Organization of the Human Mitochondrial Hydrogen Sulfide Oxidation Pathway**

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Background: H2S levels can be regulated by oxidation via sulfide quinone oxidoreductase (SQR).
Results: Human SQR uses glutathione as an acceptor forming glutathione persulfide (GSSH), which is preferentially converted to thiosulfate by human rhodanese.
Conclusion: At physiologically relevant concentrations, sulfide oxidation proceeds via GSSH to sulfite and thiosulfate.
Significance: Our combined experimental and simulation studies reveal the organizational logic of the sulfide oxidation pathway.

Sulfide oxidation is expected to play an important role in cellular switching between low steady-state intracellular hydrogen sulfide levels and the higher concentrations where the physiological effects are elicited. Yet despite its significance, fundamental questions regarding how the sulfide oxidation pathway is wired remain unanswered, and competing proposals exist that diverge at the very first step catalyzed by sulfide quinone oxidoreductase (SQR). We demonstrate that, in addition to sulfite, glutathione functions as a persulfide acceptor for human SQR and that rhodanese preferentially synthesizes rather than utilizes thiosulfate. The kinetic behavior of these enzymes provides compelling evidence for the flow of sulfide via SQR to glutathione persulfide, which is then partitioned to thiosulfate or sulfite. Kinetic simulations at physiologically relevant metabolite concentrations provide additional support for the organizational logic of the sulfide oxidation pathway in which glutathione persulfide is the first intermediate formed.

Hydrogen sulfide (H2S)3 is a biological signaling molecule that is produced by organisms ranging from bacteria to man (1–3). Long known as a toxic gas, sulfide like cyanide targets cellular respiration by reversible inhibition of cytochrome c oxidase (4). Cells have therefore evolved strategies for handling the twin challenges of averting toxicity while exploiting the signaling potential of H2S, which elicits profound physiological effects, including regulation of vascular tone, the cellular stress response, apoptosis, and inflammation (3, 5). The steady-state concentration of H2S is a product of the metabolic flux through the sulfide synthetic and the sulfide oxidation reactions. Hence, strategies for regulating cellular H2S levels likely involve enzymes responsible for its biogenesis and its clearance.

The enzymes in the trans-sulfuration (cystathionine β-synthase and γ-cystathionase) and cysteine catabolic (mercaptopyruvate sulfurtransferase) pathways catalyze H2S biogenesis (2, 6), although it is cleared, at least in part, by the mitochondrial sulfide oxidation pathway (7). The sulfide oxidation pathway couples sulfide catabolism to oxidative phosphorylation, making sulfide the first known inorganic substrate for the human electron transfer chain (8). The major oxidation products of H2S are thiosulfate and sulfate whose ratio and production rate vary in a tissue-specific manner (9). The sulfide oxidation pathway begins with sulfide quinone oxidoreductase (SQR) and includes a sulfur dioxygenase (also known as ETHE1 or persulfide dioxygenase), rhodanese, and sulfite oxidase (Fig. 1). It is believed that O2-dependent sulfide consumption is primarily controlled by its efficient catabolism via SQR and sulfur dioxygenase. Indeed, SQR and sulfur dioxygenase might be key regulators functioning to switch off sulfide signaling by consuming H2S and its persulfide product (10). In addition to connecting sulfide metabolism to both ATP and reactive oxygen species formation, H2S might mediate O2 sensing because, under hypoxic conditions, the activities of SQR and sulfur dioxygenase are inhibited leading to increased H2S levels, inhibition of cytochrome c oxidase, and consequent triggering of the hypoxic response (11).

Despite the recent profusion of literature on H2S and the critical importance of the sulfide oxidation pathway in governing cellular H2S levels, biochemical studies on the constituent enzymes are few. In fact, beyond the first step catalyzed by SQR, the very sequence of reactions in the pathway is uncertain as both persulfide and thiosulfate are reported to be products of the SQR reaction, leading to contradictory depictions of sulfide oxidation (7, 12). Although it is generally accepted that sulfide is first oxidized to zero-valent sulfur via the action of SQR (Fig. 1), the physiological sulfur acceptor in this reaction and its fate are unknown. The persulfide acceptor of SQR is postulated to be either GSH (Fig. 2, path1) or sulfite (path 2) or is unknown (path 3). Thus, the sulfane sulfur is transferred either directly...
Human Sulfide Oxidation Pathway

FIGURE 1. Overview of the enzymology of H2S homeostasis. Scheme showing enzymes involved in the mitochondrial sulfide oxidation pathway. 

