Hydrogen Sulfide Biochemistry and Interplay with Other Gaseous Mediators in Mammalian Physiology

Alessandro Giuffrè and João B. Vicente

CNR Institute of Molecular Biology and Pathology, Rome, Italy
Instituto de Tecnologia Química e Biomolecular António Xavier, NOVA University of Lisbon, Av. da República (EAN), 2780-157 Oeiras, Portugal

Correspondence should be addressed to Alessandro Giuffrè; alessandro giuffre@uniroma1.it
and João B. Vicente; jvicente@itqb.unl.pt

Received 1 December 2017; Accepted 13 March 2018; Published 27 June 2018

Hydrogen sulfide (H₂S) has emerged as a relevant signaling molecule in physiology, taking its seat as a bona fide gasotransmitter akin to nitric oxide (NO) and carbon monoxide (CO). After being merely regarded as a toxic poisonous molecule, it is now recognized that mammalian cells are equipped with sophisticated enzymatic systems for H₂S production and breakdown. The signaling role of H₂S is mainly related to its ability to modify different protein targets, particularly by promoting persulfidation of protein cysteine residues and by interacting with metal centers, mostly hemes. H₂S has been shown to regulate a myriad of cellular processes with multiple physiological consequences. As such, dysfunctional H₂S metabolism is increasingly implicated in different pathologies, from cardiovascular and neurodegenerative diseases to cancer. As a highly diffusible reactive species, the intra- and extracellular levels of H₂S have to be kept under tight control and, accordingly, regulation of H₂S metabolism occurs at different levels. Interestingly, even though H₂S, NO, and CO have similar modes of action and parallel regulatory targets or precisely because of that, there is increasing evidence of a crosstalk between the three gasotransmitters. Herein are reviewed the biochemistry, metabolism, and signaling function of hydrogen sulfide, as well as its interplay with the other gasotransmitters, NO and CO.

1. Introduction

1.1. Brief Historical Account of Hydrogen Sulfide. Hydrogen sulfide (H₂S) is known in popular culture as a toxic poisonous gas and a source of foul smell, such as that of rotten eggs, sewers, swamps, and volcanic sources. Ancient alchemic practices produced several preparations now known to release hydrogen sulfide, for example, the H₂S- and ammonia-rich gases released upon distillation to obtain Liqueur Hepatis (Balsam of the Soul), made from limestone and camel dung. In his treaty on workers’ diseases “De Morbis Artificum,” the 17th–18th century Italian physician Bernardino Ramazzini first described painful eye inflammation often leading to secondary bacterial infection and eventually blindness in workers who cleaned privies and cesspits [1]. Ramazzini hypothesized that, when the workers stirred the excrements, an unknown volatile compound was released which, besides having the undesired health effects, was also responsible for turning black their copper and silver coins on the surface. The same toxic volatile substance, originated in the Paris sewers, was likely responsible for a series of incidents in the late 18th century, ranging from mild eye and mucous membrane inflammation to severe asphyxia. Almost at the same time, in 1777, the Swedish chemist Carl Wilhelm Scheele described the stinking substance (sulfur air) resulting from the reaction of pyrite (ferrous disulfide) with a mineral acid [2].

While long considered a poisonous substance, not only hazardous for workers but also burdensome for some industrial processes (e.g., “oil souring” in oil extraction), hydrogen sulfide was in the late 20th century discovered to be endogenously formed in humans, and in the early 21st century, it...
was recognized as an extremely relevant signaling molecule in physiology and general biology.

1.2. General Chemistry of Hydrogen Sulfide. Hydrogen sulfide is a colorless flammable gas. It is a weak acid, existing in aqueous solution in equilibrium with hydrosulfide (HS⁻) and sulfide (S²⁻), according to pKₐ1~7.0 (H₂S/HS⁻) and pKₐ2~19 (HS⁻/S²⁻), at 25°C ([3] and references therein). At physiological pH values, the S²⁻ concentration in solution is therefore negligible. According to this equilibrium, at the physiological pH 7.4, c.a. 70% of hydrogen sulfide is in its HS⁻ form, the remainder occurring as H₂S. In the alkaline mitochondrial matrix (pH 8.0), HS⁻ reaches 92%, while the remaining 8% corresponds to H₂S. Contrarily, under the acidic conditions in lysosomes (pH 4.7), >99% of hydrogen sulfide is in its H₂S form and is slightly polar, allowing it to freely diffuse across and accumulate in aqueous or hydrophobic milieu such as biological membranes. Herein, unless otherwise stated, the terms “hydrogen sulfide” or “sulfide” and the abbreviation “H₂S” henceforth collectively designate the pool of the H₂S, HS⁻, and S²⁻ species.

H₂S is the sulfur species corresponding to the lowest sulfur oxidation state (−2). Sulfur, with its electronic configuration [Ne] 3s² 3p⁴, is a highly redox versatile element, with oxidation states ranging from −2 to +6 (as in sulfate), due to the six valence electrons. This versatility certainly accounts for its biological usefulness and is probably related to the major role of sulfur in the emergence and evolution of life on Earth ([4]). H₂S is a reducing species, displaying a reduction potential of −280 mV (pH 7.0, against the standard hydrogen electrode) for the two-electron HS⁻/S⁰ redox couple (−230 mV for H₂S/S⁰) ([5]), close to the values for the glutathione disulfide/glutathione (GSSG/GSH) and the cystine/cysteine redox couples.

Presently, despite major advances, the physiological H₂S concentrations are still a matter of debate, and the posited levels have been decreasing with the increasing sophistication and accuracy of the detection methods. Currently, free H₂S is reported to be submicromolar, although it may exist in equilibrium with a pool of labile sulfur-containing molecules that can liberate H₂S “on demand,” that is, under particular physiological conditions ([6]). To add more complexity to the role of H₂S in human (patho)physiology, it has become clear that a great part of the signaling effects attributed to H₂S (see below) actually results from the occurrence of persulfides and polysulfides, among other sulfur-containing molecules, that have been collectively termed “reactive sulfur species” (RSS). More details on the formation of persulfides and polysulfides, and their physiological relevance intertwined with that of hydrogen sulfide, are given below.

2. Hydrogen Sulfide Metabolism in Human Physiology

The reactivity and potential toxicity of hydrogen sulfide demand its levels to be kept under strict control. Indeed, even temporary imbalances in local hydrogen sulfide concentrations can trigger a cascade of cellular events with pathological consequences. The production and breakdown of H₂S is thus essentially ensured by specialized enzymes, tightly regulated and compartmentalized. Although H₂S is freely permeable to membranes and thus highly diffusible inside the cell, compartmentalization of its synthesis or breakdown could be relevant to ensure local effects in cellular organelles. A clue for this has been for instance provided by showing that the mitochondria-targeted H₂S donor AP39 ([10-oxo-10-(4-(3-thioxo-3H-1,2-dithiol-5yl)phenoxy)decyl] triphenylphosphonium bromide), consisting of the H₂S-releasing moiety ADT-OH (5-(4-hydroxyphenyl)3H-1,2-dithiole-3-thione)coupled to the mitochondria-targeting triphenylphosphonium (TPP⁺) moiety, protects mitochondria in oxidatively stressed endothelial cells more effectively than ADT-OH itself, not coupled to TPP⁺ and thus unable to specifically target mitochondria ([7]).

2.1. Human H₂S-Synthesizing Enzymes. The biogenesis of hydrogen sulfide in human physiology occurs via two major routes: by endogenous specialized enzymes and as an end-product or intermediate of microbial metabolic pathways within the gut microbiota, particularly in sulfate-reducing bacteria. Interestingly, by comparing germ-free versus conventional mice, it was shown that the intestinal microbiota regulates H₂S homeostasis not only in the gut but also systemically in various tissues and organs ([8]). Namely, the presence of microbiota was found to be associated with higher levels of free H₂S not only in some intestinal tracts (colon and cecum) but also in plasma. Moreover, compared to germ-free mice, the conventional animals displayed higher levels of bound sulfane sulfur in plasma, fat, and lung, and higher CSE activity and reduced cysteine levels in most organs and tissues ([8]). Another secondary source of H₂S is proposed to be persulphides and polysulfides, either endogenously generated or derived from dietary intake (reviewed in, e.g., [9]).

The three human enzymes reported to endogenously generate hydrogen sulfide are cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE, also known as cystathionase), and 3-mercaptopyrurate sulfurtransferase (MST) (Figure 1) ([10]).

2.1.1. Expression in Cells and Tissues and Cellular Localization. The occurrence of all three H₂S-synthesizing enzymes in cells, tissues, and organs and their cellular distribution remain evolving subjects, particularly taking into account that these may vary under different (patho)physiological conditions. It is generally considered that CBS regulation occurs at the protein level, the enzyme being a target of multiple posttranslational modifications and having two regulatory domains that respond to different stimuli (reviewed, e.g., in [11]), unlike CSE, for which no posttranslational regulatory mechanisms are known ([10]). Moreover, since CBS and CSE employ various combinations of
physiology. (a) Transsulfuration branch of the methionine cycle. L-Methionine (Met) is converted by L-methionine adenosyltransferase to S-adenosyl-L-methionine (AdoMet), which is used by methyltransferases in methylation reactions, generating S-adenosyl-L-homocysteine (AdoHcy). AdoHcy hydrolase then converts AdoHcy into L-homocysteine (Hcy), which can either be converted back to L-methionine through the remethylation cycle (remet), or enter the transsulfuration branch. Cystathionine β-synthase (CBS) converts Hcy and L-serine into L-cystathionine (Cysth), which is taken up by cystathionine γ-lyase (CSE) to generate L-cysteine (Cys). Hydrogen sulfide (H2S) is synthesized by CBS and CSE in several alternative reactions (see Figure 2). (b) H2S synthesis by 3-mercaptoppyruvate sulfurtransferase (MST). Cys is converted by cysteine aminotransferase (CAT) into 3-mercaptoppyruvate (3-MP), which is used by MST to synthesize H2S along with its cosubstrates thioredoxin (Trx), Cys, Hcy, and L-glutathione (GSH).

the same substrates in their H2S-generating reactions (Figures 1 and 2), regulation of one enzyme will likely affect the other, due to increased or decreased substrate availability (e.g., in [11]).

The tissue and organ distribution of the H2S-synthesizing enzymes has been studied for different organisms, employing immunohistochemical detection and analyzing transcriptional levels and H2S-generating activity. In man and rodents, CBS is mostly abundant in the liver, pancreas, kidney, brain, and nervous system [9, 12–14]. Recently, CBS expression in the carotid body, as well as in uterine and mesenteric and umbilical arteries, was reported [15]. As for CSE, it is mostly expressed in the liver, kidney, and smooth muscle (both vascular and nonvascular), its expression and H2S-generating activity being negligible in the brain, heart, and spleen [9, 13, 16, 17]. MST tissue distribution has been systematically studied for different organisms. The activity of bovine MST has been reported to be highest in the adrenal cortex, followed by the liver, heart, and kidney [18]. Abundant rat MST expression has been detected in the kidney (proximal tubular epithelium), liver (pericentral hepatocytes), aorta, and brain glial cells [19]. Recently, Tomita et al. detected abundant murine MST in the brain (neural and glial cells), liver, kidney, testes, and endocrine organs (particularly in pancreatic islets) and lower levels in bronchiolar cells, spleen, thymus, and small intestine [20]. Finally, Western blot analysis and enzymatic assays revealed the presence of active MST, but undetectable CBS and CSE, in red blood cells [21].

In terms of cellular localization, the classical view is that both CBS and CSE are cytosolic enzymes, whereas MST localizes to the mitochondria (e.g., [9, 10, 22]). However, several examples show that this classical view may be challenged. It has been reported that when blood homocysteine levels rise, microvascular endothelial cells and hepatocytes may secrete CBS and CSE, thus circulating as part of the plasma proteome and contributing to H2S generation [23]. Extracellularly synthesized H2S was shown to contribute to increased cell viability and decreased oxidative damage to DNA after serum starvation and hypoxia/reoxygenation. Furthermore, immunoprecipitation of circulating CBS and CSE from serum prior to its supplementation with homocysteine enhanced serum-induced stress towards endothelial cells. Altogether, these observations suggest that secreted CBS and CSE have a protective role in the endothelium by both producing H2S and clearing excess homocysteine. Another notable characteristic of CBS is its ability to be SUMOylated leading to protein accumulation in the nucleus [24]. Perhaps of greatest significance for their role as H2S-synthesizing enzymes is the observation that both CBS and CSE can translocate to the mitochondria under various (patho-)physiological conditions. This becomes particularly relevant taking into account that H2S can not only stimulate mitochondrial bioenergetics (i) by supplying electron equivalents to the quinol pool via sulfide:quinone oxidoreductase (reviewed in [10, 25]), (ii) by activating the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase [26], and (iii) by persulfidation of ATP synthase [27, 28] but also block mitochondrial respiration by tight inhibition of cytochrome c oxidase (CcOX, reviewed in [29–31]). CBS has been observed to partially localize to liver mitochondria, where the protein transiently accumulates under hypoxia/ischemia, with the resulting increased H2S generation protecting mitochondria from oxidative stress [32]. Once cells return to normoxia, CBS levels are restored via protein degradation by mitochondrial Lon protease, which recognizes and targets specifically the oxidized form of the regulatory heme at the CBS N-terminal domain [32].

In the colorectal cancer cell line HCT 116, a significant fraction of CBS localizes to the mitochondria, being associated with the outer mitochondrial membrane. The resulting increased H2S levels are proposed to stimulate cancer cell energy metabolism, particularly oxidative phosphorylation and glycolysis [33]. In line with this observation, CBS has been shown to contribute in various cancer cells to a shift in the fate of glucose utilization from glycolysis to the pentose phosphate pathway [34] (detailed in Section 4.3.1). By also promoting tumor angiogenesis, the increased H2S availability may contribute to increased oxygen supply to tumor cells.

![Figure 1: Hydrogen sulfide biosynthetic pathways in mammalian physiology](image-url)
In a mouse model of amyotrophic lateral sclerosis, CBS accumulation in mitochondria isolated from the spinal cord and the resulting increased H\textsubscript{2}S generation are proposed to be related with impaired cytochrome c oxidase-dependent respiration [35].

CSE has been reported to translocate to the mitochondria of vascular smooth muscle cells (SMCs) in response to stimuli that increased intracellular calcium levels. This translocation appears to be mediated by the mitochondrial membrane translocase of the outer membrane 20 (Tom20).
Whereas SMCs display impaired ATP production in hypoxic conditions, the presence of CSE inside the mitochondria contributed to cysteine catabolism, H\textsubscript{2}S synthesis, and stimulated ATP synthesis under such conditions, suggesting that CSE translocation may sustain mitochondrial bioenergetics in response to certain stresses [28].

MST is generally regarded as a mitochondrial enzyme. However, Fräsdorf et al. have shown that there are actually two splice variants of the protein, MST-Isol and MST-Iso2, both of which localize to the cytosol, while only MST-Iso2 also localizes to the mitochondria in HEK293a and HeLa cells [36].

2.1.2. Associated Metabolic Pathways. CBS and CSE participate in the transsulfuration branch of the methionine malthionyl/remethylation cycle (Figure 1(a)), a key pathway which generates extremely relevant signaling molecules for mammalian physiology. In this cycle, methionine (Met) derived essentially from dietary intake is converted to \( \text{s-adenosyl-l-methionine (AdoMet)} \) by methionine adenosyltransferase ([37] and references therein). AdoMet is the methyl group donor for virtually all methylation reactions in mammalian physiology, through the action of methyltransferases. Concurrently with methylation, \( \text{s-adenosyl-l-homocysteine (AdoHcy)} \) is generated, which is used by AdoHcy hydrolase (SAHH) to produce homocysteine (Hcy) and adenosine. Homocysteine then either enters the transsulfuration branch or proceeds through the remethylation pathway to revert to methionine, depending on different metabolic fluxes and other physiological conditions. The metabolites AdoMet, AdoHcy, and Hcy participate in the regulation of numerous physiological processes. As detailed below, AdoMet is an allosteric regulator of CBS, increasing its enzymatic activity by 2–5-fold upon binding. This allosteric activation results in an increase in \( V_{\text{max}} \), without any notable effect on the substrate affinity \( K_M \) [38, 39]. The “canonical” reactions attributed to CBS and CSE within the transsulfuration pathway result in conversion of homocysteine into cysteine as follows (Figure 2): CBS catalyzes the \( \beta \)-replacement of serine by homocysteine, yielding cystathionine, and CSE then proceeds by converting cystathionine to cysteine, \( \alpha \)-ketobutyrate, and ammonia in an \( \alpha,\gamma \)-elimination reaction. The role of CBS and CSE in hydrogen sulfide production results from an extensive repertoire of alternative reactions that these enzymes are able to catalyze (Figure 2) [39, 40], possibly related to the fact that both are pyridoxal 5’-phosphate (PLP-) dependent enzymes. This may be perceived as catalytic promiscuity or versatility-based robustness [11]. A notable thorough in vitro kinetic characterization of CBS- and CSE-catalyzed H\textsubscript{2}S-generating reactions combined with kinetic simulations has been carried out by Singh et al., allowing to envisage which reactions are relevant in vivo under different (patho)physiological conditions [39]. Both CBS and CSE are able to synthesize H\textsubscript{2}S through cysteine \( \beta \)-elimination or \( \beta \)-replacement, respectively employing one or two cysteine molecules (Figure 2, reactions (3) and (4)), or through \( \beta \)- or \( \gamma \)-replacement using cysteine and homocysteine (Figure 2, reaction (7)). CSE alone is able to synthesize H\textsubscript{2}S via homocysteine \( \alpha,\gamma \)-elimination or \( \gamma \)-replacement, respectively employing one or two homocysteine molecules (Figure 2, reactions (5) and (6)). Both CBS and CSE also catalyze cysteine \( \alpha,\beta \)-elimination to yield cysteine persulfide, which ultimately may generate H\textsubscript{2}S (Figure 2, reaction (8)). Besides H\textsubscript{2}S synthesis, some of these reactions originate thioether metabolites such as lantionine and homolanthionine, which may have been suggested as biomarkers of hydrogen sulfide generation in homocystinuric patient samples [41]. Under substrate saturating conditions, the turnover number of recombinant human CBS for the H\textsubscript{2}S-generating cysteine and homocysteine \( \beta,\gamma \)-replacement reaction (reaction (7) in Figure 2) is 3.7-fold higher than that of the cystathionine-generating canonical reaction [39]. Kinetic simulations show that this difference drops to 1.3-fold at physiologically relevant substrate concentrations, which still indicates that CBS-catalyzed H\textsubscript{2}S synthesis at the expense of homocysteine and cysteine accounts for \( \sim 56\% \) of cystathionine production. Notably, reaction (7) is responsible for 96% of the net CBS-catalyzed H\textsubscript{2}S production, whereas the cysteine-alone-based reactions (reactions (3) and (4) in Figure 2) account for a mere 1.6–2.6% of H\textsubscript{2}S synthesis. Still, the H\textsubscript{2}S-producing capacity of CBS appears to be insensitive to the systemic homocysteine levels, possibly related to the fact that homocysteine can only bind to CBS after serine or cysteine. The same kinetic simulations show that, contrarily to CBS, CSE catalyzes the canonical reaction of cysteine synthesis via cystathionine \( \alpha,\gamma \)-elimination (reaction (2) in Figure 2) with a much higher (20–30 fold) catalytic efficiency \( k_{\text{cat}}/K_M \) than determined for the H\textsubscript{2}S-synthesizing reactions (reactions (3–6) in Figure 2). At physiologically relevant substrate concentrations (10 \( \mu \)M homocysteine, 100 \( \mu \)M cysteine, and 5 \( \mu \)M cystathionine), the canonical reaction turnover is still 5-fold or 12-fold higher than those for H\textsubscript{2}S-generating cysteine or homocysteine cleavage reactions, respectively. Under those conditions, approximately 70% of CSE-catalyzed H\textsubscript{2}S production derives from cysteine and 29% from homocysteine. Also in contrast with CBS, the CSE-catalyzed H\textsubscript{2}S generation depends on homocysteine availability. Indeed, kinetic simulations assuming equimolar CBS (fully activated by AdoMet) and CSE at “normal” homocysteine concentrations (10 \( \mu \)M) reveal that CSE contributes to 32% of the synthesized H\textsubscript{2}S, whereas the CSE contribution increases to 45% and 74% under conditions of moderate and severe hyperhomocysteinemia, respectively. This indicates that CSE may have a significant role in homocysteine clearance at physiological elevated homocysteine levels, particularly in tissues or organs where CBS is absent or poorly expressed.

