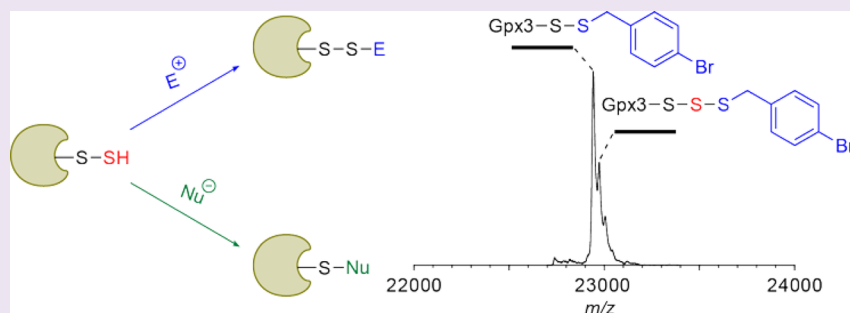


Persulfide Reactivity in the Detection of Protein S-Sulphydration

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Supporting Information



ABSTRACT: Hydrogen sulfide (H_2S) has emerged as a new member of the gaseous transmitter family of signaling molecules and appears to play a regulatory role in the cardiovascular and nervous systems. Recent studies suggest that protein cysteine S-sulphydration may function as a mechanism for transforming the H_2S signal into a biological response. However, selective detection of S-sulphydryl modifications is challenging since the persulfide group (RSSH) exhibits reactivity akin to other sulfur species, especially thiols. A modification of the biotin switch technique, using S-methyl methanethiosulfonate (MMTS) as an alkylating reagent, was recently used to identify a large number of proteins that may undergo S-sulphydration, but the underlying mechanism of chemical detection was not fully explored. To address this key issue, we have developed a protein persulfide model and analogue of MMTS, S-4-bromobenzyl methanethiosulfonate (BBMTS). Using these new reagents, we investigated the chemistry in the modified biotin switch method and examined the reactivity of protein persulfides toward different electrophile/nucleophile species. Together, our data affirm the nucleophilic properties of the persulfide sulfane sulfur and afford new insights into protein S-sulphydryl chemistry, which may be exploited in future detection strategies.

Oxidative post-translational modification (oxPTM) of protein cysteines plays an important regulatory role in many physiological systems.^{1–3} Of these, protein S-sulphydration has recently received attention in the context of hydrogen sulfide (H_2S) mediated signaling pathways^{4–7} involved in cardiovascular and cerebral vasodilatory responses.^{8,9} To date, the precise mechanisms of H_2S action are still the subject of active investigation. A significant challenge in protein S-sulphydration research is that the persulfide group (RSSH) exhibits reactivity akin to other sulfur species, in particular thiols,^{10–12} which compounds difficulties in developing selective detection methods.

In regards to detection, our interest was piqued when a commonly used alkylating agent, known as S-methyl methanethiosulfonate (MMTS),¹³ was reported to differentiate thiols and persulfides using a modified biotin switch technique (BST),⁴ originally designed to study protein S-nitrosylation.¹⁴ As shown in Figure 1a, in the modified BST thiols are first alkylated with MMTS, and persulfides were postulated to remain unreacted and be available for subsequent conjugation to N-[6-(biotinamido) hexyl]-3'-(2'-pyridyl)dithio) propionamide (biotin-HPDP). Using this modified version of the BST, a large number of proteins were reported as targets of H_2S in the mouse liver, and the basal sulphydration level of some proteins was estimated to be as high as 25%.⁴ Despite these intriguing

results, the structure of reaction products in each step of the modified BST and the underlying mechanism of thiol vs persulfide MMTS selectivity was not investigated.

Herein, we report several persulfide models and new MMTS analogue, S-4-bromobenzyl methanethiosulfonate (BBMTS). Using these reagents, we investigated the chemistry in the modified BST and examined the reactivity of protein persulfides toward different electrophile/nucleophile species. These data reaffirm the nucleophilic properties of the persulfide sulfane sulfur and afford new insights into protein S-sulphydryl chemistry, which may be exploited in future detection strategies.

