Effects of the Position of Internal Histidine Residues on the Collision-Induced Fragmentation of Triply Protonated Tryptic Peptides

Belinda B. Willard and Michael Kinter

Lerner Research Institute Mass Spectrometry Laboratory for Protein Sequencing, Department of Cell Biology, Cleveland Clinic Foundation, Cleveland, OH, USA

The collision-induced dissociation spectra of a series of synthetic, tryptic peptides that differed by the position of an internal histidine residue were studied. Electrospray ionization of these peptides produced both doubly and triply protonated molecular ions. Collision-induced fragmentation of the triply protonated peptide ions had better efficiency than that of the doubly protonated ions, producing a higher abundance of product ions at lower collision energies. The product ion spectra of these triply protonated ions were dominated by a series of doubly charged y-ions and the amount of sequence information was dependent on the position of the histidine residue. In the peptides where the histidine was located towards the C-terminus of the peptide, a more extensive series of sequence specific product ions was observed. As the position of the histidine residue was moved towards the N-terminus of the peptide, systematically less sequence information was observed. The peptides were subsequently modified with diethylpyrocarbonate to manipulate the product ion spectra. Addition of the ethoxyformyl group to the N-terminus and histidine residue shifted the predominant charge state of the modified peptide to the doubly protonated form. These peptide ions fragmented efficiently, producing product ion spectra that contained more sequence information than could be obtained from the corresponding unmodified peptide. (J Am Soc Mass Spectrom 2001, 12, 1262–1271) © 2001 American Society for Mass Spectrometry

The introduction and development of electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) have made mass spectrometry the dominant analytical tool for sequencing and identifying proteins. The combination of high sensitivity, rapid analyses, and extensive sets of data that can be used by effective database search programs have provided new methods for the characterization of proteins. The availability of these methods has, in turn, fostered new and broader interest in the characterization of proteins in the biological system. These experiments are broadly classified as proteomics and include ambitious plans to identify all proteins expressed by different cell types and tissues [1–5].

The most common protein identification experiment that is carried out is the identification of proteins separated as bands in polyacrylamide gels. These gels generally result from either two-dimensional (2-D) electrophoresis experiments or the more common sodium docecylsulfate-polyacrylamide gel electrophoresis experiments (SDS-Page or 1-D electrophoresis experiments). In each case, the protein band of interest is cut from the gel and digested with a protease to produce a series of peptides prior to mass spectrometric analysis of those peptides. This mass spectrometric analysis takes either of two general forms; peptide mass mapping using MALDI-time-of-flight mass spectrometry or amino acid sequence analysis using ESI-tandem mass spectrometry, including the related techniques of nanospray ionization-tandem mass spectrometry and microspray ionization-tandem mass spectrometry.

The amino acid sequence analysis carried out using ESI-tandem mass spectrometry is dependent on the fragmentation of the peptide ions by collision-induced dissociation (CID) to give product ion spectra that are indicative of the amino acid sequence of the peptide. Investigations from a number of laboratories have shown that the fragmentation mechanism of protonated peptides ions is consistent with the so-called mobile proton model of fragmentation [6–14]. According to this model, an ionizing proton is transferred under low energy CID conditions to different positions along the length of the peptide, weakening the amide bond at that position and facilitating the cleavage of that amide bond. The acidic conditions of electrospray ionization

Published online October 9, 2001

Address reprint requests to Dr. Michael Kinter, Department of Cell Biology/NC10, Lerner Research Institute, Cleveland Clinic Foundation, 9500 Euclid Ave, Cleveland, OH 44195, USA. E-mail: kinterm@ccf.org

1044-0305/01/$20.00
PII S1044-0305(01)00312-9

Received February 6, 2001
Revised July 27, 2001
Accepted August 13, 2001

produce multiply charged peptide ions by associating a proton with multiple basic sites in a peptide. The mobility of each proton associated with a peptide can be roughly classified according to the basicity of the moiety with which it is associated. Strongly basic sites such as lysine, arginine, and histidine residues do not allow efficient proton migration while the moderate basicity of the N-terminal amine does allow efficient proton migration. If protons are associated with only strongly basic sites, then no mobile proton is available to drive the fragmentation reactions and fragmentation of the peptides is affected.

In general, and particularly for the experiments using ESI-tandem mass spectrometry, the protease of choice for the protein digestion is trypsin. This enzyme can be purchased at relatively low cost from a number of commercial sources in a purified form that has high specific activity and has been modified to reduce both nonspecific protease activity and autolysis. In addition, digestion of a typical protein with trypsin will produce an extensive series of peptides with molecular weights in the 500 Da to 2000 Da range. This sized peptide is not only within the mass range of all relevant mass analyzers but can also be efficiently fragmented by CID using routine collision conditions.

More importantly, however, digestion of a protein with trypsin produces peptides, which we will refer to as tryptic peptides, with a structure that is particularly amenable to informative fragmentation reactions according to the mobile proton model. Trypsin cleaves proteins by hydrolyzing amide bonds at the C-terminal side of arginine or lysine residues, except when those amide bonds are lysine–proline or arginine–proline bonds. As a result, typical tryptic peptides have strongly basic sites at the C-terminus of the peptide (the C-terminal lysine or arginine) and a moderately basic site at the N-terminus (the N-terminal amine). The acidic conditions used with ESI then produce peptide ions with a proton associated with both the C-terminus, via the side chain of the lysine or arginine, and the N-terminus, via the N-terminal amine moiety. Upon CID, the proton located at the N-terminus is sufficiently mobile to be transferred to each of the different amide linkages and direct a series of fragmentation reactions that form predictable and complementary b- and y-ions [15]. The *m/z* of these b- and y-ions are then used to calculate the residue masses of the amino acid at each position. The amino acid sequence of the peptide can then be either deduced by interpretation of this product ion spectrum or by direct comparison, by computer, of the experimental product ion spectrum with what are essentially predicted product ion spectra that are based on the amino acid sequences found in the sequence databases [16, 17].

A significant proportion of tryptic peptides, however, will contain additional charge sites because of the occurrence of the other basic amino acids within the peptide sequence. Because of the specificity of the trypsin, these amino acids will generally be either histidine residues, lysine, or arginine residues that are adjacent to a proline residue, or proline residues. As one studies the product ion spectra of these types of peptides, it is clear that additional basic residues within a peptide significantly affect the fragmentation of that peptide. In particular, CID fragmentation of triply charged (and greater) peptides with internal histidine, lysine, or arginine sites often yield product ion spectra that contain little clear amino acid sequence information. It is not known exactly why these product ion spectra are less informative than the spectra obtained from other tryptic peptides.

