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**Measurement of *S*-Nitrosylation Occupancy in the Myocardium With Cysteine-Reactive Tandem Mass Tags : Short Communication**

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# Cellular Biology

# Measurement of *S*-Nitrosylation Occupancy in the Myocardium With Cysteine-Reactive Tandem Mass Tags

## Short Communication

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***Rationale:*** S-nitrosylation (SNO) is a reversible, thiol-based protein modification that plays an important role in the myocardium by protecting critical cysteine residues from oxidation. However, little is known with regard to the percentage of a given protein that is modified by SNO (ie, SNO occupancy). Current methods allow for the relative quantification of SNO levels, but not for the determination of SNO occupancy.

***Objective:*** To develop a method for the measurement of SNO occupancy, and apply this methodology to determine SNO occupancy in the myocardium.

***Methods and Results:*** We developed a differential cysteine-reactive tandem mass tag (cysTMT) labeling procedure for the measurement of SNO occupancy. To validate this cysTMT labeling method, we treated whole-heart homogenates with the S-nitrosylating agent S-nitrosoglutathione and determined maximal SNO occupancy. We also examined SNO occupancy under more physiological conditions and observed that SNO occupancy is low for most protein targets at baseline. Following ischemic preconditioning, SNO occupancy increased to an intermediate level compared to baseline and S-nitrosoglutathione treatment, and this is consistent with the ability of SNO to protect against cysteine oxidation.

***Conclusions:*** This novel cysTMT labeling approach provides a method for examining SNO occupancy in the myocardium. Using this approach, we demonstrated that IPC-induced SNO occupancy levels are sufficient to protect against oxidation. (*Circ Res*. 2012;111:1308-1312.)

**Key Words:** *S*-nitrosylation ■ oxidation ■ occupancy ■ ischemic preconditioning

S-nitrosylation (SNO) is a reversible, thiol-based modification that can modulate the activity of myocardial proteins, including those involved with $Ca^{2+}$-handling and mitochondrial energetics.[1–4] Additionally, we and others demonstrated that SNO can protect against cysteine oxidation.[4,5] This is important in the setting of ischemia–reperfusion (IR) injury, in which the burst of reactive oxygen species generated at the onset of reperfusion can lead to protein oxidation and degradation. Myocardial ischemic preconditioning (IPC) has been shown to increase protein SNO,[2,4] and thus protection from oxidation is expected to be proportionate to the percentage of protein SNO (ie, SNO occupancy). Therefore, it is important to develop a method to determine SNO occupancy.

### In This Issue, see p 1249
### Editorial, see p 1253

Cysteine-reactive tandem mass tags (cysTMT) confer the advantage of multiple isobaric tags with reporter ions between 126 and 131 Da. These labels have been used to measure SNO levels in human pulmonary arterial endothelial cells[6] and to profile thiol redox sensitivity.[7] There are also a number of additional methods that have been used to quantify relative amounts of SNO,[2–4,8,9] but these methods did not measure total free thiols, and therefore did not measure occupancy. Herein, we utilized a novel differential cysTMT labeling strategy to provide a measure of SNO occupancy. In the same sample, free thiols were labeled with one isobaric tag (ie, $cysTMT_x$), while SNO thiols were subsequently labeled with a second isobaric tag (ie, $cysTMT_y$). Free and SNO thiols were then quantified via mass spectrometry. By quantifying both free and SNO thiols, the percentage of a given cysteine residue that was modified via SNO could then be calculated.

## Methods

Male C57BL/6 mouse hearts were Langendorff-perfused (Figure 1A) and homogenized as previously described.[1–4] Homogenates

| Non-standard Abbreviations and Acronyms | |
| --- | --- |
| cysTMT | cysteine-reactive tandem mass tag |
| DTT | dithiothreitol |
| GSNO | *S*-nitrosoglutathione |
| IPC | ischemic preconditioning |
| IR | ischemia–reperfusion |
| SNO | *S*-nitrosylation |

were then subjected to differential cysTMT labeling (Figure 1B). Please see the Online Supplement for additional materials and methods related to this study.

## Results

### Validation of cysTMT Labeling Procedure With S-Nitrosoglutathione

GSNO treatment was used to validate our cysTMT labeling procedure and determine maximal SNO occupancy. Whole-heart homogenates were incubated with a supraphysiological concentration of GSNO (1 mmol/L) in the presence of 2.5% SDS, and subjected to differential cysTMT labeling (Figure 1B). As expected, SNO occupancy greatly increased following GSNO treatment (Figure 2, Online Table I). Phosphoglycerate kinase 1 (Cys316) increased from 1.8%±0.1% at baseline to 64.6%±6.3% following GSNO treatment. Similarly, creatine kinase (Cys90) increased from 1.9%±0.2% to 61.5%±9.1%, while mitochondrial malate dehydrogenase (Cys89) increased from 4.5%±0.7% to 56.4%±7.0%. Proteins with high baseline SNO occupancy also showed increased SNO with GSNO treatment. Cytochrome c oxidase 6b1 (Cys65) increased to 63.3%±8.8% from 14.9%±2.6% at baseline, and dihydropyrimidinase-related protein 2 (Cys248) increased to 57.9%±11.3% from 13.6%±2.7%. Although there were several examples of proteins that showed high levels of SNO occupancy at baseline, the majority of proteins showed low levels (1%–10%), and this is consistent with physiological levels of nitric oxide. These results demonstrate that this differential cysTMT labeling procedure can be used for determining SNO occupancy. However, the maximal SNO occupancy observed with GSNO was only 60% to 70%, suggesting that a substantial percentage (≈30%–40%) of this labile modification was lost during labeling. This appears to be the case, as 40% of peptides from GSNO-treated samples incubated for an additional period of 2 hours (at 25°C) prior to cysTMT labeling showed a decrease in SNO occupancy of more than 10% compared to samples that were labeled immediately following GSNO treatment (Online Figure I).

