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Thiosulfate Reduction in *Salmonella enterica* Is Driven by the Proton Motive Force

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Thiosulfate respiration in *Salmonella enterica* serovar Typhimurium is catalyzed by the membrane-bound enzyme thiosulfate reductase. Experiments with quinone biosynthesis mutants show that menaquinol is the sole electron donor to thiosulfate reductase. However, the reduction of thiosulfate by menaquinol is highly endergonic under standard conditions ($\Delta E^{\circ\prime} = -328$ mV). Thiosulfate reductase activity was found to depend on the proton motive force (PMF) across the cytoplasmic membrane. A structural model for thiosulfate reductase suggests that the PMF drives endergonic electron flow within the enzyme by a reverse loop mechanism. Thiosulfate reductase was able to catalyze the combined oxidation of sulfide and sulfite to thiosulfate in a reverse of the physiological reaction. In contrast to the forward reaction the exergonic thiosulfate-forming reaction was PMF independent. Electron transfer from formate to thiosulfate in whole cells occurs predominantly by intraspecies hydrogen transfer.

Thiosulfate is a potential respiratory electron acceptor for bacteria which live in anoxic environments or at the anoxic/oxic interface (6). The ability to respire thiosulfate is conferred by the enzyme thiosulfate reductase which catalyzes the reaction $S_2O_3^{2-} + 2H^+ + 2e^- \rightarrow HS^- + HSO_3^-$.

Thiosulfate is a significant intermediate in the sulfur cycle of anoxic marine and freshwater sediments, where it is involved in reduction, oxidation, and disproportionation pathways (34). The net effect of these reactions is to keep the thiosulfate concentration in these environments relatively low (submicromolar to ca. 10 μM). Thiosulfate reduction in sediments is primarily carried out by sulfate-reducing bacteria. Indeed, sulfate-reducing bacteria preferentially use thiosulfate over sulfate as an electron acceptor (35). In sulfate-reducing bacteria, the sulfite produced in the thiosulfate reductase reaction is further reduced to sulfide by sulfite reductase. Sulfite reduction is an energy-yielding reaction that is also the final step in sulfate respiration. Certain sulfate-reducing bacteria are able to grow by thiosulfate disproportionation to sulfide and sulfate (20), a pathway in which the first step is proposed to be thiosulfate reduction by thiosulfate reductase. Thiosulfate reductase activity is not restricted to sulfate-reducing bacteria but can be found in other types of environmentally abundant bacteria, such as *Shewanella* species (12).

Thiosulfate can also be found in the mammalian gut. Bacteria present in the lumen of the large intestine produce sulfide by reduction of dietary sulfate and sulfite, by fermentation of sulfur-containing amino acids, and by metabolism of sulfated mucopolysaccharides. To protect the animal from the toxic effects of this microbially produced sulfide, mitochondria in the colonic mucosa catalyze the oxidation of sulfide to thiosulfate (25, 29, 47). The thiosulfate produced is then available as a respiratory substrate for colonic bacteria. The ability of certain enteric pathogens to produce sulfide from thiosulfate has been know for almost one hundred years (69) and is the basis of some commercial tests used for strain differentiation in clinical diagnostic laboratories. Genera of enteric bacteria that typically reduce thiosulfate include *Salmonella*, *Proteus*, *Citrobacter*, and *Edwardsiella*.

The molecular basis of thiosulfate respiration has been best studied in the pathogen *Salmonella enterica* serovar Typhimurium. The electron transfer chain of *S. enterica*, like that of the closely related bacterium *Escherichia coli*, possesses three different membrane quinones that mediate electron transfer between respiratory dehydrogenases and terminal reductases (Fig. 1) (49, 76). Ubiquinone (UQ) is the major quinone during aerobic growth, whereas the naphthoquinones menaquinone (MK) and demethylmenaquinone (DMK) are produced and used mainly during anaerobic respiration (for a review, see reference 75). Thiosulfate respiration in *S. enterica* is blocked in strains that cannot synthesize naphthoquinones, suggesting that MKH_2 and/or $DMKH_2$ are the electron donors to thiosulfate reductase (44, 45). Thiosulfate reductase in *S. enterica* is encoded by the *phsABC* operon (28). All three subunits of thiosulfate reductase exhibit sequence similarity to those of *E. coli* formate dehydrogenase-N, an enzyme for which a crystal structure has been obtained (36). By analogy to formate dehydrogenase-N, the PhsA subunit of thiosulfate reductase is predicted to be a peripheral membrane protein containing an active site bis(molybdopterin guanine dinucleotide) molybdenum (MGD) cofactor (Fig. 2) (30). Similarly, PhsC is an integral membrane protein that anchors the other two subunits to the membrane. PhsC is also predicted to contain the site for naphthoquinol oxidation and two heme cofactors located at opposite sides of the membrane (8). PhsB is predicted to possess four iron-sulfur centers that transfer electrons between PhsC and PhsA. In *S. enterica*, the sulfite produced in the thiosulfate reductase reaction can be further reduced by an NADH-linked cytoplasmic sulfite reductase (26, 32). Intriguingly, *S. enterica* has recently been shown to use colonic thiosulfate to support respiratory metabolism during infection by an addi-

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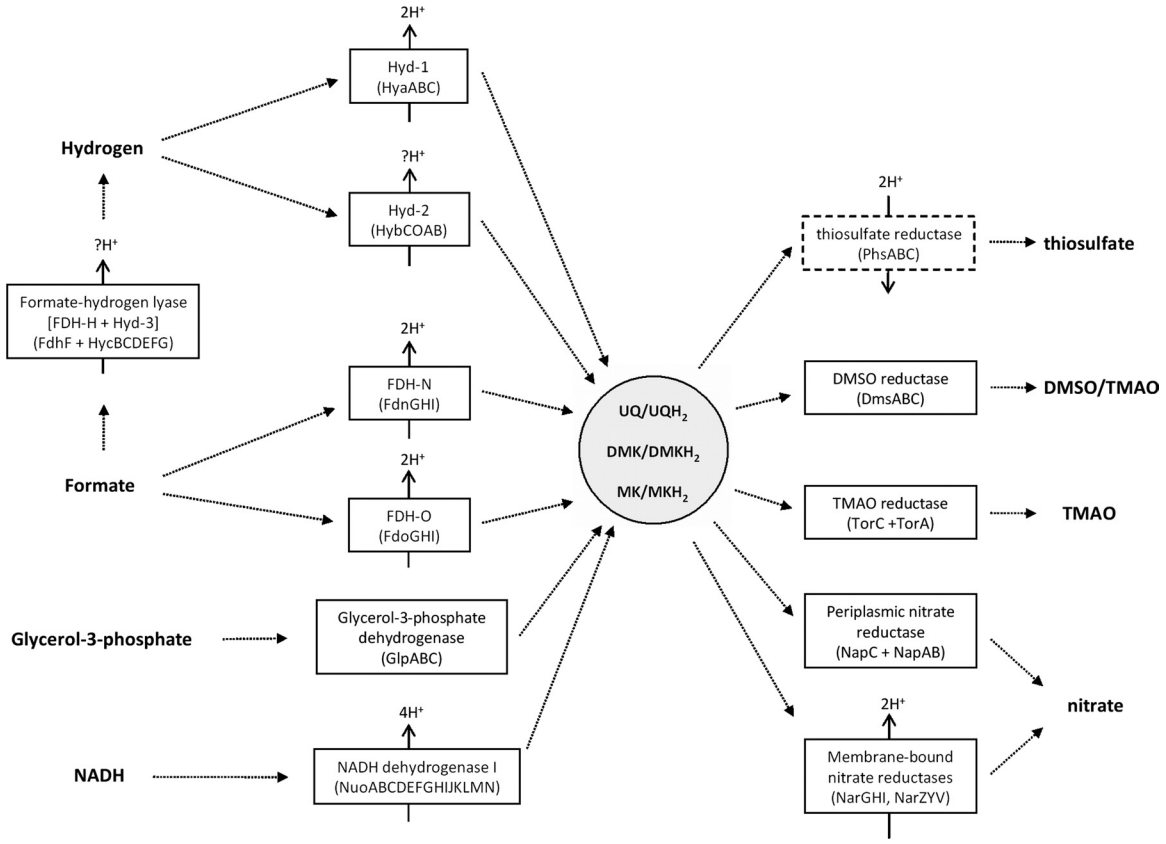

