Identification and Quantification of S-Nitrosylation by Cysteine Reactive Tandem Mass Tag Switch Assay*\[5\]

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Redox-switches are critical cysteine thiols that are modified in response to changes in the cell’s environment. S-nitrosylation (SNO) is emerging as an important modulator of these regulatory switches; however, much remains unknown about the nature of these specific cysteine residues and how oxidative signals are interpreted. Because of their labile nature, SNO-modifications are routinely detected using the biotin switch assay. Here, a new isotope coded cysteine thiol-reactive multiplex reagent, cysTMT$^6$, is used in place of biotin, for the specific detection of SNO-modifications and determination of individual protein thiol-reactivity. S-nitrosylation was measured in human pulmonary arterial endothelia cells in vitro and in vivo using the cysTMT$^6$ quantitative switch assay coupled with mass spectrometry. Cell lysates were treated with S-nitrosoglutathione and used to identify 220 SNO-modified cysteines on 179 proteins. Using this approach it was possible to discriminate potential artifacts including instances of reduced protein disulfide bonds and S-glutathionylation as well as diminished ambiguity in site assignment. Quantitative analysis over a range of NO-donor concentrations revealed a continuum of reactivity to SNO-modification. Cysteine response was validated in living cells, demonstrating a greater number of less sensitive cysteine residues are modified with increasing oxidative stimuli. Of note, the majority of available cysteines were found to be unmodified in the current treatment suggesting significant additional capacity for oxidative modifications. These results indicate a possible mechanism for the cell to gauge the magnitude of oxidative stimuli through the progressive and specific accumulation of modified redox-switches. Molecular & Cellular Proteomics 11: 10.1074/mcp.M111.013441, 1–12, 2012.

Changes in the oxidative balance can affect many aspects of cellular physiology through redox-signaling (1, 2). Oxidative species modify critical cysteine thiols, known as redox-switches, which sense and respond to the cell’s fluctuating environment (3, 4). Depending on the magnitude, these fluctuations can affect normal metabolic processes, activate protective mechanisms or be cytotoxic. Redox-signaling is thought to derive from the integration of the type and concentration of oxidizing species, their associated chemical biology and cellular localization (5–7). However, less is known about the nature of the cysteine residues targeted or how oxidative signals are interpreted within the cell.

S-nitrosylation (SNO), also known as S-nitrosation, is emerging as an important regulatory post-translational modification in many cellular processes (8). This modification is the result of the covalent addition of an NO group to a cysteine thiol; however, the specific mechanism of this addition has not been fully determined (9). SNO possesses the essential criteria for a signaling modification including a rapid reaction, specificity and enzymatic reduction (10). SNO has been associated with a variety of diseases making it the subject of intensifying research interest (8). Nitric oxide stimulation has been found to generate a multitude of biological responses from protective to cytotoxic which can be stratified based on concentration (7) suggesting that the reactivity of specific redox-switches may play a role in regulating these effects.

Because of their labile nature, SNO-modifications can be difficult to study with traditional biochemical techniques. In 2001, Jaffrey and Snyder introduced the biotin switch assay which utilizes a replacement strategy to stably label SNO-modified cysteines allowing their detection and identification (11, 12). Replacement is achieved by first blocking free thiols with an alkylating agent then reducing SNO-modifications from protective to cytotoxic which can be stratified based on concentration (7) suggesting that the reactivity of specific redox-switches may play a role in regulating these effects.

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previously SNO-modified thiols (Fig. 1A). Once labeled, biotinylated proteins can be easily detected or enriched with streptavidin. Labeled peptides can also be captured and analyzed by MS, providing large-scale SNO-site identification (13, 14). Despite the popularity of this technique, limitations have been identified which have been reviewed in (15, 16). Common critiques include the use of ascorbate as the specific reducing agent which is suspected of reducing disulfide bonds or other oxidative modifications and the lack of a permanent label at the modified cysteine residue that is detectable by MS analysis which can lead to ambiguity in site identifications. Each of these concerns has the potential to increase the incidence of false-positive results. Different variations of the assay offer several accommodations for these issues (13, 17–22); however, there is currently no unified solution.

Here, we present a new approach to the biotin switch assay using the cysteine reactive tandem mass tag (cysTMT) reagent to specifically detect, identify, and quantify SNO-modified sites. CysTMT\textsuperscript{6} is a thiol reactive version of tandem mass tag that has been established for multiplex mass spectrometry analysis (23). This new reagent fulfills the requirements for a biotin switch label and offers some distinct advantages, including a permanent mass tag and the fragmentation of up to 6 isotopically balanced reporter ions between 126–131 Da permitting multiplex quantification. Using this technique we demonstrate specific detection of SNO-modified sites and quantify the response of individual cysteine residues to GSNO treatment by mapping the continuum of protein thiol-reactivity to SNO-modification.