### Myocardial Ischemic Preconditioning Invcreases SNO Occupancy

We were also interested in determining SNO occupancy with IPC. Hearts were subjected to IPC (Figure 1A), homogenized, and labeled (Figure 1B). We identified 275 SNO peptides in at least 2 of 7 samples for control and 2 of 5 samples for IPC (Online Table II). Of these peptides, 44 showed a 2-fold or greater increase in SNO with IPC compared to control, with 5 peptides exhibiting an increase in SNO with *P*<0.05 and an additional 8 peptides with
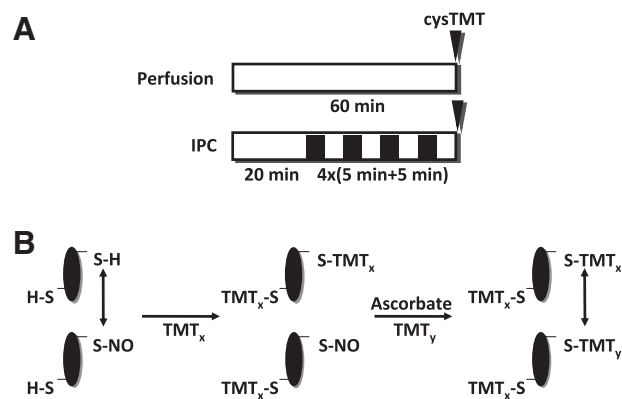
**Figure 1. Perfusion protocols and labeling methodology. A**, Hearts were randomly subjected to 1 of 2 perfusion protocols. Periods of perfusion and ischemia are indicated by open and solid boxes, respectively. **B**, Differential cysTMT-labeling strategy used for determining SNO occupancy. IPC indicates ischemic preconditioning; SNO, S-nitrosylation; cysTMT, cysteine-reactive tandem mass tag.

*P*<0.1. As shown in Figure 3, these peptides included hexokinase-1 (Cys662), short-chain acyl-CoA dehydrogenase (Cys109), mitochondrial malate dehydrogenase (Cys89), and mitochondrial aspartate aminotransferase (Cys106). Hexokinase-1 showed a 4-fold increase in SNO occupancy with IPC, increasing from 3.8%±0.4% to 14.9%±2.4%, while short-chain acyl-CoA dehydrogenase showed a 5-fold increase in SNO occupancy and increased from 4.5%±1.4% to 23.1%±8.4%. These data are consistent with our previous studies showing a ≈2- to 3-fold increase in SNO of selected proteins with IPC.[2,4] Previously, we showed a 2.7-fold increase in the SNO level of mitochondrial malate dehydrogenase with IPC,[2] and in the current study, mitochondrial malate dehydrogenase showed a 2-fold increase in SNO occupancy. Aspartate aminotransferase and citrate synthase were also observed in previous studies. Interestingly, we detected 12 SNO peptides with occupancies greater than 20% that were only observed with IPC. These identifications included several peptides of titin (Cys14224, Cys21689), dihydrolipoyl dehydrogenase (Cys477), and cysteine- and histidine-rich domain-containing protein 1 (Cys211). Thus, the SNO occupancy levels observed for many proteins with IPC are consistent with the ability of SNO to protect against oxidation.

### Incorporation of Additional Oxidative Modifications

An IPC-induced increase in oxidative modifications (ie, disulfides, sulfenic acids, etc.) could lead to the overestimation of SNO occupancy with the above approach due to the exclusion of oxidized cysteines from the total cysteine value used as the denominator. Since the cysTMT reagents label cysteine residues through the formation of a disulfide bond, oxidative modifications cannot simply be reduced and labeled with a third cysTMT reagent. Therefore, we measured oxidation in parallel samples using a modified approach (Online Figure II). We identified 187 oxidized peptides in at least 2 of 3 samples for both control and IPC (Online Table III). Under control
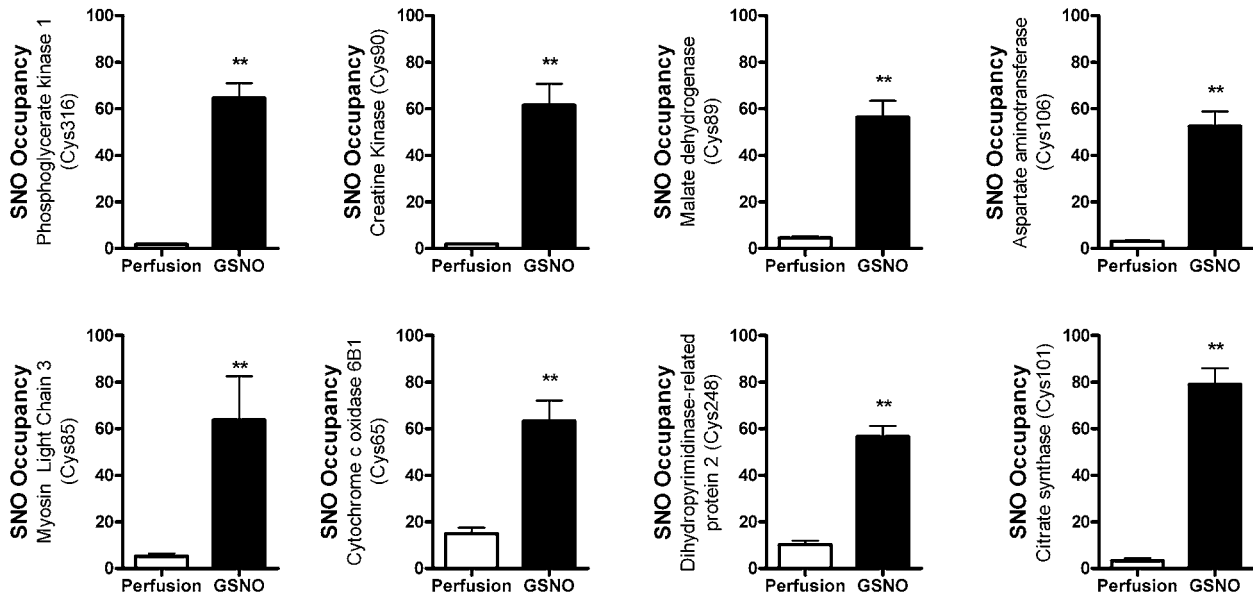
**Figure 2. Maximal SNO occupancy with GSNO treatment.** SNO occupancy (percentage of total) for representative proteins/cysteine residues in whole-heart homogenates treated with GSNO ($n=7$ per group for perfusion [open bars], 4 per group for GSNO solid bars). Representative proteins/cysteine residues: phosphoglycerate kinase 1/Cys316; creatine kinase/Cys90; malate dehydrogenase/Cys89; aspartate aminotransferase/Cys106; myosin light chain 3/Cys85; cytochrome c oxidase 6B1/Cys65; dihydropyrimidinase-related protein 2/Cys248; citrate synthase/Cys101 (**$P<0.05$ versus perfusion). SNO indicates S-nitrosylation; GSNO, *S*-nitrosoglutathione.

