



## The reactions of hydropersulfides (RSSH) with myoglobin

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### ABSTRACT

Hydropersulfides are reported to be good biological reductants, superior to thiols and akin to selenols. As such, they have been previously shown to reduce metalloproteins such as ferric myoglobin and ferric cytochrome *c* to their ferrous forms under conditions where little or no reduction from corresponding thiols is observed. Not surprisingly, the reduction of ferric myoglobin to ferrous myoglobin under aerobic conditions results in the generation of oxymyoglobin (dioxygen bound ferrous myoglobin). Previous studies have demonstrated that oxymyoglobin can also act as an oxidant with highly reducing species such as hydroxylamine and ascorbate. Considering the reducing properties of hydropersulfides, it is possible that they can also react with oxymyoglobin similarly to hydroxylamine or ascorbate. Herein, this reaction is examined and indeed hydropersulfides are found to react with oxymyoglobin similarly to other reducing species leading to a fleeting ferric myoglobin which is rapidly reduced to the ferrous form also by hydropersulfide.

### 1. Introduction

Nitric oxide (NO), carbon monoxide (CO) and hydrogen sulfide (H<sub>2</sub>S) are all endogenously generated species with important physiological functions [e.g., Ref. [1–3]]. Due to their similar size and low molecular weights, collectively they are often referred to as the small-molecule signaling species or “gasotransmitters” (although they are not gases when acting physiologically, they are solutes). The biological functions of H<sub>2</sub>S are numerous and include roles as a vascular agent [4], protectant from ischemia-reperfusion injury [5] and in the etiology of cancer [6], just to name a few. Recently it has been postulated that at least a portion of the biological actions of H<sub>2</sub>S can be attributed to the generation of hydropersulfide (RSSH, R = alkyl, H) species rather than H<sub>2</sub>S itself [e.g., Ref. [7–10]]. The basis for the idea that some of the presumed H<sub>2</sub>S biology can instead be the result of RSSH generation is that H<sub>2</sub>S and RSSH species are intimately linked via an equilibrium involving

H<sub>2</sub>S/disulfide (RSSR) and RSSH/thiol (RSH) (**Reaction 1**) [11–13] indicating that they will both be present in a biological milieu, making it difficult to discern the identity of the effector species.



Furthermore, RSSH species have been shown to possess enhanced chemical reactivity compared to the parent H<sub>2</sub>S and RSH species (*vide infra*), possibly justifying their generation as a response to biological stresses and/or fulfilling biological needs not met by simple thiols [e.g., Ref. [14]].

As alluded to above, previous studies by us and others report that RSSH species are significantly more nucleophilic and reducing compared to the corresponding RSH (including H<sub>2</sub>S) [e.g., Ref. [12, 15–17]]. Since much of the biological utility of thiols is due to their nucleophilicity and reducing abilities (as well as their metal-coordinating/Lewis base properties), it is reasonable to speculate

**Abbreviations:** RSSH, hydropersulfide; RSSR, disulfide; RSOH, sulfenic acid; RSNO, S-nitrosothiol; MCPD, methoxycarbonyl penicillamine disulfide; DIP, diazenedicarboxylic acid bis-N'-methylpiperazine; Fe<sup>III</sup>Mb, ferric myoglobin; O<sub>2</sub>Fe<sup>III</sup>Mb, oxymyoglobin; DTPA, diethylenetriamine pentaacetic acid; HPE-IAM, β-(4-hydroxyphenyl)ethyl iodoacetamide; UPLC-MS, ultra-performance liquid chromatography mass spectrometry; RSSSR, trisulfide; RSSSSR, tetrasulfide; AH<sup>-</sup>, ascorbate; A<sup>-</sup>, ascorbyl radical anion; Fe<sup>III</sup>Cyt c, ferric cytochrome *c*; Fe<sup>II</sup>Cyt c, ferrous cytochrome *c*.

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that RSSH species can also perform these duties as well, but possibly with enhanced or different reactivity. To date, there has been an emphasis on examining the redox interactions of RSSH with other sulfur species (e.g. RSSR, sulfenic acids (RSOH), S-nitrosothiols (RSNO), etc.) [e.g., Ref. [15,18,19]] and less reported regarding the interactions of RSSH with metalloproteins. Since thiols as well as H<sub>2</sub>S are known to interact extensively with certain metals and metalloproteins [e.g., Refs. [20–23]], it would not be surprising, if not expected, that the chemical biology of RSSH species involves interactions with metals and metalloproteins as well, similar to what has been previously observed for NO and CO. Thus, the reactions of RSSH with myoglobin are examined herein to extend our understanding of the chemical biology of RSSH with an important class of metalloproteins, heme proteins.

## 2. Materials and methods

**Instrumentation:** UV–vis spectra were obtained using a Shimadzu 2501 UV–vis spectrophotometer and mass spectra obtained using a Thermo TSQ Quantum electrospray Triple Quadrupole Mass Spectrometer controlled with XCalibur 2.1 via syringe pump or a Waters Acquity/Xevo-G2 UPLC-MS system coupled with a high-resolution Q-TOF MS/MS detector. O<sub>2</sub> Consumption was monitored using a YSI Oxygen Monitor (Clarke-type electrode).

**Chemicals:** Methoxycarbonyl penicillamine disulfide (MCPD) was synthesized according to a published procedure [24]. Diazenedicarboxylic acid bis-N'-methylpiperazine (DIP) was synthesized according to the method of Kosower and Kanety-Londner [25]. The inorganic polysulfide Na<sub>2</sub>S<sub>2</sub> was purchased from Dojindo Molecular Technologies (Kumamoto, Japan). Myoglobin (horse heart) and cytochrome *c* (bovine heart) were purchased from Sigma Aldrich (St. Louis, MO). All other chemicals were purchased from commercial sources and were of the highest purity available.

**UV–Vis Analysis of the reaction of ferric myoglobin (Fe<sup>III</sup>Mb) and oxymyoglobin (O<sub>2</sub>Fe<sup>II</sup>Mb) with MCPD:** The reaction of Fe<sup>III</sup>Mb with MCPD was monitored by measuring the loss in absorbance of Fe<sup>III</sup>Mb at 408 nm ( $\epsilon = 188000 \text{ M}^{-1} \text{ cm}^{-1}$ ). A 27  $\mu\text{M}$  stock solution of Fe<sup>III</sup>Mb was prepared by dissolving 0.5 mg first in 1 mL of DI water and then taking 100  $\mu\text{L}$  of this solution up in 1 mL of 50 mM phosphate buffer (pH 7.4). The concentration was determined by the absorbance at 408 nm. A 4 mM stock solution of MCPD was prepared in DI water. For the reaction, the Fe<sup>III</sup>Mb stock solution was diluted in a cuvette containing 50 mM phosphate buffer (pH 7.4) to a final concentration of 9  $\mu\text{M}$ . The UV–Vis spectrum was measured to verify the concentration. Next, an aliquot of the MCPD stock solution was added to the cuvette to give a final concentration of 90  $\mu\text{M}$  and the UV–Vis spectrum recorded every 90 s for 45 min from 300 to 700 nm. In order to prepare a stock solution of O<sub>2</sub>Fe<sup>II</sup>Mb, approximately 1 mg of Fe<sup>III</sup>Mb was dissolved in 1 mL of water and reacted with excess sodium dithionite (Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>) to fully reduce the heme protein to the ferrous state. The reduced, oxygenated O<sub>2</sub>Fe<sup>II</sup>Mb stock solution was diluted in a cuvette containing 50 mM phosphate buffer (pH 7.4) to a final concentration of 10  $\mu\text{M}$ . The UV–Vis spectrum was measured to verify the concentration (542 nm,  $\epsilon = 13,900 \text{ M}^{-1} \text{ cm}^{-1}$ ). Next, an aliquot of the MCPD stock solution was added to the cuvette to give a final concentration of 90  $\mu\text{M}$  and the UV–Vis spectrum recorded every 90 s for 45 min from 300 to 700 nm.

