Site-specific and redox-controlled S-nitrosation of thioredoxin

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Protein S-nitrosation on cysteine residues has emerged as an important posttranslational modification in mammalian cells. Previous studies have suggested a primary role for thioredoxin (Trx) in controlling protein S-nitrosation reactions. Human Trx contains five conserved Cys, including two redox-active catalytic Cys (Cys32 and Cys35) and three non-active-site Cys (Cys62, Cys69, and Cys73), all of which have been reported as targets of S-nitrosation. Prior reports have studied thermodynamic end points of nitrosation reactions; however, the kinetics of Trx nitrosation has not previously been investigated. Using the transnitrosation agent, S-nitrosogluthationine, a kinetic analysis of the selectivity and redox dependence of Trx nitrosation at physiologically relevant concentrations and times was performed, utilizing a mass spectrometry-based method for the direct analysis of the nitrosated Trx. Reduced Trx (rTrx) was nitrosated 2.7 times faster than oxidized Trx (oTrx), and rTrx was nitrosated selectively on Cys62, whereas oTrx was nitrosated only on Cys73. These sites of nitrosation were confirmed at the peptide level using a novel modification of the biotin-switch technique called the reductive switch. These results suggest separate signaling pathways for Trx-SNO under different cellular redox states.

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Detection of protein SNOs is difficult. The commonly used methods suffer from low sensitivity and high background. The biotin-switch method and variations on the original procedure (19, 25, 27–34) involve denaturation of the protein, alkylation of free Cys, and precipitation of the protein. SNOs are then reduced with ascorbate followed by the reaction of the newly liberated Cys residues with a reactive group containing a tag for later identification. The biotin-switch method is limited by high background, low sensitivity, and nonspecific loss of protein during sample workup (35). Interpretation of results is complicated by differential ascorbate reduction and labeling rates of different Cys-SNOs and Cys, respectively (35, 36). Furthermore, the sites of nitrosation may be scrambled or lost upon denaturation. The biotin switch and alternate methods (e.g., MS or UV detection) (3, 7), are typically performed at supraphysiological concentrations of protein (10–100 μM) and GSNO (100 μM–1 mM) to aid detection. Additionally, long reaction times (>10 min) are used, which likely drives the reaction to a thermodynamic equilibrium; thus, kinetic information on nitrosation is lost.

To gain a better understanding of the Trx-GSNO reaction under biologically relevant conditions, we developed an in vitro, MS-based methodology for the rapid and direct detection of protein-S-nitrosation, allowing kinetic analysis of the transnitrosation reaction between GSNO and Trx while simultaneously monitoring Trx redox status. We studied the selectivity and redox dependence of this reaction under relevant cellular concentrations of Trx and GSNO. rTrx and oTrx not only show different rates of nitrosation with GSNO, but also strikingly different reaction selectivities, with nitrosation of oTrx on Cys73 and rTrx on Cys2. We confirmed these findings with a peptide-level analysis, using a modification of the biotin-switch technique that we call the reductive switch, in which labeling is performed on native protein to prevent loss or scrambling of nitrosation upon denaturation. Together, these techniques allowed a thorough analysis of both the kinetics and the selectivity of Trx nitrosation by GSNO. These results may have implications regarding selective signaling under oxidative stress/hypoxic conditions.

**Results**

**Direct Detection of Trx Nitrosation and Redox State with TOF-MS.** The redox state of the Trx active-site Cys may affect nitrosation (3, 7). To explore the effect of the Trx redox state on the GSNO-dependen
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t rate of nitrosation, conditions were first developed to fully oxidize and reduce the Trx active-site Cys prior to reaction with GSNO. The oxidation state of recombinant human Trx1 was determined by TOF-MS analysis by deconvolution of the protein envelope to determine protein mass. Treatment of humanTrx (recombinant histidine-tagged protein) with Tris(2-carboxyethyl)phosphine (TCEP) produced rTrx with a mass of 14,126.6 (Fig. 1 A and D). Oxidized Trx is often prepared by treatment with H2O2 (3, 7); however, incubation of Trx with 1 mM H2O2 resulted in apparent thiol oxidation beyond the active-site Cys (m/z of 14,124.1) as well as in intermolecular disulfide bonds, yielding a dimeric oTrx (Fig. 1 B and E). Therefore, more moderately oxidizing conditions were investigated. Treatment of Trx with 1.2 equivalents of insulin (a natural Trx substrate) yielded oTrx (m/z of 14,124.7) that remained monomeric (Fig. 1 C and F). Insulin treatment is a physiologically relevant way of generating oTrx in vitro. This 2-Da shift between rTrx and oTrx (loss of two protons upon formation of the disulfide) was readily observed by TOF-MS analysis. When uniformly [15N]-labeled oTrx (molecular weight (MW) of 14,297.4) was mixed in a 1:1 ratio with [15N]-rTrx (MW of 14,126.6), both [15N]-oTrx and [15N]-rTrx underwent 1-Da mass shifts to 14,298.3 and 14,125.6, respec-