Recently, Majtán et al. reported on a thorough kinetic analysis of CBS-catalyzed reactions combined with simulations [38]. This study demonstrated the competitive advantage of serine over cysteine as a substrate for its condensation with homocysteine (to yield H\textsubscript{2}O or H\textsubscript{2}S, resp.), with a 2–5-fold higher catalytic efficiency of the canonical reaction compared to the H\textsubscript{2}S-generating reaction. However, despite this apparent advantage at saturating substrate concentrations, the H\textsubscript{2}S-producing reaction is able to compete with the canonical reaction under physiologically relevant conditions [38]. Indeed, one of the key findings is that a main determinant
of CBS-catalyzed H₂S production is the cysteine to serine ratio, adding another layer of complexity to be taken into account when considering the in vivo role of each H₂S-producing enzyme.

Despite the remarkable value of these kinetic studies, H₂S generation obviously also depends on the relative expression levels of each enzyme in each cell/tissue and on the systemic and local availability of substrates (cysteine, homocysteine, cystine, and serine), regulatory metabolites (e.g., AdoMet), and other effectors which negatively modulate enzymatic activity (e.g., NO and CO inhibit CBS; detailed below).

The role of 3-mercaptopyruvate sulfurtransferase (MST) is also tightly associated with cysteine metabolism. 3-Mercaptopyruvate (3-MP) is generated by cysteine (or aspartate) aminotransferase (CAT, Figure 3(a)) through cysteine deamination, using α-ketoglutarate as cosubstrate and yielding glutamate as coproduct [10]. An additional pathway whereby 3-MP is generated has been recently demonstrated to be present mostly in the cerebellum and the kidney, involving D-cysteine and a D-amino acid oxidase [42].

The interaction between MST and its 3-MP substrate gives rise to an enzyme-bound cysteine persulfide intermediate at Cys₂₄₈ (MST* in Figure 3(b)), concomitantly with pyruvate release [43]. H₂S can then be released by transfer of the active site cysteine persulfide to physiologic small thiols like cysteine, homocysteine, and glutathione or reducing molecules such as thioredoxin (Trx) or dihydrolipoic acid (DHLA) or by reaction with nonphysiological reductants like 2-mercaptoethanol and dithiothreitol [43]. The reaction of MST* with reduced thioredoxin (Trx) proceeds similarly to that with LMW RSH molecules. The MST cysteine persulfide is initially transferred to Trx, yielding CysSSH, which is then attacked by a nearby cysteine thiol, releasing H₂S and yielding oxidized Trx. The MST persulfide intermediate transfers its persulfide to an acceptor R-SH, forming an R-S-SH intermediate, which then reacts with another acceptor RSH molecule to yield a disulfide (RSSR) and H₂S. From the tested physiological acceptor substrates, thioredoxin displays the highest catalytic efficiency, orders of magnitude higher than those for DHLA, cysteine, homocysteine, and glutathione [43]. This observation is consistent with the observation that some protozoan parasites encode MST variants with a fused thioredoxin domain.

The broad impact of H₂S on mammalian physiology has been widely assessed in numerous in vivo studies,
CSE and CBS, H₂S has been shown to take part in a huge down. Making use of these models, particularly the ones for 
encephalopathy protein 1 (ETHE1, also known as persulfide dioxygenase or sulfur dioxygenase), implicated in H₂S breakdown. For a comprehensive overview of the wide spectrum of studies carried out on these models, the readers can refer to [9], a recent review specifically addressing this topic.

2.1.3. Structural and Functional Properties of H₂S-Generating Enzymes

(1) Cystathionine β-Synthase (CBS). Human cystathionine β-synthase (CBS) is considered to assemble as a homotetramer of 551 amino acid-long (~61 kDa) monomers, each consisting of three domains (Figure 4(a)): an N-terminal domain (residues 1–70) that binds a noncatalytic heme cofactor, a central catalytic PLP-binding domain (residues 71–381), and a C-terminal regulatory domain (residues 412–551, also known as Bateman module, comprising two motifs, CBS1 and CBS2) responsible for enzyme activation upon AdoMet binding. Structural studies on the human enzyme have revealed that the catalytic domain has a highly conserved structural fold of the β-family of PLP-dependent enzymes, consisting of thirteen α-helices and two β-sheets composed of four and six strands [44, 45]. Several intrinsically flexible regions that were initially not visible in the structure of a truncated human CBS lacking the C-terminal domain became visible in the structure of a “full-length” CBS construct (with a 10-residue deletion at the C-terminus, Figure 4(a)). These include the strands β4, β5, and β6 preceding the loops L145–148, L171–174, and L191–202, which are proposed to constitute the catalytic site entrance (together with loop L295–316) and are stacked between the catalytic core and the C-terminal domain, as well as helix α7 following the L191–202 loop. The active site PLP moiety is bound to the catalytic core through a Schiff base bond to the ε-amino group of lysine 119 (Figure 4(b)). A long 30-residue α-helical stretch (residues 382–411; helices α15 and α16) tethers the catalytic core to the C-terminal AdoMet-binding domain. The latter actually comprises two so-called CBS domains which share a common structural fold despite the poor sequence identity (~7% over 133 residues). The key finding in the structure of full-length CBS was the revelation that, in AdoMet-free CBS, the C-terminal domain from one monomer blocks the substrate entry site of the opposing monomer within one dimer (Figure 4(c)) [44]. This blockage is accomplished by stabilization of the above mentioned intrinsically flexible region composed by the β4, β5, and β6 strands preceding the loops L145–148, L171–174, and L191–202. Indeed, the interaction between the core and regulatory domains has been shown to involve (i) hydrophobic interactions with residues from the C-terminal domain CBS2 motif and (ii) H-bonds with residues from the C-terminal domain CBS1 motif [44, 46]. Notably, in the presence of AdoMet, the two C-terminal domains assemble utilizing knockout mouse models of the three H₂S-synthesizing enzymes CBS, CSE, and MST and of ethylmalonic encephalopathy protein 1 (ETHE1, also known as persulfide dioxygenase or sulfur dioxygenase), implicated in H₂S breakdown.

![Figure 4: Crystallographic structure of human cystathionine β-synthase (CBS).](image-url)
together in a "disc"-like form, similarly to the constitutively activated *Drosophila melanogaster* AdoMet-free CBS [47], the substrate entrance opens, and access to the active site is enhanced (Figure 4(c)), thus increasing enzymatic activity [44, 46, 48]. Each Bateman module contains two putative AdoMet-binding cavities (S1 and S2) with different binding affinities [49]. One of these cavities (S2) is exposed and thus likely represents the primary AdoMet binding site, while the other one (S1) appears to be hindered by bulky hydrophobic residues from the catalytic core [46, 48, 49]. The AdoMet-induced association between adjoining Bateman modules disrupts the interactions between the catalytic and C-terminal regulatory domains and opens the catalytic core to the substrates.

(2) Cystathionine γ-Lyase (CSE). Cystathionine γ-lyase (CSE) is a homotetrameric enzyme constituted by 405-amino-acid-long ~44 kDa monomers, each consisting of two structural domains (Figure 5(a)) [50]. The larger PLP-binding catalytic domain (comprising residues 9–263) assembles as an α/β/α fold consisting of a seven-stranded β-sheet (6 parallel and 1 antiparallel) flanked by eight α-helices. The smaller C-terminal domain (residues 264–401) is composed of a β-sheet (4 antiparallel strands) with three helices on one side. Similarly to CBS, the PLP in CSE is anchored through a Schiff base bond between the PLP carbonyl and the ε-amino group of a lysine residue (Lys212) (Figure 5(b)), although other forces are at play to stabilize PLP: π-stacking interactions between the Tyr114 phenyl moiety and the PLP pyridine ring, and H-bonds between the PLP phosphate moiety and residues Gly90, Leu91, Ser209, and Thr211 from the same subunit and Tyr60 and Arg62 from the adjacent subunit. Structural studies on CSE in the presence of its inhibitor propargylglycine (PPG) attempted to explain its mode of action [50]. While the lysine-PLP bond appears to remain unaffected, PPG is proposed to covalently bind to Tyr114, becoming a vinyl ether, while also forming H-bonds between its amino group and Gly33, and between its carboxyl moiety and Arg119 and Arg62 from the other monomer. This static position of the covalently bound PPG vinyl ether is such that it extends towards the internal PLP aldimine, thus blocking the cofactor reactivity [50].

(3) Mercaptopyruvate Sulfurtransferase (MST). Human mercaptopyruvate sulfurtransferase (MST) is composed of 297 amino acid residues and assembles as a ~33 kDa monomer consisting of two structurally related domains with a rhodanese-like fold (Figure 6(a)) [43]. The N-terminal (residues 1–138) and C-terminal (residues 165–285) domains are tethered by a 26-amino acid linker that strongly interacts with both domains. The assembly of these structurally related domains could have arisen from gene duplication. Structural studies of MST in the presence of its 3-MP substrate have shown that it binds to cysteine 248 in the active site, which is located in a cleft between the two domains (Figure 6(b)) [43]. The reaction originates a persulfidated Cys248 and the inhibitors’ pyrimidone-like aromatic rings [51], without major structural changes.

2.2. **H₂S Catabolism**

2.2.1. **H₂S: A “Self-Regulatory” Janus Molecule for Cellular Bioenergetics.** As mentioned above, while playing a key signaling role only at relatively low, physiological concentrations, at higher concentrations H₂S is potentially toxic, and without bound 3-MP reveals that no major structural differences occur [43]. In a recent report on a compound screening campaign targeting MST, the structures of MST in complex with hit compounds revealed a strong interaction between the persulfidated Cys248 and the inhibitors’ pyrimidone-like aromatic rings [51], without major structural changes.
being able to impair cell respiration through inhibition of CcOX [52]. To prevent toxicity, H$_2$S bioavailability must therefore be finely and differently regulated in different tissues and organs, depending on specific physiological demands. Control of H$_2$S bioavailability is exerted not only at the level of its biosynthesis but also through enzymatic disposal of such a potentially toxic molecule. In mammals, the ability to detoxify H$_2$S to thiosulfate and sulfate was demonstrated by metabolic labelling in early studies [53]. More recently, it was discovered that H$_2$S breakdown is mainly afforded by a mitochondrial enzymatic system (reviewed in [10, 25]), currently designated as “sulfide-oxidizing unit” [54] or “sulfide-oxidizing pathway”. Following its initial identification in the lugworm Arenicola marina [55], this mitochondrial system has been investigated in more detail. According to current views, this pathway comprises four distinct, yet functionally associated enzymes that together cooperate to catalyze the breakdown of H$_2$S to thiosulfate and sulfate, the main sulfide catabolites: a sulfide:quinone oxidoreductase (SQR), a persulfide dioxygenase (also known as ETHER1 or sulfur dioxygenase), a thiosulfate sulfurtransferase (rhodanese), and a sulfite oxidase (SOx) (Figure 7). It is to be noted that H$_2$S oxidation to polysulfide can also be catalyzed by globins (see below) and other proteins, such as catalase and superoxide dismutase, utilizing O$_2$ or H$_2$O$_2$ as electron acceptor [56–59].

From several perspectives, it is fascinating that H$_2$S oxidation by mitochondria is coupled to energy production, namely, ATP synthesis, as initially demonstrated by investigating the invertebrate Solemya reidi [60]. Electrons derived from sulfide oxidation are indeed injected into the respiratory chain at the level of coenzyme Q, thus promoting O$_2$ consumption and, in turn, energization of the inner mitochondrial membrane. On this basis, H$_2$S has been recognized as the first inorganic respiratory substrate discovered in mammals [61]. This makes H$_2$S a very peculiar molecule from a bioenergetic point of view [62], with a dual effect on mitochondrial respiration: stimulatory at low (nanomolar) concentrations when the mitochondrial sulfide oxidation pathway is fully operative or inhibitory at higher (micromolar) concentrations when CcOX activity is impaired and reduced coenzyme Q accumulates, leading to blockage of H$_2$S detoxification. As remarked recently [63], the system is therefore finely regulated through positive feedback loops so that at lower concentrations sulfide oxidation prevents its own accumulation and consequent inhibition of respiration, whereas at higher concentrations sulfide impairs its own detoxification via inhibition of CcOX. That H$_2$S can act as an effective substrate of the mitochondrial respiratory chain is in full agreement with the observation that, despite the low $K_i$ value measured with isolated CcOX ($K_i = 0.2 \mu M$...
at pH 7.4 [52]), in isolated mitochondria or intact cells, inhibition of respiration only occurs if much higher concentrations of H$_2$S are administered (micromolar to tens of micromolar; see, for instance, [64, 65]).

The dual effect of H$_2$S on cell respiration makes investigation of the mitochondrial sulfide-oxidizing activity somewhat challenging from a methodological point of view. Sulfide stimulation of mitochondrial oxygen consumption and consequent energization are indeed best appreciated at low sulfide concentrations, unable to inhibit CcOX. For this reason, most of studies on mitochondrial sulfide oxidation and related bioenergetic effects have been carried out by continuous supply of sulfide at given infusion rates, rather than by sulfide addition as a single bolus (method reviewed in [66]). Using this approach, sulfide oxidation has been quantitatively investigated in several systems, including isolated mitochondria and permeabilized or intact cells from humans and other higher organisms [54, 61, 63, 67–72]. Notably, remarkable differences in terms of H$_2$S oxidation ability have been reported between human cell lines, spanning from cells in which this catalytic activity is undetectable (as, e.g., in neural cells) to others remarkably active in H$_2$S removal, like colonocytes [54]. The latter cells are indeed physiologically exposed to massive amounts of H$_2$S produced by the intestinal microbiota and are therefore likely to have undergone adaptive mechanisms to prevent H$_2$S toxicity. In this regard, it is interesting that colonocytes were found to ensure a prominent H$_2$S-catabolyzing activity even through activity reversal of complex I in the respiratory chain [54].