FIG 1 Organization of the anaerobic electron transfer pathways of *S. enterica* LT2 and *E. coli* K-12. Only pathways relevant to this work are depicted. Electron donor molecules are shown on the left of the figure, with arrows indicating possible pathways of electron flow to electron acceptors at the right of the figure. Glyerol-3-phosphate is produced from glycerol by glycerol kinase. Enzymes are boxed. The protein subunits of each enzyme are given in parentheses. All enzymes shown are integral or peripheral membrane proteins with the exception of TorA and NapAB, which are soluble periplasmic enzymes. The H^+:$2e^-$ coupling stoichiometry of each enzyme is shown by an arrow through the box. An upward arrow indicates generation of a PMF, and a downward arrow indicates consumption of the PMF. ?H^+ indicates that PMF generation by the enzyme is likely but has not been experimentally demonstrated. The PMF-generating mechanism of FDH-N is given in Fig. 2. FDH-O, HYD-1, NarGHI, and NarZYV generate a PMF by analogous redox loop mechanisms (8, 76). The dashed box around thiosulfate reductase indicates that this enzyme is present only in *S. enterica* but can be functionally expressed in *E. coli* as in this work. *S. enterica* possesses an additional hydrogen:quinone oxidase reductase that is homologous to HYD-1 but expressed under aerobic conditions and not depicted here (81). UQ, ubiquinone; DMK, demethylmenaquinone; MK, menaquinone; DMSO, dimethyl sulfoxide; TMAO, trimethylamine *N*-oxide. Note that not all enzymes shown interacting with the quinone/quinol pool are able to use all three types of quinone (76).

tional mechanism in which induction of the host macrophage respiratory burst results in oxidation of thiosulfate to the superior electron acceptor tetrathionate (77).

A remarkable aspect of thiosulfate reduction in *S. enterica* is that under standard conditions, the reduction potential of the electron acceptor couple ($E^{\circ\prime}$ [$S_2O_3^{2-}$/HS^- + SO_3^{2-}] = −402 mV) is considerably lower than that of the electron donor couple ($E^{\circ\prime}$ = −74 mV [MK/MKH_2] or +25 mV [$DMK/DMKH_2$]) (64, 70). This results in an unfavorable $\Delta E^{\circ\prime}$ of −328 mV for the reaction when MKH_2 is employed as the electron donor. With DMK as the electron donor, the $\Delta E^{\circ\prime}$ of the reaction becomes 99 mV more unfavorable. The reaction catalyzed by thiosulfate reductase is, therefore, highly endergonic and must be linked to an exergonic process in order to operate in the observed direction. The structural similarity between thiosulfate reductase and formate dehydrogenase-N allows us to propose a possible mechanism by which this could be achieved (Fig. 2) (76). In formate dehydrogenase-N, formate is oxidized at the periplasmic side of the membrane. The electrons released in this reaction are transferred across the membrane by means of the hemes in the membrane subunit to the site of MK reduction, which is located at the cytoplasmic face of the membrane. This mechanism generates a proton motive force (PMF) because electrons have been moved across the membrane from periplasm to cytoplasm (8, 33, 41). The structural similarity of thiosulfate reductase to formate dehydrogenase-N suggests that the routes of electron transfer are the same in the two enzymes (Fig. 2). However, the direction of this electron transfer is reversed in the thiosulfate reductase, with electrons being moved from the cytoplasmic side to the periplasmic side of the membrane. As a consequence, the transmembrane electron movements consume, rather than contribute to, the PMF. The homology model thus suggests a possible mechanism by which the transmembrane PMF is coupled to electron transfer to drive the otherwise endergonic reduction of thiosulfate by the electrons derived from naphthoquinone oxidation.

In this study, we investigate the bioenergetics of thiosulfate respiration in *S. enterica* and test the hypothesis that thiosulfate reduction is driven by the PMF.

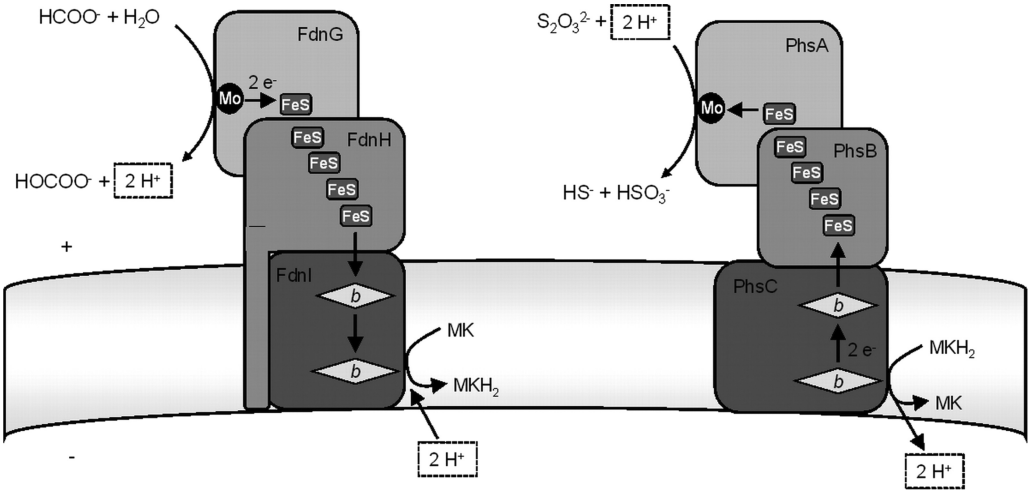

FIG 2 A mechanistic and structural model that accounts for the PMF dependence of thiosulfate reduction in *S. enterica*. The model is based on the sequence similarity between the FdnGHI subunits of *E. coli* formate dehydrogenase-N (left) and the PhsABC subunits of thiosulfate reductase (right). Although formate dehydrogenase-N contains three copies of each subunit, the electron transfer reaction occurs within a single FdnGHI unit. Thus, for simplicity, we depict the enzyme as a heterotrimer. The periplasmic location of the thiosulfate reductase active site is supported by the presence of a Tat signal peptide (7) on the PhsA subunit and by a requirement for the Tat protein transport system for the functional expression of thiosulfate reductase (A. Hinsley and B. C. Berks, unpublished observations). As discussed elsewhere (68), the carboxy-terminal transmembrane helix found on the FdnH subunit of formate dehydrogenase-N is often transferred to the amino terminus of the integral membrane subunit in related proteins, including thiosulfate reductase. The following cofactors are indicated: FeS, iron sulfur cluster; Mo, *bis*(molybdopterin guanine dinucleotide) molybdenum (or MGD) cofactor; *b*, heme b.

MATERIALS AND METHODS

Strains and growth conditions. The strains and plasmids used in this study are listed in Table 1. *S. enterica* and *E. coli* strains were cultured on Luria-Bertani (LB) medium at 37°C (53). For experiments in which induction of the *phsABC* operon was desired, cells were grown to an optical density at 578 nm (OD_{578}) of 0.6 in anoxic liquid culture supplemented with 50 mM sodium thiosulfate together with the electron donor to be used in subsequent experiments with the cells (25 mM sodium formate or 50 mM glycerol or 100% hydrogen in the gas phase). Anoxic conditions were obtained by sparging the medium with oxygen-free nitrogen (or hydrogen as appropriate) for 5 min following inoculation. Antibiotics were used at the following concentrations: ampicillin (Ap), 50 to 100 μg ml^{-1}; kanamycin (Km), 25 to 50 μg ml^{-1}.

