Chemical Biology of Reactive Sulfur Species: Hydrolysis-Driven Equilibrium of Polysulfides as a Determinant of Physiological Functions

Tomohiro Sawa,1 Tsuyoshi Takata,2,1 Tetsuro Matsunaga,2 Hideshi Ihara,3,ii Hozumi Motohashi,4,iii and Takaaki Akaie2,iv

Abstract

Significance: Polysulfide species (i.e., R-Sn-R’, n > 2; and R-Sn-H, n > 1) exist in many organisms. The highly nucleophilic nature of hydropersulfides and hydropolysulfides contributes to the potent antioxidant activities of polysulfide species that protect organisms against oxidative and electrophilic stresses.

Recent Advances: Accumulating evidence suggests that organic polysulfides (R-Sn-R’) readily undergo alkaline hydrolysis, which results in formation of both nucleophilic hydrosulfide/polysulfide (R-Sn-H) and electrophilic sulfenic acid (R’SOH) species. Polysulfides maintain a steady-state equilibrium that is driven by hydrolysis even in aqueous physiological milieus. This unique property makes polysulfide chemistry and biology more complex than previously believed.

Critical Issues: The hydrolysis equilibrium of polysulfides shifts to the right when electrophiles are present. Strong electrophilic alkylating agents (e.g., monobromobimane) greatly enhance polysulfide hydrolysis, which leads to increased polysulfide degradation and artifactual formation of bis-S-bimane adducts in the absence of free hydrogen sulfide. The finding that hydroxyl group-containing substances such as tyrosine efficiently protected polysulfides from hydrolysis led to development of the new alkylating agent, N-iodoacetyl l-tyrosine methyl ester (TME-IAM). TME-IAM efficiently and specifically traps and stabilizes hydropolysulfides and protects polysulfide chains from hydrolysis, and, when used with mass spectrometry, TME-IAM allows speciation of the reactive sulfur metabolome. In addition, the polyethylene glycol-conjugated maleimide-labeling gel shift assay, which relies on unique hydrolysis equilibrium of polysulfides, will be a reliable technique for proteomics of polysulfide-containing proteins.

Future Directions: Using precise methodologies to achieve a better understanding of the occurrence and metabolism of polysulfide species is necessary to gain insights into the undefined biology of polysulfide species. Antioxid. Redox Signal. 36, 327–336.

Keywords: reactive sulfur species, persulfide, polysulfide, hydrolysis equilibrium, electrophile, nucleophile

1Department of Microbiology, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan.
2Department of Environmental Medicine and Molecular Toxicology, Tohoku University Graduate School of Medicine, Sendai, Japan.
3Department of Biological Sciences, Graduate School of Science, Osaka Prefecture University, Osaka, Japan.
4Department of Gene Expression Regulation, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan.

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Introduction

Many investigations have confirmed the presence of hydropersulfides (RSSH) and higher order polysulfide species \( (i.e., R-S_{2-n}R', n>2; \) and \( R-S_{n}H, n>1, \) with \( R = \text{cysteine, glutathione, or proteins} \) in various organisms, including bacteria and mammals \( (2, 14, 24, 26, 30, 34, 35, 37, 39, 40) \). Accumulating evidence has suggested that these reactive sulfur species are involved in regulation of diverse physiological and pathological phenomena \( (14, 39, 40) \). For instance, cysteine persulfide (CysSSH) can serve as an important intermediate in the biosynthesis of sulfur-containing molecules, such as iron–sulfur clusters, biotin, and lipoic acid, by donating its sulfane sulfur atom \( (23, 47) \). CysSSH is also reportedly involved in regulation of tRNA methylthiolation and insulin secretion \( (44) \). Reactive sulfur species also exist as glutathione-bound forms \( (e.g., \text{glutathione persulfide} [GSSH]) \) and protein-bound forms \( (2, 24) \). Because of their strong nucleophilicity and reducing ability, reactive sulfur species can act as powerful antioxidants and play important roles in cells by regulating oxidative stress and redox signaling \( (15, 16, 24) \). Recent studies also suggested that reactive sulfur species can have potent anti-inflammatory activity in cells and \textit{in vivo} \( (45, 48, 49) \). In bacteria, persulfides and polysulfides offer protection from external bactericidal insults caused by immune cell responses and antibiotics \( (29, 36) \). Thus, bacteria-specific persulfide-producing machinery may become an attractive target for development of antibacterial drugs \( (29, 36) \).

Cysteinyl-tRNA synthetase (CARS) has been demonstrated to act as an effective CysSSH-producing enzyme \( (\text{as CPERS}) \) \( (2) \). This CysSSH-producing ability is conserved from bacteria to mammalian cells \( (2) \). In mammalian cells, two different CARSs exist: cytosolic CARS1 and mitochondrial CARS2 \( (9, 18) \). Biochemical analyses with recombiant enzymes showed that both CARS1 and CARS2 possess potent CysSSH-producing activity \( (2) \). As an intriguing finding, CysSSH derived from CARS2 may play an important role in maintaining the mitochondrial membrane potential by acting as an electron acceptor in the electron transport chain \( (2, 14, 33) \). During this process, CysSSH is suggested to be metabolized to hydrogen sulfide \( (H_2S) \), sulfite, sulfate, and other oxidized sulfur species, with this process depending on the catalytic actions of sulfide:quinone oxidoreductase and ethylmalonic encephalopathy protein 1 \( (\text{ETHE1}; \text{also known as persulfide dioxygenase}) \) in mitochondria \( (2, 14) \). Sulfide metabolism is thereby tightly controlled by the activity of these different enzymes. A recent study demonstrated that impairment of sulfide metabolism and concomitant persulfide accumulation in mitochondria led to mitochondrial dysfunction associated with hypoxia-induced brain injury \( (32) \).

