Direct Proteomic Mapping of Cysteine Persulfidation

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Abstract

Aims: Cysteine persulfidation (also called sulphydration or sulfuration) has emerged as a potential redox mechanism to regulate protein functions and diverse biological processes in hydrogen sulfide (H₂S) signaling. Due to its intrinsically unstable nature, working with this modification has proven to be challenging. Although methodological progress has expanded the inventory of persulfidated proteins, there is a continued need to develop methods that can directly and unequivocally identify persulfidated cysteine residues in complex proteomes.

Results: A quantitative chemoproteomic method termed as low-pH quantitative thiol reactivity profiling (QTRP) was developed to enable direct site-specific mapping and reactivity profiling of proteomic persulfides and thiols in parallel. The method was first applied to cell lysates treated with NaHS, resulting in the identification of overall 1547 persulfidated sites on 994 proteins. Structural analysis uncovered unique consensus motifs that might define this distinct type of modification. Moreover, the method was extended to profile endogenous protein persulfides in cells expressing H₂S-generating enzyme, mouse tissues, and human serum, which led to additional insights into mechanistic, structural, and functional features of persulfidation events, particularly on human serum albumin.

Innovation and Conclusion: Low-pH QTRP represents the first method that enables direct and unbiased proteomic mapping of cysteine persulfidation. Our method allows to generate the most comprehensive inventory of persulfidated targets of NaHS so far and to perform the first analysis of *in vivo* persulfidation events, providing a valuable tool to dissect the biological functions of this important modification. *Antioxid. Redox Signal.* 33, 1061–1076.

Keywords: chemical proteomics, cysteine, hydrogen sulfide, persulfidation/sulphydration, persulfide, mass spectrometry

Introduction

CYSTEINE PERSULFIDATION (also called sulphydration or sulfuration) is a reversible post-translational modification of protein cysteinyl thiols (-SH) to persulfides (-SSH), which can be formed in various routes, such as reaction between hydrogen sulfide (H₂S), more precisely HS⁻, and oxidized protein thiols, reaction between inorganic polysulfides and protein thiolates, and radical reaction by other reactive sulfur species (RSS) (22). Like other cysteine redox modifications, persulfidation has emerged as an important mechanism

to fine tune protein function, interaction, and localization in cells (54). It has also been demonstrated the important roles of this modification in regulating a variety of redox-dependent biological processes, such as autophagy, cellular metabolism, inflammation, cell cycle, and cell death under physiological and pathological contexts (54).

Due to its intrinsically unstable nature and greater reactivity than thiols, working with this modification, especially on a proteome-wide scale, has proven to be challenging. Snyder and colleagues first introduced an approach based on the modified biotin-switch technique (BST) for this purpose,

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Innovation

There is a continued need to develop methodology that can directly and unbiasedly identify proteome-wide persulfidation at the site level. Here, we present a new chemoproteomic workflow, called low-pH quantitative thiol reactivity profiling (QTRP), which represents the first attempt to address the need. Using low-pH QTRP, we generate so far the most comprehensive inventory of persulfidated targets of sodium hydrogen sulfide, providing a valuable resource for future investigation of the functional roles of persulfidation in cellular processes. The low-pH QTRP analysis of mouse tissues and human serum offers new insights into mechanistic, structural, and functional insights into *in vivo* persulfidation events, particularly on human serum albumin.

which employ methyl methanethiosulfonate (MMTS) to differentiate thiols and persulfides (Supplementary Fig. S1A). By using this approach, dozens of protein targets of persulfidation were identified by mass spectrometry (MS) in mouse liver lysates exposed to sodium hydrogen sulfide (NaHS) (47, 61). In a chemical aspect, however, the use of this approach remains controversial owing to the lack of selectivity of MMTS for thiol *versus* persulfide. Thereafter, many other groups independently developed several similar approaches to globally profile cysteine persulfidation, in which free thiols or persulfides in proteins are first alkylated by thiol-reactive biotin reagents (Supplementary Fig. S1B). After tryptic digestion, the persulfide-tagged biotinylated peptides are captured by streptavidin beads and eluted by reducing reagents, leaving the nonreducible thiol-tagged biotin conjugates bound to the beads. The eluted free thiol-containing peptides are further alkylated and subjected to MS analysis. By using such approaches, hundreds of protein persulfides have been identified in bacteria (56), plants (4), mouse (25), and human cells (43). In addition, Xian and colleagues innovated the tag-switch approach for more reliable detection of this modification (Supplementary Fig. S1C), which employs an electrophile called methylsulfonyl benzothiazole (MSBT) to tag all protein thiol species (-SH and -SSH) and then chemoselectively switch the MSBT tag on targeted persulfides with a biotinylated cyanoacetate probe (CN-biotin) (53, 82). This approach, in combination with

MS-based proteomics, reveals dozens of protein persulfides in A549 cells overexpressing cystathionine γ -lyase (CSE).

Without any doubt, aforementioned methodological progress has expanded the inventory of persulfidated proteins and revealed many biologically or physiologically significant persulfidation events (72, 77). However, the major limitation of current indirect approaches for protein- or site-level analysis of persulfidation is the potential of introducing false-positive identifications due to incomplete alkylation or reduction. Only direct and site-specific mapping of cysteine persulfidation in either an individual protein or a complex proteome can provide definitive confirmation.

Guided by the pioneering works by Cravatt and colleagues (20, 71, 73), we have recently developed a chemoproteomic method termed as quantitative thiol reactivity profiling (QTRP) that can profile thousands of proteomic thiols in a single experiment (24, 57, 64, 66). QTRP relies on the ability of an electrophilic thiol-reactive probe iodo-N-(prop-2-yn-1-yl) acetamide (IPM) [iodo-N-(prop-2-yn-1-yl) acetamide] (Fig. 1A) to covalently label and enrich reactive cysteine residues. In principle, persulfides can also be labeled and enriched by this method. However, the small percentage of protein persulfides in proteome, compared with much higher abundant protein cysteinyl thiols, has limited the use of QTRP for efficient enrichment and detection of IPM-labeled persulfides.

Here, we adapted our QTRP method to enable direct and site-specific mapping of cysteine persulfidation in cell lysates treated with NaHS, cultured cells heterogeneously expressing CSE, mouse tissues, and human serum. The method can provide up to hundreds of protein persulfide identifications for a single cellular condition or tissue type. Moreover, the method enables reactivity profiling of proteomic thiols and persulfides in parallel, thereby offering a chance to predict the biological activity of a detected persulfidation event. Building upon the high-quality data sets generated in this study, bioinformatic and structural analyses offer many new mechanistic, structural, and functional insights into this important cysteine modification.

