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Biogenesis of reactive sulfur species for signaling by hydrogen sulfide oxidation pathways

Tatiana V Mishanina, **Marouane Libiad**, and **Ruma Banerjee***

Department of Biological Chemistry, University of Michigan Medical School, Ann Arbor, Michigan, USA

Abstract

The chemical species involved in H_2S signaling remain elusive despite the profound and pleiotropic physiological effects elicited by this molecule. The dominant candidate mechanism for sulfide signaling is persulfidation of target proteins. However, the relatively poor reactivity of H_2S toward oxidized thiols, such as disulfides, the low concentration of disulfides in the reducing milieu of the cell and the low steady-state concentration of H_2S raise questions about the plausibility of persulfide formation via reaction between an oxidized thiol and a sulfide anion or a reduced thiol and oxidized hydrogen disulfide. In contrast, sulfide oxidation pathways, considered to be primarily mechanisms for disposing of excess sulfide, generate a series of reactive sulfur species, including persulfides, polysulfides and thiosulfate, that could modify target proteins. We posit that sulfide oxidation pathways mediate sulfide signaling and that sulfurtransferases ensure target specificity.

Sulfide chemistry is intimately interwoven with the emergence of life on this planet. It is posited that the interaction between sulfide spewing from alkaline submarine hydrothermal vents and the acidic iron-containing waters of the Hadean ocean led to formation of catalytic colloidal iron-sulfur membranes that promoted the synthesis of the first organic compounds[1]. Fossils of sulfur-metabolizing microbes dating back almost 3.5 billion years provide some of the earliest evidence for a sulfur-based chemolithoautotrophic lifestyle[2]. Indeed, the composition of sulfur isotopes in biogenic sedimentary sulfides provides a useful record of biospheric oxygenation and the coevolution of life and the environment[3]. Sulfide continued to influence evolution in later eons, and it is postulated that the presence of a metastable sulfidic oceanic zone limited metazoan colonization of the continental shelf[4], while oceanic sulfide toxicity and hydrogen sulfide (H_2S) emissions into the atmosphere have been implicated as important drivers of the Permian-Triassic mass extinction[5]. As sulfur is the sixth most abundant element in the microbial biomass[6], its metabolism is critically important to the global biogeochemical sulfur cycle.

*Correspondence should be addressed to R.B.: rbanerje@umich.edu.

Competing financial interests

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What is the key to the versatility of sulfur in metabolism? The answer lies in its ability to cycle through a variety of biologically relevant oxidation states ranging from −2, as in H_2S, to +6, as in sulfate (SO_4^{2-}) (Fig. 1). The higher valence states of sulfur are obtained through oxidation of sulfide or thiols to compounds such as thiyl radical ($RS^{\bullet}$, −1, where the number refers to the formal oxidation state of the sulfur atom(s)), hydropersulfide (RS-S^-, −1, −1), disulfide (RSSR, −1, −1) and sulfenic acid (RSOH, 0). In parallel to the usage for reactive oxygen and nitrogen species, the term ‘reactive sulfur species’ (RSS) is used here to refer collectively to reactive sulfur chemotypes (both organic and inorganic) that, under physiological conditions, can react with, oxidize or reduce other molecules[7–9]. Oxidized sulfur species can be catenated as in hydropolysulfides (RS-S-S^-, −1, 0, −1), have interchalcogen bonds as in sulfite (SO_3^{2-}, +4) and sulfate (SO_4^{2-}, +6), or have both as in thiosulfate ($S_2O_3^{2-}$, −1, +5). For simplicity, hydropersulfide and hydropolysulfides will be referred to as persulfides and polysulfides, respectively.

The cellular capacity for redox signaling via different site-specific cysteine modifications allows an oxidant signal to be transmuted into a biological response. However, unlike with reactive oxygen and nitrogen species, whose roles in cellular signaling and in varied physiological processes have been extensively studied, the role of RSS in redox homeostasis, mammalian metabolism and signaling has only recently begun to attract attention[7–10]. Central to the growing interest in RSS biochemistry is the realization both of the mammalian capacity to produce and oxidize H_2S and of the ability of H_2S, or a downstream product, to elicit varied and profound physiological effects[11]. Some important missing pieces in this emerging story are the mechanisms by which sulfur signals are conveyed and whether H_2S or an RSS derived from it (or both) constitute the signaling molecule(s). In this article, we propose that the canonical mitochondrial[12] and the newly described heme-dependent[13] sulfide oxidation pathways are important sources of RSS and that enzyme-catalyzed transpersulfidation reactions are an important mechanism for ensuring target specificity of reversible post-translational persulfide modification.

Properties of H_2S

To evaluate the varied roles ascribed to H_2S, such as its reactivity toward amino acid side chains to effect protein modification and its antioxidant properties, it is important to first examine its chemical attributes. A colorless gas with the smell of rotten eggs, H_2S is highly soluble in water (~80 mM at 37 °C) and can permeate lipid bilayers with facility[14,15]. H_2S is a weak diprotic acid with pK_{a1} and pK_{a2} values of 7 and 12–15 (ref. 16), respectively, and thus exists predominantly (72%) as the sulfide anion at the physiological pH of 7.4. By comparison, the pK_a values for cysteine and glutathione, two small-molecule thiols, are 8.3 and 8.8, respectively. H_2S is used in this article to refer collectively to all three species that exist in solution: the diprotonated (H_2S), monoanion (HS^-) and dianion (S^{2-}) forms.

