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Published on Web 04/14/2007

Neutral Fragment Mass Spectra via Ambient Thermal Dissociation of Peptide and Protein Ions

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Abstract: A novel method for the fragmentation of peptide and protein ions at atmospheric pressure outside the mass spectrometer is described. Peptide/protein ions generated by electrosonic spray ionization (ESSI) are carried through a heated coiled metal tube where they fragment. Fragment ions of types *a*, *b*, and *y* are observed for peptides such as angiotensin II and bradykinin. In the case of phosphopeptides, informative *b* and *y* ions which preserve the labile phosphate groups are observed in the negative ion mode, which is potentially useful in the location of phosphorylation sites in proteins through chemical analysis of phosphopeptides. The thermal dissociation method extends to proteins such as ubiquitin and myoglobin, giving rise to *y*-type and other fragment ions. The most important feature of this method is that it also allows characterization of the neutral fragments arising from thermal dissociation by use of on-line corona discharge ionization. This neutral re-ionization experiment is much easier to perform outside the mass spectrometer than as conventionally done, in vacuum. It yields increased structural information from the resulting mass spectra in both the positive and the negative ion modes.

Introduction

Activation and dissociation of gas-phase ions forms the basis for tandem mass spectrometry and hence for its applications in ion structural characterization and chemical analysis. The fragmentation of peptide/protein ions is a central topic in proteomics and there is strong interest in novel dissociation methods such as electron capture dissociation[1] and electron-transfer dissociation.[2] These new methods can provide structural information that complements, and often exceeds, that obtained by traditional collisional activation. Ionic dissociation, both by the traditional and newer methods is implemented in vacuum rather than at atmospheric pressure. The latter experiment, which is discussed here, is challenging because rapid thermalization of activated ions occurs via third-body collisions and because it is not readily adaptable to dissociation of mass-selected ions.

Ion thermal dissociation has been known for some years. An investigation of the thermal fragmentation of gaseous carbenium ions, such as $C_7H_{15}^+$, in a pulsed high-pressure mass spectrometer (0.5−3.0 Torr pressure) was reported by Meot-Ner and Field.[3] Subsequently, the pyrolysis of protonated ether ions was examined under similar conditions.[4] Thermally activated isomerization of gaseous cyclohexylium ions generated by radiolysis over a wide pressure range (106−1480 Torr) was studied in detail by Cacace.[5] McLuckey and co-workers have examined the dissociation of larger ions (e.g., bradykinin ions) trapped in a quadrupole ion trap, under collisions with a hot-bath gas at 1 m Torr helium.[6] This and similar studies[7] were used to distinguish "slow heating" activation and dissociation, which occurs under equilibrium conditions, from nonequilibrium dissociation. Thermal activation is the key aspect of the blackbody infrared dissociation (BIRD) method[8] and it has been used effectively to measure true thermodynamic quantities using gas-phase ion reactions, even those reactions carried out at very low pressure.[8d] Thermally induced dissociation of ions at the atmosphere/vacuum interfaces of mass spectrometers under near atmospheric pressures was introduced in the early 1990s by Smith and co-workers.[9] In these experiments, ion fragmentation was induced while the ions passed through a resistively heated capillary interface after formation by electrospray ionization (ESI). The experiment has found applications in the dissociation of multiply charged protein/peptide ions[9,10] and noncovalently bound complex ions[11] as well as in the study of the activation energy requirements for ion dissociation.[9a,10]

Building on this earlier work, we report a novel thermal dissociation method for peptide/protein ions which operates

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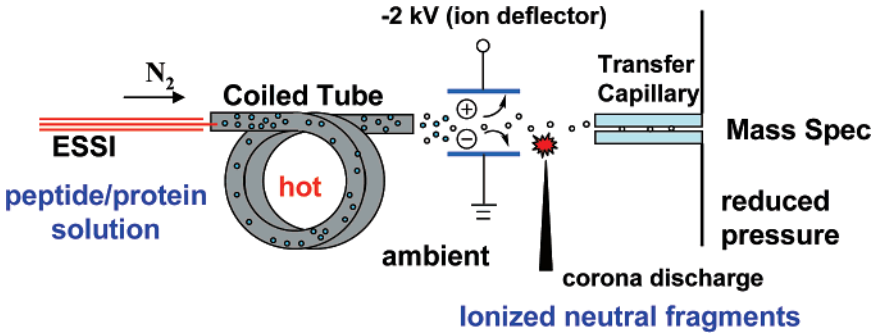
Scheme 1. Experimental Apparatus for Recording Mass Spectrum of Ionized Neutral Fragments after Thermal Dissociation of Peptide/Protein Ions



outside the mass spectrometer at atmospheric pressure. In preliminary experiments to test the feasibility of thermal dissociation at atmospheric pressure, we passed a nitrogen gas stream bearing gaseous peptide ions generated by electrosonic spray ionization (ESSI, a soft ionization method[12]) through a straight heated tube in the ambient environment, but failed to see a significant yield of fragment ions. To improve the dissociation yield, we coiled the tube to enhance turbulence and so increase precursor ion wall collisions and ion residence times. Extensive fragmentation was observed with the coiled heater. More significantly, atmospheric pressure ion dissociation allows a new experiment in which mass spectra are recorded on the neutral fragments arising from ionic dissociation. This neutral re-ionization experiment is much easier to perform outside the mass spectrometer than in vacuum and provides increased structural information.

