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Proteomic Profiling of Nitrosative Stress: Protein *S*-Oxidation Accompanies *S*-Nitrosylation

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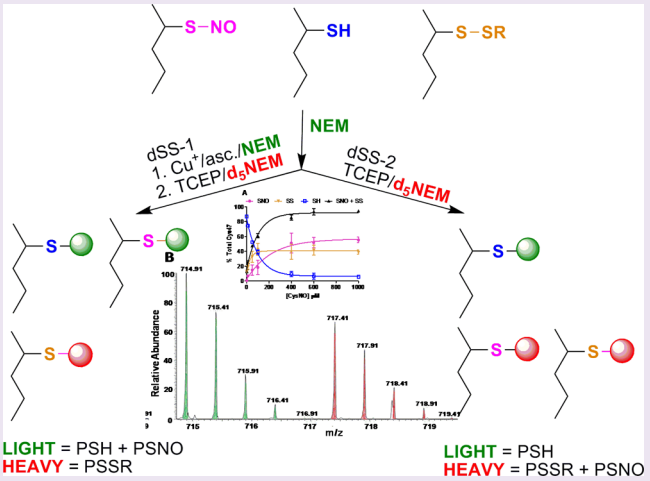
Supporting Information

ABSTRACT: Reversible chemical modifications of protein cysteine residues by *S*-nitrosylation and *S*-oxidation are increasingly recognized as important regulatory mechanisms for many protein classes associated with cellular signaling and stress response. Both modifications may theoretically occur under cellular nitrosative or nitroxidative stress. Therefore, a proteomic isotope-coded approach to parallel, quantitative analysis of cysteome *S*-nitrosylation and *S*-oxidation was developed. Modifications of cysteine residues of (i) human glutathione-S-transferase P1-1 (GSTP1) and (ii) the schistosomiasis drug target thioredoxin glutathione reductase (TGR) were studied. Both *S*-nitrosylation (SNO) and *S*-oxidation to disulfide (SS) were observed for reactive cysteines, dependent on concentration of added *S*-nitrosocysteine (CysNO) and independent of oxygen. SNO and SS modifications of GSTP1 were quantified and compared for therapeutically relevant NO and HNO donors from different chemical classes, revealing oxidative modification for all donors. Observations on GSTP1 were extended to cell cultures, analyzed after lysis and in-gel digestion. Treatment of living neuronal cells with CysNO, to induce nitrosative stress, caused levels of *S*-nitrosylation and *S*-oxidation of GSTP1 comparable to those of cell-free studies. Cysteine modifications of PARK7/DJ-1, peroxiredoxin-2, and other proteins were identified, quantified, and compared to overall levels of protein *S*-nitrosylation. The new methodology has allowed identification and quantitation of specific cysteome modifications, demonstrating that nitroxidation to protein disulfides occurs concurrently with *S*-nitrosylation to protein-SNO in recombinant proteins and living cells under nitrosative stress.



Cysteine residues in proteins play important functions including control of conformation, catalysis of enzymic reactions, and regulation of protein responses in redox signaling. Cysteine thiol groups are intrinsic targets in proteins for electrophilic attack and are sensors responding to perturbation in cellular redox balance. Reversible modifications of cysteine residues under nitrosative and oxidative stress have emerged as important mechanisms for post-translational regulation of a broad range of signaling events and cellular responses.[1,2] The most extensively studied post-translational modification linked to NO signaling and to nitrosative stress is protein *S*-nitrosylation, resulting from the chemical nitrosation of a cysteine thiol with a nitrosonium (NO^+) equivalent, readily provided by endogenous nitrosothiols, such as *S*-nitrosoglutathione and *S*-nitrosocysteine (CysNO), N_2O_3, or Lewis acid catalyzed reaction of NO and NO_2^-.[3] It has been proposed that *S*-nitrosylation forming protein-SNO is a major mechanism of NO-mediated signaling involved in cell stress response, cell proliferation, and apoptosis.[4] *S*-Nitrosylation of a variety of proteins has been described, and dysregulation of protein *S*-nitrosylation has been implicated in a number of disease states.[5]

Oxidation of protein thiols under nitrosative stress has been proposed to be the dominant protein modification caused by reactive nitrogen species formed from NO and NO_2^-, such as N_2O_3, NO_2, and peroxynitrite.[6,7] These "nitroxidation" products include reversible disulfide (SS) and sulfenic acid (SOH) modifications and irreversible sulfinic and sulfonic acid forms. (Common nomenclature does not always accurately describe the biological chemistry. Nitroxidative stress is often used to describe cellular conditions for tyrosine nitration. Similarly, nitrosation is the chemically correct description of the process widely referred to as protein nitrosylation.) Such protein thiol modifications regulate protein folding, function, and thiol-based redox signaling, with disulfide formation of particular structural and

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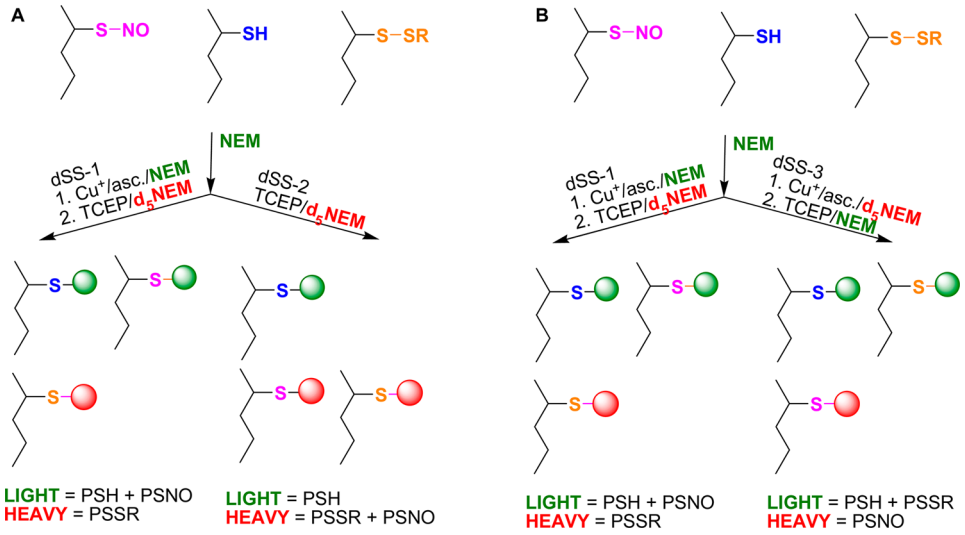

