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The Redox Chemistry and Chemical Biology of H_2S, Hydropersulfides and Derived Species: Implications to Their Possible Biological Activity and Utility

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Abstract

Hydrogen sulfide (H_2S) is an endogenously generated and putative signaling/effector molecule. In spite of its numerous reported functions, the chemistry by which it elicits its functions is not understood. Moreover, recent studies allude to the existence of other sulfur species besides H_2S that may play critical physiological roles. Herein, the basic chemical biology of H_2S as well as other related or derived species is discussed and reviewed. A particular focus of this review are the per- and poly-sulfides which are likely in equilibrium with free H_2S and which may be important biological effectors themselves.

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Introduction

Hydrogen sulfide (H_2S) has been reported to be an important, endogenously generated, small-molecule signaling agent with numerous physiological functions [for recent reviews, see 1-4]. It is proposed to be one of the so-called "gasotransmitters"[1] along with nitric oxide (NO), carbon monoxide (CO), dioxygen (O_2) and derived species [for example, 5,6]. Although the reported functions of H_2S are numerous, the chemical mechanisms associated with its biological activity are not established. Moreover, the actual levels of H_2S present in biological tissues and fluids have been a matter of some contention and controversy [7,8] with early reports of levels as high as 20-80 μM in plasma. However, it is becoming increasingly clear that concentrations of "free" H_2S are low (sub-micromolar) but that there is a labile "pool" of sulfur-containing species, in equilibrium with free H_2S, that can liberate H_2S [9]. The fact that there is this large labile pool of H_2S-releasing species indicates likely biological significance to these H_2S precursor molecules (*vide infra*).

As with all biological signaling species, the utility of the small-molecule signaling agents is due to their unique chemistry and physical properties. That is, the signaling properties of these species are due to their specific/selective chemical interactions with biological targets and subsequent effects on the function of these target molecules [for example, 10]. The best and most established example of this (at least among the small-molecule signaling agents) is the interaction of NO with its primary biological target, the enzyme soluble guanylate cyclase (sGC). The regulatory heme of sGC binds NO, which presumably results in the loss of an iron heme axial histidine ligand leading to activation of the enzyme. Significantly, NO appears unique in its ability to elicit this type of chemistry (dissociation of the axial ligand upon ligand-binding [11]). Unlike NO, the exact nature of the chemistry associated with the biological activity of H_2S is not firmly established. However, considering the fact that H_2S is first and foremost a simple thiol, it may be expected that the chemical biology of H_2S involves, at least in part, its properties as a nucleophile/reductant which can lead to changes in sulfur oxidation state. Afterall, much of the biological utility of thiols relies on these properties and interaction with other thiol species or metals. Thus, this review will focus on the biological chemistry of H_2S (and more importantly, species derived from H_2S or possibly those that can serve as sources of H_2S such as enzyme-mediated persulfide formation, *vide infra*) as a means to begin to understand its biological function and utility.

Although one of the focuses of this review is H_2S chemical biology, readers will note that the majority of the discussion presented herein relates to other sulfur species. This may seem curious considering the title of this review and the prevalence of the recent literature that mostly focuses primarily on H_2S. Therefore, before continuing further, it is worth presenting one of the tenets of this review: Some of the biological signaling/effects commonly associated with H_2S may be due to other sulfur containing species which can degrade to release H_2S (making H_2S a possible marker for these species). In studies involving

[1]The term "gasotransmitter" is a poor description of these small-molecule signaling agents since it misrepresents their chemical state when they are acting biologically. They do **not** exist as gases, but as solutes when they are acting as physiological effector/signaling agents. This term is only mentioned here due to its unfortunate prevalence in the current literature.

pharmacological or experimental addition of H_2S (or H_2S-donors), the primary effector molecule may also not be H_2S, but rather these same species made via reaction of exogenous H_2S with oxidized thiol precursor species (*vide infra*). This is, of course, speculative at this time but justifies the time spent discussing other chemical species besides H_2S. This is not to say that H_2S is biologically innocuous. On the contrary, it seems likely that biological H_2S generation has function. What is being considered herein, however, is that other sulfur-containing molecules have unique and biologically important properties that allow them to be specific effector/signaling species. The fact that they may release H_2S under certain conditions may be biologically relevant since, considering the parsimony of Nature, it seems probable that H_2S release is also purposeful. Regardless, discussions of the chemistry of many of the sulfur species besides H_2S is given to provide the basis of future work/discussion of this signaling.

Nomenclature

As with many fields, unwieldy, inappropriate or inconsistent terminology can be a major obstacle to understanding the chemistry being described. This seems especially true when describing the chemistry/biology of sulfur-containing molecules. For example, the terms thiol, sulfhydryl, and mercaptan have been used to denote essentially the same functional group, -SH. Although there are published rules or guidelines for the naming of sulfur-containing molecules [for example, 12] they often don't cover all the possible sulfur-containing species and many of these rules are not rigorously followed or are cumbersome. To be sure, this review is not intended to serve as a treatise of sulfur nomenclature. However, it is worthwhile to define the terms that will be used herein to avoid confusion. Consistent with most of the current literature, the terms hydrogen sulfide, thiol and disulfide will be used to describe H_2S, RSH and RSSR, respectively. Hydrosulfide and sulfide are often used to specifically denote the anionic species HS^- (i.e. sodium hydrosulfide, NaHS) and S^{2-} (i.e. sodium sulfide, Na_2S). When the general term hydrogen sulfide is used, especially pertaining to its presence in biological systems, this will include all protonation states (H_2S, HS^- and S^{2-}). Confusion can occur when referring to multi-sulfur species. The term alkyl hydropersulfide denotes a molecule whereby a disulfide is substituted on one end with an alkyl group and the other end with a hydrogen atom (RSSH). The term “alkyl hydrodisulfide” could have been used here (consistent with the naming of RSSR a disulfide) but since it is important to draw some structural and chemical analogy to peroxides (dialkyl peroxide, ROOR; alkyl hydroperoxide, ROOH and hydrogen peroxide, HOOH) the term “persulfide” was selected (note that “perthiol” can also be used here, but for the sake of consistency this term will not be used – however, when referring to the radical RSS·, “perthiyl” is used). The term alkyl hydropolysulfides is used to describe species of the general form RSS_nH ($n > 1$). When referring to a specific polysulfide, the number of sulfur atoms can be indicated (e.g. RSSSH can be referred to as an alkyl hydrotrisulfide). A similar system for dialkyl polysulfides will be adopted herein: dialkyl polysulfides will be generally referred to as RSS_nR ($n > 1$) and specific dialkyl polysulfides such as RSSSR will be termed dialkyl trisulfide. Especially problematic are the oxidized forms of hydropersulfides and hydropolysulfides. For example, RSSOH cannot be named a “persulfenic acid” since this term has been used to describe a molecule of the type RSOOH. In order to distinguish

between RSSOH and RSOOH, a species of the type RSSOH will be referred to herein as an alkyl perthiosulfenic acid. Due to the possible confusion and ambiguity associated with adopting any convenient nomenclature scheme, chemical formulas will be given as much as possible.

The Biological Chemistry of H_2S

Basic chemical properties

Herein, only a brief review of fundamental H_2S chemistry is given. Other, more comprehensive reviews are available [for example, 13,14,15]. Unlike the other small-molecule signaling agents NO, CO or O_2, H_2S is ionizable. The pK_a of H_2S is 6.8 and the pK_a of HS^- is approximately 14. Thus, at physiological pH, the monoanion HS^- is the predominant species with negligible concentrations of S^{2-}. The pK_a values for many thiols (RSH) are significantly higher than that of H_2S (although there are numerous thiols in proteins that have very low pK_a values). Therefore, H_2S generally has a higher equilibrium concentration of the anionic species, HS^-, compared to most "free" thiols. The reduction potential for HS· (the thiyl radical) is reported to be approximately 1 V (pH 7, vs a normal hydrogen electrode (NHE)) [16,17]. Therefore, H_2S is not a particularly good one-electron reductant and the oxidized thiyl species, HS·, is a good oxidant. Overall, the one-electron chemistry of HS^-/HS· does not appear to be significantly different from that of normal biological thiols/thiyls. The one chemical property that distinguishes H_2S from other biologically relevant alkyl thiol species (such as glutathione (GSH) or cysteine (Cys-SH)) is the fact that it has two dissociable protons that can be readily substituted with other atoms/functional groups (alkyl thiols, on the other hand, have only one dissociable proton). This is an important property since it allows H_2S to form bridges between atoms (i.e. thioethers (RSR), metal sulfides (metal-S-metal). Moreover, this property of H_2S also allows for the formation of per- and poly-sulfides (i.e. RS_nH, n>1 and RS_nR, n >2). The prevalence, relevance and chemical properties of these polysulfur species will be discussed in more detail later.