SO2^- 

SDO 

GSH 

SO4^-2 

Sulfite oxidase 

Complex III 

Complex IV 

intermembrane space 

mitochondrial matrix 

Rhodanese 

Cysteine sulfinate 

SO4^-2 

Sulfite oxidase 

FIGURE 2. Alternative wiring modes in the sulfide oxidation pathway. Path 1, GSH is the acceptor. Path 2, sulfite is the acceptor and possibly originates via the cysteine catabolic pathway in which cysteine sulfenic acid formed in the cytoplasm is converted to β-sulfanylpyruvate in the mitochondrial. Path 3, an unknown acceptor couples to SQR. Rhd, TST, and SO refer to rhodanese, a thiol sulfurtransferase, and sulfite oxidase, respectively.

The expression construct for human SQR in the pET23a vector was obtained from Dr. Marilyn Jorns (Drexel University, Philadelphia). Recombinant SQR was expressed in Escherichia coli and purified as described previously (12) with the following modifications. The expression plasmids for human SQR and for the cold-adapted chaperones were co-transformed in BL21 cells. The transformed cells were grown overnight at 37 °C in 200 ml of Luria Bertani (LB) medium containing ampicillin (60 μg/ml) and gentamycin (10 μg ml^-1). The following day, the starter culture was used to inoculate six flasks each containing 1 liter of terrific broth medium and the same two antibiotics. Cells were grown with shaking (250 rpm) at 37 °C until A600 reached 1.2, after which the temperature was reduced to 15 °C, and growth was continued until A600 reached 1.6. The cultures were induced with 0.5 mM isopropyl β-D-thiogalactopyranoside, and the cells were harvested 20 h after induction. The cell pellet was stored at −80 °C.

The cell pellets were suspended in 200 ml of 50 mM Tris acetate buffer, pH 7.6, containing 0.5 mM sucrose, 20 mM imidazole, 5 mM MgCl2, lysozyme (200 mg), DNase (10 mg), and a single protease inhibitor mixture tablet (Roche Applied Science). The cell suspension was stirred at 4 °C for 30 min and sonicated (power setting = 7) for 12 min at 30-s intervals separated by 1-min cooling periods. The cell suspension was centrifuged at 10,000 × g for 10 min, and the supernatant was diluted 1:1 with 50 mM potassium phosphate buffer, pH 7.4, containing 4% Triton X-100 (v/v), 10% glycerol (v/v), and 100 mM NaCl. The mixture was incubated for 2 h on a rocking platform shaker at 4 °C and then centrifuged at 38,000 × g for 30 min. Tris buffer, pH 8.0, NaCl, and imidazole were added to the supernatant to obtain final concentrations of 100, 300, and 20 mM, respectively. The mixture was loaded onto a nickel-nitrilotriacetic acid-agarose column (2.5 × 15 cm) pre-equilibrated with 50 mM Tris, pH 8.0, containing 0.5% Triton X-100, 300 mM NaCl, and 20 mM imidazole. The column was washed with 500 ml of equilibration buffer, and proteins were eluted with 300 mM imidazole in 50 mM Tris, pH 8.0, containing 0.5% Triton X-100 and 300 mM NaCl. The eluted protein was concentrated to ~20 ml and dialyzed overnight against 4 liters of 50 mM Tris buffer, pH 8.0, containing 0.3% Triton X-100. The dialyzed protein was loaded onto a 20-ml Mono Q column (GE Healthcare) that was pre-equilibrated with 50 mM Tris buffer,
pH 8.0, containing 0.03% DHPC and 2% glycerol. The column was washed with 100 ml of equilibration buffer, and the protein was eluted with 200 ml of a linear gradient ranging from 0 to 1 M NaCl in the equilibration buffer. SQR-containing fractions were pooled, concentrated, and stored at −80 °C. The protein purity was evaluated on a 10% SDS-polyacrylamide gel.

**Spectral Analysis of SQR**

The UV-visible absorption spectrum of purified human SQR was recorded in 50 mM Tris, pH 8.0, containing 0.03% DHPC, 200 mM NaCl, and 1% glycerol. The flavin content of purified SQR was estimated using an extinction coefficient of 11,500 M⁻¹ cm⁻¹ at 451 nm (12).

**SQR Activity Assays**

SQR activity was estimated by monitoring the reduction of CoQ₃ at 278 nm (Δε_{ex,αx} = 12,000 M⁻¹ cm⁻¹) as described previously (12) with the following modifications. SQR activity was tested in the presence of various acceptors, including sulfite, GSH, cysteine, homocysteine, sulfide, DHLA, cysteamine, coenzyme A, hypotaurine, cysteine sulfenic acid, and thioredoxin. The reactions were performed aerobically, and the reaction mixture contained 100 mM potassium phosphate buffer, pH 7.4, 0.03% DHPC, 60 μM CoQ₃, 0.1 mg ml⁻¹ BSA, 150 μM sulfide (except when sulfide was used as acceptor in the reaction), and varying concentrations of the acceptor. The reaction mixture was preincubated at 25 °C for 3 min, and the reaction was initiated by the simultaneous addition of 200 μM sodium sulfite and 0.05 μg of SQR. Control reactions were performed without addition of SQR. When sulfite was used as both donor and acceptor, its concentration was varied from 0 to 0.9 mM. One unit of activity is defined as the consumption of 1 μmol of CoQ₃ oxidized min⁻¹ mg⁻¹ of protein at 25 °C. The K_M and V_max values were obtained by fitting the data sets to the Michaelis-Menten equation. Stock solutions of CoQ₃ were prepared aerobically in DMSO.

