**Functional Diversity of the Rhodanese Homology Domain**

**THE ESCHERICHIA COLI ybbB GENE ENCODES A SELENOPHOSPHATE-DEPENDENT tRNA 2-Selenouridine Synthase**<sup>7</sup>

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_Selenocysteine_ has eight genes predicted to encode sulfurtransferases having the active site consensus sequence Cys-Xaa-Xaa-Gly. One of these genes, _ybbB_, is frequently found within bacterial operons that contain _selD_, the selenophosphate synthetase gene, suggesting a role in selenium metabolism. We show that _ybbB_ is required _in vivo_ for the specific substitution of selenium for sulfur in 2-thiouridine residues in _E. coli_ tRNA. This modified tRNA nucleoside, 5-methylaminomethyl-2-selenouridine (mnm5se2U), is located at the wobble position of the anticodons of tRNA<sup>Lys</sup>, tRNA<sup>Glu</sup>, and tRNA<sub>1</sub>. Nucleoside analysis of tRNAs from wild-type and _ybbB_ mutant strains revealed that production of mnm5se2U is lost in the _ybbB_ mutant but that 5-methylaminomethyl-2-thiouridine, the mnm5se2U precursor, is unaffected by deletion of _ybbB_. Thus, _ybbB_ is not required for the initial sulfurtransferase reaction but rather encodes a 2-selenouridine synthase that replaces a sulfur atom in 2-thiouridine in tRNA with selenium. Purified 2-selenouridine synthase containing a C-terminal His<sub>6</sub> tag exhibited spectral properties consistent with tRNA bound to the enzyme. In _vitro_ mnm5se2U synthesis is shown to be dependent on 2-selenouridine synthase, SePO<sub>3</sub>, and tRNA. Finally, we demonstrate that the conserved Cys<sup>97</sup> (but not Cys<sup>96</sup>) in the rhodanese sequence motif Cys<sup>96</sup>-Cys<sup>97</sup>-Xaa-Xaa-Gly is required for 2-selenouridine synthase _in vivo_ activity. These data are consistent with the _ybbB_ gene encoding a tRNA 2-selenouridine synthase and identifies a new role for the rhodanese homology domain in enzymes.

Selenium is present as a selenocysteine residue in many selenoproteins from prokaryotic and eukaryotic sources (1) and is also found in the wobble position of several bacterial tRNAs as the modified base 5-methyl-amino-methyl-2-selenouridine (mnm5se2U)<sup>1</sup> (2). Isoaccepting species of lysine, glutamate, and glutamine tRNA are the most abundant seleno-tRNAs present in several anaerobic and facultative bacteria (3–5). Specific incorporation of selenium into tRNA and selenoproteins requires selenophosphate (SePO<sub>3</sub>) (6). Mutants of _Escherichia coli_ or _Salmonella typhimurium_ containing a defective _selD_ gene, which encodes selenophosphate synthetase, are unable to incorporate selenium into proteins or tRNAs (7, 8). Although the pathway of selenium incorporation into protein is known in _E. coli_ and related bacteria (1), the steps or enzymes involved in the incorporation of selenium into tRNAs are not completely defined. It is known that 2-selenouridine is derived from 2-thioridine by replacement of the sulfur with selenium (Scheme 1) (9, 10). A partially purified enzyme from _S. typhimurium_, termed tRNA 2-selenouridine synthase, was shown to catalyze the ATP-independent conversion of 2-thioridine in tRNA to 2-selenouridine when supplied with selenophosphate (11). However, neither the gene encoding tRNA 2-selenouridine synthase nor the 2-selenouridine synthase reaction mechanism has been defined.

Sulfurtransferases containing a rhodanese homology domain are ubiquitous proteins that catalyze the transfer of a sulfur atom from thiosulfate (rhodanese) or from 3-mercaptopropionate (mercaptopyruvate sulfurtransferase) to thiophilic sulfur acceptors such as cyanide (12). The rhodanese homology domain has a conserved cysteine residue that participates in catalysis (12). The ~110-amino acid rhodanese domain may comprise the entire protein, as in the GlpE and PspE rhodanases of _E. coli_ (13, 14), or the domain may be fused to other protein domains of known or unknown function. Other rhodanases and mercaptopyruvate sulfurtransferases contain two rhodanese homology domains, but only the C-terminal domain contains an active site cysteine that participates in catalysis (12). Certain ThiI proteins, including that of _E. coli_, contain a C-terminal rhodanese homology domain fused to an N-terminal domain that is involved in biosynthesis of the thiazole ring of thiamin and 4-thioridine of tRNAs (15–18).

Recent investigations aimed at defining the physiological functions of sulfurtransferases suggested involvement of these enzymes in selenium metabolism. Interestingly, it was found that incubation of bovine rhodanese with selenite in the presence of glutathione resulted in formation of an equimolar rhodanese-selenium adduct that was capable of serving as a selenium donor for selenophosphate synthetase (19). In addition,
we have observed that the selD operon of certain bacteria such as Pseudomonas aeruginosa contains a second gene homologous to E. coli ybbB\(^2\) (see Fig. 1), one of eight E. coli genes predicted to encode proteins with a rhodanese homology domain.