**UV–Vis Analysis of the reaction of oxymyoglobin (O<sub>2</sub>Fe<sup>II</sup>Mb) with MCPD under N<sub>2</sub>:** A stock solution of O<sub>2</sub>Fe<sup>II</sup>Mb, was prepared as described above and diluted in a septum-capped cuvette containing 50 mM phosphate buffer (pH 7.4) to a final concentration of 19  $\mu\text{M}$ . The O<sub>2</sub>Fe<sup>II</sup>Mb solution and the MCPD stock solution were deoxygenated via sparging with N<sub>2</sub> for 30 min (conditions that allowed O<sub>2</sub>Fe<sup>II</sup>Mb to persist). The UV–Vis spectrum of O<sub>2</sub>Fe<sup>II</sup>Mb solution was then measured to verify the concentration. An aliquot of the MCPD stock solution (described above) was then added to the cuvette to give a final concentration of 190  $\mu\text{M}$ . The reaction was kept under positive N<sub>2</sub> pressure atmosphere by passing N<sub>2</sub> through the reaction headspace. The UV–Vis

spectrum was then recorded after 30 min from 300 to 700 nm.

**ESI-MS Analysis of polysulfides from reactions of MCPD with myoglobin:** The reaction products derived from the MCPD reaction with myoglobin were monitored using single ion monitoring (SIM) ESI-MS. The reaction with Fe<sup>III</sup>Mb or O<sub>2</sub>Fe<sup>II</sup>Mb and MCPD was performed using the same concentrations as described above but in a different MS-compatible buffer (50 mM ammonium carbonate buffer containing 50  $\mu\text{M}$  DTPA (pH 7.4)). The reaction was allowed to proceed for 30 min and then the MCPD products were separated from the myoglobin by using a G-25 Sephadex column (monitoring the different aliquots by UV–vis spectroscopy). Myoglobin-free fractions were concentrated using lyophilization and then re-dissolved in water. Then, the samples were injected directly into a Thermo TSQ Quantum Triple Quadrupole Mass Spectrometer controlled with XCalibur 2.1 via syringe pump. Spectra were acquired in positive ion mode with a spray voltage of 3500 V, a capillary temperature of 250 °C and, a flow rate of 1  $\mu\text{L}/\text{min}$ . Ion optics were optimized for all ions of interest.

**Monitoring dioxygen consumption by the reaction of O<sub>2</sub>Fe<sup>II</sup>Mb and MCPD:** The reaction of MCPD and O<sub>2</sub>Fe<sup>II</sup>Mb was measured by monitoring the rate of loss of oxygen in the presence of added MCPD using a Clark-type electrode. A 10 mL two-neck round bottom flask fitted with a septum and an oxygen Clark-type electrode was filled with 21 mL of 0.1 mM of O<sub>2</sub>Fe<sup>II</sup>Mb in phosphate buffer containing 50  $\mu\text{M}$  DTPA (pH 7.4) to limit headspace. A stock solution of MCPD (0.2 M) was prepared in DI water. For monitoring dioxygen consumption, the electrode was first allowed to equilibrate under ambient conditions until no change in O<sub>2</sub> levels were observed (approximately 10–12 min). After this equilibration period, dioxygen readings were recorded every minute for the next 10 min. Then, 200  $\mu\text{L}$  of the MCPD stock solution was added to the three-neck flask (final [MCPD] = 1 mM) via syringe and dioxygen readings continued to be recorded another 45 min.

**Reaction of H<sub>2</sub>S<sub>2</sub> with Fe<sup>III</sup>Mb:** Approximately 1.5 mg of myoglobin was taken up in 5 mL of 100 mM phosphate buffer (pH 7.4). One mL from the myoglobin stock was diluted into 2 mL of buffer in a cuvette and then the absorbance at 408 nm was used to calculate the concentration of Fe<sup>III</sup>Mb in the cuvette ( $\epsilon = 188000 \text{ M}^{-1} \text{ cm}^{-1}$ ). The Fe<sup>III</sup>Mb concentration in the cuvette was determined to be 3.6  $\mu\text{M}$ . A stock solution of Na<sub>2</sub>S<sub>2</sub> was made up by taking 2.6 mg (0.024 mmol) of Na<sub>2</sub>S<sub>2</sub> up in 3 mL of DI water for a concentration of approximately 8 mM. Then, 15  $\mu\text{L}$  of the Na<sub>2</sub>S<sub>2</sub> solution was added to the cuvette containing Fe<sup>III</sup>Mb giving a final Na<sub>2</sub>S<sub>2</sub> concentration of approximately 60  $\mu\text{M}$ . The reaction was monitored in the UV–Vis for 2 h and took a spectra every 15 min.

**Reaction of H<sub>2</sub>S<sub>2</sub> with cytochrome *c*:** The procedure for examining the reaction of cytochrome *c* (bovine heart) with Na<sub>2</sub>S<sub>2</sub> is the same as that described above for Fe<sup>III</sup>Mb. The concentration of cytochrome *c* in the cuvette was approximately 7.6  $\mu\text{M}$  (410 nm,  $\epsilon = 120000 \text{ M}^{-1} \text{ cm}^{-1}$ ). Preparation and handling of the Na<sub>2</sub>S<sub>2</sub> stock solution is as described above for MbFe(III). The reaction was monitored by UV–Vis for 30 min taking one scan every minute.

**DIP-Mediated conversion of H<sub>2</sub>S to polysulfides (e.g., H<sub>2</sub>S<sub>2</sub>):** A stock solution of Na<sub>2</sub>S (5 mM) was prepared by dissolving Na<sub>2</sub>S salt in DI water. DIP was dissolved in DMSO to afford a 2.5 mM stock solution. Similarly,  $\beta$ -(4-hydroxyphenyl)ethyl iodoacetamide (HPE-IAM) was dissolved in DMSO to obtain a 100 mM stock solution. In a 20 mL scintillation vial, Na<sub>2</sub>S solution (50  $\mu\text{M}$ ) was incubated without DIP or with DIP (25  $\mu\text{M}$ ) in pH 7.4 ammonium bicarbonate buffer (50 mM, 3 mL total volume) at room temperature for 10 min, followed by derivatization with HPE-IAM (1 mM) for 30 min. The polysulfide composition was analyzed by ultra-performance liquid chromatography mass spectrometry (UPLC-MS) using a Waters Acquity/Xevo-G2 UPLC-MS system coupled with a high-resolution Q-TOF MS/MS detector. Mobile phase A - water, B - acetonitrile, and C - 1% formic acid in water. An ACQUITY UPLC BEH C18 column (2.1 mm  $\times$  50 mm, 1.7  $\mu\text{m}$ ) was used for the separation using a chromatographic gradient 0–1 min 90% water + 0% ACN + 10% formic acid in water; 1–7.5 min gradient up to 10% water + 80% ACN + 10% formic acid in water; 7.5–8.4 min 10% water + 80%

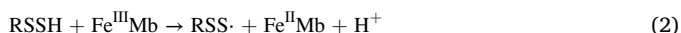
ACN + 10% formic acid in water; 8.4–8.5 min gradient up to 90% water + 0% ACN + 10% formic acid in water, 8.5–10 min 90% water + 0% ACN + 10% formic acid in water. Flow rate = 0.3 mL/min and the injection volume was 5  $\mu$ L.

