Characterization of PspE, a secreted sulfurtransferase of *Escherichia coli*

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ABSTRACT

PspE, encoded by the last gene of the phage shock protein operon, is one of the nine proteins of *Escherichia coli* that contain a rhodanese homology domain. PspE is synthesized as a precursor with a 19-amino acid signal sequence and secreted to the periplasm. Mature PspE is the smallest rhodanese of *E. coli* (85 amino acids) and catalyzes the transfer of sulfur from thiosulfate to cyanide forming thiocyanate and sulfite. Cation exchange chromatography of a freeze-thaw extract of a PspE-overexpressing strain yielded two major peaks of active, homogeneous PspE. The two peaks contained two forms of PspE ($PspE_1$ and $PspE_2$) of distinct size and/or charge that were distinguished by native polyacrylamide gel electrophoresis and gel chromatography. $PspE_2$ was converted to the more compact $PspE_1$ by treatment with thiosulfate, which suggested that $PspE_1$ is the persulfide form. One equivalent of cyanizable sulfur was associated with $PspE_1$, with much less present in $PspE_2$. Consistent with the conclusion that the single active site cysteine of $PspE_1$ contains a persulfide sulfur was the observation that this form was much more tolerant to chemical inactivation by thiol-specific modifying reagent DTNB (5,5'-dithiobis(2-nitrobenzoic acid)). Rhodanese activity was subject to inhibition by anions (sulfite, sulfate, chloride, phosphate and

arsenate), suggesting PspE has a cationic site for substrate binding. Kinetic analysis revealed that PspE employs a double-displacement mechanism, as is the case for other rhodaneses. The K_ms for SSO_3^{2-} and CN^- were 3.0 and 43 mM, respectively. PspE exhibited a k_{cat} of 72 s^{-1}. To aid in understanding the physiological role of PspE, a strain with a *pspE* gene disruption was constructed. Comparison of rhodanese activity in extracts of wild-type and mutant strains revealed that PspE is a major contributor of rhodanese activity in *E. coli*. The *pspE* mutant displayed no obvious growth defect or auxotrophies, and was capable of molybdopterin biosynthesis, indicating that *pspE* is not essential for production of sulfur-containing amino acid or cofactors. Growth of wild-type and mutant strains deficient in *pspE* and other sulfurtransferase paralogs in medium with cyanide or cadmium was compared. The results indicated that neither PspE nor other *E. coli* rhodanese paralogs play roles in cyanide or cadmium detoxification. The physiological role of PspE remains to be determined.

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CHAPTER ONE

Introduction

1.1. Sulfur: an element essential for life

Among the elements essential for life, sulfur is the eighth most abundant element in the human body after hydrogen, carbon, oxygen, nitrogen, calcium, phosphorus and potassium (1). Sulfur's biochemical importance stands out because of its chemical versatility. Electronic expansion into the *d*-orbitals allows sulfur to assume several valencies at a number of oxidation states ranging from –2 (e.g. thiols, sulfide) to +6 (e.g. sulfate). This permits sulfur to form a series of oxyanions, and compounds at these intermediate oxidation states are chemically and biologically active. Oxidation of sulfur from the –2 oxidation state to that of the most stable state, +6, gives a free energy release (ΔG) of ~700-800 kJ mol^{-1}(1;2). Sulfur in lowest oxidation state (–2) plays essential roles. Amino acids methionine and cysteine are needed for protein synthesis. Sulfhydryl groups of proteins provide binding sites for enzymes, and are essential for certain drug receptors and transport systems. Reduced sulfur is employed in the activation of substrates as, for example, with the coenzyme A and acyl carrier protein derivatives. Sulfur is an element present in molecules such as protective agent glutathione. It is also a constituent of iron-sulfur clusters or of essential coenzymes such as thiamin, biotin, lipoic acid and molybdopterin. It also belongs to many of the modifications of the nucleotides that constitute transfer RNA (1-3).

1.2. Sulfate reduction and biosynthesis of L-cysteine in *E. coli*

Sulfate uptake and reduction require a large number of transport and enzyme activities. Most of the machinery of cysteine biosynthesis is dedicated to conversion of sulfate to sulfide. Sulfate is first transported into *E. coli* cells through a single transport system that utilizes a periplasmic sulfate-binding protein and sulfate permeases, then it is activated to a phosphosulfate mixed anhydride (Fig. 1.1.) (3;4). This activation is achieved by the ATP sulfurylase-catalyzed reaction of sulfate with ATP to give adenosine 5'-phosphosulfate (APS) and PPi. A second enzyme, APS kinase, phosphorylates APS with another ATP to give PAPS, which is then enzymatically reduced by PAPS sulfotransferase to sulfite. Thioredoxin or glutaredoxin is believed to serve as the physiologic reductant for this step (4). The enzyme mechanism for PAPS sulfotransferase is thought to involve transfer of a sulfo moiety from PAPS to one of the two redox-sulfhydryl groups of thioredoxin to give an organic thiosulfate, thioredoxin-S-SO_3^-. Once formed, thioredoxin-S-SO_3^- rearranges to give free sulfite and oxidized thioredoxin, which is regenerated by thioredoxin reductase. The reduction of sulfite to sulfide is catalyzed by NADPH-sulfite reductase, an enzyme comprising two subunits arranged with a stoichiometry of $\alpha_8\beta_4$, where α is 66-kDa flavoprotein and β is a 64-kDa hemoprotein. The role of the flavoprotein is to accept electrons from NADPH and transfer them to the hemoprotein, which then reduces sulfite. L-cysteine biosynthesis is a relatively simple process when sulfide is available in the environment. Serine transacetylase catalyzes the acetylation of L-serine by acetyl-CoA to give *O*-acetylserine,

the direct precursor of L-cysteine. Synthesis of L-cysteine from *O*-acetylserine and sulfide is catalyzed by two distinct *O*-acetylserine (thiol)-lase isozymes designated –A and –B. Both *O*-acetylserine (thiol)-lases use sulfide as a nucleophile, but *O*-acetylserine (thiol)-lase B also possesses a characteristic feature, the ability to use thiosulfate in the place of H_2S, leading to the production of *S*-sulfocysteine. The conversion of *S*-sulfocysteine into cysteine has not been characterized in *E. coli* but could involve hydrolysis to cysteine and sulfate.

L-Cysteine is one of twenty common protein amino acids. The side chain (-CH_2SH) contains a reactive sulfhydryl as functional group with pKa value of 8.3. Cysteine is essential for the structure and function of proteins, e.g. to stabilize the protein's overall structure through disulfide bond formation, to provide ligands for binding of substrates and iron sulfur clusters, or to provide a unique active site residue involved in catalysis (1). In addition, the amino acid cysteine is the major sulfur source for the synthesis of sulfur-containing compounds (5;6).

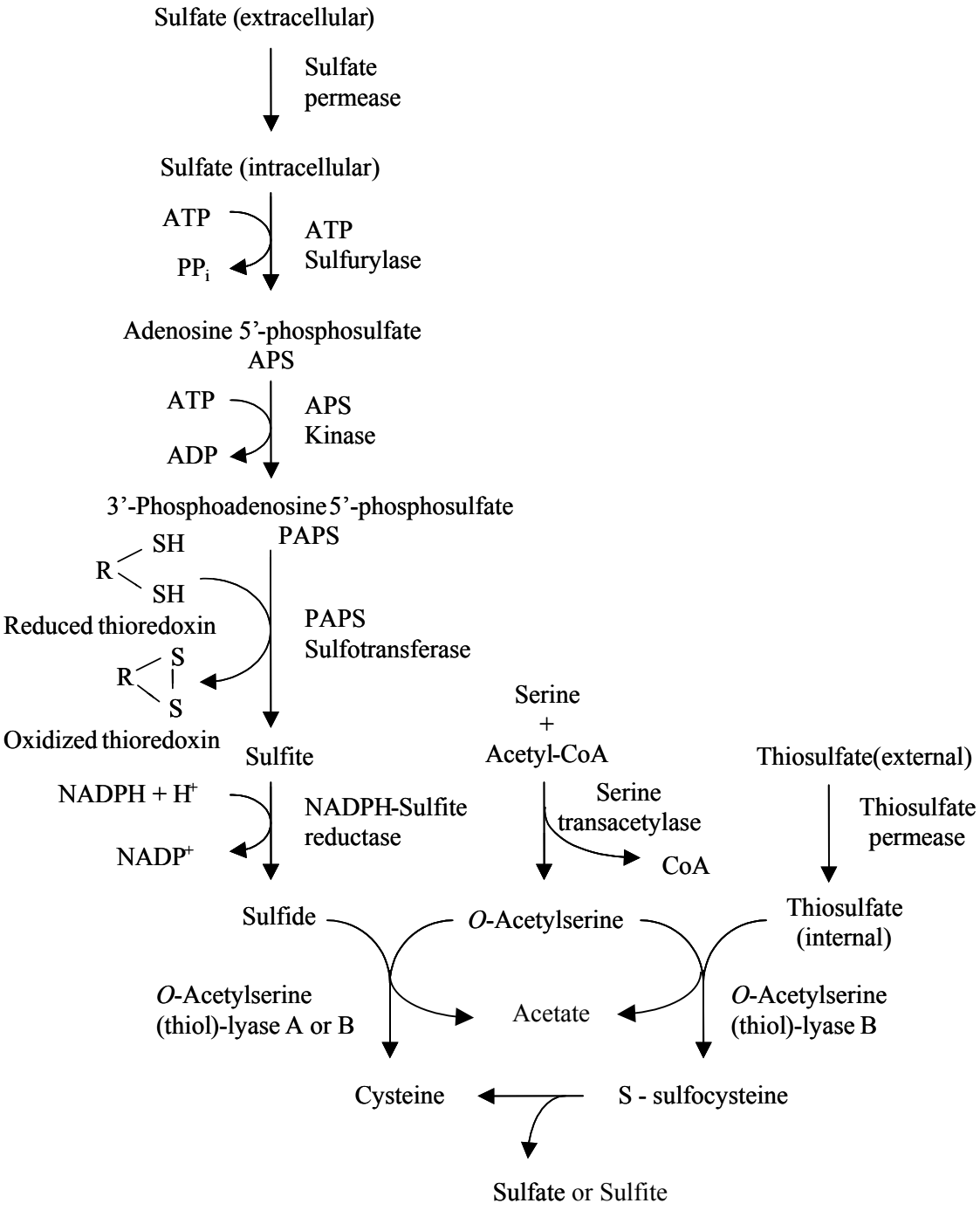


Figure 1.1. Sulfate reduction pathway and cysteine biosynthesis in *Escherichia coli*.

1.3. Sulfur containing compounds: function and biosynthesis

1.3.1. Thiamin

Thiamin (vitamin B_1), an essential component of the human diet, is known to prevent beriberi, a nervous system disease that has occurred in the Far East for centuries. It occurs in mammals as mixture of the free form (Fig. 1.2.) and the monophosphate, diphosphate (pyrophosphate) and triphosphate derivatives. Thiamin-dependent enzymes play an important role in carbohydrate metabolism and include tranketolase, α-ketoacid decarboxylase, and α-ketoacid dehydrogenase (6-8).

The thiamin biosynthetic pathway involves the separate synthesis of the thiazole and the pyrimidine moieties, which are then coupled to give thiamin phosphate (9). The biosynthesis of the thiazole moiety involves the oxidative condensation of deoxy-D-xylulose-phosphate, tyrosine, and cysteine (8). The *de novo* biosynthesis of thiazole is complex and involves at least six gene products (ThiFSGH, ThiI and IscS). ThiF catalyzes the adenylation of ThiS to form ThiS-COAMP, IscS and ThiI catalyzes the sulfur transfer from cysteine to ThiS-COAMP to form a thiocarboxylate (ThiS-COSH), which has been identified as an intermediate sulfur carrier (6;8;10). Kambampati and Lauhon (11;12) isolated IscS and found that this cysteine desulfurase was specifically involved in the transfer of sulfur from cysteine to tRNA via IscS-SSH and ThiI-SSH yielding 4-thiouridine. ThiI has been shown to be required for the synthesis of both the thiazole moiety of thiamin and the 4-thiouridine modification of tRNA (11;13;14).

1.3.2. Biotin

Biotin (vitamin H) (Fig 1.2.), as a water-soluble vitamin, is produced by bacteria, plants, and a few fungi. It plays a very important physiological role as the prosthetic group of several carboxylases involved in central metabolism, including gluconeogenesis and fatty acid biosynthesis. Biotin has been added to animal food since some cases of biotin deficiency related to diet were observed in animals. Although no severe biotin deficiency has been observed in human, it proved to be beneficial where biotin is used in fermentation broths and in cosmetics (15). The industrial production of biotin by fermentation using recombinant strains (16) has stimulated a great deal of work on the genetic and enzymology of biotin biosynthesis.

The last step required for biotin synthesis is the conversion of dethiobiotin (DTB) to biotin and requires a complex mixture of small molecules and accessory proteins for activity *in vitro* (17). The obviously necessary components include L-cysteine, biotin synthase, DTB, AdoMet (S-adenosylmethionine), NADPH, dithiothreitol (DTT), Fe^{2+}, flavodoxin and flavodoxin reductase (15;17;18). Cysteine is the initial source of the sulfur atom for the reaction. Biotin synthase (BioB) catalyzes the final step of the biotin biosynthetic pathway, where a sulfur atom is inserted between the unactivated methyl and C-6 methylene carbon atoms of the dethiobiotin. It is now established that biotin synthase belongs to the family of AdoMet-dependent iron-sulfur enzymes, defined on the basis that (i) they use a reduced iron-sulfur center and AdoMet for catalysis and (ii) they all contain a CXXXCXXC sequence providing the cysteine ligands to the cluster (5;19). Biotin synthase purified aerobically or semi-anaerobically from *E. coli* and *Bacillus*

sphaericus is a homodimer and contains a $[2Fe\text{-}2S]^{2+}$ cluster. When the enzyme is reduced, the clusters dimerize to give a $[4Fe\text{-}4S]^{+}$ cluster, which is likely to be the catalytically important species (5;6;20). Recent studies indicate that the biotin synthase Fe-S cluster is the immediate sulfur donor for biotin, which was demonstrated using biotin synthase containing isotopically-labeled sulfide in the Fe-S cluster and explains why biotin synthase functions as reagent rather than as a catalyst (6;20). This result suggests that the [Fe-S] cluster of the enzyme must be regenerated after each reaction. It has been proposed that a NifS-like enzyme, similar to the *Azotobacter vinelandii* NifS, may be required for construction of the [Fe-S] cluster *in vivo* and regeneration *in vitro* (18;21). Marquet et al reported that NifS protein from *A. vinelandii* and C-DES from *Synechocystis* as well as rhodanese from bovine liver can mobilize the sulfur, repectively, from cysteine and thiosulfate for the formation of a [2Fe-2S] cluster in the apoprotein for *E. coli* biotin synthase (20). A recent report indicates that biotin synthase displays a significant cysteine desulfurase activity, providing it with the ability to mobilize sulfur directly from free cysteine (19).