In this work, an E. coli mutant deficient in ybbB was constructed and was found to be incapable of incorporating selenium into tRNA. However, synthesis of 2-thiouridine in tRNA by the mutant was unaffected in vivo. Purified YbbB protein contained tightly bound tRNA and exhibited tRNA 2-selenouridine synthase activity. The rhodanese homology domain of the 2-selenouridine synthase has two adjacent cysteine residues at its putative active site. The second cysteine residue (Cys\(^97\)) of the rhodanese homology domain of the ybbB gene deletion was found to be essential for activity in vivo. These results indicate that the rhodanese homology domain functions in selenium transfer from selenophosphate during conversion of 2-thiouridine to 2-selenouridine in bacterial tRNA.

**EXPERIMENTAL PROCEDURES**

**Chemicals, Strains, Media, and Growth Conditions—**[\(^{75}\text{SeO}_3\)]\(^2\) was purchased from the University of Missouri Research Reactor Facility (Columbia, MO). P1 nuclease was from Roche Applied Sciences, and E. coli and shrimp alkaline phosphatases were purchased from Amersham Biosciences. RNase A and RNase-free DNase I were purchased from Sigma-Aldrich and Ambion, respectively. The bacterial strains used in this study are described in Table I. All strains are K-12 derivatives except for BL21 Star(DE3), which is derived from E. coli B. DH5\(a\) (20) was used as a host during plasmid construction. In general, the cultures were grown in LB broth supplemented with the appropriate antibiotic (200 \(\mu\)g/ml ampicillin, 25 \(\mu\)g/ml kanamycin, or 10 \(\mu\)g/ml tetracycline). All of the cultures were grown at 37°C unless indicated otherwise.

**Construction of a Chromosomal ybbB Gene Disruption—**Construction of a ybbB deletion was carried out essentially by the method of Datsenko and Wanner (21). Gene disruption targeting vector pFRT-K was constructed by introducing a kanamycin resistance cassette (\(K\_\text{\text{*}}\)) flanked by FRT sites (FLP recombinase site) from pCP20 (22) into the EcoRI-HindIII sites of pBlueprints-K\(^*\) (Stratagene). Thus, pFRT-K contains multiple cloning sites on each side of the \(K\_\text{\text{*}}\) cassette for cloning chromosomal DNA sequences that target the gene disruption. The \(K\_\text{\text{*}}\) cassette can be excised from chromosomal insertion sites upon introduction of pCP20, which provides FLP recombinase upon thermal induction (22).

For construction of the ybbB knockout strain, a 182-bp fragment ending 52 bp upstream of the ybbB initiation codon was amplified by PCR using primers BCF1 (GATCGACTCATATGCTG) and BBCS-1 (AATTGC-CACGGGCGCAAGCGGATAC), which generated PCR products of the expected size for the wild-type and ybbB-deleted strain, respectively. The chromosomal location of ybbB::K\_\text{\text{*}} was verified by showing the anticipated 43% cotransduction frequency of ybbB::K\_\text{\text{*}} with a nearby Tn10 insertion (purK79::Tn10). The purK79::Tn10 marker was introduced into strain FA026 by P1 transduction creating strain FA029 (Table I). A P1 lysate of strain FA029 and ybbB::K\_\text{\text{*}} was subsequently used to transduce strain FA031 by P1 transduction, with selection for tetracycline resistance, generating FA031. The \(K\_\text{\text{*}}\) cassette was excised from strain FA031 by FLP recombinase, creating strain FA034 (\(\Delta\text{ybbB}\)).

**Construction of the YbbB Expression Plasmid—**The gene for ybbB was amplified by PCR using forward and reverse primers ybbBF (TCTTGGATATGCAAGAGAGACAC) and ybbBH (TCCCGCGCCCGCTTTAACCCCATTC), respectively (see Fig. 1). After PCR, the Ndel-Sall fragment was cloned into pGZ117 (23) between the T\(_\text{\text{e}}\)-promoter and the region encoding a His\(_\text{\text{a}}\) affinity tag and thrombin cleavage site generating pFA204. The cloning procedure resulted in expression of YbbB-H with the C-terminal amino acid sequence ARNNACPRGSHL, where the vertical line indicates the C-terminus of YbbB and the asterisk indicates a thrombin cleavage site. The ybbB region of pFA204 was sequenced and found to be entirely correct.

**Construction of Cys-Ser Variants of YbbB—**Expression vectors for production of C96S and C97S variants of YbbB were constructed by PCR amplification of the region containing the two cysteines using pFA204 as the template and ybbBF and BCCS-1 (AATTGC-CACCGGGCGCAAGCGGATAC) (italicized bold serine codon) or ybbBF and BCCS-2 (AATTGGACGGGGGCGCAAGAGAGACAC) as primers (see Fig. 1). After amplification, PCR products were digested with Ndel and Bg11 and cloned into the same sites of pFA204, replacing the wild-type sequence. The 300-bp Ndel-Bg11 regions of the resulting products were subsequently sequenced and found to be the same. The C96S and C97S variants of YbbB are encoded by pFA218 and pFA210, respectively.