### 3. Results and discussion

The reaction of RSSH with  $\text{Fe}^{\text{III}}$ Mb: The reaction of a hydropersulfide donor (methoxycarbonyl penicillamine disulfide, MCPD [16,24]) with  $\text{Fe}^{\text{III}}$ Mb was examined. Scheme 1 depicts the chemistry of MCPD decomposition and generation of a hydropersulfide (to be abbreviated as RSSH) as well as the pathways for formation of the corresponding trisulfide (RSSSR) and tetrasulfide (RSSSSR) species (*vide infra*) from this decomposition. It is important to note that the trisulfide, RSSSR, can readily form from the reaction of RSSH with the starting MCPD disulfide. However, the primary mechanism for tetrasulfide, RSSSSR, formation requires one electron oxidation of RSSH to the perthiyl radical, RSS $\cdot$ , followed by dimerization [16].

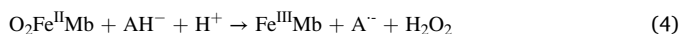
As shown in Fig. 1, when  $\text{Fe}^{\text{III}}$ Mb is reacted with MCPD under aerobic conditions it is readily converted to  $\text{O}_2\text{Fe}^{\text{II}}$ Mb.

These data are consistent with a previous report from our lab which showed identical results [16]. Galardon and coworkers [26] also report that reaction of  $\text{Fe}^{\text{III}}$ Mb with hydropersulfides results in the generation of  $\text{O}_2\text{Fe}^{\text{II}}$ Mb. These findings indicate that RSSH (from MCPD decomposition), is capable of reducing  $\text{Fe}^{\text{III}}$ Mb to  $\text{Fe}^{\text{II}}$ Mb with subsequent binding of  $\text{O}_2$  to  $\text{Fe}^{\text{II}}$ Mb (Reactions 2,3).



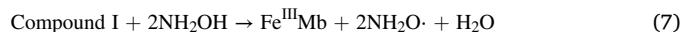
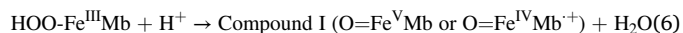
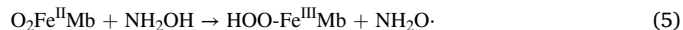
Importantly, it has already been shown that RSH species will not readily perform this chemistry (i.e. RSH will not readily reduce  $\text{Fe}^{\text{III}}$ Mb under the conditions of these experiments) [16]. Also, the fate of the oxidized RSSH species, RSS $\cdot$ , was shown to be rapid dimerization to the tetrasulfide, RSSSSR [16] (Scheme 1). An important observation in the spectra of Fig. 1 is the growth of an absorbance at approximately 616 nm. This will be discussed later.

The reaction of RSSH with  $\text{O}_2\text{Fe}^{\text{II}}$ Mb: It is well established that  $\text{O}_2\text{Fe}^{\text{II}}$ Mb is capable of oxidizing specific compounds that are reasonable one-electron reductants. For example, ascorbate ( $\text{AH}^-$ ) reacts with  $\text{O}_2\text{Fe}^{\text{II}}$ Mb to give ascorbyl radical anion ( $\text{A}^{\cdot-}$ ),  $\text{Fe}^{\text{III}}$ Mb and  $\text{H}_2\text{O}_2$  (Reaction 4) [27].

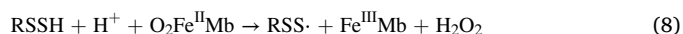


Also, hydroxylamine ( $\text{NH}_2\text{OH}$ ) and related species react with  $\text{O}_2\text{Fe}^{\text{II}}$ heme (such as  $\text{O}_2\text{Fe}^{\text{II}}$ Mb and oxyhemoglobin,  $\text{O}_2\text{Fe}^{\text{II}}$ Hb), to give

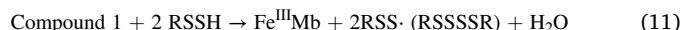
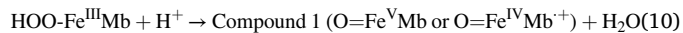
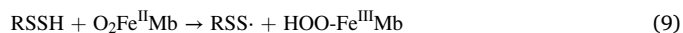
initially a fleeting  $\text{HOO-Fe}^{\text{III}}$  species (Reaction 5) [28]. The chemistry of  $\text{NH}_2\text{OH}$  differs from that of ascorbate as further chemistry appears to involve the intermediacy of a compound I-type ferryl-oxo intermediate that is capable of further oxidation of the reductant (Reaction 6, 7).



Ultimately, in the cases of ascorbate and  $\text{NH}_2\text{OH}$  this chemistry leads to the net reductive conversion of  $\text{O}_2\text{Fe}^{\text{II}}$  to  $\text{Fe}^{\text{III}}$  and concomitant generation of  $\text{H}_2\text{O}_2$  or  $\text{H}_2\text{O}$  from the bound  $\text{O}_2$ . The crucial step in these reactions (Reactions 4 and 5) requires  $\text{O}_2\text{Fe}^{\text{II}}$ Mb to act as a one-electron oxidant. This may be best understood by considering that  $\text{O}_2\text{Fe}^{\text{II}}$ Mb possesses significant  $\text{O}_2^{\cdot-}\text{Fe}^{\text{III}}\text{Mb}$  character (i.e.  $[\text{O}_2\text{Fe}^{\text{II}}\text{Mb} \leftrightarrow \text{O}_2^{\cdot-}\text{Fe}^{\text{III}}\text{Mb}]$ ) and that the bound superoxide ( $\text{O}_2^{\cdot-}$ )-like ligand is serving as the one-electron oxidant [29]. The chemical similarity between  $\text{NH}_2\text{OH}$ , ascorbate and RSSH (all good H-atom donors and reductants) [16], indicates that RSSH may react analogously with  $\text{O}_2\text{Fe}^{\text{II}}$ Mb as reported for  $\text{NH}_2\text{OH}$  or ascorbate. If RSSH reacts analogously to ascorbate,  $\text{H}_2\text{O}_2$  and  $\text{Fe}^{\text{III}}$ Mb will be formed (Reaction 8).

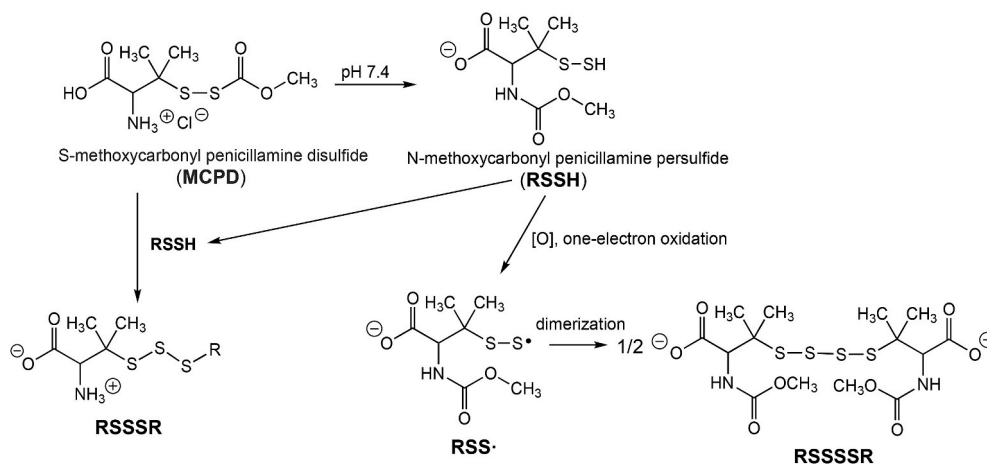


If RSSH reacts analogously to  $\text{NH}_2\text{OH}$ , then a series of reactions shown below can be proposed (Reactions 9, 10 and 11).

