ChemPubSoc Europe

DOI: 10.1002/cbic.201700137

CHEMBIOCHEM Communications

Profiling Protein S-Sulfination with Maleimide-Linked Probes

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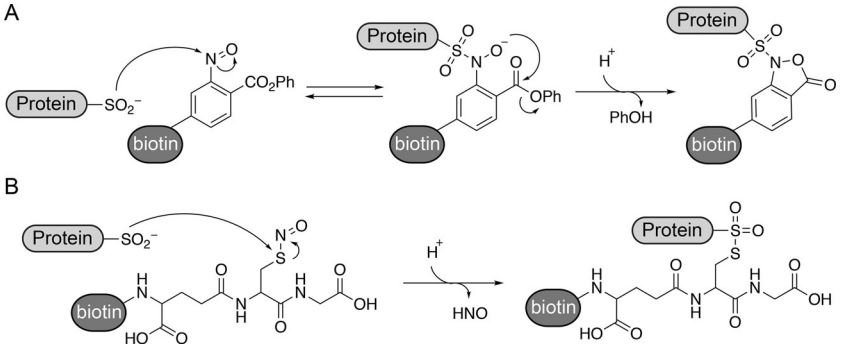
Cysteine residues are susceptible to oxidation to form *S*-sulfinyl ($R\text{-}SO_2H$) and *S*-sulfonyl ($R\text{-}SO_3H$) post-translational modifications. Here we present a simple bioconjugation strategy to label S-sulfinated proteins by using reporter-linked maleimides. After alkylation of free thiols with iodoacetamide, S-sulfinated cysteines react with maleimide to form a sulfone Michael adduct that remains stable under acidic conditions. Using this sequential alkylation strategy, we demonstrate differential S-sulfination across mouse tissue homogenates, as well as enhanced S-sulfination following pharmacological induction of endoplasmic reticulum stress, lipopolysaccharide stimulation, and inhibitors of the electron transport chain. Overall, this study reveals a broadened profile of maleimide reactivity across cysteine modifications, and outlines a simple method for profiling the physiological role of cysteine S-sulfination in disease.

In select cellular environments and physiological states, certain redox-active cysteine residues are susceptible to oxidation to *S*-sulfenylcysteine (Cys-SOH). This transient modification typically reacts with a second thiol to form a disulfide,[1] but when it is inaccessible to cellular reductants or if the oxidative load in the local environment is too high, sulfenyl-cysteine can undergo further oxidation to a sulfinic acid ($Cys\text{-}SO_2H$).[2] This intermediate oxidation state exists as a kinetically long-lived species, as further oxidation proceeds 25–50 times more slowly than either thiol or sulfenic acid oxidation.[3] Sulfinic acid lifetimes can be further extended by the local protein environment. Indeed, sulfinic acids are estimated to occupy 5% of soluble protein thiols,[4] thus providing a significant mechanism for basal oxidative damage and enzyme inactivation across the proteome.[5]

Despite the predicted prevalence of S-sulfination, until recently, there have been no methods for its direct analysis or enrichment. In two distinct methods, nitroso-linked probes provide unique strategies for direct covalent labeling of endogenous S-sulfinated proteins (Scheme 1). The first strategy begins by addition of sulfinic acid to an aryl-nitroso linked probe, followed by attack of the transient oxyanion on an *ortho*-ester to form a stable benzioxazolone ring.[6] The second strategy uses *S*-nitrosothiol-linked probes to form a thiosulfonate with S-sulfinylated proteins.[5] Mammalian cell lysates were labeled with a biotin-conjugate of *S*-nitrosoglutathione (GSNO-biotin), enriched on streptavidin beads, and analyzed by mass spectrometry. Hundreds of S-sulfinated proteins were identified, including peroxiredoxins, DJ-1, and many metabolic enzymes. This method is not ideal, as the GSNO-biotin probe oxidizes relatively quickly to form an inert sulfonic acid. Importantly, both strategies require complete alkylation of cellular thiols to prevent nonspecific reactions. In both cases, S-sulfonated cysteines ($R\text{-}SO_3H$) are chemically inert, and do not crossreact with any of the electrophilic probes in either procedure.



