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Human Sulfide:Quinone Oxidoreductase Catalyzes the First Step in Hydrogen Sulfide Metabolism and Produces a Sulfane Sulfur Metabolite

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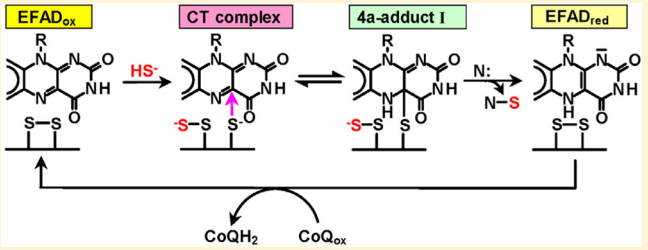
Supporting Information

ABSTRACT: Sulfide:quinone oxidoreductase (SQOR) is a membrane-bound enzyme that catalyzes the first step in the mitochondrial metabolism of H_2S. Human SQOR is successfully expressed at low temperature in *Escherichia coli* by using an optimized synthetic gene and cold-adapted chaperonins. Recombinant SQOR contains noncovalently bound FAD and catalyzes the two-electron oxidation of H_2S to S^0 (sulfane sulfur) using CoQ_1 as an electron acceptor. The prosthetic group is reduced upon anaerobic addition of H_2S in a reaction that proceeds via a long-wavelength-absorbing intermediate (λ_{max} = 673 nm). Cyanide, sulfite, or sulfide can act as the sulfane sulfur acceptor in reactions that (i) exhibit pH optima at 8.5, 7.5, or 7.0, respectively, and (ii) produce thiocyanate, thiosulfate, or a putative sulfur analogue of hydrogen peroxide (H_2S_2), respectively. Importantly, thiosulfate is a known intermediate in the oxidation of H_2S by intact animals and the major product formed in glutathione-depleted cells or mitochondria. Oxidation of H_2S by SQOR with sulfite as the sulfane sulfur acceptor is rapid and highly efficient at physiological pH ($k_{cat}/K_{m,H_2S}$ = 2.9 × 10^7 M^{-1} s^{-1}). A similar efficiency is observed with cyanide, a clearly artificial acceptor, at pH 8.5, whereas a 100-fold lower value is seen with sulfide as the acceptor at pH 7.0. The latter reaction is unlikely to occur in healthy individuals but may become significant under certain pathological conditions. We propose that sulfite is the physiological acceptor of the sulfane sulfur and that the SQOR reaction is the predominant source of the thiosulfate produced during H_2S oxidation by mammalian tissues.



Hydrogen sulfide is the most recently identified member of a small family of labile biological signaling molecules, termed gasotransmitters, that includes nitric oxide and carbon monoxide. In this paper, we will use H_2S and sulfide interchangeably to refer to the total sulfide pool (H_2S, HS^-, and S^{2-}),[a] unless otherwise noted. The recent dramatic proliferation of research and growing appreciation of the importance of H_2S signaling is underscored by the more than 250 reviews on this subject that have appeared in the past two years. The multiple physiological effects of H_2S in mammals include its documented ability (i) to function as a neuromodulator and a neuroprotectant,[3−5] (ii) to afford protection from ischemia/reprofusion injury,[6] (iii) to serve as an oxygen sensor in the carotid body,[7] (iv) to mediate vasodilation and blood pressure regulation,[8] (v) to promote angiogenesis,[9] (vi) to induce hibernation-like states in mice,[10] and (vii) to act as both a pro- and anti-inflammatory agent.[11,12] The emerging paradigm is that H_2S signaling is mediated by protein sulfhydration, a covalent modification in which cysteine is converted to a persulfide derivative, thiocysteine ($CysSS^-$). A wide variety of proteins are regulated by sulfhydration, including actin, tubulin, transcription factor NF-κB, the K_{ATP} ion channel, glyceraldehyde-3-phosphate dehydrogenase, and the tyrosine phosphatase, PTP1B.[13−16]

Cystathionine β-synthase and cystathionine γ-lyase, first recognized for their role in the transsulfuration pathway, are generally acknowledged as the principle biological sources of H_2S in mammals. γ-Cystathionase produces H_2S from cysteine. Formation of H_2S by cystathione β-synthase occurs primarily by condensation of cysteine and homocysteine. A third source may be provided from cysteine by the combined action of cysteine aminotransferase and 3-mercaptopyruvate sulfotransferase.[17]

Mitochondrial metabolism of H_2S is coupled to the synthesis of ATP.[18] The first step of this pathway is catalyzed by sulfide:quinone oxidoreductase (SQOR), a poorly characterized inner mitochondrial membrane-bound flavoenzyme that is ubiquitously expressed in animals and also found in some lower eukaryotes.[19−24] SQOR catalyzes a two-electron oxidation of H_2S to sulfane sulfur (S^0) using coenzyme Q as the electron acceptor. The enzyme also appears to require an acceptor for the sulfane sulfur. Cyanide has been used in vitro as a substitute for the currently unknown acceptor.[19,21,22] The sulfane sulfur

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produced in the SQOR reaction is a metabolic precursor of substrates for better-characterized downstream enzymes, such as sulfite oxidase. The identity of the sulfane sulfur-containing product of the SQOR reaction is necessary to address a major gap in our understanding of the mitochondrial pathway(s) for H_2S metabolism. It is worth noting that a sulfane sulfur donor is required for conversion of cysteine to thiocysteine, suggesting that a possible link may exist between H_2S metabolism and signaling.

SQOR has been purified from several bacteria.[25−27] Unlike eukaryotic SQOR, the bacterial homologues produce sulfane sulfur in the form of polysulfide chains or cyclooctasulfur rings and do not require an acceptor molecule. Characterization of eukaryotic SQOR has proven to be far more challenging, as judged by difficulties encountered in attempts to isolate recombinant forms of the lugworm or yeast enzyme.[22,24] In this paper, we describe the expression and characterization of human SQOR and the identification of the previously unknown physiological acceptor of the sulfane sulfur. Our studies suggest the enzyme may produce the sulfur analogue of hydrogen peroxide (H_2S_2) under conditions where the physiological acceptor is limiting. To the best of our knowledge, this is the first successful purification of a eukaryotic SQOR.

EXPERIMENTAL PROCEDURES

Materials. Sodium sulfite was obtained from Fluka. Potassium cyanide was purchased from Fisher. Sodium sulfide was obtained from Alfa Aesar. CoQ_1 was purchased from Sigma-Aldrich. Glutathione was obtained from Acros. DHPC was purchased from Avanti Polar Lipids. Isopropyl β-D-thiogalactopyranoside was purchased from Gold Biotechnology. Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs. PFU turbo was obtained from Agilent Technologies. Polymerase chain reaction mix was purchased from Amresco.

Expression of Human SQOR in *Escherichia coli*. A synthetic version of the gene encoding human SQOR (*sqrdl*) was obtained from Blue Heron Biotechnology, Inc. (Bothell, WA). The synthetic gene (i) contained a single Met in place of an N-terminal mitochondrion-targeting presequence (41 amino acids),[b] (ii) was optimized for expression in *E. coli*, and (iii) was flanked by unique *Nde*I and *Xho*I sites, a feature achieved by introduction of a silent mutation to remove an internal *Nde*I site (see Figure S1 of the Supporting Information for the sequence of the synthetic gene). The synthetic gene was subcloned between the *Nde*I and *Xho*I sites of plasmid pET23a (Novagen) to yield plasmid pET23a_MATopzSQOR. A pACYC-based plasmid (pCPN10/60) was isolated from ArcticExpress (DE3) Competent Cells (Agilent Technologies). Plasmid pCPN10/60 contains genes (*cpn10* and *cpn60*) for cold-adapted chaperonins from *Oleispira antarctica* (Cpn10 and Cpn60) and a gentamycin resistance gene. Plasmid pET23a_MATopzSQOR was used to transform *E. coli* BL21(DE3) cells to ampicillin resistance. Plasmid pCPN10/60 was then used to transform BL21(DE3)/pET23a_MATopzSQOR cells to gentamycin resistance. A starter culture was prepared by overnight growth of *E. coli* BL21(DE3)/pET23a_MATopzSQOR/pCPN10/60 cells at 37 °C in LB medium containing gentamycin (20 μg/mL) and ampicillin (100 μg/mL). The starter culture was used to inoculate TB medium containing the same two antibiotics. Cells were grown with shaking in 2 L flasks containing 500 mL of medium at 15 °C until A_{595} reached 1.1. SQOR expression was induced with isopropyl β-D-thiogalactopyranoside (0.5 mM). Cells were harvested 20 h after induction. The cell pellets (∼100 g from 9 L of culture) were stored at −80 °C.

