Investigation of the Tris(trimethoxyphenyl)phosphonium Acetyl Charged Derivatives of Peptides by Electrospray Ionization Mass Spectrometry and Tandem Mass Spectrometry

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Charged derivatives of peptides are useful in obtaining simpler collision-activated dissociation (CAD) mass spectra. An N-terminal charge-derivatizing reagent capable of reacting with picomole levels of peptide has been recently reported (Huang et al. *Anal. Chem.* **1997,** *69*, 137–144) in the contexts of analyses by fast atom bombardment (FAB) and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. Electrospray ionization (ESI) mass spectrometric investigation of these tris(trimethoxyphenylphosphonium) acetyl derivatives are described in this article, including studies by in-source fragmentation (ISF) and tandem mass spectrometry (MS/MS). Results from ISF are compared with those from MS/MS. Similarities and differences between ESI-ISF, MALDI-post-source decay (PSD), and FAB-CAD data are presented. Differences in fragmentation of these charged derivatives in the triple quadrupole and ion trap mass spectrometers also are discussed. Application of this derivatizing procedure to tryptic digests and subsequent analysis by liquid chromatography-mass spectrometry is also shown. (J Am Soc Mass Spectrom 2000, 11, 107–119) © 2000 American Society for Mass Spectrometry

Determining the amino acid sequence of proteins continues to be of great interest in today's scientific community especially because of the rapidly progressing human-genome project [1]. In addition, it is of importance to the pharmaceutical industries where recombinant proteins are being developed for therapy, diagnosis, and prophylaxis of human diseases [2]. Usually, protein sequences are either deduced from the cDNA sequence, or determined by Edman sequencing. Although the DNA sequence provides the primary amino acid sequence, initiated at the methionine codon and terminated at the stop codon, it does not give any information regarding the posttranslational modifications of the protein, which occur in the cytoplasm [3]. Phosphorylation, disulfide bond formation, deamidation, and glycosylation are several such important posttranslational events that modify the protein structure outside the nucleus. The Edman degradation method is very sensitive, but is only unidirectional; it is also difficult to obtain sequence information if the N-terminus is acylated or blocked because of other modifications. In cases where results from the previously mentioned techniques are ambiguous or incomplete, mass spectrometric information can be complementary.

The nomenclature for fragmentation of peptides under mass spectrometric ionization methods has been clearly defined [4]. When a peptide is subjected to collision-activated dissociation (CAD), the fragments produced are dependent on the position of the most basic residue (if any) in the sequence or protonation of any given amide group. In the latter case, due to charge-mediated fragmentation, a mixture of ion types is produced, predominantly **b** and **y** ions, and some **a**, **c**, **x**, **z** ions. In cases where there is a β-methylene unit in side chain, **d** and **w** ions are also possible. This multitude of ion types complicates the spectrum, and it requires a great scrutiny to interpret it. In an effort to obtain a simple spectrum, so called charged derivatives of the peptides can be prepared and subjected to fragmentation to generate predominantly one type of ions. This generally occurs via charge-remote fragmentation, which is also likely when a basic residue is located on either terminus. Most of the charged derivatives studied so far have been shown to produce $^*\mathbf{a}_n$ ions [5].

The results presented in this article are those of N-terminally derivatized peptides. The fragment ions contain a charged group at the N-terminus, and will be referred to as $\mathbf{a}_n$-type ions ($^*\mathbf{a}_n$) instead of $\mathbf{a}_n$ ions.

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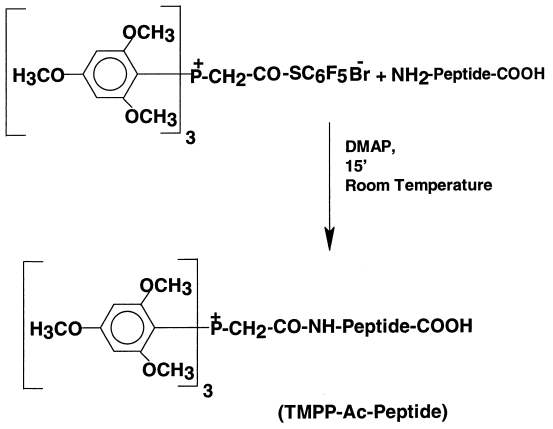

Scheme 1. Reaction of TMPP-Ac reagent with the N-terminus of a peptide.

Similarly, the other N-terminal containing fragments will be denoted as $^*\mathbf{b}_n$, $^*\mathbf{c}_n$, and $^*\mathbf{d}_n$ ions.

Although a number of charge-derivatizing reagents have been described, most of them have disadvantages [5]. One of the charge-derivatizing reagents which was developed to avoid the disadvantages of other existing reagents, consists of a tris[2,4,6-trimethoxyphenyl]phosphonium acetyl (TMPP-Ac) cation [6]. The structure of the TMPP-Ac reagent and its coupling reaction with the N-terminus of a peptide are shown in Scheme **1**; the reagent selectively derivatizes the N-terminus of the peptides at the picomole level, with only a five-fold molar excess, at room temperature. Studies of TMPP-Ac-peptides by fast atom bombardment-CAD (FAB-CAD) [6] and matrix-assisted laser desorption/ionization–post-source decay (MALDI-PSD) [7] demonstrated that it is possible to correlate the CAD spectrum of a TMPP-Ac-peptide to its structure. TMPP-Ac derivatives of peptides have also been studied by FAB-CAD in an ion trap mass spectrometer [8].

TMPP-Ac derivatization can be applied to protein digests. The lypophilized tryptic digest of proteins like cytochrome *c* can be treated directly with the TMPP-Ac reagent, and analysis of derivatized tryptic fragments by MALDI-PSD can be used to obtain sequence information [9]. Whereas a simple mixture of TMPP-Ac tryptic peptides can be analyzed directly by MALDI-PSD, preliminary separation is usually required for proteins larger than 10 kDa because a larger excess of the TMPP-Ac reagent is used for derivatization. This excess reagent may suppress the signal in MALDI, and hence HPLC is necessary. Such practical issues create a pressing need for the analysis of these charged derivatives by electrospray ionization (ESI). An obvious advantage is the possibility of LC-MS based on ESI. Hence, it was of great interest to study the fragmentation pattern of the TMPP-Ac-peptides using ESI-MS.

There have been many studies on the fragmentation pattern of charged derivatives by FAB [6, 8, 10–18] and MALDI [7, 19–23]. However, there are only a few studies based on ESI of charged derivatives [24–28]. There have been several high-energy studies of peptide fragmentation with ESI. High-energy CAD of multiple-charged ions from ESI has been used to study the parameters of charge location and charge state [29]. Wysocki et al. have reported on ESI-surface induced dissociation (SID) studies of peptides [30]. Because ESI is more commonly used with triple quadrupole and ion trap mass spectrometers, it is of interest to study the low-energy CAD of such ESI-generated ions. This paucity of such studies by ESI prompted our investigation of the fragmentation of TMPP-Ac derivatives by ESI via in-source fragmentation (ISF) and MS/MS (low-energy CAD) with a comparison to results from FAB-CAD and MALDI-PSD. Such studies would also provide insight to the fragmentation pathways of charged derivatives when ionized by ESI and fragmented under low-energy conditions.