Although selective (albeit slow) methods for H_2S detection have rapidly accumulated,^{15–18} methods for detecting protein persulfide modifications have been slower to advance. In this case, the challenges are 2-fold: (i) the chemical reactivity of the terminal sulfane sulfur in the protein persulfide is similar to the thiol and (ii) few examples of robust S-sulphydryl protein models have been reported. To this end, we initiated studies to explore the generation and reactivity of protein persulfides. Our second objective was to understand the chemical mechanisms

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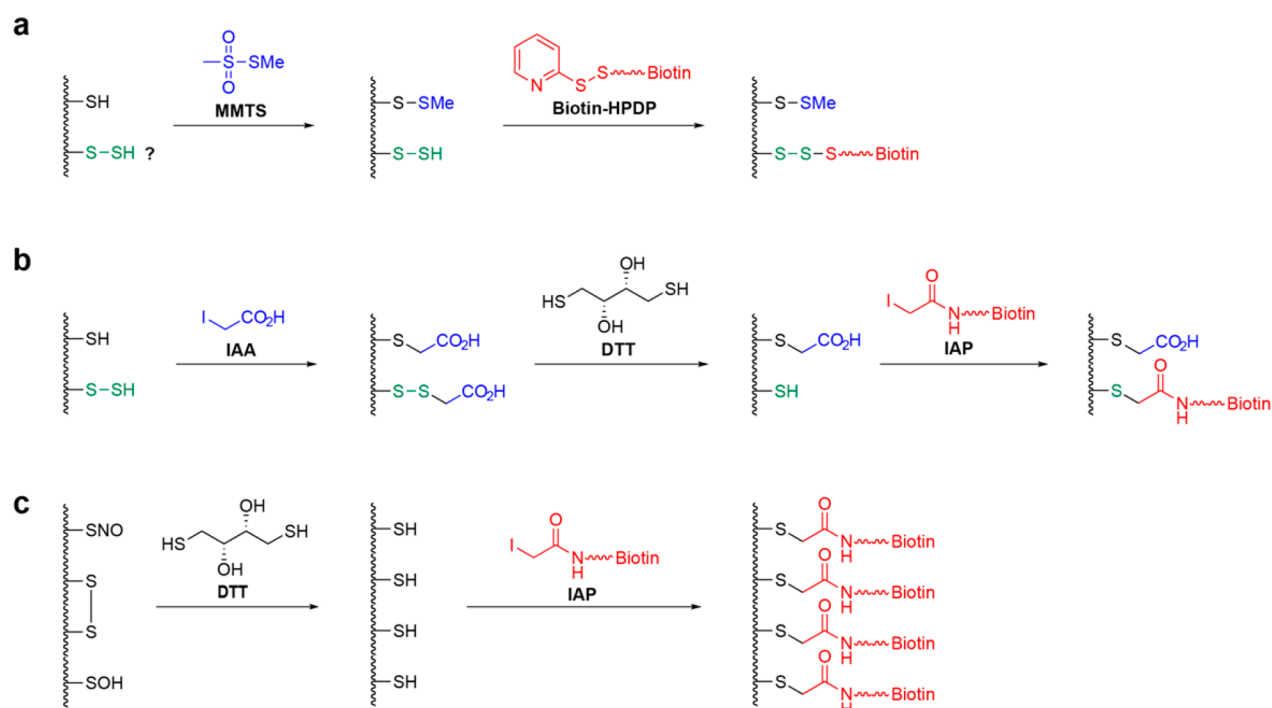


Figure 1. Reported methods for detecting protein persulfide modifications. (a) Modified biotin switch technique (BST): protein samples are alkylated by MMTS to block free thiols, while sparing persulfides. In the next step, biotin-HPDP is applied to alkylate persulfides and form protein-biotin conjugates. (b) Thiols and persulfides are reacted with iodoacetic acid (IAA). The persulfide is then reduced with dithiothreitol (DTT) and conjugated to biotinylated IAP. (c) Other cysteine oxPTMs that may be identified using the method in b.

