Characterization of Patient Mutations in Human Persulfide Dioxygenase (ETHE1) Involved in H₂S Catabolism*

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Background: ETHE1 converts persulfides to sulfite in the mitochondrial sulfide oxidation pathway.

Results: The kinetics of human ETHE1 and two mutants described in patients have been characterized.

Conclusion: Both glutathione and coenzyme A serve as persulfide carriers for ETHE1, albeit with different efficacies.

Significance: Low steady-state H₂S levels are maintained by its efficient oxidation, and patient mutations in ETHE1 impair this activity.

Hydrogen sulfide (H₂S) is a recently described endogenously produced gaseous signaling molecule that influences various cellular processes in the central nervous system, cardiovascular system, and gastrointestinal tract. The biogenesis of H₂S involves the cytoplasmic transsulfuration enzymes, cystathionine β-synthase and γ-cystathionase, whereas its catabolism occurs in the mitochondrion and couples to the energy-yielding electron transfer chain. Low steady-state levels of H₂S appear to be controlled primarily by efficient oxygen-dependent catabolism via sulfide quinone oxidoreductase, persulfide dioxygenase (ETHE1), rhodanese, and sulfite oxidase. Mutations in the persulfide dioxygenase, i.e. ETHE1, result in ethylmalonic encephalopathy, an inborn error of metabolism. In this study, we report the biochemical characterization and kinetic properties of human persulfide dioxygenase and describe the biochemical penalties associated with two patient mutations, T152I and D196N. Steady-state kinetic analysis reveals that the T152I mutation results in a 3-fold lower activity, which is correlated with a 3-fold lower iron content compared with the wild-type enzyme. The D196N mutation results in a 2-fold higher Km for the substrate, glutathione persulfide.

More than 20 pathogenic mutations in ETHE1 have been described in EE patients (3). ETHE1 belongs to the metallo-β-lactamase superfamily (4) and is most similar to the glyoxylase II proteins (5), involved in detoxification of 2-oxoaldehydes. However, unlike glyoxylase II, which contains a dinuclear metal center that binds zinc, iron, or manganese (6–10), ETHE1 has a mononuclear iron in its active site (5).

The existence in microbes of ETHE1 variants fused to rhodanese or thiosulfate thioreductase (5) and the elevated extracellular concentration of thiosulfate in ETHE1−/− mice and in EE patients (11) suggest involvement of this protein in sulfur catabolism. Indeed, recombinant human ETHE1 expressed in human HELa cells or in Escherichia coli exhibits sulfur-dependent oxygenase activity as evidenced by oxygen consumption in the presence of the co-substrate, glutathione persulfide (GSSH) (equation 1) (11).

\[
\text{RSSH} + \text{O}_2 + \text{H}_2\text{O} \rightarrow \text{RSH} + \text{SO}_3^2^- + 2\text{H}^+ \quad (\text{Eq. 1})
\]

Based on these results, ETHE1 was proposed to be a sulfur dioxygenase, which catalyzes the second step in the mitochondrial sulfide oxidation pathway downstream of sulfide quinone oxidoreductase (Fig. 1) (12). The latter oxidizes H₂S to persulfide and transfers electrons to the electron transport chain via reduced quinone (Fig. 1). Transfer of the persulfide from sulfide quinone oxidoreductase to glutathione or some other acceptor provides ETHE1 with its persulfide substrate that is oxidized to sulfite. Next, rhodanese catalyzes the conversion of 2 mol of sulfite to thiosulfate. Alternatively, sulfite can be oxidized to sulfate via the action of sulfite oxidase.