**Expression and Purification of Human Rhodanese**

A synthetic gene encoding human rhodanese, which was codon-optimized for expression in E. coli, was purchased from GeneScript (Piscataway, NJ). The cDNA was inserted into the pET-28b vector using a 5′-Ndel and a 3′-SacI site. BL21 (DE3) One Shot chemically competent E. coli cells (Invitrogen) were transformed with the rhodanese expression plasmid. The bacterial culture (1 × 6 liters) in LB medium was supplemented with 50 μg ml⁻¹ kanamycin and grown at 28 °C. After 4 h, when the A_600 was 0.5−0.6, 0.1 mM isopropyl β-D-thiogalactopyranoside was added, and the temperature was reduced to 16 °C. The cells were harvested by centrifugation after 16 h, resuspended in the lysis buffer (50 mM Tris, pH 8, containing 500 mM NaCl, 80 mg of lysozyme, and 1 tablet of protease inhibitor (Roche Applied Science)), and sonicated on ice using a macrotip with a pulse sequence of 30 s burst, 1 min rest, with a 10-min total burst time. The cell lysate was centrifuged at 17,000 × g for 50 min, and the supernatant was loaded onto a 20-ml nickel-nitri-lotriacetic acid fast flow column (Qiagen) pre-equilibrated with 50 mM Tris, pH 8.0, containing 500 mM NaCl (Buffer A). Rhodanese was eluted with Buffer A using a linear gradient ranging from 0 to 500 mM imidazole. Fractions containing rhodanese were identified by SDS-PAGE analysis, pooled, concentrated, and applied to a Superdex 200 gel filtration column (GE Healthcare) and eluted with 100 mM HEPES, pH 7.4, containing 150 mM NaCl. Fractions containing rhodanese were pooled, concentrated, and stored at −80 °C until use. Protein concentration was determined using the Bradford reagent (Bio-Rad) with BSA as a standard.

**Preparation of Glutathione Persulfide**

GSSG was prepared nonenzymatically by reacting NaHS and oxidized glutathione, and the GSSG concentration was determined using the cold cyanolysis assay as described previously (16).

**Rhodanese Activity Assays**

The following assays were used to determine the activity of rhodanese.

**H₂S Production by Rhodanese in the Presence of Thiosulfate and GSH**—Formation of H₂S was measured at 390 nm using the lead acetate assay as described previously (17). The reaction mixture containing thiosulfate (0.1−20 mM), GSH (50 mM), cysteine (50 mM) or homocysteine (50 mM), and 0.4 mM lead acetate in 100 mM HEPES, pH 7.4, and 150 mM NaCl was preincubated at 37 °C for 4 min. The reaction was initiated by addition of 1 or 10 μg of rhodanese. A molar extinction coefficient of 5,500 M⁻¹ cm⁻¹ was used to calculate the lead sulfide concentration. The K_M values for GSH, cysteine, and homocysteine were determined in the presence of 1 mM sodium thiosulfate, and the K_M value for sodium thiosulfate was determined in the presence of 50 mM GSH, cysteine, or homocysteine (Hcys). Initial velocity data were fitted to the Hill Equation 1 or to Equation 2, which includes the K_I term for substrate inhibition (seen with thiosulfate). S denotes substrate concentration, and n is the Hill coefficient.

\[
V = \frac{V_{max}S^n}{K_M + S} \quad \text{(Eq. 1)}
\]

\[
V = \frac{V_{max}S}{(K_M + S\left(1 + \frac{S}{K_I}\right))} \quad \text{(Eq. 2)}
\]

