Published in final edited form as:

J Inorg Biochem. 2014 April ; 133: 78–86. doi:10.1016/j.jinorgbio.2014.01.013.

Hydrogen Sulfide Activation in Hemeproteins: The Sulfheme Scenario

Bessie B. Ríos-González^a, Elddie M. Román-Morales^a, Ruth Pietri^a, and Juan López-Garriga^{a,*}

^aDepartment of Chemistry, University of Puerto Rico, Mayagüez Campus, PO Box 9019, Mayagüez, Puerto Rico 00681-9019

Abstract

Traditionally known as a toxic gas, hydrogen sulfide (H₂S) is now recognized as an important biological molecule involved in numerous physiological functions. Like nitric oxide (•NO) and carbon monoxide (CO), H₂S is produced endogenously in tissues and cells and can modulate biological processes by acting on target proteins. For example, interaction of H₂S with the oxygenated form of human hemoglobin and myoglobin produces a sulfheme protein complex that has been implicated in H₂S degradation. The presence of this sulfheme derivative has also been used as a marker for endogenous H₂S synthesis and metabolism. Remarkably, human catalases and peroxidases also generate this sulfheme product. In this review, we describe the structural and functional aspects of the sulfheme derivative in these proteins and postulate a generalized mechanism for sulfheme protein formation. We also evaluate the possible physiological function of this complex and highlight the issues that remain to be assessed to determine the role of sulfheme proteins in H₂S metabolism, detection and physiology.

Keywords

Sulfhemoglobin; Hydrogen sulfide; Sulfheme protein; Sulfperoxidase; Histidine; Ferryl intermediate

1. Introduction

Hydrogen sulfide (H₂S), historically viewed as a toxic gas, has emerged as a biologically relevant molecule with significant therapeutic potential [1–3]. Endogenous H₂S in humans is produced by cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfur transferase (3MST) [4]. Once generated, H₂S penetrates cell membranes by simple diffusion and acts on target biomolecules to modulate numerous biological responses. Several molecular targets have been identified, including proteins, enzymes, transcription factors, and membrane ion channels [1]. As with nitric oxide and carbon monoxide, the other two bioactive gases, H₂S can also interact with heme-containing proteins to regulate biological activities [5–7]. Remarkably, the reactivity of hemeproteins toward H₂S has been associated with relevant physiological processes in many invertebrate systems living in sulfide-rich environments.

© 2014 Elsevier Inc. All rights reserved.

*Corresponding author: Juan López Garriga, juan.lopez16@upr.edu, Tel: +1-787-265-5453, Fax: +1-787-265-5476.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

The clam *Lucina pectinata*, which lacks a mouth and gut, lives in sulfide-rich mangroves and its nutritional needs are met by a symbiotic relationship with sulfide oxidizing bacteria. Bacteria living inside the gill oxidize H_2S in the presence of oxygen (O_2) and this energy is utilized to synthesize organic nutrients for the invertebrate. The protein responsible for delivering H_2S to the bacteria is a hemeprotein called hemoglobin I (HbI) [8], which binds H_2S in the ferric heme iron with very high affinity (Figure 1a). The orientation of the vinyl and the flexibility of the propionate peripheral groups of the heme, together with its dynamic behavior facilitate H_2S binding in this hemeprotein [9–11]. Interaction of H_2S with nearby residues also contributes to the affinity of HbI for H_2S [8, 12]. This protein has a glutamine (Gln) residue at the distal ligand-binding site, instead of the equivalent histidine (His) typically found in many invertebrate and vertebrate organisms. We have shown that H_2S is stabilized in part by a hydrogen bonding interaction with Gln. H_2S release is dictated by two competing processes involving slow dissociation of H_2S from the ferric adduct and heme iron reduction followed by H_2S liberation [13, 14]. Heme reduction by H_2S has also been observed in porphyrinates [15] and in phosphodiesterase (Ec DOS-PAS) [16]. We have also shown that replacing Gln with His in HbI, H_2S not only induces heme reduction [17] but it also generates a sulfheme derivative where another sulfur atom is incorporated in one of the pyrrole rings of the heme [18]. It is plausible that His plays an essential role in sulfheme formation.

The giant tubeworm *Riftia pachyptila* is another invertebrate that lives in the sulfide-rich hydrothermal vents and is also characterized by the presence of symbiotic sulfide-oxidizing bacteria that need to be supplied with both H_2S and O_2 [19]. *Riftia* supplies H_2S and O_2 to the endosymbionts by binding both ligands simultaneously at two different sites in its extracellular hemoglobins (Hbs). The worm binds O_2 in the iron heme groups and H_2S in other sites of the proteins. It has been suggested that binding of H_2S occurs at “free cysteine” or cysteine residues (Cys) not involved in disulfide bonds [19]. Likewise, the marine worm *Oligobranchia mashikoi*, which harbors endosymbiotic bacteria and lives in a sulfide rich environment, have Hbs that bind O_2 and H_2S and similar to *Riftia* it can transport both ligands simultaneously [20]. Structural analyses of *Oligobranchia* and *Riftia* Hbs showed that their sulfide-binding Cys residues are well conserved and that these residues play a principal role in sulfide binding (Figures 1b and 1c). These conserved Cys residues are found in other animals living in sulfide-rich habitats. These facts support the notion that Cys residues are involved in sulfide binding and that this interactions would have the benefit of avoiding sulfheme formation. Although the hemoglobins in *Riftia* and *Oligobranchia* have a His residue near the heme (Fig 1b and 1c), formation of a sulfheme complex has not been detected [19, 21]. In contrast, sulfheme formation has been observed in the hemoglobins from *Lumbricus terrestris*, which inhabits sulfide-free environments and contains His at the ligand-binding site. Structural studies demonstrated that “free Cys residues” are absent in these Hbs (Figure 1d), implying that in the presence of O_2 , H_2S diffuses through these proteins without encountering alternative binding sites and once at the heme site, it can interact directly with the heme forming the sulfheme derivatives. These observations strongly suggest that His is required for the formation of the sulfheme derivatives. In *R. pachyptila* and *O. mashikoi*, the Cys residues transport H_2S to the symbiotic bacteria and protect the worms from sulfheme formation, which can limit O_2 transportation.

Human hemoglobin and myoglobin have His at the heme distal site and also form sulfheme in the presence of H_2S and O_2 or hydrogen peroxide (H_2O_2). Indeed, formation of these sulfheme complexes at moderate levels and their destruction by the physiologic turnover of red blood cells have been considered to be one of the pathways for H_2S degradation in humans [22]. The presence of these sulfheme derivatives has also been used as a marker of endogenous H_2S synthesis [23, 24]. Remarkably, human catalases and peroxidases have His

at their active site and also generate these sulfheme derivatives [25]. Thus, it is plausible that sulfheme derivatives might play a physiological role *in vivo*. This review focuses on the structural and functional characteristics of human sulfhemoglobin, sulfmyoglobin and other sulfheme proteins. We discuss the role of the distal His as a unique amino acid that triggers the formation of the sulfheme species and propose a mechanism that involves interaction of H₂S with ferric hydroperoxide and compound II intermediates.

2. Interaction of H₂S with myoglobin and hemoglobin

2.1. General Aspects of sulfhemoglobin and sulfmyoglobin

Hemoglobin and myoglobin are globular heme proteins whose functions involve the binding of O₂ to the ferrous iron (Fe^{II}) of the heme group (protoporphyrin IX). Hemoglobin (Hb) is a tetrameric protein that transports O₂ with cooperative structure fluctuations from lungs to muscles, while myoglobin (Mb) is monomeric and stores O₂ in the muscle cells. In the presence of sulfide, both Mb and Hb undergo two different chemical reactions. Both proteins are capable of binding H₂S in the open sixth position of the ferric heme iron (Fe^{III}) but with very low affinity [8, 10]. H₂S can also react rapidly with the oxy and ferryl forms of Mb and Hb producing the so-called sulfheme derivatives.