Scheme 1. Nitroso-directed methods for labeling endogenous S-sulfinated cysteine residues in proteins. A) Aryl-nitroso probes react with protein sulfinic acids to form stable benzioxazolone rings. B) *S*-nitrosoglutathione probes react with protein sulfinic acids to form thiosulfonate linkages, likely by nucleophilic displacement of HNO.

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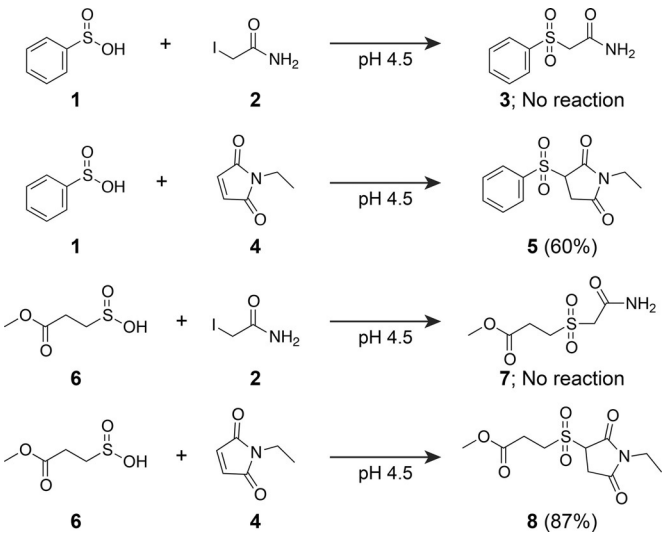
Supporting information and the ORCID identification numbers for the authors of this article can be found under https://doi.org/10.1002/cbic.201700137.

We previously reported that sulfinic acids do not react with 2-iodoacetamide (IAM) in aqueous buffers,[5] thus allowing orthogonal alkylation to reporter-linked *S*-nitrosothiols. Sulfinic acids are reported to participate in Michael additions, yet the reaction has not been translated to biological systems.[7] Building from these studies, we found that *N*-ethyl maleimide (NEM) reacts with both aryl (sodium phenylsulfinate) and alkyl (sodium 3-methoxy-3-oxopropane-1-sulfinate; SMOPS) sulfinic acids in aqueous buffer (Scheme 2 and Figure S1 in the Supporting Information). When maleimide/SMOPS were incubated at a 10:1 ratio, we observed ≈90% conversion of SMOPS to the corresponding sulfonyl-succinimide. As IAM only reacts with thiols, we reasoned that IAM and maleimide could be used sequentially for selective sulfinic acid detection. Furthermore, the pK_a of cysteine sulfinate (≈2) is 6 pH units below that of cysteine thiol (pK_a = 8.3).[2] Accordingly, we devised a sequential labeling strategy for proteome-wide analysis of S-sulfination. Reduced cysteines are first labeled with IAM at neutral pH, then the pH is reduced for orthogonal cysteine sulfinic acid labeling with NEM (Figure 1 A).



Scheme 2. Sulfinic acids react with NEM but not IAM. Reactions were carried out in 10:1 (electrophile/nucleophile) in degassed urea/citrate-phosphate buffer and assayed by HRLC-MS. Similar yields were achieved in phosphate buffer. The values represent the conversion efficiencies of the reaction mixtures, as measured by LC-MS. Supporting LC-MS extracted ion chromatographs are given in Figure S2.

For maleimide-linked probes to succeed as useful tools for S-sulfination profiling, the sulfonyl succinimide conjugate must be stable and irreversible in biocompatible buffers. To explore the product stability, *N*-ethyl-3-(phenylsulfonyl)succinimide was incubated across a panel of buffers at different pH values (Figure S3). At neutral or basic pH, the sulfonyl succinimide conjugate slowly degrades. Alternatively, when buffered below pH 6, no significant decomposition of the sulfonyl succinimide product (**5**) was observed, thus demonstrating sufficient stability for biochemical analysis.

