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SHORT COMMUNICATION

Electrospray Photochemical Oxidation of Proteins

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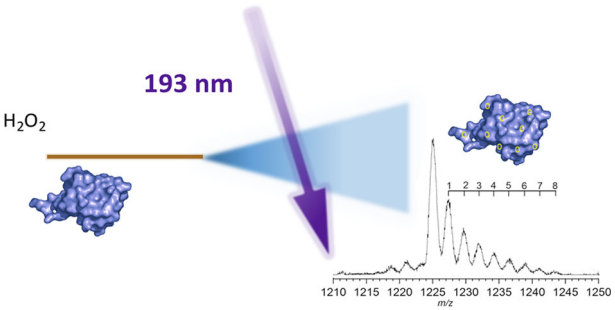

Abstract. Photooxidation of peptides and proteins by pulsed ultraviolet laser irradiation of an electrospray in the ion source of a mass spectrometer was demonstrated. A 193-nm excimer laser at 1.5-mJ pulse energy was focused with a cylindrical lens at the exit of a nanoelectrospray capillary and ions were sampled into a quadrupole time-of-flight mass spectrometer. A solution containing a peptide or protein and hydrogen peroxide was infused into the spray at a flow rate of 1 μL/min using a syringe pump. The laser creates OH radicals directly in the spray which modify biomolecules within the spray droplet. These results indicate that photochemical oxidation of proteins can be initiated directly within electrospray droplets and detected by mass spectrometry.

Keywords: Photochemical oxidation, FPOP, Electrospray, Excimer Online, Ultraviolet

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Introduction

Electrospray droplets can be used both as a source of ions as well as for compound extraction and chemical reactions. The highly charged electrospray droplets serve as small reaction vessels that can be probed by mass spectrometry. With extractive electrospray ionization (EESI), the charged electrospray droplets merge with neutral aerosol particles containing analyte molecules that are extracted to form ions [1–5]. Reactions can be induced in electrospray droplets by exposing them to gaseous reagents. For example, hydrogen/deuterium exchange is observed in proteins within electrospray droplets when the spray is exposed to a deuterating reagent such as D_2O [6, 7] and exposure of electrospray droplets to gas-phase acid or base can be used to manipulate protein charge state distribution [8, 9]. Solutions can be mixed in the electrospray cone using microfluidic channel sprayers [10] or dual-channel electrospray emitters such as theta capillaries [1] or coaxial fluidic devices [11]. Reactions with lifetimes as short as 1 μs can be monitored [12] and examples include protein folding [12–14], thermal denaturation [15], and H/D exchange [1, 11, 16]. Photochemical reactions can be initiated in the electrospray using a continuous light source directed at the spray tip [17–19].

Protein footprinting is the process of chemically modifying solvent-exposed regions of proteins in their native state followed by digestion and analysis by mass spectrometry to probe the higher-order structure [20]. Proteins can be oxidized in an electrospray using high voltage and an oxygen nebulizing gas [21, 22]. The resulting spray contains modified proteins that can be analyzed directly in the mass spectrometer or collected for digestion and off-line tandem mass spectrometry. Alternatively, fast photochemical oxidation of proteins (FPOP) for protein footprinting uses a pulsed UV laser to produce hydroxyl radicals from hydrogen peroxide [23–25]. The OH radicals rapidly oxidize the protein and subsequent digestion and mass spectrometry can reveal the portion of the molecule that was exposed in solution. With the use of radical scavengers, the lifetime of the footprinting process can be as low as microseconds, which allows the probing of protein structure in the native state. Protein footprinting has been used for a number of applications including epitope mapping and the analysis of conformations for protein therapeutics [26].

In this work, we describe the photochemical oxidation of peptides and proteins directly in the electrospray ion source of a mass spectrometer using a 193-nm excimer laser. The

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electrospray contained peptide or protein standards and hydrogen peroxide, and was irradiated with the laser to produce hydroxyl radicals in the spray droplet that subsequently oxidized the electrosprayed biomolecules. Spectra from oxidized and non-oxidized molecules were obtained from the infusion of the same sample.

Experimental

A quadrupole time-of-flight mass spectrometer (QStar-XL, Applied Biosystems, Framingham, MA) equipped with a nanoelectrospray ion source and a custom spray tip was used for mass spectrum acquisition in positive ion mode. A schematic of the system is shown in Figure S1 of the supplemental information. The electrospray emitter was made from a 50 μm ID, 360 μm OD fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA) pulled manually after exposure to the flame of a butane torch to a ca. 10-μm inner diameter tip. The emitter was positioned 5 mm from the MS inlet and a potential of 4500 V was applied to achieve a stable electrospray. The analyte solution was infused through the capillary using a syringe pump (Cole-Parmer, Vernon Hills, IL, USA) at a flow rate of 1 μL/min unless otherwise indicated. A 193-nm wavelength ArF excimer laser (ExciStar 200, Coherent, Santa Clara, CA) was directed into the electrospray and focused using a 25-mm focal length cylindrical lens to a 0.5 mm × 8 mm line perpendicular to the spray axis. The laser pulse energy was 1.5 mJ and the resulting laser fluence was 400 J/m^2. For each experiment, the electrospray was irradiated with 100 laser pulses at 20 Hz, leading to a laser irradiation time of 5 s per event.

