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Direct measurement of S-nitrosothiols with an Orbitrap Fusion mass spectrometer: S-nitrosoglutathione reductase as a model protein

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Abstract

Recent studies suggest cysteine S-nitrosation of S-nitrosoglutathione reductase (GSNOR) could regulate protein redox homeostasis. "Switch" assays enable discovery of putatively S-nitrosated proteins. However, with few exceptions, researchers have not examined the kinetics and biophysical consequences of S-nitrosation. Methods to quantify protein S-nitrosothiol (SNO) abundance and formation kinetics would bridge this mechanistic gap and allow interpretation of the consequences of specific modifications, as well as facilitate development of specific S-nitrosation inhibitors. Here, we describe a rapid assay to estimate protein SNO abundance with intact protein electrospray ionization mass spectrometry. Originally designed using recombinant GSNOR, these methods are applicable to any purified protein to test for or further study nitrosatable cysteines.

Keywords

S-nitrosation; S-nitrosothiol (SNO); GSNOR; mass spectrometry; Orbitrap Fusion; redox switch; cysteine modification

1. Introduction

Cysteine S-nitrosation is a reversible post-translational redox modification relevant to nitric oxide (NO) signal transduction in both plants and animals. Cysteine S-nitrosothiols (SNOs) form when cysteine thiolates nucleophilically attack nitroso groups, the latter afforded by NO auto-oxidation. Nitrosation is not to be confused with nitrosylation, in which oxidized sulfur reacts with a nucleophilic nitroxyl (NO^-). Both processes yield SNOs, but nitrosation is more likely since oxidized divalent sulfur is rare (see ref. [1] for a discussion on nomenclature). SNOs modulate enzyme activity, inhibit irreversible cysteine oxidation, and regulate intracellular trafficking [2–4]. SNOs are labile in vivo [5]. However, "switch" protocols have enabled identification of protein SNOs. Thiol reducing agents such as methyl methanethiosulfonate (MMTS) are used to block free cysteines, after which ascorbate semi-

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selectively reduces SNOs over other cysteine oxidative modifications in cell lysates. Subsequent biotin or tandem mass tag conjugation to the ascorbate-reduced cysteines facilitates immunoprecipitation and proteomics for identification of SNO-modified proteins [6,7]. Site-specific cysteine SNOs have been inventoried in *Arabidopsis thaliana* cell suspensions and rosette leaves [8,9] and mouse liver and brain [10].

Hydrogen deuterium exchange mass spectrometry (HDX-MS) and intrinsic tryptophan fluorometry are common techniques to measure the impact of S-nitrosation on protein structure and function [11,12]. Neither of these procedures is ideal to measure novel protein S-nitrosation and cannot determine number of SNO groups formed. HDX-MS entails non-trivial data analysis, and not all proteins exhibit differential fluorescence upon S-nitrosation.

Using intact protein electrospray ionization mass spectrometry (IP-ESI-MS), we demonstrated S-nitrosation of purified plant, mammalian, and yeast S-nitrosogluthathione reductase (GSNOR) orthologues [13]. These findings lay a foundation for a simple quantitative estimation of protein SNO content. It is tempting to interpret differential protein behavior after nitroso donor treatment as resulting from S-nitrosation. Ideally, one should first ascertain the existence of protein SNOs before investing in a labor-intensive biotin switch protocol. IP-ESI-MS of S-nitrosated and unmodified GSNOR could serve as a benchmark for S-nitrosation kinetics, as well as assure quality control for proteomic experiments. Nitrosation increases protein mass by 29 Da per modification (~ 1 m/z for a +29 charge state), a mass signature unique to S-nitrosation [14]. Trace alkali metals and detergents significantly impair protein signal detection by conventional direct infusion-electrospray mass spectrometry [15]. Zech et al. resolved protein SNOs by nanospray with a triple quadrupole (TQ) mass spectrometer [16]. However, nanospray emitters are more technically challenging to install, and results are less reproducible. The higher resolving power of Orbitrap mass spectrometers (>100 times that of a TQ) [17] makes direct protein infusion fast and reliable. In addition to containing nitrosatable cysteines capable of simultaneous modification [13,18], GSNORs also express well as soluble proteins in *E. coli* and tolerate buffer exchange and detergent removal (unpublished observations). Using cysteine to alanine (C $\rightarrow$ A) mutants with reduced or abolished capacity for S-nitrosation as negative controls increases the power of the assay. Considered together, IP-ESI-MS of nitroso donor-treated GSNOR is a time- and cost-effective quality control assay applicable to any study of protein SNOs.

