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Mass Spectrometric Features of S-Nitrosylated Peptides

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The S-nitrosylation of cysteine thiols participates in the trafficking of nitric oxide (NO) in the intra- and extra-cellular milieu. To establish a mass spectrometric method to detect protein S-nitrosylation, an S-nitrosylated peptide was analyzed using different ionization modes. Electrospray ionization generated intact molecular ions, and in-source fragmentation gave rise to a loss of 30 mass units for the NO moiety. On the other hand, matrix-assisted laser desorption/ionization using an ultraviolet laser produced ions with a mass that was 29 units smaller and represented an unmodified molecule, probably due to reductive cleavage during the ionization process. All of these mass spectrometric features are diagnostic for protein S-nitrosylation.

1. Introduction

Phosphorylation and nitrosylation of proteins are kinds of posttranslational modification that are important in molecular signaling systems.¹⁾ A recent set of studies has suggested that there is site-specificity of nitrosylation at cysteine thiols,^{2)–5)} but the dynamics of protein S-nitrosylation are poorly understood. This modification most likely occurs in a non-enzymatic fashion following the generation of nitric oxide (NO) by NO synthases. The process by which this modification is reversed is unclear. The current level of understanding of nitrosylation is very different from that of phosphorylation, for which extensive studies have elucidated the enzymatic systems involved in the modification at tyrosine, serine, and threonine residues.

To scrutinize the biology of protein S-nitrosylation, one must be able to detect this modification in a sensitive manner. The current methods for detecting protein S-nitrosylation are mostly based on the measurement of NO released from proteins. For example, chemical oxidation of NO by ozone electronically excites an NO₂ radical, which manifests chemiluminescence upon decay to the ground state that is directly proportional to the NO concentration. Alternatively, the “NO electrode,” which is configured in series with an S-NO reducing (upstream) Au electrode and an SH oxidizing Hg (downstream) electrode, is used. Recently, an antibody specific for S-nitrosocysteine was used for histochemical detection or immunoblot assays of S-nitrosylated proteins.⁶⁾ For a more precise analysis at the molecular level, electrospray ionization (ESI) mass spectrometry was used to detect an increase of 29 mass units corresponding to NO adducted to proteins and peptides.^{7)–11)} However, mass spectrometry of S-nitrosylated proteins and peptides has not been systematically carried out. Here, using an *in vitro*-nitrosylated peptide, we report the characteristic features of S-nitrosylation, including loss of NO and reduction, observed in mass spectra from different ionization modes.

2. Experimental

2.1 Sample preparation

A thiol-bearing peptide LQQCPFEDH (monoisotopic mass of 1115.4), corresponding to residues 31–39 of human serum albumin, was synthesized. It was S-nitrosylated by an NO-generating reagent “NONOate” (NOC12, nacalai-tesque, Japan). The reaction was carried out in a solution of 75% methanol and 1% acetic acid at pH 3.5. After the modification, excess reagents were removed by reverse phase chromatography.

2.2 Mass spectrometry

All measurements were carried out in a positive ion mode.

ESI mass spectra were collected using an LCQ ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA) or a Mariner time-of-flight (TOF) mass spectrometer (Applied Biosystems, Foster City, CA, USA). The peptide samples were dissolved in a solution of 75% methanol and 1% acetic acid and directly infused into the ESI-MS instrument at a flow rate of 3 μ L min^{–1} using a syringe pump.

For measurements using LCQ, the spray needle voltage was maintained at +4.5 kV and the capillary (desolvation) temperature was 200°C. The ion trap analyzer was operated at a pressure of 1.0×10^{-5} Torr (1 Torr = 133.3 Pa) as measured using a remote ion gauge. Helium gas, admitted directly into the ion trap, was used as the bath/buffer gas to improve trapping efficiency and as the collision gas for dissociation experiments. The activation amplitude (proportional to the collision energy) for collision-induced dissociation (CID) experiments was expressed as the percentage of the maximum tickle energy available.

In measurements using the Mariner, a standard setting of 100 V for the nozzle potential was increased to 200 V to induce in-source fragmentation. The nozzle (desolvation) temperature was 140°C.

Matrix-assisted laser desorption/ionization (MALDI) TOF mass spectra were obtained on a Voyager DE-RP mass spectrometer (Applied Biosystems) fitted with a pulsed nitrogen laser (337 nm) with a 3-ns pulse duration. The minimum laser fluence required to produce a detectable signal for protonated molecular ions was applied. The ion acceleration voltage was 20 kV. The

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peptide was dissolved at 10 μ M in a solution of 50% acetonitrile and 0.1% TFA (AcN/TFA), and mixed with an equal volume of saturated α -cyano-4-hydroxycinnamic acid in the AcN/TFA solution.

3. Results and Discussion

The protonated molecule $[M+H]^+$ of the S-NO peptide was detected at m/z 1145.2 in the ESI mass spectrum (Fig. 1). The in-source fragmentation was then applied by increasing the nozzle potential. A similar experiment was carried out by Ferranti *et al.* using a low resolution single quadrupole mass spectrometer, and an ion with a mass smaller by 29 mass units than the $[M+H]^+$ was reported.⁷⁾ However, in the present study, the ions generated at m/z 1115.2, which increased as the nozzle potential was raised, were 30 mass units smaller than the molecule at m/z 1145.2 (Fig. 2). The decrease of 30 mass units suggested the neutral loss of NO. The same result was obtained by a CID experiment using helium as the collision gas (data not shown).

The same sample was then analyzed using MALDI-TOF-MS with a UV laser. The $[M+H]^+$ for the S-nitrosylated peptide was not detected, instead an ion with a mass smaller by 29 units, $[M+H-29]^+$, was observed (Fig. 3). The same result was obtained when

Fig. 1. ESI-TOF mass spectra of a synthetic peptide before (upper) and after (lower) modification. The doubly charged ion of a disulfide dimer, which was formed during modification, is observed at m/z 1115.4 in the lower mass spectrum.

Fig. 2. In-source fragmentation of an S-nitrosylated peptide. The nozzle potential was 100 volts (upper) and 200 volts (lower). The doubly charged ion of a disulfide dimer contaminant (see legend for Fig. 1) is observed in both mass spectra.

Fig. 3. MALDI-TOF mass spectra of an S-nitrosylated peptide before (upper) and after (lower) S-nitrosylation. The ions at m/z 1138.2 are sodium-adducted. The mass spectra were acquired in reflector mode.

the MS was operated in a linear mode. The decrease most likely represented the reductive cleavage of S-NO to SH during the ionization process, since cleavage of the S-NO bond by UV irradiation has been reported.¹²⁾ Following cleavage, the hydrogen was probably added in the ion source. This kind of photo-induced chemical reaction has been reported in the MALDI-MS of nitrotyrosine as well.^{13, 14)}

A consistent result was obtained when human transthyretin (M_r 13761) was S-nitrosylated in vitro and analyzed. The protonated molecule of the S-nitrosylated transthyretin was detected by ESI-MS. On the other hand, in the MALDI-TOF mass spectrum, an ion corresponding to the unmodified protein, the product of reductive cleavage, was observed instead of the S-nitrosylated species (data not shown).

In conclusion, S-nitrosylated proteins and peptides can be measured as protonated molecules by ESI-MS and undergo a loss of 30 mass units by CID. Only non-nitrosylated forms are observed owing to reduction in the UV MALDI mass spectra.

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