Experimental

Preparation of Derivatives

Peptides and proteins used in the experiments were purchased from Sigma and were dissolved in acetonitrile/water (1:1 v/v). Peptides were N-terminally derivatized using TMPP-Ac-SC_6F_5 bromide reagent synthesized as described elsewhere [6]. The peptide solution was mixed with a five-fold molar excess of the derivatizing reagent in the presence of dimethyl amino pyridine (DMAP). The reaction was allowed to occur at room temperature for 15 min. After derivatization, the reaction mixture was analyzed by HPLC to separate the derivatized peptide from any underivatized peptide and hydrolyzed derivatizing reagent. A C_{18} column (4.8 × 250 mm) from Vydac was used. Separation was performed using a gradient elution program from 25% to 60% of acetonitrile/water/TFA (90%/10%/0.1% v/v/v) (solvent B) over 30 min at 1 mL/min using a Waters Millenium system. Solvent A was water/0.1% TFA (v/v). Fractions corresponding to the derivatized peptides were collected manually, and dried under reduced pressure; they were reconstituted in acetonitrile/water (1:1 v/v) when needed.

Proteins were digested using 10% (w/w) of trypsin (sequencing grade) to protein, at 37 °C for periods of 30 min to 2 h in a phosphate buffer (50 mM) at pH 8.2. Once digested, the mixture of peptide fragments was derivatized in situ, using the TMPP-Ac derivatizing reagent at a 10-fold molar excess over the estimated number of peptide fragments. For example, if 1 nmol of a protein were digested, and three fragments were produced, then there would be 3 nmol of peptides, so 30 (3 × 10) nmol of the derivatizing reagent would be used. The mixture was allowed to react at room temperature for 15 min. The derivatized tryptic fragments were separated by HPLC (gradient program used was the same as described previously). The fractions were collected manually (the R_t of the TMPP-Ac-peptides were between 28 and 37 min, while that of the underivatized peptides were between 12 and 20 min), and subsequently analyzed by ESI-ISF-MS on the Fison's

platform. Alternatively, the derivatized digest mixture was analyzed directly by LC-MS using the LCQ.

Mass Spectrometry

In-source Fragmentation Mass Spectrometry

The purified TMPP-Ac-peptides were analyzed by a Fison's VG platform ESI-MS. Samples (10 pmol/μL) were flow injected with acetonitrile/water (1:1) as the mobile phase (10 μL/min). Nitrogen was used as drying gas, and the capillary was typically held at 3.5 kV. The quadrupole mass analyzer was operated at 3 scans/s over a range of *m/z* 200–2000. Increasing the cone voltage in the ESI source to 100 V induced fragmentation of the peptide derivatives. This is referred to as ISF. When no fragmentation was desired, the cone voltage was maintained at 37 V.

ISF was also performed in the Finnigan LCQ, where TMPP-Ac derivatives were fragmented in the atmospheric region by increasing the capillary and the tube lens offset voltages simultaneously to 59 and 40 V from initial values of 25 and 10 V, respectively.

MS/MS

CAD-MS/MS of the TMPP-Ac derivatized peptides was performed using two different instruments. A triple quadrupole mass spectrometer, API 2000, manufactured by PE-SCIEX, with an ESI source was used in one case. The derivative solution (acetonitrile/water/0.1% formic acid, 10 pmol/μL) was infused at a flow rate of 5 μL/min into the ion spray source held at 5 kV. The precursor ion selected in the first quadrupole was collisionally activated in the second stage. Nitrogen was used as the collision gas. Product ions were scanned in the third quadrupole, in steps of 0.3 *m/z* for a dwell time of 1 ms. Optimum conditions for efficient fragmentation were determined by using a "ramp parameter" feature of the software. Fragmentation of single-charged ions of the TMPP-Ac derivatives occurred at accelerating potential differences (corresponding to lab frame collision energies in eV) of 50–70 V.

A Finnigan LCQ, an ESI-ion trap mass spectrometer, was also used to obtain CAD information on the TMPP-Ac derivatives (acetonitrile/water/1% acetic acid, 10 pmol/μL) both by infusion of the sample (3 μL/min) and by LC-MS. The source was held at 4.3 kV, and helium was used as the CAD gas. MS^n was performed, where n = 1, 2, 3. Fragmentation of single-charged TMPP-Ac derivatives occurred at relative collision energies of 60%–70% of a maximum of 5 V. LC instrumentation included the Alliance system from Waters corporation, and the Symmetry (from Waters) HPLC column (2.1 × 150 mm). Solvents were pumped at a flow rate of 200 μL/min. The solvent composition and gradient are the same as described earlier. Approximately 10 pmol of the derivatized-digested protein were loaded on the HPLC column for LC-MS.

MALDI-MS

MALDI spectra were obtained with a Voyager Elite reflectron time-of-flight mass spectrometer (Perseptive Biosystems) using an accelerating voltage of 22 kV. The mass spectrometer was equipped with a 337-nm nitrogen laser. A saturated solution of α-cyano-4-hydroxycinnamic acid, prepared in acetonitrile/water (1:1, v/v), was used as the matrix. A 1-μL aliquot of the sample (pmol/μL) was mixed with an equal volume of the matrix solution on the sample plate, and the mixture dried in air. PSD data were obtained when the mass spectrometer was operated in the reflectron mode. Several PSD spectra, each optimized for a different range of *m/z* values for the fragment ions, were obtained and "stitched" together to yield the composite spectrum.

Results and Discussion

In-source Fragmentation of the TMPP-Ac Derivatives of Peptides

Infusion of the TMPP-Ac derivatives of the peptides into the ESI source, at a cone voltage of 37 V, usually produced double-charged ions. This is not unusual because, along with the fixed charge on the TMPP-Ac head group, there could be protonation on a basic residue in the peptide. In larger peptides, we also observed triple-charged species. However, in the case of TMPP-Ac-VGVAPG, we only observed a single-charged ion (the charge is the one carried by the phosphonium moiety), due to the presence of amino acids containing nonpolar side chains. ISF of the charged derivatives of peptides was observed at elevated cone voltages. In our studies, a cone voltage setting of 100 V was sufficient to fragment peptide derivatives with 6–12 amino acids to yield discernible $*\mathbf{a_n}$ ions. Peptide derivatives with fewer amino acids fragmented at a lower cone voltage, and those with more than 12 amino acids required a higher cone voltage.

Although ISF of the underivatized peptide yields mostly $\mathbf{b_n}$ and $\mathbf{y_n}$ ions due to charge-mediated mechanisms, ISF of the TMPP-Ac derivatized peptide produces a series of $*\mathbf{a_n}$ ions, with a few $*\mathbf{b_n}$ ions and $*\mathbf{c_n}$ ions. Derivatized peptides containing amino acid residues with side chains tend to yield $*\mathbf{d_n}$ ions in some cases. An example depicting ISF of the TMPP-Ac derivatives of peptides is shown in Figure 1a. The spectrum consists of peaks corresponding to the ions (mostly $*\mathbf{a_n}$) formed when TMPP-Ac-GMDSLAFSGGL-NH_2 fragments at a cone voltage of 100 V in the VG-platform mass spectrometer.