In 1866, Hoppe-Seyler observed the formation of a green product after reacting Hb-O₂ with H₂S and called this green derivatives sulfhemoglobin (sulfHb). Michel et al. showed the formation of an analogous compound upon interaction of Mb-O₂ with H₂S and termed the complex sulfmyoglobin (sulfMb) [25–27]. These derivatives have a sulfur atom incorporated in the heme pyrrole B with a characteristic optical band around 620 nm [26, 28–31]. The displacement of the band depends on heme-Fe oxidation and ligation states, as well as the type of the sulfheme isomer [26, 32–34]. These sulfheme derivatives exhibit lower affinity towards O₂ compared to the native proteins [33, 35, 36] and cannot be reverted to the normal functional proteins by natural mechanism in the red cells. High levels of sulfHb and sulfMb can be poisonous since they alter the protein O₂ transport or storage functionalities, causing a cyanosis known as sulfhemoglobinemia [14]. Possible symptoms are chocolate brown arterial blood, fatigue, chest pain and tightness, dizziness, pallor, livid discoloration of the skin and lips, bluish stain on finger tips, foul urine and breath, and tremor of the upper and lower extremities [37–39]. It is very common for sulfhemoglobinemia to be initially misdiagnosed as methemoglobinemia [37, 38, 40]. A helpful note for its diagnostic is the lack of respiratory distress for the degree of cyanosis, [37] and near normal O₂ tension [41]. It can lead to end-organ damage and death when high sulfHb levels are reached (approximately 60%) [40]. In order to be clinically detectable the sulfHb concentration should be more than 5 g/L (approximately 80 μM) [37, 38] while normal concentrations are below 0.37 g/L (approximately 5.8 μM) [38]. Its identification is usually performed by spectrophotometry, co-oximetry, gas chromatography or HPLC [37, 40]. Treatment involves suspected chemical agent suspension, external O₂ supply [37] and in severe cases, blood transfusion [40].

2.2. Sulfhemoglobin and sulfmyoglobin formation

The mechanism for sulfHb and sulfMb formation is still unclear, however, it can be generated from the reaction of the Hb-O₂ and Mb-O₂ derivatives with H₂S, as well as in the presence of H₂O₂ and other thiol compounds [42]. In the case of H₂O₂, the heme ferryl species have been implicated in the formation of the sulfheme derivatives [18]. Figure 2 shows that interaction of H₂O₂ with Hb and Mb leads to the formation of ferric hydroperoxide and the ferryl species compound I (Fe^{IV}=O Por ^{•+}) and compound II (Fe^{IV}=O), which generate reactive O₂ species that can damage cellular processes.

OxyHb and oxyMb can also generate these ferryl species. It has been suggested that the reaction of H₂S with ferryl compound II is crucial for sulfMb and sulfHb formation and two major overall reactions have been proposed [25, 27]. In 1961, Nicholls proposed that sulfMb can be formed stoichiometrically by the reaction of heme ferryl compound II with H₂S and that a mixture of ferric sulfMb and small quantities of ferrous sulfMb were generated as final products (Reaction 1) [25, 43]. He also observed that ferric sulfMb could then be reduced to ferrous sulfMb by H₂S excess. On the other hand, Berzofsky et al. observed that ferrous sulfMb (77%) was the major product of the reaction between 1 mol of H₂S and 1 mol of Mb ferryl compound II [44]. As shown in Reaction 2, Berzofsky also suggested that the hydrosulfide ion (HS⁻) may be the initial reactant species since at physiological pH, 30% of H₂S is undissociated and ~70% dissociates to HS⁻ (pK_a of 7.04) [44].

Nonetheless, in 1938, Michel demonstrated that at pH 6 about 20% of Hb was converted to sulfHb, while at pH 8 only 9% of the sulfHb product was obtained. In addition, it has been shown recently that the rate of sulfMb formation decreases 250-fold at basic pH values where the predominant species is HS⁻ [45]. Thus, in both Hb and Mb the effect of increasing the pH is to decrease the rate and amount of sulfheme formation. This inverse correlation between pH and sulfheme formation, suggests that at basic conditions (pH > 7) formation of the sulfheme product is slow and restricted, which reduces the possibility of HS⁻ as the reactive sulfur species. Accordingly, it is plausible that the initial reactants involved in sulfheme formation are ferryl compound II and undissociated H₂S.

In addition, we have proposed that His plays an essential role in sulfheme formation and that the interaction of H₂S with the His-ferryl-heme ternary complex triggers the formation of the sulfheme derivative [18]. As discussed in detail latter, we have observed that hemoglobins lacking a corresponding distal His are able to form the ferryl compound I and compound II species in the presence of H₂S but sulfheme formation is not seen. Thus, formation of sulfheme requires a concerted interaction between the reactants and the distal His. On these bases, sulfheme formation can be rationalized by the reaction scheme shown in Figure 3a, which takes into account the role of His in sulfheme formation. Incorporating the His interaction, the first step is the formation of a ternary complex intermediate involving His, ferryl compound II and the heme-protein moiety. His donates a proton to the ferryl species, generating a highly reactive protonated ferryl compound II, which interacts with H₂S yielding in turn a sulfide radical (HS•) and ferric heme Mb or Hb. The HS• then attacks the porphyrin ring, producing the final sulfheme derivative. Romero and coworkers showed that Mb compound II also reacts with some thiols, yielding thiyl radical species and ferric Mb with the subsequent formation of sulfMb. It is important to mention that Nicholls, Berzofsky and others used catalase in their experiments to remove excess of H₂O₂ and to prevent the cyclic process shown in Figure 2 [26, 43, 44]. In the absence of catalase, the cyclic reaction in Figure 2 is operative and depending on the H₂O₂ concentration, ferric hydroperoxide can represent an additional initial reactant center for sulfheme complex formation. In this scenario, sulfheme formation can be described by the reaction scheme shown in Figure 3b. Here, the ternary complex intermediate involves His, ferric hydroperoxide and the heme-protein moiety. The His-ferric hydroperoxide ternary complex in the presence of H₂S forms the {Fe(III)OOHH₂S} intermediate, which can trigger heterolytic or homolytic cleavage of the O-O bond. Heterolysis yields heme ferryl

compound I and SH^- , while homolysis generates ferryl compound II and the $\text{HS}^\bullet$ species. Since the rate of sulfMb formation decreases with the hydrosulfide HS^- ion, we suggest that the ferryl compound II and the $\text{HS}^\bullet$ species are the predominant intermediates in the reaction of sulfheme formation. The $\text{HS}^\bullet$ then attacks the ferryl compound II, producing the final sulfheme derivative. Curiously, Romero et al. also observed that in the absence of catalase the reaction of Mb compound II with thiols generated higher concentration of thiyl radicals [42], which accumulates during the cyclic reaction in Figure 2 and can react with either the ferric hydroperoxide or the ferryl centers.