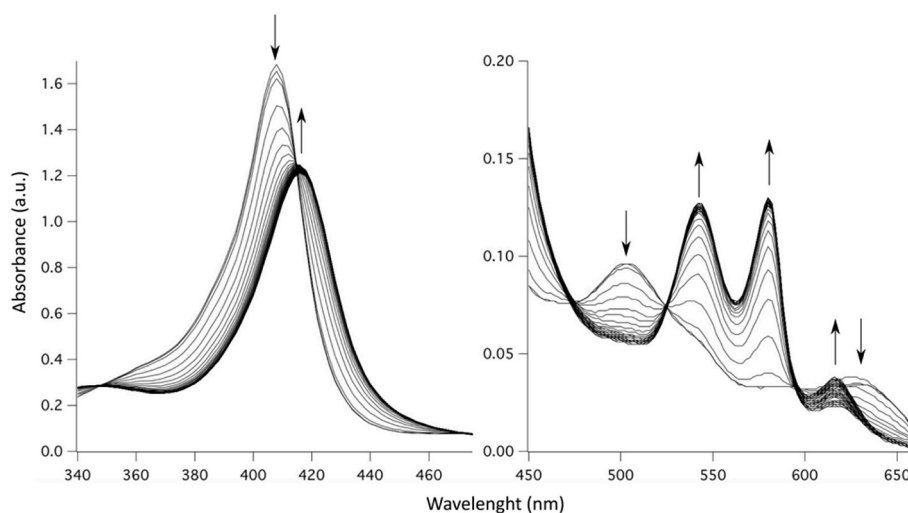


Thus, the reaction of MCPD with  $\text{O}_2\text{Fe}^{\text{II}}$ Mb was examined in greater detail. As shown in Fig. 2A the reaction of MCPD with  $\text{O}_2\text{Fe}^{\text{II}}$ Mb exhibits little change in the absorbance spectrum except for a continuous rise in the absorbance at 616 nm (Fig. 2B). On the surface, these results appear to indicate that Reaction 8 (or Reactions 9, 10 and 11) do not readily occur over the time frame of the experiment (1 h).

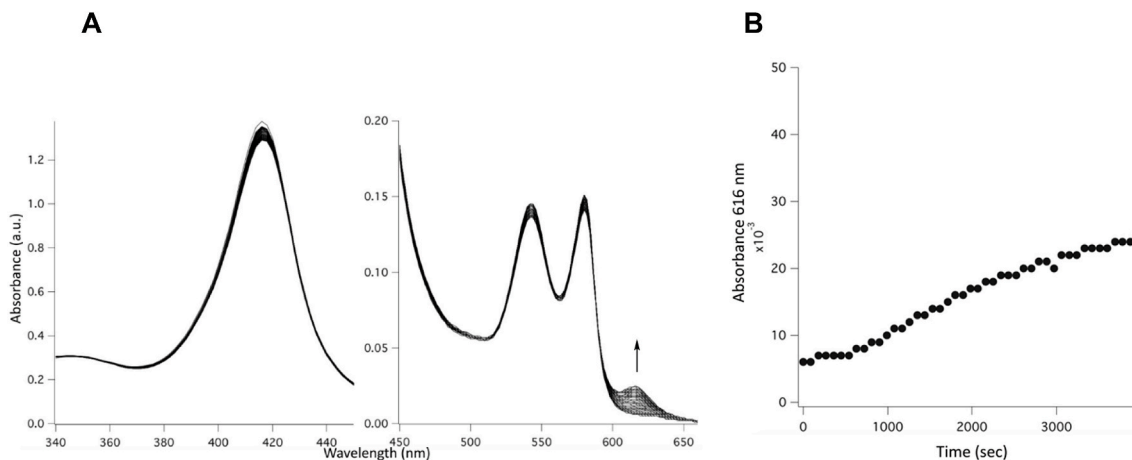
However, the results shown in Fig. 2 do not necessarily indicate a lack of reactivity between RSSH (from the hydropersulfide donor MCPD) and  $\text{O}_2\text{Fe}^{\text{II}}$ Mb. That is, if the reduction of  $\text{Fe}^{\text{III}}$ Mb by RSSH to  $\text{Fe}^{\text{II}}$ Mb and the binding of  $\text{O}_2$  to  $\text{Fe}^{\text{II}}$ Mb to give  $\text{O}_2\text{Fe}^{\text{II}}$ Mb (Reactions 2,3) are both fast compared to the reaction of RSSH with  $\text{O}_2\text{Fe}^{\text{II}}$ Mb (Reactions 8 or 9), it is possible that the initially formed  $\text{Fe}^{\text{III}}$ Mb product may be rapidly consumed and not detected. Combining Reactions 2, 3, and 8) gives Reaction 12 and combining Reactions 2, 3, 9, 10 and 11 gives Reaction 13, both of which represents a myoglobin catalyzed reduction of  $\text{O}_2$



Scheme 1. MCPD decomposition to the hydropersulfide and subsequent pathways for the formation of RSSSR and RSSSSR species.

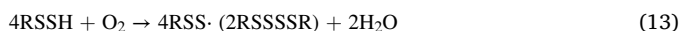
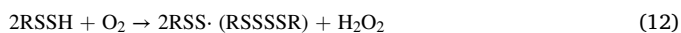


**Fig. 1.** Soret absorption (A) and Q-bands (B) changes for the reaction of  $\text{Fe}^{\text{III}}\text{Mb}$  ( $9\ \mu\text{M}$ ) with  $90\ \mu\text{M}$  MCPD at pH 7.4,  $25\ ^\circ\text{C}$ . Spectra at 0 s were obtained for  $\text{Fe}^{\text{III}}\text{Mb}$  in the absence of MCPD, and others were recorded during the reaction until 1 h after the initiation of the reaction. The directions of the absorption changes are indicated by arrows.



**Fig. 2.** A) Soret absorption and Q-bands changes for the reaction of  $\text{O}_2\text{Fe}^{\text{II}}\text{Mb}$  ( $9\ \mu\text{M}$ ) with  $90\ \mu\text{M}$  MCPD at pH 7.4,  $25\ ^\circ\text{C}$ . Data collected for 1 h after the initiation of the reaction. B) Time-dependent increase in the absorbance at 616 nm for the reaction between MCPD and  $\text{O}_2\text{Fe}^{\text{II}}\text{Mb}$ .

by RSSH (to either  $\text{H}_2\text{O}_2$  or  $\text{H}_2\text{O}$ ).

