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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_2S) 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_2S 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_2S can be attributed to the generation of hydropersulfide (RSSH, R = alkyl, H) species rather than H_2S itself [e.g., Ref. [7–10]]. The basis for the idea that some of the presumed H_2S biology can instead be the result of RSSH generation is that H_2S and RSSH species are intimately linked via an equilibrium involving H_2S/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.

$$RSSR + H_2S \rightleftharpoons RSSH + RSH \quad (1)$$

Furthermore, RSSH species have been shown to possess enhanced chemical reactivity compared to the parent H_2S 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_2S) [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 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

Abbreviations: RSSH, hydropersulfide; RSSR, disulfide; RSOH, sulfenic acid; RSNO, S-nitrosothiol; MCPD, methoxycarbonyl penicillamne disulfide; DIP, diazenedicarboxyic acid bis-N′-methylpiperazinide; Fe^{III}Mb, ferric myoglobin; O_2Fe^{II}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^-, ascorbate; $A^{\cdot-}$, ascorbyl radical anion; Fe^{III}Cyt c, ferric cytochrome *c*; Fe^{II}Cyt c, ferrous cytochrome *c*

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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_2S 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, hemeproteins.

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_2 Consumption was monitored using a YSI Oxygen Monitor (Clarke-type electrode).

Chemicals: Methoxycarbonyl penicillamine disulfide (MCPD) was synthesized according to a published procedure [24]. Diazenedicarboxyic acid bis-N′-methylpiperazinide (DIP) was synthesized according to the method of Kosower and Kanety-Londner [25]. The inorganic polysulfide Na_2S_2 was purchased from Dojindo Molecular Technologies (Kumamoto, Japan). Myogobin (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 ($Fe^{III}Mb$) and oxymyoglobin ($O_2Fe^{II}Mb$) with MCPD: The reaction of $Fe^{III}Mb$ with MCPD was monitored by measuring the loss in absorbance of $Fe^{III}Mb$ at 408 nm (ε = 188000 M^{-1} cm^{-1}). A 27 μM stock solution of $Fe^{III}Mb$ was prepared by dissolving 0.5 mg first in 1 mL of DI water and then taking 100 μL 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 $Fe^{III}Mb$ stock solution was diluted in a cuvette containing 50 mM phosphate buffer (pH 7.4) to a final concentration of 9 μM. 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 μM 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 $O_2Fe^{II}Mb$, approximately 1 mg of $Fe^{III}Mb$ was dissolved in 1 mL of water and reacted with excess sodium dithionite ($Na_2S_2O_4$) to fully reduce the heme protein to the ferrous state. The reduced, oxygenated $O_2Fe^{II}Mb$ stock solution was diluted in a cuvette containing 50 mM phosphate buffer (pH 7.4) to a final concentration of 10 μM. The UV–Vis spectrum was measured to verify the concentration (542 nm, ε = 13,900 M^{-1} cm^{-1}). Next, an aliquot of the MCPD stock solution was added to the cuvette to give a final concentration of 90 μM 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 ($O_2Fe^{II}Mb$) with MCPD under N_2: A stock solution of $O_2Fe^{II}Mb$, 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 μM. The $O_2Fe^{II}Mb$ solution and the MCPD stock solution were deoxygenated via sparging with N_2 for 30 min (conditions that allowed $O_2Fe^{II}Mb$ to persist). The UV–Vis spectrum of $O_2Fe^{II}Mb$ 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 μM. The reaction was kept under positive N_2 pressure atmosphere by passing N_2 through the reaction headspace. The UV–Vis spectrum was then recorded after 30 min from 300 to 700 nm.