2.3. Structural aspects of sulfHb and sulfMb

In sulfHb and sulfMb the sulfur atom is incorporated in one of the pyrrole rings of the heme, which lowers their affinity for O_2 . The chemical nature of the modified heme in both sulfHb and sulfMb was a matter of debate for many years and the precise physiological structure of these derivatives remains elusive. It is now well accepted that sulfHb and sulfMb consist of a modified heme in which a sulfur atom adds to the pyrrole B of the porphyrin system. Using labeled sulfur (^{35}S) experiments it was first determined that only one atom of sulfur was incorporated into the heme group of sulfMb [26–28, 44, 46]. Later, proton NMR studies of sulfMb identified the sulfur atom on pyrrole B by analyzing the chemical shifts of the heme 1,3,5,8-methyls and the 2,4-vinyl substitutions [31, 34, 47]. Based on NMR and resonance Raman data it was then proved that the sulfur atom is incorporated across the double β - β bond of pyrrole B either as episulfide or thiochlorin, with a heme-chlorin type structure [26, 28, 30, 31, 34, 44, 47, 48]. The band at 620 nm observed in the optical spectra of sulfHb and sulfMb reflects the nature of the chlorin structure. Heme-chlorin structure and other metallo-chlorin derivatives are characterized by a saturated C_β - C_β bond in one of the pyrrole ring of the porphyrin, which reduces the symmetry of heme porphyrin from D_{4h} , typical of native Hb and Mb, to a C_{2v} system (Figure 4a). This reduction induces a distortion of the macrocycle planarity and changes in the heme electronic properties, resulting in the activation of other electronic transitions at higher wavelengths. When a sulfur atom adds to the pyrrole B, the C_β - C_β bond becomes saturated, adopting a chlorin like structure with a visible band at 620 nm. Therefore, it is clear that the incorporation of the sulfur atom involves disruption of the ring conjugation to that of chlorin, which reduces O_2 affinity in both sulfHb and sulfMb [18, 33, 35, 36, 48]. NMR and crystallographic studies demonstrated that in fact, conversion of Mb to sulfMb did not affect the heme cavity or the overall protein structure and that the observed changes in O_2 binding arise entirely from the local electronic changes of heme-chlorin structure [34, 47, 49].

Proton NMR studies of several sulfMbCN complexes revealed that there are at least three isomeric forms of the chlorin structure that may affects O_2 affinity [29, 30]. As shown in Figure 4b, each sulfheme isomer, named sulfMbA, sulfMbB and sulfMbC, have different chemical properties and hence, different reactivity and stability [29, 34]. These differences reside in the porphyrin periphery alterations where the incorporated sulfur lays since all three isomers were observed at different pH, temperature and protein concentration [29]. In general, the NMR spectra of all isomers showed symmetry reduction of the prosthetic group and alteration of the π conjugation of pyrrole B [34]. The sulfMbA structure is an episulfide across the β - β bond, which is rapidly reconverted to the protoheme when the heme is extracted from the protein [28, 34, 50, 51]. SulfMbB is characterized by a “ring opened episulfide” [34], while sulfMbC is described as a thiochlorin structure. SulfMbA is converted to both sulfMbB and sulfMbC, but conversion from sulfMbB to sulfMbC has not been observed, indicating that sulfMbA is the precursor of the other two sulfhemes species [28, 34]. SulfMbC is the most stable isomeric form and is only observed when the 4-vinyl is on heme [30, 52]. Replacing this vinyl group with hydrogen only yields sulfMbA and sulfMbB and two other red sulfMb isomers (D and E), which apparently forms from the

former two isomers [52]. It is worthwhile to mention that the predominant isomeric species depends on experimental conditions such as pH and the nature of the oxidation and coordination states of the heme iron [28, 34]. Nonetheless, the sulfMbC isomer is the terminal sulfMb product and it appears to predominate at physiological conditions, implying that the stability and reactivity of this compound may account for the reduced O₂ affinity in sulfMb.

SulfHb on the other hand, shows mainly the sulfHbA isomer with only a 10% of the sulfMbB counterpart [34]. In addition, sulfHbA does not go through isomeric interconversion probably due to internal flexibility limitation [28]. SulfMb and sulfHb isomers have different chemical reactivity, which can in principle influence O₂ affinity in both proteins.

2.4. Structural and functional Implications of sulfHb and sulfMb

Similar to the native proteins, sulfMb and sulfHb bind numerous ligands at distal site of the heme iron [25, 26, 29, 31–33, 35]. Spontaneous autoxidation as well as oxidation state interconversion with different reducing and oxidation reagents are also maintained in these sulfheme derivatives without disturbing the sulfur ring. However, O₂ affinity is substantially reduced in both sulfMb and sulfHb. In this section we evaluate O₂ binding in sulfMb and sulfHb and the factors that may influence O₂ affinity in these proteins. Reconversion of the native proteins from the sulfheme derivatives and the possible physiological implications of sulfMb and sulfHb are also discussed.

The reversible reaction of O₂ with sulfMb was first recognized by Berzofsky et al., who observed that the 616 nm band, characteristic of ferrous sulfMb, shifted to 624 nm when deoxy sulfMb was exposed to O₂. Also, a shift of the Soret band from 424 nm (deoxy sulfMb) to 408 nm was detected. The authors observed that the shifts were completely reversed when the same sample was exposed to an argon atmosphere, and attributed the spectral displacements to the reversible binding of O₂ [33]. They also showed that the oxygen-binding curve of sulfMb was characterized by a rectangular hyperbolic shape, similar to native Mb, which led them to conclude that sulfMb was in fact able to bind O₂ reversibly in a concentration dependent manner. However, the concentration of O₂ required to saturate half of the sulfMb molecules, known as $p^{1/2}$ in the binding curve, was ~ 2,500 higher than that of the native protein (530 mmHg for sulfMb and 0.21 mmHg for Mb), indicating much lower O₂ affinity for the former [33].

Following Berzofsky approach, Carrico and coworker [35] studied the reversible binding of O₂ in sulfHb and observed that when the ferrous sulfHb complex was exposed to O₂, the band at 619 nm was displaced to 623 nm. They assigned these optical changes to formation of the oxy sulfHb derivative (sulfHb-O₂). As with native Hb, the O₂ binding curve was found to be sigmoidal, which supported the fact that sulfHb was able to reversibly bind O₂. The sigmoidal shape, a characteristic of cooperative behavior, also indicated that sulfHb retained some cooperative character of the native protein. Nonetheless, the $p^{1/2}$ of sulfHb was 63mmHg, while that of native Hb under similar conditions was 0.46mmHg. On this bases, it was concluded that the affinity of sulfHb for O₂ was 135 lower that the native protein [35].

The substantial decrease in O₂ affinity, in both sulfMb and sulfHb, has been attributed to the electron withdrawing character of the heme-chlorin structure. Incorporation of the sulfur atom to the β - β double bond of the pyrrole B removes electron density from the iron towards the periphery of the chlorine ring, reducing the ability of the iron to donate electron density back to the bound ligand and as a consequence, the iron affinity for O₂ decreases. It is well known that when O₂ binds to the ferrous heme in Mb and Hb, it donates two electrons to the

heme iron, increasing complex, the iron donates electron density to the in turn the electron density on the iron. To stabilize the π orbital of the O_2 , producing ferric superoxide ($Fe^{III}-OO\bullet^-$) [33]. Hence, the inability of sulfMb and sulfHb to effectively transfer electron density to O_2 , destabilizes the complex and lowers their affinity for O_2 . Although this interpretation sheds light on the reduced affinity of sulfMb and sulfHb for O_2 , it does not explain the differences of O_2 affinity between sulfMb and sulfHb. As described above, sulfMb was shown to be ~2500-fold lower, whereas O_2 binding in sulfHb was reduced by a factor of ~135. The higher affinity of sulfHb for O_2 can be rationalized by the fact that not all four hemes are modified by H_2S and the protein is a partially sulfurated tetramer, leaving hemes available for normal O_2 binding [36]. The chemical nature of the sulfHb isomeric structure can also explain the observed higher O_2 affinity. The episulfide structure of the sulfHb isoform (sulfHbA) is more nucleophilic and less electronegative than the thiochlorin of sulfMb (sulfMbC), which can in principle decrease the electron withdrawing character of the chlorin structure in the former, resulting in higher O_2 affinity. This is consistent with the reduced intensity of the 620 nm band in the optical absorption spectra of sulfHb as opposed to sulfMb. This band arises from the forbidden electronic transition in the visible or Q region of the spectra that becomes partially allowed due to configuration interactions between the heme-chlorin orbitals. Distortion of the heme planarity increases the energy of heme orbitals and as a consequence the intensity of the band associated with the forbidden transition also increases. In sulfHb, the small intensity of the 620 nm band reflects less distortion of the heme macrocycle, which decreases the electron withdrawing characteristic of the typical chlorin derivative [18].