| Fig. 1. Direct detection of rTrx, oTrx, and Trx-SNO. (A) rTrx (TCEP treated) protein envelope. (B) oTrx (H2O2 treated) showing evidence for dimeric protein in the protein envelope (dimer exhibits double the number of peaks within a given interval due to higher charge states). (C) oTrx (insulin treated) protein envelope. (D) Deconvoluted spectrum of rTrx. (E) Deconvoluted spectrum of oTrx (H2O2 treated). (F) Deconvoluted spectrum of oTrx (insulin treated). (G) Deconvoluted spectrum of [15N]-rTrx treated 1:1 with [15N]-oTrx, showing the shift of both [15N]-rTrx (Left) and [15N]-oTrx (Right) to a 50% reduced state (MW of 14,125.6 and 14,298.3, respectively). Black lines represent 50% oxidized, dashed gray lines represent fully oxidized, and solid gray lines represent fully reduced. (H) Deconvoluted spectrum for a representative time course of GSNO (10 μM) incubation with rTrx (1 μM) showing the singly nitrosated (+29) peak and lack of double nitrosation (+58). The peak at 14,167 (+41) did not increase over time upon GSNO treatment; it is likely the Trx:acetonitrile adduct. (I) Deconvoluted spectrum of polynitrosated Trx (treatment of 100 μM Trx with 1 mM GSNO for 15 min). |
tively, after 1 min of reaction (Fig. 1C). This result demonstrates that the average mass of the deconvoluted Trx peak represents the relative percentage of rTrx and oTrx in a mixture. Furthermore, oTrx is a substrate for rTrx, and in solution these species are in rapid equilibrium.

Having determined that oTrx and rTrx could be quantitatively differentiated, the transnitrosation of Trx by GSNO and the redox state of Trx over the course of reaction were simultaneously measured. The RapidFire DS Module™ (Agilent Technologies), a dual-needle, desalting system (Fig. S1) was used for rapid removal of buffer salts, GSH, GSH disulfide (GSSG), and GSNO from the reaction mixtures, followed by direct injection for analysis by TOF-MS. The rapid and in-line desalting allowed the monitoring of the reaction at the anticipated physiological concentrations of both Trx (1 μM) and GSNO (10 μM) at short time points (1–4 min). The resolution and sensitivity of the TOF-MS system were sufficient to detect nitrosated proteins above 2% of total Trx. During the reaction time course, the deconvoluted base peak for rTrx (14,126.6) decreased in intensity, while a +29 peak emerged (MW of 14,155.4, indicating the gain of NO and loss of hydrogen) (Fig. 1F). The peak at 14,167 (+41) did not increase over time upon GSNO treatment; it is likely the acetocyanitride adduct of Trx. Under these conditions, there was no evidence for glutathionylation [+306, which has been detected by others using aged GSNO stocks that contain decomposition products (37)] or for doubly (+58) or triply (+87) nitrosated Trx, which is consistent with the specificity implied by selective GSNO binding and transfer.

Reactions carried out at higher concentrations of GSNO (1 mM) and rTrx (100 μM) for longer periods of time (15 min) produced increased amounts of polynitrosation (Fig. 1J) and led to complete disulfide formation at the active site (rTrx to oTrx). The dominant Trx species was doubly nitrosated, and significant proportions of protein with three, four, and five NO additions were also observed. Because Trx contains only five Cys, even the active-site Cys (thought to be involved in denitrosation) became nitrosated under these conditions, possibly due to the simultaneous oxidation of both Cys32 and Cys35 before the disulfide could form. At shorter times (4 min, Fig. S2), significant amounts of single, double, and triple nitrosation were observed.