**In Vivo Analysis of \(^{75}\text{Se} \) Labeling—**Incorporation of selenium into protein and tRNA was determined by growing 12-ml anaerobic cultures of E. coli strains in LB-Amp supplemented with 1% glucose and 0.1 \(\mu\)g/ml Na\(_2\)SeO\(_3\) (20 \(\mu\)Ci of \(^{75}\text{Se} \)). The cultures were grown overnight at 37°C, and the cells were harvested by centrifugation. The cell pellets were resuspended in 100 \(\mu\)l of 50 mm Tris-HCl, pH 8.5, disrupted by sonication, and the cell debris was removed by centrifugation. Selenium incorporation was analyzed by electrophoresis of the extracts on 12% SDS-polyacrylamide gels using Phosphofrager detection. Bulk tRNA was isolated from the same extracts using the method previously described (9), and nucleoside analysis was performed as described (24) with the following modifications. Isolated tRNA (50 \(\mu\)l) was first digested at pH 5.3 with 6 units of P1 nuclease at 37°C for 2 h in the presence of 0.65 mm ZnSO\(_4\). The pH of the reaction was then adjusted to pH 8.5 by the addition of Tri-HCl buffer, and the sample was brought to 50 mm MgSO\(_4\) and 10 mm dithiothreitol. Five units of alkaline phosphatase (bacterial or shrimp) were added, and the sample was incubated at 37°C for an additional 3 h. Digestion was terminated by incubation at 95°C for 10 min followed by centrifugation and removal of precipitated protein. Nucleoside separation was performed by HPLC using an analytical reversed phase C\(_\text{\text{18}}\) column (Vydac) and the following mobile phase: 0–25 min 0–3% methanol in 10 mm ammonium acetate, pH 5.3, 25–35 min 3.5–15% methanol, and 35–45 min 100% methanol wash). The nucleosides were detected by electronic absorption at 254 and 313 nm, and \(^{75}\text{Se} \) was measured using a Radiolabel Detector LB508 (EG&G Berthold) placed in-line immediately after the optical detection unit.

**Expression and Purification of Enzymes—**Selenophosphate synthase was overexpressed in E. coli BL21(DE3) cells and purified as described (25). Because selenophosphate synthetase requires K\_ and is inhibited by Na\_\text{\text{+}}, all of the buffers were adjusted using HCl and KOH.

2-Selenouridine synthase and its Cys\_Ser variants were expressed in E. coli BL21 Star(DE3). Competent cells were transformed with pFA218, or pFA210, producing His-tagged 2-selenouridine synthase or its variants. Overnight cultures grown in Luria broth medium supplemented with ampicillin (100 \(\mu\)g/ml) were used to inoculate a fermentor containing 10 liters of the same medium. After growth at 37°C to an A\(_\text{\text{600}}\) of 0.5, the temper-
tRNA 2-Selenouridine Synthase of E. coli

Table I

| Strain | Genotype/property | Derivation or reference |
|-------|------------------|-------------------------|
| MG1655 | F rph-1 delG rfp-50 | Ref. 48 |
| TL524  | MG1655 del(aroZTA-argF) U169 | Ref. 49 |
| BL21(DE3) | F ompT hsdS2 F rK177 M15 | Ref. 50 |
| BL21 Star(DE3) | BL21(DE3) recA131 | In Vitrogen |
| BW25113 | lacI1 NtrC17A8 D180A | Ref. 21 |
| CAG12171 | MG1655 purK79::Tn10 | Ref. 51 |
| MC1061 | F araD139 ara-leu7696 lacY74 galK galK hsdR7 rpsL | Ref. 32 |
| CL250  | MC1061 ΔmutαA | This work |
| FA026  | BW25113 (∆ybbB::Km) | FLP removal of Km<sup>r</sup> (FA026) |
| FA027  | BW25113 (∆ybbB::FRT<sup>r</sup>) | F1 (CAG12171) → FA026 |
| FA029  | BW25113 (∆ybbB::Km<sup>r</sup>) purK79::Tn10 | F1 (FA029) → TL524 |
| FA031  | TLL254 (∆ybbB::Km<sup>r</sup>) purK79::Tn10 | FLP removal of Km<sup>r</sup> (FA031) |
| FA034  | TLL254 (∆ybbB::FRT purK79::Tn10) | FLP removal of Km<sup>r</sup> (FA031) |

* FLP-mediated excision of the Km<sup>r</sup> cassette from ∆ybbB::Km<sup>r</sup> leaves an 88-bp FRT scar.

nature was reduced to 23 °C, and 2-selenouridine synthase synthesis was induced by the addition of isoprropyl-β-D-thiogalactopyranoside (0.4 mM). The cells were harvested after 3 h of induction. The cell pellet was resuspended in 50 ml of buffer A (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM 2-mercaptoethanol, 5% glycerol) containing 10 mM imidazole and 0.5 mM phenylmethylsulfonfluoride, frozen in liquid N<sub>2</sub>, and stored at −80 °C.

For purification, the frozen cell suspension was thawed, DNaSe I was added, and the cells were disrupted by sonication. All subsequent steps were performed at 4 °C. After centrifugation for 1 h at 33,000 × g, the clarified extract was mixed with 1.5 mL of Ni<sup>2+</sup>-nitrilotriacetic acid resin (Qiagen) equilibrated in buffer A containing 10 mM imidazole. The slurry was stirred slowly for 1 h, transferred to a column, and washed with buffer A with 10 mM imidazole followed by a second wash with 20 mM imidazole. Each wash was maintained until the optical absorption at 280 nm reached base line. 2-Selenouridine synthase was eluted by application of buffer A containing 100 mM imidazole. The eluted protein was dialyzed against buffer B (50 mM Tricine-KOH, pH 8.0, 1 mM dithiothreitol, 10% glycerol) and applied to a 10-ml DEAE-Sepharose Fast Flow (Amersham Biosciences) column equilibrated in buffer B. The column was washed with 20 ml of buffer B, and adsorbed protein was eluted by elution of a 0.2-liter linear gradient of 0–0.5 M KCl in buffer B. The fractions containing pure 2-selenouridine synthase (>95% pure determined by SDS-PAGE) were pooled and concentrated using a Centricon 30 (Amicon). Following dialysis against 50 mM Tricine-KOH, pH 8.0, 1 mM dithiothreitol, 10% glycerol, the aliquots were frozen in liquid N<sub>2</sub>, and stored at −80 °C. Approximately 50 g of wet cell paste yielded 10 mg of pure 2-selenouridine synthase.

