

The Rhodanese Domain of ThiI Is Both Necessary and Sufficient for Synthesis of the Thiazole Moiety of Thiamine in *Salmonella enterica*[∇]

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Received 17 May 2011/Accepted 24 June 2011

In *Salmonella enterica*, ThiI is a bifunctional enzyme required for the synthesis of both the 4-thiouridine modification in tRNA and the thiazole moiety of thiamine. In 4-thiouridine biosynthesis, ThiI adenylates the tRNA uridine and transfers sulfur from a persulfide formed on the protein. The role of ThiI in thiazole synthesis is not yet well understood. Mutational analysis described here found that ThiI residues required for 4-thiouridine synthesis were not involved in thiazole biosynthesis. The data further showed that the C-terminal rhodanese domain of ThiI was sufficient for thiazole synthesis *in vivo*. Together, these data support the conclusion that sulfur mobilization in thiazole synthesis is mechanistically distinct from that in 4-thiouridine synthesis and suggest that functional annotation of ThiI in genome sequences should be readdressed. Nutritional studies described here identified an additional cysteine-dependent mechanism for sulfur mobilization to thiazole that did not require ThiI, IscS, SufS, or glutathione. The latter mechanism may provide insights into the chemistry used for sulfur mobilization to thiazole in organisms that do not utilize ThiI.

Thiamine pyrophosphate (TPP) is an essential coenzyme used by pyruvate dehydrogenase, α -ketoacid decarboxylase, α -ketoacid oxidase transketolase, and other enzymes, where it stabilizes acyl carbanion intermediates through dissociation of the proton at C-2 of the thiazolium ring (9). Though thiamine was isolated in the 1930s and its chemical synthesis was achieved 4 years later, significant questions remain about the biochemical details of TPP synthesis. Genetic studies, primarily in *Salmonella enterica* and *Escherichia coli*, have defined 10 gene products involved in TPP synthesis in bacteria (Fig. 1) (10). With the exception of ThiI, a role for each of these gene products in TPP synthesis has been reconstituted *in vitro* (3, 7, 12, 13, 20, 24, 29). Microbes and plants synthesize thiamine *de novo* with separate pathways for the formation of the thiazole (2-carboxy-4-methyl-5- β -hydroxyethylthiazole phosphate [THZ-P]) and pyrimidine (4-amino-5-hydroxymethyl-2-methylpyrimidine pyrophosphate [HMP-PP]) moieties (1, 5, 18, 22). In bacteria, the two moieties are joined by thiamine phosphate synthase, the product of the *thiE* gene, to form thiamine monophosphate. The biologically active form of the coenzyme, thiamine pyrophosphate (TPP), is then generated by a single phosphorylation catalyzed by thiamine monophosphate kinase (ThiL) (14, 29).

The *thiI* locus was defined by lesions that caused a thiazole auxotrophy (28) and was subsequently found to be allelic with *nuvA*, a locus required for 4-thiouridine modification of tRNA

(15). Synthesis of 4-thiouridine has been reconstituted *in vitro*, and our current understanding is summarized in Fig. 2. Experiments *in vitro* demonstrated the transfer of sulfur from the source cysteine via an IscS persulfide to form a ThiI persulfide (11) located at residue Cys456 (19). In 4-thiouridine biosynthesis, ThiI activates the tRNA uridine as its adenylate (30, 32), which reacts with the Cys456 persulfide (11, 19). Residue Cys344 then acts as a nucleophile, forming a Cys456-Cys344 disulfide and releasing 4-thiouridine. The ThiI disulfide is then reduced by cellular reductants to complete the reaction cycle (17, 27). The ThiI protein in *E. coli/S. enterica* contains three distinct domains, and each has been implicated in the mechanism of 4-thiouridine synthesis (Fig. 3). The THUMP domain is thought to bind the tRNA, the adenine nucleotide alpha-hydroxylase domain (AANH) is responsible for uridine adenylation, and the C-terminal rhodanese domain participates in sulfur transfer (2, 6, 27, 32). Five ThiI residues have been shown to be important for 4-thiouridine biosynthesis: Asp189, Lys321, Cys207, Cys344, and Cys456. Asp189 and Lys321 are conserved residues in the AANH domain and are required for adenylation of the uridine (16). Cys207 is also in the AANH domain, and the ThiI^{C207A} variant showed intermediate activity *in vivo* and *in vitro* (17). Cys456 is conserved in the rhodanese domain and carries the nucleophilic cysteine persulfide that is essential for the transfer of sulfur to uridine (19). Cys344 is a highly conserved residue located in the AANH domain that forms a disulfide bond with Cys456, releasing 4-thiouridine (17).