H_2S is a relatively stable sulfur species with a half-life on the order of minutes[10]. H_2S shows low reactivity toward disulfides such as glutathione disulfide (GSSG) and cystine, and its rate of disappearance in buffer at pH 7.4 is comparable in the absence or presence of these disulfides[17]. H_2S shows slightly higher reactivity toward the disulfide cystamine[17]. Careful studies on the kinetics of H_2S reaction with disulfides versus cysteine sulfenic acid or

cysteine nitrosothiols are needed to evaluate their kinetic competence in the context of persulfide modification of proteins. A mixture of unstable intermediates and reaction products are formed during the uncatalyzed oxidation of H_2S in aerobic solutions, including elemental sulfur (S_8), polysulfanes (S_n^{2-}, n = 2–7), sulfite and thiosulfate[18].

The bimolecular rate constants for the reaction of HS^- with two-electron oxidants such as hydrogen peroxide (H_2O_2, 0.73 M^{-1} s^{-1}), peroxynitrite (4.8×10^3 M^{-1} s^{-1}) and hypochlorite (8×10^7 M^{-1} s^{-1}) at pH 7.4 and 37 °C reveal a wide range of reactivity (Table 1) and are comparable to the rate constants reported for cysteine and glutathione[19]. However, the pH-independent rate constants for HS^-, a measure of its intrinsic reactivity, are lower by 3-fold (with hypochlorous acid), 6-fold (with peroxynitrous acid) and 25-fold (with H_2O_2) as compared to cysteine and still lower than for glutathione. This difference in intrinsic reactivity is likely due to the absence of an inductive effect on sulfide and to the lower Brønsted basicity of HS^- as compared to the thiolates of cysteine and glutathione[19]. The modest bimolecular rate constant for the uncatalyzed oxidation of H_2S by H_2O_2, and the low intracellular concentration of H_2S (discussed below), make it unlikely that this reaction is physiologically significant, unless methods exist for concentrating H_2S and oxidants (for example, H_2O_2 or peroxynitrite) in certain cellular locales.

In comparison to glutathione and cysteine, which function as redox buffers in the intracellular compartment, H_2S is predicted to be a minor antioxidant. The standard two-electron redox potential for the H_2S/S^0 couple is estimated to be −280 mV (versus the normal hydrogen electrode) at pH 7.0 (based on the value of +140 mV reported in acidic solution)[7] and is similar to those for the glutathione disulfide/glutathione ($E^{\circ\prime}$ = −262 mV) and the cystine/cysteine ($E^{\circ\prime}$ = −245 mV) redox couples[20]. However, because the intracellular steady-state concentration of H_2S (~10–30 nM)[17,21] in mammals is several orders of magnitude lower than those of cysteine (~0.1–1 mM)[22] or glutathione (1–10 mM)[22], it is unlikely that H_2S has a quantitatively significant role in oxidant defense except perhaps in specific cellular niches.

The sulfhydryl radical ($HS^\bullet$) produced by one-electron oxidation of sulfide is a strong oxidant and, once generated, can be involved in a series of radical chain reactions (Table 1). The standard reduction potential for the $SH^\bullet/HS^-$ redox couple is +920 mV[23]. $HS^\bullet$ is reactive and can react with HS^- to form the hydrodisulfide radical anion ($HSSH^{\bullet-}$), which can further react with O_2 to generate HSS^- and $O_2^{\bullet-}$. Reaction of the $HS^\bullet$ with O_2 gives the sulfur dioxide radical anion ($SO_2^{\bullet-}$)[23].

Two mechanisms that have been discussed in the context of sulfide-based signaling are persulfidation forming protein persulfides and interaction of sulfide with metalloproteins and are described below.

Metal sulfide complexation

The role of metals as sulfide carriers has been known for a while. For example, ferric ion in hemoglobin Hb1 in *Lucina pectinata*[24], a clam found in sulfide-rich mangroves, and zinc ions in with sulfide-oxidizing bacteria. H_2S binds to the open coordination site in ferric hemoglobin to generate low-spin six-coordinate ferric sulfide heme, in equilibrium with the

ferrous sulfide radical tautomer (Fig. 2a). The fate of ferric sulfide hemoglobin is determined by various factors including sulfide concentration and the dielectric constant of the distal heme pocket, with a polar environment favoring heme reduction concomitant with sulfide oxidation[26]. Sulfide can also react with ferrous-oxy heme, leading, in a series of steps, to sulfur addition to the porphyrin ring, forming sulfhemoglobin[27]. The latter is an irreversible modification also seen with myoglobin[28] and is therefore unlikely to be useful in a signaling context.

In principle, sulfide can bind to an open metal-coordination site or displace a weak ligand, such as water. In fact, the primary basis of H_2S toxicity is via metal coordination, that is through its binding to the binuclear Cu–heme iron complex in cytochrome *c* oxidase, resulting in reversible inhibition of this terminal station in the electron transfer chain where O is reduced to water[29]. Sulfide anion coordination to model ferrous iron porphyrinate complexes (Fig. 2b) has been characterized[30]. The corresponding ferric compounds are rapidly reduced by sulfide, suggesting that heme groups in polar protein environments might be susceptible to similar chemistry following sulfide binding. It has been speculated that the binding of sulfide to neuroglobin, a heme protein relatively abundant in neurons and retina, plays a protective role under conditions of oxygen deprivation, such as stroke, when sulfide concentrations reportedly increase[31]. The physiological relevance of sulfide binding to neuroglobin and to other heme proteins in a protective or signaling capacity remains to be assessed.

In order for sulfide coordination to a metallocenter to serve in a signaling pathway leading to a cellular response, it must elicit an effector function either within the same protein or with a downstream partner protein. A specific receptor for sulfide is not known, unlike with •NO, for which soluble guanylate cyclase serves as a heme protein sensor, activating production of the second messenger cGMP[32]. It remains to be seen whether sulfide binding can modulate the functions of zinc, mononuclear iron, copper or heme-containing metalloproteins in the context of signal transduction.