**Thiosulfate Production by Rhodanese in the Presence of Sulfite and GSSH**—An HPLC assay was used to estimate the rate of thiosulfate formation by rhodanese in the presence of sulfite and GSSH. The concentration of thiosulfate was determined following monobromobimane derivatization and a calibration curve using thiosulfate standards of known concentrations. Briefly, the reaction mixture contained sulfite (25 μM to1 mM) and GSSH (0.1−2 mM) and 1 μg of rhodanese in 100 mM HEPES, pH 7.4, and 150 mM NaCl. After a 5-min incubation period, monobromobimane was added to a final concentration of 1 mM, and the incubation was continued for 10 min before acidification with 0.2 mM sodium citrate, pH 2.0. A control reaction lacking rhodanese was prepared in parallel. The derivatized samples were centrifuged at 10,000 × g for 10 min at 4 °C, and
50 μl of the supernatants were injected onto a C8 reverse phase HPLC column (4.6 × 150 cm, 3 μm packing, Phenomenex), pre-equilibrated with 80% Solvent A (10% methanol and 0.25% acetic acid) and 20% Solvent B (90% methanol and 0.25% acetic acid). The column was eluted using the following gradients: Solvent B: 20% from 0 to 10 min; 20–40% from 10 to 25 min; 40–90% from 25 to 30 min; 90–100% from 30 to 32 min; 100% from 32 to 35 min; 100 to 20% from 35 to 37 min; and 20% from 37 to 40 min. The flow rate was 0.75 ml min⁻¹. The bimane adducts were detected by excitation at 340 nm and emission at 450 nm. To determine the $K_M$ value for sulfite, its concentration was varied from 25 μM to 1 mM, and the concentration of GSSH was fixed at 2 mM. To determine the $K_M$ value of GSSH, its concentration was varied from 0.1 to 2 mM, and the concentration of sulfite was fixed at 1 mM. The $K_M$ and $V_{max}$ values were obtained by fitting the data sets to the Michaelis-Menten equation.

**Simulation of SQR Reaction Rates**

The equations describing the SQR reaction rate were developed based on the SQR kinetic scheme published earlier (12) (Fig. 3). It was assumed that the sulfane sulfur acceptors (GSH, cysteine, homocysteine, and SO₃⁻) share a common binding site. The following set of Equations 3–9 describes the quasi steady-state conditions for the SQR reaction (i.e. constant concentrations of all enzyme forms at given concentrations of all substrates and products).

$$E = E_1 + E_2 + E_{31} + E_{32} + E_{33} + E_{34} + E_4$$  \hspace{1cm} \text{(Eq. 3)}$$

$$\frac{dE_1}{dt} = E_1Q - E_1H = 0$$  \hspace{1cm} \text{(Eq. 4)}$$

$$\frac{dE_2}{dt} = E_1H - E_1(R_{GSH} + R_{Cys} + R_{Hcys} + R_{SO_3}) = 0$$  \hspace{1cm} \text{(Eq. 5)}$$

$$\frac{dE_{31}}{dt} = E_2R_{GSH} - E_{31}k_{3,GSH} = 0$$  \hspace{1cm} \text{(Eq. 6)}$$

$$\frac{dE_{32}}{dt} = E_2R_{Cys} - E_{32}k_{3,Cys} = 0$$  \hspace{1cm} \text{(Eq. 7)}$$

$$\frac{dE_{33}}{dt} = E_2R_{Hcys} - E_{33}k_{3,Hcys} = 0$$  \hspace{1cm} \text{(Eq. 8)}$$

$$\frac{dE_{34}}{dt} = E_2R_{SO_3} - E_{34}k_{3,SO_3} = 0$$  \hspace{1cm} \text{(Eq. 9)}$$

Here, $E_1$ is the concentration of free (unbound) enzyme, $E_2$, $E_{31}$, $E_{32}$, $E_{33}$, $E_{34}$, and $E_4$ are the concentrations of enzyme complexes with different intermediates (Fig. 3), and $E$ is the total enzyme concentration. The reaction rate constants $k$ correspond to the individual reaction steps (Fig. 3). $H$ is [HS⁻][k; Q is [CoQ]k; and $R_{X}$ is [X]k₂X, where $X$ is sulfur acceptor.

Using the above set of Equations 3–9, the rate of H₂S consumption in the SQR reaction was derived as described in Equation 10.

$$V = E_1H$$

$$= \frac{EHQ(R_{GSH} + R_{Cys} + R_{Hcys} + R_{SO_3})}{(Q + H)(R_{GSH} + R_{Cys} + R_{Hcys} + R_{SO_3}) + HQ(1 + \frac{R_{GSH}}{k_{3,GSH}} + \frac{R_{Cys}}{k_{3,Cys}} + \frac{R_{Hcys}}{k_{3,Hcys}} + \frac{R_{SO_3}}{k_{3,SO_3}})}$$  \hspace{1cm} \text{(Eq. 10)}$$

The values of the rate constant for the individual steps were obtained using the experimental values for $K_M$ and $K_{cat}$ (Table 1). Specifically, values for $k_{cat,X}$ and $k_{X}^{-1}$ were obtained using Equations 11 and 12, where $X$ denotes the sulfur acceptor (GSH, cysteine, homocysteine, or SO₃⁻).

$$k_{cat,X} = k_{X}^{-1}$$  \hspace{1cm} \text{(Eq. 11)}$$

$$K_{M,X} = \frac{k_{X}}{k_{cat,X}}$$  \hspace{1cm} \text{(Eq. 12)}$$

These equations are derived from Equation 10 for an individual acceptor, $X$ (e.g. GSH), at zero concentrations of all other sulfur acceptors, and at saturating concentrations of H₂S and CoQ₁.