**EXPERIMENTAL PROCEDURES**

**Reagents**—cysTMT\textsuperscript{6}, cysTMT\textsuperscript{0}, N-[6-(Biotinamido)hexyl]-3’-[[2-pyridyldithio)]propionamide (biotin-HPDP), TMT affinity resin, Streptavidin Plus UltraLink Resin and Zeba desalt spin columns were from Thermo Fisher Scientific (www.thermoscientific.com). Stock solutions of S-nitrocysteine (SNO-Cys) were prepared fresh before each experiment according to the protocol outlined by Park and Kosta (24) scaled up 10 fold. Prepared solutions were found to contain 6–7 mM SNO-Cys. All other reagents including 3-nitrosoglutathione (GSNO) and other chemicals were obtained from Sigma-Aldrich (www.sigmaaldrich.com).

**Cell Culture**—Human pulmonary arterial endothelia cells (HPAEC) (Lonza, www.lonza.com) were grown in EGM®-2 Endothelial Cell Growth Medium-2 (Lonza). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO\textsubscript{2} and used between passages 6 and 9.

**Western Blot Analysis**—Proteins were separated by SDS-PAGE NuPage 4–12%, 1-mm gels (Invitrogen, www.invitrogen.com) with 2-(N-morpholino)ethanesulfonic acid running buffer. Proteins were transferred to nitrocellulose membranes which were blocked for 1 h with 5% (w/v) nonfat milk powder in TBS-t (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% (v/v) tween 20). CysTMT labeled proteins were detected by incubating with anti-TMT primary antibody (Thermo Fisher Scientific) followed by anti-mouse alkaline phosphatase conjugated secondary antibody (Jackson ImmunoResearch Inc., www.jacksonimmuno.com) in blocking solution. Biotin labeled proteins were detected by incubation with streptavidin conjugated alkaline phosphatase (Jackson ImmunoResearch Inc.). Blots were washed overnight at 4 °C and developed using immun-star substrate (Bio-Rad, www.Bio-Rad.com).

In *Vitro Detection of S-nitrosylated Proteins by Biotin/cysTMT\textsuperscript{6} Switch Assay*—SNO-modifications were detected in GSNO treated HPAEC lysates using the biotin switch assay (12, 14) (discussed in 14, 16, 25), see Fig. 1A for reaction schema). HPAECs were lysed in 20 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EDTA containing 1.0% (v/v) triton X-100 using a probe sonicator and centrifuged for 10 min at 2000 × g. The protein concentration of the resulting supernatant was determined by bicinchoninic acid assay and diluted to 0.8 μg/μl in HEN (250 mM HEPES pH 7.4, 1 mM EDTA and 0.1 mM neocuproine) including 0.4% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate. 200 μg of cell lysate were treated with 2, 10 or 20 μM GSNO or three different control treatments (untreated vehicle, 20 μM reduced glutathione (GSH) or 10 μM oxidized glutathione (GSiGG)) for 15 min at 37 °C (n = 3). GSNO is commonly used as the NO-donor for *in vitro* investigations due to its relative ease of use and because small thiol-containing molecules including glutathione are thought to play an important role in intracellular SNO-modification (8, 26–29). To control for the possibility that the glutathione carrier may modify some cysteine thiols, reduced and oxidized glutathione were used as donor controls. All steps were performed in the dark or protected from light. Treatment compounds were removed using an HEN equilibrated Zeba desalt spin column according to the manufacturer’s protocol. The remaining free thiols were blocked with 20 mM N-ethylmaleimide (NEM) in the presence of 2.5% (w/v) SDS and incubated for 20 min at 50 °C. Excess NEM was removed by acetone precipitation. As a positive control, an additional untreated sample was processed but not blocked with NEM. This allowed for the labeling of all the endogenously available cysteine residues in the lysate. SNO-modified thiols were reduced and labeled by resuspending pellets in HENS (HEN containing 1.0% (w/v) SDS), 1 mM ascorbate, 1 mM CuSO\textsubscript{4} and 0.8 mM Biotin-HPDP, cysTMT\textsuperscript{6} or, in the case of the positive controls, cysTMT\textsuperscript{0} reagent and incubated at room temp for 2 h. CuSO\textsubscript{4} was included as it has been found to increase the sensitivity but not affect specificity of the ascorbate reduction of SNO-modifications (30). TMT samples were labeled with cysTMT\textsuperscript{126–131} in the order; untreated, GSH, GSSG, 2, 10 and 20 μM GSNO for two of the replicates and labeled in reverse order for the third. Data obtained from reverse TMT labeling have been reordered in the data table for ease of analysis. Excess label was removed by acetone precipitation (2 volumes) and the resultant pellets were carefully washed with an additional volume of acetone. Pellets were resuspended to 5 μg/μl with HENS. For gel electrophoresis analysis, 200 μg of biotin-HPDP labeled protein were diluted 20 fold in neutralization buffer (20 mM HEPES, 150 mM, NaCl 1 mM EDTA, 0.5% (v/v) triton X-100). Biotinylated proteins were captured by incubation with 15 μl of washed, packed ultrakink immobilized streptavidin beads for 1 h at room temperature. Beads were washed with 4 × 50 bead volumes of wash buffer (20 mM HEPES, 600 mM NaCl 1 mM EDTA, 0.5% (v/v) triton X-100) and 2 × with elution buffer (20 mM HEPES pH 7.7, 100 mM NaCl, 1 mM EDTA). Captured proteins were eluted with 40 μl of elution buffer containing 100 mM dithiothreitol. Eluted samples were mixed with 15 μl of 4 × LDS sample buffer, boiled, separated by SDS-PAGE and silver stained according to the protocol described in (31).