1.3.3. Lipoic Acid

Lipoic acid is a sulfur-containing coenzyme (Fig 1.2.) that is synthesized in a large number of organisms including bacteria, plants, yeast, and animals. It is essential for the activity of a variety of enzyme complexes that catalyze oxidative decarboxylation. In *E. coli*, the lipoate-dependent multienzyme complexes are pyruvate and α-ketoglutarate dehydrogenases and glycine decarboxylase (1;15).

Octanoic acid, the specific precursor of lipoic acid can be derived from the fatty acid biosynthesis pathway, so the biosynthesis of lipoic acid consists of two steps in which two sulfur atoms are introduced into the nonactivated positions C-6 and C-8 (15). Experiments with 8-thiooctanoic acid and 6-thiooctanoic acid as possible intermediates indicate that sulfur is very likely first inserted into the methyl carbon and that 8-thiooctanoic acid is a probable intermediate (15). Three genes of the biosynthesic pathway for lipoic acid have been identified: *lipA, lplA* and *lipB*. LipB and LplA have been identified as lipoate ligases responsible for attaching lipoic acid to an ε-amino group of target proteins. LipA is lipoate synthase and catalyzes the formation of the two C-S bonds (3;15). Like BioB, LipA also belongs to the family of AdoMet-dependent iron-sulfur enzymes. It has been demonstrated that the chemical transformation catalyzed by lipoate synthase is very similar to the one catalyzed by biotin synthase. The sequence alignments show that both BioB and LipA have two conserved motifs, CX_3CX_2C and F(Y)NHNL, which provide strong additional arguments that both enzymes share the same mechanism (15;22).

1.3.4. Molybdopterin (MPT)

Molybdopterin (MPT) (Fig 1.2.) is a pyranopterin with a unique dithiolene group coordinating molybdenum (Mo) to form molybdenum cofactor (Moco) (23). Moco, a highly conserved pterin compound of all organisms, is required for the enzymatic activities of all molybdenum enzymes except nitrogenase (24;25). Molybdoenzymes participate in essential redox reactions in global carbon, nitrogen, and sulfur cycles (26). In humans, Moco deficiency is a rare inborn error characterized by the loss of activity of

sulfite oxidase, xanthine oxidase, and aldehyde oxidase. Affected patients die early postnatally because no therapy is available (23-25).

Moco biosynthesis is an evolutionarily conserved pathway present in archaea, eubacteria, and eukaryotes, and the pathway is a multistep process with two characterized intermediates. (i) Early step in which a guanosine derivative, most likely GTP, is rearranged into precursor Z. (ii) Transformation of precursor Z into MPT, generating the dithiolene group necessary for Mo-coordination. (iii) Metal incorporation into MPT, which requires the functions of MoeA and MogA (23;27;28). The transfer of sulfur in the biosynthesis of MPT is very similar to that in thiamin biosynthesis. In *E. coli*, MPT is formed by incorporation of two sulfur atoms into precursor Z, which is catalyzed by MPT synthase. The recently solved crystal structure of MPT synthase (23) shows the heterotetrameric nature of the enzyme that is composed of two small (MoaD) and two large subunits (MoaE). In the activated form of MPT synthase, the C terminus of MoaD is converted to a glycine thiocarboxylate that acts as the sulfur donor for the conversion of precursor Z to MPT. MoeB protein is the MPT synthase sulfurase responsible for regenerating the thiocarboxylate group at the C terminus of MoaD in an ATP-dependent reaction (23;28;29). Resent studies indicate that in an *in vitro* system of *E. coli*, three NifS-like proteins, IscS, CSD (CsdA), and CsdB (SufS) can transfer sulfur from L-cysteine for the activation of inactive MPT synthase in an ATP-dependent reaction, with CSD being the most effective of the three (5;28). Particularly noteworthy is the sequence and structural similarities between MoaD and ThiS, a thiamin biosynthesic protein. MoaD and ThiS contain the same Gly-Gly dipeptide at their C-terminal end. In both

cases, sulfur is transferred in the same way by adenylation followed by formation of the thiocarboxylate (3;23).

1.3.5. Iron-sulfur Clusters

Iron-sulfur (Fe-S) clusters are cofactors of proteins that perform a number of biological roles, including electron transfer, redox and non-redox catalysis, and sensing for regulatory processes (30). The most familiar iron-sulfur clusters are the dinuclear Fe_2S_2 and the cubane Fe_4S_4 types (Fig. 1.2.) commonly found in a class of simple biological electron carrier proteins called ferredoxins.

The assembly of iron-sulfur (Fe-S) clusters is mediated by complex machinery. Three distinct systems have been identified, termed NIF (nitrogen fixation), ISC (iron-sulfur cluster) and Suf. The *A. vinelandii nifS* gene product (NifS), which is required for full activation of the nitrogenase component proteins, is a pyridoxal phosphate (PLP) enzyme and the first discovered cysteine desulfurase. NifS is able to transfer the sulfur from cysteine to an active site cysteinyl residue of NifS, forming an enzyme-bound persulfide. Upon reduction and addition of an iron source, the persulfide sulfur can be released and efficiently incorporated into the cluster of the Fe-S protein of nitrogenase (31;32). NifU serves as a scaffold for the assembly of transient Fe-S clusters before delivery to other apoFe-S proteins (33).

In contrast to the NIF machinery that specifically deals with the maturation of nitrogenase, the ISC machinery is involved in the general pathway of biosynthesis of numerous Fe-S proteins in virtually all organisms (30;34). Several similarities have been identified when comparing the ISC and NIF systems. First, IscS and NifS are similar in

their sequences and functions as cysteine desufurases. Second, IscU corresponds to the N-terminal domain of NifU and contains three conserved cysteine residues that are essential for its function as a scaffold for intermediate Fe-S clusters. Third, IscA is closely related to its NIF counterpart (^{Nif}IscA), which is an alternate scaffold protein (30;35). Noteworthy is the versatile functions of IscS. Besides serving as cysteine desulfurase in the biosynthesis of Fe/S clusters, IscS also participates in thiamine and thionucleoside biosynthesis (12;36).

Recent studies have identified more proteins that are able to carry out cysteine desulfurase activity. *E. coli* contains three ORFs that encode proteins homologous to *A. vinelandii* NifS/IscS. They are named *iscS*, *csdA* and *csdB* (*sufS)*. IscS is 40% identical to *A. vinelandii* NifS, and this homology exists through the entire protein. The two remaining NifS homologs, CSD (CsdA) (cysteine sulfinate desulfinase, encoded by *csdA*) and CsdB (encoded by *csdB*/*sufS*), represent a separate class in that they are both 24% identical to NifS(34;37). Research has indicated that IscS is a cysteine desulfurase and has a major role in *in vivo* Fe-S cluster formation in *E. coli* (34).

1.3.6. 4-thiouridine in tRNA

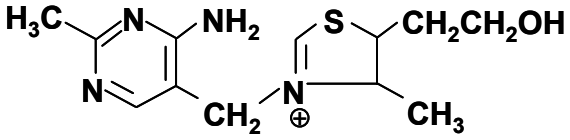
Base modification in tRNA is found in all organisms and represents the fine-tuning of the many functions of tRNA in protein translation (11;38). One modified base of known function is 4-thiouridine (s^4U, Fig. 1.2.) at position 8 of some bacterial tRNAs and serves as a photosensor for near-UV light (36). When bacteria are exposed to this light, a photochemically induced 2+2 cycloaddition occurs between s^4U-8 and cytidine-

13, followed by successive elimination reactions to afford a tRNA with a carbon-carbon bond cross-link. Such cross-linked tRNAs are inefficient substrates for some aminoacyl tRNA synthetases, and the accumulation of uncharged tRNA triggers growth arrest (14;36).

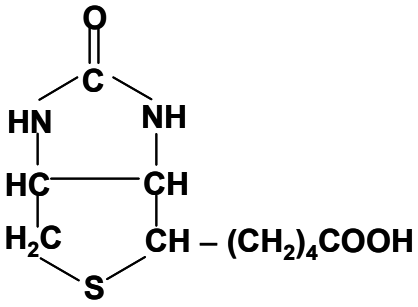
The formation of s^4U requires IscS and ThiI as well as Mg-ATP and L-cysteine as the sulfur donor (12). Early work showed that two factors, A and C, mediated s^4U synthesis (11). The *thiI* and *iscS* genes, both required for thiamin and 4-thiouridine synthesis, were characterized as a result of these initial studies (12;14). Comparison of the ThiI sequence with protein sequences in the databases revealed that ThiI has a motif shared with the sulfurtransferase rhodanese (14). It turned out that ThiI was able to catalyze the transfer of sulfur from thiosulfate to cyanide, like rhodanese (14). This result suggests that a persulfide may be formed on the active-site cysteine of ThiI during catalysis. ThiI has five cysteine residues. Sequence alignments reveal that Cys-456 of ThiI aligns with the active site cysteine residue of rhodanese (14), while Cys-344 is absolutely conserved among all sequenced ThiI orthologs (13). Analysis of ThiI variants in which each cysteine was substituted with alanine and the effect showed that both Cys-344 (13) and Cys-456 (14) of ThiI are critical for activity. Based on the current work, a scheme for sulfur mobilization and transfer in the biosynthesis of s^4U in *E.coli* is proposed (13;38). The mechanism of sulfur transfer (Fig. 1.3.) initially involves mobilization of sulfur from cysteine resulting in the formation of S^0 covalently bound to cysteine at the active site of IscS as a persulfide (IscS-SSH). This persulfide sulfur is then

transferred to Cys-456 on ThiI, which is in turn transferred to the tRNA by a mechanism that likely involves disulfide formation between Cys-456 and Cys-344 of ThiI.

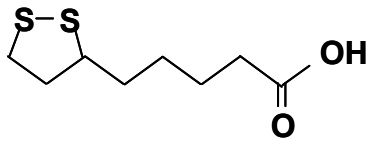
In addition to *iscS*, *E. coli* contains two other *nifS* homologs, *csdA* and *csdB*, each of which has cysteine desulfurase activity and could potentially donate sulfur for thionucleoside biosynthesis. Four thionucleosides are naturally occurring in the tRNAs of *E. coli* and five are present in the tRNAs of *Salmonella enterica*. Recent studies showed that the *iscS* gene is required for the biosynthesis of all four thionucleosides in *E. coli* (38) and all five thionucleosides in *S. enterica* (39).



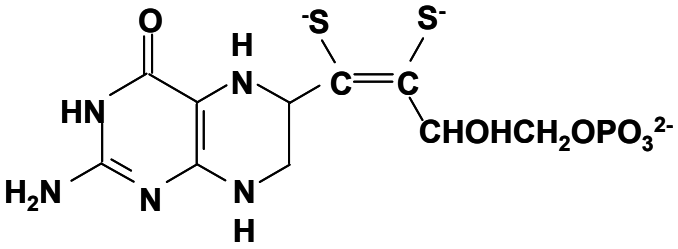
Thiamin



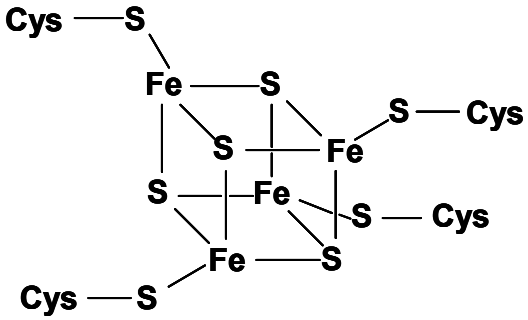
Biotin



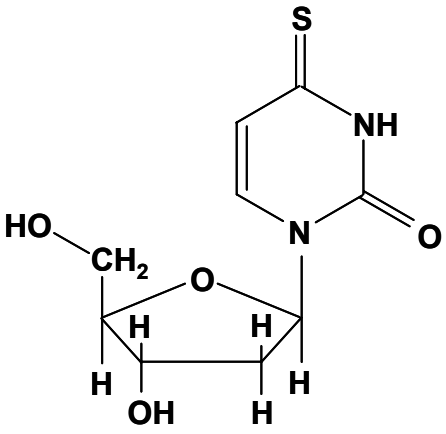
Lipoic Acid



Molybdopterin



Fe_4S_4 cluster



4-Thiouridine

Figure 1.2. Structures of sulfur containing cofactors and 4-thiouridine.

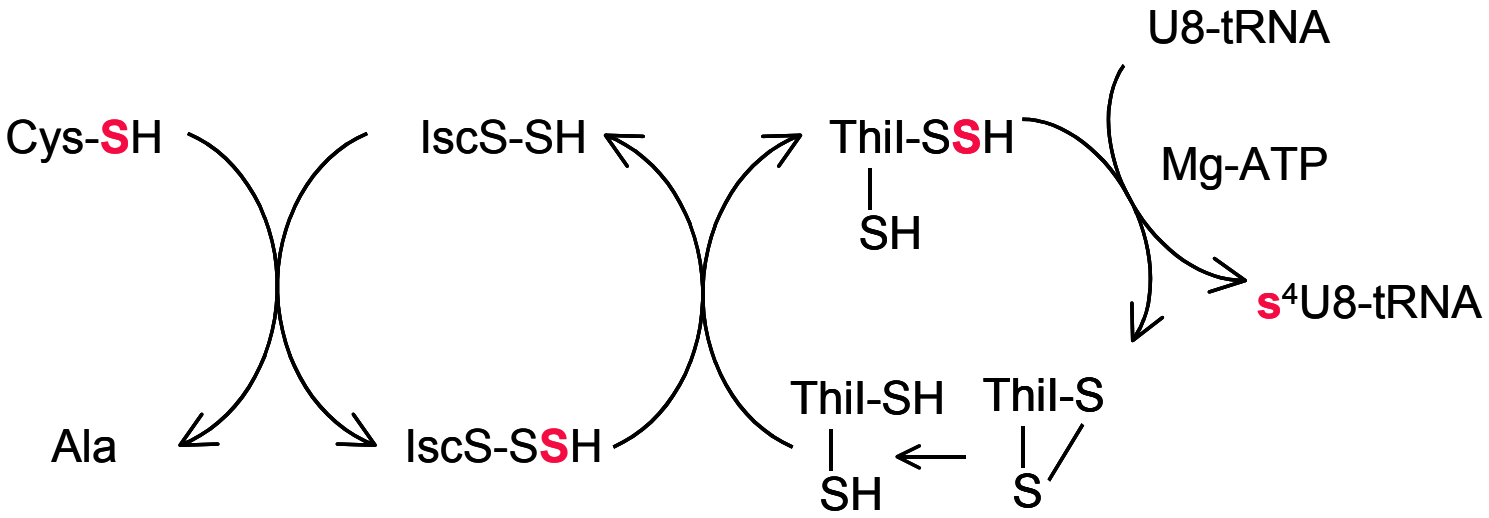


Figure 1.3. Proposed scheme for the mobilization and transfer of sulfur during s^4U biosynthesis in *E. coli*.