2.2.2. Components of the Mitochondrial Sulfide-Oxidizing Pathway. The first enzyme operating in the mitochondrial sulfide oxidizing pathway is sulfide:quinone oxidoreductase (SQR, Figure 7). SQR is a member of the protein family of disulfide oxidoreductases, which has been identified in all life domains [73]. It is a membrane-associated protein located at the periplasmic side of the cytoplasmic membrane in prokaryotes and at the inner mitochondrial membrane in eukaryotes. Although no structure of a eukaryotic SQR has thus far been obtained, crystallographic structures of prokaryotic SQRs have been reported [74–76], enabling the construction of a structural model of human SQR (Figures 8(a) and 8(b)). Several structural and amino acid sequence features linked to sulfide oxidation and quinone reduction have allowed for the classification of the SQR family into six subclasses, with human SQR classified as a type II SQR [77]. The main features of type II SQRs, particularly as compared to type I SQRs, are essentially the lack of any extended loops, the poor conservation of quinone-interacting residues, and the substitution of the conserved cysteine covalently linking
the FAD cofactor for a tyrosine (Tyr170 according to human SQR numbering), which probably establishes a covalent link with FAD in human SQR via an 8-α-O-tyrosyl bond [77]. SQRs can be monomeric or assemble into dimers or trimers of ~50 kDa subunits [78], each harboring a cysteine disulfide in its active site and a noncovalently bound flavin adenine dinucleotide (FAD) moiety implicated in electron transfer [77]. In mammals, SQR couples H₂S oxidation to the reduction of coenzyme Q, with the concomitant transfer of a sulfur atom to an acceptor that has been proposed to be either sulfite- (SO₂⁻) yielding thiosulfate (S₂O₅²⁻) [79–81] or reduced glutathione (GSH) yielding glutathione peroxidase (GSSH) [82]. Although the protein displays a higher catalytic efficiency using as cosubstrate sulfite (k_{cat}/K_M = 2.5 × 10⁶ M⁻¹·s⁻¹) as compared to GSH (k_{cat}/K_M = 1.6 × 10⁴ M⁻¹·s⁻¹) [83], at physiological concentrations of both cosubstrates, GSH was suggested to be the preferential sulfur acceptor [82–84], at variance from [79–81]. In contrast to the mammalian protein, bacterial SQRs directly release the oxidized sulfur as a soluble polysulfide with up to ten sulfur atoms, instead of reacting with a sulfur acceptor [75, 85]. The reaction mechanism of SQR has been investigated with the isolated protein not only in detergent solution [80, 82, 84] but also after incorporation into nanodiscs [83], where slightly higher catalytic rates have been measured. The postulated mechanism involves (i) reaction of the active site cysteine disulfide with H₂S associated with persulfidation of Cys379 (human SQR numbering) to form CysSSH and release of the Cys320 thiolate that in turn forms a charge transfer complex with FAD, (ii) transfer of the sulfane sulfur from the persulfidated Cys379 to the acceptor with the concomitant reduction of FAD to FADH₂, and (iii) oxidation of FADH₂ by coenzyme Q. According to kinetic data, the sulfane sulfur transfer step is the rate-limiting one in the reaction.

The next step in the sulfide oxidation pathway is catalyzed by ETHE1 (Figure 7). Mutations in the gene encoding this protein were found to result into an autosomal recessive disorder, known as ethylmalonic encephalopathy, characterized by a severe clinical picture and leading to death during early childhood [86, 87]. The crystallographic structure of human ETHE1 was recently solved (Figure 9) [88]. ETHE1 is a dimeric enzyme, each monomer comprising an αββα fold structurally related to dinic classical metallo-β-lactamases, harboring a mononuclear nonheme iron octahedrally coordinated by two histidines, one aspartate, and three water molecules [88]. The enzyme is proposed to catalyze the oxygenation of the sulfane sulfur atom in the GSSH released from SQR, yielding sulfite [55]. It is important to note that one O₂ molecule is consumed in the reaction.

GSSH and sulfite are further converted into thiosulfate and GSH by rhodanese (Rhod, Figure 7). No structural studies of the human enzyme have been reported, yet a structural model can be obtained based on the ~90% identical bovine homologue (Figures 8(c) and 8(d)) [89]. This protein has a redox-active cysteine (Cys248) in the active site and is highly promiscuous in that it can effectively react with several different substrates. Yet, at physiologically relevant substrate concentrations, the most likely reaction catalyzed by rhodanese was suggested to be the transfer of a sulfur atom from GSSH to sulfite to form thiosulfate [82]. Sulfite oxidase (SOx, Figure 7) catalyzes the last step in the sulfide oxidation pathway. SOx is a multidomain cytochrome b₅ with a molybdendenum cofactor [90] and a heme mediating the intramolecular electron transfer from sulfite to cytochrome c. Concomitantly, an oxygen atom is supplied by an H₂O molecule and sulfate (SO₄²⁻) forms as the end-product of the reaction [90].

Overall, the mitochondrial sulfide-oxidizing pathway couples the oxidation of H₂S to thiosulfate and sulfate with the injection of electrons into coenzyme Q, leading to consumption of 0.79 O₂ per H₂S molecule oxidized, as reported by Goubern and coworkers [61], which is compatible with the predicted value of 0.75. Indeed, based on the postulated...
architecture of the mitochondrial sulfide oxidation pathway presented above, the oxidation of 2 H2S molecules (involving 2 electrons) is expected to require the consumption of 1.5 O2 molecules (0.5 by CcOX plus 1 by ETHE1). As O2 is required for sulfide removal, the efficiency of mitochondrial sulfide oxidation is expected to depend on O2 availability, clearly vanishing under anoxic conditions. Evidence for a decline of mitochondrial sulfide oxidation at lower O2 concentrations was preliminarily provided in [69] and, then, substantiated in a later study [63]. To be noted that at very low O2 concentrations (down to 0.73 ± 0.04 μM), given a fixed amount of sulfide, a faster onset of cell respiration inhibition was observed at decreasing O2 concentrations [69], which is consistent with a lower efficacy of sulfide removal but also with a higher control of the respiratory electron transfer chain by GcOX at low O2 concentration.

Our knowledge of H2S catabolism and, particularly, of its implication in human physiology and pathophysiology is still rather elusive. Current views of the mitochondrial sulfide oxidation pathway are yet rather patchy and represent a matter of debate. It is likely that the physiological function of this pathway goes well beyond the mere removal/detoxification of H2S. The impact of this pathway on cell bioenergetics under pathophysiological conditions needs to be better established, as well as its ability to produce bioactive sulfur metabolites (e.g., thiosulfate) whose physiological role has only recently begun to emerge. Finally, it can be anticipated that the mitochondrial sulfide oxidation pathway plays a key role in mediating the intricate interplay between sulfide, O2, and the other gasotransmitters, but more studies are needed in this direction.

3. Signaling Mediated by Hydrogen Sulfide

The relevance of hydrogen sulfide in mammalian physiology results essentially from its role as a second messenger that transduces signals by interaction with target proteins. The initial classical view of H2S-mediated signaling initially merely considered the inhibitory properties of hydrogen sulfide towards respiratory CcOX (detailed below) leading to a state of suspended animation. Presently, the signal transducing function of hydrogen sulfide is known to occur via at least two major mechanisms, namely, through (i) interaction with protein metal centers, particularly heme moieties, and (ii) protein persulfidation (detailed below).

3.1. H2S and Heme Proteins. Besides being able to promote thiol persulfidation, H2S can react with protein hemes. As reviewed in Bianco et al. [91], the mechanism and fate of the reaction of H2S with a heme protein largely depend on several factors, such as the redox and ligation state of the heme iron, the environment in the heme pocket, the protonation state of bound sulfide, and the presence/absence of O2 or reducing agents in solution. Heme-Fe(III) can bind H2S as such or as HS− (Figure 10). Stability of one or the other heme adduct generated by reaction with hydrogen sulfide (heme-Fe(III)-H2S or a heme-Fe(III)-HS−) depends on the protein residues in the heme surroundings and, particularly, on the presence of either basic residues able to accept protons from bound H2S to yield the heme-Fe(III)-HS− species or, alternatively, nonpolar residues stabilizing the fully protonated state of the ligand. Owing to its reducing character, bound sulfide can reduce the heme iron, yielding a heme-Fe(II)-HS− radical adduct that can further react with excess H2S/HS− or O2/H2O leading, respectively, to formation of polysulfides or thiosulfate. In contrast to these reactions, H2S can also promote a covalent modification of the heme, yielding the so-called sulfheme derivative with a sulfur atom incorporated into one of the porphyrin pyrrole rings (Figure 10). The mechanism of sulfheme formation is still unclear. Sulfheme can be formed by reaction of H2S with either the ferrous oxygenated (heme-Fe(II)-O2) [92] or the ferryl heme (heme-FeV=O [93, 94]). However, also in the former case the reaction requires H2O2 and thus, likely, the formation of higher valent heme iron intermediates [95, 96]. As reviewed in [5, 91, 95–97], the reactions above have been described by several groups working on numerous hemeproteins, including, among others: globins from mammals, invertebrates, and bacteria; heme-based sensor proteins of diatomic molecules such as O2, NO, and CO; cytochrome c oxidase; catalase; and peroxidases (lactoperoxidase, myeloperoxidase and thyroid peroxidase).

Because heme proteins can usually react not only with H2S but also with NO, CO, and O2, they can play a key role in mediating the crosstalk between these gaseous ligands, namely, controlling each other’s biological function. A paradigm of this concept is CcOX. The enzyme, highly reactive with O2, is indeed a recognized target for all three gasotransmitters (NO, CO, and H2S). Each of these species can inhibit CcOX, though with markedly different kinetics and mechanisms [29]. While CO can only bind to the fully reduced heme a2-Cu2 active site, leading to competitive inhibition of the enzyme [98], CcOX inhibition by NO can proceed through two alternative reaction pathways (reviewed in [99–104]): an O2-competitive pathway, favoured at lower O2 tension and higher electron flux, leading to a nitrosyl adduct of CcOX with NO bound to ferrous heme a3, and an O2-competitive one, prevailing at lower electron flux and higher O2 tension, whereby an inhibited adduct of enzyme forms with nitrite bound at ferric heme a3 [29, 105–107]. The mechanism of CcOX inhibition by H2S has not been investigated as thoroughly as for NO and CO.
Differently from these two species, H$_2$S does not bind ferrous heme $a_2$. Based on the postulated mechanism, when the enzyme in turnover with O$_2$ is exposed to H$_2$S oxidized or reduced Cu$_{O}$ is the primary target site in CcOX. Afterwards, sulfide is thought to be transferred intramolecularly to the nearby ferric heme $a_2$, resulting in enzyme inhibition [30]. H$_2$S-inhibited CcOX thus exhibits one sulfide molecule bound to ferric heme $a_2$ [108, 109] and, possibly, a second one bound to cuprous Cu$_{O}$, as revealed by electron paramagnetic resonance (EPR) spectroscopy [110]. Regardless of the exact molecular mechanism, CcOX inhibition by H$_2$S was demonstrated to be relatively fast (initial rate constant of $2.2 \times 10^{4}$ M$^{-1}$ s$^{-1}$, as measured at pH 7.4 with the enzyme in turnover with O$_2$ and cytochrome c [30]), effective ($K_i = 0.2$ μM at pH 7.4), independent of O$_2$ concentration, and reversible [52]. Similarly to other heme proteins, CcOX inhibition by H$_2$S was found to be greatly dependent on pH. Acidic conditions remarkably enhance the inhibitory action of sulfide, and consistently, as the pH shifts from 8.05 to 6.28, $K_i$ decreases from 2.6 μM to 0.07 μM [111]. The enhanced efficacy of sulfide inhibition at lower pH suggests that sulfide binds to CcOX either as H$_2$S (e.g., in fully protonated state) or as HS$^-$ with the concomitant protonation by a protein residue. This is consistent with the active site of the enzyme being located in a nonpolar environment, therefore facilitating the binding of electroneutral or proton-neutralized anionic species [112].

H$_2$S can also act as a reducing substrate for CcOX [30, 111, 113], when the enzyme is in the so-called pulsed state [30], but the reaction products are yet to be characterized. By reacting with H$_2$S at relatively low concentrations, heme $a_2$ in the active site of the enzyme is promptly reduced and, by further reacting with O$_2$, leads to a ferryl species with optical features indistinguishable from those of the “P” catalytic intermediate [30]. Afterwards, the “P” ferryl intermediate spontaneously forms on a longer timescale, but it is unclear if the reaction results from auto reduction or electron donation from residual sulfide [30]. CcOX is therefore in principle able to catalyze the oxidative breakdown of H$_2$S, but the reaction is probably too slow to be physiologically relevant, particularly when compared with the high H$_2$S-metabolizing activity of SQR.

Another group of proteins whose reactivity with H$_2$S has been investigated in detail is the globin family. These studies focused primarily, but not exclusively, on human hemoglobin (Hb) and myoglobin (Mb). The reaction of sulfide with Hb has long been known to lead to irreversible covalent conversion of the protein heme to sulfhememe (reviewed in [96]); as described above, the modification consists in the incorporation of a sulfur atom into the heme pyrrole B. The resulting protein derivative, named “sulfehemoglobin,” exhibits a characteristic green color and lower O$_2$ affinity as compared to the native protein. Accumulation of sulfhemoglobin is therefore associated to toxicity, leading to a rare pathological condition known as “sulfehemoglobinemia.” A similar reactivity has been demonstrated also for Mb, leading to formation of “sulffmyoglobin” (Figure 10). Whereas sulffhemoglobinemia likely represents a form of sulfide toxicity, methemoglobinemia, that is, the accumulation of ferric hemoglobin (metHb) in the blood, has been suggested to protect against sulfide toxicity in mice [114]. In these in vivo studies, 2–4 mol of H$_2$S was inferred to be inactivated per mol of metHb [114]. This superstoichiometric value suggests that metHb is able not only to bind H$_2$S but also to promote its catalytic breakdown. Consistently, under aerobic conditions metHb was recently demonstrated to bind H$_2$S, oxidizing it to a mixture of thiosulfate and polysulfides [21]. The reaction, though proceeding at relatively low rates (3 min$^{-1}$ in the presence of 0.5 mg·mL$^{-1}$ metHb, at pH 7.4 and 25°C [21]), could be of physiological relevance, as Hb-rich red blood cells lack mitochondria and therefore cannot dispose H$_2$S via the mitochondrial sulfide-oxidizing pathway.

Sulfide is a relatively low-affinity ligand for metHb; at pH 7.4 and 37°C, it binds with a $k_{on}$ of $3.2 \times 10^{4}$ M$^{-1}$ s$^{-1}$ and dissociates from the oxidized heme with a $k_{off}$ of 0.053 s$^{-1}$, yielding a $K_D$ of 17 μM (whose value may be overestimated, given that the H$_2$S species represents only a fraction of sulfide at pH 7.4). The rate constant for H$_2$S binding to metHb was found to increase with decreasing pH, and on this basis, H$_2$S rather than the HS$^-$ species, more abundant at pH 7.4, initially binds to the heme. After binding to the heme iron, H$_2$S is proposed to deprotonate to HS$^-$.

The postulated Fe(III)-HS$^-$ species has been recently detected by X-ray crystallographic analysis of the sulfide-reacted metHb (Figure 10) [115].

Possible mechanisms for polysulfide and thiosulfate formation by metHb have been proposed in [21]. Interestingly, the newly formed polysulfide remains bound to the protein upon heme iron reduction by methionine sulfoxide reductase (MSR). In contrast, it reacts with physiologically relevant, low-molecular-weight (LMW) thiol compounds, such as reduced glutathione (GSH) or cysteine (CysSH), yielding the corresponding persulfides, GSSH and CysSSH, that are eventually released into the solution. In light of the high intracellular concentration of GSH, Hb can act as a source of GSSH that in turn could serve as a persulfide donor in protein persulfidation (detailed below). Therefore, based on its intricate chemistry with sulfide, depending on conditions, Hb can mediate sulfide toxicity via formation of sulfhemoglobin or be protective by promoting sulfide disposal, a particularly relevant function in red blood cells lacking mitochondria. Furthermore, Hb can be viewed as a source of physiologically relevant sulfide oxidation products, namely, thiosulfate and glutathione persulfide (GSSH), whose impact on human physiology is emerging. A somewhat different scenario occurs in atherosclerotic lesions, particularly when they are infiltrated by red blood cells. Indeed, in those lesions, oxidized forms of Hb with the heme in the ferric and ferryl state accumulate and mediate toxicity by promoting radical reactions leading to formation of crosslinked Hb species and lipid modification. Under these circumstances, H$_2$S was recently reported to exert protective effects, acting as a reductant of ferryl Hb [116].

Another heme protein whose reactivity with sulfide was investigated in depth is human leukocyte myeloperoxidase (MPO). Based on recent studies [117, 118], upon reaction with sulfide, the enzyme does not lead to formation of the sulfheme derivative, at variance with other
heme proteins. Of interest, MPO was reported to catalyze the oxidation of sulfide to sulfane sulfur species both in the presence and in the absence of $\text{H}_2\text{O}_2$ [117, 118]. The reaction involves as a central intermediate compound III, a resonance form between the ferrous/dioxygen ($\text{Fe(II)}\cdot\text{O}_2$) and ferric/superoxide ($\text{Fe(III)}\cdot\text{O}_2^-$) complex, it is facilitated by ascorbate or SOD and might be of physiological relevance as the formed sulfane sulfur species were proven to oxidize protein cysteine residues to their corresponding per/polysulfide derivatives [117].

