Mass Spectrometry

DOI: 10.1002/anie.200803477

Peptide Fragmentation Assisted by Surfaces Treated with a Low-Temperature Plasma in NanoESI**

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Herein, we discuss the mechanistic implications of peptide fragmentation observed under special conditions in nanoelectrospray ionization (nanoESI), a widely used soft ionization method. The development of electrospray ionization (ESI) has significantly enlarged the scope of mass spectrometry (MS).[1] The area most favorably impacted is peptide and protein analysis. The mass of a protein or peptide can be determined in a straightforward manner from the abundant multiply protonated or deprotonated forms of the molecule that are observed in the positive- or negative-ion mode of ESI. The characterization of the primary structure of a peptide or protein, now one of the central activities in proteomics,[2] is possible when ESI is coupled with any of a number of activation methods in the context of tandem mass spectrometry.[3,4]

Besides the wide adoption of ESIMS for biochemical analysis, there is a strong continuing interest in understanding the fundamental aspects of ESI. Although debate continues as to whether the final stages of ion formation can be explained by the charge-residue[5] or ion-evaporation model[6] for certain types of molecules, it is widely accepted that ESI is a soft ionization method, and that the ions formed are not excited significantly under typical conditions.[7] As ion internal energies are highly dependent upon the environment in which they are formed, even a "soft" ionization process can be tuned to give rise to excited ions under appropriate conditions. Examples include the use of reagents for controlled ionization and fragmentation in chemical ionization[8] and the selection of either "cold" or "hot" matrices[9] in matrix-assisted laser desorption/ionization (MALDI). Herein, we report the observation of peptide fragmentation in nanoESI after pretreatment of the nanoESI emitter with a helium low-temperature plasma (LTP) at atmospheric pressure. We studied this new fragmentation phenomenon for a range of standard peptides, including phosphopeptides, for which retention of the modification was observed, and investigated the associated mechanism.

The experimental arrangement is shown in Figure 1. A T-shaped glass tube was placed in line with the heated capillary inlet of an LTQ mass spectrometer. A nanoESI emitter[10] was positioned inside the glass tube by inserting it through a rubber stopper from the back end of the tube with the emitter tip 2 cm from the mass-spectrometer inlet. Helium discharge gas was introduced from the bottom arm into the glass tube at a low flow rate (5 mL min^{-1}). A low-temperature plasma was generated inside the glass tube by using a dielectric barrier discharge (DBD);[11] in this experiment, an alternating current (ac, 30 kHz, 3 kV_{p-p}) was applied to two electrodes attached to the outer wall of the glass tube. The LTP was turned on for 2 min with the nanoESI emitter inside the tube but not operating. After the plasma had been turned off, a high voltage (HV, 1–2 kV) was applied to the nanoESI emitter containing the peptide solution, and the mass spectrum was recorded.

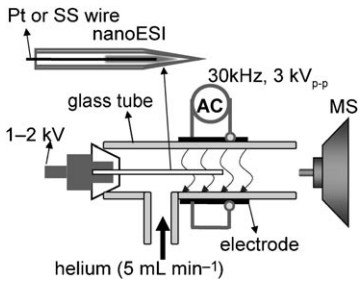

Figure 1. The experimental setup, which consists of a T-shaped glass tube containing a nanoESI emitter. A helium low-temperature plasma is generated inside the tube at atmospheric pressure. Ions are produced by nanoESI and analyzed by MS after the discharge is turned off. SS = stainless steel.

Figure 2 compares the nanoESI mass spectra recorded for bradykinin 2–9 (single-letter sequence: PPGFSPFR) before and immediately after a plasma-discharge period of 2 min. The mass spectrum before discharge (Figure 2a) is a typical nanoESI mass spectrum for this peptide: No obvious fragment ions are observed, and the protonated ($[M+2H]^{2+}$, $[M+H]^{+}$) and sodium-adduct ions ($[M+H+Na]^{2+}$, $[M+Na]^{+}$) are the major ionic species. In contrast, a series of amide-backbone fragment ions ($\mathbf{y_2}^{+}$–$\mathbf{y_7}^{+}$) characterizes the spectrum recorded after turning the plasma on for 2 min and then off again (Figure 2b). The identities of the fragment ions were verified by examining the ion-trap collision-induced dissociation (CID) data. Besides the fragment ions, small amounts of

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[**] This research was supported by the US National Science Foundation (CHE04-12782). We thank Arlene Rothwell and Karl Wood of the Campus-Wide Mass Spectrometry Center at Purdue University for ICPMS analysis.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200803477.

