

## Characterization of a 12-Kilodalton Rhodanese Encoded by *glpE* of *Escherichia coli* and Its Interaction with Thioredoxin

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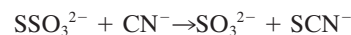
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Rhodanases catalyze the transfer of the sulfane sulfur from thiosulfate or thiosulfonates to thiophilic acceptors such as cyanide and dithiols. In this work, we define for the first time the gene, and hence the amino acid sequence, of a 12-kDa rhodanese from *Escherichia coli*. Well-characterized rhodanases are comprised of two structurally similar ca. 15-kDa domains. Hence, it is thought that duplication of an ancestral rhodanese gene gave rise to the genes that encode the two-domain rhodanases. The *glpE* gene, a member of the *sn*-glycerol 3-phosphate (*glp*) regulon of *E. coli*, encodes the 12-kDa rhodanese. As for other characterized rhodanases, kinetic analysis revealed that catalysis by purified GlpE occurs by way of an enzyme-sulfur intermediate utilizing a double-displacement mechanism requiring an active-site cysteine. The  $K_m$ s for  $\text{SSO}_3^{2-}$  and  $\text{CN}^-$  were 78 and 17 mM, respectively. The apparent molecular mass of GlpE under non-denaturing conditions was 22.5 kDa, indicating that GlpE functions as a dimer. GlpE exhibited a  $k_{\text{cat}}$  of  $230 \text{ s}^{-1}$ . Thioredoxin 1 from *E. coli*, a small multifunctional dithiol protein, served as a sulfur acceptor substrate for GlpE with an apparent  $K_m$  of 34  $\mu\text{M}$  when thiosulfate was near its  $K_m$ , suggesting that thioredoxin 1 or related dithiol proteins could be physiological substrates for sulfurtransferases. The overall degree of amino acid sequence identity between GlpE and the active-site domain of mammalian rhodanases is limited (~17%). This work is significant because it begins to reveal the variation in amino acid sequences present in the sulfurtransferases. GlpE is the first among the 41 proteins in COG0607 (rhodanese-related sulfurtransferases) of the database Clusters of Orthologous Groups of proteins (<http://www.ncbi.nlm.nih.gov/COG/>) for which sulfurtransferase activity has been confirmed.

Genes of known function belonging to the *glp* regulon of *Escherichia coli* encode proteins that are responsible for the metabolism of *sn*-glycerol 3-phosphate (glycerol-P) and its precursors, glycerol and glycerophosphodiester (38). The genes comprising this regulon belong to five operons. Transcription of all but the *glpEGR* operon is negatively regulated by the *glp* repressor GlpR, a member of the DeoR family of transcriptional regulators (34, 65, 69–72). Operon *glpACB*, encoding the subunits of the anaerobic glycerol-P dehydrogenase, is located near min 51 of the *E. coli* genome (38). Divergently transcribed from *glpACB* is *glpTQ*. The genes *glpT* and *glpQ* encode glycerol-P permease and periplasmic glycerophosphodiesterase, respectively (38). The *glpFKX* operon, at min 89, encodes glycerol diffusion facilitator, glycerol kinase, and a fructose 1,6-bisphosphatase (38; J. L. Donahue, J. L. Bownas, W. G. Niehaus, Jr., and T. J. Larson, unpublished data). The genes *glpE* and *glpG*, together with the gene encoding the transcriptional repressor, *glpR*, form a complex operon at min 77 that is divergently transcribed from *glpD* (71). The gene *glpD* encodes the aerobic glycerol-P dehydrogenase (38).

Prior to this study, the functions of the GlpE protein and the cytoplasmic membrane-associated GlpG protein were unknown (71, 72). The function of GlpG remains unknown. However, as reported by Tatusov et al. (58) and more recently by

Hofmann et al. (23), GlpE exhibits sequence similarity to a superfamily of transfer proteins including the sulfurtransferases and the tyrosine and dual-specificity phosphatases. In this work we show that GlpE is a thiosulfate:cyanide sulfurtransferase (EC 2.8.1.1), an enzyme traditionally given the name rhodanese. Rhodanases catalyze the transfer of the sulfane sulfur from thiosulfate to cyanide, forming thiocyanate and sulfite:



Although sulfurtransferases are present in many types of organisms from all three domains of life (57), their physiological roles are still in question. Proposed roles include cyanide detoxification (61), sulfur metabolism (15, 66), and mobilization of sulfur for iron-sulfur cluster biosynthesis or repair (9, 10, 51, 52).

At least two distinct rhodanases and the related enzyme mercaptopyruvate sulfurtransferase have been described in *E. coli*, but the genes for these enzymes have not been mapped nor have the sequences for the proteins been determined (3, 60, 62). In this work, we define for the first time the gene, and hence the amino acid sequence, of a rhodanese from *E. coli*. In the database Clusters of Orthologous Groups of proteins (57), there are 41 proteins in COG0607 (rhodanese-related sulfurtransferases). To our knowledge, GlpE is the first of these proteins for which sulfurtransferase activity has been confirmed.