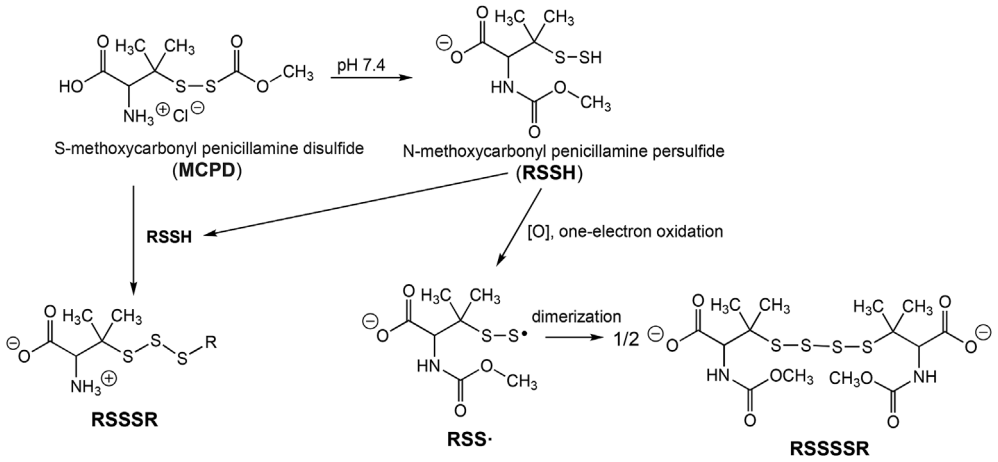
ESI-MS Analysis of polysulfides from reactions of MCPD with myoglobin: The reaction products derived from the MCPD reaction with myoglobin were monitored using single ion monitoring (SIM) ESI-MS. The reaction with $Fe^{III}Mb$ or $O_2Fe^{II}Mb$ and MCPD was performed using the same concentrations as described above but in a different MS-compatible buffer (50 mM ammonium carbonate buffer containing 50 μM 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 μL/min. Ion optics were optimized for all ions of interest.

Monitoring dioxygen consumption by the reaction of $O_2Fe^{II}Mb$ and MCPD: The reaction of MCPD and $O_2Fe^{II}Mb$ 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 $O_2Fe^{II}Mb$ in phosphate buffer containing 50 μM 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_2 levels were observed (approximately 10–12 min). After this equilibration period, dioxygen readings were recorded every minute for the next 10 min. Then, 200 μL 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_2S_2 with $Fe^{III}Mb$: 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 $Fe^{III}Mb$ in the cuvette (ε = 188000 M^{-1} cm^{-1}). The $Fe^{III}Mb$ concentration in the cuvette was determined to be 3.6 μM. A stock solution of Na_2S_2 was made up by taking 2.6 mg (0.024 mmol) of Na_2S_2 up in 3 mL of DI water for a concentration of approximately 8 mM. Then, 15 μL of the Na_2S_2 solution was added to the cuvette containing $Fe^{III}Mb$ giving a final Na_2S_2 concentration of approximately 60 μM. The reaction was monitored in the UV–Vis for 2 h and took a spectra every 15 min.

Reaction of H_2S_2 with cytochrome *c*: The procedure for examining the reaction of cytochrome *c* (bovine heart) with Na_2S_2 is the same as that described above for $Fe^{III}Mb$. The concentration of cytochrome *c* in the cuvette was approximately 7.6 μM (410 nm, ε = 120000 M^{-1} cm^{-1}). Preparation and handling of the Na_2S_2 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_2S to polysulfides (*e.g.*,H_2S_2): A stock solution of Na_2S (5 mM) was prepared by dissolving Na_2S salt in DI water. DIP was dissolved in DMSO to afford a 2.5 mM stock solution. Similarly, β-(4-hydroxyphenyl)ethyl iodoacetamide (HPE-IAM) was dissolved in DMSO to obtain a 100 mM stock solution. In a 20 mL scintillation vial, Na_2S solution (50 μM) was incubated without DIP or with DIP (25 μM) 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 × 50 mm, 1.7 μm) 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;



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

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 μL.

3. Results and discussion

The reaction of RSSH with $Fe^{III}Mb$: The reaction of a hydropersulfide donor (methoxycarbonyl penicillamine disulfide, MCPD [16,24]) with $Fe^{III}Mb$ 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·, followed by dimerization [16].

As shown in Fig. 1, when $Fe^{III}Mb$ is reacted with MCPD under aerobic conditions it is readily converted to $O_2Fe^{II}Mb$.

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 $Fe^{III}Mb$ with hydropersulfides results in the generation of $O_2Fe^{II}Mb$. These findings indicate that RSSH (from MCPD decomposition), is capable of reducing $Fe^{III}Mb$ to $Fe^{II}Mb$ with subsequent binding of O_2 to $Fe^{II}Mb$ (**Reactions 2**,**3**).

$$RSSH + Fe^{III}Mb \rightarrow RSS\cdot + Fe^{II}Mb + H^+ \quad (2)$$

$$Fe^{II}Mb + O_2 \rightarrow O_2Fe^{II}Mb \quad (3)$$

Importantly, it has already been shown that RSH species will not readily perform this chemistry (i.e. RSH will not readily reduce $Fe^{III}Mb$ under the conditions of these experiments) [16]. Also, the fate of the oxidized RSSH species, RSS·, 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 $O_2Fe^{II}Mb$: It is well established that $O_2Fe^{II}Mb$ is capable of oxidizing specific compounds that are reasonable one-electron reductants. For example, ascorbate (AH^-) reacts with $O_2Fe^{II}Mb$ to give ascorbyl radical anion ($A^{\cdot-}$), $Fe^{III}Mb$ and H_2O_2 (**Reaction 4**) [27].