conditions, oxidation occupancy was between 1% and 15% for more than 65% of the identified cysteine residues. Following IPC, 113 peptides showed increased oxidation, but the majority of these cysteine residues still showed

oxidation occupancies of 15% or less. These low levels of oxidation have only a modest effect on the calculation for SNO occupancy. For example, myosin binding protein C (Cys439) showed no change in oxidation occupancy (control:
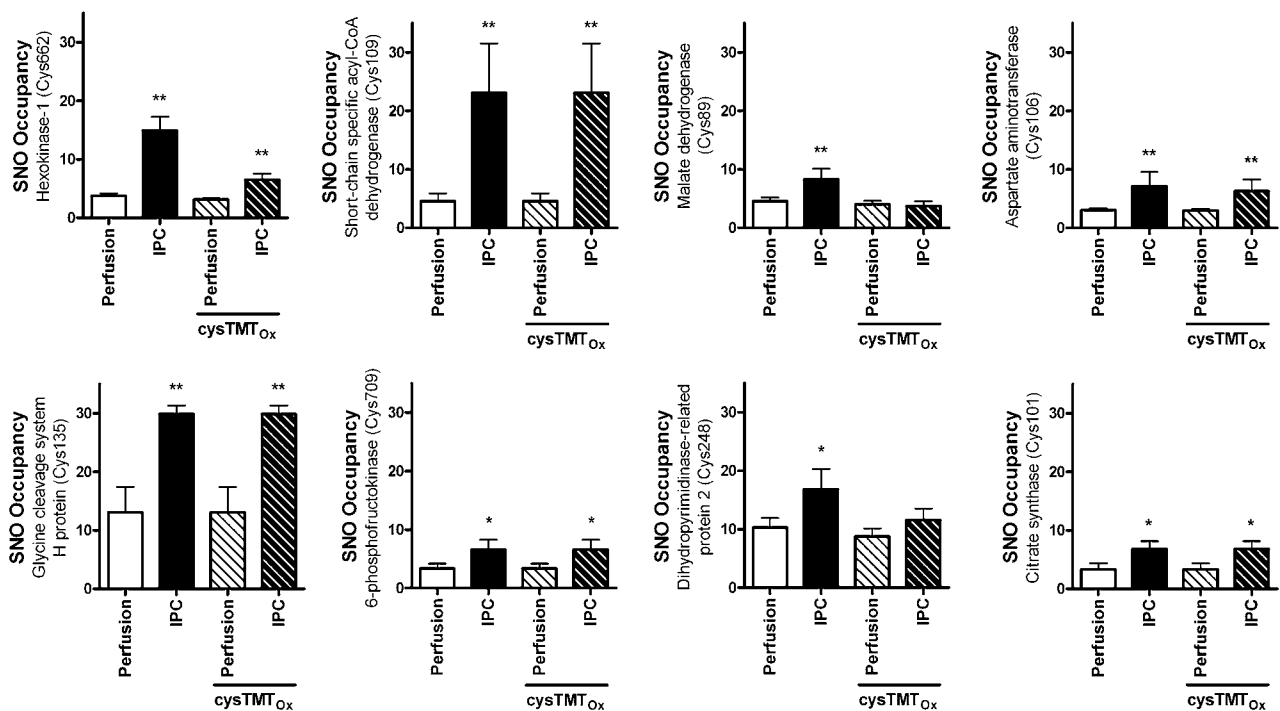


**Figure 3. SNO occupancy following IPC**. SNO occupancy (percentage of total) for representative proteins/cysteine residues in perfused hearts subjected to IPC, without cysTMT$_{Ox}$ (**left**): $n=7$ per group for perfusion [open bars], 5 per group for IPC [**solid bars**]; with cysTMT$_{Ox}$ (**right**): $n=3$ per group for perfusion [open hatched bars], 3 per group IPC [solid hatched bars]). Representative proteins/cysteine residues: hexokinase-1/Cys662; short-chain specific acyl-CoA dehydrogenase/Cys109; malate dehydrogenase/Cys89; aspartate aminotransferase/Cys106; glycine cleavage system H protein/Cys135; 6-phosphofructokinase/Cys709; dihydropyrimidinase-related protein 2/Cys248; citrate synthase/Cys101 (**$P<0.05$, *$P<0.1$ versus perfusion). IPC indicates ischemic preconditioning; SNO, S-nitrosylation; cysTMT, cysteine-reactive tandem mass tag.

13.2%±6.5%, versus IPC: 14.1%±10%). Factoring oxidation into the equation for SNO occupancy effectively decreased the calculation of SNO occupancy in IPC from 4.1%±2.0% without cysTMT$_{Ox}$ to 3.4%±1.4% with cysTMT$_{Ox}$, but this change is minimal. There was a small population of peptides that showed larger increases in oxidation with IPC, and these changes have the potential to alter the calculation of SNO occupancy. For example, cytochrome C oxidase 6B1 (Cys65) showed a 9% increase in oxidation with IPC (control: 52.1%±13.9%, versus IPC: 57.0%±30.6%). Factoring oxidation into the equation for SNO occupancy effectively decreased the calculation of SNO occupancy in IPC from 16.8%±8.5% without cysTMT$_{Ox}$ to 7.3%±5.2% with cysTMT$_{Ox}$. These results suggest that oxidative modifications have the potential to alter the calculation of SNO occupancy for certain protein targets (Figure 3), and should be included in order to accurately determine SNO occupancy.

LC-MS/MS summary data for all peptide identifications can be found in Online Table IV and Online Table V. Raw mass spectrometry data can be accessed at Peptide Atlas (http://www.peptideatlas.org/PASS/PASS00085).

