Characterization of Recombinant QueA

tRNA Modification by S-Adenosylmethionine:tRNA Ribosyltransferase-isomerase (QueA): Assay Development and Characterization of the Recombinant Enzyme*

Steven G. Van Lanen; Sylvia Daoud Kinzie; Sharlene Matthieu; Todd Link; Jeff Culp and Dirk Iwata-Reuyl‡
Department of Chemistry, Portland State University, PO Box 751, Portland, Oregon, 97207.

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‡To whom correspondence should be addressed: Department of Chemistry, Portland State University, PO Box 751, Portland, OR 97207-0751. Phone: (503) 725-5737. FAX: (503) 725-9525. E-mail: iwatareuyld@pdx.edu.
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SUMMARY

The enzyme S-adenosylmethionine:tRNA ribosyltransferase-isomerase catalyzes the penultimate step in the biosynthesis of the hypermodified tRNA nucleoside queuosine (Q), an unprecedented ribosyl transfer from the cofactor S-adenosylmethionine (AdoMet) to a modified-tRNA precursor to generate epoxyqueosine (oQ). The complexity of the reaction makes it an especially interesting mechanistic problem, and as a foundation for detailed kinetic and mechanistic studies we have carried out the basic characterization of the enzyme. Importantly, to allow for the direct measurement of oQ formation we have developed protocols for the preparation of homogeneous substrates; specifically an over-expression system was constructed for tRNA^Tyr in an *E. coli* queA deletion mutant to allow for the isolation of large quantities of substrate tRNA, and [U-ribosyl-¹⁴C]AdoMet was synthesized. The enzyme shows optimal activity at pH 8.7 in buffers containing various oxyanions, including acetate, carbonate, EDTA, and phosphate. Unexpectedly, the enzyme was inhibited by Mg²⁺ and Mn²⁺ in millimolar concentrations. The steady-state kinetic parameters were determined to be $K_M^{AdoMet} = 101.4 \, \mu M$, $K_M^{tRNA} = 1.5 \, \mu M$, $k_{cat} = 2.5 \, \text{min}^{-1}$. A short minihelix RNA was synthesized and modified with the precursor 7-aminomethyl-7-deazaguanine, and this served as an efficient substrate for the enzyme ($K_M^{RNA} = 37.7 \, \mu M$ and $k_{cat} = 14.7 \, \text{min}^{-1}$), demonstrating that the anticodon stem-loop is sufficient for recognition and catalysis by QueA.
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The post-transcriptional processing of transfer RNA (tRNA) involves a number of functionally distinct events essential for tRNA maturation (1-4). The phenomenon of nucleoside modification is perhaps the most remarkable of these events, imparting a rich structural diversity to tRNA not observed in other RNAs (4). Over 80 modified nucleosides have been characterized in tRNA (4), many of which are conserved across broad phylogenetic boundaries. In the main, the biochemical function of nucleoside modification in tRNA remains poorly understood, although roles from structural stabilization to modulation of translational fidelity have been revealed in a few cases (4).

The most structurally complex of these modified nucleosides is the hypermodified nucleoside queuosine (Q, Fig. 1),¹ which contains a cyclopentenediol group appended to a (7-aminomethyl)-7-deazaguanine core structure, and in some mammalian tRNA can be further modified by glycosylation with galactose or mannose (5). Queuosine and its derivatives occur exclusively at position 34 (the wobble position) in the anticodons of tRNA’s coding for the amino acids asparagine, aspartic acid, histidine, and tyrosine (6). Each of these tRNAs possess the genetically encoded anticodon sequence GUN, where N can be any nucleotide. Although otherwise ubiquitous throughout Eukarya and Bacteria, queuosine is inexplicably absent from the tRNA of yeast and mycoplasma, and is not found in the tRNA of Archaea.

