IscS Is a Sulfurtransferase for the *in Vitro* Biosynthesis of 4-Thiouridine in *Escherichia coli* tRNA†

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ABSTRACT: We have improved the in vitro assay for 4-thiouridine (s^4U) biosynthesis in *Escherichia coli* tRNA by substituting an unmodified tRNA transcript as substrate and including recombinant ThiI protein, a known factor required for s^4U synthesis. Using this assay, we have purified an enzyme from wild-type *E. coli* that is able to provide sulfur for s^4U synthesis in vitro. The purified protein has a molecular weight of 45 kDa and contains pyridoxal phosphate as a cofactor. This protein catalyzes sulfur transfer from cysteine to tRNA and is analogous to factor C previously reported (Lipsett, M. N. (1972) *J. Biol. Chem. 247,* 1458−1461). UV spectroscopy and HPLC analysis of thiolated tRNA and its digests confirm that the product of the in vitro reaction is s^4U. N-Terminal sequence analysis of the purified protein identifies it as IscS, a recently characterized NifS-like cysteine desulfurase that mobilizes sulfur for the synthesis of [Fe−S] clusters. We have cloned and overexpressed *iscS* and show that the recombinant protein displayed tRNA sulfurtransferase activity equal to that of the native protein. We also show that, of the multiple proteins in *E. coli* with cysteine desulfurase activity as observed by native gel staining, only IscS is able to mobilize the sulfur for transfer to tRNA. Our identification of IscS as a tRNA sulfurtransferase provides support for this activity in vivo and further expands the role for NifS proteins as versatile sulfur-carrying enzymes.

Transfer RNA of all organisms contains a variety of chemical modifications that serve to modulate its interactions with both proteins and other RNAs, such as mRNA (*1*, *2*). A number of these modified bases are found to contain sulfur (*3*). The predominant thionucleotide in *Escherichia coli* tRNA is 4-thiouridine (s^4U),[1] formed posttranscriptionally by modification of the nearly invariant uridine at position 8. Substantial evidence exists to support the function of s^4U as a cellular sensor for near-UV stress. Upon irradiation, the s^4U in certain tRNAs forms an internal cross-link to give tRNAs that are poor substrates for aminoacylation (*4*). This cross-linking results in initiation of an adaptive response (*5*) and ultimately a slow-growth phenotype (*6*), which has been used to isolate mutants defective in the synthesis of s^4U (*5*, *7*, *8*).

In their initial studies, Lipsett and co-workers (*9*) found that s^4U synthesis is mediated by two factors, A and C, that could be separated chromatographically (Figure 1). The formation of s^4U requires Mg-ATP, and cysteine as the sulfur source. In addition, factor C was found to require PLP as a cofactor (*9*). *E. coli* mutants deficient in s^4U biosynthesis were generated by exposing cells to near-UV radiation at 340 nm (*7*, *8*). Two genes, *nuvA* and *nuvC*, were identified and correlated with factors A and C, respectively (*7*, *10*). Surprisingly, *nuvC* mutants devoid of s^4U were also auxotrophic for the thiazole moiety of thiamin (*7*). Addition of 5-(hydroxyethyl)-4-methylthiazole to the growth medium relieved the thiamin requirement but did not restore s^4U in tRNA.

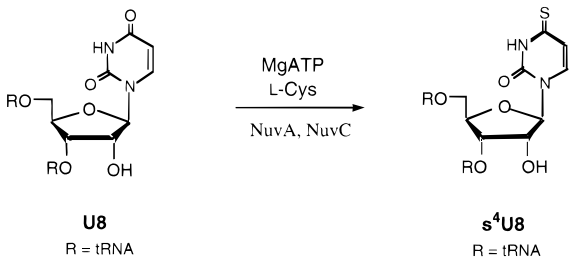

FIGURE 1: Requirements for the biosynthesis of 4-thiouridine (s^4U) in *E. coli*.

Recently, homologous genes, termed *thiI*, were found to be required for both thiazole biosynthesis in *Salmonella typhimurium* (*11*) and s^4U synthesis in *E. coli* (*12*). ThiI was also recently shown to be necessary for the conversion of the thiamin pathway enzyme ThiS into a C-terminal thiocarboxylate (*13*). Although both *thiI* and *nuvC E. coli* mutants are auxotrophic for thiazole, the ThiI protein does not bind PLP (*12*). Therefore, it is unclear whether *thiI* corresponds to the *nuvA* or *nuvC* locus.

Efforts to further purify these enzymes from wild-type strains in a number of laboratories have not been successful, as the enzymes become unstable during purification (*9*, *14*). Previous purification attempts used either bulk *E. coli* or *Saccharomyces cerevisiae* tRNA as substrate, the latter of

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[1] Abbreviations: s^4U, 4-thiouridine; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; IPTG, isopropyl 1-thio-β-galactopyranoside; Tris, tris(hydroxymethyl)aminomethane; PAGE, polyacrylamide gel electrophoresis; PLP, pyridoxal 5-phosphate; PMSF, phenylmethanesulfonyl fluoride; DEAE, diethylaminoethyl; PCR, polymerase chain reaction; TCA, trichloroacetic acid; PVDF, poly(vinylidenedifluoride).

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which is devoid of s^4U. We have observed low tRNA sulfurtransferase activity in wild-type cell-free extracts using either of these substrates. In our efforts to isolate these enzymes, we have reconstituted tRNA thiolation using an unmodified *E. coli* $tRNA^{Phe}$ transcript as substrate and purified recombinant ThiI in the in vitro reaction. Using this assay, we have purified a protein from wild-type *E. coli* and identified it as IscS, and we show that it requires ThiI (in addition to Mg-ATP and cysteine) for sulfur transfer to tRNA.