Protein modification by persulfidation

Persulfidation of proteins

Cavallini and co-workers demonstrated in 1970 that persulfides form upon addition of sodium sulfide to proteins containing disulfide bonds, such as insulin and RNase[33]. Studies by Massey and co-workers on xanthine oxidase and aldehyde oxidase[34,35] are often cited as evidence for the importance of stabilized persulfides to enzymatic activity. However, spectroscopic and structural studies have since demonstrated that the labile sulfur in these enzymes is a sulfido ligand to molybdenum and not a persulfide[36–38]. Persulfidation at a single cysteine residue in superoxide dismutase was established by mass spectrometry and shown to stabilize the enzyme against oxidation-induced aggregation without affecting catalytic activity[39]. The opposite effect, inhibition following persulfidation, has been observed with a number of erythrocytic enzymes, including glucose 6-phosphate dehydrogenase and adenylate kinase[40], and with protein tyrosine phosphatase 1B (PTP1B)[41]. In PTP1B, the active site cysteine was specifically modified, lending confidence about the potential relevance of the persulfide modification as a regulatory mechanism. *In*

vivo, persulfidation reportedly enhances the catalytic activity of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and the polymerization of actin[42]. Persulfidation of parkin, an E3 ubiquitin ligase, increases its activity[43], and this modification is depleted in brain of individuals with Parkinson's disease.

Despite the handful of examples, whether persulfidation of proteins by H_2S is important in a signaling context and by what mechanism this modification is introduced remain open questions. As H_2S cannot react directly with thiols and its rate of auto-oxidation might be too slow to be biologically relevant (Table 1)[16], reactions involving the addition of protein thiolates to either HSOH or HSSH to give the persulfide modification (Fig. 3a) might not be physiologically relevant. In contrast, proteins with reactive cysteines that have become oxidized might be susceptible to persulfidation by sulfide. A subset of all protein cysteines are reactive, and these generally have low pK_a values. Reactive cysteines are often found in protein microenvironments with a positive electrostatic potential that stabilizes the thiolate anion[44]. Their reactivity makes them susceptible to oxidative modifications including disulfide formation, S-glutathiolation, sulfenic acid formation or S-nitrosylation that lead to altered protein structure and/or function (Fig. 3b). In proteins containing a disulfide bond, a nucleophilic attack by the sulfide anion would yield the persulfide modification, although its lifetime could be short owing to elimination by the resolving cysteine. Considering the low concentration of disulfides in the reducing milieu of the cell and the modest reactivity of sulfide toward disulfides[17], the physiological relevance of this persulfidation mechanism remains to be assessed. In the absence of disulfide bonds, an alternative route to cysteine activation for sulfide attack is necessary; possibilities include glutathiolated cysteine (Cys-SSG), cysteine sulfenic acid (Cys-SOH) and S-nitrosylated cysteine[45,46]. The mechanisms for persulfidation of proteins shown in Figure 3b potentially connect sulfide-based signaling to pathways involving oxidative or nitrosative stress. Persulfidation is a reversible modification that can be removed by reducing agents such as glutathione[47], proteins such as thioredoxin[48] or glutaredoxin or via re-formation of a disulfide bond initiated by nucleophilic attack by a resolving cysteine (Fig. 3c).

An obvious limitation of the solution-based persulfidation mechanisms described in Figure 3a,b is their lack of specificity. In principle any reactive cysteine on a protein could attack oxidized sulfide, or reactive cysteines that have themselves become oxidized can be attacked by sulfide. Furthermore, multiple cysteines can be modified on the same protein, as reported for serine and homoserine dehydratases, alcohol dehydrogenase and 3-hydroxybutyrate dehydrogenase[49,50]. Unregulated persulfidation would lead to undesired changes in protein function and could be averted by the low steady-state concentration of sulfide and/or, as discussed later, by enzyme-catalyzed transpersulfidation[51].

Protein modification by polysulfides

An alternative hypothesis for sulfide-based post-translational protein modification is that H_2S is not the sulfurating species at all; rather, the S-sulfurating agent is an oxidation product of sulfide—that is, a polysulfide—that is more reactive than H_2S. In fact, it has been argued that the varied effects of H_2S can be attributed to polysulfide species that contaminate commercially available sulfide salts[52], and polysulfides show substantially

lower IC_{50} values than H_2S when the direct comparison is made[53]. Polysulfanes, such as elemental sulfur, can sulfurate cysteine thiols[49,50]. However, the limited solubility of elemental sulfur and the relative instability of polysulfides pose obvious challenges for their handling within the cell. Furthermore, the potential source of elemental sulfur and/or polysulfide species in mammals is unclear. In bacteria, polysulfides and elemental sulfane sulfur can be produced during sulfide oxidation by sulfide quinone oxidoreductase (SQR)[54]. In contrast, mammalian SQRs do not catalyze multiple rounds of H_2S oxidation and polymerization before releasing catenated sulfur products into solution. Instead, the oxidized sulfur is transferred to a small-molecule acceptor such as glutathione in each round of the catalytic cycle, forming a persulfide (rather than polysulfide) product[12,47]. The concentration of free polysulfide is reportedly ~25 nmol/g of wet mouse brain tissue[53] and ~0.3 μM in mouse blood[55].

Polysulfides can modulate protein functions such as activation of transient receptor potential (TRP) A1 channels[53], inhibition of the lipid phosphatase PTEN[52] and activation of the transcription factor Nrf2 by modification of Keap1 (ref. 56). The reactivity of polysulfides raises questions about how specificity in protein modification is achieved, as in principle a reactive cysteine on a protein can attack at multiple sites along the polysulfide chain, generating a heterogeneous mix of products (Fig. 4a). Furthermore, the resultant product mixture can undergo further reactions with either the same or a different protein or with small-molecule thiols, forming sulfur-bridged homo- or heterodimeric species, respectively (Fig. 4b). Although the effects of polysulfide exposure on cellular responses (for example, Ca^{2+} influx by TRPA1 channel activation)[53] have been reported, the nature of the resulting modification has not been directly established. With PTEN, exposure to polysulfides resulted in an intramolecular disulfide bond that was identified by mass spectrometric analysis[52], indicating loss of the initial per- or polysulfide modification, probably by the mechanism shown in Figure 4b. Polysulfide exposure of cells resulted in homo- and heterodimerization of Keap1 (ref. 56). An inter- or intramolecular disulfide link could be a signature of an initial and transient polysulfide modification.