Figure 1. Schematic depicting the d-SSwitch methodology for quantitative determination of protein modification by *S*-nitrosylation and *S*-oxidation: (A) methodology used for GSTP1 and living cells; (B) methodology used for *S. mansoni* TGR analysis.

functional importance.[2,8] Furthermore, protein *S*-nitrosylation can lead to disulfide formation and may serve as a functional precursor to regulate disulfide formation including glutathionylation.[9,10] Clearly, there is a need to measure SNO and SS modifications of the cysteome in both individual proteins and cells. The biotin switch technique (BST), most widely used to study protein-SNO, neglects unmodified (SH) and oxidized (SS) proteins.[11,12] Other methods exist to separately quantify protein disulfide formation.[13,14] A novel proteomic method, d-Switch, introduced by us to identify and quantify cysteome *S*-nitrosylation, is adapted herein to measure both SNO and SS modifications in parallel and is coined d-SSwitch.

The SNO and SS functionalities of modified proteins can be reduced to free thiol (SH) by selective chemical reactions, and therefore using two isotopologues to probe one split sample in parallel can lead to simultaneous quantitation of protein thiols in the SNO, SS, and SH chemical states (Figure 1). Using liquid chromatography tandem mass spectroscopy (LC−MS/MS), in addition to quantitation, the d-SSwitch approach allows identification of each individual modification site in the cysteome.

Human glutathione-*S*-transferase P1 (GSTP1) was used in method development and to provide comparison with the previously reported d-Switch approach.[15] GSTP1 is important in regulating cell response to NO and nitrosative stress[16] and in cancer drug resistance[17] and also plays a regulatory role in cellular signaling and stress response *via* reversible intra- and intermolecular disulfide formation.[18] *S*-Nitrosylation and nitroxidation of GSTP1 were measured in response to CysNO, NO, and several therapeutically relevant NO donor classes and an HNO donor. The d-SSwitch method was also tested in thioredoxin glutathione reductase from *Schistosoma mansoni* (TGR), a potential drug target for schistosomiasis, important in maintaining redox homeostasis in the parasite. [19]

Observations on recombinant GSTP1 were extended into a cellular context by subjecting living SH-SY5Y neuroblastoma cells to nitrosative stress *via* CysNO treatment followed by d-SSwitch analysis. Further probing of the cellular cysteome with d-SSwitch revealed proteins implicated in pathophysiological conditions associated with nitrosative and oxidative stress, including Parkinson's disease protein 7 (PARK7/DJ-1) and peroxiredoxins 1 and 2 (PRDX1, 2). In both recombinant proteins and those treated in living cells, cysteine residues sensitive to *S*-nitrosylation, under conditions of nitrosative stress or on NO donor treatment, were universally observed to be oxidized to disulfides. Nitroxidation was the major cysteome modification in all cases, and the d-SSwitch methodology was capable of identifying and quantifying modification of specific cysteine residues.

RESULTS AND DISCUSSION

Protein post-translational modification *via* *S*-nitrosylation, glutathionylation, and formation of other protein disulfides is widely held to play important roles in cell signaling.[1,2,5,15,20] The relevant chemical reactions of cysteine residues are nitrosation and oxidation, converting free cysteines (RSH) to nitrosothiol (RSNO) and disulfide (SS) functional groups, respectively. Selective cysteine modification is expected for controlled cell signaling processes; however, under conditions of nitrosative stress, it is likely that cysteine modification will be widespread and uncontrolled. We introduced a quantitative proteomics methodology to identify nitrosated cysteine residues using isotope-coded *N*-ethylmaleimide (NEM).[15] An adaptation of this method was used by Tannenbaum and Marletta,[21] and Carroll introduced a comparable, isotope-coded methodology to elegantly identify sulfenic acid modifications (RSOH).[22] Formation of a sulfenic acid is often the initial step in post-translational modification leading to formation of protein disulfides.

The rationale for development of the isotope-coded NEM approach was to allow future extension to measurement in parallel of both RSNO and SS protein modifications.[15] This is not possible with the qualitative and widely used biotin-switch technique (BST), since the methodology requires *formation* of a disulfide to label *S*-nitrosocysteines prior to analysis. Several clever and sometimes quantitative BST adaptations have been reported.[23] Alternative approaches to detect *S*-oxidation rely on the differential labeling of unmodified and oxidized thiols upon reduction, one approach using iodoacetate-based isotopologues.[13] Simultaneous quantitation of the cysteome inventory (RSH + RSNO + SS) of specific cysteine residues remains an important goal. Since we coined the approach to measurement of RSNO *versus* RSH, d-Switch, we use d-SSwitch herein to identify a new methodology measuring RSH and RSNO and disulfide (SS) modifications to specific cysteine residues.

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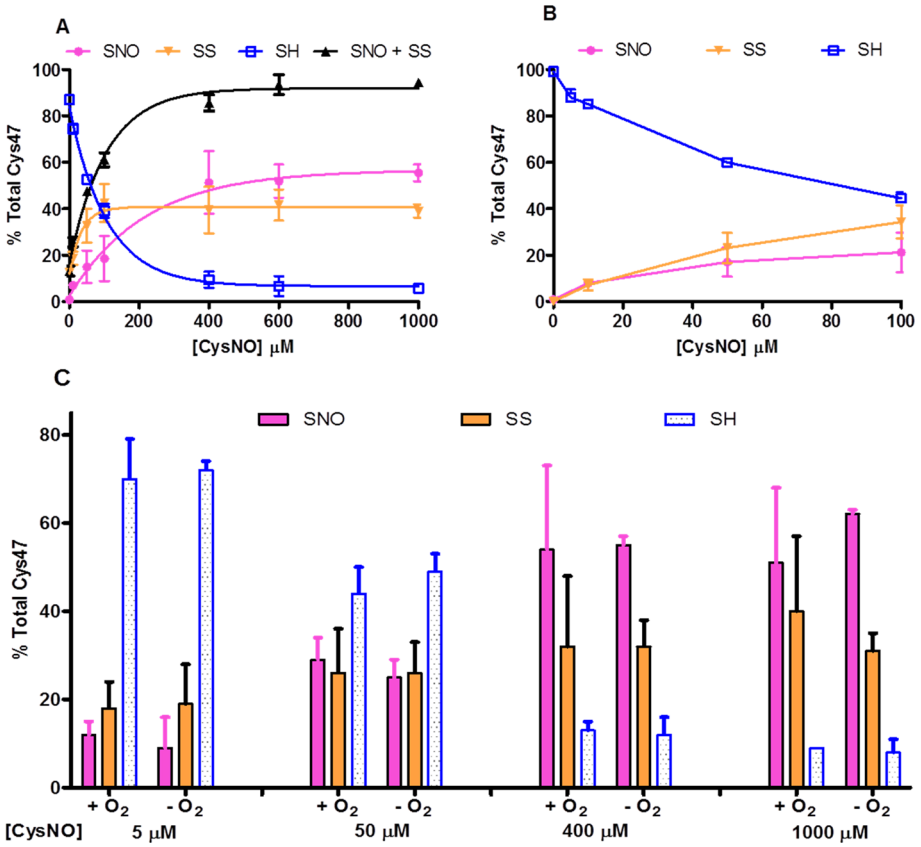