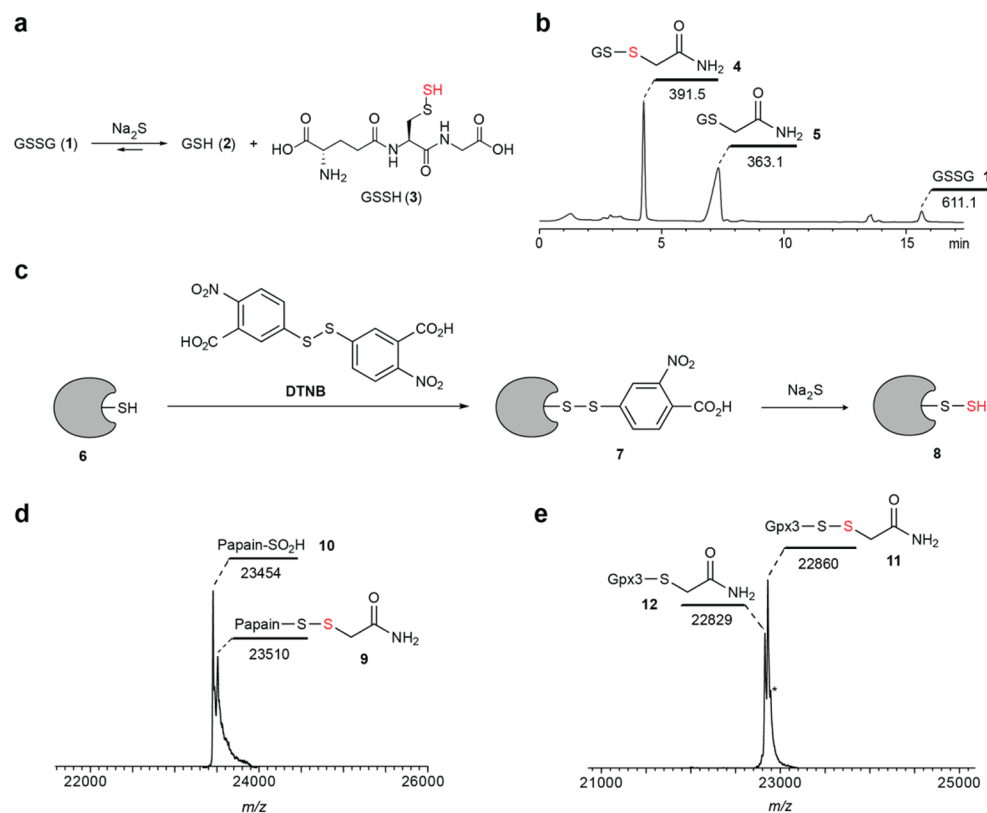


Figure 2. Preparation and verification of persulfide formation in GSH and protein models. (a) Formation of glutathione (GSH) persulfide. (b) HPLC UV trace at 254 nm and corresponding masses from the reaction in a. (c) Formation of protein persulfides. Mass analysis of the reaction between iodoacetamide (IAM), (d) papain persulfide, and (e) Gpx3 persulfide [*indicates the product derived from alkylation of Gpx3-S-S-SH (i.e., trisulfide)].

underlying current chemoproteomic methods of persulfide detection.

In an effort to identify suitable models, we first investigated the reported method¹⁹ for preparing persulfide derivatives of the tripeptide glutathione. As illustrated in Figure 2a, the GSH persulfide was obtained as an equilibrium mixture of glutathione thiol (GSH, 2) and persulfide (GSSH, 3) after the reaction of glutathione disulfide (GSSG, 1) with Na₂S. Though GSH persulfide 3 could not be directly observed by liquid chromatography–mass spectrometry (LC–MS), its formation was confirmed through conjugation with alkylating agents such as iodoacetamide (IAM) and *N*-ethylmaleimide (NEM), which yielded detectable products in LC–MS analyses (Figure 2b and Supplementary Figure S1). The inability to directly observe the persulfide species likely stems from the instability of the underivatized modification in acidic conditions and/or poor ionization properties.

In parallel investigations, single protein persulfide modifications were generated *via* a stepwise procedure in the cysteine protease, papain, and the C64,82S thiol peroxidase, Gpx3. In this method, reduced thiol 6 was first reacted with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB or Ellman's reagent) to form activated disulfide 7, which was then cleaved by Na₂S to generate persulfide 8 (Figure 2c). After conditions such as the equivalents of DTNB and Na₂S were optimized, papain and Gpx3 persulfides were successfully prepared and confirmed by intact MS analysis post-trapping with IAM or NEM (Figure 2d). In addition to the desired papain persulfide derivative 9, we observed signals consistent with papain sulfenic acid 10 (as-isolated from the original commercial sample) as well as persulfide oxidation. Of note, these species were also observed in the reaction with NEM (Supplementary Figure S2), indicating these mass signals were not unique to a particular alkylating reagent. Interestingly, in the case of Gpx3, the alkylation reaction revealed the presence of both the persulfide (major derivative) and the trisulfide (minor derivative; Figure 2e and Supplementary Figure S3).

Next, we tested the reaction of the persulfide models with MMTS. As shown in Figure 3a, the reaction of glutathione persulfide with MMTS gave a small amount of GSSH derivative 14, with major product 15 derived from GSH, and other byproducts GSSG (1) and GSSSG (13). The desired papain persulfide 16 and Gpx3 persulfide 18 products were clearly observed as the major species, albeit with some overlap between protein sulfonates and thiosulfates (i.e., papain 17 in Figure 3b; Gpx3 20 and 21 in Figure 3c). These analyses also highlight the small mass difference (~2 Da; Figure 3d) between byproducts from the oxidation of thiols and persulfides, relative to the MMTS-modified sulfur, which makes it difficult to distinguish between these chemically distinct species by intact protein MS analysis.