Steady-state tissue concentrations of H₂S are generally in the low nanomolar range (13, 14). One mechanism by which these low concentrations are maintained is via the efficient removal of H₂S by the sulfide oxidation pathway (15). It is estimated that H₂S concentrations ≥10–20 µM in the vicinity of mitochondria or 0.5–1 µM outside the cell stimulate its catabolism (15). By contrast, inhibition of cytochrome c oxidase by H₂S occurs at substantially higher H₂S concentrations: ~0.3 µM in cell homogenates and ~20 µM in intact cells (15, 16). Under hypoxic conditions, H₂S levels are expected to rise as oxygen-dependent catabolism slows. Conversely, high H₂S is expected to stimulate oxygen consumption leading to reduced oxygen concentrations. The interconnection between H₂S catabolism and oxy-

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2 The abbreviations used are: EE, ethylmalonic encephalopathy; GSSG, oxidized glutathione; GSSH, glutathione persulfide.
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FIGURE 1. Scheme showing pathways for H2S biogenesis and degradation. The transsulfuration pathway enzymes, cystathionine β-synthase (CBS) and γ-cystathionase (CSE) are primarily responsible for production of H2S whereas the mitochondrial sulfide oxidation pathway handles its catabolism. SQR denotes sulfide quinone oxidoreductase, which oxidizes H2S to persulfide, which is initially bound to an active site cysteine (denoted as SQR-SSH). It is not known if sulfide quinone oxidoreductase either directly, or via a small molecule acceptor e.g. glutathione or coenzyme A, transfers the persulfide to the next enzyme, persulfide dioxygenase.

gen has led to the proposal that H2S is important for oxygen sensing (17, 18).

The structure of ET1E1 from Arabidopsis thaliana (58% sequence identity and >70% similarity with human ET1E1) reveals a dimeric protein with iron anchored via two histidines, one aspartate, and three water molecules (5). This coordination geometry resembles the 2-His-1-carboxylate facial triad seen in mononuclear nonheme iron(II) oxygenases (19). Although the residues that ligate the second metal in the dinuclear site of glyoxylases are also present in ET1E1, structural rearrangements appear to preclude their use for metal binding. Glyoxylases I and II catalyze the oxidation of 2-oxoaldheydes to hydroxycids using glutathione as a cofactor. Like glyoxylase II, which binds S-D-lactoylglutathione (and hydrolyzes it to lactate and glutathione (8)), the active site of ET1E1 appears to be commodious enough for glutathione. However, it is substantially less spacious than the glyoxylase II active site particularly in the region that binds the thioester (5), consistent with the need to accommodate the smaller persulfide substituent. Indeed, ET1E1 does not hydrolyze S-D-lactoylglutathione and other glutathione thioesters (3, 20).

Given the importance of the sulfide oxidation pathway to H2S homeostasis and possibly, the hypoxic response, it is important to understand the reaction mechanisms and regulation of the component enzymes. In this study, we report the first steady-state kinetic characterization of wild-type human persulfide dioxygenase (ET1E1) and two pathogenic variants, T152I and D196N, reported in EE patients (21).

EXPERIMENTAL PROCEDURES

Expression and Purification of Human Persulfide Dioxygenase—Recombinant human ET1E1 missing the N-terminal mitochondrial leader peptide was expressed in the E. coli strain BL21(DE3), using an expression plasmid (11) generously provided by Dr. Valeria Tiranti (IRCCS Foundation, Milan). The expressed protein contains a His6 tag at the C terminus. An overnight 200-ml Luria Bertani (LB) culture, grown at 27 °C, was used to inoculate 6 liters of LB medium, and the cultures were supplemented with ferrous sulfate to a final concentration of 250 μM. When the A595 reached 0.4, ET1E1 expression was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside and cultured for an additional 12 h at 27 °C. Cells were harvested by centrifugation at 10,000 × g for 15 min at 4 °C. ET1E1 was purified as follows. A cell pellet from a 6-liter culture was resuspended in 400 ml of 25 mM sodium phosphate buffer, pH 7.4 (Buffer A), supplemented with 0.5 mM NaCl and 0.1% Tween 20, 1 tablet of complete protease inhibitor (Roche Applied Science), and 50 mg of lysozyme (Sigma). To the cell suspension, 35 mg of Dnase (Sigma) and MgCl2 to a final concentration of 10 mM were added and stirred at 4 °C for 30 min followed by sonication (Ultrasonic Processor XL) on ice using a macrotip with a pulse sequence of 30-s burst, 1-min rest with an output power setting of 7 (6-min total burst time). The cell lysate was centrifuged at 10,000 × g for 20 min. The supernatant was diluted 2-fold with Buffer A and loaded on a nickel-nitrilotriacetic acid fast flow column (Qiagen) equilibrated with the same buffer. After washing with 500 ml of Buffer A containing 20 mM imidazole, ET1E1 was eluted with a linear gradient ranging from 20 to 400 mM imidazole. Fractions containing ET1E1 at a purity of >95% were pooled and dialyzed overnight against 50 mM Tris, pH 8, and stored at −80 °C. Protein concentrations were determined using the Bradford reagent (BioRad) with bovine serum albumin as a standard.