**TABLE 1**

| Metabolite | $K_M$ (μM) | $K_{cat}$ (s⁻¹) | Ref. | $k_2$ (s⁻¹) | $k_3$ (μM s⁻¹) | $k_1$ and $k_4$ (μM s⁻¹) | Concentrations used for simulation |
|-----------|------------|----------------|-----|-------------|---------------|------------------------|----------------------------------|
| H₂S       | 0.013      | 12             |     | 5.1         | 113           | 27,769                 | 0.1–10 μM                        |
| CoQ₁      | 0.019      | 12             |     | 4.1         | 94            | 19,000                 | 100 μM                           |
| GSH       | 22         | 113            | This study | 4.2         | 92            | 4 μM                   | 7 μM                             |
| Cys       | 23         | 94             | This study | 4.2         | 92            |                        |                                  |
| Hcys      | 22         | 92             | This study | 4.2         | 92            |                        |                                  |
| SO₃⁻      | 0.22       | 361            | This study | 1641        | 361           | 0.1–100 μM             |                                  |
The values for $k_1$ and $k_4$ were obtained using Equation 12, which was derived from Equation 10 at a saturating concentration of $\text{SO}_3^{2-}$, zero concentrations of all other sulfur acceptors and at saturating concentrations of $\text{H}_2\text{~S}$ or CoQ$_1$. The equation uses the $K_M$ values for $\text{H}_2\text{~S}$ and CoQ$_1$ that were obtained using $\text{SO}_3^{2-}$ as the sulfane sulfur acceptor (12).

$$K_{M,\text{H}_2\text{~S}} = \frac{k_{\text{cat},\text{SO}_3}}{k_1}$$

$$K_{M,\text{CoQ}_1} = \frac{k_{\text{cat},\text{SO}_3}}{k_4} \quad \text{(Eq. 13)}$$

Calculations were performed using the Wolfram Mathematica 9.0 software (Wolfram Research Inc., Champaign, IL).

**RESULTS AND DISCUSSION**

Sulfide oxidation is critical for cellular sulfide homeostasis, and targeting the first step in the pathway catalyzed by SQR holds pharmaceutical promise for modulating $\text{H}_2\text{~S}$ levels. Sulfite has recently been proposed to be a physiological acceptor of persulfide generating thiosulfate in the SQR-catalyzed reaction (12). However, if sulfite is the acceptor in the SQR reaction, it raises the following questions: (a) what is the source of sulfite that supports the SQR reaction and (b) what are the roles of sulfur dioxygenase and rhodanese in the sulfide oxidation pathway? Although the clinical data on ethylmalonic encephalopathy patients harboring mutations in sulfur dioxygenase strongly support its role in the sulfide oxidation pathway (18), varied functions have been ascribed to rhodanese over the years. The operonic association of rhodanese and sulfur dioxygenase in bacteria and the occurrence of variants in Nature, in which sulfur dioxygenase and rhodanese are fused (18), strongly suggest a functional association between the two proteins and therefore a role for rhodanese in the sulfide oxidation pathway.

In principle, sulfite can be produced via cysteine oxidation, a highly regulated pathway that is activated under conditions of cysteine excess (19). In this pathway, cysteine is initially converted to cysteine sulfinate in the cytoplasm, and a minor fraction (~15%) is subsequently converted to β-sulfanylpyruvate, which decomposes spontaneously to sulfite and pyruvate (Fig. 2) (20). The mitochondrial availability of sulfite derived from this pathway is uncertain because cysteine sulfinate, the product of the cytoplasmic cysteine dioxygenase, is either partitioned to taurine or to sulfite via the activities of cysteine sulfinic acid, cysteine, and homocysteine (in the presence of NADPH and human thioredoxin reductase), which did not show any activity. The $K_M$ values for sulfite (218 ± 20 µM) and GSH (22 ± 3 mM) are >200–2000- and ~2–20-fold higher than the reported intracellular concentrations of these metabolites. Hence, at a concentration of 0.1 µM sulfite and 1 mM GSH, the estimated catalytic efficiencies for the two acceptors are $0.17 \times 10^6 \text{~s}^{-1}$ (for sulfite) and $5.1 \times 10^3 \text{~s}^{-1}$ (for GSH). Tissue GSSH concentration was reported to range from ~4 to 150 µM, and its formation was ascribed to nonenzymatic sulfane sulfur exchange between cysteine persulfide and oxidized glutathione (22). Our study demonstrates that GSSH can be formed via the enzymatic activity of SQR. The dominant sulfide oxidation products vary in a tissue-specific manner and is sulfate in liver and kidney (23) and thiosulfate in colonic mucosa (24). [35S]-sulfide labeling studies have demonstrated its rapid conversion to sulfate by rat tissues indicating the existence of a pathway for the eight-electron oxidation of $\text{H}_2\text{~S}$. If the first step of the sulfide oxidation pathway catalyzed by SQR consumes sulfite and produces thiosulfate, it raises the question as to how mitochondrial GSSH and sulfite are generated to support the actions of sulfur dioxygenase and sulfite oxidase, respectively, to generate sulfate. Rhodanese or an alternative thiol sulfurtransferase could possibly fill this role by utilizing thiosulfate as a persulfide donor and GSH as an acceptor to generate sulfate and GSSH, the latter being a substrate for sulfur dioxygenase (Fig. 2). Notably, this reaction represents the reverse of the well known thiosulfate-generating reaction catalyzed by rhodanese (Reaction 1).