The experiments described in this report have focused on tryptic peptides that contain an internal histidine residue. Histidine has been shown to be one of the most basic amino acids having a basicity that is lower than that of arginine and similar to that of lysine [18]. Under ESI conditions, tryptic peptides that contain histidine residues are observed predominantly in the triply protonated form, presumably with protons associated with the histidine residue, in addition to the N-terminal amine and C-terminal lysine and arginine. We have observed that the product ion spectra of triply charged ions from histidine-containing tryptic peptides give varying amounts of sequence information. This sequence information is often in the form of a series of doubly charged y-ions (y^{+2}-ions) with the amount of information highly dependent on the position of the histidine residue. These observations have led us to speculate that, according to the mobile proton model, the histidine residue is controlling the fragmentation of the triply charged ion because a fixed proton associated with the histidine residue is impeding the movement of the mobile proton associated with the N-terminal amine.

The purpose of this investigation was to systematically test the effects of histidine residues on the fragmentation of tryptic peptides under low energy conditions. In these experiments, the product ion spectra of a series of synthetic peptides have been studied. The results demonstrate that the placement of the histidine residue has a strong effect on the fragmentation patterns observed for these ions. In peptides where the histidine is farthest from the mobile proton associated with the N-terminal amine (i.e., towards the C-terminus of the peptide), a series of doubly charged y-ions are produced with good efficiency. As the position of the histidine is moved towards the N-terminus, systematically less sequence information is observed, supporting the deleterious effect of the fixed proton associated with the histidine. Further, derivatization of the peptides with diethylpyrocarbonate (DEPC) modified the N-terminal amine and the histidine residue and shifted the most abundant charge state to the double charged molecular ion. Subsequent fragmentation of this ion gave product ion spectra that contained both b- and y-ions and contained significantly more sequence information than fragmentation of the corresponding triply charged ions from the unmodified peptide. As a result,

this derivatization reaction appears to be a useful method for modifying the fragmentation chemistry of histidine-containing tryptic peptides and enhancing the sequence information obtained from such peptides.

Experimental

The peptides were synthesized with a model 431A Peptide Synthesizer (Applied Biosystems, Foster City, CA) and purified by reversed-phase HPLC prior to use. Bovine serum albumin and diethylpyrocarbonate were purchased from Sigma Chemical Company (St. Louis, MO) and used without further purification. The trypsin used for the protein digestion was a sequencing grade, modified trypsin purchased from Promega Corporation (Madison, WI).

The electrospray ionization-tandem mass spectrometry experiments were performed on a Finnigan (San Jose, CA) LCQ-Deca ion trap mass spectrometer equipped with a Protana nanospray ionization source. The ionization source was operated under microspray conditions with total flow rates of approximately 0.2 μL/min, without sheath liquid or gas, at a spray voltage of approximately 1.4 kV. The peptides were introduced by either direct infusion or reversed-phase HPLC through a 50 μm i.d. capillary with a 10 μm i.d. tip obtained from New Objective Corporation, Woburn, MA (the PicoFrit Column). For the HPLC experiments, the capillary was packed with Phenomenex (Torrance, CA) Jupiter C18 packing material and the peptides were eluted with a gradient of increasing acetonitrile in 50 mM acetic acid.

The fragmentation efficiency curves were obtained by determining the percent fragmentation of each peptide as a function of collision energy and these values were calculated as described by Wysocki and coworkers [6]. Briefly, the collision energy used for fragmentation was varied by changing the amplitude of the RF voltage applied to the endcap electrodes. At each collision energy, five spectra were acquired and averaged. The total fragment ion current was calculated as the sum of the abundance of all fragment ions, the total ion current was calculated as the sum of the abundance of all ions, and the fragmentation efficiency was calculated by dividing the total fragment ion current by the total ion current, expressing that value as a percent.

Modification of the peptides was performed with a 10-fold excess of diethylpyrocarbonate (DEPC) in a 50 mM phosphate buffer at pH 6.0. The reactions were carried out at 35 °C for 20 min. The final reaction mixture was taken to dryness and reconstituted in the either 1% acetic acid (for HPLC introduction) or 70% water/30% acetonitrile/1% acetic acid (for infusion).

Prior to digestion, a 20 pmol aliquot of BSA was run into a 12% polyacrylamide gel in a standard SDS-PAGE experiment. The protein band was detected by Coomassie blue staining and cut from the gel with a scalpel. The protein band was digested using a modified, sequencing grade trypsin using an in-gel digestion protocol

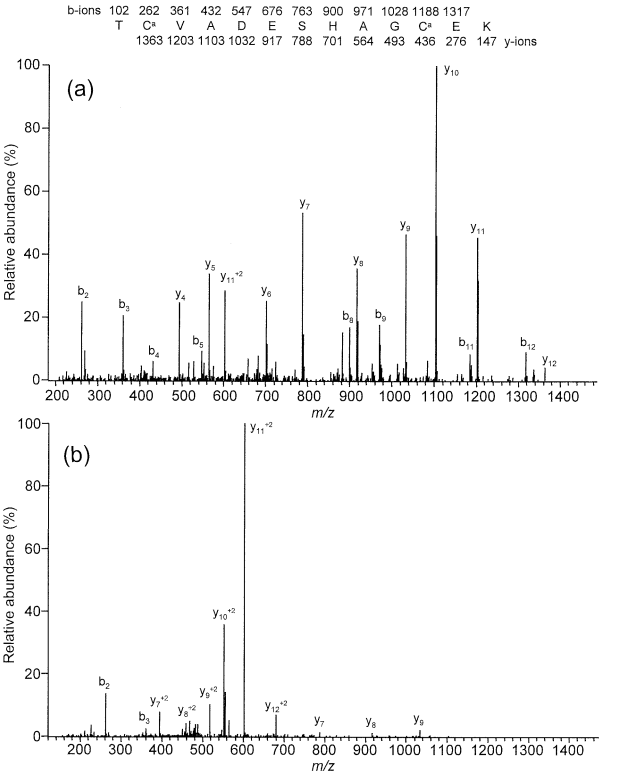

Figure 1. Product ion spectra for the peptide TC^{a}VADESHAGC^{a}EK from a tryptic digestion of bovine serum albumin. (**a**) The product ion spectrum of the doubly protonated molecular ion. (**b**) The product ion spectrum of the triply protonated molecular ion. The interpretation of these spectra is shown at the top of the figure with the calculated *m/z* of the b- and y-ions rounded to the nearest integer. The notation used is the one-letter amino acid code with the C^a designating alkylated cysteine residues. The sequence-specific ions in the spectrum are labeled as either b- or y-ions. Both spectra were recorded using a collision energy of 30% in the ion trap detector.