Cells were harvested by centrifugation, washed two times in ice-cold 10 mM sodium phosphate (pH 7.4) and 1 mM dithiothreitol (DTT), then resuspended in 50 mM sodium phosphate (pH 7.4) and 1 mM DTT, and used immediately for activity assays. No attempt to maintain anaerobic conditions during cell preparation was made.

To prepare spheroplasts, washed cells were resuspended to an OD_{578} of 3 in 200 mM Tris-HCl (pH 7.4), 20% sucrose, 10 mM EDTA, 1 mM DTT, and 0.6 mg/ml lysozyme, followed by an incubation for 30 min at 37°C. Spheroplasts were pelleted, resuspended in 50 mM sodium phosphate buffer (pH 7.4), 20% sucrose, and 1 mM DTT, and used immediately.

Analytical methods. Respiratory chain activity assays were performed in 50 mM sodium phosphate (pH 7.4) at 37°C. Assays were carried out in

TABLE 1 Strains and plasmids

Strain or plasmid	Relevant genotype	Relevant phenotype	Source or reference
Salmonella enterica			
LT2a			2
EB303	LT2a *cysI68 asr-1*::Tn*5* (Km^r)	Sulfite reductase deficient	32
Escherichia coli			
DH5α	*fhuA2* Δ(*argF-lacZ*)*U169 phoA glnV44* ϕ80 Δ(*lacZ*)M15 *gyrA96 recA1 relA1 endA1 thi-1 hsdR17*		27
BW25113	$lacI^q$ $rrnB_{T14}$ $\Delta lacZ_{WJ16}$ *hsdR514* $\Delta araBAD_{AH33}$ $\Delta rhaBAD_{LD78}$		16
JW5581	BW25113 Δ*ubiE*::Km^r	UQ and MK deficient	Keio collection (4)
JW5713	BW25113 Δ*ubiC*::Km^r	UQ deficient	Keio collection (4)
JW3901	BW25113 Δ*menA*::Km^r	MK and DMK deficient	Keio collection (4)
MC4100	F^- Δ*lacU169 araD139 rpsL150 relA1 ptsF rbs flbB5301*		13
FTD89	MC4100 Δ*hyaB* Δ*hybC*	Hydrogenase-1 and -2 deficient	54
FTD147	MC4100 Δ*hyaB* Δ*hybC* Δ*hycE*	Hydrogenase-1, -2, and -3 deficient	17
HD705	MC4100 Δ*hycE*	Hydrogenase-3 deficient	56
Plasmid pAH2	*phsABC* (−48 bp before transcript start site to 3′ NsiI site) in pUC18 (Ap^r)	Thiosulfate reductase expression plasmid	A. Hinsley (unpublished)

TABLE 2 Effect of quinone biosynthesis mutations on thiosulfate reduction in *E. coli* expressing *S. enterica* thiosulfate reductase[a]

Bacterial strain	Quinone(s) present	Thiosulfate reductase sp act (nmol thiosulfate reduced/min/mg protein) with electron donor:		
		Hydrogen (H)	Glycerol (G)	$MV^{\cdot+}$ (G)
BW25113(pAH2)	UQ, MK, DMK	28 ± 9 (2)	27 ± 4 (3)	246 ± 27 (2)
BW25113	UQ, MK, DMK	ND	0 ± 0 (2)	21 ± 8 (2)
JW5713(pAH2)	MK, DMK	20 ± 10 (2)	30 ± 2 (3)	218 ± 42 (2)
JW5581(pAH2)	DMK	3 ± 1 (2)	8 ± 4 (3)	242 ± 7 (2)
JW3901(pAH2)	UQ	0 ± 0 (2)	1 ± 0 (3)	343 ± 4 (2)

[a] The indicated strains were transformed with plasmid pAH2 carrying *S. enterica phsABC*. The bacteria were cultured anaerobically in LB medium supplemented with 50 mM sodium thiosulfate and either 100% hydrogen (H) in the gas phase or 50 mM glycerol (G). After harvesting and washing, the bacteria were assayed for sulfide production from thiosulfate with hydrogen or glycerol as the electron donor or assayed for thiosulfate-dependent $MV^{\cdot+}$ oxidation. The standard deviations of the measured activities are given. The numbers of biological repeats are shown in parentheses. UQ, ubiquinone; MK, menaquinone; DMK, demethylmenaquinone; ND, not determined.

glass vials sealed with a butyl rubber septum and rendered anoxic by sparging with oxygen-free nitrogen. Samples were removed at different time points and assayed for sulfide or nitrite production or thiosulfate consumption. Due to the presence of an induction phase for thiosulfate reductase activity in some experiments (see Fig. 3 for an example), specific activities were calculated from the linear part of the curve. Sulfide concentrations were determined by the methylene blue method of Fogo and Popowsky (21) as described in Hallenbeck et al. (26). All measurements of thiosulfate reduction in *S. enterica* by using the sulfide production assay were carried out with strain EB303, which lacks dissimilatory sulfite reductase (Asr). This ensured that only sulfide production by thiosulfate reductase, and not from further reduction of sulfite by sulfite reductase, contributed to the measured sulfide concentration. Thiosulfate concentrations were determined by high-performance liquid chromatography (HPLC) with an Aminex HPX87H column (300 by 7.8 mm; Bio-Rad) at 60°C, in a liquid phase containing 6.5 mM H_2SO_4 and at a flow rate of 550 μl/min. Thiosulfate was quantified by absorbance at 215 nm relative to known standards. Nitrite concentrations were determined by the method of Smarelli and Campbell (66). The assay was performed in 25 mM potassium phosphate buffer (pH 7.3) containing 0.05 mM EDTA, 10 mM KNO_3, and a 0.42 mM concentration of the electron donor at 30°C. The reaction was stopped with 200 μl 58 mM sulfanilamide in 3 N HCl and 200 μl 0.77 mM *N*-(1-naphthyl)ethylenediamine dihydrochloride. The samples were incubated for 10 min at room temperature and centrifuged at 20,000 × *g* for 2 min, and the absorbance of the supernatant was measured at 540 nm. Protein concentrations were determined by the method of Schmidt et al. (63). All specialty chemicals were obtained from Sigma with the exceptions of TCS (3,3′,4′,5-tetrachlorosalicylanilide; Kodak) and DMN (2,3-dimethyl-1,4-naphthoquinone; synthesized according to the method of Kruber [43]; gift from the late A. Kröger, Frankfurt, Germany).

Spectrophotometric enzyme assays. Methyl viologen (MV), benzyl viologen (BV), and DMN-linked assays were carried out in quartz (for DMN-linked assays) or optical glass (for BV/MV-linked assays) cuvettes sealed with a butyl rubber stopper and rendered anoxic by sparging with oxygen-free nitrogen. The assay solution contained 50 mM sodium phosphate (pH 7.4) and 1 mM BV^{2+}, 1 mM MV^{2+}, or 0.2 mM DMN, and the respective substrates (10 mM sodium thiosulfate, 2 mM sodium sulfide plus 2 mM sodium sulfite, or 100% hydrogen at 1 atm in the gas phase). For experiments measuring $MV^{\cdot+}$ oxidation, MV^{2+} was first reduced with sodium dithionite titrated into the cuvette from a 1 mg/ml stock. The oxidation or reduction of the dyes was determined spectrophotometrically using the following changes in extinction coefficients: ε_{600} ($MV^{\cdot+} - MV^{2+}$), 13 mM^{-1} cm^{-1}; ε_{546} ($BV^{\cdot+} - BV^{2+}$), 19.5 mM^{-1} cm^{-1}; ($DMN - DMNH_2$) $\varepsilon_{270-290}$, 15 mM^{-1} cm^{-1} (42, 46).