### MATERIALS AND METHODS

**Materials.** Unless listed below, the reagents used were purchased from Sigma Chemical Company or Fisher Scientific. Synthetic oligonucleotides were prepared with an Applied Biosystems DNA synthesizer (model 381A) using reagents supplied by Cruachem. New England Biolabs supplied restriction endonucleases and reagents for PCR and cloning. Ammonium thiosulfate and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Aldrich. Ferric nitrate and the sodium salt of mercaptopyruvate were purchased from ICN

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TABLE 1. *E. coli* strains and plasmids

Strain or plasmid	Description	Reference
<b>Strains</b>		
BL21(DE3)	<i>hsdS gal</i> ( $\lambda$ clts857 <i>ind-1</i> Sam7 <i>nin-5 lacUV5-T7</i> gene 1)	55
DH5 $\alpha$ Z1	( $\phi$ 80d <i>lacZ</i> $\Delta$ M15) <i>endA1 recA1 hsdR17 supE44 thi-1 gyrA relA1</i> $\Delta$ ( <i>lacZYA-argF</i> )U169 ( $\lambda$ att <i>lacI</i> <sup>q</sup> <i>tetR</i> Sp <sup>r</sup> )	42
<b>Plasmids</b>		
pT7-7	ColE1 origin Ap <sup>r</sup> T7 promoter	56
pGZ105	<i>glpE</i> in <i>NdeI/HindIII</i> fragment of pT7-7	72
pZE2 P <sub>N25</sub> - <i>luc</i>	ColE1 origin Km <sup>r</sup> P <sub>N25</sub> - <i>luc</i>	42
pZA3 P <sub>N25</sub> - <i>luc</i>	p15A origin Cm <sup>r</sup> P <sub>N25</sub> - <i>luc</i>	42
pGZ154	<i>glpE</i> in <i>EcoRI/XbaI</i> fragment of pZA3 P <sub>N25</sub> - <i>luc</i>	This study
pGZ132	<i>glpE</i> in <i>EcoRI/XbaI</i> fragment of pZE2 P <sub>N25</sub> - <i>luc</i>	This study
pATCBO <sub>A</sub> 2+6	ColE1 origin Ap <sup>r</sup> $\Phi$ ( <i>glpT-phoA</i> )	34

Pharmaceuticals, Inc. Promega supplied thioredoxin 1 from *E. coli*. Thioredoxin reductase purified from *E. coli* was a generous gift from C. H. Williams, Jr. (68).

**Bacterial strains and plasmids.** The bacterial strains and plasmids used or constructed are listed in Table 1. BL21(DE3) harboring pGZ105 was used to overexpress GlpE for purification. Plasmids pGZ154 and pGZ132, in which *glpE* is controlled by the tetracycline-inducible P<sub>N25</sub> promoter, were used to overexpress GlpE in DH5 $\alpha$ Z1. To construct these plasmids, the *glpE* gene was amplified from pGZ105 by PCR with the primers 5'-acgAAttcccGctagCaat-3' and 5'-tcactagtgtgacagcttacc-3', where uppercase letters indicate mismatches used for the creation of restriction sites. After cleavage with *EcoRI* and *XbaI*, the amplified product was cloned into the same sites of either pZA3 P<sub>N25</sub>-*luc* or pZE2 P<sub>N25</sub>-*luc* (42) to create pGZ154 and pGZ132, respectively. Plasmid pATCBO<sub>A</sub>2+6 (34) was used for constitutive expression of the periplasmic marker alkaline phosphatase.

**Growth media and conditions.** Cultures were grown in Luria-Bertani broth (43) at 37°C. Antibiotics were included at 100  $\mu$ g/ml for ampicillin, 50  $\mu$ g/ml for kanamycin, 25  $\mu$ g/ml for chloramphenicol, and 20  $\mu$ g/ml for spectinomycin where appropriate. Overexpression of GlpE in cells carrying pGZ105 was induced in mid-log phase by the addition of 0.5 mM isopropylthio- $\beta$ -D-galactopyranoside (IPTG) and in cells carrying pGZ154 or pGZ132 by 0.1  $\mu$ g of tetracycline per ml. Cultures were grown for 2 to 4 h after induction and then harvested in late log phase as described below.

**Cell fractionation procedures.** DH5 $\alpha$ Z1 harboring pATCBO<sub>A</sub>2+6 (*glpT-phoA* transcriptional fusion) and pGZ154 was grown, and expression of GlpE was induced by using tetracycline as described above. Cells were harvested, and two fractionation procedures were used for preparation of cell extracts.