Transpersulfidation of proteins

A number of sulfur-containing cofactors and modified thionucleosides obtain their sulfur atom by persulfide transfer[57]—that is, via transpersulfidation. The pK_a of persulfides is ~1–2 units lower than for the corresponding thiols[58], and the terminal sulfur can act as a nucleophile or an electrophile in the unprotonated and protonated states, respectively[59]. Persulfides are reactive and are inherently unstable and disproportionate to RSH and S° in solution. In light of their dual reactivity and instability, the report that cellular and tissue concentrations of small-molecule persulfides are of the same order of magnitude as for thiols such cysteine[60] is surprising. Sulfurtransferases are found in archaea, bacteria and eukaryotes and contain the rhodanese homology domain fold with an α/β topology (Fig. 5a), which can be present in a single copy, in tandem repeats or fused with other proteins[61]. Rhodanese homology domain proteins have consensus sequence motifs at the N and C termini and a catalytic cysteine embedded in an active site loop. The rhodanese homology domain proteins are often found encoded in multiple copies in genomes and can function as persulfide carriers, as in the biosynthesis of 4-thiouridine[62] and molybdopterin[63]. A sulfur

relay system, in which a persulfide group is shuttled from a cysteine desulfurase through a succession of carrier proteins, is employed for synthesis of the 2-thiouridine modification in tRNA[64,65].

In mammals, sulfurtransferases involved in sulfide synthesis and oxidation form active site persulfides (Fig. 5b). These include mercaptopyruvate sulfurtransferase, which transfers the sulfur from 3-mercaptopyruvate to an active site cysteine, forming a persulfide intermediate[48,66]. Human rhodanese uses GSSH as a sulfur donor, forming an active site persulfide[47]. It can also employ thiosulfate as a sulfur donor, albeit less efficiently than GSSH by a factor of $>2 \times 10^5$-fold[47]. A third human rhodanese domain–containing sulfurtransferase, TSTD1, utilizes thiosulfate as a sulfur donor, although the kinetics of this reaction have only been characterized at an alkaline pH[67]. Whereas the majority of sulfurtransferases are found in the cytoplasm, rhodanese is localized to mitochondria and mercaptopyruvate sulfurtransferase is found in both the cytoplasm and mitochondria. Finally, the mitochondrial enzyme SQR, involved in the sulfide oxidation pathway (discussed below), forms a persulfide intermediate[47,68]. Enzymes involved in H_2S biogenesis, cystathionine β-synthase and γ-cystathionase, can synthesize cysteine persulfide from cystine[60].

The persulfide intermediate formed in these enzymes can be subsequently transferred to a protein such as glutaredoxin or to a small-molecule acceptor such as cysteine, hypotaurine or glutathione[33,47,48,66,68]. In principle, sulfurtransferases could modify target proteins either directly or indirectly via a secondary carrier, which in turn transfers the persulfide to a client protein (Fig. 5c). The use of secondary carriers such as glutaredoxin, thioredoxin or other rhodanese homology domain proteins would permit a signaling pathway to fan out from a single persulfide generating enzyme and also achieve targeting specificity. Persulfides transferred to two-cysteine thioredoxins and glutaredoxins are likely to be short-lived owing to the presence of resolving cysteines, setting up a competition between H_2S elimination (Fig. 3c) and transpersulfidation. Evidence in support of transpersulfidation as a mechanism for sulfur transfer include data from studies of rhodanese, which reportedly mediates persulfidation of malate dehydrogenase *in vitro*, resulting in increased activity[69], and from the *Marina arenicola* SQR, which transfers persulfide to thioredoxin[70], although direct evidence for this transfer was not provided.

Mitochondrial H_2S oxidation pathway: a source of RSS?

The potential for the mitochondrial sulfide oxidation pathway present in most mammalian cells to serve as a source of RSS, and therefore, as a mediator of sulfide-based signaling, has not been appreciated. Instead, the pathway is generally viewed as one that exists to convert H_2S to a mixture of innocuous products, thiosulfate and sulfate (Fig. 6a), which are subsequently eliminated. The first step in the sulfide oxidation pathway is catalyzed by the flavoprotein SQR, which catalyzes a two-electron oxidation of H_2S and transiently forms an enzyme-bound persulfide intermediate, which is then transferred to a small-molecule acceptor. The electrons released in this reaction are passed via ubiquinone to complex III, making sulfide an inorganic substrate for mammalian oxidative phosphorylation[71]. The glutathione persulfide (GSSH) product of the reaction[12,47] is a substrate for the nonheme

iron–containing persulfide dioxygenase[72] and for rhodanese[12,47]. Sulfite oxidase, present in the intermitochondrial membrane space, oxidizes sulfite to sulfate. Organization of the sulfide oxidation pathway can vary in different archaea, bacteria and plants, variously including components that are homologous to the mammalian enzymes or different from them[73–75].

At least four RSS are produced during mitochondrial sulfide oxidation. The first is the SQR-bound persulfide, which in principle can be transferred to another protein or to any of a number of small-molecule acceptors[47,68]. SQR is anchored to the inner membrane with its catalytic domain protruding into the matrix. Its ability to donate sulfane sulfur to protein acceptors in the mitochondrial membrane or in the matrix needs to be assessed. GSSH is the second RSS produced in the sulfide oxidation pathway and is predicted to be the predominant product of the human SQR reaction at physiologically relevant concentrations of small-molecule acceptors[47]. GSSH is reactive and can transfer its sulfane sulfur group to a target protein (Fig. 5b) as demonstrated with human rhodanese, which subsequently transfers the sulfane sulfur to sulfite, forming thiosulfate (Fig. 6a)[12,47].