Results

Development of a low-pH QTRP method for direct analysis of cysteine persulfidation

While persulfides have characteristics in common with thiols, the pKa of a persulfide is significantly lower than that

FIG. 1. Development of the low-pH QTRP method for direct analysis of cysteine persulfidation. (A) Chemical structures of the IPM probe and the isotopically labeled UV-cleavable azido-biotin. (B) Schematic reactions of the electrophilic IPM probe with protein persulfides or thiols. (C) General workflow for the low-pH QTRP method. Proteomic thiols and persulfides are labeled with IPM and then digested into tryptic peptides. Equal amounts of peptide mixtures were reacted with light- and heavy-tagged UV-cleavable azido-biotin, respectively, mixed in a ratio of 1:1, and subjected to streptavidin enrichment, photorelease, and LC-MS/MS analysis. The light/heavy ratios were calculated for IPM-tagged disulfide or thioether peptides. (D) Identification of C247 of GAPDH as a target site of persulfidation in A549 cells. *Upper left*: MS1 spectrum of an IPM-tagged disulfide peptide containing GAPDH C247. Doubly charged monoisotopic precursors of light and heavy peptides are observed at m/z 879.441 (red) and 882.451 (blue), respectively, with mass errors less than 10.0 ppm. *Upper right*: XIC showing the profiles for the same light and heavy peptides. *Lower*: fully annotated HCD MS/MS spectra of the light and heavy IPM-modified disulfide peptides. (E) In comparison with regular QTRP at physiological pH, low-pH QTRP achieves dramatically higher enrichment specificity for proteomic persulfides. Enrichment specificity (%) = $100 \times \text{number of PSMs of the modified peptides} / \text{number of PSMs of all the identified peptides}$. (F) Box plot of the CV values of all the light to heavy ratios for proteomic thiols and persulfides determined by low-pH QTRP. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCD, higher energy collision dissociation; IPM, iodo-N-(prop-2-yn-1-yl) acetamide; MS, mass spectrometry; PSM, peptide-spectrum match; QTRP, quantitative thiol reactivity profiling; XIC, extracted ion chromatograms. Color images are available online.

of the corresponding thiol. Specifically, as suggested by a computational study (19), the pKa of cysteine persulfide is 4.3, which is ~ 4 U lower than that of cysteinyl thiol (pKa = 8.29). At physiological pH, the persulfide is estimated to be fully deprotonated, whereas the thiol group is only partially deprotonated. Hence, persulfides are better nucleophiles than thiols because of the greater availability of RSS^- versus RS^- (22). In theory, lowered pH can maintain relatively high reactivity of persulfides with carbon electrophiles, like the “clickable” IPM probe, while inhibiting deprotonation of thiols to form thiolates, thereby minimizing the IPM labeling of abundant proteomic thiols and enhancing the signals of IPM-labeled persulfides. Therefore, to enable the more efficient detection of persulfides, the first and foremost modification that we made in our regular QTRP workflow is to perform IPM labeling at pH 5.0 rather than physiological pH. In contrast to protein thiols, which form thioethers with this probe, persulfides form reducible disulfides (Fig. 1B). Hence, the second methodological adaptation is to remove the dithiothreitol-based reduction step to retain the probe-labeled disulfide peptides. In a proof-of-principle analysis, A549 cell lysates treated with 0.5 mM NaHS, sodium salt that dissolves to give the HS^- anion at physiological pH, were subjected to buffer exchange and then labeled with IPM at pH 5.0. The probe-labeled proteome was alkylated directly with iodoacetamide (IAA) and digested into tryptic peptides. The resulting probe-labeled peptides were conjugated with both light and heavy azido-UV-cleavable-biotin (Az-UV-biotin, Fig. 1A) reagents (1:1) *via* copper-catalyzed alkyne-azide cycloaddition (CuAAC). The biotinylated peptides were captured with streptavidin and photoreleased for MS-based shotgun proteomics. It is expected that there will be two types of probe-modified peptides, including the disulfide form and the thioether form for mapping -SSH and -SH sites, respectively (Fig. 1C). Moreover, probe-peptide adducts covalently conjugated with light and heavy tags can yield an isotopic signature (L:H = 1:1) that increases the confidence of modified peptide identifications (Fig. 1C). In this regard, only those modified peptide assignments whose MS1 intensity reflected an L/H ratio between 0.67 and 1.5 (1.5-fold change) were considered to be true identifications. For example, we identified a probe-modified disulfide peptide bearing glyceraldehyde-3-phosphate dehydrogenase (GAPDH) C247, a known persulfidation site (33), as demonstrated by the characteristic isotopic envelopes in the representative MS1 spectrum, the light to heavy ratio calculated from the extracted ion chromatogram peaks (XIC), and the fully annotated higher energy collision dissociation (HCD)-MS/MS spectrum (Fig. 1D). Also, we identified a probe-modified thioether peptide with the same sequence, of which MS1 intensity was, however, lower than that of the disulfide peptide ($4.9\text{E}6$ vs. $1.2\text{E}7$), indicating the enrichment of the latter (Supplementary Fig. S2). In total, the low-pH QTRP workflow identified and quantified 295 unique disulfide peptides, assigning to 285 -SSH sites on 209 proteins, and 1532 thioether peptides, assigning to 1471 -SH sites on 965 proteins (Supplementary Table S1). Notably, 11.7% of identified peptide-spectrum matches (PSMs) in the low-pH analysis could be assigned to probe-modified disulfide peptides, while the regular QTRP analysis only achieved 0.3% (Fig. 1E). In contrast, the percentage of identified PSMs for probe-modified thioether peptides in the low-pH QTRP analysis was ~ 1.5 -fold lower than that obtained from the

regular QTRP analysis of the same sample (Fig. 1E). Accordingly, the number of identified -SSH sites in the low-pH QTRP analysis was 13.6-fold greater than that obtained from a regular QTRP analysis, whereas ~ 2.8 -fold less -SH sites were identified in the former analysis. These results demonstrated that the low-pH setting can reach relatively high efficiency for the enrichment and identification of IPM-modified persulfides. Furthermore, the majority of coefficient of variation (CV) values for the determined $R_{\text{H:L}}$ values were less than 20% (Fig. 1F), demonstrating the reproducibility of our method. Taken together, the modified QTRP method provided an unprecedented means for the direct and unbiased identification of cysteine persulfidation in a complex proteome.