Here we explain how to obtain electrophoretically-pure, detergent-free GSNOR samples, conduct in vitro S-nitrosation of proteins, and quantify the relative SNO content by IP-ESI-MS. We also discuss common pitfalls of protein mass spectrometry, such as ion suppression and adduct formation by ubiquitous salt and detergent contaminants, and strategies for their avoidance. The goal is to use unmodified and modified GSNOR samples as controls for nitroso donor-induced S-nitrosation of other proteins.

2. Materials

2.1. Expression of His-tagged, recombinant GSNOR (see **Note 1**)

Make all aqueous solutions from water of double distilled purity or higher as indicated.

1. Antibiotic: 100mg/mL kanamycin (or alternative antibiotic at stock concentration recommended for the expression vector).
2. Lysogeny broth (LB): 25mL, autoclave before use. Add 25µl antibiotic after cooling to 60°C.
3. LB: 2.5L, autoclave before use. Add 250µl antibiotic after cooling to 60°C.
4. Orbital shaker with 0-50 °C temperature control.
5. 2M Isopropyl-β-thiogalactoside (IPTG) in H₂O. Sterilize with a 0.22µm filter and store at -20 °C.
6. UV/VIS spectrophotometer with cuvette and microtiter plate functions.
7. Refrigeration-capable centrifuges and rotors with maximum RCF 20,000 × *g*.
8. Detergent-free cell lysis buffer (30mL per liter of cells): 20mM Tris-HCl pH 7.5, 300mM KCl, 30mM imidazole, 10% (v/v) glycerol, 1mM dithiothreitol (DTT), 0.33× Roche EDTA-free protease inhibitors (*see* ^{Note 2}). Adjust pH before addition of DTT and protease inhibitor, sterilize with a 0.22µm filter and store at 4 °C. Add DTT and protease inhibitor directly before use.
9. Sonicator: Sonics Vibra cell VCX750 with a 19mm × 127mm tip for cell lysis.
10. *Optional*: Microfluidizer (Microfluidics) for further cell lysis.
11. Microcentrifuge and conical tubes: 1.5, 15, and 50mL.
12. Barrier pipet tips: 10, 200, and 1000µl, all colorless (*see* ^{Note 3}).
13. Fast Flow nickel sepharose resin beads (GE Healthcare): 200µL per liter of culture: Gently rotate container to re-suspend resin and aliquot beads into a new tube. Use the same day.
14. Elution buffer: 13.3mM Tris-HCl pH 7.5, 200mM KCl, 353mM imidazole, 6.7% (v/v) glycerol, 0.67mM DTT, 0.22× Roche EDTA-free protease inhibitors. Prepare by mixing 6.67mL lysis buffer with 3.33mL 1M imidazole. Scale volume up as needed. Use the same day.

¹We designed this protocol for polyhistidine-tagged proteins under IPTG expression control via pET28 and pET16 vectors. Typically, a 1 L culture yields ~100nmol (~4.5 mg) monomeric wildtype Arabidopsis GSNOR (AtGSNOR) and ~15nmol (~0.7 mg) monomeric AtGSNOR^{C3→A3} (cysteines 10, 271, and 370 mutated to alanine) as >85% electrophoretically pure protein. If downstream techniques require more protein, grow at least 2 L of cells for wild-type AtGSNOR.

²Aliquot 1M DTT stocks and store at -80 °C. Do not reuse DTT aliquots, as DTT is prone to oxidation. Foil- wrapped, 0.22µm-filtered 1M imidazole is stable at 25 °C for 12 months.

³Polyethylene glycol (PEG) and related compounds are insidious contaminants because they are difficult to remove and are usually only diagnosed with a mass spectrometer. High ionization efficiency by electrospray allows PEG to outcompete proteins for relevant charge states even at submicromolar concentrations. This severely suppresses protein signal [20]. Exclude Triton × 100, Nonidet P-40 and other nonionic detergents from purification buffers, as they contain or are made from ethylene glycol [21]. Rinse all glassware (used during protein expression and purification) three times with distilled water and once with ethanol or isopropanol to remove residual PEG left by dishwashing detergents. Thoroughly wash dialysis membranes and centrifugal concentrators (e.g., Amicon Ultra filtration devices). Along with these precautions, we recommend exclusive use of new, colorless, and non-autoclaved Fisherbrand or USA Scientific plastics, from cell lysis to sample injection on the mass spectrometer. Colorless barrier pipet tips are recommended but not strictly necessary until part 3.2 of this protocol. Use nitrile gloves since latex may be a contamination source [22]. Even following the aforementioned precautions, we found AtGSNOR^{C3→A3}, HsGSNOR^{C195A}, and HsGSNOR^{C268A} to copurify with some PEG. In contrast, wild-type isoforms purified the same day with the same sonicator or microfluidizer exhibited no apparent contamination.