Experimental Section

The apparatus used for ion thermal dissociation at atmospheric pressure (refer to Scheme 1) consisted of an ESSI ion source, a stainless steel tube (3.2 mm o.d., 1.6 mm i. d., length 22 cm) coiled to form two loops with a diameter of ca. 2.5 cm, and a LTQ mass spectrometer (Thermo Fisher, San Jose, CA) fitted with an atmospheric pressure interface including a heated transfer capillary. The coiled tube was wrapped with heating tape and placed between the ion source and the transfer capillary of the mass spectrometer, and the distance between the tube and the transfer capillary was varied over the range 1 mm to 4 cm. Higher signal intensity was observed at smaller distances, probably because more ions from the coiled tube outlet were collected into the MS inlet. The ESSI ion source silicon capillary tip was inserted into the coiled tube (i.e., the ions and/or microdroplets generated in the ESSI process were directly sprayed into the tube) to maximize the signal. This did not have any effect on the vacuum system of the mass spectrometer because the outlet of the coiled tube was also in air. The tube temperature was controlled by adjusting the voltage applied to the heating tape, and the temperature of the outside wall of the tube (i.e., the tape temperature) was monitored by an Omega thermocouple sensor. Temperatures used ranged from 230 to 380 °C, as indicated below. The resulting thermally generated fragment ions emerging from the tube were monitored using the LTQ mass spectrometer. The temperature of the LTQ transfer capillary was kept at 150 °C throughout the experiments. Sample solutions used were typically 0.1∼2.0 mg/mL of peptide/proteins in a solvent consisting of 50:50 methanol/water containing 0.1∼1% acetic acid; no acid was added in the case of the phosphopeptide solutions since they were examined in the negative ion mode. The sample solutions were sprayed using the high nebulizer gas flow rates typical of ESSI experiments (optimized nebulizer gas pressure: 175 psi), and the sample injection rate for ESSI was fixed at 10 μL/min for all experiments.

In the neutral fragment re-ionization experiments, a corona discharge needle was placed between the coiled tube heater and the transfer capillary of the LTQ (Scheme 1). To remove all fragment ions being carried in the gas stream and emerging from the tube prior to the neutral fragment ionization step, a pair of parallel metal plates served as an "ion switch".[13] One plate was grounded while the other was supplied with a potential bias to deflect ions; it was found that a −2 kV bias served to completely remove the signal of the ionic species as monitored by the LTQ mass spectrometer. After removal of all ions, a mass spectrum was recorded with a +2.6 kV or −2.6 kV voltage (depending on whether the positive or negative ionization mode was chosen) applied to the needle while the gas stream passed through the ion switch.

Results and Discussion

Figure 1 panels a and b show the atmospheric pressure thermal activation mass spectra of angiotensin II and bradykinin; various *a*, *b*, and *y* ions from peptide backbone cleavage as well as fragment ions due to loss of water and ammonia are observed. This observation is similar to the ion dissociation behavior in conventional collision-induced dissociation (CID) or the so-called "slow heating" techniques like sustained off-resonance irradiation collision-induced dissociation (SORI-CID).[14] Interestingly, unique dissociation behavior which is different from CID is also observed, as exemplified by the base peak at *m*/*z* 971 in the thermal dissociation spectrum of bradykinin (Figure 1b). This fragment ion (*m*/*z* 971) is the result of side-chain cleavage; however, it was not observed in the product ion spectra of the singly, doubly, or triply charged bradykinin ions under normal CID conditions (Figure 2). Mechanistically, singly protonated bradykinin (*m*/*z* 1061) dissociates into *m*/*z* 1031 (loss of HCHO from the side chain of the serine residue, a well-known pyrolysis process[15] in solution); subsequent water loss yields *m*/*z* 1013 and is followed by side-chain cleavage (loss of NH═C═NH from an arginine residue) to *m*/*z* 971. The reaction sequence is supported by the fact that *m*/*z* 971 was observed as the base peak in the product ion CID spectrum of *m*/*z* 1013. The unique dissociation behavior might be due to the possibility that in our experiments activation of peptide ions during thermal dissociation at atmospheric pressure commences

