DOI: 10.1002/cbic.200800033

Direct Evidence that ThiI is an ATP Pyrophosphatase for the Adenylation of Uridine in 4-Thiouridine Biosynthesis

Delin You,* Tiegang Xu, Fen Yao, Xiufen Zhou, and Zixin Deng*[a]

Over a hundred post-transcriptional modifications are made to nucleosides in RNA, including the best characterized 4-thiouridine (s4U) at position 8 in bacterial tRNA. The s4U serves as a near-UV photosensor, undergoing a photoinduced cross-linking reaction with cytidine-13 when exposed to near-UV light.[1] The cross-linked tRNAs are inefficient aminoacylation substrates,[2] and therefore protein synthesis stops, triggering entry into a controlled growth arrest.[3,4] Two enzymes, ThiI[5] and IscS,[6] are proposed to be involved in the modification of uridine to 4-thiouridine. IscS removes a sulfur atom from free cysteine, and the terminal sulfur is transferred to ThiI,[7] where a persulfide is likely formed at cysteine-456 prior to ThiI's effect on tRNA modification.[8] Two chemical mechanisms were proposed to account for the generation of s4U from the persulfide group and activated uridine residue,[9] but the activation of uridine by adenylation has not been proven in either of the two mechanisms. In this paper, we provide direct evidence for the formation of an adenylation intermediate in the modification process using electrospray ionization tandem mass spectrometry (LC-ESI-MS-MS), ATP pyrophosphatase activity analysis, and an isotopic tracer method.

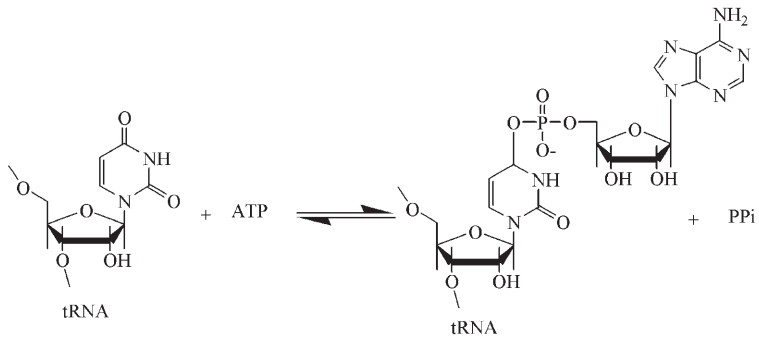
ThiI was shown previously as a sulfurtransferase, which was responsible for the transfer of a persulfide to tRNA.[8,10] It shares an adenylation-specific P-loop motif (SGGFDS) within the PPi synthetase family, seemingly required for the activation of uridine by adenylation (Scheme 1). In order to examine and prove the potential adenylation role of the ThiI protein in 4-thiouridine biosynthesis, a recombinant protein was expressed (see the Supporting Information) and assayed in vitro for its proposed ATP pyrophosphatase activity by monitoring the production of PPi using the EnzCheck pyrophosphate assay kit (Producer).[11] The kinetic constants observed for this reaction are summarized in Table 1. The specific activity of ThiI toward ATP (1.9 ± 0.1 nmol min^{-1} mg^{-1}) was comparable with that toward GTP (1.29 ± 0.07 nmol min^{-1} mg^{-1}), but the K_m value for ATP was approximately 3.5 times lower than that for GTP. Thus, the specific activity of ThiI is established to serve as an ATP pyrophosphatase which can catalyze the adenylation or guanylation of tRNA.

Table 1. Substrate specificity of ThiI and kinetic constants of the enzyme reactions.