$$O_2Fe^{II}Mb + AH^- + H^+ \rightarrow Fe^{III}Mb + A^{\cdot-} + H_2O_2 \quad (4)$$

Also, hydroxylamine (NH_2OH) and related species react with O_2Fe^{II}heme (such as $O_2Fe^{II}Mb$ and oxyhemoglobin, $O_2Fe^{II}Hb$), to give initially a fleeting HOO–Fe^{III} species (**Reaction 5**) [28]. The chemistry of NH_2OH 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**).

$$O_2Fe^{II}Mb + NH_2OH \rightarrow HOO\text{-}Fe^{III}Mb + NH_2O\cdot \quad (5)$$

$$HOO\text{-}Fe^{III}Mb + H^+ \rightarrow \text{Compound I } (O=Fe^{V}Mb \text{ or } O=Fe^{IV}Mb^{\cdot+}) + H_2O \quad (6)$$

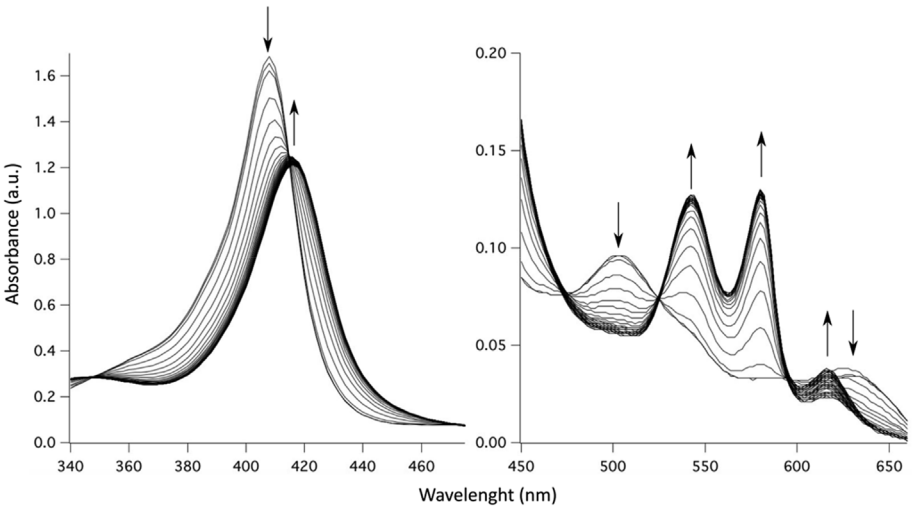


Fig. 1. Soret absorption (A) and Q-bands (B) changes for the reaction of $Fe^{III}Mb$ (9 μM) with 90 μM MCPD at pH 7.4, 25 °C. Spectra at 0 s were obtained for $Fe^{III}Mb$ in the absence of MCPD, and others were recorded during the reaction until 1 h after the initiation of the reaction. The directions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

Compound I + $2NH_2OH \rightarrow Fe^{III}Mb + 2NH_2O^{\cdot} + H_2O$ (7)

Ultimately, in the cases of ascorbate and NH_2OH this chemistry leads to the net reductive conversion of O_2Fe^{II} to Fe^{III} and concomitant generation of H_2O_2 or H_2O from the bound O_2. The crucial step in these reactions (**Reactions 4** and **5**) requires $O_2Fe^{II}Mb$ to act as a one-electron oxidant. This may be best understood by considering that $O_2Fe^{II}Mb$ possesses significant $O_2^{\cdot-}$-$Fe^{III}Mb$ character (i.e. [$O_2Fe^{II}Mb \longleftrightarrow O_2^{\cdot-}$-$Fe^{III}Mb$]) and that the bound superoxide ($O_2^{\cdot-}$)-like ligand is serving as the one-electron oxidant [29]. The chemical similarity between NH_2OH, ascorbate and RSSH (all good H-atom donors and reductants) [16], indicates that RSSH may react analogously with $O_2Fe^{II}Mb$ as reported for NH_2OH or ascorbate. If RSSH reacts analogously to ascorbate, H_2O_2 and $Fe^{III}Mb$ will be formed (**Reaction 8**).

RSSH + H^+ + $O_2Fe^{II}Mb \rightarrow RSS^{\cdot}$ + $Fe^{III}Mb$ + H_2O_2 (8)

If RSSH reacts analogously to NH_2OH, then a series of reactions shown below can be proposed (**Reactions 9**, **10** and **11**).