$$\text{GSSH} + \text{SO}_3^{2-} \leftrightarrow \text{GSH} + \text{S}_2\text{O}_3^{2-} \quad \text{REACTION 1}$$

To test the plausibility of this model (Fig. 2, path 2), recombinant human rhodanese was expressed and purified (Fig. 4A), and the ability of thiosulfate to serve as a persulfide donor was tested in the presence of various acceptors (Table 3 and Fig. 6). Compared with the $k_{\text{cat}}/K_M$ value for thiosulfate formation from sulfite and GSSH ($6.5 \times 10^6 \text{~s}^{-1}$, Fig. 7), the $k_{\text{cat}}/K_M$ value for the reverse reaction was ~217,000-fold lower ($0.03 \times 10^3 \text{~s}^{-1}$). Two other thiol acceptors, cysteine and homocysteine, showed ~13-fold higher efficiency than GSSH (~0.4 ×
10^3 M^{-1} s^{-1}) primarily due to differences in their \(k_{cat}\) values. However, unless mitochondrial concentrations of cysteine and homocysteine are significantly higher than their bulk intracellular concentrations (<10 \(\mu\)M for homocysteine and ~100 \(\mu\)M for cysteine), the likelihood of them serving as significant acceptors in most tissues is low, due to their high \(K_M\) values (~20 mM). However, in tissues such as kidney, where the intracellular cysteine concentration is higher (~0.5–0.8 mM (25, 26)), the contribution of cysteine as a sulfane sulfur acceptor from thiosulfate could be more significant. The \(K_M\) value for thiosulfate is ~0.3 mM, and substrate inhibition was observed in the presence of each of the thiol acceptors above 1 mM thiosulfate (Fig. 6).

Sulfite formation via a thiosulfate reductase activity (Reaction 2) has been postulated previously in which 2 mol of GSH are consumed (12). For sulfite to form via this mechanism, sulfur transfer occurs in two half-reactions. First, the sulfane sulfur from thiosulfate is transferred to GSH to form sulfite and GSSH (Reaction 3) followed by the attack of a 2nd mol of GSH on the persulfide releasing H$_2$S and GSSG (Reaction 4), which is likely to be nonenzymatic.

\[
\begin{align*}
2\text{GSH} + \text{S}_2\text{O}_3^2^- &\rightleftharpoons \text{GSSG} + \text{SO}_2^3^- + \text{H}_2\text{S} \\
\text{GSH} + \text{S}_2\text{O}_3^- &\rightleftharpoons \text{GSSH} + \text{SO}_2^3^- \\
\text{GSSH} + \text{GSH} &\rightleftharpoons \text{GSSG} + \text{H}_2\text{S}
\end{align*}
\]

The unfavorable kinetics of GSSH formation (Reaction 3) by rhodanese (Table 3) would limit the overall reaction (Reaction 2). Additionally, the involvement of reaction (7), which leads to H$_2$S production in a pathway dedicated to H$_2$S clearance, poses an obvious logical problem.

A human thioltransferase, TSTD1, was recently characterized for thiosulfate:glutathione transferase activity (27). The steady-state kinetics were characterized in Tris acetate buffer at an unphysiological pH value of 9. The \(K_M\) values for GSH (1 ± 0.2 mM) and thiosulfate (14 ± 2 mM) were 20-fold lower and 46-fold higher, respectively, than observed by us with human rhodanese at a physiologically relevant pH 7.4 (Table 3), although the \(k_{cat}\) value (2.7 ± 0.1 s$^{-1}$) was 4-fold higher. In general, the pH optimum for thiol transferases tends to be on the alkaline side and for the yeast enzyme (also referred to as thiosulfate reductase) that is relatively well characterized, the pH optimum is ~9 (28, 29). The activity of the yeast enzyme is ~3-fold lower at pH 7.4 (29). Coupling of the TSTD1 reaction to sulfur dioxygenase, diminished the \(K_M\) value for GSH significantly and decreased \(k_{cat}\) 1.5-fold, although the \(K_M\) value for thiosulfate, the sulfur donor, remained high and virtually unchanged (10.7 ± 0.5 mM (27)). Although the kinetics of the reverse reaction were not characterized, it is likely that TSTD1, like rhodanese, catalyzes thiosulfate formation more efficiently than GSSH particularly at a physiologically relevant pH and metabolite concentrations (see below).