Substrate	K_m [mM]	V_{max}	k_{cat}	k_{cat}/K_m [mM^{-1} s^{-1}]
ATP	0.24 ± 0.05	1.9 ± 0.10	0.145	0.604
GTP	0.84 ± 0.16	1.29 ± 0.07	0.095	0.113

The formation of adenylated, but not the guanylated intermediate in s4U formation was further examined using α-^{32}P-labeled ATP and unmodified tRNA obtained from *E. coli thiI* mutant VJS2890 (DE3) as substrate. Apparently, α-^{32}P-labeled tRNA was observed (Figure 1 A), whose labeling efficiency was found to decrease in the presence of unlabeled ATP in the reaction mixture. In addition, α-^{32}P-labeled tRNA was found to approach a diminishing level with increasing concentrations of unlabeled ATP in a kinetic assay, but a high concentration of unlabeled GTP, UTP, and CTP was found not to compete with the formation of α-^{32}P-labeled tRNA. When α-^{32}P CTP was used for the substitution of α-^{32}P ATP for the isotope tracer test, the lack of signal appearance clearly excluded the possibility that AMP was incorporated into the 3′-end of tRNA by CCA nucleotidyl transferase when ThiI was co-purified (data not shown). The results presented above, the detected K_m values at a remarkably distant ratio of 3.5:1 between ATP and GTP, and the physiological intracellular 3 mM ATP and 100 μM GTP concentrations[12] in *Escherichia coli*, strongly suggest that ThiI catalyzes the adenylation, but not the guanylation for the addition of AMP to tRNA in vivo.

To provide further evidence that ThiI catalyzes formation of a modification intermediate, both unmodified tRNA and adenylate tRNA were digested with BAL 31 nuclease. The nucleotides produced were separated by HPLC on a C8 column and analyzed by electrospray ionization tandem mass spectrometry



Scheme 1. Conversions catalyzed by the ThiI protein.

[a] *Dr. D. You, T. Xu, F. Yao, Prof. X. Zhou, Z. Deng*
Laboratory of Microbial Metabolism
and College of Life Science and Biotechnology
Shanghai Jiaotong University
Shanghai 200030 (China)
Fax: (+86) 21-62932418
E-mail: dlyou@sjtu.edu.cn
zxdeng@sjtu.edu.cn

Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.