RSSH + $O_2Fe^{II}Mb \rightarrow RSS^{\cdot}$ + HOO-$Fe^{III}Mb$ (9)

HOO-$Fe^{III}Mb$ + $H^+ \rightarrow$ Compound 1 ($O=Fe^{V}Mb$ or $O=Fe^{IV}Mb^{\cdot+}$) + H_2O (10)

Compound 1 + 2 RSSH $\rightarrow Fe^{III}Mb + 2RSS^{\cdot}$ (RSSSSR) + H_2O (11)

Thus, the reaction of MCPD with $O_2Fe^{II}Mb$ was examined in greater detail. As shown in Fig. 2A the reaction of MCPD with $O_2Fe^{II}Mb$ 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 $O_2Fe^{II}Mb$. That is, if the reduction of $Fe^{III}Mb$ by RSSH to $Fe^{II}Mb$ and the binding of O_2 to $Fe^{II}Mb$ to give $O_2Fe^{II}Mb$ (**Reactions 2,3**) are both fast compared to the reaction of RSSH with $O_2Fe^{II}Mb$ (**Reactions 8** or **9**), it is possible that the initially formed $Fe^{III}Mb$ 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 O_2 by RSSH (to either H_2O_2 or H_2O).

2RSSH + $O_2 \rightarrow 2RSS^{\cdot}$ (RSSSSR) + H_2O_2 (12)

4RSSH + $O_2 \rightarrow 4RSS^{\cdot}$ (2RSSSSR) + $2H_2O$ (13)

Thus, if O_2 is consumed by the reaction of $O_2Fe^{II}Mb$ and RSSH, this would be consistent with an initial reaction between RSSH and $O_2Fe^{II}Mb$ and a catalytic process, either **Reaction 12** or **13**, that represents catalytic consumption of O_2 by RSSH. Indeed, as shown in Fig. 3A, the addition of MCPD to a solution of $O_2Fe^{II}Mb$ results in the consumption of 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 O_2, also measured using a Clarke-type electrode [16]. Thus, the 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/$O_2Fe^{II}Mb$ 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 $O_2Fe^{II}Mb$ to give $RSS^{\cdot}$ (which dimerizes to RSSSSR) and the RSSH that does not react with $O_2Fe^{II}Mb$ gives the expected trisulfide MCP–SSS–MCP species (m/z = 387.27). It is important to note that no reaction between $Fe^{III}Mb$ (or $O_2Fe^{II}Mb$) 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 $Fe^{III}Mb$ (data not shown).

These data are consistent with the idea that RSSH reacts with $O_2Fe^{II}Mb$ and that myoglobin is capable of catalyzing the O_2-dependent degradation of RSSH. Whether the reaction of RSSH with $O_2Fe^{II}Mb$ is akin to the reaction of NH_2OH or of ascorbate with $O_2Fe^{II}Mb$ is not 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 $O_2Fe^{II}Mb$ is considerably slower than the rate of RSSH reduction of $Fe^{III}Mb$ and the rate of $Fe^{II}Mb$ oxygenation. The rate constant for MCPD decomposition (to give an RSSH species) is approximately 0.3 min^{-1} at physiological pH [24] and the RSSH formed from MCPD decomposition can react with either $O_2Fe^{II}Mb$ or MCPD (Scheme 1). Under the conditions of these experiments these reactions are clearly competitive.