To address the above issue and clarify whether the ~80 Da mass increase corresponds to –SSMe or –SSO₃[−] forms of GSH, papain, and Gpx3, we designed and synthesized an analogue of MMTS, *S*-4-bromobenzyl methanethiosulfonate (BBMTS; Figure 4a). Compared to MMTS, the advantage of this reagent is 2-fold: (i) a larger mass change results from the thio-BBMTS adduct (+200 Da), and (ii) BBMTS exhibits the telltale bromine mass pattern (i.e., strong peaks at *M* − 1 and *M* + 1), which can be discerned in low-molecular-weight peptides, including GSH. Initially, we verified the reactivity of BBMTS, which proved comparable to MMTS (Supplementary Tables S1 and S2). We then tested the BBMTS reagent in

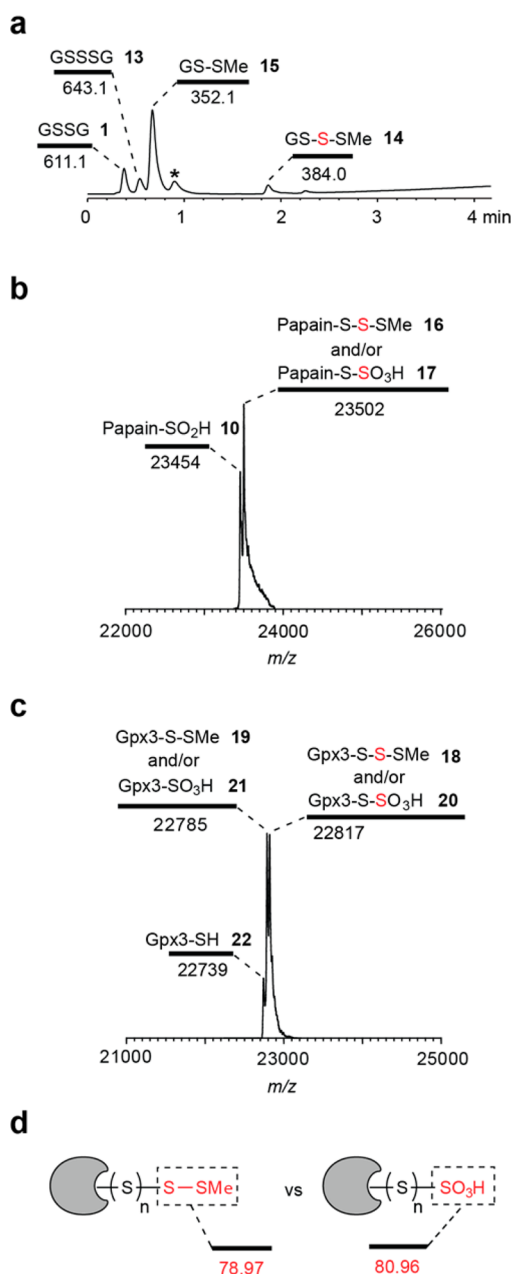


Figure 3. Reactivity of GSH and protein persulfide models with MMTS. (a) HPLC UV trace at 254 nm and corresponding masses from the reaction of GSH persulfide and MMTS (*the mass in this peak could not be assigned to a chemical structure). Mass analysis of the reaction between (b) papain persulfide and MMTS and (c) Gpx3 persulfide and MMTS. (d) Increase in molecular mass that results from MMTS modification (–SSMe) or oxidation to the thiosulfate (–SO₃[−]).

reactions with the GSH and protein persulfide models. As shown in Figure 4b, treatment of glutathione persulfide with BBMTS, gave a small amount of the desired persulfide derivative 25 and a large peak of the thiol derivative 26 (both confirmed using the bromine signature afforded by the new reagent), as well as the thiosulfate byproduct 27 (also detailed in Supplementary Figures S4–7 and Table S3). However, the reaction between protein persulfides and BBMTS clearly showed the formation of the desired persulfide derivatives, as well as alkyl polysulfides (Figures 4c,d). Taken together, these data unequivocally establish that the nucleo-