In Vitro Assay of 5-Methylaminomethyl-2-selenouridine Formation—2-Selenouridine synthase assays were performed in an anaerobic chamber (Coy Laboratories) maintained at <5 μM O<sub>2</sub>. All of the reaction mixtures (200 μl) contained 50 mM Tris-HCl, pH 7.2, 20 mM KCl, 4 mM MgCl<sub>2</sub>, 2 mM ATP, 100 mM H<sub>2</sub>S<sup>−</sup> (10 μCi of [H<sup>35</sup>Se<sup>−</sup>]), 10 μM selenophosphate synthetase, and 50 μM bulk tRNA isolated from strain FA034 (∆ybbB) as indicated. The reaction mixtures were incubated with H<sub>2</sub>S<sup>−</sup> initially at ambient temperature (∼23 °C) for 10 min. Then 2-selenouridine synthase (or variant enzyme) was added to 5 μM, and incubation was continued for an additional 30 min at ambient temperature. The reactions were terminated by the addition of an equal volume of Tris-buffered phenol followed by the addition of dithiothreitol and NaCl to 0.05 and 1.0 μl, respectively. The samples were thoroughly vortexed, and the phases were separated by centrifugation. The product, [75Se]methyls<sup>−</sup>U, was identified by HPLC as described above for in vivo experiments. Routine measurements of selenium incorporation into tRNA were performed by first precipitating RNA from the aqueous phase and thoroughly washing the tRNA pellets with cold 70% ethanol. The pellets were dried and resuspended in 25 μl of H<sub>2</sub>O<sub>2</sub>, and radioactivity was measured by liquid scintillation spectroscopy. H<sub>2</sub>S<sup>−</sup> was prepared from Na<sub>2</sub>SeO<sub>3</sub>, in an anaerobic chamber by reduction with NaBH<sub>4</sub>, according to a published procedure (26). H<sub>2</sub>S<sup>−</sup> containing H<sup>35</sup>Se<sup>−</sup> was prepared by reducing a neutralized stock solution of [75Se]S<sup>−</sup> with 80 μM dithiothreitol and dilution into H<sub>2</sub>S<sup>−</sup>. Formate dehydrogenase activity was determined using the benzyi viologen overlay assay as described (27). Methods for the isolation and quantitation of unfraccionated tRNA for mm<sup>r</sup>U analysis by HPLC were performed as previously described (28). Protein was estimated using the Bio-Rad protein reagent and bovine serum albumin as the standard. Protein purity was judged by SDS-PAGE (10 or 12%) using Gelcode Blue stain (Pierce) for detection. tRNA staining was performed by soaking SDS gels in a 0.5 μg/ml solution of ethidium bromide for 30 min at room temperature. The concentration of denatured 2-selenouridine synthase was determined by first precipitating the protein by heating at 95 °C for 5 min followed by centrifugation. The supernatant containing tRNA was removed, and the protein pellet was resuspended in 50 mM sodium phosphate, pH 7.5, containing 6 μg of ribonucleic HCl. The UV spectrum of this sample was recorded, and the molar extinction coefficient determined from the protein sequence (57,300 m<sup>−1</sup> cm<sup>−1</sup>) was used to calculate the 2-selenouridine synthase concentration. The spectrum of the native enzyme was recorded in 50 mM Tricine-KOH, pH 8.0, 20 mM KCl. The samples were subjected to DNase and RNase digestion in 10 mM Tris-Cl, pH 8.0, 5 mM MgCl<sub>2</sub> using 2 units of RNase-free DNAse I (Ambion) and 5 μg/ml bovine pancreatic RNase A (Sigma), respectively, at 37 °C for 30 min and quenched by the addition of SDS-PAGE loading buffer. All HPLC procedures were performed using a Hewlett Packard 1100 series system. Electronic absorption measurements were made using a Cary Bio 100 spectrophotometer.

RESULTS

Identification and Cloning of ybbB—With the exception of the ThiI protein of E. coli, which is involved in thiamin and 4-thiouridine biosynthesis, the physiological functions of bacterial proteins containing a rhodanese homology domain are presently unknown. The genome sequence of E. coli predicts the presence of eight proteins with the rhodanese homology domain. One of these proteins, YbbB, is a multidomain protein consisting of an N-terminal rhodanese homology domain and a second domain containing a P-loop (Walker A) motif (Fig. 1A). The P-loop motif is found in proteins that bind ATP or GTP (29, 30). There is also a region at the extreme C terminus of YbbB (residues 324–356) that has sequence similarity to a portion of the so-called helical region of isoleucyl tRNA synthetases. The helical region contributes to binding of the anticodon loop of the tRNA (31).

Evaluation of the genomic context of ybbB in sequenced genomes revealed that this gene is occasionally present as the second gene in an operon that also contains selD, encoding selenophosphate synthetase (Fig. 1B). This discovery suggested that YbbB also participates in selenium metabolism and provided a rationale for the genetic and biochemical analysis reported here.

