

Direct Evidence for Enzyme Persulfide and Disulfide Intermediates during 4-Thiouridine Biosynthesis

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General. Unless otherwise specified, all materials were purchased from either Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) and used as provided. Sephadex G-25 (DNA grade) and L-[³⁵S]cysteine were purchased from GE Healthcare (formerly Amersham Biosciences Picataway, NJ). 5-(((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid (I-AEDANS) was purchased from Molecular Probes (Eugene, OR). Ribonuclease T₁, ribonuclease A, and trypsin (sequencing grade) were purchased from Roche Molecular Biochemicals (Indianapolis, IN). Competent BLR(DE3) pLysS *Escherichia coli* cells were purchased from Novagen (Madison, WI). Ion exchange chromatography was accomplished using a BioCAD® SPRINT® system and POROS™ resins from Applied Biosystems (Cambridge, MA). The “tRNA substrate” was the *in vitro* transcript of *E. coli* tRNA^{Phe}, the preparation of which we have described elsewhere.¹ The “39-mer substrate” is a minimal RNA substrate in which both the D-loop and the anticodon stem-loops of tRNA have been deleted and the TΨC loop is replaced with a GAAA tetraloop²; the sequence is:

GCCCGGAUAGUGUCCUUGGGAAACCAAGUCCGGGCACCA.

The 39-mer substrate was obtained from Dharmacon, Inc. (Lafayette, CO) in the 2'-ACE protected form and deprotected according to the manufacturer's protocol. The dialysis of protein samples for mass spectrometric analysis was achieved using Slide-a-Lyzer® mini dialysis units (Pierce, Rockford, IL). Ni-NTA superflow resin and QIAprep Spin Miniprep kits were purchased from QIAGEN (Chatsworth, CA). QuikChange™ site directed mutagenesis kits were purchased from Stratagene (La Jolla, CA), and a Robocycler Gradient 96 Thermocycler (Stratagene) was used for the PCR component of the site-directed mutagenesis. DNA sequencing was performed at the University of Delaware DNA Sequencing & Genotyping Center using a 3130XL Genetic Analyzer from Applied Biosystems (Foster City, CA). Mass spectrometry was performed on either an OMNI-

FLEX® or Biflex III MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA) with a minimum of 250 shots collected in reflectron mode using positive ion detection.

Generation, over-expression, and purification of altered ThiI. To substitute cysteine residues in ThiI with alanine or lysine residues with arginine, the QuikChange™ mutagenesis protocol was used as described elsewhere.^{1,3} All of the ThiI variants in this paper contain the 20-amino acid N-terminal His₆ tag encoded by pET15b (Novagen), but all amino acid positions are numbered in terms of the native ThiI (with no N-terminal His₆ tag). Table S1 contains the oligonucleotides used to generate each mutant and the names of the resulting plasmid. For the construction of the C108/202/207A “triple mutant” ThiI, the parent plasmid was pBH145, which encodes C108A ThiI.⁴ An intermediate plasmid (pBH146) with the additional C202A change was made and then used to generate pBH147, the final triple mutant ThiI construct (Table S1). That plasmid was the parent plasmid for the mutagenesis to substitute Lys-349 with Arg and Cys-344 with Ala (Table S1). The over-expression, purification, and storage of each altered ThiI were accomplished using methods that we have described elsewhere.⁵ Briefly, protein expression was induced by addition of IPTG to the growth medium, and cells were harvested 3 h later. Chromatography of cell extracts over Ni-NTA resin yielded essentially homogeneous ThiI, which was changed into appropriate buffer by dialysis. IscS was further purified by chromatography over POROS™ HQ anion exchange resin, eluting with a linear gradient of potassium chloride (0 – 1.5 M) in 50 mM Tris•HCl buffer, pH 7.5. Fractions containing IscS were combined and concentrated using a Centriplus-30 ultrafiltration device. All ThiI variants were subjected to chromatography over a column of POROS™ SP cation exchange resin, eluting with a linear gradient of potassium chloride (0.05 – 1.5 M) in 50 mM potassium phosphate buffer, pH 7.5. Fractions containing ThiI were combined and concentrated using