Reaction of RSSH with $O_2Fe^{II}Mb$ under O_2-depleted conditions: The

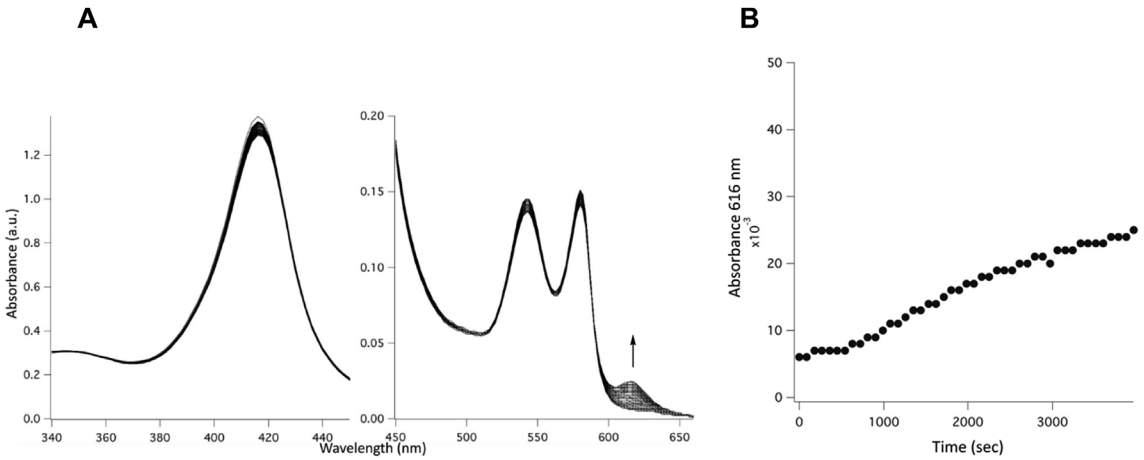


Fig. 2. A) Soret absorption and Q-bands changes for the reaction of $O_2Fe^{II}Mb$ (9 μM) with 90 μM MCPD at pH 7.4, 25 °C. Data collected for 1 h after the initiation of the reaction. **B**) Time-dependent increase in the absorbance at 616 nm for the reaction between MCPD and $O_2Fe^{II}Mb$.

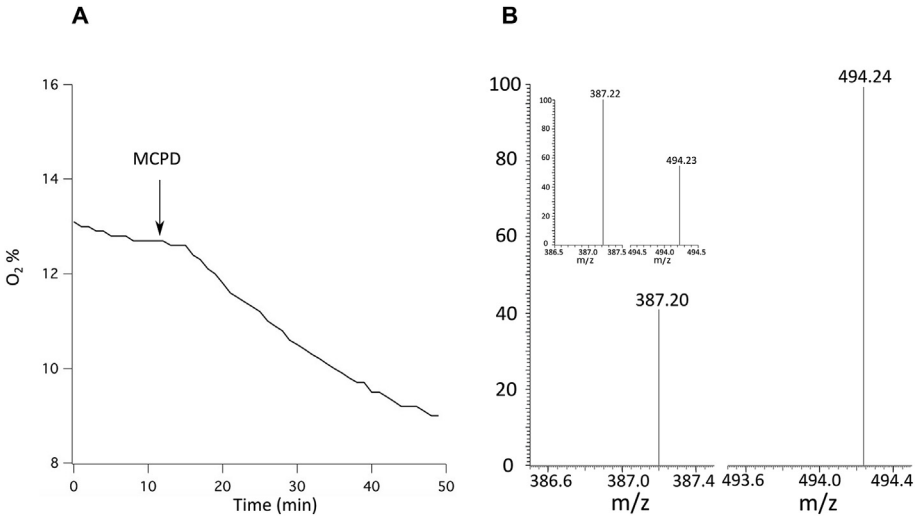


Fig. 3. A) O_2 consumption as monitored by a Clarke electrode. The arrow denotes the addition of MCPD. **B**). ESI-MS analysis of the sulfur products from the reaction of MCPD and $O_2Fe^{II}Mb$ (MCP–SSSS–MCP·NH_4^+, m/z = 494.28, MCP–SSS–MCP, m/z = 387.27 [16]). The inset show the ESI-MS analysis of the decomposition of MCPD alone in 50 mM ammonium phosphate buffer (pH 7.4) containing 50 μM DTPA.

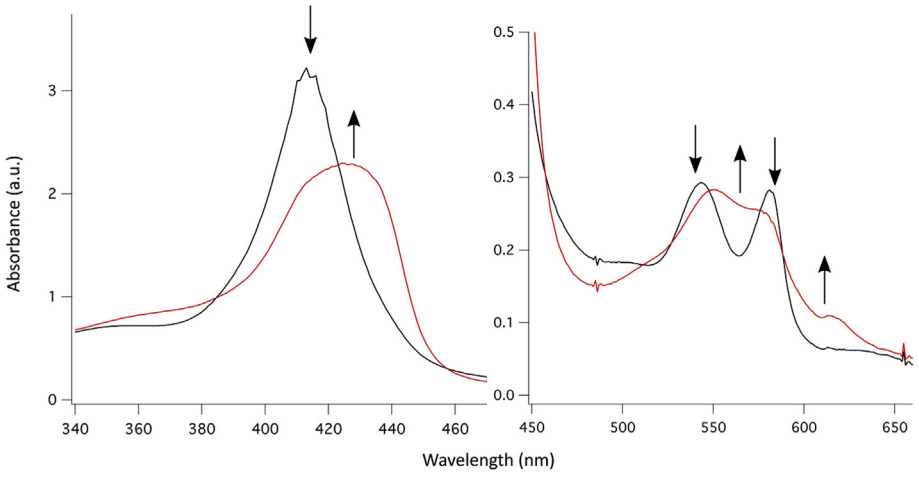


Fig. 4. Soret absorption (**A**) and Q-band (**B**) changes for the reaction of $O_2Fe^{II}Mb$ (19 μM) with 190 μM MCPD at pH 7.4, 25 °C under an N_2 atmosphere. Spectrum at 0 s (black line) was obtained for $O_2Fe^{II}Mb$ in the absence of MCPD. Spectrum in red was collected after 30 min after the initiation 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.)