Purification of Recombinant Human SQOR. All steps of the purification were conducted at 4 °C. Cell pellets (∼50 g) were thawed and suspended in 75 mL of Tris-acetate buffer (pH 7.6) containing 0.5 M sucrose and 0.1 mM EDTA. The cell suspension was mixed with lysozyme (0.5 mg/mL) and a cocktail of nucleases and protease inhibitors (20 μg/mL DNAase, 20 μg/mL RNAase, 5 mM magnesium sulfate, 12.6 μg/mL soybean trypsin inhibitor, 2 μg/mL aprotinin, 25 μg/mL phenylmethanesulfonyl fluoride, and 3 μg/mL tosyllysine chloromethylketone). The suspension was incubated with stirring for 20 min and then sonicated (Branson model 350, power setting of 6, duty cycle of 40%) for a total of 450 s in 30 s intervals, separated by 30 s cooling periods. Cell debris was removed by centrifugation at low speed (10 min at 10000*g*). Membrane-bound SQOR is found in the supernatant that contains membrane fragments generated during sonication. The low-speed supernatant was diluted 1:1 with 50 mM potassium phosphate buffer (pH 7.4) containing 1% DHPC, 10% glycerol, and a cocktail of protease inhibitors and DNAase, as described above. The sample was incubated for 2 h on a rocking platform shaker to solubilize SQOR and then centrifuged at 120000*g* for 1 h. The high-speed supernatant was collected, and its buffer was modified to contain 200 mM Tris-HCl (pH 8.0), 200 mM sodium chloride, and 40 mM imidazole. The high-speed supernatant was loaded onto a 5 mL HiTrap IMAC column (GE Healthcare), previously equilibrated with 40 mM Tris-HCl buffer (pH 8.0) containing 150 mM sodium chloride and 40 mM imidazole hydrochloride. The column was washed with 50 mM Tris-HCl buffer (pH 8.0) containing 150 mM sodium chloride, 10% glycerol, 80 mM imidazole hydrochloride, and 0.05% DHPC. SQOR was eluted with 50 mM Tris-HCl buffer (pH 8.0) containing 150 mM sodium chloride, 10% glycerol, 160 mM imidazole hydrochloride, and 0.1% DHPC and stored at −80 °C. For the final step of the purification, eight batches of IMAC-purified SQOR obtained from ∼400 g of cells were thawed, pooled, and dialyzed for 2 h versus an ∼200-fold excess of 50 mM Tris-HCl buffer (pH 8.0) containing 50 mM sodium chloride and 5% glycerol. The sample was centrifuged for 10 min at 30000*g*. The supernatant was loaded onto a 50 mL HiLoad 26/10 Q Sepharose High Performance anion exchange column (GE Healthcare), previously equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 2% glycerol and 0.03% DHPC. The column was washed with 50 mM Tris-HCl buffer (pH 8.0) containing 2% glycerol, 100 mM sodium chloride, and 0.06% DHPC (buffer A). SQOR was eluted with a 100 mL linear gradient consisting of buffer A and buffer B [50 mM Tris-HCl buffer (pH 8.0) containing 2% glycerol, 1.0 M sodium chloride, and 0.06% DHPC]. SQOR-containing fractions were pooled, concentrated using a 10K Macrosep Advance Centrifugal Device (Pall Life Sciences), and stored in aliquots at −80 °C.

Flavin Analysis. SQOR was denatured by being heated for 5 min at 100 °C. The flavin was separated from the denatured protein by microfiltration (VWR centrifugal filter) and identified as FAD, as previously described.[28] The stoichiometry of flavin incorporation and the extinction coefficient of SQOR at 451 nm were determined after denaturation of the enzyme with 3 M guanidine hydrochloride, as described by Wagner et al.[29]

Activity and Protein Assays. Except as indicated, assays were conducted under anaerobic conditions at 25 °C using a cuvette (Spectrocell) with a screw-cap equipped with a Teflon–silicon membrane. Buffers used to prepare stock solutions of sodium sulfide, sodium sulfite, or potassium cyanide were bubbled with argon for at least 20 min. Sodium sulfite and potassium cyanide (≤2 mM) stock solutions were prepared directly in the assay buffer. To prepare more concentrated potassium cyanide solutions, a 1 M solution of the substrate was neutralized to the desired pH with monobasic potassium phosphate and then diluted with assay buffer. Stock solutions of sodium sulfide were prepared in 50 mM potassium carbonate/bicarbonate buffer (pH 9.6) containing 250 mM EDTA. The sulfide concentration was determined on the basis of its absorbance at 230 nm (ε = 7200 M^{-1} cm^{-1}).[30] Stock solutions of CoQ_1 were prepared in DMSO. Reagents were added to the cuvette using argon-purged gastight Hamilton syringes. Cuvettes containing buffer, CoQ_1, and, where indicated, sulfite or cyanide were incubated at 25 °C for 2 min. An aliquot of SQOR was added, and the reaction was initiated immediately thereafter by addition of sodium sulfide. Reaction rates were determined by monitoring the reduction of CoQ_1 at 278 nm ($\Delta\varepsilon_{ox-red}$ = 12000 M^{-1} cm^{-1}) and are corrected for the corresponding blank rate observed in the absence of SQOR. The value for $\Delta\varepsilon_{ox-red}$ was determined using the reported extinction coefficient for oxidized CoQ_1 (ε_{278} = 14500 M^{-1} cm^{-1})[31] and the absorption spectrum of reduced CoQ_1 (λ_{max} = 287 nm; ε_{287} = 3340 M^{-1} cm^{-1}) observed 2 s after addition of sodium sulfide to assays containing sodium sulfite and a large excess of SQOR. Unless otherwise indicated, the concentration of SQOR used in steady-state kinetic studies was estimated on the basis of the absorbance of the enzyme at 451 nm (ε = 11500 M^{-1} cm^{-1}).

Enzyme activity during purification was monitored using a routine assay that contained 100 mM potassium phosphate buffer (pH 7.5), 0.5 mM EDTA, 80 μM CoQ_1, 600 μM sodium sulfite, and 200 μM sodium sulfide. Similar rates were observed in control studies when assays at these substrate concentrations were performed under aerobic or anaerobic conditions. Accordingly, the routine assay is conducted using aerobic buffer and uncapped 1 mL cuvettes. Stock solutions of sodium sulfide and sodium sulfite are, however, prepared and stored on ice under anaerobic conditions. During enzyme purification, protein was assessed using the Pierce BCA (bicinchoninic acid assay) protein assay kit.

Product Analysis. To determine the sulfide oxidation product formed in the presence of sulfite, assays were conducted at 25 °C in 25 mM Tris-HCl (pH 8.0) containing 400 μM sodium sulfide, 1.0 mM sodium sulfite, and 77.6 or 194 μM CoQ_1. Reactions were monitored at 278 nm until reduction of CoQ_1 was complete. Aliquots were then withdrawn and analyzed for thiosulfate by cold cyanolysis in the presence of copper chloride, as previously described.[32] To determine the product of sulfide oxidation generated in the presence of cyanide, assays were conducted at 25 °C in 100 mM potassium carbonate buffer (pH 9.0) containing 0.5 mM EDTA, 400 μM sodium sulfide, 1.0 mM potassium cyanide, and 277 μM CoQ_1. Aliquots were withdrawn when reduction of CoQ_1 was complete and analyzed for thiocyanate according to the procedure described by Wood.[33] The product of sulfide oxidation produced in the absence of sulfite or cyanide was characterized as described in Results.

Spectroscopy. All spectral data were recorded using an Agilent Technologies 8453 diode array spectrophotometer. The reaction of substrate amounts of SQOR with sulfide was monitored under anaerobic conditions at 4 °C using screw-cap cuvettes. Reaction buffer containing 50 mM sarcosine was bubbled with argon for 20 min prior to addition of SQOR and an "oxygen sponge" (0.74 μM monomeric sarcosine oxidase, ~10 units/mL of catalase). The samples were incubated for at least 20 min at 4 °C to scavenge trace amounts of oxygen prior to reaction with sulfide. Anaerobic stock solutions of sarcosine and sulfide were prepared in a manner similar to that described above.