Table S1: Generation and catalytic activity of altered ThiI

plasmid	amino acid substitutions	catalytic activity ^a	primers ^b
<i>triple mutant ThiI</i>			
pBH146	C108/202A	—	ATG TTG ATG CGT CGC GGC <u>GCC</u> CGC GTG CAT TAC GTA ATG CAC GCG <u>GCC</u> GCC GCG ACG CAT CAA CAT
pBH147	C108/202/207A	2.5 nM/min 1 nM ThiI	C CGC GTG CAT TAC <u>GCC</u> TTC TTT AAC CTC GGC GGC GCC GCC GAG GTT AAA GAA <u>GCC</u> GTA ATG CAC GCG G
<i>K349R triple mutant ThiI</i>			
pBH191	C108/202/207A K349R	3.4 nM/min 1 nM ThiI	T TGT GGT GTG ATC TCC <u>CGA</u> AGC CCG ACG GTG AAA G C TTT CAC CGT CGG GCT <u>TCC</u> GGA GAT CAC ACC ACA A
<i>C456only ThiI</i>			
pBH148	C108/202/207/344A	4.9 nM/min 1.1 μM ThiI	CGC ACG ATG CCG GAA TAT <u>GCT</u> GGT GTG ATC TCC AAA AGC GCT TTT GGA GAT CAC ACC <u>AGC</u> ATA TTC CGG CAT CGT GCG

^aThe rates of s⁴U generation were determined with the full-length tRNA substrate. Wild-type ThiI (1 nM) supports s⁴U generation at 11 nM/min under the same conditions.⁴

^bThe primers are split into codons (*upper sequence*) and their complements (*lower sequence*) with changed nucleotides underlined.

a Centriplus-30 device. Pure protein was used immediately or precipitated by addition of solid ammonium sulfate (to nominal 70% saturation) for storage at 4 °C.

As described in detail elsewhere⁶, extinction coefficients were calculated using A_{280} under native conditions and a protein concentration determined by biuret assay with bovine serum albumin as the standard. The extinction coefficient for triple mutant ThiI (64,000 $M^{-1}cm^{-1}$) was within 2% of that reported for wild-type ThiI (63,100 $M^{-1}cm^{-1}$)⁴, and the extinction coefficient for K349R triple mutant ThiI (67,200 $M^{-1}cm^{-1}$) differed by only 6%. The extinction coefficient for C456only ThiI (55,764 $M^{-1}cm^{-1}$) unexpectedly varied by somewhat more (13%), so the extinction coefficient was checked by other means. A large conformational change could account for the variation of the extinction coefficients for wild-type and C456only ThiI, but the far-UV CD spectra for these two proteins were identical within 5% (data not shown), demonstrating that they shared the same overall fold (the CD spectrum of K349R triple mutant also varied less than 5% from the wild-type spectrum). The concentration of C456only ThiI was also determined by A_{280} under the stipulated denaturing conditions using the extinction coefficient calculated from the protein sequence by the program ProtParam on the ExPasy Proteomics Server (<http://ca.expasy.org>); that value agreed within 1% of the value used to calculate the native ϵ_{280} , validating the value of 55,800 $M^{-1}cm^{-1}$ reported above for native C456only ThiI.

Activity assay. The rate of each mutant ThiI was determined by monitoring the incorporation of ³⁵S into RNA by using L-[³⁵S]cysteine as the substrate.^{3,4} A typical reaction mixture (150 μ L) was 50 mM Tris•HCl buffer, pH 8.5, containing MgCl₂ (5 mM), ATP (4 mM), PLP (20 μ M), L-[³⁵S]cysteine (500 μ M; 150 Ci/mol), DTT (1 mM), tRNA (20 μ M), IscS (4 nM) and ThiI (1 nM). Reactions were initiated by the addition of recombinant ThiI and incubated at 37 °C for 3 hr. At various times (15, 30, and 60 min), aliquots (100 μ L) of the reaction mixture were subjected to spin size exclusion chromatography over a column of Sephadex G-25 to remove unreacted L-[³⁵S]cysteine. The proteins were extracted from the eluate with an equal volume of phenol:chloroform (1:1). The layers were separated by centrifugation for 5 min, and the top (aqueous) layer was removed and passed over an additional spin column of Sephadex G-25. The ³⁵S-containing tRNA eluate (80–100 μ L) was mixed with ScintiSafe™ Econo 2 scintillation fluid (5 mL) and counted using a Tri-Carb 2900TR liquid scintillation analyzer (Packard, Meriden, CT). As a background control, tRNA substrate was omitted from a reaction mixture and analyzed by the same method; the radioactivity in the background control was subtracted from each data point to determine the amount of [³⁵S]s⁴U generated. The generation of [³⁵S]s⁴U in 39-mer substrate occurred at 65% the rate as in tRNA substrate, which is comparable to the data of Lauhon and co-workers in their initial report of the shortened substrate.²

