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Large-Scale Capture of Peptides Containing Reversibly Oxidized Cysteines by Thiol-Disulfide Exchange Applied to the Myocardial Redox Proteome

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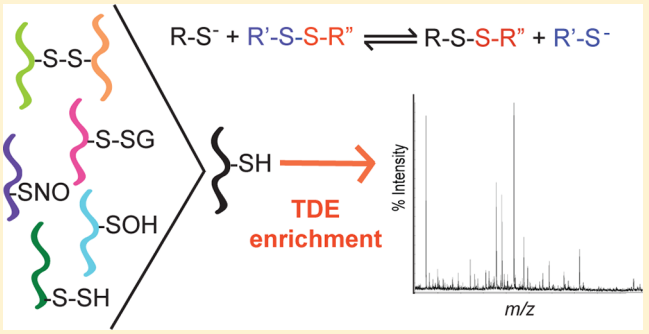
Supporting Information

ABSTRACT: Redox regulation is emerging as an important post-translational modification in cell signaling and pathogenesis. Cysteine (Cys) is the most redox active of the commonly coded amino acids and is thus an important target for redox-based modifications. Reactions that oxidize the Cys sulfur atom to low oxidation states (e.g., disulfide) are reversible, while further reactions to higher oxidation states (e.g., sulfonic acid) may be irreversible under biological conditions. Reversible modifications are particularly interesting as they mediate redox signaling and regulation of proteins under physiological conditions and during adaptation to oxidant stress. An enrichment method that relied on rapid and specific alkylation of free Cys, followed by thiol-based reduction and resin capture by thiol-disulfide exchange chemistry was applied to isolate reversibly modified Cys-containing peptides. Chromatographic conditions were optimized to provide increased specificity by removal of noncovalent interactions. The technique was highly efficient, based on near equimolar reactions with the resin, reproducible and linear for peptide elution, as quantified by label-free mass spectrometry. The method was applied to a complex protein lysate generated from rat myocardial tissue and 6559 unique Cys-containing peptides from 2694 proteins were identified. Comparison with the rat database and previous studies showed effective enrichment of proteins modified by S-nitrosylation, disulfide formation, and Cys-sulfenic acid. Analysis of amino acid sequence features indicated a preference for acidic residues and increased hydrophilicity in the regions immediately up- or downstream of the reactive Cys. This technique is ideally suited for the enrichment and profiling of reversible Cys modifications on a proteome-wide scale.



Reactive oxygen and nitrogen species (ROS and RNS) are involved in the generation of oxidative stress and physiological redox signaling via the oxidation of biomolecules, in particular proteins. There are several oxidant-sensitive amino acids that can propagate the ROS/RNS signal or be targeted during stress;[1,2] however, foremost among these is cysteine (Cys), which demonstrates high reactivity with a large range of oxidants and is targeted by numerous oxidative post-translational modifications (PTMs).[3,4] The Cys sulfur atom can exist in oxidation states between −II and +IV. Reactions that mildly oxidize the native thiol ($R-S^{-II}-H$) to low oxidation states, such as the disulfide ($R-S^{-I}-S^{-I}-R$) or sulfenic acid ($R-S^{0}-OH$), are reversible; however, higher oxidation states such as sulfinic ($R-S^{II}-O_2H$) or sulfonic ($R-S^{IV}-O_3H$) acid are generally biologically irreversible (with the exception of peroxiredoxins[5]). Oxidative PTMs may ultimately facilitate signal propagation and/or influence the protein structure/function relationship, leading to dysfunction and targeted degradation during oxidative stress. Since these PTM appear critical in pathophysiological conditions (e.g., cardiovascular diseases, including atherogenesis and ischemia/reperfusion injury[6−8]) as well as physiological signal propagation, there is a need for large-scale identification of reversibly modified Cys at the proteome level that exploits the speed and resolution of mass spectrometry (MS).[9,10]

Classically, disulfides are crucial in the establishment and maintenance of protein tertiary structure[11] and, along with other reversible Cys PTM, can influence protein function, subcellular localization, and signaling. Reversible oxidative Cys modifications also include S-thiolation (between protein and nonprotein Cys), S-sulfhydrylation (between Cys and hydrogen sulfide; H_2S), S-nitrosylation (R-SNO), and Cys-sulfenic acid. Sulfenic acid is the most unstable of the reversible Cys PTM[12] and is unique in the ability to react as both a nucleophile and electrophile,[13] often making it the reactive intermediate to other oxidation states (a proximal thiol may reduce a sulfenic acid to a disulfide, or H_2S may react to form sulfhydrylated Cys). Disulfides may form in the absence of ROS activation via thiol-disulfide exchange (TDE), where a thiolate anion ($R-S^{-}$)

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attacks an intact disulfide and substitutes itself for one of the constituents; this allows interchange between thiol and disulfide, which can propagate a signal. The formation of nitrosothiols is less straightforward as they may form by either RNS-mediated oxidative nitrosation/nitrosylation [nitric oxide (NO) itself or higher NO oxides; NO_x] or trans-nitrosylation by reactive/activated nitrosothiols (a process that also generates disulfides).[14,15] Once such reversible Cys modifications are formed, they can be recycled by thiol reductants (e.g., thioredoxin; Trx).

