The Role of the Cysteine Residues of ThiI in the Generation of 4-Thiouridine in tRNA*

Eugene G. Mueller‡, Peter M. Palenchar‡, and Christopher J. Buck
From the Department of Chemistry and Biochemistry, the University of Delaware, Newark, Delaware 19716

The enzyme ThiI is common to the biosynthetic pathways leading to both thiamin and 4-thiouridine in tRNA. We earlier noted the presence of a motif shared with sulfurtransferases, and we reported that the cysteine residue (Cys-456 of *Escherichia coli* ThiI) found in this motif is essential for activity (Palenchar, P. M., Buck, C. J., Cheng, H., Larson, T. J., and Mueller, E. G. (2000) J. Biol. Chem. 275, 8283–8286). In light of that finding and the report of the involvement of the protein IscS in the reaction (Kambampati, R., and Lauhon, C. T. (1999) Biochemistry 38, 16561–16568), we proposed two mechanisms for the sulfur transfer mediated by ThiI, and both suggested possible involvement of the thiol group of another cysteine residue in ThiI. We have now substituted each of the cysteine residues with alanine and characterized the effect on activity in vivo and in vitro. Cys-108 and Cys-202 were converted to alanine with no significant effect on ThiI activity, and C207A ThiI was only mildly impaired. Substitution of Cys-344, the only cysteine residue conserved among all sequenced ThiI, resulted in the loss of function in vivo and a 2700-fold reduction in activity measured in vitro. We also examined the possibility that ThiI contains an iron-sulfur cluster or disulfide bonds in the resting state, and we found no evidence to support the presence of either species. We propose that Cys-344 forms a disulfide bond with Cys-456 during turnover, and we present evidence that a disulfide bond can form between these two residues in native ThiI and that disulfide bonds do form in ThiI during turnover. We also discuss the relevance of these findings to the biosynthesis of thiamin and iron-sulfur clusters.

The metabolism of many sulfur-containing biomolecules remains incompletely understood. Among the metabolic pathways requiring further elucidation are those leading to iron-sulfur clusters (1–5), biotin (6–8), molybdopterin (9), lipoic acid (10), thiamin (8, 11), and sulfur-containing bases in RNA (12). The sulfur-containing nucleosides include 4-thiouridine (s4U),1 which is found at position 8 of some bacterial tRNA (Fig. 1) and serves as a photosensor for near-UV light (12). The s4U undergoes a photoactivated 2 + 2 cycloaddition with cytidine 13 when the tRNA is exposed to light of a wavelength similar to the 334 nm absorbance maximum of s4U (13–15). The resulting cross-linked tRNA are poor aminocyclation substrates (16), and the accumulation of uncharged tRNA arrests growth by triggering the stringent response (17, 18). Lipsett and co-workers (19, 20) investigated the enzymology of s4U biosynthesis in *Escherichia coli* and reported that the overall reaction utilized cysteine as the sulfur donor and required ATP as a substrate. Lipsett and co-workers (20) concluded that two enzymes were required and that one of them also plays a role in thiamin biosynthesis and requires the cofactor PLP for activity (21, 22). By using a genetic screen based on the role of s4U as a photosensor (18, 22–24), the genetic loci of two genes required for s4U biosynthesis (named *nuvA* and *nuvC*) were mapped (22–24).

By using the same genetic screen, we identified the gene *thiI* as essential for s4U formation (25) shortly after Downs and co-workers (26) identified the same gene as essential for thiamin biosynthesis. We have cloned and overexpressed thiI from *E. coli* (25). Kambampati and Lauhon (27) isolated another enzyme, IscS, that sufficed along with ThiI for in vitro generation of s4U in tRNA, and they have since confirmed (28) that *iscS* mutants lack s4U and are thiamin auxotrophs.2 IscS is a NifS-like protein that functions in iron-sulfur cluster formation, is PLP-dependent, and proceeds through an enzymatic persulfide intermediate (29, 30). Based on sequence alignments that revealed similarity between the segment of ThiI around Cys-456 and other sulfurtransferases, we investigated the importance of Cys-456 for the function of ThiI and found that C456A ThiI was inactive both in vivo and in vitro (31). The report of the role of IscS in s4U biosynthesis fit nicely with our independent conclusion that ThiI would proceed through a persulfide intermediate on Cys-456 by providing a source of S0 to form that persulfide group. Since our report, Kambampati and Lauhon (32) have established that sulfur flows from cysteine to IscS to ThiI to s4U in tRNA.