Interaction of H_2S with Metals (Iron Hemes)

A major target for H_2S biology/toxicology has been proposed to be metals. As with the other established small-molecule signaling agents (i.e. NO, CO, O_2 and derived species) whose primary biological receptors are likely metalloproteins, H_2S as well as hydropersulfides (*vide infra*) have the ability to interact with metalloproteins and these intertactions may also be important to their biological function. Although there are numerous metals (i.e. copper, iron, manganese, etc.) and types of metalloproteins (iron-sulfur proteins, non-heme iron proteins, zinc fingers, etc.), the emphasis herein will be on iron heme proteins. The decision to focus primarily on this class of metalloprotein is not because the other types are not biologically relevant but rather more due to the fact that the interactions of H_2S (and related species) with iron heme proteins have been the most studied (albeit, somewhat sparsely).

One of the earliest studies of the interaction of H_2S with a heme protein is by Keilin [18] where it was demonstrated that the reaction of H_2S with methemoglobin ($MbFe^{III}$) resulted in a spectral change caused by a presumed reversible generation of an H_2S-$MbFe^{III}$ adduct.

Much more recently, it was shown that other ferric heme proteins can react directly with H_2S to give an Fe^{III}-SH complex with the same spectral changes observed in the initial study by Keilin [19,20]. One of the most studied of all H_2S-heme protein interactions is that of H_2S with a hemoglobin of the bivalve mollusc, *Lucina pectinata*, that inhabits H_2S-rich environments [for example, 21-26]. Interest in this specific interaction stems from the fact that *Lucina pectinata* possesses a specialized H_2S-binding hemoglobin (HbI) used to transport H_2S to symbiotic chemoautotrophic bacteria living in its gills. These bacteria utilize H_2S to fix CO_2 into hexoses that are subsequently used by the mollusc. Based on X-ray crystallographic and mutation studies, it appears that stable H_2S binding to ferric HbI is facilitated by several interactions between the bound H_2S and specific distal residues - hydrogen bonding between a glutamine carbonyl and bound H_2S, a hydrophobic “cage” made up of phenylalanines and favorable electrostatic interactions between phenylalanines and the H_2S ligand [for example, 23,27]. Moreover, the non-polar binding pocket appears to disfavor deprotonation of the ferric H_2S complex contributing to its stability [26].

Based on the discussion above, heme proteins can be designed to reversibly bind/carry H_2S. However, the interaction of H_2S with other ferric heme proteins or model porphyrin systems (not specially designed to bind H_2S) can undergo further chemistry after initial H_2S binding. For example, Pietri and coworkers [26] report that mutation of the important non-polar residues in the ferric HbI binding site to polar residues can facilitate deprotonation of the bound H_2S ligand to form an Fe^{III}-HS^- complex, which can result in ferric to ferrous reduction. It was also proposed that this reduction was facilitated by excess H_2S (**Reaction 1**, HbI_m = mutated HbI).

$$Hbl_{\mathrm{m}}-Fe^{III}-\mathrm{H_2S} \rightarrow \left[Hbl_{\mathrm{m}}-Fe^{III}-HS^{-} \longleftrightarrow Hbl_{\mathrm{m}}-Fe^{II}-HS\cdot\right]+\mathrm{H_2S} \rightarrow\rightarrow Hbl_{\mathrm{m}}-Fe^{II} \quad (1)$$

The mechanism by which excess H_2S facilitates reduction of the ferric-H_2S complex (or ferric-HS^- complex) is intriguing and has recently been demonstrated for myeloperoxidase (MPO) using spectroscopic measurements on the Fe^{II}-sulfide complex, which was also independently synthesized by the reaction of reduced MPO with sulfide [20]. The sulfur-containing product of this reaction has been proposed to be dihydrogen persulfide (H_2S_2) [26]. A recent study by Pavlik and coworkers [28] examined the reaction of H_2S with model ferric and ferrous porphyrins and found that HS^- readily binds ferrous species and reduces ferric porphyrins (although the possible role of excess H_2S was not specifically addressed). This result is consistent with the idea that H_2S binding/carrying ferric porphyrins require special ligand binding pockets to accommodate H_2S binding and avoid HS^- binding/ formation and subsequent reduction.

One of the earliest reports of a direct reaction between H_2S and heme proteins was the finding that exposure of oxyhemoglobin ($HbFe^{II}$-O_2) to H_2S resulted in a green pigment [18 and references therein]. It was later found that the green pigment was an H_2S-modified heme referred to generally as sulfhemoglobin (sulfHb) and could only be generated when hemoglobin was exposed to both H_2S and O_2 (the same process for myoglobin was also observed making sulfmyoglobin (sulfMb)) [for a review, see 29]. The generation of sulfHb or sulfMb is considered irreversible and deleterious since the H_2S-mediated modification of

the porphyrin lowers the O_2 binding affinity of both proteins [30]. The mechanism of formation of the sulfHb/sulfMb is currently unknown but thought to involve reaction of H_2S with a high-valent iron-oxo species leading eventually to covalent incorporation of a sulfur atom into the pi system of one of the pyrole rings of the heme [29,30]. To be sure, formation of sulfhemes is not restricted to hemoglobin and myoglobin, as other proteins such as catalase and lactoperoxidase can also form sulfhemes when exposed to H_2S [29]. Since H_2S is generated endogenously (as a presumed signaling species made by mammalian cells and by colonic bacteria), the formation of sulfhemes may be prevalent under normal (non-pathological) conditions. Whether these basal levels of sulfheme proteins have physiological function remains to be determined.

From a purely toxicological perspective, the interaction of H_2S with cytochrome c oxidase (CcO) has been of great interest since H_2S toxicity is generally thought to be due, at least in part, to inhibition of mitochondrial respiration. Indeed, inhibition of CcO activity is proposed to be a sensitive biomarker for H_2S exposure [31]. However, a watershed paper from the Roth lab [32] reported that 80 ppm exposure of mice to H_2S put them in a state of suspended animation akin to a hibernative state. This state of suspended animation was presumably due to an inhibition of CcO. Significantly, cessation of H_2S exposure led to full metabolic recovery with no measureable deficit. The Roth group also showed that placement of mice into an H_2S-mediated state of suspended animation also protected them from lethal hypoxia [33]. Thus, exposure of mice to pharmacological levels of H_2S that presumably interact with CcO can have non-toxic and even beneficial effects. For these reasons, examination of the interaction of H_2S with CcO may be important in the elucidation of H_2S-mediated biological activity.

Although H_2S can clearly inhibit CcO, this effect is complex and highly dependent on the levels of H_2S [for example, 34]. For example, the mitochondria of marine invertebrate living in an H_2S-rich environment can utilize H_2S at low levels (< 20 μM) as an electron source for oxidative phosphorylation [35]. Moreover, this same phenomenon can be observed in chicken liver mitochondria (at H_2S levels < 5 μM) [36] and in mammalian cells (at low μM concentrations of H_2S) [37]. In mammalian cells, it is proposed that H_2S can reduce a mitochondrial element (e.g. coenzyme Q) between complexes I and III via the actions of sulfide:quinone oxidoreductase (SQR) (*vide infra*). However, at high concentrations of H_2S, inhibition of mitochondrial activity is observed, possibly via interaction at a second mitochondrial site (likely CcO) [37]. A recent study examining the interaction of H_2S with CcO by Collman et al. [38] utilizes a model system consisting of a synthetic molecule that replicates the iron heme-copper O_2 binding site of CcO. Importantly, this model system avoids interactions of H_2S with other mitochondrial components. It was found that H_2S rapidly reduces the oxidized ferric or cupric species of CcO to their active ferrous and cuprous, O_2-binding forms. This indicates that H_2S can serve as a source of electrons for CcO-mediated O_2 reduction. It appears that low levels H_2S will not effectively compete with O_2 binding. However, at high levels of H_2S, it can compete with O_2 binding, thus inhibiting CcO activity. These results are consistent with the idea that H_2S can support CcO activity at low concentrations since it can serve as reducing equivalents but at high concentrations can compete with O_2 at the ferrous-cuprous O_2-binding site and inhibit CcO activity. A recent

review by Pietri et al. [30] proposes a slightly more detailed explanation whereby at low H_2S concentrations the polar environment of the heme a_3 center (the heme center that is normally involved with O_2 binding) promotes heme a_3 reduction. However, at higher levels of H_2S, reduction of the Cu_B center (also involved in O_2 binding) occurs resulting in the formation of a stable, reduced Cu_B-H_2S complex as well as an unstable inhibitory Fe^{II}-H_2S complex of heme a_3. With an eventual exhaustion of H_2S, formation of a stable Fe^{III}-H_2S complex inhibits the system. Regardless, it is clear that the inhibition of CcO is complex, H_2S concentration-dependent and involves numerous reactive sites and intermediate species.

Based on *in vitro*, isolated mitochondria and purely chemical studies, it is clear that the effect of H_2S on mitochondrial function (and CcO activity) is at least biphasic and potentially complex. What is also clear is that high levels of H_2S are potentially deleterious with regards to inhibition of mitochondrial respiration. In mammalian cells, levels higher than low μM may adversely affect mitochondrial activity. This, of course, would predict that the extremely high levels of H_2S originally reported (levels as high as 100 μM) are likely incorrect [for example, 39], and if anything, reflects the large size of an H_2S-releasing pool [9] of which a significant part could represent persulfide/polysulfide compounds recently found to be at high physiological concentrations [40] (*vide infra*).