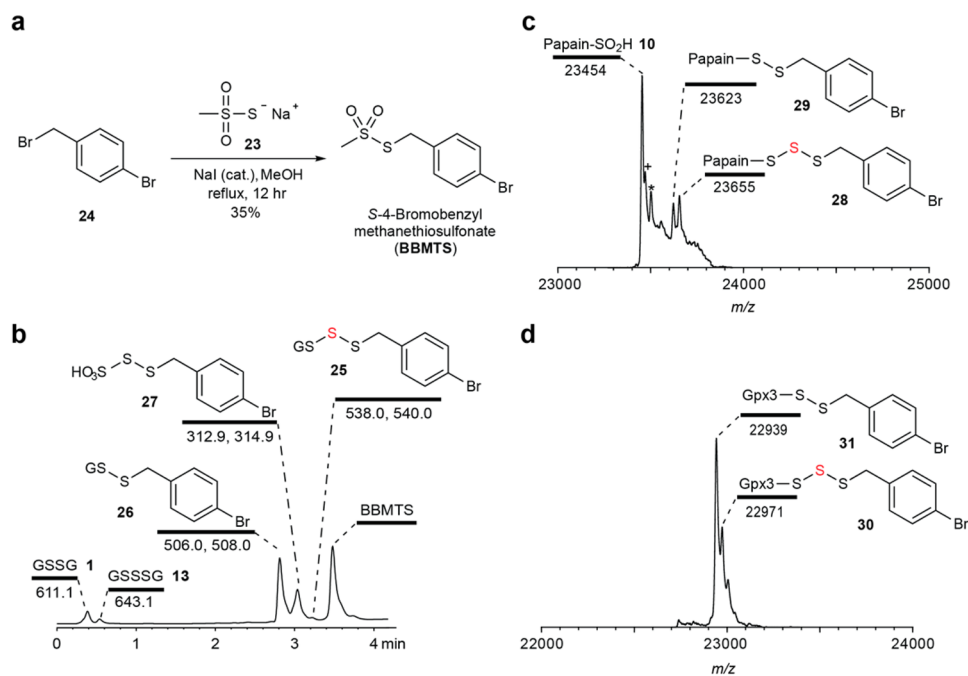


Figure 4. Reactivity of the GSH and protein persulfide models with BBMTS. (a) Synthesis of *S*-4-bromobenzyl methanethiosulfonate (BBMTS). Mass analyses of the reaction between (b) GSH persulfide and BBMTS, (c) papain persulfide and BBMTS [⁺papain-SO₃⁻ (sulfonic acid); *papain-S-SO₃⁻ (thiosulfate)], and (d) Gpx3 persulfide and BBMTS.

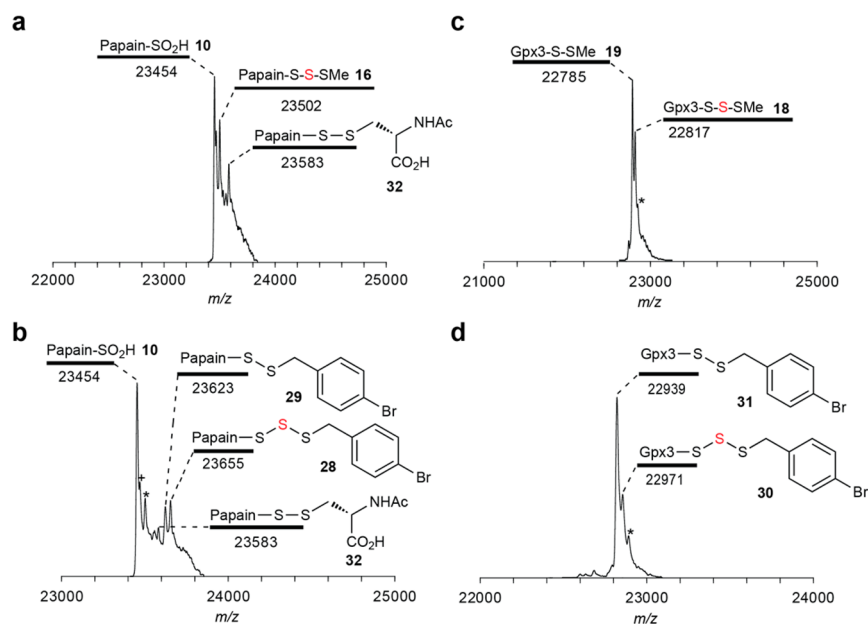


Figure 5. Sequential labeling reactions of protein persulfides with MMTS or BBMTS and biotin-HPDP analogue, *N*-acetylcysteine pyridyldisulfide (NACP). Mass analyses of the reaction between (a) papain persulfide, MMTS and then NACP [⁺papain-SO₃⁻ (sulfonic acid); *papain-S-SO₃⁻ (thiosulfate)], (c) Gpx3 persulfide, MMTS, and then NACP [*Gpx3-S-S-S-SMe (tetrasulfide)], and (d) Gpx3 persulfide, BBMTS, and then NACP as described in the Methods section [*Gpx3-S-S-S-SCH₂C₆H₄Br (tetrasulfide)].