MS/MS of the TMPP-Ac Derivatives of Peptides

Triple quadrupole mass spectrometer. The ESI source of the API/SCIEX instrument produced both double- and single-charged ions of the intact peptide derivative under the given conditions, and CAD was performed on both types of ions. Regardless of whether the single- or the double-charged species was selected as the precursor, the

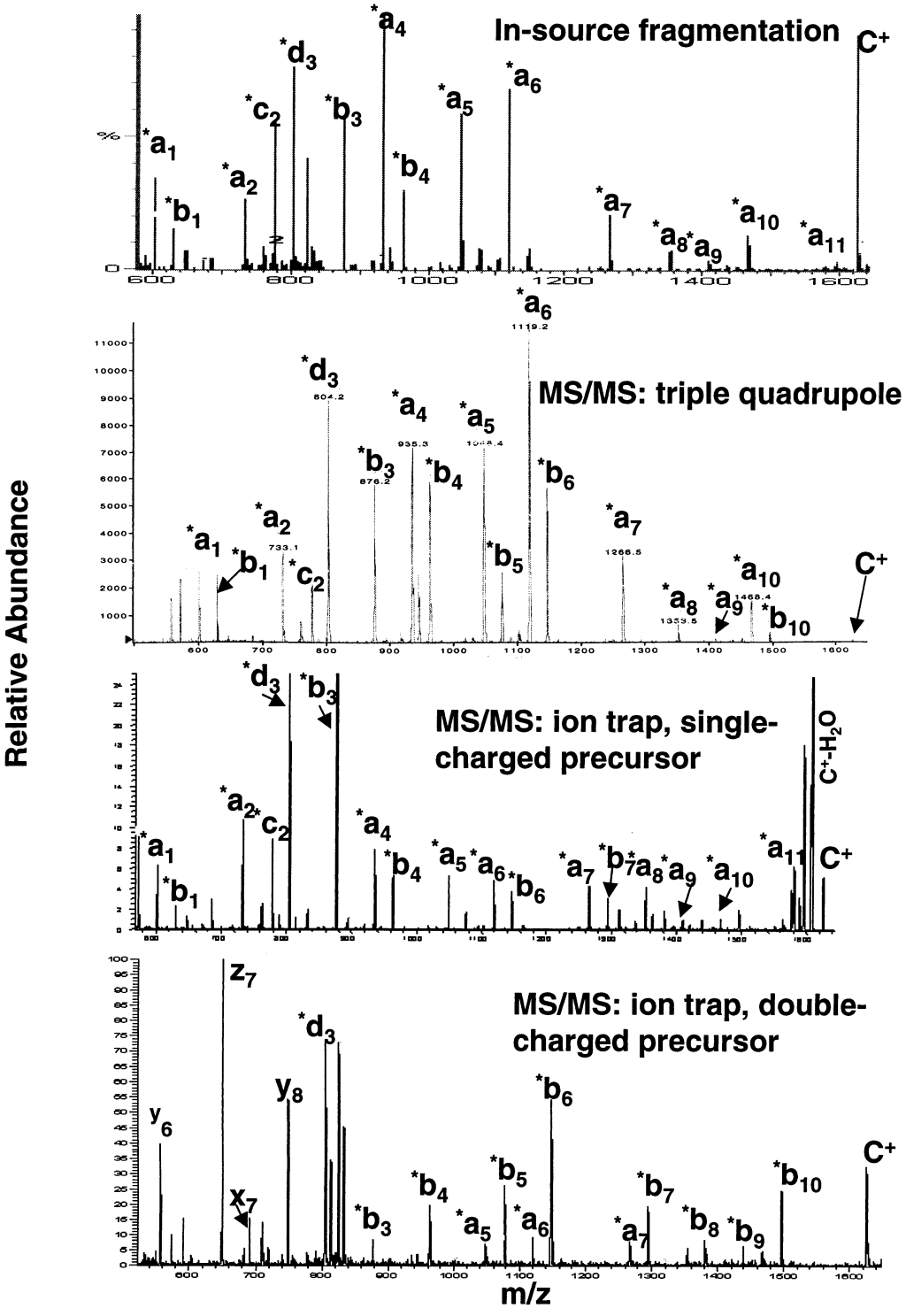

Figure 1. ESI-CAD of TMPP-Ac-GMDSLAFSGGL-NH_2 as obtained by (**a**) in-source fragmentation in the VG-platform, (**b**) MS/MS of a double-charged precursor in the triple quadrupole, (**c**) MS/MS of a single-charged precursor in the ion trap, and (**d**) MS/MS of a double-charged precursor in the ion trap.

product ion spectrum was the same, i.e., in both cases peaks were observed at the same *m/z* values, although the peak intensities differed by as much as 40% at a given *m/z* value. Because the ion count for the double-charged species was higher than that for the single-charged species, the MS/MS spectrum of the former had better S/N. Fragmentation of the precursor ions occurred at lab-frame collision energies of 60–70 eV. A series of $*\mathbf{a}_n$ ions was observed from fragmentation of either the single- or double-charged ions, along with a few $*\mathbf{b}_n$ and $*\mathbf{c}_n$ ions.

A representative spectrum, for the CAD of TMPP-Ac-GMDSLAFSGGL-NH_2 in the triple quadrupole, as observed from the double-charged precursor (*m/z* 813) is shown in Figure 1b. It is readily noticeable that a complete series of $*\mathbf{a_n}$ ions is formed, along with some $*\mathbf{b_n}$ ions.

Ion trap mass spectrometer. Fragmentation of the TMPP-Ac charged derivatives of peptides in the LCQ mass spectrometer was quite different from that described above. The pattern of fragmentation was different depending on selection of either the single- or the double-charged intact species as the precursor. Double-charged ions produced a series of $*\mathbf{b_n}$ ions, whereas the single-charged ions produced a major series of $*\mathbf{a_n}$ ions. It may be that the fragmentation pathways available to the double-charged ion are charge mediated by the proton, which is mobile, whereas fragmentation of the single-charged ion (the fixed charge) occurs by a charge-remote mechanism. It has also been reported by Lin and Glish [8] that a series of $*\mathbf{a_n}$ ions is observed for TMPP-Ac derivatized peptides when a single-charged precursor generated by FAB is fragmented in an ion trap.

An example spectrum for the fragmentation of the TMPP-Ac derivatives of peptides (single-charged precursor) in the ion trap is shown in Figure 1c. Here, we see an uninterrupted series of $*\mathbf{a_n}$ ions along with $*\mathbf{b_n}$ ions, as opposed to the case of the fragmentation of the double-charged precursor, which gives a series of $*\mathbf{b_n}$ ions and some C-terminal ions noticeable in the lower *m/z* region of the mass spectrum (Figure 1d).

Comparison of the Fragmentation of the TMPP-Ac Derivatives of Peptides by ISF vs. MS/MS

Although fragmentation of the TMPP-Ac derivatives of peptides by MS/MS in triple quadrupole and ion trap mass spectrometers is more specific because of precursor selection, essentially the same information can be obtained from ISF. Figure 1 shows the results from analyzing TMPP-Ac-GMDSLAFSGGL-NH_2 by ISF and MS/MS. A complete series of $*\mathbf{a_n}$ ions is obtained in each case. A peak for the molecular cation (C^+) is obtained with ISF (Figure 1a) indicating that this process is not as efficient for fragmentation as conventional CAD. MS/MS studies provided the opportunity to compare the fragmentation pattern originating from a single-charged ion with that from a double-charged ion. They also allowed us to observe the fragmentation of these precursors at various collision energies.

Comparison of the Fragmentation of the TMPP-Ac Derivatives of Peptide in the Triple Quadrupole vs. Ion Trap

It appears that the TMPP-Ac derivatives of peptides fragment through the charge-remote pathways (based on the formation of a-type ions) when subjected to CAD-MS/MS in the triple quadrupole mass spectrometer both as double- and single-charged precursor, and as a single-charged precursor in the ion trap. However, during the fragmentation of a double-charged precursor (one charge on the derivatized moiety, the second due to a proton along the backbone) in the trap, some C-terminal ions were detected along with the N-terminal ion types. These results suggested to us that perhaps because of the long (tens of ms) residence time in the trap, the large number of low-energy collisions promote both charge-mediated and charge-remote fragmentation. In-direct evidence for these phenomena was acquired by ISF where the trap was used only as a *m/z* analyzer (in which case the residence time of the ions was in the order of tens of microseconds), rather than as a CAD device with subsequent *m/z* analysis. The result of ISF with *m/z* analysis by the trap is shown in Figure 2, where the mass spectrum essentially represents $*\mathbf{a_n}$ ions, because processes leading to charge-mediated fragmentation did not have sufficient time to occur.

