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Mechanism of N6-Threonylcarbamoyladenonsine (t^6A) Biosynthesis: Isolation and Characterization of the Intermediate Threonylcarbamoyl-AMP

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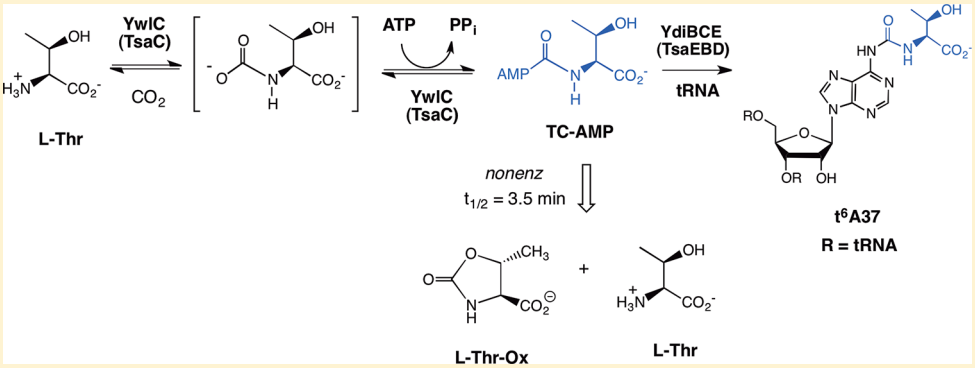
Supporting Information



ABSTRACT: Genetic and biochemical studies have recently implicated four proteins required in bacteria for the biosynthesis of the universal tRNA modified base N6-threonylcarbamoyl adenosine (t^6A). In this work, t^6A biosynthesis in *Bacillus subtilis* has been reconstituted *in vitro* and found to indeed require the four proteins YwlC (TsaC), YdiB (TsaE), YdiC (TsaB) and YdiE (TsaD). YwlC was found to catalyze the conversion of L-threonine, bicarbonate/CO_2 and ATP to give the intermediate L-threonylcarbamoyl-AMP (TC-AMP) and pyrophosphate as products. TC-AMP was isolated by HPLC and characterized by mass spectrometry and ^1H NMR. NMR analysis showed that TC-AMP decomposes to give AMP and a nearly equimolar mixture of L-threonine and 5-methyl-2-oxazolidinone-4-carboxylate as final products. Under physiological conditions (pH 7.5, 37 °C, 2 mM $MgCl_2$), the half-life of TC-AMP was measured to be 3.5 min. Both YwlC (in the presence of pyrophosphatase) and its *Escherichia coli* homologue YrdC catalyze the formation of TC-AMP while producing only a small molar fraction of AMP. This suggests that CO_2 and not an activated form of bicarbonate is the true substrate for these enzymes. In the presence of pyrophosphate, both enzymes catalyze clean conversion of TC-AMP back to ATP. Purified TC-AMP is efficiently processed to t^6A by the YdiBCE proteins in the presence of tRNA substrates. This reaction is ATP independent *in vitro*, despite the known ATPase activity of YdiB. The estimated rate of conversion of TC-AMP by YdiBCE to t^6A is somewhat lower than the initial rate from L-threonine, bicarbonate and ATP, which together with the stability data, is consistent with previous studies that suggest channeling of this intermediate.

Cells devote significant energy and information content to the modification of nucleosides in their tRNA. These modified bases are essential for proper codon recognition, aminoacylation, and reading frame maintenance during translation.[1,2] The modified nucleoside N6-threonylcarbamoyl adenosine (t^6A, Scheme 1)[3] is universally conserved in nature and found at position 37 in nearly all cytosolic tRNAs that recognize 5′-ANN-3′ codons. The importance of the biological role for t^6A has been related *in vitro* to increased stability of adjacent base pairing in the anticodon−codon interaction, as well as maintenance of anticodon loop shape and flexibility.[4−9] *In vivo*, t^6A has been shown to be important for start codon recognition and reading frame maintenance.[10,11] Early work on t^6A biosynthesis eliminated the involvement of carbamoyl phosphate and pointed toward ATP and bicarbonate as sources of the activated carbamoyl carbon (Figure 1).[12−14] Impressive initial work to isolate the biosynthetic enzymes involved led to the enrichment of t^6A activity and partial characterization of proteins required.[15,16] However, complete characterization proved challenging because of the number and likely low expression levels of the proteins involved.

A recent flurry of genetic and biochemical work in *Escherichia coli* and yeast has identified multiple genes required for t^6A formation in these strains. Initially, the *E. coli yrdC* gene and its yeast homologue SUA5 were demonstrated to be required for t^6A formation.[17] YrdC is indispensable for growth in *E. coli*, while yeast ***sua5Δ*** null strains can be constructed, but the mutation results in a severe growth defect. Consistent with the

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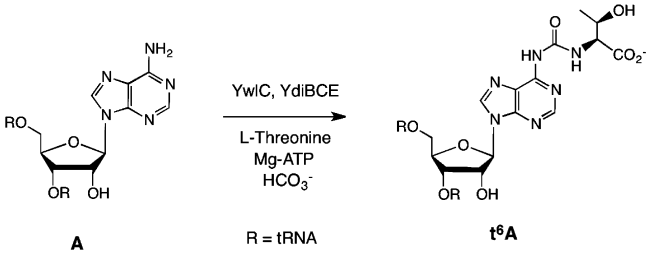
Scheme 1. Requirements for the Biosynthesis of t^6A in *B. subtilis*



universal presence of t^6A in tRNA, homologues of this gene family have been identified in every complete genome sequenced to date.[17] More recently, the *E. coli ygjD* gene[19] and the yeast homologue KAE1[18−20] were also found to be required. Initial structural work of Kae1 homologues in archaea showed it to be a novel iron-containing ATPase with metalloendonuclease activity.[21] Subsequently, additional required genes have been identified that reveal a functional divergence in t^6A biosynthesis between bacteria and eukaryotes. In yeast, Kae1 is part of the EKC/KEOPS complex, which contains five proteins, three of which (Kae1, Bud32, and Pcc1) are required for the formation of t^6A.[19] The biological activities linked to this complex are wide ranging — from telomere stability[22,23] to transcription defects[24] and genomic instability.[25,20] In *E. coli*, Handford, et al. found evidence that YgjD functions as a complex with the YjeE and YeaZ proteins.[26] YeaZ was suspected to be involved in proteolytic degradation of YgjD.[26] YjeE and its *H. influenza* ortholog have low but measurable ATPase activity,[27] and both *yeaZ* and *yjeE* are listed as essential in *E. coli*.[28] The bacterial system differs from that in yeast and other eukaryotes since no homology exists between YjeE and YeaZ and the proteins of the EKC/KEOPS complex, although it was shown that two other proteins in the yeast complex, Pcc1[18,19] and Bud32[19] are required for t^6A modification *in vivo*. Armed with this information, Deutsch et al recently reported the first *in vitro* reconstitution of t^6A biosynthesis using purified components in *E. coli*.[29] The *in vitro* modification reaction requires YrdC, the putative protein complex of YgjD, YeaZ and YjeE, in addition to L-threonine, ATP and bicarbonate. The authors used ATPase assays to show that YrdC catalyzes L-threonine dependent AMP formation and that both AMP and ADP are produced during the reaction. The genes have therefore been renamed using the abbreviation *tsa* (yeaZ = tsaB, yrdC = tsaC, ygjD = tsaD, and yjeE = tsaE).[29]

Finally, recent structural studies have shed light on homologous systems that catalyze the transfer of carbamoyl groups to both small molecule and protein acceptors. First, the structure of a YrdC/SUA5 homologue from *S. tokodaii* was solved in a complex with L-threonine and an analogue of ATP.[30] Subsequently, the structures of HypF,[31,32] an enzyme required for hydrogenase maturation, and TobZ,[33] a carbamoyltransferase involved in secondary metabolism show a mechanism for activation and transfer of a carbamoyl moiety starting with carbamoyl phosphate. In both HypF and TobZ, a carbamoyl adenylate intermediate is formed by initial dephosphorylation of carbamoyl phosphate, followed by attack of the carbamate anion on ATP. These structures are highly relevant to t^6A biosynthesis because this enzyme is a fusion of both YrdC and YgjD type proteins. The structure reveals that the carbamoyladenylate intermediate formed by the YrdC-domain is likely channeled to the YgjD domain where it is carbamoyl group is transferred to the substrate in a reaction that utilizes a novel mode of iron chelation in the active site.

Despite the recent studies on these systems, several questions remain, including the mechanism of formation of the putative carbamoyl adenylate intermediate, the characterization of its reactivity, and the precise role of each bacterial protein in t^6A biosynthesis. For example, because of it is demonstrated binding to RNA,[34] YrdC was proposed to catalyze the addition of either activated bicarbonate or an activated threonylcarbamoyl group to the tRNA. Because of the noted difficulty in expression and stability of *E. coli* YgjD, the *Bacillus subtilis* homologues were targeted in this work as a possible tractable system to reconstitute and study the mechanism of t^6A biosynthesis *in vitro* (Figure 1). In *B. subtilis*, the homologues for *E. coli yjeE/tsaE*, *yeaZ/tsaB* and *ygjD/tsaD* (*ydiB*, *ydiC* and *ydiE*, respectively) are found as sequential genes in a single operon. The *yrdC/tsaC* homologue *ywlC* is located at a separate site in the genome. The *ydiB*, *ydiC* and *ywlC* genes were initially categorized as essential in *B. subtilis*;[35] however a reinvestigation showed that deletion mutants for *ydiB* and *ywlC* can be prepared, but the mutants share a serious growth defect.[36] The phenotypes for the two mutants are very similar, which was an early clue that they may both be involved in the same pathway. Similar to its *E. coli* homologue YjeE, YdiB has measurable ATPase activity that is required for normal growth.[37] Preliminary work in this laboratory found that a *B. subtilis ywlC* conditional mutant dependent on IPTG for expression produced diminished levels of t^6A when grown in the absence of the inducer. YwlC, YdiC and YdiE have not been purified and characterized to date. In this paper, the expression, purification and reconstitution of t^6A biosynthesis in *B. subtilis* is reported with a particular focus on the characterization of L-threonylcarbamoyl AMP (TC-AMP) as the product of the YwlC/TsaC protein.