Preparation of Persulfide Substrates—Glutathione persulfide was prepared nonenzymatically by reacting NaHS and oxidized glutathione (GSSG) in a Coy anaerobic chamber (with a 95:5 atmosphere of N2:H2) (Equation 2).

\[
\text{NaHS} + \text{GSSG} \rightarrow \text{GSSH} + \text{GSH}
\] (Eq. 2)

An anaerobic solution of GSSG (20–50 mM in 300 mM sodium phosphate, pH 7.4, was mixed with a 4-fold excess of solid NaHS. The vial containing the reaction mixture was sealed immediately to prevent escape of H2S and incubated at 37 °C for 30 min. The GSSH concentration was determined by cyanolysis of sulfane sulfur using the cold cyanolysis assay and colorimetric detection of the resulting ferric thiocyanate complex, as described previously (22). The persulfides of cysteine,
homocysteine, and coenzyme A were prepared using the same strategy.

**Enzyme Assays**—ETHE1 activity was measured using a polarographic oxygen consumption assay. The complete reaction mixture contained 100 mM sodium phosphate buffer, pH 7.4, 700 μM GSSH (standard assay), and ETHE1 (1–3 μg) in a total volume of 1.5 ml in a Gilson type chamber equipped with a Clark oxygen electrode and a magnetic stirrer at room temperature. The reactions were started by injection of substrate, and oxygen consumption was recorded on a Kipp and Zonen BD single channel chart recorder. The rate of oxygen consumption was expressed as μmol of O₂ min⁻¹ mg of protein⁻¹ at 22 °C. We note that all assays had GSH at a concentration equal to GSSG as a consequence of the method of substrate preparation (Equation 2). The effect of GSH on the activity of ETHE1 was assessed in a separate assay by adding GSH to a final concentration of 5 mM. Alternative sulfur-containing compounds were tested as possible substrates at the following concentrations: 700 μM cysteine persulfide (+ 700 μM cysteine), 700 μM homocysteine persulfide (+ 700 μM homocysteine), 360 μM CoA persulfide (+ 360 μM CoA), 5 mM thiosulfate, and 5 mM GSH.

**Metal Analysis**—Plasma emission spectroscopy was used to analyze preparations of wild-type and mutant persulfide dioxygenases for their total metal content at the Chemical Analysis Laboratory, University of Georgia, Athens. Twenty metal ions, including the common transition metals, are detected by this method.

**Gel Filtration Analysis**—The native molecular mass of human ETHE1 was determined using a Sephadex G-250 column calibrated with standards from Bio-Rad. ETHE1 (0.5 ml of 12 mg/ml in 20 mM sodium phosphate buffer, pH 7.4) was loaded on the column equilibrated with the same buffer at 4 °C, and eluted at a flow rate of 0.5 ml min⁻¹. Protein was detected by absorption at 280 nm.

**Thermal Denaturation Assays**—The relative stabilities of the wild-type and mutant ETHE1 were assessed by thermal denaturation experiments. For this, 0.5 mg/ml purified protein in 100 mM HEPES, pH 7.4, in a total volume of 200 μl was placed in a quartz cuvette, and the temperature was increased from 25 to 70 °C in 5 °C increments, in a Cary 100 Bio UV/visible spectrophotometer equipped with a heating block connected to a water bath (Fisher Scientific Isotemp 3016S). Denaturation was monitored by an increase in absorbance at 600 nm at a given temperature until no further change in the optical density was observed.