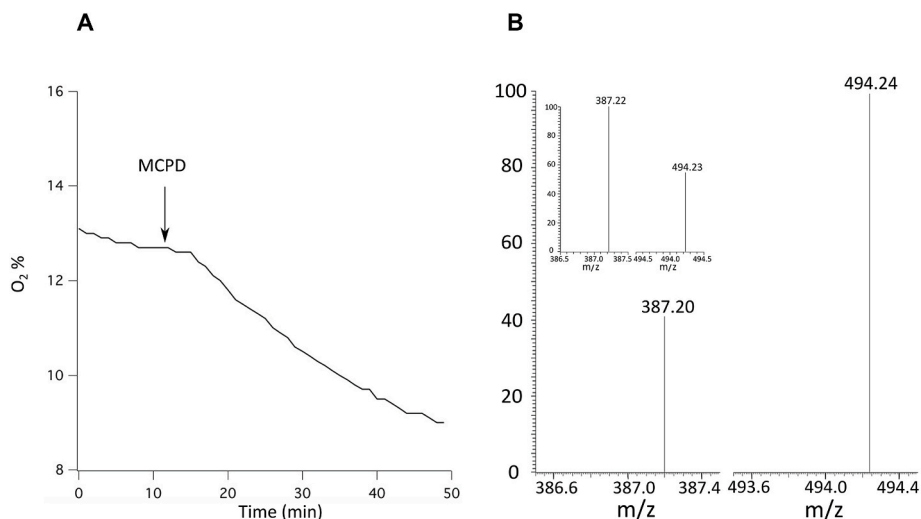


Thus, if  $\text{O}_2$  is consumed by the reaction of  $\text{O}_2\text{Fe}^{\text{II}}\text{Mb}$  and RSSH, this would be consistent with an initial reaction between RSSH and  $\text{O}_2\text{Fe}^{\text{II}}\text{Mb}$  and a catalytic process, either **Reaction 12** or **13**, that represents catalytic consumption of  $\text{O}_2$  by RSSH. Indeed, as shown in **Fig. 3A**, the addition of MCPD to a solution of  $\text{O}_2\text{Fe}^{\text{II}}\text{Mb}$  results in the consumption of  $\text{O}_2$  as measured by a Clarke electrode. It is important to note that previous work from this lab has shown that a decomposing solution of MCPD alone does not react with and consume  $\text{O}_2$ , also measured using a Clarke-type electrode [16]. Thus, the  $\text{O}_2$  consumption observed here is clearly due to the presence of myoglobin.

Another aspect of **Reactions 12** or **13** is the generation of the RSSSSR tetrasulfide species. As mentioned previously, the generation of RSS $\cdot$  can be conferred from this reaction via the detection of RSSSSR [16] (**Scheme 1**). Analysis of the MCPD/ $\text{O}_2\text{Fe}^{\text{II}}\text{Mb}$  reaction mixture shows the formation of the RSSSSR species ( $m/z = 494.28$ ) (**Fig. 3B**). It is important to note that decomposition of MCPD in the absence of any other reactant (i.e. oxidant) leads to the formation of the corresponding

trisulfide species (RSSSR) as the predominant product since the RSSH generated reacts with the precursor disulfide MCPD, giving the mostly RSSSR (**Fig. 3** inset, [16]). However, one-electron oxidation of RSSH results in significantly greater amounts of RSSSSR formation [16] (**Scheme 1**). These results are consistent with a previous paper from this lab that showed that MCPD decomposition in the absence of other reactants (e.g. oxidants) leads to predominantly greater levels of RSSSR over RSSSSR (RSSSR/RSSSSR ratio  $> 1$ ). However, when a one-electron oxidant is present the RSSSR/RSSSSR ratio flips to become  $< 1$  [16]. Thus, the results of this experiment are consistent with the idea that RSSH generated *in situ* reacts with  $\text{O}_2\text{Fe}^{\text{II}}\text{Mb}$  to give RSS $\cdot$  (which dimerizes to RSSSSR) and the RSSH that does not react with  $\text{O}_2\text{Fe}^{\text{II}}\text{Mb}$  gives the expected trisulfide MCPD-SSS-MCP species ( $m/z = 387.27$ ). It is important to note that no reaction between  $\text{Fe}^{\text{III}}\text{Mb}$  (or  $\text{O}_2\text{Fe}^{\text{II}}\text{Mb}$ ) with Cys-SH, Cys-SS-Cys and Cys-SSSCys was observed (data not shown). Also, the products of the reaction of a solution of MCPD and TEMPOL, a mixture that generates significant levels of RSSSSR species (**Scheme 1**) [16] also did not react with  $\text{Fe}^{\text{III}}\text{Mb}$  (data not shown).

These data are consistent with the idea that RSSH reacts with  $\text{O}_2\text{Fe}^{\text{II}}\text{Mb}$  and that myoglobin is capable of catalyzing the  $\text{O}_2$ -dependent degradation of RSSH. Whether the reaction of RSSH with  $\text{O}_2\text{Fe}^{\text{II}}\text{Mb}$  is akin to the reaction of  $\text{NH}_2\text{OH}$  or of ascorbate with  $\text{O}_2\text{Fe}^{\text{II}}\text{Mb}$  is not



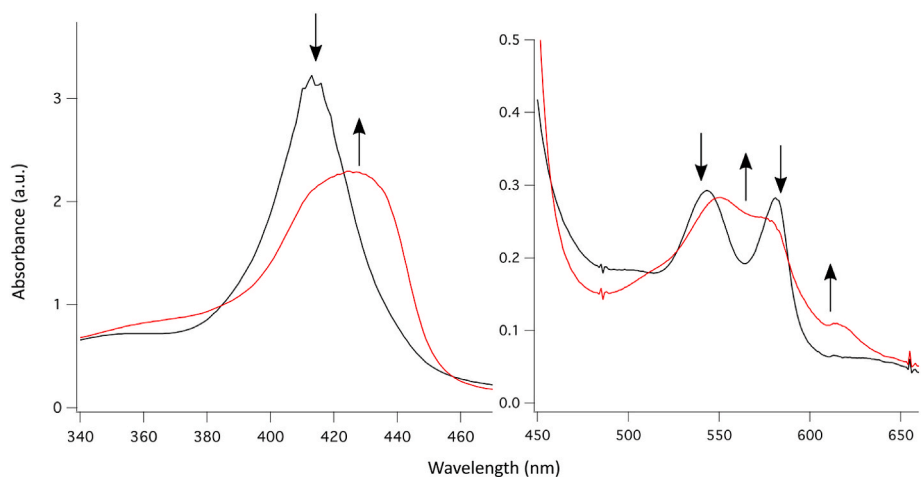
**Fig. 3.** A) O<sub>2</sub> consumption as monitored by a Clarke electrode. The arrow denotes the addition of MCPD. B). ESI-MS analysis of the sulfur products from the reaction of MCPD and O<sub>2</sub>Fe<sup>II</sup>Mb (MCP-SSSS-MCP-NH<sub>4</sub><sup>+</sup>, *m/z* = 494.28, MCP-SSS-MCP, *m/z* = 387.27 [16]). The inset show the ESI-MS analysis of the decomposition of MCPD alone in 50 mM ammonium phosphate buffer (pH 7.4) containing 50 μM DTPA.

known at this time (i.e., with regards to the nature of the reaction intermediates). It is worth re-emphasizing that the reaction of RSSH with O<sub>2</sub>Fe<sup>II</sup>Mb is considerably slower than the rate of RSSH reduction of Fe<sup>III</sup>Mb and the rate of Fe<sup>II</sup>Mb oxygenation. The rate constant for MCPD decomposition (to give an RSSH species) is approximately 0.3 min<sup>-1</sup> at physiological pH [24] and the RSSH formed from MCPD decomposition can react with either O<sub>2</sub>Fe<sup>II</sup>Mb or MCPD (Scheme 1). Under the conditions of these experiments these reactions are clearly competitive.

