursor m/z values on a priority list. However, since a significant number of peptide pairs were already detected, this was not deemed to be necessary.

Figure 2 displays the tandem mass spectra of four doubly (a–d) and two triply (e, f) charged electrosprayed peptides that may be considered typical. Spectra of unmodified peptides are in the left column and the acetamidinated peptides are shown on the right. In each comparison the precursor ions differed only by the presence of amidine groups at their N-termini and lysine residues. It is apparent from these examples that the addition of amidine labels induces significant changes in fragmentation. The most striking of these is the strongly increased production of $y_{n-1}$ fragment ions from cleavage of the N-terminal residue. These $y_{n-1}$ fragment ions are labeled with bold characters and are
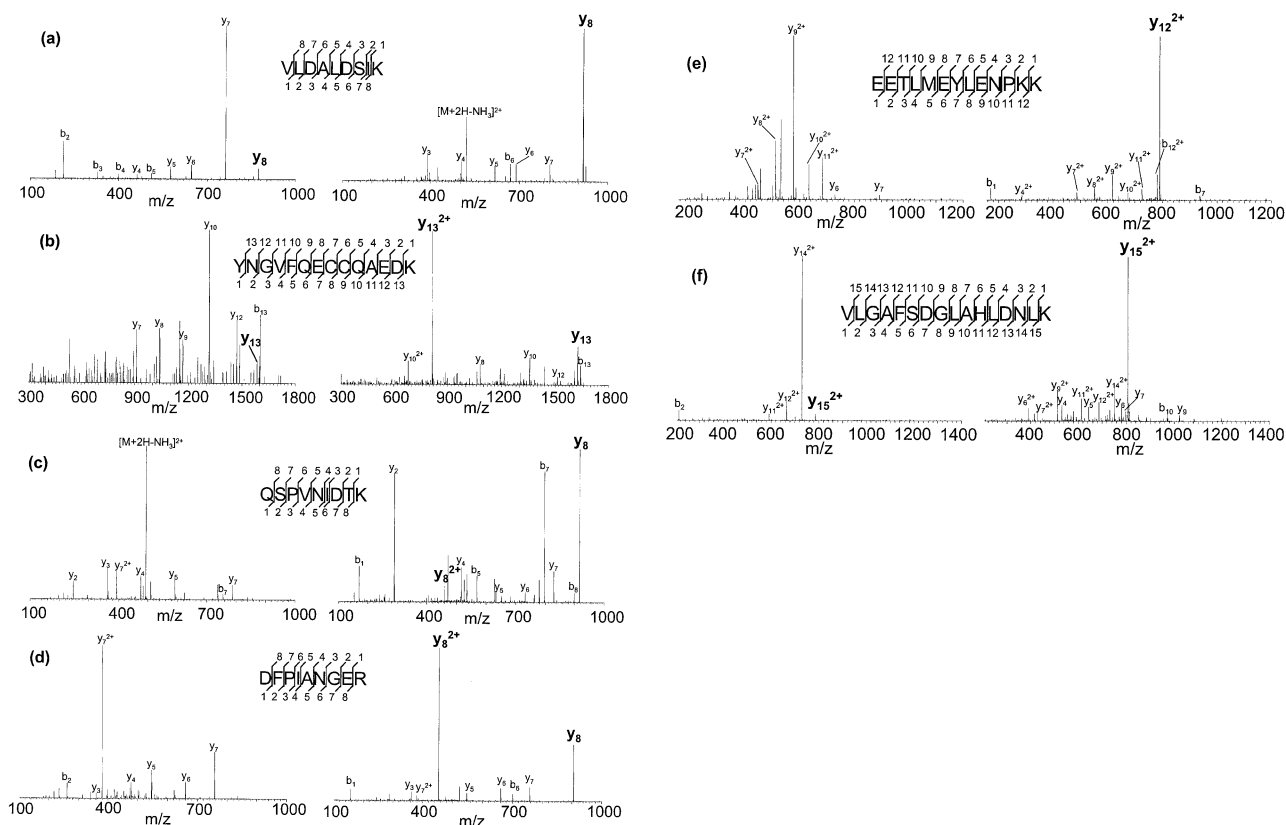
**Figure 2.** ESI ion trap tandem mass spectra of unmodified and acetamidinated tryptic peptides. Unmodified and labeled peptides are shown in the left and right column respectively. Spectra in (**a**)–(**d**) are of doubly charged precursor ions whereas those in (**e**) and (**f**) are of triply charged peptides. In each spectrum only b- and y-type ions are labeled and the $y_{n-1}$ fragment is highlighted.