results thus far indicate that RSSH is indeed capable of reacting with $O_2Fe^{II}Mb$ to give, initially $Fe^{III}Mb$ (**Reaction 8** or **9–11**) which is then quickly reduced by RSSH to $Fe^{II}Mb$ which then rapidly binds O_2 to give back $O_2Fe^{II}Mb$ (**Reactions 2** and **3**). It would be expected that this series of reactions carried out under O_2-depleted conditions could result in the detection of the $Fe^{II}Mb$ intermediate once O_2 is consumed (via **Reactions 12** or **13**). This experiment is not straightforward since rigorous degassing of a solution of $O_2Fe^{II}Mb$ can lead to simple dissociation of O_2 from $O_2Fe^{II}Mb$ to give directly $Fe^{II}Mb$. However, O_2 can be significantly depleted from solution and still allow $O_2Fe^{II}Mb$ to remain. Thus, an O_2-depleted solution of $O_2Fe^{II}Mb$ (degassed via N_2 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_2 depletion) shows the formation of the $Fe^{II}Mb$ intermediate (increase in absorbances at 434 nm and 560 nm [30]). Thus, reaction of $O_2Fe^{II}Mb$ with RSSH leads to the generation of $Fe^{III}Mb$ (via **Reaction 8** or **Reactions 9–11**), which is then reduced by RSSH to $Fe^{II}Mb$ (**Reaction 2**). With little or no O_2 in solution (consumed via **Reactions 12** or **13**), $Fe^{II}Mb$ 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 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 H_2S_2 was examined. As shown in Fig. 5B, the addition of Na_2S_2 to a buffered solution of $Fe^{III}Mb$ 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 H_2S_2 is oxidized with respect to H_2S. This is not necessarily surprising since other previous studies have shown that sulfheme formation from H_2S requires an oxidant to convert H_2S to a reactive oxidized form (such as HS·) [32]. One way to convert H_2S to polysulfur species (H_2S_n, n ≥ 2), without the intermediacy of HS·, is to utilize diamide-type molecules which can, in an O_2-independent fashion, convert thiols to disulfides [36]. If the thiol used is H_2S, the immediate product will be H_2S_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 H_2S was monitored (at 616 nm) in the presence and