MATERIALS AND METHODS

Frozen *E. coli* B cells were purchased from the University of Alabama Fermentation Facility and stored at −70 °C. [^{35}S]-L-Cysteine was purchased from NEN Life Science Products. Nuclease P1 was purchased from Boehringer-Mannheim. *Pfu* DNA polymerase was from Stratagene. Plasmid pCF23 encoding wt *E. coli* $tRNA^{Phe}$ was a gift from Dr. Rachel Green. Bacterial alkaline phosphatase, DNase I, lysozyme, pyridoxal 5-phosphate, ATP, L-cysteine, hydroxyapatite (HA-Ultragel), and all other chemicals were purchased from Sigma unless otherwise mentioned. DNA oligos were purchased from Integrated DNA Technology and purified by denaturing PAGE before use.

Buffers. The following buffers were used: buffer A, 50 mM Tris-HCl, pH 7.5, 10 mM $Mg(OAc)_2$, 50 mM KCl, 10% glycerol, 1 mM PMSF, and 14 mM 2-mercaptoethanol; buffer B, 50 mM Tris-HCl, 10% glycerol, 1 mM PMSF, and 14 mM 2-mercaptoethanol; buffer C, 10 mM potassium phosphate buffer, pH 7.1, 10% glycerol, 1 mM PMSF, and 14 mM 2-mercaptoethanol; buffer D, 1 mM potassium phosphate, 1 mM NaCl, 10% glycerol, 1 mM PMSF, and 14 mM 2-mercaptoethanol; lysis buffer, 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5% glycerol, 1 mM DTT, and 1 mM PMSF.

Sulfurtransferase Assay. tRNA sulfurtransferase assays were performed by the method of Harris and Kolanko (*14*) with several modifications. Reactions were carried out in a 50 μL volume of 50 mM Tris-HCl, pH 7.5, 10 mM $Mg(OAc)_2$, 50 mM KCl, 1.0 mM ATP, 5 μg of *E. coli* $tRNA^{Phe}$ transcript, 3% glycerol, 1 mM DTT, 10 μM [^{35}S]cysteine (1500−3000 cpm/pmol), 2.7 μg of ThiI, and indicated amounts of overexpressed IscS or *E. coli* crude protein. The reaction mixtures were incubated at 37 °C for 30 min. Samples were applied to DEAE 81 filters (2.4 cm, Whatman). The disks were washed once with 0.5 M Tris-HCl, pH 9.0, and 1.0 mM DTT for 30 min and three times with 0.3 M KCl and 20 mM cysteine for 5 min each to remove nonspecifically bound material as reported (*14*). The disks were finally placed in a Buchner funnel and washed three times with H_2O, air-dried, and counted in a liquid scintillation counter.

Isolation of [^{35}S]*-Labeled tRNA*. Large-scale sulfurtransferase reactions were carried out in a volume of 20 mL as outlined above. tRNA was isolated by phenol extraction and ethanol precipitation. The tRNA precipitate was redissolved in water and passed over a Sephadex G-50 column.

Preparation of tRNA Substrate. Unmodified *E. coli* $tRNA^{Phe}$ was prepared by runoff transcription of *Bst*N1-linearized pCF23, which contains the wild-type sequence behind the phage T7 promoter. The conditions for transcription with T7 RNA polymerase were essentially as described (*15*). The resulting tRNA was purified by electrophoresis on 8% denatured gels. tRNA was eluted from crushed gel slices with 0.5 M NaCl, phenol-extracted, and precipitated with ethanol. The tRNA was dissolved in 5 mM $MgCl_2$, denatured at 65 °C for 5 min, and then renatured by slow cooling to room temperature (15−20 min).

Construction of thiI Expression Vector and Purification of Recombinant ThiI. The *E. coli thiI* gene was amplified from *E. coli* K-12 (ATCC 25404) genomic DNA. The three base pairs upstream of the *thiI* start codon were mutated in the 5′-primer to give an *NdeI* site and the 3′-primer contained a *Hin*dIII site downstream of the stop codon for cloning into the expression vector. The 5′ primer had the sequence 5′-TGC CAA CGA ACC ATT GCC CAT ATG AAG TTT ATC ATT AAA TTG-3′ and the 3′ primer had the sequence 5′-CGT AAA AAC AAG CTT TTA CGG GCG ATA TAC CTT CAC ATT-3′ (restriction sites underlined). The PCR reaction mixture contained 1 μg of genomic DNA, 0.5 μM each primer, 0.2 mM each dNTP, and 5 units of native *Pfu* DNA polymerase in buffer supplied by the manufacturer. The expected 1.4-kb PCR product was purified on a 1% agarose gel and isolated on Qiaex-II resin (Qiagen) and digested overnight with *Nde*I and *Hin*dIII. The resulting fragment was gel-purified and ligated to digested and dephosphorylated pET-22c (Novagen). The ligation mixture was used to transform NovaBlue (Novagen) cells to ampicillin resistance. Plasmid DNA from a number of transformants was isolated and screened for insert by PCR. Sequencing of one of the isolated plasmids revealed no deviation from the *thiI* sequence previously reported (*12*). This plasmid (pCL102) was used to transform BL21(DE3) cells (Novagen) for the overexpression of ThiI.

A 1% overnight culture of cells carrying pCL102 was used to inoculate 1.0 L of LB medium containing ampicillin (100 μg/mL) and grown to an A_{600} of 0.6. Expression was induced by adding IPTG to a final concentration of 0.3 mM and growth continued for 3 h. Cells were harvested and stored at −70 °C. The frozen cell pellet from 1.0 L of culture (4.0 g) was resuspended in lysis buffer (1 mL/g of cells) and treated with lysozyme (0.3 mg/mL, 1 h, 0 °C). DNase I (1 μg/g of cells) was added and further incubated for 30 min at 0 °C. The cell lysate was centrifuged at 30000*g* for 1 h. The S-30 supernatant was diluted with buffer B and applied to a DEAE-Sephacel column (50 mL of settled gel) equilibrated with buffer B containing 50 mM KCl. The column was washed with buffer B containing 150 mM KCl and eluted with buffer B containing 0.3 M KCl. The DEAE eluate was applied on Cibacron Blue-agarose column (5 mL of settled gel) equilibrated with buffer B containing 0.3 M KCl. The column was washed with 20 mL of buffer B containing 0.4 M NH_4Cl. Bound protein was eluted with buffer B containing 1.0 M NH_4Cl. Peak fractions having ThiI protein were pooled and dialyzed against buffer A. The dialyzed protein was then applied to a Sephadex G-200 column (230 mL of settled gel) and eluted in buffer A containing 200 mM KCl. Fractions were analyzed by SDS−PAGE prior to combining and were concentrated with an Amicon PM10 membrane.