In Vivo Analysis of YbbB Activity—To test the hypothesis that the ybbB gene product functions in selenium metabolism, we generated an E. coli ybbB deletion mutant (strain FA034). The ybbB mutant had no obvious growth defect when tested on rich or minimal growth medium. Both the wild-type (TL524) and knock-out strains were grown under anaerobic conditions in media supplemented with 0.1 μM selenite (20 μCi of [75Se]<sup>−</sup>) and analyzed for selenium-containing proteins and tRNA. Fig. 2 shows electrophoretic analysis of soluble extracts from wild-type and ∆ybbB strains (lanes 1 and 2, respectively). Both strains incorporated [75Se]<sup>−</sup> into formate dehydrogenase H, a
Previous studies have shown that sulfur-modified tRNA (2-thiouridine) is required as a substrate for the selenotransferase reaction (9). To test the possibility that YbbB participates in the sulfurtransferase step of mmn\textsuperscript{5}se\textsuperscript{2}U assembly and that its deletion prevents production of substrate for the selenotransferase reaction, production of mmn\textsuperscript{5}se\textsuperscript{2}U in tRNA was compared in the wild-type and ΔybbB mutant strains. Fig. 3 shows the results of tRNA nucleoside analysis from wild-type (Fig. 3A) and ΔybbB (Fig. 3C) strains, which show identical patterns of production of mmn\textsuperscript{5}se\textsuperscript{2}U, indicating that disruption of ybbB has no affect on 2-thiouridine generation. Also shown is the nucleoside analysis of a ΔybbB ΔybbS strain, which is defective in the production of mmn\textsuperscript{5}se\textsuperscript{2}U (32).

Purification and Physical Characterization of 2-Selenouridine Synthase—The in vivo results described above strongly suggest that the ybbB gene product is tRNA 2-selenouridine synthase. To further characterize the enzyme, a ybbB overexpression system was constructed to generate sufficient quantities for purification and subsequent in vitro analysis of mmn\textsuperscript{5}se\textsuperscript{2}U formation. All of the purification steps required the presence of a reducing agent, either 2-mercaptoethanol or dithiothreitol, to prevent irreversible precipitation of the enzyme. The SDS gel electrophoretic analysis shown in Fig. 4A (panel a) indicates that purified 2-selenouridine synthase migrates as a 43-kDa monomer under reducing and denaturing conditions, consistent with the molecular mass of the monomer predicted from the ybbB gene sequence (42.8 kDa). Purified 2-selenouridine synthase had an unusual electronic absorption spectrum with λ\textsubscript{max} = 258 nm (Fig. 4B). The determined ε\textsubscript{250}, based on the total protein concentration determined by a colorimetric assay was 820 ± 90 m\textsuperscript{-1} cm\textsuperscript{-1}. No peak at 260 based on the MW of the protein was evident, and the observed ε\textsubscript{260} ~ 400 m\textsuperscript{-1} cm\textsuperscript{-1} was still much larger than ε\textsubscript{280} = 57.3 m\textsuperscript{-1} cm\textsuperscript{-1} calculated...
from the amino acid composition. The data are consistent with nucleotide absorption dominating the optical spectrum. The inset in Fig. 4B shows that there is also a weak absorption at 335 nm in the 2-selenouridine synthase spectrum that is identical to a feature in the bulk E. coli tRNA spectrum reflecting the 4-thiouridine content of tRNA. The presence of tRNA in purified 2-selenouridine synthase samples also was indicated by staining of an SDS gel with ethidium bromide (Fig. 4A, panel b). Fig. 4C shows the nuclease sensitivity of the polynucleotide detected by ethidium bromide fluorescence. The staining patterns of the enzyme sample and bulk E. coli tRNA were similar, and no bands representing high molecular mass RNA were present. Lanes 3 and 6 show that treatment of both 2-selenouridine synthase and E. coli tRNA with DNase I had no effect on the polynucleotides present. However, treatment with RNase A (lanes 4 and 7) resulted in degradation of polynucleotide in both 2-selenouridine synthase and E. coli tRNA samples and loss of ethidium bromide fluorescence. These data strongly suggest that 2-selenouridine synthase, as purified, contains tightly bound tRNA.

Three different methods were utilized for determining the stoichiometry of tRNA bound to 2-selenouridine synthase. Using the electronic absorption spectrum of the native protein and an average $\epsilon_{260} = 450 \text{ mM}^{-1} \text{ cm}^{-1}$ determined from bulk E. coli tRNA, $-1.8 \text{ tRNA}$ were present per enzyme monomer. The measurement was then repeated after first removing 2-sel-
was digested with nuclease P1 and alkaline phosphatase, and the concentrations of the major nucleosides adenine, guanosine, cytosine, and uridine were determined using bulk \textit{E. coli} tRNA as a standard. In this case, ~1.9 tRNA bound to each enzyme monomer. All three methods of quantifying tRNA gave nearly identical results. However, the stoichiometries are based on protein concentrations derived from a standard colorimetric assay using bovine serum albumin as a protein standard, which may introduce large errors. Therefore, the protein concentration was determined from the electronic absorption spectrum of denatured 2-selenouridine synthase in the absence of tRNA (see “Experimental Procedures”) and found to differ from the colorimetric method by less than 5%, indicating that the protein determination method is not likely a cause of error in the stoichiometry calculation. These data indicate that 2-selenouridine synthase monomers tightly bind two tRNA molecules.