Based on all the evidence, we proposed two alternative mechanisms for the biosynthesis of s4U (Fig. 2), and both immediately suggest a role for another cysteine residue in ThiI (31). *E. coli* ThiI has four cysteine residues other than Cys-456, and sequence alignments of known ThiI proteins (all from prokaryotes) reveal that only one cysteine residue, Cys-344 in the *E. coli* enzyme, is completely conserved,3 suggesting that this amino acid serves a critical role. We now report that this supposition is borne out by our investigations of the role of the cysteine residues in ThiI.

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‡ To whom correspondence should be addressed: Dept. of Chemistry and Biochemistry, University of Delaware, Newark, DE 19716. Tel.: 302-831-2738; Fax: 302-831-4355; E-mail: emueller@udel.edu; www.udel.edu/chem/mueller.
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1 The abbreviations used are: s4U, 4-thiouridine; PLP, pyridoxal 5’-phosphate; DTT, dithiothreitol; DTNB, 5,5’-dithiobis(2-nitrobenzoic acid) (Ellman’s reagent); His6, His6, IscS bearing the C-terminal His6 tag encoded by pET29b; PCR, polymerase chain reaction; Ni-NTA, nickel-nitritriacetic acid.

* The congruence of *nuvA* and *nuvC* to *thiI* and *iscS* remains unclear due to several apparent discrepancies between the chromosomal locations of the genes and the phenotypes of *nuvA* and *nuvC* mutants relative to *thiI* and *iscS* mutants.
3 P. Palenchar and E. Mueller, unpublished observations.
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Experimental Procedures

General—Unless otherwise stated, all materials were purchased from either Sigma or Fisher and used as provided. Sephadex G-10, Sephadex G-25 (DNA grade), and [58]sulfate were obtained from Amersham Pharmacia Biotech, Nuclease P1, dithiothreitol (DTT), chloramphenicol, kanamycin, and ATP were purchased from Roche Molecular Biochemicals. Wizard Genomic DNA Purification and pGEM®-T Easy Vector System II kits, E. coli JM109 cells, calf intestinal alkaline phosphatase, and Tag DNA polymerase were purchased from Promega Corp. (Madison, WI). Competent BLR(DE3) pLysS cells were supplied previously (31), this N49S mutation arose spontaneously during propagation of pBH113, which is wild-type thiI in E. coli (33). A Higgins analytical CLIPEUS C18 5-μm column (50 × 4.6 mm) was purchased from Bodman Industries (Aston, PA). The tRNA substrate was the l-cysteine residue of ThiI on its activity in vivo was assessed in two ways. First, the thiI mutant VJS2890(DE3) was transformed with a plasmid encoding an altered ThiI, and the transformants were subjected to near-UV screening. The screening of cells producing each altered ThiI was performed twice, except for the cells expressing C202A and C207A ThiI, which were performed four times; qualitatively identical results were obtained in every case. Second, the UV spectrum of tRNA isolated from saturated cultures of the transformants was recorded and examined for the characteristic peak due to s4U (λmax ~ 334 nm). The procedures for both of these in vivo characterizations have been described previously (31).

Assay for s4U Generation—This assay is essentially the one that we have described previously (31) except that recombinant IscS(His6) was substituted for cell extracts. The generation of s4U was monitored by following the incorporation of [35S] into s4U from L-[35S]cysteine. A typical assay mixture (350 μL) was 50 mM Tris-HCl buffer, pH 8.5, containing ATP (4 mM), pyridoxal 5′-phosphate (40 μM), magnesium chloride (5 mM), in vitro transcript of E. coli tRNAψ35S (20 μM), l-[35S]Cysteine (484 μM; 123 Ci/mmol), DTT (1 mM), IscSHis6 (4 nM), and ThiI (1 nM). For the C344A ThiI, the assays contained higher concentrations of the proteins, 4.8 μM IscSHis6 and 1.2 μM C344A ThiI. Reactions were initiated by the addition of recombinant ThiI and incubated at 37°C. At various times, aliquots (100 μL) were removed, and [35S]s4U was quantitated by the method that we have described in detail elsewhere (31). In this method, unreacted l-[35S]Cysteine is removed by size-exclusion chromatography, and the tRNA is digested to nucleosides, which are resolved by reverse phase high pressure liquid chromatography (36, 37); the amount of s4U in the s4U is determined by scintillation counting.