Chemical biology studies of hydopersulfides and polysulfides have revealed that hydopersulfides are superior to thiols as nucleophiles and reducing agents \( (15, 16) \). Hydopersulfides can be readily deprotonated so that the \( pK_a \) values of the corresponding thiols: the typical \( pK_a \) values of thiol species are \( 8–9 \), whereas \( pK_a \) values of hydopersulfide species are \( 4–7.5 \) \( (11, 42) \). A deprotonated persulfide anion is significantly more nucleophilic compared with the corresponding thiolate anion \( (24, 38, 42) \). Polysulfide species may also be more susceptible to alkaline hydrolysis of their sulfur–sulfur bonds compared with disulfide species \( (2, 8, 19, 28) \). This unique property makes polysulfide chemistry and biology more complex than previously believed. In this review article, we discuss the hydrolysis-driven equilibrium of polysulfide species, with particular emphasis on its modulation by electrophiles. Full understanding of the chemistry and chemical biology of the equilibrium of polysulfide hydrolysis is indispensable not only for development of analytical methods for polysulfide species but also for elucidation of the fundamental impacts and physiological roles of polysulfide species formed endogenously.

Alkaline Hydrolysis of Disulfides

Hydrolysis of disulfide bonds \( (\text{evident primarily under strong alkaline conditions}) \) was studied with cystine, oxidized glutathione \( (\text{GSSG}) \), and proteins, and findings showed that hydrolysis of disulfides \( (RSSR) \) produces reduced thiols \( (\text{RS}) \) and sulfenic acid \( (\text{RSOH}) \) species, as shown in Reaction (1) \( (5, 10, 12) \):

\[
\text{RSSR} + H_2O \rightleftharpoons \text{RSH} + \text{RSOH} \quad (1)
\]

At physiological or neutral \( pH \), equilibration in Reaction (1) shifted far to the left. Hydrolysis could be accelerated and driven to completion by including thiol-reactive reagents such as \( \text{HgCl}_2 \) and \( p\)-hydroxymercuribenzoate in the solution \( (5) \). For example, hydrolysis of the disulfide bond of GSSG occurs only when the \( pH \) is higher than 9 and the solution contains thiol-reactive reagents such as \( p\)-hydroxymercuribenzoate \( (5) \).

Hydrolysis-Driven Equilibrium of Sulfur–Sulfur Bonds in Polysulfides

Consistent with this disulfide reactivity, sulfur–sulfur bonds in symmetrical disulfides are reportedly stable in aqueous media with neutral \( pH \) even in the presence of strong electrophilic thiol-reactive reagents such as iodoacetamide \( (\text{IAM}) \), monobromobimane \( (\text{MBB}) \), and \( N\)-ethylmaleimide \( (\text{NEM}) \) \( (2, 8) \). According to careful and detailed studies with glutathione polysulfides \( (\text{GS}_n, n>2) \) \( (2) \) and alkylated polysulfides \( (\text{RS}_n\text{R}, n>2) \) \( (8) \), however, sulfur–sulfur bonds in polysulfides become susceptible to hydrolysis even under weak alkaline and physiological conditions, compared with such bonds in disulfides. The hydrolysis of polysulfides, which already occurs at physiological \( pH \), results in formation of reduced thiols or hydropolysulfides \( (\text{RS}_{n-1}\text{H}) \) and \( \text{RSOH} \) or polysulfenic acid \( (\text{RS}_{n-1}\text{OH}) \) \( (\text{Reaction} 2; \text{Figs. 1 and 2}) \) \( (19) \).

\[
\text{RS}_n\text{R} + H_2O \rightleftharpoons \text{RSH} + \text{RS}_{n-1}\text{OH} \text{ or } \text{RS}_{n-1}\text{H} + \text{RSOH} \quad (2)
\]

Electrophilic thiol-reactive reagents react with and capture thiols and hydropolysulfides, which drive hydrolysis (hydrolysis equilibrium shifts to the right). In fact, we observed that the hydrolysis of glutathione polysulfides was greatly enhanced by addition of strong electrophiles such as NEM, MBB, and \( 4\)-chloro-7-nitrobenzofurazan \( (\text{NBF-Cl}) \), with concomitant formation of glutathione–electrophile adducts.
FIG. 1. Hydrolysis equilibrium of polysulfides and its modulation by electrophiles and nucleophiles. Polysulfide species (RSₙR, n > 2) maintain hydrolysis equilibrium with concomitant formation of various derivatives with different redox status, such as thiols or sulfhydryls (RSH), hydropolysulfides (RSₙ₋₁H), and sulfenic acid (RSOH) or polysulfenic acid (RSₙ₋₂OH). Hydrolysis equilibrium shifts to the right in the presence of electrophiles (E) or nucleophiles (N), with concomitant product formation of a complex reaction profile. ESE, bis-sulfur–electrophile adduct; ESH, electrophile–sulfide adduct; HSₙ₋₁H, hydrogen polysulfides; RSE, electrophile–thiol adduct; RSₙ₋₁E, electrophile–polysulfide adduct; RSₙ₋₂OH, polysulfenic acids; R’Sₙ₊₁R, longer chain polysulfide; R’S’S, hydopersulfide. Color images are available online.