The similar reduction conditions of many Cys PTM and the wide range of Cys reactivity make any selective reduction chemistry problematic. For example, the reaction of ascorbic acid with SNO can be utilized;[16−18] however, the reaction conditions and products are highly variable across laboratories, likely due to the inefficiency of this reaction, and the multiple decomposition pathways that may occur (i.e., photodissociative, thermal, copper-dependent and independent).[19−22] Inefficient reduction may also generate false positive/negative protein identifications and poor reproducibility. Coupling of free thiols by alkylation chemistry (e.g., nucleophilic substitution with haloalkane-based tags) to chemical handles for enrichment and/or immunodetection[16,23−35] may further confound results due to the incomplete incorporation or overincorporation of the tags at other nucleophilic sites (e.g., primary amines).[36,37] Methods to isolate thiols without prior chemical labeling include sequential nonreducing/reducing diagonal gel electrophoresis,[38,39] organomercurial ion affinity,[40,41] and TDE chromatography. Chromatographic methods offer vastly improved resolution and site detection over gel-based techniques, while TDE offers lower reagent toxicity compared to mercury ion procedures. As only Cys may undergo TDE chemistry, the selectivity of this technique is high, as long as sufficient steps are taken to prevent noncovalent interactions. A number of studies have applied immobilized mutant Trx resins[42] or thiopropyl-sepharose 4B[43−51] to isolate proteins via TDE, but in general, better site identifications, sensitivity, and selectivity can be achieved by employing a peptide-centric approach. However, peptide TDE has been limited to studies concerned with increasing proteome coverage[52−54] and has not been applied to a global study of reversible Cys modifications.

Peptide TDE applied to complex biological samples must consider the differentiation of free and oxidized thiols, the prevention of artifactual thiol oxidation, and loss of modifications during the protocol. Furthermore, for proteomics applications, such methods must be compatible with both label-based and label-free approaches to quantitation. We optimized a method for the enrichment of reversibly oxidized Cys that utilizes rapid and selective Cys alkylation, thiol-based reduction of Cys modifications, and TDE capture. We utilized this technique for the enrichment of reversibly oxidized Cys-containing peptides from myocardial tissue. These large-scale data contained a total of 6559 unique Cys peptides from over 2500 unique protein groups with >90% enrichment efficiency. These data enabled interrogation of the physicochemical properties of the sequence space surrounding modified Cys.

EXPERIMENTAL SECTION

Preparation of Peptide Standard. Bovine serum albumin (BSA) was resuspended in 25 mM Tris, pH 7.5, and digested with 20 μg/mg BSA of trypsin (Promega, Madison, WI) at 37 °C for 12−16 h. Peptides were reduced with 10 mM dithiothreitol (DTT) for 30 min followed by concentration and desalting by solid phase extraction (SPE) tC_{18} columns (Waters Corp., Milford, MA) and solvent evaporation. Peptides were analyzed by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) as described in the Supporting Information.

Preparation of Myocardial Proteins. All animal studies were approved by the Animal Care and Ethics Committee, the University of Sydney. Lewis rats were euthanized with pentobarbital (200 mg kg^{-1}), the heart rapidly excised and subjected to Langendorff perfusion for 20 min with non-recirculating Krebs buffer, as previously described.[55] Tissue was homogenized (Omni International, Kennesaw GA) in 100 mM NaH_2PO_4, pH 6, 5 mM *N*-ethylmaleimide (NEM), 1% (w/v) sodium dodecylsulfate (SDS), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μM aprotinin, 20 μM leupeptin and 2 mM diethylenetriamine pentaacetic acid (DTPA) on ice. The homogenate was centrifuged at 13000*g* at 4 °C for 15 min and the supernatant removed. Additional homogenization buffer was added to the pellet, and the pellet underwent 2 × 30 s cycles of tip-probe sonication (Branson, Darbury, CT), followed by centrifugation at 13000*g* and 4 °C for 15 min. The supernatants were combined and an aliquot taken for protein concentration determination by Quant-iT assay (Invitrogen, Carlsbad, CA). The mixture was heated at 80 °C to denature the extracted proteins before cooling and incubation (room temperature) for 5 min at a final concentration of 40 mM NEM. Proteins then immediately underwent chloroform/methanol precipitation[56] for SDS and unreacted NEM removal before resuspension, trypsin digestion, reduction and SPE cleanup, as detailed above.