Cloning of iscS—Genomic DNA was purified from E. coli JM109 cells (Promega) using the Wizard Genomic DNA Purification protocol (Promega) according to the manufacturer’s instructions. PCR amplification of iscS in the genomic DNA was achieved using appropriate primers (Table I), Tag DNA polymerase (Promega), and HotStart tubes (Molecular Bio-Products Inc., San Diego) according to the HotStart protocol. The primers (Table I) specified an Ndel site that includes the start ATG of iscS and an EcoRI site that follows the TAA that terminates iscS. A Robocycler Gradient 96 thermal cycler (Stratagene) was used for the PCR, as described elsewhere (25). The PCR product was purified by agarose gel electrophoresis and recovered using the QIAquick gel extraction protocol (Qiagen). The isolated PCR product was ligated into the pGEM-T Easy Vector System II (Promega) as specified by the manufacturer, and restriction analysis confirmed the generation of the target plasmid. This plasmid was digested with Ndel and EcoRI (New England Biolabs, Beverly, MA), and the iscS fragment was isolated by agarose gel electrophoresis and ligated into pET29b (Novagen) that had been opened with the same enzymes. The success of the construction was confirmed by restriction analysis, and the plasmid was named pBH400.

Sequencing of iscS in pBH400 revealed two discrepancies from the published sequence as follows: a T → C transition in codon 244 that results in the substitution of proline for leucine and a deletion of the T in the penultimate codon that moves the stop codon specified by the “reverse” PCR primer out of frame and brings the C-terminal His6 tag encoded by pET29b into frame. IscS bearing the C-terminal His6 tag was denoted IscSHis6. Both alterations of iscS in pBH400 were corrected using the QuikChange™ protocol (Stratagene) with appropriate primers (Table I) as we have described previously (34). The plasmid encoding L244P IscS (with no C-terminal His6 tag) is pBH401; the plasmid encoding IscSHis6 (with Leu-244 restored) is pBH402; the plasmid encoding native IscS (with Leu-244 restored and no C-terminal His6 tag) is pBH403.
Overexpression and Purification of IscS

The procedure for overexpression and purification of IscS was identical to that for the overexpression and purification of ThiI except that cultures of BLR(DE3) pLysS/pBH402 or BLR(DE3) pLysS/pBH400 were grown and induced in LB medium containing kanamycin (30 μg/ml) and chloramphenicol (34 μg/ml). Isolated IscS was dialyzed against 50 mM potassium phosphate buffer, pH 7.5, containing magnesium chloride (5 mM), potassium chloride (100 mM), and EDTA (0.1 mM). The enzyme remains stable for weeks to months when stored at 4°C in the same buffer at moderate concentration (3–4 mg/ml). By comparing A_{280 nm} and the concentration of IscS measured by the biuret assay, we calculated ε_{280 nm} = 25,400 M⁻¹ cm⁻¹, which we now use to determine the concentration of IscS.

Joint Overexpression of ThiI and IscS—BLR(DE3) pLysS/pBH402 (encoding IscS) and BLR(DE3) pLysS/pBH403 (encoding native ThiI) were transformed with pBH113 (encoding wild-type ThiI) using the TransformAid™ protocol (MBI Fermentas, Hanover, MD). The joint overexpression of ThiI and IscS was accomplished as described for the overexpression of either protein alone. ThiI overexpressed with native IscS was cleanly separated from the latter by chromatography over Ni-NTA resin. ThiI overexpressed with IscS was co-purified with the latter by chromatography over Ni-NTA resin; the two proteins were then separated by chromatography over Poros 20 HS resin, eluting with a linear gradient (over 13 column volumes) of potassium chloride (0–1.5 M) in 50 mM potassium phosphate buffer, pH 7.5, containing DTT (1 mM). The UV-visible spectrum was recorded after exchange of isolated ThiI into 50 mM potassium phosphate buffer, pH 7.5, containing magnesium chloride (5 mM), potassium chloride (100 mM), and EDTA (0.1 mM) by size-exclusion chromatography over a spin column of Sephadex G-25 equilibrated in the same buffer.

DTNB Titrations—Samples of ThiI were subjected to DTNB titrations under both native and denaturing conditions. The enzyme samples were fresh preparations that had been isolated using buffers to which no reductant had been added. Any thiol-bearing components of the cell extracts (either small or macro-molecules) should have been removed by the chromatography over Ni-NTA and the exchange (by size-exclusion chromatography or dialysis) of ThiI-bearing column fractions into 50 mM potassium phosphate buffer, pH 7.5, containing magnesium chloride (5 mM), potassium chloride (100 mM), and EDTA (0.1 mM) by size-exclusion chromatography over a spin column of Sephadex G-25 equilibrated in the same buffer.
the A_{142} codon, of the control incubation from the A_{142} codon of each sample, and using an extinction coefficient of 13,600 M^{-1} cm^{-1} (38). To denote the wild-type thiI, solid guanidinium chloride (0.25 g, ~2.4 mmol) was added to the samples and dissolved to achieve a final guanidinium concentration of ~4.6 M; the volume and A_{142} values were measured after 20 min at room temperature. All titrations were performed in duplicate except for the C456A thiI under native conditions, which was performed in quadruplicate. Titrations of thiI with 1 eq of DTNB were performed under native conditions as described for titration with 20 eq of DTNB. All replicate experiments returned values within 5% of each other.