Bradykinin acetate salt, insulin from bovine pancreas, ubiquitin from bovine erythrocytes, horse heart cytochrome c, hydrogen peroxide, and LC-MS grade water were purchased from Sigma-Aldrich (St. Louis, MO, USA). Formic acid (99.5%, LC-MS grade) was obtained from Thermo Fisher Scientific (Waltham, MA, USA) and methanol (LC-MS grade) was purchased from EMD Millipore (Burlington, MA, USA). Analytes were dissolved in water to a concentration of 1 mg/mL and insulin solutions contained 0.1% formic acid to facilitate dissolution. The samples were vortexed until complete dissolution. All samples were further diluted to the desired concentration with a solution of methanol and water (1:1) containing 0.1% formic acid. The resulting sample solution had a pH of approximately 1.4 and all experiments were conducted under denaturing conditions. To each sample, hydrogen peroxide was added to a final concentration of 20 mM immediately before mass spectrum acquisition.

Results and Discussion

Initial experiments were conducted using a 10 μM solution of bradykinin containing hydrogen peroxide that was infused at a flow rate of 1 μL/min. Mass spectra obtained with and without laser irradiation are shown in Figure 1; five individual spectra

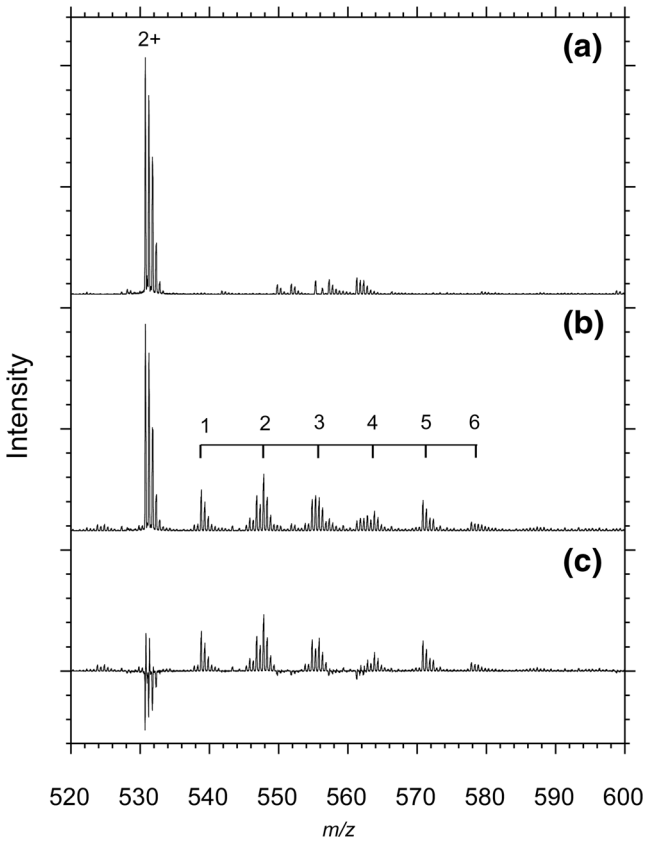

Figure 1. Representative mass spectra of +2 charge state bradykinin containing H_2O_2 acquired with (**a**) electrospray only and (**b**) electrospray with laser irradiation with number of oxygen addition indicated; (**c**) laser off spectrum subtracted from laser on spectrum

were averaged to produce the final spectrum. The mass spectrum in Figure 1a was obtained without irradiation and shows the *m/z* region with the peak corresponding to the doubly protonated peptide at 530.7 *m/z* and its sodium and potassium adducts at 541.8 and 549.8 *m/z* respectively. The mass spectrum in Figure 1b was obtained with UV irradiation. The spectrum was obtained using 20-Hz repetition rate, 1.5-mJ energy, and 20 shots per mass spectrum, and five individual spectra were averaged to produce the final spectrum. The intensity of the signal associated with $[M+2H]^{2+}$ in Figure 1b is 20% lower compared with Figure 1a. Signals associated with the addition of between 1 and 6 oxygen were detected in Figure 1b and correspond to the oxidation of the phenylalanine and proline sites on the peptide [23, 24]. These peaks can be seen in Figure 1c which was obtained by subtracting Figure 1a from Figure 1b. The extracted ion current for the signal corresponding to the doubly protonated molecule $[M+2H]^{2+}$ and the signal corresponding to the oxidized peptide $[M+O+2H]^{2+}$ in Figure 1b is shown in Figure S2. The signal from the doubly protonated molecule decreases when the laser is on accompanied with a new peak associated with the oxidized molecule which is observed until the laser is shut off and the signal returns to baseline. Additionally, the intensity of the peak associated with the addition of two oxygens is greater than that

from the addition of a single oxygen atom. This may result from oxidation of the two phenylalanines, which are easily oxidized by hydroxyl radicals [27] that are likely to be at a high concentration given the 2000-fold excess of hydrogen peroxide with respect to the analyte.