H_2S and RSSH formation

H_2S is a fully reduced sulfur species with a formal oxidation state of 2-. As such, in biological systems, it can only be oxidized. Since both thiols (RSH) and H_2S are formally at the same oxidation state, they will not react with each other. Thus, fully reduced thiols are not expected to be direct targets of H_2S reactivity [41]. However, oxidized thiol species such as dialkyl disulfides (RSSR) or sulfenic acids (RSOH), do react with H_2S. For example, H_2S has been shown to react with disulfides to give the corresponding thiol and RSSH (**Reaction 2**) [for example, 42,43].

$$\mathrm{H_2S + R'SSR \rightleftharpoons R'SSH + RSH} \quad (2)$$

As mentioned above, the existence of H_2S in biology allows for the generation of RSSH (since thiols can only make RSSR via analogous chemistry). The reaction of H_2S with a dialkyl disulfide can be viewed simply as a reaction of a nucleophile, H_2S/HS^-, with an electrophile, RSSR analogous to the classical cysteine thiol/disulfide exchange reactions [44]. Although the equilibrium constant for **Reaction 2** is not known and likely to be highly dependent on the nature of “R” and reaction conditions (e.g. pH), the possibility of this reaction having biological relevance has been reported [43]. To be sure, it has also been stated that the reaction of H_2S with oxidized sulfur species like RSSR is not likely to occur due to the low concentration and relatively unfavorable reduction potential for H_2S and competition with other thiol species [for example, 45] (it should be noted, however, that the idea that an unfavorable reduction potential opposes this reaction has been questioned [13]). However, the reactivities of RSSR moieties towards thiol nucleophiles vary on a large scale [44] and therefore it would not be unreasonable to expect that biological H_2S exists in redox equilibrium with other reduced and oxidized thiol species (as depicted in **Reaction 2**), although the position of these equilibria are not known and may vary greatly depending on

the oxidized thiol species. Thus, the relevance of **Reaction 2** in forming biological persulfides remains to be established.

Alkyl Hydropersulfide Formation, Chemistry and Biological Relevance

RSSH Biosynthesis

It is intriguing to imagine that the biological actions of H_2S could be, to a significant degree, the result of interactions with oxidized thiols in proteins or peptides. That is, reaction of H_2S with an oxidized thiol species (RSSR, RSOH) associated with a protein or peptide results in the generation of an RSSH species in the protein/peptide, which may be the actual biological effector species. Importantly, biological RSSH generation can occur enzymatically to give directly an RSSH species, which can then go on to set up RSSH/H_2S equilbria (**Reaction 2**) (both or either of which may be important signaling species). For example, cystine (Cys-SS-Cys) can be converted to cysteine hydropersulfide (Cys-SSH) via the actions of cystathionine γ-lyase (CSE) [46] or cystathionine β-synthase (CBS) [40] (**Figure 1**).

Interestingly, the K_m for the reaction of Cys-SS-Cys with CSE is reported to be lower than that of other known substrates [47], implying that Cys-SS-Cys is a preferred substrate.

As mentioned immediately above, alkyl hydropersulfide biosynthesis does not require initial H_2S generation. Since hydropersulfides can be degraded in biological systems to generate H_2S (for example, via the reverse of **Reaction 2**), it is proposed that H_2S can serve as a "marker" for hydropersulfides [40]. Indeed, it has been further considered that hydropersulfides can be the actual major effector species in biological signaling [40,45].

If hydropersulfides are biologically important effector species and if a major source of biological hydropersulfide (i.e. Cys-SSH) is from Cys-SS-Cys via CSE or CBS catalytic activity, then the levels and regulation of Cys-SS-Cys may be an important issue regarding RSSH formation. Currently, it is known that the predominant form of cysteine in extracellular space/fluids is the oxidized Cys-SS-Cys form [for example, 48] although both reduced and oxidized forms exist. Interestingly, cysteine (Cys-SH) and Cys-SS-Cys are taken up by cells via distinct transporters. Cys-SH is taken up by a Na^+-dependent neutral amino acid transporter. On the other hand, Cys-SS-Cys is taken up by a Na^+-independent transporter, designated x^-_c, that exchanges Cys-SS-Cys and glutamate [49,50]. The x^-_c transport system is composed of two disulfide-linked subunits: xCT, which mediates the transport and 4F2hc, which is required for cell surface expression of the complex [reviewed by 51]. Initial studies focused on the import of cystine into cells due to its presumed importance in GSH production, however more recently, attention has focused more on the efflux of glutamate due to its potential role in excitatory signaling in the central nervous system. Interestingly, mice deficient in xCT are healthy and fertile with no signs of neurologic defects reported, however analysis of amino acids in the plasma revealed approximately twice the concentration of cystine [52]. Additionally, fibroblasts and macrophages derived from xCT deficient mice failed to survive in culture without a reducing agent in the media such as 2-mercaptoethanol [52,53]. Deficient macrophages also showed impaired survival following activation by lipopolysaccharide (LPS), presumably due to increased sensitivity to oxidative stress caused by the respiratory burst. Importantly,

this transporter is induced by a variety of cellular stresses including oxidative stress (i.e. H_2O_2) [54]. Uptake of Cys-SS-Cys into cells may be expected to lead to higher Cys-SH levels due to rapid intracellular reduction. However, it is reported that Cys-SS-Cys concentrations can be relatively high (approximately 30 μM in HT29 colon epithelial cells) and that the Cys-SH/Cys-SS-Cys ratio is only around 4 (130 μM Cys-SH/30 μM Cys-SS-Cys) compared to a GSH/GSSG ratio in the same cells of over 150 [55]. (Significantly, more recent studies indicate that the GSH/GSSG ratio can be much higher than generally reported [56]). It was also observed that the Cys-SH/Cys-SS-Cys and GSH/GSSG redox systems are independent of each other and not metabolically coupled (that is, changes in GSH/GSSG ratios are not mirrored by a change in Cys/Cys-SS-Cys ratios) [55,57]. Moreover, it is also reported that the Cys-SH/Cys-SS-Cys redox couple is independent of thioredoxin (Trx1) [55]. These may be important considerations in evaluating the role of Cys-SS-Cys in thiol redox physiology. Since it appears that significant intracellular concentrations of Cys-SS-Cys are maintained in a manner that does not depend on the redox status of GSH/GSSG or Trx1 this then leaves the question of what is important in regulating Cys-SS-Cys levels (or the Cys-SH/Cys-SS-Cys ratio)? That is, what is primarily responsible for converting Cys-SS-Cys to Cys-SH in a cell? Currently, the primary mechanism by which Cys-SS-Cys is reduced to Cys-SH in cells is not established. Reduction of Cys-SSCys by GSH, glutaredoxin or thioredoxin, although possible, is likely to be much too slow to be primary reduction pathways [55]. Thus, if Cys-SS-Cys levels are found to be important/regulatory for the generation of Cys-SSH, then this question will need to be answered.

As discussed above, alkyl hydropersulfides, whether from Cys-SS-Cys metabolism or the reaction of H_2S with disulfides, are likely to be biologically relevant. This idea is supported by a recent report indicating significant levels of Cys-SSH and glutathione persulfide (GSSH) along with protein-persulfides in human and mouse tissues and cells in culture [40]. Of special note is the extremely high levels of GSSH (as high as 150 μM in mouse brain) and Cys-SSH (1-4 μM in mouse tissues) found in mammalian systems. Thus, it is useful to carefully consider the chemistry and biochemistry of persulfides as possible biological effector species. At the cellular level, H_2S is rapidly consumed in mitochondria by SQR, which results in persulfide intermediates [58]. The K_m for this process is 100 nM while mitochondrial consumption by another persulfide (i.e. GSSH) metabolizing enzyme, ETHE1 (also known as persulfide dioxygenase) has a higher K_m [59], thus predicting a biological "equilibrium" between H_2S and GSS^- to be in favor of persulfides. Persulfide intermediates can also be produced by 3-mercaptopyruvate sulfurtransferase (3-MST) from cysteine/ketoglutarate [for example, 60], which is higher in the brain and may account for the finding of higher persulfide in this tissue bed [40]. Thus, it appears that the presence of persulfides can be maintained by a balance of metabolic processes that seems to favor their formation. The presence of oxidants (i.e. H_2O_2) and CysSSCys and the recycling of H_2S suggests that persulfides may be the predominant species with H_2S serving as a unique dosimeter for these species.

Alkyl Hydropersulfide Chemistry: Nucleophilic and Electrophilic Properties

The biological chemistry of RSSH has not been examined extensively (at least compared to thiols). Perhaps the lack of description of RSSH chemical biology is at least partially due to

the fact that the general biological relevance of these species has only recently been reported (although they have been known to be biologically relevant in specific systems for some time, *vide infra*). Also, RSSH possess an inherent instability that makes them somewhat difficult to study – alkyl hydropersulfides will spontaneously decompose in a pH-dependent manner to give numerous products including the corresponding thiol, disulfide, sulfide and elemental sulfur (S^0 which often seen as insoluble S_8) (**Reaction 3**) [for example, 61].

$$RSSH \rightarrow RSH + S^0 + RSSR + H_2S \quad (\text{not balanced}) \quad (3)$$

Although the details of the mechanism of this degradation are not established, this inherent instability is thought to prohibit the long-term storage of alkyl hydropersulfides. Thus, examination of RSSH chemistry/biochemistry is typically performed via *in situ* generation either chemically [for example, 43,62] or enzymatically [40].