Sulfur mobilization in thiazole synthesis shares many features with those described above for 4-thiouridine synthesis. In thiazole synthesis, ThiFS forms a stable complex, and ThiF catalyzes the adenylation of the terminal glycine residue of ThiS (31). It is the acyladenylate on ThiS that accepts sulfur from a persulfide donor (24). It has been suggested that sulfur mobilization in thiazole synthesis utilizes ThiI as a persulfide

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[∇] Published ahead of print on 1 July 2011.

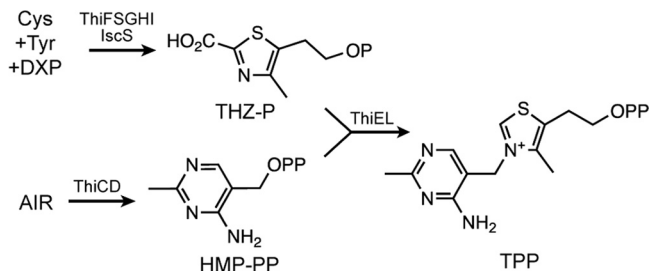


FIG. 1. Thiamine biosynthesis in *S. enterica*. A schematic of the thiamine biosynthetic pathway in *S. enterica* is shown. The enzymes involved in each step are indicated above the relevant arrow. Abbreviations: Cys, cysteine; DXP, deoxyxylulose phosphate; Tyr, tyrosine; AIR, 5-aminoimidazole ribotide; THZ-P, 2-carboxy-4-methyl-5-β-hydroxyethylthiazole phosphate; HMP-PP, 4-amino-5-hydroxymethyl-2-methylpyrimidine pyrophosphate.

carrier between IscS and the ThiS-acyladenylate (17, 24). Indeed, *in vivo* results suggest that *thiI* is required for the formation of the ThiS thiocarboxylate (24), although ThiI was not required for the *in vitro* formation of the ThiS thiocarboxylate (31). The role of ThiI in sulfur mobilization in thiazole synthesis has not been directly tested by mutational analyses or *in vitro* studies.

This study was initiated to probe the role of ThiI in thiazole synthesis in the context of what is known about sulfur mobilization in 4-thiouridine biosynthesis. The results here showed that ThiI residues critical for 4-thiouridine biosynthesis were not required for thiazole synthesis and that the rhodanese domain of ThiI was sufficient for synthesis of thiazole *in vivo*. Nutritional studies showed that there was an alternative mechanism of sulfur mobilization to thiazole that depends on exogenous cysteine. These results provide insights into sulfur mobilization in thiazole synthesis and described important criteria for the appropriate annotation of ThiI in genomic data.

MATERIALS AND METHODS

Strains, media, and chemicals. Minimal medium was NCE (26) supplemented with MgSO₄ (1 mM), trace minerals (0.1×) (4), and glucose (11 mM). The final concentration of thiamine or thiazole was 100 nM. Rich medium was Difco nutrient broth (NB; 8 g/liter) with NaCl (5 g/liter). Solid medium contained 1.5% agar. Antibiotics were added at the following concentrations in rich and minimal media, respectively: chloramphenicol (Cm), 20 and 5 g/liter; ampicillin (Ap), 150 and 30 g/liter; tetracycline (Tc), 20 and 10 g/liter; kanamycin (Km), 50 and 12.5 g/liter. All chemicals were purchased from Sigma-Aldrich, St. Louis, MO. The strains used in this study were derivatives of *S. enterica* strain LT2, and their

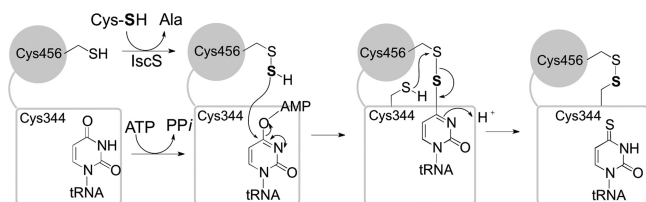
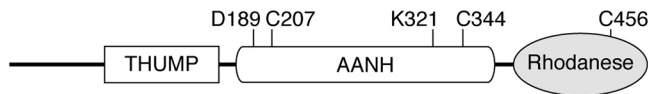


FIG. 2. Sulfur mobilization by ThiI in 4-thiouridine biosynthesis. Events depicted occur in the active site of ThiI. The boxes represent the THUMP and AANH domains of ThiI, while the gray circles represent the rhodanese domain. Critical cysteine residues 344 and 456 are shown. tRNA represents the rest of the tRNA molecule containing the uridine being acted on by ThiI.