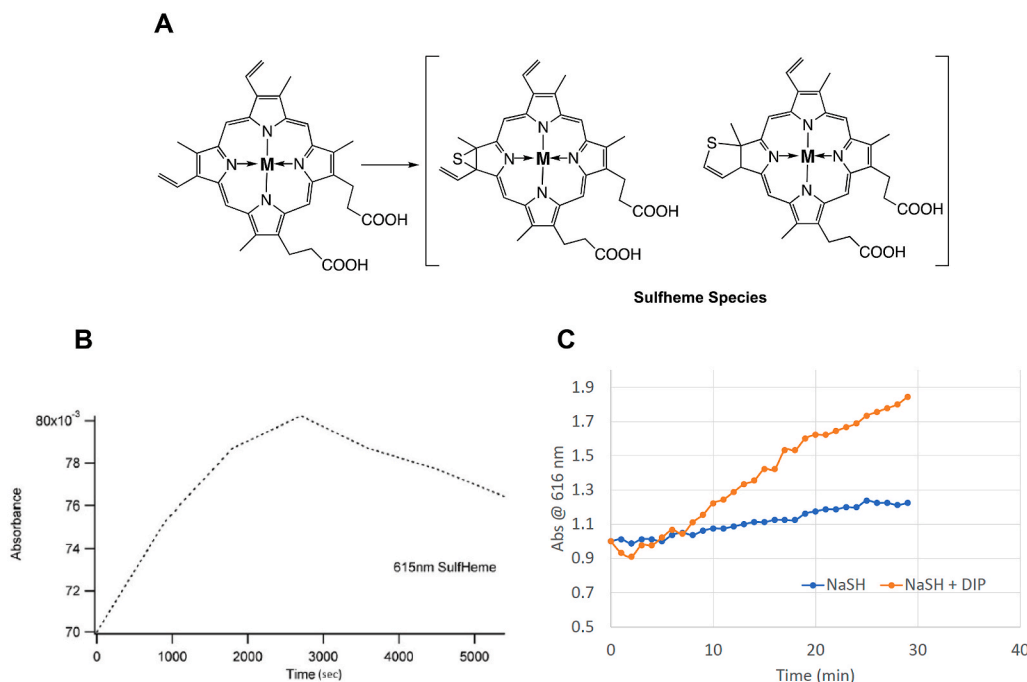
Reaction of RSSH with O<sub>2</sub>Fe<sup>II</sup>Mb under O<sub>2</sub>-depleted conditions: The results thus far indicate that RSSH is indeed capable of reacting with O<sub>2</sub>Fe<sup>II</sup>Mb to give, initially Fe<sup>III</sup>Mb (Reaction 8 or 9–11) which is then quickly reduced by RSSH to Fe<sup>II</sup>Mb which then rapidly binds O<sub>2</sub> to give back O<sub>2</sub>Fe<sup>II</sup>Mb (Reactions 2 and 3). It would be expected that this series of reactions carried out under O<sub>2</sub>-depleted conditions could result in the detection of the Fe<sup>II</sup>Mb intermediate once O<sub>2</sub> is consumed (via Reactions 12 or 13). This experiment is not straightforward since rigorous degassing of a solution of O<sub>2</sub>Fe<sup>II</sup>Mb can lead to simple dissociation of O<sub>2</sub> from O<sub>2</sub>Fe<sup>II</sup>Mb to give directly Fe<sup>II</sup>Mb. However, O<sub>2</sub> can be significantly depleted from solution and still allow O<sub>2</sub>Fe<sup>II</sup>Mb to remain. Thus, an O<sub>2</sub>-depleted solution of O<sub>2</sub>Fe<sup>II</sup>Mb (degassed via N<sub>2</sub> sparging) was treated with MCPD in excess and the absorbance changes monitored. As seen in

Fig. 4, the absorbance spectrum taken after 30 min (allowing for complete O<sub>2</sub> depletion) shows the formation of the Fe<sup>II</sup>Mb intermediate (increase in absorbances at 434 nm and 560 nm [30]). Thus, reaction of O<sub>2</sub>Fe<sup>II</sup>Mb with RSSH leads to the generation of Fe<sup>III</sup>Mb (via Reaction 8 or Reactions 9–11), which is then reduced by RSSH to Fe<sup>II</sup>Mb (Reaction 2). With little or no O<sub>2</sub> in solution (consumed via Reactions 12 or 13), Fe<sup>II</sup>Mb becomes stable and detectable.

The formation of sulfheme species: As shown in Figs. 1 and 2, the reaction of RSSH with myoglobin results in the generation of a species with an absorbance at approximately 616 nm. This is consistent with the generation of sulfheme, a modified porphyrin whereby a sulfur atom is appended to one of the pyrrole moieties of the porphyrin [e.g., see Ref. [31], [32]]. Sulfhemes are reported to consist of several structures, all possibly derived from the initial formation of an episulfide-type species [33,34] (Fig. 5A). The mechanism of sulfheme formation is not firmly established and has been proposed to occur via an oxidizing sulfur species, such as a hydrothiyl radical (HS·) [32,34,35]. It remains possible that sulfheme generation observed herein could be the result of HS· formation, although the presence of an oxidant that would readily generate HS· from an RSSH species is not immediately evident. However, it was also considered that the sulfheme generated under the



**Fig. 4.** Soret absorption (A) and Q-band (B) changes for the reaction of O<sub>2</sub>Fe<sup>II</sup>Mb (19 μM) with 190 μM MCPD at pH 7.4, 25 °C under an N<sub>2</sub> atmosphere. Spectrum at 0 s (black line) was obtained for O<sub>2</sub>Fe<sup>II</sup>Mb in the absence of MCPD. Spectrum in red was collected after 30 min after the initiation of the reaction. Arrows indicate the directions of the absorption changes. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)



**Fig. 5.** A) Structures of sulfheme species generated from porphyrins. B) Sulfheme formation via the reaction of  $\text{Na}_2\text{S}_2$  with  $\text{Fe}^{\text{III}}\text{Mb}$  (absorbance at 616 nm). C) The reaction of  $\text{H}_2\text{S}$  with  $\text{Fe}^{\text{III}}\text{Mb}$  in the presence and absence of DIP and the formation of sulfheme.

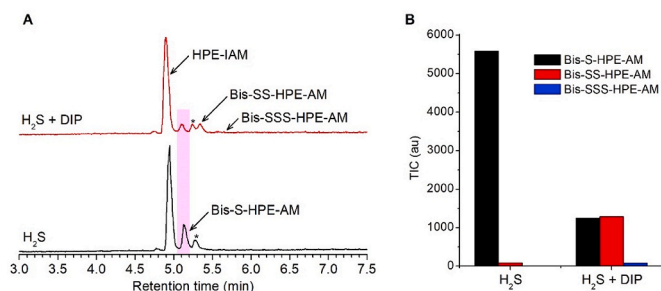
conditions of the experiments described could instead be due to the formation of polysulfur species since the hydropersulfide generated from MCPD is capable of sulfheme formation. In order to determine whether a purely inorganic polysulfide can generate sulfheme, authentic  $\text{H}_2\text{S}_2$  was examined. As shown in Fig. 5B, the addition of  $\text{Na}_2\text{S}_2$  to a buffered solution of  $\text{Fe}^{\text{III}}\text{Mb}$  results in immediate formation of sulfheme (as evidenced by the rise in absorbance at 616 nm).