Thiosulfate is the third RSS product in the sulfide oxidation pathway and, although relatively stable in solution, is a substrate for thiosulfate:glutathione sulfurtransferases, such as rhodanese[47] and TSTD1 (ref. 67). Its solution stability combined with its ability to serve as a source of sulfane sulfur makes thiosulfate a potentially ideal ‘Trojan horse’ RSS that, upon activation by a specific sulfurtransferase, can mobilize its terminal sulfur for transfer. The role of thiosulfate in sulfide-based signaling needs to be assessed, particularly in light of recent reports of its therapeutic potential in acute lung injury[76] and hypertensive cardiac disease[77]. The archaeon *Metallosphaera cuprina* utilizes tetrathionate as an energy source and, interestingly, transfers the thiosulfate group between cysteines in proteins involved in dissimilatory sulfur metabolism[78]. Although an enzyme that catalyzes the oxidative condensation of 2 mol of thiosulfate to form tetrathionate has not been described in mammals, such an activity, if present, would open up yet another RSS-based post-translational modification.

Sulfite, the fourth RSS produced during sulfide oxidation, is readily oxidized by enzymes such as myeloperoxidase, prostaglandin H synthase and eosinophil peroxidase generating highly reactive products including the sulfite radical anion ($^{\bullet}SO_3^-$) and the peroxymonosulfate ($^-O_3SOO^{\bullet}$) and sulfate ($SO_4^{\bullet-}$) radical anions[79]. It is likely that cells limit the damaging potential of sulfite by efficiently oxidizing it to sulfate by means of sulfite oxidase, or through its utilization by other enzymes, such as rhodanese. The toxicity of sulfite is consistent with the fact that free serum sulfite concentrations are below detection limits[80], while free tissue sulfite concentrations have not been reported to our knowledge. Interestingly, the antimicrobial and antioxidant properties of sulfite have led to its use as a preservative in the food and pharmaceutical industries, and sulfite sensitivity is observed in a subset of asthmatics[81]. Sulfite reductase found in archaea, bacteria and plants represents an alternate route for sulfite removal[82,83].

As a source of RSS, the sulfide oxidation pathway bears similarity to the mitochondrial electron transfer pathway, a quantitatively significant source of ROS[84]. A role for

mitochondrial ROS has been demonstrated in bacterial clearance by macrophages[85], inflammasome activation[86] and induction of a cellular hormetic response[87]. Mechanisms of dynamic regulation of ROS and RSS production are poorly understood, and it is not known whether an increase in the mitochondrial proton motive force, known to intensify ROS production[88], affects RSS output.

Metal-catalyzed H_2S oxidation: another source of RSS?

Oxidation of sulfide to thiosulfate by hemoglobin[89] and ferritin[90] has been reported, and protection against sulfide toxicity by induced methemoglobinemia has been known for many years[91]. However, until recently, the mechanism of heme-dependent sulfide oxidation remained a mystery. Numbering ~5 trillion per liter of blood, red blood cells are expected to influence sulfide homeostasis in the circulation, particularly in light of their capacity to generate H_2S[92]. Recently, catalytic sulfide oxidation by human methemoglobin has been shown to yield thiosulfate and metal-bound polysulfide[13] (Fig. 6b). The factors governing partitioning of the initially formed ferric sulfide hemoglobin between thiosulfate and polysulfide formation are not presently known. The postulated chemical mechanism of sulfide oxygenation is complex, and thiosulfate production is predicted to predominate when the flux of H_2S is low and oxygen tension is high. It is likely that other heme-containing proteins such as myoglobin and cytochrome P_{450} are capable of catalyzing similar sulfide oxidation chemistry in other tissues. Although heme protein–dependent formation of polysulfides describes a route for their biogenesis in mammalian cells, it also raises questions regarding polysulfide management via sequestration or transfer given the inherent reactivity of the product.

Sulfide rapidly reduces the ferric forms of cytochrome *c* oxidase and cytochrome *c*. The product of cytochrome *c* oxidase reduction under aerobic conditions has not been characterized but is predicted to be elemental sulfur[93,94]. Sulfide binds to the heme iron in myeloperoxidase[95] and lactoperoxidase[96]. Although it has been observed that re-formation of the iron–sulfide complex of myeloperoxidase from compound I is oxygen dependent, possible sulfide oxidation products were not characterized[95]. In addition to its reversible inhibition of myeloperoxidase, it is speculated that sulfide could modulate inflammation[95]. Sulfide coordinates to copper in superoxide dismutase, which converts it to elemental sulfur[97]. Careful characterization of sulfide affinity and reaction kinetics, along with product analysis, during catalytic sulfide oxidation by these and other metalloproteins is needed to assess their physiological relevance to RSS production and H_2S clearance.

Future directions

Still in its infancy, the field of H_2S chemical biology is ripe for investigation, with many more questions open than answered. At the most fundamental level is the issue of whether H_2S or an RSS derived from it is pertinent to sulfide-based signaling or whether, as with superoxide and H_2O_2 in ROS signaling, multiple species are involved. Furthermore, the chemical nature of the interaction of H_2S or RSS with other signaling molecules, particularly $^{\bullet}NO$, needs to be addressed in light of the growing evidence for their crosstalk[55,98,99]. Our hypothesis that sulfide oxidation pathways are a source of RSS with

signaling potential and our discovery of a novel heme-dependent catalytic sulfide oxidation pathway in red blood cells[13] raise questions about the fate of the resulting RSS and the involvement of other heme proteins in RSS generation in other tissues. Finally, studies aimed at unraveling cellular strategies for regulating RSS production, sequestration and mobilization will be critical for understanding sulfide signaling and crosstalk with other signaling pathways.