(i) **Periplasmic fraction.** Spheroplasts were prepared by incubation of cells with lysozyme and EDTA essentially as described by Kaback (27). Cells were washed twice with 0.5 volume of 10 mM Tris-HCl (pH 8) and then resuspended in 30 mM Tris-HCl (pH 8)–20% sucrose–10 mM EDTA at an absorbance at 600 nm of approximately 3. Lysozyme was added (0.5 mg/ml) using a freshly prepared stock solution of 25 mg/ml in 10 mM Tris-HCl (pH 8). After incubation at room temperature for 30 min with gentle agitation, spheroplasts were removed by centrifugation for 5 min at 10,000  $\times$  g. The supernatant was decanted and saved as the periplasmic fraction. Cell pellets were resuspended in 10 mM Tris-HCl (pH 8) and lysed by sonication (cytoplasmic fraction).

(ii) **Freeze-thaw fraction.** The second fractionation procedure combined freeze-thaw treatment (26) and incubation with EDTA to disrupt the outer membrane. Cells were harvested by centrifugation (6,000  $\times$  g for 5 min at 4°C) and washed with 0.5 volume of 25 mM Tris-acetate (pH 8.6)–10 mM ammonium thiosulfate precooled to 4°C. The cell pellet was stored at –70°C. The frozen cells were thawed on ice, resuspended in 1/50 the original volume of buffer A (50 mM Tris-HCl [pH 7.2], 3 mM EDTA), and incubated on ice for 30 min. Cells were collected by centrifugation, and the supernatant fraction was saved. Incubation in buffer A was repeated twice, and the three supernatant fractions were combined (freeze-thaw extract). After isolation of the freeze-thaw extract, the remaining cells were resuspended in 10 mM Tris-HCl (pH 8) and lysed by sonication (cytoplasmic fraction).

Rhodanese, alkaline phosphatase, and glucose 6-phosphate dehydrogenase activities were determined for the periplasmic, freeze-thaw, and cytoplasmic fractions. Total cellular enzyme activities were determined by using an aliquot of whole cells that was pelleted, resuspended in 10 mM Tris-HCl (pH 8), and sonicated as described above.

**Protein purification.** BL21(DE3) harboring pGZ105 was grown in 500 ml of Luria-Bertani broth, and expression of GlpE was induced by the addition of IPTG as described above. Freeze-thaw extraction was performed as described above. The freeze-thaw extract was loaded onto a prepacked Waters quaternary

methylamine (Q)-polymethacrylate anion-exchange column (10 by 100 mm; Protein-Pak Q15HR 1000 $\text{\AA}$ ) equilibrated at room temperature with buffer A. The flow rate was maintained at 1.5 ml/min. After being washed with 180 ml of buffer A, the column was developed with a 45-ml gradient from 0 to 105 mM NaCl in buffer A, followed by a 112.5-ml gradient from 105 to 255 mM NaCl in buffer A. Fractions containing rhodanese activity, eluted between 150 and 190 mM NaCl, were pooled. Rhodanese was concentrated by reapplying the pooled fractions diluted twofold with buffer A to the anion-exchange column. The enzyme was eluted with buffer A containing 750 mM NaCl. Fractions containing rhodanese activity were pooled, brought to 15% glycerol, and stored in aliquots at –70°C. Protein concentrations were determined by the method of Bradford (11) with bovine serum albumin as the standard, using materials purchased from Pierce Chemical Company and the manufacturer's microassay method.

**Estimation of subunit and native molecular masses.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (33) on 15% polyacrylamide gels. A modified sample loading buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.02% bromophenol blue, and 1.5 M  $\beta$ -mercaptoethanol was prepared immediately prior to use. Use of the less-reducing buffer described by Laemmli (33) resulted in migration of GlpE as a doublet. Protein was visualized with Fast Stain from Zoion Biotech, Inc.

Gel filtration chromatography was used to determine the apparent molecular mass of GlpE under nondenaturing conditions. Samples of 0.1 ml containing from 40 to 80  $\mu$ g of GlpE or various standards were applied to a Waters glass Protein-Pak 300SW column (8 by 300 mm). The column was equilibrated at ambient temperature with 20 mM Tris-HCl (pH 7.2)–1 mM EDTA–100 mM NaCl at a flow rate of 0.8 ml/min. Void volume and total volume were determined by using blue dextran and vitamin B<sub>12</sub>, respectively. The protein standards used for generation of the standard curve were bovine serum albumin, ovalbumin, soybean trypsin inhibitor, myoglobin, and RNase.

**Assay of sulfurtransferase activity.** Two methods were used to measure the sulfurtransferase activity of GlpE. Assays were performed at ambient temperature (approximately 22°C).

(i) **First method.** During purification of GlpE and for characterization of the enzyme, the assay used was essentially that described previously to quantify thiocyanate (3, 66). Assays contained 100 mM Tris-acetate (pH 8.6), 50 mM ammonium thiosulfate, 50 mM KCN, and enzyme in a final volume of 0.5 ml. Reactions were initiated by the addition of KCN and terminated, after 0.5 to 2 min, by the addition of 0.25 ml of 15% formaldehyde. Color was developed by the addition of 0.75 ml of ferric nitrate reagent [100 g of Fe(NO<sub>3</sub>)<sub>3</sub> · 9H<sub>2</sub>O and 200 ml of 65% HNO<sub>3</sub> per 1,500 ml]. Assays were clarified by centrifugation, and the absorbance at 460 nm was determined (66). One unit of enzyme is defined as the amount that catalyzes the production of 1  $\mu$ mol of thiocyanate per minute and corresponds to an absorbance change at 460 nm of 2.8 in this system.