## Discussion

The cysTMT labeling protocol described herein provides a novel approach for determining SNO occupancy. Previous studies have developed methods for the relative quantification of SNO, but these methods do not quantify SNO occupancy.[2–4,6,8,9] This method was validated using GSNO to induce maximal SNO occupancy (Figure 2) and was then applied to a physiological model of cardioprotection. Consistent with previous studies, we found a ≈2- to 3-fold increase in SNO with IPC.[2,4] We identified a number of proteins that showed a significant increase in SNO occupancy with IPC, including mitochondrial malate dehydrogenase, aspartate aminotransferase, and citrate synthase (Figure 3). We also found numerous SNO peptides with occupancies ranging from 20% to 55% that were only present with IPC. These SNO occupancy levels are consistent with the ability of SNO to shield proteins from oxidation. Additionally, the protection afforded by SNO may prevent the degradation of proteins that are excessively oxidized, and therefore, protein function is preserved as we showed in our previous study.[4] Indeed, IPC has been shown to prevent the degradation of many mitochondrial proteins following IR injury.[10] Interestingly, several of the protein targets that showed decreased degradation following IPC also showed high levels of SNO in our model of cardioprotection, including malate dehydrogenase, α-ketoglutarate dehydrogenase, and voltage-dependent anion channel protein 2.[2,4] Although SNO occupancy levels did not exceed 50% for most proteins, the preservation of 25% to 50% of protein function is likely adequate for cardioprotection. For example, consider that heterozygosity is often insufficient to cause disease. The potential also exists for underestimation of SNO occupancy using this approach; we find that even with GSNO treatment,

occupancy levels do not exceed 60% to 70%, suggesting that a minimum of 30% to 40% of SNO is lost during labeling. Furthermore, nitric oxide signaling is known to be compartmentalized. This study was performed using whole-heart homogenates, but certain cellular compartments may have enhanced nitric oxide or SNO signaling. Thus, low occupancy does not preclude an important role for SNO in cardioprotection.

The loss of SNO during labeling is a potential confounding factor with this approach, thus leading to the possible underestimation of SNO occupancy. Concurrent cysteine modifications, such as oxidation, are an additional confounding factor. Structural disulfides and other oxidative modifications at baseline are unlikely to affect SNO occupancy, but the stimulation of oxidative modifications could lead to the oxidation of free thiols, and this may alter the calculation for SNO occupancy (Figure 3). Therefore, the inclusion of oxidized thiols is necessary for an accurate determination of SNO occupancy. Protein degradation may be another confounding factor.[10]

In conclusion, our cysTMT labeling procedure provides a novel approach for the measurement of SNO occupancy in the myocardium. This method was validated using GSNO-treated whole-heart homogenates, and was also used to demonstrate that IPC-induced SNO is sufficient to shield cysteine residues against oxidation.

## Disclosures

None.

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# Novelty and Significance

### What Is Known?

- Ischemic preconditioning (IPC) is a cardioprotective mechanism.
- S-nitrosylation (SNO) is a reversible posttranslational modification that is increased with myocardial IPC.
- IPC-induced SNO has been shown to shield cysteine residues against oxidation, but the percentage of a given cysteine that is modified by SNO with IPC is not known.

### What New Information Does This Article Contribute?

- A new cysteine-reactive tandem mass tag (cysTMT) method for determining the percentage of a given cysteine residue that is modified by SNO (ie, SNO occupancy).
- SNO occupancy levels with IPC are consistent with the hypothesis that SNO provides cardioprotective effects by shielding cysteine residues from oxidation, as occurs during ischemia–reperfusion injury.

Herein we examined whether IPC-induced SNO was sufficient to shield cysteine residues from oxidation. To test this hypothesis, we developed a novel differential cysTMT-labeling procedure for determining the percentage of a given cysteine residue that is modified by SNO (ie, SNO occupancy). Using this methodology, we determined that with IPC, SNO occupancy increased to levels that are consistent with the ability of SNO to protect against oxidation. Previous studies have developed methods for the relative quantification of SNO, but this is the first study to examine SNO occupancy. This method can also be adapted to examine other redox-based modifications.

**SUPPLEMENTAL MATERIAL**

*Detailed Materials and Methods*

**Animals**

   Male C57BL/6 mice (n = 21) were obtained from Jackson Laboratories (Bar Harbor/ME). All animals utilized in this study were between the ages of 12-15 weeks. Prior to myocardial excision, mice were anesthetized with pentobarbital sodium (50-100 mg/kg) via intraperitoneal injection. This investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996) and was approved by the Institutional Laboratory Animal Care and Use Committee.

**Solutions and drugs**

   Krebs-Henseleit buffer (KHB) consisted of (in mmol/L): NaCl (120), KCl (4.7), $NaH_2PO_4$ (1.2), $NaHCO_3$ (25), $MgSO_4$ (1.2), Glucose (10), and $CaCl_2$ (1.75); pH 7.4. KHB was bubbled with 95% $O_2$/5% $CO_2$. S-nitrosoglutathione (GSNO; Sigma, St. Louis/MO) was used as an *S*-nitrosylating agent. Ascorbate (Sigma) was used as an *S*-nitrosylation (SNO)-specific reducing agent. All solutions were made fresh on the day of experimentation.

**Heart perfusion protocols**

   Hearts were Langendorff-perfused in the dark as previously described;[1-4] treatment protocols are shown in Fig. 1a. Hearts were randomly subjected to a perfusion protocol (60 minute perfusion period) or an IPC protocol (20 minute equilibration period, 4 cycles of 5 minutes ischemia and 5 minutes reperfusion). Hearts were snap frozen in liquid nitrogen immediately following the treatment protocol.

**Whole-heart homogenate preparation**

   Whole-heart homogenates were prepared as described previously.[1-4] All subsequent procedures were performed in the dark. Hearts were powdered on liquid nitrogen with a mortar and pestle, and resuspended in 1.0 mL of homogenization buffer containing (in mmol/L): sucrose (300), HEPES-NaOH 8.0 (250), EDTA (1), and Neocuproine (0.1). An EDTA-free protease inhibitor tablet (Roche Diagnostics Corporation, Indianapolis/IN) was introduced just before use. Samples were then homogenized via Dounce glass homogenization on ice and centrifuged at 1,000 g for 2 minutes. The supernatant was recovered as total crude homogenate. Protein concentration was determined using the Bradford protein assay.

***S*-nitrosoglutathione incubation**

   For GSNO treatment, whole-heart homogenates (1 mg) were incubated directly with 1mmol/L GSNO for 30 min at 25°C in homogenization buffer with 2.5% SDS. GSNO was removed via acetone precipitation.