1.4. Sulfane sulfur and sulfurtransferases

The term sulfane designates sulfur atoms that are bonded covalently in chains only to other sulfur atoms (40). Examples are the outer sulfurs of the inorganic thiosulfate anion (SSO_3^{2-}), its organic analog, the thiosulfonate ion (RSO_2S^-), persulfides (RSS^-), the internal chain sulfurs of organic and inorganic polysulfides (RSS_xSR) and polythionates ($^-O_3SS_xSO_3^-$), and all the sulfurs in the staggered eight-membered ring of elemental sulfur (S_8) (shown in the sulfane pool of Fig. 1.4.) (2;40;41).

Sulfane atoms are somewhat unstable, readily oxidizing in air, reducing with thiols, and decomposing slowly in dilute acid to release free sulfur. Most sulfane-containing compounds are stable in alkali, but polythionates decompose in strong alkali to form various products including thiosulfate, sulfite, sulfate and elemental sulfur (40). Persulfide groups on proteins are protected by the neighboring groups and transported to sites of biosynthesis. Evidence for persulfide groups can be obtained from a broad band of absorbance at 335-350. But the absorption coefficient is only about 310 M^{-1} at 335 nm. Practically, sulfane sulfurs are identified by susceptibility to cyanolysis, which involves nucleophilic attack on the sulfur-sulfur bond by cyanide, with formation of thiocyanate (2;40):

$$RSS^- + CN^- \longrightarrow RS^- + SCN^-$$

Thus, reactive sulfane sulfur is also called cyanolyzable sulfur. Sulfane sulfur is the precursor of the sulfur that becomes incorporated into some sulfur-containing

cofactors and molecules. It is the transport form for reactive sulfur and the 'currency' for sulfur trafficking in many organisms (2).

Sulfurtransferases are a group of proteins that catalyze the formation, interconversion and reactions of compounds containing sulfane sulfur atoms (41). Fig. 1.4 shows the sulfane pool in biological systems and the sulfurtransferases' probable participation in sulfane metabolism (41).

Rhodanese (thiosulfate : cyanide sulfurtransferase, EC 2.8.1.1) (designated "1" in Fig. 1.4.) is the longest-known, most studied and best understood sulfurtransferase (41-43). Further details about this enzyme will be provided later. Rhodanese is known mostly for its catalysis of the transfer of sulfane sulfur directly to cyanide. Less well known is the fact that rhodanese is also able to catalyze the rapid interconversions of all of the sulfane pool components (41).

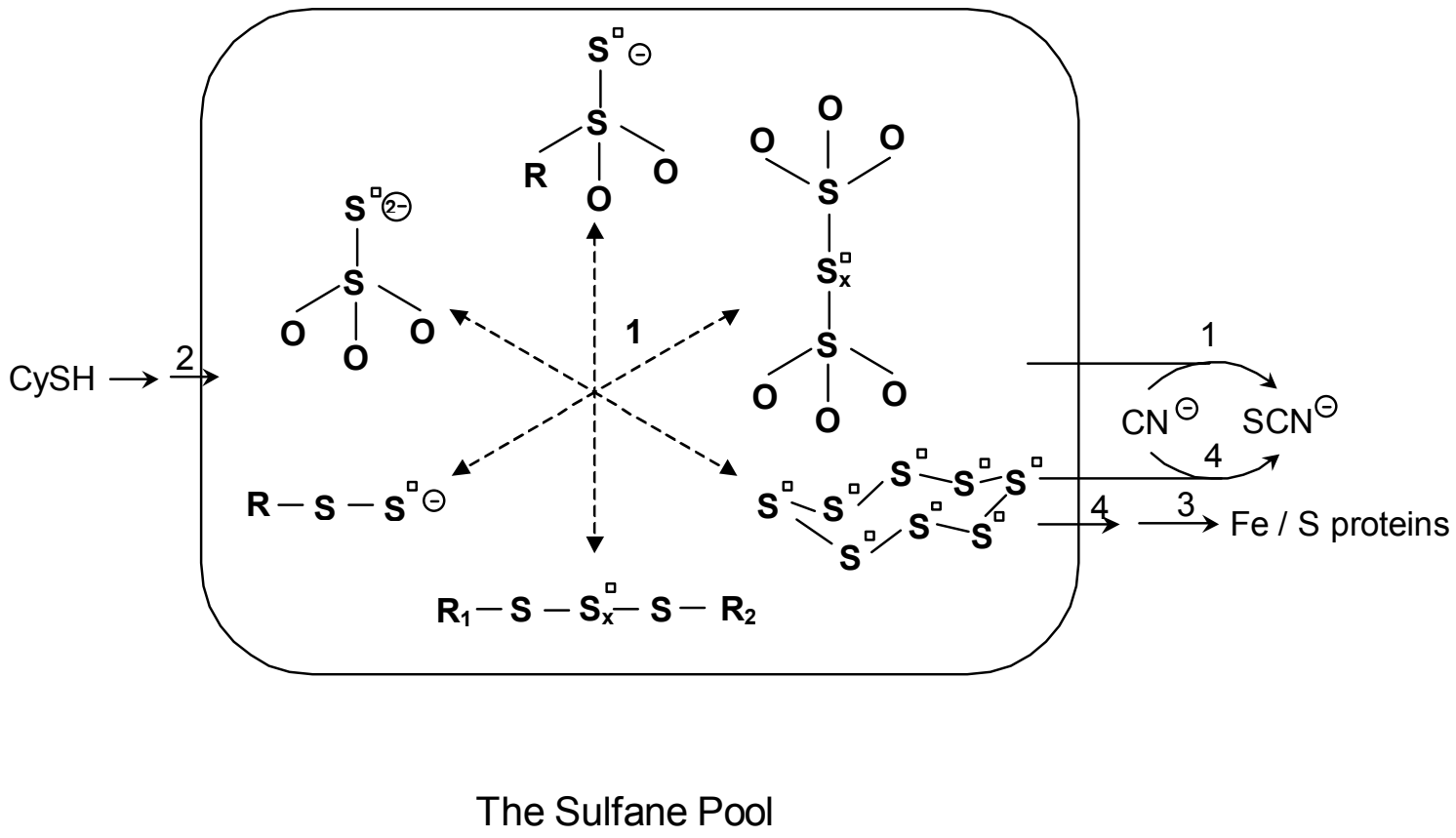
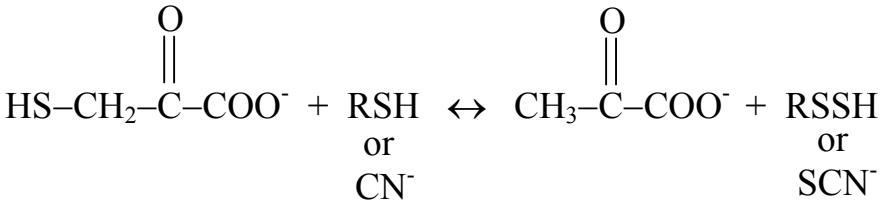


Figure 1.4. Biological materials containing sulfane sulfur.

Sulfane atoms are marked with the symbol □. Numbers over reaction arrows indicate the participation of the following proteins: 1, rhodanese; 2, mercaptopyruvate sulfurtransferase; 3, thiosulfate reductase; 4, serum albumin.

1.4.1. Mercaptopyruvate sulfurtransferase (MST) (EC 2.8.1.2)

Mercaptopyruvate sulfurtransferase (MST) (EC 2.8.1.2) (designated "2" in Fig. 1.4.) catalyzes the cleavage of a carbon-sulfur bond and transfer of the sulfur atom from 3-mercaptopyruvate to any of a variety of thiophiles, including thiols, cyanide, sulfite, and sulfinates (42;44).



MST has been highly purified from rat liver, *E. coli* and bovine kidney (42;43;45). Recent studies show that MST is also found in plants (46-48). MST and rhodanese are evolutionarily related enzymes (49) based on the following findings: the alignment of the amino acid sequence reveals 60% identity between MST and rhodanese; MST and rhodanese possess both MST and rhodanese activities, but the ratios of their activities differ greatly; in a mutagenesis study with rat rhodanese cDNA, replacement of the two amino acid residues in the active site of rhodanese (Arg and/or Lys) with MST type residues (Gly and/or Ser) increased MST activity and decreased rhodanese activity, indicating that rhodanese was partly converted to MST (50). The *E. coli sseA* gene was found to encode MST (51). SseA is one of the few prokaryotic proteins containing the active site motif CGSGVTA that in eukaryotic sulfurtransferases specifies for MST activity over rhodanese activity (52). The physiological role for MST is not clear yet.

Proposed MST functions include detoxification of cyanide and degradation of cysteine (41;48).

1.4.2. Thiosulfate reductase (EC 2.8.1.3)

The third of the sulfurtransferases, thiosulfate reductase (EC 2.8.1.3) (designated "3" in Fig. 1.4.), occurs in both prokaryotic and eukaryotic organisms. It uses electrons from thiols, like glutathione (GSH), to reduce the sulfane sulfur atoms of inorganic thiosulfate and organic thiosulfonate to the sulfide level, probably for use in the synthesis of iron/sulfur proteins (41;53). But this hypothesis has not been proven.

$$SSO_3^{2-} + GSH \leftrightarrow SO_3^{2-} + GSSH$$

$$(GSSH + GSH \leftrightarrow H_2S + GSSG)$$

The thiosulfate reductase catalyzed reaction is similar to the rhodanese reaction in the cleavage of the sulfur-sulfur bond of the sulfur-donor substrate with transfer of the sulfane sulfur to a sulfhydryl nucleophile. However, the enzymes differ in both acceptor substrate specificity and mechanism: unlike both rhodanese and MST, thiosulfate reductase will use GSH or cysteine as an acceptor substrate, but will not use cyanide. Incidentally, thiosulfate reductase contains one cysteine residue, but the sulfhydryl group of this cysteine residue is not directly involved in the catalytic mechanism (41;53).

Thiosulfate reductase is encoded by the *phsABC* operon in *S. enterica* (54). It is proposed that thiosulfate reductase contains a molybdopterin cofactor and is capable of oxygen atom transfer, because this enzyme is able to reduce chlorate to chlorite (55). Recent studies indicate that thiosulfate reductase may be involved in heavy metal

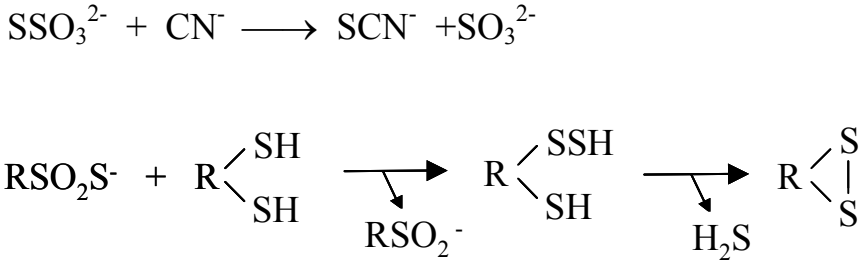
detoxification in microorganisms based on the fact that expression of the thiosulfate reductase gene (*phsABC*) from *S. enterica* in *E. coli* caused cadmium removal by precipitation with hydrogen sulfide, the product of the reaction catalyzed by thiosulfate reductase (54).

Surprisingly, serum albumin (labeled "4" in Fig. 1.4.) is able to catalyze the cyanolysis of elemental sulfur (41). Based on the observation that it was easy to load serum albumin with elemental sulfur *in vivo* and *in vitro* and the sulfur bound to albumin was quite reactive with cyanide, it was proposed that serum albumin is a sulfane sulfur carrier, used to transport sulfur from the liver, where it is formed, to the other organs, where it can be used for the synthesis of iron/sulfur centers (41).

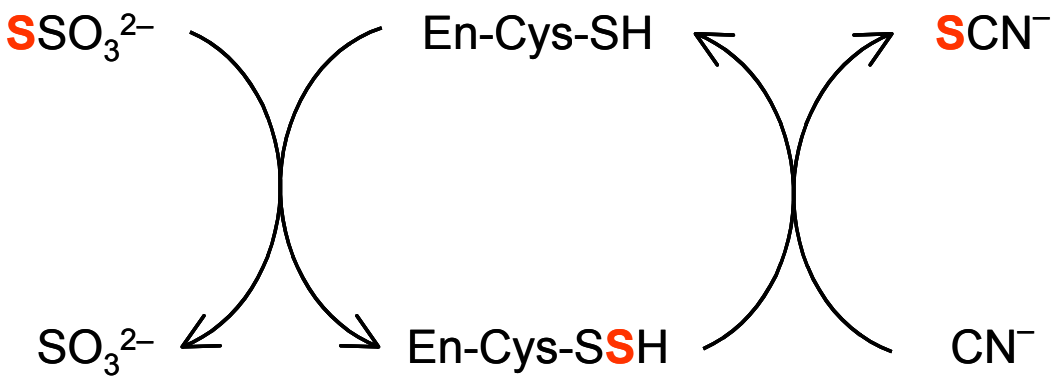
1.4.3. Thiosulfate: cyanide sulfurtransferase (rhodanese)

Rhodanese (thiosulfate: cyanide sulfurtransferase, EC 2.8.1.1) is ubiquitous biologically, found in most mammalian tissues, in frogs, birds and plants, in some insects and in several microorganisms (56). Rhodanese is the best-characterized sulfurtransferase, and catalyzes the transfer of a sulfane sulfur atom from sulfane sulfur-donor substrates to thiophilic acceptor substrates (57).

Donors of sulfane sulfur to rhodanese include thiosulfate, thiosulfonates (RSO_2S^-), persulfide (RSS^-), and thiocystine (CySSSCy) (58). Sulfur acceptor substrates include cyanide, sulfite, sulfinate and dithiols (43). The general equations are:



Bovine liver rhodanese has been the subject of extensive study in both its mechanism of action (59-61) and its structure (62-64). As is indicated below, bovine rhodanese is shown to catalyze the reaction using a double displacement (ping-pong) mechanism. In the course of the reaction, the enzyme cycles between two catalytic intermediates, the sulfur-free (En-Cys-SH) and the sulfur-substituted (En-Cys-SSH, persulfide-containing) forms (60).



The sulfur-donor substrate forms a kinetically significant complex with enzyme and is cleaved by the enzyme to form a covalently substituted sulfur-enzyme (En-Cys-SSH), with discharge of the first product. En-Cys-SSH is then attacked by the sulfur-acceptor substrate to produce the final product and regenerate the free enzyme (41;60). A

later purified rhodanese from *Acinetobacter calcoaceticus lwoffi* showed varied mechanisms when acceptor substrates were a thiol or dithiothreitol (65).