Persulfide trapping. To trap Cys-456 of ThiI in its persulfide form, it was treated with I-AEDANS, a fluorescent derivative of iodoacetamide. C456onlyThiI (Table S1) was added (11 μ M final conc) to a solution (150 μ L) of L-cysteine (400 μ M), PLP (160 μ M), and IscS (10 μ M) in 50 mM Tris•HCl buffer, pH 8.5. After 1 h at 37 °C, an aliquot (100 μ L) was removed and freed of cysteine by spin size exclusion chromatography over a column of Sephadex G-25. To the eluate containing ThiI and IscS or to control samples containing either ThiI or IscS alone, I-AEDANS (5 eq relative to total Cys in IscS and ThiI) was added. After 30 min at room temperature, aliquots of each sample were mixed with an equal volume of either reducing or non-reducing 2× Laemmli sample buffer. Samples were heated to 100 °C and analyzed by SDS-PAGE (10% gel). Fluorescence was observed using a Gel Doc 1000 gel documentation system equipped with a UV transilluminator and running Quantity One, version 4.2.3 software (BioRad, Hercules, CA). Protein was visualized using Coomassie Brilliant Blue R-250 stain (Fig. 2 of the communication).

The trapped persulfide form of ThiI was separated from IscS and analyzed by MALDI-MS. The reaction mixture (2 mL) was 50 mM potassium phosphate buffer, pH 8.5, containing MgCl₂ (5 mM), PLP (250 μ M), L-cysteine (250 μ M), IscS (125 μ M), and C456only ThiI (33 μ M). The reaction was initiated by the addition of recombinant ThiI and incubated

at 37 °C for 1 min. The reaction mixture was treated for 1 h at room temperature with I-AEDANS (2 eq relative to L-cysteine and total Cys in IscS and ThiI). ThiI and IscS were separated by chromatography over a column of POROS™ SP cation exchange resin, eluting with a linear gradient of potassium chloride (0.05 – 1.5 M) in 50 mM potassium phosphate buffer, pH 7.5. Fractions containing ThiI were combined and concentrated using a Centriplus-30 ultrafiltration device (~330 μ L final volume, 9.8 μ M ThiI; 50% of ThiI injected). An aliquot (100 μ L) of the recovered ThiI was dialyzed against 25 mM ammonium bicarbonate buffer (3 × 1 L) and then digested with trypsin (1:30 trypsin:ThiI) overnight at 37 °C. An aliquot (30 μ L) of the digestion was removed for MALDI-MS analysis. To check for the presence of disulfide bonds, DTT (10 mM final conc) was added to a second aliquot (30 μ L) and incubated at 50 °C for 20 min. Both samples were separately concentrated and desalted using a ZipTip C₁₈ (Millipore) following the manufacturer's protocol except that the peptides were eluted with matrix solution (3–4 μ L), which is saturated α -cyano 4-hydroxy cinnamic acid in aqueous acetonitrile (70%) containing trifluoroacetic acid (0.03%). An aliquot (0.3 – 0.5 mL) of each sample was spotted on a MALDI plate and air dried. The mass spectra were obtained on the OMNI-FLEX MALDI-TOF mass spectrometer (Fig. 2 of the communication). Similar results were obtained with the unpurified mixture of ThiI and IscS except that the C-terminal-most tryptic peptide of IscS partially overlapped with the ThiI peptide bearing the alkylated persulfide group of Cys-456 (data not shown).