[15]. The digest was either analyzed directly, injecting 1 μL for capillary column LC-ESI-tandem mass spectrometry or modified with DEPC. For the modification, the entire mixture was taken to dryness, reconstituted in 100 μL of 50 mM phosphate buffer, pH 6 and derivatized with 10 μl DEPC at 35 °C for 20 min. The reaction mixture was taken to dryness and reconstituted in 20 μl 1% acetic acid, injecting 1 μl for the capillary column LC-ESI-tandem mass spectrometry analysis.

Results and Discussion

The product ion spectra shown in Figures 1 and 2 are derived from two histidine-containing peptides formed by a tryptic digestion of BSA. The mass spectra of these peptides contained significant amounts of both doubly- and triply-protonated molecular ions (data not shown), and these ions were fragmented to produce the spectra shown in the figures. The product ion spectra of the doubly charged peptide ions shown in part A of these figures are relatively informative. The spectrum shown in Figure 1a, for example, contains an extensive series of

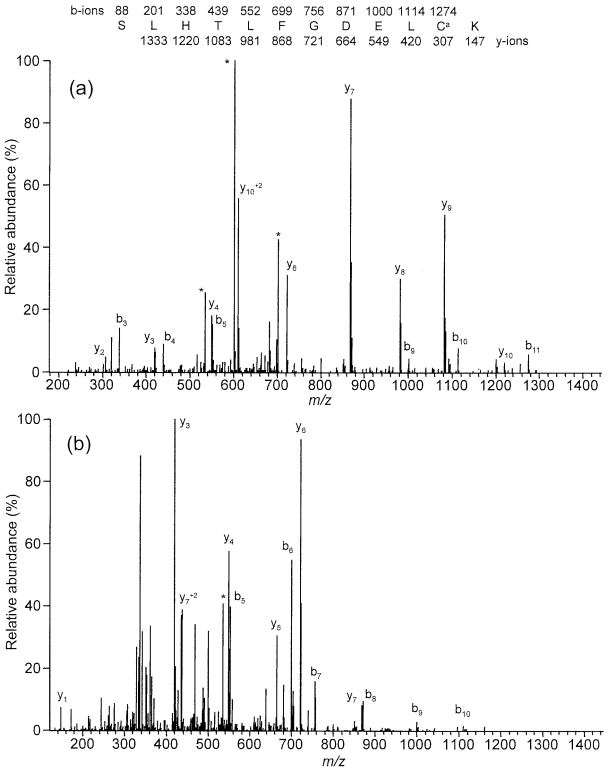

Figure 2. The product ion spectra for the peptide SLHTLFGDELC^{a}K from a tryptic digestion of bovine serum albumin. (**a**) The product ion spectrum of the doubly protonated molecular ion. (**b**) The product ion spectrum of the triply protonated molecular ion. The interpretation of these spectra is shown at the top of the figure with the calculated *m/z* of the b- and y-ions rounded to the nearest integer. The notation used is the one-letter amino acid code with the C^a designating alkylated cysteine residues. The sequence-specific ions in the spectrum are labeled as either b- or y-ions. Both spectra were recorded using a collision energy of 30% in the ion trap detector.

y-ions and complementary b-ions from which a significant amount of amino acid sequence information can be derived.

The product ion spectra of the triply charged peptide ions shown in Figures 1b and 2b, respectively, are not as informative. The product ion spectrum of the triply charged TC^{a}VADESHAGC^{a}EK peptide ion shown in Figure 1b contains a series of doubly charged y-ions that would reveal the TC^{a}VADE portion of the amino acid sequence. On the other hand, the product ion spectrum of the triply charged SLHTLFGDELC^{a}K peptide ion shown in Figure 2b contains no doubly charged y-ions but does contain a limited series of singly charged y- and b-ions that would reveal the −FGDE portion of the amino acid sequence. This spectrum also contains a number of ions with relative abundances in the 5% to 25% range between *m/z* 200 and 600 of unknown origin that produce a level of chemical noise in the spectrum that might confound both manual interpretation strategies and automated database search programs. Based on these product ion spectra, one can observe that the additional proton associated with these

Table 1. The synthetic peptides

Synthetic peptide[a]	Amino acid sequence
Control	ITDFNDFNDFNDFNTR
H4	ITD**H**NDFNDFNDFNTR
H7	ITDFND**H**NDFNDFNTR
H10	ITDFNDFND**H**NDFNTR
H13	ITDFNDFNDFND**H**NTR

[a] A series of peptides were synthesized. All peptides are based on a typical tryptic peptide structure with a C-terminal arginine residue. The control peptide has no internal histidine residue. The other peptides contain an internal histidine, with the position of that histidine varied along the length of the peptide.

histidine-containing peptides significantly reduces the amount of sequence information present in the product ion spectra of these peptides. Further, it also appears based on these spectra, that the effect of the histidine residue is dependent on the position of the histidine in the peptide sequence. The purpose of the experiments described in this report was to study these effects.

A series of five synthetic peptides, shown in Table 1, were designed to study the effects of internal histidine residues on the product ion spectra of tryptic peptides. The control peptide was designed as a typical tryptic peptide containing a C-terminal arginine residue with no internal basic residues. The other peptides contain an internal histidine residue, inserted in the place of a phenylalanine residue such that the position of the histidine is varied along the length of the peptides. As noted in the table, these peptides are designated according to the position of the histidine residue relative to the N-terminus of the peptide as H4, H7, H10, and H13, respectively.

Product ion spectra of the doubly charged molecular ions of the control and H4 peptide, recorded under the same collision conditions, are shown in Figure 3. The product ion spectrum of the control peptide is consistent with the general appearance of the product ion spectra of tryptic peptides, where a series of complementary y- and b-ions are observed. In the product ion spectrum of this doubly charged peptide ion, extensive sets of each ion series are recorded and these product ions reveal the entire peptide sequence. In contrast, the product ion spectrum of the doubly charged peptide ion of the H4 peptide contains significantly less amino acid sequence information because fewer product ions are observed. Further, the ions that are observed have lower relative abundances than the product ions from the control peptide shown in Figure 3a. Similar results were observed for the other histidine-containing peptides.