A definitive picture of the biochemical function or functions of queuosine has yet to emerge, but it has been correlated with eukaryotic cell development and proliferation (7-10), neoplastic transformation (8,11-13), tyrosine biosynthesis in animals (14), translational frameshifts essential to retroviral protein biosynthesis (15-17), and with the ability of pathogenic bacteria to invade and proliferate in human tissue (18). Underlying most, if not all, of these phenomena is a role in modulating translational fidelity, consistent with queuosines location in the anticodon. However,
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evidence also exists that implicates queuosine (or the free base queuine) in signal transduction (19-26), potentially by tRNA-independent mechanisms.

FIGURE 1

Although present in both Eukarya and Bacteria, only Bacteria are capable of de novo queuosine biosynthesis (Fig. 2). Eukaryotes utilize a salvage system and acquire queuosine as a nutrient factor and from intestinal flora (27), and insert queuine directly into the appropriate tRNA by the enzyme tRNA-guanine transglycosylase (TGT) (28). A related TGT is present in Bacteria (29), but in this case the substrate is the queuosine precursor 7-aminomethyl-7-deazaguanine (preQ₁, Fig. 2), which appears to be derived from GTP (30). Considerable effort has been directed over the past decade at elucidating the catalytic mechanism (31,32) and RNA specificity of the bacterial TGT enzymes (33,34), and high-resolution X-ray crystal structures have been solved for the enzyme from *Zymomonas mobilis* (35,36). The only other enzyme identified in the pathway is S-adenosylmethionine:tRNA ribosyltransferase-isomerase (QueA), which catalyzes the penultimate step in the de novo biosynthesis of queuosine, the formation of epoxyqueuosine (oQ) via the addition of an epoxycyclopentadiol ring to preQ₁ modified tRNAs (Fig. 2). Remarkably, the epoxycyclopentadiol moiety of oQ originates from the ribosyl portion of S-adenosylmethionine (AdoMet) (37,38), the first example of the stoichiometric use of AdoMet as a ‘ribosyl’ donor in an enzymatic reaction. The overall reaction involves the elimination of both methionine and adenine from AdoMet, the transfer of the ribosyl moiety to tRNA, and its rearrangement to form an epoxy-carbocycle.

FIGURE 2

FIGURE 3

QueA has garnered considerable interest for its central role in the biosynthesis of queuosine, the unique utilization of AdoMet, and as a fascinating problem in fundamental mechanistic
Characterization of Recombinant QueA enzymology. The reaction catalyzed by QueA is unprecedented in biological systems, and the results of preliminary mechanistic studies are consistent with a catalytic mechanism involving sulfonium ylide and vinyl sulfonium intermediates (39), species never before implicated in an enzymatic reaction. However, in contrast to TGT, the lack of an easily accessible activity assay has stymied the basic characterization and quantitative analysis of this remarkable enzyme, and precluded detailed kinetic studies. We report here a solution to this problem with protocols for the preparation of appropriate substrates, the development of a quantitative assay for the reaction with short RNA substrates, the elucidation of optimal reaction conditions, the measurement of the steady-state kinetic parameters, and a preliminary evaluation of the importance of RNA structural information for recognition and catalysis by QueA.