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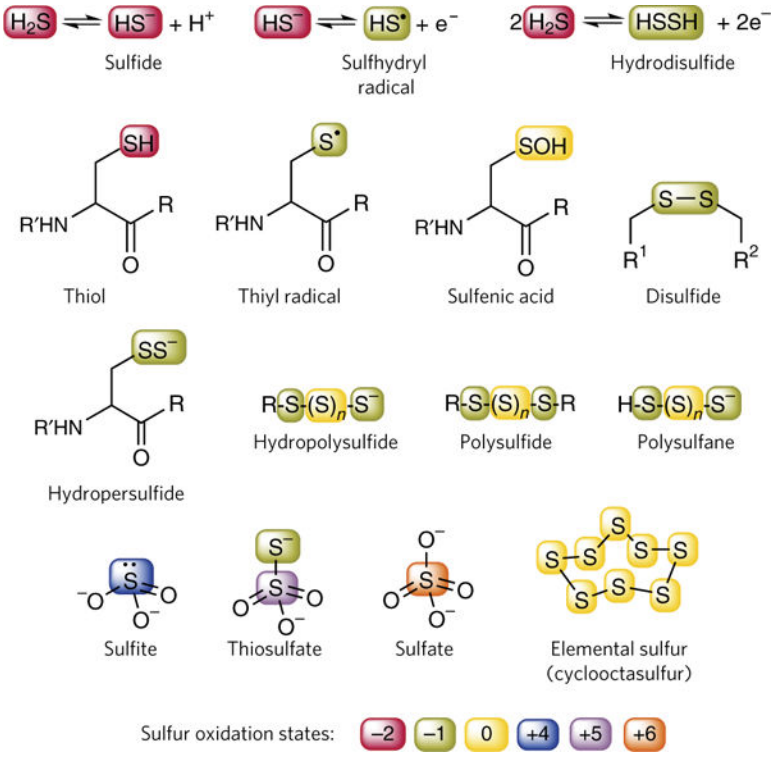

Figure 1. Structures of some biologically relevant RSS chemotypes

The use of the term ‘sulfane sulfur’ in the literature is sometimes confusing. Here, the IUPAC nomenclature for sulfane sulfur—sulfur-bonded sulfur—is used. The red (−2), green (−1), yellow (0), blue (+4), purple (+5) and orange (+6) rectangles are used to designate the valence states of sulfur as specified. In molecules containing catenated sulfurs, $n \geq 2$ and R ≠ H.

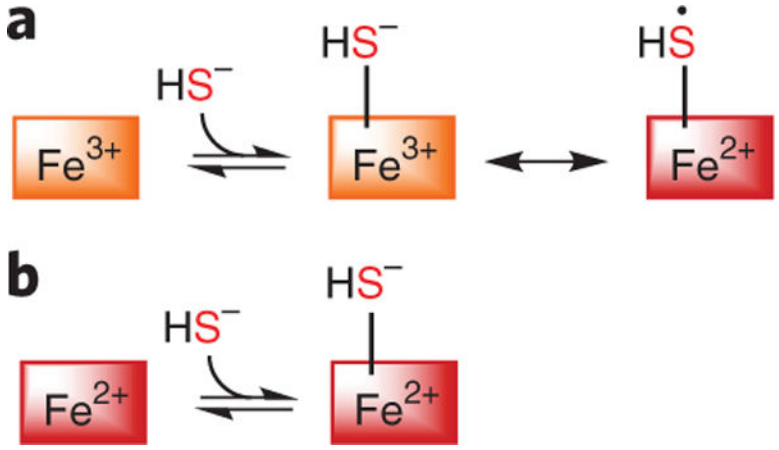

Figure 2. Models for H_2S interaction with heme proteins

(**a**) Binding of sulfide anion to ferric heme results in the formation of ferric sulfide complex, which, depending on the polarity of the distal heme pocket, could lead to heme iron reduction and formation of the sulfur radical. Depending on the heme protein, either H_2S or HS^- can bind ferric heme. (**b**) In principle, H_2S can also bind ferrous hemes, as seen with model porphyrinate complexes.

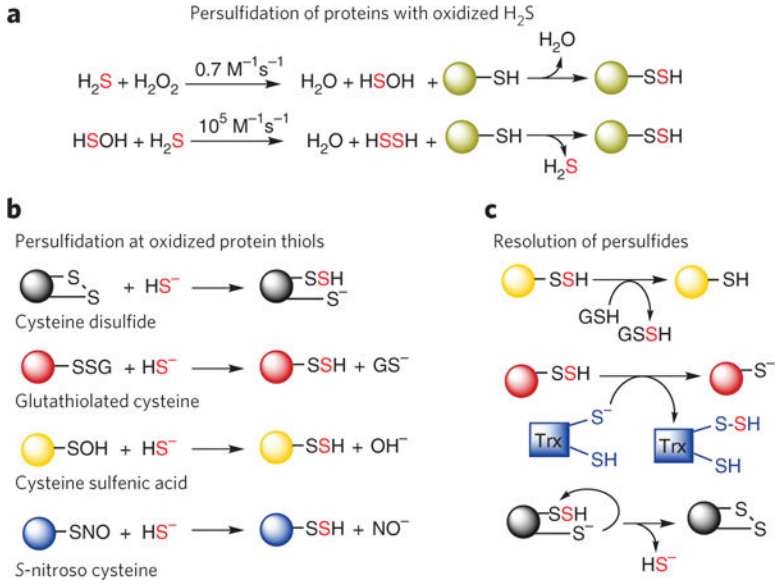

Figure 3. Potential mechanisms for persulfidation and the resolution of this modification