MATERIALS AND METHODS

Materials and General Methods. DNA oligonucleotides were from IDT Technologies (Coralville, IA) and purified on polyacrylamide gels before use. Biochemicals and enzymes were from Sigma-Aldrich, except for L-threonine, which was from ChemImpex. Radiolabeled α-^{32}P-ATP (3000 Ci/mmol) was from Perkin-Elmer Life Sciences. T7 RNA polymerase was purified from strain BL21(pAR219), which was a generous gift of F. Studier. *B. subtilis ywlC* mutant strain BFS215 was a generous gift of P. Stragier. Mass spectrometry and NMR spectroscopy was performed at the Analytical Instrumentation Center at the University of Wisconsin School of Pharmacy. DNA sequencing was performed at the University of Wisconsin Biotechnology Center.

Cloning, Expression and Purification of Proteins. The *ywlC* and *ydiB* genes were amplified by PCR from *B. subtilis* 168 genomic DNA, digested and ligated into pET15b. The ligation mixtures were used to transform NovaBlue cells (Novagen) to give N-terminal His_6 fusion expression plasmids pCL219 (YwlC) and pCL223 (YdiB). Oligonucleotides used for cloning *ywlC* were 5′- GTT TGG TCACAT ATG AAAACG AAA AGATGG TTT-3′ (N-terminal) and 5′- ATC TGA GGATCC TCA GCGAAT CAC TCTTCC TCC-3′ (C-terminal) and for *ydiB* 5′- CGG GTG ACTCAT ATG AAGCAA TTA AAATGG AGA-3′ (N-terminal) and 5′- TAA TTT GGATCC TCA ATTGCT AAT ATTGTC ATG-3′ (C-terminal). Plasmids were isolated using a spin miniprep kit (Qiagen), verified for sequence and used to transform *E. coli* chemically competent

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BL21(DE3) cells. Protein was overproduced by growing cells at 37 °C to A_{595} = 0.6, then adding IPTG to 1 mM and harvesting cells after 5 h additional growth at the same temperature. Cells from 100 mL cultures were collected by centrifugation and resuspended in 2 mL of lysis buffer (10 mM Tris pH 7.5, 1 mM $MgCl_2$) containing 0.3 mg/mL lysozyme. After incubation at rt for 20 min, cells were cooled on ice and disrupted by sonication (3 × 20 s), and cell debris was pelleted by centrifugation (12800*g* for 30 min). Supernatant was loaded onto a 1 mL column of HisBind resin (EMD Biosciences) equilibrated in binding buffer (50 mM NaPi pH 8, 300 mM NaCl, 25 mM imidazole). Columns were washed with 10 volumes binding buffer, 4 volumes wash buffer (binding buffer with 40 mM imidazole) and fusion proteins eluted with 3 column volumes of elution buffer (binding buffer with 250 mM imidazole). The main elution fraction (1 mL) was loaded onto a 10 mL Sephadex G-50 column equilibrated and eluted with 50 mM sodium phosphate buffer, pH 7.5, 300 mM NaCl, 5% glycerol. Fractions containing protein were pooled, aliquotted and stored at −78 °C.

Because of solubility issues of the His_6-fusion proteins, expression plasmids for both *ydiC* and *ydiE* (pCL242 and pCL 245) were constructed as the N-terminal thioredoxin (trxA) fusions using the ligation independent cloning vector pET-32 Xa/LIC (Novagen/EMD Biosciences) according to the manufacturer protocol. Oligonucleotides used for PCR amplification for *ydiC* were 5′-GGTATTGAGGGT CGCATGACAATATTAGCAATTGAT-3′(N-terminal), and 5′-AGA GGAGAGTTAGAGCCCTACTTTTGACTTTCAATCCA-3′(C terminal), and for *ydiE* 5′-GGT ATTGAGGGTCGCATGAGTGAGCAAAAAGACATG-3′ (N-terminal) and 5′-AGAGGA GAGTTAGAGCCTTATCTCGTGAGACTTTGATA-3′ (C-terminal). To increase expression level and solubility, the fusion proteins were expressed by growing at 37 °C to A_{595} = 0.6, then cooling to 21 °C over 20 min, followed by addition of IPTG to 1 mM and incubating 12 h at the reduced temperature. Cells were collected by centrifugation (5000*g* 20 min at 4 °C), disrupted by sonication at a concentration of 1 mL in lysis buffer (10 mM Tris pH 7.5) per 50 mL culture. Fusion protein was purified on the basis of the His_6-tag using HisBind NTA resin similar to the procedure described above for YwlC and YdiB. The trx-YdiE fusion protein was soluble above 1 mg/mL, whereas the concentration of the trx-YdiC protein was kept below 0.7 mg/mL to prevent precipitation and aggregation.

RNA Substrates. RNA substrates were obtained by runoff transcription using T7 RNA polymerase as described previously.[38] DNA templates for the preparation of short stem-loop RNAs were single stranded oligodeoxynucleotides comprising the antisense of the desired RNA sequence and a 17-nt region that is the antisense of the T7 promoter. For example, the DNA template for *B. subtilis* $tRNA^{Thr}$ anticodon stem loop was 5′- ACTGATTACAAGTCAGTC CTATAGTGA GTC GTATTA-3′. These templates were used in transcription reactions with the 17 nt primer (5- TAATACGACTCACTATAG −3′) containing the T7 promoter sequence. To prepare unmodified tRNAs, two overlapping DNA oligonucleotides were used as templates after conversion to double stranded DNA by reverse transcriptase as previously described.[39] For example, DNA oligonucleotides used for *B. subtilis* $tRNA^{Thr}$ were 5′-TAATAC GACTCA CTATAG CCG GTGTAG CTC AATTGGTAG AGC AACTGA CTT GTA ATC −3′ and 5-TGGTGC CGG CAA GAG GACTTG AAC CCC CAA CCT ACT GAT TAC AAGTCA −3′. Transcription reactions contained 50 mM Tris, pH 7.8, 50 mM KCl, 10 mM DTT, 1 mM spermidine, 5 mM each of ATP, UTP,CTP, GTP, 35 mM $MgCl_2$, 1 μM template DNA, RNasin (0.02 U/μL), and T7 RNA Polymerase 0.1 U/μL. Reactions were incubated at 37 °C for 12−16 h. Template DNA was digested by the addition of RQ1 DNase (0.1 U/mL) for 1 h at 37 °C, and an equal volume of 7.5 M urea containing 0.5% bromophenol blue was added. This mixture was heated to 90 °C for 2 min and the RNA was purified on a 8% polyacrylamide slab gel. Bands containing RNA of interest were cut from the gel and eluted in 0.5 M NaCl at RT for 4 h. The gel slices were removed by filtration, the RNA was precipitated with 2.5 volumes of cold ethanol and after centrifugation (10000*g*, 30 min), the RNA was dissolved in water and its concentration measured by absorbance at 260 nm.

HPLC Assay for t^6A formation. Complete reaction mixtures (50 μL) contained 50 mM Tris pH 7.2, 20 mM $MgCl_2$, 25 mM KCl, 5 mM L-threonine, 2 mM ATP, 20 mM $NaHCO_3$, 20 μM tRNA substrate, and 1 μM of each enzyme when present. Reactions were typically incubated for 20 min at 37 °C, then stopped by addition of an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). The mixture was vortexed for 30 s and layers were separated by centrifugation at 14000*g* for 4 min. The top aqueous layer was added to a 1 mL column of Sephadex G-50 which had been drained by centrifugation at 7000*g* for 1 min. After addition of the aqueous layer to the column, the column was eluted by centrifugation as before. The resulting purified RNA was then digested to nucleosides with nuclease P1 and bacterial alkaline phosphatase and analyzed by HPLC according to the procedure of Gerhke et al.[40]

HPLC Assays for YwlC and YrdC Activity. Reaction mixtures typically contained 50 mM MOPS pH 7.5, 20 mM $MgCl_2$, 25 mM KCl, 2 mM ATP, 10 mM L-threonine, and 20 mM $NaHCO_3$ with varying amounts of YwlC or YrdC. After incubating at 25 °C, the reaction mixture was analyzed by HPLC on a Shimadzu HPLC with using a Supelco LC-18T column (25 mm × 4.6 mm) and UV detection at 260 nm. For analytical measurements of product concentrations, a phosphate buffer system was employed consisting of 20 mM potassium phosphate pH 6 (Buffer A) and Buffer A with 20% methanol (Buffer B). The gradient used was 100% A from 0 to 12 min followed by a linear gradient of 0−100% B from 12 to 30 min. Product concentrations were measured by peak integration and compared to a standard curve of ATP or AMP to convert to the number of moles. For kinetic assays, product concentrations were measured during the linear part of the progress curve (typically 30 s) during which less than 10% product has formed. Plots of rate vs substrate concentration were plotted and fit to Michaelis−Menten kinetics using Kaleidagraph graphics software (Synergy). Each rate measurement was the average of two or more measurements.

Preparative Scale Reactions for the Production of TC-AMP. For the preparation of TC-AMP, 400 μL reaction mixtures described above containing 90 μg of YwlC and 15 units of inorganic pyrophosphatase were incubated at 25 °C for 11 min and purified by a single injection using the HPLC system described for the assays. Collected samples were immediately frozen on dry ice and could be stored at −78 °C . When samples devoid of salt were required (e.g., for mass spectrometry), repurification of carefully concentrated samples was carried out by HPLC using volatile buffers. Buffer A

contained 2 mM triethylammonium bicarbonate (TEAB), pH 7 while Buffer B consisted of Buffer A containing 20% methanol. Typical yields of isolated TC-AMP were ca. 40 nmol for each 400 μL reaction.