3.2. Persulfidation and Persulfide (Bio)chemistry. As mentioned above, many of the signaling functions attributed to $\text{H}_2\text{S}$ have been shown to be tightly associated with formation of persulfides through the modification of specific cysteine side chains from target proteins, often involving free reactive LMW persulfides (RSSH), particularly cysteine persulfide (CysSSH) and glutathione persulfide (GSSH). Polysulfides are also considered as potentially relevant from a physiological viewpoint, as reviewed elsewhere [119]. However, because they are contaminants of most inorganic $\text{H}_2\text{S}$ donors employed in research and therefore possibly responsible for some of the effects attributed to $\text{H}_2\text{S}$, their (bio)chemistry is still far from being fully understood and will not be detailed herein. The relevance of persulfidation is attested by the fact that deficiency in persulfidated proteins has been associated with different pathologies, including cardiovascular and neurodegenerative (Parkinson’s) diseases [120, 121]. Besides its signaling function, persulfidation may also constitute a mechanism to prevent thiol oxidation or electrophilic modification and irreversible damage [122]. The ability of LMW persulfides to readily donate one electron and form a stable perthiyl radical (RSS•), unreactive towards oxygen or NO, led to the proposal of a possible role in redox processes [123]. Recently, the oxidation of cysteine persulfides to perthiosulfenic acid (CysSSOH) observed in different proteins as a consequence of NADPH oxidase activation led to the proposal of this modification as an additional redox-based signaling event [124]. The chemical and biochemical properties, biosynthesis and natural occurrence, analytical detection methods, and cellular longevity of persulfides and polysulfides have all been covered in excellent articles and reviews (e.g., [3, 6, 120, 121, 125–129]). As in other fields focused on reactive molecules, there seem to be controversies as to which species and reactions are more plausible and relevant from a chemical-to-physiological viewpoint. Presently, there is however a consensus that $\text{H}_2\text{S}$ cannot react directly with protein cysteine thiols. Protein persulfidation, in order to occur, has special requirements both at a “local” (the environment surrounding the target cysteine) and a “global” (redox status, free glutathione/cysteine/$\text{H}_2\text{S}$ availability) level. Currently, although several possibilities for the formation of persulfidated proteins through posttranslational modification can be envisaged, only four appear to be more plausible (Figure 11(a)). Two of these possibilities involve previous oxidation of the protein target cysteine thiol to a sulfenic moiety (CysSOH), for example, by reaction with hydrogen peroxide, or to a disulfide (CysSSR) by reaction with an oxidized sulfur-containing LMW molecule, for example, oxidized glutathione (GSSG). Both of these oxidized cysteine residues can then be targeted directly by $\text{H}_2\text{S}$, resulting in a persulfidated protein. Reduced cysteine residues (e.g., protein thiols) can be modified to their persulfide derivatives through reaction with a free LMW persulfide (RSSH), such as GSSH or (homo)cysteine persulfide (derived from the $\text{H}_2\text{S}$ biosynthetic or catabolic pathway [125, 126]), or via reaction with HS−, generated upon reaction of $\text{H}_2\text{S}$ with metal centers, particularly oxidized protein heme iron. The protein cysteine thiol reaction with HS− should yield a transient CysSSSH− that, upon reaction with oxygen, yields the protein-bound CysSSH and superoxide anion, similar to that reported for HSSH− [3, 130]. In addition to post-translational persulfidation, it has been quite recently posited that in mammals a major source of persulfidated (and polysulfidated) proteins in vivo are actually cysteinyl-tRNA synthetases (CARSs, particularly the mitochondrial CARS2), through cotranslational incorporation of cysteine persulfide or polysulfide at cysteine sites into the nascent polypeptide [131] (Figure 11(b)). From a cellular signaling perspective, the advantage of protein persulfidation by $\text{H}_2\text{S}$ and/or persulfides is that the resulting species can be reverted to the thiol moiety in different ways, such as direct reaction with reduced glutathione (GSH) or through the thioredoxin system [126], thus affording a reliable switching modification.

As for the LMW persulfides, which are key molecules in protein persulfidation, they are increasingly coming into the limelight due to their intrinsic reactivity (e.g., [125]). Until recently, the two major sources of these persulfides, particularly cysteine persulfide (CysSSH) and glutathione persulfide (GSSH), were considered the enzymes involved in $\text{H}_2\text{S}$ metabolism. CysSSH is readily generated by the $\text{H}_2\text{S}$-synthesizing CBS and CSE, using cystine (CysSSCys) as substrate [125]. Notably, in the course of this reaction, longer polythiolated products with catenated sulfur atoms, such as CysSSSSH (synthesized by both CBS and CSE) and CysSSSSSSH (synthesized by CSE), have been detected. The enzymatic generation of these species further results in appreciable amounts of oxidized polysulfide species like CysSSSCys, CysSSSSSCys, and CysSSSSSSSCys. The newly formed CysSSH and longer cysteine persulfides can then react with reduced glutathione to yield GSSG and GS(S)H. It should however be noted that, at physiologically relevant substrate concentrations, $\text{H}_2\text{S}$ rather than CysSSH is expected to be the major product of the transsulfuration pathway enzymes [132], also taking into account that in the cytoplasm, cysteine and reduced glutathione are much more abundant than their oxidation products cystine and GSSG [132]. Recently, MST was also recognized as a source of CysSSH and GSSH, as well as of longer polysulfides, related with its ability to generate other RSS, such as hydrogen persulfide ($\text{H}_2\text{S}_2$) and hydrogen trisulfide ($\text{H}_2\text{S}_3$) [133, 134]. MST-derived persulfides and polysulfides have been associated with higher cellular levels of protein-bound sulfane sulfur [134, 135]. The MST-catalyzed CysSS(o)H and GSS(o)H production was demonstrated with recombinant MST (wild-type and site-directed variants), MST-expressing COS cells lysates, and mouse brains (cell suspensions and whole
showing that in the brain LMW persulfinides and protein persulfidation are essentially generated via MST and not CBS or CSE [133]. A principal role in CysSSH (and CysSOH) can react directly with H$_2$S (derived from its synthesizing enzymes CBS, CSE, and MST) to yield a protein cysteine persulfide (CysSSH). Proteins with reduced cysteine residues (CysSH) can react with free LMW persulfides (RSSH, such as glutathione persulfide and cysteine persulfide, products of H$_2$S biosynthetic—CBS, CSE, and MST—or catabolic—SQR—enzymes) or with the HS$^-$ radical formed by reaction of H$_2$S with metal centers (M$^{n+}$). The resulting protein-bound cysteine perthiyl radical (CysSSH$^-$) can then react with oxygen to yield CysSSH and superoxide anion. 

(b) Generation of protein cysteine persulfides through cotranslational incorporation of CysSSH. Mammalian cysteinyl-tRNA synthetases (CARSs, dark blue box, which also synthesize free CysSSH from cysteine) catalyze the synthesis of tRNA-bound cysteine persulfides (light blue line), which incorporate CysSSH into the nascent polypeptide (dark blue line) at cysteine sites upon translation (ribosome depicted in green and mRNA in grey).

Besides the LMW persulfides derived from the H$_2$S-synthesizing enzymes CBS, CSE, and MST [125, 132, 133], GSSH is a metabolic intermediate of the mitochondrial sulfide-oxidizing pathway [126] (Figure 7). Indeed, GSH has been suggested as the preferential sulfur acceptor in the H$_2$S oxidation reaction catalyzed by SQR, yielding GSSH [82]. This persulfide is, in turn, the preferential substrate (with sulfite as cosubstrate) for rhodanese to generate thiosulfate as the final oxidation product of sulfide oxidation, together with sulfate [82].

Regardless of how LMW persulfides are generated, their reactivity prompts them to be major signal-transducing species linked to H$_2$S (patho)physiology. In a nutshell, persulfides are stronger nucleophiles than their thiol counterparts are (yet displaying also a weak electrophilic character). Despite this assumption, only recently it was clearly demonstrated by Cuevasanta et al. [136] that a human serum albumin persulfide derivative displays 20-fold increased reactivity with respect to its thiol counterpart. Persulfides have lower pK$_a$ values than the analogous thios, thus existing mostly in their anionic nucleophilic RSS$^-$ form [3, 6]. Conversely, their weak electrophilicity is exhibited solely in their protonated RSSH form. Interestingly, both the outer sulphydryl and inner sulfenyl sulfurs have

![Diagram](image-url)
electrophilic character, the reaction occurring on either one depending on factors such as steric hindrance or acidity of the leaving group [3]. The ambiguous nucleophilic/electrophilic nature of LMW persulfides, while at the basis of their physiological relevance, accounts for their instability. These species indeed quickly decay or react with other species, particularly oxidants, hampering their characterization both in vitro and in vivo. This notwithstanding, the molecular details and functional consequences of persulfidation at specific cysteine residues have been demonstrated for a number of targets and shown to regulate several physiological processes (reviewed in, e.g., [127]), such as glycolysis [26], ER stress [137], apoptosis [138], vasorelaxation, and vasodilation [139] (detailed in Table 1). Regarding the latter, H₂S was the third gasotransmitter, following NO and CO, to be regarded as an endothelium-derived relaxing factor (EDRF) as NO [147], and an endothelium-derived hyperpolarizing factor (EDHF), thus regulating vasodilation (detailed below). Despite the relatively few examples available in the literature, protein persulfidation is likely prevalent, yet technically challenging to be detected due to the intrinsic reactivity of persulfides. Using an adaptation of the biotin switch assay modified to detect persulfidated rather than nitrosated proteins, Mustafa et al. reported that 25–50% of hepatic proteins are persulfidated [26]. The extent of protein persulfidation in vivo has been further demonstrated by increasingly elaborate and quantitative methodologies [125, 140–143]. It is expected that the number of identified new targets of protein persulfidation will continuously increase, pairing this posttranslational modification with other highly relevant cellular switches.

4. Interplay between Gasotransmitters

4.1. Brief Historical Account of Gasotransmitters. The term “gasotransmitters” [144] has entered the biochemistry and physiology jargon to designate essentially the three small molecules gaseous in nature with demonstrated roles in signal transduction: nitric oxide (NO; although the formally correct abbreviation should be NO), herein NO will be used for simplicity), carbon monoxide (CO), and hydrogen sulfide (H₂S). Arguably, molecular oxygen (O₂), although not synthesized endogenously in mammalian cells, could be considered a gasotransmitter due to its gaseous nature, reactivity, and physiological relevance. Besides, O₂ is a substrate of the mammalian enzymes that synthesize NO and CO, so O₂ levels dictate the bioavailability of the latter. Gasotransmitter-mediated signaling is an intricate and integrated process involving not only NO, CO, H₂S, and O₂ but also the collective pool of reactive species (reactive oxygen species (ROS), reactive nitrogen species (RNS), and reactive sulfur species (RSS) resulting from their cross-reactions, establishing a so-called reactive species interac-tome (RSI) [145]. Irrespectively of the identity of the gasotransmitter molecule or derived species, its signaling mode of action can be narrowed down essentially to two possibilities: interaction with metal centers or modification of cysteine residues in proteins, both resulting either in the activation or in the inactivation of the target protein. The three gasotransmitters NO, CO, and H₂S share a common history in that they were initially merely regarded as toxic and poisonous molecules, until the discovery that virtually all life forms, from bacteria to man, are endowed with dedicated synthesizing and detoxifying enzymes and produce or scavenge NO, CO, and H₂S to accomplish specific functions. Indeed, in the late 1980s and early 1990s, major advances concerning the role of NO in the regulation of cardiovascular function led to NO being considered Molecule of the Year in 1992 and to the Nobel Prize in Physiology or Medicine being awarded in 1998 to Robert Furchgott, Ferid Murad, and Louis Ignarro Jr “for their discoveries concerning nitric oxide as a signaling molecule in the cardiovascular system” [146]. One of the key findings was the identification of the endothelium-derived relaxing factor (EDRF) as NO [147, 148]. This observation, which revealed beneficial rather than deleterious effects of an otherwise considered toxic molecule, triggered a subfield of biology dedicated to signaling by gaseous second messengers. Indeed, besides other regulatory roles attributed to NO, similar findings were demonstrated for CO, which was also shown to have a role in vasorelaxation [149]. The same function came to be assigned to H₂S [17], which also led it to be labeled as an EDRF [127]. The multiple functions of gasotransmitters in human physiology are well attested by the “explosion” of interest that arose since the discovery of NO as a signaling molecule in the mid-1980s, CO in the mid-1990s, and H₂S in the 2000s. This is well illustrated by carrying out a Pubmed search querying “gasotransmitters,” “nitric oxide,” “carbon monoxide,” and “hydrogen sulfide” (Figure 12). Altogether, the three gasotransmitters regulate, often through similar molecular processes, vasodilation, energy metabolism, redox homeostasis, apoptosis, cell cycle, reproduction, neuronal

| Protein Target | Cysteine | Physiological Process | Consequences of Persulfidation | Refs. |
|---------------|---------|----------------------|-------------------------------|-------|
| Protein Tyr phosphatase 1B (PTP1B) | Cys₂¹₅ | ER stress response | PTP1B is inhibited, enhancing PERK-mediated phosphorylation of eIF2α, suppressing protein translation. | [137] |
| p65 subunit of nuclear factor κB (NF-κB) | Cys₅₈ | NF-κB signaling; apoptosis | Stimulates binding of p65 to ribosomal protein S3, resulting in increased translation of prosurvival genes. | [138] |
| Inwardly rectifying potassium channel subunit Kir6.1 | Cys₄₁₃ | Vasorelaxation and vasodilation | Enhances channel activity through decreased affinity for ATP and increased binding of PtdIns(4,5)P₂. | [139] |

*Protein kinase RNA-like ER kinase; beukaryotic translational initiation factor 2α; phosphatidylinositol-4,5-bisphosphate.

Table 1: Protein targets for cysteine persulfidation and molecular and cellular consequences.
function, and so on, and dysregulation of the metabolism and/or levels of NO, CO, or H₂S is associated with several pathological conditions, from cardiovascular and neurodegenerative diseases to diabetes and cancer, among others.

A surprising element in gasotransmitter history was the finding that mammals, among many other organisms, encode specialized enzymes that synthesize these previously considered noxious gaseous molecules. NO is enzymatically produced by NO synthases, of which three isoforms have been described and historically classified according to their location or regulation, yet presently considered to be ubiquitous and constitutively expressed: inducible NO synthase (iNOS), endothelial NO synthase (eNOS), and neuronal NO synthase (nNOS) (reviewed, e.g., in [150]). Regardless of the isoform and its localization or regulation, NO synthases catalyze the conversion of arginine and O₂ into NO and citrulline, using electron equivalents derived from NADPH. Besides being synthesized on demand by NO synthases, NO is endogenously generated (i) under hypoxic conditions through nitrite reduction by several heme proteins (including hemoglobin, as reviewed in [151]), (ii) in the digestive tract as an intermediate of bacterial nitrogen metabolism (particularly in the mouth and the gut), and (iii) chemically from nitrite under the acidic environment in the stomach (reviewed, e.g., in [152]). As for CO, it is generated by either isoform of heme oxygenase, HO-1 and HO-2, which catalyze the conversion of heme, with O₂ as cosubstrate, into biliverdin and CO, employing NADPH-derived electron equivalents (reviewed, e.g., in [153]). H₂S synthesis is catalyzed by CBS, CSE and MST, as described above.

As mentioned before, it has become increasingly clear that no gasotransmitter should be regarded as an isolated entity with an independent cellular function and lifespan. Rather, gasotransmitters interact with each other not only by direct cross-reactions that generate other reactive species but also by having cooperative or opposite molecular and cellular consequences towards their targets. Moreover, each gasotransmitter in many ways is able to modulate the synthesis of the others, which represents an additional intricate level of regulation. An example of this modulatory action is described below, concerning the regulation of CBS-catalyzed H₂S production by NO and CO.

4.2. Chemical Bases of the Interplay between H₂S and NO. The biological effects of H₂S and NO are intimately interconnected. The interplay between these two gaseous molecules takes place through numerous mechanisms acting at different levels (reviewed in [154]). Many studies aimed at gaining insight into the chemical foundation of the interaction between H₂S and NO. An extensive analysis has been carried out of the reactivity of sulfide with several NO metabolites, including nitrite [155], peroxynitrite [156, 157], and nitrosothiols [158–163], and with NO releasers such as nitroprusside [163–167]. Despite these efforts, the picture is currently far from being completely clear. As discussed in [5, 168–171], some remarkable discrepancies have indeed emerged, mainly regarding the nature of the species generated in these reactions and their suitability as signaling molecules under physiological conditions.

The first S/N hybrid species suggested to form by reaction of nitrosothiols (e.g., S-nitrosothiocarbanion (GSNO)) with sulfide was thionitrous acid/thionitrite (HSNO/ONS⁻) [161]. In line with previous studies by Whiteman et al. [172, 173], Filipovic et al. [161] reported the formation of HSNO by reaction of H₂S with either GSNO or acidified nitrite, as well as by investigation of the reaction of NO with HS⁻ generated by pulse radiolysis. In addition to HSNO, the reaction of GSNO with sulfide was found to yield also a yellow product assumed to be a mixture of polysulfides. Of interest regarding its potential physiological implications, HSNO was proposed to serve not only as a source of both NO and nitrosonium ion (NO⁺) but also as a donor of nitrosonium ion (NO⁺) to thiol, thereby promoting transnitrosation reactions even across biological membranes [161]. On these bases, HSNO was suggested to be a key species in the interplay between H₂S and NO, sitting at the crossroad between the H₂S and NO signaling pathways.

This view was challenged in subsequent studies by Cortese-Krott et al. [145, 155, 159, 160, 169, 174], who failed to detect HSNO accumulation during the reaction of GSNO with H₂S and claimed HSNO to be too short-lived to accumulate and be identified under physiological conditions, in agreement with Munro and Williams [175] and Williams [176]. Compared to other physiologically relevant nitrosothiols, HSNO was indeed suggested to be much more unstable as it undergoes a facile isomerization by hydrogen shift, leading to formation of four distinct isomers. The yellow product generated by reaction of GSNO with H₂S initially described in [161] was assigned by Cortese-Krott et al. [159] to nitropersulfide (SSNO⁻), a compound discovered and chemically characterized in the 80s [177]. The same research groups reported later [160] that, under physiologically relevant conditions, the reaction between sulfide and NO generates not only SSNO⁻ but also polysulfides and SULFI/NO [ON(NO)SO₄⁻], an additional bioactive...
compound consisting of an adduct of two molecules of NO with sulfite. Based on these and further investigations [174], Cortese-Krott et al. reported that SSNO− is stable in the presence of thiols and cyanides and is able to release NO upon decomposition, targeting the Keap1/Nrf2 redox system, among other possible ones. Overall, this led to the proposal that SSNO−, rather than HSNO, plays a key role in the interplay between NO and H2S under physiological conditions [159]. In line with this proposal, SSNO− was reported to be very stable under anaerobic conditions, to decay slowly (half life of about 40 minutes) in constant aerobic buffer under aerobic conditions, and to directly react with deoxygenated methHb with a rate constant of 11 M−1 s−1, yielding ferrous nitrosyl Hb (HbNO) presumably via NO release [178]. In contrast, by characterizing pure crystalline SSNO− prepared as a bis(triphenylphosphine)iminium (PNP+) salt according to [179], Filipovic et al. reported that SSNO− is a highly unstable species, leading to rapid decomposition with formation of HNO/NO− in the presence of water, light, or acid and producing HSNO by reaction with H2S, cyanide, and GSH [180, 181].