(**a**) H_2S is relatively stable and is oxidized slowly by H_2O_2 to give HSOH. Either HSOH or HSSH formed from HSOH in the presence of a second equivalent of H_2S can be attacked by a reactive cysteine on a protein to generate the persulfide modification. The bimolecular rate constants for HSOH and HSSH formation in solution at pH 7.4 and 37 °C are noted. (**b**) Persulfidation can result from the nucleophilic attack of a sulfide anion on an oxidized protein thiol (such as disulfide, mixed disulfide, cysteine sulfenic acid or S-nitrosylated cysteine). (**c**) Persulfide modifications on proteins are reversible and, unless sequestered, labile. They can be removed via persulfide interchange reactions involving glutathione (GSH), thioredoxin (Trx) or a cysteine on the same or a different protein. In all cases, the product is ultimately H_2S, formed upon reduction of the persulfide moiety by either a second mole of GSH or the NADPH–thioredoxin reductase system.

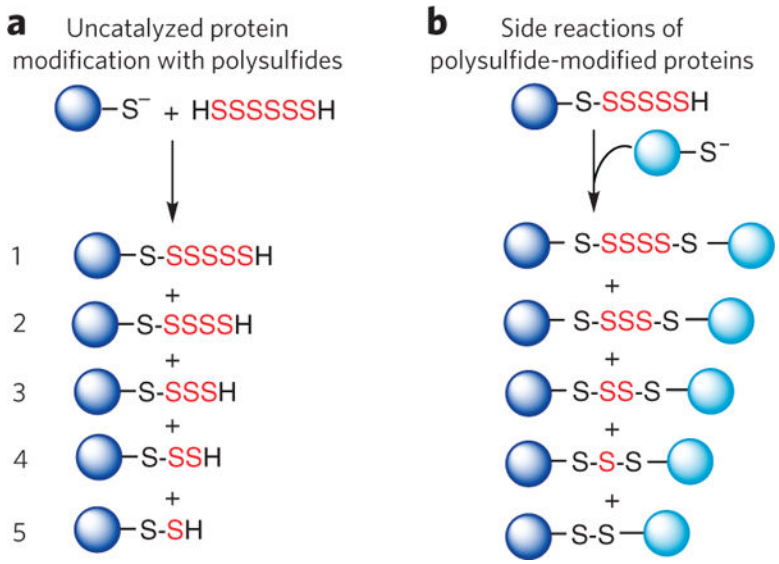

Figure 4. Potential complexity associated with uncatalyzed protein modification by polysulfides

(**a**) A reactive thiol on a protein (dark blue sphere) could attack one of several sulfurs in a polysulfide chain generating a series of protein modifications (1–5) and eliminating H_2S or polysulfides with varying number of sulfur atoms, which are omitted for clarity of presentation. This reaction complexity could be averted in an enzyme-catalyzed persulfidation reaction in which nucleophilic attack on a specific sulfur atom in the polysulfide chain is promoted. (**b**) Further reactions with persulfide- or polysulfide-modified proteins with a cysteine on the same or a different (light blue sphere) protein (or a small-molecule thiol) could result in homo- or heterodimerization in which the protein subunits are linked via bridging sulfur atoms. Alternatively, a vicinal cysteine residue on the modified protein could give rise to an intramolecular sulfur linkage.

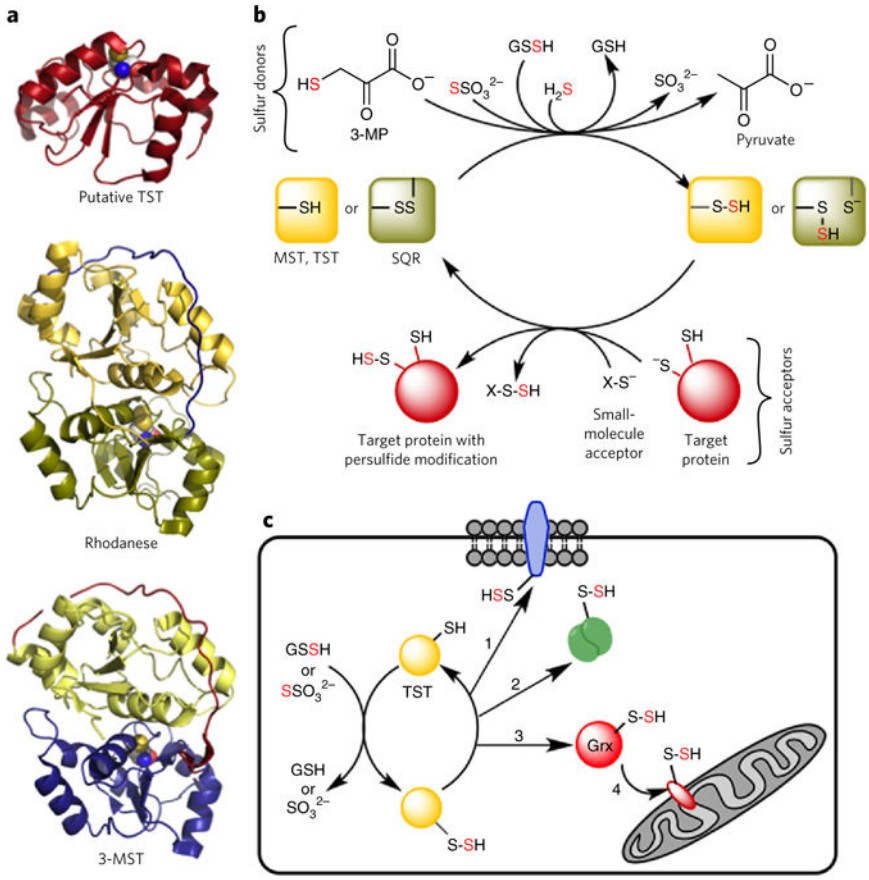

Figure 5. Rhodanese homology domain proteins and persulfide relay system for transpersulfidation