Figure 2. Quantitative analyses of GSTP1 *S*-nitrosylation and *S*-oxidation: (A) nitrosated (SNO), oxidized (SS), or unreacted (SH) Cys47 as a function of CysNO concentration; (B) Cys47 modification at lower CysNO concentrations, with subtraction of vehicle control levels of each of SNO and SS; (C) dependence of *S*-nitrosylation and *S*-oxidation upon O_2. Data show mean ± SD (n = 4).

***S*-Nitrosylation and Oxidation Quantified by d-SSwitch: GSTP1.** Study of human GSTP1, used in method development for d-Switch, allows comparison with the d-SSwitch approach.[15] GSTP1 is also a highly relevant protein for study with importance in regulating cell response to NO and nitrosative stress, cell signaling, and stress response *via* reversible disulfide bond formation.[16−18] GSTP1 has major roles in cellular response to oxidative and nitrosative stress. Cysteine modifications are proposed to have functional roles in catalysis of glutathionylation and control of oligomerization and dissociation with key partners, such as c-Jun NH_2-terminal kinase (JNK) and PRDX, events that signal cellular response to stress.[24,25] Cys-47, the most reactive of the four cysteine residues, was observed by d-Switch to be *S*-nitrosated by CysNO in a concentration-dependent manner.[15] However, GSTP1 is sensitive to oxidation *via* formation of intramolecular and intermolecular disulfide bonds, the products of which have been analyzed previously.[26]

GSTP1 was treated with CysNO, an effective transnitrosating agent to simulate nitrosative stress. As depicted (Figure 1A), free thiols were blocked with *N*-ethylmaleimide (NEM) before sample splitting to give two samples, denoted dSS-1 and dSS-2. Sample dSS-1 was treated with Cu^I/ascorbate to selectively reduce RSNO groups to free thiols that were labeled with NEM. The remaining stable disulfides were reduced with TCEP to free thiols that were labeled with d_5-NEM. Therefore in dSS-1, all RSH + RSNO groups were labeled with the light isotopologue (NEM) and all SS groups were labeled with the heavy isotopologue (d_5-NEM). Sample dSS-2 was treated with TCEP to reduce both RSNO and disulfides in the presence of d_5-NEM, thus labeling RSH with the light d_0 isotopologue and all RSNO + SS groups with the heavy d_5 isotopologue. The formation of GST(C101A) dimers at two different CysNO concentrations and their efficient reduction to monomers by dSS-2 treatment was confirmed in Coomassie-stained SDS−PAGE gels (Supplementary Figure 1). In addition, the phosphine TPPTS was explored in place of Cu^I/ascorbate, giving similar final results (Supplementary Figure 2). A more detailed workflow is provided in Supporting Information (Supplementary Figure 3).

After in-gel digest, LC−MS/MS analysis was used to quantify d_0- and d_5-labeled peptides that have identical retention time and ionization efficiency, using a methodology similar to d-Switch. Simple algebraic derivation using $d_5/(d_0 + d_5)$ ratios for split dSS-1 and dSS-2 samples provides the quantitative inventory for the modified cysteome. In the case of GSTP1, Cys47 was observed to undergo *S*-nitrosylation *with* dependence on CysNO concentration, as was observed with d-Switch; however, the extent of Cys47-SNO formation was greatly overestimated by d-Switch, which was anticipated, because d-Switch neglects *S*-oxidation. The complete neglect of cysteine oxidation in BST and most other methods for identification or measurement of *S*-nitrosylation can be seen to be a serious flaw in overestimation of protein-SNO formation.

The C101A mutant of GSTP1 was used to focus on reaction of Cys47, removing the influence of the next reactive cysteine residue, Cys101. Loss of Cys47 free thiol by *S*-oxidation and *S*-nitrosylation was dependent on CysNO concentration and was complete in the presence of 0.4 mM CysNO (Figure 2A). Approximately 50% of Cys47 free thiol was lost in the presence of 100 μM CysNO, converted to Cys47-disulfide and Cys47-SNO in a 3:2 ratio (Figure 2B). Protein *S*-nitrosylation in the presence

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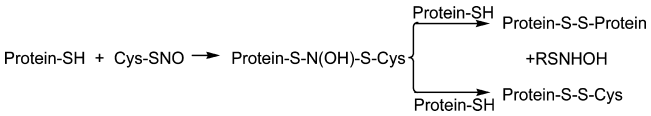
of excess nitrosating agent (CysNO) can theoretically follow pseudo-first-order kinetics; however, disulfide formation (in the absence of GSH) requires Cys47-SS-Cys47 disulfide bond formation, and therefore the relative amount of *S*-oxidation was lower at high [CysNO] as protein free thiol was depleted.

The effect of oxygen on reaction of Cys47 in the presence of CysNO was studied in order to test for the intermediacy of N_2O_3, known as both a nitrosating and oxidizing agent and formed from the reaction of NO with O_2. No dependence on O_2 was observed. The reaction of GSTP1(C101A) with CysNO was also studied in the presence of dimedone to test for the intermediacy of Cys47-SOH in formation of protein disulfides, but again no significant dependence was observed (Supplementary Figure 4). The conclusion for GSTP1 is that at all concentrations of CysNO, protein *S*-oxidation was concomitant with *S*-nitrosylation; whether at lower concentrations that model endogenous low-MW nitrosothiols or at higher concentrations relevant to nitrosative stress. Furthermore, disulfide formation independent of dimedone (RSOH) and independent of O_2 indicates oxidation *via* reaction of Cys47 with CysNO, a speculative general mechanism for which is shown in Scheme 1. Mechanisms

Scheme 1. Mechanism for Disulfide Formation Independent of O_2 and a Sulfenate Intermediate



for GSSG disulfide formation *via* reaction of GSH with GSNO have been proposed previously;[27] however, these mechanisms are dependent on O_2 or require millimolar concentrations of GSH.