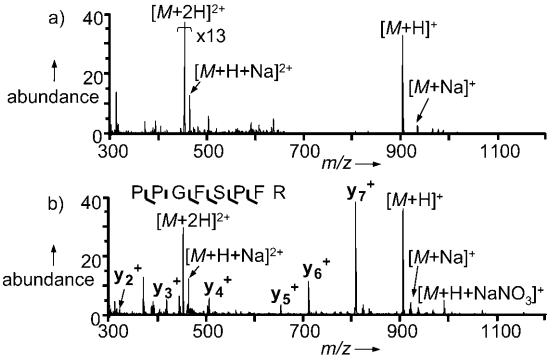

Figure 2. Positive-mode nanoESI mass spectra recorded for bradykinin 2–9 (PPGFSPFR; 20 μM in MeOH/H_2O/HOAc (50:49:1, v/v/v)) a) before generation of the plasma and b) immediately after exposure of the emitter to the plasma for 2 min.

adduct ions with masses 63 Da greater than those of the protonated or sodiated peptide ions were also observed after operating the plasma. These adduct ions might be formed by clustering with plasma-generated HNO_3 (formed from nitrogen oxidation during discharges in air).[12] Peptide fragmentation was also observed when nanoESI was conducted with the plasma turned on. However, as a result of the strong interference of ions produced in the LTP with peptide ions, all nanoESI mass spectra reported were recorded with the LTP turned off.

The nanoESI of more than 30 peptides with different sequences and various sizes (5–26 amino acid residues) was studied with LTP-treated surfaces. For most of the peptides, a-, b-, and y-type fragment ions due to amide-bond cleavage were observed (see Table 1S in the Supporting Information). The fragmentation patterns observed after LTP treatment, however, differed markedly from the CID data recorded for any of the protonated or sodiated molecular ions. For example, Figure 3 shows the nanoESI data collected for a phosphopeptide (TRDIYETDYpYRK) after plasma treatment. A rich spectrum of fragments is present, including a consecutive series of y ions ($\mathbf{y_3}^+$–$\mathbf{y_{11}}^+$) and several a-type ions. Most notably, there is little loss of the labile phosphate groups

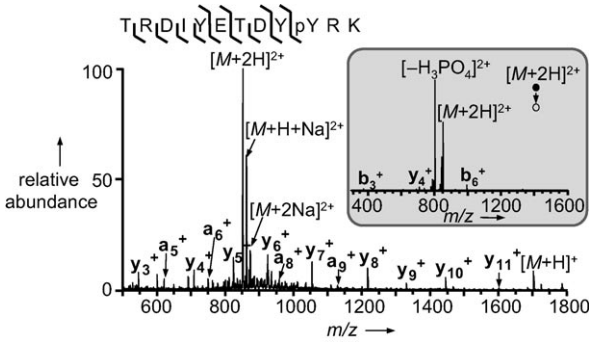

Figure 3. Mass spectrum derived from positive nanoESI of a phosphopeptide (TRDIYETDYpYRK; 10 μM in MeOH/H_2O/HOAc (50:49:1, v/v/v)) immediately after LTP operation for 2 min. The inset shows data acquired from conventional ion-trap CID of the $[M+2H]^{2+}$ peptide ions.

either from the molecular ion or from the fragments that contain the phosphorylation group. The extensive backbone fragmentation that occurs after plasma operation, together with little loss of phosphorylation, makes it quite straightforward to pinpoint the phosphorylation site on Tyr[10]. Note that there are five amino acid residues in this peptide that could be modified (Thr[1], Tyr[5], Thr[7], Tyr[9], and Tyr[10]). For comparison, conventional ion-trap CID data for the doubly protonated peptide ions are shown in the inset of Figure 3. The base peak corresponds to the neutral loss of phosphoric acid (loss of 98 Da), and a limited number of backbone fragments are observed at low relative abundances. For several phosphopeptides with phosphorylation at either Ser, Thr, or Tyr, extensive backbone fragmentation is generally observed with no or very little loss of phosphoric acid (see Table 1S in the Supporting Information).