FIG. 2. Polysulfide hydrolysis occurring at physiological pH and accelerated by dimedone. (A) A schematic of the reaction of GSSSSG and dimedone taking place effectively upon hydrolysis of polysulfide of GSSSSG. (B) GSSSSG decay clearly observed at physiological pH 7.5 and prompted by dimedone. GSSSSG (10 μM) was reacted with or without various concentrations of dimedone under physiological and alkaline conditions (0.1 M sodium phosphate buffer, pH 7.5–8.0, at 37°C for 90 min), followed by sulfur analysis by LC-MS/MS. Data are mean ± SD (n = 3); *p < 0.05 and **p < 0.01; one-way ANOVA with Turkey’s test. (C) A representative LC-MS/MS profile and each fragmentation pattern of GSₙ-dimedone adducts generated from the reaction in (B). GSSSSG, glutathione tetrasulfide; LC-MS/MS, liquid chromatography–tandem mass spectrometry; SD, standard deviation. Color images are available online.
As with symmetrical polysulfides, hydrolysis of asymmetrical polysulfides \( (RS_nR', n > 2) \) was also noted (2, 8). \textit{Ab initio} calculations suggested that the RSOH-reactive reagent, dimedone, does not react directly with polysulfide species, but only with RSOH/RS\(_{n-1}\)OH (22). Consistent with this notion, formation of RSOH/RS\(_{n-1}\)OH was supported by the finding that addition of dimedone could also shift the hydrolysis equilibrium equation to the right (Fig. 2) (2, 8, 19).

RSOH can react with thiols to form disulfides (3, 4). In eukaryotic and prokaryotic cells, appreciable amounts of GSSH have been found. For instance, high micromolar levels of GSSH were detected in mouse tissues, including the brain, liver, and lung (2, 24). The reaction between RS\(_{n-1}\)OH and such hydopersulfides (R'S\(_{n+1}\)R) will result in formation of longer chain polysulfides (R'S\(_{n+1}\)R) (Fig. 1). Thus, polysulfide hydrolysis may play an important role in initiating sulfur chain elongation (sulfur catenation), as Figure 1 illustrates.

Electrophilic thiol-reactive reagents (E) can react with thiols to form an electrophile–thiol adduct (RSE) (Fig. 1). The RSE thus formed is stable, and no additional reaction with electrophiles and nucleophiles will occur. Electrophile–polysulfide adducts (RS\(_{n-1}\)E), however, particularly those with more than two sulfur atoms, are susceptible to alkaline hydrolysis, which leads to formation of secondary metabolites, as shown in Figure 1. Electrophile–sulfide adducts then react with electrophiles to form bis-sulfur–electrophile adducts (ESEs). ESEs also form in the reaction of electrophilic thiol-reactive reagents with H\(_2\)S. For instance, the strong electrophile, MBB, was used to detect H\(_2\)S by means of measuring the formation of the ESE of MBB (41, 46). However, as just mentioned, ESEs may form in the absence of free H\(_2\)S in reactions of polysulfides with electrophiles. Thus, analysis using strong electrophiles, such as MBB, NEM, and NBF-Cl, to speciate a complex mixture of reactive sulfur species is quite difficult (Figs. 1 and 2) (2, 8, 19, 28). While the harsh electrophiles are not suited to assess free sulfide levels, a value for total sulfide is reportedly obtained using a strong electrophilic probe such as NEM whenever persulfides and polysulfides are present, as pointed out by Sutton \textit{et al.} (43). Thiol-reactive reagents that are suitable for the precise analysis of reactive sulfur species are discussed in detail below.

**Polysulfide Bond Stabilization by Hydroxyl Group-Containing Compounds**

As described above, the hydrolysis equilibrium of polysulfides can be shifted to the right by the presence of electrophiles (Fig. 1). Hydrolysis-driving potentials varied...
among the electrophiles studied; NBF-Cl, MBB, and NEM were the strongest (hydrolysis accelerators; Fig. 3), whereas IAM derivatives that contain tyrosine-like residues, such as β-(4-hydroxyphenyl)ethyl (HPE)-IAM and N-iodoacetyl l-tyrosine methyl ester (TME)-IAM, were the weakest (Fig. 4) (2, 19, 28). As Figure 4 shows, the electrophilic moiety of HPE-IAM and TME-IAM is the same as that of IAM. However, of interest is that hydrolysis of polysulfides was significantly reduced in the presence of HPE-IAM or TME-IAM compared with that in the presence of IAM (19, 28). The same study also demonstrated that spontaneous hydrolysis of polysulfides in the absence of electrophiles was markedly suppressed by addition of tyrosine, a phenolic (i.e., hydroxyphenyl containing) amino acid (19). Other chemicals and endogenous compounds containing a hydroxyphenyl or hydroxyl group, such as 4-acetamidophenol, glycerol, sucrose, and serine, also possessed polysulfide-stabilizing activities, as reported earlier (19) and shown here in Figure 3. These observations suggest that a hydroxyphenyl or hydroxyl group antagonizes to prevent the hydrolysis of polysulfides so that the compounds containing hydroxyl groups may function as hydrolysis protectors. It is thus speculated that the phenolic moiety in HPE-IAM may protect against spontaneous hydrolysis of polysulfides as well as IAM-induced hydrolysis of polysulfides (19). We performed an extensive study of hydroxyl moiety-containing substances in terms of protective effects, which revealed that tyrosine possessed the strongest protective activity against polysulfide hydrolysis (19) (Fig. 3). Furthermore, tyrosine partially protected polysulfides against hydrolysis induced by alkylating reagents and dimedone (19). Tyrosine-dependent protection of polysulfides from hydrolysis was clearly observed in the presence of all electrophiles tested. We should emphasize here that the polysulfide stabilization mediated by hydroxyl group-containing compounds is a completely new mode of regulation of polysulfide stability, homeostasis, or metabolism; it may even be involved in physiological processes and may become a specific target in pharmacological modulation of reactive sulfur species. Additional studies of polysulfides and the metabolism of reactive sulfur species regulated by hydrolysis in vivo and in humans are therefore warranted.

Hydropolysulfide Alkylation in the Analysis of Reactive Sulfur Species

As discussed earlier, hydropolysulfides are reported more acidic than the corresponding thiols. For example, the \( pK_a \) value of GSSH has been determined to be 5.45, which is significantly lower than the \( pK_a \) value of glutathione—8.94 (7). Because of this acidity, hydropolysulfides exist in their deprotonated state (polythiol anions, RS\(_n^-\)) at the physiological pH range (7, 15, 17). In addition, RS\(_n^-\) thus formed can be more nucleophilic compared with the corresponding thiol anion because of an alpha effect (6). Such strong nucleophilicity and reducing ability of hydropolysulfides may contribute to their potent antioxidant and protective activities against cellular insults caused by oxidative and electrophilic stresses (1, 3, 21, 24, 25).