EXPERIMENTAL PROCEDURES

General. Buffers and salts of highest quality grade were purchased from Sigma unless otherwise noted. DTT, IPTG, and ampicillin were from US Biologicals. [U-14C]glucose was from ICN (46 mCi/mmol) and [8-14C]guanine was from Sigma (45.3 mCi/mmol). Bulk yeast tRNA Type X and Escherichia coli tRNA tyrosine specific I (HPLC standard) were from Sigma. Glutathione Sepharose 4B, Sephadex G-25 (superfine, DNA grade), and nucleotides were purchased from Amersham Biosciences. POROS chromatography resins were from PerSeptive Biosystems. Benzoylated-naphthoylated DEAE cellulose (BND-cellulose) was from Sigma, and NACS resin was from BRL Life Technologies, Inc. Whatman GF-C disks were from Fisher. Plasmid Midi Kits and Ni-NTA agarose were from Qiagen, and PUREscript RNA isolation kits were from Gentra Systems, Inc. Centriprep YM-30, Centricon YM-10, and Centricon YM-3 units were from Amicon. Dialysis was performed in Slide-A-Lyzer cassettes from Pierce. Oligonucleotides were from Operon Technologies and IDT. The plasmid pET-11a was from Novagen, pBluescript II SK(+) from Stratagene, and pTrc99B from Pharmacia. DNA from
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restriction digests and PCR was purified with GeneClean III (Bio 101) kits following the manufacturers’ instructions. Buffers made for RNA work were prepared with DEPC-treated water and filtered through Cameo 25ES nitrocellulose filters from Osmonics, Inc. Protein concentrations were based on the Bradford dye-binding procedure (BioRad). Cytoscint ES liquid scintillation cocktail was from ICN.

*Instrumentation.* Centrifugation was performed with an Avanti J-20 XP centrifuge using JA-25.50 and JA-10 rotors, an LE-80K ultracentrifuge using a Ti-60 rotor, and a Model TJ-6 swinging bucket centrifuge, all from Beckman Coulter, Inc. UV/Vis spectrophotometry was performed with the Varian Cary 100 Bio. HPLC was carried out with a Hitachi system consisting of the LACHROM software, the D-7000 System Manager, an L-7100 pump, and an L-4500A diode array detector. Continuous elution PAGE was carried out with a BioRad 491 Prep Cell. PCR and enzyme assays were carried out on the GeneAmp PCR System 2400 from Perkin Elmer. Sonication was done with a Model W-375 sonicator from Heat Systems-Ultrasonics, Inc. Protein and nucleic acid PAGE was carried out with the Mini Protean III from BioRad. Radioactivity from enzyme assays was quantitated with a Beckman LS 6500 liquid scintillation counter. A Molecular Dynamics Typhoon 9200 variable mode imager with ImageQuant 5.2 software was used to measure and analyze radioactivity in gels, and for fluorescence detection of ethidium bromide or SybrGreen stained nucleic acids in PAGE and agarose gels.

*Bacterial Strains, Plasmids, and Enzymes.* *E. coli* strain DH5α (F φ80dlacZΔM15 Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rK-,mK+) phoA supE44 λ- thi-1 gyrA96 relA1) was from Gibco BRL Life Technologies. *E. coli* strain BL21(DE3) (F dcm ompT hsdS(rK-,mK+) galλ) was from Stratagene. The *E. coli* queA deletion mutant K12QueA (37), and plasmids pHH1 (40) and pGEX-QA (37), were generous gifts from Helga Kersten (Universität Erlangen). Restriction
Characterization of Recombinant QueA enzymes were from MBI Fermentas and New England Biolabs, Inc. T₄ kinase and T₄ ligase were from New England Biolabs. Pfu and Pfu Turbo Polymerase were from Stratagene. Factor Xa was from Promega. 6-Phosphogluconate dehydrogenase type V from torula yeast was purchased from ICN as an ammonium sulfate suspension. L-Glutamate dehydrogenase type I from bovine liver, pyruvate kinase type II from rabbit muscle, glucose-6-phosphate dehydrogenase type VII from Bakers yeast, and myokinase from rabbit muscle were from Sigma, also as ammonium sulfate suspensions. Phosphoriboisomerase from torula yeast and hexokinase type F-300 from Bakers yeast were from Sigma as lyophilized powders and were stored in 50% glycerol and the appropriate buffer at −90°C. Recombinant adenine phosphoribosyltransferase (APRTase) was overproduced from pQEAPT1 in E. coli strain B25 (B25/pQEAPT1, a generous gift from Dr. Milton Taylor, Indiana University). Expression and purification of APRTase was essentially as described (41,42). The enzyme was judged ~95% pure by SDS-PAGE analysis. Activity was measured as described (43). Recombinant S-adenosylmethionine synthetase (MAT) from E. coli was overproduced from pK8 (44) in E. coli DH5α (pK8, a generous gift of Dr. G. Markham, Fox Chase Cancer Center). Expression and streptomycin sulfate and ammonium sulfate purification steps were as described (45). The enzyme was enriched to ~80% as judged by SDS-PAGE; activity was measured as described (45). Recombinant 5-phosphoribosyl-1-pyrophosphate (PRPP) synthetase from Salmonella typhimurium was overproduced from pBRS11R in E. coli DH5α (pBRS11R, a generous gift from Dr. Vern L. Schramm, Albert Einstein University), and purified by ammonium sulfate fractionation. The enzyme was enriched to ~80% as judged by SDS-PAGE. Activity was assayed as described (46). Recombinant T₇ RNA Polymerase was overproduced from pT7-911Q (a generous gift from Dr. Thomas E. Shrader, Albert Einstein University) as a 6xHis fusion protein in E. coli DH5α and purified on Ni-NTA agarose (Qiagen). All recombinant enzymes were over-produced using standard
Characterization of Recombinant QueA procedures, and cell lysis was achieved with sonication to give cell free extracts containing the relevant recombinant proteins.