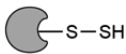
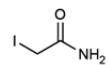
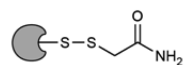
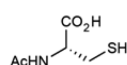

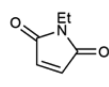
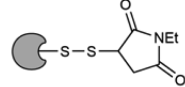
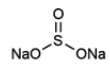

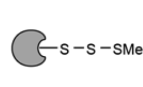

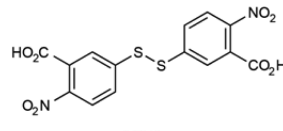
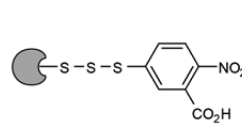
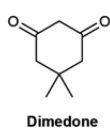
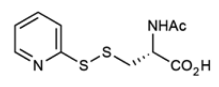
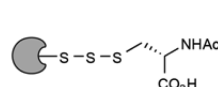

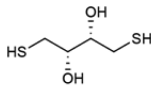

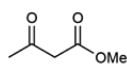
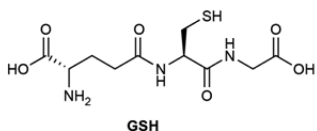

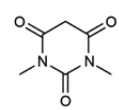
philic sulfane sulfur of protein persulfides reacts with electrophilic MMTS and BBMTS reagents.

Since the MMTS and BBMTS electrophiles react with protein persulfides, an important question arises: what does the biotin signal in the modified BST stem from? To address this issue, we treated papain and Gpx3 products of the MMTS/BBMTS alkylation step with the biotin-HPDP analogue, *N*-acetylcysteine pyridyldisulfide (NACP). Ensuing intact MS analysis of these reactions indicated the following: (i) no

product corresponding to a NACP-labeled persulfide was detected in papain or Gpx3, (ii) a small amount of NACP-labeled thiol was observed in papain (32; Figures 5a,b), and (iii) no NACP-labeled thiol was observed in Gpx3 (Figures 5c,d). These studies suggest that the protein-NACP labeling product is derived from thiol alkylation.

Finally, since little has been reported about the chemical reactivity of protein persulfides, we investigated the behavior of the Gpx3-SSH sulfane sulfur with various nucleophilic and

Table 1. Reactivity of Gpx3 Persulfide Towards Electrophilic and Nucleophilic Reagents

		 $\xrightarrow{\text{Reagent}}$ Product			
Entry	Reagent	Product	Entry	Reagent	Product
1	 IAM		8	 NAC	
2	 NEM		9	 Sulfite	No reaction
3	 MMTS		10	 Methylsulfinate	No reaction
4	 DTNB		11	 Dimedone	No reaction
5	 NACP		12	 Malononitrile	No reaction
6	 DTT		13	 Methyl acetoacetate	No reaction
7	 GSH		14	 N,N'-Dimethylbarbituric acid	No reaction

electrophilic reagents (Table 1 and Supplementary Figure S8). The nucleophilicity of the sulfane sulfur was reaffirmed in reactions with electrophilic alkylating reagents such as IAM, NEM, and MMTS (Entry 1–3), as well as disulfide-containing compounds, like DTNB and NACP (Entry 4–5). As an electrophile, the sulfane sulfur could be reduced to the free thiol form by DTT (Entry 6), GSH (note free thiol and mixed disulfide forms; Entry 7), and NAC (Entry 8). By contrast, weaker sulfur-based nucleophiles like sulfite or methyl sulfinate did not reduce the Gpx3 persulfide (Entry 9–10). We also tested the reaction of Gpx3 persulfide with carbon nucleophiles known to react with sulfenic acid (RSOH), but not disulfides or nitrosothiols (RSNO). These studies show that dimedone, malononitrile, methyl acetoacetate, and barbituric acid carbon nucleophiles exhibited no reactivity toward the Gpx3 persulfide (Entries 11–14). As before, thiosulfate formation was also noted in the persulfide samples, most likely resulting from oxidation by trace metal ions in the presence of oxygen.²⁰ The thiosulfate species was stable and also did not react with the

carbon nucleophiles (Supplementary Figures S9 and S13F–K and Table S4).