Reactivity profiling of proteomic thiols and persulfides

Although the reactivity of low-molecular-weight persulfides toward oxidants and electrophiles has been extensively measured (22), the intrinsic reactivity of proteomic persulfides has yet to be explored. With the low-pH QTRP method in hands, we first sought to simultaneously quantify the intrinsic reactivity of proteomic thiols and persulfides using a similar strategy described by us and others (Fig. 2A and Supplementary Table S2) (45, 57, 73). Specifically, we employed a dose-dependent labeling of the NaHS-treated A549 proteome with low ($10\text{ }\mu\text{M}$) and high ($100\text{ }\mu\text{M}$) levels of IPM at pH 5.0. After tryptic digestion, the resulting IPM-modified peptides were further conjugated to light and heavy Az-UV-biotin, respectively, *via* CuAAC. Then, the light- and heavy-labeled samples were mixed, captured with streptavidin, and photoreleased for liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis. Hyperreactive thiol species (-SH and -SSH) are expected to label to completion at low probe concentrations ($10\text{ }\mu\text{M}$), and less reactive thiol species should show concentration-dependent increases in IPM labeling. Accordingly, the thiol species quantified in this analysis could be classified into three groups. Those with calculated ratio of heavy to light ($100\text{ }\mu\text{M}$ vs. $10\text{ }\mu\text{M}$, $R_{10:1}$) lower than 3.0 were defined to be hyperreactive; those within the range from 3.0 to 5.0 were considered to be moderately reactive, whereas the rest were classified as weakly reactive. In total, we identified and quantified 84 -SSH sites and 1224 -SH sites with average $R_{10:1}$ value of 3.44 and 4.61, respectively (Fig. 2B). 35.7% of the identified persulfides were quantified with $R_{10:1}$ value below 3.0, indicating hyperreactivity, whereas such a percentage of the corresponding thiols was only 23.4% (Fig. 2C). Notably, the identified persulfidated sites were 100% covered in the mapped cysteinyl sites, allowing for a side-by-side comparison of persulfide activity with a subset of thiols that retain reactivity at low pH. Overall, 91.7% of persulfides exhibited higher or equivalent reactivity than that of the corresponding cysteinyl thiols ($R_{10:1}^{-\text{SH}} \geq R_{10:1}^{-\text{SSH}}$) (Fig. 2D), such as the active or redox-sensitive sites of cofilin-1 (CFL1), pyruvate kinase (PKM), peroxiredoxin (PRDX) 1, and ubiquitin-conjugating enzyme E2N (UBE2N) (Supplementary Fig. S3). Nonetheless, interestingly, a few persulfides showed slightly higher $R_{10:1}$ compared with the corresponding thiols. For example, the cysteinyl thiol of the active site of GAPDH (C152) and its persulfide form had the determined $R_{10:1}$ value of 5.60 and 8.07, respectively, indicating the persulfidation-dependent loss of chemical reactivity (Fig. 2E).

FIG. 2. Reactivity profiling of proteomic thiols and persulfides at low pH. (A) Workflow for reactivity profiling of proteomic thiols and persulfides at low pH. (B) Box plot showing the distribution of heavy (100 μ M)/light (10 μ M) ratios ($R_{10:1}$) of proteomic thiols and persulfides determined from the low-pH QTRP analysis of the NaHS-treated A549 cell lysates. Widths of boxes are proportional to square roots of the number of identifications. (C) Pie charts showing the distribution of persulfide and thiol reactivity of the NaHS-treated A549 proteome at low pH. (D) A side-by-side comparison of the persulfide and thiol on the same cysteine residues. Those with $R_{10:1}^{-SH} \leq R_{10:1}^{-SSH}$ are depicted in red color; the rest is depicted in gray. (E, F) XICs of IPM-tagged disulfide (-SSH) and thioether (-SH) peptides from GAPDH (E) or TXN (F) from the low-pH QTRP analysis of the NaHS-treated A549 cell lysates. The profiles for light- and heavy-labeled peptides are shown in red and blue, respectively. $R_{10:1}$ values calculated from two independent experiments are displayed below the individual chromatograms. TXN, thioredoxin. Color images are available online.

In general, the enhanced chemical reactivity of persulfidated cysteines may be reflected in increased biological activity, *vice versa*. This finding therefore supports the recent proposal that persulfidation of GAPDH at C152 is inhibitory (33), although it has been previously suggested that this modification positively regulates the GAPDH activity (47). In another interesting example, the determined $R_{10:1}$ of the active site C43 of thioredoxin (TXN) domain-containing protein 17 (NDC17) (also known as thioredoxin-related protein of 14 kDa, TRP14), a protein with polysulfide- and protein persulfide-reducing properties (21), was 1.45-fold higher for persulfide than for the thiol (Fig. 2F).

Furthermore, we applied the regular QTRP workflow to the same sample for measuring proteomic thiols and persulfides by reactivity at pH 7.6. As a result, the hyperreactive protein thiols ($R_{10:1} < 3.0$) at this physiological pH appeared to have significantly lower $R_{10:1}$ values than those obtained in the low-pH QTRP data set (Supplementary Fig. S4A), in accordance with a previous report (6). Likewise, we noted a general loss in reactivity of persulfides with decreased pH (Supplementary Fig. S4B). For example, persulfides on

all three sites of GAPDH showed the increases in $R_{10:1}$ value by at least twofold (for C152, $R_{10:1}^{pH5.0} = 8.07/R_{10:1}^{pH7.6} = 3.69$; for C156, $R_{10:1}^{pH5.0} = 8.75/R_{10:1}^{pH7.6} = 3.62$; for C247, $R_{10:1}^{pH5.0} = 5.93/R_{10:1}^{pH7.6} = 2.05$, Fig. 2E and Supplementary Fig. S4C). These findings rationalize the ability of low pH to stabilize not only hyperreactive thiols but also persulfides. Moreover, the percentage of hyperreactive and moderately reactive persulfides ($R_{10:1}^{-SSH} < 5.0$) appeared to be constantly high at different pH (85.7% for pH 5.0 and 83.3% for pH 7.6), whereas the percentage of cysteinyl thiols with at least moderate reactivity ($R_{10:1}^{-SH} < 5.0$) decreased at reduced pH (52.2% for pH 5.0 vs. 66.4% for pH 7.6), which might attribute to the relative enrichment of probe-modified persulfides in the low-pH QTRP workflow (Fig. 2C and Supplementary Fig. S4D).

Deep profiling of protein persulfides formed in the NaHS-treated cell lysates

Theoretically, low pH attenuates deprotonation and oxidation of free thiols, thereby making less oxidized thiols to

react with HS⁻ derived from NaHS. On contrary, low pH should considerably prevent persulfides from further reactions or degradation. Thus, we set out to process the lysates from five commonly used cell lines in a low-pH buffer from the beginning. Specifically, cells were harvested in the lysis buffer (pH 5.0) containing 0.5 mM NaHS, incubated at 37°C for 1 h, and then labeled with the IPM probe. The probe-labeled proteomes from two biological replicates were processed and analyzed as described above. We observed an excellent degree of overlap within Venn diagrams between two biological replicates (Supplementary Fig. S5A). The quality of this data set was further demonstrated by the high reproducibility of quantification, with the average CV values of all the measured L/H ratios lower than 20% for each cell line (Supplementary Fig. S5B). As expected, the analytic performance was facilitated by relatively high enrichment specificity (9.3–52.7% identified PSMs were assigned to probe-modified disulfide peptides, Supplementary Fig. S5C). The analysis resulted in the identification of up to 988 unique persulfidation sites from a single cell type (Fig. 3A). Overall, a total of 1547 unique persulfidation sites were identified across five cell lines, which were mapped to 994 proteins (Fig. 3B and Supplementary Table S3).