The nucleophilicity of hydropolysulfides (and also RS\(_n^-\)) is also an important issue to consider when one aims to quantitate, speciate, and visualize the presence of hydropolysulfides in biological samples. In many previous studies, thiol-reactive agents, including NBF-Cl, MBB, NEM, and IAM, have been used to alkylate terminal hydropersulfide and hydropolysulfide moieties to form alkylated polysulfides (RS\(_n\)E) to prevent oxidation of reactive thiols or labile RS\(_n^-\) during extraction or analysis (13, 24, 50). However, as alluded to earlier, those electrophilic alkylating agents (E) have promoted the alkaline hydrolysis of polysulfur chains, which resulted in degradation of both RS\(_n\)E polysulfide species (Figs. 1 and 3). In addition, those reagents can degrade RS\(_n\)R in biological samples (Fig. 3B) (2, 19, 28). We should also note that treatment with alkylating agents can result in formation of ESEs from degradation of polysulfides even in the absence of free H\(_2\)S (Fig. 1). Although the formation of ESEs has been used as an indicator of the presence of H\(_2\)S (41, 46), more careful attention should be paid to formation of ESEs via hydrolysis-coupled degradation of polysulfides with alkylating agents (2, 8, 19, 28).

As described above, our recent study clearly showed that incorporation of a phenol (hydroxyphenyl) moiety into an alkylating agent (e.g., HPE-IAM, Fig. 2) markedly reduced the hydrolysis-dependent degradation of polysulfides compared with the corresponding alkylating agent without the phenol moiety (e.g., IAM) (Figs. 4 and 5) (2, 8, 19). On the basis of this observation, recently we successfully developed a new IAM-based alkylating agent (Figs. 4 and 5) (28). We synthesized TME-IAM by using carbodiimide-mediated condensation of iodoacetic acid and TME (Fig. 4) (28).

During polysulfide hydrolysis promoted by an electrophile, once an electrophile reacts with one of the sulfur residues in a polysulfide chain, then that sulfur becomes a good leaving group (since it is positively charged) (Fig. 6). At this point, any nucleophile, such as water, could react with a sulfur atom adjacent to the sulfur bound to an electrophile (Fig. 6). This would cause cleavage of the S-S bond and
generate sulfenic acid and an electrophile–sulfur adduct (8). The S-S bond cleavage by water occurs in a polysulfide chain, although to a lesser extent in the absence of an electrophile. Therefore, the compounds that possess hydroxyl moieties may compete with the nucleophilic attack by water to reduce hydrolysis-dependent degradation of polysulfides regardless of the apparent involvement of exogenous electrophiles (Figs. 5 and 6).

Accordingly, TME-IAM can markedly attenuate the hydrolysis-driven degradation of polysulfides (28). For example, a loss of ~40% of glutathione tetrapsulfide (GSSSSG) was determined after incubation in 100 mM phosphate buffer (pH 7.4) at 37°C for 1 h, and addition of MBB increased degradation, with the total loss being ~70% (28). In contrast, in the presence of TME-IAM, GSSSSG degradation was almost completely suppressed (28). As an important result, TME-IAM retained its efficient alkylation of hydropersulfide and hydropolysulfide moieties through the IAM functional group. Sulphydryl alkylation by TME-IAM led to increased hydrophobicity of analytes and thereby sufficient retention during reverse-phase liquid chromatography (LC) separation. In fact, the use of TME-IAM in mass spectrometry-based sulfur metabolomics allowed clear detection of hydro polysulfides such as GSSH and CysSSSH in mouse tissues (e.g., liver and brain). When compared with data obtained using HPE-IAM, data obtained with the use of TME-IAM showed significantly smaller amounts of bis-S adducts (partly indicative of H2S), higher amounts of various persulfides and polysulfides as well as GSSG, and higher amounts of polysulfides such as GSSSSG (28). These data suggest that speciation of reactive sulfur species is largely influenced by the particular thiol-reactive alkylating agents used (2, 8, 19, 28). Extensive and careful sulfur metabolomic investigations with the use of alkylating agents such as TME-IAM therefore allow us to achieve a more precise understanding of the complex metabolome of reactive sulfur species in biological systems.

For rigorous and quantitative measurement of metabolites by means of liquid chromatography–tandem mass spectrometry (LC-MS/MS), isotope-labeled standards are spiked into the samples and are necessary to standardize the ionization efficiency of various endogenous metabolites (as in isotope dilution mass spectrometry) (20, 31). One advantage of utilizing TME-IAM as the alkylating agent is that we can readily synthesize isotope-labeled TME-IAM by using commercially available isotope-labeled tyrosine as the starting material (28).