*Cloning and Construction of an Homologous Over-Expression System for E. coli tgt.* Template DNA for the PCR-based amplification of the tgt structural gene from *E. coli* was prepared by isolation of the 1.8kb *Sal* I/Bam HI fragment from the *E. coli* genomic clone pHH1 (40). Amplification of tgt was carried out with Pfu polymerase directed by the primers tgtSTART (5’-CGGTCGACC**CATATG**AAATTTGAACCTGGACACCACCG-3’) and tgtSTOP (5’-GCGCAGGATCCTTTATTAATATTACGTCAACTGAAGG-3’). Designed into tgtSTART were the restriction enzyme sites *Sal* I (italics) and *Nde*I (underlined), the later as part of the translation initiation codon (bold) for the tgt gene, while tgtSTOP contained a *Bam*HI site (underlined) downstream from the translation termination codon. The PCR program included an initial hold for 5 min at 95 °C, followed by 10 cycles of 95 °C for 2 min, 55 °C for 2 min, and 75 °C for 2.5 min, followed by a 7 min hold at 75 °C. After amplification the PCR product was gel purified (0.8% agarose), subjected to restriction digest with *Sal*I/BamHI, and ligated into the *Sal*I/BamHI sites of pBluescript II SK(+) to give the plasmid pBlue-TGT. The structure of the tgt gene was confirmed by sequencing and subcloned as an *Nde*I/BamHI fragment into the *Nde*I/BamHI sites of the *E. coli* expression vector pET-11a to give the expression plasmid pET-TGT.

*Purification of Recombinant E. coli tRNA-guanine transglycosylase (TGT).* To a cell free extract of *E. coli* BL21(DE3)/pET-TGT in 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 2 mM DTT and 1 mM PMSF was added 1/10 volume of streptomycin sulfate (10% w/v) and the solution stirred for 30 min at 4 °C. After centrifugation at 50,000g for 4 h, the supernatant was recovered and dialyzed against 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 5 mM DTT, and 50 µM ZnSO₄. Approximately 5 mg of crude protein was then loaded onto a POROS HQ column (4.6 mm x 100
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mm) equilibrated with 20 mM Tris-HCl (pH 7.5), and 0.5 mM DTT (buffer A). A series of linear gradients were developed from buffer A to 2.0 M KCl in buffer A (buffer B) with a flow rate of 5 ml/min, and TGT elution was determined by activity assays and SDS-PAGE. Fractions containing TGT, which eluted at 0.24 M KCl, were pooled, concentrated with Centriprep YM-30 units, and dialyzed against 20 mM Tris-HCl (pH 7.5), 1.0 mM MgCl2, and 0.5 mM DTT, and 50 µM ZnSO4. The TGT sample was then subjected to cation exchange chromatography on POROS HS (4.6 mm x 100 mm) equilibrated in 20 mM Tris-HCl (pH 7.5), 1.0 mM MgCl2 and 0.5 mM DTT (buffer C). A series of linear gradients were developed from buffer C to 2.0 M KCl in buffer C (buffer D) with a flow rate of 5 ml/min, and TGT elution was determined by activity assays and SDS-PAGE. Fractions containing TGT were concentrated with Centriprep YM-30 units and dialyzed against 20 mM Tris-HCl (pH 7.5), 50 mM KCl, 50 µM ZnSO4, and 2 mM DTT, and stored as a 40% glycerol stock at –90°C. The TGT was judged to be >95% pure by SDS-PAGE.