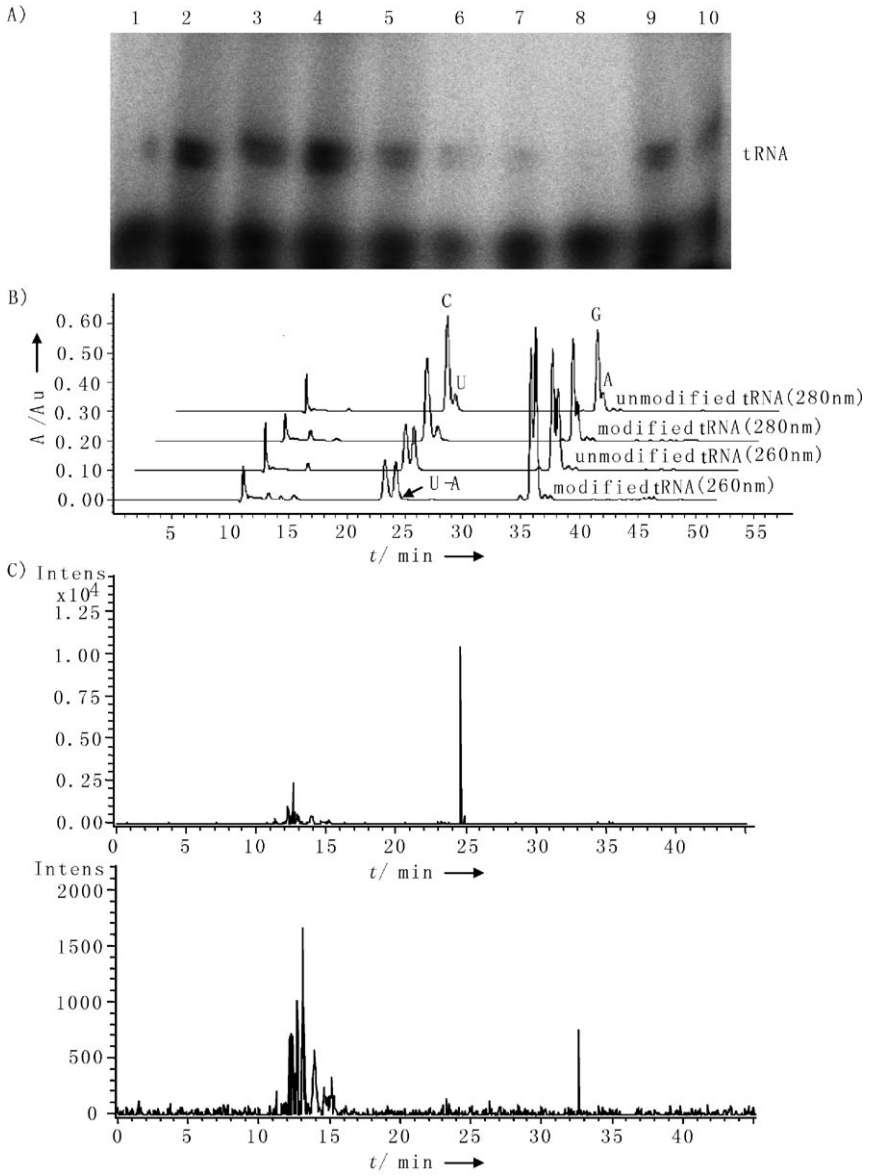


Figure 1. Detection of an adenylated intermediate by PAGE and HPLC-MS. A) Formation of adenylated tRNA (U8) in the presence of [α-^{32}P] ATP without unlabeled ATP (lane 2), with 0.05 mM GTP (lane 3), with 0.25 mM GTP (lane 4), with 1.25 mM GTP (lane 5), with 1.25 mM CTP (lane 9), or with 1.25 mM UTP (lane 10). The radioactivity of the AMP bound tRNA decreased by 0.01 mM ATP (lane 6), 0.05 mM ATP (lane 7), or 0.25 mM ATP (lane 8). A tRNA without ThiI treatment as a negative control (lane 1). B) The adenylated uridine intermediate could be detected by HPLC from BAL 31 nuclease and CAP digests of modified tRNA, but not unmodified tRNA. The retention time of cytidine is 23.2 min (C), uridine is 24.2 min (U), guanosine is 35.9 min (G), adenosine is 36.3 min (A), and that of adenylated uridine is 24.6 min (U-A). C) An anticipated adenylated uridine intermediate with a monoisotopic mass of *m/z* 576.3 $[M+H]^+$ was identified in the total ionization of the BAL 31 nuclease and CAP digest of adenylated tRNA (upper panel) by comparison with the total ionization of a digest of unmodified tRNA (lower panel).

(LC-ESI-MS-MS). An anticipated adenylated uridine intermediate (Scheme 1) with a monoisotopic mass of *m/z* 576.3 $[M+H]^+$ was identified in the total ionization of the BAL 31 nuclease digest of adenylated tRNA, by comparison with the total ionization of a digest of unmodified tRNA (Figure 1C). The elution positions of nucleotides are shown in Figure 1B. Cytidine, uridine, guanosine, and adenosine in the digested product were identified by mass spectrometry, and the UV spectra of peaks were obtained by a photodiode array detector, which are consistent with the results reported by C. W. Gehrke.[13] A monoisotopic mass of *m/z* 576.3 $[M+H]^+$ was identified immediately after uridine components in the HPLC trace of the digest of tRNA, which has a characteristic absorption spectrometry different from adenosine, cytidine, guanosine, and uridine (Figure 2). An additional attempt was made to prepare the pure unmodified tRNA by in vitro transcription in order to prove the exact location of adenylation by sequencing, but no conclusive result has been obtained yet, likely due to the low quantity and/or instability of the modification intermediate.

This communication not only provided evidence that the activation of uridine by adenylation is the first reaction, but also provided strong support for the recently proposed catalytic mechanism for ThiI in 4-thiouridine biosynthesis.

Experimental Section

Cloning and expression of the *thil* gene and purification protocols of ThiI are described in detail in the Supporting Information. ATP pyrophosphatase activity was monitored as previously described.[11] In this assay, PPi is converted to Pi by inorganic pyrophosphatase. The substrate 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG) is enzymatically converted by purine nucleoside phosphorylase (PNP) to ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine. Enzymatic conversion of MESG results in a shift in absorbance maximum from 330 nm for the substrate to 360 nm for the product. One unit of enzyme was defined as the amount of enzyme that catalyzes the formation of 1 nmol of the product (PPi) min^{-1}. Specific activity was expressed as units per mg of protein. The pyrophosphate concentration was determined based on a standard $Na_4P_2O_7$ curve. For kinetic measurements, a solution of 0.2 mM MESG, 1 U purine nucleoside phosphorylase, and the appropriate concentration of ATP or GTP in Tris (50 mM pH 7.5), $MgCl_2$ (1 mM), and sodium azide (0.1 mM) was incubated at 22 °C. After 5 min, unmodified tRNA (2.5 μM), ThiI