**Mass Spectrometry**—For MS studies and quantification, 200 μg of each cysTMT\textsuperscript{6} sample were combined (1200 μg total), diluted to 1.8 ml with TBS pH 7.0, passed through a Zeba desalt spin column equilibrated with TBS. The resulting samples were adjusted to 0.02% (v/v) Rapigest\textsuperscript{TM} SF (Waters, www.waters.com) and digested overnight with 30 μg of trypsin (Promega, www.promega.com) at 37 °C. Digestions were halted by 0.25 mM phenylmethylsulfonil fluoride and then TMT labeled peptides were captured by 2 h incubation with 600
μl of TMT affinity resin slurry at room temperature. Unlabeled peptides were removed by washing with 3 × 5 ml of TBS, 3 × 5 ml TBS containing 4 μl urea followed by 3 × 5 ml of TBS. Beads were then incubated for 2 h at room temperature followed by 3 × 5 ml washes of TBS and then 2 × 5 ml ddm-H2O to reduce buffering capacity and salt content. CysTMT labeled peptides were eluted with 2 × 600 μl portions of 50% (v/v) ACN/0.4% (v/v) trifluoroacetic acid and combined. Samples were dried in a speed vac and then cleaned using a detergent removal spin column (Thermo Fisher Scientific) and C18 UltraMicroSpin™ column (Nest Group, www.nestgrp.com) both according to the manufacturer’s protocol.

Peptide identification by liquid chromatography/tandem mass spectrometry (LC/MS/MS) analysis was performed using an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) interfaced with a NanoAcuity UPLC system (Waters, www.waters.com) using Xcalibur 2.1. Peptides were loaded on to a hand packed column consisting of 75 μm × 15 cm of Michrom Magic C18 (5 μm particles with 100 Å pore size) at 750 nL/min for 15 min at 3% B (B = 90% (v/v) ACN 0.1% (v/v) formic acid, A = 0.1% (v/v) formic acid). Elution was performed at 300 nL/min by increasing the gradient to 55% B over 95 min before a bump to 100% B for 4 min followed by and re-equilibration back to 3% B for 10 min. Precursors were acquired at 30,000 linear response and greater than 1.1 were reactive but considered less sensitive to the NO-donor. Nonlinear regression was not performed for the most insensitive sites, where signal was only detected in the 20 μM GSNO, instead a reactivity value of 3.5 was assigned.

**Consensus Sequence Analysis**—Twenty-five target cysteine residues were selected from each of the response groups and 20 residues on either side of each site were submitted for consensus sequence analysis. After alignment with ClustalW2 (www.ebi.ac.uk/Tools/clustalw2/index.html), the sequences were screened for conserved motifs using the CONSENSUS, TEIRESIAS, and PRATT algorithms (http://ccct.cit.nih.gov/Alignment/consenus.html; http://cbcsrv.watson.ibm.com/Tspd.html; www.ebi.ac.uk/Tools/pratt/index.html), respectively (35). Frequencies of flanking residues were computed with WebLogo (http://weblogo.berkeley.edu). For secondary structure and solvent accessibility predictions the NetSurfP server was employed (www.cbs.dtu.dk/services/NetSurfP) (36). Prediction of pKₐ for SNO-modified cysteine residues was performed using PROPKA 3.0 (http://propka.ki.ku.dk/) for those target cysteine residues where high quality crystal structures were available from the Protein Database (PDB) (supplemental Table S7) (37, 38). Hydrobicity analysis was performed on the 10 residues flanking the modified cysteine using the hydrophobicity plotter from InnoRagen (www.innovagen.se). Disulfide bond prediction was performed using protein FASTA sequences and the DIANNA prediction program (39).