Treatment of oTrx and rTrx with GSNO. Having determined that Trx nitrosation could be directly detected without derivatization, separate kinetic analyses on the rate of nitrosation for rTrx and oTrx by GSNO were carried out. Both rTrx and oTrx (1 μM) were readily nitrosated upon treatment with 10 μM GSNO. The rate of nitrosation for rTrx was greater than that for oTrx, with pseudo-first-order rate constants of 3.53 ± 0.15 and 1.33 ± 0.05 s⁻¹, respectively (Fig. 2A). No evidence of polynitrosation was observed. oTrx-SNO was 2 Da lower in mass from rTrx-SNO (Fig. 2C), indicating that the initial redox state of Trx was reflected in the nitrosated species, without interconversion between rTrx to oTrx (as would be expected if rTrx first oxidized to oTrx and was then nitrosated). Rather, oTrx-SNO and rTrx-SNO are two distinct species that form at different rates.

Over the course of the reaction, rTrx partially oxidized, forming low amounts of oTrx (<10% of total unmodified Trx, Fig. 2B). Oxidation of the rTrx active site following reaction with GSNO has been reported previously (3). On examination of the rTrx active-site oxidation with GSNO, GSSG, and GSH, it appears that GSSG, a contaminant in commercially available GSNO, is the relevant oxidant in vitro (Fig. 3 and Figs. S3 and S4).

Nitrosation Specificity of rTrx Versus oTrx. Given the striking differences in nitrosation rate between rTrx and oTrx, the redox-dependent specificity of Cys nitrosation was determined. A library of Trx Cys mutants was designed to remove possible sites of nitrosation. The mutants made included C62S, C69S, C73S, C69S/C73S, and C23S/C35S and a series of kinetic experiments with GSNO were performed. Pseudo-first-order rate constants (Fig. 3C) were calculated based on the percent of nitrosated Trx. As previously described, mutant Trx (1 μM) was reacted with GSNO (10 μM) for 1, 2, 3, and 4 min, desalted using the RapidFire DS Module™, and analyzed by TOF-MS. For all analyzed proteins, only a single NO addition was observed under these conditions.

The rTrx mutants C73S (3.26 s⁻¹) and C69S (3.59 s⁻¹), both nitrosated at rates similar to wild type (3.53 s⁻¹), with the double mutant C73S/C69S (2.92 s⁻¹) slightly slower (Fig. 3A, black bars; Fig. 3C contains rate constants with error for each). Strikingly, rTrx-C62S (0.56 s⁻¹) nitrosated significantly more slowly (6-fold), suggesting that the primary site of nitrosation for rTrx is Cys62. The oTrx mutant C73S demonstrated the slowest nitrosation rate (0.73 s⁻¹), with C69S (1.13 s⁻¹) also nitrosating slightly slower than wild type (1.33 s⁻¹) (Fig. 3A, gray bars). C69S/C73S double mutant showed no detectable nitrosation, and C62S nitrosated slightly faster than wild type (1.92 s⁻¹). Based on these observations, Cys73 is the primary site of nitrosation for oTrx, with a secondary site at Cys69. Cys62, the presumed site of nitrosation for rTrx, does not appear to be nitrosated on oTrx. The specificity of oTrx nitrosation is markedly different from rTrx. The active-site mutant C23S/C35S was used to ensure that the use of insulin and TCEP did not influence the rate of nitrosation; indeed, there was no significant difference between rates of the insulin treated (1.53 s⁻¹) and TCEP treated (1.64 s⁻¹) C23S/C35S mutant (Fig. 3B).

Peptide-Level Confirmation of Cys Selectivity for Nitrosation of oTrx and rTrx. To confirm the nitrosation selectivity suggested by the TOF-MS analysis of intact Trx and Trx mutants, a modified version of the biotin-switch assay, dubbed the dextricutive switch, was employed to identify the individual sites of nitrosation (Fig. S5). Briefly, the reaction was initiated by incubating Trx (1 μM) with GSNO (10 μM), and quenched over time (3, 4, 5 min) with deuterium-labeled N-ethyl maleimide (d₄-NEM, heavy label). Nonheavy labeled Cys were subsequently reduced with DTT and counterlabeled with nonisotopic NEM (light label). Samples were then trypsinized and analyzed by liquid chromatography (LC)-TOF-MS to determine ratios of heavy to light label, and C23S/C35S and a series of kinetic experiments with GSNO were performed. Pseudo-first-order rate constants (Fig. 3C) were calculated based on the percent of nitrosated Trx. As previously described, mutant Trx (1 μM) was reacted with GSNO (10 μM) for 1, 2, 3, and 4 min, desalted using the RapidFire DS Module™, and analyzed by TOF-MS. For all analyzed proteins, only a single NO addition was observed under these conditions.