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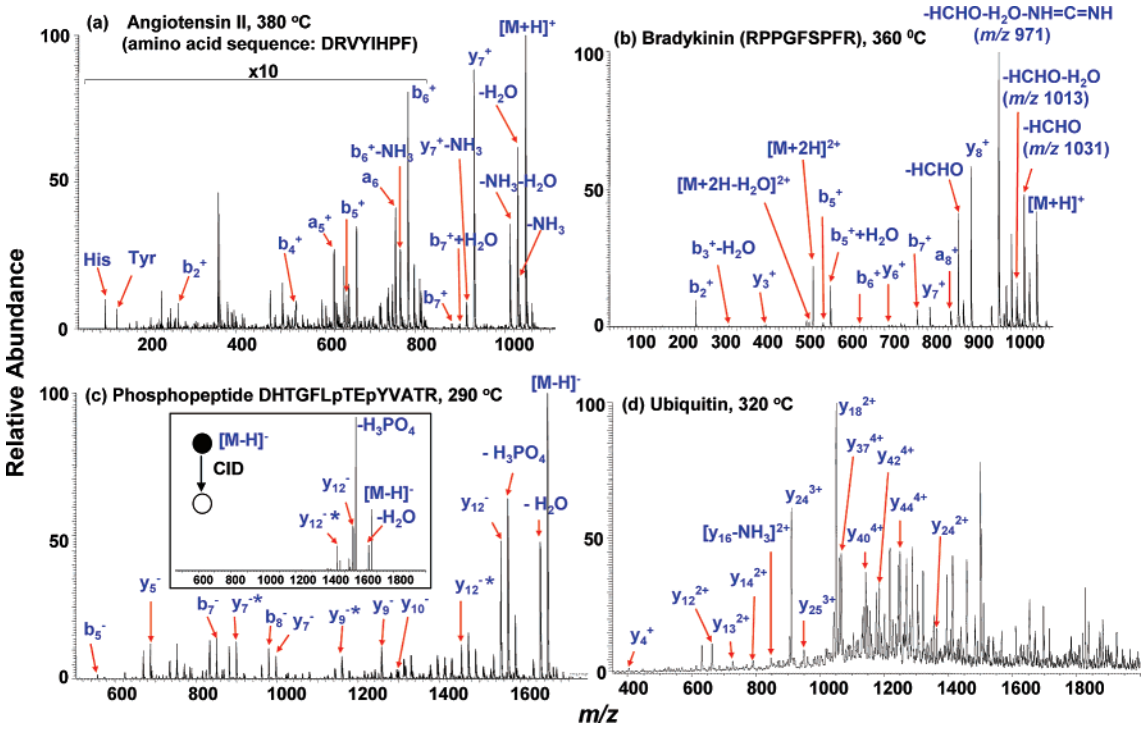

Figure 1. Mass spectra showing atmospheric pressure thermal dissociation of (a) angiotensin II at 380 °C, (b) bradykinin at 360 °C, (c) phosphopeptide DHTGFLpTEpYVATR at 290 °C (*y* ions with loss of one phosphate group are labeled with an asterisk), and (d) ubiquitin at 320 °C.

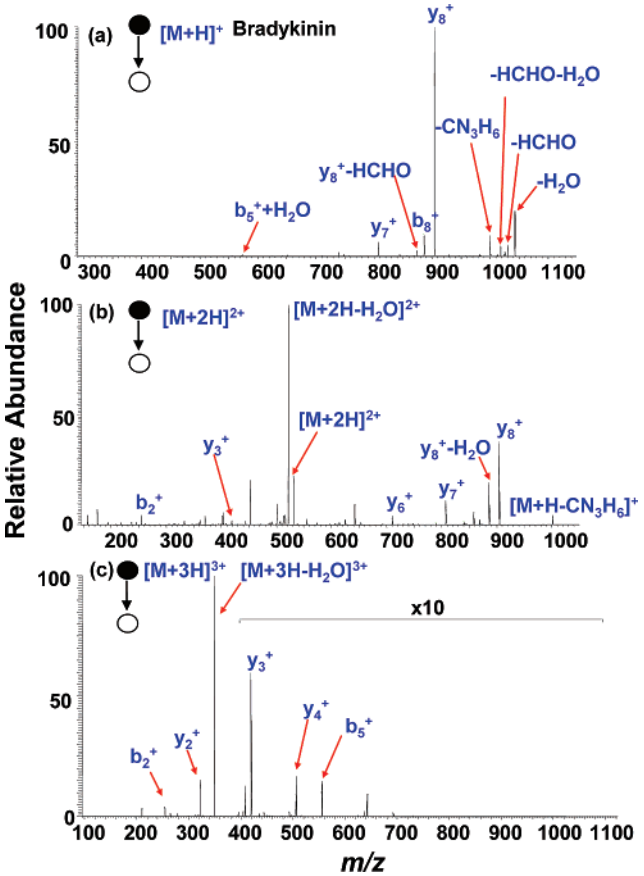

Figure 2. CID mass spectra of (a) singly (*m*/*z* 1061), (b) doubly (*m*/*z* 531), and (c) triply (*m*/*z* 354) charged bradykinin (RPPGFSPFR) ions at collision energy of 25%. Note the fragment ion of *m*/*z* 971 in the thermal dissociation of bradykinin is missing in the CID spectra.