**In Vivo Detection of SNO-Modifications**—For in vivo experiments, HPAECs were grown as described above and used between passages 7–9. When the cells reached ~95% confluence, the growth media was removed followed by two washes with PBS and replaced with serum free endothelial cell basal medium. Cells were treated with 1 mM L-NG-Nitroarginine methyl ester (L-NAME, a nitric oxide synthase inhibitor) (90 min), 200 μM Cys (20 min), 50 μM SNO-Cys (20 min), 200 μM SNO-Cys (20 min), or left untreated. SNO-Cys was used as the NO-donor for the in vivo experiments because GSNO does not readily cross cell membranes. SNO-Cys has been found to enter the cell via amino acid transporters to induce SNO-modified thiols, possibly by transfer of its NO group to an intracellular glutathione molecule (13, 40). Following treatments, media was aspirated and cells were washed once with PBS and then lysed by scraping in 250 μl HEPES, 1 mM EDTA, 0.1 mM neocuprine, 1.0% (w/v) Triton X-100, 20 mM NEM. Lysates were briefly sonicated and then centrifuged for 5 min at 3000 × g. Resulting supernatants were adjusted to 2.5% (w/v) SDS and incubated for 20 min at 50 °C. As a blocking control, untreated samples were lysed in the absence of NEM, treated with 5 mM dithiotreitol (DTT) for 20 min to reduce all cysteine residues and then blocked with NEM. After blocking, excess NEM was removed by acetone precipitation. Pellets were resuspended in HENS buffer and divided, 15% was labeled with biotin–HPDP and 85% with cysTMT® each according to the procedure described above. TMT samples were labeled with cysTMT®128–131 in the order; untreated/DTT, L-NAME, untreated, 200 μM Cys, 50 and 200 μM SNO-cys for two of the replicates and in reverse order for the third. Reporter ion intensities obtained from reverse TMT labeling have been reordered in the data table for ease of analysis. Samples were normalized by bicinchoninic acid assay before combining and then processed, captured and analyzed as described above. For in vivo experiments, data were searched using Mascot (Matrix Science, www.matrixscience.com) with Proteome Discoverer version 1.2 (Thermo Fisher Scientific).
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**RESULTS**

Comparison of cysTMT Reagent to Biotin-HPDP in Biotin Switch Assay—The suitability of the cysTMT reagent for SNO detection was initially assessed by comparison with the widely used biotin-HPDP. Both reagents have the same pyridyldithiol reactive group and are a similar size suggesting they should label available thiols equivalently (Fig. 1B and C). Western blot analysis of HPAEC lysates subjected to the biotin switch demonstrated that cysteine labeling is essentially equivalent between the two reagents for each condition (Fig. 1D).

Detection and Site Mapping of SNO-modifications In Vitro—The standard biotin switch assay was used to reveal the presence of NO-induced modifications in GSNO-treated HPAEC lysates (Fig. 2A). Silver stain gel analysis shows a clear increase in the amount of protein labeled following GSNO treatment when compared with the untreated, GSH or GSSG treated samples (n = 3), indicating these proteins are modified by NO. Samples treated with 2, 10 and 20 µM GSNO displayed an increasing level of SNO-modification (Fig 2A, lanes 5–7). Faint background bands could be observed in the controls lanes (Fig. 2A, lanes 2–4), and are likely the result of incomplete NEM blocking. Untreated and unblocked samples resulted in the labeling of all available cysteine residues. A marked increase in the extent and number of proteins labeled was seen in this condition compared with SNO-modified proteins (Fig. 2A, lanes 5–7 versus 9).