**HPLC Analysis of the ETHE1 Reaction**—Stock solutions of the following standards: glutathione, thiocitrate, sulfate, sodium citrate, and GSSH, were prepared in 20 mM sodium phosphate buffer, pH 7.4. A stock solution of 60 mM monobromobimane (Invitrogen) was prepared in dimethyl sulfoxide and stored at −20 °C. Bimane adducts of standards were prepared in 20 mM sodium phosphate buffer, pH 7.4 (200-μl total volume), containing a 250 μM concentration of the standard compound and 1 mM monobromobimane incubated at room temperature for 10 min, and the reaction was terminated with 100 μl of 0.2 N sodium citrate, pH 2.0. Samples were protected from light during all the steps. The products of the ETHE1-catalyzed reaction were analyzed using the following reaction mixture: 250 μM GSSH, 250 μM GSH, and 2 μg of purified ETHE1 in 20 mM sodium phosphate buffer, pH 7.4 (total volume, 200 μl). The reaction mixture was incubated at room temperature for 5 min. Monobromobimane was then added to a final concentration of 1 mM, and incubation was continued for 10 min before acidification with sodium citrate as described above. A control reaction lacking ETHE1 was prepared in parallel. The derivatized samples were centrifuged at 10,000 × g for 10 min at 4 °C, and the supernatants were separated on a C8 reverse phase HPLC column (4.6 × 150 cm, 3 μm packing, Phenomenex) at room temperature using an Agilent 1100 series HPLC system equipped with a multisignal fluorescence detector. The sample (50 μl) was injected into the column equilibrated with a solution containing 80% Solvent A (100 ml of methanol, 897.5 ml of water, and 2.5 ml of acetic acid) and 20% Solvent B (900 ml of methanol, 97.5 ml of water, and 2.5 ml of acetic acid) and resolved using a gradient of Solvent B (20% from 1 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, 100–8% from 35 to 37 min, 8% from 37 to 45 min). The flow rate was 0.75 ml/min. Bimane adducts were detected by excitation at 340 nm and emission at 450 nm.

**Electrospray Ionization Mass Spectroscopy Analysis**—HPLC fractions corresponding to the elution peaks of interest were collected and submitted to the Mass Spectrometry Core Facility, Chemistry Department, University of Michigan.

**RESULTS**

**Purification and Characterization of Persulfide Dioxygenase**—We used a one-step protocol to purify recombinant human ETHE1 to >95% purity (Fig. 2A). The yield was ~75 mg of purified protein/liter of culture for both wild-type and mutant (T152I and D196N) proteins. The molecular mass of the recombinant proteins lacking the N-terminal 11-amino acid leader sequence was 25 kDa, based on SDS-PAGE analysis, consistent with the size predicted from its sequence (27.8 kDa). Wild-type and mutant purified persulfide dioxygenases eluted as single peaks from a size exclusion column with a retention time corresponding to 28 kDa, indicating that it exists as a monomer in solution (Fig. 2B).

**Metal Analysis**—The metal content of the wild-type and mutant proteins was determined by plasma emission spectroscopy. For wild-type protein, 0.82 mol of iron/mol of enzyme was obtained (Table 1). Both patient mutations resulted in
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TABLE 1
Comparison of the kinetic properties of wild-type and mutant ETHE1

| ETHE1    | Iron content | $V_{\text{max}}$ | $K_m$ | $k_{\text{cat}}$ | $k_{\text{cat}}/K_m$ |
|----------|--------------|------------------|-------|------------------|----------------------|
| Wild type| 0.82         | 113 ± 4          | 0.34  | 47               | 140                  |
| Wild type*| 0.82         | 251 ± 11         | 0.25  | 104              | 416                  |
| T152I    | 0.33         | 29.0 ± 1.3       | 0.31  | 12               | 39                   |
| D196N    | 0.60         | 99.0 ± 3.8       | 0.74  | 41               | 56                   |

*5 mM GSH.