We first compared persulfidated sites/proteins identified in the present analysis with those identified by another proteome-wide analysis of persulfidation in human cells (43). More than 200 persulfidated sites identified previously were also observed in our data set, whereas the majority of the sites identified here (87%) represent novel persulfidation targets (Supplementary Fig. S6). Additionally, we observed a higher overlap at the protein level compared with the site level (Supplementary Fig. S6). Our analysis also confirmed several functional persulfidation sites reported in the literature *via* low-throughput approaches. For example, we confirmed a persulfidation event on C63 of L-lactate dehydrogenase A (LDHA), which has been suggested to regulate cellular bioenergetics in HCT116 colon cancer cells (67). Moreover, we found that the resolving cysteines of the typical 2-Cys peroxiredoxins (PRDX1-4) underwent persulfidation after NaHS treatment. Orthogonally, we verified these targets of persulfidation by a combination of a well-established tag-switch approach and immunoblotting detection (Supplementary Fig. S7).

To gain better understanding of the systems impact of cysteine persulfidation, we performed Gene Ontology (GO) classification of the human protein persulfides mapped across five cell lines. As shown in Figure 3C, the persulfidated proteins are involved in many important biological processes and/or pathways, including oxidation–reduction process, glycolysis/gluconeogenesis, NF- κ B signaling, and Parkinson's disease. Interestingly, cysteine persulfidation has previously been reported to play functional roles in these processes (17, 58, 61, 69). Moreover, our analysis indicates that the roles of this modification in cellular processes are much broader than previously anticipated. For instance, we noted that RNA binding represents the most significantly enriched GO term for persulfidated proteins. Of particular interest, in a mostly recent report, human antigen R, an RNA binding protein, was shown to be persulfidated by H₂S generated by CSE, thereby preserving endothelial cell function and delaying atherogenesis (10).

Relation of persulfidation to other cysteine modifications

Not unexpectedly, the majority of persulfidated proteins had only one or two modified sites, whereas only 2.4% of all identified proteins had more than five modified cysteines (Fig. 3D). Interestingly, we identified 31 proteins containing “orphan” cysteines, which are highly prone to overoxidation (Supplementary Table S3). Persulfidation on these sites are likely to serve a protective function. Moreover, we noted that persulfidation exhibited the remarkably site selectivity on a selected group of proteins with multiple cysteines being 100% persulfidated. For example, among all three modified cysteines on GAPDH, persulfidation almost predominantly occurred at C156 and C247 (Fig. 3E), in accordance with a recent finding (33). Interestingly, these two sites were also the preferential targets of sulfenylation (1) (Fig. 3E). A similar correlation could also be observed for persulfidation and sulfenylation of CFL1 C80 (Fig. 3E). This finding is not unreasonable, as it has been suggested that persulfidation chemistry involves the preferential addition of SH⁻ to a sulfenic acid (22). To examine this premise more systematically, we compared the human persulfidated cysteines profiled in this study to thousands of sulfenylation sites identified previously *via* chemoproteomics (1, 23, 28, 79, 80). Interestingly, 57.9% of persulfidated cysteine residues can also be comodified by sulfenylation (Supplementary Fig. S8A), further confirming the intrinsic relationship between these two modifications.

Many of known persulfidated cysteine residues have also been found to undergo S-nitrosylation. The reciprocal interplay between persulfidation and S-nitrosylation has also been elicited in many previous studies (2, 60, 61). We compared our data sets with dbSNO 2.0 (16), a curated online resource that records 1247 S-nitrosylated cysteines on 720 human proteins. Of these, 276 sites were included in our data set (Supplementary Fig. S8B). Notably, many previously unknown co-targets of persulfidation and S-nitrosylation are functionally of importance, indicating the potential crosstalk of these two modifications on target proteins. For example, persulfidation and S-nitrosylation may competitively modulate the activity of their enzymatic targets by modifying the catalytic cysteines, such as PRDX6 C47 and ubiquitin-like modifier-activating enzyme 1 (UBA1) C632, or, alternatively, affect protein–protein interactions and protein stability by modifying the critical regulatory cysteines, such as TXN C73 (7), protein/nucleic acid deglycase DJ-1 (PARK7) C46 (32), and ubiquitin carboxyl-terminal hydrolase isozyme L1 (UBCHL1) C152/C220 (38). Nonetheless, >80% of identified persulfidation sites were not found previously as comodification targets of S-nitrosylation. We further examined the flanking sequences of persulfidated cysteine residues for the presence of linear motifs and compared these with motifs associated with S-nitrosylation. As shown in Supplementary Figure S9A, persulfidated cysteines resided within linear motifs that mainly consist of basic and hydrophobic amino acids. Although these types of amino acids were also overrepresented in the flanking sequences of S-nitrosylated sites, the presence of acidic amino acids represented an additional feature that may define S-nitrosylation (Supplementary Fig. S9B). Indeed, the acid–base motif has been well demonstrated as a site for forming S-nitrosylation (34). These findings suggest that persulfidation is associated with sequence motifs that are distinct from those for

FIG. 3. Deep profiling of protein persulfides formed in the NaHS-treated cell lysates. (A) Number of persulfidation sites identified per cell line by the low-pH QTRP workflow. (B) Venn diagram of persulfidation sites identified in different cell lines. (C) Significantly ($p < 0.01$, Fisher test) enriched GO and KEGG terms. Each point is proportional in size to the number of persulfidated proteins associated with the term. (D) Schematic overview of the number of persulfidation sites per protein (red) and the modification stoichiometry per protein (blue). Modification stoichiometry of a protein is represented as the percentage of all cysteines in target proteins being persulfidated, which is determined by dividing the number of persulfidated cysteines by the total Cys number. (E) Analysis of the site-specific target preference of persulfidation and S-sulfenylation for GAPDH (left) and CFL1 (right). BP, biological process; CFL1, cofilin-1; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; MF, molecular function. Color images are available online.

S-nitrosylation, challenging the notion of a shared specificity for persulfidation and S-nitrosylation. However, interestingly, we found that acidic amino acids (Glu/Asp) were underrepresented at the ± 1 and ± 2 positions of nonpersulfidated cysteines from the same proteins (Supplementary Fig. S9C). It has been dem-

onstrated that protonated Glu (E) is capable of reducing the pKa of an adjacent thiol *via* hydrogen bond formation (74). Hence, underrepresented Glu at adjacent positions of nonpersulfidated cysteines should increase their pKa in turn, thereby possibly prohibiting deprotonation and persulfide formation.