**Simulation of the SQR Reaction Kinetics**—The dependence of the SQR reaction rate on physiologically relevant concentra-
tions of various acceptors was examined using kinetic simulations. Because hepatic coenzyme Q concentration (100 μM (30)) is \( \gg K_M, \text{CoQ1} \) for human SQR (19 μM (12)), its concentration for the purposes of simulations was fixed at 10× \( K_M \). At intracellular concentrations of hepatic GSH (7 mM (25)) and sulfite (0.1 or 1 μM), GSH is predicted to be the dominant con-

![Graphs and tables](https://example.com/graphs-and-tables)

**FIGURE 5.** Kinetic characterization of \( H_2S \) oxidation by SQR in the presence of varying concentrations of small molecule acceptors. The reaction mixtures contained 0–0.6 mM sulfite (A), 0–80 mM GSH (B), 0–0.9 mM sulfide (C), 0–70 mM L-homocysteine (D), and 0–75 mM cysteine (E) in 100 mM potassium phosphate buffer, pH 7.4, 0.03% DHPC, 0.1 mg ml\(^{-1}\) BSA, 150 μM sulfide (except when sulfide was used as acceptor in the reaction), and 0.05 μg of SQR at 25 °C. The data are representative of three independent experiments.

**TABLE 2**
Kinetic parameters for \( H_2S \) oxidation by human SQR

| Acceptor | \( K_{M, \text{sulfide}} \) | \( K_{M, \text{acc}} \) | \( V_{\max} \) | \( k_{\text{cat}} \) | \( k_{\text{cat}}/K_{M, \text{sulfide}} \) | \( k_{\text{cat}}/K_{M, \text{acc}} \) |
|----------|----------------|--------------------|----------------|----------------|-----------------|----------------|
| Sulfite  | 0.013 ± 0.003\(^a\) | 0.22 ± 0.02 | 476 ± 16 | 373 ± 13 | 2.9 × 10\(^7\) | 1.7 × 10\(^6\) |
| GSH      | 0.01 ± 0.001 | 22 ± 3 | 144 ± 12 | 113 ± 9 | 1.1 × 10\(^7\) | 5.1 × 10\(^6\) |
| Cysteine | 0.005 ± 0.001 | 23 ± 4 | 120 ± 5 | 94 ± 4 | 1.9 × 10\(^7\) | 4.1 × 10\(^6\) |
| Homocysteine | 0.007 ± 0.001 | 22 ± 3.5 | 117 ± 6 | 92 ± 5 | 1.3 × 10\(^7\) | 4.2 × 10\(^6\) |
| Sulfide  | 0.32 ± 0.04 | 95 ± 5 | 74 ± 4 | | | |

\(^a\) Data are from Jackson et al. (12).

**TABLE 3**
Kinetic parameters for sulfur transferase reactions catalyzed by human rhodanese

| Persulfide donor | Acceptor | \( K_{M, \text{donor}} \) | \( K_{M, \text{acc}} \) | \( V_{\max} \) | \( k_{\text{cat}} \) | \( k_{\text{cat}}/K_{M, \text{donor}} \) | \( k_{\text{cat}}/K_{M, \text{acc}} \) |
|-----------------|----------|----------------|--------------------|----------------|----------------|----------------|----------------|
| GSSH | Sulfite | 0.450 ± 0.004 | 0.06 ± 0.01 | 609 ± 25 | 389 | 0.86 × 10\(^6\) | 6.5 × 10\(^6\) |
| Thiosulfate | Cysteine | 0.35 ± 0.06 | 20.0 ± 0.5 | 13.0 ± 0.3 | 7.4 | 21 × 10\(^3\) | 0.37 × 10\(^3\) |
| Thiosulfate | GSH | 0.3 ± 0.1 | 20.5 ± 1.9 | 15.5 ± 0.9 | 8.7 | 29 × 10\(^3\) | 0.4 × 10\(^3\) |

\(^a\) \( K_{M, \text{acc}} \) denotes the \( K_M \) for the persulfide acceptor, and Hcy is homocysteine.

\(^b\) \( V_{\max} \) was estimated from data sets in which the acceptor concentration was varied.
tributary to the SQR reaction rate, whereas cysteine and homocysteine (100 and 4 μM (21, 25, 31, 32)) make negligible contributions (Fig. 8, A and B). The contribution of sulfite to the SQR reaction rate increases monotonically with increasing concentrations, with $V_{\text{GSH}}$ at 20 mM and exceeding $V_{\text{GSH}}$ at concentrations >30 μM (Fig. 8C). To our knowledge, hepatic sulfite concentration has not been reported but is expected to be very low due to its high reactivity. Free serum sulfite was reportedly below detection limits (20 nM), and bound sulfite was reported to be 0.47 ± 0.25 μM in one study (33), and total (free and protein-bound) serum sulfite was reported to vary from 0 to 11 μM (mean = 4.6 ± 2.3 μM, n = 76 donors) in humans (34). Hence, assuming an intracellular concentration of 0.1 μM for free sulfite, GSH is predicted to be the dominant acceptor with cysteine (at 0.1 mM) and sulfite contributing ~1 and ~0.5% of the total SQR reaction rate, respectively (Fig. 8A).