FIGURE 3. Kinetic analysis of ETHE1. A, dependence of the reaction velocity on substrate concentration for wild-type ETHE1. These data were obtained in the standard assay as described under “Experimental Procedures” with varying GSSH concentrations (in the presence of an equimolar GSH concentration). B, the activity of wild-type and mutant proteins was monitored by the rate of oxygen consumption in the presence of 500 μM GSSH and 500 μM GSH using an oxygen electrode.

The kinetic parameters were determined by monitoring oxygen consumption in the presence of GSSH and either an equimolar amount of GSH (or 5 mM GSH) as described under “Experimental Procedures.” Reactions were performed in 100 mM sodium phosphate buffer, pH 7.4, and contained 1–3 μg of enzyme.

FIGURE 4. Thermal stabilities of wild-type and mutant ETHE1. Thermal melting curves for wild-type ETHE1 (circles), T152I (triangles), and D196N (squares). Protein unfolding was monitored spectrophotometrically at 600 nm as described under “Experimental Procedures.”

The substrate concentrations used are described under “Experimental Procedures.” Whenever a persulfide substrate was tested, an equimolar concentration of the corresponding thiol was present in the reaction mixture.

Effect of GSH on ETHE1 Activity—Because the GSSH samples used in the enzyme assays bring in an equal concentration of GSH, the effect of the latter on ETHE1 was assessed. GSH is not a substrate for ETHE1, and oxygen consumption was not observed in its presence (Table 2). To assess the alternative possibility that GSH is an inhibitor of ETHE1, enzyme activity was measured at varying concentrations of GSSH and a single high, but physiologically relevant, concentration of GSH (5 mM). Under these conditions, a 2.2-fold increase in ETHE1 activity was observed compared with the standard assay containing 700 μM GSH and a saturating and equal concentration (700 μM) of GSH. The $K_m$ for GSSH was slightly lower (0.25 ± 0.03 mM) resulting in a 3-fold higher $k_{\text{cat}}/K_m$ value. The activating effect of GSH on ETHE1 is presently not understood.

Stability of Wild-type and Mutant ETHE1—During purification, the T152I and D196N mutants appeared to be less stable than wild-type ETHE1, as evidenced by the propensity for precipitation during the concentration and dialysis steps. Comparison of the thermal denaturation profiles confirmed that both mutants are less stable than wild-type ETHE1 (Fig. 4). The $T_m$ value of wild-type ETHE1 (59.8 ± 0.5 °C) was significantly higher than for the T152I (44.8 ± 0.05 °C) and D196N (49.8 ± 0.7 °C) enzymes.

Identification of Sulfite as the Product of the ETHE1-catalyzed Reaction—The derivatized reaction mixture was separated by HPLC, and peaks of interest were subjected to mass spectrometric analysis. In the presence of ETHE1, a new peak was observed with a retention time of 18 min was observed (Fig. 5A), which was absent in the control sample lacking enzyme (Fig. 5B). The migration of the new peak coincided with the bimane adduct of a standard sulfite sample (Fig. 5C). The identity of sulfite was confirmed by mass spectrometric analysis in the negative ion mode that yielded a parent ion with an $m/z$ value of 271.04,
which was identical to that obtained for the bimane adduct of a sulfite standard (data not shown). The retention times for the bimane adducts of GSH and sulfide which are also present in the reaction mixture were 7.0 and 29.6 min, respectively. The GSH-bimane peak (Fig. 5, A and B) co-elutes with a contaminant present in the derivatization mixture (Fig. 5 C, retention time of 6.9 min).

Modeled Structure of Human Persulfide Dioxygenase—The structure of the Arabidopsis ETHE1 was used to generate a model of the human protein to obtain structural insights into the T152I and D196N mutations (Fig. 6). The modeled structure reveals that the active site iron is ligated by two histidines (His-79 and His-135 human sequence numbering) and one aspartate (Asp-154) (Fig. 6 A). Whereas one of the residues that is mutated in a patient, Thr-152, is proximal to an iron ligand, Asp-154, the other, Asp-196 is distant from the active site. Both mutated residues reside on extended loops. The side chain of Thr-152 is engaged in a hydrogen bond interaction with the backbone carbonyl of Leu-156 stabilizing a bend in the loop (Fig. 6 B). This interaction might be important for the optimal positioning of Asp-154, an iron ligand. Mutation of Thr-152 to isoleucine is predicted to lead to loss of this stabilizing interaction.