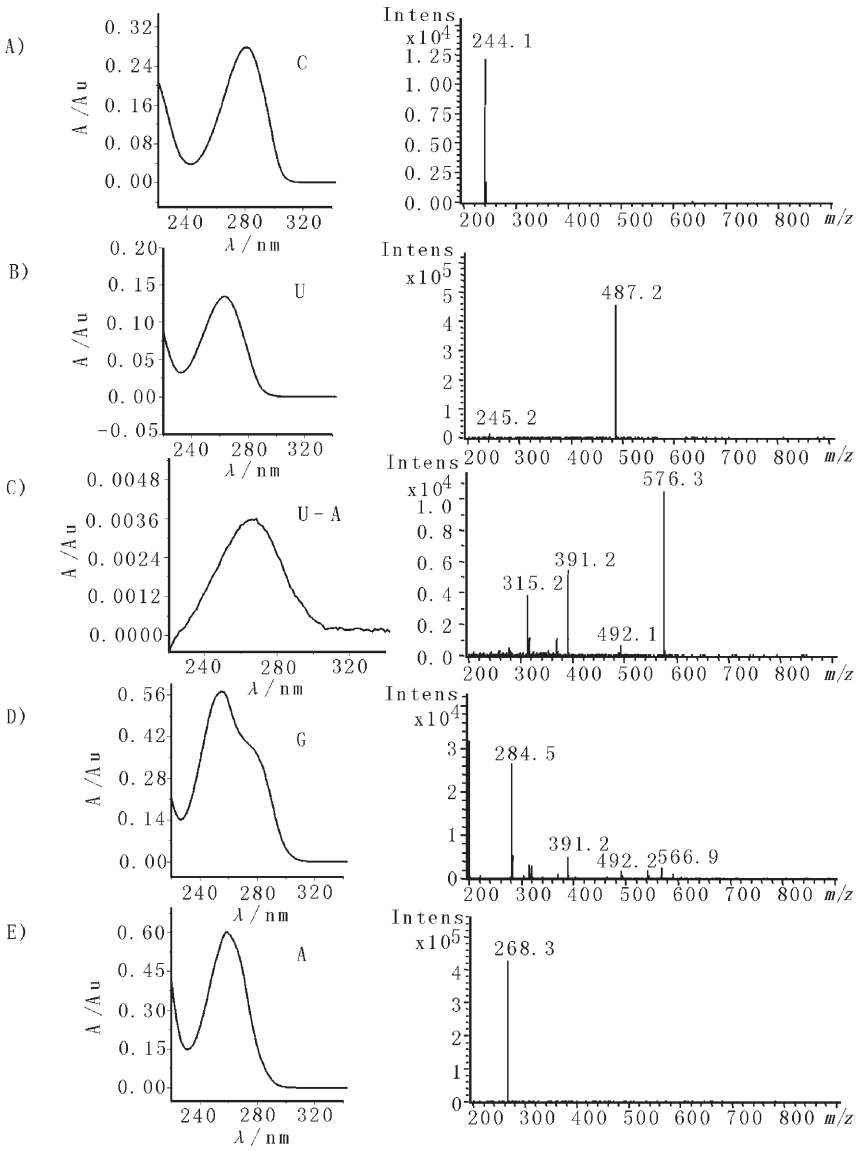


Figure 2. UV spectrum and LC-MS analysis of nuclease digests of tRNA. Modified tRNA digests were identified by UV spectra and ESI-MS spectrum. A) Positive ion ESI-MS spectrum of cytidine at *m/z* 244.1 (right) and the UV spectrum λ_{max} at 279.2 nm (left); B) uridine at *m/z* 245.2 (right) and λ_{max} at 262.6 nm (left); D) guanosine at *m/z* 284.5 (right) and λ_{max} at 254.4 nm (left); E) adenosine at *m/z* 268.3 (right) and λ_{max} at 257.9 nm (left). which are consistent with the results reported by C. W. Gehrke.[13] C) A monoisotopic mass of *m/z* 576.3 was identified immediately after uridine components in the HPLC trace of the digest of tRNA (right), and exhibits a characteristic UV spectrum (λ_{max} = 267.4 nm; left), which is not only different from adenosine, cytidine, guanosine, and uridine, but also different from a similar analogue 4-methoxy-1-β-D-ribofuranosyl-2-pyrimidinone whose maximal UV absorption spectrum is at 274 nm,[16] and 4-thiouridine which is at 340 nm.[13]

(4.4 μM), and inorganic pyrophosphatase (0.03 U) were added, and the reaction was followed by monitoring the increase of absorbance at 360 nm. All assays were run in triplicate. Kinetic parameters k_{cat} and K_m were calculated by fitting to a Lineweaver–Burk plot.

The unmodified tRNA was isolated as previously described.[14] A single colony of VJS2890 (DE3) was used to inoculate LB medium (1000 mL) and this culture was incubated overnight at 37 °C with moderate shaking. The cells were harvested by centrifugation for 10 min at 5000 *g*. The supernatant was decanted and the pellet was resuspended in 10 mL of Tris (10 mM pH 7.4), $MgCl_2$ (1 mM). Using a vortex mixer, the cells were vigorously agitated for 1 min with water-saturated phenol (10 mL) and the mixture was subjected to centrifugation for 30 min at 10000 *g*. The aqueous phase was decanted and the cells were resuspended, extracted, and subjected to centrifugation as described above. The two aqueous phases were combined (13 mL final volume). To precipitate the tRNA, 0.8 mL of sodium acetate (5 M pH 