Site-mapping of SNO-modifications was performed using the cysTMT\textsuperscript{6} switch assay (Fig. 2B for experimental schema). The 6-plex MS analysis revealed a total of 220 SNO-modified cysteine residues on 179 different proteins (Fig. 2D for example spectra and Figs. 2E–2G for reporter ion clusters and supplemental Table S1 for all site information and detailed MS data). The specificity of SNO-modification by GSNO was also assessed by comparison to a site-mapping analysis of untreated and unblocked samples (n = 3) (Fig. 2C). This analysis identified a pool of 691 available cysteine residues; 191 of which correspond with SNO-sites indicating that only ∼28% of the available protein thiols are targets of SNO-modification.
under the current conditions (Fig. 2H and supplemental Table S2 for detailed MS data). Although proteome contains many more cysteine residues, the current study was performed under nonreducing and largely non-denaturing conditions and therefore targeted only the accessible and available sites. The majority of cysteine residues are engaged in disulfide bonds, inaccessible, or are otherwise modified and not available for SNO-modification with any NO stimulus. False discovery rates for the in vitro and positive control MS experiments were determined to be 0.5% across all experiments at a minimum protein probability of 0.95. All spectra meeting the minimum identification scores were deposited in the PRIDE public database (http://www.ebi.ac.uk/pride/, accession numbers: 16868–16870) (41).

cysTMT® Provides Detection and Discrimination of S-Nitrosylated Thiols—A persistent criticism of the biotin switch assay has been the use of ascorbate as the SNO-specific reducing agent. Some studies have suggested that it can potentially reduce protein disulfide bonds or other oxidative modification such as glutathionylation, leading to false-positive site identifications (42–44). Using the cysTMT® switch assay, reduced disulfide bonds can be detected by the presence of six approximately equal reporter ion intensities (Fig. 3A). A total of six out of 228 identified cysteine residues displayed a reporter ion signature suggestive of the unintended reduction of a disulfide bond (supplemental Table S3). Of those six cysteine residues, five have been previously implicated in disulfide pairs, either in the literature or by se-
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Fig. 3. cysTMT<sup>6</sup> switch assay enables detection of non-SNO cysteine modifications. Example of average reporter ion intensities for the reduction of a (A) disulfide bond (CD59 glycoprotein Cys 70, n = 2) and (B) donor induced glutathionylation (Dihydropyrimidinase-like 2 Cys 436, n = 1). The disulfide bond is discriminated by the presence of similar reporter ion intensities across all six conditions. In the current study six sites possessed this signature and one site indicated significant glutathionylation (supplemental Table S3). Additionally, examples of possible minor donor glutathionylation are presented in (C) and (D). Insets highlight reporter ion intensity for GSH (127 Da) and GSSG (128 Da) compared with untreated (126 Da) (n = 6 and 9, respectively, * = p < 0.05). Error bars indicate S.E.

Quantification of Thiol-reactivity to S-Nitrosylation—Individual cysteine reactivity was determined by treating cell lysates with a range of GSNO concentrations (2, 10, 20 μM). Analysis of the reporter ion intensities (cysTMT<sup>129–131</sup>) indicated thiols had differential responses to GSNO (supplemental Table S4). Nonlinear regression was performed on the reporter ion intensities across the range GSNO treatments to calculate a slope (m), representing each thiol’s reactivity to SNO-modification (Figs. 4A–4C). Modified cysteine residues were found to respond along a continuum of reactivity from sites which react more readily to SNO-modification to those that are less receptive (Fig. 4D). For the purposes of analysis, modified sites were grouped into three categories. Cysteines with a GSNO-reactivity slope close to 1 (0.9 < m < 1.1, straight line), were considered to have a linear response (Fig. 4B). Sites that reacted more readily (m < 0.9, convex line) with GSNO, were labeled as sensitive (Fig. 4A), while sites that were more resistant (m > 1.1, concave line) were considered less sensitive (Fig. 4C). The majority of modified cysteine residues were in the less sensitive group (126/222) whereas relatively few (32) were assigned as sensitive. It should be noted that in the silver stain analysis, higher abundance proteins in the cell may be preferentially detected by the biotin switch assay (Fig 2A). To determine if this is the case, the differences in thiol-reactivity were compared with the average reporter ion intensities for protein and peptide abundance detected in the MS analysis and no correlation was identified (supplemental Fig. S1A and S1B).

Given the additional information for each GSNO reactive thiol, we considered that the differences in reactivity could be related to a specific physical property of the individual cysteine residues: predicted pKa, solvent exposure, local hydrophobicity, or its linear amino acid sequence (supplemental Fig. S2). Analysis of these structural properties de-
pended on the presence of 3-dimensional x-ray crystallographic structures in the PDB database (www.pdb.org). Since a comprehensive list was not available, a survey of 24 proteins was used, which comprised modified cysteine residues that spanned the range of thiol-reactivity (supplemental Table S7). In the case of a linear amino acid consensus sequence, protein structure was not required. Instead, the 40 flanking amino acid residues from 25 modified cysteine residues were selected from each of the reactivity groups; sensitive, linear, and insensitive. From this sampling, no correlation or consensus sequence emerged for any of the physical properties tested even when thiol-reactivity was considered.