RESULTS

Menaquinol is the electron donor to thiosulfate reductase. *S. enterica* possesses three respiratory quinones, namely, ubiquinone (UQ) and the naphthoquinones menaquinone (MK) and demethylmenaquinone (DMK). Previous studies have demonstrated that naphthoquinones, but not UQ, are involved in thiosulfate respiration (44, 45). However, it was not established whether both or only one of the naphthoquinols is able to serve as the electron donor to thiosulfate reductase. As the $E^{\circ\prime}$ for DMK reduction is much higher than that for MK reduction ($E^{\circ\prime}$ [MK/MKH_2] = −74 mV; [DMK/$DMKH_2$] = +25 mV) (64, 70, 76), thiosulfate reduction by $DMKH_2$ would be thermodynamically even more unfavorable than MKH_2-mediated reduction.

The model bacterium *Escherichia coli* is closely related to *S. enterica* but is unable to respire thiosulfate. However, *E. coli* cells transformed with a plasmid bearing the *S. enterica phsABC* operon show high levels of thiosulfate reduction with either physiological or artificial electron donors (22). This recombinant *E. coli* system allows the behavior of *S. enterica* thiosulfate reductase to be probed using *E. coli* genetic backgrounds and is used in this work for all experiments employing *E. coli* strains.

The abilities of the two respiratory naphthoquinols to act as electron donors to *S. enterica* thiosulfate reductase were assessed using an *E. coli ubiE* mutant that synthesizes DMK but not MK or UQ (78). High rates of thiosulfate reduction with either hydrogen or glycerol as the electron donor were observed for the parental strain but not for the DMK-only (*ubiE*) mutant (Table 2). Control experiments using methyl viologen radical ($MV^{\cdot+}$) as a direct electron donor to thiosulfate reductase verified that thiosulfate reductase was expressed at equivalent levels in the two strains. Since both hydrogen and glycerol-3-phosphate are able to reduce DMK (78), the very low rates of thiosulfate reduction exhibited by the *ubiE* mutant imply that $DMKH_2$ is a poor substrate for thiosulfate reductase. By inference, MKH_2 is the principal electron donor to thiosulfate reductase. The residual levels of hydrogen or glycerol-dependent thiosulfate reductase activity in the *ubiE* mutant may be due to trace amounts of MK produced by the mutant strain (<1% of the MK found in the parental strain in the study of Wissenbach et al. [78]) or may reflect a low thiosulfate reductase activity with $DMKH_2$ as the electron carrier. By use of a qualitative iron agar growth assay, thiosulfate reduction in cells metabolizing a complex carbon source was also found to depend on MK (data not shown). Experiments using a *menA* strain that produces UQ, but not MK or DMK, confirm that UQ is not an electron donor to thiosulfate reductase (Table 2).

Thiosulfate reduction requires the proton motive force. The proposal that the thiosulfate reductase reaction is driven by the

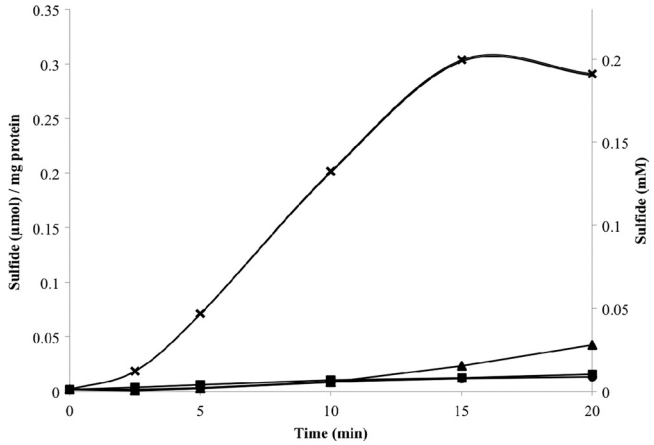

FIG 3 The effect of protonophores on sulfide production from thiosulfate in a cell suspension of *Salmonella enterica* serovar Typhimurium. *S. enterica* EB303 was cultured anaerobically in LB medium with 25 mM sodium formate and 50 mM sodium thiosulfate. Washed cells (0.66 mg protein/ml) were incubated under anoxic conditions at 37°C in 10 mM sodium phosphate (pH 7.4) and 1 mM DTT with 10 mM sodium thiosulfate and 4 mM sodium formate. Samples were removed at the indicated time points and assayed for sulfide formation. ×, no addition; ●, 10 μM CCCP; ■, 20 μM FCCP; ▲, 50 μM TCS.

proton motive force (PMF) was tested by investigating the effects of protonophores and ionophores on the reduction of thiosulfate. Intact cells of *S. enterica* were able to produce sulfide from thiosulfate under anaerobic conditions when formate, glycerol, or hydrogen was present as an electron donor (Fig. 3 and Table 3). Sulfide production was nearly completely inhibited (<5% residual activity) when low concentrations of the protonophore carbonyl cyanide-3-chlorophenylhydrazone (CCCP), carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP), or 3,3′,4′,5-tetrachlorosalicylanilide (TCS) was added to the bacterial suspension (Fig. 3 and Table 3). Control experiments showed that cell swimming (observed by light microscopy), which depends upon PMF-driven flagellar rotation, was blocked at the protonophore concentrations used. Valinomycin is an ionophore that disrupts the electrical component of the PMF. Addition of 10 μM valinomycin reduced sulfide production from thiosulfate with formate as the electron donor by *S. enterica* spheroplasts by about 70% (spheroplasts were used to facilitate ionophore access to the cytoplasmic membrane). A combination of 10 μM valinomycin together with 5 μM nigericin, an ionophore that dissipates the proton concentration gradient component of the PMF, inhibited sulfide production in spheroplasts by 80%. In *E. coli* expressing *S. enterica* thiosulfate reductase [*E. coli* DH5α(pAH2)], the protonophore CCCP inhibited the reduction of thiosulfate by formate, hydrogen, or glycerol by at least 90% (Table 3). Thus, in both *S. enterica* and recombinant *E. coli*, thiosulfate reduction with physiological electron donors is strongly inhibited by agents that degrade the PMF.

To identify the site responsible for the PMF dependence of thiosulfate reduction, different sections of the respiratory chain were tested for their sensitivity to protonophores. Electron transport to nitrate from hydrogen, formate, or glycerol was not inhibited by the addition of CCCP (Table 3), even though a PMF is essential for the reduction of thiosulfate by the same electron donors. For each of these electron donors, the respiratory chains to thiosulfate and nitrate diverge only after the quinone/quinol pool (Fig. 1) (67, 76). It can, therefore, be inferred that the PMF-dependent site in thiosulfate respiration lies between MKH_2 and thiosulfate and, thus, that the PMF acts directly on thiosulfate reductase. To test this proposal, individual oxidoreductase activities were assayed using the MK analogue 2,3-dimethyl-1,4 naphthoquinone (DMN) (74). The reduction of DMN by hydrogen or glycerol was insensitive to the presence of the protonophore CCCP (Table 3), confirming that the PMF-dependent step in thiosulfate respiration is located after these dehydrogenases. The assay of menaquinol:thiosulfate oxidoreductase with $DMNH_2$ is complicated by the low-level activity of the reaction

TABLE 3 Effect of the protonophore CCCP on thiosulfate reduction in *S. enterica* and recombinant *E. coli*[a]