RESULTS

Expression and Purification of Recombinant Human SQOR. Major barriers are frequently encountered when attempting to produce even modest amounts of a functional, recombinant, membrane-bound mammalian protein like SQOR. In an attempt to overcome these obstacles, we surveyed a wide range of expression vectors, prokaryotic and eukaryotic host cells, and growth conditions. We succeeded in expressing mature SQOR (47 kDa) in *E. coli* by using a synthetic version of the human gene that lacked the N-terminal mitochondrion-targeting presequence and had been optimized for expression in *E. coli*. The synthetic gene was subcloned into plasmid pET23a to introduce a C-terminal $(His)_6$ tag. *E. coli* BL21(DE3) cells were sequentially transformed with the resulting SQOR expression plasmid and a second compatible (pACYC-based) plasmid containing two genes for cold-adapted chaperonins.[34] SQOR is expressed as a catalytically active, membrane-bound protein when the clone harboring both plasmids is grown at 15 °C, conditions that minimize inclusion body formation. Optimal solubilization of SQOR was achieved using 0.5% 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine (DHPC), a mild, short-chain, phospholipid detergent.[35] The solubilized enzyme was purified to >95% homogeneity (Figure 1) in two steps by using metal (Ni^{2+}) affinity and anion exchange chromatography (Table 1).

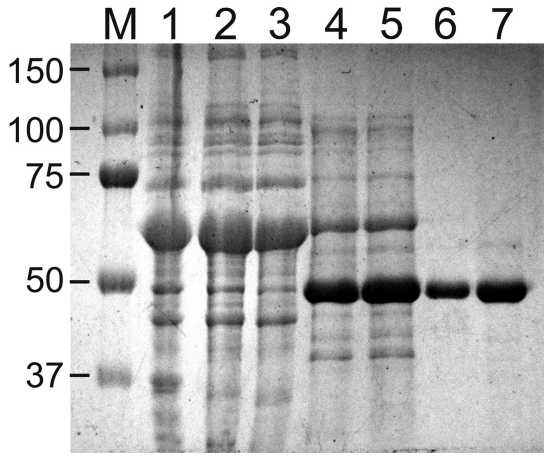

Figure 1. Expression of recombinant human SQOR. The SDS–12% polyacrylamide gel was stained for protein with ProSieve Blue Protein Staining Solution (Lonza): lane M, molecular markers; lane 1, crude cell lysate; lane 2, high-speed supernatant; lane 3, IMAC wash; lane 4, IMAC eluate; lane 5, dialyzed IMAC eluate; lane 6, Q Sepharose eluate; lane 7, concentrated Q Sepharose eluate.

Table 1. Purification of Recombinant SQOR from *E. coli*[a]

purification step	total activity (units)[b]	total protein (mg)	specific activity (units/mg)	yield (%)
high-speed supernatant	32200	27600	1.17	100
IMAC	11100	89.8	124	34.5
Q Sepharose	8330	14.3	581	25.9

[a]SQOR was purified from ~400 g of cells, as detailed in Experimental Procedures. [b]A unit of activity is defined as the formation of 1 μmol of reduced CoQ_1 at 25 °C using a routine aerobic assay, as described in Experimental Procedures.

Table 2. Spectral and Catalytic Properties of Recombinant Human SQOR

λ_{max} (nm)	451, 385, and 277
ε_{451} (M^{-1} cm^{-1})	11500
A_{280}/A_{451}	9.24
mol of FAD/mol of protein	0.82
catalytic activity	$k_{cat,app}$ (s^{-1})[a]
$HS^- + CN^- + CoQ_1$	82 ± 6
$HS^- + SO_3^{2-} + CoQ_1$	251 ± 9
$HS^- + CoQ_1$	18.5 ± 0.9
$HS^- + GSH + CoQ_1$	19 ± 3

[a]Reaction rates were measured at 25 °C by monitoring CoQ_1 reduction at 278 nm in anaerobic 100 mM potassium phosphate buffer (pH 8.0) containing 0.5 mM EDTA, 200 μM sulfide, 80 μM CoQ_1, and, where indicated, 600 μM sulfite, 1.0 mM cyanide, or 1 mM glutathione (GSH).

Recombinant human SQOR is stable and contains an approximately stoichiometric amount of FAD (Table 2). The enzyme exhibits a typical flavoprotein visible absorption spectrum with peaks at 451 and 385 nm and a pronounced shoulder at 473 nm that is eliminated upon denaturation with guanidine hydrochloride (Figure 2). The yellow flavin color of the denatured enzyme is recovered in the filtrate obtained after microfiltration. The data indicate that FAD is noncovalently bound to human SQOR. The results fail to support a proposal that the coenzyme would be covalently attached to a highly conserved tyrosine residue in eukaryotic SQORs.[23]

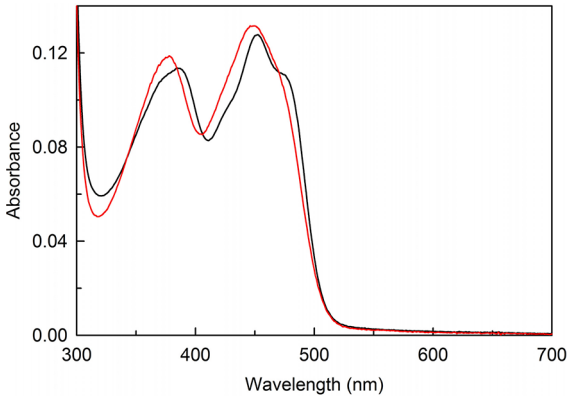

Figure 2. Absorption spectra of native and denatured SQOR. The black curve is the absorption spectrum of SQOR in 100 mM potassium phosphate buffer (pH 7.4) containing 0.04% DHPC. The red curve was recorded after denaturation with 3 M guanidine hydrochloride.

Sulfide Oxidation with Cyanide as the Acceptor of the Sulfane Sulfur. An initial assessment of the catalytic activity of human SQOR was performed at pH 8.0 in assays containing cyanide as the acceptor of the sulfane sulfur and CoQ_1, a water-soluble ubiquinone derivative, as the electron acceptor. Sulfide oxidation is readily detected under these conditions by monitoring the reduction of CoQ_1 at 278 nm ($k_{cat,app}$ = 82 ± 6 s^{-1}) (Table 2). The reaction is accompanied by the formation of a stoichiometric amount of thiocyanate (Table 3), as observed with rat and lugworm SQOR.[19,22] The rate of sulfide oxidation observed with human SQOR is ~20-fold faster than that reported for lugworm SQOR under similar conditions ($k_{cat,app}$ = 4.5 s^{-1}).[22]

Table 3. Stoichiometry of Products Formed in the SQOR Reaction with Different Acceptors of the Sulfane Sulfur[a]

		[product] (μM)	
acceptor	[CoQ_1] (μM)	thiosulfate	thiocyanate
cyanide	277		243 ± 6
sulfite	77.6	77.4	
	194	182	

[a]Assays were conducted in the presence of a limiting amount of CoQ_1, 400 μM sulfide, and 1.0 mM cyanide or 1.0 mM sulfite, as described in Experimental Procedures. Aliquots were withdrawn when reduction of CoQ_1 was complete and analyzed for thiocyanate or thiosulfate using previously described methods.[32,33]

Identification of a Putative Physiological Acceptor of the Sulfane Sulfur. The reaction with cyanide is clearly not biologically relevant. A clue regarding the possible physiological acceptor of the sulfane sulfur in the SQOR reaction was provided by an intriguing metabolic abnormality exhibited by patients suffering from ethylmalonic encephalopathy and the corresponding mouse model. The patients and the knockout mice harbor a defective gene, *ETHE1*, that encodes sulfur dioxygenase (SDO).[1] This enzyme produces sulfite by catalyzing the oxidation of the sulfane sulfur in glutathione persulfide (GSS^-).[1,19] Wild-type levels of SQOR activity are observed with liver homogenates from *Ethe1*$^{-/-}$ knockout mice in assays using cyanide as the acceptor of the sulfane sulfur. Nevertheless, both the mice and the ethylmalonic encephalopathy patients exhibit extremely high, toxic, levels of H_2S.[1] The observed impairment of H_2S metabolism suggested that sulfite might be the physiological acceptor of the sulfane sulfur in the SQOR reaction.