**Determination of SNO occupancy with cysteine-reactive tandem mass tags**

   SNO occupancy was determined using the **cys**teine-reactive **T**andem **M**ass **T**ag (cysTMT) labeling and enrichment kit (Thermo Fisher Scientific, Waltham/MA); see Fig. 1b for depiction of labeling protocol. All subsequent procedures were performed in the dark. Homogenates (1000 μg) were diluted in HEN buffer containing (in mmol/L): **H**EPES-NaOH 8.0 (250), **E**DTA (1), and **N**eocuproine (0.1) with 2.5% SDS and an EDTA-free protease inhibitor tablet (Roche Diagnostics Corporation, Indianapolis/IN). Samples were incubated with 5 mmol/L $cysTMT_x$ for 2 hours at 25°C (per manufacturer's instruction) to block non-modified (i.e., free) thiol groups. Following removal of $cysTMT_x$, samples were incubated with 20 mmol/L ascorbate and 5 mmol/L $cysTMT_y$ for 2 hours at 25°C to label SNO thiol groups. Excess cysTMT label was

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removed via acetone precipitation; protein pellets were washed with acetonitrile, followed by two additional acetone washes to ensure that all excess cysTMT label was removed. Samples were then resuspended in buffer containing (in mmol/L): $NH_4HCO_3$ (50), EDTA (1) and 0.05% SDS, and subjected to trypsin digestion (Promega, Madison/WI) overnight at 37$^o$C with agitation. Samples were concentrated by SpeedVac (Thermo Fisher Scientific), diluted in Tris buffered saline (TBS), and incubated with anti-cysTMT resin overnight at 4$^o$C with rotation. The supernatant was removed and the resin was washed with 3x0.5 mL TBS, 3x0.5 mL 0.05% CHAPS in TBS, 3x0.5 mL 4M urea in TBS, and 3x0.5 mL HPLC-grade water. CysTMT-labeled peptides were eluted using 3x0.4 mL 0.4% trifluoroacetic acid/50% acetonitrile. All fractions were combined, resuspended in 0.1% formic acid, and cleaned with a $C_{18}$ column (ZipTip; Millipore, Billerica/MA). Liquid chromatography tandem mass spectrometry was performed using an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific), as described below. One sample from each treatment group was run in triplicate in order to increase protein/peptide identifications. The MASCOT database search engine was used for protein identification and Proteome Discoverer v1.3 (Thermo Fisher Scientific) was used for cysTMT quantification. SNO occupancy was calculated using the ratio of $cysTMT_y/(cysTMT_x+cysTMT_y)$. Peptides were filtered at a false discovery rate of 5%. Any sample containing fewer than 150 unique peptide identifications was excluded from analysis for perfusion and IPC groups. Additionally, peptides containing more than one cysTMT-labeled cysteine residue were excluded from analysis for all groups.

**Determination of oxidation occupancy with cysteine-reactive tandem mass tags**

Oxidation occupancy was determined using the cysTMT labeling and enrichment kit (Thermo Fisher Scientific); see Online Fig. II for depiction of labeling protocol. Homogenates (1000 $\mu$g) were diluted in HEN buffer with 2.5% SDS and an EDTA-free protease inhibitor tablet (Roche Diagnostics Corporation). Samples were then split in half. One half of the sample was incubated with 5 mmol/L $cysTMT_x$ for 2 hours at 25$^o$C (per manufacturer's instruction) to block non-modified (i.e., free) thiol groups. Following removal of $cysTMT_x$, samples were incubated with 20 mmol/L ascorbate and 5 mmol/L $cysTMT_y$ for 2 hours at 25$^o$C to label SNO thiol groups. The second half of the sample was incubated with 20 mmol/L ascorbate for 2 hours at 25$^o$C to reduce SNO thiol groups. Non-modified (i.e., free) and ascorbate-reduced thiols were then blocked with 25 mmol/L NEM for 20 minutes at 50$^o$C. Following removal of the NEM, samples were incubated with 10 mmol/L dithiothreitol for 2 hours at 25$^o$C to reduce oxidative modifications. Following removal of the dithiothreitol, samples were incubated with 5 mmol/L $cysTMT_z$ for 2 hours at 25$^o$C to label oxidized thiol groups. Excess cysTMT label was removed via acetone precipitation; protein pellets were washed with acetonitrile, followed by two additional acetone washes to ensure that all excess cysTMT label was removed. Samples were then recombined and resuspended in buffer containing (in mmol/L): $NH_4HCO_3$ (50), EDTA (1) and 0.05% SDS, and subjected to trypsin digestion (Promega) overnight at 37$^o$C with agitation. Samples were concentrated by SpeedVac (Thermo Fisher Scientific), diluted in TBS, and incubated with anti-cysTMT resin overnight at 4$^o$C with rotation. The supernatant was removed and the resin was washed with 3x0.5 mL TBS, 3x0.5 mL 0.05% CHAPS in TBS, 3x0.5 mL 4M urea in TBS, and 3x0.5 mL HPLC-grade water. cysTMT-labeled peptides were eluted using 3x0.4 mL 0.4% trifluoroacetic acid/50% acetonitrile. All fractions were combined, resuspended in 0.1% formic acid, and cleaned with a $C_{18}$ column (ZipTip; Millipore, Billerica/MA). Liquid chromatography tandem mass spectrometry was performed using an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific), as described below. One sample from each treatment group was run in triplicate in order to increase protein/peptide identifications. The MASCOT database search engine was used for protein identification and Proteome Discoverer v1.3 (Thermo Fisher Scientific) was used for cysTMT quantification. Oxidation occupancy was calculated using the ratio of $cysTMT_z/(cysTMT_x+cysTMT_y+cysTMT_z)$; SNO occupancy was

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calculated using the ratio of $cysTMT_y/(cysTMT_x+cysTMT_y+cysTMT_z)$. Peptides were filtered at a false discovery rate of 5%. Any sample containing fewer than 150 unique peptide identifications was excluded from analysis for perfusion and IPC groups. Additionally, peptides containing more than one cysTMT-labeled cysteine residue were excluded from analysis for all groups.