E. coli/S. enterica type (58/207)

B. subtilis type (149/207)

FIG. 3. Structural domains of *S. enterica* ThiI. Motifs in the *S. enterica* ThiI are based on those suggested previously (27), and the residue numbers are those of *E. coli*. Residues 1 to 163 contain an N-terminal ferredoxin-like (NFLD) domain and a THUMP domain (2), both of which are thought to interact with the tRNA (27). Residues 173 to 400 contain the adenylation domain (AANH). The C-terminal portion contains the rhodanese-like domain.

genotypes are listed in Table 1. All strains were generated for this study or were part of the laboratory collection.

Mutant construction. (i) **Local mutagenesis.** A P22 lysate (HT *int201* [21, 23]) was generated on strain DM12255, which contains a Tn10d (Tc) insertion in *phnU*, linked to the *thiI* locus. This lysate was mutagenized by hydroxylamine (8) and used to transduce a wild-type strain (DM10000) to Tc^r. Tc^r transductants were screened for those that required exogenous thiamine when grown on glucose minimal medium (Thi⁻). The causative mutations were transduced into a new genetic background to confirm causation of the thiamine requirement, and the lesions in *thiI* were identified by sequence analysis. All DNA sequencing was performed at the University of Wisconsin—Madison Biotechnology Center.

(ii) **Site-directed mutagenesis.** A plasmid (pDM1322) containing *thiI* ligated into the NdeI and BamHI sites of pET-14b (Novagen) was constructed. Relevant *thiI* mutants were generated by site-directed mutagenesis with the QuikChange XL site-directed mutagenesis kit according to the manufacturer's protocol (Agilent Technologies, Santa Clara, CA). Sequences of primers used are available from the corresponding author upon request. The presence of the relevant alleles in the resulting constructs was confirmed by sequence analysis.

Expression of the C-terminal domain of ThiI. The C-terminal domain of ThiI (residues 385 to 482) was amplified by PCR using chromosomal DNA of wild-type *S. enterica* as a template and using primers SalthiI-R385for and SalthiI-endrev. The primers introduced an initiating methionine and restriction sites (BamHI and HindIII) to the amplified sequence. The amplified DNA was cloned as a blunt-end fragment into plasmid pSC-B-amp/kan (Stratagene) to give plasmid pDM1316. Plasmid pDM1316 was digested with BamHI and HindIII at the sites engineered in the primers, and the resulting 312-bp C-terminal *thiI* fragment was cloned into pSU19 to give plasmid pDM1320. Plasmids containing the C-terminal variants, C456S and R414H, were constructed by cloning a PCR amplification product from pDM1329 DNA or DM12302 chromosomal DNA, respectively. In each case, the constructs were confirmed by sequence analyses.

Nutritional analysis. Nutritional requirements were determined by liquid growth analysis and in solid medium by using soft agar overlays. For liquid growth analysis, strains were grown overnight in NB (antibiotic was present for plasmid-containing strains). Cells were pelleted and resuspended in NaCl (0.85%). A 0.2-ml aliquot of this suspension was used to inoculate 5 ml minimal medium containing glucose. Cell growth was measured by monitoring absorbance at 650 nm (*A*₆₅₀) while strains were incubated with shaking (200 rpm) at 37°C. Alternatively, 5 μl of cell suspension was used to inoculate 195 μl of the appropriate minimal medium in each well of a 96-well microtiter plate. Growth at 37°C with shaking at intensity level 2 was monitored using a microplate spectrophotometer (Bio-Tek Instruments). The specific growth rate was determined as μ = ln(*X*/*X*₀)/*T*, where *X* is the *A*₆₅₀ value during the exponentially linear portion of growth (routinely between *A*₆₅₀ 0.2 and 0.7) and *T* is time in hours. For soft agar overlays, cells were grown overnight in NB. An 0.2-ml aliquot of the overnight culture was used to seed 3.5 ml of 0.75% agar, which was overlaid on a minimal medium plate. Nutritional supplements were then spotted, and growth was assessed after 18 h of incubation at 37°C.