(ii) **Second method.** Assays to measure the ability of GlpE to transfer sulfur from thiosulfate to thioredoxin 1 were performed essentially as described previously for the bovine liver rhodanese (48). Each assay (1 ml, final volume) contained 50 mM potassium phosphate (pH 8.2), 0.1 U of thioredoxin reductase per ml, 50  $\mu$ M NADPH, and the indicated amount of thioredoxin 1. Cuvettes containing all reagents except NADPH were used as blanks. NADPH was added, and the reaction mixtures were allowed to equilibrate for at least 1 h. Periodic measurements of the absorbance at 340 nm were used to ensure that the mixtures had reached equilibrium. After equilibrium had been reached, either purified GlpE (4  $\mu$ M monomer, 48  $\mu$ g/ml), ammonium thiosulfate (80 mM), or both were added.

The basis for this assay is that when thioredoxin 1, which contains the active-site motif WCGPC, accepts a sulfane sulfur from rhodanese, a persulfide is formed at the N-terminal cysteine within the active site. The C-terminal cysteine within the active site then reacts with the persulfide, yielding sulfide and oxidized thioredoxin 1. Thioredoxin reductase acts to reduce the disulfide bond of oxidized thioredoxin 1, with the concomitant oxidation of NADPH (48). The decrease in absorbance at 340 nm was measured to determine the rate of NADPH oxidation.

**Other enzyme assays.** Alkaline phosphatase activity was determined by utilizing the substrate *p*-nitrophenylphosphate and monitoring the increase in absorbance at 410 nm (34). Glucose 6-phosphate dehydrogenase activity was determined by following the increase in absorbance at 340 nm due to reduction of NADP in the presence of glucose 6-phosphate (21).

## RESULTS

**GlpE is a sulfurtransferase.** A PSI-BLAST search (4) using GlpE as the query revealed that GlpE exhibits sequence similarity to the family of sulfurtransferases that includes rhodanases and mercaptopyruvate sulfurtransferases and to the family of tyrosine and dual-specificity phosphatases (23, 58). Together, these two families of proteins constitute a superfamily of transfer proteins (23, 58). Figure 1 illustrates alignment of GlpE with the conserved regions of several representative





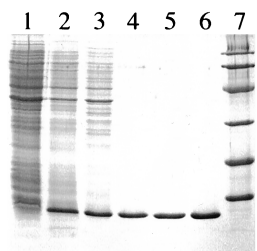


FIG. 2. SDS-PAGE illustrating the overexpression and purification of GlpE. Lane 1, total cellular protein, BL21(DE3) harboring pT7-7 (10  $\mu$ g). Lane 2, total cellular protein, BL21(DE3) harboring pGZ105 (10  $\mu$ g). Lane 3, proteins released from intact cells [BL21(DE3) harboring pGZ105] by the modified freeze-thaw method (10  $\mu$ g). Lane 4, pooled fractions following the first anion-exchange chromatographic step (2  $\mu$ g). Lanes 5 and 6, pooled fractions following the second anion-exchange chromatographic step (2 and 4  $\mu$ g, respectively). Lane 7, molecular mass markers, corresponding to 97, 66, 45, 30, 21, and 14 kDa.

sulfate were excluded. Figure 3B is a secondary double-reciprocal plot of the apparent maximum velocities in Fig. 3A versus cyanide concentration. The regression of the data to the equation describing a double-displacement mechanism yielded  $K_m$  values for thiosulfate and cyanide of 78 and 17 mM, respectively. The  $k_{cat}$  based on GlpE functioning as a dimer was 230  $s^{-1}$ .

Although rhodanases are related to mercaptopyruvate sulfurtransferases (Fig. 1) (45, 46), substitution of mercaptopyruvate in the standard sulfurtransferase assay resulted in less than 1% of the thiocyanate formation obtained with an equivalent concentration of thiosulfate. Cysteine, thioglycerol, dithiothreitol, and  $\beta$ -mercaptoethanol were unable to replace thiosulfate as the sulfur donor for GlpE (data not shown).

Several previously characterized rhodanases, including the bovine liver enzyme (64) and the accessible rhodanase of *E. coli*, are specifically inhibited by certain anions (3). In contrast, little or no inhibition by anions was observed for GlpE. Addition of sodium phosphate, sodium acetate, sodium chloride, sodium sulfate, or potassium phosphate at an ionic strength of 0.3 resulted in 20% or less inhibition of rhodanase activity (data not shown). However, as shown in Fig. 3A, substrate inhibition by thiosulfate was observed, particularly at low cyanide concentrations. This type of inhibition was previously described for the bovine liver rhodanase (64) and the accessible rhodanase of *E. coli* (3).