\textbf{In Vitro Analysis of the 2-Selenouridine Synthase Reaction—}

2-Selenouridine synthase activity was demonstrated using a coupled assay system in which [\textsuperscript{75}Se]selenophosphate was generated from H\textsuperscript{75}Se\textsuperscript{2-} using selenophosphate synthetase, and the formation of \textsuperscript{75}Se-labeled tRNA was measured. Table II lists the results of \textit{in vitro} assays performed under various conditions. In the presence of all components of the coupled assay (see “Experimental Procedures”), 2-selenouridine synthase catalyzed the formation of 5.6 \( \mu \text{M} \) \textsuperscript{75}Se-labeled tRNA. Production of the correct product was confirmed by HPLC analysis of tRNA digests isolated from the \textit{in vitro} reactions. Fig. 5 shows a typical chromatogram from this analysis and includes plots of 254- and 313-nm absorption and \( \gamma \)-radiation detected during separation of the tRNA digests (Fig. 5, panels a–c, respectively). The major radioactivity peak found in the reaction having both selenophosphate synthetase and 2-selenouridine synthase has a retention time consistent with that of authentic mnm\textsubscript{5}se\textsubscript{2}U. Moreover, the \textit{inset} in Fig. 5 shows the electronic absorption spectrum of the \textsuperscript{75}Se-labeled compound that is identical to the spectrum of authentic mnm\textsubscript{5}se\textsubscript{2}U, thus confirming the identity of the modified nucleoside in these samples. A smaller peak of radioactivity was also observed eluting just prior to mnm\textsubscript{5}se\textsubscript{2}U that is consistent with hypomodified mnm\textsubscript{5}se\textsubscript{2}U such as nm\textsubscript{5}se\textsubscript{2}U present in the sample. Table II also shows that about half as much mnm\textsubscript{5}se\textsubscript{2}U was formed in the absence of added tRNA compared with the complete reaction, consistent with the observation that 2-selenouridine synthase is purified with tRNA bound to the enzyme (see above). Given the relatively high yield of labeled tRNA in the absence of externally added tRNA, it is likely that a substantial fraction of the tRNA bound to 2-selenouridine synthase serves as a substrate.

The data provided in Table II and Fig. 5 (panels d and e) indicate that the modification of tRNA with selenium requires...
both 2-selenouridine synthase and selenophosphate synthetase. The requirement for selenophosphate synthetase and its substrate ATP in the coupled assay indicates that HSe\(^-\) alone does not support mnm\(^5\)se\(^2\)U synthesis but that the more reactive SePO\(_3\) product generated by selenophosphate synthetase is required in the 2-selenouridine synthase-catalyzed reaction, as shown earlier with an impure enzyme system (11). This conclusion is supported by the experiment in which the addition of 500 \(\mu\)M unlabeled SePO\(_3\) inhibited formation of \(^{75}\)Se-labeled tRNA from \(^{75}\)SePO\(_3\) generated \textit{in situ} (Table II). The inhibition is caused by dilution of the \(^{75}\)SePO\(_3\) rather than product inhibition of selenophosphate synthetase because SePO\(_3\) at this concentration has previously been shown not to inhibit the reaction (25).

As shown in Table II, in an assay mixture containing 5 \(\mu\)M 2-selenouridine synthase (10 \(\mu\)M tRNA bound to the enzyme) about 2.7 \(\mu\)M mnm\(^5\)se\(^2\)U was generated without the addition of tRNA. In the reaction mixture containing 50 \(\mu\)M bulk tRNA, the mnm\(^5\)se\(^2\)U produced was increased by 2.9 \(\mu\)M, but because the total yield was similar to the amount of enzyme present in the reaction, it is possible that under these experimental conditions, 2-selenouridine synthase is capable only of a single turnover. Alternatively, the mnm\(^5\)se\(^2\)U substrate may be limiting in the reaction mixture.3 The results in Table III show that small but significant increases in mnm\(^5\)se\(^2\)U production were detected upon increases in added tRNA. At the highest tRNA concentration tested, 2-selenouridine synthase performed about three turnovers, indicating that the enzyme is capable of multiple turnovers. Further analysis of the data shows that about 4–6% of the mixture of tRNAs added was modified with selenium during the reaction. This yield is roughly comparable with the amount of tRNA present in bulk \textit{E. coli} tRNA capable of being modified with selenium (27) and likely represents the amount of the sulfur-modified tRNA (mnm\(^5\)s\(^2\)U) that serves as a substrate for 2-selenouridine synthase \textit{in vivo}.

Mutagenesis of the Rhodanese Active Site Motif—2-Selenouridine synthase contains the rhodanese sequence motif Cys-Xaa-Xaa-Gly within the protein sequence Cys\(^96\)-Cys\(^97\)-Xaa-Xaa-Gly. From a search of known bacterial genome sequences, we found that Cys\(^97\) but not Cys\(^96\) in this motif is strictly conserved in open reading frames with YbbB-like sequences. Hence, the ability of \textit{E. coli} to generate \(^{75}\)Se-labeled tRNA was tested when either Cys\(^96\) or Cys\(^97\) was mutated to serine. Fig. 2B shows the results of complementation of strain FA034 (ΔybbB) with 2-selenouridine synthase containing C96S or C97S (\textit{lanes} 4 and 5, respectively). As expected, neither

\[ \text{Table III} \]

| tRNA added | \(^{75}\)Se incorporation into tRNA |
|-----------|---------------------------------|
|           | \(^{75}\)Se | tRNA added \(a\) |
| None      | 2.7     | 5.2 |
| 50 \(\mu\)M | 5.3     | 5.2 |
| 100 \(\mu\)M | 6.7     | 4.0 |
| 150 \(\mu\)M | 8.5     | 3.9 |
| 200 \(\mu\)M | 14.1    | 5.7 |

\(a\) Calculated after accounting for 2-selenouridine synthase-bound tRNA.

\[ ^{3} \text{It important to note that the tRNA substrate is bulk tRNA isolated from the } \Delta ybbB \text{ strain (FA034) and does not contain mnm}^{5}\text{se}^{2}\text{U.} \]
mutation prevented $^{75}\text{Se}$ incorporation into formate dehydrogenase H. However, changing Cys$^{97}$ to Ser completely eliminated $^{75}\text{Se}$ modification of tRNA, indicating that the conserved Cys$^{97}$ is required for mmn$^{5}\text{Se}^2\text{U}$ synthesis in vivo.