The product ion spectra of the doubly protonated forms of these peptides were studied as a function of collision energy, as shown in Figure 4. This figure illustrates the relationship between the fragmentation efficiency of the doubly charged molecular ions of the control and histidine-containing peptides, showing the poorer efficiency of the histidine-containing peptides. That is, the histidine-containing peptides require more

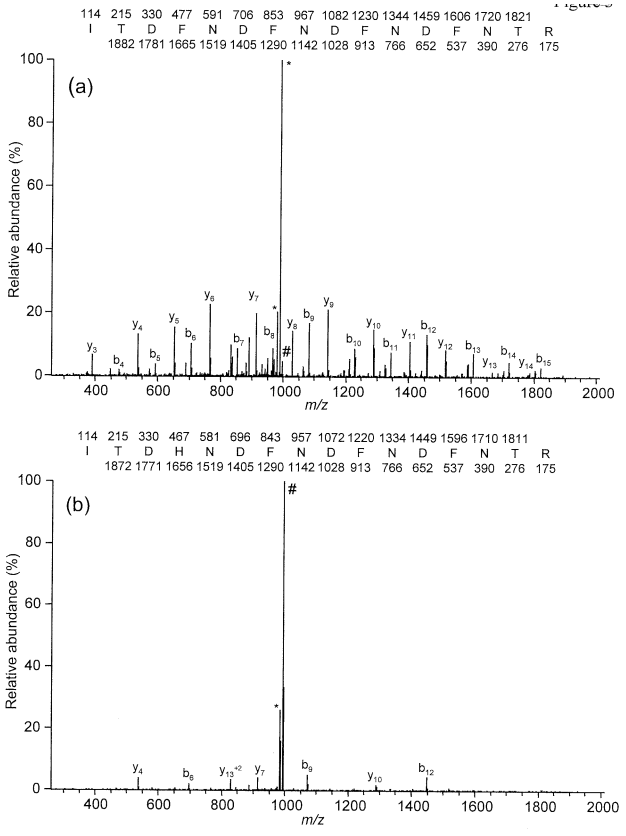

Figure 3. The product ion spectra for the doubly protonated molecular ions of the control (**a**) and H4 (**b**) peptides. The interpretations of these spectra are shown at the top of each spectrum with the calculated *m/z* of the b- and y-ions rounded to the nearest integer. The sequence-specific ions in the spectrum are labeled as either b- or y-ions. The ions labeled with a # are the molecular ions and the ions labeled with an asterisk are doubly-charged ions produced by loss of water from the molecular ion.

energy input in order to achieve the same amount of fragmentation, relative to the control peptide. One should be aware that in these experiments the collision energy is varied as a percentage of the voltage applied to the end cap electrodes in a quadrupole ion trap and the collision gas pressure in the ion trap is high enough to assure multiple collisions. Also, several low mass ions including the y_1-, b_2-, and immonium-ions are not detected in the quadrupole ion trap because of the low mass cut off of this instrument. These ions generally represent only a small portion of the total ion current and as a result their exclusion from these analyses would not have a significant effect on the fragmentation efficiencies that were measured.

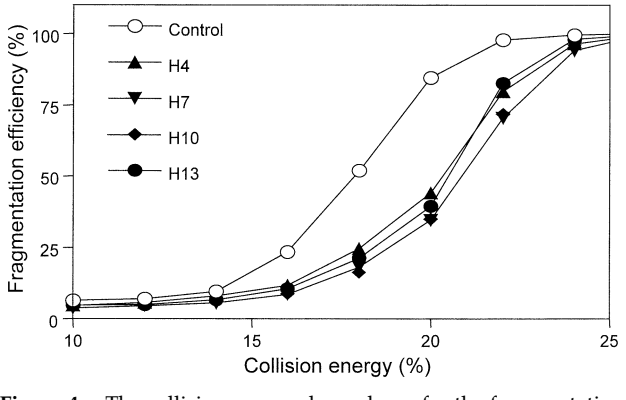

Figure 4. The collision energy dependence for the fragmentation of the doubly protonated molecular ions of the five synthetic peptides. As described in the methods section, the collision energy used for the fragmentation reactions was varied and the integrated abundances of the product ions determined as a measure of fragmentation efficiency. The amino acid sequences of the synthetic peptides are given in Table 1.

The fact that the doubly protonated peptide ions from histidine-containing peptides require higher collision energies to achieve a desired degree of fragmentation is consistent with preferential protonation of the C-terminal arginine and the internal histidine residue rather than the N-terminal amine. As a result, the availability of a mobile proton is diminished by the higher basicity of the histidine relative to the N-terminal amine. It is certainly possible to use the higher collision energies shown in the figure when fragmenting peptides such as these. However, an undesirable effect of using higher collision energies can be an increased frequency of other types of less predictable peptide fragmentation reactions, such as rearrangement reactions, that can increase the amount of chemical noise in the spectra.

An alternative to using higher fragmentation energies with the doubly protonated peptide ions of histidine-containing peptides would be to fragment the triply protonated species that are also observed for such peptides. One might expect that fragmentation of the triply protonated peptide ions would be preferable to fragmentation of the doubly charged species because the higher charge state requires protonation of the N-terminal amine along with protonation of the C-terminal arginine and internal histidine. As a result, this protonation state includes a more mobile proton on the N-terminal amine so that fragmentation should be facilitated.

The product ion spectra of the triply protonated peptide ions of two of the histidine-containing peptides, the H13 and H4 peptides, are shown in Figure 5a and 5b, respectively. The product ion spectrum for the H13 peptide is dominated by a series of doubly charged y-ions (y^{+2}-ions). The y_6^{+2}- to y_{15}^{+2}-ions are detected in this spectrum and a significant portion of the peptide sequence, a series of 10 amino acids, could be deduced from these ions. The product ion spectrum for the H4 peptide (Figure 5b) is strikingly different from that of the H13 peptide. No y^{+2}-ions are observed in this spectrum and relatively few singly charged y- or b-ions are observed. Further, a high degree of chemical noise is observed and, although not apparent in this presentation of the spectrum, the absolute intensity of the product ions is lower for the H4 peptide than the H13 peptide. Increasing or decreasing the collision energy used for these spectra did not change the product ions