**Chemical inactivation of GlpE by DTNB.** Catalysis by the bovine liver rhodanase requires a cysteine residue. The cysteine forms a persulfide linkage to the sulfane sulfur from thiosulfate in an enzyme-sulfur intermediate (63, 67). GlpE contains two cysteine residues, one of which has been identi-

TABLE 3. Localization of GlpE to the cytoplasm<sup>a</sup>

Fraction	% of activity released		
	Alkaline phosphatase	Glucose-6-phosphate dehydrogenase	Rhodanase
Spheroplasts	69	5	5
Freeze-thaw	76	<1	97

<sup>a</sup> Each cell fractionation procedure was performed at least twice. Reported results are from typical experiments. Total enzyme activities recovered by the preparation of spheroplasts from 33 ml of logarithmically growing culture were 0.03 U of alkaline phosphatase, 0.3 U of glucose-6-phosphate dehydrogenase, and 1 U of rhodanase. Total enzyme activities recovered by the freeze-thaw fractionation method from 25 ml of logarithmically growing culture were 12 U of alkaline phosphatase, 0.1 U of glucose-6-phosphate dehydrogenase, and 10 U of rhodanase.

TABLE 4. Purification of *glpE*-encoded rhodanase from *E. coli*

Purification step	Volume (ml)	Amt of protein (mg)	Activity (U)	Sp act (U/mg)	Yield (%)
Whole cells <sup>a</sup>	500	120	1,300	11	100
Freeze-thaw extract	30	9.6	860	90	66
Anion exchange 1	32	1.6	350	220	27
Anion exchange 2	2	1.3	350	270	27

<sup>a</sup> Activity was determined using sonicated samples of liquid cell culture.

fied as the active site of GlpE, based on sequence alignment with other sulfurtransferases (Cys-65) (Fig. 1). GlpE was incubated with the cysteine-specific modifying reagent DTNB to verify that a cysteine residue is required for rhodanase activity. Incubation of GlpE with DTNB in a 1:2 molar ratio resulted in a greater-than-90% loss of activity (Fig. 4).

**Thioredoxin acts as a sulfur acceptor substrate for GlpE.** It has been shown that reduced dithiols such as dithiothreitol (1, 53), dihydrolipoate (50), and *E. coli* thioredoxin 1 (48) serve as

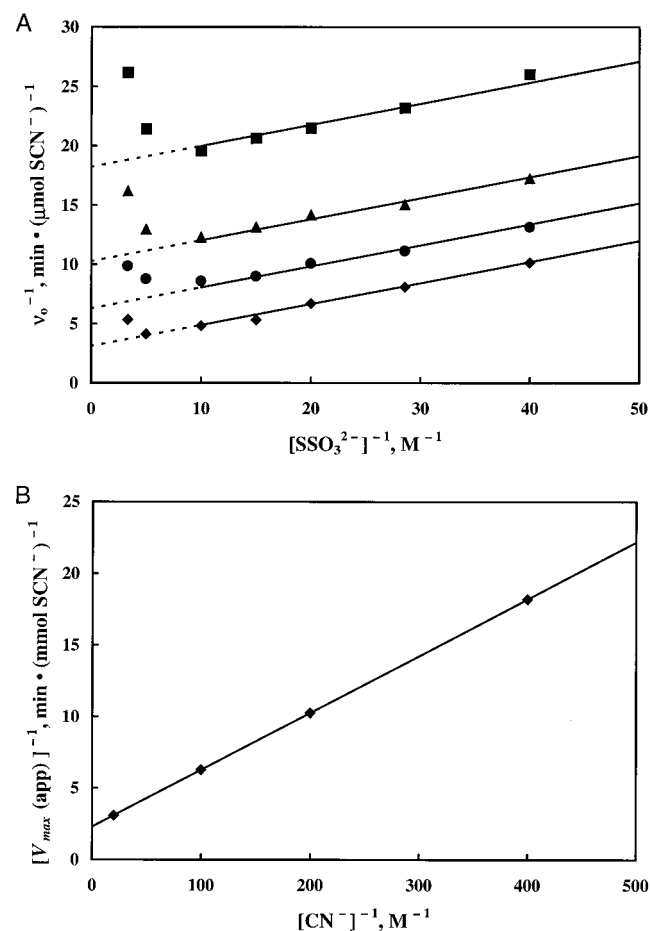


FIG. 3. Kinetic characterization of the thiosulfate:cyanide sulfurtransferase reaction catalyzed by GlpE. Each assay, performed as described in the text, contained 10  $\mu$ l (0.75  $\mu$ g) of purified GlpE in 100 mM Tris-acetate (pH 8.6)–6 mM cysteine. (A) Double-reciprocal plot of the rate of thiocyanate formation versus thiosulfate concentration at various fixed concentrations of cyanide: 2.5 mM ( $\blacksquare$ ), 5 mM ( $\blacktriangle$ ), 10 mM ( $\bullet$ ), and 50 mM ( $\blacklozenge$ ). (B) Secondary double-reciprocal plot of apparent  $V_{max}$  from the data in panel A versus cyanide concentration. Data, excluding the points exhibiting substrate inhibition, were fit to the equation describing a double-displacement mechanism.