**Assay for Disulfide Bond Formation during Turnover**—To test for disulfide bond formation in thiI during turnover, the sU generation assay was run without DTT, with a reduced concentration of cysteine (which can reduce disulfide bonds), and in phosphate buffer to allow separation of IscS and thiI by cation-exchange chromatography. The assay (1 ml) consisted of 150 mM potassium phosphate buffer, pH 8.5, containing ATP (4 mM), PLP (40 μM), tRNA (18 μM), cysteine (70 μM), IscS (24 μM), and thiI (6 μM). After 5 h at 37 °C, thiI was separated from IscS and other reaction components by chromatography over Poros 20 HS resin as described above except that DTT was omitted from the buffers. The thiI eluted in two fractions, which were combined (1 ml; 2–3 μM), concentrated in a Microcon-10 device, and subjected to DTNB titrations under denaturing conditions as described above. As a control for oxidation under these conditions, thiI was also incubated in the absence of tRNA and cysteine and subjected to the same work up. To measure the extent of sU formation under these conditions, a duplicate reaction containing [35S]cysteine was run in parallel; 0.73 (3 thiol groups) was added to the samples and dissolved to achieve a final guanidinium concentration of ~4.6 M; the volume and A_{142} values were measured after 20 min at room temperature. All titrations were performed in duplicate except for the C456A thiI under native conditions, which was performed in quadruplicate. Titrations of thiI with 1 eq of DTNB were performed under native conditions as described for titration with 20 eq of DTNB. All replicate experiments returned values within 5% of each other.

**RESULTS**

**Cloning and Overexpression of IscS**—The PCR-based cloning of iscS from E. coli genomic DNA resulted in the generation of an IscS overexpression plasmid based on pET29b (pBH400). Sequencing revealed a deletion in the penultimate codon of iscS during the PCR, resulting in a frameshift that fused a C-terminal His_6 tag to IscS (denoted IscS-His_6). In addition, a proline residue was encoded by codon 244 rather than the leucine specified by the sequence in the genomic data base. Overexpressed and purified L244P IscS-His_6 proved fully competent for in vitro assay of thiI activity. Site-directed mutagenesis was used to generate overexpression plasmids for IscS with Leu-244 restored (pBH402) and with both Leu-244 and the penultimate codon restored (pBH403), which brings the stop codon back into frame and eliminates the C-terminal His_6 tag. IscS-His_6 and L244P IscS-His_6 behaved identically in our assays of thiI activity; since they are easily purified, we used them in preference to native IscS (L244P IscS-His_6 was used in earlier experiments, and IscS-His_6 was used in later experiments).

**Generation, Overexpression, and Physical Characterization of Altered ThiI**—Site-directed mutagenesis afforded thiI with each cysteine residue singly changed to alanine. All of the thiI variants discussed below also contain a serine in place of the wild-type Asn-49. We discovered this mutation only after the generation of the altered thiI, and we determined that the N49S thiI is insignificantly (15–20%) more active than the wild-type enzyme (with Asn-49) in our current assay system. We did not restore Asn-49 in each of the variant thiI, for the existing protein context provided an internally consistent evaluation of the effects of altering the cysteine residues. ThiI variants with one cysteine residue replaced with alanine were identical to wild-type thiI regarding overexpression, purification, and storage. Extinction coefficients at 280 nm were determined for each altered thiI, and they varied less than 5% from that of wild-type enzyme (63,100 M^{-1} cm^{-1}) as follows: C108A, 64,500 M^{-1} cm^{-1}; C202A, 64,000 M^{-1} cm^{-1}; C207A, 66,000 M^{-1} cm^{-1}; C344A, 63,900 M^{-1} cm^{-1}. The far-UV CD spectrum of each altered thiI was essentially identical to the spectrum of wild-type enzyme (data not shown). Together, these data indicate that the substitutions of alanine for cysteine did not disrupt the global protein fold.