S-Nitrosylation and S-Oxidation Quantified by d-SSwitch: TGR. TGR is another enzyme closely associated with response to oxidative stress and a therapeutic target for the parasitic diseases commonly known as schistosomiasis.[28] TGR plays a significant role in maintaining redox homeostasis in the parasite, performing and combining the roles of two individual human enzymes, glutathione reductase and thioredoxin reductase.[28,29] The 65 kDa TGR, with 15 Cys residues, is a dimeric flavoprotein, each subunit having the components of both a thioredoxin reductase (TrxR) domain and a glutaredoxin (Grx) domain.[19] The active site of the TrxR domain is composed of residues from both subunits: FAD and a redox-active Cys154/Cys159 pair from one subunit, and a redox-active Cys596/Sec597 pair from the other. The active site of the Grx domain contains a redox-active Cys28/Cys31 pair. TGR is shown to have several external pockets and cavities, characterized by distinct surface electrostatic potentials,[30] and hence varying reactivity is expected for the cysteine residues.

TGR(U597C) was used as a second model protein to assess d-SSwitch. Protein (15 μM) was treated with CysNO (50 and 500 μM) in the presence of NADPH (100 μM) followed by NEM blocking and d-SSwitch analysis using similar in-gel digest and LTQ-FT-ICR LC−MS/MS approaches as applied to GSTP1 (Figure 1B). Representative MS spectra are shown for the peptide fragments containing NEM and d_5-NEM labeled Cys402 (Figure 3 A,B). Peptides were identified and quantified, containing Cys154/Cys159, Cys347, Cys402, Cys417, Cys520, Cys574, and Cys596/Cys597 (Figure 3C).

Cys154/Cys159 and Cys596/Sec597 are catalytically important and switch oxidation state during the TGR catalytic cycle. In our study of mutant TGR, the trypsinized peptide fragments, TAVLDYVEPTPIGTTWGLGGTC^{154}VNVGC^{159}IPK and KSGVSPIVSGC^{596}C^{597}G containing these dithiol/disulfide pairs, were found largely in the oxidized disulfide form in the resting state, in accord with previous reports.[30] The remaining free thiol (4−15%) was consumed by addition of CysNO. Angelucci *et al.* speculated that Cys520 and Cys574 might also form a dithiol−disulfide redox couple. The evidence from d-SSwitch is that CysNO does not induce intramolecular Cys520-Cys574 disulfide formation, since at lower CysNO concentrations only Cys574 is oxidized.

Not all cysteines are reactive; for example, Cys347 in the NADPH-binding domain,[30] was insensitive to nitrosative stress. However, for cysteine residues sensitive to nitrosative stress, such as Cys417 and Cys402, both in the FAD-binding domain, *S*-oxidation accompanied *S*-nitrosylation.

Study of TGR demonstrates d-SSwitch as a quantitative tool for discerning reactivity of individual cysteine residues in a cysteine-rich, redox-active protein. Observation of both unreactive and reactive cysteines, at high CysNO concentration, demonstrates that even under nitrosative stress, selective modification of the cysteome is expected. Furthermore, oxidation of reactive cysteines always accompanies and usually dominates over *S*-nitrosylation.

Protein Modification Elicited by NO and HNO Donors Quantified by d-SSwitch. In this study, we used CysNO to induce nitrosative stress, because CysNO acts primarily as a nitrosating agent.[31] However, CysNO and other small molecule nitrosothiols are often referred to as NO donors. d-SSwitch was used to compare GSTP1 modification by NO donors from different chemical classes (Scheme 2). Therapeutically important NO donor nitrates, such as nitroglycerin (GTN), contain an organic nitrate group that is known to be a chemical oxidant. Reaction with thiols yields sulfenic acid or thionitrate, both of which react further with thiol to yield disulfides.[32] For NO and other NO donor classes *S*-oxidation has been proposed.[10,33−35] In the case of thiophilic HNO donors (*e.g.*, AcOM-IPA/NO), *S*-oxidation is expected.[36] Finally, two examples of hybrid NO donor NSAIDs with therapeutic potential were tested, GT-094 and NOSH-aspirin, the latter a hybrid NO donor and H_2S donor.[37,38]

In the presence of all NO and HNO donors tested, *S*-oxidation rather than *S*-nitrosylation was the dominant modification of reactive cysteine residues of GSTP1 (Figure 4). DEA/NO is a controlled source of NO, which in the presence of O_2 causes *S*-oxidation and limited *S*-nitrosylation *via* N_2O_3 formation, consistent with previous observations using d-Switch.[15] GTN caused significant *S*-oxidation, whereas GT-094 and the HNO donor, AcOM-IPA/NO, caused almost complete oxidation of Cys47 to disulfide. Only marginal reaction was observed for NOSH-aspirin. The chemistry of the NO and HNO donors tested dictates that only CysNO is capable of a direct nitrosation reaction, and even for this compound, oxidation of Cys47 to disulfide was dominant. A mechanism of *S*-oxidation *via* RSNO formation and HNO release has been proposed[39] but is disfavored in the reaction of CysNO with GSTP1, since the production of HNO would lead to total *S*-oxidation, as seen for the HNO donor AcOM-IPA/NO, which was not observed for CysNO treatment.