Mapping cysteine persulfidation induced by endogenous H₂S

Encouraged by the unprecedented ability of the low-pH QTRP method for in-depth proteomic mapping of cysteine persulfidation, we then extended our method to identify protein persulfides induced by endogenous H₂S. To this end, we first expressed Flag-tagged CSE in HEK293 cells (Supplementary Fig. S10A) supplemented with 5 mM L-cysteine and then applied low-pH QTRP to profile protein persulfides in these cells. As a result, we identified a total of 26 persulfidated cysteine sites on 24 proteins (Supplementary Table S4). Notably, one-fourth of the identified proteins bearing endogenous persulfidation sites are involved in the glycolytic pathway (Supplementary Fig. S10B and Supplementary Table S4), including fructose-bisphosphate aldolase A, enolase, GAPDH, LDHA, phosphoglycerate kinase 1, and PKM. Indeed, H₂S has emerged as an important regulator of cellular metabolism, especially glycolysis (29, 68). Our identification of endogenous persulfidation sites on glycolytic enzymes provides further support for this notion.

Furthermore, we applied low-pH QTRP to three major organs of mice, liver, brain, and heart, which have been widely used for the investigation of physiological and pathophysiological H₂S signaling (44, 55). Specifically, mouse tissues were harvested from wild-type C57BL mice, frozen in liquid nitrogen immediately, mechanically homogenized, and lysed into protein mixtures at low pH. The protein lysates were then processed and analyzed in three independent low-pH QTRP experiments. In total, we mapped a total of 131 endogenous persulfidated cysteine residues on 92 proteins in mouse liver, brain, and heart (Supplementary Fig. S11A and Supplementary Table S5). By comparing these endogenous sites with persulfidated sites identified in NaHS-treated human cell lysates, we found that 75 persulfidated cysteine sites exactly match to the mouse–human orthologs, indicating that these sites are evolutionarily conserved (Supplementary Table S5). It is not unexpected that there are more persulfidated sites/proteins identified in the heart (91/59) compared with the liver (29/24) and brain (28/23), as the steady concentration of free and acid-labile H₂S in the heart is believed to be remarkably higher than that in the latter (39). Moreover, only one persulfidated site, C154 of malate dehydrogenase, could be found in all three data sets (Supplementary Fig. S11B), probably due to the dramatic difference in protein expression between these organs (26). Accordingly, the functional impact of persulfidation on the hepatic, brain, and cardiac proteomes may also be different. In fact, bioinformatic analysis revealed the highest enrichment of persulfidated hepatic and brain proteins involved in oxidation–reduction process and glycolysis, respectively, whereas persulfidated cardiac proteins were mostly enriched in tricarboxylic acid (TCA) cycle (Supplementary Fig. S11C). Our analysis therefore provides further support for several previous findings. For example, the roles of H₂S in the regulation of redox status have been reported in hepatocytes (36, 44). Moreover, it has been previously shown that H₂S can increase the efficiency of the TCA cycle in hypertrophic cardiomyocytes by activating several key enzymes in this process, although the detailed molecular mechanism remains largely unknown (41).

Gaining mechanistic and structural insights into persulfidation of human serum albumin

Given that mounting evidence points to important roles of H₂S in vascular physiology and pathophysiology, we sought to explore endogenous persulfidation events in human serum. The human serum samples collected from six healthy subjects were diluted with the low-pH buffer, pooled, processed, and analyzed by low-pH QTRP, which allowed us to map 24 endogenous persulfidated sites on 11 proteins. Interestingly, in addition to the single, freely accessible cysteine residue C58 of human serum albumin (mostly known as C34 in the signal sequence-free HSA), cysteine residues in the 10 of 17 HSA disulfide bonds could also be persulfidated (Fig. 4A, B). This may be explained by the mechanism proposed by a previous study (70), in which persulfidation is derived from the reaction between H₂S and disulfide bond through a nucleophilic displacement (Fig. 4C, reaction 1). It is important to note that these HSA persulfidation events may occur at a low percent site occupancy, as it has been well demonstrated that most HSA cysteines exist predominantly in disulfide forms *in vivo* (12, 14). However, it has been proposed that the persulfide can be reduced to free thiol by sulfide and yield the polysulfide precursor HSSH (Fig. 4C, reaction 2) (70). In that way, all the identified persulfidated sites in the disulfide bonds of HSA should also provide freely accessible thiols for the IPM-based labeling. In other words, there will presumably be multiple freely accessible thiols on HSA, in addition to that of C58. In fact, we did identify 13 IPM-tagged cysteinyl thiols of HSA (Supplementary Table S6). Moreover, the MS intensity of the IPM-tagged thiols and persulfides on the same disulfide-forming cysteine residues exhibited a good correlation (Pearson correlation = 0.927), further supporting our hypothesis (Fig. 4D).

We next asked whether these modified cysteines were structurally distinct from their unmodified partners in the disulfide bonds. Both modified and unmodified disulfide-forming cysteines of HSA mainly resided in the secondary structure of helix (Fig. 4E). However, the modified ones had remarkably higher relative residue solvent accessibility (RSA) values than those calculated from their unmodified counterparts (Fig. 4F). Furthermore, such a difference was found to be statistically significant (Fig. 4G). These findings suggest that persulfidation is favored on protein surfaces, at least for HSA.

Discussion

In recent years, chemical probes and MS-based proteomics have made it possible to globally and site-specifically map distinct types of cysteine modifications (1, 63, 79–81). In contrast, cysteine persulfidation has mainly been indirectly profiled through proteomics at the protein level (3, 4, 17, 31, 47), whereas peptide-centric approaches have started to emerge only recently (25, 43, 56). Furthermore, current methodologies based on the modified BST or the tag-switch strategy rely on the selectivity and potency of reducing or alkylating agents. However, it has proven difficult to achieve a 100% reduction and/or alkylation. In support of this notion, re-analysis of a human proteome data set acquired from >2000 LC-MS/MS runs revealed that ~10–20% of free cysteines in all the identified peptides were not modified by the commonly used alkylating reagent IAA (18). To overcome the limitations of indirect approaches, here, we present

FIG. 4. Persulfidation of human serum albumin. (A) A schematic view showing the single, freely accessible cysteine and 17 disulfide bonds of HSA. Persulfidated sites in the disulfide bonds and their unmodified counterparts are shown in red and blue, respectively. M: modified; U: unmodified. (B) The identified persulfidation sites are mapped onto the crystal structure of HSA (PDB #: 4EMX) and visualized with Discovery Studio 2.5. (C) A plausible mechanism of persulfide formation in the presence of H_2S . (D) Correlation analysis of MS1 intensity of IPM-labeled thiols and persulfides in the disulfide bonds of HSA. (E) Stack column plot shows that both persulfidated and unmodified HSA cysteines are mainly localized at helix. (F) Relative RSA of persulfidated cysteines in the disulfide bonds of HSA in comparison with their unmodified counterparts. (G) Box plot showing a statistically significant difference of relative RSA between persulfidated and unmodified cysteines in the disulfide bonds of HSA. Significance is calculated by two sample t test, $n=10$. HAS, human serum albumin; RSA, residue solvent accessibility. Color images are available online.

a modified QTRP method based on low-pH electrophile labeling, facilitating the unbiased and site-specific mapping of cysteine persulfidation in complex proteomes. The initial benchmarking analysis demonstrates that the low-pH QTRP method can provide a remarkably higher number of persulfide identifications than that generated from the regular QTRP workflow. Interestingly, two similar low-pH-based chemoproteomic approaches were also applied to enhance identification of selenocysteine sites, which also occur at low stoichiometry like persulfides (6, 27).