**Synthesis of [U-ribosyl-14C]ATP.** An enzymatic synthesis was used to produce [U-ribosyl-14C]ATP from [U-14C]glucose. The synthesis was carried out according to Parkin et al. (47) with slight modifications. Purification of [U-ribosyl-14C]ATP was carried out by HPLC as described by Lim et al. (48) with a semipreparative Hypersil 5 C18 column (250 x 10 mm, 5 µm). The fractions containing the purified ATP were pooled, lyophilized to dryness, and dissolved in water. Concentration was determined by UV spectroscopy (ε260 = 15.4 x 10³ M⁻¹ cm⁻¹). After correcting for the loss of 14CO₂, the radiochemical yield was typically 85-95%.

**Synthesis of [U-ribosyl-14C]S-Adenosylmethionine.** The purified [U-ribosyl-14C]ATP was used in an enzymatic reaction to produce [U-ribosyl-14C]AdoMet essentially as described by Park et al. (49). [U-ribosyl-14C]AdoMet was purified by HPLC using a semipreparative Hypersil 5 C18 column (250 x 10 mm, 5 um). The column was developed under isocratic conditions of 20 mM
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ammonium acetate (pH 6.0) and 2% methanol at a flow rate of 3 ml/min, and the fractions containing [U-\(^{14}\)C-ribosyl]AdoMet were pooled, lyophilized to dryness, and dissolved in 10 mM H\(_2\)SO\(_4\). The radiochemical yields were typically >90%. Concentration was determined by UV spectroscopy (\(\varepsilon_{260} = 14.9 \times 10^3 \text{M}^{-1}\text{cm}^{-1}\)). The purity of the [U-ribosyl-\(^{14}\)C]AdoMet was checked by comparing with authentic material (Sigma) by TLC and HPLC.

**Construction of an Homologous Over-Expression System for E. coli tRNA\(^{Tyr}\).** A synthetic gene for *E. coli* tRNA\(^{Tyr}\) was constructed from four oligonucleotides using standard procedures (50) such that the 5' and 3'-ends of the gene could be ligated into the EcoR1 and BamHI sites, respectively, of pTrc99B. The 2 oligonucleotides comprising the sense strand of *E. coli* tRNA\(^{Tyr}\) gene were:

5'-AATTCGGTGTTGGGTTCCGGAGCGCCAAAGGGAGCAGACTGT-3' (Tyr-S1), 5'-AAATCTGCCGTCATCGACTTCGAAGGTTCGAATCCTTCCCACCACCTATTATTAAG-3' (Tyr-S2),

The 2 oligonucleotides comprising the antisense strand were:

5'-GATCCTTAATAAGTGGTGTTGGGGAAGGATTCGAACTTCCAAGAGCTAATCTTG-3' (Tyr-AS1), 5'-TCGATCACCGCAGATTTACAGTCTGCTCCCTTTGGCCTCGGGAACCCCACCACCG-3' (Tyr-AS2).

The structure of the tRNA\(^{Tyr}\) gene of the resultant plasmid pTrc-Tyr was confirmed by sequencing.