Enzyme-ion pair formation experiments (61) and anion inhibition studies (66) indicate a cationic site used for binding of the substrate thiosulfate. This suggestion is consistent with the crystal-structure studies of bovine liver rhodanese (62-64). The three-dimensional structure of this enzyme reveals that the 293-residue single polypeptide chain is folded into two domains of roughly equal size, with nearly identical conformations but very dissimilar amino acid sequences. The native protein has four cysteine residues and no disulfides. The active site sulfhydryl group of Cys-247 is located at the bottom of a pocket between the two domains and binds the transferred sulfur in a persulfide linkage during catalysis. Crystallographic analysis also showed that the side chains of Phe-212, Phe-106, Tyr-107, Trp-35, and Val-251 provide a hydrophobic wall of the active site and that the guanidine and ε-NH_2 groups of Arg-186 and Lys-249 may participate in binding thiosulfate in the correct orientation and act as an "electrophilic assistant" (67;68).

The sulfur free form (En) and persulfide form (En-S) have been shown to have differing stabilities with respect to various chemical modifying reagents, selected oxidants and reductants (69;70). When En-S form rhodanese is incubated with 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), only 0.6 equivalent of sulfhydryl groups reacts with DTNB. After removal of persulfide sulfur, a further 0.6 equivalent of sulfhydryl groups becomes accessible to the reagent (71). It is proposed that the presence of persulfide sulfur of sulfur-containing rhodanese protects the enzyme against the

inactivating agents to a large extent (72). This result can be explained in view of the conformational changes that rhodanese undergoes when the enzyme cycles between the En-S and En forms. Studies showed that the formation of persulfide at the active site triggers a conformational change and exerts a stabilizing influence on the structure (69;73).

It is generally believed that the primary physiological role of rhodanese is in the conversion of inorganic cyanide to the less toxic thiocyanate anion (56). However, the widespread distribution and abundance of rhodanese as well as its subcellular localization in the matrix of liver and kidney mitochondria suggest additional functions (41;56). A recently discovered rhodanese, ThiI of *E. coli* has been found to be involved in thiamin and 4-thiouridine biosynthesis (14). The reports that rhodanese will utilize physiological dithiols, dihydrolipoate (59;66;74) and thioredoxin (75;76) yielding sulfide, sulfite and oxidized dithiol as products indicate that rhodanese may be involved in the formation or repair of iron-sulfur centers.

The most highly conserved regions of the rhodanese sulfurtransferases are the active site and two structural motifs, designated CH2A and CH2B. BLAST searches indicate that there are nine predicted proteins in *E. coli* that display sequence similarity to bovine rhodaneses (Fig. 1.5.). The data from Dr. Larson's lab indicate that four of them have rhodanese activity: GlpE (76), PspE (this study) (77), ThiI (14), and YgaP (unpublished data from F. Ahmed). Another protein, SseA is a mercaptopyruvate sulfurtransferase (51;52).

In early studies, two *E. coli* rhodaneses of less than 20,000 molecular weight were identified (66). One of the enzymes is localized in the periplasmic space and the other within the cytoplasm as inferred from their ease of extraction and accessibility to substrates in whole cells. Two recently characterized rhodaneses in *E. coli* possess similar features, GlpE (encoded by *glpE*) and PspE (encoded by *pspE*), of which GlpE is localized in the cytoplasm (76) and PspE may be in the periplasmic space (78-80). Other features of GlpE and PspE do not correspond directly to the rhodaneses characterized earlier.

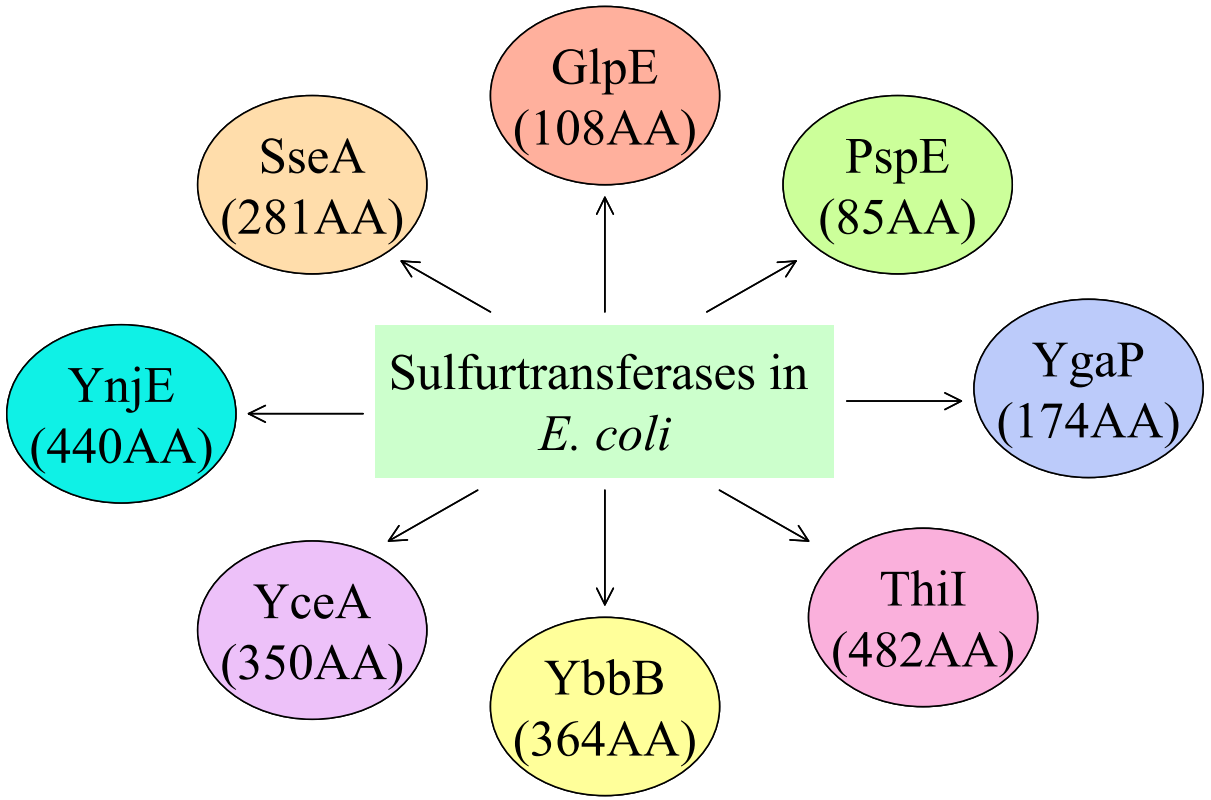


Figure 1.5. Proteins of *E. coli* possessing rhodanese domains revealed by BLAST searches.

The indicated proteins each contain one or more rhodanese homology domains. An additional protein (YibN) displays sequence similarity to rhodanese, but contains an aspartate residue at the position corresponding to the active site cysteine Data from Dr. Larson's lab. Figure courtesy of F. Ahmed.

GlpE, a 12-kDa rhodanese encoded by the *glpE* gene, is a member of the *sn*-glycerol 3-phosphate (*glp*) regulon of *E. coli*. GlpE catalyzes the rhodanese reaction using a double-displacement mechanism requiring an active-site cysteine. The apparent molecular mass of GlpE under nondenaturing conditions is 22.5 kDa, indicating that GlpE functions as a dimer. GlpE is capable of transferring sulfur from thiosulfate to thioredoxin with relatively high affinity (76). The crystal structure of GlpE revealed that this enzyme is a single domain protein and belongs to the rhodanese homology superfamily. The active-site main chain conformation is strongly preserved in GlpE and bovine rhodaneses despite the fact that the overall amino acid sequence identity between GlpE and the active-site domain of bovine rhodanese is only about 17% (81). GlpE displays α/β topology based on five β strands and five α helices. The GlpE catalytic Cys residue is persulfurated and enclosed in a structurally conserved 5-residue loop in a region of positive electrostatic field.

PspE is the smallest sulfurtransferase discovered so far (85 amino acids) in *E. coli.* It is encoded by *pspE*, part of a phage shock protein operon that is induced in response to heat, ethanol, osmotic shock and infection by filamentous bacteriophages (78;79). The operon includes five genes: *pspA*, *B*, *C*, *D* and *E.* *pspE*, the last gene of the operon, is expressed in response to stress as part of the *psp* operon, but is also transcribed independently. The N-terminal region of PspE has a signal peptide, which is cleaved after PspE is synthesized (80). This indicates that PspE may be a periplasmic protein (78;79). A recent study has shown that PspE is a rhodanese and the catalysis utilizes a

double displacement mechanism. The K_ms for SSO_3^{2-} and CN^- were 4.6 and 27 mM, respectively (77).

Despite the explicit characterization of the above rhodaneses, the physiological functions of these proteins are still generally unknown. The rhodanese homology domain is frequently found fused to other protein domains of known or unknown function, which provides us clues regarding the physiological functions of rhodaneses (unpublished data of Dr. Larson's lab). While performing BLAST searches using GlpE as query sequence, we found that some proteins of known or predicted function contain a fused rhodanese domain, generally at their carboxy termini (Fig. 1.6.). Some examples include ThiI of *E. coli* and the MoeB proteins of mycobacteria, fungi, plants and mammals, both of which are known to be involved in biosynthesis of sulfur-containing cofactors or tRNAs. A rhodanese domain was also found fused to a variety of other proteins, such as members of the NADH-disulfide oxidoreductase family and HesB proteins. These findings not only provide a rationale for the involvement of rhodanese domains in the biosynthesis of sulfur-containing cofactors, but also indicate that rhodaneses may participate in other biosynthetic pathways or cellular processes, such as oxidation/reduction of disulfide bonds and the assembly and/or repair of Fe-S clusters.

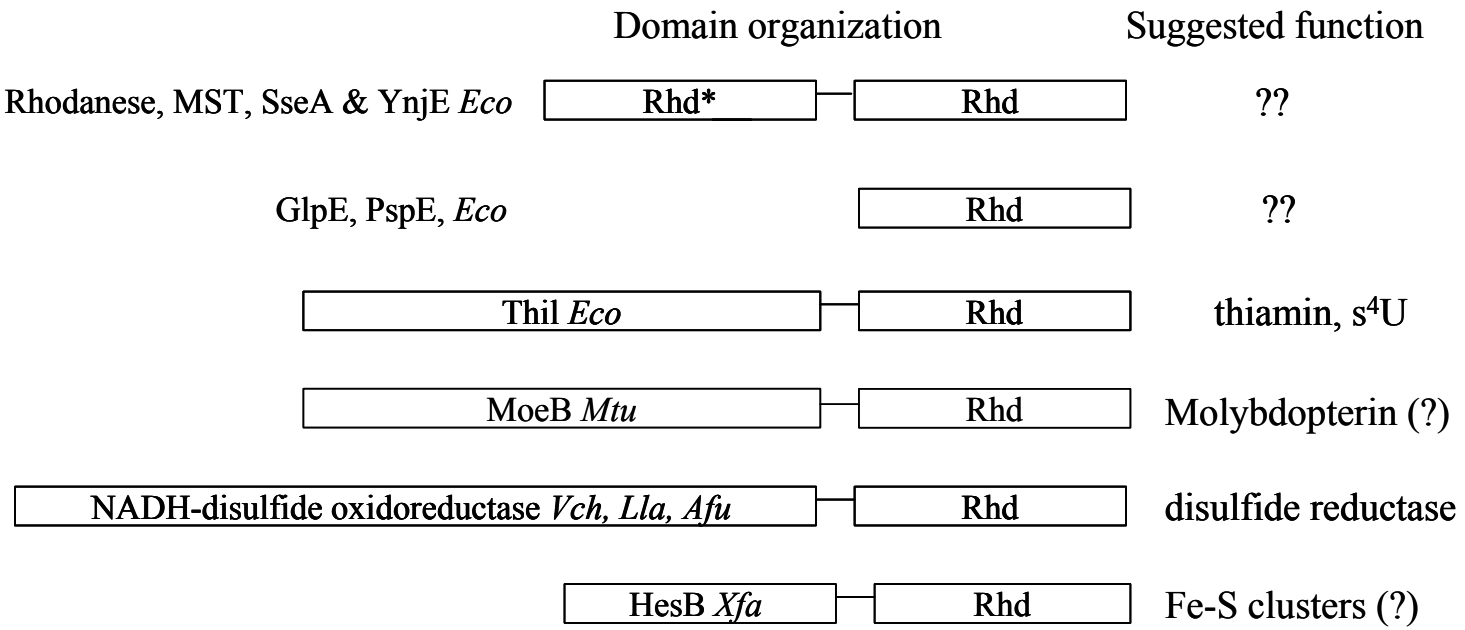


Figure 1.6. Examples of proteins with fused rhodanese domains.

Rhodanese represents bovine rhodanese and other two domain rhodaneses. Abbreviations: Rhd, rhodanese homology domain; Rhd*, Rhd with aspartate in place of the active site cysteine; MST, mercaptopyruvate sulfurtransferase; ThiI, enzyme for thiamin and 4-thiouridine synthesis; MoeB, molybdopterin synthase sulfurylase; HesB, protein related to IscA/IscU. Organisms include: *Eco*, *E. coli*; *Mtu, Mycobacterium tuberculosis*;*Vch*, *Vibrio cholerae*; *Lla*, *Lactococcus lactis*; *Afu*, *Archaeoglobus fulgidus; Xfa, Xylella fastidiosa*.

The focus of this research project was to characterize PspE, the secreted and smallest sulfurtransferase of *E. coli*. From the freeze-thaw extract of a PspE-overexpressing strain, PspE was purified and compared to other characterized rhodaneses. Two forms of PspE were characterized by native gel electrophoresis. Determining the cyanolyzable sulfur in purified PspE characterized the two peaks of PspE yielded by cation exchange chromatography. The stability of the two forms of PspE toward thio-specific reagent DTNB and anion inhibition experiments provided insight into the active site of PspE. Through kinetic analysis, the reaction mechanism and k_{cat} of PspE and the K_ms for SSO_3^{2-} and CN^- were determined and compared with other rhodaneses. A *pspE*-deletion strain was constructed, and its rhodanese activity, as well as its cyanide and cadmium tolerance were tested and compared with the wild-type strain. The results provide clues and a foundation for studies aimed at determining the physiological role of PspE in *E. coli*.

CHAPTER TWO

Materials and methods

2.1. Materials

Unless listed below, the reagents used were purchased from Sigma Chemical Company or Fisher Scientific. Oligonucleotides for PCR were synthesized by DNAgency. New England Biolabs supplied restriction endonucleases and reagents for PCR and cloning. Ammonium thiosulfate was purchased from Aldrich.

2.2. Bacterial strains and plasmids

The bacterial strains and plasmids used or constructed are listed in Table 2.1.

2.3. Media and growth conditions

E. coli cells were grown aerobically at 37^{o}C in Luria-Bertani broth (LB (82)). Cells containing plasmids were grown in the presence of antibiotic as appropriate (100 μg/ml ampicillin (Ap), 25 μg/ml kanamycin (Km) or 10 μg/ml tetracycline (Tc)). Overexpression of PspE in strains BL21(DE3)(pHC4.1) was induced in mid-log phase (OD_{600} of 0.4 to 0.6) by the addition of 0.5 mM isopropylthio-β-D-galactopyranoside (IPTG) (76). Cultures were grown for 2 to 4 h after induction and then harvested when OD_{600} reached 1.0.