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In the (nondenatured) state, local protein structure improved SNO stability (as it is thought to in vivo) during the initial alkylation (heavy label) step; even in the folded state, nearly complete d5-NEM labeling was seen in control experiments with rTrx (>90%), whereas the reduced proteins (wild type, C62S, or C73S) all had rates <0.9/s, whereas the reduced proteins (wild type, C73S) nitrosated readily on the Cys62/Cys69-containing peptide (2.77/s and 3.58/s respectively, Fig. 4, white bars). Considerable attenuation of the nitrosation rate on the Cys62/Cys69-containing peptide of rTrx-C62S mutant (0.73/s) supports the finding that Cys62, and not Cys69, is the target of nitrosation on rTrx.

Unexpectedly, the rate of nitrosation on the Cys73-containing peptide of rTrx (2.23/s) was faster overall than the nitrosation of the Cys73-containing peptide of oTrx (1.47/s) (Fig. 4B). For the C62S mutant, this selectivity was reversed, and nitrosation on the Cys73-containing peptide was faster for oTrx-C62S (2.8/s) than

Fig. 3. Nitrosation rates of Cys mutants of Trx. (A) Plot of calculated rates of nitrosation for rTrx (black) and oTrx (gray). Rates were calculated assuming pseudo-first-order kinetics for Trx with an excess of GSNO, as the negative of the linear slopes of the plot of ln (Trx consumption) versus time. Trx consumption was calculated as the average (with standard deviation) of 100-% nitrosation (determined by the ratio of the +29 (singly nitrosated) peak to total Trx). Rates were calculated from a single experiment with n = 5 at each data point (C69S, C73S/C69S, C32S, C32S/C35S), or as the average from injections performed over multiple days (wild-type, C62S, C73S). (B) Plot of calculated nitrosation rates for the active-site mutant C32S/C35S, which served as a control for the reduction/oxidation conditions used in A. (C) Table of the rates shown in A and B.

Fig. 4. Reductive-switch assay for nitrosation. (A) Rates of nitrosation for rTrx and oTrx on the peptides containing Cys73 (gray) and Cys62/Cys69 (white). C62S+C73S (mix) is a 1:1 mixture of the two mutant proteins (final concentration 1 μM). All data points (0.1, 3, 4, and 5 min) were collected in triplicate from independently prepared samples. All samples were subjected to the reductive-switch protocol, and nitrosation at each data point was determined as the ratio of heavy labeled to light-labeled peptide. Rates were calculated assuming pseudo-first-order kinetics, as the negative of the linear slopes of the plot of ln (Trx consumption) versus time. (B) Table of the rates shown in A.
for rTrx-C62S (1.29/s). Furthermore, the rTrx-C73S mutant protein nitrosated slightly faster than wild-type rTrx on the Cys62/Cys69-containing peptide. Taken together with the intact protein results, these data indicate that Cys62 is key to the nitrosation of rTrx, but that downstream reactions of rTrx-Cys62-SNO can occur, possibly via inter- or intra-Trx transnitrosation from Cys62 to Cys73, explaining the nitrosation observed on Cys73 of wild-type rTrx. If intermolecular transnitrosation was occurring, we would expect that Cys62 on one molecule of Trx would compensate for the mutation in rTrx-C62S, allowing faster nitrosation on Cys73; whereas if intramolecular transnitrosation from Cys62 to Cys73 was occurring, the addition of Cys62 on another Trx molecule would not change the rate of nitrosation on Cys73. To determine which of these possibilities were most likely, a 1:1 mixture of the C62S and C73S Trx proteins was assayed. Both the reduced and oxidized forms of this mixture (rC62S + rC73S and oC62 + oC73S, respectively) had rates identical to wild type on both the Cys73-containing peptide and the Cys62/Cys69-containing peptides (Fig. 4), consistent with intermolecular transnitrosation, where rTrx-Cys62-SNO transfers NO to Cys73 on another rTrx molecule (Fig. 5, Lower Left). The in vivo relevance of this SNO-Trx mediated transnitrosation of Trx remains unclear.

