Sulfide Oxidation by a Noncanonical Pathway in Red Blood Cells Generates Thiosulfate and Polysulfides

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Background: RBCs produce H$_2$S but, lacking mitochondria, are devoid of the canonical sulfide oxidation pathway.

Results: RBCs utilize methemoglobin to catalyze H$_2$S oxidation producing thiosulfate and polysulfide.

Conclusion: In the presence of NADPH and a reductase, ferric sulfide hemoglobin is converted to oxyhemoglobin, completing the sulfide oxidation cycle.

Significance: We describe a novel mechanism for H$_2$S oxidation that may be pertinent to other hemeproteins.

A cardioprotectant at low concentrations, H$_2$S is a toxin at high concentrations and inhibits cytochrome c oxidase. A conundrum in H$_2$S homeostasis is its fate in red blood cells (RBCs), which produce H$_2$S but lack the canonical mitochondrial sulfide oxidation pathway for its clearance. The sheer abundance of RBCs in circulation enhances the metabolic significance of their clearance strategy for H$_2$S, necessary to avoid systemic toxicity. In this study, we demonstrate that H$_2$S generation by RBCs is catalyzed by mercaptopyruvate sulfurtransferase. Furthermore, we have discovered the locus of sulfide oxidation in RBCs and describe a new role for an old protein, hemoglobin, which in the ferric or methemoglobin state binds H$_2$S and oxidizes it to a mixture of thiosulfate and hydropolysulfides. Our study reveals a previously undescribed route for the biogenesis of polypeptides, which are increasingly considered important for H$_2$S-based signaling, but their origin in mammalian cells is unknown. An NADPH/flavoprotein oxidoreductase system restores polysulfide-carrying hemoglobin derivatives to ferrous hemoglobin, thus completing the methemoglobin-dependent sulfide oxidation cycle. Methemoglobin-dependent sulfide oxidation in mammals is complex and has similarities to chemistry reported for the dissolution of iron oxides in sulfidic waters and during biodegradation of metal sulfides. The catalytic oxidation of H$_2$S by hemoglobin explains how RBCs maintain low steady-state H$_2$S levels in circulation, and suggests that additional hemeproteins might be involved in sulfide homeostasis in other tissues.

Biotic sulfide oxidation is an important limb of the global sulfur cycle and may have influenced early symbiotic relationships in the evolution of the eukaryotic lineage (1). However, the bacterially derived sulfide oxidation pathway that resides in mitochondria is missing in RBCs, which as producers of H$_2$S must rely on an alternative strategy for its clearance. This problem is magnified by the sheer abundance of RBCs in circulation, which in the absence of an H$_2$S clearance mechanism, would lead to certain death due to the buildup of toxic levels of H$_2$S and its facile permeation across lipid bilayers (2, 3). As a signaling molecule, H$_2$S elicits profound physiological effects ranging from cardioprotection to being an anti-inflammatory mediator and a neuromodulator (4, 5). Three enzymes, cystathionine β-synthase (CBS), γ-cystathionase (CSE), and mercaptopyruvate sulfurtransferase (MST) are primarily responsible for cellular H$_2$S biogenesis, and their relative contributions vary in a tissue-specific manner (6, 7). Sulfide homeostasis is contributed in part by its proficient removal via enzymes in the mitochondrial sulfide oxidation pathway, which convert it to thiosulfate and sulfate, the primary forms of sulfur excreted from the body. Circulating levels of H$_2$S in plasma are low (~150 nm) (8) and are expected to be critically influenced by sulfide metabolism in red blood cells (RBCs), which number ~5 billion/milliliter of blood in a normal adult. In principle, RBCs might influence blood vessel tension by regulating local H$_2$S levels, which in turn, regulate their own microcirculation. However, little is known about H$_2$S homeostasis in RBCs, i.e. which enzymes contribute to its production and how H$_2$S is removed. Although MST activity in RBCs was determined with its true substrate, 3-mercaptopuruvate (9), the activity of CSE was measured with the substrate analog β-chloroalanine (10), which also reacts with other pyridoxal phosphate-containing enzymes, making the reported CSE activity in RBCs uncertain.

Because the toxicity of H$_2$S results from its inhibition of cytochrome c oxidase (11), organisms have developed strategies for averting a buildup of H$_2$S. An obvious candidate for sulfide oxidation in RBCs is hemoglobin (Hb) present at an intracellular concentration of ~5 μM. Several lines of evidence support the feasibility of Hb-dependent sulfide oxidation, which could be important, in addition to the canonical mitochondrial sulfide oxidation pathway (12). The reaction of sulfide with Hb was first reported in 1863 under conditions that led to the formation of sulfhemoglobin, with a characteristic green color (13). This reaction is observed with oxyferrous Hb, results in covalent