Table 2.1. *E. coli* strains and plasmids

Strain or plasmid	Description	Reference
Strains		
MG1655	Wild-type isolate (F^- *rph*-1 λ^-)	(83)
BL21(DE3)	*hsdS gal* (λ*cIts857 ind-1 Sam7 nin-5 lacUV5-T7* gene 1)	(84)
DH5αZ1	*(Φ80d lacZΔM15) endA1 recA1 hsdR17 supE44 thi-1 gyrA relA1 Δ(lacZYA-argF)U169 (λatt lacI tetR* Sp^r*)*	(85)
CAG12028	MG1655 *zcj-233*::Tn*10*	(86)
BW25113	*lacI*^{q} *rrnB*$_{T14}$ *ΔlacZ*$_{WJ16}$ *hsdR514 ΔaraBAD*$_{AH33}$ *ΔrhaBAD*$_{LD78}$	(87)
TL524	MG1655 Δ(*lacZYA-argF*)*U169*	(88)
HC1.1	BW25113 Δ*pspE*::Km^r *FRT*	This work
HC6.1	TL524 Δ*pspE*::Km^r *FRT*	This work
HC7.1	TL524 Δ*pspE*::*FRT*	This work
FA036	TL524 *ΔpspE*::*FRT sseA*::Sp^r *ΔygaP*::*FRT ΔynjE*::*FRT ΔglpE*::*FRT* Δ*yceA*::*FRT ΔybbB*::Km^r *purK-79*::Tn*10*	This work
JLD26503	TL524 *ΔpspE*::*FRT sseA*::Sp^r *ΔygaP*::*FRT ΔynjE*::*FRT ΔglpE*::*FRT* Δ*yceA*::*FRT thiI*::Km^r	This work
Plasmids		
pBS-KS^+	ColE1 origin Ap^r	Stratagene®
pCP15	pUC19 derivative carrying a FRT-flanked Km^r cassette	(89)
pCP20	FLP^+ Ap^r Cm^r *rep*A^{ts}	(89)
pKD46	*oriR101 repA101*(ts) P_{araB}-*gam-bet-exo*	(87)
pPS-3	pBluescript carrying the *psp* operon	(78)
pT7-7	ColE1 origin Ap^r T7 promoter	(90)
pFRT-K	pBS-KS^+ carrying a FRT-flanked Km^r cassette between *Eco*RI and *Hin*dIII	This work
pSB1	pBS-KS^+ carrying *pspE* on a *Sal*I-*Eco*RI fragment	This work
pHCE1	pFRT-K carrying a 5'-flanking region of *pspE* between *Xho*I and *Hin*cII	This work
pHCE2	pHCE1 carrying a 3'-flanking region of *pspE* between *Sma*I and *Bam*HI	This work
pHC4.1	pT7-7 carrying *pspE* between *Nde*I and *Eco*RI	This work

2.4. General methods

Polymerase chain reactions (PCR) were done using the reagents from New England Biolabs, *Taq* DNA polymerase from Promega, and a GeneAmp PCR System 9600 thermocycler. Approximately 0.5 μg of chromosomal DNA or a suspension of an isolated colony in water heated at 95^{o}C for 30 minutes was used as a template. DNA was extracted from agarose gel through use of the GFX™ PCR DNA and Gel Band Purification reagents (Amersham Pharmacia Biotech) following the instructions provided by the manufacturer. Ligation reactions were performed as suggested by New England Biolabs using their T4 DNA ligase. Plasmid DNA was isolated by the QIAprep Minipreps reagents from QIAGEN following the instructions provided by the manufacturer. Analysis of DNA by gel electrophoresis with 0.8 to 1.2% agarose was performed using TBE buffer (0.05 M Tris, 0.05 M boric acid, 0.01 M Na_2EDTA), containing 0.5 μg/ml ethidium bromide, as described by Sambrook et al. (82). Competent cells were prepared by the $CaCl_2$ method, with transformation performed using plasmid DNA as described by Maniatis et al. (91). P1 transductions were performed as described by Silhavy et al. (92). Protein concentrations were determined by the method of Bradford (93) with bovine serum albumin as the standard using reagents purchased from Piece Chemical. SDS-PAGE was performed as described by Laemmli (94) with 15% polyacrylamide gels.

2.5. Construction of plasmid pFRT-K and pSB1

Plasmid pFRT-K was developed by Janet L. Donahue. The Km^r FRT cassette (*Eco*RI-*Hin*dIII fragment) derived from pCP15 (89) was cloned into the same sites of pBluescript-KS^+ to create pFRT-K, which contains a 1.5 kb kanamycin-resistance (Km^r) gene flanked by FRT sites and multiple cloning sites. FRT sites contain directly repeated sequences that are the targets for yeast Flp recombinase recognition (89). Plasmid pPS-3 kindly provided by Peter Model (The Rockefeller University, New York) contains the *psp* operon cloned as a 4.5 kb *Eco*RI restriction fragment into the pBluescript (78). The *pspE* gene was subcloned by digesting pPS-3 with *Sal*I and *Eco*RI and cloning the resulting 715 bp fragment into the same sites of pBluescript-KS^+ to create pSB1 (work done by Scott Battle).

2.6. Deletion of the chromosomal *pspE* gene

Plasmid pHCE2 in which most of the *pspE* gene was replaced by a Km^r FRT cassette was constructed by cloning DNA fragments that flank *pspE* into pFRT-K such that these regions flank the Km^r FRT cassette. A total of 207 bp of the 315 bp *pspE* gene was deleted (codons 3-71). The deletion/insertion was constructed as follows: A 413 bp region from the 5' flanking region of *pspE* was obtained from pSB1 by digestion with *Xho*I and *Dra*I and ligation into the *Xho*I-*Hin*cII sites of pFRT-K, creating pHCE1. Subsequently, a 127 bp 3' flanking region of *pspE* was obtained on a *Psh*AI-*Bam*HI fragment from pSB1 and inserted into the *Sma*I-*Bam*HI sites of pHCE1 to create pHCE2.

pHCE2 was linearized by digestion with *Xho*I and *Bam*HI. The DNA fragment carrying Δ*pspE*::Km^r was introduced into the chromosome by transformation of BW25113(pKD46), with selection for kanamycin resistance. Plasmid pKD46 encodes lambda Red recombinase, which facilitated recombination of the linear DNA into the chromosome to create strain HC1.1 (87). Using bacteriophage P1-mediated transduction, the *pspE* deletion was moved into the wild-type strain TL524 for testing of potential phenotypes (92). The isolated strain was named HC6.1. Excision of the Km^r gene from Δ*pspE*::Km^r was done by transformation of HC6.1 with pCP20, a plasmid that has a temperature-sensitive origin of replication and thermal induction of yeast Flp recombinase synthesis. Ampicillin-resistant transformants were selected at 30^{o}C. Some were colony-purified once nonselectively at 43^{o}C and tested for loss of all antibiotic resistances. A Km^s and Ap^s isolate was sought and named HC7.1 (89).

2.7. Construction of pHC4.1 for overexpression of PspE

The *pspE* gene was amplified from genomic DNA of strain MG1655 by PCR using the primers pspE1H1, ccatagaaggacgctCaTatgtttaa and pspE2H1, cgctcatggtgaattcttttaacct (pspE2H1 includes stop codon), where the underlined letters indicate restriction sites for *Nde*I and *Eco*RI, and the uppercase letters are mismatches. After cleavage with *Nde*I and *Eco*RI, the amplified product was cloned into the same sites of pT7-7, yielding pHC4.1.

2.8. Purification of PspE

BL21(DE3)(pHC4.1) was grown in 500 ml of LB, and expression of PspE was induced by the addition of IPTG as described above. Freeze-thaw extraction of PspE was based on the same method used for purification of GlpE (76). Cells were harvested by centrifugation (6,000 × g for 5 min at 4 ^{o}C) and washed with 20 ml of 20 mM sodium acetate (pH 5) (buffer A). The cell pellet was stored at –70 ^{o}C. To release PspE from the cells, the frozen cells were thawed on ice, resuspended in 10 ml of buffer A, and incubated on ice for 30 min. Cell debris was removed from the extract by centrifugation at 40,000 × g for 90 min. The freeze-thaw extract was loaded onto a prepacked Waters propylsulfonic acid (SP) cation-exchange column (1 × 10 cm) equilibrated at room temperature with buffer A. The column was washed with the same buffer until no more protein came off the column. Then the column was developed with a 55-ml gradient from 0 to 825 mM NaCl in buffer A. Two peaks containing rhodanese activity, eluting between 330 and 600 mM NaCl, were pooled separately, adjusted to pH 7.0 by addition of 20 µl of 1 M Tris-HCl (pH 8.0) per ml of collected fraction, and stored in aliquots at 4 ^{o}C.

2.9. Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (94) on 15% polyacrylamide gels. Non-denaturing polyacrylamide gel electrophoresis was performed by a modification of the original

Laemmli method (SDS was omitted from all reagents) (94). The acrylamide concentration of the separation gel was 12% or 15% (w/v). The sample loading buffer contained 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 0.03% bromophenol blue. Gels were pre-electrophoresed for 1h. Protein was visualized with Fast Stain from Zoion Biotech, Inc.

2.10. Assay of rhodanese

During purification of PspE and for characterization of the enzyme, the assay used was essentially that described previously to quantify thiocyanate (43). Reaction mixtures contained 100 mM Tris-acetate (pH 8.6), 10 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 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 $Fe(NO_3)_3 \cdot 9H_2O$ and 200 ml of 65% HNO_3 per 1,500 ml]. The absorbance at 460 nm was determined using a Shimadzu UV 160U spectrophotometer. One unit of enzyme is defined as the amount that catalyzes the production of 1 μmol of thiocyanate per min at 25 °C and corresponds to an absorbance change at 460 nm of 2.8 in this system.

2.11. Determination of cyanolyzable sulfur of PspE

The persulfide sulfur (sulfane sulfur) bound to cysteine residue of proteins can be quantitatively released by treatment with CN^- and quantified as SCN^- (40). The two peaks of PspE obtained from cation-exchange column chromatography were adjusted to pH 7.0 by addition of 1 M Tris-HCl pH 8.0, because persulfide sulfur is unstable in acid solution (42). Various amounts of PspE were treated with 100 mM cyanide for 5 min at room temperature. Thiocyanate was then quantified as the ferric thiocyanate complex as is done for the rhodanese assay.

CHAPTER THREE

Results

3.1. Construction of a Δ*pspE* strain

To address the function of PspE in *E. coli*, 207 bp of coding sequence of *pspE* were replaced with a 1.5 kb Km^r cassette flanked by FRT sites (87;89). The *pspE* deletion was moved into the MG1655 genetic background by P1 transduction (92). In addition, a strain in which the Km^r cassette was eliminated by Flp-mediated recombination was constructed (92). Strains harboring multiple deletions of genes for predicted sulfurtransferases were constructed using the same methods. Strains FA036 and JLD26503 (Table 2.1.) were constructed by Farzana Ahmed and Janet L. Donahue respectively.

3.2. Verification of *pspE* disruption

Two methods were used to verify that the desired *pspE* disruption had been constructed. First, as shown in Fig. 3.1., the gene deletion was verified by PCR analysis of genomic DNA with primers flanking the *pspE* gene. Lane 1 shows the expected 350-bp PCR product corresponding to the wild-type *pspE* gene. Lane 2 shows the expected 1.7-kb product corresponding to Δ*pspE*::Km^r, and lane 3 shows the 230-bp product expected for the *pspE* deletion following removal of the Km^r cassette. Secondly, genetic

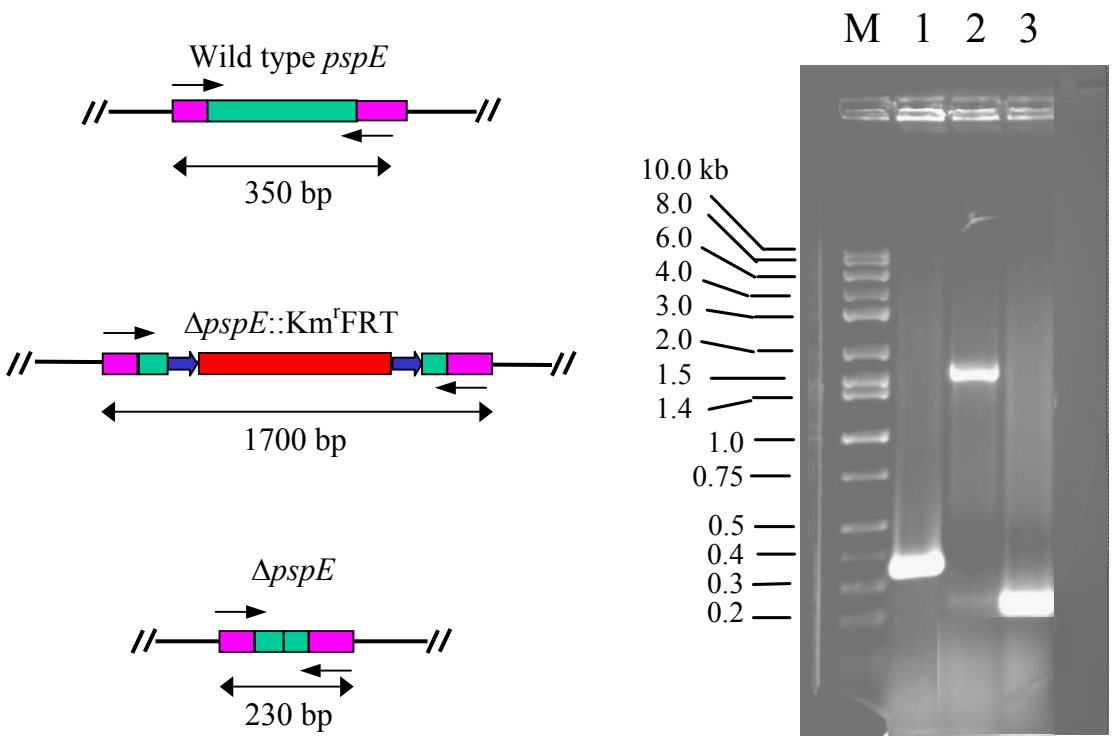


Figure 3.1. Verification of *pspE* deletions.

Left panel: schematic of the templates used for PCR. Top, middle, and bottom indicate DNA from the wild type, HC6.1 (Δ*pspE*::Km^r), and HC7.1 (Δ*pspE)* strains, repectively. The small arrows facing toward each other on each template indicate the primers. Two-headed arrows under the template indicate the expected sizes in base pair (bp) of PCR products. Right panel: agarose gel electrophoresis of PCR products obtained from genomic DNA from strains TL524 (wild type) (lane 1), HC6.1 (Δ*pspE*::Km^r) (lane 2) and HC7.1 (Δ*pspE*) (lane 3). Lane M, DNA size markers. Primers pspE1H1 and pspE2H1 were used for amplification in all reactions.

mapping of the *pspE* deletion was performed by P1 transduction. *pspE* and *zcj-233*::Tn*10* (86) are expected to be highly co-transducible, being separated by 0.026 min (1187 bp) on the chromosome. Strain HC6.1 (Δ*pspE*::Km^r) was transduced with a P1 lysate prepared on strain CAG12028 (*pspE*$^+$ *zcj-233*::Tn*10*). Tc^r colonies were selected and then scored for Km^r. Of the 50 Tc^r colonies scored, 49 became Km^s (98%). The high observed co-transduction frequency between *pspE* and *zcj-233*::Tn*10* (expected 96%) indicates that the targeted deletion was in the *pspE* locus.