**In Vivo Characterization of Altered ThiI**—The thiI mutant E. coli VJS28890(DE3) (33) was transformed with plasmids expressing wild-type and altered thiI, and the transformants were screened for near-UV sensitivity. VJS28900(DE3) is near-UV-resistant because it lacks a functional thiI and so fails to generate sU in tRNA (the photosensor). VJS28890(DE3) was fully complemented by plasmids encoding wild-type, C108A, and C202A thiI, but VJS28890(DE3) harboring the plasmid encoding C344A thiI retained the near-UV-resistant phenotype. The plasmid encoding C207A thiI conferred an intermediate phenotype; only one-third to one-half the number of colonies formed with each plasmid as compared to VJS28890(DE3) expressing wild-type thiI.

**TABLE I**

| Purpose                                      | Primers                  |
|----------------------------------------------|--------------------------|
| **ThiI**                                     |                          |
| C108A (pBH144)                               | G GAA GGC AAA ACC TTC GCC GTA CGC GTG AAG CGC |
| C202A (pBH143)                               | GCG CTG CAT GCC TAC GCC GAA GGT TTT GCC TTC C |
| C207A (pBH142)                               | ATG TTG ATG CGT CGC GGC GCC GGT CAT TAC AC |
| C344A (pBH141)                               | GTC CAT GCG CAT TAC GCC GTC GTC CAT CAA CAT |
| Correct penultimate codon                    | C CGC GGC GAT GTC CAT CAA CAT GAC CAT CAG GCG |
| Correct P244L                                 | GCC GCG GAG GTT AAA GAA GGC GTA ATG CAC GGG G |
| Cloning                                      | CGC AGC ATG CGG GAA TAT GCT GTG GTC ATC TTC AAA AGC |
| Thiamin                                        | GCT TTT GGA GAT CAC ACC AGC GAA TAT CCG CAT GCG |
| IscS                                         |                          |
| Cloning                                      | AGA CAT ATG AAA TTA CCG ATT TAT CTC |
| Correct P244L                                 | G CTT GCA GGC ACT CTG CCT GCT CAC ACG ATC G |
| Correct penultimate codon                    | C GAT CTG GTG AAC AGG AGG AAT GCC GGA ACG C |
|                 | GCC ATC GAA TGG GCT CAT CAT TAA GAA TTA GAG CTC CTG CG |
|                 | CG AC8 GAG CTC GAA TIC TTA ATG ATG ACC GCA TCA TTG CAT GAC |

VJS28890(DE3) expressing C202A thiI appears to become inviable more quickly than the same cells expressing other thiI variants when stored at 4 °C on LB agar containing kanamycin and carbenicillin. This conclusion is based on the repeated observation that a much lower degree of dilution of a resuspended colony is required to generate the same colony density on a selection plate as compared to VJS28890(DE3) expressing wild-type thiI variants. We can offer no rational explanation for this behavior in light of the essentially wild-type character of C202A thiI in every other regard.
onies appeared on the near-UV-exposed side as appeared on the masked side of the plate, and all of the colonies on the exposed side were very small; after 24 h further incubation in the dark, both sides of the selection plate were indistinguishable regarding colony number and size. The UV-visible spectra were recorded for tRNA isolated from VJS2890(DE3) harboring plasmids encoding wild-type and altered ThiI (Fig. 3). The levels of s\(^{4}U\) in tRNA correlate well with the selection results, with little or no s\(^{4}U\) indicated in the tRNA from VJS2890(DE3) expressing C344A ThiI and a reduced amount of s\(^{4}U\) in the tRNA from VJS2890(DE3) expressing C207A ThiI.

**Activity Assays**—The assay procedure was changed from our previous report (31) by inclusion of purified IscS\(_{\text{His6}}\) rather than cell extracts. A molar ratio of 4:1 IscS\(_{\text{His6}}\):ThiI was used in the assays because it affords a linear response in rate when the concentration of ThiI is varied, and additional IscS\(_{\text{His6}}\) does not increase the reaction rate. No s\(^{4}U\) generation was observed when ThiI was omitted from the reaction mixture, even with elevated concentrations of IscS\(_{\text{His6}}\) (4.8 \(\mu M\)). The rates of s\(^{4}U\) formation catalyzed by wild-type, C108A, and C456A ThiI make it unlikely that the activity of C344A ThiI was impaired for s\(^{4}U\) generation by 2700-fold (Fig. 4 and Table II). C456A ThiI (31) was used along with duplicate runs to generate the data in Table II. The ThiI variants were denoted: □, wild-type; ○, C108A; △, C202A; ◊, C207A; ×, C344A. All of the variants were present at a concentration of 1 \(\text{nm}\) in the assay except for C344A ThiI, which was present at 1.2 \(\mu M\).