$\text{Hb} + \text{H}_2\text{S} \rightarrow \text{Hb-SH} + \text{O}_{2}$

2 The abbreviations used are: CBS, cystathionine β-synthase; H$_2$S, hydrogen sulfide; CSE, γ-cystathionase; MetHb, methemoglobin; MSR, methionine synthase reductase; MST, mercaptopyruvate sulfurtransferase; oxy-Hb, oxyhemoglobin.
modification of the tetrahydropyrole (14), and is not pertinent to catalytic sulfide oxidation. Stoichiometric binding of H₂S to ferric or methemoglobin (MetHb) to give sulfide-MetHb was first reported in 1933 (15). MetHb is formed by auto-oxidation of ferrous Hb and represents ~1–3% of total Hb. Methemoglobinemia protects against sulfide toxicity and a ratio of ~3 mol of H₂S inactivated per mol of MetHb heme has been reported, suggesting turnover rather than simply sulfide binding by heme (16). Free hemin converts sulfide to thiosulfate under aerobic conditions in an ~50% yield (17). Some invertebrates living in sulfide-rich environments use Hb as a sulfide carrier to deliver H₂S to symbiotic bacteria that use it as an energy source (18).

Spectroscopic and structural studies on Hb suggest that several factors control the reactivity of H₂S with Hb including the orientation of side chains and the dielectric constant in the vicinity of the distal ligand (19). Collectively, these observations provide evidence for heme-dependent sulfide chemistry, albeit a role for Hb in catalytic sulfide oxidation in higher organisms has not been considered. In this study, we report that sulfide homeostasis in RBCs involves its generation by MST and its oxidative removal by MetHb, resulting in the production of thiosulfate and hydropolysulfides (hereafter referred to as polysulfides).

**EXPERIMENTAL PROCEDURES**

**Materials**—Human RBCs, obtained from normal healthy volunteers, were kindly provided by Dr. David A. Fox (University of Michigan). Human MetHb and sickle cell HbS (ferrous form), Na₂S nonahydrate, 2,3-diphosphoglycerate, inositol hexaphosphate, iodoacetamide, cysteine and d,L-homocysteine, and Clostridium kluyveri diaphorase were purchased from Sigma. The following reagents were obtained from the indicated vendors: monobromobimane (FluoroPure grade, Molecular Probes, Grand Island, NY), 3-mercaptopyruvate (Research Organics, Cleveland, OH), and DTT (Gold Biotechnology, St. Louis, MO). Recombinant human CBS (20, 21), CSE (22, 23), MST (23), and methionine synthase reductase (MSR) (24) were purified as described previously.

**Production of RBC Samples**—RBC were washed three times by resuspension in 3 volumes of PBS containing 5 mM glucose and centrifuged for 10 min at 2000 × g at 4 °C. The packed cells were diluted in 9 volumes of PBS containing 5 mM glucose and immediately used in experiments. For lysate preparation, RBCs were washed with and then resuspended in an equal volume of PBS. The suspension was mixed with 4 volumes of distilled water to give a final 1:9 dilution of RBC lysate. The lysate was kept frozen at −80 °C until use. To prepare the cytoplasmic and membrane fractions of RBCs, 1 ml of packed cells was mixed with 4 ml of distilled water and placed on ice for 2 min. Then, 4 ml of distilled water was added, and the mixture was centrifuged at 3200 × g for 10 min at 4 °C. The supernatant (8 ml) was carefully aspirated, mixed with 0.8 ml of 1 M HEPES, pH 7.4, and centrifuged again to remove remaining RBC ghosts and membranes. The supernatant obtained after the second centrifugation represents the cytoplasmic fraction of RBC diluted 1:9 in 100 mM HEPES buffer, pH 7.4. The sediment of RBC ghosts obtained after centrifugation of RBC lysates (1 ml) was diluted with 8 ml of distilled water and centrifuged at 3,200 × g for 10 min at 4 °C. The supernatant was removed, distilled water was added to a total volume of 9 ml, and 1 ml of 1 M HEPES, pH 7.4, was added. This mixture represents RBC membrane fraction corresponding to a 1:9 dilution of RBC with 100 mM HEPES buffer, pH 7.4. Cytoplasmic and membrane fractions of RBC were used immediately or were stored at −80 °C until further use.

**Production of RBCs Containing MetHb**—A suspension of RBCs (10% (v/v in PBS containing 5 mM glucose) was incubated with NaNO₂ (20 mM for 60 min or 50 mM for 10 min) at 25 °C. Then, RBC suspension was diluted by the addition of 20 volumes of PBS followed by centrifugation (10 min at 2000 × g). The washing procedure was repeated three times resulting in an NaNO₂ concentration of <6 μM. The RBCs were resuspended in PBS containing 5 mM glucose (to give 10% v/v) and used for anaerobic H₂S clearance analysis at 25 °C. A solution of NaNO₂ (5 μM) in PBS was used as a blank.