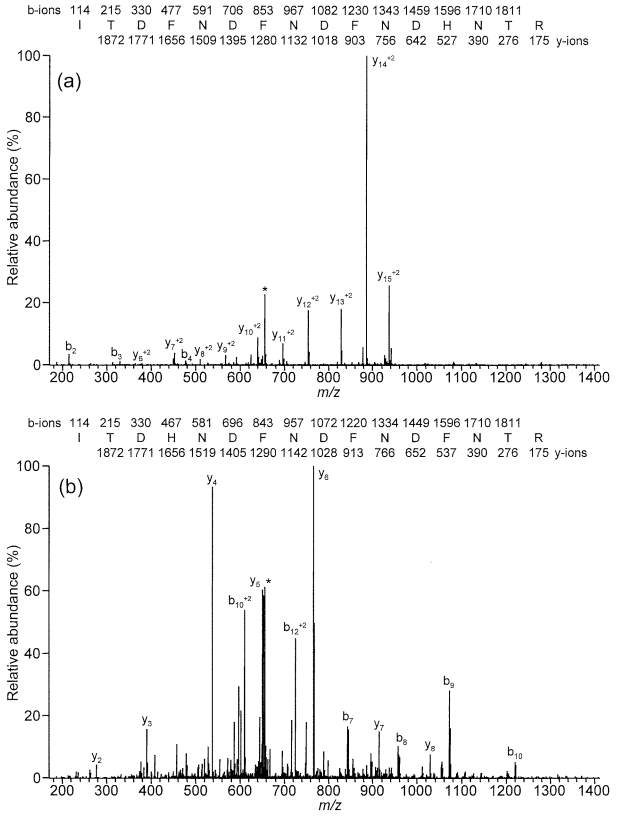

Figure 5. The product ion spectra for the triply charged molecular ions of the H4 (**a**) and H13 (**b**) peptides. The interpretations of these spectra are shown at the top of each spectrum with the calculated *m/z* of the b- and y-ions rounded to the nearest integer. The sequence-specific ions in the spectrum are labeled as either b- or y-ions. The ions labeled with an asterisk are doubly-charged ions produced by loss of water from the molecular ion.

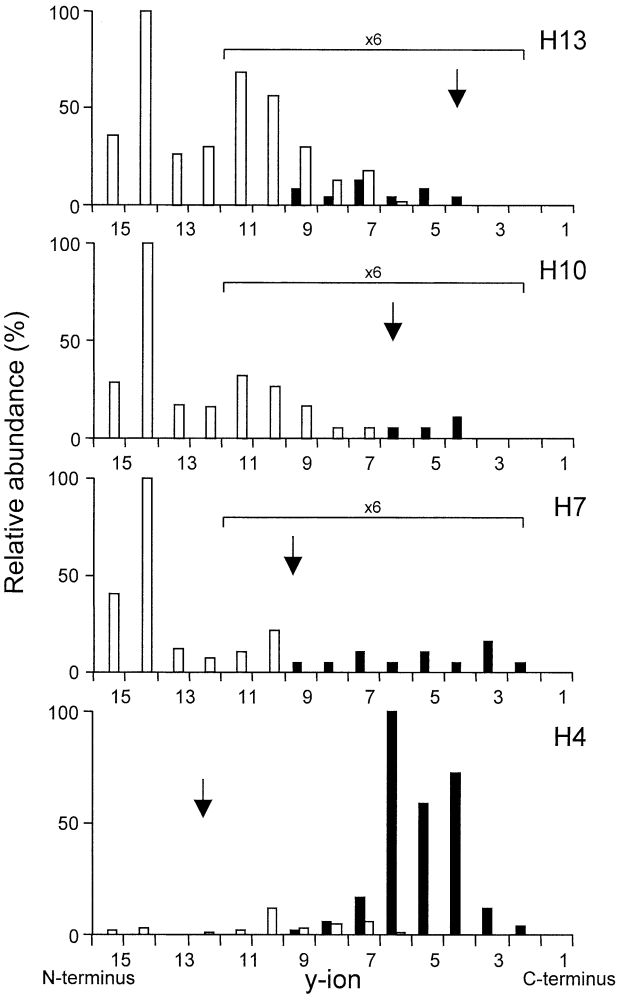

Figure 6. Histograms of the abundance of the y-ions in the product ion spectra of the triply charged molecular ions of the histidine-containing synthetic peptides. These data are extracted from the product ion spectra of each peptide to focus of the relative abundance of the y-ions. The open bars represent the doubly charged y-ions and the closed bars represent the singly charged y-ions. The position of the histidine residue in each peptide is given by the arrow.

that were observed, although changes in the relative abundance of those ions were observed.

To compare the sequence ions present in the product ion spectra of each triply protonated peptide ion, the relative abundance of the y^{+2}- and y-ions are plotted in Figure 6, with the position of the histidine residue shown with the arrow. The data in this figure show that as the histidine residue is moved closer to the N-terminus, the number and intensity of the y^{+2}-ions decreases. In the case of the H10 peptide, the y_7^{+2}- to y_{15}^{+2}-ions are still observed, however the abundances of the y_7^{+2}- and y_8^{+2}-ions are reduced relative to the H13 peptide and the y_6^{+2}-ion is no longer detected. When the histidine is moved to position 7 in the H7 peptide, the y_7^{+2}-, y_8^{+2}-, and y_9^{+2}-ions are no longer observed. Finally, in the case of the H4 peptide, the y^{+2}-ions are not detected and only a limited number of y-ions are observed.

The trend that is observed in Figure 6 is that the extent of the y^{+2}-ion series increases as the internal histidine is moved farther away from the N-terminus. This trend is consistent with a fragmentation scheme in which the protons on the triply protonated peptide ions are fixed at the C-terminus and the internal histidine and a mobile proton resides on the N-terminal amine. With CID, the mobile proton is able to migrate to the different amide bonds and direct facile fragmentation reactions, up to the proton fixed on the internal histidine residue. In the case of the H13 peptide, the histidine is considerably removed from the N-terminus and a relatively large number of amide bonds are accessible. This accessibility allows formation of an extensive series of y^{+2}-ions. In the case of the H4 peptide, however, the histidine is in close proximity to the N-terminus and few amide bonds are accessible. As a result, no facile fragmentation reactions are available and the few product ions that are observed are the result of relatively unfavorable pathways.

A common method for enhancing the fragmentation of peptides is to chemically derivatize them in a manner that promotes certain dissociation pathways [19–22].