The results shown in Fig. 5B are consistent with the idea that an oxidized sulfur species is responsible for sulfheme formation since  $\text{H}_2\text{S}_2$  is oxidized with respect to  $\text{H}_2\text{S}$ . This is not necessarily surprising since other previous studies have shown that sulfheme formation from  $\text{H}_2\text{S}$  requires an oxidant to convert  $\text{H}_2\text{S}$  to a reactive oxidized form (such as  $\text{HS}^\cdot$ ) [32]. One way to convert  $\text{H}_2\text{S}$  to polysulfur species ( $\text{H}_2\text{S}_n$ ,  $n \geq 2$ ), without the intermediacy of  $\text{HS}^\cdot$ , is to utilize diamide-type molecules which can, in an  $\text{O}_2$ -independent fashion, convert thiols to disulfides [36]. If the thiol used is  $\text{H}_2\text{S}$ , the immediate product will be  $\text{H}_2\text{S}_2$  (Fig. 6).

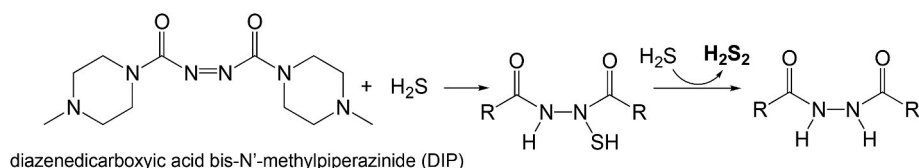
In an attempt to determine if the sulfheme-forming species can be a polysulfur compound (e.g. RSSH, R = alkyl or H), the generation of sulfheme from  $\text{H}_2\text{S}$  was monitored (at 616 nm) in the presence and absence of the established diazenedicarboxylic acid (diamide) thiol oxidant diazenedicarboxylic acid bis-*N'*-methylpiperazine (DIP) [25]. Fig. 5C shows the rate of sulfheme formation from the reaction of  $\text{H}_2\text{S}$  with  $\text{Fe}^{\text{III}}\text{Mb}$  in the presence (orange trace) and absence (blue trace) of the thiol oxidant DIP. It is clear that the rate of formation of sulfheme dramatically increases in the presence of the DIP thiol oxidant, implicating  $\text{H}_2\text{S}_2$ , and possibly higher order polysulfides, as the sulfheme-forming species.

To verify further that  $\text{H}_2\text{S}_2$  (or a higher order polysulfide derived from  $\text{H}_2\text{S}_2$ ) can be responsible for sulfheme formation under these

conditions, the DIP-mediated  $\text{H}_2\text{S}$  oxidation to higher order polysulfides was examined independently. HPE-IAM was used to trap polysulfides generated from the  $\text{H}_2\text{S}/\text{DIP}$  reaction (Fig. 6).  $\text{H}_2\text{S}$  alone will be trapped by HPE-IAM to give the thioether bis-S-HPE-AM whereas  $\text{H}_2\text{S}_2$ , if formed, will be trapped by HPE-IAM to give the dialkyl disulfide bis-SS-HPE-AM. As shown in Fig. 7A,  $\text{H}_2\text{S}$  incubation with HPE-IAM (20 equiv.) in pH 7.4 ammonium bicarbonate buffer produces bis-S-HPE-AM



**Fig. 7.** DIP-mediated conversion of  $\text{H}_2\text{S}$  to polysulfides (e.g.  $\text{H}_2\text{S}_2$ ). (A) Total ion chromatograms of  $\text{H}_2\text{S}$  (50  $\mu\text{M}$ ) incubated with and without DIP (25  $\mu\text{M}$ ) in pH 7.4 ammonium bicarbonate buffer (25 mM) containing DTPA (100  $\mu\text{M}$ ) at room temperature for 10 min, followed by trapping with HPE-IAM (1 mM) for 30 min. The asterisk indicates the presence of small amount of impurity in the commercial HPE-IAM sample. (B) Comparison of polysulfur species such as  $\text{H}_2\text{S}$ ,  $\text{H}_2\text{S}_2$  and  $\text{H}_2\text{S}_3$  (measured by the trapped HPE-AM species bis-S-HPE-AM, bis-SS-HPE-AM, and bis-SSS-HPE-AM, respectively) from the  $\text{H}_2\text{S}$ , and  $\text{H}_2\text{S} + \text{DIP}$  reaction mixtures.



**Fig. 6.** The conversion of  $\text{H}_2\text{S}$  to  $\text{H}_2\text{S}_2$  by DIP.

as a major product along with a minor bis-SS-HPE-AM impurity, suggesting that the commercial  $\text{Na}_2\text{S}$  salt has low level of  $\text{Na}_2\text{S}_2$ . In the presence of DIP, however, a significant increase in bis-SS-HPE-AM formation with concomitant decrease in bis-S-HPE-AM (Fig. 7B) is observed, indicating that DIP oxidizes  $\text{H}_2\text{S}$  into  $\text{H}_2\text{S}_2$  (Fig. 6). Interestingly, a small amount of bis-SSS-HPE-AM is also generated in the  $\text{H}_2\text{S}/\text{DIP}$  reaction.

Of course, these results do not necessarily preclude  $\text{HS}^-$  as the ultimate sulfheme-forming species since  $\text{H}_2\text{S}_2$  can conceivably be an  $\text{HS}^-$  precursor via simple homolysis or other possible mechanisms. However, it is also possible that polysulfur species such as  $\text{H}_2\text{S}_2$  are directly responsible for reacting with porphyrins to generate sulfheme. It is worth remembering that the hydropersulfide donor MCPD also reacts with myoglobin to generate sulfheme (Figs. 1 and 2), indicating that hydroalkylpersulfides (i.e. RSSH) are capable of generating sulfhemes (not just inorganic polysulfur species such as  $\text{H}_2\text{S}_2$ ). RSSH species can form numerous sulfur species *in situ* via a series of equilibrium reactions [19] thus making assignment of the reactive species responsible for sulfheme formation difficult. However, considering that polysulfur species, such as  $\text{RSS}^-$  ( $\text{R} = \text{alkyl or H}$ ) are potent nucleophiles (superior to the corresponding thiolate), it is intriguing to speculate that sulfheme formation can occur via nucleophilic attack on a pyrrole of the porphyrin ring, eventually leading to incorporation of a sulfur atom (Fig. 8). As mentioned above, several sulfheme adducts have been reported and include an episulfide and a thiochlorin species (Fig. 5A) [32].