**H₂S Production by RBC Lysate**—The rate of H₂S production by RBC lysate was monitored as described previously (25). Briefly, 100 μl of RBC lysate was incubated with the chosen substrate in a total volume of 0.5 ml of 100 mM HEPES, pH 7.4, in a 20-ml polypropylene syringe barrel. The syringe was sealed with a plunger. The syringe headspace was flushed five times with N₂ using an attached three-way stopcock and then filled with N₂ to a total volume (liquid + gas) of 20 ml. The syringe then was incubated at 37 °C with rocking. At the desired time points, 200-μl aliquots were collected from the gas phase and injected into a gas chromatograph equipped with a model 355 sulfur chemiluminescence detector (Agilent) for H₂S analysis as described (25). The reaction mixture contained 10 mM cysteine for CSE activity (26), 10 mM cysteine + 10 mM d,l-homocysteine for CBS activity (26), and 0.5 mM 3-mercaptopruvate + 20 mM DTT to monitor MST activity (23).

**Western Blot Analysis of H₂S-producing Enzymes in RBCs**—Expression of CBS, CSE, and MST in RBC was assessed by Western blot analysis using custom-made primary antibodies as described previously (26).

**Sulfide Clearance Assay**—The kinetics of H₂S clearance was monitored as described previously (25). Briefly, 0.5 ml of the sample (RBC lysate, RBC cytoplasmic or membrane fraction, or MetHb solution) was placed inside a 20-ml polypropylene syringe barrel, and the syringe was sealed with a plunger. For anaerobic assays, the syringe headspace was flushed five times with N₂ and then filled with N₂ to a total volume (liquid + gas) of 10 ml. Then, 10 ml of 40 ppm H₂S in N₂ was added to the syringe to give a final volume of 20 ml. For aerobic assays, the syringe headspace was filled with air to a total volume (liquid + gas) of 10 ml, and 10 ml of 40 ppm H₂S in N₂ was added, using a three-way stopcock, to give a final volume of 20 ml. The syringes were incubated at 25 °C with rocking, and 200-μl aliquots were collected from the gas phase and injected into the gas chromatograph at the desired times. The concentration of MetHb in 100 mM HEPES buffer, pH 7.4, was determined spectrophotometrically (ε₁₄₅ nm = 179 M⁻¹ cm⁻¹).

**Stopped-flow Spectroscopy**—The kinetics of interaction between H₂S and MetHb under anaerobic conditions were performed using an Applied Photophysics stopped-flow spectrophotometer (SX.MV18) placed inside an anaerobic chamber (Vacuum Atmospheres Co., Hawthorne, CA) filled with N₂.
$\text{H}_2\text{~S Oxidation by Hemoglobin}$

containing $\sim 0.2$—$0.5$ ppm O$_2$. A solution of MetHb (2 \text{ \mu M}) before mixing in 100 \text{ mM} HEPES buffer, pH 7.4) was mixed rapidly with various concentrations of Na$_2$S in the same buffer. The pH dependence of the reaction was monitored under aerobic conditions using a Hi-Tech Scientific stopped-flow spectrophotometer (SF-61DX). MetHb (2.5 \text{ \mu M}), prepared in 100 mM buffers with pH values ranging from 5.5 to 9.0, was rapidly mixed with 2 mM Na$_2$S solutions prepared in the same buffers. Borate, Tris, HEPES, and citrate buffers were used for pH 9.0, 8.0, 7.4, and 6.0, respectively. Citrate was also used for pH 5.5. The kinetics of absorbance changes at 405 nm was monitored at 25 °C. The data were fitted to a single exponential function.

**Product Analysis**—MetHb (25 \text{ \mu M} in 100 \text{ mM} HEPES buffer, pH 7.4) was incubated at 25 °C under aerobic (air) or strictly anaerobic (N$_2$) conditions in an anaerobic chamber (Vacuum Atmospheres Co.) in closed sample tubes with liquid nitrogen phase at 2.1. Samples lacking MetHb were used as controls. Na$_2$S in 100 \text{ mM} HEPES buffer, pH 7.4, was added to give the desired concentration ($\sim 2000$—$1600$ \text{ \mu M}), and samples were removed at the desired time points for HPLC or cyanolysis analyses as described below. Approximately 5—7% of the sample comprised thiosulfate and sulfite. At the end of the incubation, the low molecular weight fraction of the reaction mixture was separated using 0.5-ml centrifugal filters with a 10-kDa cutoff (Milipore), centrifuged for 6 min at 10,000 × g, and the filtrate was collected and used for cyanolysis analysis.