Batch-Based TDE Chromatography. Thiopropyl-sepharose 6B beads were rinsed in Milli-Q water (6 mL per 30 mg beads) and then with 100 mM Tris-HCl, pH 7.5 (3 mL). Samples were resuspended in 100 mM Tris-HCl, pH 7.5, 0.5% (w/v) SDS and 1 mM DTPA, applied to the beads and allowed to bind at room temperature for 2 h with gentle tumbling. The resin and captured peptides were washed twice with 0.5 mL of 100 mM Tris-HCl, pH 7.5, 0.5% (w/v) SDS for 10 min and then 7 times with 1 mL 100 mM Tris-HCl, pH 7.5, for 5 min each. Captured peptides were eluted with 5 × 200 μL of 10 mM DTT in 10 mM Tris-HCl, pH 7.5, each for 15 min. The unbound fraction and first two washes were combined, desalted by SPE, and either analyzed or applied again to the TDE method. Elution fractions were pooled and desalted by SPE. Those samples destined for two-dimensional liquid chromatography (2DLC) fractionation underwent additional alkylation (with 30 mM iodoacetamide) before SPE desalting.

LC−MS/MS. First dimension fractionation for 2DLC tandem mass spectrometry (2DLC−MS/MS) was performed offline by hydrophilic interaction LC (HILIC as described in the Supporting Information. LC−MS/MS was performed using a Dionex UltiMate 3000 High-Performance Liquid Chromatography (HPLC) system (Dionex, Sunnyvale, CA) coupled online to an LTQ Orbitrap Velos (Thermo Scientific, Weltham, MA) mass spectrometer as detailed in the Supporting Information. Raw data were viewed in Xcalibur (Thermo Scientific) and scripted to .mgf in Mascot distiller (Matrix Science, London, U.K.).

Data Analysis. BSA peptide data were searched against the Uniprot database using an in-house Mascot server, with the variable modification methionine sulfoxide, two possible missed cleavages and peptide, and fragment tolerances of 10 ppm and 0.8 Da, respectively. Myocardial peptide data were searched

against the UniProt *Rattus norvegicus* database (taxonomy no. 10116; release date 09/21/11) as above with the additional variable modifications, carbamidomethyl (C) and NEM (C), using an in-house Mascot server and X!Tandem through Scaffold 3 (Proteome Software, Portland, OR). A reversed *R. norvegicus* database was generated and used to filter identifications to a <2% false discovery rate (FDR).

Label-Free Quantitation. Data were scripted to mzXML using msconvert (http://proteowizard.sourceforge.net/index.shtml), and peaks were extracted and integrated using an in-house script (Wolfram Mathematica, Champaign, IL). A tolerance of 0.05 Da was applied for all m/z ion values to generate extracted ion chromatograms (EIC), followed by numerical integration with a tolerance of 30 s surrounding the maximum intensity value of the EIC (designated as retention time).

Bioinformatics. Gene Ontology (GO) terms were obtained via input of gene names into the DAVID bioinformatics tool[57] with a background reference of all GO terms from *R. norvegicus*. Values were obtained for over-representation analysis and modified Fisher Extract calculation.[58] Sequence annotation features were obtained for reversible Cys modifications from the UniProt database and over-representation analysis performed against a background of all *R. norvegicus* UniProt annotated sequences. The analysis of the amino acid sequences surrounding identified reversibly oxidized Cys residues used 13-mer sequences (6 amino acids up and downstream of the Cys) generated from experimentally captured Cys-containing peptides and the *R. norvegicus* database. The frequency of each amino acid was measured at each position and compared to the background to generate a ratio representing the relative frequency in comparison to the background. Kyte-Doolittle hydropathy was calculated on the aligned 13-mers.[59] Motif analysis was performed using the Motif-X tool[60] on all Cys-peptide sequences identified in the elution of TDE chromatography, with the search parameters: central character C, width 13, occurrence 20, significance 1×10^{-6}, and background and foreground rat proteome.

RESULTS AND DISCUSSION

The objective was to develop an efficient TDE chromatography-based method for specific large-scale enrichment of peptides containing reversibly oxidized Cys (Figure 1). The key areas affecting the performance of the method were (i) TDE capture onto the resin, (ii) prevention of noncovalent interactions during binding and washing, and (iii) elution of covalently bound peptides.