It has been proposed that alkyl hydropersulfides can be a “hyperactivated” form of a thiol [10,43,63]. That is, some of the biologically relevant chemistries associated with thiol function could be accentuated when it is converted to a hydropersulfide. For example, thiol conversion to a hydropersulfide can increase the nucleophilicity of the reactive sulfur atom [40], make the sulfur a better reductant [64] and, although not yet confirmed, likely forms a better metal ligand (most probably with “soft” metals). It is worth noting, however, that these statements are primarily based on species “free” in solution and/or for thiols whose pK_a is > 7 (at pH 7). Protein thiols can be extremely reactive (as nucleophiles, reductants, metal ligands) due to the protein environment. Alkyl hydropersulfides are more acidic than the corresponding thiol by 1-2 pK_a units, indicating that a greater proportion of the anionic species (RSS^-) will be present when thiols are converted to the corresponding hydropersulfide. Although it can be envisioned that hydropersulfide formation on a protein active-site cysteine can lead to increased protein activity (due to, for example, increased nucleophilicity for thiols with $pK_a > 7$ at pH 7), this is not firmly established. Mustafa and coworkers [63] reported that hydropersulfide formation, via reaction of H_2S with the enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) led to an increase in enzyme activity as evidenced by an increase V_{max} (but no change in the K_m). It needs to be mentioned, however, that an increase in V_{max} with no change in K_m does not necessarily reflect the presence of a kinetically distinct enzyme but instead may be more consistent with the idea that H_2S simply reduced oxidized/inactive enzyme leading to an increase in the amount of total active enzyme (thus an increase in V_{max} and no change in K_m). Consistent with this idea is the finding that hydropersulfide formation in a cysteine protease leads to an inhibited enzyme but further treatment with high levels of H_2S eventually leads to the fully reduced, active enzyme [43]. Regardless, the idea of “hyperactivated” activity in proteins remains to be established (at least with regards to the “normal” or established function of proteins). Indeed, currently most studies indicate that protein persulfide formation leads to an inhibition of thiol protein activity [for example, 65].

The fact that alkyl hydropersulfides could be trapped by electrophiles and characterized indicates that they have nucleophilic character [for example, 43,62]. The fundamental reactivity of a hydropersulfide would be expected to be a function of its protonation state.

As the anion (RSS^-), the terminal, negatively charged sulfur atom should be nucleophilic due to the negative charge and an α-effect (increase in reactivity due to adjacent electron pairs [for example, 66]). The nucleophilicity of a hydropersulfide (or persulfide anion) versus the corresponding thiol was clearly demonstrated recently when it was shown that significant reaction with H_2O_2 was only seen under conditions where GSSH was formed, not GSH or HS^- [40]. Also, it was reported that GSSH reacts with the biological electrophile 8-nitro-cyclic guanosine monophosphate (8-NO_2-cGMP) to displace the nitro group, eventually forming the 8-SH-cGMP [40]. Under the conditions of this study, other thiols, including H_2S did not react with 8-NO_2-cGMP, consistent with the idea that free alkyl hydropersulfides are significantly more nucleophilic than free thiols. The observed increase in nucleophilicity with persulfides compared to the corresponding thiols for the above mentioned studies may be due simply to the fact that a larger percentage of the persulfide anion is present compared to the thiolate anion under the experimental conditions. Whether persulfide anions are inherently more nucleophilic than the corresponding thiolate anions (possibly due to an α-effect) remains to be determined.

The protonated alkyl persulfide species (RSSH) would not be expected to be as nucleophilic as the persulfide anion species. However, RSSH would be expected to have electrophilic character. That is, nucleophilic attack on either of the sulfur atoms is possible. The electrophilic character of RSSH has been proposed in the reaction of cyanide ion (CN^-) with RSSH to give the corresponding thiol and thiocyanate (^-SCN) (**Reaction 4**) [for example, 67,68].

$$RSSH + CN^- \rightarrow RSH + {}^-SCN \quad (4)$$

Importantly, the reaction of CN^- with the RSSH species to give thiocyanate (sometimes referred to as “cyanolysis”) has been used as evidence for the presence of a hydropersulfide in proteins [for example, 69,70,71] (more recently, a unique biotin switch assay was developed by Zhang et al. [72], which was successfully applied for the detection of protein-bound cysteine persulfides in cells [40]). The mechanism of thiocyanate formation from the reaction of CN^- and hydropersulfide is intriguing and potentially important since it represents a fundamental reaction of hydropersulfides. This reaction can be envisioned to occur in several ways (**Figure 2**).

Direct attack at the sulfane sulfur by CN^- (**Figure 2a**) would lead directly to the established thiocyanate and thiol products. (Note: sulfane sulfur is defined as a sulfur-bonded sulfur atom formally with six-electrons [73] and in this case the terminal sulfur atom). This mechanism would imply greater electrophilicity associated with the sulfane sulfur atom. Alternatively, attack of CN^- can occur reversibly at either sulfur atom eventually leading to the thermodynamically most stable products, which in this case would be ^-SCN and the corresponding thiol (**Figure 2b**). Finally, tautomerization of a persulfide to give a thiosulfoxide (RS(S)H) would generate a reactive “singlet sulfur” sulfane species which would be expected to be very electrophilic and reactive towards nucleophiles such as CN^- (**Figure 2c**) [73]. The electrophilic character of sulfane sulfur species was recently used in the development of reaction-based fluorescent probes for sulfane sulfur [74].

The mechanisms shown in **Figure 2** are perhaps problematic when considered in a biological context. For example, when comparing RS^- versus HS^- as possible leaving groups, it may be predicted that HS^- will be superior to RS^- in this regard (since the pK_a of a typical, free RSH is > 7 and the pK_a of H_2S is approximately 7, indicating that the HS^- anion is more stable than the RS^- anion and therefore a better leaving group). Discounting other factors, this predicts a reaction at the internal (rather than the terminal) sulfur atom at pH 7. Of course, in proteins, the pK_a values of the conjugate acids of the leaving RS^- groups may vary significantly, making it hard to predict which would be a better leaving group, RS^- (or Protein-S^-) or HS^-. For the analogous reaction of CN^- with unsymmetrical disulfides (RSSR'), the products are primarily based on the formation of the most stable thiolate anion [75], which would predict predominant nucleophilic attack at the internal sulfur atom for RSSH. It can be argued that the terminal sulfur atom is less sterically hindered, therefore favoring reaction at that site. Indeed, sterics have been reported to play a role in this reaction [76]. Although the reaction could be under thermodynamic control (as would be the case for reaction **b** in **Figure 2**) predicting that the product would be ^-SCN and the thiol, the relevance of this mechanism to a biological system may be questionable since many other competing reactions associated with the products are possible, and this may preclude setting up equilibrium processes. Finally, the tautomerization of a hydropersulfide to a thiolsulfoxide has been examined theoretically and the favorability of this process appears to depend significantly on the molecule [77-80]. Generally, using HSSH as a model hydropersulfide, it appears that the thiosulfoxide tautomer (H_2SS) is higher in energy than the corresponding hydropersulfide (HSSH) by approximately 20-40 kcal/mol. Interestingly however, for disulfide difluoride (FSSF) the thiosulfoxide (F_2SS) is reported to be more stable than the disulfide by a few kcal/mol indicating the relative stability of these two isomers can be significantly altered by its elemental composition (and possibly a protein environment). Regardless, it is clear that RSSH possesses an electrophilic sulfur atom that is capable of reacting readily with nucleophiles (such as CN^-).

One-Electron Redox Chemistry of Persulfides

Thiyl radicals (RS·) are common in biological systems, mostly present in oxidizing enzymes such as ribonucleotide reductase [for example, 81]. The reduction potentials for thiyl radicals have been reported to be slightly less than 1 volt (i.e. for the $GS\cdot,H^+/GSH$ couple, $E°' = 0.92$ V, pH 7.4 [82]) indicating that thiyl radical species can be reasonable one-electron oxidants (and that thiols/thiolates are only moderate one-electron reductants). Although the reduction potential for RSS· is not reported, it has been shown that $RSSH/RSS^-$ is more reducing than RSH/RS^-. The S-H bond of RSSH is approximately 21-22 kcal/mole weaker than the S-H bond of RSH [64] (approximately 70 kcal/mole for RSS-H versus 92 kcal/mole for RS-H [83]). The reason for the decrease in bond strength for RSSH can be attributed to the increased stability of the perthiyl radical (RSS·) compared to the thiyl radical (RS·). The odd electron of RSS· is resonance stabilized by the adjacent sulfur atom, an affect unavailable to the odd electron of a thiyl radical (**Figure 3**). The resonance effect responsible for the stability associated with the perthiyl radical is akin to the effect that stabilizes, for example, nitroxide radicals ($R_2NO\cdot$).