(**a**) Structures of three rhodanese homology domain proteins in which the active C-terminal domain is shown in red (top, *S. cerevisiae* putative single-domain thiosulfate sulfurtransferase (TST) YOR285, PDB 3D1P), green (middle, bovine rhodanese, PDB 1RHD) or blue (bottom, human 3-mercaptopyruvate sulfurtransferase (MST), PDB 4JGT), and the inactive N-terminal domain, when present, is shown in yellow. The active site cysteines are in ball representation, and a persulfide intermediate is seen in the rhodanese structure. (**b**) Sulfurtransferases such MST and TSTs (for example, rhodanese and TSTD1) and sulfide quinone oxidoreductase (SQR) accept sulfur atoms from their respective substrates (3-mercaptopyruvate for MST, thiosulfate or GSSH for TSTs and H_2S for SQR) and form a persulfide intermediate. The outer sulfur can be transferred to a protein or small-molecule acceptor, resulting in a persulfide product. (**c**) Specificity in transpersulfidation can be achieved by enzyme-catalyzed transfer of the persulfide group. In the example shown here, either GSSH or thiosulfate transfers the outer sulfur to TST, forming a persulfide intermediate, which can be transferred to protein targets (1, 2) or to an intermediate carrier such as glutaredoxin (Grx, 3), which, in turn, transfers the persulfide to a target protein (4). Because two-cysteine-containing thiroredoxins and Grxs have vicinal resolving cysteines, the resulting persulfide modification on them is expected to be short-lived. In contrast, in carriers with a single active site cysteine, such as a subset of Grxs, the lifetime of the persulfide modification will be longer.

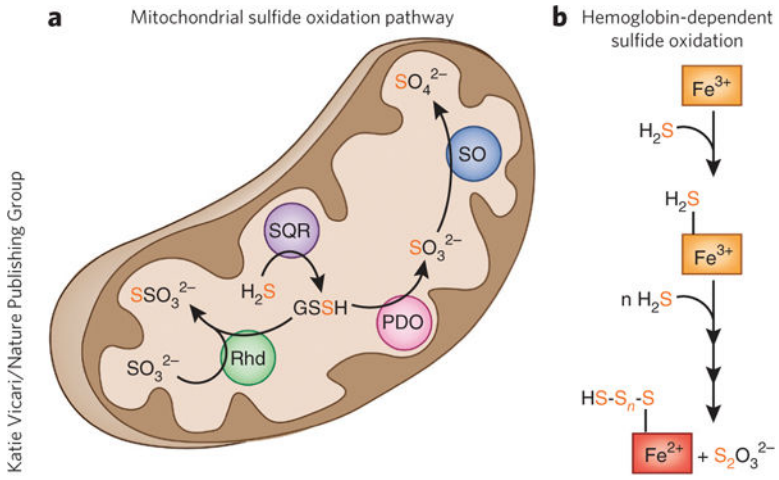

Figure 6. Sulfide oxidation pathways

(**a**) The canonical sulfide oxidation pathway found in most tissues resides in the mitochondrion and involves four enzymes. In the first step of the pathway, sulfide quinone oxidoreductase (SQR) oxidizes sulfide to persulfide, which is transferred from the active site of SQR to a small molecule acceptor, such as glutathione (GSH). The glutathione persulfide (GSSH) product can be oxidized by persulfide dioxygenase (PDO) to sulfite or can be used in a sulfurtransferase reaction catalyzed by rhodanese (Rhd) in the presence of sulfite, to form thiosulfate. In the final step, sulfite is oxidized to sulfate by sulfite oxidase (SO). (**b**) Heme-dependent sulfide oxidation pathway. An alternative pathway for sulfide oxidation involves ferric heme–dependent conversion of H_2S to a mixture of thiosulfate and polysulfides. This newly discovered mechanism has been established for human hemoglobin and could be an activity of other heme proteins as well. For clarity, the fate of the H_2S sulfur atom is highlighted in red and other reactants and reaction stoichiometries are omitted.

Table 1

Reactivity of H_2S and its oxidation products

Oxidant	k (M^{-1} s^{-1})[a]	Temperature (°C)
$H_2S + O_2^{\bullet -} \rightarrow H_2O_2 + S^{\bullet -}$	2.0×10^2	37
$HS^- + H_2O_2 \rightarrow HSOH + OH^-$	0.73	37
$HS^- + ONOOH \rightarrow HSOH + NO_2^-$	4.8×10^3	37
$HS^- + HOCl \rightarrow HSCl + OH^-$	8.0×10^7	37
$HSOH + HS^- \rightarrow HSSH + OH^-$	1.0×10^5	37
$HS^- + S^{\bullet -} \rightarrow HSS^{\bullet 2-}$	5.4×10^9	37
$HSS^{\bullet 2-} \rightarrow HS^- + S^{\bullet -}$	5.3×10^5 s^{-1} [b]	37
$HSS^{\bullet 2-} + O_2 \rightarrow HSS^- + O_2^{\bullet -}$	4.0×10^8	37

[a] H_2S, which is fully reduced, shows a spectrum of reactivity with oxidants ranging from sluggish (with H_2O_2) to high (with HOCl). The initial products are either one ($S^{\bullet -}$) or two electron (for example, HSOH) more oxidized than H_2S and represent reactive sulfur species, which can undergo further reactions. The rate constants for the reaction of GSH and cysteine, where available, show that they are very similar to those for H_2S at the same pH and temperature. The values in this table are from ref. 19 and denote bimolecular rate constants, with the one exception noted.

[b] The rate constant for this unimolecular reaction has units of s^{-1}.