Hydrophobicity Largely Mediates Noncovalent Interactions. A previous approach for peptide TDE by thiopropyl-sepharose chromatography[52] involved acetonitrile (MeCN) and salt washes to aid in the removal of noncovalently bound (non-Cys) peptides, and a single, low volume (100−200 μL) elution of Cys-peptides with DTT. We assessed this method using tryptic peptides generated from BSA. MALDI-TOF MS showed that while Cys-containing peptides were present in the elution (Figure S1a of the Supporting Information), the dominant peaks could be attributed to non-Cys peptides (61% of assignable peaks; n = 11/18). The use of a detergent (0.5% SDS) at low concentration in the binding and wash steps (Figure S1b of the Supporting Information) reduced the intensity of these contaminant peaks by approximately 50% (based on MS peak intensity) and allowed the observation of 16 eluted Cys-containing peptides; however, specificity

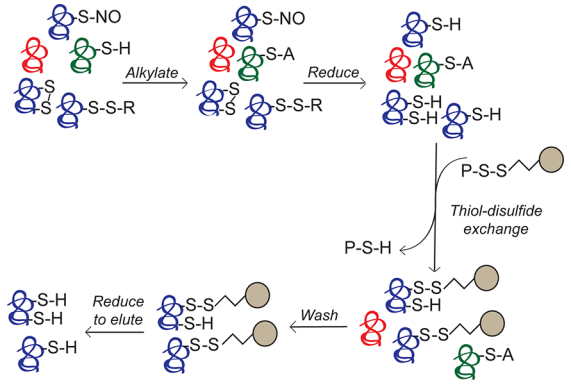

Figure 1. Schematic of TDE chromatography method. In the initial step, thiols are alkylated to allow for the differentiation of free and reversibly oxidized thiols. Samples are then reduced to convert reversibly oxidized Cys to free thiols. These are immobilized onto resin functionalized with a protected disulfide via TDE. Noncovalently bound proteins/peptides are removed by a stringent wash protocol and covalently bound peptides eluted by reduction of disulfide bonds. R denotes either another protein (interdisulfide) or nonproteinaceous group (e.g., glutathione) involved in S-thiolation of Cys residues; A denotes the addition of an alkylating group to Cys (e.g., maleimide), and P denotes the pyridine-based protecting group for which peptide Cys are substituted.

remained poor with 20 non-Cys peptides also identified. We next employed a batch-based TDE method of binding and washing, in the presence of 0.5% SDS and increased the volume of the DTT elution (1 mL using 5 × 200 μL elution steps). This resulted in greatly improved specificity with Cys-containing peptides accounting for 92% of those ionized by MALDI-MS (n = 23/25; Figure S1c of the Supporting Information). This improvement in nonspecific binding was not observed when the batch-based method was employed in the absence of SDS (42% non-Cys peptides; Figure S1d of the Supporting Information).

Our results indicated that previous methods[52] were less efficient at removing interfering noncovalent interactions and elution of covalently bound peptides. This is perhaps less surprising as the original method was intended to increase peptide sequence % coverage by inclusion of Cys peptides for improved protein identifications, rather than be optimized for specific enrichment of reversibly oxidized Cys-containing peptides alone. Our method included (i) addition of a hydrophobic modifier to the aqueous wash steps (replacing a polar organic phase), (ii) use of a batch-based, rather than column-based method, which will increase the efficiency of resin washing, and (iii) a higher volume elution step.

TDE Chromatography Efficiently and Specifically Captures Cys Peptides. LC−MS/MS analysis of tryptic BSA peptides eluted from batch-based TDE chromatography allowed the identification of all 35 Cys residues in the predicted BSA sequence (Figure S2 of the Supporting Information). Of the 72 nonredundant peptides confidently identified (Table S1 of the Supporting Information), 59 contained at least 1 Cys residue. In addition, no Cys-containing peptides were identified in the unbound fraction (data not shown). To assess the efficiency of capture, spectrophotometric measurement of the TDE protecting group (2-thiopyridine, 2-TP) release versus Cys loading was undertaken (Figure 2a). A strongly positive linear correlation (r > 0.999) was observed with a low variance (R^2 = 0.999) and a slope close to unity (1.186 ± 0.015),