Although the mechanisms shown here are highly speculative and based solely on the idea that a higher order, nucleophilic polysulfide (e.g.  $\text{H}_2\text{S}_2$ ) is the sulfheme-forming species, it is worth noting that nucleophilic attack on the beta-carbon of a pyrrole has been previously proposed as a mechanism of sulfheme formation in myoglobin [37]. In that report, it was postulated that hydrosulfide ( $\text{HS}^-$ ) could attack the beta-carbon of a porphyrin pyrrole when myoglobin was in a high-valent ferryl oxo form ( $\text{OFe}^{\text{IV}}\text{Mb}$ ) to form a ferrous-pyrrole episulfide. Although the overall process proposed by Berzofsky and coworkers [37] differs substantially from the mechanism postulated herein, it does suggest a possible initial nucleophilic attack by a sulfur species on a porphyrin pyrrole as an important first step, akin to the proposed pathway herein. Importantly, all mechanisms, including the one proposed here, require an oxidation step for  $\text{H}_2\text{S}$ -mediated sulfheme formation. In the

mechanism depicted in Fig. 8,  $\text{H}_2\text{S}$  would need to be oxidized to  $\text{H}_2\text{S}_2$  (or other polysulfide) prior to sulfheme formation. In other proposed mechanisms  $\text{H}_2\text{S}$  is converted to  $\text{HS}^-$  [32] or  $\text{H}_2\text{S}$  reacts with an oxidized ferryl form of myoglobin [37], thus accommodating the need for an oxidant. It can also be speculated that sulfheme formation occurs via a polysulfur radical species (e.g.  $\text{RSS}^\cdot$  or  $\text{HSS}^\cdot$ ) that adds to the pyrrole analogously to what has already been proposed for  $\text{HS}^-$ . Again, discriminating between these and other mechanism is the topic of future work.

Sulfheme does not form with all hemoproteins and occurs most notably with myoglobin and hemoglobins. Structural studies indicate that sulfheme is formed in heme proteins with an iron binding distal histidine and in proteins capable of forming high valent peroxy- or oxo-iron species when reacted with, for example,  $\text{H}_2\text{O}_2$  (thus possessing an open coordination site) [32,34,38]. Consistent with this idea, the reaction of ferric cytochrome *c* ( $\text{Fe}^{\text{III}}\text{Cyt c}$ , a coordinately saturated, substitutionally inert heme protein that does not form peroxy- or oxo complexes) with  $\text{H}_2\text{S}_2$  results in only reduction to  $\text{Fe}^{\text{II}}\text{Cyt c}$  with no sulfheme formation (data not shown). Interestingly, the mechanisms proposed herein (Fig. 8) would still be possible with  $\text{Fe}^{\text{III}}\text{Cyt c}$  since metal-oxo formation or coordination to the metal center are not required (provided there is no steric restriction to nucleophilic attack). Thus, the reason for the lack of sulfheme formation with  $\text{Fe}^{\text{III}}\text{Cyt c}$  via reaction with  $\text{H}_2\text{S}_2$  is not currently evident.

As mentioned previously, the mechanisms proposed above for sulfheme formation are speculative (and not the primary focus of this study). However, sulfheme formation in the reactions examined herein may be a very important observation in that it further supports the idea that RSSH reacts with  $\text{O}_2\text{Fe}^{\text{II}}\text{Mb}$ , leading to a catalytic cycle that converts RSSH to higher order polysulfides at the expense of  $\text{O}_2$ . The rationale for indicating this is that in all mechanisms proposed to date, sulfheme formation occurs via an intermediary  $\text{Fe}^{\text{III}}\text{Mb}$  or ferryl-oxo (via Reaction 10) species. Since sulfheme continuously forms in the reaction between  $\text{O}_2\text{Fe}^{\text{II}}\text{Mb}$  and RSSH (Fig. 2A), this likely indicates that the  $\text{Fe}^{\text{III}}\text{Mb}$  or ferryl-oxo species is continuously generated during the reaction, consistent with the idea of the existence of Reaction 8 or Reactions 9–11 (i.e.  $\text{O}_2\text{Fe}^{\text{II}}\text{Mb}$  reacting with RSSH), thus providing further evidence for a reaction between RSSH and  $\text{O}_2\text{Fe}^{\text{II}}\text{Mb}$ .

It is worth pointing out that the sulfheme absorbance disappears over time (Fig. 5B). This is not unexpected since it has been reported that sulfheme formation is reversible [39] and it is conceivable that the mechanism of this reversion can simply be the microscopic reverse of the mechanisms of formation proposed herein (Fig. 8).

#### 4. Summary/Conclusion

The results of this study indicate that RSSH is a potent enough reductant to not only reduce  $\text{Fe}^{\text{III}}\text{Mb}$  but react with  $\text{O}_2\text{Fe}^{\text{II}}\text{Mb}$  as well. These reactions allow the  $\text{O}_2$ -binding myoglobin to catalyze the  $\text{O}_2$ -dependent oxidation of RSSH. Also, sulfheme production was observed during these reactions, implicating hydropersulfides as possible agents in sulfheme formation. It needs to be emphasized, however, that the mechanisms discussed herein for sulfheme formation are speculative and require further investigation. Consistent with previous reports, it is clear that sulfheme formation requires an oxidation event starting from  $\text{H}_2\text{S}$ . Importantly, sulfheme formation starting with  $\text{O}_2\text{Fe}^{\text{II}}\text{Mb}$  and RSSH further supports the idea of a reaction between RSSH and  $\text{O}_2\text{Fe}^{\text{II}}\text{Mb}$  with an intermediary  $\text{Fe}^{\text{III}}\text{Mb}$  or oxo-ferryl species. The physiological relevance of this chemistry is, at this time, difficult to predict since the rate constant for the reaction of RSSH with  $\text{O}_2\text{Fe}^{\text{II}}\text{Mb}$  (Reaction 8 or Reaction 9) is unknown. It is clear that this reaction is slower than the rate of reduction of  $\text{Fe}^{\text{III}}\text{Mb}$  by RSSH (Reaction 2) or the rate of association of  $\text{O}_2$  and  $\text{Fe}^{\text{II}}\text{Mb}$  (Reaction 3), under the experimental conditions employed herein. Regardless, the studies herein further support the idea that RSSH is a potent reductant and a potent nucleophile, both chemical properties that likely dominate its chemical biology.

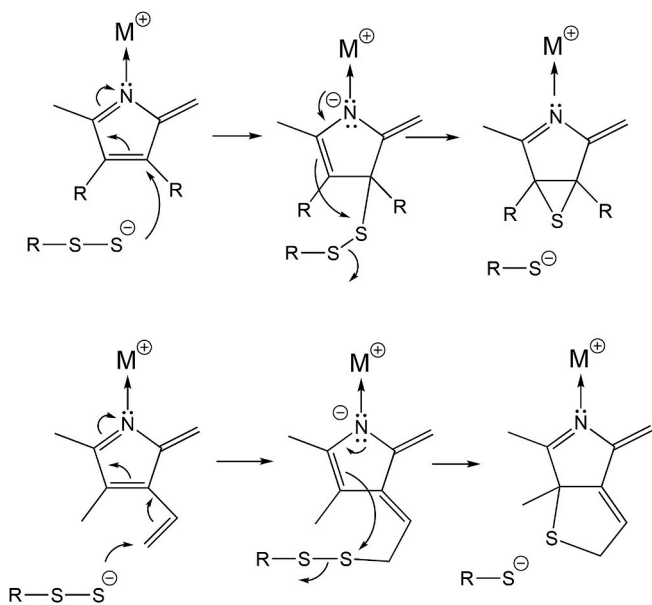


Fig. 8. Possible mechanisms for the generation of sulfheme species (episulfide – top reaction and thiochlorin – bottom reaction) via nucleophilic addition to a porphyrin pyrrole by a persulfide ( $\text{R} = \text{alkyl or H}$ ).

## Declaration of competing interest

None.

## Acknowledgements

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