Indeed, we discovered that human SQOR will readily oxidize sulfide in assays containing sulfite in place of cyanide. The rate of sulfide oxidation in the presence of 600 μM sulfite ($k_{cat,app}$ = 251 ± 9 s^{-1}) is 3-fold faster than that observed for the reaction with 1.0 mM cyanide in an otherwise identical assay at pH 8.0 (Table 2). Significantly, the reaction with sulfite is accompanied by the formation of a stoichiometric amount of thiosulfate (Table 3). The results show that sulfite can act as the acceptor of the sulfane sulfur produced during sulfide oxidation by SQOR. It is noteworthy that numerous studies have shown that the observed product, thiosulfate, plays a central role in mammalian metabolism of H_2S, as will be discussed.

Sulfide Oxidation in the Absence of Cyanide or Sulfite. Studies with rat and lugworm SQOR lead us to expect that sulfide oxidation by human SQOR would be difficult to detect in the absence of an acceptor for the sulfane sulfur, such as cyanide.[19,22] However, we found that sulfide oxidation by

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human SQOR is readily detectable in assays containing only sulfide and CoQ_1. The observed rate of sulfide oxidation in this apparently "acceptor-free" reaction at pH 8.0 ($k_{cat,app}$ = 18.5 ± 0.9 s^{-1}) is, however, considerably slower than that seen for the reaction in the presence of sulfite or cyanide (13.6- or 4.5-fold, respectively) (Table 2). It is worth noting that a similar acceptor-free reaction has been detected with yeast SQOR.[24]

Various groups have postulated that SQOR might use glutathione as the acceptor of the sulfane sulfur in a reaction that would produce GSS^-,[1,19] the persulfide substrate for SDO. In a preliminary test of this hypothesis, we sought to determine whether glutathione could accelerate the rate of sulfide oxidation by human SQOR, as observed with sulfite or cyanide. However, we found that the rate of sulfide oxidation in the presence of 1 mM glutathione was, within experimental error, identical to that observed for the acceptor-free reaction with only sulfide and CoQ_1 (Table 2).

Identification of the Sulfide Oxidation Product Formed in the Acceptor-Free Reaction. A working mechanism for SQOR catalysis predicts that turnover of the enzyme will occur only in the presence of an acceptor for the sulfane sulfur, as will be discussed. The putative acceptor-free reaction would appear to be incompatible with this mechanism, unless sulfide itself can act as the sulfane sulfur acceptor. In this case, the acceptor-free reaction should produce hydrogen disulfide, H_2S_2, the sulfur analogue of hydrogen peroxide. The pK_a values estimated for hydrogen disulfide (pK_{a1} = 5.0; pK_{a2} = 9.7)[36] suggest that the compound will exist predominantly as the monoanion, HS_2^-, at pH 8.0. The absorption spectrum of hydrogen disulfide is highly sensitive to its protonation state. The un-ionized H_2S_2 molecule exhibits a maximum at 258 nm, whereas a maximum at 358 nm is estimated for the dianion, S_2^{2-}. The dramatic 100 nm bathochromic shift caused by the removal of two protons is attributed to resonance delocalization of electrons in the dianion that are localized to S−H σ bonds in the neutral molecule.[37,38] Intermediate spectral properties are expected for the monoanion, HS_2^-, but, to the best of our knowledge, have not previously been described.

On the basis of the considerations described above, we hypothesized that the acceptor-free SQOR reaction would generate the monoanion of hydrogen disulfide at pH 8.0, a species likely to absorb in the near-UV region. Evidence for evaluating this hypothesis was sought by monitoring the spectral course of assays conducted in the presence or absence of sulfite or cyanide. The reaction in the presence of 400 μM sulfite results in a progressive loss of the intense absorption band of oxidized CoQ_1 at 278 nm and its tail of absorbance at longer wavelengths (λ > 300 nm). The end of the reaction is signaled by the appearance of a stable, relatively weak absorption band at 287 nm due to reduced CoQ_1 (Figure 3A). A single minimum at 272 nm is seen in difference spectra calculated by subtracting the final spectrum of reduced CoQ_1 from spectra observed during the reaction (data not shown). It is worth noting that similar kinetics are observed for the decrease in absorbance at 272 or 317 nm (Figure 3A, inset).

The same spectral course is observed for assays containing 1.0 mM cyanide instead of sulfite (see Figure S2 of the Supporting Information). A striking difference is, however, observed when assays are conducted in the absence of sulfite or cyanide. In this case, the loss of the 278 nm absorption band of oxidized CoQ_1 is accompanied by the formation of a species absorbing in the 300−400 nm region (Figure 3B). The corresponding difference spectra exhibit a minimum at 272 nm and a maximum at 317 nm (data not shown). Except for a small initial lag, the increase in absorbance at 317 nm exhibits kinetics similar to that observed for the decrease in absorbance at 272 nm (Figure 3B, inset).

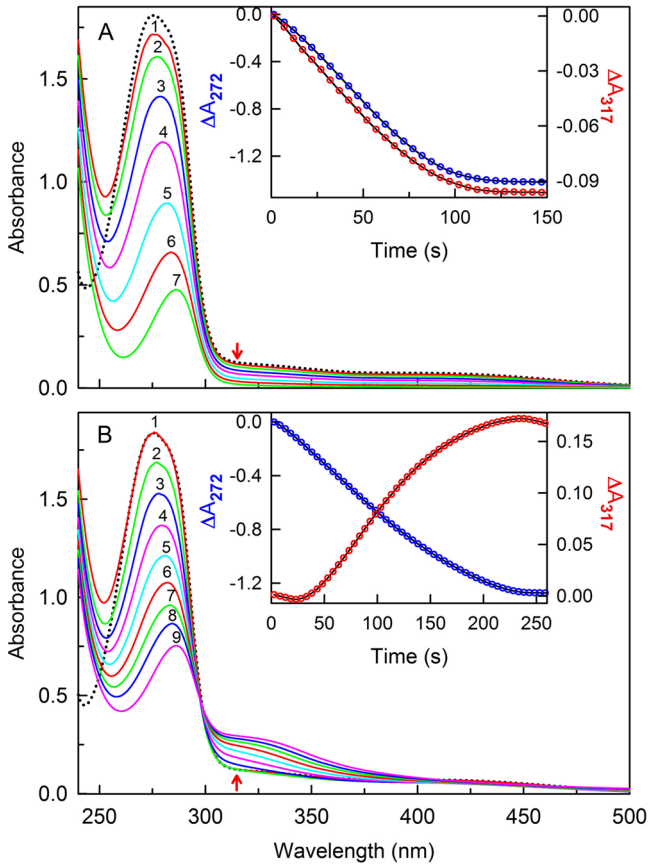

Figure 3. Effect of sulfite on the spectral course of SQOR catalytic assays. Reactions were conducted in 100 mM potassium phosphate (pH 8.0) containing 0.5 mM EDTA at 25 °C in the presence (A) or absence (B) of sulfite. Red arrows indicate the direction of the spectral changes observed at λ > 300 nm. (A) The dotted black line was recorded before addition of 300 μM sulfide to an assay mixture containing 160 μM CoQ_1, 400 μM sulfite, and 7.2 nM SQOR. Curves 2−7 were recorded 1.4, 12, 27, 42, 62, 82, and 132 s, respectively, after sulfide addition. (B) The dotted black line was recorded before addition of 300 μM sulfide to an assay mixture containing 160 μM CoQ_1 and 35.6 nM SQOR. Curves 1−9 were recorded 2.6, 33.2, 58.2, 88.2, 108, 133, 158, 183, and 233 s, respectively, after sulfide addition. The inset in each panel shows the time course of absorbance changes at 272 and 317 nm plotted according to the left and right *y*-axes, respectively.