Cellular Nitrosative Stress: Is Dominant S-Oxidation an Artifact of a Cell Free System? The applications of d-SSwitch, described above, to GSTP1 and TGR under nitrosative stress

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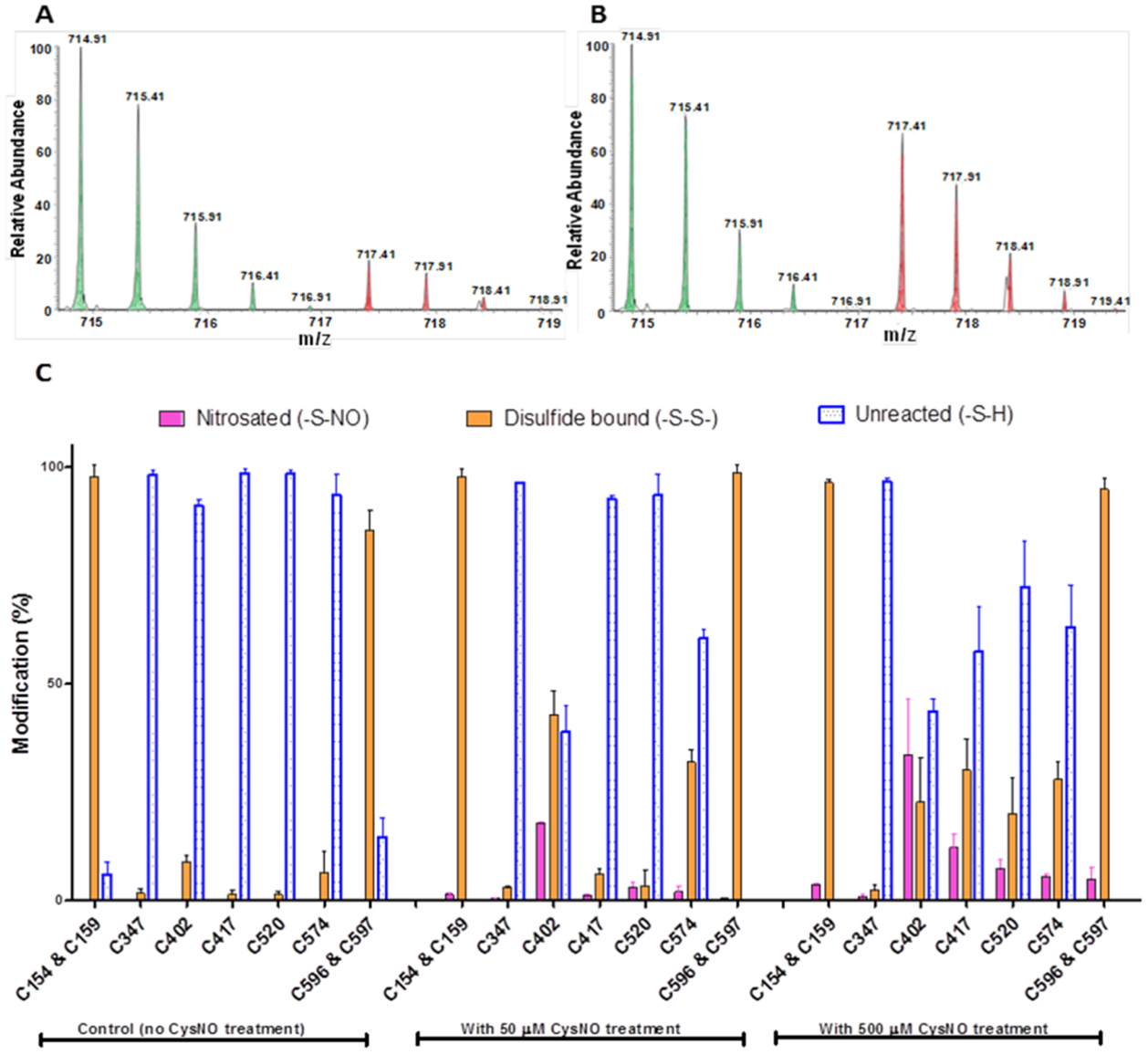
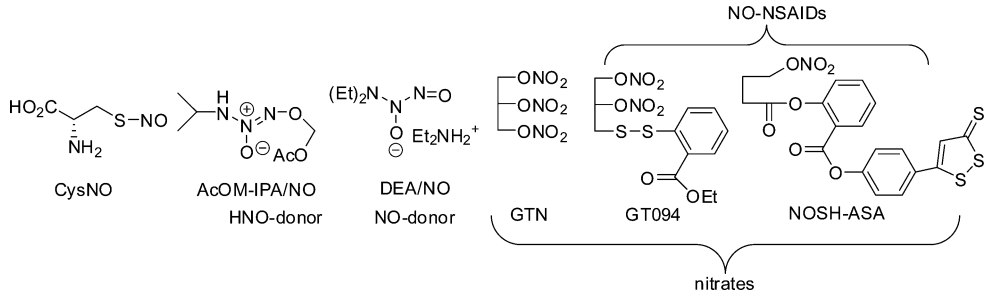


Figure 3. Quantitative analysis of *S. mansoni* TGR *S*-nitrosylation and *S*-oxidation. (A, B) Spectra for the Cys402-containing peptide from TGR treated with CysNO (50 μM) and analyzed by d-SSwitch (see Figure 1B): (A) dSS-3 fraction showing unreacted (RSH) and oxidized (SS) Cys402 (green) and nitrosated (SNO) Cys402 (red); (B) dSS-1 fraction showing unreacted and nitrosated Cys402 (green) and oxidized Cys402 (red). (C) Measurement of nitrosated (SNO), oxidized (SS), or unreacted (SH) TGR cysteine residues as a function of CysNO concentration (0, 50, 500 μM) by quantitative d-SSwitch analysis of 7 peptide fragments. Data show mean ± SD (n = 4).

Scheme 2. Chemical Structures of NO/HNO Donors



indicate that *S*-nitrosylation to protein-SNO is not the major cysteine modification observed. The term nitroxidative stress, introduced by Jack Lancaster, appears much more appropriate, since relatively little nitrosation is observed under conditions of nitrosative stress.[7,33,40] However, before jumping to this conclusion it was important to consider if the reductive intracellular environment would limit *S*-oxidation or nitroxidation.

Neuroblastoma SH-SY5Y cell cultures were subjected to nitrosative stress with CysNO. This neuronal cell line has the advantage of expressing GSTP1, allowing correlation with

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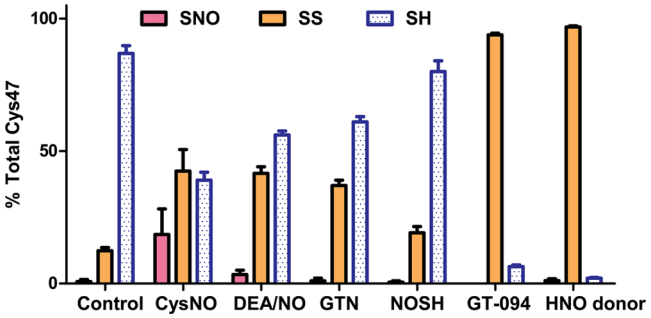

Figure 4. Quantitative comparison of GSTP1 modification by NO and HNO donors. GSTP1(C101A) was treated with donors (100 μM). Cys47 modification was assayed by d-SSwitch. Data show mean ± SD (n = 4).

cell-free experiments. Living cells were incubated with CysNO before lysis and analysis by d-SSwitch. The MS and MS/MS data were analyzed for protein identification using the MassMatrix search engine against the UniProt humanV57-p10 database,[41] followed by tracing the full scan MS spectra of the d_0- and d_5-labeled peptides. Representative MS spectra are shown in Supplementary Figure 5.