We first utilized the low-pH QTRP method to globally and simultaneously profile reactivity of proteomic thiols and persulfides. This analysis provided the first experimental evidence for the widely recognized premise that protein cysteines equipped with a persulfide group appear to exhibit increased chemical reactivity when compared with base cysteines with sulfur in the thiol state. Nonetheless, the extent of changes in reactivity between persulfides and the corresponding thiols varied considerably in a site-dependent manner, and a few persulfides became even less reactive than the corresponding thiols. Accordingly, persulfidation-induced diverse changes in chemical reactivity may reflect the complexity of its biological activities. Although persulfidation is often suggested to activate protein function (54), the inhibitory roles of this modification have also been observed in many functionally important proteins, such as actin (40), androgen receptor (83), aquaporin-8 (9), Ca^{2+} /calmodulin-dependent protein kinase I (65), GAPDH (33), human antigen (10), krueppel-like factor 5 (46), SHC-transforming protein 1 (76), PKM (43), tyrosine-protein phosphatase non-receptor type 1 (37), protein phosphatase-1 (78), and proline-rich tyrosine kinase 2 (11). In addition, reactivity profiling generated another interesting insight into persulfide activity. We observed that up to half of the measured persulfides showed at least twofold loss of reactivity at decreased pH (5.0 vs. 7.6), suggesting that some persulfides are likely to be stabilized and deactivated by relatively low pH of the protein microenvironment. Hence, the local or global protein conformational changes may have a considerable impact on the activity of these persulfides.

To achieve a proteome-wide depth greater than that provided by indirect approaches, we applied the low-pH QTRP method to NaHS-treated lysates from five commonly used human cell lines, resulting in the unequivocal identifications of 1547 persulfidated cysteine residues on 994 proteins. This data set not only encompasses a subset of known persulfidation sites (Supplementary Fig. S6) but also confirms many H_2S -regulated protein targets, on which specific modification sites remain largely unknown, such as actin, cytoplasmic 1 (47), mitogen-activated protein kinase 3 (15), P53 (8), superoxide dismutase (59), tubulin beta (47), and UDP-glucose 6-dehydrogenase (47). Interestingly, we also noted that persulfidation occurs at the active or regulatory cysteines of many proteins involved in RSS metabolism and depersulfidation process, such as Cat (catalase) (52), glutathione reductase (21, 51), PARK7 (5), TXN (35), and TXNDC17 (21), implying the existence of feedback loops in these cellular processes. It is important to stress that the persulfidation formed in the presence of a high amount of exogenous NaHS may not be physiologically relevant. Nevertheless, our analysis provides a wealth of site information for predicting and interrogating functionally important protein persulfides and their physiological mechanisms. Moreover, bioinformatics

analysis reveals many potential biological processes and pathways for persulfidation, providing more interesting leads for the future functional studies. For example, among persulfidated proteins bioinformatically enriched in protein-folding process (Fig. 3C), 60kDa heat shock protein, mitochondrial (HSPD1) (also known as HSP60) contains a hotspot (C442) of persulfidation across five tested cell lines, suggesting a ubiquitous role of this modification in regulation of HSPD1-mediated protein folding, since this site is crucial for HSPD1 chaperone activity (48). In another example, bioinformatics analysis suggests that protein ubiquitination and sumoylation may also be influenced by persulfidation (Fig. 3C). Of note, persulfidation occurs at the active sites of several key enzymes involved in these processes, such as UBA1 C632, UBE2L5 C86, and UCHL1 C90, which can possibly lead to direct modulation of their enzymatic activity. In addition, our site-level data sets of persulfidation were compared with other cysteine modifications recorded either in previous reports or in a web portal database. Although persulfidation shares many co-targets with sulfenylation or *S*-nitrosylation, it is associated with unique consensus motifs that may define this distinct type of cysteine modification.

Furthermore, we illustrate the utility of our method by identifying endogenous protein persulfides in cells expressing CSE, mouse tissues, and human serum. Although the depth of mapped endogenous persulfidation sites is significantly lower than that achieved in NaHS-treated cell lysates, we gain additional insights into mechanistic and functional features of persulfidation events *in vivo*, particularly on HSA. The presence of endogenous persulfidated HSA in human blood samples is a controversy on debating (21, 62, 75). Taking advantage of our method, the analysis of human serum samples provides a confirmatory evidence showing that endogenous persulfidation occurs not only at the single, freely accessible C58 but also at multiple cysteine residues in the disulfide bonds of HSA, although the stoichiometry of these modifications may be low. Considering that persulfidation is an equilibrium process (Fig. 4C) (70), these HSA persulfides can be further reduced to free thiols by endogenous H₂S, thereby contributing to the maintenance of redox balance in the vascular fluid to some extent. This hypothesis can be further supported by a recent report, in which *S*-thiolations (*S*-cysteinylation and *S*-homocysteinylation) are observed at multiple cysteine residues of endogenous HSA (49).

It is important to note that there are some potential shortcomings of the low-pH QTRP method. First, it can only be used for proteomic applications, although the low-pH labeling strategy in combination with MS can be adopted for orthogonally validating persulfide formation on individual proteins discovered by traditional indirect approaches. Second, our analysis failed to identify IPM-labeled protein polysulfides ($-S_nH$, $n \geq 3$), likely because of the low stoichiometry of these species or the potential loss caused by sample preparation (*e.g.*, IAA-mediated cleavage of polysulfide chains) (13). Last but not the least, because this method relies on negative selection of highly abundant probe-modified thioether peptides for enrichment, it remains difficult to achieve an ideal efficiency for analyzing endogenous persulfidation. Hence, advances of positive selection approaches based on persulfide-specific reagents are eagerly awaited.

In summary, the low-pH QTRP method, as an efficient alternative to conventional indirect approaches, provides a valuable tool for direct and unbiased proteomic mapping of

cysteine persulfidation. In general, this low-pH setting can be easily adopted in other electrophile-based labeling approaches, such as isotope-coded affinity tags (29), cysteine-based tandem mass tags (65), and stable isotope cysteine labelling with iodoacetamide (68), for the same type of proteomic analysis of persulfidation. More importantly, this analytical platform can also be used to screen versatile chemicals and to identify persulfide-specific warheads for the future development of positive selection approaches.

Materials and Methods

Chemicals

IPM, light- and heavy-labeled UV-cleavable azido-biotin reagents were purchased from KeraFast (Boston, MA). MSBT and CN-biotin used for tag-switch assay were synthesized as described previously (82) and kindly provided by Prof. Ming Xian. DMEM medium, fetal bovine serum (FBS), penicillin–streptomycin, high performance liquid chromatography-grade water, formic acid, acetonitrile, and methanol were purchased from Thermo Fisher Scientific (Waltham, MA). Other chemicals were obtained from Sigma–Aldrich (St. Louis, MO), unless otherwise indicated.