It is important to note that the perthiyl radical has an absorbance at 374 nm (ε_{374} = approx. 1700 $M^{-1}cm^{-1}$) that is distinct from those typically observed for thiyl radicals, which allows facile detection of perthiyl intermediates in reactions [64]. The more stable perthiyl radical is responsible for the increased propensity for H-atom donation and reductive electron transfer to oxidants by hydropersulfides compared to the analogous thiol species [64] thus allowing hydropersulfides to be superior antioxidants. That is, reaction of hydropersulfides with one-electron oxidants (such as an organic radical species, R·) is more favorable than the analogous reaction with thiols (**Reaction 5**) and due to the relative stability of the perthiyl radical, further oxidation chemistry via perthiyl-mediated oxidation is diminished.

$$RSSH/RSS^{-} + \mathrm{R}\cdot(\mathrm{oxidant}) \rightarrow RSS\cdot + RH/\mathrm{R}^{-} \quad (5)$$

As expected, single electron reduction of metalloproteins is also significantly more prevalent with persuflides than with thiols. For example, hydropersulfides can interact with ferric heme proteins resulting in reduction to the ferrous species [i.e. 43,64,84]. Although H_2S and thiols can also serve as reducing agents, it appears that hydropersulfides are far superior in this regard. An alkyl hydropersulfide was also reported to inhibit the activity of the heme protein cytochrome P450 by altering the heme moiety by an as yet undetermined fashion [85].

As shown above, one-electron oxidation of hydropersulfides/persulfide anion generates the perthiyl radical and, generally, perthiyl radicals are presumed to be less oxidizing than the corresponding thiyl radicals. However, perthiyls will react with O_2 ($k = 5 \times 10^6\ M^{-1}s^{-1}$) to give initially a perthioperoxyl radical (RSSOO·) and ultimately to sulfate (SO^{2-}_4) [86]. It is presumed that sulfate formation results from rearrangement of RSSOO· to give the sulfonyl-type radical (RSS(O)O·. In a purely chemical system, SO^{2-}_4 generation is presumed to result from bimolecular dimerization of RSS(O)O· species followed by further degradation. The bimolecular nature of this process clearly limits its relevance in biological systems. Perthiyl radicals will also react with biological reductants such as ascorbate ($RSS\cdot + AH^- \rightarrow RSSH + A^{\cdot-}$, $k = 4.1 \times 10^6\ M^{-1}s^{-1}$), although expectedly at a decreased rate compared to the more reactive thiyl radicals ($RS\cdot + AH^- \rightarrow RSSH + A^{\cdot-}$, $k = 4.9 \times 10^8\ M^{-1}s^{-1}$) [86]. Although speculative at this time, it is also worthwhile to mention that akin to thiyl radical species [87], perthiyl radicals may also react rapidly with thiolates present in a biological milieu to give a trisulfide radical anion ($RSSSR^{\cdot-}$). In the case of disulfide radical anions ($RSSR^{\cdot-}$, formed from the reaction of $RS^{\cdot-}$ with RS^-), these species are very good reductants, capable of reducing O_2 to superoxide (O^{-}_2). Whether the equivalent chemistry can occur with putative $RSSSR^{\cdot-}$ species remains to be determined.

Two-Electron Redox Chemistry of Hydropersulfides

Oxidation of alkyl hydropersulfides with $2e^-$ oxidants such as hydrogen peroxide (H_2O_2) occurs readily. Indeed, RSSH species are reported to react with H_2O_2 significantly faster than thiols and overexpression of the persulfide synthesizing enzyme CSE affords protection of cells from H_2O_2 toxicity [40]. Although not established, it appears likely that reaction of alkyl hydropersulfides/persulfide anion with H_2O_2 will give an alkyl perthiosulfenic acid (not to be confused with a persulfenic acid, RSOOH, *vide supra*) (**Reaction 6**).

$$RSSH + H_2O_2 \rightarrow RSSOH + H_2O \quad (6)$$

Furthermore, analogous to thiol chemistry where sulfenic acids react with thiols to give H_2O and disulfides [for example, 88], perthiosulfenic acids are likely unstable with respect to reaction with excess persulfides or thiols to give a di-substituted tetrasulfide/trisulfide species (**Reaction 7,8**).

$$RSSOH + R'SH \rightarrow RSSSR' + H_2O \quad (7)$$

$$RSSOH + R'SSH \rightarrow RSSSSR' + H_2O \quad (8)$$

Interestingly, significant levels of tri-, tetra- and higher polysulfides have been detected in mammalian tissues and plasma, alluding to the possible and significant relevance of oxidative polysulfide generation from initial hydropersulfide biosynthesis [40].

Figure 4 schematically depicts all of the chemistry discussed for hydropersulfides. To be sure, some of this chemistry is speculative or currently unknown and, for certain aspects, based on extending known thiol chemistry. Regardless, this scheme can serve as a starting point and basis of further investigation and, at the very least, depicts the diverse chemistry of hydropersulfides.

The Chemistry of Dialkylpolysulfides and Alkyl Hydropolysulfides

The formation of H_2S in biological systems allows for the formation of alkyl hydropersulfides/persulfide anions, which in turn allows for the formation of dialkylpolysulfides (R-S_n-R, n>2) (**Reactions 2,6,7,8**) (although as mentioned above, alkyl hydropersulfides can be made via H_2S-independent mechanisms, which may have greater biological importance). Moreover, it appears that dialkylpolysulfides are biologically prevalent [40]. Therefore, it is worth considering the chemistry of dialkylpolysulfides that may be responsible for their biological fate/function (if any). Previous interest in dialkylpolysulfides, at least from a biological perspective, is due to the fact that they are major components of garlic and other foods with presumed health benefits [for example 89]. Interestingly, dialkylpolysulfides have also been implicated as toxic species capable of eliciting hemolytic anemia in certain animals [for example, 90]. Regardless, the chemistry responsible for either the presumed beneficial or deleterious effects of dialkylpolysulfides is not established.

Like hydropersulfides, dialkylpolysulfides are capable of acting as electrophiles. Reaction of a dialkylpolysulfide (e.g. glutathione trisulfide, GSSSG) with a nucleophilic thiol results in a substitution reaction of the type shown below for a dialkyltrisulfide (**Reaction 9,10**).

$$RSSSR + R'SH \rightarrow RSSH + R'SSR \quad (9)$$

$$RSSSR + \mathrm{R}'SH \rightarrow RSH + \mathrm{R}'SSSR \quad (10)$$

The factors that govern the position of the nucleophilic attack, in the case of a dialkyltrisulfide, have not to our knowledge been established. It is likely that steric effects would favor nucleophilic attack on the sulfur atom(s) furthest away from the R-group and are bound to two sulfurs with large electronegativities (as in **Reaction 10**). However, since RSSH is more acidic than RSH (making RSS^- a better leaving group than RS^-) it may be expected that this will favor nucleophilic attack on a sulfur atom that would allow expulsion of an alkyl hydropersulfide/persulfide anion (or alkyl hydrogenpolysulfide, RS-S_n-H as in **Reaction 9**). Significantly, Massey and coworkers [84] reported that **Reaction 9** was responsible for an interesting effect whereby GSSG was able to enhance the rate of reduction of ferric cytochrome c by GSH. This enigmatic finding initially indicated that an oxidant, GSSG, was able to assist the reductant, GSH, in reducing ferric cytochrome c. Massey was able to explain this phenomenon by showing that commercial GSSG contained significant amounts of the polysulfide GSSSG, which can react with GSH to give GSSH (via **Reaction 9**). As discussed previously, hydropersulfides/persulfide anions are superior one-electron reductants compared to thiols, therefore allowing more facile ferric cytochrome c reduction. Reduction of ferric cytochrome c by an alkyl hydropersulfide/persulfide anion would generate the corresponding alkyl perthiyl radical (RSS·) (**Reaction 11**).

$$RSS^-/RSSH + Fe^{III} - cyt \quad \mathrm{c} \rightarrow Fe^{II} - cyt \quad \mathrm{c} + RSS\cdot + \left(\mathrm{H}^+\right) \quad (11)$$

In a simple chemical system, RSS· may have the opportunity to dimerize to give, in this case, the dialkyltetrasuflide (RSSSSR) (**Reaction 12**) (as mentioned above, reaction with O_2 is possible as well).

$$RSS\cdot + RSS\cdot \rightarrow RSSSSR \quad (12)$$

Further reaction of RSH with the dialkyltetrasulfide would then generate, among other possible products, the dialkyltrisulfide and an alkyl hydropersulfide (**Reaction 13**).

$$RSSSSR + \mathrm{R}'SH \rightarrow \mathrm{R}'SSSR + RSSH \quad (13)$$

Therefore, **Reactions 12** and **13** allows GSSSG to be catalytic in facilitating the overall reduction of ferric cytochrome c by GSH (via hydropersulfide formation). Of course, in a biological system, many other processes/reactions may intercept reactive intermediates like alkyl perthiyl radicals or even hydropersulfides. However, this example is important in illustrating the redox exchange chemistry that exists for dialkylpolysulfides.

The above discussion indicates that dialkylpolysulfides can act as electrophiles. Indeed, due to the enhanced ability for hydropolysulfides/polysulfide anions (such as an alkyl hydropersulfide) to act as leaving groups in substitution reactions, dialkylpolysulfides are assuredly more electrophilic than the corresponding disulfides.