15. Storage buffer: 20mM Tris-HCl pH 7.5, 300mM KCl, 1mM DTT. Add DTT immediately before use. Filter (0.22µm) and store at 4 °C.
16. 10kDa molecular weight cut-off centrifugal concentrators: Ultra-0.5mL, 4mL, and 15mL (Amicon).
17. *Optional*: HiPrep 26/60 S200-HR Sephacryl preparative size exclusion column (GE Healthcare). Calibrate with 43-669kDa high molecular weight mass standards (GE Healthcare).
18. *Optional*: FPLC system (AKTA prime) with a fraction collector and in-line A₂₈₀ and conductivity meters, all at 4 °C. Use as needed for additional protein purity. Depending on the nature of impurities, other chromatographic methods (i.e. ion exchange) may also be appropriate.
19. SDS-PAGE precast gels: 4-15% acrylamide Mini-PROTEAN TGX Stain-Free (Biorad).
20. SDS-PAGE gel rigs and power supply: Mini-PROTEAN Tetra cell and PowerPac (Biorad).
21. SDS-PAGE running buffer: 10× Tris-Glycine (Biorad). Mix 100mL 10× stock with 900mL water.
22. Coomassie stain: 1g Coomassie R250, 100mL acetic acid, 300mL methanol, 600mL water.
23. Protein concentration assay reagents: bicinchoninic acid (BCA) reagents A and B (Pierce): Mix 10mL reagent A with 200µl reagent B. Use the same day.
24. Protein standards: bovine serum albumin (BSA) dilutions at 0.6, 0.4, 0.2, 0.1, and 0.06 mg/mL.
25. 96-well clear polystyrene microtiter plates.
26. Li-Cor Odyssey flatbed scanner or CCD camera-equipped LED dark box to image gels.

2.2. In vitro nitrosation

1. LC-MS-grade water (MS-water). Use for all aqueous solutions in parts 3.2 and 3.3.
2. Colorless barrier pipet tips (Thermo ART or equivalent). Use for all pipetting in parts 3.2 and 3.3.
3. Colorless microcentrifuge tubes: 0.5 and 1.5mL (Fisherbrand or USA Scientific). Use to make all solutions in parts 3.2 and 3.3. Avoid glassware after purification since it is a source of metal ions.
4. Cysteine (CysSH) solution: 400mM CysSH, 100mM diethylenetriaminepentaacetic acid (DTPA), 285mM HCl: Dissolve 7.9mg DTPA in 185µL MS-water + 4.9µL concentrated HCl and incubate at 70 °C with

occasional vortexing. Cool to room temperature, add 4.9mg L-cysteine, and adjust volume to 200µL with MS-water. Use the same day.

5. 200mM sodium nitrite (NaNO_2): Dissolve 13.8mg NaNO_2 in 1mL MS-water. Use the same day (*see* Note 4).
6. 20mM Tris-HCl pH 8.0. Prepare with MS-water, filter (0.22µm), and store at room temperature.
7. 20mM ammonium acetate (NH_4Ac): Prepare with MS-water, filter (0.22µm), and store at room temperature. Do not adjust the pH of NH_4Ac (*see* Note 5).

2.3. Mass spectrometry of intact protein

1. Mass spectrometer: Thermo Orbitrap Fusion Tribrid or comparable (resolution 20,000) equipped with a heated electrospray ion source, 1.6mm OD 0.125mm ID PEEK tubing and column connectors, and a syringe pump capable of flow rates in the range 1-10 µL/min. (*see* Note 6)
2. 250µL Hamilton Gastight syringe equipped with flat tipped (point style 3) needle.
3. LC-MS-grade methanol (CH_3OH).
4. Acidified CH_3OH : Combine MS-water, MS-grade CH_3OH , and glacial acetic acid at a ratio of 49/47/4 (v/v/v) in a new microcentrifuge tube.
5. Deconvolution software: Thermo XCalibur and Protein Deconvolution 3.0.