**Hydrolysis Equilibrium in Protein Polysulfides and Its Application to the Sulfur Proteome**

The hydrolysis-driven polysulfide equilibrium discussed here can be applied for the sulfur proteome to probe into the exact presence and profile of polysulfide chains formed in many proteins. Hydropersulfide and hydropolysulfide residues in proteins (Prot-S)n are labeled with polyethylene glycol (PEG)-conjugated maleimide to increase the apparent molecular size of proteins (2, 8, 27). On electrophoresis, a mobility shift occurs that depends on the number of PEG labels introduced. Molecular size alterations that are induced

![FIG. 5. Dual functions of TME-IAM in precise polysulfur metabolomics. TME-IAM efficiently alkylates labile polythiolate anions (RSn−, n > 1) derived from deprotonation of hydropolysulfides (RSnH, n > 1) through the IAM moiety. TME-polysulfide adducts resist hydrolytic degradation because of TME-IAM-mediated protection against alkaline hydrolysis. TME-IAM also suppresses the hydrolysis of polysulfur species (RSnR, n′ > 2). RSn−, hydropolysulfides; RSn−OH, poly-sulfenic acids. Color images are available online.](image)

![FIG. 6. Polysulfide hydrolysis promoted by electrophiles (E), but suppressed by hydroxyl groups in various chemicals and metabolites. Formation of an electrophile–sulfur adduct may initiate the heterolytic cleavage of the S-S bond mediated via the nucleophilic attack by water, which is most likely competed with nucleophilic hydroxyl residues of various compounds and metabolites (e.g., tyrosine and serine), thereby leading to the reduction and prevention of polysulfide hydrolysis.](image)
by the PEG-maleimide reaction and electrophile treatment can be determined by gel electrophoresis; this protocol is called the PEG-conjugated maleimide-labeling gel shift assay (PMSA) (Fig. 3B) (2, 8, 27). Strong electrophiles such as NBF-Cl, MBB, NEM, and p-chloromercuribenzoic acid readily react with Prot-S⁻\(_n\)-H that forms on hydrolysis and thus cause a reduction in the apparent molecular sizes of proteins (Fig. 3B) (2, 8, 27). In fact, extensive hydrolysis and decomposition of protein polysulfidation by harsh electrophiles such as NBF-Cl were clearly observed, as evidenced by the PMSA for alcohol dehydrogenase 5 (ADH5) protein-bound polysulfides (Fig. 3B).

Our seminal work performed earlier established that protein persulfidation and polysulfidation occur not only via post-translational modifications but also via direct incorporation of precursor CySS\(_s\)-H produced by CARS into tRNA during translation as a part of the translation process (2). Thus, as expected, many types of proteins are endogenously persulfidated and polysulfidated (24). This was unequivocally supported by the finding that all proteins that were investigated, including glyceraldehyde-3-phosphate dehydrogenase, ETHE1, ADH5, and aldehyde dehydrogenase 1 family, member A1, were identified as endogenously polysulfidated by means of the PMSA (2, 8, 27).

**Concluding Remarks**

Polysulfide species (i.e., R-S\(_n\)-R', n > 2; and R-S\(_n\)-H, n > 1) occur in a wide variety of organisms as low-molecular-weight forms and as proteinaceous forms. The highly nucleophilic nature of hydrosulfide and hydropolysulfide species contributes to the potent antioxidant activities of polysulfide species that protect organs against oxidative and electrophilic stresses. Recent studies discovered that polysulfide species (R-S\(_n\)-R') readily undergo alkaline hydrolysis, which results in formation of both nucleophilic hydrosulfide and polysulfide species (R-S\(_n\)-H) and electrophilic R'SOH species. As a more important feature, polysulfides can maintain a steady-state equilibrium that is driven by hydrolysis even under physiological conditions at neutral and weak alkaline pH values in vivo. This unique property makes polysulfide chemistry and biology more complex than previously believed. This hydrolysis equilibrium of polysulfides shifts to the right in the presence of electrophiles. In fact, electrophilic alkylating agents that are frequently used for sulfide analysis, such as MBB, greatly enhance the hydrolysis of polysulfides, which leads to increased polysulfide degradation as well as artificial formation of bis-S-bimane adducts in the absence of free H\(_2\)S. The finding that hydroxyl group-containing substances such as tyrosine quite efficiently protected polysulfides from hydrolytic degradation led to the development of the new alkylating agent, TME-IAM. TME-IAM efficiently and specifically traps and stabilizes hydroperoxysulfides and hydropolysulfides and protects polysulfide chains from hydrolysis, and, when used with mass spectrometry, TME-IAM allows successful speciation of the reactive sulfur metabolome. PMSA, the assay that relies on the unique hydrolysis equilibrium of polysulfide species, will be a reliable technique for the proteomics of polysulfide-containing proteins. Better understanding of the occurrence as well as metabolism of polysulfide species by means of precise methodologies is necessary to obtain insights into the unclarified biology of polysulfide species.

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**Authors’ Contributions**

T.S., H.M., and T.A. conceived and wrote the review. T.T., T.M., and H.I. helped in the literature search and drawing of figures. All the authors read and approved the article.

**Author Disclosure Statement**

No competing financial interests exist.

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**References**

1. Abiko Y, Ishii I, Kamata S, Tsuchiya Y, Watanabe Y, Ihara H, Akaike T, and Kumagai Y. Formation of sulfur adducts of N-acetyl-p-benzoquinoneimine, an electrophilic metabolite of acetaminophen in vivo: participation of reactive persulfides. *Chem Res Toxicol* 28: 1796–1802, 2015.

2. Akaike T, Ida T, Wei FY, Nishida M, Kumagai Y, Alam MM, Ihara H, Sawa T, Matsunaga T, Kasamatsu S, Nishimura A, Morita M, Tomizawa K, Nishimura A, Watanabe S, Inaba K, Shima H, Tanumia N, Jung M, Fujii S, Watanabe Y, Ohmuraya M, Nagy P, Feilsch M, Fukuto JM, and Motohashi H. Cysteinyl-tRNA synthetase governs cysteine polysulfidation and mitochondrial bioenergetics. *Nat Commun* 8: 1177, 2017.

3. Alcock LJ, Perkins MV, and Chalker JM. Chemical methods for mapping cysteine oxidation. *Chem Soc Rev* 47: 231–268, 2018.

4. Allison WS. Formation and reactions of sulfenic acids in proteins. *Acc Chem Res* 9: 293–299, 1976.

5. Andersson LO and Berg G. Hydrolysis of disulfide bonds in weakly alkaline media. I. Oxidized glutathione. *Biochim Biophys Acta* 192: 534–536, 1969.