	Sp act (nmol electron acceptor reduced/min/mg protein)			
	S. enterica EB303		*E. coli* DH5α(pAH2)	
Reaction (growth medium)	No addition	10 μM CCCP	No addition	10 μM CCCP
Electron transport chains				
Formate → thiosulfate (A)	29.1 ± 10.8 † (6)	0.8 ± 0.1 † (3)	13.5 ± 1.2 † (3)	1.4 ± 0.5 † (3)
Glycerol → thiosulfate (B)	14.1 ± 2.0 † (2)	0.6 ± 0.1 † (2)	13 ± 4 ‡ (2)	1 ± 1 ‡ (2)
Hydrogen → thiosulfate	52.2 ± 10.4 † (4) (A)	0.7 ± 0.5 † (4) (A)	6.7 ± 3 † (2) (C)	0.7 ± 0 † (2) (C)
No added donor → thiosulfate (A)	4.2 ± 0.6 † (2)	ND	3 ± 3 † (2)	ND
Formate → no added acceptor (A)	0 ± 0 † (2)	ND	ND	ND
Hydrogen → nitrate (A)	7.1 ± 3.6 § (2)	6.0 ± 1.1 § (2)	2.8 ± 0.4 § (2)	6.7 ± 0.6 § (2)
Formate → nitrate (A)	0.8 ± 0.3 § (2)	4.9 ± 0.4 § (2)	1.1 ± 0.0 § (2)	2.1 ± 0.4 § (2)
Glycerol → nitrate (A)	3.8 ± 0.3 § (2)	4.6 ± 0.3 § (2)	0.9 ± 0.0 § (2)	1.5 ± 0.2 § (2)
Partial reactions				
Hydrogen → DMN (A)	64 ± 15 ¶ (4)	69 ± 6 ¶ (4)	57 ± 8 ¶ (3)	55 ± 6 ¶ (2)
Glycerol → DMN (A)	39 ± 8 ¶ (2)	38 ± 7 ¶ (3)	52 ± 4 ¶ (7)	45 ± 11 ¶ (3)
$MV^{\cdot+}$ → thiosulfate (A)	308 ± 48 ‖ (5)	317 ± 32 ‖ (4)	574 ± 70 ‖ (4)	589 ± 18 ‖ (4)

[a] Bacteria were cultured anaerobically in LB medium supplemented with 50 mM sodium thiosulfate and 25 mM sodium formate (A), 50 mM glycerol (B), or 100% hydrogen in the gas phase (C). Specific activities of whole cells were determined by the production of sulfide (†), consumption of thiosulfate (‡), production of nitrite (§), reduction of 2,3-dimethyl-1,4-naphthoquinone (DMN) (¶), or oxidation of methyl viologen radical ($MV^{\cdot+}$) (‖). Electron donors and acceptors for the electron transport chain assays were used at the following concentrations: 4 mM sodium formate, 4 mM glycerol, 1 atmosphere hydrogen, 2 mM sodium thiosulfate, and 0.04 mM potassium nitrate. The standard deviations of the measured activities are given. The number of biological repeats is shown in brackets. ND, not determined.

and by interference from other enzymes maintaining MKH_2 (and $DMNH_2$) in the reduced state (74). Therefore, only the partial reaction of thiosulfate reductase with the dye methyl viologen could be tested. Methyl viologen-linked thiosulfate reduction in *S. enterica*, or in the recombinant *E. coli* strain, was unaffected by the addition of CCCP (Table 3). The methyl viologen radical ($MV^{\cdot+}$) has a significantly lower reduction potential ($E^{\circ\prime}$ [MV^{2+}/$MV^{\cdot+}$] = −440 mV) than MK ($E^{\circ\prime}$ [MK/MKH_2] = −74 mV) and so might bypass the MKH_2-oxidizing site of thiosulfate reductase. If this is the case, then the PMF-sensitive site in thiosulfate reductase lies between the sites of electron donation by MKH_2 and $MV^{\cdot+}$. Alternatively, the electrons from $MV^{\cdot+}$ may take the physiological electron transfer route within the enzyme but be of sufficiently low potential that they are able to overcome the requirement for a PMF in thiosulfate respiration. The first explanation is supported by the carbon monoxide inhibition data reported below. In either case, the data indicate that catalysis at the thiosulfate reductase active site is PMF independent.

In summary, the data presented here confirm the prediction that thiosulfate respiration in *S. enterica* is driven by the PMF. They further show that the PMF acts on the electron transfer pathway within thiosulfate reductase but prior to the thiosulfate-reducing catalytic site, consistent with the hypothesis that the PMF is required to drive transmembrane electron transfer within thiosulfate reductase (Fig. 2).

Thiosulfate respiration in *S. enterica* is close to thermodynamic equilibrium. Sulfide production from thiosulfate by cell suspensions of *S. enterica* slows over time and then ceases (Fig. 3 and 4). This behavior is characteristic of a reaction that is close to thermodynamic equilibrium. Figure 4 compares levels of sulfide production by *S. enterica* EB303 at two initial thiosulfate concentrations (2 mM and 10 mM). *S. enterica* EB303 lacks sulfite reductase and is therefore unable to convert the sulfite produced by thiosulfate reduction to sulfide. The initial reaction velocities are similar at both thiosulfate concentrations, demonstrating thiosulfate saturation of the enzyme active site in both cases. In the experiments with 2 mM and 10 mM initial thiosulfate concentrations, sulfide production ceased at final sulfide concentrations of 0.21 mM and 0.27 mM, respectively (Fig. 4). When a 200-fold excess of MKH_2 over MK is assumed and the Nernst equation is applied, these sulfide concentrations yield respective $\Delta E'$ values of −208 mV and −192 mV. For aerobically growing *E. coli*, PMF values around −200 mV have been reported (24, 83), and the PMF in anaerobically respiring *E. coli* and *S.* Typhimurium is 15 to 20 mV less negative (65, 72), suggesting a PMF of up to −180 mV for the anaerobically respiring bacteria. Therefore, the $\Delta E'$ values for thiosulfate respiration are in a similar range to the reported PMF values and are consistent with the hypothesis that the physiological thiosulfate reductase reaction is close to equilibrium with the PMF. Further evidence for this hypothesis comes from an experiment in which 0.19 mM sulfide was added to the cells at the same time as 2 mM thiosulfate. Due to the high concentration of sulfide present at the start of the experiment, the thiosulfate reductase reaction was already close to equilibrium, and only limited additional sulfide was produced from thiosulfate (measured final sulfide concentration of 0.24 mM corresponding to a $\Delta E'$ of −189 mV; Fig. 4). Conversely, addition of 2 mM zinc acetate to precipitate free sulfide from the reaction as insoluble zinc sulfide allowed the reaction to continue (Fig. 4). Similarly, lowering the potential of the direct electron donor to thiosulfate reductase by using $MV^{\cdot+}$ ($E^{\circ\prime}$ [MV^{2+}/$MV^{\cdot+}$] = −440 mV) instead of supplying electrons by the physiological route through MKH_2 overcame the inhibition of thiosulfate reduction by 0.2 mM sodium sulfide ($MV^{\cdot+}$-dependent rate without sulfide, 230 ± 45 nmol thiosulfate reduced/min/mg protein; $MV^{\cdot+}$-dependent rate in the presence of 0.2 mM Na-sulfide, 220 ± 35 nmol thiosulfate reduced/min/mg protein). This experiment also rules out the possibility of an inhibitory effect of sulfide on the thiosulfate-reducing catalytic site.

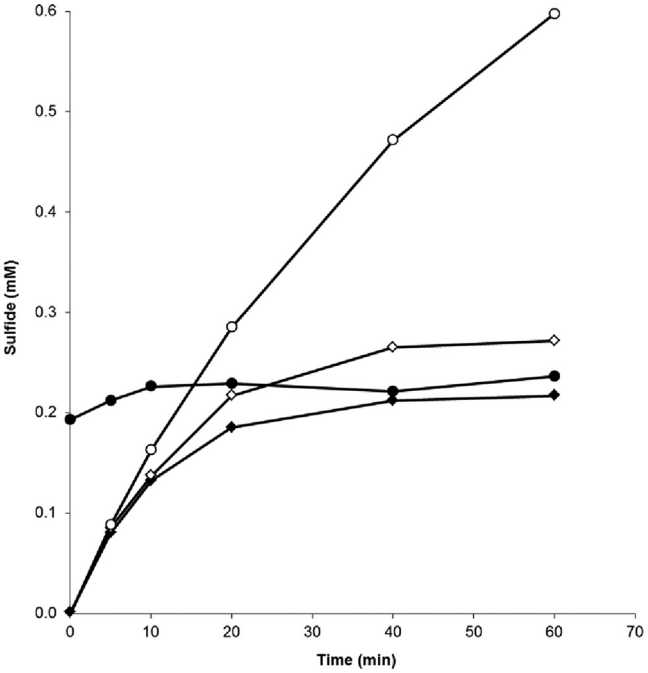

FIG 4 Thiosulfate reduction in *S. enterica* is close to thermodynamic equilibrium. *S. enterica asr* mutant EB303 was cultured anaerobically in LB medium supplemented with 50 mM sodium thiosulfate and 25 mM sodium formate. Washed cells (0.95 mg protein/ml) were incubated under anoxic conditions at 37°C with 4 mM sodium formate and 2 mM sodium thiosulfate (◆), 10 mM sodium thiosulfate (◇), 2 mM sodium thiosulfate and 0.19 mM sodium sulfide (●), or 2 mM sodium thiosulfate and 2 mM zinc acetate (○). Samples were removed at the indicated time points and assayed for sulfide concentration.