For HPLC analysis of products, the samples (45 \mu l) were mixed with 2.5 \mu l of a 1 \text{ M} Tris and 2.5 \mu l of 60 \text{ mM} monobromobimane in dimethyl sulfoxide and incubated for 10 min at 25 °C. Then, 10 \mu l of metaphosphoric acid solution (16.8 mg/ml) was added, the sample was stirred and centrifuged (3 min at 13,000 × g), and the supernatant was separated and frozen until use. The samples were protected from light during the derivatization procedure. Samples were separated using a Zorbax Eclipse XDB-C18 column (5 \text{ \mu M}, 4.6 × 150 mm, Agilent) with the following gradient and a flow rate of 1 ml min$^{-1}$. Solution A contained 100 \text{ mM} ammonium acetate, pH 4.75, and 10% methanol and Solution B contained 100 \text{ mM} ammonium acetate, pH 4.75, and 90% methanol. The percentage of B increased as follows in the gradient: 0—10 min, linear 0 to 20%; 10—15 min, linear 20 to 50%; 15—20 min, isocratic 50%; 20—22 min, 50 to 100%; 22—27 min, isocratic 100%; 27—29 min, linear 100% to 0%; 29—35 min, isocratic 0%. Peaks were detected using excitation at 390 nm and fluorescence emission at 490 nm. The column was calibrated with known concentrations of sodium sulfide, sodium sulfite, and sodium thiosulfate.

Hot and cold cyanolysis was used to detect other catenated sulfur products (27). Briefly, for cold cyanolysis 0.5 ml of sample was mixed with 0.25 ml of solution containing 62.5 \text{ mM} potassium cyanide and 125 \text{ mM} ammonium hydroxide, and the mixture was incubated for 45 min at 25 °C. Then, 0.75 ml of Goldstein’s reagent was added, the mixture was vortexed and centrifuged for 3 min at 13,000 × g, and absorbance of the supernatant was measured at 460 nm. Goldstein’s solution is prepared by dissolving 1.25 g of Fe(NO$_3$)$_3$·9H$_2$O in 12.5 ml water to which 13.1 ml of concentrated HNO$_3$ and water to a final volume of 50 ml are added. A calibration curve was prepared using sodium thiocyanate of known concentrations.

For hot cyanolysis, the sample (0.5 ml) was mixed with 0.25 ml of a solution containing 200 \text{ mM} potassium cyanide and 1 \text{ M} ammonium hydroxide, and the mixture was incubated for 40 min at 99 °C. Then, the samples were centrifuged for 3 min at 13,000 × g, and the supernatant was mixed with an equal volume of ferric nitrate reagent (made by dissolving 5 g of Fe(NO$_3$)$_3$·9H$_2$O in 15 ml water to which 10 ml of concentrated HNO$_3$ and water to a final volume of 50 ml are added). The absorbance was measured at 460 nm. Solutions of sodium thiosulfate processed in the same way as samples were used for calibration. The sensitivity of the hot and cold cyanolysis method is $\sim 10$ \text{ \mu M}, and the assay was linear from 0.01 to 1 \text{ mM}.

To probe the role, if any, of free cysteines in MetHb for sulfide oxidation, they were blocked with iodoacetamide by mixing MetHb (250 \text{ \mu M}) in 100 \text{ mM} Tris buffer, pH 8.0, and 10 \text{ mM} iodoacetamide (prepared in the same buffer) followed by incubation for 1 h at 25 °C in the dark. Then, the sample was applied to a PD-10 desalting column (GE Healthcare), and MetHb was eluted with 100 \text{ mM} HEPES buffer, pH 7.4. Iodoacetamide-treated MetHb was diluted to a concentration of 25 \text{ \mu M} with 100 \text{ mM} HEPES buffer, pH 7.4, and incubated aerobically for 1 h with 800 \mu M Na$_2$S at 25 °C. Then, the level of cyanolyzable sulfur was measured using the cold cyanolysis method.

**O$_2$ Consumption Assay**—O$_2$ consumption was measured using an O$_2$ electrode. For this assay, MetHb (25 \text{ \mu M}) in 100 \text{ mM} HEPES buffer, pH 7.4, at 25 °C was placed in a 1.5-ml Gilson-type chamber equipped with a Clark-type oxygen electrode and a magnetic stirrer. After stabilization of the background signal, a solution of Na$_2$S in the same buffer was injected into the chamber, and O$_2$ consumption was recorded using a chart recorder.