Hydrogen disulfide should readily react with nucleophiles, such as sulfide or cyanide, as judged by results obtained with other persulfides.[33] To investigate its chemical reactivity, the near-UV-absorbing product was generated by allowing the acceptor-free SQOR assay with limiting CoQ_1 to proceed until CoQ_1 had been completely reduced. Subsequent addition of 400 μM sulfite results in a rapid loss of the absorbance attributed to the putative hydrogen disulfide product in a reaction that is complete in less than 5 min. The spectrum observed at the end of the sulfite reaction coincides with that expected for reduced CoQ_1 (Figure 4). The final spectrum was subtracted from the spectrum observed prior to sulfite addition. The resulting difference spectrum exhibits maxima at 269 and 315 nm (Figure 4, inset). The maximum at 315 nm falls within

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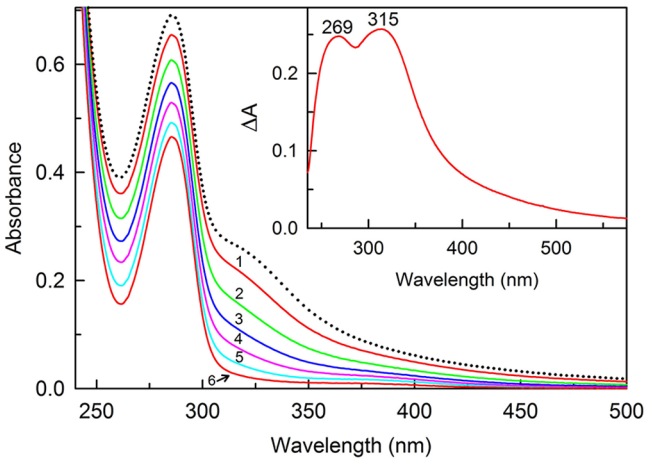

Figure 4. Reaction of sulfite with the sulfur oxidation product formed during the apparent acceptor-free oxidation of sulfide by SQOR. Reactions were conducted in 100 mM potassium phosphate (pH 8.0) containing 0.5 mM EDTA at 25 °C. The dotted black line was recorded after maximal formation of the sulfur oxidation product in an assay mixture containing 300 μM sulfide, 160 μM CoQ_1, and 35.6 nM SQOR. Curves 1–5 were recorded 22, 42, 62, 92, and 292 s, respectively, after addition of 400 μM sulfite. The inset shows a difference spectrum calculated by subtracting the spectrum observed at the end of the sulfite reaction from the spectrum observed before sulfite addition.

the range expected for the hydrogen disulfide monoanion. A similar reaction is observed when sulfite is replaced with cyanide. However, a considerably higher cyanide concentration (56 mM) is required to achieve a comparable reaction rate (data not shown). The observed difference in the reactivity of the two nucleophiles is attributed to the fact that only a small amount of the added cyanide (<5%) will exist as the reactive cyanide anion in solution at pH 8.0 (pK_a = 9.31), whereas most of the sulfite (>90%) will be present as the reactive SO_3^{2-} dianion (pK_a = 6.9) at this pH.

In addition to nucleophiles, hydrogen disulfide is expected to be consumed by thiols in a reduction reaction that produces H_2S (λ_{max} = 230 nm).[30,33] Accordingly, studies were conducted to determine whether dithiothreitol (DTT) or glutathione could reduce the product formed in the acceptor-free SQOR reaction. In fact, the near-UV absorbance of the product was eliminated within 15 min of addition of 1 mM DTT (see Figure S3 of the Supporting Information) or 1 mM glutathione (data not shown). The reaction of hydrogen disulfide with DTT is expected to generate an organic persulfide intermediate, $HS\text{-}CH_2\text{-}(CHOH)_2\text{-}CH_2\text{-}SS^-$, that will undergo a rapid intramolecular reaction to produce oxidized DTT and H_2S. We reasoned that a slower intermolecular reaction might permit detection of the persulfide intermediate formed with glutathione, GSS^- (λ_{max} ~ 340 nm).[39] However, GSS^- was not detected in the glutathione reaction, which exhibited a spectral course very similar to that observed with DTT.

Addition of 1 mM glutathione to SQOR assays containing only sulfide and CoQ_1 did not affect the rate of sulfide oxidation (see Table 2). The spectral course of these assays is, however, very similar to that seen in the presence of sulfite or cyanide (see Figure S4 of the Supporting Information). The observed reduction of the near-UV-absorbing product with thiols can account for the failure to detect formation of this species when SQOR assays are conducted in the presence of glutathione.

Effect of pH on Catalysis by Human SQOR. We sought to evaluate the catalytic efficiency of SQOR with different sulfane sulfur acceptors by comparing sulfide oxidation rates under optimal conditions for each acceptor, including reaction pH. A bell-shaped pH–activity profile is observed for sulfide oxidation with cyanide as the sulfane sulfur acceptor. The

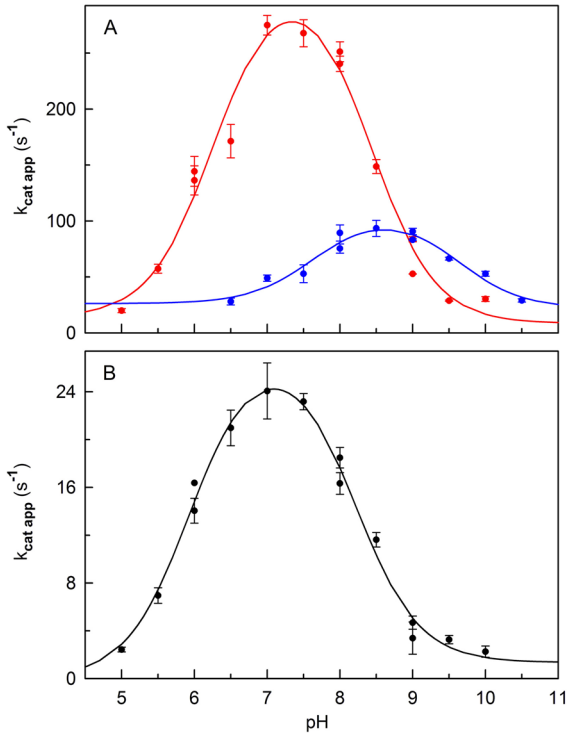

Figure 5. Effect of pH on the rate of sulfide oxidation observed with SQOR in the presence or absence of sulfite or cyanide. The red and blue curves in panel A were obtained for the reactions in the presence of sulfite and cyanide, respectively. Panel B shows the results obtained for the reaction in the absence of sulfite and cyanide. Reactions were monitored at 25 °C by measuring the reduction of CoQ_1 at 278 nm, as described in Experimental Procedures. Assays contained 200 μM sulfide, 80 μM CoQ_1, and, where indicated, 600 μM sulfite or 1.0 mM cyanide. All reaction mixtures contained 0.5 mM EDTA and one of the following buffers: 100 mM potassium citrate (pH 5–6), 100 mM potassium phosphate (pH 6–8), 100 mM potassium pyrophosphate (pH 8–9), and 100 mM potassium carbonate/bicarbonate (pH 9–10.5). The solid lines were obtained by fitting eq 1 to the data (●).

reaction exhibits an optimum at pH 8.5 (Figure 5A). The data could be fit to a double-ionization titration curve (eq 1).

$$V_{obs} = ([H^+]^2 V_{AH2} + [H^+]K_1 V_{AH} + K_1K_2V_A) / (K_1K_2 + [H^+]K_1 + [H^+]^2) \quad (1)$$

The results indicate that maximal activity is observed when one group is unprotonated ($AH_2 \leftrightarrows AH + H^+$) ($pK_{1app}$) and a second group is protonated ($AH \leftrightarrows A + H^+$) ($pK_{2app}$) (Table 4). A similar optimum at moderately alkaline pH (9.0) is observed for the reaction of lugworm SQOR with cyanide.[22] Sulfide oxidation by human SQOR with sulfite as the sulfane sulfur acceptor also exhibits a bell-shaped pH–activity profile. However, the optimum is at pH 7.5, close to physiological pH

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Table 4. Effect of pH on Oxidation of Sulfide by SQOR with Different Acceptors of the Sulfane Sulfur[a]

acceptor	pH optimum	pK_{1app}	pK_{2app}
cyanide	8.5	7.6 ± 0.3	9.6 ± 0.4
sulfite	7.5	6.3 ± 0.2	8.4 ± 0.1
sulfide	7.0	5.9 ± 0.1	8.22 ± 0.09

[a]Parameters were determined as described in the legend of Figure 5.

and 1 pH unit lower than that observed for the cyanide reaction (Figure 5A). The values estimated for pK_{1app} and pK_{2app} in the reaction with sulfite exhibit a similar shift to a more acidic pH (Table 4). The acceptor-free SQOR reaction exhibits a pH optimum at 7.0 (Figure 5B) and apparent pK_a values that are most similar to those observed for the reaction with sulfite (Table 4).