Characterization of TCAMP by Mass Spectrometry. Samples of TC-AMP were obtained as follows. Product collections from HPLC purification of four separate 400 μL reactions using TEAB buffers were pooled to give approximate 8 mL of 20 nmol of product. This solution was transferred to a 50 mL round-bottom flask on ice and solvent was removed by lyophilization on ice over 4 h. Cold water (0.5 mL) was added to the flask to dissolve the residue followed by another round of lyophilization on ice. A final 100 μL of cold water was added and the solution was stored at −78 °C for analysis by mass spectrometry. A portion of the final sample was analyzed by HPLC and found to be 80% TC-AMP with 20% AMP. This material was diluted 20-fold with a 1:1 mixture of acetonitrile/water and analyzed by mass spectrometry using a MaXim ultra high resolution QTOF mass spectrometer in negative ion mode.

^{1}H NMR Characterization of TC-AMP. For NMR characterization, TC-AMP isolates (12 × 400 μL reactions) from HPLC using the phosphate buffer system described above were pooled (approximately 35 mL) in a 100 mL pear shaped flask and concentrated by careful rotary evaporation using a vacuum pump. The solution of TC-AMP was kept cold but not frozen during the concentration. After removal of the solvent, cold deuterium oxide (D_2O, 4 mL) was added to dissolve the resulting solid and the evaporation repeated. Finally the solid was dissolved in 0.4 mL of cold D_2O. The final solution contained 0.4 mL of a 0.64 mM solution that also contained ca. 0.5 M KPi at pH 6. HPLC analysis showed this material to be 96% TC-AMP and 4% AMP. An initial ^{1}H NMR spectrum (128 transients) of this solution was obtained using a Varian Inova-500 instrument at 500 MHz using a HCX probe running at 10 °C. This material gave the following ^{1}H NMR signals that have been attributed to TC-AMP (H-4′ of the ribose is obscured by the solvent peak): 1.072 (d, 3H, CH_3, J = 6.5 Hz), 3.659 (d, 1H, H-β, J = 4.0 Hz), 4.191 (m, H-5′, 2H), 4.120 (m, H-α, 1H), 4.334 (m, H-3′, 1H), 4.441 (app t, H-2′, 1H), 6.089 (d, 1H, H-1′, J = 6 Hz), 8.226 (s, 1H, H-2), 8.409 (s, 1H, H-8).

Characterization of TC-AMP Decomposition. The NMR sample described above was allowed to warm to 25 °C and the decomposition was monitored by periodic analysis by both HPLC and ^{1}H NMR. After 44 h, decomposition was complete and final products were identified by the addition of authentic samples. Separate solutions of L-threonine and (4S, 5R)-5-methyl-2-oxazolidinone-4-carboxylic acid (4 μL of 50 mM solutions in 0.5 M potassium phosphate pH 6 in D_2O) were added to the decomposed TC-AMP sample in the NMR tube. After the addition of each authentic sample, a spectrum was acquired for comparison with the original decomposed sample. The rate of TC-AMP decomposition was measured by integration of peaks for TC-AMP using HPLC conditions described above.

Preparation of Putative TC-AMP Decomposition Products. *trans*-(4*S*,5*R*)-5-Methyl-2-oxazoldinone-4 carboxylic acid (L-Thr-ox) was prepared as previously described[41] and the ^{1}H NMR data (DMSO-d_6) matched reported values:[34] 1.33 (d, 3H, J = 6.4 Hz), 3.92 (dd, 1 H, J = 0.8, 5.2 Hz), 4.53 (app hextet, 1 H), 8.04 (bs, 1 H).

L-Threonine *N*-carboxyanhydride (L-Thr-NCA) was prepared in two steps. In step one, the *t*-butyl ether of L-Thr-NCA was prepared as described[42] by refluxing L-Thr (250 mg, 1.42 mmol) and triphosgene (338 mg, 1.14 mmol) in THF (12.5 mL) for 3 h and then precipitating the concentrated residue with hexane. This procedure produced a 5:1 mixture of the desired L-Thr-NCA *tert*-butyl ether (major) and L-Thr-ox (minor) via loss of the *tert*-butyl ether. A portion of this material (86 mg) was dissolved in 10% trifluoroacetic acid in dichloromethane (3 mL) and stirred for 1 h at room temperature to give a similar mixture of L-Thr-NCA and L-Thr-ox that could be used as a standard. ^{1}H NMR data for L-Thr-NCA (DMSO-d_6): 1.10 (d, 3H, J = 6.4 Hz), 3.93 (m, 1 H, overlaps with L-Thr-ox signal), 4.29 (dd, 1H, J = 1.0, 2.0 Hz), 9.02 (bs, 1 H).

Measurement of TC-AMP stability. Reaction mixtures (400 μL) containing 50 mM Tris pH 7.3, 20 mM $MgCl_2$, 25 mM KCl, 1.5 mM ATP, 8 mM L-Thr, and 28 μg YwlC were incubated at 25 °C for 20 min then injected onto HPLC using the phosphate buffer system described above. TC-AMP was collected in a typical volume of ca. 0.7 mL (ca. 5 μM) and was immediately incubated in a water bath at the desired temperature. The composition of the HPLC buffer eluent containing the purified TC-AMP was 20 mM potassium phosphate pH 6.5 containing approximately 20% methanol. Aliquots (50 μL) of the incubation mixture were withdrawn at various time points and frozen immediately on dry ice for later quantitation by HPLC. Concentrations of TC-AMP and AMP were measured by integration of peaks in the HPLC chromatograms detected by absorbance at 260 nm and calibrated using known AMP standards. The value of A_{260} for TC-AMP solutions was found to change by less than 1% after hydrolysis to AMP. Plots of ln [TC-AMP] vs time were linear, and (pseudo) first order rate constants were obtained from the slope of the linear fit. Subsequent stability studies used purified and concentrated TC-AMP in which the pH was adjusted with 1 N KOH and/or buffer and/or enzyme added to the desired concentrations. In these latter experiments, reaction mixtures (final volume 150 μL) without added TC-AMP were equilibrated to the required temperature in a circulating water bath and decomposition reactions were initiated by addition of 8.5 μL of an 88 μM solution of TC-AMP. Aliquots of 25 μL were removed and frozen as described above.

Conversion of TC-AMP into t^{6}A by YdiBCE. Reaction mixtures contained 50 mM Tris pH 7.5, 20 mM $MgCl_2$, 25 mM KCl, 20 μM TC-AMP and 16 μM unmodified *B. subtilis* $tRNA^{Thr}$ transcript in a final volume of 30 μL. When included in the reaction, ATP was 2 mM and YdiB, YdiC, and YdiE each were 5 μM. Reactions were incubated at 37 °C for 20 min, then stopped by addition of 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1) and vortexed for 30 s. RNA was isolated and analyzed for the presence of t^{6}A as described above for the t^{6}A HPLC assay.

RESULTS

Cloning and Purification of *B. subtilis* Proteins Required for t^{6}A Biosynthesis. Cloning and expression of *ywlC* and *ydiB* as the His_6 N-fusion proteins were efficient, and these proteins are highly expressed and soluble in *E. coli* BL21(DE3). Unfortunately, expression of *ydiC* and *ydiE* gave protein that was found largely in inclusion bodies. After much effort, solubility problems were finally solved by the construction of N-terminal thioredoxin (trxA) fusions, which allowed good expression and sufficient solubility for *in vitro* studies. Even with this tag, expression had to be performed at

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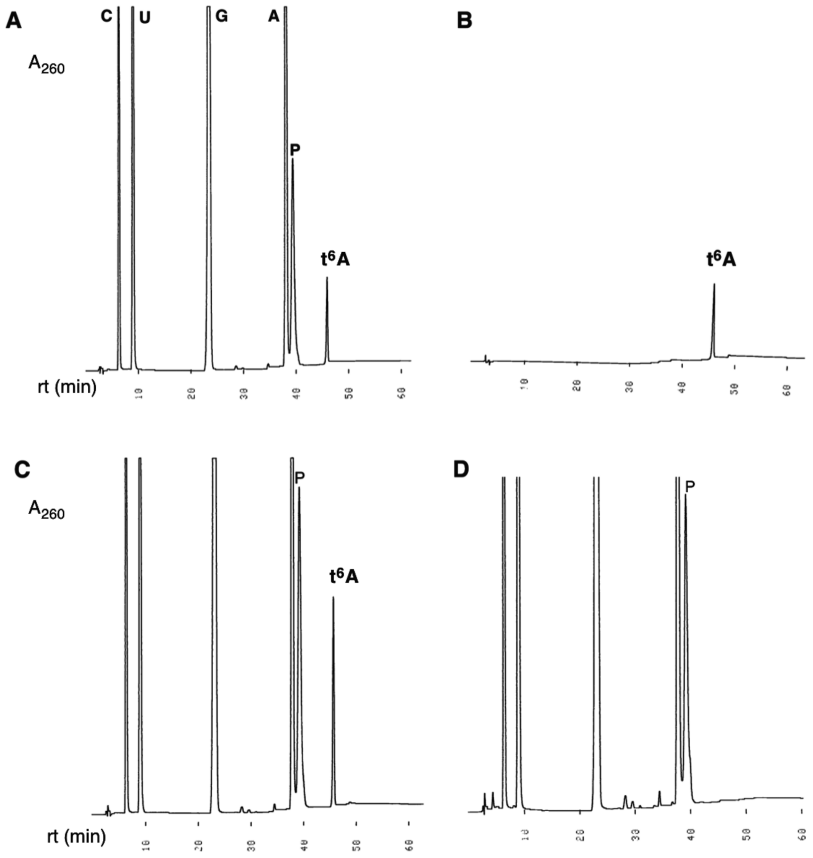

Figure 1. HPLC analysis of t^6A formation *in vitro*. (A) complete reaction mixture with unmodified $tRNA^{Thr}$ substrate (B) authentic sample of 0.5 nmol synthetic t^6A (D) coinjection of full reaction mixture with authentic t^6A. (D) complete reaction mixture with A37C mutant $tRNA^{Thr}$. The peak labeled "P" corresponds to a variable amount of phenol which passes through the G-50 spin column.