**Quantitation of Thiol Groups in ThiI**—Under denaturing conditions (~4.6 \(M\) guanidinium chloride), DTNB titrations revealed 5.1 ± 0.2 free thiol groups in wild-type ThiI. Under native conditions, DTNB titrations revealed 3.9 ± 0.3 and 3.3 ± 0.3 reactive thiol groups, respectively, in wild-type and C344A ThiI. The variation from integral values is within the uncertainty of the method. To determine whether or not a disulfide bond forms during turnover, ThiI was isolated after s\(^{4}U\) generation in the absence of an exogenous reductant other than the substrate cysteine. DTNB titrations under denaturing conditions revealed 4.1 ± 0.3 free thiol groups in ThiI (determined in triplicate). A parallel assay (performed in quadruplicate) of s\(^{4}U\) generation showed that 73% of ThiI turned over under these reaction conditions, so 3.5 free thiol groups were expected. Although the observed 4.1 free thiol group is significantly higher than the expected value, this discrepancy likely arises from the obligatory presence of cysteine, which can reduce disulfide bonds. As a control for oxidation unrelated to catalytic activity, ThiI was incubated under identical conditions except that cysteine and tRNA were omitted. DTNB titrations under denaturing conditions of the isolated ThiI revealed 4.9 ± 0.2 thiol groups (determined in triplicate), demonstrating that s\(^{4}U\) generation significantly decreased the number of free thiol groups in ThiI, consistent with the formation of a disulfide bond during turnover.

**Evidence of a Disulfide Bond between Cys-344 and Cys-456**—To test whether or not Cys-344 and Cys-456 can form a disulfide bond, titrations of ThiI with 1 eq of DTNB were performed; if a disulfide bond forms, then one expects 2 eq of the chromophore 5-thio-2-nitrobenzoate to be released. DTNB will first react with the free thiol group of one cysteine residue to release 1 eq of chromophore and form a mixed disulfide between the cysteine residue and “half” of DTNB. A second cysteine residue can then displace another equivalent of chromophore to

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\(5\) We have observed a similar phenotype with K321R thiI (35) and rationalized it on the basis of stochastic differential expression from the same promoter in different colonies, consistent with the findings of Siegele and Hu (39).
form the purely enzymic disulfide. When wild-type ThiI was treated with 1 eq of DTNB, 1.8 ± 0.2 eq of chromophore were produced. With C456A ThiI, 1.3 ± 0.2 eq of chromophore were generated, whereas 0.9 ± 0.1 eq of chromophore resulted from treatment of C344A ThiI with 1 eq of DTNB. ThiI, then, can readily form a disulfide bond unless either Cys-344 or Cys-456 is replaced with alanine, which is most simply explained by a disulfide bond between Cys-344 and Cys-456.

**DISCUSSION**

Based on sequence alignments with sulfurtransferases, we previously investigated (31) the role of Cys-456 of ThiI in the biosynthesis of s^4U and found that residue to be essential. The catalytically essential Cys-456 is found on a C-terminal extension of ~100 amino acids that was then found only in ThiI from *E. coli* and its close relatives *Salmonella typhimurium* and *Hemophilus influenzae* among the organisms for which a ThiI sequence was known (31). Currently, sequences of ThiI from 23 organisms (all prokaryotes) are known, and 8 have the C-terminal extension. Some of the bacteria with the shorter ThiI have open reading frames that are highly similar to the C-terminal extension found in the *E. coli* ThiI, which suggests that this C-terminal extension may function as an autonomous sulfurtransferase domain. Consistent with our proposed domain structure, Kambampati and Lauhon (32) report that light trypsinolysis cleaves ThiI into two major fragments, the larger of which is inactive for sulfur transfer and of a size (Mr ~45,000) consistent with our postulated N-terminal domain.