the most intense peaks in each of the acetamidinated MS/MS spectra. In contrast, $y_{n-1}$ ions were often not even observed from unmodified peptides. These ions were detected from three of the unmodified peptides shown here, but in each case the $y_{n-1}$ peak was not very intense.

Interestingly, the charge state of the $y_{n-1}$ ion varied from one peptide to another. Predominantly singly charged $y_{n-1}$ ions appear in Figure 2a and c whereas doubly charged fragments are most intense in Figure 2b and d–f. As evidenced by the data of Figure 2a–d, doubly protonated precursors may yield either singly or doubly charged $y_{n-1}$ fragment ions. The four examples of amidinated $[M + 2H]^{2+}$ precursor ions in Figure 2a–d illustrate this variability. In one example, Figure 2a, only the 1+ charge state of the $y_{n-1}$ fragment ion is observed. In the other spectra of doubly protonated precursor ions both 1+ and 2+ charge states of this fragment were observed. In contrast, triply protonated precursors such as those in Figure 2e and f produce $y_{n-1}$ ions that are predominantly doubly charged. This effect was consistently observed with other triply charged precursor ions that were analyzed. The observation of doubly charged $y_{n-1}$ fragment ions is analogous to the production of doubly charged ions from triply charged unmodified precursors. Furthermore, destabilizing

Coulombic repulsion is expected to be weaker in the doubly charged $y_{n-1}$ fragment than in its complementary $b_1$ fragment. Therefore, two of the three protons should remain with the $y_{n-1}$ fragment in most cases. Although far less intense than $y_{n-1}$, the singly charged $b_1$ ion was observed for acetamidinated EETLMEYLEN PKK. This ion could not be observed for VLGAFSDG LAHLDNLK since its *m/z* was below the low-mass cutoff in that mass scan.

Of the 51 acetamidinated peptides that were analyzed a $y_{n-1}$ fragment ion was observed for *every* case. Furthermore, this ion was the base peak of its spectrum in 32 out of 51 cases (63%). By comparison, 18 out of 41 (44%) of the unmodified peptides also yielded $y_{n-1}$ fragment ions, and in only one case (2%) was it the most intense peak in its spectrum. Despite the increased efficiency of N-terminal residue cleavages, the number of other peaks in MS/MS spectra of labeled peptides are comparable to that observed with unmodified peptides. For example, the spectrum of Figure 2a displays a contiguous series of y-ions spanning $y_3$ to $y_8$. The fact that other sequence ions are observed from amidinated peptides should facilitate protein identifications. If one exploits the information that is available from the enhanced fragmentation of the N-terminal peptide bond, it is possible to increase the confidence of peptide

assignments from database searches. The identification of the N-terminal residue provides a database searching constraint. We simulated the application of this constraint using the translated genome of *Caulobacter crescentus* and a database analysis program (PRODIGIES) that was written in house [34, 35]. When candidate sequences are limited by both the mass of a precursor ion and the identity of the N-terminal residue, the number of candidate sequences is reduced by approximately one order of magnitude. This simplification will reduce the occurrence of false positive sequence matches, thus generally improving the confidence of protein assignments. Furthermore, with fewer viable candidates, the amount of time required for database searches should decrease.