From the observations reported above, it is clear that additional investigations are needed to reconcile the discrepancies present in the literature and gain further insight into the chemical foundation of the interplay between NO and H2S.

4.3. Modulation of H2S Metabolism by NO and CO. Besides the chemical reactions that define new players in gasotransmitter-mediated signaling, there is an intricate web of cross-regulatory mechanisms whereby each gasotransmitter regulates the homeostasis of the others. While part of that cross-regulation may occur at the transcriptional and translational level, herein we will mainly cover the part of that cross-regulation may occur at the transcriptional and translational level, herein we will mainly cover the molecular mechanisms that result from direct interactions between the gasotransmitters and their target proteins, focusing on the modulation by NO and CO of H2S biosynthesis and breakdown.

4.3.1. Modulation of H2S-Generating Enzymes by NO, CO, and Derived Species. The most thoroughly studied H2S-generating enzyme in terms of regulatory mechanisms has certainly been CBS. In what regards its modulation by the other gasotransmitters NO and CO, CBS regulation is centered at its noncatalytic heme bound to the N-terminal domain. This interplay between gasotransmitters results essentially from the fact that H2S production by CBS is reversibly inhibited by NO and CO. The direct effect of this inhibitory mechanism would be that local increases in NO or CO result in decreased H2S synthesis, which has been demonstrated in different cellular models and tissues [182–185]. However, recent evidence suggests that CBS inhibition may in fact result in overall increased H2S production, due to a metabolic switch in the transsulfuration pathway (Figure 1(a)) [11, 186]. In this perspective, inhibiting CBS deviates the transsulfuration branch from cysteine to H2S production via CSE, which has a higher H2S-synthesizing activity, particularly using the accumulated homocysteine also resulting from CBS inhibition [11, 186]. Moreover, the pathophysiological significance of CBS inhibition by gasotransmitters may extend beyond "merely" affecting H2S homeostasis. CBS acts as a metabolic hinge determining the ratio between the remethylation and transsulfuration steps of the methionine cycle (Figure 1(a)). CO inhibition of CBS in various cancer cells has been shown to affect the methylation state of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3), suppressing 6-phosphofructo-2-kinase, resulting in a shift of glucose from glycolysis to the pentose phosphate pathway [34, 187].

The regulatory heme-binding domain may be considered a hallmark of CBS evolution, since it is absent from CBS from prokaryotes or unicellular eukaryotes. The low-spin hexacoordinate β-type heme is axially bound to the His65 imidazole and Cys83 thiolate moieties (human CBS numbering; Figure 4(b)). Besides its regulatory role, the CBS heme has been proposed to have a structural stabilizing effect and contribute to improve CBS folding [188]. After initial submission of this review, a quite interesting observation has been reported, which will once again stir the field of CBS heme regulation. Kumar et al. observed that the N-terminal residues 1–40, so far absent from all CBS crystallographic structures, constitute an intrinsically disordered region that is able to bind heme with Cys and His as axial ligands, in a cysteine-proline-based motif [189]. This observation, deserving further exploration, constitutes an additional level of complexity regarding all the literature on the CBS heme regulation (see below).

The heme regulatory role is essentially related to its redox state and the nature of its endogenous and exogenous ligands. The initial designation of the CBS heme as a redox sensor stemmed from its sensitivity to reducing conditions. Indeed, heme reduction by excess sodium dithionite at 37°C slowly (>20 minutes) inactivates the enzyme, which has been assigned to a ligand switch process in which one of the endogenous ligands of heme iron is replaced by another as yet unknown ligand (Figure 13 [190]. The resulting species has been termed "C424" due to its characteristic UV-visible absorption spectrum, in which the Soret band obtained for reduced CBS with its  max at 449 nm is shifted to a lower-intensity band centered at 424 nm. Irrespective of the bound endogenous ligands, once reduced, the heme is able to bind the gasotransmitters CO and NO, which results in reversible enzyme inhibition (Figure 13) [191–195]. Although the low reduction potential (~350 mV, [196]) measured for the CBS heme raised some doubts about the occurrence of reduced CBS heme in vivo, the human enzyme methionine synthase reductase (MSR) has been shown to be able to catalyze CBS reduction in vitro at the expense of NADPH oxidation. Moreover, MSR is able to generate both the ferrous-CO and the ferrous-NO species of human CBS [191, 197]. Despite the significant distance between the heme and PLP cofactors (~11 Å from edge to edge and ~20 Å from the Fe ion to the pyridoxine ring), communication between them is ensured by a network of molecular interactions occurring at helix 8 (depicted by the yellow arrow in Figure 4(b)) [198, 199]. While at the PLP end of helix 8 the residues Thr257 and Thr260 establish hydrogen bonds with the PLP...
phosphate group, at the heme extremity of this helix Arg265 is in electrostatic contact with the heme ligand Cys52 thiolate moiety. The relevance of these residues in the allosteric communication between the active (PLP) and regulatory (heme) sites has been further demonstrated in clinically relevant variants [199, 200].

The kinetic characterization of the interplay between the three gasotransmitters centered at CBS has been made possible by taking advantage of the distinct characteristic UV-visible absorption spectra displayed by the heme cofactor in different oxidation and ligation states. Most studies on the CBS redox state and ligand reactivity have thus employed static and time-resolved (stopped-flow) absorption spectroscopy [192–195, 199–204].

The reaction of reduced CBS with CO has been thoroughly characterized in vitro and shown to result in a hexacoordinate species in which the endogenous Cys52 ligand has been replaced by CO, with no evidence for stable intermediates. Indeed, the reaction is limited by the slow dissociation of Cys52 (0.003–0.017 s⁻¹ [192, 195, 197, 204]), as confirmed by laser flash photolysis and time-resolved Raman spectroscopy [192]. Equilibrium titrations revealed that CO binds to ferrous CBS according to two dissociation constants ($K_1 = 0.7–1.5 \mu M$ and $K_2 = 45–68 \mu M$; $C_{CO} = 30 \mu M$), which has been assigned to anticooperativity between paired monomers [192] or differences in the heme microenvironment [194]. The inhibitory effect of CO binding to the ferrous heme in human CBS is characterized by $K_i$ values of 5.6–9.5 \mu M [194, 204].

Recently, our groups have reported that a clinically relevant variant with a mutation in the N-terminal domain, p.P49L, displays a markedly higher affinity for CO with respect to WT, while showing an essentially WT-like behavior in other functional and structural terms [203]. While this mutation may induce increased flexibility of the loop containing both heme endogenous ligands, the overall higher proneness to become inhibited by CO may represent a new pathogenic mechanism in classical homocystinuria. Current efforts to develop alternative therapies for classical homocystinuria have essentially focused on chemical and pharmacological chaperones to recover misfolded protein variants originating from missense mutations [205–211] and recently on the development of a PEGylated CBS for enzyme replacement therapy [212–214].

NO binding to reduced CBS is remarkably faster and displays much higher affinity than CO binding. However, initial reports on the reaction between ferrous CBS and NO indicated otherwise. Very low affinity for NO was reported ($K_d = 281 \mu M$), employing a high excess of sodium dithionite to keep CBS reduced throughout the NO titrations and using a slow NO releaser (diethylamine NONOate) as NO source [193]. Under these conditions, free NO, either unreacted or dissociated from the ferrous-NO CBS adduct, reacts with the excess dithionite and leads to an overestimation of the dissociation constants, as later shown even with minute amounts of excess dithionite [195]. Recently, our groups have shown that NO binding to ferrous CBS occurs with an affinity compatible with a physiological role of NO in controlling CBS function [195]. An apparent $K_d$ of 0.23 \mu M was determined at the lowest dithionite concentration used in that study, which implies that the actual affinity is even higher than measured. Contemporarily, a $K_d = 30 \mu M$ was determined and still was considered an upper limit for this dissociation constant, as it was measured using NADPH and human MSR as reduction system, therefore involving multiple equilibria (NADPH ↔ MSR, MSR → CBS, and CBS ↔ NO) [191]. As compared to CO, NO binding to reduced CBS not only occurs with markedly higher affinity but is also orders of magnitude faster, as investigated by stopped-flow absorption spectroscopy. Differently from

**Figure 13:** Mechanisms of heme reduction and ligand binding to human CBS.
CO, NO binding results in a pentacoordinate ferrous-NO adduct, where both endogenous ligands have been displaced and a single NO molecule is bound. No reaction intermediates have been detected upon mixing NO with reduced CBS, at least within the time resolution of the stopped-flow equipment. Moreover, also differently from CO, NO binding proceeds with rate constants linearly dependent on NO concentration (up to 800 μM), that is, unlimited by dissociation of the endogenous ligands, yielding a bimolecular rate constant of ~8 × 10^3 M^-1 s^-1 (at 25°C). In line with the higher affinity and faster binding of NO with respect to CO, NO dissociation from ferrous CBS is slower than CO dissociation. In mechanistic terms, since NO binding is not rate-limited by the displacement of Cys52 (which instead limits CO binding), it has been proposed that NO may initially displace the His65 heme ligand (Figure 13). In turn, the absence of evidence for a hexacoordinate intermediate suggests that Cys52 is immediately displaced upon NO binding. Finally, although it has been established that the final NO adduct is a pentacoordinate species, it remains to be demonstrated whether NO ends up on the His65 or Cys52 side of the heme, since both residues are supposed to dissociate from the heme iron. It should be noted that if NO ends up on the Cys52 side, the binding mechanism must involve the formation of a transient bis-NO intermediate (Figure 13). Irrespectively of the mechanistic details, the kinetic and spectroscopic investigations of CBS reduction and CO and NO binding have determined parameters compatible with an in vivo role of this regulatory mechanism.

The reaction of reduced CBS with O₂ was also investigated by stopped-flow absorption spectroscopy and shown to be reasonably fast, occurring with a bimolecular rate constant of ~1 × 10^5 M^-1 s^-1 (25°C) [202], that is, approximately 10-fold faster than the reaction with NO. Although this reaction reverts CBS back to its active ferric state, it also generates superoxide anion as a reaction product, which may have damaging effects. This oxidation process has indeed been reported to involve a partial heme loss. Superoxide anion promptly reacts with NO (at rates close to the diffusion limit) to yield the powerful oxidant peroxynitrite. Interestingly, it has been shown that CBS heme not only is inactivated by peroxynitrite but also may be a source of it. The reaction of oxidized CBS with peroxynitrite has been shown to result in enzyme inactivation with an IC₅₀ of 150 μM, occurring according to a bimolecular rate constant of 2.4–5 × 10^4 M^-1 s^-1 and leading to nitration of Trp₂₀₈, Trp₄₃₀, and Trp₂₂₁, as well as damaging the heme moiety [215]. Intriguingly, a recent report demonstrated that the reaction between ferrous-NO CBS and O₂, previously shown to revert the heme to its ferric state, yields peroxynitrite [201] (Figure 13). Moreover, the reaction of reduced CBS with nitrite may itself constitute a source of NO [191, 201], which further adds to the complex heme-mediated regulation of H₂S synthesis by CBS (Figure 13).

4.3.2. Effect of AdoMet on CO/NO Binding and Inhibition of H₂S Production by CBS. CBS continues to surprise researchers in the field since regulation of the protein activity not only is intricate but also at times appears odd. It has long been established that AdoMet is an activator of CBS, increasing its enzymatic activity by 2- to 5-fold. Moreover, AdoMet binding also has a stabilizing effect, particularly under “normal” (nonpathological) conditions [216]. Recently, the structural details of this regulatory mechanism have been elucidated. While the C-terminal regulatory domain from one monomer blocks the substrate entry site in the catalytic core of an adjacent monomer, AdoMet binding to the C-terminal domain (also called Bateman module) causes a domain shift and leads to the association of two C-terminal domains in a disk-like form, unblocking the substrate entrance and thus “activating” (or derepressing) the enzyme [44, 46]. The relevance of this regulatory mechanism is well attested by the fact that some homocystinuric patients express CBS variants with mutations in the C-terminal domain, that are naturally activated (more active than WT) and have limited or absent AdoMet response as the molecular basis of disease [217, 218]. While working on the kinetic characterization of NO and CO binding to human CBS, we noticed that AdoMet has the intriguing effect of eliciting faster and tighter binding of both CO and NO, thus making the enzyme more prone to inhibition by either gasotransmitter [204]. This observation is directly related to the binding of exogenous ligands, since AdoMet has been shown to have no effect on the heme redox properties [196]. Under the same reaction conditions, AdoMet makes CO inhibition >10 times more potent than in its absence (Kᵢ(CO, AdoMet) = 0.7 μM versus Kᵢ(CO, AdoMet) = 9.5 μM) [204]. The kinetic details of this functional effect have been investigated, and it was shown that AdoMet increases CO affinity by ~5-fold and association kinetics by ~10-fold, whereas the effect on NO binding is less drastic (~2-fold higher affinity with respect to AdoMet-free CBS). The latter observation also enforces the previous mechanistic proposal that CO and NO “attack” different sides of the heme. Recently, this observation has been extended to a clinically relevant variant, p.P49L, which displays the same AdoMet response in terms of becoming more sensitive to CO, while being slightly impaired in the AdoMet activation of H₂S production, compared to WT [203]. Irrespectively of the mechanistic details, the AdoMet-induced enhanced CO and NO binding appears as an intricate regulatory mechanism which adds to the complexity of CBS regulation.

4.3.3. Modulation of Sulfide Oxidation by NO. Whereas the effect of CO and NO on H₂S biosynthesis has been investigated in detail, much scarcer is the information on the effect of these two gaseous molecules on H₂S catabolism. Because CO and NO, similarly to cyanide, can both potently inhibitCcOX through different mechanisms (see above), relatively low concentrations of these gasotransmitters are expected to affect mitochondrial sulfide oxidation. Following cell exposure to CO or NO, a transient inhibition of CcOX is indeed expected to occur and lead to electron accumulation in the mitochondrial respiratory chain, eventually impairing quinol oxidation. As a result, SQR-mediated oxidation of sulfide is expected to be impaired as long as CcOX inhibition persists. Accordingly, addition of the NO releaser MAMA NONOate (≥20 μM) or cyanide (260 μM) to colonocytes...
was recently reported to inhibit cellular respiration and its stimulation by 20 µM NaHS [219]. Furthermore, it is worth noting that long-term intraperitoneal or intravenous administration of nitroglycerine in rats was shown to induce decreased rhodanese activity and diminished levels of sulfane sulfur in liver extracts [220]. The observed inhibition of rhodanese was proposed to arise from $S$-nitrosation of the catalytic cysteine in the protein active site. A cysteine in the isolated rhodanese from bovine liver was indeed shown to be susceptible to $S$-nitrosation by the NO donors $S$-nitroso-$N$-acetylpenicillamine (SNAP) and GSNO, leading to enzyme inhibition [220].

The preliminary information reported above unveils an additional level at which the interplay between the gasotransmitters can be established. A full description of these molecular mechanisms awaits further investigations.

**4.3.4. Modulation of NO and CO Synthesis by Hydrogen Sulfide.** In light of the crosstalk between gasotransmitters, hydrogen sulfide has been shown to have a role in the control of NO and CO bioavailability under specific (patho)physiological conditions.

As mentioned above, CO is generated by either isoform of heme oxygenase, HO-1 and HO-2 [153]. Several lines of evidence point to a role of hydrogen sulfide in increasing the expression of heme oxygenase, particularly HO-1, and consequently CO levels (mostly evaluated from the abundance of carboxylated hemoglobin), thereby exerting either protective or deleterious effects (e.g., [221–227]).

The underlying regulatory mechanism is proposed to involve the Keap1/Nrf2 system. In particular, persulfidation of Keap1 Cys$_{151}$, by reaction either of H$_2$S with oxidized Cys$_{151}$ or of reduced Cys$_{151}$ with H$_2$S-derived HS, causes its dissociation from Nrf2 that, in turn, translocates into the nucleus, ultimately resulting in increased HO-1 transcription and protein levels (Figure 14) [225]. Another possibility whereby CO production may be modulated resides in the fact that a thiol/disulfide redox switch comprising three Cys-Pro signatures in HO-2 regulates heme binding [228, 229]. The sensitivity of this thiol/disulfide redox switch to cellu- lar redox state and possibly to the intracellular sulfide levels may afford another level of H$_2$S-mediated control of CO levels. Interestingly, the beneficial health effects of certain garlic-derived organosulfur compounds, particularly diallyl disulfide and trisulfide, have been proposed to be related with the modulation of CO production by H$_2$S via the Keap1/Nrf2 pathway [230–232].

The control of NO biosynthesis by hydrogen sulfide has been investigated in more detail and appears to occur at the transcriptional, translational, and posttranslational levels, involving other signaling pathways, besides a direct inhibition of all NOS isoforms by H$_2$S. The abundance of data in the literature highlights the fact that the effect of H$_2$S on the expression and activity of each NOS isoform can vary—sometimes yielding conflicting results—with the model organ, tissue, or cell type investigated and with the nature, concentration, and potency of the employed sulfide donor. Moreover, whereas in some studies “only”
the levels of NO (or nitrite/nitrate) are measured as a function of sulfide exposure, other studies further investigate the NO-mediated cellular and physiological effects of sulfide exposure.