We then examined whether the conjugate can undergo a retro-Michael displacement of phenylsulfinate.[8] If the sulfonyl-succinimide conjugate is reversible, the released phenylsulfinate could react with excess maleimide to form a scrambled species. However, we observed no NEM-maleimide scrambling after two hours at pH 4.5; this confirms that under these conditions, the sulfonyl-succinimide is stable and not reversible (Figure S1). Based on these findings, we achieve orthogonal maleimide conjugation to sulfinic acids at low pH, while simultaneously limiting lysine cross-reactivity and minimizing succinimide hydrolysis.

Next, the sequential-labeling protocol was adapted for analysis of sulfinic acids in mammalian cell lysates (Figure 1 A). HEK-293T cells were lysed in 6 M urea to denature the proteins and incubated with dithiothreitol (DTT) to reduce any disulfides, S-nitrosothiols, persulfides, or other reversible cysteine modification. Importantly, pre-alkylation of cysteine with either IAM or maleimide in denaturing phosphate-buffered saline

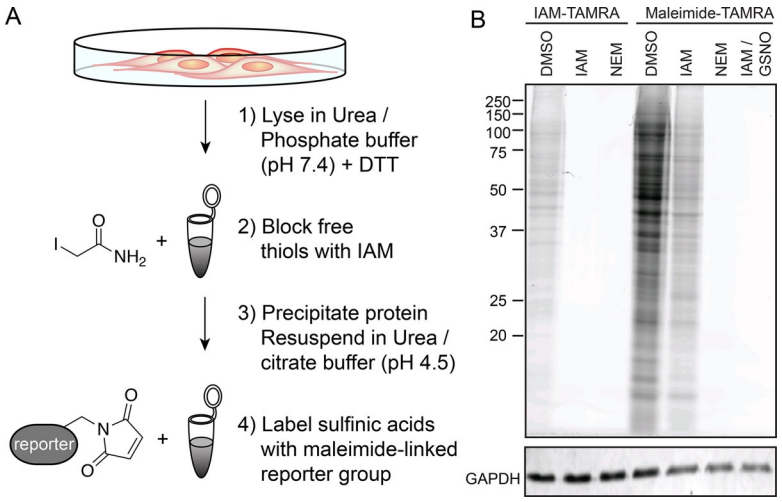


Figure 1. Sequential alkylation strategy for detecting endogenous S-sulfination. A) Schematic of the sequential alkylation strategy for labeling endogenous S-sulfinated proteins. Proteins are denatured in urea/PBS buffer for IAM labeling, then precipitated and resuspended in urea/citrate-phosphate buffer for maleimide labeling. B) IAM-TAMRA and maleimide-TAMRA labeling of HEK-293T cell lysates after pre-treatment with various electrophiles; IAM-TAMRA (50 μM), IAM (50 mM), NEM (50 mM), and GSNO (200 μM).

(6 M urea, PBS, pH 7.4) blocked all iodoacetamide-tetramethylrhodamine (IAM-TAMRA) labeling (Figure 1 B). Once the lysate was pre-blocked with IAM, addition of maleimide-TAMRA at pH 4.5 led to a broad profile of labeled proteins. Importantly, pre-incubation with IAM alkylates all cellular thiols, including free cysteine and glutathione. In combination with the additional protein-precipitation step, there is little chance for thiol interference with the sulfonyl succinimide product. We also found that 2,2′-dipyridyldisulfide (DPS; aldrithiol) reacts efficiently with both phenylsulfinate and SMOPS (93%; Figure S4). Moreover, GSNO addition blocked all maleimide labeling, either by *trans*-nitrosation of unreacted thiols or by thiosulfonate formation with sulfinic acids.[5] Thus, even after denaturation, reduction, and alkylation with IAM, a persistent profile of proteins react with maleimide at pH 4.5. Based on our studies with synthetic standards, these data are consistent with maleimide conjugation to sulfinic acids.