Detection of S-Nitrosylation In Vivo—HPAECs in culture were treated with increasing SNO-Cys concentrations or control compounds. Silver stain analysis of the biotin switch assay revealed an increase in SNO-modified proteins for 50 to 200 μM SNO-Cys treated samples over the control DTT, L-NAME, untreated or Cys treatments (n = 3) (Fig. 5A). CysTMT6 switch assay coupled with MS analysis identified 25 SNO-modified cysteine residues for the 200 μM and 11 for the 50 μM treatments (Fig. 5B and supplemental Table S5 and S6). The number of modified sites is somewhat less than detected in the in vitro analysis and could reflect their availability in vivo or the relatively small amount of starting material and the labile nature of these modifications. Modified sites were compared with the in vitro data set demonstrating that thiol-reactivity predicted modification depending on the magnitude of oxidative stimuli (Figs. 5C and 5D). Modifications induced by 50 μM SNO-Cys were found to have SNO-reactivities between m = 0.7871 - 1.184 whereas 200 μM SNO-Cys affected sites between m = 0.7871 - 2.332 (Fig. 5E). SNO-modified cysteine residues were also obtained from the literature and compared with the SNO-reactivity curve. Lam and colleagues exposed cells to 1 mM SNO-Cys and identified SNO-modifications using the biotin switch assay (13). From their study, 19 modified cysteine residues were matched to the GSNO-response data set and were found to modify a wide range of sites including those with greater insensitivity than in the 200 μM treatment, m = 0.6166 - 3.5 (supplemental Fig. S3). Of note, additional analysis of the unmodified but available cysteine residue data set revealed a total of 11 SNO-sites induced by 1 mM SNO-Cys treatment obtained from the literature were present compared with only 1 site from the 200 μM treatment (supplemental Fig. S4).

DISCUSSION
Since its introduction, many incarnations of the biotin switch assay have made incremental improvements to the technique (15, 16). Here, we describe a variation that achieves a simple solution to multiple limitations which involves using the cysTMT6 reagent. The addition of cysTMT6 provides an enrichable, permanent mass tag at the modified cysteine residue removing the ambiguity of site assignment and reducing the potential false-positives that can occur for peptides with multiple cysteine residues or with nonspecific binding during capture. The reporter ions facilitate the simultaneous analysis of up to six samples, improving the detection of potential artifacts and providing relative quantification at individual SNO-modified sites. Previously quantification of SNO-modifications has been achieved using the iTRAQ or SNO-CAP (SNO-capture) techniques (20–22). iTRAQ is a commonly used method of peptide quantification in which isotopically balanced labels react with a peptide’s N-terminus allowing for 8-plex analysis. iTRAQ labeling is unrelated to the
SNO-modification resulting in no signature mass to indicate the modified cysteine residue. Labeling is performed after the elution of captured peptides, requiring an extra step which can also introduce additional error. SNOCAP is an isotope coded thiol reactive reagent with a biotin group for enrichment that results in a signature mass tag at the modified cysteine. SNOCAP does not have reporter ions; instead, quantification is measured at the parent ion level and offers only 2-plex analysis which limits experimental design options. Also, this reagent is not commercially available.

Relative quantification using the cysTMT6 reagent improved identification of potential ascorbate artifacts. Some studies have reported that it can also reduce disulfide bonds or other oxidative modifications producing incorrect identifications (42–44). However, Forrester and colleagues suggest that ascorbate reduction of protein or mixed disulfides is thermodynamically unfavorable and that the majority of the reported artifacts are the result of accidental exposure to light or other contaminants (16, 45). Our data supports this as out of the 228 cysteine residues identified, only six displayed a reporter ion signature suggestive of the unintended reduction of a disulfide bond and 5 indicated some level of potential glutathionylation. From the current data it is not possible to determine a specific mechanism for the reduction of these modifications. These sites may represent a small group of ascorbate sensitive thiols or the reduction could be the result of inadvertent light or other exposure during sample processing. Whatever the origins of these potential artifacts, our results indicate these events are rare but readily discriminated using this quantitative strategy. The cysTMT6 switch technique coupled with the correct control conditions offers a simplified solution for the specific detection of SNO-modifications including better observation of background modifications and methodological artifacts.