The presence of extended dialkylpolysulfides (i.e. dialkyltetrasulfides, dialkylpentasulfides, etc.) allows for the formation of alkyl hydropolysulfides with more than two sulfur atoms. For example, reaction of a dialkyltetrasulfide with a thiol can give, among other possible products, an alkyl hydrotrisuflide (**Reaction 14**).

$$RSSSSR + \mathrm{R}'SH \rightarrow RSSSH + \mathrm{R}'SSR \quad (14)$$

The factors that make hydropersulfides more reactive than the corresponding thiols (as nucleophiles, acids, reductants, *vide supra*) should also make alkyl hydrotrisulfides (RSSSH) more reactive than alkyl hydropersulfides. For example, it is known that hydropersulfides are more acidic than the corresponding thiol (*vide supra*). It is also reported that for a series of dihydropolysulfides (HS_nH, n = 1-5), increasing the number of sulfur atoms leads to decreasing pK_a (H_2S pK_a = 7.0; H_2S_2 pK_a = 5.0, H_2S_3 pK_a = 4.2, H_2S_4 pK_a = 3.8, H_2S_5 pK_a = 3.5) [91]. It seems likely that increasing the number of consecutive sulfur atoms will also lead to increased nucleophilicity and one-electron reducing capability as well.

Comparison of Persulfide Chemistry with Peroxide Chemistry

Clearly, much is known regarding the chemistry and biological activity of peroxides (ROOH, ROOR, HOOH) [for example, see 92]. Based on their structural similarity there may be the tendency to extrapolate known peroxide (e.g. ROOH) chemistry to that of persulfide chemistry. To some extent, this seems reasonable. For example, H_2O_2 acts as a nucleophilic oxidant under basic conditions via HOO^- chemistry and an electrophilic oxidant under acidic conditions via $HOOH\ ^+{}_2$ (or the equivalent) chemistry (**Figure 5**).

As mentioned above, the same scenario is proposed for hydropersulfides (i.e. as the anion it is a nucleophilic oxidant and under acidic conditions the neutral (or protonated) form is an electrophilic oxidant, **Figure 4**). It is worth mentioning, however, that the pK_a of RSSH is in the range of 6-8 (in the physiological pH range) whereas the pK_a of H_2O_2 is 11.8 and far removed from physiological pH. Thus, the anionic chemistry of hydropersulfides is clearly more accessible under biological conditions compared to H_2O_2 (and other alkyl hydroperoxides, ROOH). Although there are some potentially important chemical similarities between persulfides and peroxides, there are also some important differences as well. These chemical differences are best illustrated by comparing some of the basic physical constants associated with these two functional groups. The O-O bond dissociation energy (BDE) of a peroxide is dependent on the substitution and in the range of 36-51 kcal/mole (the BDE for HO-OH is 51 kcal/mole and the BDE for CH_3O-OCH_3 is 36 kcal/mole). On the other hand, the BDE for an S-S bond in a persulfide is significantly higher. The BDE for HS-SH is 66 kcal/mole and for CH_3S SCH_3 the BDE is 74 kcal/mol [83]. The low BDE for peroxides (at least compared to persulfides) can be explained by the weakening of the O-O bond due to repulsion between the adjacent lone pairs of electrons on the adjacent oxygen atoms. Since the S-S bond is significantly longer compared to the O-O bond of a peroxide (2.05 angstroms versus 1.48 angstroms, respectively) and the electron pairs are in larger, more diffuse orbitals, the electron-pair repulsion is lessened in the case of the persulfides.

Significantly, increasing the number of sulfur atoms (to make polysulfides) decreases the S-S BDE. For example, the S-S BDEs for the series HS-SH, HS-SSH and HSS-SSH are 66, 50 and 33.6 kcal/mole, respectively [83]. The rationale for this is the same as was given previously for explaining the stability of RSS· compared to RS· (**Figure 3**). Thus, any reaction whose product is a perthiyl radical will be more favorable than a corresponding reaction that generates a simple thiyl radical product. By analogy, polyoxides should follow the same trends. However, the stability of the polyoxides (i.e. H_2O_3, etc.) is significantly lower with only scant and controversial reports of their biological relevance [for example, 93,94]. To illustrate this, consider that the HO-OH, HO-OOH and HOOOOH BDEs are reported to be only 51, 25 and 7-12 kcal/mole, respectively [95]. Thus, it appears that polysulfide species are biologically accessible whereas polyoxides, if formed, would be considerably less stable.

The reactivity of the perthiyl radical and the peroxyl radical are also quite different. For example, the S-H BDE for HSS-H is approximately 70 kcal/mol, whereas the O-H BDE for HOOH is 90 kcal/mol [83]. Thus, HOO· (which is simply protonated superoxide, $O^{-}_{2} + H^{+}$) is a significantly better one-electron oxidant compared to HSS· (or RSS·). This also implies that RSSH can act as an anti-oxidant (as mentioned previously) via H-atom donation, whereas ROOH is never considered an antioxidant and has always been viewed as an oxidant. That is, reaction of RSSH with a strong one-electron oxidant will quench the oxidant and generate a fairly stable radical, RSS· (RSSH + R· → RSS· + R-H), which is a poor oxidant. On the other hand, reaction of a strong one-electron oxidant with ROOH in the equivalent manner will give another strong oxidant ROO·.

One of the most studied reactions of peroxides is the Fenton reaction whereby a peroxide (such as H_2O_2) is reduced by a metal to generate the oxidized metal and the very strong one-electron oxidant, hydroxyl radical (HO·) (**Reaction 15**) [92].

$$\begin{gathered} \mathrm{H_2O_2 + M^n \rightarrow HO\cdot + M^{n+1} + HO^-} \\ \left(\mathrm{M^n} = Fe^{2+}, Cu^{1+}; Mn^{+1} = Fe^{3+}, Cu^{2+}\right) \end{gathered} \quad (15)$$

From a pathophysiological/toxicological perspective, the Fenton reaction may be important since it represents a counter intuitive process whereby reductants (e.g. reduced metals and biological species capable of reducing metals) are able to create a potent oxidant, HO·. The potential for the H_2O_2,H^+/HO·,H_2O (pH 7, vs NHE) couple is 0.320 V [96]. Presumably, the relative favorability of this process is due in part to the breaking of a weak O-O bond and the generation of the extremely stable H_2O molecule. The reduction potential for HSSH has not, to our knowledge, been reported so a direct comparison to H_2O_2 is not yet possible. However, the reduction potential for the RSSR/$RSSR^{\cdot-}$ couple is reported to be approximately -1.4 V, or extremely unfavorable [97,98]. It should be noted that the product of the one-electron reduction of RSSR is not chemically analogous to that shown for the Fenton reaction (HO· + HO^-). The product of RSSR reduction is typically viewed as being the disulfide radical anion ($RSSR^{\cdot-}$) as opposed to the thiyl radical and thiolate/thiol species (RS· + RS^-). It has been shown that the equilibrium constant for the reaction of thiyl radical

and thiolate (**Reaction 16**) is in the range of 33-2000, depending on the R group, indicating a predominance of the radical anion dimer [99].

$$RS\cdot + RS^- \rightleftharpoons RSSR^{\cdot -} \quad (16)$$

$RSSR^{\cdot -}$, is a strong one-electron reductant as the extremely negative reduction potential for RSSR indicates, (as opposed to the strong oxidant, HO·, formed form the Fenton reaction). Thus, an oxidizing "Fenton-like" reaction associated with persulfides appears unlikely. Finally, the two-electron reduction potential for H_2O_2 is 1.32 V [92] and for ROOH approximately 1.28 V (both pH 7) [100], both of which are positive and relatively favorable. Thus, reactions whereby substrates are oxidized by 2-electrons can occur readily with peroxides (although there can be kinetic restrictions to this chemistry). On the other hand, the 2-electron reduction potential for RSSR is only approximately 0.08 V [98] (note: the 2-electron reduction potential for H_2S_2 is not reported), indicating that direct two-electron oxidations via persulfides are not as favorable as it is with peroxides. Based on this brief discussion, hopefully it is evident that there are similarities between persulfides/polysuflides and peroxides/polyoxides but that the biological relevance or implications of these chemistries can be distinct and even opposite. Thus, care must be taken when extrapolating peroxide chemistry to explain the biological mechanisms of persulfides.

Possible Biological Implications of Persulfide and Polysulfide Generation

The chemical properties of persulfides and polysulfides discussed above indicate enhanced and distinct reactivity compared to thiols. Moreover, it is now clear that relatively high levels of these species are present in mammalian tissues and plasma. The overriding question that remains is: What are the functions/roles of per- and polysulfides present in biology? That is, why has Nature chosen to generate these species? Of course, it is far too early to present any definitive answers to this question. However, it is at least worthwhile to speculate on the biological functions and roles of these species since something is known about their chemical properties and it is reasonable to contend that their functions are based on their unique or novel chemistries.