Our findings concerning Cys-456 coupled with the report of Kambampati and Lauhon (27) that IscS was involved in s^4U biosynthesis led us to offer two mechanisms for s^4U biosynthesis (Fig. 2). The key features of these two mechanisms are formation of a persulfide on Cys-456 by trans-persulfidation at the expense of the persulfide on IscS and the liberation of the terminal sulfur of the Cys-456 persulfide as a formal equivalent of S^2^- rather than S^0_. Subsequently, Kambampati and Lauhon (32) confirmed the transfer of sulfur from free cysteine to IscS to ThiI to rRNA, which led those authors to propose (independently of our results) a sulfur transfer scheme that is similar to our mechanisms although less detailed. Our mechanisms (Fig. 2) both postulate the participation of a thiol group to liberate the formal S^2^- equivalent (in s^4U itself or as hydrogen sulfide), which immediately suggests the possible participation by another enzymic cysteine residue. As observed by us and Kambampati and Lauhon (32), sequence alignments reveal that only Cys-344 is absolutely conserved among all ThiI for which sequences are known. To test the role of the conserved Cys-344 and the other three cysteine residues, we separately substituted each one with alanine in *E. coli* ThiI and measured the effect on ThiI function both in *vivo* and in *vitro*. All of the altered ThiI appear to maintain the global fold of wild-type enzyme as judged by overexpression and storage behavior, extinction coefficients, and far-UV CD spectra that do not vary significantly from those of wild-type ThiI. The substitution of Cys-108 and Cys-202 had negligible effects on the measured activity of ThiI both in *vivo* (Fig. 3) and in *vitro* (Table II). In agreement with the prediction from sequence alignments, Cys-344 proved critical for ThiI activity both in *vivo* (Fig. 3) and in *vitro* (Table II), although C344A ThiI retains some activity under our assay conditions. C207A ThiI is an intermediate case, impaired but functional in *vivo* (Fig. 3) and in *vitro* (Table II). This mild effect on activity could arise from a local conformational change upon substitution of Cys-207 that does not significantly perturb the solution behavior or CD spectrum. These findings, however, are also consistent with a more direct role for Cys-207 in s^4U generation that is either nonessential or may be assumed by some other component in the cell or the assay mixture. In either case, detailed kinetic studies, a structure, or both will be required to determine the role of Cys-207.

We are left, then, with a need to accommodate a critical but not absolute role for Cys-344. One explanation for the roles of IscS and the cysteine residues in ThiI would be formation of an iron-sulfur cluster in ThiI (IscS (29) was named for its role in iron-sulfur cluster formation). The hypothetical ThiI iron-sulfur cluster might serve as an intermediate in the transfer of sulfur from free cysteine to rRNA, similar to the proposals for biotin (6, 7) and lipoic acid biosynthesis (10). In this case, a small amount of iron-sulfur cluster would be generated in the assay mixture, producing highly active ThiI. No evidence has been presented to suggest that ThiI bears such a cluster, but only overexpressed ThiI has been examined, leaving open the possibility that the high level of ThiI in the overexpression cells swamps the capacity to generate iron-sulfur clusters. Although we did not consider the requirement of an iron-sulfur cluster likely, the experiment was straightforward: we overexpressed IscS and ThiI in the same cells. Even though IscS was present at roughly 4-fold higher concentration than ThiI, isolated ThiI displays no spectroscopic evidence of an iron-sulfur cluster (or any other cofactor).

The main premise that led us to undertake the substitution of each cysteine with alanine was that an enzymic disulfide bond might form during catalysis (Fig. 2). Because the resting state of ThiI could be the form with the disulfide bond, we titrated the wild-type enzyme with DTNB in the presence of guanidinium chloride, and the denatured wild-type ThiI showed 5 thiol groups. Since ThiI has five cysteine residues, we conclude that ThiI is fully reduced in its resting state (at least in cells that overexpress it).

By having ruled out a disulfide bond in resting ThiI, we sought evidence that a disulfide bond forms during turnover. If true, omitting reductant would limit the enzyme to a single turnover, for the ThiI would be left in the inactive disulfide-bonded state. Quantitation of the s^4U formed and titration of free thiol groups in the resultant ThiI would allow assessment of the proposed disulfide bond. In practice, the substrate cysteine is also a reductant for disulfide bonds, so we are hampered in attaining the desired single turnover conditions. We proceeded by omitting DTT from the assay mixture and lowering the concentration of cysteine. Under these conditions, less than 1 eq of s^4U was formed relative to ThiI, supporting the need for exogenous reductant for multiple turnovers. Titration of ThiI isolated from the reaction mixture revealed fewer thiol groups than before the reaction. Both results are in agreement with disulfide bond formation during turnover, but does the disulfide bond form between Cys-456 and Cys-344? Titration of native, wild-type ThiI with 1 eq of DTNB produced 2 eq of the thiolate product 5-thio-2-nitrobenzoate, which requires formation of a disulfide bond on ThiI. When C456A and C344A ThiI were subjected to titration with 1 eq of DTNB, only 1 eq of the thiolate product formed (the other “half” of DTNB was left as a mixed disulfide with ThiI). The simplest explanation of these results is formation of a disulfide bond between Cys-456 and Cys-344.