Parkinson's disease protein 7 (DJ-1/PARK7) contains a network of hydrogen bonds that stabilize sulfinic acid modification at Cys106, presumably inactivating its deglycase activity.[9] DJ-1 has three conserved cysteines (Cys46, Cys53, and Cys106), but only Cys106 forms a stable sulfinic acid[5,10] (Figure 2 A and B). Interestingly, oxidized DJ-1 (Cys106-SO_2H) is reported to impart additional antioxidant properties.[10] Thus, we prepared purified, recombinant DJ-1 protein to validate the chemical identity of the IAM-resistant maleimide protein conjugate. DJ-1 protein was treated with increasing concentrations of hydrogen peroxide, followed by sequential treatment with IAM and NEM. In the absence of peroxide, DJ-1 Cys106 is primarily reduced and alkylated by IAM, leaving only residual NEM labeling by in-gel fluorescence analysis (Figure 2 C). Incubation with 5 or 100 equivalents of hydrogen peroxide increased maleimide labeling; this indicates increased sulfinic acid formation.

As the sulfonyl-succinimide is only stable in acidic buffers, the peroxide-treated DJ-1 samples were digested with pepsin at pH 1.5 and analyzed by HRMS. As with the gel-based analysis, we observed robust peroxide-dependent sulfonyl-succinimide labeling on Cys106 (Figure 2 D). Furthermore, no succinimide or sulfonyl-succinimide adducts were detected on either Cys46 or Cys53, which lack the stabilizing hydrogen-bond network of Cys106. More detailed analysis of the MS/MS spectra confirmed the accurate annotation of the sulfonyl succinimide conjugate, as well as a minor, but reproducible neutral loss corresponding to release of succinimide sulfinic acid (Figure 2 E). Based on these findings, the sequential-labeling strategy captures endogenous sulfinic acids as sulfonyl succinimide conjugates, which are sufficiently stable for site-specific mass spectrometry analysis.

Next, we profiled the pharmacological induction of redox stress in RAW 264.7 cells. The mitochondrial electron-transport-chain inhibitors oligmoycin, rotenone, and paraquat all induced minor increases in maleimide-TAMRA labeling (Figure 3 A). Interestingly, potassium cyanide induced more significant labeling, thus suggesting it is a strong inducer of sulfinic acid formation. Alternatively, antimycin A reduced labeling; this could signify further oxidation of sulfinic acids to unreactive sulfonic acids. Lipopolysaccharide (LPS) and tunicamycin