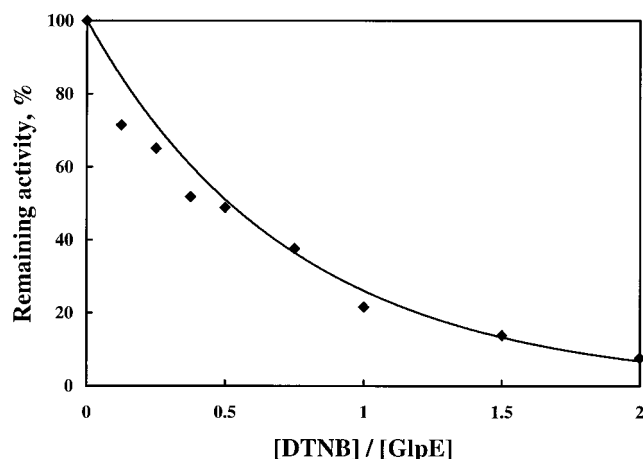


FIG. 4. Inactivation of GlpE by DTNB. Purified GlpE (60  $\mu\text{g}$ ) was incubated overnight at an ambient temperature in 0.2 ml of 100 mM Tris-acetate (pH 8.6)–150 mM NaCl with various molar ratios of DTNB. Remaining rhodanase activity was determined and compared with that for GlpE incubated without DTNB.

sulfur-acceptor substrates for bovine liver rhodanase. Thioredoxin 1 acts as a substrate with an affinity near that determined for cyanide (apparent  $K_m$ s of 18.5  $\mu\text{M}$  [48] and 63  $\mu\text{M}$  [64], respectively). To test thioredoxin 1 as a sulfur acceptor substrate for GlpE, the assay method described for bovine rhodanase was used (48). (See Materials and Methods for the basis for this assay.) A mixture containing thioredoxin 1, thioredoxin reductase, and NADPH was allowed to equilibrate. Then GlpE, ammonium thiosulfate, or both were added. Addition of either GlpE or thiosulfate alone resulted in no significant oxidation of NADPH. However, addition of both GlpE and thiosulfate resulted in a marked increase in the rate of NADPH oxidation (Fig. 5A). Oxidation of NADPH was dependent on the presence of thioredoxin 1 and thioredoxin reductase as well as GlpE and thiosulfate. To quantify the affinity of thioredoxin 1 for the sulfur transferred from GlpE, the thioredoxin 1 concentration was varied while the thiosulfate concentration was held at 80 mM (Fig. 5B). Regression of the resulting data yielded an estimate of the apparent  $K_m$  for thioredoxin 1 of 34  $\mu\text{M}$  and an apparent  $V_{\text{max}}$  of 1 nmol of NADPH oxidized per min, almost 15 times faster than the maximum rate of NADPH oxidation observed with the bovine liver rhodanase (48). This apparent maximum rate corresponds to a turnover number of 0.5 mol of NADPH oxidized per mol of GlpE dimer per min.

## DISCUSSION

**Comparison of GlpE with other sulfurtransferases.** The well-characterized rhodanase from bovine liver mitochondria is approximately twice the size of GlpE and functions as a monomer. The bovine liver enzyme, however, contains two approximately 15-kDa domains. The two domains are structurally similar, although they exhibit only limited sequence similarity to each other (54). Hence, it is thought that duplication of an ancestral rhodanase gene has given rise to the genes that encode the two-domain rhodanases. This hypothesis is supported by the identification of GlpE and Sud, a polysulfide sulfurtransferase from *Wolinella succinogenes* (29, 30), as single-domain sulfurtransferases having sequence similarity to the catalytic domain of the bovine liver enzyme (Fig. 1). Like the bovine enzyme, GlpE and Sud require a cysteine residue

for activity and utilize a double-displacement reaction mechanism.

Although GlpE possesses some of the characteristics of the previously identified accessible rhodanase from *E. coli* (molecular weights of 12,000 and 14,000, accessibility of enzyme to substrates added to intact cells, and release from cells by freeze-thaw) (3), it is not likely that these two enzymes are identical. First, the catalytic properties of the two enzymes are distinct. The  $K_m$ s for  $\text{SSO}_3^{2-}$  are 78 and 5 mM for GlpE and the accessible rhodanase, respectively. The  $K_m$ s for  $\text{CN}^-$  are about the same (17 and 24 mM, respectively), but the  $k_{\text{cat}}$ s differ (115  $\text{s}^{-1}$  per GlpE monomer versus 260  $\text{s}^{-1}$  per monomer for the accessible rhodanase) (3). Also, GlpE does not exhibit anion-specific inhibition as described for accessible rhodanase and bovine liver rhodanase.  $K_i$  values for sulfate and acetate versus thiosulfate were 36 and 45 mM, respectively, for accessible rhodanase (3) and 40 and 167 mM, respectively, for bovine rhodanase (64). Specific inhibition of bovine rhodanase by anions has been linked to two positively charged residues (R248 and K249) located adjacent to the active-site cysteine and an arginine residue (R186) within the CH2A motif (36). None of these residues are conserved in GlpE (see Fig. 1).