Also, the double-charged ion did not seem to fragment by charge-mediated pathways during MS/MS in the triple quadrupole mass spectrometer (Figure 1b). The quadrupole ion trap and transmission quadrupole mass spectrometers are distinctly different, i.e., in the transmission quadrupole the residence time of the ions is short (<100 μs). Thus, considerably more collisions will occur in the ion trap and, in principle, higher internal energies can be deposited in an ion, but in smaller increments. In addition, because of the longer reaction times, an ion that has accumulated sufficient internal energy to decompose by a low-energy process may not survive to undergo another activating collision [31]. Thus, it is not surprising that the mass spectra for the fragmentation of double-charged ions in the trap have peaks corresponding to fragment ions produced from charge-mediated processes.

Speculation on the Fragmentation Pathways of TMPP-Ac-Peptides

The major pathways by which an $\mathbf{a_n}$ ion can be formed during fragmentation of the peptide backbone is by loss of CO from a $\mathbf{b_n}$ ion [13]. In the case of charged derivatives, 1,2-elimination is responsible for $*\mathbf{a_n}$ ion formation [32], which is a charge-remote mechanism. Recently, a mechanism for $*\mathbf{a_n}$ ion formation in the case of TMPP-Ac derivatives of peptides has been proposed by Liao et al. [7], through the shift of an amide hydrogen.

Although in FAB-CAD (high-energy), $*\mathbf{b_n}$ ions were not commonly observed, they are observed during our low-energy CAD studies by ESI-MS. Higher laser powers in MALDI-PSD also produced $*\mathbf{b_n}$ ions. Charge-remote mechanisms for $\mathbf{b_n}$ ion formation in multiple-charged ions by low-energy CAD have been proposed earlier [33]. Wagner proposed charge-remote mechanisms for $*\mathbf{b_n}$ ion formation in charge-derivatized peptides when studied by FAB-CAD [34]. Thus, formation

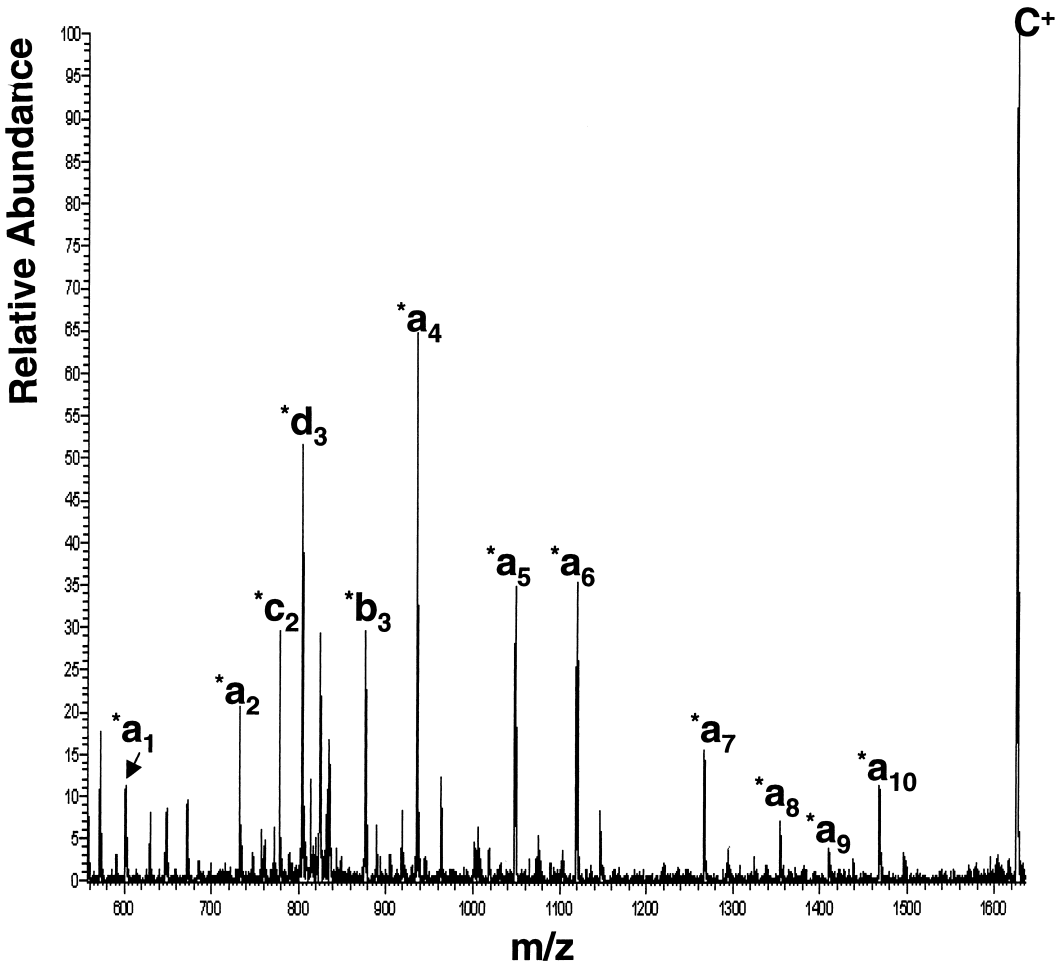

Figure 2. In-source fragmentation of TMPP-Ac-GMDSLAFSGGL-NH_2 in the ion trap mass spectrometer (LCQ).

of $^*\mathbf{b}_n$ ions by charge-remote mechanisms in TMPP-Ac-peptides could be from α-hydrogen shifts.

During the fragmentation of double-charged ions of TMPP-Ac-peptides (hence protonated along the backbone) by low-energy CAD (other than in the ion trap), if charge-mediated processes occur predominantly, there should be some characteristic evidence in the spectrum. For example, if a $^*\mathbf{b}_n$ ion is formed by a charge-mediated process, it should carry a charge on its carbonyl end (C-terminus). This process will make it a double-charged ion (as there is already a fixed charge on the N-terminus), which would be represented by a peak in the lower *m/z* range. This was not observed during CAD of any of the 10 model peptides (ranging from 5 to 15 residues) that we investigated. Further, no complementary **y**-ion signals were widely observed. Hence, charge-mediated pathways can be ruled out as producing the $^*\mathbf{b}_n$ ions. Conceivably, prior to the fragmentation of the double-charged species, charge stripping (removal of the additional proton) may occur yielding a species with a fixed charge (C^+), which undergoes further fragmentation (presumably via charge-remote pathways). However, during fragmentation of the double-charged ions in the ion trap mass spectrometer, a mixture of N-terminal and C-terminal ions was observed which led us to conclude that charge-mediated pathways may also be available.