We investigated possible mechanisms that could account for the fragmentation observed after plasma treatment of the surfaces. We found that the discharge surface of the glass tube was not involved directly in inducing peptide fragmentation, as fragmentation was observed with the same nanoESI emitter after its removal from the tube, where it had been subjected to LTP treatment. Deionized water contained in a nanoESI emitter and subjected to LTP treatment was analyzed subsequently by inductively coupled plasma mass spectrometry (ICPMS). The elemental analysis showed increases in concentration for a variety of metals, including Na, Ca, and Fe (see Table 2S in the Supporting Information). Reports have shown that electrolytes inside bulk glass can be released at the surface during plasma discharges.[13] For the same reason, the LTP may cause electrolytes to leak from the wall surface of the nanoESI emitter (made from borosilicate glass) into the spray solution. In an attempt to confirm the role of the electrolyte, we added different kinds of electrolytes, including ammonium acetate (NH_4OAc), ammonium bicarbonate (NH_4HCO_3), cesium iodide (CsI), sodium iodide (NaI), and sodium chloride (NaCl), to the peptide solution individually and tested the effects over a range of concentrations (0.1–500 mM) by nanoESI. In general, very similar fragmentation patterns to that obtained after exposure to the LTP were observed with all electrolytes, provided the concentration of the electrolyte was relatively high (>50 mM for weak electrolytes and >10 mM for strong electrolytes). Figure 4 shows nanoESI spectra recorded for an aqueous solution of bradykinin with NH_4OAc (100 mM) without plasma treatment. At a relatively low spray voltage (1.2 kV, Figure 4b), backbone-fragment ions ($\mathbf{y_4}^+$–$\mathbf{y_8}^+$) are evident. Interestingly, when a relatively high voltage (1.5 kV) was applied, fragmentation was no longer observed (Figure 4c). This "on/off" character of the fragmentation can be manipulated by switching the spray voltage between low and high settings, as demonstrated in the total-ion chronogram (Figure 4a).

We investigated the fragmentation process further by collecting the condensate of the spray on a grounded gold counter electrode under different nanoESI conditions. The condensates were each dissolved in MeOH/H_2O/HOAc (50:49:1, v/v/v; 20 μL) and examined by using nanoESI. Fragments were only observed in the mass spectra of

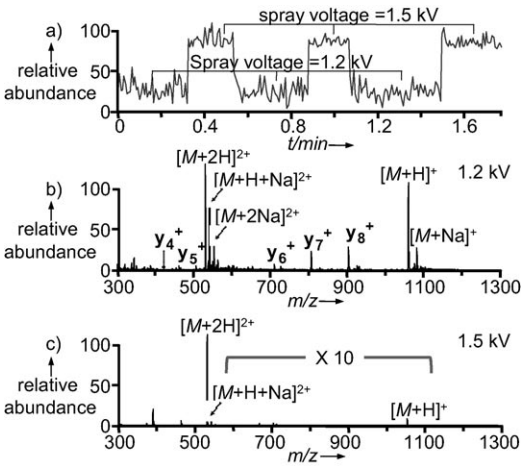

Figure 4. Positive-mode nanoESI of bradykinin (RPPGFSPFR; 20 μM aqueous solution containing NH_4OAc (100 mM)): a) total-ion chronogram; b) mass spectrum with spray voltage at 1.2 kV; c) mass spectrum with spray voltage at 1.5 kV. All experiments were carried out without plasma activation.

condensates derived from peptide solutions sprayed at relatively low voltage that contained electrolytes in high concentrations. This observation suggests that fragmentation occurs at atmospheric pressure, before any ions or charged droplets enter the mass spectrometer. This phenomenon is different from the "prefolding dissociation" reported by Han et al., who added electrolytes to the ESI spray solution to weaken electrostatic intramolecular interactions within a protein and thus improve backbone cleavage upon collisional activation inside the mass spectrometer.[14] We also found similarities between peptide fragmentation observed after plasma operation and the fragmentation induced in the skimmer-tube lens region (referred to as in-source CID) in terms of fragment identity and the relative abundance of fragments. Furthermore, the degree of preservation of phosphorylation was higher for fragmentation in nanoESI after plasma processing than for in-source CID, whereas ion-trap CID showed the lowest degree of phosphorylation preservation (see Figure 2S in the Supporting Information). The similarities and differences in peptide fragmentation are the combined result of the activation process and the nature of species that undergo activation. Although multiple collision events are involved in collisional activation for both in-source CID and ion-trap CID, higher collision energies might be involved under the former conditions. Notably, whereas just one selected ionic species is activated in ion-trap CID, multiple ionic species, including solvated ions and charged droplets, are subjected to activation and subsequent fragmentation during in-source CID. It is quite possible that similar ion activation and a similar population of species are involved in peptide fragmentation after plasma treatment as in in-source CID. That is, solvated ions or charged droplets are collisionally activated. The preservation of phosphorylation may simply result from a higher degree of solvation around the charged phosphate group than on the peptide backbone.