Discussion

In the studies reported here we have undertaken a kinetic analysis of the redox dependence on Cys selectivity of Trx following exposure to GSNO. To this end, two previously undescribed methods for the analysis of Trx nitrosation were developed. The first, using a dual-valve rapid desalting device, followed by TOF-MS, allowed for the direct detection of Trx nitrosation and redox state at short time intervals. By analyzing the intact protein without derivatization, SNO loss and intra- or intermolecular scrambling of the nitrosation site was minimized. This technique allowed for analysis at time points as short as 1 min, allowed many replicates in a single experiment, was highly sensitive (approximately 1 pmol protein could be detected), and was highly reproducible. The second methodology, the reductive switch, was complementary to the online-desalting/TOF-MS intact protein experiments. Analysis of peptides following the reductive-switch procedure allowed the assignment of nitrosation sites to single peptides via TOF-MS. Taken together, these two techniques revealed the kinetics and selectivity of Trx nitrosation by GSNO.

Of the five cysteine residues in human Trx1 (Fig. 5A), only two were selectively nitrosated by GSNO, Cys62 and Cys73. rTrx nitrosated rapidly and primarily at Cys62, whereas oTrx nitrosated more slowly overall with selectivity toward Cys73 (Fig. 5B). Cys69, Cys32, and Cys35 did not appear to be selective targets for nitrosation; nitrosation occurred only at high concentrations of GSNO (1 mM). At biologically relevant concentrations, Trx was nitrosated at only a single Cys (Cys62 or Cys73). This striking specificity preference in Cys reactivity between oTrx and rTrx is what would be expected for a modification involved in signal transduction. The differences in rates of nitrosation (with rTrx nitrosation >2-fold faster than oTrx) imply that, in the case of a mixture of rTrx and oTrx, rTrx nitrosation would predominate under kinetically controlled conditions.

The concentrations of protein and GSNO had dramatic effects not only on the rates but also on the selectivity of Trx nitrosation. At the high concentrations of GSNO often used for in vitro experiments (100 μM–1 mM), nucleophile–electrophile reactivity drives the reaction leading to full nitrosation of all cysteines in the protein. This intrinsic reactivity, and the high concentrations needed for clear signal when using many standard nitrosation assays, provides a basis for the conflicting data in the literature regarding Trx nitrosation and highlights the importance of physiological conditions and concentrations for studying the selectivity of transnitrosation reactions. In sharp contrast, the results when the reaction is carried out at concentrations comparable to measured physiological concentrations of Trx (2–12 μM (38) and GSNO (1–10 μM (16, 17, 39)) showed clear selectivity for specific Cys and was redox-dependent.

We hypothesize that the selectivity of nitrosation is controlled by the conformation of the protein, either by conformational changes affecting GSNO binding (binding near Cys73 in oTrx and Cys62 in rTrx) or by different protein microenvironments in rTrx and oTrx that either activate the nucleophilicity of the respective Cys (Cys62 and Cys73) or that stabilize the corresponding SNOs. Although rTrx and oTrx are structurally similar (40) according to crystallographic measurements, the local dynamics of the protein may well be different in the two redox states, because the active-site disulfide bond of oTrx is predicted to reduce movement of the loops surrounding the active site. Cys73, the Cys nearest the active site (Fig. 5A), would be predicted to be particularly susceptible to conformational changes around the active site.

The peptide-level analysis using the reductive-switch assay supports the nitrosation of rTrx on Cys62 and of oTrx on Cys73; however, these data revealed an additional mechanistic insight: Cys62 is required for rTrx nitrosation on Cys73, but not for oTrx nitrosation on Cys73. oTrx appears to react with GSNO directly at Cys73, whereas rTrx appears to react with GSNO directly at Cys62, making rTrx-Cys62-SNO the direct kinetic product of the reaction of rTrx with GSNO. However, nitrosation was observed on Cys73 of wild-type rTrx, but not Cys62-rTrx, and the reaction of a mixture of C62S-rTrx and C73S-rTrx showed wild-type nitrosation rates on Cys73, implying that intermolecular transnitrosation is possible. It appears that rTrx-Cys62-SNO may subsequently react with another molecule of rTrx to form rTrx-Cys73-SNO (Fig. 5B, Lower Left), and thus rTrx itself can be considered a target for transnitrosation reactions with rTrx-Cys62S. The in vivo significance of this is unknown, as it would be dependent on transient Trx-Trx interactions in areas of high localized Trx concentration. It is possible that for rTrx, Cys62-SNO dominates at shorter time points (at 5 min or less) and is the kinetically favored product, whereas Cys73-SNO is the more stable, thermo-
of oxidative stress, oTrx (nitrosation). Under cellular reducing conditions, rTrx (and rTrx-SNO) likely predominate in vivo under normal conditions. Because both rTrx and oTrx are NADPH-dependent, it is reasonable to assume that oTrx and rTrx-SNO may accumulate under conditions of oxidative stress or during depletion of NADPH. The differences in Cys selectivity for nitrosation between rTrx and oTrx may represent a redox-dependent mechanism for signal transduction via cascading transnitrosation reactions. Because Cys73 and Cys62 are on opposite faces of the protein (Fig. 5A), it is possible that oTrx-SNO and rTrx-SNO have different binding partners and thus different downstream transnitrosation targets (Fig. 6). This would provide an alternate mechanism of signal transduction during periods of nitrosative or oxidative stress.