Characteristic Behavior of Proline and Aspartic Acid Residues

In our study of a variety of derivatized peptides, whenever proline was present in the amino acid sequence, there was no conspicuous signal due to cleavage at that position during ISF in the VG platform mass spectrometer. Figure 3 shows the ESI-ISF mass spectrum for TMPP-Ac-VGVAPG; it is clear that a series of $^*\mathbf{a}_n$ ions is produced except for $^*\mathbf{a}_5$, which corresponds to cleavage at the position of the proline residue. It has been proposed by Liao et al. [7] that the lack of an amide hydrogen on a proline residue does not allow the mechanism for $^*\mathbf{a}_n$ ion formation in the TMPP-Ac derivatives when analyzed by MALDI-PSD. However, a weak signal corresponding to cleavage at the proline residue was observed during our ESI-CAD-MS/MS

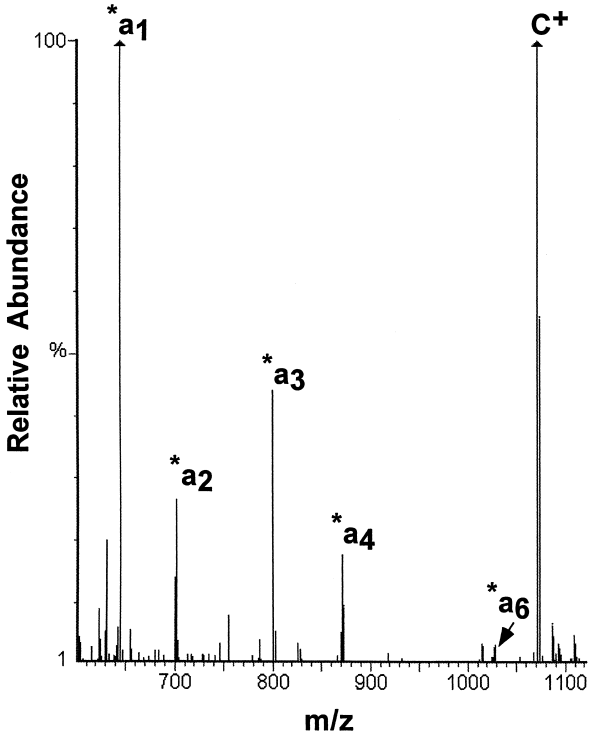

Figure 3. ESI-ISF mass spectrum of TMPP-Ac-VGVAPG.

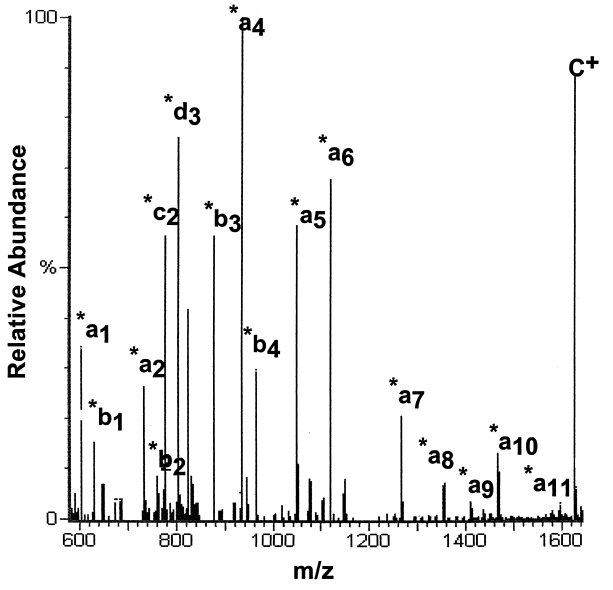

Figure 4. ESI-ISF mass spectrum of TMPP-Ac-GMDSLAFSGGL-NH_2.

study of the same peptide derivative. Also, a weak signal was obtained in MALDI-PSD for cleavage at proline at higher laser powers [7]. Thus, the mechanistic aspects are unclear.

Aspartic acid does not form an $*\mathbf{a}_n$ ion; it forms a $*\mathbf{d}_n$ ion, due to the loss of the side chain. It has been proposed that the $*\mathbf{d}_n$, $*\mathbf{b}_n$, and $*\mathbf{c}_{n-1}$ ions are formed via cyclic intermediates involving the side chain, and hence are formed preferentially over the $*\mathbf{a}_n$ ion [7]. Figure 4 is the ESI-ISF mass spectrum of TMPP-Ac-GMDSLAFSGGL-NH_2 showing a prominent peak for the $*\mathbf{d}_3$ ion (no $*\mathbf{a}_3$ ion) along with peaks for a $*\mathbf{b}_3$ ion and a $*\mathbf{c}_2$ ion; peaks for $*\mathbf{a}_n$ ions are discernible for fragmentation at all other residues. Thus, cleavage at the site of the aspartic acid residue yields a signature pattern ($*\mathbf{c}_{n-1}$, $*\mathbf{b}_n$, $*\mathbf{d}_n$), which, even though it disrupts the $*\mathbf{a}_n$ series, should not be difficult to identify because of the characteristic presence of the three peaks as a cluster.

Comparison of the Fragmentation of TMPP-Ac-Peptides by ESI-ISF vs. by MALDI-PSD and FAB-CAD

TMPP-Ac-peptides have been thoroughly studied for their fragmentation behavior by MALDI-PSD [7] and FAB-CAD [6]. Hence, it is meaningful to compare the results from ESI-ISF with those from the other two methods. The results from ESI-ISF match more closely with those from MALDI-PSD than those from FAB-CAD. For example, analysis of a derivatized peptide containing proline by FAB-CAD always yields an $*\mathbf{a}_n$ ion due to cleavage at the site of the proline residue, whereas in ESI-ISF the signal for this ion is weak and, in MALDI-PSD, the signal is laser power dependent [7]. The formation of a $*\mathbf{d}_n$ ion from fragmentation exclusively at the position of aspartic acid is observed with both ESI-ISF and MALDI-PSD. In FAB-CAD, although a $*\mathbf{d}_n$ ion is observed, there is also an $*\mathbf{a}_n$ ion without the loss of side chain.

These variations in results can be attributed to the differences inherent in these methods of analysis. High-energy CAD spectra are usually the result of single collisions between precursor ions and the inert gas-phase atoms, whereas low-energy CAD results from multiple collisions and reflects multistep cleavage reactions [35]. In FAB-CAD (high-energy), dissociation is induced by imparting excess energy (keV) to a selected precursor ion, promoting high-energy fragmentation. In MALDI-PSD, although a precursor can be selected, CAD is not promoted intentionally. The fragments formed either during desorption (prompt fragmentation) or from metastable decay of the precursor during flight are identified using the reflectron. Because it is possible to control the fragmentation by adjusting the laser power, probably most fragmentation occurs during the desorption/ionization (D/I) process. Therefore, it cannot be called a true CAD process. Although the energy imparted in the process is quite enough to promote fragmentation, it is not like the high-energy CAD in FAB. Similarly, in ESI-ISF, the increase in cone voltage promotes more energetic ion/molecule collisions, but the amount of energy transferred to the fragmenting ion is unknown. However, because ISF is a low-energy process, fragmentation is not expected to be similar to that in high-energy FAB-CAD. Figure 5