The reversion of the sulheme products to the native functional proteins has been studied for many years. In 1961, Nicholls [25] was the first to observe the reversion of sulfMb to native Mb, when he exposed the sulfMb sample to H_2O_2 . Similar results were obtained when he exposed sulfMb to dithionite in the presence of O_2 or cyanide (CN^-), which also produces H_2O_2 . Therefore, the reversion of sulfMb to Mb appears to be mediated by H_2O_2 in a reaction that may involve products associated with the spontaneous oxidation of H_2O_2 . Photo-excitation of the sulfMb-CO complex also induced reversion of the sulheme derivative to the normal Mb-CO species. Berzofsky et al. [32] observed that when a sulfMb-CO sample was exposed to successive periods of irradiation, the Soret band shifted from 413 nm, typical of the sulfMb-CO complex, to 422 nm with a progressive disappearance of the 612 nm band. The results were explained in terms of the differences in activation energy, both in the ground and excited state of the chlorin structure. In the ground state, large activation energy is required to release the bound sulfur and to unsaturate the pyrrole ring. During photo-excitation, the structure reaches an electronic excited state that reduces the activation energy, resulting in a thermodynamically favored reversion of the native Mb-CO complex [32].

The sulfMb reversion process was further studied by extracting the sulheme prosthetic group of under various conditions [44]. The sulhemin complex was unstable at all conditions and was rapidly converted to the protohemin and a mixture of sulfur containing molecules in which 50% was found to be SO_4^{2-} . The presence of peroxides increases the decomposition rate of sulhemin, supporting the notion that H_2O_2 or products associated with its oxidation are probably required to initiate the reversibility process [25, 44]. In addition to the peroxides, $HgCl_2$ and $Hg(OAc)_2$ were shown to decompose the sulhemin derivative as well as. It appears that Hg^{2+} removes the sulfur atom from the sulhemin molecule, generating the intact porphyrin system and a stable HgS compound [44]. Although reversion of the sulheme products to the native derivatives has been observed under several experimental conditions, the analogous process has not been detected *in vivo*. This suggests that the only mechanism of sulfHb and sulfMb removal in our body is through the natural decomposition of these molecules in tissues and cells. Curiously, it was

demonstrated recently that H₂S diffuses rapidly across the red blood cell and that erythrocytes and plasma consume endogenous H₂S [53]. Klingerman et al. also showed that once in the blood, H₂S interacts primarily with Hb and not with proteins in the plasma [54]. Thus, it is plausible that oxyHb interacts with H₂S to form sulfHb, which is then eliminated by the natural destruction of red blood cells. Furthermore, the long normal life span of erythrocytes indicates that sulfHb can be detected and that indeed, it can be used as a biomarker for H₂S generation in cells.

3. Interaction of H₂S with enzymes

Enzymes are found in bacteria, fungi, plants and animals and their function is to serve as biological catalysts. They accelerate reactions that are necessary to sustain life but some inhibitors decrease the reaction rate and turnover of enzymes. Similar to human Hb and Mb, there is a set of some enzymes like lactoperoxidase and catalase that react with H₂S, forming analogous sulfheme derivatives. Nonetheless, published information about the interaction of H₂S with these enzymes is limited [25, 43]. In this section we discuss the current knowledge about the interaction of H₂S with some heme peroxidases and catalase and correlate these interactions with human Hb and Mb.

3.1 Heme peroxidase (Lactoperoxidase and Myeloperoxidase)

Heme peroxidases are enzymes that catalyze the oxidation of a number of inorganic and organic compounds using H₂O₂ as the primary substrate. These enzymes turn reactive O₂ species like H₂O₂, which damage cell structure, to harmless product by adding hydrogen from a donor molecule. Lactoperoxidase (LPO) and myeloperoxidase (MPO) are two important heme-containing peroxidases involved in the immune defense system [55–58]. Both defend the system from invading microorganism by the bactericidal activities of the oxidized substrates [56, 59]. For example, MPO catalyzes the formation of hypochlorous acid (HOCl) from chloride species and H₂O₂, which has antimicrobial activity and kills other pathogens in humans [59]. In the resting form, the heme groups of LPO and MPO are penta-coordinated with the iron in the high spin ferric state. As shown in Figure 5, the distal sites of both enzymes have His and arginine (Arg), [57, 60, 61] which are important in their catalytic activities, specifically in the formation and stabilization of ferryl intermediates [62]. The generalized mechanism is similar to that shown in Figure 2. Interaction of the enzymes with H₂O₂ generates the ferryl compound I and compound II intermediates. As opposed to Hb and Mb, compound I is quite stable in these enzymes. Compound II is generated by the donation of an electron from a reducing substrate to the porphyrin cation radical (Por^{•+}) or compound I. The enzymes then return to their resting state by another electron transfer reaction from a second substrate, which reduces compound II with the concomitant formation of the ferric enzyme, a second radical substrate and a water molecule. The electron donor molecules vary from inorganic compounds such, as halides (X⁻), to organic (AH₂) substrates, which are oxidized to hypohalous acids (HOX) and organic radicals (•AH), respectively [63].

The catalytic activity of LPO is inhibited by sulfhydryl compounds such as methylmercaptoimidazole (MMI) and thiouracil [64, 65]. Ohtaki and coworkers suggested that the irreversible inactivation of the enzyme was induced by the reaction of compound II with the antithyroid drug MMI [66]. In 1984, Nakamura and coworkers also evaluated the interactions of LPO with H₂S, MMI, cysteine and dithiothreitol in the presence of H₂O₂ [43]. They found that as Hb and Mb, the reaction of H₂S with the enzyme in the presence of H₂O₂, yielded a sulfheme derivative, which they named sulflactoperoxidase. The ferrous sulflactoperoxidase complex has a characteristic band at approximately 638 nm whereas the ferric derivative has bands at 605 nm and 727 nm [43]. Reaction of MMI with resting LPO yielded a spectrum with visible band at 592 nm and 635 nm and based on the similarity with

the one obtained with H₂S, the authors suggested that sulflactoperoxidase was also formed with MMI.