Purification of tRNA Sulfurtransferase. All procedures were carried out at 0−8 °C. Frozen *E. coli* B cells (180 g) were ground with twice the weight of alumina according to

ref *7* and extracted with 250 mL of buffer A. The crude cell extract was centrifuged for 30 min at 30000*g* to obtain an S-30 extract. The S-30 supernatant was centrifuged at 100000*g* for 4 h to pellet ribosomes. The clear S-100 supernatant was loaded onto a column of DEAE-Sephacel (Pharmacia) (225 mL of settled gel) equilibrated with buffer B containing 50 mM KCl. The column was washed with 400 mL of buffer B and then eluted with a linear salt gradient (50−500 mM KCl) in 500 mL of buffer B. Selected fractions were assayed for protein and tRNA sulfurtransferase activity as described above. Fractions having peak enzyme activity were pooled. The eluted protein from the DEAE-Sephacel column was diluted with buffer B and applied to a Cibacron Blue 3GA-fast flow agarose column (50 mL of settled gel) equilibrated with buffer B containing 50 mM KCl. The column was washed with 300 mL of equilibration buffer and the bound protein was eluted with a linear gradient of 50−500 mM KCl in 500 mL of buffer B. Peak fractions having tRNA sulfurtransferase activity were pooled and frozen at −70 °C. The frozen solution of the Blue eluate is carefully thawed and diluted with buffer C and applied onto a heparin−agarose column (30 mL of settled gel) previously equilibrated with buffer C. After the column was washed with 125 mL of equilibration buffer, protein was eluted with a linear KCl gradient (50−500 mM) in 300 mL of buffer C. Fractions having peak tRNA sulfurtransferase activity were pooled and dialyzed for 4 × 1 h against buffer D. The dialyzed protein was applied to a hydroxyapatite column (10 mL of settled gel) equilibrated in buffer D. The column was eluted with a linear gradient of potassium phosphate buffer, pH 7.0 (1−100 mM), in 150 mL of buffer D. Hydroxyapatite eluate fractions 8−10 were pooled. A portion (2 mL) of the pooled fractions was brought to 50 mM Tris-HCl, pH 7.5, and 50 mM KCl and applied on the SP-Sepharose fast-flow column (5 mL of settled gel). Flowthrough fractions were analyzed by SDS−PAGE and sulfurtransferase activity.

Construction of iscS Expression Vector and Purification of Recombinant IscS. The *iscS* gene was amplified from *E. coli* K-12 as described above using a 5′-primer with the sequence 5′- TAC GGA GTT TAT AGA CAT ATG AAA TTA CCG ATT TAT CTC-3′ and a 3′-primer with the sequence 5′-TCC TGA TTC CGA GAA TTC TTA ATG ATG AGC CCA TTC GAT-3′ (restriction sites underlined). The PCR product (1.2 kb) was digested with *Nde*I and *Eco*RI and ligated to digested and dephosphorylated pET-22c. Transformation of the ligation mixture into NovaBlue cells gave recombinants, from which plasmid DNA was isolated. One plasmid, pCL010, was used to transform BL21(DE3) cells for the overproduction of protein. Conditions used for the overexpression of IscS were similar to ThiI. A frozen cell pellet from 2.0 L of culture (5.1 g) was thawed and treated with lysozyme as described above. The S-30 lysate was centrifuged at 100000*g* for 4 h. The S-100 supernatant was directly applied on the DEAE-Sephacel (50 mL of settled gel) equilibrated with buffer B containing 50 mM KCl. The column was washed with 200 mL of equilibration buffer and eluted with a linear salt gradient (50−500 mM KCl) in 400 mL of buffer B. Fractions containing IscS were pooled, diluted with buffer B, and loaded onto a Cibacron Blue-agarose column (50 mL of settled gel) equilibrated in buffer B containing 50 mM KCl. The column was washed with 175 mL of equilibration buffer and eluted with a KCl gradient (50−500 mM) in 500 mL of buffer B. The column fractions were analyzed by SDS−PAGE prior to combining.

Determination of Cysteine Desulfurase Activity. Sulfide liberated from cysteine was measured by its conversion to methylene blue (*16*). Reactions were performed under anaerobic conditions as previously described (*19*). The reaction mixture consisted of 50 mM Tris-HCl, pH 8.0, 10 mM $Mg(OAc)_2$, 5 mM DTT, 10 μM PLP, 3% glycerol, 1 mM PMSF, 2.5 mM cysteine, and 0.17 μM IscS. The sulfide concentration was determined from a standard Na_2S curve.

Protein Determination. Protein concentration was determined by a modified Bradford procedure (Bio-Rad catalog no. 500-0006.)

Polyacrylamide Gel Electrophoresis. Polyacrylamide gels (10% and 7%) were used for SDS and native gels, respectively. Coomassie brilliant blue was used for protein staining. Electrophoresis was performed according to ref *17*. Gel staining for the enzymes that liberate sulfide was a modified procedure previously reported (*18*, *19*). The native gels were stained in a buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM $Mg(OAc)_2$, 10 mM cysteine, 0.5 mM lead nitrate, 5.0 mM DTT, and 20 μM PLP.