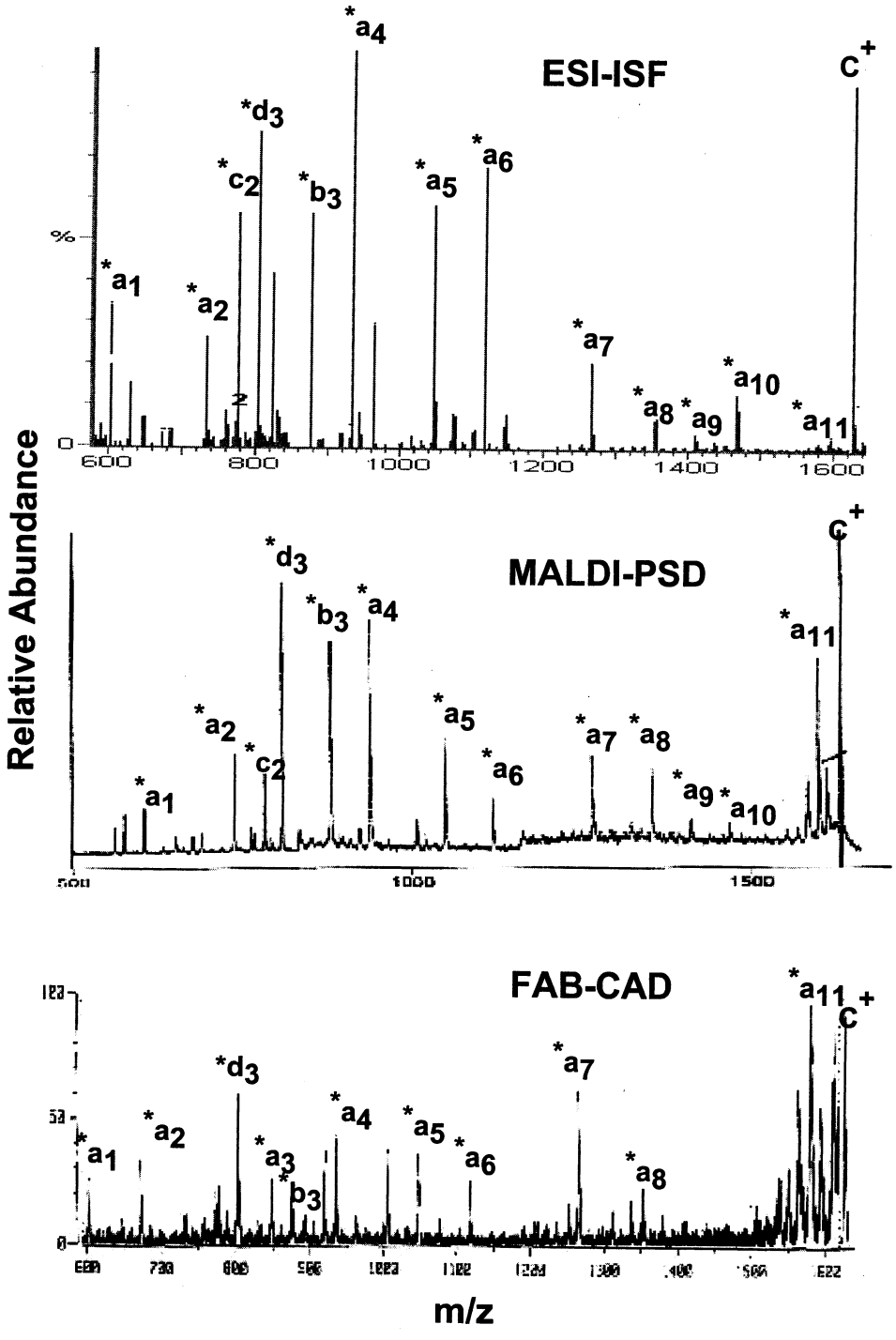

Figure 5. Fragmentation of TMPP-Ac-GMDSLAFSGGL-NH_2 by ESI-ISF, MALDI-PSD, and FAB-CAD, respectively.

compares the spectra of TMPP-Ac-GMDSLAFSSGL-NH_2 as obtained by all three ionization techniques. All three spectra show a complete series of $*\mathbf{a}_n$ ions; a few $*\mathbf{b}_n$ ions and $*\mathbf{c}_n$ ions are observed in all of them especially in the lower *m/z* region. Note that because of cleavage at the aspartic acid residue, $*\mathbf{a}_3$ is seen in the FAB-CAD spectrum in addition to $*\mathbf{d}_3$, while in MALDI-PSD and ESI-ISF spectra, only $*\mathbf{d}_3$ is seen.

ESI-MS Study of TMPP-Ac Derivatized Tryptic Digests

We have extended the study of derivatized synthetic peptides to derivatized peptide fragments produced during tryptic digests of proteins. Advantages due to TMPP-Ac derivatization are explicit during the chromatographic separation of digestion products. Certain

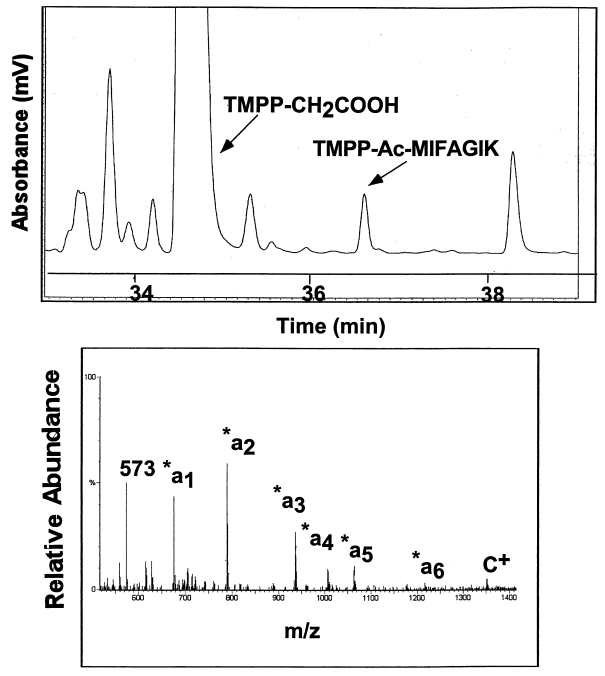

Figure 6. (**a**) Chromatogram of the TMPP-Ac derivatized cytochrome *c* digest (peaks corresponding to TMPP-Ac peptides and reagent). (**b**) The ESI-ISF mass spectrum corresponding to one of the HPLC fractions, TMPP-Ac-MIFAGIK, from the digest.

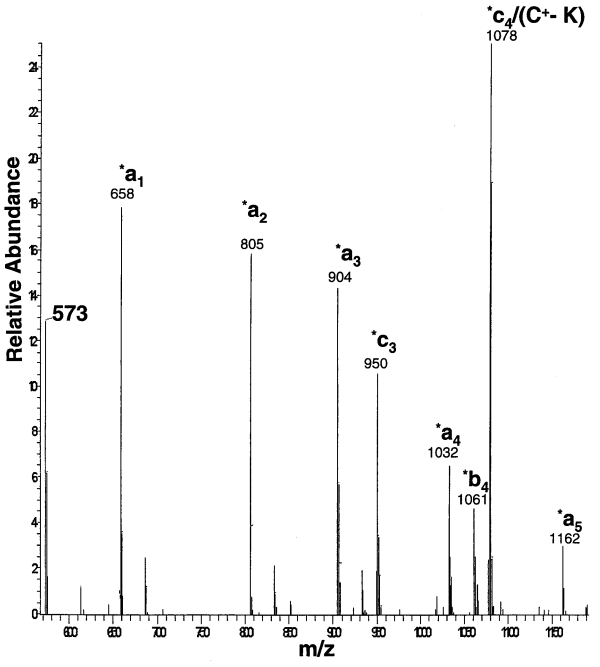

Figure 7. ESI-CAD of TMPP-Ac-IFVQK (residues 9–13 of cytochrome *c*) in LCQ. MS/MS of precursor (*m/z* 1209) at relative collision energy 65%.

peptides, which are of short length, have very short retention times; after TMPP-Ac derivatization, the retention times are increased, thereby permitting better separation and detection of these species. Also, the detection limit for fragments in protein digests on a conventional reverse phase column can be increased by derivatization because the molar absorptivity of the TMPP-Ac derivatized peptides is much higher than that for underivatized peptides. Hence, even if picomoles of the protein are digested, TMPP-Ac derivatized tryptic peptides can be separated and detected by conventional HPLC using an absorbance detector.