The profoundly reduced activity of C344A ThiI (2700-fold, Table II) is also consistent with formation of a disulfide bond between Cys-456 and Cys-344 during turnover. But if this catalytically critical disulfide bond exists, why does C344A ThiI display residual activity? If the side chain of Cys-344 were paired but functional, a disulfide bond might form between Cys-456 and Cys-344. But if this mixed disulfide with ThiI). The simplest explanation of these results is formation of a disulfide bond between Cys-456 and Cys-344.
Wild-type ThiI registered 4 thiol groups, and C344A ThiI registered 3. These results indicate that the side chains of Cys344 is among the four (of five) cysteine side chains in ThiI that appear to be surface-accessible, which supports the residual in vitro activity of C344A ThiI arising from rescue by thiol groups of DTT. All of our results, then, are consistent with our hypothesis that Cys344 forms a disulfide bond with Cys456 during turnover of ThiI (Fig. 2).

We now consider the extrapolation of our results and proposals to the biosynthesis of other sulfur-containing molecules. Our mechanistic possibilities accommodate the dual function of ThiI in sU and thiamin biosynthesis, especially considering the evidence that the C terminus of E. coli ThiI constitutes a sulfurtransferase domain of ~100 amino acids that may behave nearly independently from the N-terminal portion of ThiI (31). The persulfide on Cys456, then, would serve as a nucleophile at either another active site on ThiI to make the RNA-disulfide intermediate (Fig. 2A) or at an active site in the complex of the proteins ThiF and ThiS to form a disulfide intermediate on the way to the thiocarboxylate of ThiS, which is the ultimate sulfur donor for thiamin biosynthesis (33). If the C-terminal domain of ThiI constitutes a hydrogen sulfide-generating domain (Fig. 2B), the dual role of ThiI in sU and thiamin biosynthesis is also readily accommodated; the hydrogen sulfide is shunted either toward the activated uridine in tRNA in another ThiI-active site or toward the adenylate of the terminal carboxylate of ThiS in an active site in the ThiI-ThiS complex. This latter possibility is attractive in light of the reports by both the groups of Begley et al. (11) and Lauhon and Kambampati (28) that ThiI stimulates but is not required for the production of the ThiI thiocarboxylate in vitro; hydrogen sulfide is generated slowly by IscS (27, 29), which would allow thiocarboxylate formation in the absence of ThiI. Furthermore, Lauhon and Kambampati (28) report that inorganic sulfide (at 5 mM) can substitute for IscS and cysteine in the in vitro generation of the thiocarboxylate of ThiS, and those authors also raise hydrogen sulfide generation as a likely role for ThiI. However, the absence of detectable activity by C456A ThiI must be accommodated because IscS should suffice if the postulated C-terminal domain of ThiI serves only to generate hydrogen sulfide. Scenarios such as an activating conformational change triggered by the formation of the Cys456 persulfide can account for the observations, but these issues clearly require further investigation.

A mechanism of hydrogen sulfide generation similar to the one we propose (Fig. 2B) may also operate in the generation of iron-sulfur clusters. IscS would again serve as an S0 donor to form a persulfide group on another protein, which could be a scaffold protein such as NifU (4) or IscU (3, 5) or a protein that make a persulfide group on another protein, which could be a protein that delivers to growing iron-sulfur clusters is chemically reasonable and consistent with current reports of cluster generation, and we offer it to encourage further experiments.

The studies presented here along with our previous report concerning the essential role of Cys456 (31) provide a solid framework for understanding the nature of sulfur transfer from cysteine through IscS and ThiI to tRNA. ThiI contains neither an iron-sulfur cluster nor a disulfide bond in its resting state. In the absence of reductants, ThiI supports sU generation but cannot achieve more than one turnover and suffers a loss of thiol groups. All of the evidence supports the formation of a disulfide bond between Cys456 and Cys344 during turnover, and indirect evidence strongly suggests that this disulfide bond forms readily in native ThiI. Since the disulfide bond between Cys456 and Cys344 is a feature of both of our proposed mechanisms (Fig. 2), we cannot yet exclude nor favor one or the other, but we have already begun studies that should allow us to do so.

Note Added in Proof—Begley and co-workers (40) have recently reported an acylsulfide linkage between the C terminus of ThiS and Cys184 of ThiF and propose that this species rather than the thiocarboxylate of ThiS is the true intermediate in thiamin biosynthesis. While this finding clouds the involvement of ThiI in thiamin biosynthesis, it does provide a gratifying example of a nucleophilic attack by an enzymic persulfide group, which was unprecedented when we offered the first mechanism proposing such an event (31).

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Eugene G. Mueller, Peter M. Palenchar and Christopher J. Buck

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