## Enhanced Neutral Loss of $NH_3$ in MALDI of Amidinated Peptides

One might expect different results for singly charged peptide ions since the charge might be sequestered on an amidino group and consequently be less mobile than in multiply protonated species. Thus we investigated the fragmentation of amidinated $[M + H]^{1+}$ ions using MALDI mass spectrometry. Data for acetamidinated tryptic digests of hemoglobin are displayed in Figure 3. Figure 3a and b display MALDI-TOF mass spectra acquired using the matrices CHCA and 2,5-DHB, respectively. The peaks of intact peptide ions and their [M + H − $NH_3$]$^{1+}$ post-source decay (PSD) products were numbered and the latter are denoted by asterisks. It is apparent that the loss of $NH_3$ occurs quite readily with CHCA matrix but not to a great extent with 2,5-DHB. The promotion of this type of fragmentation must be related to the addition of amidine labels since these results were not observed from this unmodified tryptic digest analyzed using the same conditions. Furthermore, the loss of $NH_3$ appears to occur independent of lysine amidination since Peptides 3 and 7 are arginine-containing tryptic peptides that were labeled at only their N-termini. Interestingly, Peptide 9 (TYFPH FDLSHGSAQVK) did not yield a [M + H − $NH_3$]$^{1+}$ PSD product. As we previously discussed [31], N-terminal threonine promotes hydrolysis of the adjacent amidine group to an acetyl moiety prior to analysis and this evidently precludes the $NH_3$ loss. The observed $m/z$ ratio of this precursor ion was consistent with N-terminal hydrolysis. The lack of PSD from this peptide, despite the presence of an amidine group on the C-terminal lysine, suggests that the neutral loss of $NH_3$ in other peptides may be exclusively from the N-terminal labels. The intensity of [M + H − $NH_3$]$^{1+}$ products was significantly decreased when 2,5-DHB was the matrix (Figure 3b). This result is consistent with the well-known attribute of 2,5-DHB being a "cooler" matrix. Lastly, a mass spectrum of this tryptic digest was acquired using AP/MALDI and an ion trap mass spectrometer with CHCA as the matrix (Figure 3c). Com-
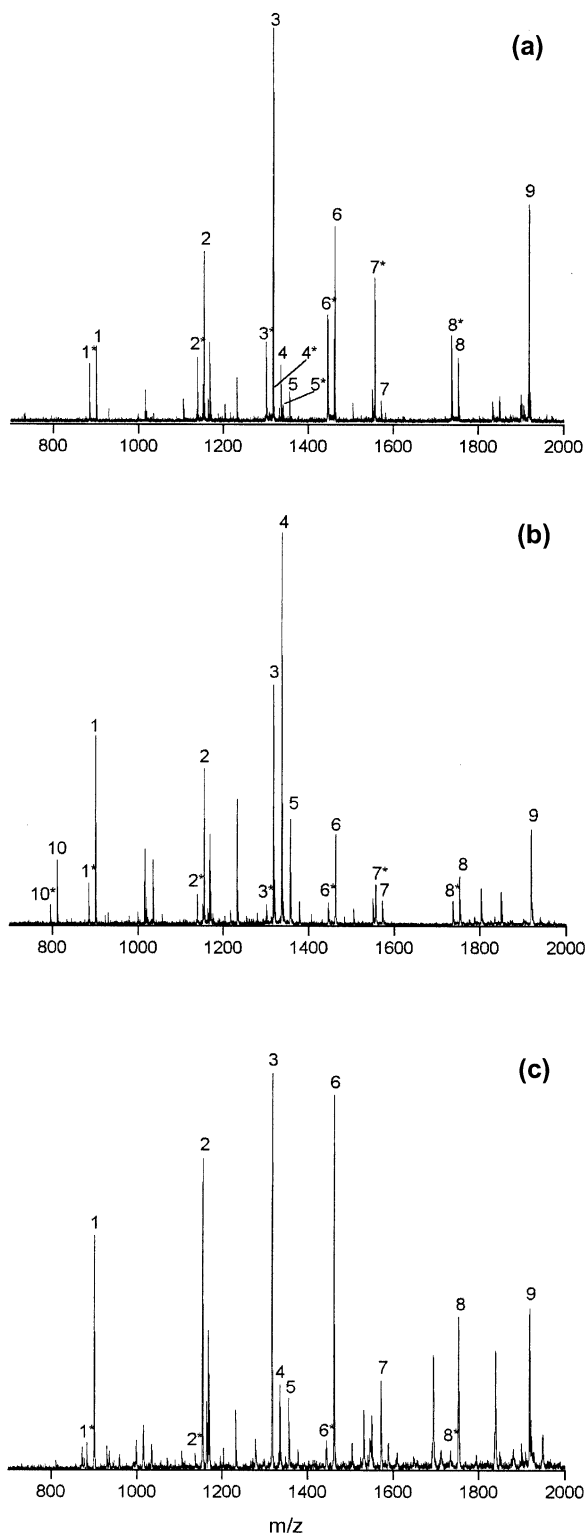