As mentioned earlier, it has been proposed that hydropersulfide generation could represent a "hyperactivation" of thiol reactivity where aspects of biological thiol chemical reactivity are enhanced. On the other hand, as mentioned above, hydropersulfide formation can also lead to an inhibition of thiol protein activity, as has been reported numerous times already. To date, protein hyperactivation has not been unequivocally demonstrated (*vide supra*). Based on the recent findings that numerous proteins contain persulfides [6,40], it is reasonable to suspect that formation of these species is a general phenomenon that alters the activity/function of numerous systems within a cell (likely as the result of a general cellular stress or signal). However, it should be noted that some methods used for identification of protein persulfides are questionable (see detailed discussions in [62,72]). It is necessary to develop more sensitive, selective, and 'easy to use' methods for persulfide detection. With respect to hydropersulfides (which are the precursor to all polysulfides and higher hydropolysulfides) it is worthwhile at this point to "step back" and examine the likely biological conditions conducive to its biosynthesis, the consequences of its formation and its possible

physiological role. First and foremost, it needs to be stressed that a hydropersulfide is OXIDIZED with respect to a thiol. That is, the formation of an -SSH group requires an oxidation of an -SH function. This is analogous to the relationship between a thiol, RSH, and a corresponding disulfide, RSSR. Thus, generation of a hydropersulfide requires an initial oxidizing event. In the case of biological hydropersulfide formation, the oxidizing event (or the oxidized species that must be present) can be the formation of Cys-SS-Cys from Cys-SH. As mentioned above, CSE-mediated conversion of Cys-SS-Cys to Cys-SSH is a major pathway of biological persulfide/polysulfide generation. The levels of intracellular Cys-SS-Cys have been reported to be in the range of 30 μM (in colon epithelial cells) [55]. Significantly, the K_m of Cys-SS-Cys for CSE is also approximately 30 μM [47], indicating that Cys-SS-Cys levels may be regulatory for Cys-SSH generation. As described previously, Cys-SS-Cys levels are regulated independently from the GSH/GSSG system, with Cys/Cys-SS-Cys ratios being significantly lower than GSH/GSSG ratios. It is tempting to speculate that maintaining higher levels of Cys-SS-Cys may be due to an important biological requirement to maintain Cys-SSH generation capabilities (with subsequent formation of other persulfide/polysulfide species). If the presence of the oxidized thiol Cys-SS-Cys is an important regulatory factor for hydropersulfide formation, then factors that promote or cause an increase in Cys-SH oxidation to Cys-SS-Cys can promote increased hydropersulfide formation (with subsequent increases in GSSH, Protein-SSH etc.). These factors can be, of course, oxidants such a peroxides or oxidizing nitrogen oxides (e.g. NO_2, N_2O_3, etc.). As described above, hydropersulfides (and their more prevalent anions) are superior one- and two-electron reductants compared to their corresponding thiols. What this indicates is that oxidizing conditions (e.g. high H_2O_2 or high Cys-SS-Cys levels) can lead to the direct formation of oxidized thiol species that are powerful reductants (e.g. Cys-SSH, GSSH, etc.). Moreover, normal cellular reducing conditions would lead to reduction back to the normal thiol. That is, the direct biosynthesis of a potent reductant, Cys-SSH and derived species, can be the result of an oxidizing environment. This represents a novel and fascinating possibility that could be an important and immediate first step in protecting cells from the immediate ravages of oxidants. If indeed persulfide/polysulfide generation represents a protective strategy, the next question would be: Why would persulfides/polysulfides be protective? As shown by Everett and Wardman [64], hydropersulfides are potent antioxidants due to their reducing capabilities. For example, GSSH could directly scavenge H_2O_2 [40] or rapidly quench one-electron oxidants (such as oxidizing radical species). It is worth pointing out a unique factor associated with the antioxidant potential of hydropersulfides – oxidation of persulfides followed by reduction can generate back the original thiol species. As shown in **Figure 6**, persulfide formation in proteins could make them especially reactive with oxidants (in this case H_2O_2 will be used as a prototypical oxidant). Step-wise oxidation of a persulfide with H_2O_2 leads to perthiosulfenic, perthiosulfinic and perthiosulfonic acids. All of these species would likely inhibit protein activity. However, under normal reducing conditions, all of these species can be reduced to give back the original thiol proteins. In the case of thiol protein oxidation, for the most part, oxidation past the sulfenic acid state would irreversibly inhibit the thiol protein and regaining biological activity would likely require *de novo* protein synthesis. Thus, the sacrificial sulfur associated with protein persulfide generation can allow oxidation and protein thiol regeneration when normal cellular redox conditions are re-established.

Indeed, protection of cysteine phosphatase activity against irreversible oxidation by H_2O_2 via presumed hydropersulfide formation has been observed, although the mechanistic details of this protection have not been discussed [65,101]. Significantly, the oxidation of alkyl hydropersulfides and eventual reductive regeneration of the corresponding thiol has further biological precedence. The sulfide-metabolizing enzyme ETHE1 has been reported to specifically oxidize hydropersulfides to the corresponding perthiosulfonic acid, which then is reduced to give sulfite (SO^{2-}_3) and thiol [59,102].

Thus, protection of cells, proteins and biological macromolecules from oxidants via persulfide formation can be due to two factors: 1) direct scavenging of oxidants, and 2) formation of a sacrificial sulfur species in proteins. **Scheme 1** depicts these possible roles of persulfide/persulfide formation in biological systems. Due to the relative newness of this field, the proposed protection afforded by biological persulfide generation currently has only scant support [40] and the ideas presented herein will need to be tested carefully. However, the idea that hydropersulfide generation can play a protective role makes chemical sense and seemingly can make physiological sense since it combines the “modified” thiol chemistry alluded to earlier with a physiological protective function (that is not related to the actual protein function). Regardless, there is little doubt that the general area of investigation of the mechanism(s) of per- and poly-sulfide function (as well as H_2S function) will be a topic of significant research interest for many years to come.

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Abbreviations

sGC	soluble guanylate cyclase
NHE	normal hydrogen electrode
$MbFe^{III}$	methemoglobin
HbI	H_2S-binding hemoglobin
MPO	myeloperoxidase
$HbFe^{II}$-O_2	oxyhemoglobin
sulfHb	sulfhemoglobin
sulfMb	sulfmyoglobin
CcO	cytochrome c oxidase
SQR	sulfide:quinone oxidoreductase
CSE	cystathionine γ-lyase

CBS	cystathionine βsynthase
Trx1	thioredoxin
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
8-NO_2-cGMP	8-nitro-cyclic guanosine monophosphate
BDE	bond dissociation energy
NHE	normal hydrogen electrode

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- Hydrogen sulfide (H_2S) is proposed to be an endogenously synthesized small-molecule signaling agent involved in a variety of physiological functions.
- Many of the biological actions proposed for H_2S may be due to the presence of other sulfur species such as per- and/or poly-sulfides.
- Persulfides (RSSH) and related species have unique chemistry distinct from other biologically relevant sulfur species.
- The biological utility of persulfides may be a result of their enhanced ability (compared to thiols) to serve as nucleophiles and reductants.

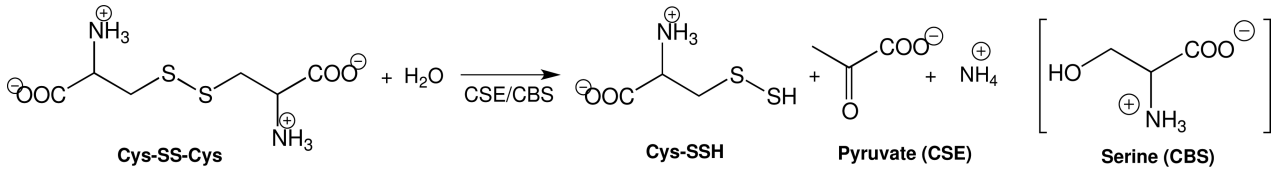


Figure 1.
Generation of Cys-SSH from Cys-SS-Cys.

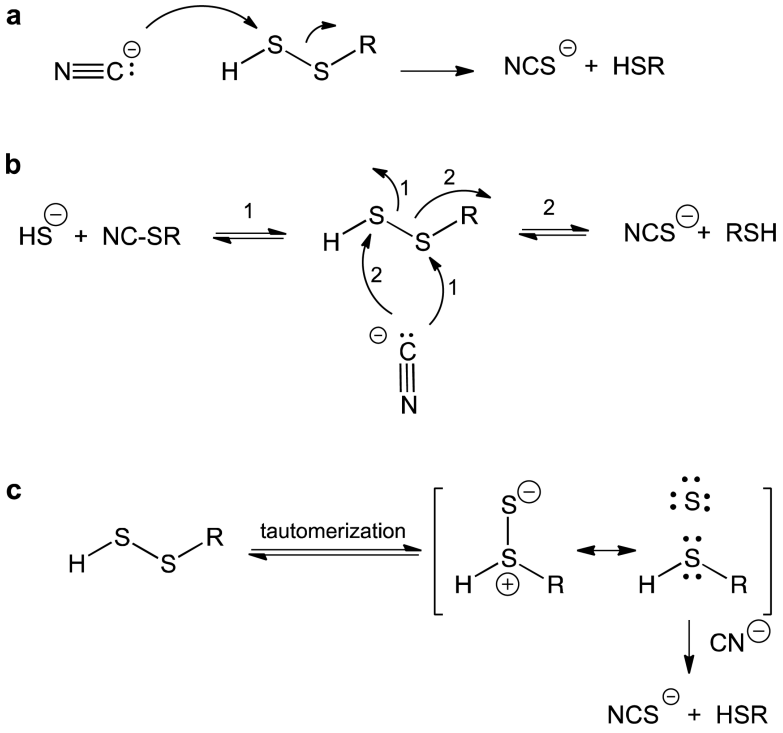


Figure 2.
Possible mechanisms of $^{-}$SCN formation from reaction of CN^{-} with a persulfide.