We have investigated GSH and protein persulfide models for chemical reactivity studies. Among these prototypes, GSH and papain persulfides have been previously described;¹⁹ however, the protein persulfides were not evaluated or confirmed by mass analysis. Using this early study as inspiration, in this work, we have examined the utility of a new persulfide protein model, based on a single-cysteine mutant of thiol peroxidase, Gpx3. This model proved quite facile for the generation of protein persulfides, and intact MS analysis was not complicated by the presence of a large fraction sulfenic acid-modified protein, in contrast to that observed for commercial sources of papain. With respect to the GSH persulfide model, we observed only a small amount of persulfide IAM and NEM alkylation products. The most likely explanation for these data is that the reaction of sterically unhindered persulfide derivatives **14** and **25** with low molecular weight thiols (i.e., GSH and GSSH) is rapid, relative to chemical alkylation. Thus, compared to protein persulfide

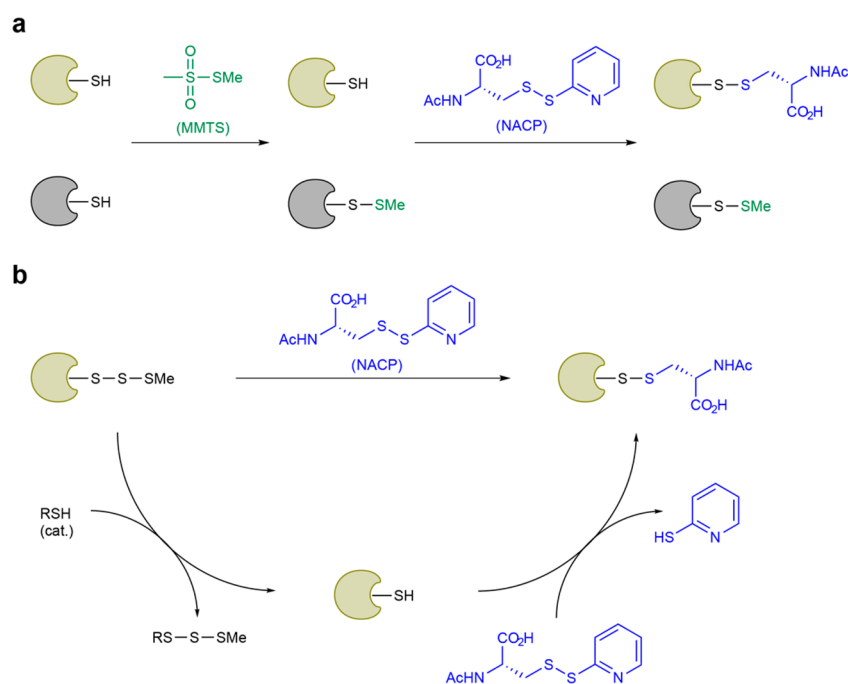


Figure 6. Proposed labeling mechanism of protein persulfide, methanthiosulfonate (MMTS or BBMTS), and pyridyldisulfide NACP. Two possible models are possible. (a) Free thiols may be incompletely blocked in the first MMTS alkylation step and subsequently react with the pyridyldisulfide biotin reagent. (b) Alternatively, or in addition, labeling may be achieved *via* stepwise thiol-disulfide exchange in a reaction catalyzed by trace free thiols (RSH).

models, the GSH persulfide may have less utility in reactivity studies.

In addition, we have examined the chemistry underlying the derivitization steps in the modified BST using the GSH and protein persulfide model systems. In contrast to earlier supposition,⁴ we demonstrate that the terminal persulfide sulfur undergoes facile reaction with both MMTS and BBMTS and that these alkylated forms do not react with the NACP pyridyldisulfide. These findings thus reaffirm the nucleophilic reactivity of the sulfane sulfur atom in protein persulfides and are consistent with physical organic studies^{10,11} and the IAM/NEM labeling observed in this work. In fact, the only NACP-labeled species that we observed in this study corresponded to the reaction product derived from thiol, as opposed to the persulfide. As shown in Figure 6, two possible models could account for this finding. In one scenario, free thiols may be incompletely blocked in the first MMTS alkylation step and subsequently react with the pyridyldisulfide biotin reagent (Figure 6a). Alternatively, or in addition, biotin labeling may be achieved *via* stepwise thiol-disulfide exchange in a reaction catalyzed by trace free thiols (Figure 6b). A common element in both models is that not all free thiols are blocked during the MMTS labeling step.