Regulation of eNOS by H₂S has been thoroughly investigated. In different cell types and under different conditions and stimuli, eNOS activation in response to sulfide exposure seems to be mediated by the PIP3/Akt (phosphatidylinositol 3,4,5-trisphosphate-serine/threonine-specific protein kinase) pathway (Figure 14). The molecular mechanism is proposed to involve the reduction by H₂S of the vascular endothelial growth factor receptor 2 (VEGFR2) Cys₃₀₂₅-Cys₃₀₄₅ disulfide that in the oxidized state represses its receptor activity. This H₂S-induced disulfide reduction thus prolongs the VEGFR2 activity and consequently the phosphatidylinositol-4,5-bisphosphate-3-kina (PI3K) activity, yielding an increase in PIP3 levels (which may also be influenced by persulfidation of Cys₁₂₄ in the phosphatase and tensin homolog (PTEN)). The resulting increased Akt activation leads to enhanced eNOS phosphorylation at Ser₁₁₇₇ and/or Ser₁₁₉₀ thereby increasing the NO-synthesizing activity. Another level of sulfide-mediated eNOS regulation concerns the direct persulfidation at Cys₄₄₃ (Figure 14). Whereas dimerization appears to be a prerequisite for optimal eNOS activity, S-nitrosation of Cys₄₄₃ induces dimer dissociation into the virtually inactive monomeric form. Sulfide-mediated persulfidation of this residue actively competes with S-nitrosation, thereby maintaining eNOS in the active homodimeric form. Direct in vitro inhibition of recombinant eNOS by H₂S is characterized by an extremely high (nonphysiological) IC₅₀ of 170 μM [233]. Regarding the sulfide-mediated modulation of eNOS expression, various reports have either shown a down- or upregulation of eNOS mRNA and protein levels in response to H₂S exposure. These somehow contradictory results obtained for different model systems (animals, organs, and cells) rule out the establishment of a clear trend, induction versus repression, for sulfide modulation of eNOS expression.

Besides its direct in vitro inhibition at nonphysiological H₂S concentrations (IC₅₀ = 210 μM) [233], sulfide-mediated modulation of iNOS is reported to occur mainly through regulation of its expression. Similarly to eNOS, initial contradictory results indicated either an increase or a decrease in iNOS-mediated NO production in response to sulfide exposure [234]. This controversy was addressed by comparatively investigating the effects of different H₂S donors [235], showing that the rate of H₂S release by the donors is a key determinant for the downstream effects. Subsequent investigations on various models commonly assigned an inhibitory effect of sulfide on iNOS mRNA expression and consequently iNOS-mediated NO production, thereby contributing to the establishment of H₂S as an anti-inflammatory molecule (reviewed, e.g., in [154]).

Currently, the information on nNOS modulation by H₂S is scarce. Besides its inhibition at high nonphysiological sulfide concentrations (IC₅₀ = 130 μM) [233, 236], nNOS expression seems to be either insensitive to sulfide exposure or decreased upon overexpression of the H₂S-synthesizing CBS in hypertensive (but not normotensive) rats [237].

Altogether, the multiple molecular mechanisms whereby each of the three gasotransmitters may control the bioavailability of the others highlight the intricate signaling mechanisms that govern mammalian (patho)physiology and offer valuable possibilities for pharmacological targetting of each pathway.

## 5. Concluding Remarks

Hydrogen sulfide evolved from being merely perceived as a toxic and poisonous molecule to a prominent signaling molecule in mammalian physiology, joining nitric oxide and carbon monoxide as the triad of gasotransmitters. The last couple of decades witnessed an accumulation of data showing that (i) H₂S is endogenously synthesized and catabolized by specialized enzymes and kept under a tight control through multiple intrinsic regulatory mechanisms, (ii) the signaling functions attributed to H₂S are mainly linked to the interaction with and modification of target proteins, via reaction with metal centers (mostly in heme proteins) and through cysteine persulfidation that alters protein structure and function, (iii) dysregulation of H₂S homeostasis is at the basis of several pathologies, including cardiovascular and neurodegenerative diseases and cancer, and (iv) possible mechanisms underlying a functional crosstalk between the three gasotransmitters have been unveiled. The clear link between H₂S metabolism and homeostasis with human health has triggered a strong interest in the discovery and development of pharmacological (synthetic and naturally derived) compounds, either to release H₂S or to modulate the H₂S metabolism enzymes. Despite the accumulated knowledge on hydrogen sulfide biochemistry over the years, all a contribution as this one can amount to be, particularly concerning a field in its infancy, is just a still picture of a fast moving field. Indeed, during the writing of this manuscript, several pieces of information even challenging previous observations arose in the literature, further contributing to the complexity of this quickly progressing field.

## Abbreviations

| Abbreviation | Definition |
|--------------|------------|
| 3-MP         | 3-Mercaptopyrurate |
| AdoHcy       | S-Adenosyl-L-homocysteine |
| AdoMet       | S-Adenosyl-L-methionine |
| ADT-OH       | 5-(4-Hydroxyphenyl)-3H-1,2-dithiole-3-thione |
| Akt          | Serine/threonine-specific protein kinase |
| AOAa         | Aminoxyacetic acid |
| AP39         | [10-Oxo-10-(4-(3-thioxy-3H-1,2-dithiol-5yl)phenoxy)decyl] triphenylphosphonium bromide |
| ATP          | Adenosine triphosphate |
| CARSs        | Cysteinyl-tRNA synthetases |
| CAT          | Cysteine (or aspartate) aminotransferase |
| CBS          | Cystathionine β-synthase |
| CccOx        | Mitochondrial cytochrome c oxidase (complex IV) |
| CO           | Carbon monoxide |
| CSE          | Cystathionine γ-lyase |
CysSOH: Cysteine residue with modified thiol to sulfenic acid
CysSSH: Cysteine persulfide
CysSS\((\text{H})\): Cysteine polysulfide
CysSOH: Cysteine perthiosulfenic acid
CysSSR: Cysteine residues with modified thiol to disulfide
DHLA: Dihydrolipoic acid
EDHF: Endothelium-derived hyperpolarizing factor
EDFR: Endothelium-derived relaxing factor
eIF2α: Eukaryotic translational initiation factor 2α
eNOS: Endothelial nitric oxide synthase
EPR: Electron paramagnetic resonance spectroscopy
ETHE1: Persulfide dioxygenase, or ethylmalonic encephalopathy protein 1
FAD: Flavin adenine dinucleotide
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
GSNO: S-Nitrosoglutathione
GSSH: Glutathione persulfide
GSS\((\text{H})\): Glutathione polysulfide
Hb: Hemoglobin
H\(_2\)S: Hydrogen sulfide
H\(_2\)S\(_2\): Hydrogen persulfide
H\(_2\)S\(_3\): Hydrogen trisulfide
HO-1: Heme oxygenase 1
HO-2: Heme oxygenase 2
HS\(^-\): Hydrosulfide
HSNO/ONS\(^+\): Thionitrous acid/thionitrite
iNOS: Inducible nitric oxide synthase
Keap1: Kelch-like ECH-associated protein 1
Kir6.1: Inwardly rectifying potassium channel subunit Kir6.1
LMW: Low-molecular-weight
metHb: Ferric hemoglobin
Mb: Myoglobin
MSR: Methionine synthase reductase
MST: 3-Mercaptopyruvate sulfurtransferase
NADPH: Nicotinamide adenine dinucleotide phos-

Conflicts of Interest
The authors declare that they have no conflicts of interest.

Acknowledgments
The iNOVA4Health Research Unit (LISBOA-01-0145-FEDE-007344), which is cofunded by Fundação para a Ciência e a Tecnologia/Ministério da Ciência, Tecnologia e Ensino Superior, through national funds, and by FEDER under the PT2020 Partnership Agreement, is acknowledged by João B. Vicente. The grants PNR-CNR Aging Program 2012–2014 and PRIN 20155EB2CM 003 by Ministero dell’Istruzione, dell’Università della Ricerca of Italy are acknowledged by Alessandro Giuffré. The authors are grateful to present and past collaborators and to Catarina Tomé for critically reading the manuscript.

References

[1] B. Ramazzini, *De Morbis Artificum Diatriba*, Baptismum Conzattum, Padova, 1713.
[2] C. W. Scheele, *Chemische Abhandlung von der Luft und dem Feuer*, Magnus Swedenus, Upsala, Sweden, 1777.
[3] E. Cuevasanta, M. N. Möller, and B. Alvarez, "Biological chemistry of hydrogen sulfide and persulfides," *Archives of Biochemistry and Biophysics*, vol. 617, pp. 9–25, 2017.
[4] K. R. Olson and K. D. Straub, "The role of hydrogen sulfide in evolution and the evolution of hydrogen sulfide in metabolism and signaling," *Physiology*, vol. 31, no. 1, pp. 60–72, 2016.
[5] Q. Li and J. R. Lancaster Jr, "Chemical foundations of hydrogen sulfide biology," *Nitric Oxide*, vol. 35, pp. 21–34, 2013.
[6] K. Ono, T. Akaike, T. Sawa et al., "Redox chemistry and chemical biology of H\(_2\)S, hydrosulfides, and derived species: implications of their possible biological activity and utility," *Free Radical Biology and Medicine*, vol. 77, pp. 82–94, 2014.
[7] B. Szczesny, K. Módis, K. Yanagi et al., "AP39, a novel mitochondria-targeted hydrogen sulfide donor, stimulates cellular bioenergetics, exerts cytoprotective effects and protects against the loss of mitochondrial DNA integrity in oxidatively stressed endothelial cells in vitro," *Nitric Oxide*, vol. 41, pp. 120–130, 2014.
[8] X. Shen, M. Carlström, S. Borniquel, C. Jäder, C. G. Kevil, and J. O. Lundberg, "Microbial regulation of host hydrogen
sulfide bioavailability and metabolism,” Free Radical Biology and Medicine, vol. 60, pp. 195–200, 2013.

[9] P. Rose, P. K. Moore, and Y. Z. Zhu, “H₂S biosynthesis and catabolism: new insights from molecular studies,” Cellular and Molecular Life Sciences, vol. 74, no. 8, pp. 1391–1412, 2017.

[10] O. Kabil and R. Banerjee, “Enzymology of H₂S biogenesis, decay and signaling,” Antioxidants & Redox Signaling, vol. 20, no. 5, pp. 770–782, 2014.

[11] R. Banerjee, “Catalytic promiscuity and heme-dependent redox regulation of H₂S synthesis,” Current Opinion in Chemical Biology, vol. 37, pp. 115–121, 2017.

[12] L. Bao, Č. Vlček, V. Pačes, and J. P. Kraus, “Identification and tissue distribution of human cystathionine β-synthase mRNA isoforms,” Archives of Biochemistry and Biophysics, vol. 350, no. 1, pp. 95–103, 1998.

[13] O. Kabil, V. Vitvitsky, P. Xie, and R. Banerjee, “The quantitative significance of the transsulfuration enzymes for H₂S production in murine tissues,” Antioxidants & Redox Signaling, vol. 15, no. 2, pp. 363–372, 2011.

[14] S. H. Mudd, J. D. Finkelstein, F. Irreverre, and L. Laster, “Transsulfuration in mammals. Microsomes and tissue distributions of three enzymes of the pathway,” Journal of Biological Chemistry, vol. 240, no. 11, pp. 4382–4392, 1965.

[15] S. Saha, P. K. Chakraborty, X. Xiong et al., “Cystathionine β-synthase regulates endothelial function via protein S-sulphydration,” The FASEB Journal, vol. 30, no. 1, pp. 441–456, 2016.

[16] Y. Ogawara, S. Isoda, and S. Tanabe, “Tissue and subcellular distribution of bound and acid-labile sulfur, and the enzymatic capacity for sulfide production in the rat,” Biological & Pharmaceutical Bulletin, vol. 17, no. 12, pp. 1535–1542, 1994.

[17] G. Yang, L. Wu, B. Jiang et al., “H₂S as a physiologic vasorelaxant: hypertension in mice with deletion of cystathionine γ-lyase,” Science, vol. 322, no. 5901, pp. 587–590, 2008.

[18] T. Taniguchi and T. Kimura, “Role of 3-mercaptopyruvate sulfurtransferase in the formation of the iron-sulfur chromophore of adrenal ferredoxin,” Biochimica et Biophysica Acta (BBA) - Enzymology, vol. 364, no. 2, pp. 284–295, 1974.

[19] N. Nagahara, T. Ito, H. Kitamura, and T. Nishino, “Tissue and subcellular distribution of mercaptopyruvate sulfurtransferase in the rat: confocal laser fluorescence and immunoelectron microscopic studies combined with biochemical analysis,” Histochemistry and Cell Biology, vol. 110, no. 3, pp. 243–250, 1998.

[20] M. Tomita, N. Nagahara, and T. Ito, “Expression of 3-mercaptopyruvate sulfurtransferase in the mouse,” Molecules, vol. 21, no. 12, article 1707, 2016.

[21] V. Vitvitsky, P. K. Yadav, A. Kurthen, and R. Banerjee, “Sulfide oxidation by a noncanonical pathway in red blood cells generates thiosulfate and polysulfides,” Journal of Biological Chemistry, vol. 290, no. 13, pp. 8310–8320, 2015.

[22] O. Kabil and R. Banerjee, “Redox biochemistry of hydrogen sulfide,” Journal of Biological Chemistry, vol. 285, no. 29, pp. 21903–21907, 2010.

[23] S. E. Bearden, R. S. Beard Jr, and J. C. Pfau, “Extracellular transsulfuration generates hydrogen sulfide from homocysteine and protects endothelium from redox stress,” American Journal of Physiology-Heart and Circulatory Physiology, vol. 299, no. 5, pp. H1568–H1576, 2010.

[24] O. Kabil, Y. Zhou, and R. Banerjee, “Human cystathionine β-synthase is a target for sumoylation,” Biochemistry, vol. 45, no. 45, pp. 13528–13536, 2006.

[25] F. Bouillaud and F. Blachier, “Mitochondria and sulfide: a very old story of poisoning, feeding, and signaling?,” Antioxidants & Redox Signaling, vol. 15, no. 2, pp. 379–391, 2011.

[26] A. K. Mustafa, M. M. Gadalla, N. Sen et al., “H₂S signals through protein S-sulphydralation,” Science Signaling, vol. 2, no. 96, p. ra72, 2009.

[27] K. Módis, Y. J. Ju, A. Ahmad et al., “S-Sulphydralation of ATP synthase by hydrogen sulfide stimulates mitochondrial bioenergetics,” Pharmacological Research, vol. 113, no. Part A, pp. 116–124, 2016.

[28] M. Fu, W. Zhang, L. Wu, G. Yang, H. Li, and R. Wang, “Hydrogen sulfide (H₂S) metabolism in mitochondria and its regulatory role in energy production,” Proceedings of the National Academy of Sciences of the United States of America, vol. 109, no. 8, pp. 2943–8, 2012.

[29] C. E. Cooper and G. C. Brown, “The inhibition of mitochondrial cystochrome oxidase by the gases carbon monoxide, nitric oxide, hydrogen cyanide and hydrogen sulfide: chemical mechanism and physiological significance,” Journal of Bioenergetics and Biomembranes, vol. 40, no. 5, pp. 533–539, 2008.

[30] P. Nicholls, D. C. Marshall, C. E. Cooper, and M. T. Wilson, “Sulfide inhibition of and metabolism by cytochrome c oxidase,” Biochemical Society Transactions, vol. 41, no. 5, pp. 1312–1316, 2013.

[31] J. B. Vicente, F. Malagrinò, M. Arese, E. Forte, P. Sarti, and A. Giuffré, “Bioenergetic relevance of hydrogen sulfide and the interplay between gasotransmitters at human cystathionine β-synthase,” Biochimica et Biophysica Acta (BBA) - Bioenergetics, vol. 1857, no. 8, pp. 1127–1138, 2016.

[32] H. Teng, B. Wu, K. Zhao, G. Yang, L. Wu, and R. Wang, “Oxygen-sensitive mitochondrial accumulation of cystathionine β-synthase mediated by Lon protease,” Proceedings of the National Academy of Sciences of the United States of America, vol. 110, no. 31, pp. 12679–12684, 2013.

[33] C. Szabo, C. Coletta, C. Chao et al., “Tumor-derived hydrogen sulfide, produced by cystathionine-β-synthase, stimulates bioenergetics, cell proliferation, and angiogenesis in colon cancer,” Proceedings of the National Academy of Sciences of the United States of America, vol. 110, no. 30, pp. 12474–12479, 2013.

[34] T. Yamamoto, N. Takano, K. Ishiwata et al., “Reduced methylation of PFKFB3 in cancer cells shunts glucose towards the pentose phosphate pathway,” Nature Communications, vol. 5, article 3480, 2014.

[35] A. Davoli, V. Greco, A. Spalloni et al., “Evidence of hydrogen sulfide involvement in amyotrophic lateral sclerosis,” Annals of Neurology, vol. 77, no. 4, pp. 697–709, 2015.

[36] B. Fräsdorf, C. Radon, and S. Leimkühler, “Characterization and interaction studies of two isoforms of the dual localized 3-mercaptopyruvate sulfurtransferase TUM1 from humans,” Journal of Biological Chemistry, vol. 289, no. 50, pp. 34543–34556, 2014.

[37] J. D. Finkelstein, “Pathways and regulation of homocysteine metabolism in mammals,” Seminars in Thrombosis and Hemostasis, vol. 26, no. 3, pp. 219–226, 2000.
Oxidative Medicine and Cellular Longevity

[38] T. Majtan, J. Krijt, J. Sokolová et al., “Biogenesis of hydrogen sulfide and thiouethers by cystathionine beta-synthase,” Antioxidants & Redox Signaling, vol. 28, no. 4, pp. 311–323, 2018.

[39] S. Singh, D. Padovani, R. A. Leslie, T. Chiku, and R. Banerjee, “Relative contributions of cystathionine β-synthase and γ-cystathionase to H₂S biogenesis via alternative transulfuration reactions,” Journal of Biological Chemistry, vol. 284, no. 33, pp. 22457–22466, 2009.

[40] T. Chiku, D. Padovani, W. Zhu, S. Singh, V. Vitvitsky, and R. Banerjee, “H₂S biogenesis by human cystathionine γ-lyase leads to the novel sulfur metabolites lanthionine and homolanthionine and is responsive to the grade of hyperhomocysteinemia,” Journal of Biological Chemistry, vol. 284, no. 17, pp. 11601–11612, 2009.

[41] V. Kožič, J. Krijt, J. Sokolová et al., “Thioethers as markers of hydrogen sulfide production in homocystinurias,” Biochimie, vol. 126, pp. 14–20, 2016.