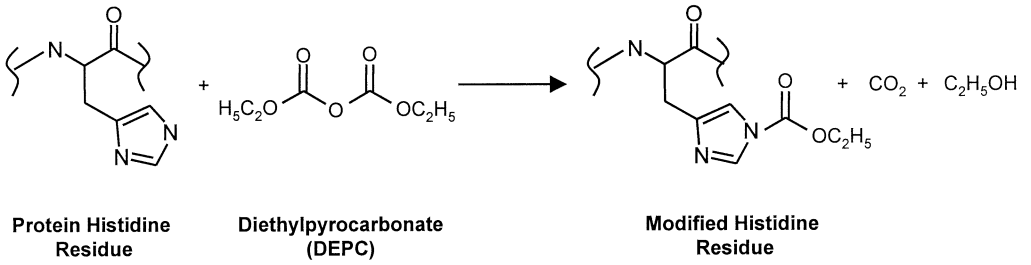

Figure 7. Reaction scheme for peptide modification with diethylpyrocarbonate. The modification of histidine residues is carried out by reaction diethylpyrocarbonate (DEPC) with the histidine to produce the modified residue. This reaction is typically carried out in a 50 mM phosphate buffer, pH 6.0, at 35 °C for 20 min.

One class of derivatization reactions modifies either the N- or C-terminus to localize a charge at the ends of a given peptide [19]. Subsequent fragmentation of these fixed-charge derivatives results in a preferential formation of either C- or N-terminal fragments that facilitate de novo sequencing. Other methods are used that modify selected residues within the peptide, such as arginine or lysine residues [20, 21]. These techniques have been used to increase both the ionization and fragmentation efficiencies in MALDI mass spectrometry. Derivatization of peptides with phenyl isothiocyanate results in the formation of N-terminally derivatized peptide which under low energy CID conditions gives a highly favored fragmentation of the first amide linkage. This results in the formation of a b_1-ion which is used to determine the identity of the first one or two amino acids in an unknown peptide sequence [22].

Considering the success of those methods, we have attempted to enhance the product ion spectrum of the histidine-containing peptides by derivatizing the peptides with DEPC. This derivatizing reagent has been used by protein chemists to selectively modify protein histidyl groups by adding an ethoxyformyl group to the ring nitrogen [23]. The modification reaction is shown in Figure 7 and produces a net increase of 72 Da per ethoxyformyl group that is added. When the synthetic peptides were reacted with DEPC under the conditions described in the methods, both the histidine residue and the N-terminal amine could be derivatized. The mass spectra of these fully-derivatized peptides showed doubly protonated peptide ions with no detectable triply protonated peptide ions (data not shown). With shorter reaction times and lesser amounts of the DEPC reagent, the derivatization reaction could be controlled so that only one ethoxyformyl group was added to the peptides. The product ion spectra of these derivatized peptides showed that this addition was at the N-terminal amine and not the histidine residue. The mass spectra of these partially-modified peptides were also dominated by the doubly protonated peptide ions.

Figure 8 shows a series of fragmentation efficiency curves for modified forms of the control and H4 peptides. As seen in Figure 8a by the shift of the fragmentation efficiency curve to lower collision energies, modification of the control peptide with DEPC results in a significant enhancement of the fragmentation. One would speculate that this enhancement is due to a decrease in the gas phase basicity of the modified N-terminal amine relative to the unmodified N-terminal amine. No such enhancement is seen in Figure 8b for the N-terminally modified H4 peptide where the fragmentation efficiency curve coincides with the curve for the unmodified peptide. One would speculate that this lack of enhancement is because the second protonation site in the doubly charged H4 peptide is the histidine residue, which is not modified, rather than the

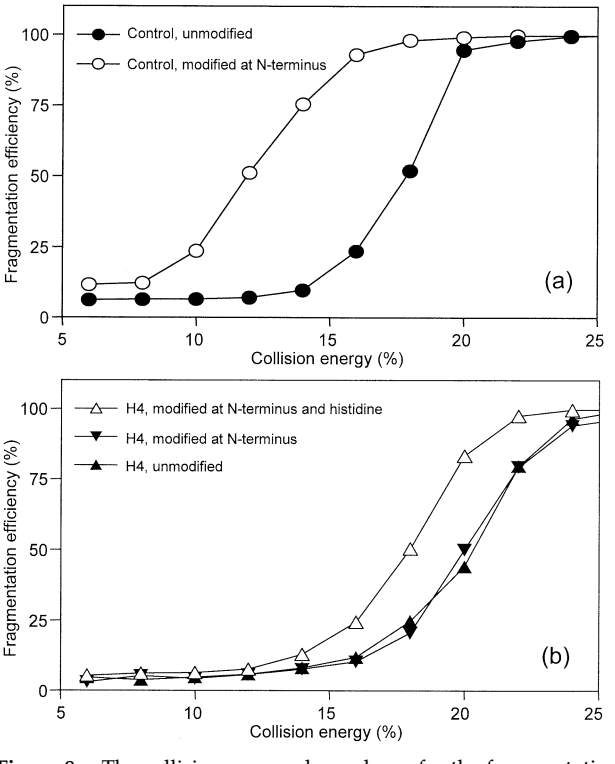

Figure 8. The collision energy dependence for the fragmentation of the doubly protonated molecular ions of the control (**a**) and H4 (**b**) peptides after derivatization with diethylpyrocarbonate. As described in the methods section, the collision energy used for the fragmentation reactions was varied and the integrated abundances of the product ions determined as a measure of fragmentation efficiency.

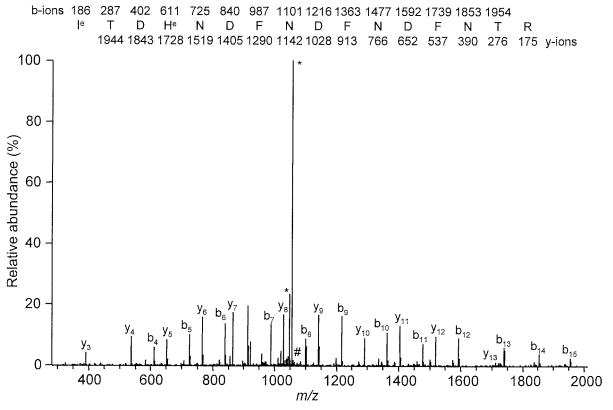

Figure 9. The product ion spectrum of the doubly charged molecular ion of the H4 peptide after derivatization with diethylpyrocarbonate. The derivatization reaction modified the N-terminus and the histidine residue in this peptide. The interpretation of this spectrum is shown at the top of the spectrum with the calculated *m/z* of the b- and y-ions rounded to the nearest integer. The sequence-specific ions in the spectrum are labeled as either b- or y-ions. The notation used is the one-letter amino acid code with the I^e and H^e designating ethoxyformyl modified isoleucine and histidine residues, respectively. The ion labeled with a # is the molecular ion and the ions labeled with an asterisk are doubly-charged ions produced by loss of water from the molecular ion.

modified N-terminal amine. In contrast, the H4 peptide that was modified at both the N-terminal amine and histidine residue shows an enhancement of the fragmentation efficiency. The enhancement of fragmentation seen for the completely modified form of H4 peptide suggests that the second proton on these doubly protonated peptide ions is localized on the modified histidine site, with the modification reducing the basicity of this protonation site and enhancing the mobility of the proton upon CID.