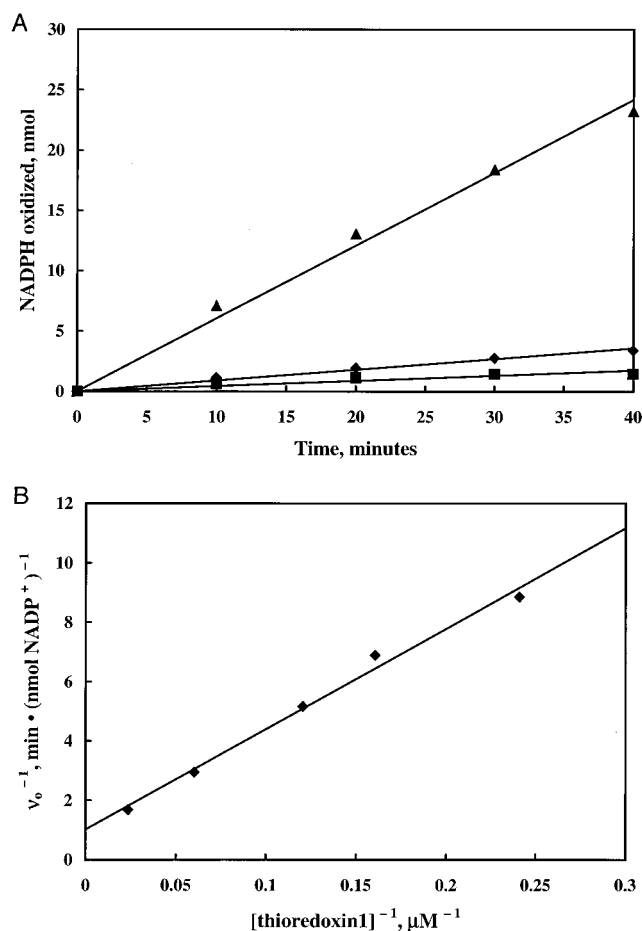


FIG. 5. Thioredoxin 1 as a sulfur acceptor substrate for GlpE. (A) Assay mixtures containing 16.6  $\mu\text{M}$  thioredoxin 1, 0.1 U of thioredoxin reductase per ml, and 50  $\mu\text{M}$  NADPH were preequilibrated. At time zero, either 4  $\mu\text{M}$  GlpE (■), 80 mM ammonium thiosulfate (◆), or both (▲) were added. The absorbance at 340 nm was periodically measured to determine the rate of NADPH oxidation. (B) Double-reciprocal plot of the rate of NADPH oxidation measured as described for panel A versus thioredoxin 1 concentration.

These basic residues have also been implicated in the recognition of thiosulfate as a substrate. Substitution of R186 in the bovine liver rhodanese with isoleucine increases the  $K_m$  for thiosulfate from 4 to 70 mM. Substitution of K249 with alanine results in a protein that has almost no activity with thiosulfate as the substrate, but the variant can utilize a number of organic thiosulfonates almost as well as the wild-type protein (41). Lack of conservation of these residues in GlpE may be the basis for the lower affinity of GlpE for thiosulfate compared with other rhodanases. The relatively low affinity of GlpE for both thiosulfate and cyanide suggests that these compounds are not the physiological substrates of the enzyme.

Finally, GlpE and accessible rhodanese differ in their response to reducing agents. Addition of cysteine not only activated oxidized accessible rhodanese but also resulted in a shift from dimeric to monomeric form (3). Although GlpE is activated by cysteine, it does not exhibit this shift to monomeric form in the presence of cysteine or dithiothreitol. The gene encoding GlpE is only one of a number of genes that have been identified in *E. coli* that encode rhodanese like proteins (23, 58). One of the other paralogs is likely the previously characterized accessible rhodanese. Deletion of the *glpE* gene did not significantly decrease total rhodanese activity measured in cell extracts, verifying that GlpE is not the only protein possessing rhodanese activity in *E. coli* (data not shown). Other organisms also contain multiple sulfurtransferase genes. For example, disruption of the *rhda* (rhodanese) gene of *Azotobacter vinelandii* did not result in a discernible phenotype or abolish all rhodanese activity (13). Finally, among the 21 completely sequenced genomes in the COG database (57), 8 genomes contain multiple sulfurtransferase genes.