**Figure 3.** MALDI-TOF (**a**) and (**b**), and AP/MALDI ion trap (**c**) mass spectra of an acetamidinated tryptic digest of hemoglobin. CHCA matrix was used for (**a**) and (**c**). 2,5-DHB was the matrix for (**b**).

pared with the CHCA/TOF data, fewer ions lose $NH_3$. This reduction of $NH_3$ loss may be explained by a combination of effects: Collisional cooling is faster
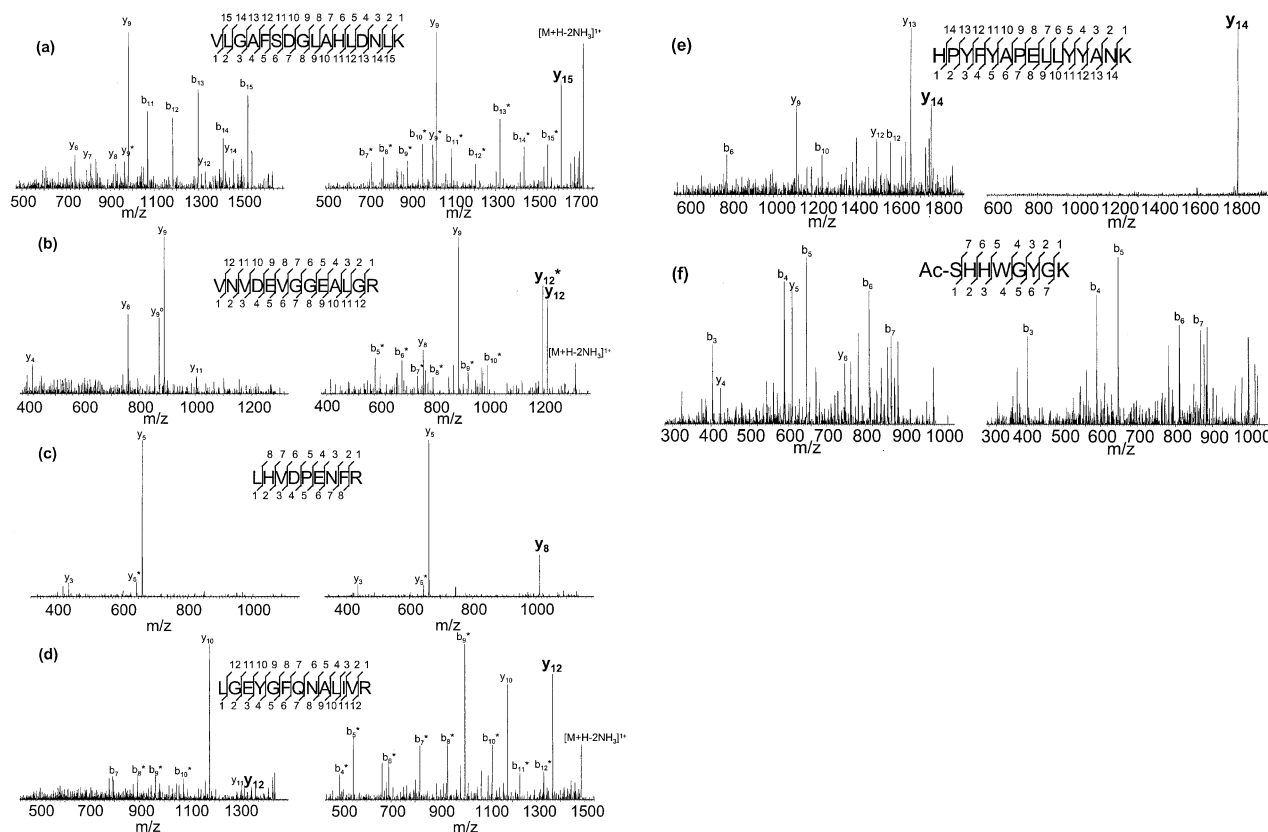
**Figure 4**. AP/MALDI ion trap tandem mass spectra of unmodified and acetamidinated tryptic peptides are displayed in the left and right column respectively. All precursor ions were singly charged.