**EPR Spectroscopy**—Samples for EPR spectroscopy were prepared under both aerobic and strictly anaerobic conditions (in an anaerobic chamber) at 25 °C. Na$_2$S was added to a 50 \text{ \mu M} solution of MetHb in 100 \text{ mM} HEPES buffer, pH 7.4, to give a final sulfide concentration of 1.2 \text{ mM}. The reaction mixture (400 \mu l) was placed in an EPR tube and sealed with a rubber stopper. After 5 min, the sample was frozen in liquid N$_2$. The EPR spectra were recorded at 14 K on a Bruker ESP 300E spectrometer equipped with an Oxford ITC4 temperature controller using the following settings: 5.00 G modulation amplitude, 100 kHz modulation frequency, 3500 G sweep width (centered at 2500 G), 9.387 GHz microwave frequency, and 0.104-milliwatt microwave power. A 10 mm copper sulfate solution was used as a standard to determine the concentration of low-spin ferric ion in samples. For high spin ferricyanide, the sample was added to a 25 \text{ \mu M} solution of MetHb in 100 \text{ mM} HEPES buffer, pH 7.4, to a final concentration of 250 \text{ \mu M}. Sodium ferricyanide was added to a final concentration of 2 \text{ mM} to fully convert the sample to the MetHbS form as confirmed by a shift in the Soret peak from 415 to 405 nm and broadening of the $\alpha$ and $\beta$ peaks. Excess ferricyanide was removed by gel filtration using a Superdex 200 column, and the sample was then used for monitoring reactivity with sulfide as described above for normal MetHb.

**Mass Spectrometric Analysis**—Mass spectrometric analysis of low molecular weight fractions obtained after incubation of MetHb with sulfide was performed at a core facility (Depart
The samples were run on an Agilent 6520 Q-TOF LCMS system with a dual ESI ion source in the negative ion mode. A mobile phase containing 70% acetonitrile, 30% water and 0.1% formic acid was used with a flow rate was 0.4 ml min\(^{-1}\). The m/z range was 50 to m/z 3200, with a scan rate of 2 spectra per sec.

RESULTS

\(H_2S\) Turnover in Human RBCs—To assess \(H_2S\) production by RBCs, we examined the presence and activity of CBS, CSE, and MST. When human RBC lysates were exposed to the \(H_2S\)-generating substrates for CBS (cysteine + homocysteine) or CSE (cysteine) \((21, 22)\), \(H_2S\) production was not observed. In contrast, in the presence of the MST substrate 3-mercaptopropionate \(\text{3-MP in Equation 1, in which DTT}_{\text{red}}\) and \(\text{DTT}_{\text{ox}}\) are reduced and oxidized DTT, respectively), a robust rate of \(H_2S\) production \((10.8 \pm 1.5 \text{ mmol h}^{-1}\text{liter cells}^{-1})\) at 37 °C was observed.

\[
\text{3-MP + DTT}_{\text{red}} \rightarrow \text{pyruvate + DTT}_{\text{ox}} + H_2S \quad (\text{Eq. 1})
\]

Western blot analysis of RBC lysates revealed the presence of MST (Fig. 1A), which co-migrated with recombinant human MST. Although the upper band seen in RBC lysates is also observed in extracts from human cell lines \((e.g.\ \text{HepG}2\ and\ \text{HEK}293,\ not\ shown)\), the lower band is only seen in RBC lysate, and its identity is currently not known. In contrast, CBS and CSE were not detected in RBC lysates (Fig. 1A). These results establish the capacity of human RBCs to produce \(H_2S\) via MST and raise the obvious question as to how it is cleared, which was examined next.

The addition of \(H_2S\) to a 10% (v/v) suspension of human RBCs resulted in its rapid disappearance under aerobic and anaerobic conditions (Fig. 1B) with a \(t_{1/2}\) of \(\approx 14\) s (normalizing for undiluted cell mass) and a rate constant of \(\approx 3.0\) min\(^{-1}\). Fractionation of RBC lysates into cytosolic and membrane components established that the cytosolic fraction houses the \(H_2S\) clearance activity. Treatment of RBCs with sodium nitrite increased, whereas treatment with DTT decreased the \(H_2S\) clearance rate (Fig. 2). Collectively, these results suggested a role for MetHb in \(H_2S\) removal by RBCs. To test this hypothesis, a solution of 0.5 mg ml\(^{-1}\) MetHb (to simulate its presence at 1.6% of total Hb in a 10% RBC suspension) was tested and found to support an almost identical rate of \(H_2S\) clearance as that of RBCs in suspension (Fig. 1C). In this experiment, the amount of sulfide \((17.9\ nmol)\) and ferric heme \((16\ nmol)\) was approximately equivalent. Hence, the observed clearance of \(H_2S\) in these experiments and by RBCs in suspension is likely to represent its stoichiometric binding to MetHb.