The ESI voltage of 4500 V produced a stable spray without oxidizing the peptide as shown in Figure 1a. Above 5000 V, some oxidation of bradykinin was observed without laser irradiation as shown in Figure S3. Voltage lower than 4000 V resulted in unstable electrospray. The laser was directed into the Taylor cone and did not visibly affect the spray. When the laser was directed a few millimeters downstream of the Taylor cone, oxidation of the analyte was still observed but the signal was not as intense. Mass spectra acquired by varying the laser beam position relative to the MS inlet are shown in Figure S4. Laser energies between 0.5 and 6 mJ were tested. Energies between 1.5 and 3 mJ energy produced results comparable with those in Figure 1b whereas energies above 4 mJ produced a lower signal from the protonated molecule without a significant change in the peak corresponding to the oxidized molecule. Below 1.5 mJ, the intensity of the signal from the oxidized molecule was lower than in Figure 1b. Representative mass spectra of bradykinin oxidation using varying laser energies are shown in Figure S5. At higher energy, the signal intensity of peaks from multiple oxygen addition is greater, which is consistent with a greater radical concentration anticipated at higher pulse energies.

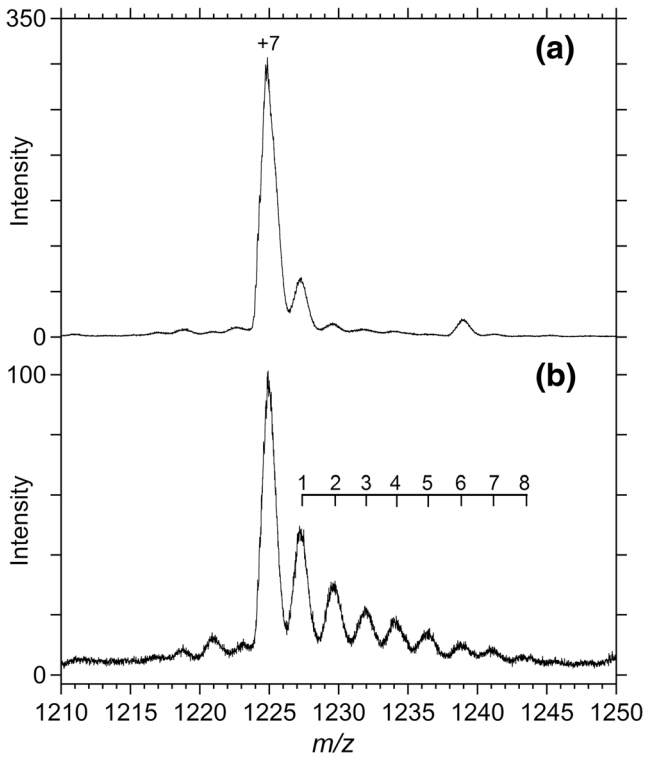

Figure 2. Mass spectra of the +7 charge state of ubiquitin electrosprayed from an aqueous solution containing H_2O_2 acquired by (**a**) electrospray only and (**b**) irradiated at 193 nm

Mass spectra of the protein ubiquitin are shown in Figure 2. A 10 μM solution of the protein in water with 20-mM H_2O_2 concentration was infused at 1 μL/min. The region of $[M+7H]^{7+}$ is shown in Figure 2a for laser off and Figure 2b was obtained with the 193-nm laser directed at the electrospray. The spectra were obtained by averaging 10 individual mass spectra. In Figure 2b, the intensity of the peak corresponding to the protonated molecule was lower by approximately 70% with the laser on and peaks corresponding to the addition of up to 8 oxygen atoms were observed. A mass spectrum of ubiquitin obtained from a solution without H_2O_2 is shown in Figure S6.

Mass spectra obtained under similar conditions for insulin and myoglobin are shown in the Supplementary Information Figures S7 and S8, respectively. The insulin mass spectra for charge state $[M+5H]^{5+}$ are similar to Figure 2 with a 40% decrease in the signal corresponding to the protonated protein and the addition of up to 11 oxygen upon 193-nm laser irradiation. The myoglobin solutions containing peroxide resulted in oxidation of the protein even in the absence of UV irradiation. The peaks associated with the oxidized protein were absent when myoglobin was electrosprayed without hydrogen peroxide (Figure S8).

Conclusions

Pulsed-laser initiated photochemical oxidation reactions within electrospray droplets with on-line mass spectrometry were demonstrated. Photochemical oxidation of peptides and proteins within charged electrospray droplets in the presence of hydrogen peroxide was accomplished by irradiating the spray just downstream of the Taylor cone with a pulsed 193-nm UV laser. Although both protein and water molecules absorb the laser energy at this wavelength, no evidence of fragmentation was observed. When combined with native electrospray and tandem mass spectrometry, this approach has the potential to enable in-electrospray protein footprinting. Furthermore, the approach is general and has the potential for application to additional wavelengths and other photochemical or photothermal processes. Reactions within electrospray droplets can be observed at time scales down to 1 μs [12], which suggests that photochemical reactions can potentially be observed on these time scales.

Acknowledgements

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