when the ions are formed at atmospheric pressure, and the ions are also cooled by He buffer gas once injected into the ion trap.

*CID of Singly Charged Acetamidinated Peptides*

Using an AP/MALDI source and a quadrupole ion trap mass spectrometer we have investigated the CID of singly protonated, acetamidinated peptides. As in the electrospray study described above, tryptic peptides from the digests of several model proteins were employed in this work and the goal here was to compare the fragmentation of amidinated and unmodified peptides. MS/MS spectra of six unmodified and acetamidinated tryptic peptides are displayed in Figure 4. Figure 4a–e involve peptides that were labeled at their N-termini and any lysine residues that might be present. The y-, and b-type ions of these peptides are labeled, and asterisks are used to denote the losses of $NH_3$. Each of the spectra shown in this figure is the average of 50 MS/MS scans. The data were processed in this manner since averaged mass spectra more reliably reflect fragmentation tendencies than do single spectra. In Figure 4 a–e the $y_{n-1}$ fragment ions (highlighted in bold) were typically among the most abundant. In contrast, this fragment was very weak or not detected from the unmodified peptides. The enhancement of this dissoci-

ation pathway is similar to that observed with electrosprayed doubly and triply charged peptide ions. However, there are also some important charge-dependent differences. Most striking is the predominance of b-$NH_3$ (b*) fragment ions from singly charged amidinated precursor ions. In some cases (Figure 4a, b, and d) a contiguous series of these ions was observed while the unmodified version of these peptides only produced fewer b-type fragments. The tendency of amidinated peptides to produce contiguous b*-ion series may be very useful in de novo sequencing experiments. Unmodified peptides often do not yield such complete and easily interpretable information. While this unique type of fragmentation may facilitate de novo sequencing, it is important to note that not every peptide generates b*-ions. The spectra of Figure 4c and e are good examples of this difference. Interestingly, the presence of proline residues in peptides, such as in these two cases, often suppresses the formation of b*-ions. Many researchers have identified the formation of y-ions via cleavage on the N-terminal side of proline residues as a very efficient fragmentation pathway [15–19]. It has been proposed that this pathway is generally favored because the proline's amide group is more efficiently protonated than others [15]. Perhaps the high fragmentation efficiency from these sites precludes the formation of b*-ions. The spectrum of the amidinated peptide

in Figure 4e is a unique example of enhanced cleavage adjacent to proline since $y_{n-1}$ is the *only* fragment ion observed. In contrast, the spectrum of the unmodified form of this peptide contained a number of other sequence ions. This dramatic case of $y_{n-1}$ formation is likely exacerbated by the fortuitous conjunction of two favored fragmentation processes at the same site: N-terminal to proline and the amidine-induced dissociation of the N-terminal bond. The elimination of other peaks from MS/MS spectra is obviously not optimal for protein identification. However, proline residues located adjacent to N-terminal peptide bonds are not common. When such a distinctive fragmentation pattern appears it strongly suggests the presence of proline at the second position from the N-terminus. It may be possible to exploit this sequence-specific information in data interpretation. The peptide featured in Figure 4f is the N-terminal fragment of carbonic anhydrase and it is acetylated in vivo. Consequently it was amidinated at only its lysine. The comparison of these spectra is a special case since those discussed thus far all contained an amidine group on their N-termini. Quite interestingly, the spectra of the amidinated and unmodified peptide are not strikingly different. The fragment ions observed and their intensity distributions are basically similar. Also, the acetamidine labeled derivative did not yield the $y_{n-1}$ or b*-ions common in the other examples. These observations implicate the *N-terminal* acetamidine group as being directly involved in these fragmentation pathways. Furthermore, the absence of y* ions in the amidinated spectrum serves as further evidence that the lysine-amidine derivative does not readily lose $NH_3$.