in the fine ESSI droplets entering the coiled heating tube, rather than occurring only in the free gas phase without solvent effects. Thus it might be the result of the combined effects of both thermal activation and numerous collisions of either naked ions or microsolvated ions in the gas stream. In a separate experiment, simply heating the peptide angiotensin I in methanol/water solution under reflux conditions under atmospheric pressure for over an hour did not produce any fragments in solution as determined by ESSI of the solution before and after heating. (See experimental details given in the Supporting Information). This result suggests that the dissociation mechanism in the present experiments is not a simple solution-phase process but rather that gaseous collisions (not excluding ion/surface collisions) play the major role in ion dissociation. Note, however, that the present study is not aimed at elucidation of mechanisms and this issue will be taken up subsequently. The observation that a much higher fragmentation yield was observed using a coiled tube compared to a straight tube under the same experimental conditions (tube temperature and length) suggests that the turbulent gas flow in the coiled tube increases the residence time of the peptide precursor ions. This is consistent with the fact that few wall collisions are expected in the straight tube arrangement at the near-supersonic velocities achieved in ESSI;[12] a residence time of about 1 ms is calculated for a carries gas with a velocity of 300 m/s.

Protein phosphorylation is among of the most widely studied post-translational modifications and it plays key regulatory roles in cellular processes.[16] It has been found that atmospheric pressure thermal dissociation is useful for the determination of phosphorylation sites in peptides, since the negative ion spectra produce many *b* and *y* ions with preservation of labile phosphate groups (e.g., the ions b_7^-, b_8^-, y_5^-, y_7^-, y_9^-, y_{10}^-, and y_{12}^- in the example shown in Figure 1c). This information is not available using CID (the inset of Figure 1c shows that few fragment ions are formed upon CID of the singly charged

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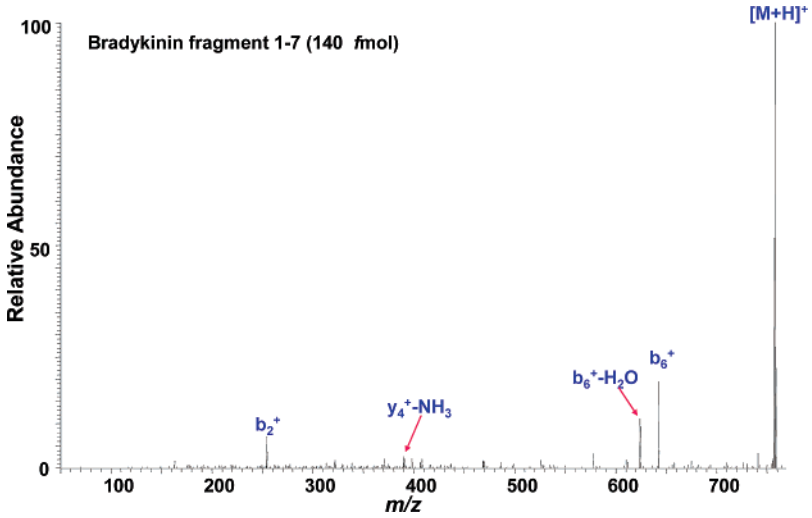

Figure 3. Atmospheric pressure thermal dissociation mass spectrum of 1 μg/mL of peptide bradykinin fragment 1−7 (**RPPGFSP**, MW 756) at 310 °C (background subtracted; the injected amount for acquiring this spectrum was 140 *f*mol of bradykinin fragment 1−7).

deprotonated phosphopeptide ion $[M-H]^-$). In another example, the insulin receptor-derived peptide TRDIpYETDpYYRK, upon thermal dissociation at 230 °C, yields the fragment ions b_6^-, b_8^-, y_4^-, y_5^-, y_6^-, and y_9^- which retain phosphate groups (see spectrum shown in Figure 1S, Supporting Information). This feature of thermal dissociation is attractive because phosphopeptides in mixtures often have greater ionization efficiencies in the negative ion mode than in the positive ion mode owing to the reduction in isoelectric point arising from phosphorylation.[17]