3. Methods

3.1. Protein expression and purification

Steps 3-14 require approximately 8 h. (*see* Note 7)

1. Inoculate 25mL LB + 100µg/ml kanamycin antibiotic with a single colony of BL21 (DE3) *Escherichia coli* that harbors the construct of interest and grow overnight at 37 °C.
2. Dilute overnight cultures 1:50 into LB + 10 µg/ml kanamycin and grow at 37 °C in an orbital shaker at 180 RPM for 2-4 h. Decrease temperature to 16 °C when optical density at 600 nm (OD_{600}) is 0.4. Add IPTG to 500µM when OD_{600} is 0.5. Incubate overnight at 16 °C (*see* Note 8). *Perform steps 3-13 at 4 °C*

⁴Sodium nitrite is a strong oxidizer. Do not dispense near flammable compounds.

⁵Alkali metal ion adducts (Na^+ and K^+) will obscure identification of protein SNOs [23].

⁶We utilized an Orbitrap Fusion Tribrid with XCalibur and Protein Deconvolution 3.0 (all from Thermo Scientific [24,17]), but any high-resolution mass spectrometer equipped for intact protein direct infusion is suitable. Protein mass increases by 29 Da per nitrosation, whereas a sodium adduct ($[\text{M} - \text{H}^+ + \text{Na}^+]$) adds 22 Da. This amounts to 0.3 m/z units for the AtGSNOR +20 charge state. Since alkali metals are ubiquitous, the mass spectrometer should have a resolving power of at least 20,000 m/z (full width at half peak maximum) to distinguish nitrosation from salt adduction. A TQ mass spectrometer with a nanospray source may also be feasible [16].

⁷We developed this protocol for GSNOR orthologues expressed from pET16 and pET28 as N-terminal hexahistidine fusions. We have subsequently adapted this procedure for purification of tag-free AtGSNOR using the pET23_SUMO/Ulp protease system [25]. Other transgene-vector combinations will entail optimization.

3. Centrifuge cultures at $10,000 \times g$ for 10 min at 4 °C, discard supernatants, and re-suspend cells in 30mL lysis buffer per liter of culture.
4. Lyse cells by sonication. Place cell suspension in a 50mL conical tube surrounded by ice. Insert sonicator probe into the cell suspension such that the bottom of the probe is 3cm from the bottom of the conical tube. Sonicate with 3s pulses at 50% maximum intensity followed by 6s of rest. Sonicate for a total of 4min and 30 s (of which 1min and 30s are sonication and 3min are rest).
5. *Optional:* Lyse cells further by microfluidic shearing. After sonication, pour cell debris suspension into the Microfluidizer. Cover the shearing cell with ice. Apply 100 MPa of pressure and collect the lysate. Pour lysate back into the Microfluidizer and repeat 2-4 more times.
6. Centrifuge lysate at $18,000 \times g$ for 15 min at 4 °C to pellet insoluble material and recover the cell-free supernatant (CFS) in a new tube.
7. Equilibrate Fast flow nickel resin with 6 column volumes (CV) lysis buffer. Mix by gently inverting the tube. Avoid pipetting the resin during equilibration. Incubate on ice for 5 min. Spin at $1,000 \times g$ for 10 s to collect resin, discard supernatant, and repeat with another 6 CV lysis buffer. Incubate on ice 5 min, spin at $1,000 \times g$ for 10 s, discard supernatant, and mix resin with 1 CV lysis buffer.
8. Gently pipet equilibrated nickel sepharose resin into the CFS and incubate at 4 °C on an orbital shaker at 100 RPM for 30-60 min. Spin at $1,000 \times g$ for 5 min and discard the supernatant.
9. Carefully re-suspend resin in 3 CV of lysis buffer. Incubate on ice for 5 min, spin at $1,000 \times g$ for 10 s, and discard the supernatant. Repeat this step 3 times for a total of 4 washes.
10. To elute protein, re-suspend resin in 3 CV of elution buffer, shake at 100 RPM for 15 min, spin at $1,000 \times g$ for 10 s, and recover the supernatant (*see*^{Note 9}).
11. Prior to buffer exchange, fill a 15mL Amicon concentrator to capacity with double distilled water. Pipet up and down with colorless barrier pipet tips, pipet off 90% of the volume, and fill again. Repeat this step two more times for a total of three rinses.
12. Fill the rinsed Amicon concentrators to capacity a fourth time and spin at $5,000 \times g$ in a fixed angle rotor centrifuge. Discard flow-through, but do not aspirate water retained on the filter. Repeat this step once for a total of two centrifugations. The concentrators are now ready to use.