In vitro studies were also performed on the C96S and C97S enzyme variants utilizing the coupled assay (Table IV). Within the error of measurement, wild-type and C96S 2-selenouridine synthase produced similar amounts of $^{75}\text{Se}$-labeled tRNA consistent with the in vivo study presented above. However, whereas a ΔybbB strain transformed with C97S 2-selenouri-
dine synthase showed no evidence of tRNA modification with $^{75}\text{Se}$ in vivo, the purified C97S 2-selenouridine synthase was capable of modifying about half as much tRNA as wild-type 2-selenouridine synthase. It important to note that the product yields represent catalytic activity occurring within an experimental reaction time of 30 min and do not reflect rates of catalysis per se. Nevertheless, these data indicate that the nonconserved Cys$^{96}$ is not necessary for 2-selenouridine synthase catalysis, but whereas Cys$^{97}$ is not absolutely required for mmn$^{5}\text{Se}^2\text{U}$ synthesis in vitro, it is required for in vivo activity.

### TABLE IV

| Enzyme | $^{75}\text{Se}$ incorporation into tRNA |
|--------|---------------------------------------|
| Wild type | 5.4 100 |
| C96S | 5.8 107 |
| C97S | 2.8 52 |

DISCUSSION

The genome of *E. coli* is predicted to encode eight proteins containing a rhodanese homology domain. One of these sequences, ybbB, encodes a protein that is predicted to play a role in selenium metabolism based on the genomic context of ybbB homologs and the known ability of rhodanese domains to bind selenium. In the current work, results of both in vivo and in vitro studies have revealed that the protein encoded by ybbB does indeed function in selenium metabolism. Although a strain deficient in ybbB was fully capable of incorporating selenium into formate dehydrogenase to yield an active enzyme, conversion of mmn$^{5}\text{Se}^2\text{U}$ to mmn$^{5}\text{Se}^2\text{U}$ at the wobble position in tRNA was prevented. Complementation of the ybbB mutant was observed upon introduction of the wild-type ybbB gene. Furthermore, the in vitro coupled assay system utilized in the present study confirms earlier results using a partially purified preparation from *S. typhimurium* that only selenophosphate and a tRNA 2-selenouridine synthase are required for catalyzing mmn$^{5}\text{Se}^2\text{U}$ synthesis in tRNA (11).

Although the determinants of 2-selenouridine synthase needed for binding of tRNA substrates have not been defined, the purified enzyme contained two molecules of tightly bound tRNA, suggesting a specific interaction occurs in vivo. Although the effects of the histidine tag on tRNA binding are currently unknown, access to tRNAs bound by 2-selenouridine synthase will facilitate identification of the specific tRNA species and the molecular protein-nucleotide contacts, which is currently in progress. Previously, selenium adducts of tRNA$^{1}\text{Asp}$, tRNA$^{1}\text{Glu}$, and tRNA$^{1}\text{Gln}$ isoaccepting species, as well as minor tRNA species, have been identified (3–5) and presumably serve as substrates for the 2-selenouridine synthase-catalyzed reaction. The fact that 2-selenouridine synthase activity is observed in vitro in the absence of added tRNA indicates that at least one of these three isoaccepting species is likely bound to purified 2-selenouridine synthase.

Besides 2-selenouridine synthase, the physiological function of only one other rhodanese-containing protein (ThiI) of *E. coli* has been determined. ThiI catalyzes the thiolation of uridine to generate $^6\text{U}$ at position 8 of some isoaccepting tRNAs and also functions in thiamin biosynthesis (16–18). In the proposed ThiI reaction mechanism, IscS catalyzes the transfer of sulfur from cysteine to ThiI, resulting in the formation of a persulfide intermediate at Cys$^{556}$ of the rhodanese homology domain (15, 16, 18). ThiI is also capable of catalyzing thiosulfate:cyanide sulfurb transferase (rhodanese) activity at a very low rate (17). Although we have not been able to detect rhodanese activity in 2-selenouridine synthase preparations, analysis of the C97S variant suggests that the rhodanese domain participates in 2-selenouridine synthase formation in vivo. Thus, it seems possible that 2-selenouridine synthase reacts with selenophosphate to generate a perselenide at Cys$^{97}$ in the rhodanese domain during the catalytic cycle. A cysteine perselenide species has also been invoked in the reaction mechanism of the *E. coli* NifS-like enzyme CsdB (34), which has a high specificity for L-selenocysteine. The presence of a P-loop motif within the C-terminal domain of 2-selenouridine synthase suggests the involvement of a nucleoside triphosphate in the reaction. However, studies on the partially purified 2-selenouridine synthase indicated that ATP is not needed for the reaction (11). Further work is needed to determine whether the P-loop motif plays a role in catalysis.