The product ion spectrum from the doubly charged peptide ion for the fully modified H4 peptide is shown in Figure 9. This spectrum was acquired under the same collision conditions used to acquire the product ion spectrum for the doubly protonated ion of the unmodified peptide shown in Figure 3b. The product ion spectrum of the modified peptide contains extensive sets of both the y- and b-ions, and these ions reveal the entire peptide sequence. Many of these sequence ions were not observed in the product ion spectrum of the unmodified peptide shown in Figure 3b. Further, the overall relative abundances of the product ions seen in Figure 9 are consistently more than four to five times higher than the product ions seen in Figure 3. These observations are consistent with an enhancement, in terms of both the intensity of the sequence-specific ions and the amount of sequence information present in the product ion spectrum, of the peptide fragmentation by the histidine modification.

In order to test the applicability of this derivatization reaction on a complex tryptic digest, a model protein, BSA, was digested with trypsin and derivatized with DEPC. Analysis of the original digest detected 36 peptides, including the peptides discussed with Figures 1 and 2, covering 61.6% of the protein sequence. Of these 36 peptides, 12 peptides contained histidine residues. After derivatization with DEPC, 29 modified peptides were detected, with nine of these peptides containing histidine residues (Table 2). The nine histidine-containing peptides that were detected were all modified at both the N-terminus and the histidine residue. In addition, several of these peptides were modified at the C-terminal lysine residue.

Table 2. Modified histidine-containing peptides detected in a digest of bovine serum albumin (BSA). An in-gel digest of BSA was modified using diethylpyrocarbonate as described in the methods. The histidine-containing peptides detected by capillary column HPLC-electrospray ionization-tandem mass spectrometry analysis are shown

Measured molecular weight of the unmodified peptide ($M + H^+$, Da)	Sequence[a]	Unmodified charge states[b]	Modified charge states[b]
898.4	$L^eC^aVLH^eEK$	+2	+1
1072.6	$S^eH^eC^aIAEVEK$	+2	+2
1249.6	$F^eK^eDLGEEH^eFK$	+3	+2
1305.6	$H^eLVDEPQNLIK$	+2	+2
1419.6	$S^eLH^eTLFGDELC^aK$	+3	+2
1440.7	$R^eH^ePEYAVSVLLR$	+3	+2
1554.7	$D^eDAPH^eC^aYSTVFDK$	+2	+2
1909.0	$L^eFTFH^eADIC^aTLPDTEK$	+3	+2

[a] The notation used is the one-letter amino acid code with the H^e, L^e, S^e, F^e, K^e, R^e, and D^e designating ethoxyformyl modified residues, and the C^a designating an alkylated cysteine residue.
[b] Most abundant charge state is given.

The histidine-containing peptides detected after DEPC modification showed a consistent shift to lower protonation states, as indicated in Table 2, with the modified histidine-containing peptides observed predominantly in their doubly protonated forms. As an example, the product ion spectra of the peptide $S^eLH^eTLFGDELC^aK$ after DEPC derivatization is shown in Figure 10. The product ion spectra from the underivatized peptide are shown in Figure 2. Fragmentation of this modified peptide gave an interpretable product ion spectrum containing more sequence information than was observed in the spectra shown in Figure 2. A similar, general enhancement was seen for the other histidine-containing peptides detected in the derivatized digest.

Conclusion

The sequencing and identification of proteins is significantly facilitated by the general structure of the peptides formed by protein digestion using trypsin. This general peptide structure contains good protonation sites at both the C-terminus and the N-terminus of the peptide. Further, the basicity of one of these sites, the N-terminal amine, is such that the associated proton is

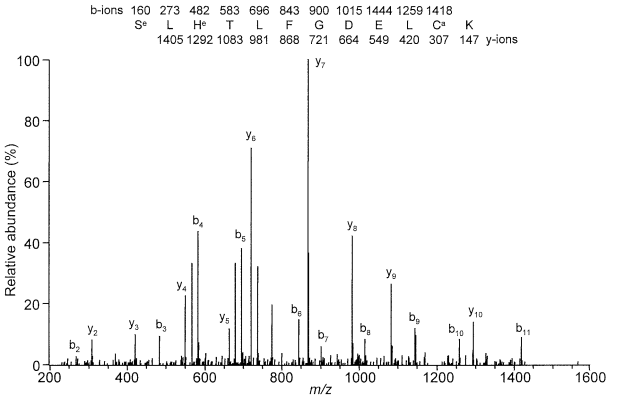

Figure 10. The product ion spectrum of the doubly charged molecular ion of the $S^eLH^eTLFGDELC^aK$ from a tryptic digestion of bovine serum albumin after derivatization with diethylpyrocarbonate. The derivatization reaction modified the N-terminus and the histidine residue. The interpretation of this spectrum is shown at the top of the spectrum with the calculated *m/z* of the b- and y-ions rounded to the nearest integer. The sequence-specific ions in the spectrum are labeled as either b- or y-ions. The notation used is the one-letter amino acid code with the S^e and H^e designating ethoxyformyl modified serine and histidine residues, respectively, and the C^a designating an alkylated cysteine residue.

efficiently mobilized by CID and able to direct fragmentation reactions which clearly reveal the amino acid sequence of the peptide through a series of complementary b- and y-ions. This idealized behavior, however, is disrupted when an additional protonation site is introduced into a peptide sequence.

In these experiments, the inclusion of a histidine residue as the additional protonation site was studied using a series of histidine-containing synthetic peptides. Each histidine-containing peptide gave both doubly and triply protonated molecular ions upon electrospray ionization. The triply protonated ions have a better fragmentation efficiency than the doubly protonated ions and give CID spectra that are dominated by y^{+2}-ions. In addition, these experiments have shown that the number and abundance of y^{+2}-ions is dependent on the placement of histidine. Specifically, the amount of sequence information that is obtained through the y^{+2}-ion series increases as the position of the histidine is moved from the N-terminus of the peptide towards the C-terminus. These results are consistent with the mobile proton model of peptide fragmentation and are consistent with a situation where the mobility of the mobile proton on the N-terminus of the peptide is significantly affected by a fixed proton on the histidine residue.