FIGURE 5. HPLC analysis of sulfite generated by ETHE1. A, a complete reaction containing 250 μM GSSH (250 μM GSH and H2S) and 2 μg of enzyme was separated by HPLC using a C18 column as described under “Experimental Procedures.” B and C, a control reaction mixture was set up as in A but lacked ETHE1 (B), and the bimane adduct of a sulfite standard was separated in C. The peak with a retention time of ~18 min represents sulfite and was seen in the reaction mixture (A) and in the sulfite standard (C), and its identity was confirmed by electrospray ionization mass spectrometry. The peaks at 29.65 min and ~7.0 min represent H2S and a mixture of GSH (A and B) and a contaminant present in the monobromobimane (C), respectively.

FIGURE 6. Modeled structure of human ETHE1. The structure was created using the Arabidopsis ETHE1 structure (Protein Database IC code 2GCU) as a template for the human ETHE1 sequence. A, the iron (orange sphere) ligands, His-79, Asp-154, and His-135, form the 2His:1Asp facial triad and are shown in stick representation. The locations of the two residues that are mutated in patients, Thr-152 and Asp-196, are indicated in stick representation. The location of the iron atom was obtained by overlaying the structure of the Arabidopsis ETHE1 on the modeled structure of the human protein. B, close-up shows the side chain interactions of Thr-152, which would be lost in the T152I mutation. The hydroxyl group of threonine interacts with the backbone carbonyl of Leu-156 stabilizing a bend in the loop. C, close-up shows the side chain interactions of Asp-196, which would be affected by the D196N mutation. One of the carboxylate oxygen atoms interacts with the backbone amide nitrogens of His-198, Gly-199, and Phe-200, whereas the other interacts with the backbone carbonyl oxygen of Phe-200. This network of electrostatic interactions is likely to important for positioning the loop.
tion and is likely to be responsible for the 67% decrease in iron content and a corresponding decrease in $V_{\text{max}}$ (Table 1).

The side chain of Asp-196, which appears to be quite distant from the active site, forms multiple interactions that appear to stabilize a turn in the loop on which it resides (Fig. 6C). Hence, one of the β-carboxylate oxygens of Asp-196 interacts with the backbone amide nitrogens of His-198, Gly-199, and Phe-200 whereas the other oxygen interacts with the backbone carbonyl oxygen of Phe-200. Mutation of Asp-196 to asparagine results in a 40% reduction in iron content and selectively impacts $K_m$ for GSSH versus $V_{\text{max}}$ (Table 1).

**DISCUSSION**

Nonheme iron dioxygenases are widely distributed in Nature and catalyze a diversity of oxidation reactions. These enzymes are important in biosynthetic, DNA repair, posttranslational modification, and catabolic pathways. The active site mononuclear iron is coordinated by 2His/1Asp(Glu) ligands, leaving open three sites that are occupied by water (Fig. 7, [1]). In ETHE1, a minimal mechanism involves displacement of water ligands upon substrate binding (shown as S and N coordination [2] followed by O$_2$ coordination to form an Fe(III)-superoxo complex [3]. The ligated sulfur acquires a partial radical cation character via resonance [4], and recombination with the Fe(II) superoxo ligand yields a cyclic peroxo intermediate [5]. Cleavage of the O-O bond forms a sulfoxyl cation, and metal-bound activated oxygen [6] and subsequent transfer of the metal-bound activated oxygen [7] and hydrolysis yield sulfite. The tenets of this mechanism are currently under investigation using spectroscopic approaches.