In summary, these experiments show that the physiological thiosulfate reductase reaction is close to thermodynamic equilibrium with the PMF.

The thiosulfate reductase reaction can be reversed under physiological conditions and is then PMF independent. Since the reduction of thiosulfate with MKH_2 is thermodynamically unfavorable, the reverse reaction (formation of thiosulfate from sulfite and sulfide with MK as an electron acceptor) should be thermodynamically favorable. The ability of thiosulfate reductase to catalyze the reverse reaction was therefore investigated.

E. coli expressing *S. enterica* thiosulfate reductase was able to catalyze the oxidation of a combination of sulfide and sulfite with benzyl viologen (BV^{2+}) or DMN as the electron acceptor (Table 4). This activity was found only in cells expressing thiosulfate reductase and required both sulfide and sulfite to be present. These experiments show that the chemistry at the thiosulfate reductase active site is reversible.

Combined sulfide and sulfite oxidation also occurred under physiological conditions with trimethylamine *N*-oxide (TMAO)

TABLE 4 Thiosulfate production from a combination of sulfide plus sulfite by *S. enterica* thiosulfate reductase[a]

Reaction	Sp act (nmol sulfide + sulfite oxidized/min/mg protein)		
	E. coli DH5α(pAH2)		
	No addition	10 μM CCCP	*E. coli* DH5α (no addition)
Sulfide + sulfite → BV^{2+} (a)	252 ± 92 (9)	240 ± 88 (4)	2 ± 2 (3)
Sulfite → BV^{2+} (a)	0 ± 1 (2)	ND	ND
Sulfide → BV^{2+} (a)	0 ± 1 (2)	ND	ND
Sulfide + sulfite → DMN (b)	55 ± 5 (8)	53 ± 7 (2)	14 ± 7 (3)
Sulfide + sulfite → TMAO (c)*	258 ± 147 (5)	274 ± 42 (2)	0 ± 0 (2)
Sulfide + sulfite (c)*	4 ± 5 (2)	ND	ND

[a] *E. coli* strain DH5α, transformed where indicated with plasmid pAH2 carrying *S. enterica phsABC*, was cultured anaerobically in LB medium supplemented with 50 mM sodium thiosulfate and 50 mM glycerol. In experiments labeled with an asterisk (*), the medium was additionally supplemented with 50 mM trimethylamine *N*-oxide (TMAO). After harvesting and washing, the bacteria were assayed for the indicated activities. Specific activities correspond to the reduction of benzyl viologen (BV^{2+}) (a) or 2,3-dimethyl-1,4-naphthoquinone (DMN) (b) or the production of thiosulfate (c). Electron donors and acceptors for the electron transport chain assays were used at the following concentrations: 2 mM sodium sulfide and 2 mM sodium sulfite, 5 mM TMAO. The standard deviations of the measured activities are given. The number of biological repeats is shown in brackets. ND, not determined.

TABLE 5 Involvement of quinones in combined sulfide and sulfite oxidation by TMAO[a]

Bacterial strain	Quinone(s) present	Sp act (nmol sulfide + sulfite oxidized/min/mg protein)
BW25113(pAH2)	UQ, MK, DMK	328 ± 36
JW5713(pAH2)	MK, DMK	388 ± 93
JW5581(pAH2)	DMK	599 ± 25
JW3901(pAH2)	UQ	0 ± 0

[a] *E. coli* strains were transformed with plasmid pAH2 carrying *S. enterica phsABC* and cultured anaerobically in LB medium supplemented with 50 mM sodium thiosulfate, 50 mM glycerol, and 50 mM trimethylamine *N*-oxide (TMAO). After harvesting and washing, the bacteria were assayed for thiosulfate formation from sulfide plus sulfite with TMAO as electron acceptor. The standard deviations of the measured activities (two biological repeats) are given.

as the electron acceptor (Table 4). Since the electron transfer pathways to TMAO receive electrons from MKH_2 and $DMKH_2$ (Fig. 1) (48, 78), it can be inferred that electrons derived from the combined oxidation of sulfide and sulfite at the thiosulfate reductase active site flow backwards through the enzyme to reduce the naphthoquinone pool. This deduction was confirmed by experiments using *E. coli* quinone biosynthesis mutants which showed that naphthoquinones are essential for electron transfer from sulfide plus sulfite to TMAO (Table 5). It is striking that this reverse thiosulfate reductase reaction can function with DMK as the electron acceptor (Table 5) while the physiological forward reaction cannot utilize $DMKH_2$ (Table 2). This suggests that the inability of $DMKH_2$ to support thiosulfate reduction is due to the unfavorable redox potential of $DMKH_2$ relative to MKH_2 rather than discrimination between the two types of naphthoquinol by the quinol-oxidizing site of thiosulfate reductase.

Combined sulfide and sulfite oxidation by bacterial cells was unaffected by the addition of the protonophore CCCP, regardless of whether BV^{2+}, DMN, or TMAO was used as the electron acceptor (Table 4). Thus, the reverse reaction of thiosulfate reductase, including the full reversal of electron flow in the enzyme that is required when MK or DMK is the electron acceptor, is independent of PMF. This is in stark contrast to the essential role of the PMF in the forward reaction with the physiological electron donor MKH_2. This comparison represents further evidence that the role of the PMF in thiosulfate reduction is to provide the thermodynamic driving force for the otherwise endergonic electron flow from MKH_2 to thiosulfate rather than being required by the catalytic chemistry of the enzyme.

Hydrogen is an intermediate in electron transfer from formate to thiosulfate. Formate is a good electron donor for thiosulfate respiration in *S. enterica* and in recombinant *E. coli* expressing thiosulfate reductase (Table 3). *S. enterica* and *E. coli* have multiple enzymes for oxidizing formate (57), and two pathways for transferring electrons from formate to thiosulfate reductase via the MK pool are conceivable (Fig. 1). In one pathway, the isoenzymes formate dehydrogenase-N (FDH-N) and formate dehydrogenase-O (FDH-O) donate electrons from formate directly to MK. In contrast, formate dehydrogenase-H (FDH-H) associates with hydrogenase-3 (HYD-3) to form the formate-hydrogen lyase (FHL) complex, in which formate reduces protons to hydrogen. Since hydrogen is also a good electron donor for thiosulfate respiration (Table 2), it is conceivable that electrons from formate could reach thiosulfate by a pathway in which hydrogen produced by FHL is subsequently oxidized by the MK-reducing hydrogenases HYD-1 and HYD-2. Such hydrogen cycling has been inferred to occur in both *S. enterica* and *E. coli* under fermentative growth conditions (1, 52, 82). The involvement of these two possible formate-oxidizing pathways in electron transfer from formate to thiosulfate was assessed.