Interestingly, the response of GSTP1 to nitrosative stress in living cells was very similar to that of the recombinant protein: *S*-oxidation of Cys47 to disulfide increased 3-fold (from 21% to 60%) and *S*-nitrosylation was observed, but at a relatively low level (10%). The extent of Cys47 *S*-nitrosylation measured by d-SSwitch is lower than that measured by d-Switch, because the latter method did not measure protein disulfides. The same shortcoming and consequent overestimation of *S*-nitrosylation is common to BST-based and most published methods[15,23,42] for identification and quantitation of protein *S*-nitrosylation.

The neuronal cysteome was further interrogated by d-SSwitch, focusing on the 20−26 kDa gel band and proteins with >25% sequence coverage (Table 1). Several cysteines were largely present as free thiol under control conditions: phosphatidylethanolamine-binding protein 1 (PEBP1) Cys168, PRDX2 Cys70, transgelin-2 (TAGLN2) Cys63, 40S ribosomal protein S5 (RPS5) Cys155, and Cys172. For these cysteines, *S*-nitrosylation induced by nitrosative stress was at the level of 1−5% of the total cysteine inventory (RSH + RSNO + RSSR). The increase in *S*-oxidation for these cysteines was modestly higher (2−8%); however, these cysteine residues remained substantially unreactive toward nitrosative stress. Two cysteine residues of PRDX1 and PRDX2, present in control samples largely (70−83%) in the oxidized form, also showed measurable but modest response to nitrosative stress. In contrast, three cysteine residues in Park7/DJ-1 were more susceptible to nitrosative stress induced modifications, undergoing 3−11% *S*-nitrosylation and 12−33% *S*-oxidation. DJ-1 is an oncogene that is causative in a subset of familial Parkinson's disease (PD).[43] The precise function of DJ-1 is not defined; however, substantial evidence has been reported for roles in transcriptional regulation, oxidative stress response, mitochondrial regulation, and chaperone interactions with apoptotic proteins.[44−46] Irreversible oxidation of the thiolate Cys106 (pK_a ~5)[47] to a sulfenate is widely seen as a regulator of function, whereas Cys46 and Cys53 are argued to act as oxidatively labile regulators of Cys106 modification.[46] The sensitivity of these residues to nitrosative stress supports a role for all 3 cysteines in coordinating stress response. One report ascribed a role for *S*-nitrosylation of Cys46 and Cys53;[48] however, d-SSwitch showed that *S*-oxidation was the major modification induced by nitrosative stress.

In cell cultures, SNO-protein formation for individual cysteines, where detected, was measured at 1−12%. SH-SY5Y cells were subjected to nitrosative stress and assayed by a biotin pull-down method paralleling d-SSwitch. Cells were incubated with CysNO, lysed, treated with NEM to block Cys free thiol, and reacted with biotin maleimide in the presence of Cu^I/ascorbate to label SNO-proteins with biotin, which were then separated with avidin magnetic beads. The remaining proteins were treated with TCEP/NEM, the TCEP reduction step assisting in the detection of homo or hetero dimerized proteins on SDS−PAGE. Coomassie Blue was used to quantify total *S*-nitrosylated protein within given gel bands relative to non-nitrosylated protein (Supplementary Figure 6). Using this method, the relative amount of *S*-nitrosated proteins was shown to increase with CysNO concentration (Figure 5A). At the CysNO concentration used in cellular d-SSwitch experiments, 10−15% of total protein and 10% of the 20−26 kDa protein was *S*-nitrosylated. Although this method does not have the quantitative rigor of d-SSwitch, the extent of *S*-nitrosylation is comparable in the two methods.

A Nitrosating Environment Induces Cellular Nitroxidative Stress. Townsend, Tew, and co-workers have extensively studied the response of cell cultures and proteins, including GSTP1, to nitrosative stress induced by the NO donor diazeniumdiolate PABA/NO, noting limited *S*-nitrosylation and

Table 1. Nitrosated (SNO) and Oxidized (SS) Thiols of Proteins Estimated by d-SSwitch for 20−26 kDa Gel Bands from Cell Lysates after Incubation of SH-SY5Y Neuroblastoma Cells with and without 1 mM CysNO (n = 3)

				control		CysNO (1 mM)	
protein	mass (kDa)	coverage (%)	peptide	SNO (%)	SS (%)	SNO (%)	SS (%)
GSTP1	23.7	71	$ASC_{47}LYGQLPK$		21.2 (±2.5)	9.6 (±1.8)	59.3 (±7.7)
Park7/DJ-1	19.9	64	$VTVAGLAGKDPVQC_{46}SR$	1.7 (±1.1)	22.9 (±5.5)	5.0 (±1.7)	35.0 (±3.9)
			$DVVIC_{53}PDASLEDAKK$		22.5 (±6.4)	11.5 (±2.6)	55.9 (±17.9)
			$GLIAAIC_{106}AGPTALLAHEIGFGSK$	1.5 (±0.6)	8.9 (±0.2)	6.3 (±3.8)	23.3 (±8.0)
PEBP1	21.1	59	$APVAGTC_{168}YQAEWDDYVPK$		17.9 (±5.0)	2.9 (±0.9)	26.1 (±3.7)
PRDX1	22.1	52	$HGEVC_{173}PAGWKPGSDTIKPDVQK$	0.2 (±0.2)	69.7 (±10.1)	2.0 (±1.2)	72.4 (±17.3)
PRDX2	21.9	45	$LGC_{70}EVLGVSVDSQFTHLAWINTPR$		12.5 (±1.2)	3.3 (±1.8)	16.4 (±1.5)
			$LVQAFQYTDEHGEVC_{172}PAGWKPGSDTIKPNVDDSK$		83.2 (±3.2)	1.8 (±0.4)	90.7 (±6.3)
RPS5	22.9	30	$VNQAIWLLC_{155}TGAR$	1.0 (±1.0)	11.1 (±2.0)	4.4 (±1.4)	17.0 (±0.3)
			$TIAEC_{172}LADELINAAK$	0.5 (±0.6)	5.6 (±1.1)	1.7 (±0.9)	7.8 (±0.5)
TAGLN2	22.4	49	$DGTVLC_{63}ELINALYPEGQAPVK$	0.8 (±0.5)	13.0 (±2.1)	4.5 (±2.2)	15.5 (±2.2)

GSTP1: glutathione S-transferase P1, Park7 DJ1: Parkinson disease protein 7, PEBP1: Phosphatidylethanolamine-binding protein 1, PRDX1: Peroxiredoxin-1, PRDX2: Peroxiredoxin-2, RPS5: 40S ribosomal protein S5, TAGLN2: Transgelin-2.