It should be noted that the TMPP-Ac reagent undergoes rapid hydrolysis to produce TMPP-CH_2COOH during its reaction with the peptides. TMPP itself is another byproduct. These products are represented by major peaks in the chromatogram. Often a major peak for TMPP-CH_2COOH occurs amidst the peaks corresponding to derivatives of some of the peptides. Fortunately, there were no cases of co-elution of the reagent-related species and derivatives of the peptides reported in this paper. Problems due to co-elution could be overcome by LC-MS using the mass spectrometer for precursor ion selection.

The chromatogram for the HPLC separation of TMPP-Ac derivatized tryptic peptides of cytochrome *c* is shown in Figure 6a. The peak at ~35 min corresponds to TMPP-CH_2COOH. It was possible to identify 15 TMPP-Ac derivatized tryptic fragments from cytochrome *c*, whereas in the underivatized mixture only 13 were identified. Although some tryptic fragments were unidentified (by MALDI-MS or ESI-MS) before derivatization, it was possible to detect them after derivatization, especially the ones with mass <500 Da.

The ESI-ISF mass spectrum of TMPP-Ac-MIFAGIK (see labeled peak in the chromatogram), representing the TMPP-Ac derivative of the tryptic fragment containing residues 80–86, is shown in Figure 6b. A series of $*\mathbf{a}_n$ ions, from which the sequence can be deduced, is seen. In some cases, the signal due to fragmentation of the last residue in a tryptic fragment is not detectable. However, because the C-terminal residue can only be lysine or arginine, absence of said peak should not preclude identification of the complete sequence. Note also that most of the fragments observed in Figure 6b are $*\mathbf{a}_n$ ions, and that no $*\mathbf{b}_n$ or $*\mathbf{c}_n$ ions are present.

Analysis of the same sample by online LC-MS was performed using a Waters chromatographic system and LCQ mass spectrometer. During the LC-MS run, the LCQ was operated in the dual scan mode (double play). A full scan was first performed, and a subsequent MS/MS scan was performed at relative collision energy of 65% on a list of precursor ions. The ESI-CAD spectrum of TMPP-Ac-IFVQK (residues 9–13) is shown in Figure 7. A series of $*\mathbf{a}_n$ ions is seen in the spectrum along with an intense peak at *m/z* 1078, corresponding to the $*\mathbf{c}_4$ ion or loss of the terminal K residue from the precursor (C^+).

The following example shows that it is possible to obtain simple and interpretable spectra from TMPP-Ac derivatives of peptides whose sequences are not known *a priori*. While analyzing the derivatized mixture of tryptic fragments of cytochrome *c* by LC-MS, MS/MS

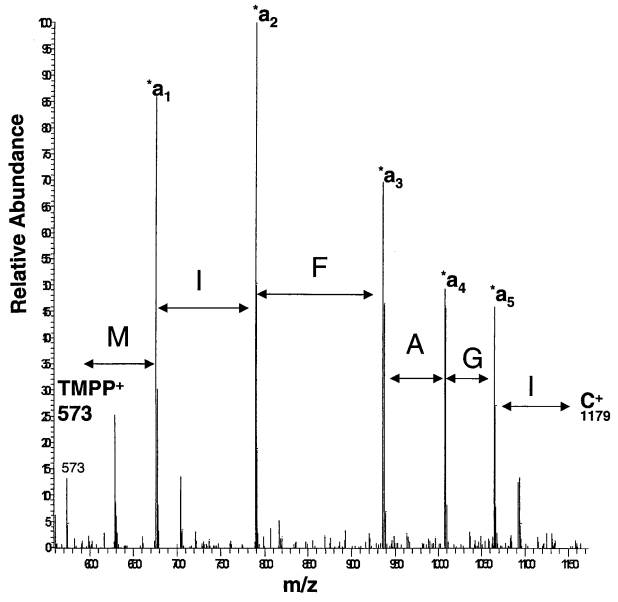

Figure 8. ESI-CAD of TMPP-Ac-MIFAGI (residues 80–85 of cytochrome *c*) in LCQ. MS/MS of precursor (*m/z* 1179) at relative collision energy 65%.

was performed on a precursor ion of *m/z* 1179.0. From the calculated masses of possible tryptic fragments and their derivatives, 1179.0 Da corresponds to TMPP-Ac-GITWK. However, the peaks in the MS/MS spectrum did not match the *m/z* of any ions that would be produced from fragmenting this derivatized peptide. Upon closer examination, it was possible to deduce the sequence of the derivatized peptide as being MIFAGI, corresponding to residues 80–85; the mass of TMPP-Ac derivative of MIFAGI is also 1179 Da. This peptide probably originated from the loss of K from the tryptic fragment MIFAGIK. Thus, this example demonstrates the applicability of correlating the MS/MS spectra of the TMPP-Ac derivatives of peptides to their amino acid sequence, due to a major series of $*\mathbf{a}_n$ ions, as shown in Figure 8.

Another protein studied was glucagon, HSQGT FTSDY SKYLDSRRAQ DFVQWLMNT, 29 residues in length, with three cleavage sites. Three tryptic fragments were obtained corresponding to residues 1–12, 13–17, and 18–29. The TMPP-Ac derivatized fragments were prepared, and then separated by HPLC. Figure 9 shows the ESI-ISF mass spectrometry (on the VG-platform mass spectrometer) of the TMPP-Ac derivative of YLDSR (residues 13–17). Note that there is a series of mostly $*\mathbf{a}_n$ ions, corresponding to the first four residues. Cleavage at the aspartic acid residue results in a $*\mathbf{d}_3$ ion rather than an $*\mathbf{a}_3$ ion, along with a $*\mathbf{b}_3$ ion and a $*\mathbf{c}_2$ ion. This result is expected from our earlier observations.