Whereas mutation of Cys$^{97}$ to Ser abolishes 2-selenouridine synthase activity in vivo, in vitro selenium incorporation is reduced by only 50%. This discrepancy might be explained by the adjacent Cys$^{96}$ partially fulfilling the role of Cys$^{97}$ under the reaction conditions. Alternatively, differences between in vitro conditions and the intracellular environment may affect the efficiency of tRNA modification. The apparent 50% in vitro activity of the C97S variant may also represent an artificially high turnover rate because of the experimental design. That is, selenium incorporation was assessed after a 30-min reaction and does not correspond to an actual rate of reaction. Because the catalytic rates of 2-selenouridine synthase and its variants have not been determined, it is possible that the $k_{cat}$ of wild-type 2-selenouridine synthase is much greater than the C97S mutant. If the wild-type enzymatic reaction has rapidly come to completion (e.g. 5 min), a much slower C97S variant may convert half as much substrate to product as the wild-type 2-selenouridine synthase in the 30-min reaction. Future detailed kinetic studies of the 2-selenouridine synthase reaction are necessary for further analysis of the role of Cys$^{97}$ in catalysis.

Multiple alignment of representative YbbB orthologs and the prototype single domain rhodanese GlpE reveals two unique features of the tRNA 2-selenouridine synthase (supplemental figure). First, the predicted active site loop, beginning at the active site cysteine, is highly conserved as CXXRGXGRS among YbbB orthologs. This sequence may be considered a signature for rhodanese domains that use the larger selenophosphate as substrate. The nature of the active site loop of the rhodanese homology domain is important for substrate specificity (12, 35). A second feature present in the rhodanese domain of all recog-

4 Whereas our preparations of 2-selenouridine synthase were purified in part using affinity chromatography resulting in >95% pure protein, biosynthesis of mmn$^{5}\text{Se}^2\text{U}$ from *S. typhimurium* has been reported to require multiple proteins (47). Low molecular mass basic ribosomal proteins L1, L3, L5, and initiation factor 3 were associated with high activity 2-selenouridine synthase fractions. Therefore, we cannot rule out the possibility that one or more additional proteins required for optimal activity is absent from the in vitro experiments that may lead to ambiguous interpretation of the residual C97S activity.
nizable YbbB orthologs is a conserved ~36-amino acid insertion relative to GhpE, the prototype single-domain rhodanese (supplemental figure) (36). This conserved sequence has the consensus P业务OXY业务ER业务X业务3业务G业务T业务X业务Y业务X业务Z业务业务XPG (where O indicates a hydrophobic amino acid) and is located between the CH2A motif (37) and the active site. This YbbB-specific motif may provide determinants for positioning of the anticodon loop of the tRNA substrate near the active site cysteine. There are other conserved residues within the C-terminal domain of YbbB (supplemental figure) that no doubt also play key roles in catalysis.

2-Selenouridine synthase orthologs are apparently encoded by the genomes of a wide variety of bacteria, with examples found in all divisions of the Proteobacteria and in some Gram-positive bacteria and cyanobacteria. In many cases, ybbB is found as the second gene in predicted selD-ybbB operons (Fig. 1). The assertion that these two genes are in fact cotranscribed is supported by the fact that the termination codon for selD overlaps the predicted initiation codon for ybbB in most situations. Alternatively, one example (Magnetococcus sp. MC-1) was found where the gene order was reversed (ybbB-selD). Finally, one member of the Archaea (Methanococcus jannaschii) is predicted to encode a YbbB-like protein, but in this case its two domains are found as two polypeptides encoded by a putative operon with the rhodanese portion encoded by the distal gene (MJ0052) and the polypeptide with the P-loop motif encoded by the promoter proximal gene (MJ0053).

Although the 2-selenouridine synthase mutant was defective in the biosynthesis of mnm3seU in tRNAs, no observable growth phenotype was associated with this mutation. The selenoselenoacetic acid base 5-methylaminomethyl-2-selenouridine (mnm3seU) is present at position 1 (wobble position) of the anticodon of tRNA34Lys, tRNA34Gin, and tRNA34Ala. Modifications at this position have been proposed to have roles in codon-anticodon interaction (38, 39), aminoaacetylation of tRNA (40–42), reading frame maintenance (43), translation rate (44), and binding of tRNA to the ribosome (45). Suppressor tRNAs have been used as a tool to study the function of these modification in tRNA-codon interaction (7, 46). In E. coli the mnmA mutant lacking the 2-thiouridine modification in the wobble position of the above tRNAs was shown to suppress nonsense mutations less efficiently than the wild-type suppressor strain, suggesting that the 2-thiouridine modification influences codon-anticodon interaction (46). A suppressor tRNA derived from RNA34U of S. typhimurium was also found to function less efficiently in a selD mutant lacking seU (7). We tested the influence of the ΔybbB mutation on the efficiency of suppression of an amber mutation in the N gene of phage lambda by a UAA/UAG suppressor tRNA derived from tRNA34Lys (supG). The supG ΔybbB strain produced a similar number of plaques compared with the supG ybbB+ parent, indicating that selection of the suppressor tRNA has no apparent effect on the efficiency of nonsense suppression in this context.

The data presented in this report establish the identity of ybbB in E. coli as a gene for SeP-O4-dependent 2-selenouridine synthase and provide a preliminary characterization of the enzymatic reaction. This ability to catalyze the transfer of selenium from SeP-O4 for replacement of sulfur in 2-thiouridine makes 2-selenouridine synthase a unique member within the family of proteins containing a rhodanese homology domain. Future studies will focus on a more thorough characterization of the enzymatic reaction including identifying other products of the reaction, which will assist in determining the mechanism of 2-selenouridine synthesis.