Protein Sequencing. Native IscS was precipitated with TCA and dissolved in SDS-gel loading buffer and electrophoresed as described above. The unstained gel was electroblotted onto a PVDF membrane following standard procedures. N-Terminal sequencing was carried out at the Department of Biochemistry, Michigan State University, East Lansing, MI.

tRNA Digestion and Nucleoside Analysis. Nuclease digestion of tRNA was performed as described previously (*20*) with minor modifications. Aliquots of 50 μg of *E. coli* $tRNA^{Phe}$, either before or after thiol modification, were dissolved in water, and heated to 90 °C for 5 min, and then cooled to room temperature. Sodium acetate, pH 5.3, and $Zn(OAc)_2$ were added to give final concentrations of 30 and 0.2 mM, respectively, in a total volume of 100 μL. Three units of nuclease P1 was added and the mixture was incubated at 37 °C for 2 h and then 8 μL of 1 M Tris pH 7.8 was added, followed by 3 units of bacterial alkaline phosphatase. The mixture was incubated for an additional 1 h. The resulting mixture of nucleosides was analyzed by reversed-phase HPLC. Aliquots of 5 μg of digested tRNA were loaded onto an analytical C-18 HPLC column (Vydac) and eluted with a linear gradient of 2−20% methanol in 10 mM ammonium phosphate, pH 5.3, over 60 min. s^4U was detected at 330 nm, while the other nucleosides were detected at 260 nm. In cases where ^{35}S-labeled tRNA was used, peak fractions were analyzed by UV absorbance at 330 nm and liquid scintillation counting.

RESULTS

Overproduction and Purification of ThiI. To use ThiI in the in vitro tRNA sulfurtransferase reaction, we cloned and overexpressed *thiI* from *E. coli* K-12. A protein with a relative mass of 55 kDa was readily visible by SDS−PAGE as the major soluble protein after cell lysis and centrifugation (Figure 2, lane 1). The observed mass of ThiI closely corresponded to its calculated predicted molecular weight. ThiI was purified sequentially on DEAE, Cibacron Blue, and Sephadex G-200 columns as shown in Figure 2 (lanes 2−4).

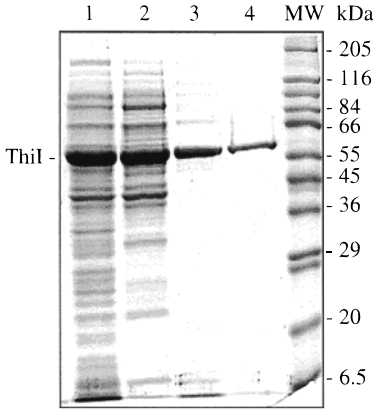

FIGURE 2: Purification of overexpressed ThiI. SDS−PAGE analysis of the column fractions at different purification stages. Lane 1, 10 μg of S-30 supernatant; lane 2, 10 μg of DEAE eluate; lane 3, 2.0 μg of Cibacron Blue eluate; lane 4, 1.0 μg of G-200 eluate (single fraction); lane MW, molecular mass markers.

Table 1: Improved tRNA Sulfurtransferase Assay[a]

reaction	pmol of [^{35}S] incorporated into tRNA
none	0.21
S-100	0.86
S-100 + ThiI	2.89
S-100 + *E. coli* tRNA	1.18
S-100 + ThiI + *E. coli* tRNA	3.16
ThiI + *E. coli* tRNA	0.27
ThiI + *E. coli* tRNA^{Phe} transcript	0.35
S-100 + *E. coli* tRNA^{Phe} transcript	3.6
S-100 + ThiI + *E. coli* tRNA^{Phe} transcript	69.8

[a] Assay conditions were as described under Materials and Methods. S-100 protein (23.2 μg), ThiI (2.7 μg), and tRNA (5 μg) were used in the reactions as indicated.

The enzyme bound to the Blue column very tightly, which enabled the removal of the majority of other cellular proteins with 0.4 M NH_4Cl. ThiI was not affinity-eluted with 2.0 mM ATP or 0.2 mM PLP or tRNA (2 A_{260}/mL) (data not shown) but could be eluted with 1.0 M NH_4Cl. Subsequent purification by gel filtration on Sephadex G-200 effectively removed all of the contaminating proteins from the Blue eluate. After this step, the enzyme gave a single band in Coomassie-stained SDS gels.

Purification of tRNA Sulfurtransferase. In our hands, the tRNA sulfurtransferase activity in wild-type *E. coli* cell extracts was very low when bulk *E. coli* tRNA was used as a substrate (Table 1). We obtained similar results with *S. cerevisiae* tRNA, which is devoid of s^4U (data not shown). By including recombinant ThiI in the in vitro reaction and using unmodified *E. coli* tRNA^{Phe} transcript as the substrate,

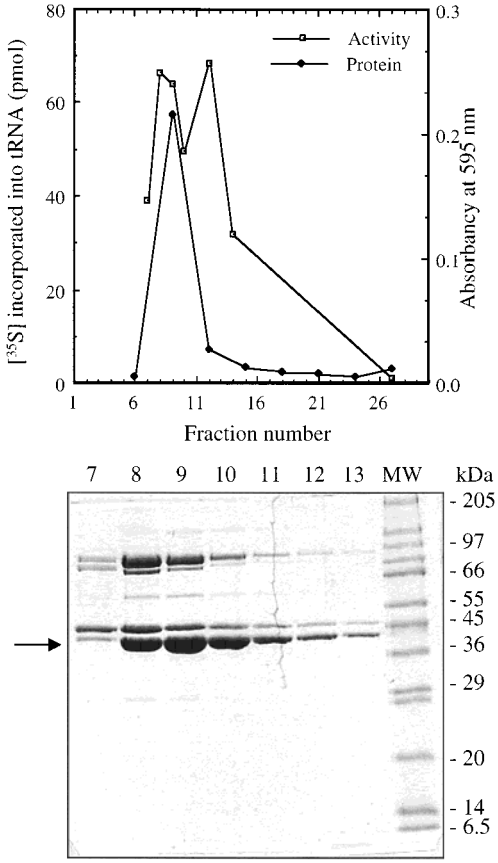

FIGURE 3: tRNA sulfurtransferase activity and protein analysis of the hydroxyapatite eluates. Column chromatography was performed as described under Materials and Methods. Upper panel, column fractions (5 μL) were assayed for protein (◆) and tRNA sulfurtransferase activity (⊡) as described under Materials and Methods. Lower panel, column fractions (15 μL) as indicated were electrophoresed on SDS−polyacrylamide gels. Arrow points to the protein whose elution paralleled the tRNA sulfurtransferase activity.