Although ETHE1 exhibits activity with GSSH in vitro, its physiological substrate is not known. In principle, the persulphide bound in the active site peptide of sulfide quinone oxidoreductase might serve directly as a substrate for ETHE1 or indirectly, following transfer to a small molecule acceptor. The active site of ETHE1 appears to be sufficiently spacious to accommodate either GSSH or a short peptide from sulfide quinone oxidoreductase. In contrast, smaller persulfide carriers, e.g. cysteine and homocysteine that were tested in this study, do not appear to function as substrates for human ETHE1. In ethe1$^{-/-}$ mice, H$_2$S and thiosulfate levels are severalfold higher than in wild-type mice, sulfit is undetectable, and the activity of rhodanese, postulated to generate thiosulfate (12), is unaffected (11). Because rhodanese is postulated to be downstream of ETHE1 in the pathway (Fig. 1), the activity of ETHE1 as a GSSH dioxygenase does not easily explain the clinical profile of EE patients, i.e. accumulation of thiosulfate and of C4 and C5 acylcarnitines. Because coenzyme A persulfide is a potent inhibitor of short chain acyl-CoA dehydrogenases (23, 24), we tested the hypothesis that it might serve as a substrate for ETHE1, thus explaining the observed accumulation of C4 and C5 acylcarnitines. Because coenzyme A persulfide was used at a final concentration of 360 μM versus 700 μM GSSH used in the standard assay. Thus, in the absence of information on the relative concentrations of GSSH versus coenzyme A persulfide in the mitochondrial matrix, the possibility that CoA serves as a physiologically relevant carrier for persulfide remains open.

Whereas a central role for thiosulfate in mammalian sulfide catabolism was suggested by early metabolic labeling studies (25), the formation and clearance of thiosulfate are poorly understood. Sulfite has been proposed to be a persulfide acceptor of sulfide quinone oxidoreductase generating thiosulfate as the product (26). Based on these studies, we tested the hypothesis that thiosulfate is an alternate substrate for ETHE1 and therefore accumulates in ethe$^{-/-}$ mice and in patients with EE. We proposed that thiosulfate can be catabolized to 2 mol of sulfite by Equation 3 or that glutathione rather than water displaces the terminal sulfur following its oxidation (Equation 4), and subsequent displacement of the second mole of sulfite from GSSO$_2$H by water (Equation 5) completes the reaction.
ever, thiosulfate did not stimulate oxygen consumption and does not appear to be a substrate for ETHE1 (Table 2).

\[
\begin{align*}
\text{S}_2\text{O}_3^{2-} + \text{O}_2 + \text{H}_2\text{O} & \rightarrow 2\text{SO}_2^{2-} + 2\text{H}^+ \quad \text{(Eq. 3)} \\
\text{S}_2\text{O}_3^{2-} + \text{O}_2 + \text{GSH} & \rightarrow \text{GSSG+H} + \text{SO}_3^{2-} \quad \text{(Eq. 4)} \\
\text{GSSG+H} + \text{H}_2\text{O} & \rightarrow \text{SO}_2^{2-} + 2\text{H}^+ + \text{GSH} \quad \text{(Eq. 5)}
\end{align*}
\]

An alternative explanation for the observed accumulation of thiosulfate in EE patients is that it is derived via the activity of cysteine dioxygenase, which oxidizes cysteine to cystein sulfinic acid (27). The latter subsequently undergoes transamination to form β-sulfinepyruvate, which decomposes to pyruvate and sulfite. We postulate that in the absence of a functional ETHE1, the persulfide generated by the activity of sulfide quinone oxireductuctase is preferentially consumed by rhodanese to form thiosulfate, explaining the clinically observed overproduction of this compound in EE patients.

In summary, we describe the first steady-state kinetic characterization of wild-type ETHE1 and two missense mutations identified in EE patients. The sulfide oxidation pathway is believed to play an important role in switching between low steady-state intracellular H2S levels and the higher concentrations at which physiological effects of H2S are elicited (28). The oxygen dependence of the ETHE1 reaction predicts that its activity will be limited under hypoxic conditions, suggesting one mechanism for accumulation of H2S. Our studies provide the framework for evaluating the kinetics of H2S clearance under normoxic versus hypoxic conditions.

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