Antibodies

Anti-PRDX1 (15816-1-AP, diluted at 1:1000; Proteintech), anti-PRDX2 (10545-2-AP, diluted at 1:1000; Proteintech), anti-PRDX3 (10664-1-AP, diluted at 1:2000; Proteintech), anti-PRDX4 (10703-1-AP, diluted at 1:1000; Proteintech), anti-CSE (A6121, diluted at 1:1000; ABclonal), anti-flag (20543-1-AP, diluted at 1:500; Proteintech), anti- β -actin (PR-0255, diluted at 1:1000; ZSGB-BIO); goat anti-mouse HRP (ZDR5307, diluted at 1:2500; ZSGB-BIO); goat anti-rabbit HRP (ZDR5306, diluted at 1:2500; ZSGB-BIO); anti-Peroxiredoxin-SO_{2/3} (ab16830, diluted at 1:2000; Abcam).

Cell culture

Human A549, A431, HEK293, MDA-MB-231, and U2OS cells (American Type Culture Collection, ATCC, Washington, DC) were maintained at 37°C in 5% CO₂, humidified atmosphere, and were cultured in DMEM medium (Cat. no. C11995500BT) supplemented with 10% (vol/vol) FBS (Cat. no. 10099141) and 1% (vol/vol) penicillin–streptomycin (Cat. no. 15070063).

Expression of Flag-tagged CSE

The construct of CSE expression plasmid (pcDNA3.1-3xFlag-*cse*) and its control (pcDNA3.1-3xFlag) was purchased from YouBao (F101694). Transfection was performed in 80% confluent HEK293 cells (10 cm dish) by addition of 25 μ g of plasmid and 225 μ L of polyethylenimine. After 6 h of transfection, cells were cultured in regular DMEM medium with 10% FBS for another 48 h. After serum deprivation overnight, cells were incubated with 5 mM *L*-cysteine for 1 h and then harvested for the low-pH QTRP analysis.

Animals

Low-pH QTRP analyses were performed on the livers, brains, and hearts removed from 4- to 6-week-old wild-type

male C57BL/6J mice. Animals were housed and maintained on a 12-h light–dark cycle at a 23°C, with free access to food and water. All animal experiments were handled in accordance with protocols approved by the Animal Care and Use Committees of the National Center for Protein Sciences, Beijing.

Probe labeling of NaHS-treated cell lysates

Cells were grown until 80–90% confluency, rinsed with cold PBS quickly, and scratched from plates. Cells were pelleted by centrifugation at 100 *g* for 3 min at 4°C and lysed by sonication on ice in either low pH buffer (50 mM sodium acetate [NaOAc], 1% Igepal, pH 5.0) or NETN buffer (50 mM HEPES pH 7.6, 150 mM NaCl, and 1% Igepal), both of which supplemented with protease-inhibitor cocktail and catalase (200 U/mL). The resulting cell lysates were treated with 0.5 mM NaHS (Cat. no. 161527) for 1 h at 37°C and then labeled with the IPM probe (Cat. no. EVU111) at room temperature (RT) (25°C) for additional 1 h with light protection and rotation.

Probe labeling of cell lysates from HEK293 expressing Flag-tagged CSE

HEK293 cells expressing Flag-tagged CSE were grown until 80–90% confluency, rinsed with cold PBS quickly, incubated with the low pH buffer (50 mM NaOAc, 1% Igepal, pH 5.0) containing 100 μ M IPM supplemented with protease-inhibitor cocktail and catalase (200 U/mL). After scratch from plates, cells were transferred into microcentrifuge tubes and further lysed by sonication on ice. The probe labeling reaction was performed for additional 1 h at RT with light protection and rotation.

Probe labeling of mouse tissue lysates

Freshly collected mouse tissues without NaHS stimulation were rinsed briefly in cold PBS, frozen in liquid nitrogen immediately, and then mechanically lysed by homogenization and sonication in the low pH buffer (50 mM NaOAc, 1% Igepal, pH 5.0) supplemented with 100 μ M IPM, protease-inhibitor cocktail, and 200 U/mL catalase. The resulting lysates were further incubated at RT for 1 h with rotation and light protection.

Probe labeling of human serum samples

Human serum samples from six healthy subjects were obtained from the Shengjing Hospital of China Medical University (Shenyang, China), with the approval of the Research Ethics Committee at this hospital. A total of 15 μ L serum without NaHS stimulation was diluted in 500 μ L (50 mM NaOAc, 1% Igepal, pH 5.0) supplemented with 100 μ M IPM, protease-inhibitor cocktail, and 200 U/mL catalase and incubated at RT for 1 h with rotation and light protection.

Preparation of probe-labeled proteomes

Probe-labeled proteome samples (at pH 5.0) were alkylated by adding the stock solution of IAM (Cat. no. I1149) at a final concentration of 40 mM. After 30 min incubation at RT with light protection, the resulting samples were then pre-

cipitated with methanol–chloroform system, as previously described (80). Protein pellets were resuspended in 50 mM ammonium bicarbonate (pH 8.5) (Cat. no. A6141) by sonication. Protein concentrations in the resuspended solution were measured with BCA assay (23225; Life Technologies) and adjusted to 2 mg/mL. For a typical low-pH or regular QTRP experiment, 1 mL of protein solution was used and digested with sequencing grade trypsin (V5113; Promega, Madison, WI) at a 1:50 (enzyme/substrate) ratio overnight at 37°C. The tryptic digests were desalted with HLB extraction cartridges (Waters; Cat. no. 186000383), as previously described (80), and evaporated to dryness under vacuum.

Click chemistry, capture, and enrichment

Desalted tryptic digests were reconstituted in a solution of 30% acetonitrile at pH ~6 and divided into two equal aliquots. Click chemistry was performed by the addition of 1 mM light or heavy UV-cleavable azido-biotin, 10 mM sodium ascorbate (Cat. no. A7631), 1 mM TBTA (Cat. no. 678937), and 10 mM CuSO₄ (Cat. no. 209198). Samples were allowed to react with rotation in the dark at RT for 2 h. Light (EVU102) and heavy (EVU151) isotopic tagged samples were mixed in a ratio of 1:1 after click chemistry. Samples were then purified by strong cation-exchange chromatography (Cat. no. SMM HIL-SCX; The Nest Group) as described previously (80) and enriched with prewashed Streptavidin-Sepharose beads (Cat. no. 17-5113-01; GE) at RT for 2 h. After capture, the Streptavidin-Sepharose beads were sequentially washed with 50 mM NaOAc, pH 4.5, and 50 mM NaOAc, pH 4.5, containing 2 M NaCl, then double-distilled H₂O (twice each) with rotation to remove nonspecifically bound peptides, and reconstituted in 25 mM ammonium bicarbonate. The streptavidin-sepharose suspension was transferred to several glass tubes (Cat. no.00265709; Thermo Scientific) and irradiated with 365 nm UV light (UVP, UVLMS-38 EL series) at RT with stirring for 2 h. The supernatant was collected, evaporated to dryness under vacuum, and stored at –20°C until LC-MS/MS analysis.