Bioinformatic analysis. The TIGRFam TIGR00342 was downloaded from the Comprehensive Microbial Resource (CMR; <http://cmr.jcvi.org>), using the CMR Gene Attribute Download tool and the JCVI/CMR's OMNIOME.pep database. Using CMR, blastp (WU-BLAST version 2.0MP) was run on

TABLE 1. Strain and plasmid list

Strain or plasmid	Genotype (strain) or vector and protein encoded (plasmid)
Strains	
DM10000	Wild type
DM269	<i>thiI887::Tn10d</i> (Tc)
DM11537	Δ <i>thiF</i>
DM12225	<i>phnU::Tn10d</i> (Tc)
DM12260	<i>thiI887::Tn10d</i> (Tc) pET-14b
DM11774	<i>thiI887::Tn10d</i> (Tc) pDM1322 (pET-ThiI)
DM11544	<i>thiI887::Tn10d</i> (Tc) pDM1324 (pET-ThiI ^{D189A})
DM11545	<i>thiI887::Tn10d</i> (Tc) pDM1326 (pET-ThiI ^{C207S})
DM11546	<i>thiI887::Tn10d</i> (Tc) pDM1327 (pET-ThiI ^{K321M})
DM11528	<i>thiI887::Tn10d</i> (Tc) pDM1328 (pET-ThiI ^{C344S})
DM11547	<i>thiI887::Tn10d</i> (Tc) pDM1329 (pET-ThiI ^{C456S})
DM13436	<i>thiI887::Tn10d</i> (Tc) pSU19
DM13437	<i>thiI887::Tn10d</i> (Tc) pDM1319 (pSUthiI)
DM13438	<i>thiI887::Tn10d</i> (Tc) pDM1320 (pSUthiI-Cter)
DM13439	<i>thiI887::Tn10d</i> (Tc) pDM1321 (pSUthiI-Cter ^{C456S})
DM13477	<i>thiI887::Tn10d</i> (Tc) pDM1331 (pSUthiI-Cter ^{R414H})
DM10959	<i>thiI1205::Tn10d</i> (Tc)
DM13162	Δ <i>iscS</i>
DM13304	<i>thiI1205::Tn10d</i> (Tc) Δ <i>iscS</i>
DM13483	<i>thiI1205::Tn10d</i> (Tc) <i>gshA102::MudJ</i> (Kn)
DM13484	<i>thiI1205::Tn10d</i> (Tc) <i>sufS::Cat</i>
Plasmids	
pDM1322 (pET-ThiI)	pET-14b (Ap ^r); ThiI
pDM1324 (pET-ThiI ^{D189A})	pET-14b (Ap ^r); ThiI ^{D189A}
pDM1326 (pET-ThiI ^{C207S})	pET-14b (Ap ^r); ThiI ^{C207S}
pDM1327 (pET-ThiI ^{K321M})	pET-14b (Ap ^r); ThiI ^{K321M}
pDM1328 (pET-ThiI ^{C344S})	pET-14b (Ap ^r); ThiI ^{C344S}
pDM1329 (pET-ThiI ^{C456S})	pET-14b (Ap ^r); ThiI ^{C456S}
pDM1318 (pSCthiI)	pSC-B (Ap ^r Kn ^r); ThiI
pDM1316 (pSCthiI-Cter)	pSC-B (Ap ^r Kn ^r); ThiI-Cter (residues 385-482)
pDM1317 (pSCthiI-Cter ^{C456S})	pSC-B (Ap ^r Kn ^r); ThiI-Cter ^{C456S} (residues 385-482)
pDM1330 (pSCthiI-Cter ^{R414H})	pSC-B (Ap ^r Kn ^r); ThiI-Cter ^{R414H} (residues 385-482)
pDM1319 (pSUthiI)	pSU19 (Cm ^r); ThiI
pDM1320 (pSUthiI-Cter)	pSU19 (Cm ^r); ThiI-Cter (residues 385-482)
pDM1321 (pSUthiI-Cter ^{C456S})	pSU19 (Cm ^r); ThiI-Cter ^{C456S} (residues 385-482)
pDM1331 (pSCthiI-Cter ^{R414H})	pSU19 (Cm ^r); ThiI-Cter ^{R414H} (residues 385-482)

TIGR00342 using *E. coli* K-12 ThiI residues 404 to 482 as the query. Default parameters were used, except that the E-value cutoff was lowered to 0.001. Members of TIGR00342 containing the rhodanese domain were separated from those without a rhodanese domain, and duplicate species entries were removed. Bioinformatic analysis was performed by the Advanced Genome Analysis Resource facility at the University of Wisconsin Biotechnology Center.