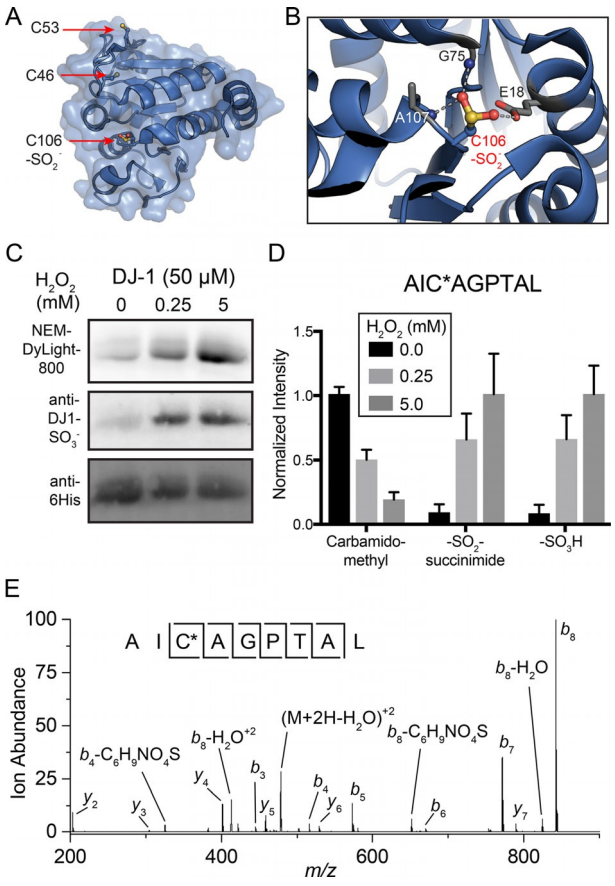


Figure 2. Analysis of sulfonyl succinimide formation on recombinant human DJ-1. A) Structure of DJ-1 (PDB ID: 1SOA) highlighting the three encoded cysteines. Cys46 and Cys53 are surface exposed; Cys106 is buried in the putative active site. B) DJ-1 Cys106 forms a stabilized sulfinic acid through a network of hydrogen bonds. C) Profiling S-sulfination of DJ-1 by sequential alkylation with IAM and maleimide-DyLight 800. Recombinant DJ-1 was pretreated with hydrogen peroxide, followed by DTT, IAM, and maleimide-DyLight 800 to detect sulfinic acids. D) Label-free MS quantitation of DJ-1 Cys106 oxidation summed and normalized from the pepsin-digested peptide AICAGPTAL. Error bars represent standard deviations from four biological replicates. E) Collision-induced dissociation MS/MS spectra of the DJ-1 *N*-ethyl-3-(sulfonyl-Cys106)succinimide-conjugated peptide (AIC*AGPTAL); *: sulfonyl-succinimide.

stimulated more labeling, thus suggesting that sulfinic acids might be more readily induced outside of the mitochondria, either through LPS-mediated induction of NADPH oxidases or through tunicamycin stimulation of endoplasmic reticulum (ER) stress. Interestingly, a single ≈75 kD protein dominated maleimide-TAMRA labeling across the proteome, thus suggesting that there is at least one major S-sulfinated protein in macrophage cells. Collectively, stimulus-dependent maleimide labeling confirms the detection of an IAM-resistant population of modified thiols, further supporting direct conjugation to sites of S-sulfination.

Similar to previous results with GSNO-biotin, maleimide-biotin labeled a distinct profile of S-sulfinated proteins across different mouse tissues (Figure 3 B).[5] Although a similar 75 kD protein is present across all tissues, there are clear differences that could reflect differential protein expression or redox

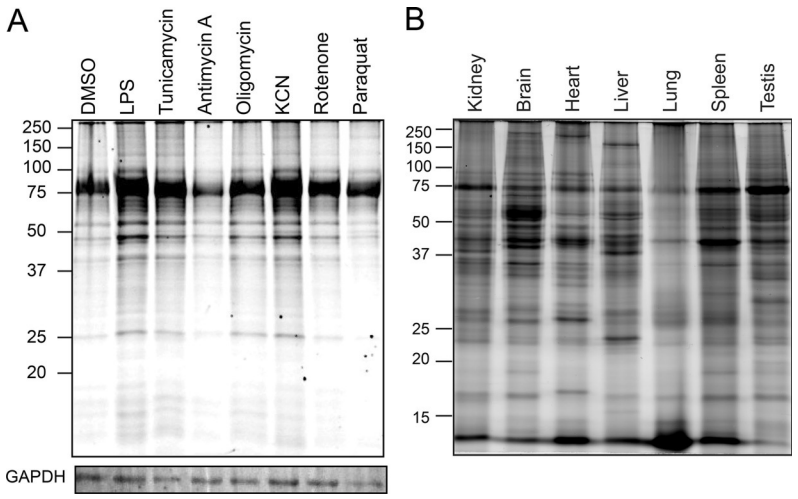


Figure 3. Profiling S-sulfination in stimulated cells and tissues by in-gel fluorescence of maleimide-TAMRA. A) Stimulus-dependent induction of protein S-sulfination in RAW 264.7 cells (24 h); LPS (100 ng mL^{-1}), tunicamycin (10 μM), antimycin A (50 μM), oligomycin (5 μM), KCN (1 mM), rotenone (1 μM), and paraquat (40 μM). B) S-sulfination profile across different mouse tissues.