**In Vivo Over-Expression of preQ\(_1\)-tRNA\(^{Tyr}\).** Production cultures of *E. coli* K12QueA/pTrc-Tyr were grown in LB/amp until the OD\(_{600}\) reached 0.9-1.0, when over-expression of the tRNA\(^{Tyr}\) gene was induced by addition of IPTG to a final concentration of 0.5 mM. To determine the appropriate harvesting times for optimal production of preQ\(_1\)-tRNA\(^{Tyr}\), 3 ml aliquots were removed immediately before and 1, 2, 4, 8, and 13 h post-induction with IPTG. The RNA was extracted from the samples using PUREscript RNA isolation kits (Gentra Systems, Inc.) according to the manufactures directions. The RNA samples and commercial *E. coli* tRNA\(^{Tyr}\) were then analyzed by HPLC on W-POREX 5 C4 column (Phenomenex, 250 x 4.6 mm) using a
Characterization of Recombinant QueA modification of the mobile phase developed by Pearson et al. (51). For the large-scale isolation of preQ₁-tRNA\textsubscript{Tyr} 500 ml cultures were allowed to grow 13 h post-induction when the cells were collected by centrifugation at 3,000 g for 10 min and flash frozen in liquid nitrogen. The cells were stored at −90 °C until further use.

The acid guanidinium thiocyanate/phenol/chloroform method was used to lyse cells and extract ribonucleic acids (52). After washing the crude RNA pellet with 70% ethanol, the RNA was dissolved in DEPC-treated water and an equal volume of 8 M lithium chloride was added. The solution was placed at −20 °C for 2 h, followed by centrifugation at 20,000 g for 20 min to remove large RNA. To the supernatant, containing preQ₁-tRNA\textsubscript{Tyr}, was added an equal volume of isopropanol and the solution cooled at −20 °C for 2 h. The precipitate was collected by centrifugation at 20,000 g for 20 min, washed with 70% ethanol, and dissolved in 3 mM sodium citrate (pH 6.3). The concentration of tRNA was determined by UV-Vis spectroscopy using ε\textsubscript{260} = 15 U/mg/ml. Approximately 25% of the total tRNA was determined to be preQ₁-tRNA\textsubscript{Tyr} by tRNA-tyrosine synthetase activity assays (53).

Purification of preQ₁-tRNA\textsubscript{Tyr}. Crude tRNA was initially fractionated by chromatography on BND-cellulose according to Gillam et al. (54). The preQ₁-tRNA\textsubscript{Tyr} containing fractions, identified by QueA and tRNA-tyrosine synthetase activity assays, were pooled and the tRNA precipitated by the addition of isopropanol and cooling. The tRNA precipitate was washed twice with 70% ethanol and dialyzed against 10 mM NaOAc (pH 4.5), 10 mM MgCl₂, and 0.2 M NaCl. The enriched preQ₁-tRNA\textsubscript{Tyr} was further purified by chromatography on NACS resin according to Thompson et al. (55). The preQ₁-tRNA\textsubscript{Tyr} containing fractions were pooled and the tRNA precipitated by the addition of isopropanol and cooling. After washing with 70% ethanol, the purified preQ₁-tRNA\textsubscript{Tyr} was dissolved in 3 mM citrate (pH 6.3).
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To remove bound metal ions from the preQ₁-tRNA_{Tyr} EDTA was added to a concentration of 5 mM and the solution was dialyzed at 80 °C for 10 min against 3000 volumes of 3 mM sodium citrate (pH 6.3), followed by dialysis at 6 °C over 5 h with 2 buffer changes. The preQ₁-tRNA_{Tyr} was then precipitated by the addition of isopropanol and cooling. After washing with 70% ethanol, the purified preQ₁-tRNA_{Tyr} was dissolved in 3 mM citrate pH 6.3. The concentration of preQ₁-tRNA_{Tyr} was determined by UV-Vis spectroscopy using ε₂₆₀ = 816,000 M⁻¹ cm⁻¹ (determined at http://paris.chem.yale.edu/extinct.html). Based on tyrosine-tRNA synthetase and QueA activity assays the tRNA was determined to be •85% preQ₁-tRNA_{Tyr} (900 pmol/A₂₆₀).