We have also shown that the fragmentation patterns of histidine-containing peptides can be altered and improved by modifying the histidine with DEPC. This modification reaction forms ethoxyformyl-modified peptides that give predominantly doubly-protonated molecular ions using electrospray ionization. The modified peptides fragmented more efficiently using CID and the product ion spectra contained a consistently interpretable series of b- and y-ions. Further, the presence and abundance of these sequence ions does not depend on the placement of the histidine residue. These observations also appear consistent with the mobile proton model, allowing one to speculate that the basicity of the modified histidine residue is sufficiently reduced to allow efficient migration of the proton associated with that site.

Finally, the utility of this modification reaction has also been tested, modifying a tryptic digest of BSA. The modification resulted in a significant increase in sequence information for several of the histidine-containing peptides. We propose that this modification would be useful in selected instances where additional sequence information is needed for histidine-containing peptides.

References

1. Yates, J. R., 3rd. Mass spectrometry and the age of the proteome. *J. Mass Spectrom.* **1998,** *33,* 1–19.
2. Chambers, G.; Lawrie, L.; Cash, P.; Murray, G. I. Proteomics: A new approach to the study of disease. *J. Pathol.* **2000,** *192,* 280–288.
3. Bichsel, V. E.; Liotta, L. A.; Petricoin, E. F., 3rd. Cancer proteomics: From biomarker discovery to signal pathway profiling. *Cancer J.* **2001,** *7,* 69–78.
4. Fields, S. Proteomics in genomeland. *Science* **2001,** *291,* 1221–1224.
5. Godovac-Zimmermann, J.; Brown, L. R. Perspectives for mass spectrometry and functional proteomics. *Mass Spectrom. Rev.* **2001,** *20,* 1–57.
6. Dongré, A. R.; Jones, J. L.; Somogyi, Á.; Wysocki, V. H. Influence of peptide composition, gas-phase basicity, and chemical modification on fragmentation efficiency: Evidence for the mobile proton model. *J. Am. Chem. Soc.* **1996,** *118,* 8365–8374.
7. Summerfield, S.; Gaskell, S. J. Fragmentation efficiencies of peptide ions following low energy collisional activation. *Int. J. Mass Spectrom. Ion Processes* **1997,** *165/166,* 509–521.
8. Gu, C.; Somogyi, Á.; Wysocki, V. H.; Medzihradszky, D. F. Fragmentation of protonated oligopeptides XLDVLQ (X = L,H,K or R) by surface induced dissociation: Additional evidence for the mobile proton model. *Anal. Chem. Acta* **1999,** *397,* 247–256.
9. Cox, K. A.; Gaskell, S. J.; Morris, M.; Whiting, A. Role of the site of protonation in the low-energy decompositions of gas-phase peptide ions. *J. Am. Soc. Mass Spectrom.* **1996,** *7,* 522–531.
10. Nari, H.; Somogyi, Á.; Wysocki, V. H. Effect of alkyl substitution at the amide nitrogen on amid bond cleavage: Electrospray ionization/surface-induced dissociation fragmentation of substance P and two alkylated analogs. *J. Mass Spectrom.* **1996,** *31,* 1141–1148.
11. Jones, J. L.; Dongré, A. R.; Somogyi, Á.; Wysocki, V. H. Sequence dependence of peptide fragmentation efficiency curves determined by electrospray ionization/surface-induced dissociation mass spectrometry. *J. Am. Chem. Soc.* **1994,** *116,* 8368–8369.
12. Burlet, O.; Orkiszewski, R. S.; Ballard, K.; Gaskell, S. J. Charge promotion of low-energy fragmentations of peptide ions. *Rapid Comm. Mass Spectrom.* **1992,** *6,* 658–662.
13. McCormack, A. L.; Somogyi, Á.; Dongré, A. R.; Wysocki, V. H. Fragmentation of protonated peptides: Surface-induced dissociation in conjunction with a quantum mechanical approach. *Analyt. Chem.* **1993,** *65,* 2859–2872.
14. Wysocki, V. H.; Tsaprailis, G.; Smith, L. L.; Breci, L. A. Mobile

and localized protons: A framework for understanding peptide dissociation. *J. Mass Spectrom.* **2000,** *35,* 1399–1406.
15. Kinter, M.; Sherman, N. E. *Protein Sequencing and Identification Using Tandem Mass Spectrometry;* John Wiley & Sons, Inc: New York, 2000; pp 64–79.
16. Eng, J. K.; McCormack, A. L.; Yates, J. R., III. An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *J. Am. Soc. Mass Spectrom.* **1994,** *5,* 976–989.
17. Perkins, D. N.; Pappin, D. J.; Creasy, D. M.; Cottrell, J. S. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* **1999,** *20,* 3551–3567.
18. Harrison, A. G. The gas-phase basicities and proton affinities of amino acids and peptides. *Mass Spectrom. Rev.* **1997,** *16,* 201–217.
19. Roth, K. D. W.; Huang, Z. H.; Sadagopan, N.; Watson, J. T. Charge derivatization of peptides for analysis by mass spectrometry. *Mass Spectrom. Rev.* **1998,** *17,* 255–274.
20. Dikler, S.; Kelly, J. W.; Russell, D. H. Improving mass spectrometric sequencing of arginine-containing peptides by derivatization with acetylacetone. *J. Mass Spectrom.* **1997,** *32,* 1337–1349.
21. Brancia, F. L.; Oliver, S. G.; Gaskell, S. J. Improved matrix-assisted laser desorption/ionization mass spectrometric analysis of tryptic hydrolysates of proteins following quanidation of lysine-containing peptides. *Rapid Comm. Mass Spectrom.* **2000,** *14,* 2070–2073.
22. Summerfield, S.; Bolgar, M. S.; Gaskell, S. J. Promotion and stabilization of b1 ions in peptide phenylthiocarbamoyl derivatives: Analogies with condensed-phase chemistry. *J. Mass Spectrom.* **1997,** *32,* 225–231.
23. Miles, E. W. Modification of histidyl residues in proteins by diethylpyrocarbonate. *Methods Enzymol.* **1977,** *47,* 431–442.