Coiled-tube atmospheric pressure thermal dissociation extends to proteins. In the case of ubiquitin, which is known to undergo almost no nozzle-skimmer dissociation (ascribed to rapid folding of the nascent gaseous ion),[18] a variety of *y* ions is generated by simple thermal dissociation (Figure 1d). Among them, y_{18}, y_{24}, y_{37}, and y_{44} ions arise from cleavage C-terminal to aspartic acid residues, a characteristic dissociation feature of protein ions observed previously under CID in ion traps.[19] While the rich protein spectrum shown in Figure 1d remains to be investigated in detail, the capability of dissociating protein ions is promising for the possible extension of this method to "top down" protein sequencing.[20]

In another example of atmospheric pressure thermal dissociation, myoglobin was ionized by ESSI and heated to 320 °C. Among the *y* ions generated, y_{34}^{4+} and y_{34}^{5+} originate from cleavage at the 119/120 (His/Pro) residue, a facile fragmentation pathway owing to the presence of proline.[21] An additional feature of atmospheric pressure thermal dissociation, besides the simplicity and speed of implementation of the method, is its sensitivity. This enhances its potential value in analytical applications. Characteristic dissociations of one peptide, bradykinin fragment 1−7, are observed using samples as small as 140 *f*mol (Figure 3). Because ion-selection at atmospheric pressure is challenging, the application of this thermal method to the analysis of peptide/protein mixtures may be best implemented in conjunction with adequate separation of either peptide/protein molecules using chromatography or of the ionized peptide/protein ions using ion mobility spectrometry.

Because thermal fragmentation proceeds outside the mass spectrometer in these experiments, examination of *neutral fragments* emerging from the coiled tube is straightforward. Using conventional dissociation methods in vacuum, specialized tandem mass spectrometers are required to perform analogous collisional re-ionization experiments.[22] In the ambient dissociation experiments, ionization of the neutral fragments in the gas flow emerging from the hot coiled tube simply uses on-line corona discharge ionization (Scheme 1). To avoid interference from the ions already present in the gas flow, an "ion switch"[13] is used to deflect these ionic fragments. A proof-of-principle experiment using the tetrapeptide Met-Arg-Phe-Ala (MRFA) was first carried out. Peptide Met-Arg-Phe-Ala (MRFA, MW 523) was chosen for this initial thermal dissociation/neutral re-ionization experiment because it yields a fragment ion b_2^+ (*m*/*z* 288) by loss of the neutral fragment Phe-Ala (MW 236) while Phe-Ala is not seen as the protonated ion [Phe-Ala+H^+] (*m*/*z* 237, i.e., the y_2 ion) in either the thermal dissociation mass spectrum (Figure 4a) or the ESSI mass spectrum (not shown) of this peptide. In the neutral fragment ionization experiment (i.e., on-line corona-discharge ionization of the gas stream emerging from the thermal dissociation coiled tube after removal of ionic species in the gas stream using the ion switch), the neutral fragment, Phe-Ala, was ionized via protonation using corona discharge (presumably ionized water vapor serves as the immediate ionizing agent) and the resulting ion $[Phe\text{-}Ala+H]^+$ appeared at *m*/*z* 237 (Figure 4b). CID of this ion $[Phe\text{-}Ala+H]^+$ (*m*/*z* 237, Figure 4c) gave rise to *m*/*z* 120 (a_1^+ ion of Phe-Ala), *m*/*z* 191 (by loss of CO and H_2O), and *m*/*z* 219 (by loss of H_2O), consistent with its structure. This assignment was confirmed by the identity of the CID spectrum shown in Figure 4c with that obtained from the ion $[Phe\text{-}Ala+H]^+$ (*m*/*z* 237, Figure 4d) generated directly by ESSI ionization of an authentic