⁸Protein yield seems to increase if the antibiotic concentration is lowered to 10% upon scale-up (unpublished observations), possibly by reducing environmental stress. Induce protein expression with less IPTG (50-200μM) and higher O.D.₆₀₀ (0.8-2.0), and lower the incubation temperature to 12-25 °C after induction to optimize yield of aggregation-prone proteins (e.g., AtGSNOR^{C3→A3}). Test multiple IPTG and antibiotic concentration combinations at different temperatures.

⁹Regenerate nickel sepharose by washing with 10 CV 50mM ethylenediaminetetraacetic acid (EDTA) followed by 10 CV milliQ water, 10 CV 10mM nickel sulfate, and 10 CV milliQ water. Store in 20% (v/v) ethanol.

13. Buffer exchange the eluted protein with storage buffer using the washed 15mL Amicon ultra concentrator. Pipet the eluted protein into the filter chamber and fill to capacity with storage buffer. Centrifuge at $5,000 \times g$ until 90% of the solvent has flowed through (13.5mL for a 15mL Amicon). Repeat this step twice for a total of three buffer exchanges.
14. Aliquot purified protein into multiple microcentrifuge tubes, flash freeze in liquid nitrogen, and store at -80°C for further use. This is a convenient stopping point for a day.
15. Pipet 10 μL of purified, buffer-exchanged protein into 90 μL of double distilled water to obtain a 1:10 dilution. Use the 1:10 dilution to prepare 25 μL each of 1:15, 1:30, 1:60, and 1:100 dilutions.
16. Determine protein concentration using the BCA method. Pipet 10 μL of five known quantities of bovine serum albumin (BSA) in duplicate in a 96-well microtiter plate. Pipet 10 μL of each purified protein dilution in duplicate in the microtiter plate. Also, pipet 10 μL of double distilled water in duplicate for the blank. Pipet 200 μL of the BCA reagent mixture onto each BSA, purified protein dilution, and water sample. Incubate the plate at 37°C for 30 minutes and record absorbance at 562nm. Subtract the water sample absorbance from all other absorbance values and plot a linear regression of absorbance as a function of BSA quantity. Use the linear regression to estimate the quantity of purified protein. Multiply each estimated purified protein quantity by its dilution factor to calculate an estimated concentration of the purified protein stock.
17. To estimate protein purity, resolve 10 μg purified protein on a 4-15% polyacrylamide SDS gel and perform densitometry after Coomassie staining (see Note 10).
18. If protein is >90% pure, validate protein concentration with a protein specific extinction coefficient (for AtGSNOR, $\epsilon_{280} = 42,400 \text{ M}^{-1} \cdot \text{cm}^{-1}$).
If samples are 80% pure, perform size exclusion chromatography (steps 19-20).
19. Equilibrate size exclusion column with DTT-free storage buffer. When A_{280} and conductivity traces have settled, inject sample from step 7. Run the column isocratically at 1mL/min (see Note 11)
20. Pool, concentrate, and buffer exchange relevant fractions (Fig. 1).

¹⁰Estimate relative purity with ImageJ/FIJI [26], available online for free. Draw a box the height and width of a gel lane and calculate the fraction of area under the curve belonging to the band of interest (monomeric AtGSNOR expressed from pET28b has a molecular mass of 42.7 kDa). For greater dynamic range, stain gels with Coomassie G-250, scan with the 700nm channel of a Li-Cor ® Odyssey, and analyze with Image Studio 4.

¹¹At 1 mL/min, the column will equilibrate in about 10 h. We observed AtGSNOR^{C3→A3} and human GSNOR mutants (HsGSNOR^{C195A} and HsGSNOR^{C268A}) to elute between 3 and 4 h after injection. Calibrate new columns with protein standards since retention times may vary slightly. Increase flow rates to as high as 3 and 2 mL/min for equilibration and analyte separation, respectively, without affecting peak separation.

3.2. In vitro nitrosation of GSNOR

SNOs are photosensitive. Minimize exposure of samples and reagents to light. Steps 1-6 will require approximately 5 h.