5.2) was added, followed by addition of 26 mL absolute ethanol and incubation for a minimum of 1 h at −20 °C. The tRNA was pelleted by centrifugation for 30 min at 10000 *g* and this pellet was redissolved in 1 mL of Tris (50 mM pH 7.6). The tRNA was purified by electrophoresis on denaturing polyacrylamide gels (8%, *v/v*) and eluted from the gel in Tris (50 mM pH 7.6) for 24 h at 4 °C. After precipitating the tRNA with ethanol, tRNA was redissolved in 1 mL of Tris (50 mM pH 7.6).

Unmodified tRNA (1.9 μM) and ThiI (10 μM) were incubated at 37 °C for 30 min in 10 μL of buffer consisting of Tris (50 mM pH 7.5), $MgCl_2$ (1 mM), and α-^{32}P-labeled ATP (0.33 μM, 10 μCi). For the competition experiments, varying amounts of unlabeled ATP (0.01-0.25 mM), varying amounts of unlabeled GTP (0.05–1.25 mM), unlabeled CTP (1.25 mM), or unlabeled UTP (1.25 mM) were added. Samples were then applied to polyacrylamide gel (8%, *v/v*) containing urea (7 M), and electrophoresis was performed using Tris (90 mM)/boric acid (90 mM) and EDTA (2 mM). The gel was then exposed on an imaging plate and visualized with a phosphoimager (Fujifilm FLA3000).

The adenylated tRNAs were prepared at 37 °C in a reaction mixture containing Tris (50 mM pH 7.5), $MgCl_2$ (1 mM), unmodified tRNA (12.8 μM), ATP (2.5 mM), and ThiI (10 μM). The adenylated and unmodified tRNA digestions were accomplished by the method of Gehrke et al.[15] with slight modification. A solution (0.2 mL) containing tRNA (5 nmol) was incubated for 2 min at 95 °C then cooled to 37 °C. Subsequently 0.2 mL of Tris (40 mM pH 7.5), NaCl (1.2 M), $CaCl_2$ (24 mM), $MgCl_2$ (24 mM), EDTA (2 mM), and BAL 31 nuclease (8 U) were added to this solution. The mixture (0.4 mL) was incubated for 2 h at 37 °C followed with the addition of 0.4 mL of Tris (500 mM pH 9.0), to adjust the pH and calf intestine alkaline phosphatase (10 U). The solution was incubated for 2 h at 37 °C, the resultant nucleosides were directly analyzed by chromatography. Otherwise the adenylated intermediate is unstable and could not be detected by LC-MS after incubation for 2 min at 95 °C.