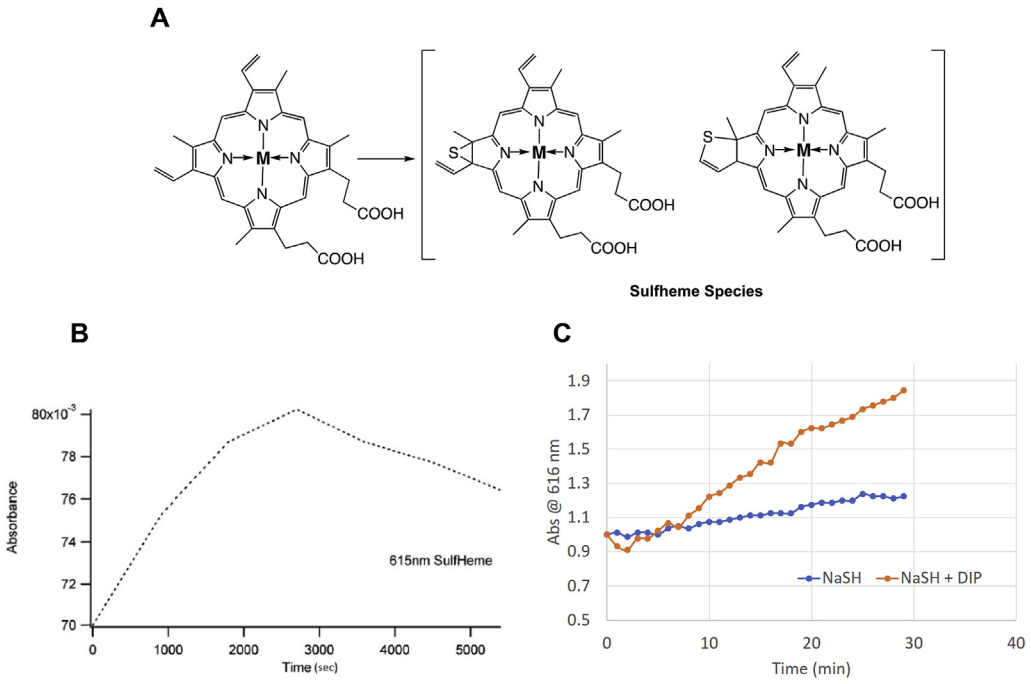


Fig. 5. A) Structures of sulfheme species generated from porphyrins. **B**) Sulfheme formation via the reaction of Na_2S_2 with $Fe^{III}Mb$ (absorbance at 616 nm). **C**) The reaction of H_2S with $Fe^{III}Mb$ in the presence and absence of DIP and the formation of sulfheme.

absence of the established diazenedicarboxylic acid (diamide) thiol oxidant diazenedicarboxyic acid bis-N′-methylpiperazinide (DIP) [25]. Fig. 5C shows the rate of sulfheme formation from the reaction of H_2S with $Fe^{III}Mb$ 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 H_2S_2, and possibly higher order polysulfides, as the sulfheme-forming species.

To verify further that H_2S_2 (or a higher order polysulfide derived from H_2S_2) can be responsible for sulfheme formation under these conditions, the DIP-mediated H_2S oxidation to higher order polysulfides was examined independently. HPE-IAM was used to trap polysulfides generated from the H_2S/DIP reaction (Fig. 6). H_2S alone will be trapped by HPE-IAM to give the thioether bis-S-HPE-AM whereas H_2S_2, if formed, will be trapped by HPE-IAM to give the dialkyl disulfide bis–SS–HPE-AM. As shown in Fig. 7A, H_2S incubation with HPE-IAM (20 equiv.) in pH 7.4 ammonium bicarbonate buffer produces bis-S-HPE-AM as a major product along with a minor bis–SS–HPE-AM impurity, suggesting that the commercial Na_2S salt has low level of Na_2S_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 H_2S into H_2S_2 (Fig. 6). Interestingly, a small amount of bis–SSS–HPE-AM is also generated in the H_2S/DIP reaction.

Of course, these results do not necessarily preclude HS· as the ultimate sulfheme-forming species since H_2S_2 can conceivably be an HS· precursor via simple homolysis or other possible mechanisms. However, it is also possible that polysulfur species such as H_2S_2 are directly responsible for reacting with porphyrins to generate sulfheme. It is worth remembering that the hydropersulfide donor MCPD also reacts with

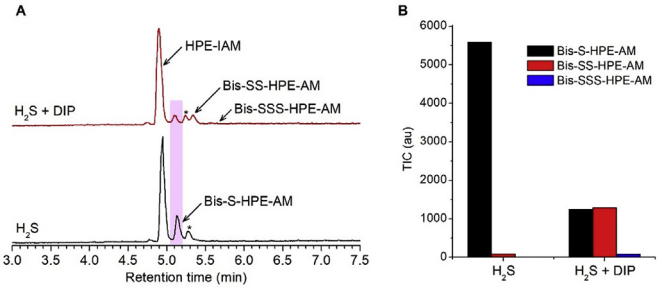


Fig. 7. DIP-mediated conversion of H_2S to polysulfides (e.g. H_2S_2). (**A**) Total ion chromatograms of H_2S (50 μM) incubated with and without DIP (25 μM) in pH 7.4 ammonium bicarbonate buffer (25 mM) containing DTPA (100 μM) at room temperature for 10 min, followed by trapping with HPE-IAM (1 mM) 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 H_2S, H_2S_2 and H_2S_3 (measured by the trapped HPE-AM species bis-S-HPE-AM, bis–SS–HPE-AM, and bis–SS–HPE-AM, respectively) from the H_2S, and H_2S + DIP reaction mixtures.

myoglobin to generate sulfheme (Figs. 1 and 2), indicating that hydroalkylpersulfides (i.e. RSSH) are capable of generating sulfhemes (not just inorganic polysulfurs species such as H_2S_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 RSS^- (R = 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

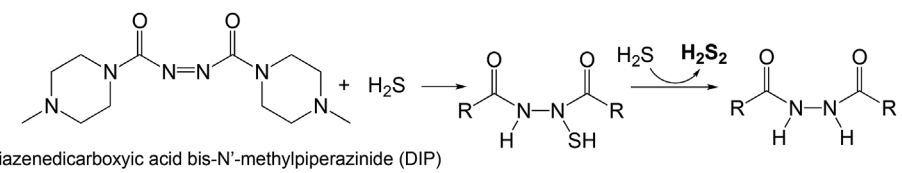


Fig. 6. The conversion of H_2S to H_2S_2 by DIP.