LC-MS/MS analysis

MS was performed on a Q Exactive plus (Thermo Scientific) instrument operated with an Easy-nLC1200 system (Thermo Scientific). All samples were reconstituted in 0.1% (vol/vol) formic acid and pressure-loaded onto a 2-cm microcapillary precolumn packed with C18 (3 μ m, 120 Å; SunChrom). The precolumn was connected to a microcapillary analytical column (12 cm, 150- μ m inner diameter) packed with C18 (1.9 μ m, 120 Å; Dr. Maisch) and equipped with a homemade electrospray emitter tip. The spray voltage was set to 2.0 kV, and the heated capillary temperature was set to 320°C. The LC gradient consisted of 0 min, 7% B; 14 min, 10% B; 51 min, 20% B; 68 min, 30% B; and 69–75 min, 95% B (where A was water with 0.1% [vol/vol] formic acid, and B was acetonitrile with 0.1% [vol/vol] formic acid) at a flow rate of 600 nL/min. HCD MS/MS spectra were recorded in data-dependent mode with a top-20 method. MS1 spectra were measured with a resolution of 70,000, an AGC target of 3×10^6 , a maximum injection time of 20 ms, and a mass range from *m/z* 300 to 1400. HCD MS/MS spectra were acquired with a resolution of 17,500, an AGC target of 1×10^6 , a maximum injection time of 60 ms, a 1.6 *m/z*

isolation window, and a normalized collision energy of 30. Peptide m/z that triggered MS/MS scans were dynamically excluded from further MS/MS scans for 18 s.

Peptide identification and quantification

Raw data files were searched against the *Homo sapiens* or *Mus musculus* universal protein resource canonical database with pFind 3.0 studio (18). The precursor-ion mass and fragmentation tolerance were set as 10 and 20 ppm, respectively, for the database search. A specific-tryptic search was used with a maximum of three missed cleavages allowed. A maximum of three modifications were allowed per peptide. Modifications of +15.9949 Da (methionine oxidation, M), +57.0214 Da (iodoacetamide alkylation, C), +284.0942 ($C_{11}H_{16}N_4O_3S$, C, for IPM-tagged persulfides), and +252.1222 ($C_{11}H_{16}N_4O_3$, C, for IPM-tagged thiols) were searched as variable modifications. No fixed modifications were searched. False discovery rates at the spectrum, peptide, and protein levels were <1%. Quantification of IPM-tagged unique peptides was performed with pQuant, as previously described (42), which directly uses the RAW files as the input. pQuant calculates $R_{H/L}$ values on the basis of each identified MS scan with a 15 ppm m/z tolerance window and assigns an interference score (confidence score σ) to each value from 0 to 1. In principle, the lower the calculated Int. Score, the less co-elution interference signal is observed in the extracted-ion chromatograms. In this regard, the median of peptide ratios with $\sigma \leq 0.5$ was considered to calculate site-level ratios.

Western blot

Protein samples were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes (IPVH00010; Merck Millipore), blocked with 5% milk in TBST at RT for 1 h, and probed with the indicated primary antibodies. After incubation at 4°C overnight, the membranes were washed three times with cold TBST and incubated with the HRP-conjugated specific secondary antibodies. The blots were processed with ECL chemiluminescence kit (CWbio, CW0049S) and visualized by chemiluminescence (Tanon 5200 scanner, Shanghai, China) using the GelCap software.

Bioinformatics

GO and Kyoto Encyclopedia of Genes and Genomes pathway enrichment ($p < 0.01$) analyses were performed by using the functional annotation tool DAVID followed by the protocol provided by developers (30).

Sequence and structural analysis

The consensus sequence motifs of persulfidated cysteines were visualized by pLogo (50), a linear sequence prediction algorithm based on their statistical significance ($p < 0.05$). The crystal structures of HSA (PDB #: 4EMX) were visualized by Discovery Studio (Version 2.5; Accelrys), and the relative RSA values for cysteine residues were calculated with a default probe radius of 1.4 Å.

Tag-switch assay

The assay was carried out as described previously (53). Briefly, A549 cell lysates treated with or without 0.5 mM NaHS were incubated with 10 mM MSBT in 1% SDS at 37°C for 30 min with occasional vortexing and followed by incubation of 2 mM CN-biotin at 37°C for another 1 h. Biotinylated proteins were then captured by streptavidin beads for 30 min at RT with rotation and then washed three times with NETN buffer. Beads were finally resuspended in loading buffer to release biotin-conjugated proteins for Western blot analysis.

Statistical analysis

Correlation analysis of MS1 intensity of IPM-labeled thiols and persulfides in the disulfide bonds of HSA was performed using Origin 8 (OriginLab Cooperation). Pearson correlation coefficient value is provided in Figure 4D. Significance of the difference in relative RSA of persulfidated and unmodified disulfide-forming cysteines of HSA was also calculated by Origin 8. Statistical values including the exact n are provided in Figure 4G.

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Author Disclosure Statement

No competing financial interests exist.

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Supplementary Material

Supplementary Table S1
Supplementary Table S2
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Abbreviations Used

BST = biotin-switch technique
Cat = catalase
CFL1 = cofilin-1
CSE = cystathionine γ -lyase
CV = coefficient of variation
GAPDH = glyceraldehyde-3-phosphate dehydrogenase
GO = Gene Ontology
H₂S = hydrogen sulfide
HCD = higher energy collision dissociation
HSA = human serum albumin
HSPD1 = 60kDa heat shock protein, mitochondrial
IAA = iodoacetamide
IPM = iodo-N-(prop-2-yn-1-yl) acetamide
LDHA = L-lactate dehydrogenase A
MMTS = methyl methanethiosulfonate
MS = mass spectrometry
MSBT = methylsulfonyl benzothiazole

NaHS = sodium hydrogen sulfide
NaOAc = sodium acetate
PARK7 = protein/nucleic acid deglycase DJ-1
PKM = pyruvate kinase
PRDX = peroxiredoxin
PSM = peptide-spectrum match
QTRP = quantitative thiol reactivity profiling
RSA = residue solvent accessibility
RSS = reactive sulfur species
RT = room temperature
TCA = tricarboxylic acid cycle
TUBB = tubulin beta
TXN = thioredoxin
TXNDC17 = thioredoxin domain-containing protein 17
UBA1 = ubiquitin-like modifier-activating enzyme 1
UBE2N = ubiquitin-conjugating enzyme E2N
UCHL1 = ubiquitin carboxyl-terminal hydrolase isozyme L1
XIC = extracted ion chromatograms