Although the molecular mechanism of sulflactoperoxidase formation from H₂S and the subsequent inactivation of the enzyme have not been described, the analogous processes have been well evaluated with the sulfur-containing compound MMI [43, 64–68]. Ohtaki in 1982 and Nakamura in 1984, suggested that LPO compound II was reduced to the ferric state by one electron transfer from MMI with the concomitant formation of a MMI radical [43, 66]. The presence of the MMI radical was later confirmed by EPR studies and it was also shown that the radical did not interact directly with the heme iron. The authors suggested that the MMI radical must bind to a specific site of heme porphyrin ring and that the unpaired electron of the radical was then transferred to the heme ferryl group [67]. It was also proposed in that study that the distal His might influence MMI oxidation and subsequent binding to the heme porphyrin. Remarkably, this supports the unique role of the distal His in sulfheme formation as suggested previously [18]. Overall, it is now believed that MMI interacts with LPO ferryl compound II, inducing the formation of a thiyl radical that inhibits LPO irreversibly by reacting directly with the heme porphyrin system. Based on the optical spectra reported by Nakamura it is plausible that the MMI radical interacts with the heme and generates sulflactoperoxidase, which inhibit the enzyme activity[43]. A similar mechanism can be invoked for the inactivation of LPO by H₂S. Moreover, the fact that the optical spectra of LPO in the presence of H₂S and H₂O₂ shows bands similar to sulfHb and sulfMb, strongly suggest that the heme group of the sulflactoperoxidase derivative is a chlorin type structure in which the sulfur atom is incorporated across the β-β double bond of the pyrrole B [44, 47, 69].

Like LPO, MPO activity is inhibited in the presence of H₂S and H₂O₂ [70, 71]. The mechanism of inhibition has not been described and warrants further investigation. It has been speculated that the inhibition may be mediated by the formation of the sulfheme derivative, which lowers the affinity of the enzyme for the substrates [25, 70, 71]. Although formation of the sulfheme derivative has not yet been demonstrated experimentally in MPO, the enzyme has a His residue in the heme distal site that in the presence of H₂S and the natural H₂O₂ substrate can contribute to sulfheme formation (Figure 5b).

It has been suggested recently that H₂S interacts with HOCl, the enzymatic product of MPO, and prevents oxidative damage *in vivo*. As mentioned before HOCl possesses antimicrobial activity in cells. Nevertheless, excessive production of HOCl can cause oxidative stress to proteins, lipids, DNA and RNA. HOCl seems to contribute to the oxidative damage of these biomolecules in patients with Alzheimer's disease (AD) since high levels of MPO are found in the prefrontal cortex, hippocampal microglia and neurons. Whiteman et al. showed that H₂S prevents oxidative damage in neuronal cells exposed to HOCl and suggested that the direct interaction of both reactants could be an important mechanism for HOCl removal *in vivo* in AD patients [72]. Likewise, it has been suggested that HOCl can also modify low-density lipoproteins (LDL) associated with the formation of arteriosclerotic plaques. In 2007 Laggner and coworkers demonstrated the ability of H₂S to prevent modification of LDL by HOCl either via direct interaction with HOCl or inhibition of MPO [71]. Nonetheless, it has been suggested recently that the reaction of H₂S with HOCl is not favorable *in vivo*, unless it could accumulate at high local concentrations [73]. Thus, it is highly possible that H₂S stops HOCl oxidative damage by limiting MPO activity via sulfheme formation.

It is evident that LPO forms sulflactoperoxidase and that MPO must probably generate the analogous compound, however, the physiological implication of these derivatives remains unknown and warrants additional examination. Moreover, interaction of H₂S and the possible physiological role of these interactions should be further evaluated in other

peroxidases, in particular those having His at the active site and are involved in H_2O_2 and O_2 chemistry. For example, thyroid peroxidase (TPO), an enzyme that participates in hormone biosynthesis and uses H_2O_2 as a substrate, is inactivated by MMI possibly through the formation of a sulfheme like derivative [43]. The sulfheme TPO complex may also be produced in the presence of H_2S . In fact, amino acid sequence alignment shows that TPO has His in the active distal site, which is involved in the enzyme catalytic activity and probably may play a crucial role in sulfheme formation [57, 74]. Although we focus in human peroxidases, the dehaloperoxidase-hemoglobin from the invertebrate *Amphitrite ornate* forms the sulfheme product and has His near the heme [75]. Indeed, as we describe next, catalase has a His in the distal active site and generates the sulfheme derivative upon reaction with H_2S .

3.2 Catalase

Catalase is a heme-containing enzyme found in many bacteria and almost all plants and animals. The protein is a tetramer and each monomer contains a heme group [76–78]. As Reaction 3 shows, the enzyme protects the cells by converting H_2O_2 into water and O_2 [79]. The proposed mechanism occurs in two steps and involves the ferryl intermediates [80]. In the first step the enzyme reacts with H_2O_2 to form compound I. In the second step, compound I reacts with another molecule of H_2O_2 and the enzyme returns to the resting state. Two electrons are transferred to one molecule of H_2O_2 and then two electrons are accepted from a second H_2O_2 . Catalase does not form compound II as part of the normal catalytic cycle but at low concentration of H_2O_2 and in the presence of one electron donor generation of compound II has been observed. The efficiency of the catalytic reactions is improved by the interaction of the active site His and Asn residues (His and Asn in the positions 74 and 147, respectively) with the ferryl intermediates.

Catalase is inhibited by ligands like cyanide, azide, 3-amino-1,2,4-triazol and nitric oxide [81–84]. Also, as Beers and Sizer proposed, H_2S could inhibit catalase in two ways: it may form an inactive compound with the primary catalase peroxide complex and that it may react directly with either the protein moiety or with the heme group [85]. Nicholls then proposed that the interaction of H_2S with catalase was similar to that of Mb in which H_2S reacts with the ferryl compound II intermediate and modifies the heme porphyrin system to produce the analogous sulfheme derivatives [25]. The product of the reaction of catalase with H_2S and H_2O_2 yielded an optical spectrum with a characteristic band at 635 nm and was therefore ascribed to ferrous sulfcatalase. Ferric sulfcatalase was also observed with absorption bands at 585 nm and 710 nm. Based on this, Bersofsky suggested that the structure of sulfcatalase should be similar to sulfMb and sulfHb [26, 44, 47, 69]. Therefore, these observations support the notion that the sulfheme product is generated in presence of O_2 and/or H_2O_2 and that the distal His residue near the iron regulates sulfheme formation. Interestingly, catalase can be regenerated from sulfcatalase by oxidizing agents like O_2 and ethyl hydroperoxide and by small molecules like cyanide and azide, and sodium dithionite [25].

4. Role of His in sulfheme protein formation

Several mechanistic aspects of sulfheme protein formation remain unknown. However, there are enough experimental evidences that can provide an insight into the characteristics of sulfheme protein generation. For example, as mentioned in this review, we have suggested that His is directly involved in sulfheme protein formation. In this respect, we have shown that the cytoplasmic HbI, HbII and HbIII from *L. pectinata*, which have Gln instead of His, do not form the sulfheme derivatives [18]. In addition to Gln, HbI has a cluster of Phe residues near the heme, while in HbII and HbIII one of the Phe is replaced with Tyr. Thus, it appears that neither Gln, Phe nor Tyr are involved in sulfheme protein formation. Moreover, as Table 1 shows, sulfheme was not detected in the HbI Gln/Val, Gln/Asn and Gln/Arg mutants, while the product was only observed in the Gln/His variant [14, 18]. Similarly, it was shown recently that the native Ec DOS-PAS heme sensor, which has Met and Arg in its distal site, does not form the sulfheme complex, supporting the role His in sulfheme formation [86]. Mutations of these residues by Ala, Ile, Leu and Glu did not induce formation of sulfheme, instead a verdoheme product was observed in the Arg/Ala and Arg/Ile mutants. Verdoheme is a modified heme that has oxygen incorporated in the α -meso heme position. It is known for being one of the heme degradation steps in heme oxygenase [86]. Thus, it is evident that amino acids with: (i) non-polar aliphatic groups like glycine, alanine, valine and isoleucine, (ii) polar uncharged groups like glutamine and asparagine, (iii) aromatic group like phenylalanine and tyrosine, and (iv) positively charged groups like lysine and arginine, are not involved in the synthesis of the sulfheme species.