RESULTS AND DISCUSSION

The functions of ThiI in 4-thiouridine biosynthesis and thiamine synthesis are genetically separable. Five residues of ThiI have been shown to be important for synthesis of the 4-thiouridine modification of tRNA *in vitro* (Fig. 3) (16, 17, 19). These residues were independently changed in *S. enterica* ThiI, and plasmids carrying each of the variants (C344S, C456S, D189A, K321M, and C207S) or the wild-type gene were constructed. The resulting derivatives of plasmid pET-14b expressing ThiI proteins were transformed into a mutant strain lacking *thiI*, DM269 (*thiI887::Tn10d*). In the resulting strains, the plasmid-encoded protein was the only source of ThiI. Each strain was monitored for growth in the absence of thiamine to assess complementation of the *thiI* lesion. All variants except ThiI^{C456S} restored growth of a *thiI* mutant strain in minimal medium. Some of these data

are shown in Fig. 4. These results indicated that of the five ThiI residues required for 4-thiouridine synthesis, only residue Cys456 was required for thiazole synthesis.

The ThiI variants that complemented a *thiI* mutant strain provided insights into the mechanism of ThiI in thiazole biosynthesis. ThiI residues Asp189, Lys321, and Cys207 lie in the adenylation domain. Substitution at any of these residues eliminated or decreased tRNA modification, consistent with the role of ThiI in adenylation of the tRNA uridine in synthesis of the 4-thiouridine (17). In contrast, ThiI variants with substitutions at any of these residues were proficient for thiazole synthesis *in vivo*. These data allow the conclusion that the adenylation activity of ThiI is not required for thiazole synthesis. Similarly, residues Cys456 and Cys344 were directly involved in sulfur mobilization for the 4-thiouridine modification of tRNA *in vitro* (17, 19). In contrast, Cys456, but not Cys344, was required for thiazole synthesis *in vivo*. These data support the conclusion that sulfur mobilization in thiazole synthesis is mechanistically distinct from that in 4-thiouridine synthesis.

The C-terminal rhodanese domain of ThiI is sufficient for thiazole synthesis. Local mutagenesis of the *thiI* gene generated 16 independent mutants that required thiamine for

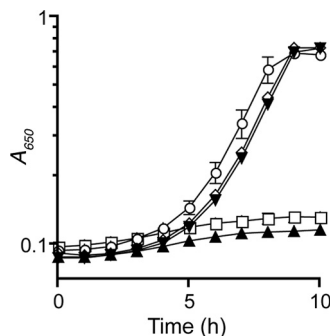


FIG. 4. ThiI variants defective in 4-thiouridine biosynthesis complement a *thiI* mutant strain. Growth of strains at 37°C with shaking was monitored over time as absorbance at 650 nm. Strains were grown in minimal NCE medium with glucose and ampicillin; all strains grew with wild-type rate and yield if thiamine was added to the medium. Strains were DM269 (*thiI87::Tn10d*) with plasmids pET-14b (□), pET-ThiI (○), pET-ThiI^{C344S} (◇), pET-ThiI^{D189A} (▽), and pET-ThiI^{C456S} (▲). Data shown are the averages and standard deviations of three independent cultures.

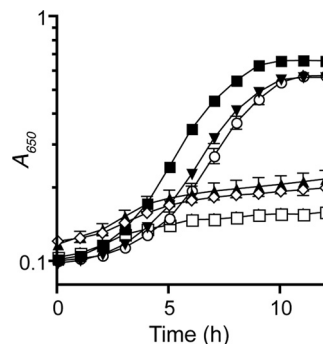


FIG. 5. The rhodanese domain of ThiI is sufficient for thiamine-independent growth. Strains were grown in NCE medium supplemented with glucose. The following plasmids were introduced into DM269 (*thiI*): pSU19 (□), pSUThiI (○), pSUThiI-Cter (▽), pSUThiI-Cter^{C456S} (▲), and pSUThiI-Cter^{R414H} (◇). Strain DM269 with pSU19 was also grown in medium supplemented with thiamine (■).

growth. The distribution of causative mutations was striking, with 14 of the 16 mutations generating a nonsense codon, two of which were isolated twice (Table 2). All of the lesions affected the rhodanese domain (residues 385 to 482). Of the two missense mutations isolated, one caused a substitution at residue Cys456 (C456Y) and the other resulted in an R414H substitution in the rhodanese domain.