Carbon monoxide is a hydrogenase inhibitor (5, 31, 59) and was used in an attempt to distinguish between hydrogen-dependent and hydrogen-independent routes of electron transfer from formate to thiosulfate. Formate-dependent thiosulfate reduction was almost completely inhibited in *S. enterica* when the assay vessels contained 100% carbon monoxide in the headspace (Table 6). However, carbon monoxide was also found to block electron transfer from glycerol to thiosulfate even though glycerol-3-phosphate dehydrogenase is not sensitive to carbon monoxide (demonstrated by the failure of carbon monoxide to inhibit nitrate reduction by glycerol) (Table 6). In addition, thiosulfate reduction by glycerol was still severely inhibited by carbon monoxide (<3% residual activity) in an *E. coli* mutant lacking all three hydrogenases (HYD-1, HYD-2, and HYD-3). Thus, the inhibitory effect of carbon monoxide on electron transport from glycerol-3-phosphate to thiosulfate does not arise from the involvement of a hydrogen intermediate. By implication, carbon monoxide acts directly on thiosulfate reductase. However, thiosulfate reduction with $MV^{\cdot+}$ as an electron donor was unaffected by carbon monoxide (Table 6). Thus, it can be inferred that the carbon monoxide-sensitive site in thiosulfate reductase lies between the site of MKH_2 oxidation and the last site at which $MV^{\cdot+}$ can act as an electron donor to the enzyme. Carbon monoxide is a weak metal ligand that would not be expected to bind to the predicted electron transfer centers of thiosulfate reductase. The inhi-

TABLE 6 Effect of carbon monoxide (CO) on sulfide production from thiosulfate[a]

Reaction (growth condition)	Sp act (nmol thiosulfate or nitrate reduced/min/mg protein)
Formate → thiosulfate (A)	34.1 ± 9.5 †
Formate → thiosulfate [+ CO] (A)	0.3 ± 0.1 †
Glycerol → thiosulfate (B)	27.8 ± 6.4 †
Glycerol → thiosulfate [+ CO] (B)	0.8 ± 0 †
$MV^{\cdot+}$ → thiosulfate (A)	155 ± 5 ‡
$MV^{\cdot+}$ → thiosulfate [+ CO] (A)	165 ± 5 ‡
Glycerol → nitrate (A)	3.8 ± 0.3 §
Glycerol → nitrate [+ CO] (A)	10.4 ± 0.7 §

[a] *S. enterica* EB303 was cultured anaerobically in LB medium supplemented with 50 mM sodium thiosulfate and 25 mM sodium formate (A) or 50 mM glycerol (B). Specific activities of washed whole cells were determined by the production of sulfide (†), oxidation of methyl viologen radical ($MV^{\cdot+}$) (‡), or production of nitrite (§). The electron donor and acceptor concentrations used were the same as those in Table 2. The indicated assays (+ CO) contained one atmosphere carbon monoxide. The standard deviations of the measured activities (two biological repeats) are given.

TABLE 7 Thiosulfate reduction in *E. coli* hydrogenase mutants expressing *S. enterica* thiosulfate reductase[a]

Electron donor	Thiosulfate reductase sp act (nmol thiosulfate reduced/min/mg protein)			
	MC4100	FTD89 (HYD-1$^-$ HYD-2$^-$)	FTD147 (HYD-1$^-$ HYD-2$^-$ HYD-3$^-$)	HD705 (HYD-3$^-$)
Formate	15 ± 3	4 ± 1	3 ± 1	3 ± 1
Glycerol	30 ± 4	31 ± 3	27 ± 2	26 ± 1
None	3 ± 1	4 ± 1	4 ± 1	4 ± 1

[a] Each strain was transformed with plasmid pAH2 carrying *S. enterica phsABC*. The strains were cultured anaerobically in LB medium supplemented with 50 mM sodium thiosulfate and 25 mM sodium formate. After harvesting and washing, the bacteria were assayed for sulfide production from thiosulfate in the presence of the indicated electron donors. Electron donors were used at a concentration of 4 mM. The standard deviations of the measured activities (two biological repeats) are given.

bition of thiosulfate reductase by carbon monoxide is, therefore, unexpected and currently unexplained.

As an alternative approach to elucidating the pathways of electron transfer from formate to thiosulfate, the effects of genetically removing components of the possible electron transport pathways were assessed using the recombinant *E. coli* model. Formate-dependent thiosulfate reduction was abolished in strains lacking the MK-reducing hydrogenases HYD-1 and HYD-2 or in a strain deficient in the hydrogen-producing hydrogenase HYD-3 (Table 7), suggesting that the bulk of the electron flux from formate to thiosulfate goes through hydrogen as an intermediate. Control experiments showed that electron transfer from glycerol to thiosulfate was unaffected in the hydrogenase mutant strains (as expected of a process not involving a hydrogen intermediate or formate), thereby confirming that thiosulfate reductase was expressed in all the mutant backgrounds (Table 7).

DISCUSSION

Thiosulfate is the lowest-potential respiratory electron acceptor used by enteric bacteria. Nevertheless, electron transfer to thiosulfate proceeds via the quinone pool, resulting in the terminal thiosulfate reductase needing to catalyze a reaction at a reduction potential considerably below that of its quinol electron donor. In this study, we showed how thiosulfate reductase is able to meet this challenge.

First, we found that thiosulfate reductase utilizes only MKH_2 as an electron donor. Since MKH_2 has the lowest potential of the three membrane quinols present in the electron transport chain, this minimizes the energetic input required to reduce thiosulfate. The idea that MKH_2 is employed as the electron donor to thiosulfate reductase for thermodynamic reasons is supported by the observation that thiosulfate reductase is capable of catalyzing the exergonic reverse reaction using higher-potential DMK as the electron acceptor.

Second, we showed that endergonic electron transfer between MKH_2 and the thiosulfate reductase catalytic site is driven by the transmembrane PMF. A structural model for thiosulfate reductase based on the homologous enzyme formate dehydrogenase-N suggests that thiosulfate reductase operates by a reverse redox loop mechanism in which the PMF drives electron transfer from the cytoplasmic to the periplasmic side of the membrane (Fig. 2). The accompanying oxidation/reduction reactions release protons to the cytoplasm and consume protons from the periplasm, leading to an overall coupling stoichiometry for thiosulfate reductase of $2{\downarrow}H^+{:}2e^-$.

An earlier study had observed that physiological thiosulfate reductase activity in *S. enterica* was abolished in strains lacking F_oF_1 ATP synthase (55). In principle, this effect might be explained by a requirement for the ATP synthase to generate the PMF required for thiosulfate reduction. However, $MV^{\cdot+}$-linked thiosulfate reductase activity, which we show bypasses the PMF-sensitive site in thiosulfate respiration, was also almost abolished in the ATP synthase mutants, suggesting that the primary effect of the lesions in these strains was on gene expression or assembly.

The use of an ion motive force to drive endergonic electron transfer is a well-established principle in microbial metabolism. The most familiar example is the synthesis of NADH from higher-potential electron donors in many autotrophic bacteria, which proceeds by reverse electron transfer through proton-translocating NADH:quinone oxidoreductase, and also in some cases through the cytochrome bc_1 complex (51). Other ion motive force-driven systems have been shown to operate by a reverse redox loop mechanism of the type proposed here for thiosulfate reductase. Such examples include electron transfer from succinate to MK in the succinate dehydrogenases of MK-dependent Gram-positive and anaerobic bacteria (62, 80) and electron transfer from nitrite to cytochrome *c* in the nitrite oxidase reaction of nitrifying bacteria (14). Further examples of redox reactions driven by an ion motive force include the reduction of ferredoxin by hydrogen in methanogens (71) and the reduction of ferredoxin by NADH by the Rnf complex in bacteria (9).