6. Benaichouche M, Bosser G, Paris J, and Pluchon V. Relative nucleophilicities of arylsulphide and thiolate ions in dimethylacetamide estimated from their reaction rates with alkyl halides. *J Chem Soc Perkin Trans 2* 2: 1421–1424, 1990.

7. Benchao D, Semelak JA, Cuevaasta E, Mastrogiavanni M, Grassano JS, Ferrer-Sueta G, Zeida A, Trujillo M, Moller MN, Estrin DA, and Alvarez B. Acidity and nucleophilic reactivity of glutathione persulfide. *J Biol Chem* 295: 15466–15481, 2020.
8. Bogdandi V, Ida T, Sutton TR, Bianco C, Ditroi T, Koster G, Henthorn HA, Minnion M, Toscano JP, van der Vliet A, Pluth MD, Feelisch M, Fukuto JM, Akaike T, and Nagy P. Speciation of reactive sulfur species and their reactions with alkylating agents: do we have any clue about what is present inside the cell? *Br J Pharmacol* 176: 646–670, 2019.

9. Coughlin CR, 2nd, Scharer GH, Friederich MW, Yu HC, Geiger EA, Creadon-Swindell G, Collins AE, Vanlander AV, Coster RV, Powell CA, Swanson MA, Minczuk M, Van Hove JL, and Shaikh TH. Mutations in the mitochondrial cysteiny-l-tRNA synthase gene, CAR2, lead to a severe epileptic encephalopathy and complex movement disorder. *J Med Genet* 52: 532–540, 2015.

10. Donovan JW and White TM. Alkaline hydrolysis of the disulfide bonds of ovomucoid and of low molecular weight aliphatic and aromatic disulfides. *Biochemistry* 10: 32–38, 1971.

11. Everett SA and Wardman P. Perthiols as antioxidants: radical-scavenging and prooxidative mechanisms. *Methods Enzymol* 251: 55–69, 1995.

12. Florence TM. Degradation of protein disulphide bonds in dilute alkali. *Biochem J* 189: 507–520, 1980.

13. Francoleon NE, Carrington SJ, and Fukuto JM. The reaction of H2S with oxidized thiols: generation of persulfides and implications to H2S biology. *Arch Biochem Biophys* 516: 146–153, 2011.

14. Fujii S, Sawa T, Motohashi H, and Akaike T. Persulfide synthases that are functionally coupled with translation mediate sulfur respiration in mammalian cells. *Br J Pharmacol* 176: 607–615, 2019.

15. Fukuto JM and Hobbs AJ. A comparison of the chemical biology of hydropersulfides (RSSH) with other protective biological antioxidants and nucleophiles. *Nitric Oxide* 107: 46–57, 2021.

16. Fukuto JM, Ignarro LJ, Nagy P, Wink DA, Kevil CG, Feelisch M, Cortese-Krott MM, Bianco CL, Kumagai Y, Hobbs AJ, Lin J, Ida T, and Akaike T. Biological hydrosulfides and related polysulfides—a new concept and perspective in redox biology. *FEBS Lett* 592: 2140–2152, 2018.

17. Fukuto JM, Vega VS, Works C, and Lin J. The chemical biology of hydrogen sulfide and related hydropersulfides: interactions with biologically relevant metals and metalloproteins. *Curr Opin Chem Biol* 55: 52–58, 2020.

18. Hallmann K, Zsarka G, Moskau-Hartmann S, Kirschner J, Korinthenberg R, Ruppert AK, Ozbekir O, Weber Y, Becker F, Lerche H, Elger CE, Thiele H, Nurnberg P, Sander T, and Kunz WS. A homozygous splice-site mutation in CARS2 is associated with progressive myoclonic epilepsy. *Neurology* 83: 2183–2187, 2014.

19. Hamid HA, Tanaka A, Ida T, Nishimura A, Matsunaga T, Fujii S, Morita M, Sawa T, Fukuto JM, Nagy P, Tsutsuki H, Ida T, Motohashi H, Akaike T, and Nishida M, Kumagai Y, and Akaike T. Exposure to electrophiles impairs reactive persulfide-dependent redox signaling in neuronal cells. *Chem Res Toxicol* 30: 1673–1684, 2017.

20. Harwood DT, Kettle AJ, Brennan S, and Winterbourn CC. Simultaneous determination of reduced glutathione, glutathione disulfide and glutathione sulphonamide in cells and physiological fluids by isotope dilution liquid chromatography-tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* 877: 3393–3399, 2009.

21. Henderson CF, Bica I, Long FT, Irwin DD, Stull CH, Baker BW, Suarez Vega V, Taagher ZM, Fletes ED, Bartleson JM, Humphrey ML, Alvarez L, Akiyama M, Kumagai Y, Fukuto JM, and Lin J. Cysteine trisulfide protects *E. coli* from electrophile-induced death through the generation of cysteine hydopersulfide. *Chem Res Toxicol* 33: 678–686, 2020.

22. Heppner DE, Hristova M, Ida T, Mijuskovic A, Dustin CM, Bogdandi V, Fukuto JM, Dick TP, Nagy P, Li J, Akaike T, and van der Vliet A. Cysteine perthiosulfenic acid (CysSOH): a novel intermediate in thiol-based redox signaling? *Redox Biol* 14: 379–385, 2018.

23. Hidese R, Mihara H, and Esaki N. Bacterial cysteine de-sulfurases: versatile key players in biosynthetic pathways of sulfur-containing biofactors. *Appl Microbiol Biotechnol* 91: 47–61, 2011.

24. Ida T, Sawa T, Ibara H, Tsuchiya Y, Watanabe Y, Kumagai Y, Suematsu M, Motohashi H, Fujii S, Matsunaga T, Yamamoto M, Ono K, Devarie-Baez NO, Xian M, Fukuto JM, and Akaike T. Reactive cysteine persulfides and S-polythiolation regulate oxidative stress and redox signaling. *Proc Natl Acad Sci U S A* 111: 7606–7611, 2014.