As shown in Table 1, His is a common feature in those proteins that generate sulfheme, reiterating the role of His in the reaction of sulfheme. Thus, with the exception of cytochrome c oxidase, horseradish peroxidase, and the phosphodiesterase His mutant (Ec DOS-PAS Met95His), distal His seems to be essential for sulfheme formation. In cytochrome c oxidase, the catalytic center includes a heme porphyrin (a_3), a copper (Cu_B) center and three His residues, which are coordinated to Cu_B . The fact that heme a_3 . Cu_B binuclear centers are directly involved in the catalytic activity of the enzyme precludes sulfheme formation. Interestingly, both LPO and horseradish peroxidase (HRP) have a His and Arg in their active site, which are important for their enzymatic activity, but only the former generates sulfheme, while the latter does not [25, 43]. This exclusive behavior can be attributed to the distance between the His and the heme iron, which is shorter in LPO (4.94 Å), as opposed to HRP (6.00 Å) (Figure 5a and 5d). On the other hand, the His residue in the Ec DOS-PAS Met95His mutant is coordinated to the heme iron, which blocks H_2O_2 binding and sulfheme formation [86]. Clearly, this shows that the orientation and position of the His residue at the heme active site are crucial for the synthesis of the sulfheme product. The fact that only proteins that have His at the distal site generate sulfheme, the reactivity of this residue should be taken into account when considering the mechanism of sulfheme protein formation.

5. Summary and Future Perspectives

In view of the current data we suggest that: (a) the formation of the sulfheme derivate requires a His residue in the heme distal site with an adequate orientation to form an active ternary complex; (b) the ternary complex intermediate involves His, ferric hydroperoxide or compound II and the heme-protein moiety; (c) interaction of this heme cluster with H_2S triggers formation of the $SH^\bullet$, yielding the final ferrous or ferric sulfheme product. These observations and the tautomeric nature [87, 88] and orientation of distal His, which can behave as an acid-base catalyst, allows suggesting the general mechanisms shown in Figure 3. Due to the cyclic nature of the reaction between hemeproteins and H_2O_2 (Figure 2), it is difficult to assign the initial peroxide adduct (ferric hydroperoxide or compound II)

responsible for sulfheme formation. Assuming that in the presence of an H_2O_2 scavenger the end product of the reaction is compound II, then the predominant reaction can be summarized as shown in Figure 3a. On the other hand if the cyclic reaction is operative, the reaction can be described as demonstrated in Figure 3b. In both scenarios the $\text{SH}\cdot$ appears to be the reactive species that attack the double β - β bond of pyrrole B.

It is clear that much remains to be investigated before implicating sulfheme proteins in any physiological function. Nonetheless, the fact that this complex is also produced during the catalytic reaction of heme-peroxidases and catalases, strongly suggests that this derivative can also be generated in other heme-containing enzymes involved in O_2 chemistry. Also, albeit the concentration of endogenous H_2S may be too low to generate sulfheme *in vivo*, exposure of H_2S by donors can induce formation of the complex, which can be used in turn to assess the presence of exogenous H_2S in tissues and cells. Key issues that requires further investigation to address the above hypotheses include: (a) the precise peroxide adduct involved in sulfheme formation, (b) the subsequent reaction of the thiyl radical with the heme ferric or ferryl compound II intermediate, (c) the final sulfheme product (ferric versus ferrous) and (d) the stability, decay and reversibility of the sulfheme complex *in vitro* and *in vivo*. Insights into these issues will determine the role of sulfheme proteins in H_2S metabolism, detection and physiology.

Acknowledgments

We thank NIH-RISE 2 Best (Grant R25SGM088023) and the National Science Foundation (Grant 0843608) for their financial support.

Abbreviations

HbI	hemoglobin I
Hbs	hemoglobins
sulfHb	sulfhemoglobin
sulfMb	sulfmoglobin
MMI	methylmercaptoimidazole
HRP	horseradish peroxidase
$\text{HS}\cdot$	sulfide radical
$(\text{Fe}^{\text{IV}}=\text{O})$	compound II
$\text{Fe}^{\text{III}}\text{-OO}\cdot$	ferric superoxide
LPO	lactoperoxidase
MPO	myeloperoxidase
$\text{Por}\cdot^+$	porphyrin cation radical

References

1. Kashfi K, Olson KR. Biochem Pharmacol. 2013; 85:689–703. [PubMed: 23103569]
2. Kimura H, Shibuya N, Kimura Y. Antioxid Redox Signal. 2012; 17:45–57. [PubMed: 22229673]
3. Olson KR. Am J Physiol Regul Integr Comp Physiol. 2011; 301:R297–312. [PubMed: 21543637]
4. Singh S, Banerjee R. Biochim Biophys Acta. 2011; 1814:1518–1527. [PubMed: 21315854]
5. Kabil O, Banerjee R. J Biol Chem. 2010; 285:21903–21907. [PubMed: 20448039]