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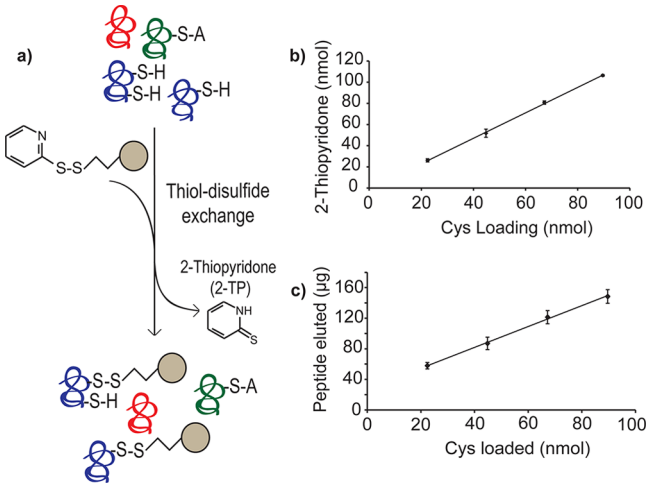

Figure 2. Efficient capture of Cys peptides assessed by peptide quantitation and spectrophotometric assay. (a) Schematic showing TDE between functionalized resin and a Cys-containing peptide to liberate the 2-thiopyridinone (2-TP) protecting group. (b) Spectrophotometric measurement (343 nm) of 2-TP release vs Cys concentration, slope = 1.186 ± 0.015, R^2 = 0.999. (c) Quantitation of total peptide eluted from TDE resin, R^2 = 0.998. Symbols are as indicated in Figure 1.

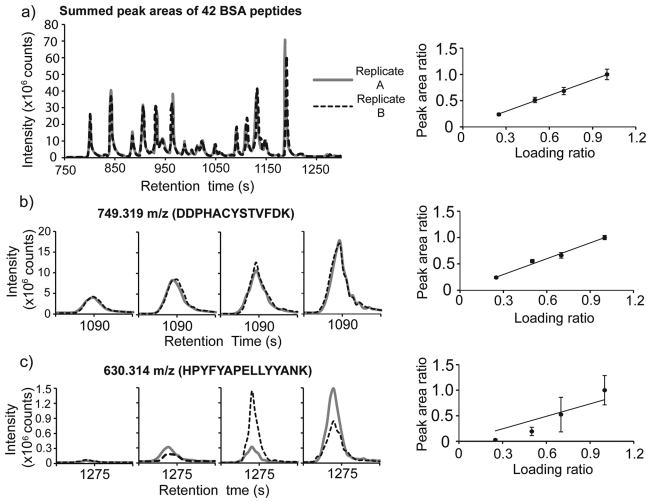

Figure 3. Label-free quantitation of peptides eluted with increased loading. (a) A representative summed MS ion spectrum of two of six experimental replicates "A" and "B" with a replicate PA ratio of 0.97 (i.e., 97% reproducible for n = 2 replicates). On the right, the PA of all loadings (6 replicates per point) are plotted as a ratio to the maximum load (linearity plot), slope = 0.995 ± 0.068, R^2 = 0.998. (b) Replicate extracted peak spectra (A and B) for the 749.319 m/z ion (Cys-containing, sequence: DDPHACYSTVFDK), indicating replicate PA ratios of 0.91, 0.94, 0.90, and 0.96 for sequential loadings, and a linearity plot for all loadings, with slope = 0.999 ± 0.054 and R^2 = 0.985. (c) As for (b) for the 630.314 m/z ion (non-Cys containing, sequence: HPYFYAPELLYYANK), replicate ratios of 0.54, 1.88, 0.29, and 1.56, with a linear regression slope = 0.815 ± 0.316 and R^2 = 0.793. All replicate PA ratios are given as A/B, and loading or PA ratios for linearity plot are divided by the maximum loading.

indicating an almost equimolar reaction between peptides and resin at all loadings (Figure 2b). The slightly higher slope (~ 1:1.2 mol Cys:2-TP) is likely due to the unbinding/rebinding of peptides containing vicinal thiols ($CX_{2-6}C$) as these are known to be highly reactive to TDE chemistry. This linear trend was also observed when the total peptide eluted from the column was measured; however, in order to assess whether this trend was indicative of linear covalent Cys binding or of increased noncovalent binding at higher loadings, label-free quantitation of eluted peptides was performed.