21 °C overnight for good yields and the YdiC fusion protein could not be stored above 0.7 mg/mL without precipitation. SDS gel analysis of the purified proteins loaded separately compared to a mixture that was incubated in the t^6A assay buffer at 37 °C for 4 h (Figure S2) showed that the *B. subtilis* enzymes appear to be stable in the reaction buffer for this length of time. Attempts to purify the native forms of YdiC and YdiE by specific cleavage of the fusion proteins with thrombin or Factor Xa protease led to multiple products, perhaps because the proteins are not completely stable in the cleavage reaction conditions. El Yacoubi et al.[18] found that *E. coli ygjD* mutants could only be complemented by expression of *B. subtilis ydiE* in combination with *ydiC*. However, attempts to express and/or purify combinations of the proteins without fusions as a soluble complex have been unsatisfactory thus far. Since the TrxA-fusion proteins were found to be catalytically active and showed the predicted RNA substrate specificity (*vide infra*) for t^6A formation, all experiments in this report are for the purified fusion proteins.

Characterization of Requirements and RNA Substrate Specificity for t^6A Biosynthesis in vitro. With purified enzymes in hand, reconstitution experiments were conducted using unmodified *B. subtilis* $tRNA^{Thr}$ as substrate. Reactions included Mg-ATP, L-threonine, bicarbonate and Tris buffer in addition to various combinations of proteins. Following reaction, the RNA was isolated, then enzymatically digested to nucleosides and fractionated on HPLC to identify t^6A levels. Figure 1 shows HPLC traces of the representative reaction mixtures. The full reaction mixture (Figure 1A) shows a peak present at 46 min in the chromatogram, which is the expected location of t^6A in results in approximately 90% modification of the tRNA transcript in 20 min at 37 °C. The peak at 46 min in the chromatogram was confirmed as t^6A by coinjection (Figure 1B,C) with an authentic sample prepared synthetically.[43] The high level of activity is somewhat surprising since *E. coli* YrdC was found to have poor affinity for unmodified $tRNA^{Thr}$ transcript.[17] Similar to the recently reported data for t^6A synthesis in *E. coli*, omission of any one of the four *Bacillus* proteins results in complete absence of t^6A formation (Table 1). Modification was also found to require L-threonine, Mg-ATP and $NaHCO_3$, although more extreme measures (degassing and addition of PEP carboxylase/PEP) were required to reduce the bicarbonate/CO_2 levels to show a significant decrease in t^6A modification activity.

A brief study of the RNA substrate specificity (Table 1) shows that the expected substrates $tRNA^{Thr}$(GGU) and $tRNA^{Lys}$(UUU) are modified, whereas $tRNA^{Phe}$(GAA) is not modified. Mutation of the normally modified A37 of substrate $tRNA^{Thr}$ to a C leads to complete loss of activity (Figure 1D), whereas a mutation of A36 to U in $tRNA^{Phe}$(GAA) to give $tRNA^{Phe}$(GAU) leads to full activity as a substrate. Small stem loop RNAs based on $tRNA^{Lys}$ or $tRNA^{Thr}$ sequences showed no activity in the *in vitro* assay even though YrdC has been shown to bind tightly to these constructs.[44] These results are consistent with the work of both Ames et al.,[45] who proposed that a major substrate determinant is a U at the last codon position 36 and Morin, et al.,[46] who found in oocytes that in addition to U36, the overall L-shaped architecture is required.

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Table 1. Requirements for *in vitro* t^6A Formation in *B. subtilis*

reaction components or RNA substrate	t^6A formation
complete reaction mixture[a]	+++
−L-threonine	(−)
−ATP	(−)
$-HCO_3^-$	+
−tRNA	(−)
−YwlC	(−)
−YdiB	(−)
−YdiC	(−)
−YdiE	(−)
$tRNA^{Lys}$	+++
$tRNA^{Thr}$A37C	(−)
$tRNA^{Phe}$	(−)
$tRNA^{Phe}$A36U	+++
$tRNA^{Thr}$ ACSL	(−)
$tRNA^{Lys}$ ACSL	(−)

[a]Complete reaction mixture contains Tris pH 7.2, 20 mM $MgCl_2$, 25 mM KCl, 2 mM ATP, 20 mM $NaHCO_3$, 20 μM $tRNA^{Thr}$, and 1 μM each of YwlC, YdiB, YdiC and YdiE. ACSL, anticodon stem loop.

Thus, the *in vitro* reconstitution experiments contained the minimum components required to mimic known *in vivo* characteristics of t^6A biosynthesis.

Characterization of the ATPase Activity of YwlC and YdiBCE. Initial experiments used TLC analysis of reactions containing α-^{32}P-labeled ATP to characterize the ATPase activity of the four individual *B. subtilis* proteins. These results corroborate similar experiments recently reported by Deutsch, et al.[29] for the biosynthesis of t^6A in *E. coli*. The results of experiments with the *B. subtilis* enzymes can be summarized briefly as follows: YwlC catalyzes L-threonine-dependent hydrolysis of ATP to give AMP. The previously documented YdiB-catalyzed hydrolysis of ATP to give ADP is significantly activated by addition of both YdiC and YdiE. When the full reaction mixture containing all components was analyzed, similar amounts of AMP and ADP were formed by the action of YwlC and YdiBCE, respectively. However, neither of the ATPase reactions appeared to be dependent on tRNA or on each other. This made formulation of a coherent initial biochemical model difficult.

YwlC Catalyzes the Formation and Release of L-Threonylcarbamoyl-AMP (TC-AMP). To better quantify the ATPase reactions catalyzed by YwlC and YdiBCE, HPLC assays were developed. An analytical HPLC system was employed that could separate ATP, AMP and ADP sufficiently for quantitation using phosphate buffer with a methanol gradient. Consistent with the TLC results, reactions of YwlC with millimolar concentrations of Mg-ATP, bicarbonate and L-threonine showed clear production of AMP; but reproducible formation of an unidentified product with a longer retention time was also observed. Figure 2A shows a representative chromatogram of the YwlC reaction indicating an unidentified peak at 21 min under the stated HPLC conditions. Formation of this product was dependent on the presence of YwlC, L-threonine and ATP, and its formation was proportional to the amount of YwlC used. Initial observations suggested this material was an adenylate species. First, when α-^{32}P-labeled ATP was used in the reaction mixture, the product collected at 21 min was radiolabeled with specific activity consistent with the amount of UV absorbance (data not shown). Second, as shown in Figure 2B, reinjection of isolated material that was stored at room temperature for varying amounts of time showed degradation to AMP.

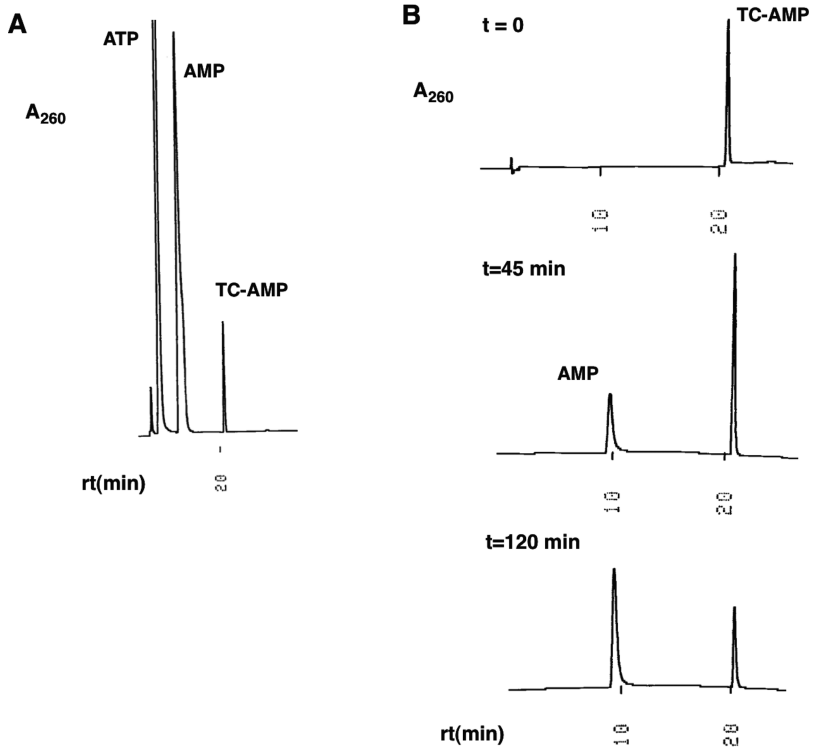

Figure 2. (A) Typical HPLC trace of YwlC catalyzed formation of AMP and TC-AMP. (B) Example time course of decomposition of freshly isolated TC-AMP to give AMP.