The above results raised the question of whether the rhodanese domain of ThiI was sufficient for synthesis of thiazole. An algorithm to detect linker regions in proteins defined such a region in ThiI from residues 381 to 390 (25). A plasmid expressing only the rhodanese domain of ThiI (residues 385 to 482) was constructed and used to test complementation of a *thiI* mutant. This construct was sufficient to restore thiamine-independent growth of a *thiI* mutant strain (Fig. 5). As shown in Fig. 5, complementation by the rhodanese domain was eliminated when residue Cys456 was changed to Ser, or when Arg414 was changed to His. Together, these results support

the hypothesis that the rhodanese domain of ThiI was sufficient for thiazole synthesis. These data further demonstrate that ThiI mediated sulfur transfer in 4-thiouridine synthesis via a different mechanism than that in the synthesis of thiazole.

A possible role for the rhodanese domain in sulfur mobilization to thiazole. The accepted mechanistic model for the role of ThiI in 4-thiouridine synthesis is shown in Fig. 2. In this model, the THUMP domain binds the tRNA and the AANH domain activates the tRNA uridine by adenylation (11, 16, 32). The ThiI persulfide on Cys456 then displaces the adenosyl group on the uridine (19, 30). Finally, Cys344 acts as a nucleophile, forming a Cys456-Cys344 disulfide and releasing the product 4-thiouridine. The resulting disulfide in ThiI can then be reduced to begin the next reaction cycle (17, 27, 30). Previously, it was proposed that ThiI served a similar role in sulfur transfer in thiazole synthesis (17, 24). Figure 6 represents a mechanism for ThiI in sulfur mobilization to thiazole that is both consistent with previous studies and supported by the data described above. The mechanism depicted implies analogous chemistry in 4-thiouridine biosynthesis and thiazole biosynthesis. In both cases, the substrate (i.e., tRNA uridine or ThiS terminal glycine) is first activated by adenylation. In the case of 4-thiouridine biosynthesis, ThiI catalyzes the substrate adenylation reaction. In thiazole synthesis, ThiF has been shown to adenylate the terminal glycine residue of ThiS, generating an acyladenylate (24). The data showing that the adenylation activity of ThiI is not required for thiazole synthesis

TABLE 2. ThiI variants that result in a thiamine requirement *in vivo*

Strain	Allele	Base change(s)	Protein variant(s) ^a
DM12300	<i>thiI1209</i>	C1172T, C1414T	T391I, Q472*
DM12302	<i>thiI1210</i>	G1241A	R414H
DM12304	<i>thiI1211</i>	C1342T	Q448*
DM12355	<i>thiI1212</i>	C1255T	Q419*
DM12357	<i>thiI1213</i>	C1255T	Q419*
DM12359	<i>thiI1214</i>	G1356A	W452*
DM12361	<i>thiI1215</i>	C886T	Q296*
DM12422	<i>thiI1216</i>	C175T	Q59*
DM12424	<i>thiI1217</i>	G1397A	C456Y
DM12426	<i>thiI1218</i>	C1342T	Q448*
DM12428	<i>thiI1219</i>	C1393T	Q465*
DM12430	<i>thiI1220</i>	C1342T	Q448*
DM12432	<i>thiI1221</i>	C1342T	Q448*
DM12883	<i>thiI1222</i>	C886T	Q296*
DM12885	<i>thiI1223</i>	G1355A	W452*
DM12889	<i>thiI1224</i>	C886T	Q296*

^a Asterisks indicate the generation of nonsense codons.

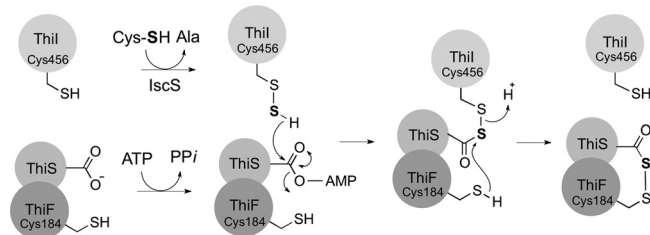


FIG. 6. Proposed model for ThiI mechanism in thiazole biosynthesis. Sulfur mobilization is proposed to proceed as described in the text. The gray circle represents the ThiI rhodanese domain. The terminal glycine carboxyl group of ThiS is depicted.