Steady-State Kinetic Parameters for Oxidation of Sulfide by SQOR with Different Acceptors of the Sulfane Sulfur. Turnover of SQOR with each acceptor was monitored at the pH optimum determined for the reaction. The reactions with sulfite or cyanide involve three different substrates. Steady-state kinetic parameters for such reactions can be estimated by varying the concentration of one substrate at a fixed, saturating concentration of the other two substrates.[40] We found that reaction rates with sulfite or cyanide as the acceptor exhibit an expected hyperbolic dependence on the concentration of each of the three varied substrates [$k_{cat,app} = k_{cat}[S]/(K_m + [S])$]. Values obtained for k_{cat} with either acceptor are independent of the nature of the varied substrate (Table 5). Interestingly, the turnover rate observed with sulfite at pH 7.5 is, within experimental error, identical to that observed with cyanide at pH 8.5, as judged by comparing the average values obtained for k_{cat} with the two acceptors ($k_{cat,avg}$ = 370 ± 14 and 345 ± 11 s^{-1}, respectively). The apparent K_m observed for sulfite is 4-fold smaller than that observed for cyanide. However, very similar apparent K_m values for sulfide and CoQ_1 are obtained with either acceptor.

The reaction with sulfide as the sulfane sulfur acceptor is essentially an oxidative dimerization of two identical substrates. According to a steady-state equation derived for this type of reaction (eq 2)[41,42]

$$V_{obs} = \frac{V_{max}}{1 + \frac{K_{m2}}{[S]}\left(1 + \frac{K_{m1}}{[S]}\right)} \quad (2)$$

the velocity observed at a fixed saturating concentration of CoQ_1 should not exhibit a simple hyperbolic dependence on the concentration of sulfide except under the limiting condition where [sulfide] ≫ K_{m1}. In fact, the latter is observed when the sulfide concentration is varied in the range from 40 to 2100 μM (see Figure S5 of the Supporting Information). Measurements at higher sulfide concentrations were not possible because of prohibitively high blank rates. The apparent K_m obtained for sulfide in this reaction (315 ± 28 μM) is ~25-fold larger than that observed with sulfite or cyanide as the sulfane sulfur acceptor (Table 5). The high K_m value precluded studies at a fixed saturating sulfide concentration and a variable concentration of CoQ_1. The turnover rate estimated for the reaction with sulfide as the sulfane sulfur acceptor (k_{cat} = 65 ± 2 s^{-1}) exhibits a relatively modest decrease (6-fold) compared with values obtained for the reactions with sulfite or cyanide. The catalytic efficiency of the reaction with sulfide as the acceptor is, however, more than 100-fold lower than that observed with sulfite or cyanide, as judged by values calculated for the ratio $k_{cat}/K_{m,H_2S}$ (Table 5).

Table 5. Steady-State Kinetic Parameters for Three Reactions Catalyzed by Human SQOR[a]

Sulfide + Sulfite + CoQ_1 ⇒ Thiosulfate + CoQ_1H_2				
variable substrate	fixed substrates[b]	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
sulfide	sulfite, CoQ_1	13 ± 3	379 ± 20	(2.9 ± 0.6) × 10^7
sulfite	sulfide, CoQ_1	174 ± 20	368 ± 14	(2.1 ± 0.3) × 10^6
CoQ_1	sulfide, sulfite	19 ± 2	364 ± 8	(1.9 ± 0.2) × 10^7
Sulfide + Cyanide + CoQ_1 ⇒ Thiocyanate + CoQ_1H_2				
variable substrate	fixed substrates[c]	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
sulfide	cyanide, CoQ_1	10.9 ± 0.7	343 ± 9	(3.1 ± 0.2) × 10^7
cyanide	sulfide, CoQ_1	650 ± 80	330 ± 12	(5.1 ± 0.7) × 10^5
CoQ_1	sulfide, cyanide	14 ± 2	360 ± 13	(2.7 ± 0.3) × 10^7
2 Sulfide + CoQ_1 ⇒ Hydrogen Disulfide + CoQ_1H_2				
variable substrate	fixed substrate[d]	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
sulfide	CoQ_1	315 ± 28	65 ± 2	(2.1 ± 0.2) × 10^5

[a]Apparent K_m values for the variable substrate were determined at saturating concentrations of the fixed substrates or at concentrations that yielded the maximal possible reaction rate in cases where excess substrate inhibition was observed. All measurements were taken in buffers containing 0.5 mM EDTA at 25 °C. Reactions with sulfite, cyanide, or sulfide as the sulfane sulfur acceptor were conducted in 100 mM potassium phosphate (pH 7.5), 100 mM potassium pyrophosphate (pH 8.5), or 100 mM potassium phosphate (pH 7.0), respectively. [b]Measurements were taken at the following fixed substrate concentrations: 83 μM sulfide, 99 μM CoQ_1, and 2000 μM sulfite. [c]Measurements were taken at the following fixed substrate concentrations: 47 μM sulfide, 72.5 μM CoQ_1, and 6000 μM cyanide. [d]Measurements were taken at 66.2 μM CoQ_1.

Anaerobic Reduction of SQOR with Sulfide. Anaerobic reaction of SQOR with 1 or 2 equiv of sulfide results in the rapid formation of an intermediate that exhibits a moderately intense long-wavelength absorption band that is centered at 673 nm and extends out to nearly 900 nm, accompanied by a loss of ~35% of the absorbance of the oxidized enzyme at 451 nm (Figure 6, curve 1). The SQOR intermediate undergoes a slow isosbestic conversion to a reduced species that exhibits a maximum at 365 nm, a plateau around 450 nm, and a tail of absorbance extending into the long-wavelength region (Figure 6, curve 6). The conversion of the intermediate to the final reduced product exhibits apparent first-order kinetics (Figure 6, inset). However, the observed rate of this reaction [k = (4.1 ± 0.2) × 10^{-3} s^{-1}] is >4 orders of magnitude slower than rates observed for turnover of SQOR with sulfite, cyanide, or sulfide as the sulfane sulfur acceptor. The possible identity and catalytic significance of the two species observed during reaction of SQOR with sulfide will be discussed. Immediate oxidation of the reduced enzyme is observed upon addition of CoQ_1 (Figure 6, curve 7). The reoxidized enzyme exhibits a similar absorption band at 450 nm but enhanced absorbance in the 380 nm region as compared with the untreated enzyme. The basis for the latter difference is unclear.

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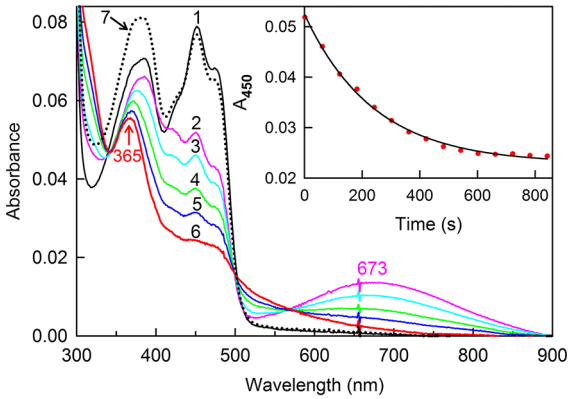


Figure 6. Anaerobic reaction of SQOR with sulfide and CoQ_1. Curve 1 is the absorption spectrum of 6.82 μM SQOR in anaerobic 25 mM Tris-HCl buffer (pH 8.0) at 4 °C. Curves 2–6 were recorded 0, 1, 3, 5, and 14 min, respectively, after addition of 16.4 μM sulfide. Curve 7 (···) was recorded 1 min after the addition of 17.5 μM CoQ_1. The inset shows a plot of the absorbance decrease at 450 nm observed after sulfide addition. The black line was obtained by fitting a single-exponential equation ($y = Ae^{-k_1t} + B$) to the data (red circles). Similar results were obtained in a separate experiment upon addition of 1.1 equiv of sulfide (data not shown).

DISCUSSION

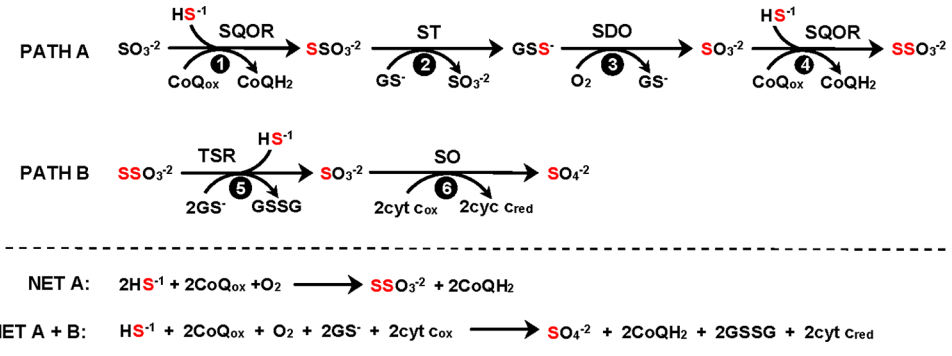
Human SQOR is expressed in *E. coli* at low temperature as a catalytically active membrane-bound protein, an outcome dependent on the use of a synthetic gene optimized for expression in a prokaryotic host and the assistance of cold-adapted bacterial chaperonins. Solubilized SQOR is readily purified to produce a stable homogeneous holoenzyme that contains a nearly stoichiometric amount of noncovalently bound FAD. SQOR is one of a small group of mammalian membrane-bound proteins that have been successfully expressed in *E. coli*. A similar approach may facilitate the expression of other intrinsic mammalian proteins.