we observed over a 20-fold enhancement in the sulfurtransferase activity (Table 1). The improved assay has enabled us to purify the factor that requires PLP and mediates the transfer of sulfur from cysteine in the biosynthesis of s^4U as outlined in Table 2 and Figures 3 and 4.

As shown in Figure 3, a polypeptide indicated by the arrow was identified (lower panel) whose staining intensity paralleled the enzyme activity (upper panel). The molecular weight of this protein from gel analysis was 45 kDa. Close examination of the intensities of eluted proteins suggested coelution of another protein of similar size along with the sulfurtransferase. The coeluted protein migrated slightly lower than the sulfurtransferase and was predominantly

Table 2: Purification of *E. coli* tRNA Sulfurtransferase[a]

fraction	total protein (mg)	total activity[b] (pmol × 10^6)	specific activity (pmol/μg)	*x*-fold purification	yield (%)
S-100	2850.25	10.83	3.8	1	100
DEAE-Sephacel	537.95	7.85	14.6	3.8	72.5
Cibacron Blue-agarose	89.27	5.0	56.0	14.7	46.2
heparin	8.98	2.81	312.9	82.3	26
hydroxyapatite 8−10	6.21	2.19	352.6	92.8	20.2
hydroxyapatite 11−15	0.61	0.31	508.2	133.7	2.9

[a] Assay was carried out as described under Materials and Methods. [b] Picomoles of [^{35}S] incorporated into tRNA were calculated after subtracting from protein + ThiI blank.

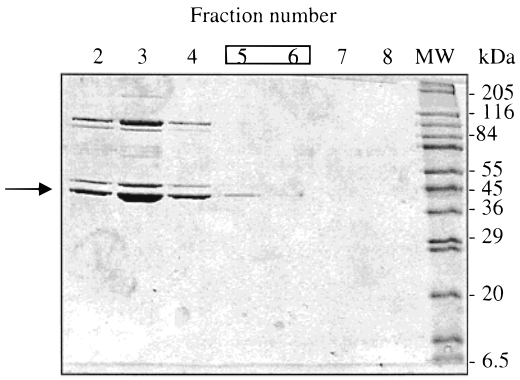

FIGURE 4: SP-Sepharose column chromatography of hydroxyapatite eluate. Column chromatography was performed as described under Materials and Methods. Column fractions as indicated (10 μL) were electrophoresed on SDS−polyacrylamide gels. Arrow and box point to the protein and column fractions used for the N-terminal sequencing.

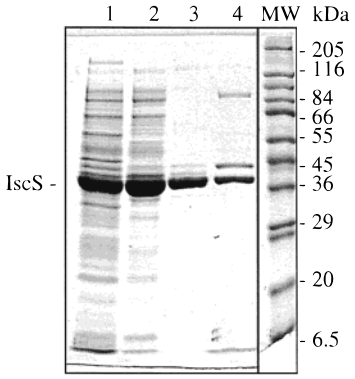

FIGURE 5: Purification of overexpressed IscS protein: SDS−PAGE analysis of the purification procedure. Lane 1, 10 μg of S-100 supernatant; lane 2, 10 μg of DEAE eluate; lane 3, 2.0 μg of Cibacron Blue eluate; lane 4, 2.0 μg of hydroxyapatite pooled fractions 11−15; lane MW, molecular mass markers.

present in hydroxyapatite fractions 8−10, and hence these fractions were combined as a separate pool. On the basis of the protein amount and enzyme activity profiles (Figure 3) coupled with the specific activities reported in Table 2, we suspected that the protein indicated by the arrow in fractions 11−15 was the desired sulfurtransferase. The enzyme was purified 134-fold at the end of the hydroxyapatite step.

The sulfurtransferase and the coeluted protein did not bind to an SP−Sepharose column. However, flowthrough fractions 5 and 6 (Figure 4) contained the putative sulfurtransferase in sufficient purity to perform N-terminal sequence analysis. Protein fractions indicated by the arrow and box (Figure 4) were concentrated and electroblotted to a PVDF membrane. N-Terminal sequencing of the protein band yielded the sequence MKLPIYLDYSATTPVD (residues 1−16). This amino acid sequence was found to be identical to that of IscS, an *E. coli* NifS-like protein previously described by Flint (*21*). This protein shows significant sequence homology to the original NifS protein encoded by the *Azotobacter vinelandii nifS* gene (GenBank accession number M20568), and also to yeast tRNA splicing factor SPL1(NFS1, GenBank accession number M98808), among other NifS-like proteins.

Overproduction of IscS. The *E. coli iscS* gene was cloned into pET-22c by standard methods. Induction with IPTG produced large amounts of a protein of about 45 kDa. The overexpressed protein is found primarily in the soluble S-100 fraction (Figure 5, lane 1) and was purified by ion-exchange chromatography on DEAE-Sephacel and affinity chromatography on Cibracron Blue agarose (Figure 5, lanes 2−3). Recombinant IscS comigrated with native IscS in the SDS gels (lane 4). In addition, the recombinant enzyme catalyzed sulfur transfer to tRNA (Table 3) with an activity similar to that of the native enzyme isolated from the wild-type strain. Purified IscS alone did not catalyze sulfur transfer to tRNA but required ThiI (Table 3). The specific activity of the purified IscS for cysteine desulfurase activity (0.091 unit/mg) was comparable to that reported previously by Flint (0.078 unit/mg) (*21*).