Label-Free Quantitation Shows Linear and Reproducible Cys-Containing Peptide Capture by TDE. Label-free quantitation was performed by MS peak area (PA) integration of 42 distinct m/z peaks, representing 33 Cys-containing and 9 non-Cys-containing BSA peptides, at 4 different peptide loadings (n = 6 experimental replicates at each loading; 24 experiments in total). The experimental variance/reproducibility at the same loading was indicated as either a PA ratio (Figure 3 and Figure S3 of the Supporting Information) or an average percentage (Table S2 of the Supporting Information). Linearity with respect to loading amount was expressed as a slope (of a least-squares regression, with the intercept fixed at zero), linear correlation (r), and variance (R^2 and %; Table S2 of the Supporting Information). Integration over the entire retention time series for the 42 distinct m/z values at each loading indicated a high overall reproducibility between experiments (91 ± 9%) and signal linearity (R^2 = 0.998) with a slope of 1.00 ± 0.07. When individual peptide signals were considered (representative shown in Figure 3, panels b and c, see Table S2 of the Supporting Information for a complete list), it was observed that all 33 Cys-containing peptides had a positive linear correlation greater than 0.97 (average: 0.99 ± 0.02) and an average slope close to unity (0.99 ± 0.05); however, only 3/9 non-Cys peptides fell within these parameters. For non-Cys-containing peptides, average values for linear correlation (0.89 ± 0.20) and slope (1.03 ± 0.23) indicated a poorer fit to a linear trend and greater variability between peptides. In addition, experimental reproducibility was higher for Cys-containing peptide PA replicates (average PA reproducibility: 87 ± 4%) than for non-Cys peptides (average PA reproducibility: 72 ± 19%), indicating that the major contributors to high linearity and reproducibility of the total summed PA (Figure 3a) were the Cys-containing peptides.

The reproducibility and capture linearity versus loading, summed MS ion integrals, and individual Cys-peptide MS ion integrals confirmed the accuracy of the technique. Decreased or poorly reproducible peak areas of noncovalently bound peptides indicated their relative depletion. The accurate relative fold estimation of the technique makes it highly applicable to label-free quantitative studies or those that chemically label postenrichment, allowing for the use of increased starting material to profile low-abundance modifications.

Reversibly Oxidized Cys in Rat Myocardium. We next attempted to determine whether the TDE batch-based method was compatible with very complex peptide mixtures at high sensitivity and selectivity. We utilized protein extraction conditions to ensure (i) the prevention of artifactual Cys oxidation/TDE and (ii) the efficient removal of free thiols in the absence of (mis-)alkylation at other sites. Extractions were performed at a pH below neutral, in the presence of a metal-ion chelator with complete first-sphere coordination properties (DTPA)[61] and in the presence of an alkylating agent (NEM). The low pH and metal ion chelator slow auto-oxidation and TDE processes while NEM will compete to "block" free thiols, forming thioethers that are less reactive to oxidation. The alkylating reagent NEM was utilized under previously optimized conditions[62] to give complete Cys alkylation, while

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limiting the levels of alkylation at other sites. By performing homogenization in 5 mM NEM under denaturing conditions and at acidic pH, we block reactive Cys and generate few mis-alkylations. A higher NEM concentration (40 mM) can be applied for a short time to alkylate buried or unreactive Cys. To the best of our knowledge, few other studies have addressed these issues, which likely result in both false positive and negative identifications due to auto-oxidation and/or incomplete/overalkylation.

To assess whether the efficiency of capture seen for BSA peptides translated to a complex peptide mixture, a sequential binding experiment was performed, where unbound peptides from a primary round of TDE were applied to a second round of TDE and the resulting eluents analyzed by LC−MS/MS (Figure 4). Both the primary and secondary rounds of TDE

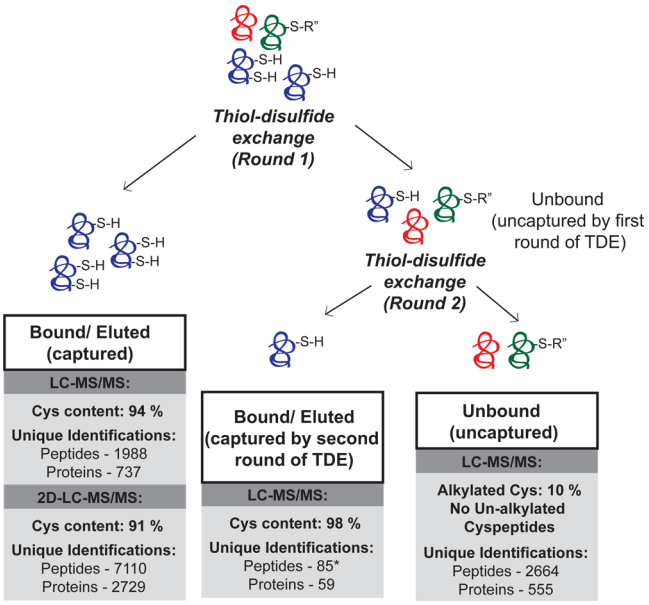

Figure 4. Schematic and results of sequential binding and 2DLC−MS/MS studies on myocardial proteins by TDE. The schematic shows the process used for sequential binding and values for Cys-peptide specificity (% of total peptides) and the number of unique proteins and peptides from each experiment. The symbols are as indicated in Figure 1.

enrichment showed >90% selectivity for Cys-containing peptides; however, while the primary round of TDE contained 1988 nonredundant peptides corresponding to 737 proteins, the second contained only 85 nonredundant peptides corresponding to 59 predicted high-abundance proteins, with only 7 additional peptides found in the secondary round of TDE compared to the primary. However, further fractionation (see below) demonstrated that these were not unique (Table S3 of the Supporting Information). In the unbound, 2664 peptides from 555 proteins (data not shown) were observed with 10% containing Cys; however, all of these peptides were NEM alkylated at Cys and could not therefore have been captured by TDE.