Disulfide Bond Localization. Indirect evidence supported the formation of a disulfide bond between Cys-344 and Cys-456 during the catalytic cycle of ThiI. To provide direct evidence for that disulfide bond, ThiI was held to a single turnover and analyzed by tryptic digestion and MALDI-MS. Triple mutant ThiI (Table S1) was added (5 μ M final conc) to a solution (150 μ L) containing IscS (20 μ M) and the activity assay components except for the omission of DTT and a lower concentration of L-cysteine (70 μ M) to limit ThiI to a single turnover, as previously described.⁴ To monitor the extent of turnover, an assay using L-[³⁵S]cysteine (70 μ M, 250 Ci/mol) as substrate was run in parallel. A typical reaction mixture (150 μ L) was 50 mM Tris•HCl buffer, pH 8.5, containing MgCl₂ (5 mM), ATP (4 mM), PLP (20 μ M), L-cysteine (70 μ M), tRNA substrate (20 μ M), IscS (20 μ M) and ThiI (5 μ M). After 3 h at 37 °C, an aliquot (100 μ L) of the reaction mixture was freed of cysteine and other small molecules by spin size exclusion chromatography over a column of Sephadex G-25 equilibrated with 50 mM Tris•HCl buffer, pH 8.5. Quantitation of [³⁵S]s⁴U from the radioassay (as described above) revealed 69% turnover of ThiI, in accord with previous results.⁴ For MALDI-MS analysis, iodoacetamide (25 mM final conc) was added to the spin column eluate in order to block the Cys from IscS and any unreacted Cys of ThiI. After 1 h at room temperature, the sample (100 μ L) was changed by dialysis into 25 mM ammonium bicarbonate buffer (3 × 1 L). The dialyzed sample was digested with trypsin (1:30 trypsin:total enzyme) overnight at 37 °C. Peptides were lyophilized to dryness and then redissolved in 25 mM ammonium bicarbonate buffer (50 μ L). One aliquot (20 μ L) was set aside for MALDI-MS analysis. A second aliquot (20 μ L) was first reduced by the addition of DTT (10 mM final conc) and incubation at 50 °C for 20 min; iodoacetamide was then added (25 mM final conc), and the sample was incubated at room temperature for 1 hr. Both peptide samples were concentrated and desalted using a ZipTip C₁₈ and subjected to MALDI-MS as described above except that the spectra were obtained with the Biflex III MALDI-TOF mass spectrometer (Fig. 3 of the communication).

Disulfide Bond localization with K349R triple mutant ThiI. To improve the detection of peptides containing Cys-344, Lys-349 was substituted with Arg in the context of triple mutant ThiI (Table S1). The procedure for disulfide bond localization (as described above) was repeated with K349R triple mutant ThiI, with mass spectra obtained on the OMNI-FLEX MALDI-TOF mass spectrometer. A parallel assay with L-[³⁵S]cysteine (70 μ M, 250 Ci/mol) as substrate showed that 80% of K349R triple mutant ThiI had turned over. As described in the communication, the Cys-344–Cys-456 disulfide-linked peptide was mass shifted as expected for the substitution of Lys with Arg. Reduction replaced the

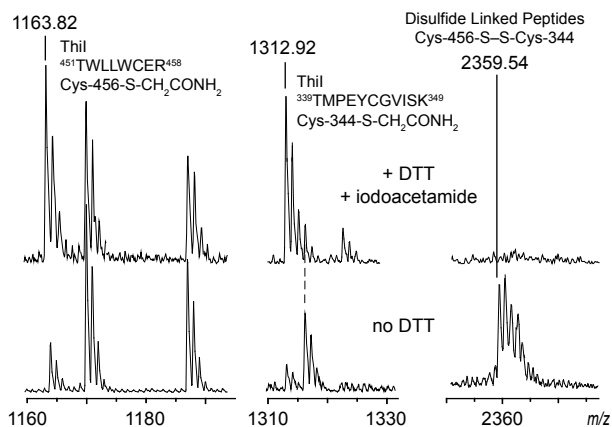


Fig. S1. Partial MALDI mass spectra showing the disulfide linkage in K349R triple mutant ThiI (*bottom*). When the same sample is treated with DTT and then iodoacetamide, the disulfide-linked peptide disappears, and the alkylated peptides containing Cys-456 and Cys-344 are detected (*top*).

disulfide-linked peptide with the unshifted Cys-456 containing peptide and the mass-shifted peptide containing Cys-344, which was more intense than its Lys-terminated cognate (Fig. S1). No other disulfide-linked peptides were detected, including the IscS peptide containing Cys-328 in a disulfide bond with the substrate cysteine that has very nearly the same mass as the Cys-344–Cys-456 disulfide-linked peptide from triple mutant ThiI.