Activation of Cysteine Desulfurase Activity of IscS by ThiI. Data shown in Figure 6 demonstrate that ThiI stimulates IscS cysteine desulfurase activity. We observed a maximum 2-fold increase in activity with stoichiometric amounts of ThiI (0.17 μM). Addition of excess ThiI (2.5 times the IscS concentration) did not result in any further stimulation. BSA, which is known to contain a reactive cysteine, and carbonic anhydrase have no effect on the desulfurase activity of IscS.

Table 3: tRNA Sulfurtransferase Activity of Native and Overexpressed IscS[a]

reaction	pmol of [^{35}S] incorporated into tRNA
overexpressed IscS	0.62
overexpressed IscS + tRNA	0.46
overexpressed IscS + tRNA +ThiI	957.2
native IscS + tRNA + ThiI	516.8
ThiI + tRNA	0.03

[a] Assay conditions were as described under Materials and Methods. ThiI (2.7 μg), IscS (1.0 μg), and tRNA (5.0 μg) were used in the reactions as indicated. Hydroxyapatite eluate 11−15 was used for native IscS. Values were subtracted from blank and protein + ThiI.

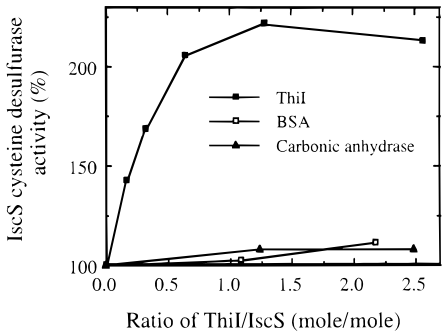

FIGURE 6: Effect of ThiI on the cysteine desulfurase activity of recombinant IscS. Increasing amounts of ThiI (■), BSA (□), or carbonic anhydrase (▲) were added to cysteine desulfurase assay reaction mixtures containing 7.5 μg of IscS. Desulfurase activity is reported relative to IscS alone.

Identification of the Product as 4-Thiouridine. To confirm that the product of the sulfurtransferase reaction is indeed s^{4}U, the UV spectra of the tRNA substrate and product were recorded and compared (Figure 7). Thiolation of uridine at the 4-position shifts the absorption maximum of the nucleoside from 262 to 334 nm. This results in the appearance of a new minor absorption peak in tRNA around 330−340 nm that uniquely identifies s^{4}U (*3*). The tRNA^{Phe} transcript used in the assay displayed no peak in this region, whereas ^{35}S-labeled tRNA showed an absorption peak at 340 nm (upper

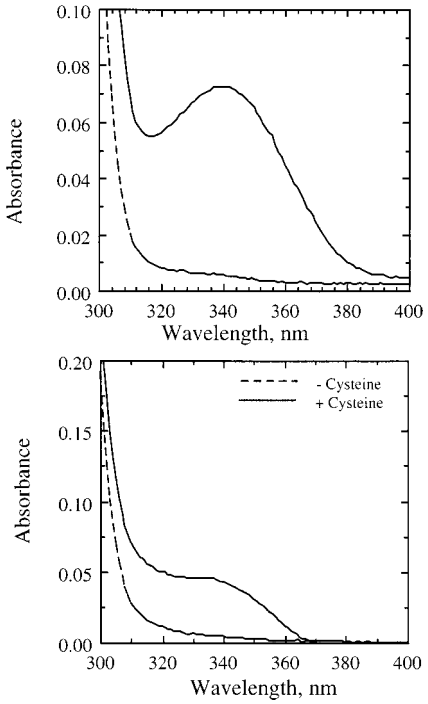

FIGURE 7: UV absorption spectra of thiolated tRNA. Upper panel, UV spectra of [^{35}S]-labeled tRNA recovered after sulfur incorporation (—) and Phe tRNA transcript (- - -). Lower panel, in vitro sulfur transfer reaction was carried in a cuvette in the presence (—) and absence (- - -) of cold cysteine. The UV spectra were recorded at the end of the reaction (30 min). Spectra were recorded with 3.4 A_{260} units of tRNA in 0.5 mL of water (upper panel) and buffer (lower panel).

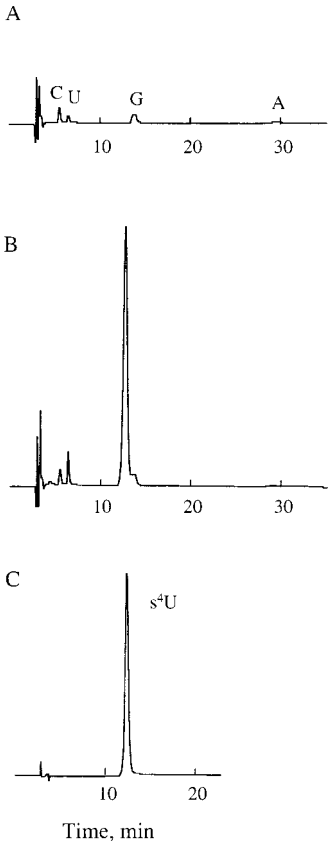

FIGURE 8: HPLC analysis of the thiolated tRNA. tRNA was digested as described under Materials and Methods. Nucleosides were detected at 340 nm (s^4U λ_{max}). A—C represent chromatograms of the tRNA transcript, thiolated tRNA, and standard s^4U, respectively.

panel).[2] This strongly suggests the presence of s^4U in the labeled tRNA product. In addition, we have also carried out the sulfurtransferase reaction in the cuvette with cold cysteine and recorded the spectra (lower panel). The shoulder in the absorption spectra of the reaction that contains cysteine is again consistent with the presence of s^4U in the tRNA product.