### Wideband Versus Narrowband Excitation

In the AP/MALDI experiments just discussed the use of wideband activation was essential for minimizing the intensities of otherwise dominant $[M + H - NH_3]^+$ product ions. The spectra displayed in Figure 5 demonstrate this need. In the first example, Figure 5a, the CID of acetamidinated VVAGVANALAHK was studied with a narrow band of resonant excitation frequencies. The dominant fragmentation pathway was found to be a neutral loss of $NH_3$ (denoted by $[M + H - NH_3]^+$). The other products observed were mostly b*-type with a weak signal from $y_{n-1}$ (i.e., $y_{11}$). The most intense of these peaks was only about 30% of the $[M + H - NH_3]^+$ ion. Narrowband excitation often produced even less of these types of ions. For example, Figure 5b displays the MS/MS spectrum of VGAHAGEYGAEALER. The most intense sequence ion in this spectrum is the $y_{n-1}$ fragment, but its abundance was only ~15% that of $[M + H - NH_3]^+$. Many other ions were detected, but these were even less abundant. This limited fragmentation from amidinated peptides was commonly observed with narrowband excitation. In contrast, the use of wideband activation provided much more complete fragmentation (Figure 5c and d). Therefore, product
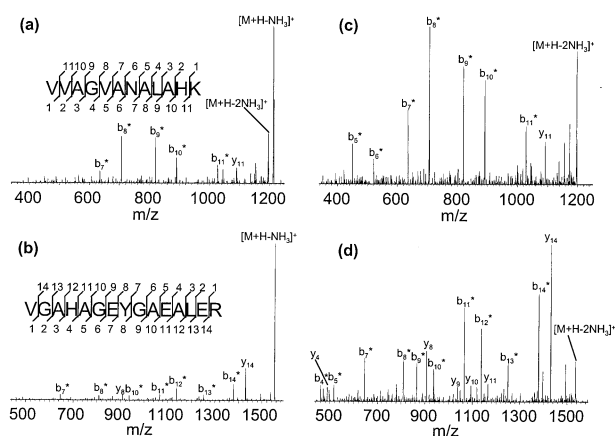


**Figure 5**. AP/MALDI ion trap tandem mass spectra of tryptic peptides with narrow (**a**) and (**b**), and wideband (**c**) and (**d**) activation. The MS/MS spectra of VVAGVANALAHK appear in (**a**) and (**c**) while the fragmentation spectra of VGAHAGEYGAE-ALER are displayed in (**b**) and (**d**).

ions resulting from neutral losses of small groups such as $NH_3$ could be further activated to produce informative sequence ions. Unlike the experiment with narrow band activation, these data were not dominated by $NH_3$ loss from the precursor ion. Wideband activation of these two peptide ions generated primarily b*- and y-type fragment ions. In fact, each peptide yielded a contiguous series of b* ions analogous to the data described in Figure 4. Furthermore, the $[M + H - NH_3]^+$ ion of each precursor was not detected when using wideband activation. The removal of this product is advantageous in protein identification experiments since it does not convey sequence-specific information. Interestingly, the types of sequence ions produced, as well as their relative intensity distributions, were similar in both narrow and wideband activation experiments. Thus, it seems that the primary effect of wideband activation is to eliminate the dominance of $[M + H - NH_3]^+$ dissociation products.