It is of interest that expression of both the accessible rhodanese and GlpE is subject to catabolite repression (3, 12, 71) and repressed during anaerobic growth (3) (data not shown).

**GlpE and thioredoxin 1.** GlpE, like bovine liver rhodanese (48), is capable of transferring sulfur from thiosulfate to thioredoxin 1 with relatively high affinity. GlpE has an apparent  $K_m$  for thioredoxin 1 of 34  $\mu$ M when thiosulfate is present near its  $K_m$ . Thus, the affinity for thioredoxin 1 is 500 times higher than that determined for cyanide (17 mM). Sulfurtransferases from other organisms may also have the ability to utilize dithiol proteins such as thioredoxin as sulfur acceptors. For example, a partially purified rhodanese isolated from *Thiobacillus novellus* was capable of producing sulfide from thiosulfate in the absence of cyanide. The physiological sulfur acceptor substrate present in the partially purified preparation had a molecular mass of between 10 and 20 kDa (22) and so may have been a protein similar to thioredoxin. Interestingly, the two structural motifs, designated CH2A and CH2B, that flank the active site in the superfamily of transfer proteins (Fig. 1) have been suggested to play a role in protein-protein interactions. These interactions may mediate recognition of protein substrates by the tyrosine and dual-specificity phosphatases (18). It is intriguing to speculate that the CH2A and CH2B motifs of the sulfurtransferases play a similar role in recognition of dithiol protein substrates. *E. coli* contains multiple dithiol proteins besides thioredoxin 1 that could fulfill this function. A second thioredoxin, the gene product of *trxC*, has recently been characterized (44). In addition to the two thioredoxins, *E. coli* possesses three isoforms of glutaredoxin, proteins structurally and functionally similar to the thioredoxins (6, 37). Given the overlap of function that has been demonstrated for the thioredoxin/glutaredoxin family of proteins, it is possible that these other proteins also serve as sulfur acceptor substrates for sulfurtransferases.

A number of cytoplasmic proteins in *E. coli*, including elon-

gation factor Tu (25), DnaK (17), and the small dithiol proteins thioredoxin 1 and 2 (40, 44), and many others (39), are released from cells by most methods used for isolating periplasmic proteins. Retention of proteins such as DnaK and thioredoxins by lysozyme-generated spheroplasts, however, indicates a cytoplasmic location (17, 40, 44, 59). GlpE also is a cytoplasmic protein, since it is retained by spheroplasts.

Recently, it was shown that release of thioredoxin, DnaK, and elongation factor Tu by mild osmotic shock required the presence of the gated mechanosensitive ion channel MscL (2, 8). Preliminary results from our laboratory suggest that MscL also facilitates release of GlpE by osmotic shock (data not shown).

**Physiological role of GlpE.** The physiological role of rhodanases is still in question. Cyanide detoxification has been proposed as a possible role (61), but given the low affinity for cyanide ( $K_m = 17$  mM), this seems unlikely for GlpE.

Rhodanases may play various roles in sulfur metabolism (66). The expression of Rhda, a rhodanese-like protein from *Synechococcus* sp. strain PCC7942, is induced by sulfur starvation (35). However, nothing has thus far implicated GlpE in metabolism of sulfur in *E. coli*. Growth on various sulfur sources or on limiting sulfur had no apparent effect on the expression of a single-copy *glpE-lacZ* transcriptional fusion (data not shown). To our knowledge, the only phenotype identified to date for a rhodanese-like enzyme is that of CysA from *Saccharopolyspora erythraea* (15). Disruption of the gene encoding CysA results in cysteine auxotrophy but, as described above for *glpE*, does not have a significant effect on the total rhodanese activity. The pathway for cysteine biosynthesis in *S. erythraea* differs from that established for *E. coli* and involves thiosulfate as an intermediate. Thus, it is possible that CysA functions to synthesize thiosulfate (15). Although thiosulfate is not an obligate intermediate in aerobic cysteine biosynthesis in enteric bacteria, thiosulfate might be an intermediate in anaerobic biosynthesis of cysteine (19, 31).

Rhodanese has also been proposed to mobilize sulfur for the formation or repair of iron-sulfur clusters. The iron-sulfur clusters of ferredoxins (10, 51), succinate dehydrogenase (9), and mitochondrial NADH dehydrogenase (52) could be reconstituted by incubation with various combinations of bovine liver rhodanese, thiosulfate, dihydrolipeate, and iron ions. Interestingly, thioredoxin 1 has also been proposed to play a role in the formation or repair of iron-sulfur clusters (14). The participation of sulfurtransferases in the formation of iron-sulfur clusters has been questioned, however, since there is strong evidence that NifS/IscS and associated proteins are involved in mobilization of sulfur from cysteine for synthesis of iron-sulfur clusters (20, 28, 47, 73).

Although GlpE has now been identified as a sulfurtransferase, its physiological function and possible association with the metabolism of glycerol-P remain to be elucidated. The relationship between GlpE and thioredoxin that was discovered during this study might help clarify these issues.

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