3.3 Comparison of rhodanese activity in wild type and Δ*pspE* strains

E. coli has nine genes predicted to encode proteins with the rhodanese homology domain. Besides PspE, GlpE and YgaP are known to have rhodanese activity. To address the phenotype of the Δ*pspE* strain, the rhodanese activity in wild-type and sulfurtransferase-deficient strains was compared. Table 3.1. shows the rhodanese activity in extracts of wild type, Δ*pspE* (HC7.1) and other multiply mutant strains. The specific activity of rhodanese in the crude extract of the Δ*pspE* strain is 55% of that of wild type. Deletion of seven additional genes encoding proteins with the rhodanese domain did not result in a further decrease in rhodanese activity. These results suggest that *pspE* is one of the major contributors of rhodanese activity in *E. coli.*

Table 3.1. Comparison of rhodanese activity in extract of wild-type and sulfurtransferase-deficient strains.

Strain	Relevant genotype	Specific activity (U/mg)[a]
TL524	Wild type	0.31
HC7.1	TL524 *ΔpspE*::*FRT*	0.17
FA036	TL524 *ΔpspE*::*FRT sseA*::Sp^r *ΔygaP*::*FRT* *ΔynjE*::*FRT ΔglpE*::*FRT ΔyceA*::*FRT ΔybbB*::Km^r	0.16
JLD26503	TL524 *ΔpspE*::*FRT sseA*::Sp^r *ΔygaP*::*FRT* *ΔynjE*::*FRT ΔglpE*::*FRT ΔyceA*::*FRT thiI*::Km^r	0.18

[a] Cultures were grown in LB at 37°C and harvested when OD_{600} reached about 0.5. Activities were determined using crude extracts of sonicated cells.

3.4. Cyanide sensitivity of *pspE* mutant

Since cyanide detoxification is thought to be a physiological function of rhodanese, the sensitivity of the *pspE* mutant toward cyanide was compared to that of the wild type. Various concentrations of cyanide (from 0.03 mM to 1 mM) were added to logarithmically growing cultures. There was no significant difference in growth pattern when comparing the *pspE* mutant and wild type (Fig. 3.2). Growth was almost stopped in the presence of 1 mM KCN. The same results were obtained when thiosulfate (1 mM) was added (data not shown). The multiple rhodanese mutant FA036 was assessed using the same growth conditions; growth of the multiple mutant was also similar to the wild type under all conditions (with or without thiosulfate), no significant difference was found either (data not shown). These results indicate that during growth of *E. coli* under the conditions tested, these rhodaneses don't play a role in cyanide detoxification.

Some other phenotypic tests were carried out using *pspE* mutant and other sulfurtransferase deficient strains (in collaboration with Janet L Donahue and Farzana Ahmed). The results indicated that other than ThiI, none of the predicted sulfurtransferases are required for production of sulfur containing amino acids (cysteine and methionine) and cofactors (thiamin, biotin, lipoate and molybdopterin).

The periplasmic Dsb (disulfide bond formation) proteins, DsbA and DsbB, are involved in Hg^{2+} and Cd^{2+} detoxification (95;96). Since DsbA is a dithiol protein, it might provide reducing potential to periplasmic PspE and further form hydrogen sulfide, which precipitates (thereby detoxifying) heavy metal cation at cell surface. Thus,

mutation of *pspE* may have an effect on the sensitivity of *E. coli* to Hg^{2+} and Cd^{2+}. Growth of wild-type and mutant strains deficient in *pspE* and other sulfurtransferase paralogs was compared on solid LB agar containing various concentrations of Cd^{2+} (0.01, 0.03, 0.1, 0.3 and 1.0 mM). Except for medium containing 1 mM Cd^{2+}, all strains grew in the presence of Cd^{2+}. No significant difference was observed for sulfurtransferase-deficient strains (including multiply mutant strains FA036 and JLD26503, data not shown).

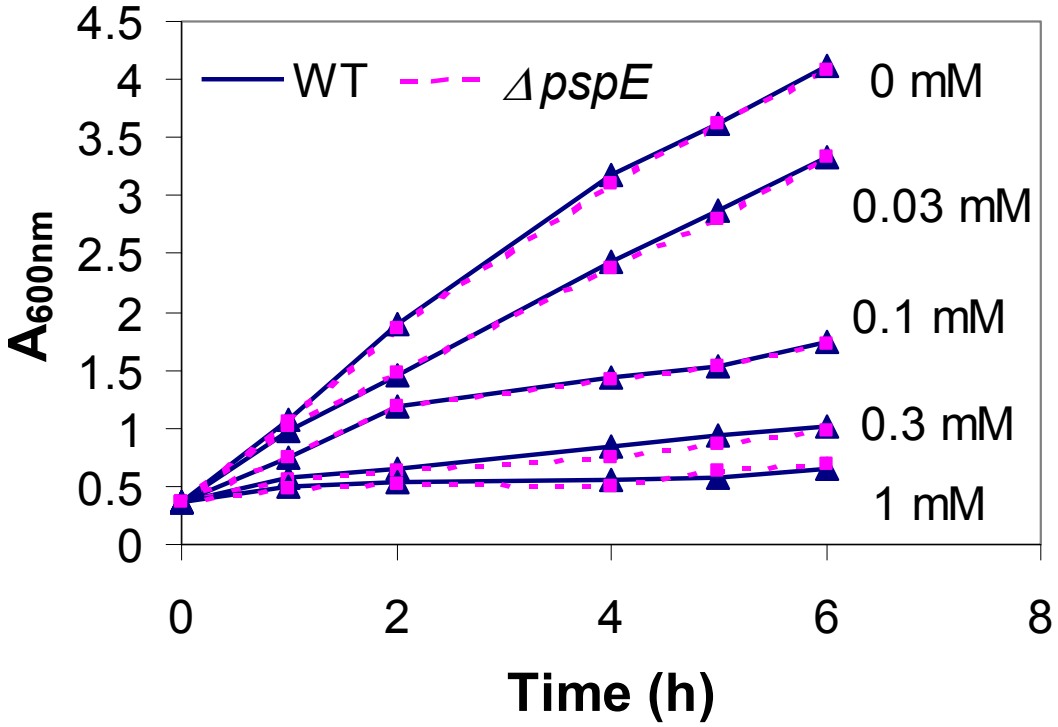


Figure 3.2. Effect of cyanide on growth of wild-type and Δ*pspE* strains.

The wild-type (TL524) (solid lines) and Δ*pspE* strain (HC7.1) (dotted lines) were grown overnight in LB medium and then diluted 50 fold into fresh LB medium. After 1h of growth, cyanide (from 0.03 to 1 mM) was added (time zero) at the indicated concentrations.

3.5. Purification of cysteine persulfide and sulfur-free forms of PspE

PspE is synthesized as a precursor with a 19-amino acid signal sequence and secreted to the periplasm (78;80). Its periplasmic location makes it easy to release PspE by using a freeze-thaw treatment (76). Thus, this single step both extracted and purified PspE (2.5-fold). According to Kurzban et al., bovine rhodanese is stabilized against inactivation processes at low pH (e.g., pH 4-6) (97). We tested the pH stability of PspE and found that PspE was also stable at low pH (pH 5-6). PspE bound to a cation exchange column at pH 5 and eluted as two distinct peaks during a salt gradient. PspE from both peaks had high specific activity and was essentially pure. From a 500-ml culture, a total of 5.4 mg of PspE was purified 4-fold to a specific activity of 130 to 150 U/mg (Table 3.2.). The major protein found in each peak was more than 95% homogeneous, as visualized by protein staining of an SDS-15% polyacrylamide gel (Fig. 3.3.), and exhibited the same apparent size (approximately 6 kDa). We designate the two peaks of PspE as $PspE_1$ and $PspE_2$ that correspond to peak 1 and peak 2 respectively.

Non-denaturing polyacrylamide gel electrophoresis (PAGE) revealed that $PspE_1$ and $PspE_2$ consisted of two dominant forms that differed in electrophoretic mobility (Fig. 3.4.). $PspE_1$ had a more compact size relative to that of $PspE_2$. Gel filtration chromatography was used to show that the differences in mobility are due to differences in their Stokes radii (data not shown). When $PspE_2$ was treated with 1 mM ammonium thiosulfate, it was largely converted to faster migrating $PspE_1$. Pretreatment with cysteine resulted in slower migration of both forms. These results suggest that $PspE_1$ has been

modified by thiosulfate at the active site cysteine to form the cysteine persulfide. This supposition was confirmed by determination of cyanolyzable sulfur associated with each of the two forms of PspE (PspE has a single cysteine at the active site). Fig. 3.5. shows that one equivalent of cyanolyzable sulfur was associated with $PspE_1$, with much less present in $PspE_2$. These results confirmed the idea that the two forms of PspE differ by the presence or absence of persulfide sulfur at the active site cysteine.

Table 3.2. Purification of PspE from *E. coli.*

Purification Step	Volume (ml)	Amt of Protein (mg)	Activity (U)	Sp act (U/mg)	Yield (%)
Whole cells[a]	750	30	1100	36	100
Freeze-thaw extract	15	11	1000	91	91
Cation exchange Peak 1	2	2.9	390	130	35
Peak 2	2	2.5	380	150	35

[a] Activity was determined using sonicated cells from a liquid culture.

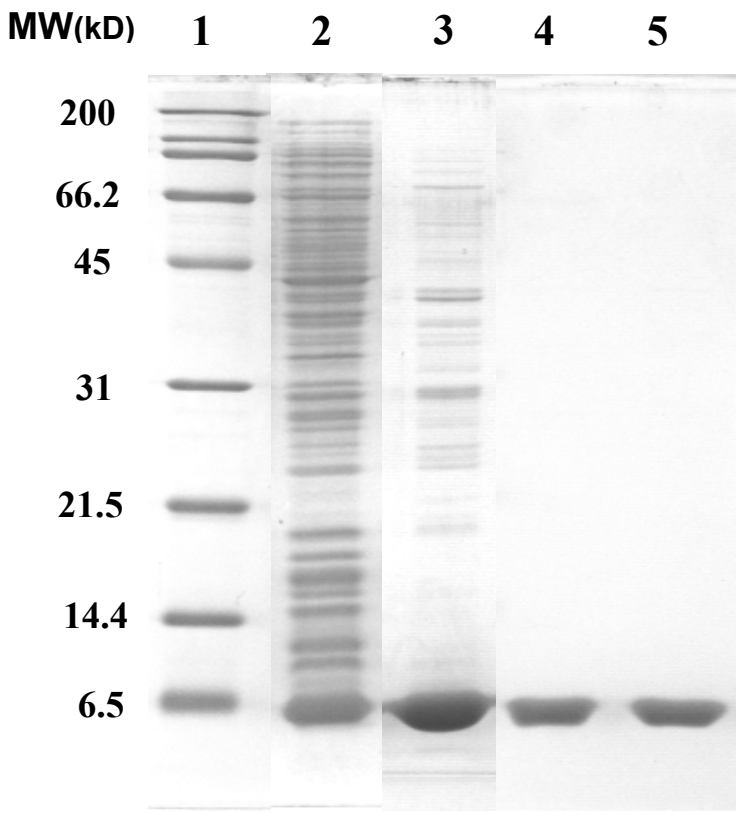


Figure 3.3. Purification of PspE. Fractions obtained during purification of PspE were analyzed using SDS-PAGE.

Lane 1, molecular mass markers. Lane 2, total cellular proteins of strain BL21(DE3)(pHC4.1) (10 μg). Lane 3, freeze-thaw extract (10 μg). Lanes 4 and 5, pooled peak 1 and peak 2 from the cation-exchange column (3 μg each lane).

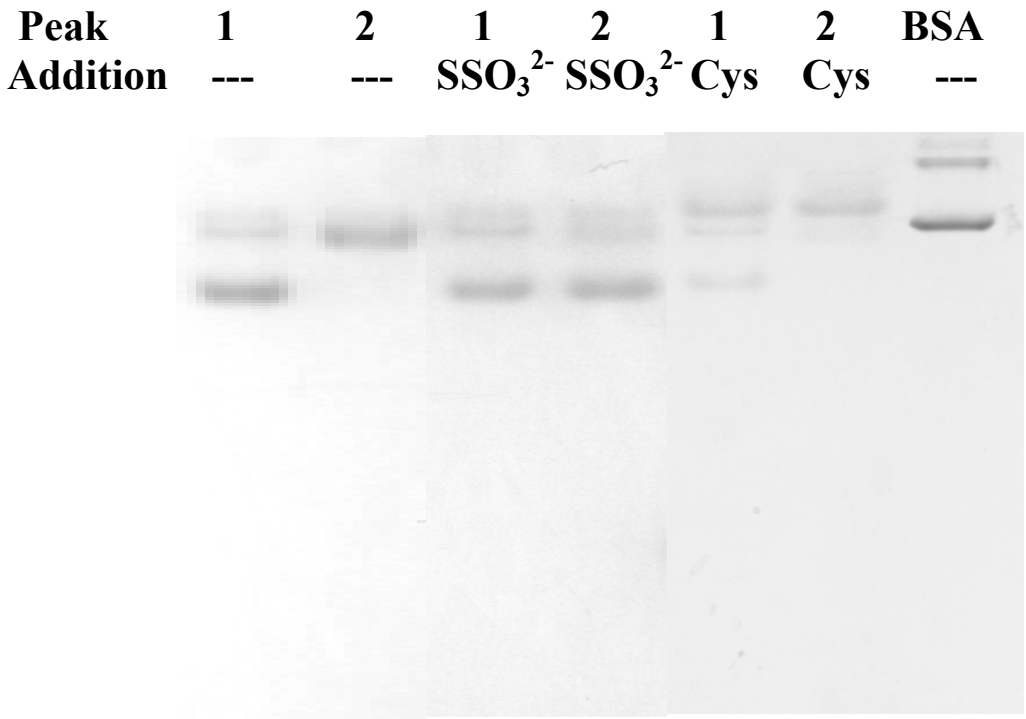


Figure 3.4. Interconversion of two forms of PspE.

$PspE_1$ and $PspE_2$ were analyzed using native-PAGE. In each lane 3 μg of PspE was analyzed. Where indicated, PspE was pre-incubated with 1 mM thiosulfate or 1 mM cysteine for 5 min at 22 °C. BSA lane, 2 μg BSA (monomeric and dimeric forms are the two major bands) was used as standard.