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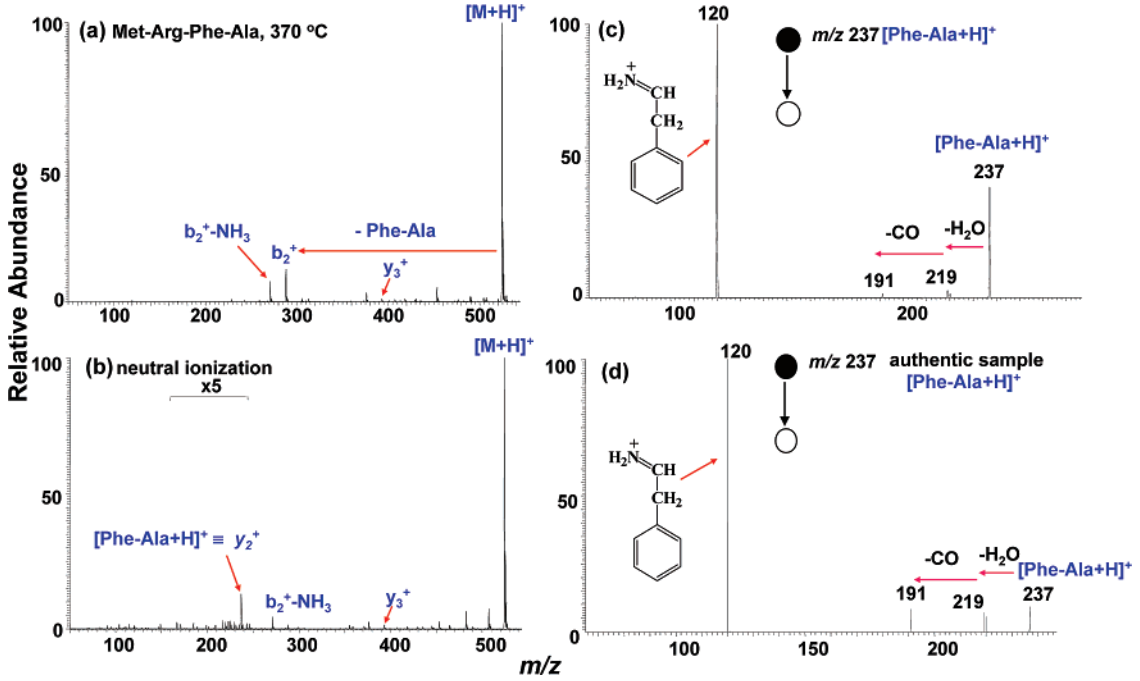

Figure 4. (a) Atmospheric pressure thermal dissociation mass spectrum of Met-Arg-Phe-Ala at 370 °C; (b) mass spectrum showing ionization of the neutral species emerging from the heated coiled tube. The protonated ion $[Phe\text{-}Ala+H]^+$ (*m*/*z* 237) arises from the ionization of the neutral fragment Pha-Ala; (c) CID of $[Phe\text{-}Ala+H]^+$ (*m*/*z* 237) generated by ionization of the neutral fragment; (d) CID of the ion $[Phe\text{-}Ala+H]^+$ generated from ESSI of the authentic dipeptide Phe-Ala. Comparison of panels c and d confirms the structure of the neutral fragment Phe-Ala generated from thermal dissociation of Met-Arg-Phe-Ala.

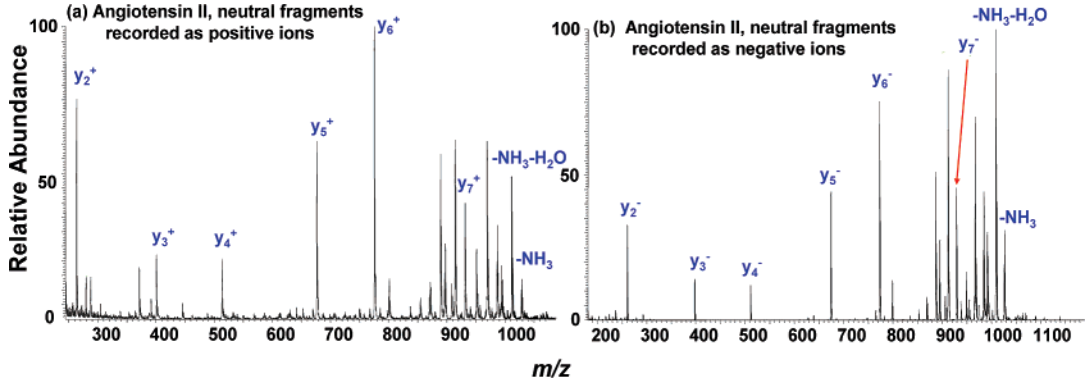

Figure 5. Mass spectra recorded by ionizing the neutral fragment generated from angiotensin II by thermal dissociation showing (a) positive and (b) negative ionization modes.

sample of the dipeptide Phe-Ala. This experiment confirms that the neutral fragment generated by thermal dissociation can be ionized by corona discharge and seen as the corresponding protonated molecule in the mass spectrum.