states. Despite continuous exposure to high concentrations of oxygen, lung homogenates show the lowest overall labeling. Indeed, the low-molecular-weight (< 15 kD) band, presumably thioredoxin, is labeled more efficiently in lung than other tissues. Clearly, site-specific MS-based proteomic annotation of these tissue-specific oxidation events will greatly enhance our understanding of cellular redox regulation and how cysteine can reach higher oxidation states in proteins. Sulfonyl-succinimide-labeled peptides have different retention times from those thiol-succinimide conjugates, and different masses due to the two additional oxygens (+32 D), thus providing clear orthogonality for MS annotation.

Cysteine thiols are both nucleophilic and sensitive to the cellular redox environment. Whereas cysteines typically form disulfide bonds when exposed to oxidants, certain thiols are further oxidized to sulfinic acids and sulfonic acids. Here we have demonstrated that maleimide reacts with cysteine sulfinic acids in proteins. By using a sequential alkylation strategy, maleimide probes can selectively label protein sulfinic acids on chemical standards, recombinant protein, and in cell homogenates from stimulated cells. The resulting sulfonylcysteine-succinimide adducts are stable in acidic buffers, thus providing a simple strategy to profile S-sulfination in biological samples.

Although this approach simplifies the direct detection of protein S-sulfination, it also demonstrates potential challenges in profiling such a transient, intermediate post-translational modification. Whereas increased labeling can be attributed to enhanced S-sulfination, decreased labeling can represent either lower S-sulfination or further conversion to S-sulfonation (R-SO_3H). Thus, changes in maleimide labeling alone cannot be used to assess redox status, and more detailed analysis will be required. Furthermore, mass spectrometry profiling studies that use this approach will require site-specific peptide analysis to avoid the detection of any thiols that might have escaped complete iodoacetamide alkylation. Acid-stable proteases, such as pepsin or Glu-C, might be necessary to stabilize the modification during proteolysis. With these points in mind, future studies will explore site-specific S-sulfination across distinct physiological states, while providing a simple and accessible method for direct S-sulfination analysis.

Experimental Section

Synthetic methods, characterization, and mass spectra are provided in the Supporting Information.

HPLC assays of sulfonyl succinimide formation and reversibility: Stock solutions of benzene sulfinic acid (20 mM), *N*-ethylmaleimide (200 mM), and iodoacetamide (200 mM) were freshly prepared in DMSO for each experiment. Benzene sulfinic acid (50 μL) and either *N*-ethylmaleimide or iodoacetamide (50 μL) were added to urea (900 μL, 6 M) in citrate-phosphate buffer (pH 4.5). After 1 h, the reaction mixture was analyzed by HPLC. Stock solutions of benzene sulfinic acid (10 mM), maleimide (100 mM), and 1-ethyl-3-(phenylsulfonyl)succinimide (100 mM) were prepared in DMSO. Benzene sulfinic acid (50 μL) and maleimide solutions (50 μL) were added to urea (900 μL, 6 M) in citrate-phosphate buffer (pH 4.5). After 30 min, the reaction mixture was analyzed by HPLC. Next, 1-ethyl-3-(phenylsulfonyl)succinimide (50 μL) and maleimide stock solution (50 μL) were added to urea (900 μL, 6 M) in citrate-phosphate buffer (pH 4.5). After 2 h, the reaction mixture was analyzed by HPLC.