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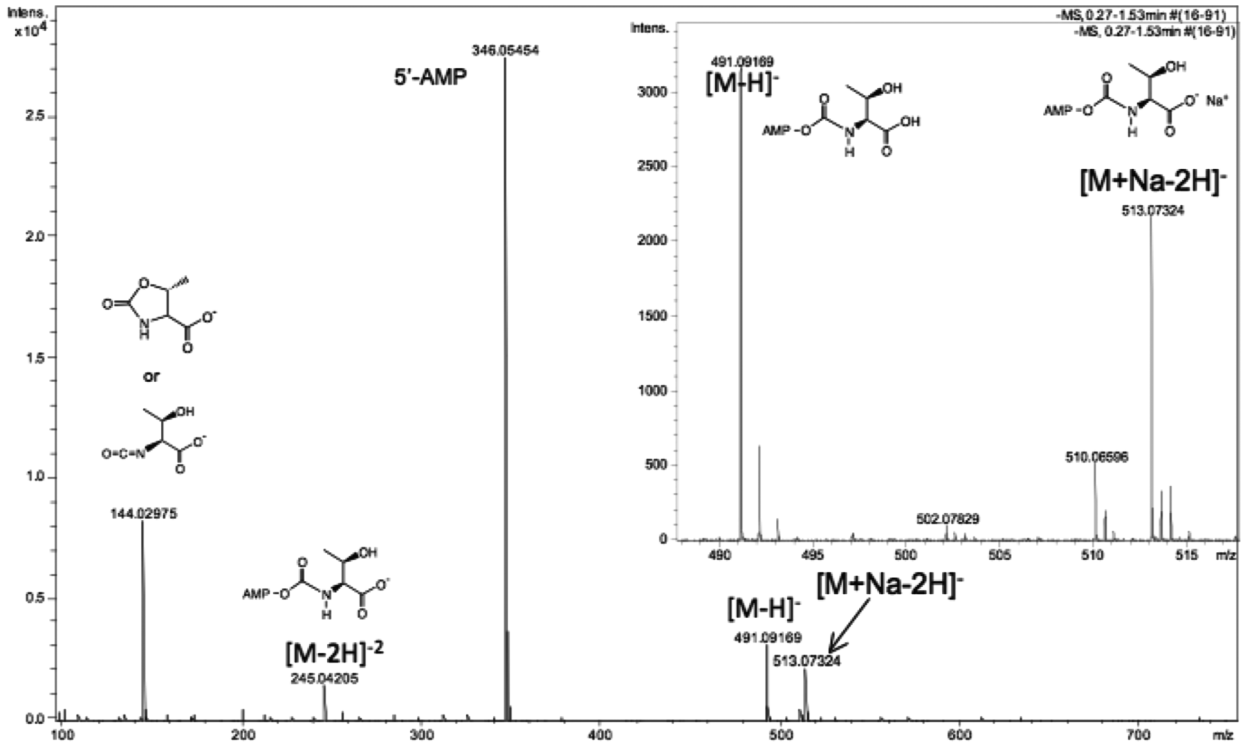

Figure 3. Mass spectrum of purified TC-AMP. Peaks are visible that are consistent for monoanion (M−H) at m/z = 491, the dianion (M−2H) at m/z = 245 and the dianion monosodium salt (M−2H Na^+) at 513. Also visible is AMP (M−H) at m/z = 346 and a degradation product at m/z = 144.

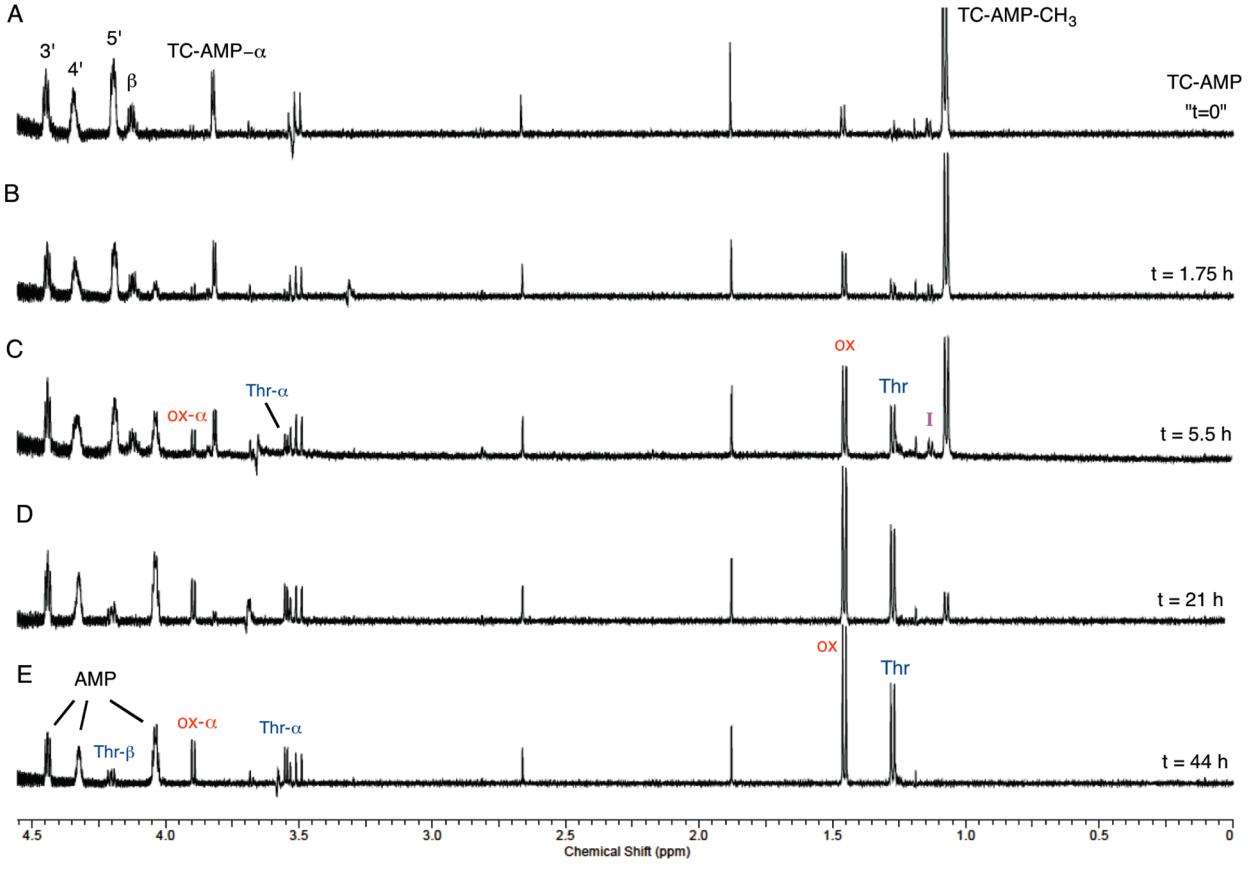

Figure 4. 500 MHz ^{1}H NMR spectrum of purified TC-AMP and its decomposition at 25 °C. The "t = 0" spectrum (A) is of the initial purified, concentrated TC-AMP sample with resonances labeled that are consistent with the proposed structure. The t = 44 h spectrum (E) shows the final decomposition products and AMP. The doublet labeled "I" at 1.1 ppm in spectrum C is a possible intermediate in the decomposition. Resonances downfield are omitted for clarity.

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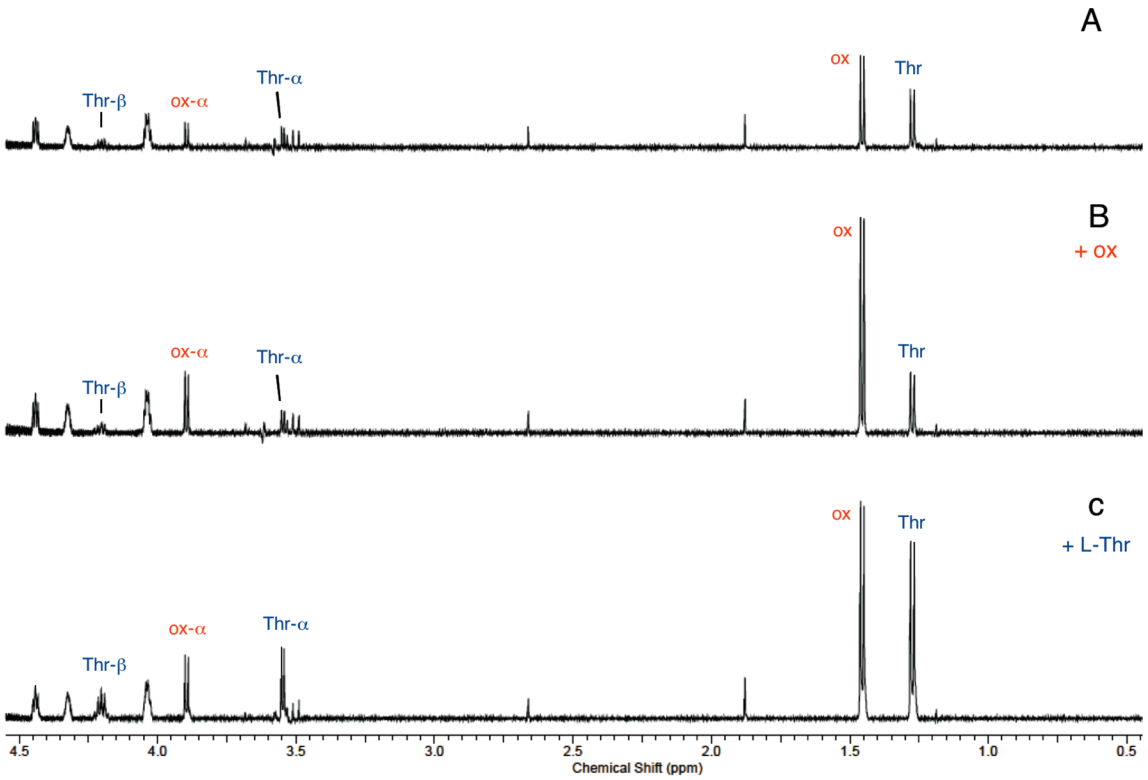

Figure 5. Identification of TC-AMP decomposition products by addition of known standards. (A) Final spectrum of decomposition of TC-AMP, (B) addition of mol of L-Thr-ox, (C) addition of mol of L-Thr.

After scaling up reactions to isolate more material, further characterization was achieved by mass spectrometry and ^{1}H NMR. Figure 3 shows the MS data for purified desalted material that was approximately 80% pure. Along with AMP (M-H m/z = 346), a known degradation product, there is observation of a negatively charged species with a measured mass consistent with the molecular formula $C_{15}H_{21}NO_6$ (theoretical 491.093316; found 491.09169; difference 3.3 ppm). This is the molecular formula for L-threonylcarbamoyl-AMP (TC-AMP). In addition to the M-H peak at m/z = 491, peaks are observed that correspond to the dianion M-2H as well as the monosodium dianion M-2H+Na. Interestingly, the mass spectrum also shows a relatively strong peak at m/z = 144.02975. This mass is consistent with a number of possible decomposition products that lack AMP. These include L-threonine isocyanate (L-Thr-NCO), the isomeric oxazolidinone, (4*S*,5*R*)-5-methyl-2-oxazoldinone-4-carboxylic acid (L-Thr-ox), and L-threonine-N-carboxyanhydride (L-Thr NCA), all of which have a calculated m/z = 144.02968. L-Threonine itself was not observed in the mass spectrum under these conditions perhaps because it exists mainly as the neutral zwitterion.