1. Prepare 100mM S-nitrosocysteine (CysNO): pipet 100 μ L 400mM CysSH into 100 μ L 200mM NaNO₂. The solution will turn bright red after 3 s. Place on ice and shield from light with a lid or opaque cloth. The reaction will reach completion in approximately 30 min [19]. For the control, prepare 200mM CysSH with MS-water instead of NaNO₂.
2. Thaw purified protein on the bench top. Once thawed but still cold, immediately spin at 16,000 $\times g$ for 5 mins at 4 °C to pellet any aggregated protein. Determine the protein concentration of the supernatant. Place the tube on ice.
3. In a microcentrifuge tube at room temperature, add the following in order: 152 μ L 20mM Tris pH 8, 6 μ L 10mM DTT, 40 μ L 100 μ M purified GSNOR. Gently pipet up and down after each addition. Add 2 μ L CysNO (or CysSH as a control) to initiate nitrosation and immediately shield samples from light. Incubate at room temperature for 30 min. As an additional control to block S-nitrosation, prepare a sample with CysNO in which the DTT concentration is 5mM (5 μ L 200mM DTT). Cool 20mM NH₄Ac to 4 °C (*see* ^{Note 12}).
4. Spin samples at 16,000 $\times g$ for 5 min at 4 °C to pellet insoluble material. Apply supernatants to pre-washed 0.5mL concentrators, add 300 μ L of 4 °C 20 mM NH₄Ac, and spin samples at 12,000 $\times g$ at 4 °C until retentate volumes are 100 μ L or less (about 10 min).
5. Discard the flow-through, add 400 μ L NH₄Ac to samples, and spin at 12,000 $\times g$ again. Repeat this step at least 5 times to afford a Tris concentration of 1.5 μ M, or a dilution factor of 15,000 for all components except the filter-retained protein.
6. Determine protein concentration using the BCA method. Proceed directly to analytical methods or flash freeze proteins in liquid nitrogen and store at -80 °C for later use (*see* ^{Note 13}).

¹²Longer incubations will increase the proportion of GSNOR that is di- and trinitrosated. Previous work with cysteine to alanine mutants suggested AtGSNOR cysteine 370 is the most kinetically efficient nitrosation target, while cysteines 10 and 271 have comparable kinetics [13]. However, the fractional distribution of Cys10-, Cys271- and Cys370- mono- and di-nitrosated AtGSNOR that comprise the two ensembles is unknown. We also have not yet been able to obtain an AtGSNOR sample that is completely trinitrosated. Prior DTT treatment of GSNOR ensures reduction of all GSNOR cysteines before CysNO addition. Samples containing a molar excess of DTT (5mM) will not exhibit GSNOR S-nitrosation—DTT will reduce all SNOs in the milieu (including CysNO). Adjust sample volumes to achieve final concentrations of 300 μ M DTT, 20 μ M GSNOR, and 1mM CysSH/NO. In addition to CysNO, try other nitroso group donors such as S-nitrosoglutathione (GSNO), S-nitroso-N-acetylpenicillamine (SNAP), and sodium nitroprusside (SNP). Note that assay time and reagent concentrations may require modification.

¹³Both the abundance of AtGSNOR SNO adducts as observed by mass spectrometry and the differential effect of nitrosation on enzymatic activity gradually diminish with repeated freeze-thaw cycles (unpublished observations). We still observe qualitative differences between S-nitrosated and control samples after two or more freeze-thaws. Nevertheless, for robust quantitative measurements, proceed directly from CysNO treatment to mass spectrometry.

3.3. Intact protein mass spectrometry

1. Ensure the mass spectrometer is calibrated. Consult the Orbitrap Fusion maintenance guide for assistance with calibration [17]. Install a heated electrospray ionization source. In the tune window, set the following parameters: Mode = intact protein, ion transfer tube = 275 °C, ion trap = orbitrap, resolution = 60,000 FWHM, spray voltage = 3500 V (positive ionization), AGC target = 4×10^5 , S lens = 60%, sheath gas = 5, auxiliary gas = 5, m/z scan range = 1000-3000. Allow the mass spectrometer source temperatures to stabilize (see Note 14).
2. Thoroughly rinse infusion tubing to remove any contaminants by manually infusing multiple syringe volumes (2-4 times) of MS-CH₃OH into the source in 10-15 s.
3. Mix 20 µL of sample protein from part 2 step 6 with 180 µL acidified CH₃OH (see Note 15).
4. Infuse 10-20 µL of denatured protein into the mass spectrometer with the Hamilton syringe. An ion series distinct from background signal should appear in the tune window. Install the syringe in the syringe pump, set the flow rate to 3 µL/min, and begin recording data.
5. Open the data file in XCalibur and observe the summed ion chromatogram after 1 min of acquisition. The ion series should begin to exhibit signal peaks at predictable m/z values (see Note 16). Continue collecting data until nitrosation peaks resolve from neighboring minor adducts, usually about 10 min per sample (Fig. 2).
6. If samples contain PEG (Fig. 3), increase the sheath and auxiliary gas flow rates and/or apply source fragmentation energy (see Note 17).