Interaction of \(H_2S\) with MetHb—The binding of \(H_2S\) with MetHb resulted in a red shift in the Soret band from 405 to 423 nm and the appearance of absorption bands at 577 and 541 nm, respectively (Fig. 3, A and B). Identical spectral changes were observed under aerobic and anaerobic conditions. The initially formed 423 nm absorbing species was tentatively identified as ferric sulfide Hb. Its formation was dependent on sulfide concentration, yielding a \(k_{\text{on}}\) of 14 ± 2 μM \((\text{Fig. 3C, inset})\). The dependence of \(k_{\text{obs}}\) on the total sulfide concentration \((\text{Fig. 3C, inset})\), yielded a \(k_{\text{off}}\) for the association of sulfide with MetHb of \(3.2 \times 10^4 \pm 100\ \text{m}^{-1}\text{s}^{-1}\), \(k_{\text{off}}\) of 0.053 ± 0.008 s\(^{-1}\), and a \(K_p\) of 17 ± 2 μM (pH 7.4 at 37 °C). The rate of formation of the 423 nm species was inversely proportional to pH, decreasing with an
increasing pH between 5.5 and 9.0 (Fig. 3D). The pH dependence suggests that \( \text{H}_2\text{S} \) rather than the sulfide anion (\( \text{HS}^- \)) initially interacts with MetHb. The \( K_d \) and \( k_{\text{on}} \) values represent the upper limits, as the \( \text{H}_2\text{S} \) concentration at pH 7.4 is \( \sim 20\% \) of the total sulfide concentration.

The 423 nm spectrum was stable for \( \sim 1 \) h but changed slowly over 48 h during which the Soret band shifted to 407 nm and the total sulfide concentration.

The 423 nm spectrum was stable for \( \sim 1 \) h but changed slowly over 48 h during which the Soret band shifted to 407 nm and the total sulfide concentration.

\( g = 5.83 \) (Fig. 7A). Treatment with excess \( \text{H}_2\text{S} \) under aerobic conditions results in the appearance of a low-spin rhombic signal with \( g \) values of 2.51, 2.25, and 1.86 \( \mu \)s, representing 65 \( \mu \)s spin or \( \sim 33\% \) of the initial heme concentration (Fig. 7B). The closely spaced \( g \) values are typical of thiolate-ligated

gest that the flavoprotein, methemoglobin reductase can also convert the 423 nm Hb species to oxy-Hb. Well known allosteric modulators of ferrous Hb that influence oxygen affinity, e.g. 2,3-diphosphoglycerate and inositol hexaphosphate, did not affect the \( \text{H}_2\text{S} \) binding or clearance kinetics by MetHb (Fig. 4, A–C). However, a small decrease in the absorbance change at 405 nm was seen in the presence of inositol hexaphosphate (Fig. 4B), the origin of which is not known. Our results are consistent with binding of \( \text{H}_2\text{S} \) to MetHb versus oxy-Hb. Similar kinetics and spectral changes were observed when the interaction of \( \text{H}_2\text{S} \) with sickle cell (Fig. 4, D and E) or normal MetHb was monitored.

Products of \( \text{H}_2\text{S} \) Oxidation by MetHb—The addition of an excess of \( \text{H}_2\text{S} \) to MetHb heme results in all the \( \text{H}_2\text{S} \) being consumed in 30–60 min under aerobic conditions (Fig. 5A). Under these conditions, thiosulfate accumulates with similar kinetics (Fig. 5B). The presence of thiosulfate was established by HPLC analysis of the monobromobimane derivative (Fig. 6A), by hot cyanolysis of the filtrate obtained using a 10-kDa cutoff filtration device (not shown) and by mass spectrometric analyses (Fig. 6, B and C). The amount of thiosulfate recovered corresponded to \( \sim 35\% \) of the \( \text{H}_2\text{S} \) present initially (800 \( \mu \)s). An additional 30–40% of the sulfur, recovered as cyanolyzable sulfur that was tightly bound to Hb, could be detected by cold cyanolysis of the entire reaction mixture (Fig. 5C). The amount of cyanolyzable sulfur varied with the \( \text{H}_2\text{S} \) to heme ratio and was not observed in control samples lacking MetHb or \( \text{Na}_2\text{S} \). Cold cyanolysis of the filtrate obtained using a 10-kDa cutoff filter demonstrated the absence of free HSSH and polysulfides in the reaction mixture. To determine whether the polysulfide (i.e., cyanolyzable sulfur) was protein-bound, mass spectrometric analysis of Hb from the reaction mixture was performed. However, evidence for polysulfide (i.e., Hb-\((\text{S})_n\text{-SH}\))-modified Hb was not obtained. Furthermore, pretreatment of MetHb with iodoacetamide to block reactive and accessible cysteines did not prevent the formation of polysulfides, consistent with their formation on the heme rather than on a cysteine residue on the protein. Approximately 12% of the sulfur remained bound to the heme iron, because cold cyanolysis releases up to the penultimate sulfur atom in a chain. Thus, the thiosulfate and iron-bound polysulfide accounted for \( \sim 77–87\% \) of the sulfur introduced into the reaction mixture as \( \text{H}_2\text{S} \). Consumption of \( \text{H}_2\text{S} \) was accompanied by \( \text{O}_2 \) consumption, which was detected only after the \( \text{H}_2\text{S} \) heme stoichiometry of the sample exceeded 2:1 (Fig. 5D).