NMR Characterization of TC-AMP and Its Decomposition Products. Final characterization of TC-AMP and its degradation products by ^{1}H NMR was achieved by purification of the product from optimized large-scale enzymatic reactions. The addition of inorganic pyrophosphatase to the YwlC reaction mixtures resulted in a several-fold increase in the final concentration of TC-AMP. The optimum YwlC concentration was 5.75 μM and the minimum amount of pyrophosphatase required was 15 units in a 400 μL reaction. Further increases in the concentration of either enzyme did not increase the yield of TC-AMP. Careful concentration of pooled collections from HPLC purification gave 0.5 mL of a 640 μM solution of TC-AMP in ca. 0.5 M KPi, pH 6 that was approximately 90% pure. The 500 MHz ^{1}H NMR spectrum of TC-AMP at 10 °C shows signals for both the threonine and AMP groups consistent with the proposed structure. All peaks have been assigned and are consistent with the TC-AMP structure except for the ribose 2′ proton, which is obscured by the solvent peak.

To identify the products of TC-AMP decomposition, the NMR sample described above was allowed to decay at 25 °C. Periodically, aliquots were removed for HPLC analysis and a ^{1}H NMR spectrum was recorded. Representative spectra of the decomposition are shown in Figure 4. The downfield region of the spectrum, which includes peaks for the ribose H1′ and adenine H2 and H8 protons are omitted for clarity. The spectra are consistent with the formation of three final products: AMP and two additional threonine-like products. The final decomposition products were identified by doping the NMR solution with authentic samples of known compounds. As mentioned above, the MS spectra of a partially degraded TC-AMP solution showed a peak at m/z = 144 suggesting the known oxazolidinone L-Thr-ox as a possible breakdown product. This compound was synthesized,[41] purified and characterized, and added to the degraded TC-AMP NMR sample. Figure 5A shows a spectrum of the final decomposition mixture, while Figure 5B shows a spectrum of the same mixture after the addition of L-Thr-ox, showing matched and increased signals of one of the major decomposition products. In a similar manner, the other product was identified as L-threonine (Figure 5C). The signals downfield in the spectrum matched an authentic sample of AMP (not shown), which is consistent with the identification of AMP by HPLC.

An additional species in the decomposition mixture, identified by a doublet at ca. 1.1 ppm (indicated by **I** in Figure 4C), is visible from the start of the decomposition and present

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at a nearly constant ratio to TC-AMP throughout the reaction before decaying completely. It is possible that this species is an intermediate in the decomposition. Possible candidates for **I** are the isocyanate L-Thr-NCO, and the *N*-carboxyanhydride, L-Thr-NCA. The isocyanate has not been described to date. L-Thr-NCA was prepared using a literature method.[42] In DMSO, L-Thr-NCA is relatively stable, decaying directly to the oxazolidinone with a half-life of about 2 days. However it was found to decompose rapidly ($t_{1/2}$ ca. 5 min) in 0.5 M KP_i pH 6 to give an approximately 60:40 mixture of the L-Thr-ox and L-threonine. Because of the short half-life, it would not be expected to accumulate in the reaction. Moreover, the ^{1}H NMR spectrum of the NCA in 0.5 M KP_i does not appear to match that of **I** (data not shown). It is possible that **I** is L-Thr-NCO, but this has yet to be confirmed. Together the data suggest that TC-AMP in phosphate buffer at pH 6 degrades to a mixture of AMP, L-threonine, and the oxazolidinone, which are formed directly or through one or more intermediates.

Kinetic Stability of TC-AMP. The rate of decomposition of TC-AMP to AMP was initially measured directly after HPLC purification from scaled up reaction mixtures. Eluted TC-AMP was incubated at the desired temperature and aliquots taken at specific time points were frozen and subsequently analyzed by HPLC (cf., Figure 2B). The HPLC data fit well to (pseudo) first order kinetics. In subsequent experiments, purified concentrated TC-AMP solution was added to temperature equilibrated reaction mixtures under a variety of conditions. Data and reaction conditions are summarized in Table 2, in

Table 2. Conditions and Reported Half-Life for Decomposition of TC-AMP

buffer	$MgCl_2$ (mM)	pH	temp (°C)	$t_{1/2}$[e]
0.5 M KPi[a]	0	6.0	25	8.5 h
20 mM KPi[b]	0	6.5	25	4.0 h
20 mM KPi[b]	0	6.5	37	1.16 h
20 mM KPi[b]	0	7.5	37	16.5 min
50 mM MOPS[c]	20	7.5	25	11.8 min
MOPS/YwlC[d]	20	7.5	25	14.4 min
MOPS/YrdC[d]	20	7.5	25	19.3 min
50 mM MOPS	2	7.5	37	3.5 min
50 mM MOPS	5	7.5	37	1.7 min
50 mM MOPS	20	7.5	37	21 s

[a]In D_2O, single measurement. [b]Contains ca. 20% methanol. [c]Reactions with MOPS contained 25 mM KCl. [d]0.5 μM enzyme. [e]Average of at least two experiments.

which the rates are given as the calculated half-life. Not surprisingly, TC-AMP is destabilized by increases in pH, temperature or Mg^{2+} concentration. Incubation of TC-AMP with either YwlC or YrdC resulted in no increase in the rate of decomposition, and in fact showed a small but statistically significant protective effect that could be due to sequestration and/or binding in a stable conformation. Thus, alone these enzymes do not appear to catalyze further transformation of TC-AMP into a more reactive product, such as the isocyanate.

Production of TC-AMP By Both YwlC and *E. coli* YrdC Does Not Proceed via An Activated Bicarbonate Intermediate. The progress curves of TC-AMP formation by YwlC and its *E. coli* ortholog YrdC were studied. As shown in Figure 6A, the reaction catalyzed by YwlC produces a significant molar excess of AMP relative to TC-AMP, even at reaction times as short as 20 s. At first, this was not surprising

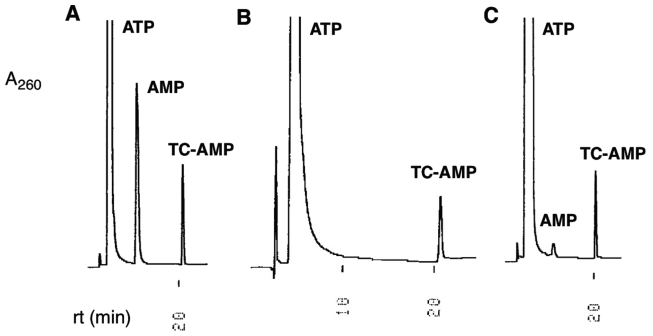

Figure 6. AMP formation during synthesis of TC-AMP by YwlC and YrdC. (A) YwlC reaction (t = 2 min) (B) YrdC reaction (t = 15 s) (C) YwlC reaction (t = 2 min) in the presence of inorganic pyrophosphatase. Concentrations of the other substrates were saturating.

because of the expected formation of an activated bicarbonate intermediate, such as carboxy-AMP, which would be easily hydrolyzed if released or exposed to water in the absence of the other t^{6}A enzymes. However, the reaction catalyzed by YrdC was found to form almost no AMP at short reaction times (Figure 6B). Moreover, addition of inorganic pyrophosphatase to the YwlC reactions drastically reduces AMP production to significantly below one molar equivalent relative to TC-AMP at reaction times of a few minutes or less (Figure 6C).

A plot of the progress curves for YwlC formation of TC-AMP in the absence and presence of inorganic pyrophosphatase illustrates the magnitude of the effect further. In the absence of pyrophosphatase, the production of TC-AMP levels off while AMP continues to be produced at an approximately linear rate (Figure 7A). Addition of pyrophosphatase (Figure 7B) inhibits AMP production to levels below that of TC-AMP for a few minutes, upon which TC-AMP production begins to level off and AMP rises. The reason for the dependence of AMP formation on pyrophosphate concentration is not clear at present.

However, the observation of substoichiometric amounts of AMP produced in the reaction by both YrdC and YwlC suggests that the formation of TC-AMP does not require an ATP-activated bicarbonate intermediate. This implies that the enzymes utilize ATP only for the formation of TC-AMP from L-threonine carbamate, the latter of which can be formed directly from the amino acid and CO_2. The very low levels of AMP produced by YrdC at short reaction times allowed an estimation of the kinetic parameters for ATP as the variable substrate. With concentrations of L-Thr at 10 mM and bicarbonate at 20 mM, values of K_m = 93 ± 15 μM and k_{cat} = 0.10 ± 0.02 s^{-1} for ATP were found.