Materials and Methods

Plasmids, Protein, and Reagents. All reagents and chemicals were purchased from Sigma unless otherwise noted. Human Trx1 was expressed as a N-terminal His6-tagged construct from the expression vector pET16b, in Escherichia coli BL21(DE3) as described previously (4). The [35S]-labeled Trx was expressed in BL21(DE3) cells grown in minimal media (M9) supplemented with 14 g/L [35S]-ammonium sulfate (Cambridge isotopes) and purified as previously described. Cys mutants were generated by QuikChange (Stratagene) mutagenesis using the manufacturer’s protocol. Trx Cys variants were confirmed using TOF-MS of the intact proteins and the tryptic-digested peptides. Protein purity was assayed by SDS-PAGE, and concentration was determined by absorbance at 280 nm.

Direct Analysis of Protein Nitrosation. TRX and oTRX were prepared as described above, then diluted to 2 μM in 50 mM potassium phosphate, pH 7.4 (PB). A fresh solution of GSNO (Cayman Chemicals) was prepared in PB and the concentration determined spectrophotometrically (ε = 0.92 at 335 nm). GSNO stocks were diluted to 20 μM and stored protected from light for no longer than 6 h. All reactions were carried out at room temperature. TRX and GSNO were loaded into separate 1-mL syringes and connected to a dual syringe pump attached to a 3-μL mixing tee. After mixing, TRX and GSNO were allowed to react in the tubing for a set time (based on flow rate and dead volume) before application to the RapidFire™ DS Module (Agilent Technologies). The system was equilibrated with water (containing 0.1% formic acid, mobile phase A) in the first valve state. During the second valve state, the sample loop (10 μL) was filled with the reaction mixture. In the third valve state, the sample (C18) was applied to the trapping cartridge (C18) and washed with mobile phase A. In the fourth valve state, the sample was eluted from the trapping cartridge with mobile phase B (acetonitrile containing 0.1% formic acid) and injected directly onto an Agilent TOF mass spectrometer (MSD TOF, Model 1969A). Following injection the device returned to valve state 1. The cycle (equilibration, loop filling, washing/desalting, and elution) was repeated 5 times for each time point. Syringe pump flow rates were changed for each time point to increase reaction time. The following instrument parameters were used for the TOF-MS: gas temperature, 350 °C; gas flow, 10 L/min; nebulizer, 30 psi; capillary voltage, 3,500 V; fragmentor, 90 V. The total ion chromatogram consisted of periodic elutions for each replicate. The extracted protein envelopes were manually examined to ensure no dimer or insulin remained. Deconvolution of the protein envelopes was performed using MassHunter Software (Agilent Technologies) in the 13,000- to 16,000-MW range, using the exact mass range of 600-1,600. The resulting deconvoluted spectra intensities were used to calculate the relative ratio of TRX to SNO-TRX for quantitative analysis of reaction rates. For denitrosation experiments, 1 μM oTRX was treated with 10 μM GSNO or 1 mM GSH for 2 min, after which 10 μM GSNO or 1 mM GSH was added via a second syringe pump and mixing tee, and the mixture incubated for 1 min before rapid desalting and TOF-MS analysis as above.