We next increased fractionation of the peptides eluted from TDE using offline HILIC coupled to online reversed phase LC−MS/MS. This approach identified 7110 nonredundant peptides from 2727 protein groups with 91% of the peptides (n = 6559 peptides from 2694 protein groups) containing Cys (Table S3 of the Supporting Information). This study was therefore the largest global site-directed profile of reversibly oxidized Cys in the myocardium or any cell or tissue type to-date. It should be noted that as these sites are observed in the absence of any exogenous oxidative/nitrosative agent, they represent a database of sites modified under physiological conditions and thus potential sites of redox regulation/signaling independent of pathological stimuli. Bioinformatics were utilized to assess the potential function, distribution, and common sequence features of these reactive/modified Cys.

GO assessment of captured proteins compared to a background reference (the prevalence of each GO term in the entire rat genome) allowed for the identification of over-represented classes of proteins. GO terms were ranked utilizing a modified Fisher's extract test (DAVID bioinformatics[57,58]) and a fold over-representation ratio to determine the most over-represented functions and subcellular locations associated with reversibly modified Cys (Figure S4 of the Supporting Information). Of the 44 over-represented functional classes, 3 clear clusters could be observed: (i) oxidoreductases (5 GO terms) and antioxidants (peroxiredoxins were the most over-represented term), (ii) extracellular matrix, receptor, and surface proteins (7 GO terms), and (iii) structural stabilization (5 GO terms, including cytoskeletal functions). GO analysis of over-represented cellular localizations/complexes revealed an association with degradation and folding machinery (signalosome and proteasome) and organelles/cell membranes (248 protein groups labeled "plasma membrane" and 117 labeled "organelle membrane"), particularly from mitochondria (Figure S4b of the Supporting Information). A similar analysis was performed on sequence annotation features, utilizing the UniProt database for the terms: "disulfide", "S-nitrosylated", "sulfenic acid", and "redox active" (Figure S4c of the Supporting Information). These sequence annotation features were over-represented within the data set and the following was observed: 179 Uniprot annotated disulfide bond-containing, 16 redox-active, 5 sulfenic acid-forming, and 12 S-nitrosylated proteins.

A literature review was undertaken to compare the enriched Cys sites to those obtained from previous analyses (Figure S5 of the Supporting Information), in particular *S*-nitrosocysteine enrichments, performed by biotin switch,[18] SNO-resin-assisted capture (SNORAC),[50] SNO site identification (SNOSID-modified biotin switch),[17] and organo-mercurial ion affinity[41] in rat or mouse tissues. Approximately 90% of the SNO-sites identified in studies utilizing rat or mouse myocardial samples[18,50] were observed here, as well as approximately 37% of those observed in the liver[41] and 55% of those in the brain[17] (Figure S5 of the Supporting Information). Protein identifications, rather than Cys sites alone, yielded even higher levels of consistency between studies (>93% in heart and >56% in other tissues), indicating these proteins may have multiple oxidatively modified Cys sites. The total overlap between these previous studies was poor (<20% shared protein identifications; Figure S5 of the Supporting Information), likely due to the specific analysis of mitochondria in[18] combined with tissue-specific protein expression.

A potential confounder in previous methods utilizing TDE for isolation of reversible Cys modifications is that they have attempted to isolate SNO following ascorbate reduction.[16,51] Ascorbate, however, is a relatively poor reducing reagent and few independently proven S-nitrosylation sites have thus been identified. Using a thiol-containing reducing reagent, we identified 14 of the 20 S-nitrosylation sites annotated in the