25. Ibara H, Kasamatsu S, Kitamura A, Nishimura A, Tsutsuki H, Ida T, Ishizaki K, Toyama T, Yoshida E, Abdul Hamid H, Jung M, Matsunaga T, Fujii S, Sawa T, Nishida M, Kumagai Y, and Akaike T. Exposure to electrophiles impairs reactive persulfide-dependent redox signaling in neuronal cells. *Chem Res Toxicol* 30: 1673–1684, 2017.

26. Ikeda M, Ishima Y, Shibata A, Chuang VTG, Sawa T, Ibara H, Watanabe H, Xian M, Ouchi Y, Shimizu T, Ando H, Ukawa M, Ishida T, Akaike T, Otagiri M, and Maruyama T. Quantitative determination of polysulfide in albumins, plasma proteins and biological fluid samples using a novel combined assays approach. *Anal Chim Acta* 26: 18–25, 2017.

27. Jung M, Kasamatsu S, Matsunaga T, Akashi S, Ono K, Nishimura A, Morita M, Abdul Hamid H, Fujii S, Kitamura H, Sawa T, Ida T, Motohashi H, and Akaike T. Protein polysulfidation-dependent persulfide dioxygenase activity of ethylmalonic encephalopathy protein 1. *Biochem Biophys Res Commun* 480: 180–186, 2016.

28. Kasamatsu S, Ida T, Koga T, Asada K, Motohashi H, Ibara H, and Akaike T. High-precision sulfur metabolism innovated by a new specific probe for trapping reactive sulfur species. *Anastox Redox Signal* 34: 1407–1419, 2021.

29. Khan S, Fujii S, Matsunaga T, Nishimura A, Ono K, Ida T, Ahmed KA, Okamoto T, Tsutsuki H, Sawa T, and Akaike T. Reactive persulfides from *Salmonella Typhimurium* downregulate autophagy-mediated innate immunity in macrophages by inhibiting electrophilic signaling. *Cell Chem Biol* 25: 1403–1413.e4, 2018.

30. Kunikata H, Ida T, Sato K, Aizawa N, Sawa T, Tawaraya H, Murayama N, Fuji S, Akaike T, and Nakazawa T. Metabolomic profiling of reactive persulfides and polysulfides in the aqueous and vitreous humors. *Sci Rep* 7: 41984, 2017.

31. Lehmann WD. A timeline of stable isotopes and mass spectrometry in the life sciences. *Mass Spectrom Rev* 36: 58–85, 2017.

32. Marutani E, Morita M, Hirai S, Kai S, Grange RMH, Miyazaki Y, Nagashima F, Traeger L, Magliocca A, Ida T,
HYDROLYSIS EQUILIBRIUM OF POLYSULFIDES

Matsunaga T, Flicker DR, Corman B, Mori N, Yamasaki Y, Batten A, Li R, Tanaka T, Ikeda T, Nakagawa A, Atochin DN, Ihara H, Olenchok BA, Shen X, Nishida M, Hanaoka K, Kevil CG, Xian M, Bloch DB, Akaike T, Hindle AG, Motohashi H, and Ichinose F. Catabolism ameliorates hypoxic brain injury. Nat Commun 12: 3108–2021.

33. Motohashi H and Akaike T. Sulfur-utilizing cytoprotection and energy metabolism. Curr Opin Physiol 9: 1–8, 2019.

34. Numakura T, Sugiura H, Akaike T, Ida T, Fujii S, Koarai A, Yamada M, Onodera K, Hashimoto Y, Tanaka R, Sato K, Shishikura Y, Hirano T, Yanagisawa S, Fujino N, Okazaki T, Tamada T, Hoshikawa Y, Okada Y, and Ichinose M. Production of reactive persulfide species in chronic obstructive pulmonary disease. Thorax 72: 1074–1083, 2017.

35. Ono K, Jung M, Zhang T, Tsutsuki H, Sezaki H, Ihara H, Wei FY, Tomizawa K, Akaike T, and Sawa T. Synthesis of t-cysteine derivatives containing stable sulfur isotopes and application of this synthesis to reactive sulfur metabolome. Free Radic Biol Med 106: 69–79, 2017.

36. Ono K, Kitamura Y, Zhang T, Tsutsuki H, Rahman A, Ihara T, Akaike T, and Sawa T. Cysteine hydropersulfide inactivates β-lactam antibiotics with formation of ring-opened carbothioic S-acids in bacteria. ACS Chem Biol 16: 731–739, 2021.

37. Peng H, Zhang Y, Palmer LD, Kehl-Fie TE, Skaar EP, Trinidad JC, and Giedroc DP. Hydrogen sulfide and reactive sulfur species impact proteome S-sulfhydration and global virulence regulation in Staphylococcus aureus. ACS Infect Dis 3: 744–755, 2017.

38. Saund SS, Sosa V, Henriquez S, Nguyen QN, Bianco CL, Soeda S, Millikin R, White C, Le H, Ono K, Tantillo DJ, Kumagai Y, Akaike T, Lin J, and Fukuto JM. The chemical biology of hydropersulfides (RSSH): chemical stability, reactivity and redox roles. Arch Biochem Biophys 588: 15–24, 2015.

39. Sawa T, Motohashi H, Ihara H, and Akaike T. Enzymatic regulation and biological functions of reactive cysteine persulfides and polysulfides. Biomolecules 10: 1245, 2020.

40. Sawa T, Ono K, Tsutsuki H, Zhang T, Ida T, Nishida M, and Akaike T. Reactive cysteine persulfides: occurrence, biosynthesis, antioxidant activity, methodologies, and bacterial persulphide signalling. Adv Microb Physiol 72: 1–28, 2018.