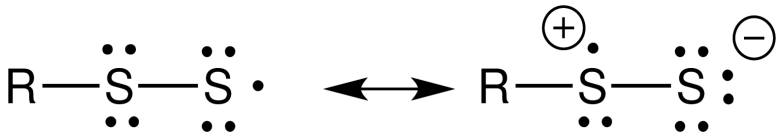


Figure 3.
Resonance stability of the perthiyl radical

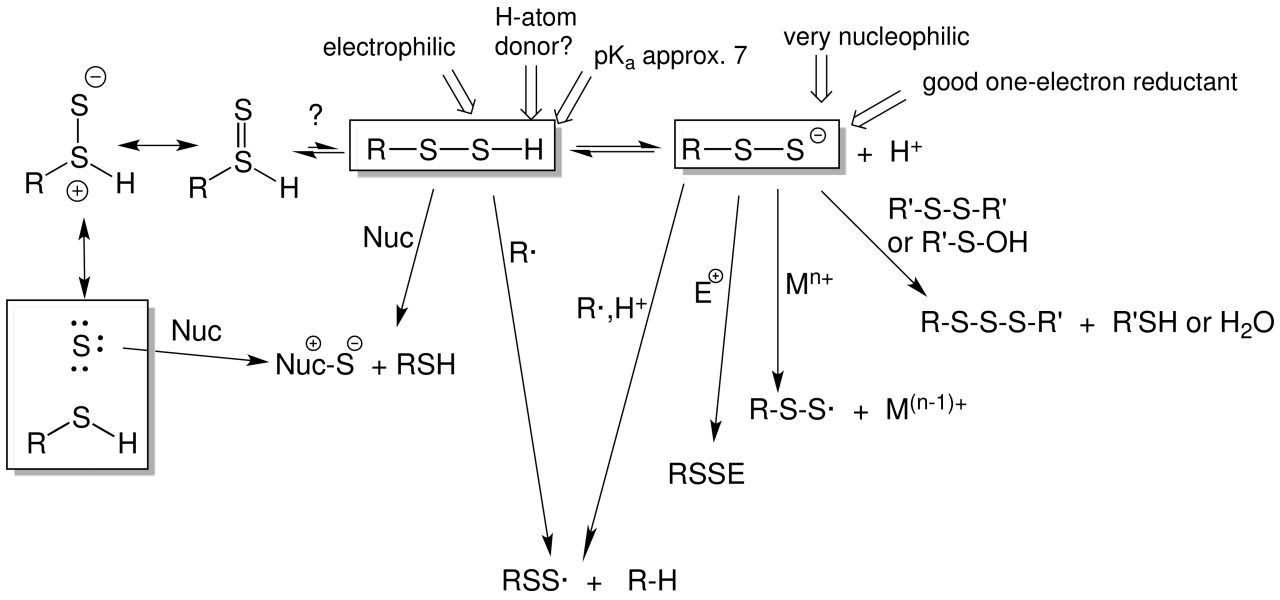


Figure 4.
Possible/putative biologically relevant reactions of persulfides.

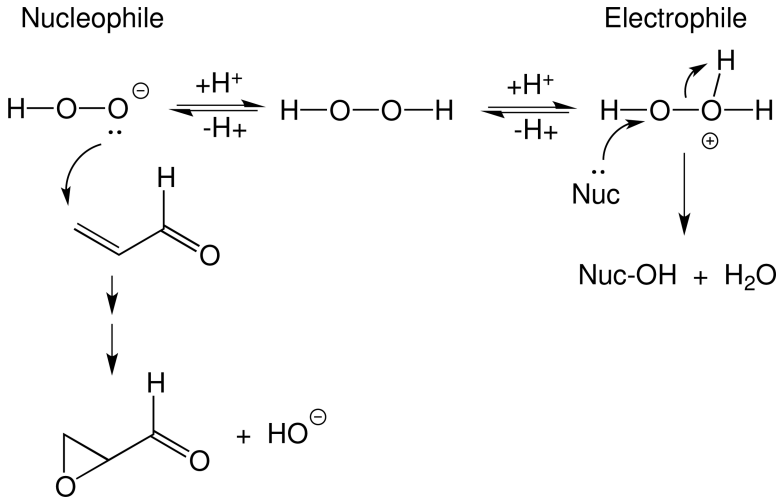


Figure 5.
H_2O_2-mediated oxidations under basic and acidic conditions.

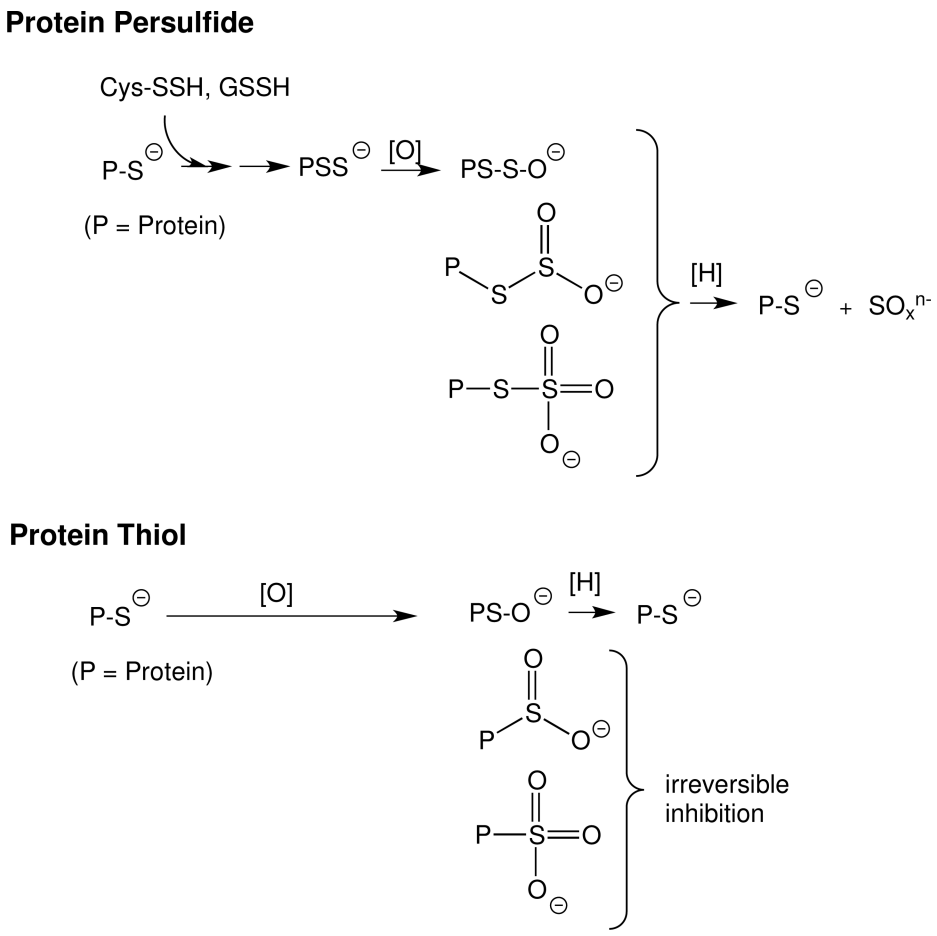
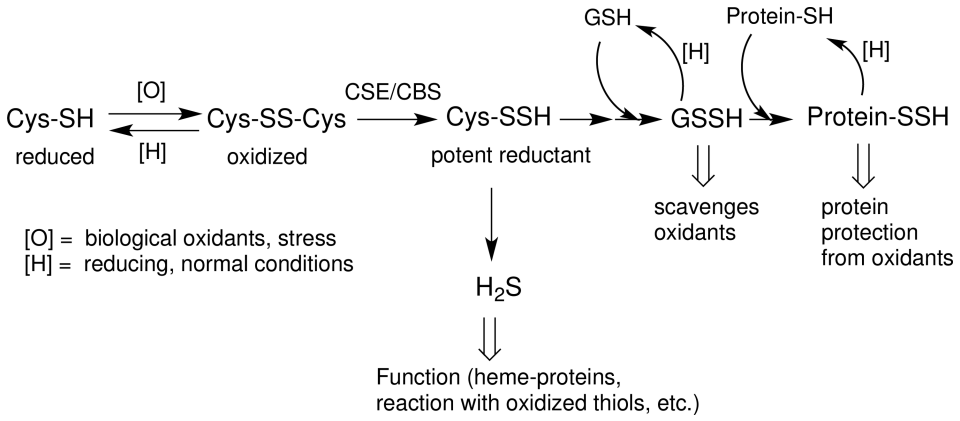


Figure 6.
Possible role of protein persulfide formation.



Scheme 1.

Possible roles of persulfide and protein persulfide generation.