Angiotensin II has also been examined in this neutral fragment ionization experiment. Figure 5 panels a and b display positive and negative ion mass spectra obtained upon ionization of the neutral fragments generated from thermal dissociation of angiotensin II, respectively. The formation of a series of positively charged *y* ions (y_2^+ to y_7^+ in Figure 5a) as well as a series of negatively charged *y* ions (y_2^- to y_7^- in Figure 5b), points to the presence of the corresponding neutrals (i.e., deprotonated forms of *y* ions) emerging from the hot coiled tube. Note that the positively charged *y* ions are 2 Da higher in mass than the corresponding negatively charged *y* ions (See systematic description of structures and relationships between neutral and ionic fragments in Supporting Information). The assignment of these as *y* ions was confirmed by CID experiments. For instance, the y_2^+ ion dissociates to $[Phe+H]^+$ and it also loses H_2O and CO upon CID, in agreement with the CID behavior of $[Pro\text{-}Phe+H]^+$ formed by ESSI of the authentic peptide Pro-Phe. These *y* ions disappeared when the tube temperature was lowered to the point where thermal fragmentation was negligible, confirming the origin of the neutrals as thermal dissociation products. It is very likely that thermal dissociation of angiotensin II involves the ion rather than its neutral form since the peptide (isoelectric value 6.7[23]) would carry net charges in the initial sample solution (1% acetic acid, pH ≈ 3) prior to spray ionization and thermal dissociation. The fact that neutrals arise from ion dissociation is also supported by the formation of complementary product ions b_2^+, b_4^+, b_5^+, and b_6^+ (but not b_1^+ and b_3^+) (see Figure 1a). Note that y_7^+ and other peaks (e.g., those due to NH_3 and $[H_2O+NH_3]$ losses) in Figure 3a are also present in Figure 1a, suggesting that these species emerge from the coiled tube in both the ionic and neutral forms. Clearly, given the high fragment coverage (i.e., the large set of

(23) Prowl. http://prowl.rockefeller.edu/prowl-cgi/sequence.exe, Oct 31, 2006.

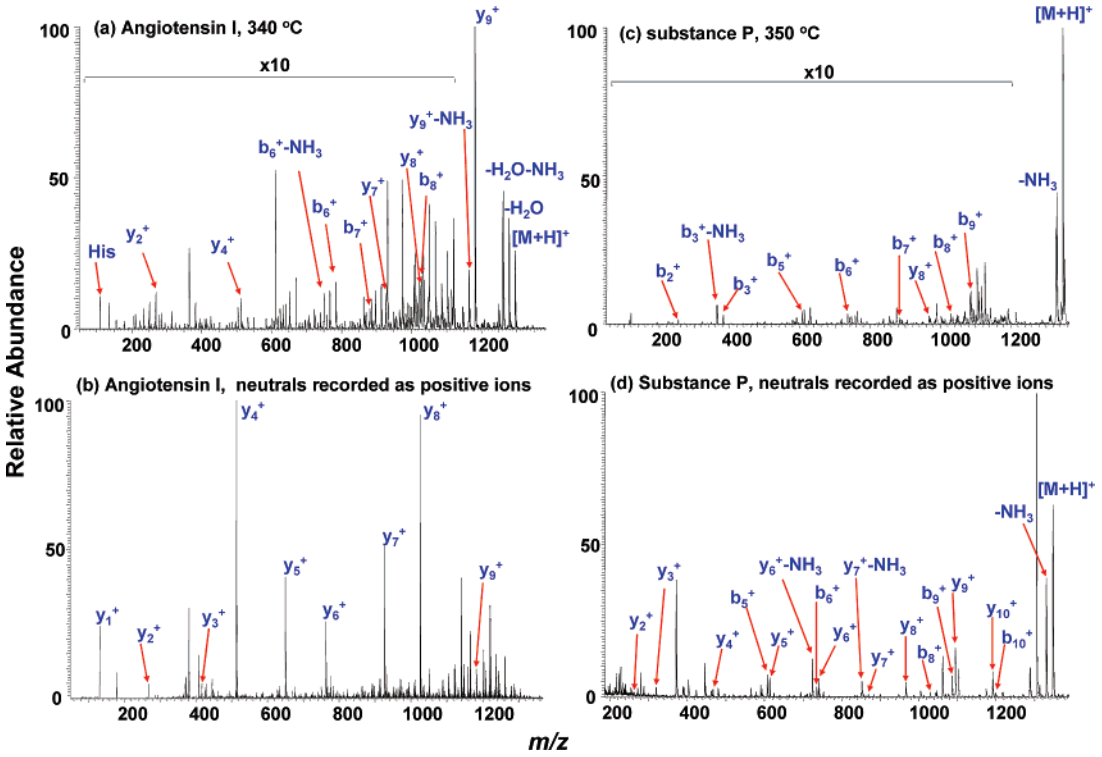

Figure 6. (a) Atmospheric pressure thermal dissociation mass spectrum of angiotensin I (DRVYIHPFHL) at 340 °C; (b) mass spectrum in the positive ion mode showing ionization of the neutral species emerging from the coiled tube (background subtracted); (c) atmospheric pressure thermal dissociation mass spectrum of substance P (RPKPQQFFGLM) at 350 °C; (d) mass spectrum in the positive ion mode showing ionization of the neutral species emerging from the coiled tube.