Intramolecular vs. Intermolecular ThiI Disulfide Bond. To generate the Cys-344–Cys-456 disulfide bond, a single turnover assay with triple mutant ThiI (Table S1) was performed as described above for disulfide bond localization. After 3 h, the assay was freed of small molecules using spin size-exclusion chromatography over a column of Sephadex G-25 equilibrated with 50 mM Tris•HCl buffer, pH 8.5. An aliquot (20 μ L) was treated with the thiol reactive compound *N*-ethylmaleimide (NEM; 20 mM final conc) to block any unreacted Cys of both ThiI and IscS. After 30 min at room temperature, solid guanidine hydrochloride (~5 M final conc.) was added to denature the enzymes, followed by additional NEM (40 mM final conc). After 1 h at room temperature, the sample was dialyzed against 50 mM Tris•HCl buffer, pH 8.5 (6 \times 1 L). After dialysis, the sample was set aside for SDS-PAGE analysis. A parallel assay with L-[³⁵S]cysteine (70 μ M, 250 Ci/mol) as the substrate showed that 64% of triple mutant ThiI had turned over.

In an attempt to form ThiI dimer for use as a gel mobility standard, C456only ThiI (Table S1) was treated with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). DTNB makes a mixed disulfide group with free thiol groups, releasing 5-thio-2-nitrobenzoic acid (TNB), a yellow chromophore that can be quantitated by its absorbance at 412 nm. In this case, the sub-stoichiometric DTNB treatment should allow the displacement of a second equivalent of TNB from the mixed disulfide groups by the Cys of another C456only ThiI to yield a disulfide-linked ThiI dimer. C456only ThiI (2 μ M) was therefore treated with DTNB (1 μ M) in 50 mM potassium phosphate buffer, pH 8.5 (100 μ L), containing MgCl₂ (5 mM), KCl (100 mM), EDTA (0.1 mM). After 1 h at room temperature, 1.02 \pm 0.1 eq of TNB (relative to DTNB) was produced, indicating very low levels of ThiI dimer formation. A small ThiI dimer band, however, was detected by nonreducing SDS-PAGE analysis.

Samples of DTNB-treated C456only ThiI, IscS alone, and the single turnover reaction were split into two equal parts. One half was mixed with an equal volume of non-reducing 2 \times Laemmli sample buffer, and the other half was treated with DTT (50 mM final conc) and then mixed with an equal volume of non-reducing 2 \times Laemmli sample buffer. After 5 min at

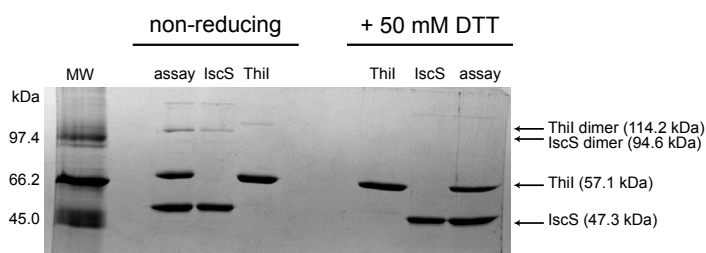


Fig. S2. SDS-PAGE gel analysis of ThiI after a single turnover. After >60% turnover of triple mutant ThiI, the amount of ThiI dimer (or higher order aggregate) is negligible. *assay*, the single turnover reaction mixtures; *IscS*, resting state of IscS; *ThiI*, DTNB-treated C456only ThiI.

100 $^{\circ}$ C, the samples were analyzed by SDS-PAGE (Fig. S2). The turnover percentage was verified using the remaining portion of the cold assay mixture and the DTNB titration method described elsewhere, which returned a value of 71% turnover. If an intermolecular disulfide bond formed during turnover, then over 60% of the ThiI should be detected as a dimer under the nonreducing SDS-PAGE conditions. However, the amount of ThiI dimer was negligible (Fig. S2), clearly indicating that the disulfide bond formed between Cys-344 and Cys-456 of the ThiI is intramolecular.

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