¹⁴Emitter age and distance from the ESI source determine the voltage required for electrospray [27]. Sheath and auxiliary gas values are given in arbitrary units and may require adjustment for different mass spectrometers. The number of charges carried by a protein dictates optimal m/z range for measurement; unfolded proteins exhibit signal at lower m/z because they can accommodate more charges than compact folded proteins.

¹⁵Dilution of protein samples to 10 µM in 50:50 water:methanol with subsequent addition of acetic acid to 3 % is the standard IP-ESI-MS methodology. We used a final volumetric ratio of aqueous/CH₃OH/acetic acid of 49/47/4. This ratio is not critical, as long as there is sufficient methanol to unfold the protein and assist in desolvation, and adequate acidity to promote protein ionization.

¹⁶For example, hexahistidine-tagged, unmodified *Arabidopsis thaliana* GSNOR (AtGSNOR, Genbank accession AED95034.1) will have sharp peaks at m/z ~1942 (+22), ~1858 (+23), and ~1780 (+24). CysNO-treated AtGSNOR will produce additional signal intensities absent from the untreated sample (e.g., m/z ~1943.5, ~1944.7, and ~1946 in the +22 charge state, indicative of mono-, di-, and tri-nitrosation, respectively). These extra signals will be absent from samples simultaneously treated with CysNO and 5mM DTT. Both samples will also contain peaks corresponding to N-gluconoylation, a glycosylation frequently observed in *E. coli*-expressed recombinant proteins (see ref. [28]). We have also recently confirmed that tag-free AtGSNOR expressed with the pET23-SUMO/Ulp protease system (See Note 7) produces a similar ion series to that of His-tagged AtGSNOR, apart from a lack of N-gluconoylated adducts.

¹⁷PEG-contaminated protein samples display one or more symmetrical, isotopically-resolved charge states with variable numbers ethylene glycol oligomers differing in mass by 44 Da (see Figure 3). While PEG suppresses protein ionization, one may still be able to optimize protein signal if PEG contamination is minor. Adjust the scan range to 1200-3000 m/z to avoid acquiring signal from PEG. Increasing sheath and auxiliary gas flow rates from 5 to 20 enhances solvent nebulization at the source, and counter-current auxiliary gas deflects low charge state PEG species, thereby increasing the protein to PEG signal ratio at the expense of total ion intensity. 30-50 eV of in-source fragmentation may promote PEG-protein ion cluster dissociation. Signal to noise ratios improve proportionally with the square root of acquisition time.

7. Once measurements are complete, empty the Hamilton syringe, rinse syringe and PEEK tubing at least three times with CH₃OH to avoid cross-contamination, and apply the next sample.
8. Deconvolve spectra to measure accurate intact protein molecular masses with Protein Deconvolution 3 (Thermo) in isotopically unresolved mode (Manual ReSpect) (*see* Note 18).
9. In the Process and Review tab, apply these thresholds: charge carrier = [H⁺], mass range = 40,000-50,000 Da, 10 charge states 100, mass tolerance = ± 20 ppm (0.855 Da for AtGSNOR), relative abundance threshold = 5%, noise reduction = 95% confidence, iterations = 3. Click “Process.” The resultant mass profile comprises peaks that represent average masses calculated from homologous signals in constituent charge states and weighted by intensity (Fig. 4). Nitrosated adducts differ from unmodified protein masses by multiples of 29 Da (*see* Note 19).
10. Check the mass profile for other possible modifications such as oxidation (+16 Da), glutathionylation (+ 306 Da) or cysteinylolation (+ 119 Da) (*see* Note 20).
11. Calculate fractional abundance by dividing the peak intensity for a particular GSNOR species (unmodified, mono-, di-, or tri-nitrosated GSNOR) by the sum of intensities for all four GSNOR species. The Process and Review tab results table contains intensity values (*see* Note 21).

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¹⁸Perform deconvolution as stated here or with other software. Baseline correction, peak smoothing, and centroiding can reduce background noise if signal is sufficiently abundant. In Protein Deconvolution 3.0 (Thermo), deconvolved peak widths represent standard error of mass determination, while peak heights are proportional to signal intensities averaged across all charge states relative to the combined ion intensity of the most abundant protein adduct.

¹⁹The calculated mass shift due to S-nitrosation may not exactly equal the theoretical mass increase (29 Da), particularly if signal intensity is low or if salt ions are present. 1 Da deviation in either direction (28 or 30 Da) is not uncommon. One can be reasonably confident that S-nitrosation explains a protein's mass increase if the mass shift is only observed in the presence of nitroso donors over replicate experiments.