The resultant nucleosides were collected in the supernatant after centrifugation at 15000 *g* for 10 min. The supernatants were analyzed by ESI LC-MS/MS (Agilent 1100 series LC/MSD Trap system) with a Kromasil C8 (4.6×250 mm, 5 μm) column. The flow rate was 0.3 mL min^{-1} at RT. Eluent A was Milli-Q water with trifluoroacetic acid (0.1%, *v*/*v*), and eluent B was acetonitrile (Merck) with trifluoroacetic acid (0.1%, *v*/*v*). The HPLC conditions were 0 to 10.00 min with 1% B, 10.00 to 30.00 min with 5% B, 30.00 to 60.00 min with 35% B, and 60.00 to 70.00 min with 80% B. The photodiode array detector (DAD) was set at 254 nm. The mass spectrometer was run in positive ionization detection mode and set to scan between 200 and 1200 *m*/*z*. The drying gas temperature was 350 °C, the drying gas flow rate was 9.0 L min^{-1}, and the nebulizer pressure was 275.8 kPa.

Acknowledgements

We thank Prof. Eugene G. Mueller (University of Louisville) for supplying the E. coli. thiI *mutant VJS2890 (DE3), Dr. Linquan Bai for critical reading of the manuscript. This work received supports from the Ministry of Science and Technology, the National Science Foundation of China, China Ocean Mineral Resources R&D Association, the Shanghai Municipal Council of Science and Technology, and the Shanghai Leading Academic Discipline Project B203.*

Keywords: adenylation · ATP pyrophosphatase · biosynthesis · ThiI · thiouridine

[1] D. E. Bergstrom, N. J. Leonard, *Biochemistry* **1972**, *11*, 1–9.
[2] D. S. Carre, G. Thomas, A. Favre, *Biochimie* **1974**, *56*, 1089–1101.
[3] T. V. Ramabhadran, J. Jagger, *Proc. Natl. Acad. Sci. USA* **1976**, *73*, 59–63.
[4] G. F. Kramer, J. C. Baker, B. N. Ames, *J. Bacteriol.* **1988**, *170*, 2344–2351.
[5] E. G. Mueller, C. J. Buck, P. M. Palenchar, L. E. Barnhart, J. L. Paulson, *Nucleic Acids Res.* **1998**, *26*, 2606–2610.
[6] R. Kambampati, C. T. Lauhon, *Biochemistry* **1999**, *38*, 16561–16568.
[7] R. Kambampati, C. T. Lauhon, *J. Biol. Chem.* **2000**, *275*, 10727–10730.
[8] P. M. Palenchar, C. J. Buck, H. Cheng, T. J. Larson, E. G. Mueller, *J. Biol. Chem.* **2000**, *275*, 8283–8286.
[9] E. G. Mueller, P. M. Palenchar, C. J. Buck, *J. Biol. Chem.* **2001**, *276*, 33588–33595.
[10] C. M. Wright, G. D. Christman, A. M. Snellinger, M. V. Johnston, E. G. Mueller, *Chem. Commun.* **2006**, 3104–3106.
[11] D. L. You, L. R. Wang, F. Yao, X. F. Zhou, Z. X. Deng, *Biochemistry* **2007**, *46*, 6126–6133.
[12] T. W. Traut, *Mol. Cell. Biochem.* **1994**, *140*, 1–22.
[13] C. W. Gehrke, K. C. Kuo, *J. Chromatogr.* **1989**, *471*, 3–36.
[14] Y. M. Hou, P. Schimmel, *Nature* **1988**, *333*, 140–145.
[15] C. W. Gehrke, K. C. Kuo, R. A. McCune, K. O. Gerhardt, P. F. Agris, *J. Chromatogr. B* **1982**, *230*, 297–308.
[16] M. J. Robins, S. R. Naik, *Biochemistry* **1971**, *10*, 3591–3597.

Received: January 18, 2008
Published online on July 4, 2008