On the basis of our experimental results, we conclude that the peptide fragmentation observed in nanoESI at relatively low spray voltages and after plasma discharge is related to the ion-formation process during ESI. Our hypothesis is that solvated ions (or very fine charged droplets) might be ejected directly with relatively high speeds, in contrast to the formation of micron-sized charged droplets with low speeds in the early stages of normal ESI.[15–17] The formation of solvated ions during the onset of ESI is facilitated by high electrolyte concentrations. Under such conditions, peptide molecules have a higher surface activity and occupy the solution surface, where they are partially solvated,[18] whereas electrolytes are in the inner regions.[19] When an appropriate voltage is applied, peptide molecules carrying a certain number of solvent molecules and charges may be ejected selectively into the gas phase with relatively high speeds similar to those in ion evaporation[20,21] or field ejection.[22] The solvated ions then undergo multiple collisions, which eventually cause fragmentation and desolvation. The reason that no fragmentation is observed in nanoESI at a high electrolyte concentration when a high spray voltage is used may be related to the change of spray mode:[23] Under these conditions, charged droplets are ejected. Thus, solvent molecules play two important roles in ESI: They decrease the internal energy of the droplets upon evaporation and shield analytes from direct collisional fragmentation. The dried peptide ions derived from charged droplets might be cooled efficiently by the time they are formed, or formed in a vacuum region, where collisional activation is greatly diminished.

In summary, unique peptide-fragmentation patterns are observed by nanoESI after subjection of the spray emitters to plasma treatment. The types of fragments include a-, b-, and y-type ions. In the case of phosphopeptides, rich backbone fragmentation is generally observed, and most noticeably, labile phosphate groups are preserved. These features are attractive for the structural characterization of phosphopeptides. Mechanistic studies suggest that the role of the plasma is to release electrolytes from the glass emitter so that they can be taken up into the spray solution to cause a high local electrolyte concentration. The fragmentation itself is related to ion formation under these special ESI conditions. These conditions, together with the relatively low spray voltage applied, enable the direct ejection of solvated ions with relatively high speeds in the early stages of the spray. The subsequent gas-phase collisional activation of the solvated ions produces the fragments observed in the mass spectra. A higher degree of solvation around the charged groups may account for the preservation of phosphate groups during activation. If this explanation is correct, then alternative and simpler methods (without plasma treatment of the surface) are possible. Further investigation by both experimental and computational approaches is needed to prove and characterize such processes. Nevertheless, this study demonstrates that under special conditions, ESI, a widely accepted "soft" ionization technique, can also be "hard" and cause significant and useful peptide fragmentation. The unique fragmentation pattern that appears to be associated with solvated peptide ions also encourages further studies on their little-explored

chemistry, including their reactivity and activation in the gas phase.

Experimental Section

All experiments were carried out with a Finnigan LTQ mass spectrometer (Thermo Electron, San Jose, CA). The following instrument conditions were used for positive-mode mass spectrometry: capillary temperature: 150 °C; heated-capillary voltage: 15 V; tube-lens voltage: 65 V. The T-shaped glass tube, with an outer diameter (o.d.) of 7.8 mm and an inner diameter (i.d.) of 4.8 mm, was purchased from Chemglass (Vineland, NJ). A compact ozonizer power supply[24] was used to provide an alternating current. The nanoESI emitters with a tip-orifice diameter of approximately 30 μm were pulled from borosilicate glass capillaries (1.5 mm o.d., 0.86 mm i.d.) by using a P87 Flaming/Brown micropipette puller (Sutter Instruments, Novato, CA). A spray voltage in the range of 1–2 kV was applied to a stainless-steel or platinum wire, which was inserted into the emitter into contact with the spray solution.

Received: July 17, 2008
Published online: September 30, 2008

Keywords: electrospray ionization · fragmentation · mass spectrometry · peptides · plasma chemistry

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