Synthesis and Purification of Minihelix RNA. An RNA 17-mer corresponding to the E. coli tRNA_{Asn} anticodon stem loop (5'-GCGGACUGUAAAUCGC-3') was synthesized by in vitro transcription with recombinant T₇ RNA polymerase (56). After elimination of the DNA template with RNase-free DNase and phenol:chloroform:isoamyl alcohol extraction, the minihelix RNA was purified by continuous elution PAGE essentially as described by Cunningham et al. (57). The fractions containing the minihelix RNA (determined by urea-PAGE and TGT activity assays) were pooled and concentrated using a Centricon YM-3. RNA concentration was determined by absorbance at 260 nm with ε=162000 M⁻¹ cm⁻¹ and MW=5442.4 Da (determined at http://paris.chem.yale.edu/extinct.html). The minihelix RNA was judged •95% pure by urea-PAGE.

Insertion of preQ₁ into Minihelix RNA. An aliquot of TGT (75 µg) was added to a 1 ml solution containing 20 mM HEPES (pH 7.5), 10 mM MgCl₂, 50 mM KCl, 2 mM DTT, 37 µM minihelix RNA, and 50 µM [8-¹³C]guanine. After 3 h at 37 °C, the reaction was terminated by the addition of one-tenth volume of 2M NaOAc (pH 4.0) and phenol:chloroform:isoamyl alcohol. The aqueous phase was recovered and applied to a Quik-Sep column (Isolab Inc.) containing 2 ml of Sephadex G-25. The sample was centrifuged (1.5 min at 700g) to separate the unreacted guanine
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from the minihelix RNA, the resin washed with 3 mM citrate (pH 6.3), and the fractions containing the minihelix RNA combined and concentrated using a Centricon YM-3 unit to give minihelix RNA with a specific activity of 2.8 mCi/mmol.

The base preQ₁, synthesized as described (58,59), was inserted into the minihelix RNA by a TGT-catalyzed reaction under the above conditions with preQ₁ in 12-fold excess over minihelix RNA. After work-up as above the concentrated minihelix RNA contained essentially no radioactivity (specific activity < 0.005 mCi/mmol), indicating that > 99.8% of the minihelix RNA contained preQ₁.

**Expression and Purification of GST-QueA fusion protein.** Recombinant GST-QueA was over-produced in *E. coli* DH5α/pGEX-QA using standard expression conditions. Affinity chromatography on Glutathione Sepharose 4B media was carried out essentially as described previously (37).

Following affinity chromatography the protein was loaded (1mg/run) onto a POROS HQ column (4.6 mm x 100 mm) equilibrated with 20 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 2 mM DTT, and 10% glycerol (buffer E). A series of linear gradients were developed from buffer E to 1M KCl in buffer E (buffer F) at a flow rate of 4 ml/min, and fractions containing GST-QueA were pooled and dialyzed against 50 mM Tris-HCl (pH 8.0), 100 mM KCl, 1.0 mM DTT, and 10% glycerol. From SDS-PAGE analysis the protein was judged to be > 95% pure.

**Cleavage of GST-QueA with Factor Xa protease.** A sample of the fusion protein was cleaved in a solution containing 50 mM Tris-HCl (pH 8.0), 100 mM KCl, 1 mM CaCl₂, 4 mM DTT, Factor Xa (1.0 µg/1.0 mg of fusion protein), and GST-QueA. The reaction was carried out overnight at room temperature, and QueA purified by HPLC under the conditions listed above.