6. Kajimura M, Fukuda R, Bateman RM, Yamamoto T, Suematsu M. *Antioxid Redox Signal*. 2010; 13:157–192. [PubMed: 19939208]
7. Li Q, Lancaster JR Jr. *Nitric Oxide*. 2013; 35C:21–34. [PubMed: 23850631]
8. Kraus DW, Wittenberg JB. *J Biol Chem*. 1990; 265:16043–16053. [PubMed: 2398044]
9. Colón JFC, Silfa E, Garriga JL. *J Am Chem Soc*. 1998; 120:9312–9317.
10. Fernandez-Alberti S, Bacelo DE, Binning RC Jr, Echave J, Chergui M, Lopez-Garriga J. *Biophys J*. 2006; 91:1698–1709. [PubMed: 16782787]
11. Silfa E, Almeida M, Cerda J, Wu S, Lopez-Garriga J. *Biospectroscopy*. 1998; 4:311–326. [PubMed: 9787907]
12. Rizzi M, Wittenberg JB, Coda A, Ascenzi P, Bolognesi M. *J Mol Biol*. 1996; 258:1–5. [PubMed: 8613980]
13. Pietri R, Leon RG, Kiger L, Marden MC, Granell LB, Cadilla CL, Lopez-Garriga J. *Biochim Biophys Acta*. 2006; 1764:758–765. [PubMed: 16380302]
14. Pietri R, Roman-Morales E, Lopez-Garriga J. *Antioxid Redox Signal*. 2011; 15:393–404. [PubMed: 21050142]
15. Pavlik JW, Noll BC, Oliver AG, Schulz CE, Scheidt WR. *Inorg Chem*. 2010; 49:1017–1026. [PubMed: 20038134]
16. Takahashi H, Sekimoto M, Tanaka M, Tanaka A, Igarashi J, Shimizu T. *J Inorg Biochem*. 2012; 109:66–71. [PubMed: 22387619]
17. Pietri R, Lewis A, Leon RG, Casabona G, Kiger L, Yeh SR, Fernandez-Alberti S, Marden MC, Cadilla CL, Lopez-Garriga J. *Biochemistry*. 2009; 48:4881–4894. [PubMed: 19368335]
18. Roman-Morales E, Pietri R, Ramos-Santana B, Vinogradov SN, Lewis-Ballester A, Lopez-Garriga J. *Biochem Biophys Res Commun*. 2010; 400:489–492. [PubMed: 20732304]
19. Bailly X, Vinogradov S. *J Inorg Biochem*. 2005; 99:142–150. [PubMed: 15598498]
20. Numoto N, Nakagawa T, Kita A, Sasayama Y, Fukumori Y, Miki K. *Proc Natl Acad Sci U S A*. 2005; 102:14521–14526. [PubMed: 16204001]
21. Arp AJ, Childress JJ, Vetter RD. *J Exp Biol*. 1987; 128:139–158.
22. Predmore BL, Lefer DJ, Gojon G. *Antioxid Redox Signal*. 2012; 17:119–140. [PubMed: 22432697]
23. Perna AF, Luciano MG, Ingrosso D, Pulzella P, Sepe I, Lanza D, Violetti E, Capasso R, Lombardi C, De Santo NG. *Nephrol Dial Transplant*. 2009; 24:3756–3763. [PubMed: 19654230]
24. Whiteman M, Le Trionnaire S, Chopra M, Fox B, Whatmore J. *Clin Sci (Lond)*. 2011; 121:459–488. [PubMed: 21843150]
25. Nicholls P. *Biochem J*. 1961; 81:374–383. [PubMed: 14479446]
26. Berzofsky JA, Peisach J, Blumberg WE. *J Biol Chem*. 1971; 246:3367–3377. [PubMed: 4324899]
27. Michel HO. *J Biol Chem*. 1938; 126:323–348.
28. Bondoc LL, Chau MH, Price MA, Timkovich R. *Biochemistry*. 1986; 25:8458–8466. [PubMed: 3828290]
29. Chatfield MJ, La Mar GN, Balch AL, Lecomte JT. *Biochem Biophys Res Commun*. 1986; 135:309–315. [PubMed: 3954774]
30. Chatfield MJ, La Mar GN, Lecomte JT, Balch AL, Smith KM, Langry KC. *J Am Chem Soc*. 1986; 108:7108–7110.
31. Timkovich R, Vavra MR. *Biochemistry*. 1985; 24:5189–5196. [PubMed: 4074688]
32. Berzofsky JA, Peisach J, Alben JO. *J Biol Chem*. 1972; 247:3774–3782. [PubMed: 5033389]
33. Berzofsky JA, Peisach J, Blumberg WE. *J Biol Chem*. 1971; 246:7366–7372. [PubMed: 4331435]
34. Chatfield MJ, La Mar GN, Kauten RJ. *Biochemistry*. 1987; 26:6939–6950. [PubMed: 3427054]
35. Carrico RJ, Blumberg WE, Peisach J. *J Biol Chem*. 1978; 253:7212–7215. [PubMed: 29895]
36. Park CM, Nagel RL, Blumberg WE, Peisach J, Magliozzo RS. *J Biol Chem*. 1986; 261:8805–8810. [PubMed: 3722175]
37. Harangi M, Matyus J, Nagy E, Nagy E, Paragh G, Balla J, Olah AV. *Clin Toxicol (Phila)*. 2007; 45:189–192. [PubMed: 17364641]
38. Noor M, Beutler E. *West J Med*. 1998; 169:386–389. [PubMed: 9866446]

39. Tangerman A, Bongaerts G, Agbeko R, Semmekrot B, Severijnen R. *J Clin Pathol*. 2002; 55:631–633. [PubMed: 12147663]
40. Baranoski GV, Chen TF, Kimmel BW, Miranda E, Yim D. *J Biomed Opt*. 2012; 17:97005. [PubMed: 22975680]
41. Gopalachar AS, Bowie VL, Bharadwaj P. *Ann Pharmacother*. 2005; 39:1128–1130. [PubMed: 15886294]
42. Romero FJ, Ordonez I, Arduini A, Cadenas E. *J Biol Chem*. 1992; 267:1680–1688. [PubMed: 1309791]
43. Nakamura S, Nakamura M, Yamazaki I, Morrison M. *J Biol Chem*. 1984; 259:7080–7085. [PubMed: 6725282]
44. Berzofsky JA, Peisach J, Horecker BL. *J Biol Chem*. 1972; 247:3783–3791. [PubMed: 5033390]
45. Libardi SH, Pindstrup H, Cardoso DR, Skibsted LH. *J Agric Food Chem*. 2013
46. Morell DB, Chang Y, Clezy PS. *Biochim Biophys Acta*. 1967; 136:121–130. [PubMed: 6032032]
47. Chatfield MJ, La Mar GN, Smith KM, Leung HK, Pandey RK. *Biochemistry*. 1988; 27:1500–1507. [PubMed: 3365403]
48. Andersson LA, Loehr TM, Lim AR, Mauk AG. *J Biol Chem*. 1984; 259:15340–15349. [PubMed: 6511796]
49. Evans SV, Sishta BP, Mauk AG, Brayer GD. *Proc Natl Acad Sci U S A*. 1994; 91:4723–4726. [PubMed: 8197124]
50. Scharberg MA, La Mar GN. *J Am Chem Soc*. 1993; 115:6513–6521.
51. Scharberg MA, La Mar GN. *J Am Chem Soc*. 1993; 115:6522–6528.
52. Chatfield MJ, La Mar GN, Balch AL, Smith KM, Parish DW, LePage TJ. *FEBS Lett*. 1986; 206:343–346. [PubMed: 3758356]
53. Jennings MJ. *Am J Physiol - Cell Ph*. 2013
54. Klingerman CM, Trushin N, Prokopczyk B, Haouzi P. *Am J Physiol Regul Integr Comp Physiol*. 2013
55. Arnhold J, Flemmig J. *Arch Biochem Biophys*. 2010; 500:92–106. [PubMed: 20399194]
56. Kussendrager KD, van Hooijdonk AC. *Br J Nutr*. 2000; 84(Suppl 1):S19–25. [PubMed: 11242442]
57. Furtmuller PG, Zederbauer M, Jantschko W, Helm J, Bogner M, Jakopitsch C, Obinger C. *Arch Biochem Biophys*. 2006; 445:199–213. [PubMed: 16288970]
58. Zamocky M, Jakopitsch C, Furtmuller PG, Dunand C, Obinger C. *Proteins*. 2008; 72:589–605. [PubMed: 18247411]
59. Stankovi S, Majki -Singh N. *J Med Biochem*. 2011; 30:230–236.
60. Fiedler TJ, Davey CA, Fenna RE. *J Biol Chem*. 2000; 275:11964–11971. [PubMed: 10766826]
61. Singh AK, Singh N, Sharma S, Singh SB, Kaur P, Bhushan A, Srinivasan A, Singh TP. *J Mol Biol*. 2008; 376:1060–1075. [PubMed: 18191143]
62. Battistuzzi G, Bellei M, Bortolotti CA, Sola M. *Arch Biochem Biophys*. 2010; 500:21–36. [PubMed: 20211593]
63. Bertini, I.; Gray, HB.; Stiefel, EI.; Valentine, JS. *Biological Inorganic Chemistry. Structure and Reactivity*. 1. University Science Books; United States of America: 2007.
64. Edelhoch H, Irace G, Johnson ML, Michot JL, Nunez J. *J Biol Chem*. 1979; 254:11822–11830. [PubMed: 91609]
65. Michot JL, Nunez J, Johnson ML, Irace G, Edelhoch H. *J Biol Chem*. 1979; 254:2205–2209. [PubMed: 429280]
66. Ohtaki S, Nakagawa H, Nakamura M, Yamazaki I. *J Biol Chem*. 1982; 257:761–766. [PubMed: 6172424]
67. Bandyopadhyay U, Bhattacharyya DK, Chatterjee R, Banerjee RK. *Biochem J*. 1995; 306(Pt 3): 751–757. [PubMed: 7702570]
68. Doerge DR. *Arch Biochem Biophys*. 1986; 244:678–685. [PubMed: 3947087]
69. Chatfield MJ, La Mar GN. *Arch Biochem Biophys*. 1992; 295:289–296. [PubMed: 1316736]
70. Claesson R, Granlund-Edstedt M, Persson S, Carlsson J. *Infect Immun*. 1989; 57:2776–2781. [PubMed: 2547720]