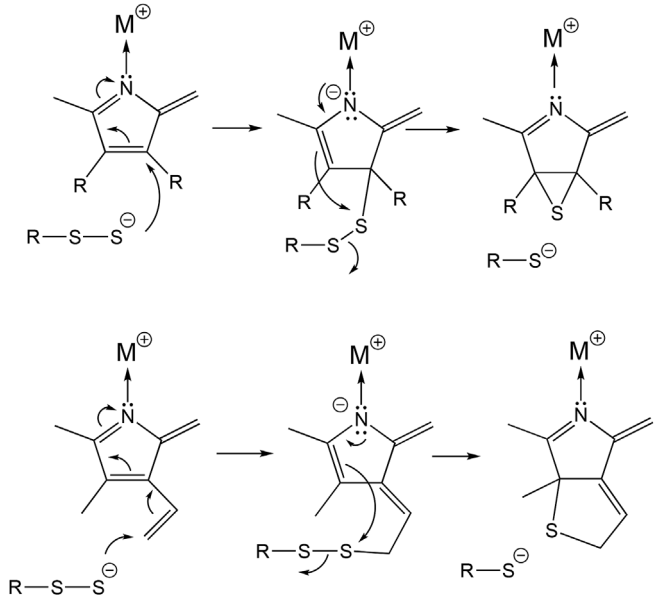


Fig. 8. Possible mechanisms for the generation of sulfheme species (episulfide – top reaction and thiochlorin – bottom reaction) via nucleophilic addition to a porphyrin pyrrole by a persulfide (R = alkyl or H).

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. H_2S_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 (HS^-) could attack the beta-carbon of a porphyrin pyrrole when myoglobin was in a high-valent ferryl oxo form ($OFe^{IV}Mb$) 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 H_2S-mediated sulfheme formation. In the mechanism depicted in Fig. 8, H_2S would need to be oxidized to H_2S_2 (or other polysulfide) prior to sulfheme formation. In other proposed mechanisms H_2S is converted to HS· [32] or H_2S 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. RSS· or HSS·) that adds to the pyrrole analogously to what has already been proposed for HS·. Again, discriminating between these and other mechanism is the topic of future work.

Sulfheme does not form with all hemeproteins 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 peroxo- or oxo-iron species when reacted with, for example, H_2O_2 (thus possessing an open coordination site) [32,34,38]. Consistent with this idea, the reaction of ferric cytochrome *c* (Fe^{III}Cyt c, a coordinately saturated, substitutionally inert heme protein that does not form peroxo- or oxo complexes) with H_2S_2 results in only reduction to Fe^{II}Cyt c with no sulfheme formation (data not shown). Interestingly, the mechanisms proposed herein (Fig. 8) would still be possible with Fe^{III}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 Fe^{III}Cyt c via reaction with H_2S_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 O_2Fe^{II}Mb, leading to a catalytic cycle that converts RSSH to higher order polysulfides at the expense of O_2. The rationale for indicating this is that in all mechanisms proposed to date, sulfheme formation occurs via an intermediary Fe^{III}Mb or ferryloxo (via **Reaction 10**) species. Since sulfheme continuously forms in the reaction between O_2Fe^{II}Mb and RSSH (Fig. 2A), this likely indicates that the Fe^{III}Mb or ferryloxo species is continuously generated during the reaction, consistent with the idea of the existence of **Reaction 8** or **Reactions 9–11** (i.e. O_2Fe^{II}Mb reacting with RSSH), thus providing further evidence for a reaction between RSSH and O_2Fe^{II}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 Fe^{III}Mb but react with O_2Fe^{II}Mb as well. These reactions allow the O_2-binding myoglobin to catalyze the 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 H_2S. Importantly, sulfheme formation starting with O_2Fe^{II}Mb and RSSH further supports the idea of a reaction between RSSH and O_2Fe^{II}Mb with an intermediary Fe^{III}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 O_2Fe^{II}Mb (**Reaction 8** or **Reaction 9**) is unknown. It is clear that this reaction is slower than the rate of reduction of Fe^{III}Mb by RSSH (**Reaction 2**) or the rate of association of O_2 and Fe^{II}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.

Declaration of competing interest

None.

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