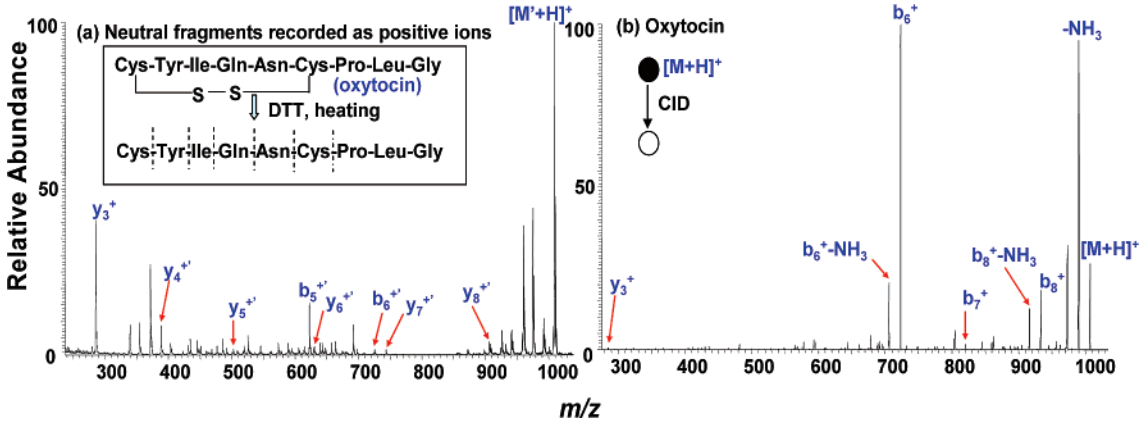

Figure 7. (a) Mass spectrum in the positive ion mode showing ionization of the neutral species arising from thermal dissociation of oxytocin at 360 °C in the presence of disulfide bond reducing reagent 1,4-dithio-DL-threitol (DTT). The concentration of DTT in the sample solution of oxytocin (1.0 mg/mL) used for ESSI prior to thermal dissociation was 3.7 mg/mL. Note that the label prime "′" indicates the reduced form. The inset shows the structure of oxytocin. (b) CID MS^2 spectrum of the protonated native oxytocin.

y ions), neutral fragment mass spectra provide additional structural information not given by the directly formed ionic fragments. Neutral fragment mass spectra were recorded for other peptides, including angiotensin I (DRVYIHPFHL) and substance P (RPKPQQFFGLM) (see mass spectra shown in Figure 6). Increased structural information (mainly based on the observed series of *y* ions) was obtained as summarized in Table 1.

Interestingly, in the case of disulfide bond-containing peptide oxytocin, with addition of the reducing reagent 1,4-thio-DL-threitol (DTT) to the peptide solution, the neutral fragment mass spectrum recorded after thermal dissociation shows the formation of characteristic fragment ions $b_5^{+\prime}$, $b_6^{+\prime}$, $y_4^{+\prime}$, $y_5^{+\prime}$, $y_6^{+\prime}$, $y_7^{+\prime}$ and $y_8^{+\prime}$ in the reduced forms (Figure 7a). By contrast, the

Table 1. Summary of *b* and *y* Ions Generated from Direct Thermal Dissociation and Neutral Fragment Re-ionization

peptides	direct thermal dissociation in the positive ion mode	neutral fragment re-ionization in the positive ion mode
angiotensin I	b_6^+, b_7^+, b_8^+, y_2^+, y_4^+, y_7^+, y_8^+, y_9^+	y_1^+, y_2^+, y_3^+, y_4^+, y_5^+, y_6^+, y_7^+, y_8^+, y_9^+
angiotensin II	b_2^+, b_4^+, b_5^+, b_6^+, b_7^+, y_7^+	y_2^+, y_3^+, y_4^+, y_5^+, y_6^+, y_7^+
substance P	b_2^+, b_3^+, b_5^+, b_6^+, b_7^+, b_8^+, b_9^+, y_8^+	b_5^+, b_6^+, b_8^+, b_9^+, b_{10}^+, y_2^+, y_3^+, y_4^+, y_5^+, y_6^+, y_7^+, y_8^+, y_9^+, y_{10}^+

CID of the protonated native oxytocin ion gives rise to the fragment ions b_6^+, b_7^+, and b_8^+ containing intact disulfide bond (Figure 7b). It can be seen that neutral fragment ionization

coupled with disulfide bond reduction provides quite different sets of fragment ions and thus complementary structural information.

In conclusion, the thermal ion dissociation method outside the mass spectrometer provides useful sequence information from both ionic and neutral fragments via direct thermal dissociation and from neutral fragment re-ionization. The experiment described here is easy to implement and should find application in proteomics.

Acknowledgment. This work was supported by the National Science Foundation (Grant CHE04-12782).

Supporting Information Available: Fragment ion nomenclature and supporting spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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