[42] N. Shibuya, S. Koike, M. Tanaka et al., “A novel pathway for the production of hydrogen sulfide from D-cysteine in mammalian cells,” Nature Communications, vol. 4, article 1366, 2013.

[43] P. K. Yadav, K. Yamada, T. Chiku, M. Koutmos, and R. Banerjee, “Structure and kinetic analysis of H₂S production by human mercaptopyruvate sulfurtransferase,” Journal of Biological Chemistry, vol. 288, no. 27, pp. 20002–20013, 2013.

[44] J. Ereño-Orbea, T. Majtan, I. Oyenarte, J. P. Kraus, and L. A. Martínez-Cruz, “Structural basis of regulation and oligomerization of human cystathionine β-synthase, the central enzyme of transulfuration,” Proceedings of the National Academy of Sciences of the United States of America, vol. 110, no. 40, pp. E3790–E3799, 2013.

[45] M. Meier, M. Janosik, V. Kery, J. P. Kraus, and P. Burkhard, “Structure of human cystathionine β-synthase: a unique pyridoxal 5’-phosphate-dependent heme protein,” The EMBO Journal, vol. 20, no. 15, pp. 3910–3916, 2001.

[46] J. Ereño-Orbea, T. Majtan, I. Oyenarte, J. P. Kraus, and L. A. Martínez-Cruz, “Structural insight into the molecular mechanism of allosteric activation of human cystathionine β-synthase by S-adenosylmethionine,” Proceedings of the National Academy of Sciences of the United States of America, vol. 111, no. 37, pp. E3845–E3852, 2014.

[47] M. Koutmos, O. Kabil, J. L. Smith, and R. Banerjee, “Structural basis for substrate activation and regulation by cystathionine beta-synthase (CBS) domains in cystathionine β-synthase,” Proceedings of the National Academy of Sciences of the United States of America, vol. 107, no. 49, pp. 20958–20963, 2010.

[48] T. J. McCordie, J. Kopec, S.-J. Hyung et al., “Inter-domain communication of human cystathionine β-synthase: structural basis of S-adenosyl-L-methionine activation,” Journal of Biological Chemistry, vol. 289, no. 52, pp. 36018–36030, 2014.

[49] A. L. Pey, T. Majtan, J. M. Sanchez-Ruiz, and J. P. Kraus, “Human cystathionine β-synthase (CBS) contains two classes of binding sites for S-adenosylmethionine (SAM): complex regulation of CBS activity and stability by SAM,” Biochemical Journal, vol. 449, no. 1, pp. 109–121, 2013.

[50] Q. Sun, R. Collins, S. Huang et al., “Structural basis for the inhibition mechanism of human cystathionine γ-lyase, an enzyme responsible for the production of H₂S,” Journal of Biological Chemistry, vol. 284, no. 5, pp. 3076–3085, 2009.

[51] K. Hanaoka, K. Sasakura, Y. Suwanai et al., “Discovery and mechanistic characterization of selective inhibitors of H₂S-producing enzyme: 3-mercaptoppyruvate sulfurtransferase (3MST) targeting active-site cysteinylpersulfide,” Scientific Reports, vol. 7, article 40227, 2017.

[52] L. C. Petersen, “The effect of inhibitors on the oxygen kinetics of cytochrome c oxidase,” Biochimica et Biophysica Acta (BBA) - Bioenergetics, vol. 460, no. 2, pp. 299–307, 1977.

[53] C. G. Curtis, T. C. Bartholomew, F. A. Rose, and K. S. Dodgson, “Detoxification of sodium S-sulphide in the rat,” Biochemical Pharmacology, vol. 21, no. 17, pp. 2313–2321, 1972.

[54] E. Lagoutte, S. Mimoun, M. Andriamihaja, C. Chaumontet, F. Blachier, and F. Bouillaud, “Oxidation of hydrogen sulfide remains a priority in mammalian cells and causes reverse electron transfer in colonocytes,” Biochimica et Biophysica Acta (BBA) - Bioenergetics, vol. 1797, no. 8, pp. 1500–1511, 2010.

[55] T. M. Hildebrandt and M. K. Grieshaber, “Three enzymatic activities catalyze the oxidation of sulfide to thiosulfate in mammalian and invertebrate mitochondria,” FEBS Journal, vol. 275, no. 13, pp. 3352–3361, 2008.

[56] K. R. Olson, Y. Gao, F. Arif et al., “Metabolism of hydrogen sulfide (H₂S) and production of reactive sulfur species (RSS) by superoxide dismutase,” Redox Biology, vol. 15, pp. 74–85, 2018.

[57] K. R. Olson, Y. Gao, E. R. DeLeon et al., “Catalase as a sulfide-sulfur oxidoreductase: an ancient (and modern?) regulator of reactive sulfur species (RSS),” Redox Biology, vol. 12, pp. 325–339, 2017.

[58] D. G. Searcy, “H₂S-O₂ oxidoreductase activity of Cu,Zn superoxide dismutase,” Archives of Biochemistry and Biophysics, vol. 334, no. 1, pp. 50–58, 1996.

[59] D. G. Searcy, J. P. Whitehead, and M. J. Maroney, “Interaction of Cu,Zn superoxide dismutase with hydrogen sulfide,” Archives of Biochemistry and Biophysics, vol. 318, no. 2, pp. 251–263, 1995.

[60] M. A. Powell and G. N. Somero, “Hydrogen sulfide oxidation is coupled to oxidative phosphorylation in mitochondria of Solemya reidi,” Science, vol. 233, no. 4763, pp. 563–566, 1986.

[61] M. Goubert, M. Andriamihaja, T. Nubel, F. Blachier, and F. Bouillaud, “Sulfide, the first inorganic substrate for human cells,” The FASEB Journal, vol. 21, no. 8, pp. 1699–1706, 2007.

[62] C. Szabo, C. Ransy, K. Modis et al., “Regulation of mitochondrial bioenergetic function by hydrogen sulfide. Part I. Biochemical and physiological mechanisms,” British Journal of Pharmacology, vol. 171, no. 8, pp. 2099–2122, 2014.

[63] A. Abou-Handam, C. Ransy, T. Roger, H. Guedouari-Bounihi, E. Galardon, and F. Bouillaud, “Positive feedback during sulfide oxidation fine-tunes cellular affinity for oxygen,” Biochimica et Biophysica Acta (BBA) - Bioenergetics, vol. 1857, no. 9, pp. 1464–1472, 2016.

[64] X. Leschelle, M. Goubert, M. Andriamihaja et al., “Adaptive metabolic response of human colonic epithelial cells to the adverse effects of the luminal compound sulfide,” Biochimica et Biophysica Acta (BBA) - General Subjects, vol. 1725, no. 2, pp. 201–212, 2005.

[65] R. Yong and D. G. Searcy, “Sulfide oxidation coupled to ATP synthesis in chicken liver mitochondria,” Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, vol. 129, no. 1, pp. 129–137, 2001.
of H₂S in mammalian cells and mitochondria,” Methods in Enzymology, vol. 554, pp. 201–228, 2015.

[67] M. Groeger, J. Matallo, O. McCoak et al., “Temperature and cell-type dependency of sulfide effects on mitochondrial respiration,” Shock, vol. 38, no. 4, pp. 367–374, 2012.

[68] N. Helmy, C. Prip-Buus, C. Vons et al., “Oxidation of hydrogen sulfide by human liver mitochondria,” Nitric Oxide, vol. 41, pp. 105–112, 2014.

[69] J. Matallo, J. Vogt, O. McCoak et al., “Sulfide-inhibition of mitochondrial respiration at very low oxygen concentrations,” Nitric Oxide, vol. 41, pp. 79–84, 2014.

[70] S. Mimoun, M. Andramihaja, C. Chaumontet et al., “Detoxification of H₂S by differentiated colonic epithelial cells: implication of the sulfide oxidizing unit and of the cell respiratory capacity,” Antioxidants & Redox Signaling, vol. 17, no. 1, pp. 1–10, 2012.

[71] K. Módis, C. Coletta, K. Erdélyi, A. Papapetropoulos, and C. Szabo, “Intramitochondrial hydrogen sulfide production by 3-mercaptopyruvate sulfurtransferase maintains mitochondrial electron flow and supports cellular bioenergetics,” The FASEB Journal, vol. 27, no. 2, pp. 601–611, 2013.

[72] D. G. Searcy, “Metabolic integration during the evolutionary origin of mitochondria,” Cell Research, vol. 13, no. 4, pp. 229–238, 2003.

[73] U. Theissen, M. Hoffmeister, M. Grieshaber, and W. Martin, “Single eukaryotic origin of eukaryotic sulfide:quinone oxidoreductase, a mitochondrial enzyme conserved from the early evolution of eukaryotes during anoxic and sulfidic times,” Molecular Biology and Evolution, vol. 20, no. 9, pp. 1564–1574, 2003.

[74] J. A. Brito, F. L. Sousa, M. Stelter et al., “Structural and functional insights into sulfide:quinone oxidoreductase,” Biochemistry, vol. 48, no. 24, pp. 5613–5622, 2009.

[75] M. M. Cherney, Y. Zhang, M. Solomonson, J. H. Weiner, and M. N. G. James, “Crystal structure of sulfide:quinone oxidoreductase from Acidithiobacillus ferrooxidans: insights into sulfidotrophic respiration and detoxification,” Journal of Molecular Biology, vol. 398, no. 2, pp. 292–305, 2010.

[76] M. Marcia, U. Ermler, G. Peng, and H. Michel, “The structure of Aquifex aeolicus sulfide:quinone oxidoreductase, a basis to understand sulfide detoxification and respiration,” Proceedings of the National Academy of Sciences of the United States of America, vol. 106, no. 24, pp. 9625–9630, 2009.

[77] M. Marcia, U. Ermler, G. Peng, and H. Michel, “A new structure-based classification of sulfide:quinone oxidoreductases,” Proteins: Structure, Function, and Bioinformatics, vol. 78, no. 5, pp. 1073–1083, 2010.

[78] M. M. Cherney, Y. Zhang, M. N. G. James, and J. H. Weiner, “Structure–activity characterization of sulfide:quinone oxidoreductase variants,” Journal of Structural Biology, vol. 178, no. 3, pp. 319–328, 2012.

[79] K. D. C. Augustyn, M. R. Jackson, and M. S. Jorns, “Use of tissue metabolite analysis and enzyme kinetics to discriminate between alternate pathways for hydrogen sulfide metabolism,” Biochemistry, vol. 56, no. 7, pp. 986–996, 2017.

[80] M. R. Jackson, S. L. Melideo, and M. S. Jorns, “Human sulfide:quinone oxidoreductase catalyzes the first step in hydrogen sulfide metabolism and produces a sulfane sulfur metabolite,” Biochemistry, vol. 51, no. 34, pp. 6804–6815, 2012.

[81] M. R. Jackson, S. L. Melideo, and M. S. Jorns, “Role of human sulfide: quinone oxidoreductase in H₂S metabolism,” Methods in Enzymology, vol. 554, pp. 255–270, 2015.

[82] M. Libiad, P. K. Yadav, V. Vitvitsky, M. Martinov, and R. Banerjee, “Organization of the human mitochondrial hydrogen sulfide oxidation pathway,” Journal of Biological Chemistry, vol. 289, no. 45, pp. 30901–30910, 2014.

[83] A. P. Landry, D. P. Ballou, and R. Banerjee, “H₂S oxidation by nanodisc-embedded human sulfide quinone oxidoreductase,” Journal of Biological Chemistry, vol. 292, no. 28, pp. 11641–11649, 2017.

[84] T. V. Mishanina, P. K. Yadav, D. P. Ballou, and R. Banerjee, “Transient kinetic analysis of hydrogen sulfide oxidation catalyzed by human sulfide quinone oxidoreductase,” Journal of Biological Chemistry, vol. 290, no. 41, pp. 25072–25080, 2015.

[85] C. Griesbeck, M. Schütz, T. Schödl et al., “Mechanism of sulfide:quinone reductase investigated using site-directed mutagenesis and sulfur analysis,” Biochemistry, vol. 41, no. 39, pp. 11552–11565, 2002.

[86] V. Tiranti, P. D’Adamo, E. Briem et al., “Ethylmalonic encephalopathy is caused by mutations in ETFE1, a gene encoding a mitochondrial matrix protein,” The American Journal of Human Genetics, vol. 74, no. 2, pp. 239–252, 2004.

[87] V. Tiranti, C. Viscomi, T. Hildebrandt et al., “Loss of ETFE1, a mitochondrial dioxygenase, causes fatal sulfide toxicity in ethylmalonic encephalopathy,” Nature Medicine, vol. 15, no. 2, pp. 200–205, 2009.

[88] I. Pettinati, J. Brem, M. A. McDonough, and C. J. Schofield, “Crystal structure of human persulfide dioxygenase: structural basis of ethylmalonic encephalopathy,” Human Molecular Genetics, vol. 24, no. 9, pp. 2458–2469, 2015.

[89] R. J. Trevino, F. Glubish, R. Berni et al., “NH₁-terminal sequence truncation decreases the stability of bovine rhodanese, minimally perturbs its crystal structure, and enhances interaction with GroEL under native conditions,” Journal of Biological Chemistry, vol. 274, no. 20, pp. 13938–13947, 1999.

[90] K. Johnson-Winters, G. Tollin, and J. H. Enemark, “Elucidating the catalytic mechanism of sulfite oxidizing enzymes using structural, spectroscopic, and kinetic analyses,” Biochemistry, vol. 49, no. 34, pp. 7242–7254, 2010.

[91] C. L. Bianco, J. P. Toscano, and J. M. Fukuto, “Chapter 2 – an integrated view of the chemical biology of NO, CO, H₂S and O₂,” in Nitric Oxide (Third Edition), L. J. Ignarro and B. Freeman, Eds., pp. 9–21, Academic Press, London, UK, 2017.

[92] H. O. Michel, “A study of sulfhemoglobin,” Journal of Biological Chemistry, vol. 126, no. 1, pp. 323–348, 1938.

[93] J. A. Berzofsky, J. Peisach, and W. E. Blumberg, “Sulfheme proteins. I. Optical and magnetic properties of sulfymoglobin and its derivatives,” Journal of Biological Chemistry, vol. 246, no. 10, pp. 3367–3377, 1971.

[94] P. Nicholls, “The formation and properties of sulphymoglobin and sulphocatalase,” Biochemical Journal, vol. 81, no. 2, pp. 374–383, 1961.

[95] P. Nagy, “Mechanistic chemical perspective of hydrogen sulfide signaling,” Methods in Enzymology, vol. 554, pp. 3–29, 2015.

[96] B. B. Ríos-González, E. M. Román-Morales, R. Pietri, and J. López-Garriga, “Hydrogen sulfide activation in hemoproteins: the sulfheme scenario,” Journal of Inorganic Biochemistry, vol. 133, pp. 78–86, 2014.
[97] R. Pietri, E. Román-Morales, and J. López-Garriga, "Hydrogen sulfide and heme proteins: knowledge and mysteries," *Antioxidants & Redox Signaling*, vol. 15, no. 2, pp. 393–404, 2011.

[98] B. Chance, M. Erecinska, and M. Wagner, "Mitochondrial responses to carbon monoxide toxicity," *Annals of the New York Academy of Sciences*, vol. 174, pp. 193–204, 1970.

[99] M. Brunori, A. Giuffrè, E. Forte, D. Mastrononila, M. C. Barone, and P. Sarti, "Control of cytochrome c oxidase activity by nitric oxide," *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, vol. 1655, no. 1–3, pp. 365–371, 2004.

[100] C. E. Cooper, "Nitric oxide and cytochrome oxidase: substrate, inhibitor or effector?," *Trends in Biochemical Sciences*, vol. 27, no. 1, pp. 33–39, 2002.

[101] C. E. Cooper and C. Giulivi, "Nitric oxide regulation of mitochondrial oxygen consumption II: molecular mechanism and tissue physiology," *American Journal of Physiology-Cell Physiology*, vol. 292, no. 6, pp. C1993–C2003, 2007.

[102] P. Sarti, M. Arese, A. Bacchi et al., "Nitric oxide and mitochondrial complex IV," *IUBMB Life*, vol. 55, no. 10–11, pp. 605–611, 2003.

[103] P. Sarti, E. Forte, D. Mastrononila, A. Giuffrè, and M. Arese, "Cytochrome c oxidase and nitric oxide in action: molecular mechanisms and pathophysiological implications," *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, vol. 1817, no. 4, pp. 610–619, 2012.

[104] P. Sarti, A. Giuffrè, M. C. Barone, E. Forte, D. Mastrononila, and M. Brunori, "Nitric oxide and cytochrome oxidase: reaction mechanisms from the enzyme to the cell," *Free Radical Biology and Medicine*, vol. 34, no. 5, pp. 509–520, 2003.

[105] M. G. Mason, P. Nicholls, M. T. Wilson, and C. E. Cooper, "Nitric oxide inhibition of respiration involves both competitive (heme) and noncompetitive (copper) binding to cytochrome c oxidase," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 3, pp. 708–713, 2006.

[106] D. Mastrononila, M. L. Genova, M. Arese et al., "Control of respiration by nitric oxide in Kellin-Hartree particles, mitochondria and SH-SYSY neuroblastoma cells," *Cellular and Molecular Life Sciences (CMLS)*, vol. 60, no. 8, pp. 1752–1759, 2003.

[107] P. Sarti, A. Giuffrè, E. Forte, D. Mastrononila, M. C. Barone, and M. Brunori, "Nitric oxide and cytochrome c oxidase: mechanisms of inhibition and NO degradation," *Biochemical and Biophysical Research Communications*, vol. 274, no. 1, pp. 183–187, 2000.

[108] P. Nicholls, "The effect of sulphide on cytochromeaa3, isos- teric and allosteric shifts of the reduced α-peak," *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, vol. 396, no. 1, pp. 24–35, 1975.