To further confirm the presence of s^4U in the tRNA product, the ^{35}S-labeled tRNA was digested to nucleosides and analyzed by HPLC as described (*20*) (Figure 8). The tRNA transcript contains no s^4U (panel A). The thiolated ^{35}S-tRNA digest contains s^4U (panel B) and more than 85% of the recovered ^{35}S label was present in this peak. The UV absorbance spectra and HPLC analysis clearly confirm that s^4U is present in the tRNA.

Specificity of IscS-Catalyzed tRNA Sulfurtransferase in E. coli. We have checked for the presence of enzymes with cysteine desulfurase activity in the column fractions of sulfurtransferase purification by native gel staining (Figure 9, see Materials and Methods). The supernatant of *E. coli* B cell lysate contains, in addition to IscS, two other separated proteins that produce sulfide from L-cysteine in the presence of DTT (lower panel, lane 1). These two proteins have greater mobility than IscS in the native gels. The protein with faster mobility gave an intense band upon activity staining and has an estimated molecular mass of 33 kDa on SDS gels. Flint et al. (*19*) reported the presence of two proteins of this mass in *E. coli*, *O*-acetylserine sulfhydrylases A and B, that mobilized sulfide from cysteine. Unlike IscS, they did not bind to the DEAE column (Figure 8, lower panel, lane 2). The DEAE flowthrough fraction had very low tRNA sulfurtransferase activity (4% of DEAE eluate). IscS was the only protein that both mobilized sulfide in all the column fractions (Figure 8, lower panel, lanes 3—8) and catalyzed sulfur transfer to tRNA (Table 2). In addition, we observed no tRNA sulfurtransferase activity in any column fractions that did not contain IscS. These findings imply that IscS may be specifically involved in the in vitro transfer of sulfur to tRNA when cysteine is used as the sulfur donor.

DISCUSSION

By using recombinant ThiI and an unmodified $tRNA^{Phe}$ transcript as substrate for an *in vitro* assay, we have isolated a PLP-containing protein from wild-type *E. coli* as a factor involved in s^4U biosynthesis. Using UV spectroscopy and HPLC analysis, we have confirmed that the product of sulfur transfer from ^{35}S-labeled cysteine is s^4U. The N-terminal sequence of this tRNA sulfurtransferase is identical to the IscS protein recently characterized by Flint (*21*, *26*). IscS is an NifS-like protein that acts as a cysteine desulfurase and can mobilize sulfur for [Fe—S] cluster reconstitution in apodihydroxyacid dehydratase in vitro (*19*). The original NifS protein was characterized from the nitrogen-fixating bacterium *A. vinelandii* and is required for the proper reconstitution of the [Fe—S] clusters in nitrogenase (*22*). NifS is also able to reconstitute the [Fe—S] clusters of other proteins both

[2] In our hands, tRNA isolated from wild-type *E. coli* has a relatively flat absorbance peak centered at 338 nm. Mueller et al. (*12*) assigned the peak for s^4U at 334 nm, and other values range from 330 to 340 nm (*1*).

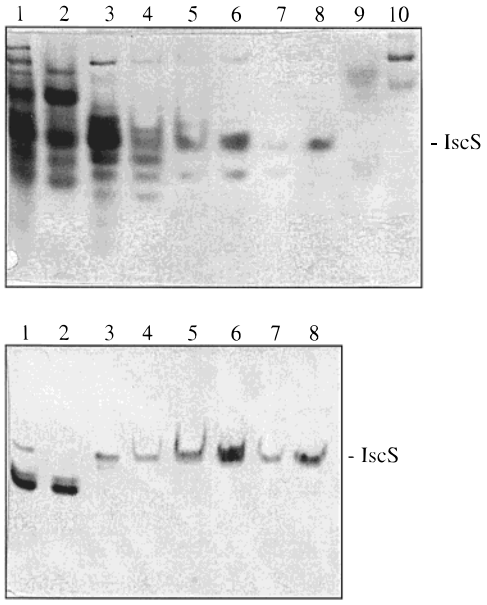

FIGURE 9: Native PAGE and staining for sulfur mobilizing enzymes. Electrophoresis and gel staining was performed as described under Materials and Methods. Upper panel, Coomassie stained gel; lower panel, the same gel stained for enzymes that liberate sulfide from cysteine. Lane 1, 40 μg of S-100 supernatant; lane 2, 40 μg of DEAE flowthrough; lane 3, 30 μg of DEAE eluate; lane 4, 10 μg of Cibacron Blue eluate; lane 5, 4.3 μg of heparin eluate; lane 6, 6.3 μg of hydroxyapatite eluate fractions 8−10; lane 7, 1.3 μg of hydroxyapatite eluate fractions 11−15; lane 8, 2.0 μg of overexpressed IscS. Lanes 9 and 10 α-lactoglobulin, carbonic anhydrase, and urease standard proteins (Sigma catalog no. MW-ND-500).

in vitro (*23*, *24*) and in vivo (*25*). Recently, Zheng et al. (*26*) have studied an *iscS*-containing gene cluster in *A. vinelandii* that is involved in [Fe−S] cluster assembly. They noted that *E. coli* also contains this cluster, as do many other bacteria that do not fix nitrogen. There is strong evidence that the *iscS* gene is essential for viability in *A. vinelandii* (*26*).