²⁰Automated deconvolution can reveal more mass variants for control samples than suggested by a Coomassie-stained gel. Background noise, salt adducts, and N-terminal gluconoylation (+178 Da)—a common modification of heterologous proteins expressed in *E. coli* [28]—are all contributing factors. These additional mass variants will not prohibit SNO identification, but we advise the researcher to determine if nitroso donors affect their abundance. To simplify presentation, one may cull low abundance adducts from mass profiles during deconvolution by increasing the relative abundance cutoff (that is, the percent of the intensity of the most abundant species beneath which no masses will be displayed).

²¹The relationship between nitroso donor concentration and relative SNO content is reproducibly precise in replicate experiments with fresh samples (*see* Note 13). We observe CysNO-treated wild-type AtGSNOR to be approximately 20% unmodified, 30% mono-, 35% di-, and 15% tri-nitrosated after a 30 min incubation at room temperature. Mutation of cysteines 10, 271, and/or 370 to alanine decreases both the number of nitrosated adducts and their relative abundance [13]. Note that abundance ratios are *relative* and depend on relative ion intensity. Absolute quantification (in moles) would require a standard curve of ion intensity as a function of increasing unmodified AtGSNOR concentrations. While theoretically possible, one must exercise caution as some SNO adducts may ionize more efficiently than others. We expect different instruments to produce similar, but not identical, results. Differences in mass spectrometer source voltages and temperatures could alter the lability of SNO adducts. To increase precision, the time between the end of CysNO treatment and the injection of samples onto the mass spectrometer should be as similar as possible in replicate experiments (between 150 and 180 minutes in our hands).

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Figure 1.

AtGSNOR^{C₃→A₃} electrophoretic purity (E.P.) improves with size exclusion chromatography (SEC). A: Coomassie-stained 10% acrylamide SDS gel of AtGSNOR wild-type and the C₃→A₃ mutant after purification with nickel sepharose (Ni) or additional SEC. E.P. was estimated by ImageJ with the straight line tool to measure area under the curve. The numbers and arrow to the left denote molecular masses of protein standards and the predicted size of monomeric AtGSNOR, respectively. B: AtGSNOR^{C₃→A₃} size exclusion chromatogram (A₂₈₀ trace) with pooled fractions indicated.

Figure 2.

AtGSNOR extracted ion chromatograms, +22 and +23 charge states. A: AtGSNOR incubated with CysSH. In addition to unmodified AtGSNOR (black arrowheads), note a gluconated form (GLC, red asterisks) and a salt adduct ($\text{Na}^+ + 2\text{K}^+$). B: AtGSNOR incubated with CysNO. In addition to unmodified AtGSNOR (black arrowheads), note mono-, di-, and tri-nitrosated adducts (SNO_x).

Figure 3.

Nebulizer gas modulation reduces ion suppression by PEG. A: AtGSNOR^{C3→A3} copurifies with 6-7 kDa PEG mixtures. B: PEG suppresses protein ionization (+22 state emphasized). C,D: Quadrupled sheath and auxiliary gas fluence improves protein signal quality.

Figure 4.

Deconvolution reveals the mass and distribution of nitrosated AtGSNOR adducts. A: Wildtype CysSH-treated AtGSNOR mass profile (5% relative abundance threshold). Peaks represent species with ion intensities $\geq 5\%$ the intensity of the most abundant peak (i.e., unmodified AtGSNOR). This electrophoretically “pure” sample (see Fig. 1A, lane 1) comprises many adducts. Culprits include source fragmentation (e.g. H₂O loss), salt cations from purification buffers (Na⁺ and K⁺), in vivo covalent modification (e.g., gluconation), or processes concomitant with CysSH treatment. Gluc: N-gluconoylation. Native AtGSNOR expressed from pET23 (see Notes 7 and 16) does not exhibit gluconoylated species. B: Mass profile after incubation with CysNO. Species separated by multiples of 29 Da correspond to nitrosated adducts. Note that while unassigned peaks (denoted by (?)) and salt adducts are common to both samples, nitrosated masses (AtGSNOR +29, 60, 90 Da) are only observed in the presence of CysNO. C and D: A and B, respectively, with 30% relative abundance cutoffs. The higher abundance threshold helps to emphasize the impact of the nitroso donor on the partitioning of unmodified AtGSNOR into mono-, di-, and trinitrosated forms. Black arrowheads denote unmodified AtGSNOR.