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The combination of individual modified cysteine residues and their relative response specifically to SNO-modification permitted the investigation into the question of thiol-reactivity being attributable to a physical property of the protein. Although many groups have looked for structural clues to predict the likelihood of SNO-modification, evidence for a signif-
icant determining factor is not conclusive. An early report of SNO-modified cysteine residues suggested that a linear acid/base motif surrounding the target cysteine facilitated modification (46). Since then, there has not been agreement on the utility of this motif; although there have been some confirmatory reports (47, 48), it has not been identified in all data sets (13, 19, 28). We had hoped that thiol-reactivity would provide some additional insight into this question. Unfortunately, no correlation emerged for any of the physical properties considered even when individual site reactivity was included. It would seem more likely that determination SNO-modification is a function of the local redox-environment, the three-dimensional arrangements and modification status of the adjacent amino acid residues (28). A recent report examined a variety of potential structural characteristics including hydrophobicity, pKₐ, as well as primary and secondary structure to identify unique subsets of SNO-modified thiols which the authors speculate forms the bases for specificity in multiple mechanisms of SNO-modification (47). Taken together, it seems unlikely that a single unified consensus sequence determines SNO-modification. Alternatively, a study assessing the structural characteristics of SNO-modified proteins suggest that the presence of an acid/base pocket near the target cysteine may facilitate modification (28). This is an interesting possibility; however a similar analysis was not performed here.

In a recent study, general thiol-reactivity was assessed using an iodoacetamide-based tag and identified a large number of cysteines in three human cell lines (49). The authors reported that cysteine reactivity predicts function, suggesting that highly reactive sites are more likely to be involved in active site chemistry and have greater regulatory potential. Unexpectedly, comparison with the current data set revealed only a 6% (67/1082) overlap in characterized cysteine residues. The sites that did match between the two studies showed no correlation in reactivity suggesting the chemistry of alkylation is significantly different and not applicable to a naturally occurring post-translational modification, like SNO (supplemental Fig. S5). Possible explanations for the disparity could be the analysis of different cell types or the use of a reducing agent during iodoacetamide-based labeling. In the present study, preparations were unreduced, leaving endogenous disulfide bonds intact. The addition of many more cysteine containing peptides would have increased the complexity in the MS analysis and could have suppressed the identification of some sites including those targeted for SNO-modification.

An important limitation to the biotin switch assays has been its sensitivity with few studies successfully reporting the detection of endogenous levels of S-nitrosylation. Many investigations have relied on the use of exogenous NO-donors to induce SNO-modification (13, 17, 19, 22, 50). Although the judicious use of exogenous donors can be informative, it is important to consider the resulting modified thiols as SNO-modifiable until more definitive information is available (28). A limiting factor in the sensitivity of the traditional biotin switch assays has been the use of ascorbate as the SNO-specific reducing agent (51). Ascorbate, although reasonably specific, has limited reducing power and, even in the presence of copper, has not achieved the levels of detection seen with other techniques (30, 51). Recently, alternate chemistries including a solid phase and biotin based organomercury reagents have been utilized to target SNO-modified thiols. Doulias and colleagues report detecting 328 endogenous SNO-modified sites from mouse liver (47). This study was performed using ~150 fold more starting material than in the current study (30 versus 0.2 mg), making an accurate comparison between the two approaches difficult. While this new approach is promising, the organomercury compounds have not been subject to the same scrutiny as ascorbate regarding specificity of SNO-modification detection (16, 45, 51).

The level of SNO-cys sites detected here are similar to, if not more sensitive than, other similar studies identifying SNO-modifications in mammalian cell types. Recent proteomic investigations using the ascorbate based switch techniques have identified between 44 and 90 S-nitrosylation sites using the NO-donor, GSNO, at concentrations between 10 and 100 μM GSNO (14, 17, 19, 29). However many more sites can be detected when greater concentrations of NO-donor are used. 951 SNO-modified sites were identified in myocardial lysates following treatment with 1 mM GSNO which is well beyond the physiological range (50). Although absolute sensitively is an important goal in SNO-detection, given the general reducing character of the cell and the labile nature of SNO-modifications it is unlikely that significant levels of S-nitrosylation exist in an unstimulated cell (22). It is more likely that the majority, and the more regulatory significant, SNO-modifications are short lived and arise in response to local or global changes in the cell’s redox state. The techniques that most adeptly balance detection sensitivity, specificity and quantitative potential will be best suited to characterize these important post-translational modifications and understand their regulatory potential in redox signaling.