**Optimization of cysteine-reactive tandem mass tag labeling procedure**

The cysTMT labeling procedure was optimized and tested under various experimental conditions. The optimal amount of protein was determined to be between 500-1000 $\mu$g per labeling reaction (Online Fig. III). At this protein concentration, the highest number of protein/peptide identifications was observed. To maximize protein and peptide identifications, a total of 1000 $\mu$g of protein was labeled for all groups. The time for cysTMT labeling was also optimized. In previous studies, a period of 20 minutes was sufficient to block free thiols in whole-heart homogenates with *N*-ethylmaleimide.[1, 2, 4] However, incubation periods of 20, 40 and 60 minutes were not sufficient to block free thiols with the cysTMT reagents. To ensure maximal labeling efficiency, an incubation time of 2 hours was chosen for all samples, as recommended by the manufacturer. Murray *et al.* also used the same two hour cysTMT incubation time.[5] Based on the number of non-labeled cysteine residues detected in our samples, the cysTMT labeling efficiency was estimated to be >98%. The high labeling efficiency also demonstrates that 5 mmol/L cysTMT reagent is sufficient to label all available thiols in 1000 $\mu$g of whole-heart homogenate. The amount of anti-cysTMT resin was also optimized in order to maximize the number of cysTMT-labeled peptide identifications. Additionally, we confirmed that abscorbate did not interfere with the cysTMT labeling process. However, treatment with stronger reducing agents such as TCEP resulted in the loss of cysTMT labels. Finally, samples were randomly labeled with the $cysTMT_{126-131}$ reagents to account for potential label bias; similar results were obtained for all samples regardless of the specific cysTMT labels used.

**Liquid chromatography-tandem mass spectrometry analysis on LTQ Orbitrap Velos**

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed using an Eksigent nano-LC 1D plus system (Dublin/CA) coupled to an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) using HCD fragmentation. Peptides were first loaded onto a Zorbax 300SB-$C_{18}$ trap column (Agilent, Palo Alto/CA) at a flow rate of 6 µL/minute for 6 minutes, and then separated on a reversed-phase PicoFrit analytical column (New Objective, Woburn/MA) using a 40-minute linear gradient of 2-40% acetonitrile in 0.1% formic acid at a flow rate of 250 nL/minute. LTQ Orbitrap Velos settings were as follows: spray voltage 1.5 kV, and full MS mass range: m/z 300 to 2000. The LTQ Orbitrap Velos was operated in a data-dependent mode (i.e., one MS1 high resolution [60,000] scan for precursor ions followed by six data-dependent MS2 scans for precursor ions above a threshold ion count of 2000 with collision energy of 35%).

**MASCOT database search**

The raw files generated from the LTQ Orbitrap Velos were analyzed using Proteome Discoverer v1.3 software (Thermo Fisher Scientific) with the NIH six-processor MASCOT cluster search engine (http://biospec.nih.gov, version 2.3). The following search criteria were used: database, Swiss-Prot (Swiss Institute of Bioinformatics); taxonomy, Mus musculus (mouse); enzyme, trypsin; miscleavages, 2; variable modifications, oxidation (M), deamidation (NQ), acetyl, (N-term), cysTMT (C); MS peptide tolerance 25 ppm; MS/MS tolerance as 0.8 Da. Peptides were filtered at a false discovery rate (FDR) of 5%. To account for protein sequence redundancy, only top ranking peptides were accepted as determined by the peptide rank filter for Proteome Discoverer v1.3; the protein grouping algorithm was used to determine the top ranking proteins for all peptide identifications. Proteome Discover v1.3 was also used to quantify cysTMT labeled cysteine residues.

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**Statistics**

   Statistical significance ($p<0.05$) was determined between groups using an ANOVA for multiple groups or a Student's $t$-test for two groups. Data are expressed as mean±SEM.

## *Supplemental References*

1. Kohr MJ, Aponte AM, Sun J, Wang G, Murphy E, Gucek M, Steenbergen C. Characterization of potential S-nitrosylation sites in the myocardium. *Am J Physiol Heart Circ Physiol.* 2011;300:H1327-H1335.
2. Kohr MJ, Sun J, Aponte A, Wang G, Gucek M, Murphy E, Steenbergen C. Simultaneous measurement of protein oxidation and S-nitrosylation during preconditioning and ischemia/reperfusion injury with resin-assisted capture. *Circ Res.* 2011;108:418-426.
3. Lin J, Steenbergen C, Murphy E, Sun J. Estrogen receptor-beta activation results in S-nitrosylation of proteins involved in cardioprotection. *Circulation.* 2009;120:245-254.
4. Sun J, Morgan M, Shen RF, Steenbergen C, Murphy E. Preconditioning results in S-nitrosylation of proteins involved in regulation of mitochondrial energetics and calcium transport. *Circ Res.* 2007;101:1155-1163.
5. Murray CI, Uhrigshardt H, O'Meally RN, Cole RN, Van Eyk JE. Identification and quantification of S-nitrosylation by cysteine reactive tandem mass Tag switch assay. *Mol Cell Proteomics.* 2011;11:M111.013441.

***Online Table Legends***

Online Table I. **SNO occupancy measurements from perfusion and GSNO-treated whole-heart homogenates as identified via differential cysTMT labeling.** LC-MS/MS derived peptide sequences and MASCOT MS2 search identifications (false discovery rate of 5%); non-cysteine containing peptides were filtered from the data set. Each peptide identification was present in at least two samples for both control and GSNO. The top rank protein is displayed in the 'Protein Accession' column for each peptide identification; all protein isoforms are listed for any peptide identifications with a shared sequence. SNO occupancy is expressed as mean±SD; fold-change represents the ratio of GSNO/Perfusion (n = 7/group for perfusion, 4/group for GSNO).

Online Table II. **SNO occupancy measurements from perfusion and IPC whole-heart homogenates as identified via differential cysTMT labeling.** LC-MS/MS derived peptide sequences and MASCOT MS2 search identifications (false discovery rate of 5%); non-cysteine containing peptides were filtered from the data set. Each peptide identification was present in at least two samples for both control and IPC. The top rank protein is displayed in the 'Protein Accession' column for each peptide identification; all protein isoforms are listed for any peptide identifications with a shared sequence. SNO occupancy is expressed as mean±SD; fold-change represents the ratio of IPC/Perfusion (n = 7/group for perfusion, 5/group for IPC).

Online Table III. **Oxidation occupancy measurements from perfusion and IPC whole-heart homogenates as identified via differential cysTMT labeling.** LC-MS/MS derived peptide sequences and MASCOT MS2 search identifications (false discovery rate of 5%); non-cysteine containing peptides were filtered from the data set. Each peptide identification was present in at least two samples for both control and IPC. The top rank protein is displayed in the 'Protein Accession' column for each peptide identification; all protein isoforms are listed for any peptide identifications with a shared sequence. Oxidation occupancy is expressed as mean±SD; fold-change represents the ratio of IPC/Perfusion (n = 3/group for perfusion, 3/group for IPC).