### Proposed Cyclic Intermediate for $y_{n-1}$ Formation

The ubiquitous formation of $y_{n-1}$ fragment ions in both ESI and MALDI can be rationalized by an intermediate structure that is similar to that associated with aspartic acid-induced cleavage. Wysocki and co-workers have proposed that the aspartic acid effect involves a seven-membered ring intermediate that facilitates the transfer of a proton from the side-chain of this residue to the carbonyl oxygen that is C-terminally adjacent to it (Figure 6) [22]. They proposed that following this transfer the deprotonated oxygen from the side-chain of aspartic acid attacks the carbonyl carbon to induce peptide bond cleavage. Likewise, Gross and coworkers investigated structures in which seven-membered ring intermediates were formed at the C-termini of peptides to which $Li^+$ was bound, instead of a proton [29]. They proposed that this intermediate led to a dissociation
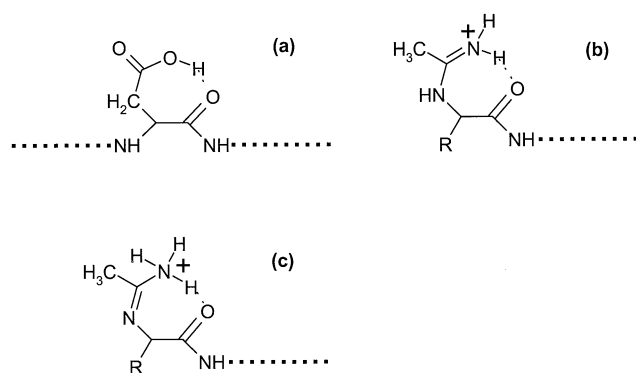
**Figure 6.** Proposed cyclic intermediates leading to ion fragmentation: (**a**) model from reference 22 that explains cleavage C-terminal to aspartic acid (**b**) analogous model that explains loss of amidinated N-terminal residue (**c**) similar model that explains facile NH$_3$ loss.

pathway similar to that described for the aspartic acid phenomenon. Similarly, a seven-membered ring intermediate may promote $y_{n-1}$ formation through a pathway that involves attack on the carbonyl carbon by an amidine nitrogen. As displayed in Figure 6b and c, such an intermediate is feasible when an amidine group is added to the N-terminus of a peptide. Furthermore, such an intermediate is suggested by preliminary calculations. Using Gaussian 03, the proposed structures were geometrically optimized by molecular mechanics with the AMBER force field, then the resulting geometries were used as the starting points for a HF/STO-3G electronic structure stationary point search. This work indicates that hydrogen bond stabilization of a cyclic intermediate as shown in Figure 6 is feasible. The protonated structure in Figure 6c may help explain the increased propensity for NH$_3$-loss from amidinated peptides that was demonstrated in Figures 3, 4, and 5. This protonated tautomer of the Figure 6b structure is more likely to lose NH$_3$ since it involves the cleavage of a single, rather than double, bond. By elimination of NH$_3$ from an amidine group a resonance-stabilized cation would be formed. In unmodified peptides this elimination should not occur so readily since the N-terminal carbocation would not be resonance-stabilized. Alternatively, the work of Schnier et al. involving the fragmentation of singly charged bradykinin (RPPGF SPFR) ions may also shed light on the facile NH$_3$-loss we observe [36]. They proposed that the most stable form of singly protonated bradykinin involves a salt bridge that facilitates loss of NH$_3$. By analogy, the facile NH$_3$ loss from amidinated peptides may be explained by a similar process since basic amidine groups are present at each terminus.

## Conclusion

In this paper, we have demonstrated that tryptic peptides labeled with amidine groups fragment quite differently from their unmodified counterparts. In both

MALDI and electrospray ionization experiments involving singly, doubly, and triply charged amidinated precursor ions, enhanced quantities of $y_{n-1}$ fragment ions are observed. Observation of this dissociation product should prove useful in protein identifications since the identity of a peptide's N-terminal residue can be used as a database searching constraint. A seven-membered cyclic intermediate structure is consistent with the observation of N-terminal amide bond cleavage. Neutral loss of NH$_3$ was observed to be enhanced for singly charged amidinated peptide ions. This effect can be rationalized by a protonated amidine tautomer. Under certain CID conditions the formation of contiguous series of b$^*$ ions was possible. The observation of these fragment ion ladders will facilitate de novo sequencing experiments.

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