41. Shen X, Peter EA, Bir S, Wang R, and Kevil CG. Analytical measurement of discrete hydrogen sulfide pools in biological specimens. Free Radic Biol Med 52: 2276–2283, 2012.

42. Steudel R and Chivers T. The role of polysulfide dianions and radical anions in the chemical, physical and biological sciences, including sulfur-based batteries. Chem Soc Rev 48: 3279–3319, 2019.

43. Sutton TR, Minion M, Barbarino F, Koster G, Fernandez BO, Cumpstey AF, Wischmann P, Madhani M, Frenneaux, MP, Postle AD, Cortese-Krott, MM, and Feelsich, M. A robust and versatile mass spectrometry platform for comprehensive assessment of the thiol redox metabolome. Redox Biol 16: 359–380, 2018.

44. Takahashi N, Wei FY, Watanabe S, Hirayama M, Ohuchi Y, Fujimura A, Kaitsuka T, Ishii I, Sawa T, Nakayama H, Akaike T, and Tomizawa K. Reactive sulfur species regulate tRNA methylthiolation and contribute to insulin secretion. Nucleic Acids Res 45: 435–445, 2017.

45. Tawarayama H, Suzuki N, Inoue-Yanagimachi M, Himori N, Tsuda S, Sato K, Ida T, Akaike T, Kunikata H, and Nakazawa T. Glutathione trisulfide prevents lipopoly saccharide-induced inflammatory gene expression in retinal pigment epithelial cells. Ocul Immunol Inflamm 1–12, 2020. [Epub ahead of print]; DOI: 10.1080/09273948.2020.1833224.

46. Wintner EA, Deckwerth TL, Langston W, Bengtsson A, Leviten D, Hill P, Insko MA, Dumpit R, VandenEkar E, Toombs CF, and Szabo C. A monobromobimane-based assay to measure the pharmacokinetic profile of reactive sulphide species in blood. Br J Pharmacol 160: 941–957, 2010.

47. Zhang B, Crack JC, Subramanian S, Green J, Thomson AJ, Le Brun NE, and Johnson MK. Reversible cycling between cysteine persulfide-ligated [2Fe-2S] and cysteine-ligated [4Fe-4S] clusters in the FNR regulatory protein. Proc Natl Acad Sci U S A 109: 15734–15739, 2012.

48. Zhang T, Ono K, Tsutsuki H, Ihara H, Islam W, Akaike T, and Sawa T. Enhanced cellular polysulfides negatively regulate TLR4 signaling and mitigate lethal endotoxin shock. Cell Chem Biol 26: 686–698.e4, 2019.

49. Zhang T, Tsutsuki H, Islam W, Ono K, Takeda K, Akaike T, and Sawa T. ATP exposure stimulates glutathione efflux as a necessary switch for NLRP3 inflammasome activation. Redox Biol 41: 101930, 2021.

50. Zivanovic J, Kouroussis E, Kohl JB, Adhikari B, Bursac B, Schott-Roux S, Petrovic D, Miljkovic JL, Thomas-Lopez D, Jung Y, Miler M, Mitchell S, Milosevic V, Gomes JE, Benhar M, Gonzalez-Zorn B, Ivanovic-Burmazovic I, Torregrossa R, Mitchell JR, Whiteman M, Schwarz G, Snyder SH, Paul BD, Carroll KS, and Filipovic MR. Selective persulphide detection reveals evolutionarily conserved antiaging effects of S-sulfhydration. Cell Metab 30: 1152–1170.e13, 2019.

Address correspondence to:
Prof. Tomohiro Sawa
Department of Microbiology
Graduate School of Medical Sciences
Kumamoto University
Kumamoto 860-8556
Japan

E-mail: sawat@kumamoto-u.ac.jp

Prof. Takaaki Akaike
Department of Environmental Medicine and Molecular Toxicology
Tohoku University Graduate School of Medicine
Sendai 980-8575
Japan

E-mail: takaike@med.tohoku.ac.jp

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### Abbreviations Used

| Abbreviation | Description |
|--------------|-------------|
| ADH5         | alcohol dehydrogenase 5 |
| CARS         | cysteinyl-tRNA synthetase |
| CPERS        | cysteine persulfide synthase |
| CysSSH       | cysteine persulfide |
| E            | electrophilic thiol-reactive reagents |
| ESEs         | bis-sulfur–electrophile adducts |
| ESH          | electrophile–sulfide adduct |
| ETHE1        | ethylmalonic encephalopathy protein 1 |
| GSSG         | oxidized glutathione |
| GSSH         | glutathione persulfide |
| GSSSG        | glutathione trisulfide |
| GSSSSG       | glutathione tetrasulfide |
| H₂S          | hydrogen sulfide |
| HPE-IAM      | β-(4-hydroxyphenyl)ethyl iodoacetamide |
| IAM          | iodoacetamide |
| LC-MS/MS     | liquid chromatography–tandem mass spectrometry |
| MBB          | monobromobimane |
| NBF-Cl       | 4-chloro-7-nitrobenzofurazan |
| NEM          | N-ethylmaleimide |
| PEG          | polyethylene glycol |
| PMSA         | PEG-conjugated maleimide-labeling gel shift assay |
| RSE          | electrophile–thiol adduct |
| RSH          | thiols |
| RSₙ⁻         | polythiolate anions |
| RSₙ⁻₁E       | electrophile–polysulfide adducts |
| RSₙ⁻₁OH      | polysulfenic acid |
| RSₙ⁻₁R       | hydro polysulfides |
| RSₙ⁻R        | polysulfide |
| RSOH         | sulfenic acid |
| RSSH         | hydropersulfides |
| SDS          | sodium dodecyl sulfate |
| TME-IAM      | N-iodoacyethyl t-tyrosine methyl ester |