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UniProt database for the 12 proteins observed (Figure S5 of the Supporting Information), which include low-abundance transcriptional regulators, oxidant sensors, and nuclear proteins. Twelve of these sites were not previously identified in *S*-nitrosocysteine proteomics enrichments.[17,18,41,50] Ascorbate may be insufficiently active to reduce these sites; they may be artificially oxidized as the reduction is performed at basic pH and occasionally in the presence of copper, or previous techniques may be insufficiently sensitive to observe these sites in complex tissue homogenates. The choice of myocardial tissue allowed us to determine the sensitivity of the technique in the context of the wide dynamic range of protein abundances. Myofibril and cytoskeletal proteins, those associated with the extracellular matrix, proteins involved in metabolism, and mitochondrial respiratory chain proteins are all abundant and heavily disulfide bonded or contain reactive catalytic Cys residues. Our method was also able to identify reversibly oxidized Cys-containing peptides from predicted low copy number proteins, such as transcription factors, nuclear proteins, and kinases. As an example of our identification of low-abundance proteins, we observed that Cys_{59} of *R. norvegicus* nuclear factor-kappa B (NF-KB) p50 subunit (F1LQH2_RAT) is redox modified (Figure S6 of the Supporting Information). This is unannotated in *R. norvegicus* but a known S-nitrosylation site in *Homo sapiens* (NFKB1_HUMAN), which influences DNA binding.[63]

A recent study also identified S-nitrosylated Cys peptides in human pulmonary arterial endothelial cells (HPAECs) through the use of Cys-reactive tandem mass tags (CysTMT[35]), following an ascorbate/$CuSO_4$ reduction strategy. We identified 154/179 (~86%) of the proteins identified in that study, as well as ~67% (n = 148/220) of the SNO sites. Endothelial cells represent only a small fraction of the cells in myocardial tissue (predominantly myocytes), further suggesting that our approach is capable of mining into the low-abundance proteome. It should be noted however, that our technique does not currently distinguish between different reversible Cys modifications, and it is therefore not yet possible to unequivocally state the proportion of S-nitrosylated sites versus other reversible Cys PTMs within our data set.

Comparison of 13-mer sequences (−6 to +6 position) surrounding the captured Cys residues to a whole genome background by either motif analysis (MoxifX[60]) or single amino acid frequency revealed an over representation of acidic (Asp/Glu) and small, aliphatic (Ala) or polar (Gly) residues up or downstream of Cys (Figure S7 of the Supporting Information). Single amino acid frequency also indicated an under representation of large/aromatic amino acids (His, Phe, Trp), as well as Cys and Ser. Calculations of the hydrophobicity of the local environment of Cys (13-mer sequences) by the Kyte-Doolittle scale[59] revealed a slightly increased hydrophilicity for the captured Cys (−0.18 ± 0.68) in comparison to the background (−0.06 ± 0.90), likely reflective of the increased prevalence of Asp/Glu and decreased prevalence of Phe/Trp. These results provide evidence that acidic residues up or downstream of Cys may predicate reversible redox modifications. While the acid−base motif was specifically suggested for S-nitrosylation,[64] this evidence indicates that an acidic residue may be relevant to a larger range of reversible Cys modifications (the role of a proximal base further than 6 positions from the Cys is also possible). The increased propensity of small amino acids and the under representation of large and other oxidizable amino acids (concurrent with a slightly more hydrophilic environment) indicated the possible preference for open sites that are accessible to oxidant species.

CONCLUSION

Cys oxidation is an increasingly important regulatory PTM in health and disease. Techniques to quantitatively identify Cys modifications are few and often poorly optimized. In consideration of (i) the interrelation of many biological oxidants (often formed by secondary reactions of another), (ii) the reactivity of Cys with a large number of oxidants, and (iii) the sheer number of modifications that can be formed, the grouping of these Cys modifications for enrichment by their reduction properties (i.e., reversible vs irreversible) may be the most practical means to globally profile the plethora of modified Cys residues present in a complex system. This assures that (i) all interrelated reversible Cys modifications may be profiled at once, allowing for the use of less material for site discovery and (ii) no assumptions are made as to the reactivity of certain sites, which may interfere with quantitative results (i.e., fold changes are related to abundance of modified sites and not due to their incomplete or nonspecific reduction and capture). TDE offers the ability to capture reversibly oxidized Cys residues, following reduction without further derivatization, by a chemistry that is specific to Cys (avoiding reaction with other nucleophilic residues) and that can be coupled to high-resolution LC−MS/MS. We have shown the parameters of our TDE chromatography technique (i.e., specificity, capture efficiency, reproducibility, and linearity) to be perfectly suited to quantitative relative fold estimation and have utilized it to gain the largest list of reversibly Cys-modified peptides/proteins generated to date. The scope for regulation of protein function by oxidative modification of Cys is growing ever larger as more studies identify proteins regulated by ROS/RNS. Our technique was shown to provide excellent profiling of known reactive and modified Cys sites, capturing sites not observed by single-modification-based studies while being able to capture other known reactive motifs (e.g., vicinal thiols), which may prove integral to redox regulation.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. 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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