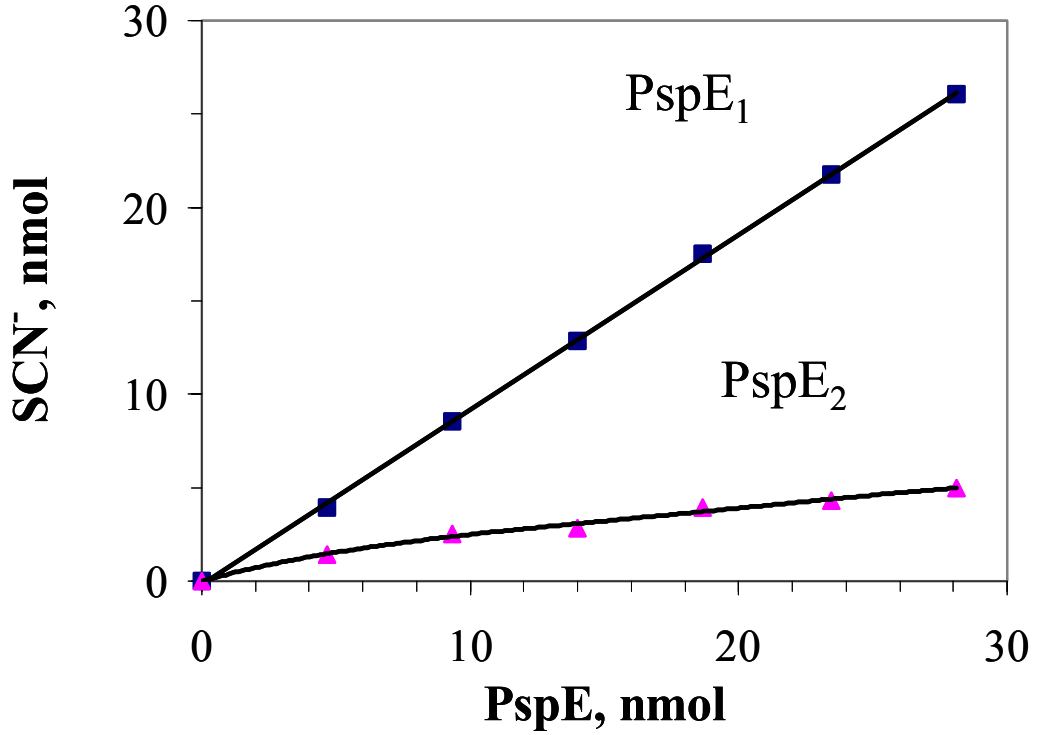


Figure 3.5. The cyanolyzable sulfur of PspE ($PspE_1$ and $PspE_2$).

Aliquots of $PspE_1$ and $PspE_2$ were adjusted to pH 7.0 by addition of 1 M Tris-HCl (pH8.0). Aliquots of PspE were treated with 100 mM cyanide for 5 min at room temperature. Thiocyanate was then quantified as the ferric thiocyanate complex as is done for the rhodanese assay. The amount of PspE was estimated using the Bradford protein assay, with bovine serum albumin as standard.

3.6. Differential sensitivity of $PspE_1$ and $PspE_2$ to chemical inactivation by DTNB

Cysteine-specific modifying reagent 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) has been used to verify that cysteine residues of various rhodaneses are required for activity (76). The sulfhydryl group of sulfur-free bovine rhodanese has twice the reactivity toward DTNB compared with that of rhodanese containing a cysteine persulfide at the active site (71). Therefore, the sensitivities of the two forms of PspE to DTNB were compared. It was found that $PspE_1$ was much more tolerant to chemical inactivation by DTNB than $PspE_2$, with twice as much activity remaining (DTNB to PspE molar ratio of 2:1) (Fig. 3.6.). These results are consistent with the conclusion that the single active cysteine of PspE eluting first from the cation exchange column contains a persulfide sulfur.

The two forms of purified PspE were relatively stable compared to GlpE when stored at 4°C (76). Addition of micromolar concentrations of cysteine or dithiothreitol was found to activate GlpE (76), and the same was found for PspE. Analysis using non-denaturing gel electrophoresis revealed that both forms of PspE activated by reductant had a slower migration (Fig. 3.4). The results indicate that activated PspE adopts a new conformation distinct from those of the two forms isolated by ion exchange column.

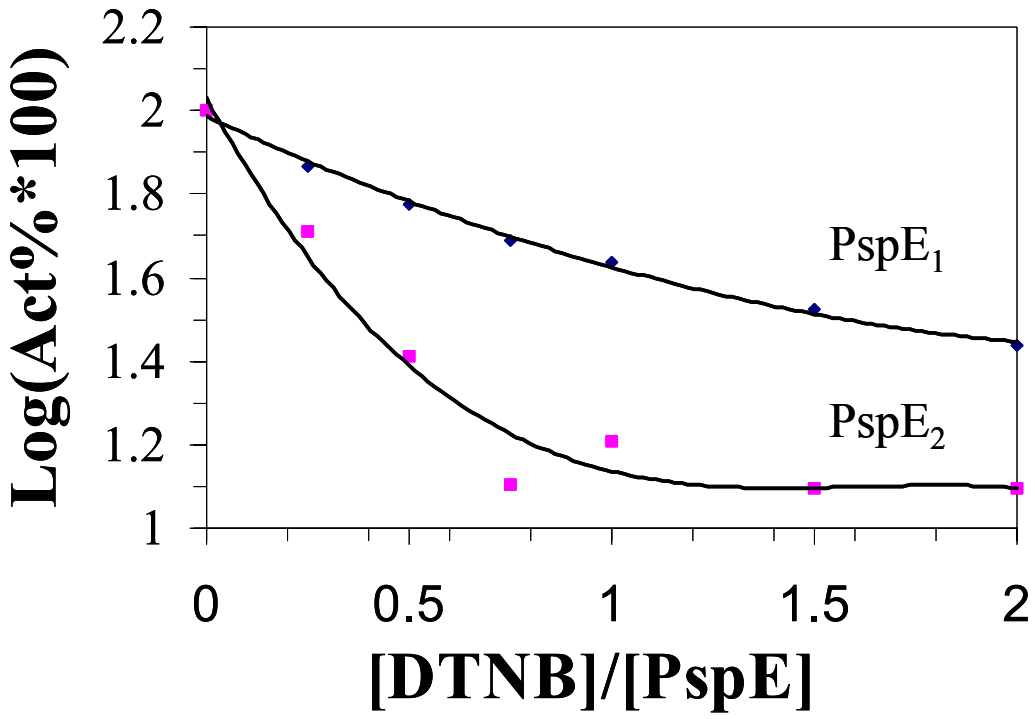


Figure 3.6. Inactivation of PspE by DTNB.

The two forms of PspE (19 μg) were incubated separately for 3 hours at an ambient temperature in 20 mM Tris-HCl (pH 7.0) with various molar ratios of DTNB. Remaining rhodanese activities were determined and compared with that for PspE incubated without DTNB.

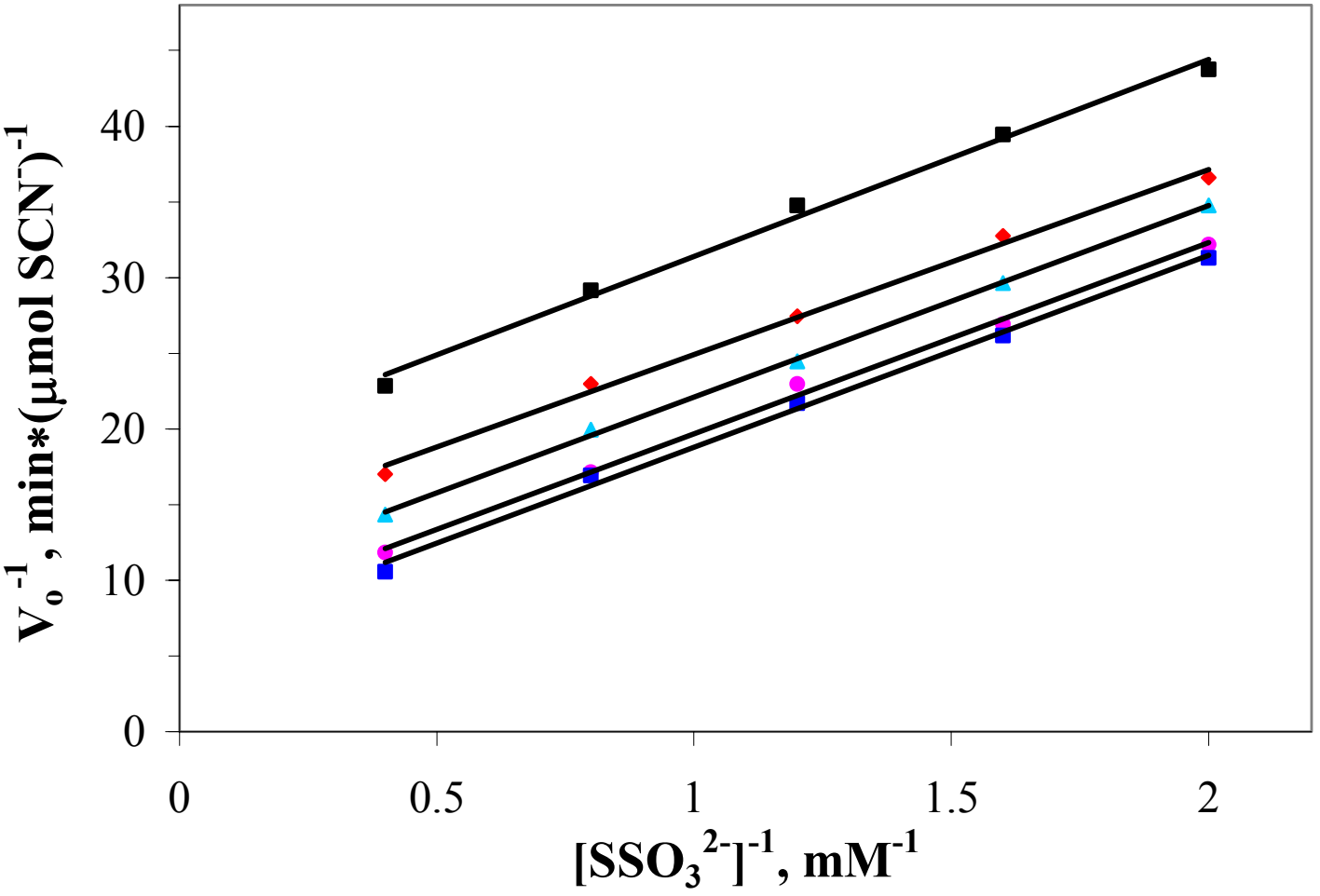
3.7. Catalytic properties of PspE

Many well-characterized rhodaneses utilize a double-displacement (ping-pong) mechanism (60;76). Adams et al. reported a double-displacement mechanism for PspE (77). The protein they used contains a N-terminal 6×His tag, which may affect the catalytic properties of PspE. Besides, the concentrations of thiosulfate chosen for kinetic analysis were very high relative to K_m (2-10 times K_m), which may have led to inaccuracy in the determination of K_m and reaction mechanism. In the kinetic study we noticed that with the cyanide concentration increasing, the pH of the reaction solution increased. Thus we doubled the concentration of Tris-acetate buffer to100 mM to stabilize the reaction pH (pH 8.6). One mM cysteine was added to the reactions to activate PspE. Since analysis by native gel electrophoresis showed that $PspE_2$ was converted to the active form more completely by addition of cysteine, this form of PspE was used to study this enzyme's kinetic properties. Data from activity measurements of purified form 2 PspE with various concentrations of thiosulfate at fixed concentrations of cyanide fit the equation describing a double-displacement mechanism. A plot of the data (Fig. 3.7.) yielded K_m values for thiosulfate and cyanide of 3.4 and 43 mM, respectively. The V_{max} value obtained was 460 μmol $SCN^- \bullet min^{-1} \bullet mg^{-1}$, yielding a k_{cat} of 72 s^{-1}.

Although previously characterized *E. coli* rhodanese GlpE is inhibited only weakly by certain anions (76), greater inhibition by anions was observed for PspE. Using the standard rhodanese assay except that thiosulfate concentration was 10 mM, addition of sodium sulfate or sodium chloride at a concentration of 0.25 M resulted in about 50%

inhibition of rhodanese activity. Addition of sodium phosphate at the same concentration resulted in about 65% inhibition. Sulfite, one of the products of the rhodanese assay reaction, strongly inhibited PspE activity. It was found that addition of 1 mM sodium sulfite resulted in more than 50% inhibition of rhodanese activity (Fig. 3.8.).

(A)



(B)

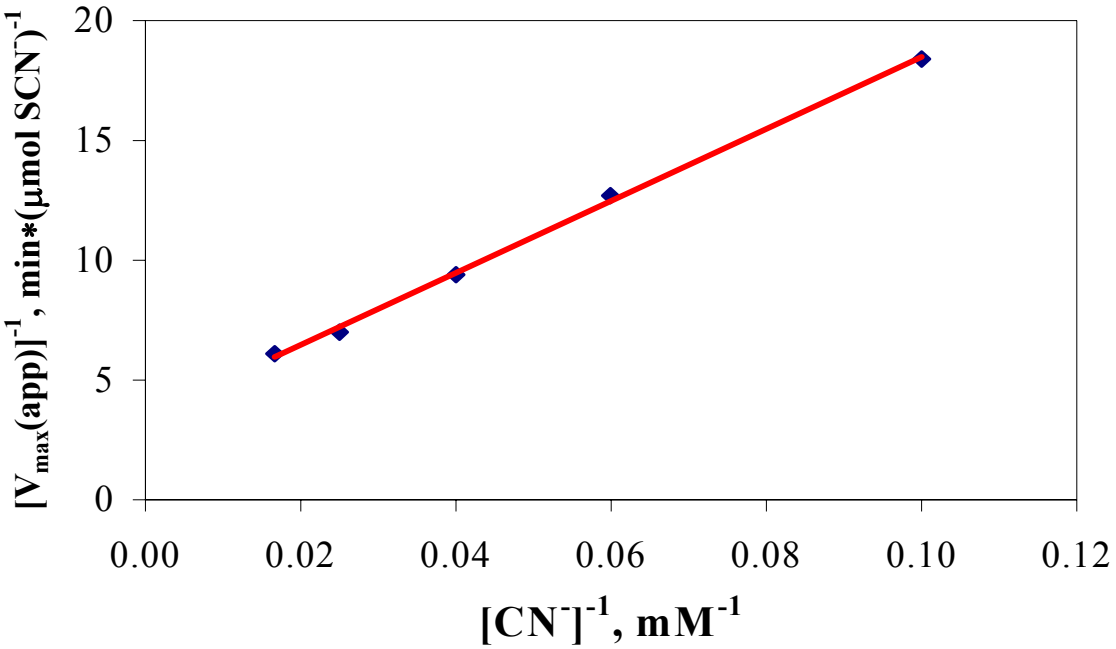


Figure 3.7. Kinetic characterization of the thiosulfate:cyanide sulfurtransferase reaction catalyzed by PspE.