Similarly, the oxidized B chain of insulin (30 residues

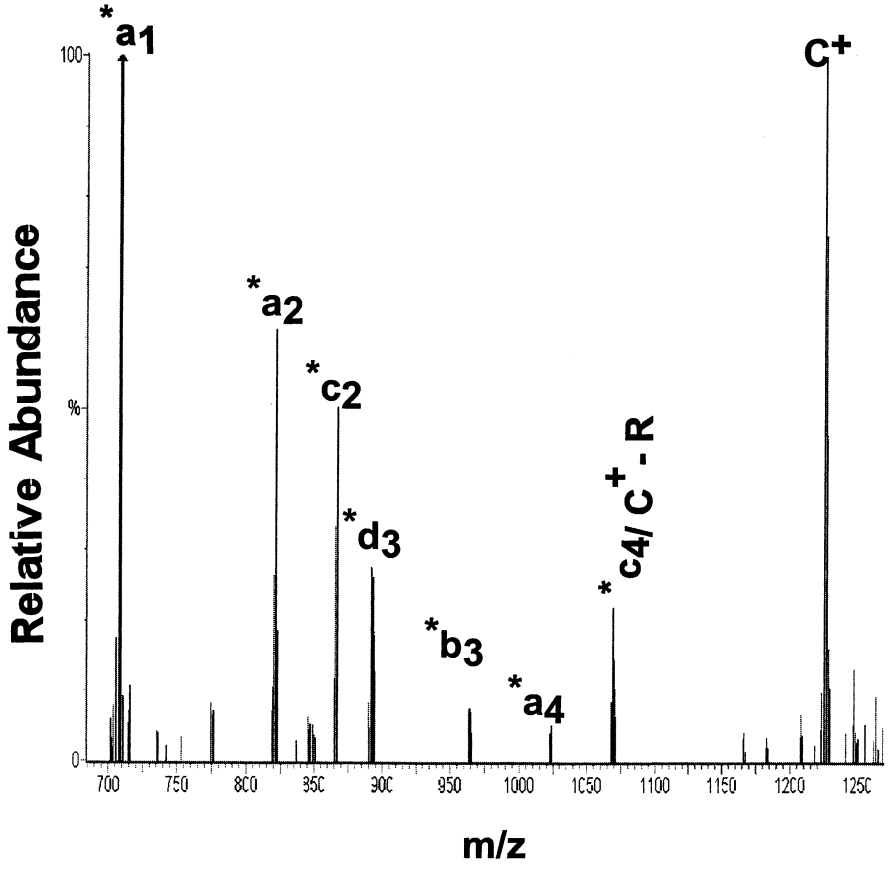

Figure 9. ESI-ISF mass spectrum of TMPP-Ac-YLDSR, residues 13–17 of Glucagon.

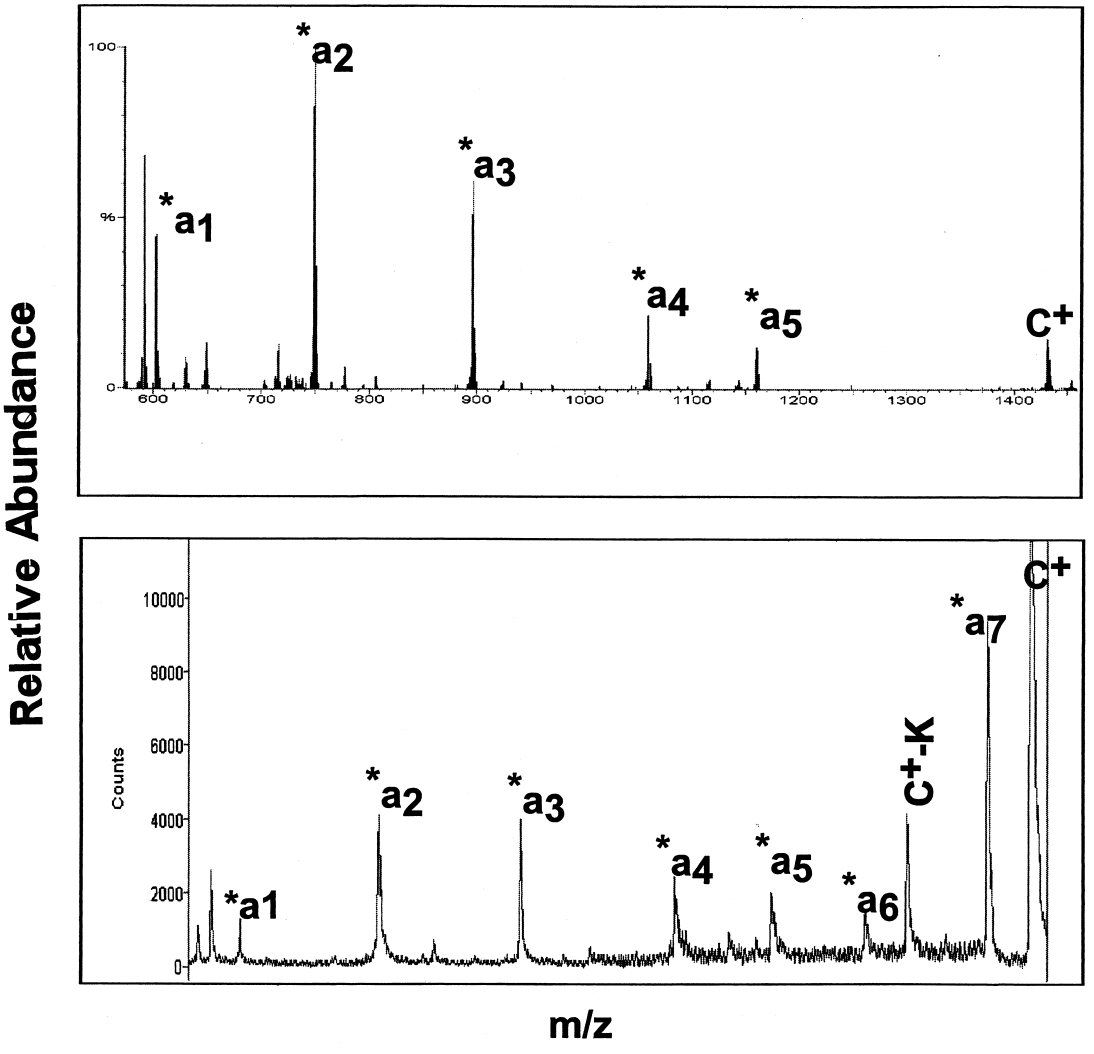

Figure 10. Comparison of ESI-ISF vs. MALDI-PSD for TMPP-Ac-GFFYTPK (insulin chain − B, residues 23–29).

and 2 cleavage sites) was digested and the resulting fragments derivatized with the TMPP-Ac reagent. The ESI-ISF and MALDI-PSD spectra of one of the derivatized fragments (residues 23–29) are shown in Figure 10. Again, a series of $*\mathbf{a}_n$ ions is observed for the first five residues in the top panel. Residue 6 is proline, and the signal due to $*\mathbf{a}_6$ is not discernible; this, again, is consistent with our earlier observations. The ESI-ISF spectrum in Figure 10 compares well with that of the MALDI-PSD spectrum of the same derivatized peptide, in that $*\mathbf{a}_n$ ions are observed in each. The difference is that $*\mathbf{a}_6$, the ion due to cleavage at the proline residue, is observable in MALDI-PSD, as is a signal due to fragmentation of the terminal lysine ($*\mathbf{a}_7$). Previous studies have shown that the signal due to cleavage at proline in MALDI-PSD is laser power dependent [7].

Although analysis of enzymatic digests of proteins by LC-MS/MS is becoming routine, preliminary TMPP-Ac derivatization would help detect the smaller peptide fragments, resulting in better coverage. In addition, the relatively simple fragmentation pattern of the TMPP-Ac-peptides, even under low-energy CAD, would help one to deduce the amino acid sequence without the aid of extensive computation.

Conclusions

This ESI-ISF/CAD-MS study of the TMPP-Ac derivatives of peptides shows that a series of $*\mathbf{a}_n$ ions is consistently produced. The fact that $*\mathbf{a}_n$ ions are observed suggests that the fragmentation is likely via charge-remote pathways. Our results from ESI-CAD-MS/MS are also similar to those observed in FAB-CAD-MS/MS and MALDI-PSD-MS for TMPP-Ac-peptides. Comparison of the results of the ESI-ISF-MS and the MS/MS study of the TMPP-Ac derivatives shows that precursor-selected CAD-MS/MS is similar to ISF-MS. Fragmentation of these charged derivatives differs in an ion trap depending on whether a single- or double-

charged precursor is selected; charge-mediated processes also seem to be present in the latter case.

The results from analyses of derivatized protein digests indicate that the behavior of individual TMPP-Ac derivatives is maintained even in the case of complicated mixtures such as those derivatized from protein digests. Sequence information is readily obtainable based on the predominance of a simple series of $^{*}\mathbf{a}_{n}$ ions.

Acknowledgments

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