TABLE 3. Cysteine allows growth of a *thiI* mutant in minimal medium^a

Strain	Relevant genotype	Specific growth rate			Final cell yield		
		Min	Cys	THZ	Min	Cys	THZ
DM10959	<i>thiI</i>	0.35 ± 0.01	0.74 ± 0.03	0.78 ± 0.04	0.45 ± 0.09	1.45 ± 0.03	1.56 ± 0.00
DM13304	<i>thiI iscS</i>	0.12 ± 0.01	0.31 ± 0.01	0.36 ± 0.01	0.36 ± 0.03	0.98 ± 0.03	1.45 ± 0.02
DM13483	<i>thiI gshA</i>	0.27 ± 0.01	0.50 ± 0.01	0.53 ± 0.01	0.39 ± 0.02	1.44 ± 0.00	1.53 ± 0.04
DM13484	<i>thiI sufS</i>	0.30 ± 0.01	0.69 ± 0.07	0.69 ± 0.01	0.43 ± 0.01	1.45 ± 0.04	1.56 ± 0.02

^a Strains were grown in NCE medium supplemented with glucose (11 mM) and the indicated additions. Min, minimal medium. Growth rate is reported as μ [$\mu = \ln(X/X_0)/T$], and the final cell yield is A_{650} after 25 h of growth. Data shown are the averages and standard deviations of three independent cultures.

support this assignment. In both pathways, IscS transfers a sulfur atom to ThiI Cys456, forming a persulfide, a proposal consistent with the requirement for Cys456 in both pathways. This persulfide then attacks the substrate (uridine or ThiS) adenylate, displacing the adenosyl group and forming a disulfide between ThiI and the 4 position of uridine or an acyl persulfide with the C terminus of ThiS. In the final step, a second cysteine (ThiI Cys344 or ThiF Cys184, respectively) attacks the disulfide bond to release either the final product (4-thiouridine biosynthesis) or ThiI (in the case of thiazole biosynthesis). The last step is consistent with the *in vivo* data showing that an *E. coli* strain with a ThiF^{C184S} variant is a thiamine auxotroph (31), which implicated the ThiFS acylpersulfide as either an intermediate or a final sulfur donor in thiazole synthesis (24, 31).

Evaluation of ThiI annotations in genome sequences. Search of the JCVI/CMR's OMNIOME.pep database for proteins with the TIGR00342 multidomain (THUMP and AANH) identified 207 proteins annotated as thiamine biosynthesis/4-thiouridine biosynthesis proteins. Of these, 58 (28%) contained a rhodanese domain, exemplified by the *E. coli*/*S. enterica*-type ThiI discussed herein. However, 159 (72%) had only the THUMP and AANH domains, exemplified by *Bacillus subtilis*. The THUMP domain, the AANH domain, and the rhodanese domain are implicated in the synthesis of 4-thiouridine (2, 16, 17). However, data in the above sections showed that the rhodanese domain alone is sufficient for ThiI to function in thiazole synthesis. The *B. subtilis* ThiI is among the 72% of the TIGR00342-containing proteins without a rhodanese domain annotated as thiamine biosynthetic proteins, and yet this protein is not required for thiazole synthesis (J. Perkins, personal communication; P. Dos Santos, personal communication). A role for the ThiI protein of *B. subtilis* in the synthesis of 4-thiouridine is presumed by the annotation but has not been reported.

S. enterica ThiI is a bifunctional enzyme that (i) binds and adenylates tRNA and (ii) mobilizes sulfur. Both functions are required for 4-thiouridine biosynthesis, while only sulfur mobilization is required for thiazole synthesis, as shown here. These findings suggest that the current annotation of the ThiI family of proteins should be revisited to better reflect their separable roles in the synthesis of 4-thiouridine and thiazole synthesis. Specifically, proteins annotated as ThiI based on containing only the AANH and THUMP domains are unlikely to be involved in thiazole synthesis and would be better annotated as 4-thiouridine sulfurtransferases. Based on the results here, only those proteins with the THUMP, AANH, and rhodanese domain are likely to be involved in both pathways. At

this point, it is unclear how organisms with ThiI proteins with only two domains mobilize sulfur for 4-thiouridine synthesis *in vivo*. It has been suggested that an orphan rhodanese domain acts with the ThiI protein in *B. subtilis* and other organisms that contain a short ThiI protein (27).