71. Laggner H, Muellner MK, Schreier S, Sturm B, Hermann M, Exner M, Gmeiner BM, Kapiotis S. *Free Radic Res.* 2007; 41:741–747. [PubMed: 17577734]
72. Whiteman M, Cheung NS, Zhu YZ, Chu SH, Siau JL, Wong BS, Armstrong JS, Moore PK. *Biochem Biophys Res Commun.* 2005; 326:794–798. [PubMed: 15607739]
73. Carballal S, Trujillo M, Cuevasanta E, Bartesaghi S, Moller MN, Folkes LK, Garcia-Bereguian MA, Gutierrez-Merino C, Wardman P, Denicola A, Radi R, Alvarez B. *Free Radic Biol Med.* 2011; 50:196–205. [PubMed: 21034811]
74. Loughran NB, O'Connor B, O'Fagain C, O'Connell MJ. *BMC Evol Biol.* 2008; 8:101. [PubMed: 18371223]
75. Nicoletti FP, Thompson MK, Franzen S, Smulevich G. *J Biol Inorg Chem.* 2011; 16:611–619. [PubMed: 21298302]
76. Schroeder WA, Shelton JR, Shelton JB, Robberson B, Apell G, Fang RS, Bonaventura J. *Arch Biochem Biophys.* 1982; 214:397–421. [PubMed: 7082009]
77. Sumner JB, Dounce AL. *Science.* 1937; 85:366–367. [PubMed: 17776781]
78. Sumner JB, Gralen N. *Science.* 1938; 87:284. [PubMed: 17831682]
79. Michiels C, Raes M, Toussaint O, Remacle J. *Free Radic Biol Med.* 1994; 17:235–248. [PubMed: 7982629]
80. Purwar N, McGarry JM, Kostera J, Pacheco AA, Schmidt M. *Biochemistry.* 2011; 50:4491–4503. [PubMed: 21524057]
81. Brown GC. *Eur J Biochem.* 1995; 232:188–191. [PubMed: 7556149]
82. Chance B. *J Biol Chem.* 1949; 179:1299–1309. [PubMed: 18134592]
83. Keilin D, Hartree EF. *Biochem J.* 1945; 39:148–157. [PubMed: 16747875]
84. Margoliash E, Novogrodsky A. *Biochem J.* 1958; 68:468–475. [PubMed: 13522646]
85. Beers RF Jr, Sizer IW. *Science.* 1954; 120:32–33. [PubMed: 13168390]
86. Du Y, Liu G, Yan Y, Huang D, Luo W, Martinkova M, Man P, Shimizu T. *BioMetals.* 2013; 26:839–852.10.1007/s10534-013-9640-4 [PubMed: 23736976]
87. Shimahara H, Yoshida T, Shibata Y, Shimizu M, Kyogoku Y, Sakiyama F, Nakazawa T, Tate S, Ohki SY, Kato T, Moriyama H, Kishida K, Tano Y, Ohkubo T, Kobayashi Y. *J Biol Chem.* 2007; 282:9646–9656. [PubMed: 17202139]
88. Li S, Hong M. *J Am Chem Soc.* 2011; 133:1534–1544. [PubMed: 21207964]

Highlights

- Sulfhemoglobin has been implicated in H₂S detection and metabolism.
- The mechanistic, structural and functional aspects of sulfheme are presented.
- Formation of sulfheme requires His, hydroperoxide or compound II and H₂S.
- This cluster triggers formation of the SH•, yielding the final sulfheme product.
- This can also be applied to other heme enzymes involved in O₂ and H₂O₂ chemistry.

Figure 1.

Distal active site of: (a) *L. pectinata* (PDB:1MOH), (b) *R. pachyptila* (PDB: 1YHU), (c) *O. mashikoi* (PDB:2ZS0), and (d) *L. terrestris* (PDB:1X9F). In *L. pectinata*, H₂S (shown as the sphere) binds to the ferric heme iron. *R. pachyptila* and *O. mashikoi* bind H₂S in the free Cys residue and O₂ in the heme iron center shown as spheres. In *L. terrestris*, the Cys residue is replaced by Leu, and in the presence of O₂, H₂S diffuses through the protein without encountering alternative binding sites and once at the heme site, it can interact directly with heme forming the sulfheme derivative.

Figure 2.
Cyclic reaction of myoglobin with hydrogen peroxide.

Figure 3.
Proposed mechanism for sulfheme protein formation.

Figure 4.

Structure of: (a) Mb with bound oxygen (green, PDB: 1MBO) and after exposure of H₂S (blue, PDB: 1YMC), and (b) SulfMb isoforms. The final sulfheme product is a modified chlorin-type heme with a sulfur atom incorporated into one of the pyrrole rings. Several isoform were identified in which sulfMbA is an episulfide, while sulfMbB and sulfMbC are characterized by a ring opened episulfide and a thiochlorin, respectively.

Figure 5.

Distal active site of: (a) LPO (PDB: 2NQX), (b) MPO (PDB: 1CXP), (c) catalase (PDB: 3RGP) and (d) HRP (PDB: 1ATJ). The structures show the distances from the iron to the NE2 of the distal His.

Table 1

Sulfheme formation in different hemeproteins

Protein	Key distal site residues	Sulfheme
Horse heart Mb	His	Yes (618 nm)
Human Hb	His	Yes (620 nm)
<i>L. pectinata</i> HbII and HbIII ^a	Gln	No
<i>L. pectinata</i> HbI	Gln	No
HbI Gln64Asn ^b	Asn	No
HbI Gln64His ^b	His	Yes (624 nm)
HbI Phe29His ^b	Gln	No
HbI Phe68His ^b	Gln	No
HbI Phe29Leu ^b	Gln	No
HbI Gln64Arg ^b	Arg	No
HbI Phe68Val ^b	Gln	No
<i>Macrobdella decora</i> Hbs ^c	His	Yes (620 nm)
<i>Lumbricus terrestris</i> Hbs ^c	His	Yes (620 nm)
Catalase	His	Yes (635 nm)
Lactoperoxidase	His	Yes (638 nm)
Dehaloperoxidase ^d	His	Yes (620 nm)
Horseradish peroxidase	Arg	No
Cytochrome c oxidase	His (cyt a ₃)	No
Heme-O ₂ sensor Ec Dos Pas	Met, Arg	No
Ec Dos Pas Met95Ala ^e	Ala	No
Ec Dos Pas Met95His ^e	His	No
Ec Dos Pas Met95Leu ^e	Leu	No
Ec Dos Pas Arg97Ala ^e	Ala	No
Ec Dos Pas Arg97Glu ^e	Glu	No
Ec Dos Pas Arg97Ile ^e	Ile	No

^a HbII and HbIII are O₂ transport proteins found in *L. pectinata* [8].

^b Point mutants of HbI from *L. pectinata*. The numbers represent the position of the amino acid in the polypeptide chain [17, 18].

^c *Macrobdella decora* and *Lumbricus terrestris* are invertebrates that live in sulfide-free environments and have large Hbs that transport O₂ [18].

^d This enzyme is found in the marine worm *Amphitrite ornate* and is believed to be involved in H₂S catabolism [75].

^e Point mutants of the heme sensor phosphodiesterase from *Escherichia coli* (Ec DOS PAS) [86].