[109] P. Nicholls, L. C. Petersen, M. Miller, and F. B. Hansen, "Ligand-induced spectral changes in cytochrome c oxidase and their possible significance," *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, vol. 449, no. 2, pp. 188–196, 1976.

[110] B. C. Hill, T. C. Woon, P. Nicholls, J. Peterson, C. Greenwood, and A. J. Thomson, "Interactions of sulphide and other ligands with cytochrome c oxidase. An electron-paramagnetic-resonance study," *Biochemical Journal*, vol. 224, no. 2, pp. 591–600, 1984.

[111] P. Nicholls and J. K. Kim, "Sulphide as an inhibitor and electron donor for the cytochrome c oxidase system," *Canadian Journal of Biochemistry*, vol. 60, no. 6, pp. 613–623, 1982.

[112] P. R. Rich, B. Meunier, R. Mitchell, and A. John Moody, "Coupling of charge and proton movement in cytochrome c oxidase," *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, vol. 1275, no. 1–2, pp. 91–95, 1996.

[113] P. Nicholls and J. K. Kim, "Oxidation of sulphide by cyto- chrome aa3," *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, vol. 637, no. 2, pp. 312–320, 1981.

[114] R. P. Smith and R. E. Gosselin, "On the mechanism of sulphide inactivation by methemoglobin," *Toxicology and Applied Pharmacology*, vol. 8, no. 1, pp. 159–172, 1966.

[115] V. Vitvitsky, P. K. Yadav, S. An, J. Seravalli, U. S. Cho, and R. Banerjee, "Structural and mechanistic insights into hemoglobin-catalyzed hydrogen sulfide oxidation and the fate of polysulfide products," *Journal of Biological Chemistry*, vol. 292, no. 13, pp. 5584–5592, 2017.

[116] L. Potor, P. Nagy, M. Méhes et al., "Hydrogen sulfide abrogates hemoglobin-lipid interaction in atherosclerotic lesion," *Oxidative Medicine and Cellular Longevity*, vol. 2018, Article ID 3812568, 16 pages, 2018.

[117] D. Garai, B. B. Rios-González, P. G. Furtmüller et al., "Mechanisms of myeloperoxidase catalyzed oxidation of H2S by H2O2 or O2 to produce potent protein Cys-polysulfide-inducing species," *Free Radical Biology and Medicine*, vol. 113, pp. 551–563, 2017.

[118] Z. Pálinkás, P. G. Furtmüller, A. Nagy et al., "Interactions of hydrogen sulfide with myeloperoxidase," *British Journal of Pharmacology*, vol. 172, no. 6, pp. 1516–1532, 2015.

[119] H. Kimura, "Hydrogen polysulfide (H2S)n signaling along with hydrogen sulfide (H2S) and nitric oxide (NO)," *Journal of Neural Transmission*, vol. 123, no. 11, pp. 1235–1245, 2016.

[120] B. D. Paul and S. H. Snyder, "H2S: a novel gasotransmitter that signals by sulfhydration," *Trends in Biochemical Sciences*, vol. 40, no. 11, pp. 687–700, 2015.

[121] B. D. Paul and S. H. Snyder, "Protein sulfhydration," *Methods in Enzymology*, vol. 555, pp. 79–90, 2015.

[122] R. Millikin, C. L. Bianco, C. White et al., "The chemical biology of protein hydropersulfides: studies of a possible protective function of biological hydropersulfide generation," *Free Radical Biology and Medicine*, vol. 97, pp. 136–147, 2016.

[123] C. L. Bianco, T. A. Chavez, V. Sosa et al., "The chemical biology of the persulfide (RSSH)/perthiyl (RSS-) redox couple and possible role in biological redox signaling," *Free Radical Biology and Medicine*, vol. 101, pp. 20–31, 2016.

[124] D. E. Heppner, M. Hristova, T. Ida et al., "Cysteine thio- sulfenic acid (Cys-SSOH): a novel intermediate in thiold-based redox signaling?," *Redox Biology*, vol. 14, pp. 379–385, 2018.

[125] T. Ida, T. Sawa, H. Ihara et al., "Reactive cysteine persulfides and S-polythiolation regulate oxidative stress and redox signaling," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 111, no. 21, pp. 7606–7611, 2014.

[126] T. V. Mihanina, M. Libiad, and R. Banerjee, "Biogenesis of reactive sulfur species for signaling by hydrogen sulfide oxidation pathways," *Nature Chemical Biology*, vol. 11, no. 7, pp. 457–464, 2015.
bioactive reaction products of the NO/H2S interaction are vasorelaxants, products of sulfation of nitric oxide metabolism, redox Biology and Medicine, vol. 554, pp. 271–279, 2015.

A. Berenyiova, M. Grman, A. Mijuskovic et al., "Evidence for the formation of a novel nitrosothiol from the gaseous mediators nitric oxide and hydrogen sulphide," Biochemical and Biophysical Research Communications, vol. 343, no. 1, pp. 303–310, 2006.

M. M. Cortese-Krott, D. Pullmann, and M. Feelisch, "Nitrosopersulfide (SSNO ) targets the Keap-1/Nrf2 redox system," Pharmacological Research, vol. 113, no. Part A, pp. 490–499, 2016.

A. P. Munro and D. L. H. Williams, "Reactivity of sulfur nucleophiles towards S-nitrosothiols," Journal of the Chemical Society, Perkin Transactions 2, no. 9, pp. 1794–1797, 2000.

D. L. H. Williams, Nitrosation Reactions and the Chemistry of Nitric Oxide, Elsevier, Amsterdam, 2004.

F. Seel and M. Wagner, "Reaction of sulfides with nitrogen monoxide in aqueous-solution," Zeitschrift für Anorganische und Allgemeine Chemie, vol. 558, no. 3, pp. 189–192, 1988.

C. Bolden, S. B. King, and D. B. Kim-Shapiro, "Reactions between nitrosopersulfide and heme proteins," Free Radical Biology and Medicine, vol. 99, pp. 418–425, 2016.

F. Seel, R. Kuhn, G. Simon, and M. Wagner, "PNP-Pthionitrit und PNP-Monothionitrit / PNP-Pentithionitrite and PNP-M onothionitrite," Zeitschrift für Naturforschung B, vol. 40, no. 12, pp. 1607–1617, 1985.

R. Wedmann, I. Ivanovic-Burmazovic, and M. R. Filipovic, "Nitrosopersulfide (SSNO ) decomposes in the presence of sulfide, cyanide or glutathione to give HSNO/SNO : consequences for the assumed role in cell signalling," Interface Focus, vol. 7, no. 2, article 20160139, 2017.

R. Wedmann, A. Zahl, T. E. Shubina et al., "Does perthionitrite (SSNO ) account for sustained bioactivity of NO? A (bio)chemical characterization," Inorganic Chemistry, vol. 54, no. 19, pp. 9367–9380, 2015.

T. Morikawa, M. Kajimura, T. Nakamura et al., "Hypoxic regulation of the cerebral microcirculation is mediated by a carbon monoxide-sensitive hydrogen sulfide pathway," Proceedings of the National Academy of Sciences of the United States of America, vol. 109, no. 4, pp. 1293–1298, 2012.

T. Shintani, T. Iwabuchi, T. Soga et al., "Cystathionine β-synthase as a carbon monoxide–sensitive regulator of bile excretion," Hepatology, vol. 49, no. 1, pp. 141–150, 2009.

C. N. Wang, G. L. Duan, Y. J. Liu et al., "Overproduction of nitric oxide by endothelial cells and macrophages contributes to mitochondrial oxidative stress in adrenocortical cells and adrenal insufficiency during endotoxemia," Free Radical Biology and Medicine, vol. 83, pp. 31–40, 2015.

M. Kajimura, R. Fukuda, R. M. Bateman, T. Yamamoto, and M. Suematsu, "Interactions of multiple gas-transducing systems: hallmarks and uncertainties of CO, NO, and H2S gas biology," Antioxidants & Redox Signaling, vol. 13, no. 2, pp. 157–192, 2010.

O. Kabil, V. Yadav, and R. Banerjee, "Heme-dependent metabolite switching regulates H2S synthesis in response to endoplasmic reticulum (ER) stress," Journal of Biological Chemistry, vol. 291, no. 32, pp. 16418–16423, 2016.

M. Suematsu, T. Nakamura, Y. Tokumoto, T. Yamamoto, M. Kajimura, and Y. Kabe, "CO-CBS-H2S axis: from vascular integration of CO signaling," Journal of Biological Chemistry, vol. 288, no. 30, pp. 21267–21276, 2013.
mediator to cancer regulator,” *Microcirculation*, vol. 23, no. 3, pp. 183–190, 2016.

[188] J. Oliveriusová, Vladimir Kery, K. N. Maclean, and J. P. Kraus, “Deletion mutagenesis of human cystathionine β-synthase. Impact on activity, oligomeric status, and S-adenosylmethionine regulation,” *Journal of Biological Chemistry*, vol. 277, no. 50, pp. 48386–48394, 2002.

[189] A. Kumar, A. Wißbrock, N. Goradia et al., “Heme interaction of the intrinsically disordered N-terminal peptide segment of human cystathionine-β-synthase,” *Scientific Reports*, vol. 8, no. 1, p. 2474.

[190] M. M. Cherney, S. Pazicni, N. Frank, K. A. Marvin, J. P. Kraus, and J. N. Burstyn, “Ferrous human cystathionine β-synthase loses activity during enzyme assay due to a ligand switch process,” *Biochemistry*, vol. 46, no. 45, pp. 13199–13210, 2007.

[191] C. Gherasim, P. K. Yadav, O. Kabil, W.-N. Niu, and M. M. Cherney, S. Pazicni, N. Frank, K. A. Marvin, J. P. Kraus, J. Oliveriusová, Vladimír Kery, K. N. Maclean, and J. P. Kraus, “Activity by second coordination sphere ligands: the role of the pyridoxal 5′-phosphate (PLP) and heme environments of human cystathionine β-synthase,” *Biochemistry*, vol. 47, no. 10, pp. 3194–3201, 2008.

[192] J. B. Vicente, H. G. Colaço, F. Malagrínó et al., “A clinically relevant variant of the human hydrogen sulfide-synthesizing enzyme cystathionine β-synthase: increased CO reactivity as a novel molecular mechanism of pathogenicity,” *Oxidative Medicine and Cellular Longevity*, vol. 2017, Article ID 8940321, 13 pages, 2017.

[193] S. Carballal, E. Cuevasanta, P. K. Yadav et al., “Kinetics of nitrite reduction and peroxynitrite formation by ferrous heme in human cystathionine β-synthase,” *Journal of Biological Chemistry*, vol. 291, no. 15, pp. 8004–8013, 2016.
[215] L. Celano, M. Gil, S. Carballal et al., “Inactivation of cystathionine β-synthase with peroxynitrite,” *Archives of Biochemistry and Biophysics*, vol. 491, no. 1–2, pp. 96–105, 2009.

[216] A. Prudova, Z. Bauman, A. Braun, V. Vitvitsky, S. C. Lu, and R. Banerjee, “S-Adenosylmethionine stabilizes cystathionine β-synthase and modulates redox capacity,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 17, pp. 6489–6494, 2006.

[217] M. I. S. Mendes, H. G. Colaço, D. E. C. Smith et al., “Reduced response of cystathionine beta-synthase (CBS) to S-adenosylmethionine (SAM): identification and functional analysis of CBS gene mutations in homocystinuria patients,” *Journal of Inherited Metabolic Disease*, vol. 37, no. 2, pp. 245–254, 2014.

[218] M. I. S. Mendes, A. S. Santos, D. E. C. Smith et al., “Insights into the regulatory domain of cystathionine beta-synthase: characterization of six variant proteins,” *Human Mutation*, vol. 35, no. 10, pp. 1195–1202, 2014.

[219] M. Beaumont, M. Andriamihaja, A. Lan et al., “Detrimental effects for colonocytes of an increased exposure to luminal hydrogen sulfide: the adaptive response,” *Free Radical Biology and Medicine*, vol. 93, pp. 155–164, 2016.

[220] I. Kwiecień, M. Sokolowska, E. Luchter-Wasylevska, and L. Włodek, “Inhibition of the catalytic activity of rhodanese by S-nitrosylation using nitric oxide donors,” *The International Journal of Biochemistry & Cell Biology*, vol. 35, no. 12, pp. 1645–1657, 2003.

[221] E. D’Araio, N. Shaw, A. Millward, A. Demaine, M. Whiteman, and A. Hodgkinson, “Hydrogen sulfide induces heme oxygenase-1 in human kidney cells,” *Acta Diabetologica*, vol. 51, no. 1, pp. 155–157, 2014.

[222] H. Jia, J. Ye, J. You, X. Shi, W. Kang, and T. Wang, “Role of the cystathionine β-synthase/H₂S system in liver cancer cells and the inhibitory effect of quinolone-indole conjugate QC2 on the system,” *Oncology Reports*, vol. 37, no. 5, pp. 3001–3009, 2017.

[223] K. Kupai, N. Almási, M. Kósa et al., “H₂S confers colonic protection against TNBS-induced colitis by HO-1 upregulation in rats,” *Inflammopharmacology*, vol. 26, no. 2, pp. 479–489, 2018.

[224] M. Shao, C. Zhuo, R. Jiang et al., “Protective effect of hydrogen sulphide against myocardial hypertrophy in mice,” *Oncotarget*, vol. 8, no. 14, pp. 22344–22352, 2017.

[225] L. Xie, Y. Gu, M. Wen et al., “Hydrogen sulfide induces Keap1 S-sulfhydration and suppresses diabetes-accelerated atherosclerosis via Nrf2 activation,” *Diabetes*, vol. 65, no. 10, pp. 3171–3184, 2016.

[226] C. Y. Zhang, X. H. Li, T. Zhang, J. Fu, and X. D. Cui, “Hydrogen sulfide upregulates heme oxygenase-1 expression in rats with volume overload-induced heart failure,” *Biomedical Reports*, vol. 1, no. 3, pp. 454–458, 2013.

[227] S. Zhang, T. Wu, T. Xia, X. Rong, M. Chu, and R. Wu, “Regulation of the heme oxygenase-1/carbon monoxide system by hydrogen sulfide in murine coxsackievirus B3-induced myocarditis,” *Cellular and Molecular Biology (Noisy-le-Grand, France)*, vol. 61, no. 2, pp. 69–73, 2015.

[228] L. Yi, P. M. Jenkins, L. I. Leichter, U. Jakob, J. R. Martens, and S. W. Ragsdale, “Heme regulatory motifs in heme oxygenase-2 form a thiol/disulfide redox switch that responds to the cellular redox state,” *Journal of Biological Chemistry*, vol. 284, no. 31, pp. 20556–20561, 2009.

[229] L. Yi and S. W. Ragsdale, “Evidence that the heme regulatory motifs in heme oxygenase-2 serve as a thiol/disulfide redox switch regulating heme binding,” *Journal of Biological Chemistry*, vol. 282, no. 29, pp. 21056–21067, 2007.

[230] N. E. B. Saidu, I. A. Asali, B. Czepukojc, B. Seitz, C. Jacob, and M. Montenari, “Comparison between the effects of diallyl tetrasulfide on human retina pigment epithelial cells (ARPE-19) and HCT116 cells,” *Biochimica et Biophysica Acta (BBA) - General Subjects*, vol. 1830, no. 11, pp. 5267–5276, 2013.

[231] I. S. Shin, J. Hong, C. M. Jeon et al., “Diallyl-disulfide, an organosulfur compound of garlic, attenuates airway inflammation via activation of the Nrf-2/HO-1 pathway and NF-kappaB suppression,” *Food and Chemical Toxicology*, vol. 62, pp. 506–513, 2013.

[232] T. Zeng, C. L. Zhang, F. Y. Song et al., “The activation of HO-1/Nrf-2 contributes to the protective effects of diallyl disulfide (DADS) against ethanol-induced oxidative stress,” *Biochimica et Biophysica Acta (BBA) - General Subjects*, vol. 1830, no. 10, pp. 4848–4859, 2013.

[233] S. Kubo, Y. Kurokawa, I. Doe, T. Masuko, F. Sekiguchi, and A. Kawabata, “Hydrogen sulfide inhibits activity of three isoforms of recombinant nitric oxide synthase,” *Toxicology*, vol. 241, no. 1–2, pp. 92–97, 2007.

[234] G. S. Oh, H. O. Pae, B. S. Lee et al., “Hydrogen sulfide inhibits nitric oxide production and nuclear factor-xB via heme oxygenase-1 expression in RAW264.7 macrophages stimulated with lipopolysaccharide,” *Free Radical Biology and Medicine*, vol. 41, no. 1, pp. 106–119, 2006.

[235] M. Whiteman, L. Li, P. Rose, C. H. Tan, D. B. Parkinson, and P. K. Moore, “The effect of hydrogen sulfide donors on lipopolysaccharide-induced formation of inflammatory mediators in macrophages,” *Antioxidants & Redox Signaling*, vol. 12, no. 10, pp. 1147–1154, 2010.

[236] S. Kubo, I. Doe, Y. Kurokawa, H. Nishikawa, and A. Kawabata, “Direct inhibition of endothelial nitric oxide synthase by hydrogen sulfide: contribution to dual modulation of vascular tension,” *Toxicology*, vol. 232, no. 1–2, pp. 138–146, 2007.

[237] X.-C. Duan, S.-Y. Liu, R. Guo et al., “Cystathionine-β-synthase gene transfer into rostral ventrolateral medulla exacerbates hypertension via nitric oxide in spontaneously hypertensive rats,” *American Journal of Hypertension*, vol. 28, no. 9, pp. 1106–1113, 2015.

[238] *The PyMOL Molecular Graphics System, Version 1.8* SchrödingerLLC.

[239] S. V. Evans, B. P. Sishta, A. G. Mausk, and G. D. Brayer, “Three-dimensional structure of cyanomet-sulfinyloglobin C,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 11, pp. 4723–4726, 1994.