At an upper limit of 1 μM for free intracellular sulfite, it contributes ~4% to the total SQR reaction rate (Fig. 8B).

Conclusions—Conversion of sulfide to GSSH, which is subsequently partitioned between sulfur dioxygenase and sulfur transferase (catalyzed by rhodanese or some other thioltransferase), to form sulfite and thiosulfate, respectively, is consistent with early labeling studies with $[^{35}S]Na_{2}S$ (9). Rat liver mitochondria convert sulfide to a mixture of thiosulfate and sulfate or predominantly to sulfate in the absence and presence of exogenous GSH, respectively. Addition of unlabeled thiosulfate at high concentrations (4 mM) did not affect oxidation of $[^{35}S]Na_{2}S$, which is inconsistent with thiosulfate being an intermediate persulfide carrier (Fig. 2, path 2) and precursor of sulfite and sulfate in the sulfide oxidation pathway. In fact, path 1 (Fig. 2) is consistent with the ratio of 1.5 O$_2$ consumed/1 sulfide added, which was observed with isolated rat mitochondria.
This ratio can be explained by the two-electron oxidation of a single mole of sulfide by SQR, which reduces half an equivalent of \( \text{O}_2 \) and the four-electron oxidation of persulfide to sulfite by sulfur dioxygenase, which reduces 1 eq of \( \text{O}_2 \).

Based on the available data, path I in Fig. 2 helps explain the clinically observed increase in thiosulfate and sulfide in sulfur dioxygenase (ETHE1)-deficient patients who show drastically reduced sulfite levels (18). We suggest that the observed increase in thiosulfate in these patients results from the lack of competition for GSSH from sulfur dioxygenase, which favors overproduction of thiosulfate by rhodanese using sulfite produced via the cysteine catabolic pathway. The increased \( \text{H}_2\text{S} \) levels in these patients might be due to inhibition of SQR either via feedback inhibition by a pathway intermediate or due to inhibition of cytochrome c with rising \( \text{H}_2\text{S} \) concentrations, leading to a further increase in sulfide concentration.

**FIGURE 7.** Kinetic analysis of thiosulfate generation by rhodanese. Dependence of the reaction velocity on the concentration of GSSH (A) or sulfite (B). The reaction mixture contained sulfite, GSSH, and 1 \( \mu \text{g} \) of rhodanese, and thiosulfate formation was monitored by HPLC as described under “Experimental Procedures.” The data are representative of three independent experiments. C. HPLC profiles of a reaction mixture containing 150 \( \mu \text{M} \) sulfite, 2 \( \text{mM} \) GSSH, and 1 \( \mu \text{g} \) of rhodanese (black line) and of a control mixture lacking rhodanese (gray line). The peak with a retention time of \(-21\) min represents thiosulfate. Sulfite (\( \text{SO}_3^{2-} \)), \( \text{H}_2\text{S} \) (present in the GSSH substrate due to the conditions employed for its synthesis, see “Experimental Procedures”), and thiosulfate (\( \text{S}_2\text{O}_3^{2-} \)) are well separated. The peak labeled with an asterisk is derived from monobromobimane. The peak area of thiosulfate formed nonenzymatically from \( \text{H}_2\text{S} \) and sulfite in the control sample was subtracted from an area in the reaction mixture, and the concentration was determined using a calibration curve generated using standards of known concentrations.

**FIGURE 8.** Simulation of SQR reaction rates at physiologically relevant concentrations of sulfane sulfur acceptors. Dependence of the SQR reaction rate on the concentration of \( \text{H}_2\text{S} \) at physiologically relevant concentrations of acceptors ([GSSH] = 7 \( \text{mM} \), [Cys] = 100 \( \mu \text{M} \), and [Hcy] = 4 \( \mu \text{M} \)) and 190 \( \mu \text{M} \) CoQ1 and 0.1 \( \mu \text{M} \) sulfite (A) or 1 \( \mu \text{M} \) sulfite (B). C, fraction of the SQR reaction rate contributed by sulfite increases monotonically at increasing concentrations of sulfite. \( \text{H}_2\text{S} \), cysteine, homocysteine, GSH, and CoQ1 concentrations were fixed at 1, 100, and 4 \( \mu \text{M} \), 7 \( \text{mM} \), and 190 \( \mu \text{M} \), respectively.
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