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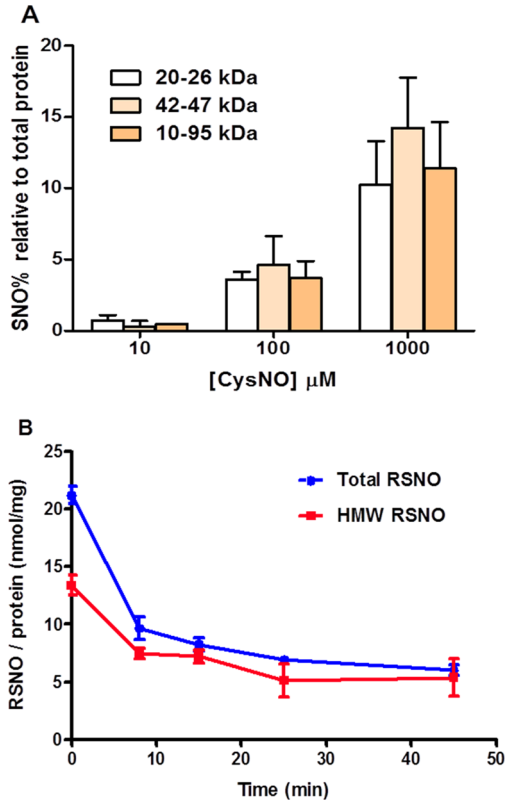

Figure 5. Cellular protein *S*-nitrosylation and denitrosylation after treatment of SH-SY5Y cells with CysNO. (A) Protein *S*-nitrosylation was measured by a biotin pull-down method using avidin beads to pull down nitrosated proteins. *S*-Nitrosylation was normalized to total *S*-nitrosylation, *S*-oxidation, and unreacted cysteine content as 100%. The intensity of each gel band (20−26, 42−47, 10−95 kDa) was quantified using ImageJ software, and the ratio of *S*-nitrosylated protein in CysNO-treated cells was normalized to the untreated control as 0%. Data show mean and SD (10 μM CysNO treatment: n = 3; 100 μM CysNO treatment: n = 8; 1 mM CysNO treatment: n = 6). (B) Time course of cellular denitrosylation after removal of nitrosating agent from SH-SY5Y cell cultures. The total amounts of nitrosothiol (total RSNO, blue) and high molecular weight nitrosothiol (HMW RSNO, red) were measured at different time points (8, 15, 25, 45 min) after removal of CysNO and cell lysis and normalized to total protein concentration. Data show mean ± SD (n = 3).

dominant *S*-oxidation to disulfide, *viz.*, *S*-glutathionylation.[2,9,24,49] The chemical reactivity of CysNO is dominated by trans-nitrosation reactions and therefore nitrosative stress induced by CysNO would be expected to cause higher levels of *S*-nitrosylation; however, in all d-SSwitch experiments we observe disulfide formation to be quantitatively dominant. Thus, the dominant chemistry under nitrosative stress is nitroxidative.

Nitrosative stress, caused by reactive nitrogen species, including nitrosating species, has been linked with many pathological conditions, mediated by post-translational modifications of the redox-sensitive cysteome. For example, nitrosative stress induced by overexpression of iNOS and impaired clearance of nitrosothiol (GSNO) has been proposed to contribute to hepatocellular carcinoma.[50,51] In a transgenic mouse model, O^6-alkylguanine-DNA alkyltransferase (AGT) was shown to be *S*-nitrosylated and deactivated, promoting mutagenesis. In contrast, nitrosative stress can be beneficial in initiating apoptosis and other cell death pathways, and this concept has been demonstrated in animal models and in cancer cells.[52,53] Induction of nitrosative stress in ovarian cancer cells caused protein glutathionylation, accompanied by the activation of unfolded protein response (UPR), leading to cell death.[9] Protein modification was causative *via* *S*-oxidation of specific cysteine residues, although in the study cited, *S*-nitrosylation was not detectable. The application of d-SSwitch to these and other systems will provide the benefit of identifying and quantifying both *S*-nitrosylation and *S*-oxidation of specific protein cysteines.

Challenges in Measurement of Reversible Cysteome Post-translational Modification. The d-SSwitch methodology presented herein represents a useful new quantitative tool for parallel measurement of protein *S*-nitrosylation and *S*-oxidation. Application to two proteins important in redox signaling and stress response and proteomic analysis of living cells under nitrosative stress demonstrate the scope of the method. Moreover, these studies clearly show that where cysteine *S*-nitrosylation is observed, *S*-oxidation is also observed and is usually quantitatively superior. Further, cellular nitrosative stress leads to selective cysteine post-translational modification. In addition to the study of two proteins that are therapeutic targets, the comparison of therapeutically relevant NO and HNO donors showed very different patterns of *S*-nitrosylation and *S*-oxidation and again dominant cysteine *S*-oxidation.

Protein disulfide and protein-SNO post-translational modifications are both reversible, with differing chemical and enzymic susceptibility. Inarguably, the post-translational modification most closely associated with cell signaling is protein phosphorylation, an enzymically reversible modification. Several methods exist for quantitation and identification of cellular protein phosphorylation; however, no researcher would conduct such experiments without treatment of cell lysates with phosphatase inhibitor cocktails to prevent dephosphorylation. This is a consideration that is seldom discussed in measurement of protein *S*-nitrosylation; therefore we measured protein denitrosylation after cell treatment.