Each assay, performed as described in the text, contained 0.63 µg of purified $PspE_2$ in 200 mM Tris-acetate (pH 8.6)-1 mM cysteine. (A) Double-reciprocal plot of the rate of thiocyanate formation versus thiosulfate concentration at various fixed concentrations of cyanide: 10 mM (■), 16.7 mM (♦), 25 mM (▲), 40 mM (●), 60 mM (■). (B) Secondary double-reciprocal plot of apparent V_{max} values from the data in panel A versus cyanide concentrations.

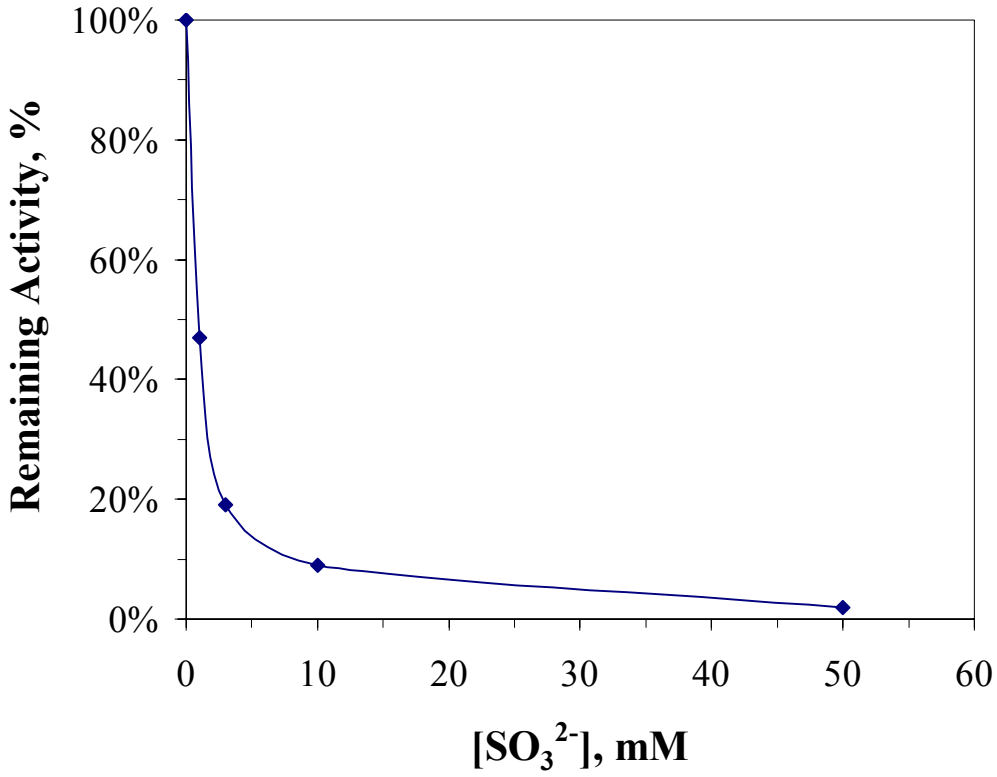


Figure 3.8. Inactivation of PspE by sulfite.

The standard rhodanese assay was used. Sodium sulfite was added at the indicated concentrations (0, 1, 3, 10, and 50 mM). Purified $PspE_2$ was used in this assay.

CHAPTER FOUR

Discussion

The fact that PspE is synthesized as precursor with a 19-amino acid signal sequence (78-80)and that it could be easily released by freeze-thaw extraction method (Fig. 3.3.) suggest that it has a periplasmic location. It is noteworthy that among the eight predicted sulfurtransferases in *E. coli*, PspE is the only periplasmic protein, which indicates that there might be a designated role for this protein. The periplasmic space lies between the inner and the outer membranes of gram-negative bacteria. A number of processes that are vital to the growth and viability of the cell occur within this compartment. Periplasmic proteins can be divided into the following categories based on their functions (98): (i) solute or ion-binding proteins that function in conjunction with ABC transporters or chemotaxis receptors for the sensing and uptake of sugars, amino acids, peptides, vitamins and ions; (ii) catabolic enzymes that degrade complex molecules into simpler ones for transport across the inner membrane; (iii) detoxifying enzymes that serve a protective role for the cell; and (iv) enzymes or proteins that promote the biogenesis of major envelope proteins or proteinaceous appendages, peptidoglycan, lipopolysaccharide, capsules, or membrane-derived oligosaccharide. Thiosulfate, one of the sulfur donor substrates of rhodanese, can serve as an alternative mineral sulfur source in some bacteria (99). When sulfane acceptors for rhodanese are dithiols, such as reduced lipoate or reduced glutathione, a persulfide is formed. Oxidation of the acceptor (via disulfide formation) permits the production of sulfide. In this case the net reaction of

thiosulfate cleavage is equivalent to a thiosulfate reductase activity (99). The periplasmic location of PspE implies that this rhodanese may function in the assimilation of thiosulfate. It was found in *S. enterica* and *E. coli* that the reduction of thiosulfate occurs in the cytoplasm through the reaction of thiosulfate with *O*-acetyl-L-serine to form the thiosulfonate *S*-sulfocysteine, which is then reduced to L-cysteine (4). *S. enterica* has a separate thiosulfate reductase (55). The reduction of thiosulfate is not completely understood and no evidence shows that PspE plays a role in this function.

Periplasmic Dsb (disulfide bond formation) proteins, DsbA and DsbB are involved in Hg^{2+} and Cd^{2+} detoxification (95;96). We hypothesized that PspE could use reduced dithiol DsbA as sulfur acceptor substrate for PspE to produce hydrogen sulfide, which then precipitates Hg^{2+} or Cd^{2+} by formation of metal sulfide. We tested the Cd^{2+} sensitivity of the *pspE* mutant and compared it with that of the wild-type strain and demonstrated that PspE isn't involved in Cd^{2+} detoxification.

Rhodanese has also been proposed to mobilize sulfur for the formation or repair of ion-sulfur cluster. The iron-sulfur clusters of ferrodoxins (56), succinate dehydrogenase (100), and mitochondrial NADH dehydrogenase (101) could be reconstituted *in vitro* by incubation with bovine liver rhodanese, thiosulfate, iron ions and a reducing agent. In *E. coli* iron-sulfur clusters are present in a number of respiratory enzymes, like NADH dehydrogenase and succinate dehydrogenase. PspE is localized in the periplasmic space, where it is also proximal to the aerobic respiratory chain and may play a role in the restoration of the activity of these iron-sulfur proteins.

GlpE is the first characterized rhodanese in *E. coli* whose gene has been defined (76). Alignment of the amino acid sequence of PspE with that of GlpE using SIM – local similarity program (102) reveals that the sequence identity is ~24% (Fig. 4.1.). Besides the highly conserved active site cysteine (Cys65 in GlpE), Glycine (Gly68 in GlpE) and Serine (Ser70 in GlpE) residues are conserved in both enzymes. It's known that both residues are present in the active-site loop (81).

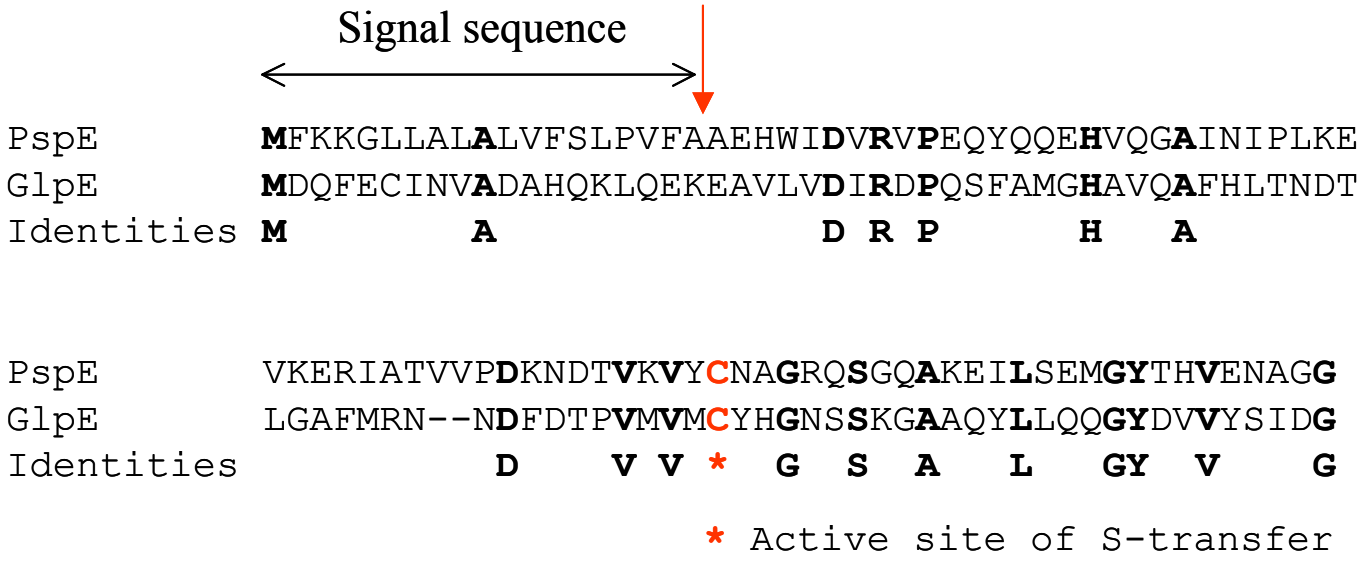


Figure 4.1. Alignment of the amino acid sequences of PspE and GlpE.

The asterisk marks the active site cysteine. About 24% identity over a 70 residue overlap was found using SIM-local similarity program. The double-headed arrow on the top indicates the signal sequence (19 amino acid) of PspE. The red arrow next to it indicates the cleavage site.

Compared to GlpE and bovine liver rhodanese, PspE is a much smaller rhodanese of calculated size 9.4 kDa (Fig. 3.3.). GlpE is a 12-kDa single domain rhodanese (76). The bovine liver rhodanese, however, contains two approximately 15-kDa domains (63). PspE possesses some of the characteristics of the previously identified accessible rhodanese from *E. coli*. For example both of them can be released by freeze-thaw treatment (66), the K_ms for SSO_3^{2-} are about the same (3.4 and 5 mM for PspE and accessible rhodanese, respectively), K_ms for CN^- are similar (43 and 24 mM for PspE and accessible rhodanese, respectively), and both enzymes exhibit anion inhibition as described for bovine liver rhodanese. There are some discrepancies between these two enzymes. For example, the apparent size of PspE (6 kDa upon SDS-PAGE analysis) is smaller than that of accessible rhodanese (14 kDa by molecular exclusion chromatography). The difference in analysis methods could contribute to this difference. k_{cat} for PspE (72 s^{-1}) is lower than that for accessible rhodanese (260 s^{-1}), which may be due to the presence of 5 to 10 fold higher concentration of cysteine in the rhodanese assay system used to assay accessible rhodnanese. Considering PspE is the only periplasmic rhodanese found in *E. coli,* it is very likely that the accessible rhodanese is PspE.

Compared to GlpE, PspE has higher affinity for thiosulfate (K_ms are 3.4 and 78 mM for PspE and GlpE respectively), which suggests that thiosulfate may be the physiological substrate for PspE. The low affinity of both PspE and GlpE for cyanide (K_ms are 43 and 17 mM respectively) indicates that this compound is not the physiological substrate for the enzymes (1 mM cyanide severely inhibited the growth of both rhodanese mutant and wild-type strains, see Fig. 3.2.). Comparison of the cyanide

sensitivity of the *pspE* mutant, multiple rhodanese mutant and wild-type strains confirmed that none of the eight predicted sulfurtransferases plays a role in cyanide detoxification in *E. coli* (Fig 3.2).

A recent study reported characterization of a purified N-terminally His-tagged PspE (77). The K_ms for thiosulfate and cyanide were 4.6 and 27 mM respectively. These data are basically consistent with the results of my work (K_ms for thiosulfate and cyanide are 3.0 and 43 mM, respectively). The specific activity (800 U/mg) of this His-tagged PspE is five times higher than that observed in my work. Since PspE is such a small protein (85 AA) and it has a cationic active site, six histidines on its N-terminus may affect its catalytic properties, and thus increase its activity.

According to the generally accepted mechanism, during catalysis rhodanese cycles between two distinct forms, the free enzyme (En), and a covalent enzyme-sulfur intermediate (En-S) (60). These two forms are usually prepared separately from stocks of enzyme either by supplying sufficient thiosulfate to insure that all the enzyme is sulfur-substituted, or by adding a small amount of cyanide, thereby stripping the enzyme of its persulfide sulfur (70). An early study indicated that a purified sulfurtransferase of *A. calcoaceticus lwoffi* migrated as a tight double band on nondenaturing gels (65). Both bands showed sulfurtransferase activity. But the molecular difference between these two bands was not characterized. PspE in my work is the first rhodanese to be purified as two distinct forms using cationic exchange chromatography in the absence of thiosulfate and cyanide. The two forms were shown to be a sulfur-free form (En) and persulfide form (En-S) by using nondenaturing gels (Fig. 3.4.) and cyanolyzable sulfur determination

(Fig. 3.5.). The differential sensitivity of two forms to chemical inactivation by DTNB is consistent with the early studies of bovine liver rhodanese (71). It is reasonable to suppose that the presence of the persulfide group in sulfur-containing rhodanese protects the enzyme against inactivation from the sulfhydryl-group-modification reagents.

The bovine liver rhodanese contains four free sulfhydryl groups, one of which is essential for thiocyanate-forming activity (62). In the stable complexes rhodanese is associated with other mitochondrial proteins through disulfide bonds. As ordinarily isolated, this enzyme is susceptible to inactivation by autoxidation (66;103), forming insoluble aggregates. The same autoxidation was observed in *E. coli* rhodanese and this enzyme inactivation could be partially or completely overcomed by including cysteine in the assay mixture (66). PspE only contains one cysteine residue that is in its active site. My observation that PspE could be activated by micromolar concentrations of cysteine or dithiothreitol (Fig. 3.4.) indicates that PspE may also undergo autoxidation, probably through intermolecular reactions.

Using the amino acid sequence of *E. coli* PspE as query, a BLAST search (104) showed that PspE is highly conserved in enteric bacteria, such as *E. coli, S. flexneri* and *S. enterica*. This result suggests that PspE might have a defined function in these bacteria. The physiological role of PspE remains to be determined. Since PspE was purifed as persulfide form (En-S) as well as sulfur free form (En) in the absence of thiosulfate and cyanide, it will be very interesting to find out where the sulfur on PspE originates and what its destiny is. Quantitation of the persulfide sulfur in LB using PspE and a rhodanese assay run to completion revealed that there was at least 13 nmol of

thiocyanate produced per 1 ml of LB. This result suggests that a small amount of thiosulfate in LB might be the sulfur source for formation of the persulfide form of PspE. Work to clarify this question is in progress.

CHAPTER FIVE

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