Recombinant SQOR catalyzes the two-electron oxidation of H_2S using CoQ_1 as the electron acceptor and one of three different nucleophiles (cyanide, sulfite, and sulfide) as the acceptor of the sulfane sulfur in reactions that exhibit pH optima at 8.5, 7.5, and 7.0, respectively. The reaction with cyanide is accompanied by the formation of a stoichiometric amount of thiocyanate. Our studies confirm the nucleophile's ability to act as an artificial acceptor for eukaryotic SQOR, in agreement with results obtained with rat and lugworm SQOR.[19,22] The reaction with sulfite produces thiosulfate, a known intermediate in H_2S metabolism. The product of the reaction with sulfide as the acceptor has been tentatively identified as hydrogen disulfide, H_2S_2, on the basis of its near-UV absorption and reactivity with nucleophiles and thiol reductants. The effect of the acceptor on the catalytic efficiency of H_2S oxidation can be assessed by comparison of values obtained for $k_{cat}/K_{m,H_2S}$ at the pH optimum of each reaction. The catalytic efficiency observed with sulfite at physiological pH (2.9×10^7 M^{-1} s^{-1}) is similar to that observed with cyanide at moderately alkaline pH (3.1×10^7 M^{-1} s^{-1}), whereas a 100-fold lower value is seen with sulfide (2.1×10^5 M^{-1} s^{-1}). The reaction with sulfide as the acceptor is unlikely to occur in healthy individuals but may become significant under certain pathological conditions. We propose that sulfite is the biological acceptor, as discussed below.

Role of SQOR in the Biosynthesis of Thiosulfate. Thiosulfate is the major product of H_2S oxidation by (i) isolated mitochondria,[19,43] (ii) hepatocytes depleted of glutathione,[44] and (iii) the colon in intact animals where large quantities of H_2S are produced by sulfate-reducing bacteria.[45] Oxidation of thiosulfate to sulfate (i) requires a glutathione-dependent thiosulfate reductase and sulfite oxidase[46,47] and (ii) is observed when isolated mitochondria are supplemented with glutathione and with untreated hepatocytes.[43,44] Additionally, the elegant experiments of Koj et al.[48] and Szczepkowski et al.[49] support a central role for thiosulfate as a key intermediate in the oxidation of H_2S by perfused liver and intact animals.

We propose that thiosulfate biosynthesis occurs in the first step of H_2S oxidation in a reaction catalyzed by SQOR with sulfite as the acceptor of the sulfane sulfur. Our studies with the recombinant human enzyme show that the reaction is rapid and highly efficient at physiological pH. In fact, the observed efficiency of SQOR is just 4-fold lower than that reported for carbonic anhydrase, one of the most potent known catalysts ($k_{cat}/K_m = 1.2 \times 10^8$ M^{-1} s^{-1}).[50] Rhodanese is a mitochondrial enzyme best known for its ability to ultilize thiosulfate as a source of sulfane sulfur in the production of thiocyanate.[51] Hildebrandt and Grieshaber postulated that thiosulfate is produced from GSS^- and sulfite in an alternate rhodanese reaction.[19] However, thiosulfate formation with human SQOR is more than 2 orders of magnitude faster than the rate of thiosulfate formation observed with bovine or rat rhodanese ($k_{cat,app}$ = 2.3 or 0.22 s^{-1}, respectively). Furthermore, the

Scheme 1. Proposed Pathways for H_2S Metabolism in (i) Glutathione-Depleted Cells or Mitochondria (path A) and (ii) Untreated Cells or Intact Animals (paths A and B)[a]



[a]Abbreviations: ST, sulfur transferase; SDO, sulfur dioxygenase; TSR, thiosulfate reductase; SO, sulfite oxidase.

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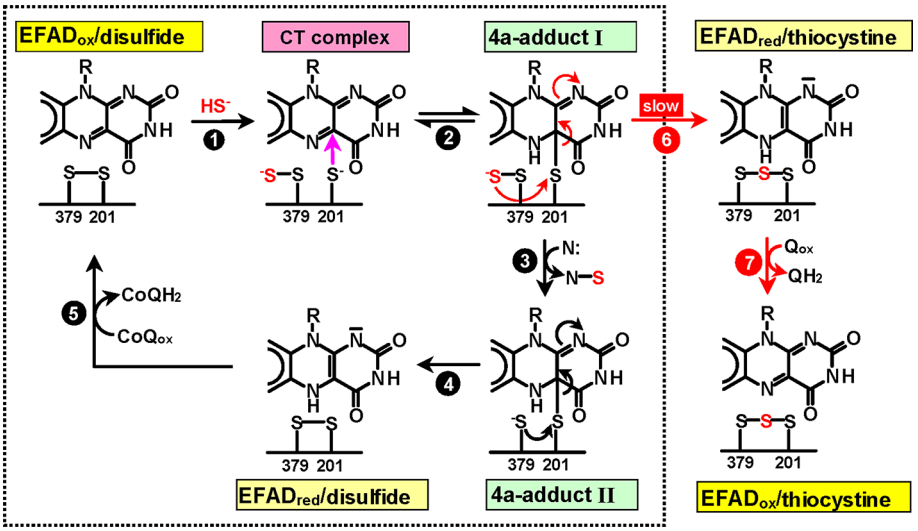
Scheme 2. Catalytic Mechanism Proposed for SQOR Indicated by Steps 1−5 Enclosed within the Dotted Rectangle[a]



[a]The complex formed in step 1 may involve CT interaction with $Cys_{201}S^-$, as indicated, or with $Cys_{379}SS^-$ (not shown). Step 6 is postulated to account for a slow step observed during the anaerobic reaction of the enzyme with sulfide, as discussed in the text.

sluggish rhodanese reaction is unlikely to effectively compete with the much faster rate of GSS^- oxidation observed with SDO ($k_{cat,app}$ = 51 s^{-1}).[c] Finally, it is worth noting that inhibitors of SQOR reduce the level of production of thiosulfate from H_2S by mouse colon.[52] Overall, the results strongly implicate the SQOR reaction as the predominant source of thiosulfate produced during H_2S oxidation by mammalian tissues.

Thiosulfate is the major product of H_2S oxidation in glutathione-depleted cells or mitochondria. Formation of 1 mol of thiosulfate requires an eight-electron oxidation of 2 mol of H_2S. A four-step pathway can account for the observed conversion of H_2S to thiosulfate under these conditions (Scheme 1, path A). Steps 1 and 4 are catalyzed by SQOR. The thiosulfate produced in step 1 is likely to act as the substrate for a sulfur transferase (ST) that regenerates the sulfite consumed in step 1 and produces GSS^-. The latter undergoes a four-electron oxidation reaction catalyzed by SDO (step 3) that produces the sulfite required for step 4 and regenerates the glutathione consumed in step 2. The ST and SDO reactions are likely to be tightly coupled to prevent decomposition of GSS^-, a labile metabolite.[d] The pathway is largely short-circuited by the absence of SDO in ethylmalonic encephalopathy patients because of their impaired ability to produce sulfite. The resulting greatly elevated H_2S levels will promote oxidation of H_2S by SQOR in an alternate reaction that uses sulfide as the acceptor and produces hydrogen disulfide instead of thiosulfate. Hydrogen disulfide is, however, likely to be reduced by glutathione in a futile cycle that regenerates H_2S and causes oxidative stress by depleting the mitochondrial pool of reduced glutathione. In this regard, it is noteworthy that *N*-acetylcysteine, an antioxidant and glutathione precursor, has been shown to reduce the severity of the pathology exhibited by ethylmalonic encephalopathy patients.[53]