Sequence homology searches have shown that many organisms contain multiple NifS proteins with significant homology to each other and to aminotransferases in general (*27*). These proteins make up a new class of PLP-utilizing enzymes, which also includes serine/pyruvate and phosphoserine aminotransferases (*27*, *28*). In addition to *iscS*, *E. coli* contains two other genes with homology to *nifS* (*29*). One of these is CSD, cysteine sulfinate desulfinase, which has a broad lyase activity on a number of cysteine analogues and appears to differ mechanistically from IscS (*29*). The other, termed *csdB*, is analogous to mammalian selenocysteine lyase and has significantly lower activity with cysteine as a substrate (*30*). In addition to the reconstitution of metalloclusters, NifS from *A. vinelandii* was identified as a selenocysteine lyase (*31*) and recently as a selenide delivery protein for the biosynthesis of selenophosphate in vitro (*32*). An *nifS* gene in *Bacillus subtilis* is required for NAD biosynthesis (*33*). A mitochondrial NifS protein in *S. cerevisiae*, SPL1(NFS1), is necessary for efficient splicing of tRNA (*34*) and was found to suppress deficiencies in superoxide dismutase by mediating [Fe−S] cluster formation (*35*). This protein has 56% amino acid sequence identity with *E. coli* IscS. Interestingly, in Lipsett's original work with s^4U biosynthesis (*9*), a factor was isolated from yeast that could substitute for the PLP-dependent factor C activity.

Analysis of *E. coli* extracts and chromatography fractions for other proteins with cysteine desulfurase activity suggests that only one such protein in *E. coli* is able to provide sulfur for the formation of s^4U in vitro. On the basis of our results, we propose that IscS may be involved in the *in vivo* delivery of sulfur for the biosynthesis of s^4U in *E. coli*. This activity would further expand the cellular role for these proteins and suggests that, as a class, they are able to mobilize sulfur from cysteine and transfer it specifically to a variety of macromolecule acceptors.

Mechanistic studies by Dean and co-workers (*31*) originally showed that NifS removes the sulfur from cysteine to produce alanine and either sulfide or a mixture of sulfide and sulfur(0), depending on whether the reaction is run in the presence or absence of DTT, respectively. Strong evidence was provided for the formation of an active-site cysteine persulfide. The mechanism of sulfur transfer to tRNA presumably would involve such a persulfide intermediate. We have confirmed earlier reports (*9*) that s^4U synthesis requires the presence of a reduced thiol. In our hands, the reaction is stimulated over 4-fold by the addition of 0.1 mM DTT and we observed no change in activity when the DTT concentration is raised to 5 mM. This suggests that if the sulfur(0) formed on IscS is transferred directly to tRNA or to another protein acceptor, it must be protected from potential reducing agents in solution. Flint (*21*) has proposed a scheme in which sulfur(0), generated at the active site of a NifS-like protein, is transferred to other carrier molecules and ultimately to an apoprotein for the production of Fe−S clusters.

The requirement for Mg-ATP in the synthesis of s^4U has mechanistic analogy with a variety of enzymes in the purine and pyrimidine biosynthesis pathways that activate oxygen for substitution by nucleophilic nitrogen species. Some examples include the phosphorylation of O-4 of UTP by CTP synthetase (*36*), the phosphorylation of IMP by adenylosuccinate synthetase (*37*) and the adenylation of XMP by GMP synthetase (*38*). For s^4U synthesis, phosphorylation or adenylation of uridine 8 at the 4-position would give an activated intermediate that could react with sulfide to give s^4U and either phosphate or AMP, respectively. BLAST (*39*) searches with putative *thiI* homologues from various sources show homology to GMP synthetase in the ATP pyrophosphatase domain.

Recently, Begley and co-workers (*13*) have shown that *thiI*$^-$ mutants cannot convert the thiamin biosynthetic protein ThiS into a C-terminal thiocarboxylate. This transformation has not yet been reconstituted in vitro and it is possible that other factors are required. The identification of IscS as the initial sulfur-mobilizing enzyme for ThiI-dependent s^4U synthesis suggests that it may also be involved in the biosynthesis of thiazole. In this hypothesis, ThiI mediates the ultimate sulfur transfer step after accepting sulfur in some form from IscS. Our finding that IscS-catalyzed cysteine desulfurase activity increases in the presence of ThiI may signify that a productive complex is formed between the two proteins. We also have recent evidence that ^{35}S from ^{35}S-labeled L-cysteine is transferred sequentially from IscS to ThiI and then to the tRNA.[3] In addition, we have found that ThiI is able to bind unmodified tRNA, whereas IscS does

[3] Manuscript in preparation.

not (data not shown). These observations would be consistent with ThiI as the ultimate sulfur transferring agent to tRNA. It would require ThiI to both activate the 4-position of uridine 8 with Mg-ATP and transfer the sulfur to give s^4U. This mechanism is unlikely in the case of thiazole biosynthesis because a different factor, ThiF, has been found that adenylates ThiS (*13*). Now that we have developed a reliable in vitro system for s^4U synthesis, we can further address these issues.

Finally, Mueller et al. (*12*) have discussed an uncertainty in the assignment of *thiI* as *nuvA* or *nuvC*. From the work of Lipsett and co-workers (*10*), *nuvA* was mapped at 9.3 min on the *E. coli* chromosome. This is very close to the location of *thiI* (9.6 min). The *nuvC* gene was mapped to 42−46 min and both *nuvC*$^-$ and *thiI*$^-$ mutants were reported to be auxotrophs for thiamin. This results in a discrepancy, since recombinant ThiI from *E. coli* does not bind PLP (*12*), which is a characteristic of the *nuvC* gene product. Our finding that a PLP-containing enzyme, IscS, is involved in sulfur transfer to give s^4U suggests that this enzyme may be the *nuvC* product and that *thiI* is equivalent to *nuvA*. However, the chromosomal location of *iscS* is 57.3 min., significantly different from the location assigned to *nuvC*. The possibility that more than two gene products are required for s^4U synthesis has not been rigorously excluded, although we have used purified components for our in vitro studies. We are attempting to prepare *iscS* deletion mutants in *E. coli* in order to confirm the relevance of *iscS* to s^4U synthesis in vivo and to assess if these mutants are viable. Characterization of the phenotype of an *iscS*$^-$ strain may give further insight into the interrelationship between the biosynthesis of s^4U and thiazole.

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