Reductive-Switch Assay. TRX and oTRX were prepared as described above, then diluted to 1 μM in 50 mM potassium phosphate, pH 7.4, with 200 mM NaCl (PBS). Fresh GSNO stocks were prepared as above in PBS. All reactions were performed in triplicate. For each reaction, the final volume was 500 μL containing 1 μM TRX and 10 μM GSNO. Reactions were quenched (Cys alkylated) by the addition of 1 mM (final concentration) d5-labeled N-methyl-maleimide (d5-NEM, CDN Isotopes) at 37 °C for 1 h. For the 0.1-min reactions, GSNO was added, tubes vortexed, then d5-NEM was immediately added. Samples were then treated with 2 mM DTT for 10 min at room temperature to reduce nitrosothiols. NEM (5 mM) was added as a counterlabel and samples were reacted for 1 h at 37 °C. Samples were concentrated over 5-kDa cutoff spin concentrators (Vivaspin) to 50 μL, followed by addition of trypsin (4 μg/μL) (Trypsin Gold, Promega). Samples were digested for 1 h at 37 °C, then analyzed via LC-MS using a Zorbax C18 (2.1 × 50 mm) column and a Waters LCT Premier XE TOF-MS. The heavy- and light-labeled peptides containing Cys32/Cys35, Cys62/Cys69, and Cys73 were quantified using MassLynx software (Waters).

Fig. 6. Hypothetical model for redox-dependent signal transduction via TRX nitrosation. Under cellular reducing conditions, TRX (left) nitrosates on Cys62 and transfers that nitroso group to protein partners (blue). Under conditions of oxidative stress, oTRX (right) nitrosates on Cys73 and transfers that nitroso group to different protein partners (red), such as Casp3.

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Supporting Information

Fig. S1. Schematic of the RapidFire DS Module™ (Agilent Technologies) rapid desalting system. The device starts by equilibrating the trapping cartridge with pump 1 (water containing 0.1% formic acid) (state #1). Samples are applied to the device by syringe infusion to fill sample loop (state #2). The sample loop is then flushed with pump 1, applied to a trapping cartridge (C18), washed, and the sample buffer is carried to waste (state #3). The trapped sample is then eluted from the trapping cartridge with pump 2 (acetonitrile containing 0.1% formic acid) and applied directly to the detection device (TOF-MS) (state #4). After injection the device returns to state #1 for reequilibration. W, waste; P1, pump 1; P2, pump 2; TC, trapping cartridge; V1, valve 1; V2, valve 2.