Under anaerobic conditions, the decrease in free \( \text{H}_2\text{S} \) concentration corresponds to the heme concentration, consistent with \( \text{H}_2\text{S} \) binding to the iron but not being oxidized further (Fig. 5E). A small accumulation of thiosulfate was observed under anaerobic conditions, which was accompanied by an equivalent decrease in the concentration of sulfite present as a contaminant in \( \text{Na}_2\text{S} \) (Fig. 5F).

EPR Characterization of \( \text{H}_2\text{S} \) Oxidation by MetHb—The EPR spectrum of MetHb shows the presence of high-spin ferric iron with \( g = 5.83 \) (Fig. 7A). Treatment with excess \( \text{H}_2\text{S} \) under aerobic conditions results in the appearance of a low-spin rhombic signal with \( g \) values of 2.51, 2.25, and 1.86 \( \mu \)s, representing 65 \( \mu \)s spin or \( \sim 33\% \) of the initial heme concentration (Fig. 7B). The closely spaced \( g \) values are typical of thiolate-ligated
hemes, and the EPR spectrum is very similar to those reported for cytochrome c variants engineered to have a cysteine ligand (28). Treatment with excess H₂S under anaerobic conditions also resulted in the disappearance of the high-spin signal and the appearance of the low-spin signal representing 102 μM spin concentration or 51% of the initial heme iron concentration (Fig. 7C). These results are consistent with the conversion of high-spin ferric MetHb to low-spin ferric sulfide MetHb followed by further conversion to diamagnetic species.

**DISCUSSION**

Curiously, despite the large volume of blood (~5 liters in an adult human) and the sheer abundance of RBCs with their...
potential to influence systemic H2S metabolism, the mechanism of sulfide homeostasis in the circulatory compartment is unknown. The problem is particularly intriguing in light of the capacity of RBCs for generating H2S and the absence of the canonical mitochondrial sulfide oxidation pathway in these cells. In this study, we have addressed this metabolic conundrum by establishing that RBCs produce H2S via the action of MST and clear sulfide via MetHb-catalyzed oxidation of H2S to thiosulfate and polysulfides (Fig. 8).

We propose that the binding of sulfide to MetHb results in the formation of H2S-FeIII Hb (in the presence of NADPH and flavin oxidoreductases, the initially formed ferric sulfide hemoglobin is converted to oxy-Hb [1] (Fig. 8)). As observed with the Lucina pectinata ferric Hb, the rate constant for binding of H2S to human MetHb increases with decreasing pH (Fig. 3D), suggesting that H2S rather than the more abundant HS- initially binds to heme (18). We propose that [1] is in equilibrium with HS-FeII Hb [2]. The latter is also proposed as an intermediate for the sulfide-carrying Hb from L. pectinata (29), and its feasibility is supported by model studies showing that HS- ligation to ferric porphyrinate generates the corresponding FeIII derivative (30). In principle, the ferrous heme-bound HS radical could react with O2 to form SO2 (rate constant in solution is 7.5 x 10^9 M^-1 s^-1 at pH 7.0 and 37 °C (31)). However, this reaction appears to be prevented in the heme pocket, because O2 consumption is not seen until the H2S:heme stoichiometry is 2:1 (Fig. 5D). Alternatively, the reaction of the second mole of sulfide with [2] might be more rapid in the heme pocket than its reaction with O2. However, in solution, the reaction of the sulfhydryl radical anion (S-). with sulfide anion (HS-) to generate the hydrodisulfide radical anion (HSS-2) is also fast, with a k on of 5.4 x 10^9 M^-1 s^-1 at pH 7.0 and 37 °C (32). Hence, we propose that a second mol of sulfide reacts with the HS-FeII Hb radical [2] to generate the HSS-FeII radical anion species [3]. Oxygenation of the ferrous hydrodisulfide radical anion [3] would generate the sulfur dioxide radical derivative [4], which could be oxidized to give [5] and O2. Oxygenation of the outer sulfur atom of [3] would explain the need for the second equivalent of sulfide prior to oxygen consumption. As noted previously, the rate constant for oxygenation of the sulfide radical anion at pH 7.0 and 37 °C is 7.5 x 10^9 M^-1 s^-1 (31). Hydrolysis of [5] yields the thiosulfate [6], which following oxidation would yield the ferric product complex [7]. Dissociation of thiosulfate from [7] regenerates MetHb.