To measure denitrosylation, the SH-SY5Y cells treated with CysNO were incubated for different time periods in fresh media prior to lysis. The lysates were separated into high and low molecular weight using a 10 kDa cutoff filter. The total amount of nitrosothiol was measured using a tri-iodide based chemiluminescence assay.[54] Protein *S*-nitrosylation (HMW > 10 kDa) fell significantly within the first few minutes after the treatment (Figure 5B), independent of the composition of the lysis buffer (data not shown). Exposure to heat, light, and metal ions can cause S-NO bond cleavage; however, d-SSwitch and other approaches to SNO quantitation take precautions against such homolytic degradation. In analogy with dephosphorylation by phosphatases, enzymes such as Trx may catalyze denitrosylation.[5] The inhibition of enzymes catalyzing such putative protein denitrosylation, without perturbation of other cysteome modifications, should be an objective of future studies. Nevertheless, the observations made herein with the novel d-SSwitch analysis reveal that, under nitrosative stress, proteins either recombinant or in living cells undergo a similar pattern of cysteome modification: reactive cysteines undergo both *S*-nitrosylation and *S*-oxidation with *S*-oxidation dominant.

METHODS

Chemicals and Reagents. All chemicals and reagents were purchased from Sigma Aldrich, Thermo Fisher Scientific, or Invitrogen unless otherwise mentioned. d_5-NEM and the cOmplete Mini protease inhibitor cocktail tablets were purchased from Cambridge Isotopes and Roche, respectively. CysNO, GTN, and GT-094 were synthesized

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by standard or published procedures.[37] AcOM-IPA/NO was kindly provided by Dr. Daniela Andrei (Dominican University, River Forest, IL).[36] NOSH-aspirin was kindly provided by Dr. Khosrow Kashfi (CUNY, NY).[38] CysNO was freshly prepared in neocuproine (100 μM) stock solution and used immediately after its concentration was determined spectrophotometrically. Stock solutions of DEA/NO and AcOM-IPA/NO were also freshly made before use. GSTP1, GSTP1-(C101A), and TGR proteins were expressed from *Escherichia coli* as previously described.[19,55]

d-SSwitch Method for Quantitation of *S*-Nitrosylation *vs* Disulfide Formation. All steps were performed in the dark in amber colored vials. Purified GSTP1 or TGR protein or cell lysate storage buffer was exchanged with reaction buffer containing 40 mM ammonium bicarbonate, 1 mM EDTA, and 0.1 mM neocuproine at pH 7.4. After incubation with the testing compound at 37 °C for 30 min, the unreacted thiols were blocked by NEM (20 mM) in the presence of 5% SDS at 55 °C for 30 min with frequent vortexing. The excess NEM was removed, and the protein was collected using a 10 kDa Amicon Ultra centrifugal filter device. Collected protein sample was divided to two equal portions, d-SS1 and d-SS2. Sample d-SS1 was treated with 5 mM sodium ascorbate, 1 μM CuCl, and 5 mM NEM at 25 °C for 60 min. Treatment was removed, and sample d-SS1 was washed with the reaction buffer using the cutoff filter. Both sample d-SS1 and sample d-SS2 were then incubated with 50 mM TCEP at 60 °C for 10 min. After removing TCEP, remaining protein in sample d-SS1 and d-SS2 were treated with 5 mM d_5-NEM at 25 °C for 1 h, respectively. The samples were then run on SDS−PAGE, and the protein bands of interest were excised and subjected to in-gel tryptic digestion using Pierce in-gel trypsin digestion kit (Thermo Scientific). Resulting digests were analyzed using either an Agilent 6310 ESI Ion Trap mass spectrometer (Agilent Technologies) or a Thermo hybrid LTQ-FT linear ion trap mass spectrometer (Thermo Electron Corp.) in positive ion mode as described in Supporting Information. The precision of the analysis is high for replicate experiments on the same batch of recombinant protein as reported. For experiments carried out under controlled oxygen level, the reaction buffer was prepared by bubbling through either O_2 or N_2 for at least 1 h prior to the experiment. During the treatment the reaction vial was sealed with a proper rubber septum, and additional reagents were transferred by a syringe.

SH-SY5Y Cell Lysate Sample Preparation. The normal growth medium was replaced by reduced serum medium (Life Technologies) 1 h prior to CysNO treatment (1 mM, 20 min), and the cell lysate was prepared as detailed in Supporting Information.

Estimation of Protein *S*-Nitrosylation with Biotin Pull-Down. Lysates from CysNO-treated (10, 100, and 1000 μM) SH-SY5Y cells were treated with NEM (20 mM) and 5% SDS and incubated for 30 min at 55 °C to label the unreacted Cys thiols. The lysate was then filtered through 10 kDa Amicon filters, and the recovered proteins were reacted with biotin maleimide (1 mM) in the presence of sodium ascorbate (5 mM) to label the nitrosylated Cys thiols. The excess reagents were removed through 10 kDa cutoff filters and the biotin maleimide labeled proteins were separated using streptavidin-coated magnatic beads (Invitrogen). The lysate fraction with the non-nitrosated proteins was reduced with TCEP (50 mM) at 60 °C for 10 min and was reacted with 5 mM NEM at 25 °C for 1 h to label the oxidized Cys residues. The biotinalated and the nonbiotinalated protein fractions were analyzed using the SDS−PAGE followed by quantitation of the Coomassie-stained gel bands with ImageJ software.[56]

Cellular Denitrosylation Study. The concentration of protein nitrosothiols induced by CysNO and cellular nitrosothiols were measured by a triiodide-dependent, ozone-based chemiluminescence assay (described in Supporting Information) using Sievers 280i nitric oxide analyzer (NOA, GE Analytical Instruments). Briefly, after the treatment (1 mM CysNO), the CysNO-containing medium was removed, the cells were washed (PBS), and the lysate was immediately collected for time point 0. For other time points, cells were maintained in fresh growth medium at 37 °C protected from light and lysed at 8, 15, 25, and 45 min. After centrifugation, each lysate supernatant was divided I half, and one portion was directly subjected to chemiluminescence assay to measure the total cellular nitrosothiols. The other portion was filtered through the 10 kDa cutoff filter to separate high molecular weight nitrosothiols (HMW RSNO) from low molecular weight nitrosothiols (LMW RSNO). HMW RSNO was then measured by chemiluminescence assay using NOA. Data were obtained from three individual experiments and triplicates for each time point.

■ ASSOCIATED CONTENT

Supporting Information

Detailed methods, supplementary figures, MS spectra, and PAGE blots. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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