Fig. S2. Reaction of 100 μM Trx with 1 mM S-nitrosoglutathione (GSNO). Deconvoluted spectra of Trx treated with GSNO at higher concentrations for 4 min. The ratio (1:10) of Trx to GSNO is the same as shown in Fig. 1H, but both reactants are 100-fold more concentrated. Significant amounts of the 1-SNO, 2-SNO, and 3-SNO species were clearly detected, with small amounts of the 4-SNO.
Fig. S3. Oxidation of rTrx. Oxidation rates (disulfide formation at the active site, Cys 32-Cys35) determined by mass shift of Trx following treatment with GSNO, glutathione (GSH), and glutathione disulfide (GSSG) at various concentrations. rGSH is depleted of residual GSSG by the addition of tris(2-carboxyethyl) phosphine (TCEP). Percent oxidation for each time point was calculated as the mass shift from fully reduced (with a 2-Da shift for fully oxidized). Rates were determined by the linear slope of a plot of % oxidation versus time. The commercially available GSNO used in these studies contained detectable amounts of GSH and GSSG (Fig. S4). Furthermore, in the cellular milieu, the Trx-GSNO reaction takes place in the presence of high concentrations of GSH and other cellular oxidants and reductants. Thus, the reduction and oxidation of Trx by GSH and GSSG was examined (Fig. S2). There was some buffer/air oxidation of rTrx, and a slight increase in oxidation rate upon treatment of GSNO. It was anticipated that GSH would act as a reductant and was therefore surprising that 1 mM GSH oxidized Trx with a rate similar to that of 1.2 equivalents of insulin (a Trx substrate). Commercially available GSH also contained a significant percentage of GSSG (approximately 25%, Fig. S2), and GSSG also oxidized Trx. The oxidation rates of rTrx by GSNO and GSH suggest that GSSG, rather than GSH or GSNO, is the relevant oxidizing agent. To confirm this, a sample of GSH was assayed and then treated with a sufficient amount of TCEP to remove GSSG, forming fully reduced GSH (rGSH). This sample was analyzed by LC-MS (Fig. S2) to ensure that all the TCEP had been consumed. rGSH was then incubated with rTrx to probe for oxidation. rGSH did not oxidize rTrx, but, rather, protected against air/buffer oxidation. Taken together, these data imply that GSSG contamination, or GSSG formed during the reaction, causes oxidation of rTrx and that this oxidation is an in vitro artifact versus a reaction relevant to nitrosation in vivo.
Fig. S4. LC-MS analysis of GSH and GSNO stocks. (Left) LC-MS analysis of GSH stock treated with TCEP to reduce GSSG to GSH. GSH stock was treated with TCEP, and the reaction was monitored over time to observe the consumption of TCEP and GSSG (90-min reaction). GSSG \([4.9 \text{ min}, m/z \ 307 (M+H^+)]\), GSH \([2.6 \text{ min}, m/z \ 308]\), TCEP-oxide \([4.6 \text{ min}, m/z \ 267]\), and TCEP \([4.2 \text{ min}, m/z \ 251]\). Total consumption of TCEP was complete after 90 min of reaction. (Right) GSNO stock was analyzed by LC-MS, which demonstrated the presence of GSSG in the GSNO stock solution (approximately 5%). A small \(<1\%\) but detectable amount of GSH was also present. GSSG \([4.9 \text{ min}, m/z \ 307 (M+H^+)]\), GSH \([2.6 \text{ min}, m/z \ 308]\), and GSNO \([5.4 \text{ min}, m/z \ 337]\). Samples were separated on a Phenomenex Columbus C18 column \((150 \times 2 \text{ mm, } 5 \mu\text{m})\) using a gradient elution of water \((A)\), containing 0.1% formic acid, and methanol \((B)\), containing 0.1% formic acid as follows: isocratic at 2% B for 2 min, linear increase to 60% B over 8 min, linear increase to 90% B in 2 min, isocratic at 90% B for 2 min, linear decrease to 2% B in 1 min, reequilibrate at 2% B for 5 min. TIC, total ion chromatogram.
Fig. S5. Schematic of the reductive-switch assay. Trx is nitrosated with GSNO and quenched at various time points by the addition of the heavy-labeled cysteines reactive reagent $d_5$-N-ethyl maleimide (NEM) (blue ovals, heavy label). Nitrosation and oxidation are reduced with DTT and the newly reduced Cys labeled with NEM (green ovals, light label). Following tryptic digest and LC-MS, the ratios of heavy-labeling to light-labeling at each peptide are calculated.

Fig. S6. Reductive-switch assay oxidation controls. “Oxidation” refers to either nitrosation or other forms of sulfur oxidation (disulfide, etc.). (A) Trx shows no increase in oxidation for either the Cys73-containing peptide or the Cys62/Cys69-containing peptide upon treatment with 10 μM GSH. (B) Trx shows no oxidation increase for either the Cys73-containing peptide or the Cys62/Cys69-containing peptide upon treatment with 10 μM GSSG. (C) Trx shows a linear increase in oxidation of Cys73-containing peptide and the Cys62/Cys69-containing peptide upon treatment with 10 μM GSNO. All data were normalized to background with no GSNO. In all cases, the percent oxidation is the ratio of light-NEM to heavy-NEM.
Fig. S7. SNO-Trx stability to GSH. GSNO (10 μM) and rTrx (1 μM) were reacted at mixing tee #1 (Mixing T1) for 2 min. GSH (1 mM) was added at mixing tee #2 (mixing T2) and allowed to react for 1 min prior to capture and desalting on the RapidFire DS Module™ (Agilent Technologies). In control experiments GSH (1 mM) was mixed with Trx (1 μM) at mixing T1 for 2 min, and GSNO (10 μM) was added at mixing T2 and allowed to react for 1 min. Additionally, a control reaction of 2 min was performed using GSNO (10 μM) and rTrx (1 μM) in the absence of mixing T2. The percent SNO present after mixing is shown. All data collected represent n = 5. ND, none detected.

<table>
<thead>
<tr>
<th>Mixing T1</th>
<th>Mixing T2</th>
<th>SNO-Trx (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rTrx + GSNO</td>
<td>-</td>
<td>11.7 ± 0.2</td>
</tr>
<tr>
<td>rTrx + GSNO</td>
<td>GSH</td>
<td>7.0 ± 0.2</td>
</tr>
<tr>
<td>rTrx + GSH</td>
<td>GSNO</td>
<td>ND</td>
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</tbody>
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