Ferrous polysulfides [8] can be formed via recombination of the radical anion intermediate [3] with a sulfhydryl radical gen-
generated via reaction of sulfide with $\text{O}_2$. The rate constant for this oxidation reaction is $200 \text{M}^{-1}\text{s}^{-1}$ at pH 7.0 and 37 °C (32). The $\text{H}_2\text{O}_2$ generated in this reaction could potentially react with sulfide generating sulfenic acid ($k_{\text{on}} = 0.73 \text{M}^{-1}\text{s}^{-1}$ in solution (32)), which could react with [3] (estimated $k_{\text{on}} = 10^5 \text{M}^{-1}\text{s}^{-1}$ (32)) leading to chain elongation. The stoichiometry of the cyanolyzable sulfur recovered under $\text{H}_2\text{S}$ turnover conditions indicates that the chain length increases in proportion to the $\text{H}_2\text{S}$ concentration and up to six sulfur atoms are bound to the heme iron when heme:$\text{H}_2\text{S}$ is 1:16. In studies on protection from sulfide toxicity in mice with nitrite-induced methemoglobinemia, a ratio of 3 mol of $\text{H}_2\text{S}$ were found to be inactivated per mol of MetHb heme and interpreted as evidence for $\text{H}_2\text{S}$ binding to additional non-heme sites on MetHb (16). In light of our results, the observed stoichiometry can be interpreted as evidence for a mixture of thiosulfate and polysulfide generated per mol of $\text{H}_2\text{S}$ cleared in sulfide-treated mice.

The proposed MetHb-catalyzed oxidation chemistry has precedence in the bacterial leaching of metal sulfides by catalytic ferric ion (33). The primary sulfur product formed during leaching of pyrite ($\text{FeS}_2$) or molybdenite ($\text{MoS}_2$) by *Thiobacillus thiooxidans* is thiosulfate, whereas polysulfides are generated during bioleaching of sphalerite ($\text{ZnS}$) and galena ($\text{PbS}$) (33). An intermediate ferric-thiosulfate complex is observed in support of the proposed product complex [7] in our sulfide oxidation scheme (Fig. 8). In contrast, the abiotic sulfide oxidation that accompanies reductive dissolution of iron oxides in sulfidic sediments and euxinic basins predominantly yields elemental sulfur (34). We speculate that RBCs clear $\text{H}_2\text{S}$ by converting sulfide to thiosulfate. Polysulfide products are more likely to be formed when the flux of $\text{H}_2\text{S}$ is high and/or $\text{O}_2$ is limiting. In solution, the concentration of sulfide and $\text{O}_2$ determine the distribution of the oxidation products; thiosulfate predominates when the sulfide concentration is low, whereas polysul-
FIGURE 6. HPLC and MS analysis of sulfur-containing compounds. A, the upper chromatogram indicates the elution times for the monobromobimane derivatives of sodium sulfite (peak 1), sodium thiosulfate (peak 2), and sodium sulfide (peak 4). Peak 3 corresponds to monobromobimane. The middle chromatogram represents the control reaction mixture containing 100 mM HEPES buffer, pH 7.4, and 1 mM Na2S following a 15-min aerobic incubation at 25 °C. The bottom chromatogram was obtained following incubation of MetHb (25 μM) under the same conditions as the control sample. Note the significant decrease in the sulfide peak (4) and increase in the thiosulfate peak (2) in the presence of MetHb.

B and C, ESI-mass spectrometric analysis of low molecular weight fractions of samples in 20 mM ammonium carbonate buffer, pH 7.4, containing 25 μM MetHb alone (B) or MetHb plus 1 mM Na2S (C). The peak with m/z = 112.938 (indicated by an arrow) corresponds to HS2O3-. The m/z = 134.92 peak could represent the monosodium salt of HS2O3-. The low molecular fractions were obtained by centrifugation using Amicon Ultra 0.5-ml centrifugal filters with a 10-kDa cutoff.

FIGURE 7. EPR spectra of MetHb treated with sulfide. Shown are EPR spectra of samples containing 50 μM MetHb in 100 mM HEPES, pH 7.4 (A), or after a 5-min incubation with 1200 μM Na2S under aerobic (B) or anaerobic (C) conditions at 25 °C. The EPR settings are described under “Experimental Procedures.”
fides and elemental sulfur are favored when the sulfide concentration is high (35).

At 1–3% of total Hb, MetHb is present at a concentration of ~25–75 μM in blood (corresponding to 100–300 μM ferric heme). Because RBCs occupy ~50% of blood volume, they are likely to play a significant role in sequestering H2S by binding and in oxidizing H2S when levels rise. As permeation of the lipid bilayer by H2S is facile and not predicted to require transporters (36), MetHb could also play an important role in clearing H2S produced by other cell types and diffusing into RBCs from blood, thus buffering H2S levels in circulation. We speculate that reducing equivalents from NADPH can be transferred via MetHb reductase to release heme iron-bound polysulfides to regenerate oxy-Hb, as demonstrated with MSR (Fig. 3F). Polysulfides, which are inherently more reactive than H2S, are increasingly considered to be important for mediating the cellular effects of H2S (37). Additionally, thiosulfate has been shown to attenuate angiotensin II-induced hypertensive cardiac disease (38) and acute lung injury (39), and a role for thiosulfate, if any, in signaling remains to be addressed. Our study demonstrates a route for Hb-dependent thiosulfate and polysulfide biogenesis in mammals and suggests the possibility that additional hemeoproteins might be involved in the generation of these reactive sulfur species in other tissues.

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