YwlC and YrdC Catalyze Efficient Formation of ATP from TC-AMP and PP_i. Purification of TC-AMP allowed the study of the reverse reaction with PP_i to form ATP. Figure 8 shows HPLC analysis of reaction mixtures containing TC-AMP, PP_i, $MgCl_2$ containing buffer and either YrdC or YwlC. Compared with the control reaction without enzyme, both YwlC and YrdC catalyze formation of ATP with no hydrolysis to AMP above background levels. This was surprising, since pyrophosphate is clearly shown to promote formation of AMP by YwlC in the presence of saturating ATP and L-threonine. In addition, the concentration of YrdC used in the reactions shown in Figure is over 600-fold less than that for YwlC, indicating a relatively large value of k_{cat} for YrdC in the reverse

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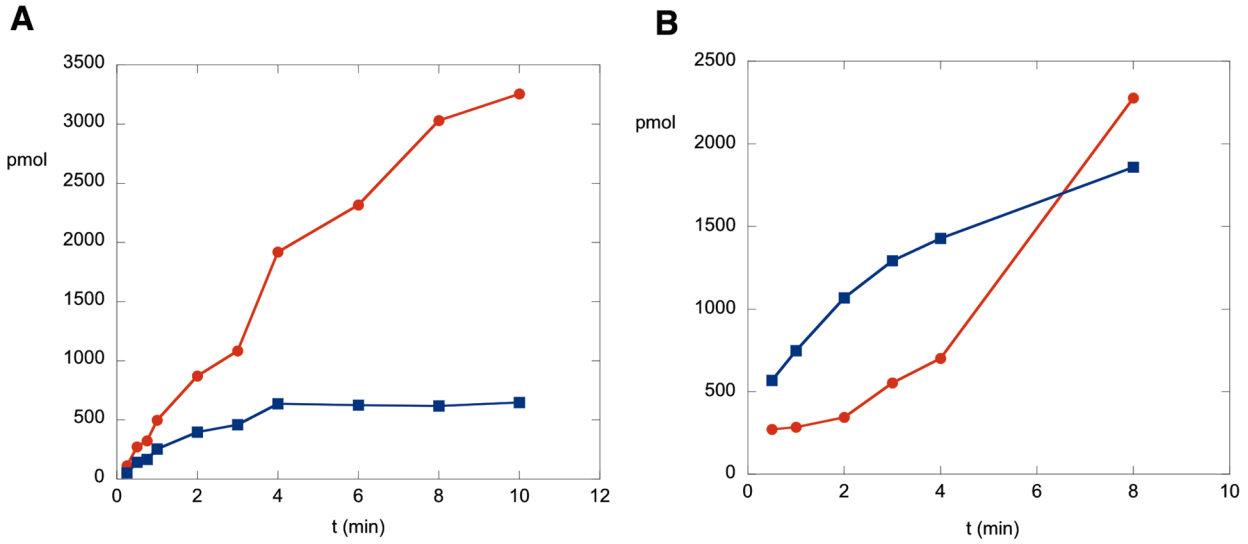

Figure 7. Progress curve plots for YwlC catalyzed formation of TC-AMP in the (A) absence of inorganic pyrophosphatase and (B) presence of pyrophosphatase. For both plots the number of pmol of AMP (●) and TC-AMP (■) are shown vs time.

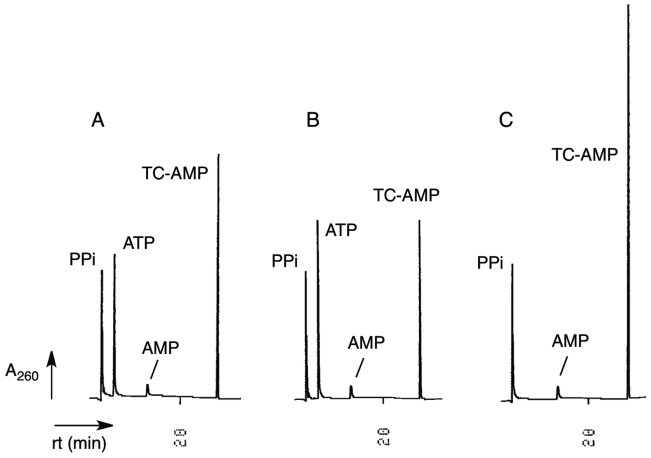

Figure 8. Formation of ATP from TC-AMP catalyzed by YwlC and YrdC. Reactions (50 μL) contained 5 μM TC-AMP and 1 mM PPi in buffer with either (A) 0.11 pmol YrdC; (B) 76 pmol YwlC; or (C) no enzyme. Reactions were incubated for 2 min at 25 °C and analyzed by HPLC.

direction. With TC-AMP as the variable substrate and 1 mM PPi with 30 s reaction times, the following kinetic constants were measured for YrdC: k_{cat} = 20 ± 2 s^{-1}; K_m = 0.68 ± 0.15 μM; k_{cat}/K_m = 3.3 ± 1.1 × 10^7 M^{-1} s^{-1}. The apparent low value for K_m for TC-AMP prevented accurate measurement of kinetic constants for YwlC.

YdiBCE Catalyzes Formation of t^6A from TC-AMP in the Absence of Both YwlC and ATP. To answer the question of whether TC-AMP is a true productive intermediate for t^6A biosynthesis, we incubated this product with the YdiBCE complex and unmodified $tRNA^{Thr}$. Reaction of 12.5 μM $tRNA^{Thr}$ with 20 μM purified TC-AMP and 5 μM YdiBCE for 20 min at 25 °C gave efficient t^6A formation as measured by HPLC analysis of the recovered and digested tRNA. Since these reactions did not contain L-threonine, this experiment is consistent with the assigned structure of TC-AMP and also supports the hypothesis that it is a productive intermediate in the biosynthetic pathway. As shown in Table 3, formation of t^6A required all three proteins of the YdiBCE complex,

Table 3. Requirements for the Synthesis of t^6A from TC-AMP

reaction components	t^6A formation (pmol)[a]
YdiBCE + ATP + TC-AMP + $tRNA^{Thr}$ (complete)	276 ± 88
complete − ATP	353 ± 58
complete − TC-AMP	nd[b]
complete − YdiB	nd
complete − YdiC	nd
complete − YdiE	nd

[a] 15 min reaction at 37 °C. [b] nd, none detected.

consistent with the overall requirements in the initial characterization experiments. Interestingly, ATP is not required for this step of the reaction, and in fact, seems to suppress the modification somewhat, although the data are not statistically significant. HPLC data confirm earlier TLC analysis that showed ATP hydrolysis to ADP occurs at a roughly similar rate to AMP production during t^6A formation in the full reaction mixture (Figure 9). An estimate of the turnover rate for YdiBCE-catalyzed conversion of TC-AMP to give t^6A gave a value of 0.19 ± 0.06 min^{-1} using 10 μM TC-AMP. This can be compared to the measured rate of 0.40 ± 0.1 min^{-1} for the overall reaction starting with L-Thr, bicarbonate and ATP. The observed slightly slower turnover could be evidence for

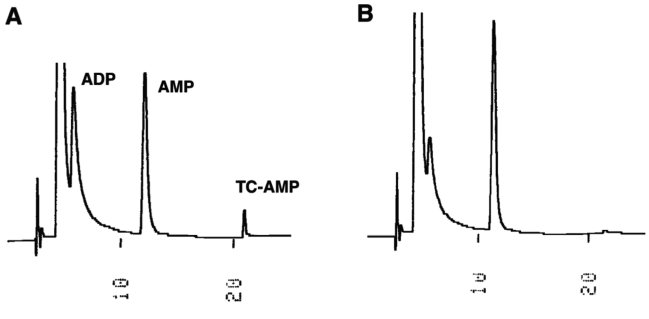

Figure 9. HPLC analysis of TC-AMP production in the full reaction mixture with and without substrate tRNA. (A) Full reaction components with $tRNA^{Thr}$A37C mutant (B) same as A, but with $tRNA^{Thr}$ wt substrate.

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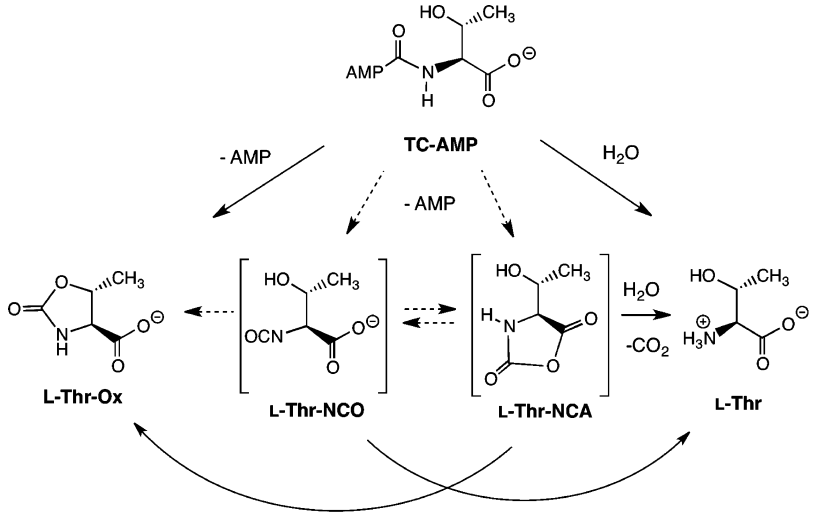
Scheme 2. Decomposition Pathways for TC-AMP in Aqueous Media



channeling, although the data must be interpreted as preliminary because of the large fusion constructs used in these experiments.

Analysis of the complete reaction mixture by HPLC (Figure 8) shows a marked effect of the presence of tRNA substrate on the appearance of TC-AMP in the reaction mixture. In the absence of tRNA (or in the presence of a $tRNA^{Thr}$ A37C mutant), there is a significant buildup and release of the intermediate (Figure 8A). Presence of substrate tRNA in the reaction suppresses this buildup and gives a noticeable increase in AMP production (Figure 8B). This is further evidence that TC-AMP is an intermediate in the modification reaction.