Online Table IV. **Summary data for LC-MS/MS-derived peptide identifications from perfusion, IPC and GSNO-treated whole-heart homogenates used for determining SNO occupancy.** Protein group accessions, MASCOT MS2-derived peptide sequences, modifications, calculated mass (MH+ [Da]), IonScore, expectation value, charge, and mass-to-charge ratio (m/z [Da]) are shown for all search identifications (false discovery rate of 5%). The top rank protein is displayed in the 'Protein Accession' column for each peptide identification; all protein isoforms are listed for any peptide identifications with a shared sequence. To view protein descriptions, coverage, number of amino acids (# AAs), molecular weight (MW [kDa]), and calculated pI, click on the '+' found on the left side of the spreadsheet; non-cysteine containing peptides were filtered from the data set.

Online Table V. **Summary data for LC-MS/MS-derived peptide identifications from perfusion and IPC-treated whole-heart homogenates used for determining oxidation occupancy.** Protein group accessions, MASCOT MS2-derived peptide sequences, modifications, calculated mass (MH+ [Da]), IonScore, expectation value, charge, and mass-to-charge ratio (m/z [Da]) are shown for all search identifications (false discovery rate of 5%). The top rank protein is displayed in the 'Protein Accession' column for each peptide identification; all protein isoforms are listed for any peptide identifications with a shared sequence. To view protein descriptions, coverage, number of amino acids (# AAs), molecular weight (MW [kDa]), and calculated pI, click on the '+' found on the left side of the spreadsheet; non-cysteine containing peptides were filtered from the data set.
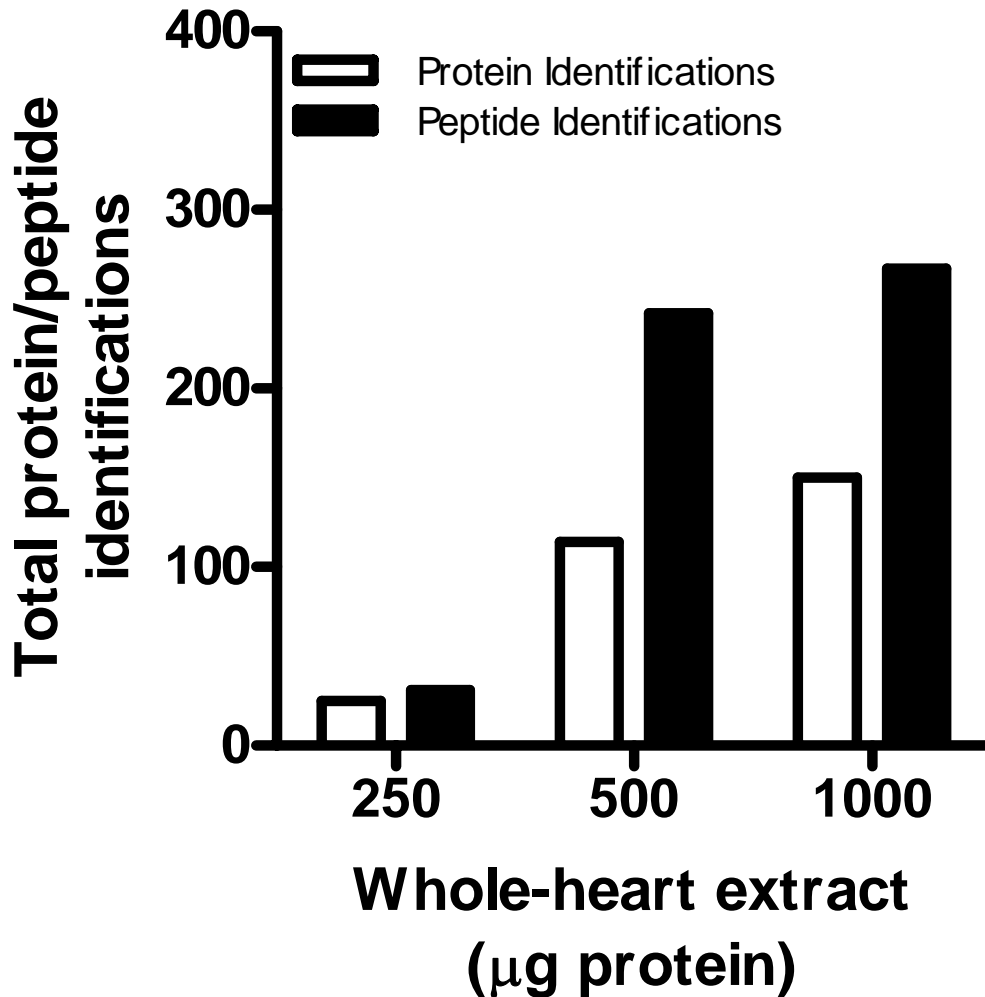
**ONLINE FIGURE I**
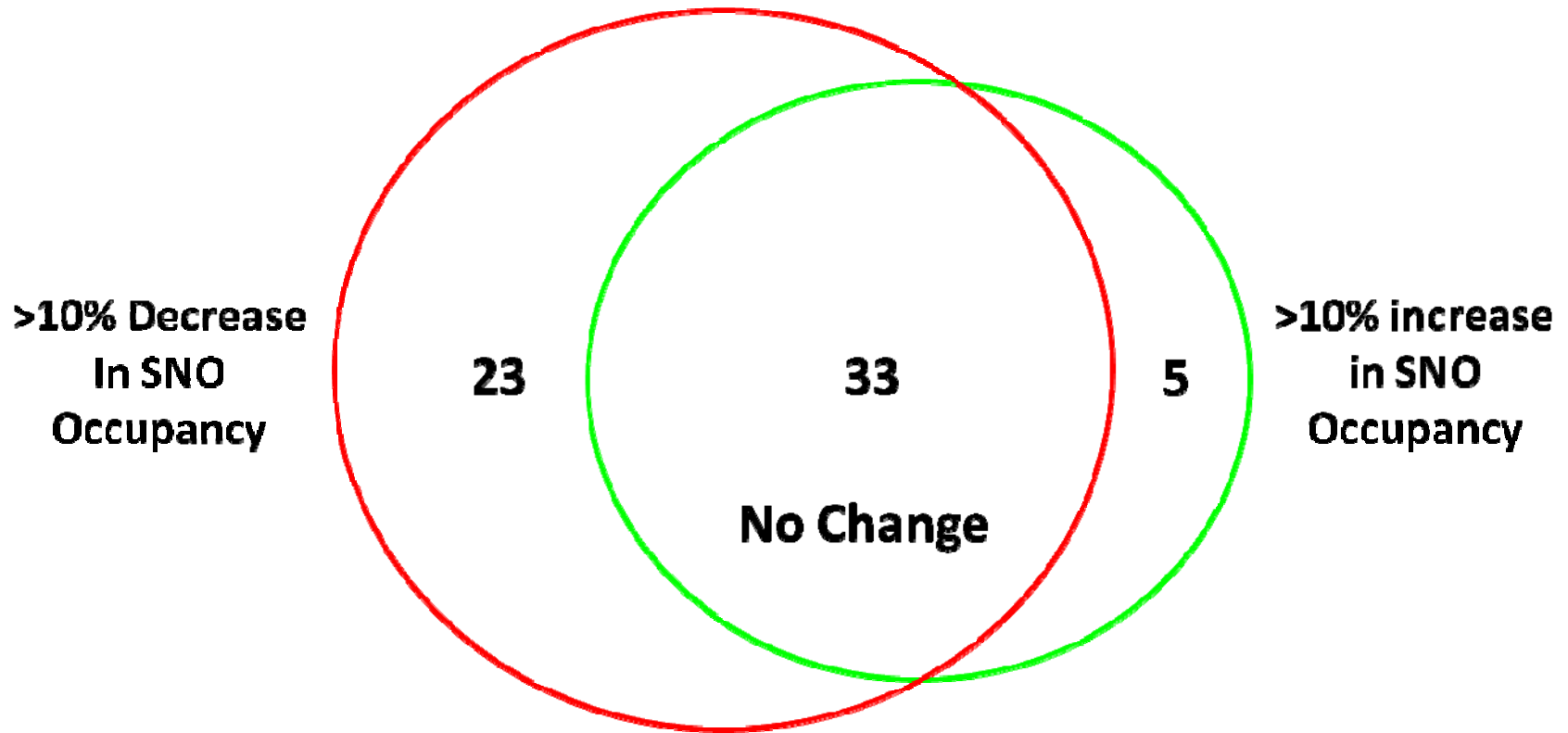
Online Figure I. **Venn diagram depicting SNO decomposition that occurs during cysTMT labeling.** Whole-heart homogenates were treated with 1 mmol/L GSNO for 30 minutes. For each sample, one half was labeled with CysTMT immediately following GSNO treatment, while the other half was incubated for an additional period of 2 hours at 25°C prior to cysTMT labeling.