Exogenous cysteine can restore thiamine-independent growth of a *thiI* mutant strain. The result that the rhodanese domain of ThiI was sufficient for thiazole synthesis, coupled with the fact that not all thiamine-producing organisms have a rhodanese domain associated with a ThiI enzyme, suggested that another paradigm(s) for sulfur mobilization to thiazole synthesis might exist. For instance, the IscS persulfide could serve as the direct sulfur donor in some metabolic networks, or organisms may employ a different mechanism altogether. It seemed plausible that both the redox environment of the cell and the intracellular concentration of cysteine or other free thiols could influence these possibilities, perhaps in organismal or environmentally specific ways. This idea is supported by the observation that *in vitro* cysteine and IscS could serve as the sole sulfur source to ThiS (12). Further, sulfide can provide the source of sulfur *in vitro* without ThiI, ThiF, or ThiS (12, 20) (N. C. Martinez-Gomez, unpublished data), suggesting that thiazole synthase, ThiG, could utilize a variety of sulfides.

In vivo, a *thiI* mutant requires thiazole, and efforts to isolate suppressor mutations by spontaneous or chemical mutagenesis were unsuccessful. As might be predicted from the *in vitro* results, sulfide provided in the form of Li₂S or Na₂S restored growth of a *thiI* mutant strain in the absence of thiamine, although this result was not considered physiologically relevant (data not shown). Unexpectedly, nutrient studies found that addition of cysteine (0.2 mM) to the medium allowed growth of a *thiI* mutant strain in the absence of thiazole (Table 3). Restoration of thiamine-independent growth was specific to the addition of cysteine; no other physiologically relevant sulfur source or reducing compound tested restored growth to a *thiI* mutant strain (data not shown). Significantly, the ability of cysteine to allow growth did not require IscS. Neither did this effect of cysteine require SufS (a cysteine desulfurase) or GshA (glutathione biosynthetic enzyme). These conclusions were based on the data in Table 3, which showed that the thiazole requirement of each double mutant strain (*thiI iscS*, *thiI gshA*, and *thiI sufS*) was satisfied by exogenous cysteine. The slightly reduced growth rate of the double mutants compared to the *thiI* mutant is consistent with the deleterious effects of these mutations on other aspects of metabolism. Significantly, the addition of cysteine fully satisfied the thiazole requirement as reflected by the similar growth rates allowed by cysteine and thiazole. Finally, cysteine did not allow growth of a *thiF* mutant

in the absence of thiazole (data not shown), indicating that cysteine did not serve as a direct sulfur source to ThiG.

Together, these data suggest that an alternative mechanism for sulfur mobilization to ThiS existed in the cell that depends on exogenous cysteine. The finding that cysteine satisfied the thiazole requirement of *iscS*, *gshA*, and *sufS* derivatives of a *thiI* mutant indicates that the alternative mechanism of sulfur mobilization does not depend on either of the dominant cysteine desulfurases in the cell or the primary thiol reductant, glutathione. It is possible that an additional cysteine desulfurase, possibly via an orphan rhodanese in the cell, can mediate sulfur transfer to ThiS. The requirement for exogenous cysteine might indicate a need to accumulate an appropriate persulfide. Such a scenario may reflect and/or mimic a paradigm for thiazole synthesis in organisms that do not require ThiI for thiazole biosynthesis.

Conclusions. In *S. enterica* and *E. coli*, ThiI is involved in the synthesis of both thiazole and 4-thiouridine (15, 28). The data here showed that the THUMP and AANH domains of this protein, which are the basis for annotation of proteins as ThiI in genomes, are not required for thiazole synthesis. This work emphasizes that caveats can exist when annotation of gene function is propagated from a single experimental organism. Finding an additional cysteine-dependent mechanism of sulfur mobilization to thiazole *in vivo* suggests that there may be diverse processes for sulfur mobilization to thiazole. Further characterization of this mechanism could improve understanding of the relationship between the thiol environment in the cell and mobilization of sulfur to thiazole synthesis.

ACKNOWLEDGMENTS

We acknowledge undergraduates James Vasta and Patrick Borchert for isolating several of the *thiI* mutants.

The National Institutes of Health grant GM47296 and the 21st Century Scientists Scholars program from the J. M. McDonnell Foundation to D.M.D. supported this work. L.D.P. was supported by NSF through Graduate Research Fellowship grant DGE-0718123.

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