**Biological Implications**—The ability to stratify protein thiol-reactivity presents an opportunity for insight into how the cell responds to redox signals. Considering the roles of reactive oxygen and nitrogen species in the cell as signaling molecule through agent of oxidative damage, the relative reactivity may facilitate the function of these sites. Because few of the SNO-modifications identified here have been functionally characterized, it is difficult to predict what impact SNO might have on a protein’s function. However, there are some specific indications for how thiol-reactivity may relate to redox-responses. One example is peroxiredoxins (Prx) family, which defend against oxidative species. Prx can neutralize an oxidant through the sequence of; reaction at its active site thiol, disulfide formation with an adjacent Prx and regeneration by the protein thioredoxin (52–54). Prx-6 was found here to be modified at its active site (C47) with a reactivity in the linear
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range (m = 1.144), indicating this thiol is reactive to relatively low concentrations and well suited as a redox scavenger (supplemental Fig. S6). Prx-1 on the other hand, has its active site at C52 but forms an interchain disulfide with C172 on its partner molecule (52). Regeneration and continued redox-defense depends on the availability of the second thiol. Here we found C172 to be among the most SNO-insensitive thiols (m = 3.5) suggesting it would be unmodified in most situations. Oxidation of C172 may also be responsible for the loss in Prx activity seen in instances of high oxidative stress (52).

The differences observed in thiol-reactivity suggest the possibility of a cellular network of redox-switches that can detect the magnitude of an oxidative stimulus to respond accordingly. It is tempting to speculate that the progressive and specific accumulation of modified sites may trigger differential responses depending on the level of oxidation. For example, the activation of the most sensitive switches would affect routine housekeeping metabolic processes whereas the modification of increasingly insensitive sites triggers protective and defensive responses. This concept can be supported by examples from the available data set. The glycolytic enzymes pyruvate kinase, phosphofructokinase and glyceraldehyde-3-phosphate dehydrogenase were all found to have highly reactive cysteines (m = 0.6985, C424; 0.8826, C411; 0.9157, C247, respectively) and are known to be redox-regulated (supplemental Fig. S7) (55–57). The protective potential of redox-signaling can be seen in the cytoprotective effects associated with low fluxes of ROS or SNO prior to subsequent ischemic insults, including the preservation of protein synthesis (58, 59). Protein synthesis can be inhibited by oxidative damage in part by modification of elongation factor 2 (EF2) (60). EF2 is also known to be S-nitrosylated and was determined here to have a reactivity at C41 (m = 1.083) (supplemental Fig. S8) (51). Given the necessity of protein synthesis for enduring an oxidative insult, the relative reactivity of C41 allows for reversible SNO-modification early in an attempt to shield that site from irreversible oxidative damage, preserving function. Additional investigation will be required to determine this potential relationship; however, the affects of oxidizing species, such as nitric oxide, can be stratified based on concentration (7) supports this potential aspect of redox-signaling.

The redox-sensor capacity of the cell extends beyond the analysis performed here. SNO-modification was observed on 220 cysteine residues, while an additional 500 sites were found to be available but unaffected by the current GSNO treatments (supplemental Fig. S4, supplemental Table S2). Of note, 11 SNO-sites induced by 1 mM SNO-Cys treatment obtained from the literature matched to the available but unmodified dataset obtained in this study while only 1 site matched from the 200 μM treatment. It seems likely that the current analysis represents one part of the thiol-reactive spectrum suggesting many additional thiols are reserved to respond to greater stimuli or other oxidative species. For example, the redox-activated chaperone DJ-1 protects cells from oxidative stress by influencing antioxidant gene regulation (61). The 2 available cysteine residues identified here (C46 and C53) were not modified by 20 μM GSNO and likely require a greater stimuli to induce the cytoprotective effects. Additionally, large amounts of oxidative damage is known to induce apoptosis, including triggering the release of apoptosis-inducing factor 1 (AIF1) (62, 63). Modification of C256 on AIF1, identified here, may be one of the many redox-switches involved in the initiation of apoptosis. As such, the relative insensitivity of this site is necessary for its function as a sensor of significant oxidative damage.

CONCLUSION

The influence of oxidative species in the cell can range from benign signaling molecules to agents of destruction; however, the mechanisms of these effects are not well understood. In the current study, we have developed a simplified and more specific technique for the detection, identification and multiplex quantification of SNO-modifications. Using this approach it was possible to readily discriminate potential false positive results including non-SNO-modifications and mis-assigned sites. In addition we characterized the continuum of thiol-reactivity to S-nitrosylation that was validated in vivo. Analysis of the dataset suggests a possible mechanism for the cell to gauge the magnitude of an oxidative stimulus. We anticipate thiol-reactivity profiles for other oxidative species; although it is not clear how they will relate to the one established here. Additionally, thiol-reactivity could be influenced through adjacent post-translational modifications, such as phosphorylation, allowing the cell to modulate its detection and response to oxidative signals.

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[5] This article contains supplemental Figs. S1 to S8 and Table S1 to S7.

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