Recently, it was also reported that the persulfide-modified active site cysteine of protein tyrosine phosphatase 1B (PTP1B) can be alkylated by iodoacetic acid (IAA). Subsequent reduction of the persulfide adducts and labeling with iodoacetamide-linked biotin (IAP) has also been proposed as an indirect approach for detecting protein S-sulfhydration (Figure 1b).⁶ However, it is not apparent how this method distinguishes persulfide modifications from other DTT-reducible modifications, such as disulfide bonds, sulfenic acids, and nitrosothiols (Figure 1c). Nevertheless, IAA derivitization of the PTP1B persulfide provides further evidence that the persulfide is nucleophilic with similar reactivity to

thiols. Likewise, the γ -sulfur of persulfide is a weak electrophile that shares some reactivity with disulfides (also alluded to by sulfur transfer mechanisms in Fe–S biosynthetic pathways¹²) but is chemically distinct from the sulfur atom in sulfenic acid, which is significantly more electrophilic in nature. Collectively, these data reaffirm the nucleophilic properties of the persulfide sulfane sulfur and afford new insights into protein S-sulfhydryl chemistry, which can serve as a resource in developing future detection strategies.

METHODS

Sodium sulfide nonahydrate ($\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$), extra pure, was obtained from ACROS. DTNB was obtained from Sigma, and the purity was $\geq 98\%$. Papain 2 \times crystallized was purchased from Sigma. All other chemicals were purchased from Sigma-Aldrich or Fisher at the highest available purity.

Preparation of Persulfides. (1). *Glutathione Persulfide.*¹⁹ To a solution of freshly prepared glutathione (oxidized form, 1 mM final concentration) in 1 mL of Tris-HCl buffer (100 mM, pH 7.4) was added freshly prepared sodium sulfide (1 mM final concentration), and the solution was stirred at 37 °C for 15 min. The resulting glutathione persulfide (1 mM final concentration) was directly used without further purification.

(2). *Papain Persulfide.*¹⁹ A solution of papain (10 mg) in 1 mL of Tris-HCl (pH 7.4, 100 mM, degassed with N_2 for 10 min) was incubated with cysteine (1.7 mg) at rt for 10 min, and the protein was purified through a PD10 column (GE Healthcare) containing G-25 Sephadex. Protein-containing fractions were pooled from this column (500 μL , 270 μM) and incubated with DTNB (50 μL , 4 mM) at rt for 20 min, and purified by passing through a PD-10 column. A fraction of the pooled protein (200 μL , 120 μM) was then incubated in a solution of Na_2S (4 μL , 30 mM) at rt for 10 min and purified by PD-10 column to give the papain persulfide, which was immediately used in further reactions.

(3). *Gpx3 Persulfide.* C64,82S Gpx3 was prepared as previously described.^{21,22} A solution of C64,82S Gpx3 (100 μM , 100 μL) was buffer exchanged through a P-30 spin filtration column (BioRad) that

had been equilibrated in Tris-HCl buffer (pH 7.4, 100 mM, degassed with N₂ for 10 min). After determination of protein concentration (80 μM), the solution was incubated with DTNB (4 μL, 4 mM) at rt for 20 min and purified again by spin filtration. The resulting protein (100 μL, 62 μM) was then incubated with a Na₂S solution (1 μL, 30 mM) at rt for 10 min and purified by spin column to give the Gpx3 persulfide, which was immediately used in downstream reactions.

Verification of Persulfide Formation and Analysis of Chemical Reactivity. Freshly prepared GSH or protein persulfide (final concentration 1 mM for glutathione; 20 μM for protein) was incubated with IAM or other nucleophilic or electrophilic reagents (final concentration 4 mM for glutathione; 2 mM for protein) at rt for 1 h. After purification, aliquots of the samples were analyzed by electrospray LC-MS on a Poroshell 120 (Agilent) or ZIC-PHILIC HPLC column (the Nest group).

Reaction of GSSH with MMTS or BBMTS. To a solution of freshly prepared glutathione persulfide (1 mM final concentration) in 1 mL of Tris-HCl buffer (100 mM, pH 7.4) was added MMTS or BBMTS (4 mM final concentration), and the solution was stirred at rt for 30 min. Sample aliquots were generated at each step and analyzed by LC-MS.

Reaction of Protein Persulfides with MMTS or BBMTS Followed by NACP. A freshly prepared protein persulfide solution (20 μM final concentration) was incubated with the methanethiosulfonate (2 mM final concentration) at rt for 30 min and then purified through a P-30 spin column. The sample solution was then incubated with NACP (2 mM final concentration) at rt for 3 h and purified as before. Sample aliquots were generated at each step and analyzed by LC-MS.

■ ASSOCIATED CONTENT

● Supporting Information

Synthesis/preparation of the materials; LC-MS analysis; NMR spectra. 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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