Gel-based profiling of protein S-sulfination: Mouse RAW 264.7 cells and human HEK-293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (*v*/*v*) fetal bovine serum (JR Scientific) and 1% (*v*/*v*) penicillin/streptomycin/glutamine (Gibco). Cells were harvested at 80% confluence with a cell scraper in urea/PBS buffer (6 M) supplemented with IAM (1 mM, pH 7.4) and lysed by sonication (4 °C, 10% duty cycle, 15 s). Protein concentrations were measured by using the BioRad DC assay, and then diluted to 2 mg mL^{-1}. Next, lysates were incubated with dithiothreitol (2.5 mM) for 30 min, followed by iodoacetamide (50 mM) with EDTA (10 mM) and 2% (*w*/*v*) SDS for 1 h in the dark. The reaction was quenched by precipitation with chloroform/methanol, and the precipitates were re-solubilized in urea (6 M) in

citrate-phosphate buffer (pH 4.5). Next, lysates were incubated with maleimide-TAMRA (50 μM) for 30 min. For tissue homogenate studies, tissues were collected from an 18-month-old C57/B6 mouse, frozen immediately in liquid nitrogen, and later Dounce homogenized in aqueous urea (6 M)/ PBS (pH 7.4) supplemented with IAM (1 mM). The homogenate was cleared by centrifugation at 3000 *g* for 5 min, then at 5000 *g* for 5 min, and the supernatant was processed as described above. Protein samples were resolved by SDS-PAGE (10 or 12% gels) and imaged by using a Typhoon flatbed fluorescence gel scanner. Next, the resolved proteins were transferred to an Immobilon-FL membrane (Millipore) and blocked with 5% bovine serum albumin (BSA, Fisher) in Tris-buffered saline-Tween 20 (TBS-T, pH 7.4) for 1 h at room temperature, followed by washing with TBS-T (3×5 min). After blocking with 5% BSA, immunoblotting was performed with the primary and secondary antibodies, and detected by using an Azure Biosystems c600 imager. For GAPDH detection, blots were probed with anti-GAPDH mouse monoclonal antibody (mAb 6C5, Calbiochem, 1 $\mu g\,mL^{-1}$, 2.5% BSA, 0.02% sodium azide, TBS-T, pH 7.0), washed, and probed with a secondary Alexa Fluor 488 donkey-anti-mouse antibody conjugate (IgG H + L, Life Technologies, 2 $\mu g\,mL^{-1}$ antibody, 0.06% sodium azide, TBS-T) for 1 h at room temperature. For His_6 tag detection, blots were probed with the anti-His_6 tag monoclonal antibody (mAb IgG2b, Thermo Fisher, 1 $\mu g\,mL^{-1}$, 2.5% BSA, 0.02% sodium azide, TBS-T, pH 7.0), washed, and probed with a secondary Alexa Fluor 488 donkey-anti-mouse antibody for 1 h at room temperature. Anti-DJ1SO_3H blots were probed with anti-DJ1(oxidized) rabbit monoclonal antibody (IgG, Abcam, 0.25 $\mu g\,mL^{-1}$ antibody, 2.5% BSA, 0.02% sodium azide, TBS-T, pH 7.0), washed, and probed with AzureSpectra 700-conjugated secondary antibody (IgG, Azure Biosystems, 1 $\mu g\,mL^{-1}$ antibody, 2.5% BSA, 0.02% sodium azide, TBS-T, pH 7.0) for 1 h.

Mouse studies were approved by the University of Michigan Institutional Animal Care and Use Committee (PRO0005 707).

Acknowledgements

We would like to thank John E. Crellin, Hashim F. Motiwala, Rachel Pricer (University of Michigan) for help with chemical synthesis, analysis, and figure preparation. Financial support was provided by the National Institutes of Health DP2 GM114848, T32 CA140044 Proteome Informatics of Cancer Training Grant (A.M.K), T32 GM008597 Chemical Biology Interfaces Training Grant (S.E.H.), S10 OD021619 (Orbitrap Fusion Lumos Tribrid Mass Spectrometer), a National Science Foundation Graduate Research Fellowship (A.M.K.), the American Heart Association 14POST20420040 (J.D.M.), and the University of Michigan.

Conflict of Interest

The authors declare no conflict of interest.

Keywords: cysteine · Michael addition · protein modifications · redox chemistry · sulfur

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Manuscript received: March 9, 2017
Revised manuscript received: June 16, 2017
Accepted manuscript online: August 14, 2017
Version of record online: September 1, 2017