**ONLINE FIGURE II**
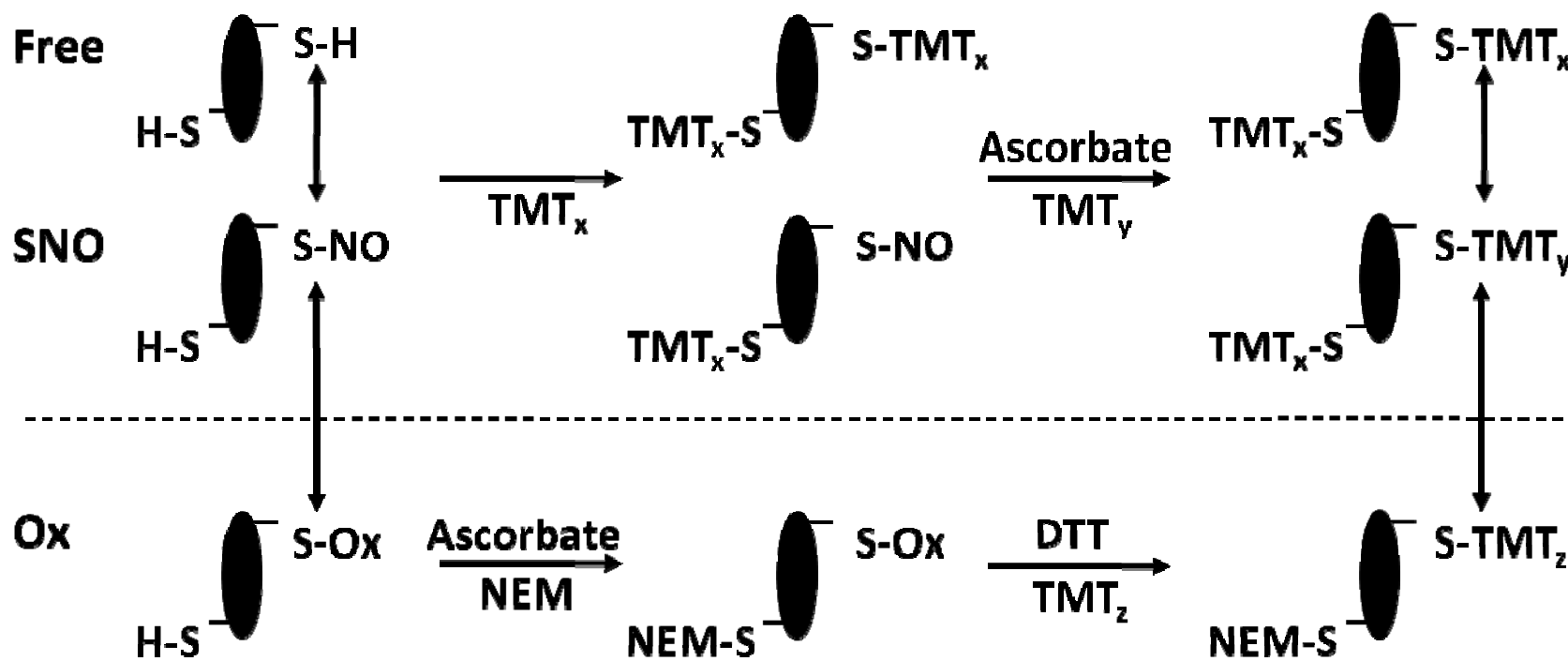
Online Figure II. **Labeling methodology.** Differential cysTMT labeling strategy used for determining oxidation occupancy; the dashed line denotes the separate processing of each half of the sample.

**ONLINE FIGURE III**



Online Figure III. **Determination of optimal protein concentration for cysTMT labeling.** Whole-heart extracts were labeled with the cysTMT labels at various protein concentrations (250 μg, 500 μg, 1000 μg) to determine the optimal protein concentration for cysTMT labeling. White bars represent MASCOT-derived protein identifications; black bars represent MASCOT-derived peptide identifications.