It is known that thiosulfate can be converted to sulfate in a glutathione-dependent pathway involving the sequential action of thiosulfate reductase (TSR) and sulfite oxidase (SO)[46,47] (Scheme 1, path B). The operation of both paths A and B in the presence of glutathione means that two pairs of enzymes, SQOR with SO and ST with TSR, will compete for substrates (sulfite and thiosulfate, respectively) that are common to both pathways. Interestingly, SQOR and SO[54] exhibit nearly identical catalytic efficiencies for sulfite utilization, as judged by values obtained for $k_{cat}/K_{m,sulfite}$ (2.1×10^6 and 2.4×10^6 M^{-1} s^{-1}, respectively). Its inner mitochondrial membrane location may favor the ultilization of sulfite by SQOR because reaction with SO requires transport of the metabolite from the matrix to the intermembrane space. However, the availability of H_2S is likely to play a decisive role in the partitioning of sulfite between the two pathways. Consistent with this hypothesis, an elevated level of urinary excretion of thiosulfate is observed under conditions associated with elevated H_2S levels, such as Down's syndrome,[55] environmental exposure to H_2S gas,[56] and ethylmalonic encephalopathy.[1,e] The partitioning of thiosulfate is clearly regulated by the availability of glutathione, probably because 2 mol of glutathione is consumed by the TSR reaction in path B whereas only catalytic amounts are required for the ST−SDO reaction in path A.

Mechanism of SQOR Catalysis. SQOR exhibits homology with flavoprotein disulfide oxidoreductases, such as glutathione reductase and flavocytochrome *c* sulfide dehydrogenase.[23] These enzymes utilize a Cys-S-S-Cys disulfide bridge as a redox center in addition to flavin. Human SQOR contains two cysteine residues (Cys201 and Cys379) that are (i) conserved in all eukaryotic homologues[23] and (ii) essential for catalytic activity.[f] Cys201 aligns with the "proximal" cysteine of the redox-active disulfide in flavocytochrome *c* sulfide dehydrogenase, i.e., the cysteine closer to the C(4a) position of FAD.[57] By analogy with mechanisms observed for other members of the flavoprotein disulfide oxidoreductase family,[58] we propose that the SQOR reaction is initiated by nucleophilic attack of HS^- (the predominant H_2S form at physiological pH) at the distal cysteine, Cys379, to produce an intermediate containing (i) a protein-bound persulfide, $Cys_{379}SS^-$, and (ii) a charge-transfer (CT) complex of FAD with $Cys_{201}S^-$ or $Cys_{379}SS^-$ (Scheme 2, step 1). Nucleophilic attack of $Cys_{201}S^-$ at the C(4a) position of the flavin ring produces a covalent flavin adduct, 4a-adduct I (step 2). Reaction of this intermediate with a nucleophilic acceptor of the sulfane sulfur (N:) generates an intermediate, 4a-adduct II, containing the thiolate form of Cys379 (step 3).

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Nucleophilic attack of $Cys_{379}S^-$ at the sulfur atom in the 4a-adduct produces an intermediate containing 1,5-dihydroFAD plus the original disulfide ($EFAD_{red}$/disulfide) (step 4). The catalytic cycle is completed upon transfer of electrons from $EFAD_{red}$/disulfide to coenzyme Q (step 5).

Consistent with the proposed mechanism, turnover of SQOR requires an acceptor (N:) that may be sulfite, cyanide, or HS^- and produces an expected sulfane sulfur-containing product, thiosulfate, thiocyanate, or hydrogen disulfide, respectively. An intermediate that exhibits a long-wavelength absorption band, centered at 673 nm and extending out to nearly 900 nm, is detected immediately after SQOR is mixed with sulfide under anaerobic conditions. Blue neutral flavin radicals absorb in the long-wavelength region but exhibit maxima at shorter wavelengths and negligible absorbance at 700 nm.[59] On the other hand, CT complexes with oxidized or 1,5-dihydroflavins exhibit maxima in the long-wavelength region that can vary over a wide range (from >500 to ~800 nm), depending on the difference in the one-electron reduction potential of the donor and acceptor.[60−63] The absorption spectrum of the SQOR intermediate is consistent with a CT complex of the oxidized flavin with $Cys_{201}S^-$ or $Cys_{379}SS^-$ that may be in equilibrium with 4a-adduct I. Studies to further characterize this intermediate are in progress.

The SQOR intermediate is slowly converted to a reduced species that exhibits a maximum at 365 nn, a broad shoulder around 450 nm, and a tail of absorbance at λ > 500 nm. The spectral properties of this species are highly suggestive of a 1,5-dihydroflavin complex with a CT acceptor. The complex is, however, formed at a rate that is much slower than turnover, indicating that it is not a kinetically competent intermediate. We propose that a slow rearrangement of 4a-adduct I produces a CT complex of 1,5-dihydroFAD with thiocystine, $Cys_{379}SSSCys_{201}$ (Scheme 2, step 6). The reaction does not require a sulfane sulfur acceptor but does produce a species, $EFAD_{red}$/thiocystine, that could react with coenzyme Q to generate $EFAD_{ox}$/thiocystine (Scheme 2, step 7). Step 7 can account for the reaction observed when the reduced species is mixed with CoQ_1. It is worth noting that thiocystine is postulated as a catalytic intermediate in the oxidative polymerization of sulfide that is observed with prokaryotic SQORs.[25−27]

CONCLUDING REMARKS

H_2S is a signaling molecule with multiple physiological functions but also a highly toxic substance that must be tightly regulated to avoid potential adverse effects, such as inhibition of cytochrome oxidase and mitochondrial respiration. The highly efficient SQOR reaction will rapidly convert H_2S to thiosulfate, a nontoxic metabolite and known intermediate in the mitochondrial oxidation of the gasotransmitter. Nevertheless, the observed metabolism of H_2S to sulfate via a thiosulfate intermediate appears to be somewhat convoluted compared with a hypothetical three-step pathway in which H_2S is directly converted to GSS^- and then sequentially oxidized to sulfite and sulfate ($H_2S \Rightarrow GSS^- \Rightarrow SO_3^{2-} \Rightarrow SO_4^{2-}$). The alternative route is, of course, ruled out by the fact that glutathione does not act as an acceptor of the sulfane sulfur in the SQOR reaction. However, the comparison suggests that the more convoluted metabolism of H_2S via a thiosulfate intermediate may have evolved because the metabolite plays an important role in H_2S signaling. Thus, the zero-valent sulfur in thiosulfate may provide a source of the sulfane sulfur that is required for sulfhydration of cysteine residues in proteins and/or act as an intracellular storage site from which H_2S can be readily mobilized by a thiosulfate reductase. Unraveling the myriad aspects of H_2S signaling will clearly require additional studies.

ASSOCIATED CONTENT

Supporting Information

Sequence of the synthetic gene used to express recombinant SQOR (Figure S1), oxidation of sulfide by SQOR in the presence of cyanide and CoQ_1 (Figure S2), reaction of dithiothreitol with the sulfur oxidation product (Figure S3), oxidation of sulfide by SQOR in the presence of glutathione and CoQ_1 (Figure S4), and effect of sulfide concentration on the observed rate of reduction of CoQ_1 (Figure S5). This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

SQOR, sulfide:quinone oxidoreductase; FAD, flavin adenine dinucleotide; DHPC, 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine; EDTA, ethylenediaminetetraacetate; IMAC, immobilized metal ion affinity chromatography; GSS^-, glutathione persulfide; DTT, dithiothreitol; CT, charge-transfer; ST, sulfur transferase; TSR, thiosulfate reductase.

ADDITIONAL NOTES

[a] At physiological pH, the pool exists as a 3:1 HS^-/H_2S mixture.
[b] G. Hauska, personal communication.
[c] M. R. Jackson and M. S. Jorns, unpublished observations.
[d] SDO has been reported to form a complex with rhodanese.[1] The ST that produces GSS^- is, however, currently unknown.
[e] A minor cysteine catabolic pathway is thought to provide the sulfite required to generate the elevated level of urinary thiosulfate observed in ethylmalonic encephalopathy patients.[1] Cysteinesulfinic acid, produced from cysteine by cysteine dioxygenase, is mainly (70−90%) converted to hypotaurine. However, a small amount of this metabolite may undergo transamination to produce β-sulfinylpyruvate, a compound that spontaneously decomposes to pyruvate and sulfite.[2]
[f] S. L. Melideo and M. S. Jorns, unpublished observations.

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