Even with MKH_2 as the electron donor, the unfavorable $\Delta E^{\circ\prime}$ of the thiosulfate reductase reaction is sufficiently large (−328 mV) that it is implausible that it could be driven by a transmembrane PMF of a magnitude unlikely to exceed 200 mV. However, the reaction will not take place under standard conditions *in vivo*. This has particularly important thermodynamic consequences for the $S_2O_3^{2-}/(HS^- + SO_3^{2-})$ couple because in a cleavage reaction the corresponding Nernst equation shows that the reduction potential of the couple increases as the absolute concentrations of the reactants decrease. Thus, if the concentrations of thiosulfate,

sulfite, and sulfide are set at a more physiologically reasonable 100 μM, the reduction potential of the $S_2O_3^{2-}/(HS^- + SO_3^{2-})$ couple becomes −308 mV and the unfavorable ΔE for the thiosulfate reductase reaction falls to −233 mV. If, in addition, we assume that the MK pool is very highly reduced, then the ΔE for the overall thiosulfate reductase reaction falls below −200 mV, allowing the reaction to be driven by the PMF. These approximate energetic calculations suggest that the PMF is only just sufficient to drive the thiosulfate reductase reaction. This supposition is borne out by our experimental data, which suggest that the thiosulfate reductase reaction in cells is close to thermodynamic equilibrium with the PMF.

Sulfur-sulfur bond formation and cleavage reactions are widespread in sulfur chemistry. Thus, the principle that the reduction potentials of cleavage reactions are considerably higher under physiological conditions (low product concentrations) than under standard conditions should be borne in mind in any consideration of the energetics of microbial sulfur metabolism. This principle may explain, for example, why thiosulfate-oxidizing bacteria feed electrons into the electron transport chain through high-potential cytochromes *c* ($\Delta E^{\circ\prime}$ in the range of +200 to 350 mV) (23) even though thiosulfate would be expected to be a considerably better electron donor based on an $\Delta E^{\circ\prime}$ ($2SO_4^{2-}/S_2O_3^{2-}$) of −245 mV (79). It could also explain why sulfate-reducing bacteria have distinct electron transfer chains leading to adenosine-5′-phosphosulfate (APS) reductase and sulfite reductase, even though the two enzymes catalyze reactions with similar standard reduction potentials ($\Delta E^{\circ\prime}$ of −60 mV [$APS^{2-}/AMP^{2-} + HSO_3^-$] compared with −116 mV [HSO_3^-/HS^-]) (38). Since APS reduction involves cleavage, APS should be a significantly better electron acceptor than sulfite under physiological conditions. APS reductase could, as a consequence, be linked to a different and more energy-conserving electron transport chain than sulfite reductase.

Operons encoding orthologs of *S. enterica* PhsABC can be found in the genomes of all enteric bacteria that respire thiosulfate, suggesting that the PMF-driven mechanism of thiosulfate reduction described here for *S. enterica* can be generalized to other thiosulfate-reducing enteric bacteria. However, while *phsA* and *phsB* orthologs can be found in the genomes of nonenteric thiosulfate-reducing organisms, these operons do not encode a PhsC ortholog, leaving open the question of how these organisms are able to provide the enzyme with electrons at a suitably low potential. A complication in addressing this issue is the high sequence similarity between the PhsAB subunits of thiosulfate reductase and the PsrAB subunits of the enzyme polysulfide reductase (30, 40), which leads to difficulty in distinguishing these two enzyme activities during genome annotation. In the best-characterized polysulfide reductase enzymes, the integral membrane subunit PsrC contains no heme groups and oxidizes MKH_2 at the periplasmic side of the membrane (37). PsrC has been proposed to be involved in PMF generation (18), a suggestion that is at variance with the low reduction potential of the polysulfide reductase reaction {$E^{\circ\prime}$ [$^-$S-$(S)_n$-S^-]/[$^-$S-$(S)_{n-1}$-S^- + HS^-] = −260 mV} (61) and evidence for significant inhibition of physiological polysulfide reduction by protonophores (18). In this context, it is intriguing that the growth of *Shewanella oneidensis* MR-1 with thiosulfate as an electron acceptor requires an operon that contains a PsrC orthologue rather than a PhsC orthologue (12), suggesting that PsrC family proteins may also be able to use the PMF to drive electrons from MKH_2 to thiosulfate.

Putative thiosulfate reductase operons in sulfate-reducing bacteria do not code for integral membrane subunits. In these bacteria, cytoplasmic thiosulfate reductases may receive electrons directly from ferredoxin, while periplasmic enzymes could take electrons from periplasmic hydrogenases.

The data reported here show that operation of the thiosulfate reductase of *S. enterica* consumes energy in the form of the PMF, posing the question of how this energetic expenditure benefits the cell. Thiosulfate respiration would be of net benefit if the overall electron transfer pathway from electron donor to thiosulfate reductase contributed more to the PMF than is consumed by thiosulfate reductase. Thiosulfate reduction takes place only in the absence of more electropositive electron acceptors and appears to function primarily as an adjunct to fermentative metabolism (6, 28). Under these conditions, NADH and fermentation products are the most likely electron donors for thiosulfate respiration. Linking NADH oxidation to thiosulfate reduction by using proton-translocating NADH dehydrogenase I (coupling stoichiometry of $\uparrow 4H^+:2e^-$) in combination with thiosulfate reductase (coupling stoichiometry $\downarrow 2H^+:2e^-$) would result in overall PMF generation of $\uparrow 2H^+:2e^-$ (Fig. 1). This pathway is possible since NADH I can be expressed under anaerobic conditions and is able to reduce MK (73). Net PMF generation by thiosulfate respiration is also plausible with formate as the electron donor. Formate is a major product of fermentation via pyruvate. Three formate oxidation pathways are present in enteric bacteria. However, our data show that electron transfer from formate to thiosulfate occurs predominantly through a mechanism involving hydrogen as an intermediate (Table 7). This pathway is proposed to involve formate-dependent hydrogen production by the formate hydrogen lyase complex. The hydrogen then acts as an electron donor to a short electron transfer chain comprising a MK-reducing hydrogenase (hydrogenase-1 or -2), the MK pool, and thiosulfate reductase (Fig. 1). Since hydrogenase-1 and thiosulfate reductase have equal and opposite coupling stoichiometries, electron transfer from hydrogen to thiosulfate using hydrogenase-1 generates no net PMF (the situation for electron transfer via hydrogenase-2 is less clear, since the hydrogenase-2 coupling stoichiometry is unknown). However, the formate-hydrogen lyase complex is almost certainly an additional coupling site because its reaction is significantly exergonic under physiological conditions (3, 39), because highly similar enzymes in methanogens generate ion motive gradients (11, 50), and because a fragment of mitochondrial complex I retaining homologues of the formate-hydrogen lyase membrane subunits is proton translocating (19). As a consequence, electron transfer from formate to thiosulfate by the formate-hydrogen lyase pathway is likely to generate a net PMF. Recycling of the hydrogen produced by fermentation has long been proposed to occur in enteric bacteria (58, 60), but evidence in support of this phenomenon has only recently started to accrue (1, 52, 82). Our demonstration of hydrogen as an intermediate in formate-dependent thiosulfate respiration provides additional evidence for hydrogen cycling in enteric bacteria.

In addition to directly contributing to PMF generation, thiosulfate respiration may confer a further energetic advantage to fermenting *S. enterica* by disposing of NADH generated by the fermentative pathways. This is because for each two moles of NADH that are reoxidized by a nonfermentative pathway, one

mole of acetyl coenzyme A can be routed through the acetate kinase pathway to generate a mole of ATP by substrate-level phosphorylation rather than being used to regenerate NAD^+ by reduction to ethanol (10). In this context, the greatest benefit of thiosulfate reduction to *S. enterica* may be the production of sulfite, which can be further reduced to sulfide by the enzyme anaerobic sulfite reductase, consuming three molecules of NADH (26).

In summary, we have suggested how thiosulfate respiration could play a positive role in the bioenergetics of *S. enterica*, despite our demonstration that the thiosulfate-reducing step in this metabolism is energy requiring. Given the recent discovery that thiosulfate is a significant host-generated electron acceptor in the mammalian gut, it is likely that thiosulfate respiration provides an important growth advantage and that thiosulfate respiration may contribute to the pathogenesis of *Salmonella* species at the stage of gut colonization.

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