DISCUSSION

The experiments described in this work focus on both the chemical and biochemical characterization of L-threonylcarbamoyl-AMP (TC-AMP), an intermediate in the biosynthesis of t^6A. Evidence has been presented in this work for the role of YwlC/TsaC (and its *E. coli* ortholog YrdC) as a TC-AMP synthetase. TC-AMP has been isolated, fully characterized and its stability measured under physiological conditions. The measured half-life for TC-AMP of 3.5 min in phosphate buffer at 37 °C, pH 7.5, 2 mM $MgCl_2$ is in the lower range of kinetic stability for other biologically relevant phosphoric anhydrides. For example, carbamoyl phosphate has a half-life of 45 min at pH 7.8 at 37 °C.[47] Since this measurement is in the absence of Mg^{2+}, this value should be compared to a half-life of 16.5 min for TC-AMP under similar conditions (Table 2). The relatively faster degradation of TC-AMP is not surprising, given its multiple possible intramolecular degradation pathways, and greater free energy ($\Delta G'^{\circ}$) of hydrolysis. Acyl adenylates are known to have greater $\Delta G'^{\circ}$ values for hydrolysis compared with the corresponding acyl phosphates.[48,49]

By measuring the values of k_{cat}/K_m for both the formation of TC-AMP and its reverse reaction to give ATP, it is possible to use the Haldane relation (eq 1):

$$K_{eq} = \frac{(k_{cat}/K_m)_{fwd}}{(k_{cat}/K_m)_{rev}} \quad (1)$$

to estimate the equilibrium constant for this reaction.[50] Using these values (k_{cat}/K_m forward = 1.1×10^3 M^{-1} s^{-1}; k_{cat}/K_m reverse = 3.3×10^7 M^{-1} s^{-1}), an estimated value for $K_{eq} = 3.3 \times 10^{-5}$ is found in favor of ATP formation. This corresponds to a value of $\Delta G = -25.6$ kJ/mol. By adding to this the reported updated value of $\Delta G'^{\circ} = -45.6$ kJ/mol for the hydrolysis of ATP to give AMP and PPi,[48] an estimated value of $\Delta G'^{\circ}$ for TC-AMP hydrolysis to AMP of −71 kJ/mol is obtained. This number is quite large, but in the correct relative range, considering the corresponding values for $\Delta G'^{\circ}$ for acetyl phosphate (−43 kJ/mol), acetyl adenylate (−55 kJ/mol) and carbamoyl phosphate (−51 kJ/mol).[49]

NMR analysis of the decomposition of TC-AMP showed that in phosphate buffer at pH 6, the final decomposition products are L-threonine and the oxazolidinone (L-Thr-ox), which are formed in approximately equal amounts. A third species observed in the NMR spectra at 5−10% of TC-AMP could be an intermediate in the degradation pathway, such as the isocyanate (L-Thr-NCO). As shown in Scheme 2, both products as well as the putative intermediates can be formed directly from TC-AMP, via either an intramolecular addition or elimination reaction or by hydrolysis. The isocyanate and the NCA would each be predicted to give both the oxazolidinone and L-threonine. This has been confirmed for the NCA, which interestingly degrades in phosphate buffer rapidly to give nearly the same ratio of the two products as in the NMR study. Thus, it is not expected to accumulate during the NMR experiment. The rearrangement of the NCA to the oxazolidinone has been previously documented under anhydrous basic conditions.[51] However, basic conditions do not appear to be required since this rearrangement occurs in DMSO, although slowly.

Support for the isocyanate as an intermediate comes from the reported decomposition of carbamoyl phosphate that involves unimolecular elimination of phosphate from the dianionic species to give isocyanate.[47] Although TC-AMP is a phosphodiester and hence only a monoanion at pH > 2, one can postulate a similar unimolecular elimination pathway for TC-AMP at pH > 4, with the ionized carboxylate acting as base to remove the proton on the carbamate nitrogen to eliminate AMP. Alternatively, enough inorganic phosphate dianion in the reaction may exist in 0.5 M KPi at pH 6 to promote the elimination.

The results of the kinetics and stability studies of TC-AMP have implications for the mechanistic role of YwlC and YrdC. Unlike proposed mechanisms to date, the data presented in this work do not support a mechanism in which t^6A biosynthesis

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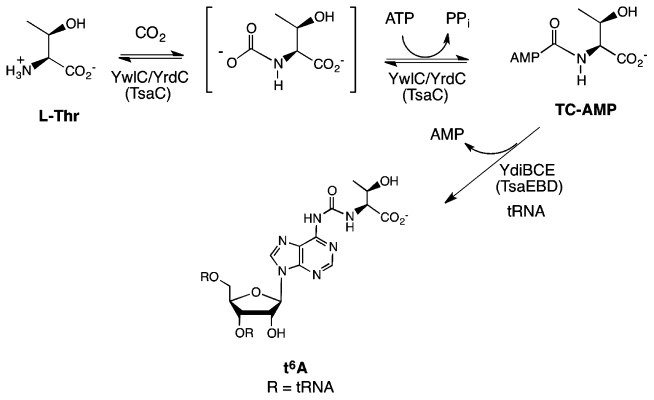
proceeds via the formation of an ATP-activated bicarbonate species. Instead, as shown in Scheme 3, these enzymes most

Scheme 3. Proposed Mechanism for t^6A Biosynthesis in Bacteria



likely directly catalyze the formation of *N*-carboxy-L-threonine, by shifting the equilibrium to favor this product from carbon dioxide and the amino acid. It is well documented that aliphatic amines, including amino acids such as threonine, form stable carbamates at high concentrations of CO_2/bicarbonate and amino acid at alkaline pH (~8.5−12).[52] Thus, the enzyme's role in forming *N*-carboxy-L-threonine (L-threonine carbamate) is essentially that of (a) approximation to increase the local concentration of reactants on the enzyme surface, and (b) increasing the effective pH in the active site. The latter is accomplished by positioning functional groups to deprotonate the L-threonine ammonium group or selectively bind the neutral amine for attack on CO_2, deprotonate the same nitrogen again after attack, and position group(s) to stabilize the resulting carbamate anion. The source of the carbamate carbon would then most likely be from the more electrophilic CO_2 rather than bicarbonate.

Once formed, the carbamate could be positioned to form the adenylate by attack on the alpha phosphate of ATP to give the product TC-AMP, as hypothesized in recent structural work.[31] This mechanism is consistent with recent crystal structure data for the carbamoylation enzymes HypF and TobZ, which also catalyze formation of carbamoyl adenylate intermediates. For both of these enzymes, the proposed substrate is the anion of carbamic acid, formed by energetically favorable hydrolysis of carbamoyl phosphate, which then attacks ATP to give the analogous adenylate species. Unlike this pathway for generating the carbamate, the YwlC reaction to form TC-AMP is quite energetically unfavorable. It is similar in some respects to the formation of aminoacyl adenylates by aminoacyl-tRNA synthetases, in which the K_{eq} for formation of the adenylate is 10^{-7}.[53] For both reactions, formation of the final products (t^6A and aminoacyl-tRNA) give release of AMP which renders the overall reaction favorable.

While the *Bacillus* results match the recent work in *E. coli*[29] in the overall requirements for TC-AMP and t^6A formation, there are significant differences between YwlC and YrdC in both the rate of AMP formation in the forward reaction and the k_{cat} for ATP formation in the reverse reaction. These differences between YwlC and YrdC may in part be because YwlC and many other SUA5 homologues contain an additional "SUA5" domain (pfam 03481) of about 100 aa at the C-terminus. This domain is absent in *E. coli* YrdC and in other organisms such as higher eukaryotes. It is possible that this extra domain contains an additional binding site for ATP and/or PPi that influences the kinetics of the forward and reverse reactions. It is clear from the effect of pyrophosphatase that PPi causes excess AMP formation in the forward reaction in the presence of ATP, but not in the reverse reaction. It is presently unclear if the source of AMP in the forward reaction is via hydrolysis of ATP directly or via TC-AMP. It is conceivable that the YwlC-TC-AMP complex may actively hydrolyze ATP to AMP via the C-terminal domain. Such reactivity has been observed in firefly luciferase, in which the bound luciferase-luciferyl-AMP complex appears to hydrolyze ATP to give AMP and PP_i.[54] In addition, it was found that both YwlC and YrdC contain anywhere from 1 to 5 mol % of bound TC-AMP after isolation by affinity chromatography and gel filtration. This is consistent with the recent reassessment of the crystal structure of *S. tadaii* SUA5 by Parthier et al.[33] in which significant electron density was found for bound TC-AMP, which they suspect remained bound throughout the protein isolation. Interestingly, whereas purified YrdC also contained bound AMP, YwlC contained only bound ATP, which is again indicative of possible mechanistic differences.

The final step in t^6A formation is the addition of TC-AMP to A37 of substrate tRNAs to give t^6A. Previous work suggested that YrdC binds RNA[35] and exerts the binding specificity required of a protein that modifies tRNA directly.[45] Structural work with YrdC homologues[31−33] suggested that this domain is involved in the activation of carbamate by ATP. The *in vitro* experiments presented here show that in *B. subtilis*, the reaction of tRNA with TC-AMP can be catalyzed by the YdiBCE (TsaEBD) complex in the absence of YwlC. Consistent with the overall reaction, each of the YdiBCE proteins is required the final step. This result suggests that one or more of the YdiBCE proteins also binds tRNA. In addition, ATP was not required, which was surprising because of the YdiBCE complex-dependent activation in the production of ADP in the complete reaction mixture. While it is clear that ATP would not be required for the chemistry of addition of TC-AMP to tRNA to give t^6A, *in vivo* data has shown that the ATPase activity of YdiB is required for normal growth.[37] The exact role for YdiB(TsaE) and YdiC(TsaB) are not yet known. It is conceivable that ATP hydrolysis by YdiBCE may be necessary for the binding of tRNA in a productive manner or for chaperone activity *in vivo* as has been proposed previously. Alternatively, ATP hydrolysis by YdiBCE may serve an additional role, in a capacity that may or may not be directly related to the chemistry of t^6A formation. Future work on the kinetics of t^6A formation and RNA binding may elucidate the role of the prokaryotic specific proteins.

Finally, these enzymes have been proposed as potential antibiotic targets. Elucidation of the structure and reactivity of the intermediate provides an initial framework for the rational design of inhibitors that may be optimized into specific antibiotic drugs. While the overall t^6A modification is required for all forms of life, there appears to be sufficient differentiation not only between bacteria and eukaryotes but even within bacteria to enable the development of specific inhibitors. Further structural and mechanistic work on these systems will be necessary to provide such targeted therapeutics.

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■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures and figures for *B. subtilis ywlC* mutant tRNA analysis, protein expression, and TLC analysis of ATPase reactions. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

PPi, pyrophosphate; TC-AMP, L-threonylcarbamoyl adenosine-5′-monophosphate; L-Thr, L-threonine; L-Thr-ox, *trans*-(4*S*,5*R*)-5-methyl-2-oxazoldinone-4 carboxylic acid; L-Thr-NCO, L-threonine isocyanate; NCA, N-carboxyanhydride

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