**QueA Activity Assays.** Routine assays of QueA activity were initially carried out in 100 mM Tris-HCl (pH 8.7), 50 mM KCl, 10 mM MgCl₂, 0.5 mM DTT, 25 µg crude preQ₁-tRNA Tyr, and
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50 μM [U-ribosyl-\(^{14}\)C]AdoMet in a final volume of 50 μL at 37 °C. Reactions were terminated at the appropriate times by the addition of 3 volumes of cold 10% TCA, and the precipitated tRNA collected on Whatmen GF-B filters by vacuum filtration, rinsed with cold 5% TCA followed by ethanol, dried, and the radioactivity quantitated by liquid scintillation counting. After optimum conditions for enzyme activity were elucidated routine assays were carried out in 100 mM glygly (pH 8.7), 100 mM EDTA, 100 mM KCl, 0.5 mM DTT, 3.75 μM preQ\(_1\)-tRNA\(^{Tyr}\), and 100 μM [U-ribosyl-\(^{14}\)C]AdoMet in a final volume of 50 μL at 37 °C. Activity assays were initiated and terminated as described above.

QueA Activity Assays with Minihelix RNA. Reactions with preQ\(_1\)-modified minihelix RNA were carried out in 100 mM glygly (pH 8.7), 100 mM EDTA (pH 8.7), 100 mM KCl, 0.5 mM DTT, with variable minihelix RNA, [U-ribosyl-\(^{14}\)C]AdoMet and GST-QueA in a final volume of 50 μL at 37 °C. Reactions were terminated at the appropriate time by decreasing the pH to 6.5 with the addition of acetic acid and heating briefly at 95 °C. The samples were loaded onto a Quik-Sep column containing 250 μL of DEAE cellulose equilibrated in 50 mM imidazole (pH 6.5). The columns were washed with 15 volumes of 50 mM imidazole (pH 6.5), and the minihelix RNA eluted with 7 column volumes of 50 mM imidazole (pH 6.5) and 1 M NaCl. The unreacted AdoMet (wash) and minihelix RNA were both collected and counted.

Ionic Strength and pH Optima. The pH profile for QueA was carried out in 100 mM buffer, 50 mM KCl, 20 mM MgCl\(_2\), 0.5 mM DTT, 1.5 μM preQ\(_1\)-tRNA\(^{Tyr}\), 100 μM [U-ribosyl-\(^{14}\)C]AdoMet, and 100 nM GST-QueA under initial velocity conditions. The pH profile was also carried out under the above conditions with 100 mM EDTA and no MgCl\(_2\). The buffers used were MES (pH 5.6-6.6), MOPS (6.7-7.7), Tris-HCl (7.5-8.7), CHES (8.7-9.3), and CAPS (9.8-11.0). Optimal
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Ionic conditions were tested in 100 mM glygly (pH 8.7), 0.5 mM DTT, 1.5 µM preQ$_1$-tRNA$^{\text{Tyr}}$, 100 µM [U-ribosyl-14C]AdoMet, and 100 nM GST-QueA under initial velocity conditions.

**Steady-State Kinetic Assays.** The steady-state kinetic parameters for GST-QueA were determined from activity assays carried out in 100 mM glygly (pH 8.7), 100 mM EDTA (pH 8.7), 100 mM KCl, 0.5 mM DTT, with variable preQ$_1$-tRNA$^{\text{Tyr}}$ (or minihelix RNA) and [U-ribosyl-14C]AdoMet in a final volume of 50 µL at 37 °C. GST-QueA had a final concentration of 200 nM with variable AdoMet and saturating preQ$_1$-tRNA$^{\text{Tyr}}$ (15 µM), and 50 nM with variable preQ$_1$-tRNA$^{\text{Tyr}}$ and saturating AdoMet (1 mM). Reactions with preQ$_1$-modified minihelix RNA were terminated by the stepwise addition of carrier yeast tRNA Type X (to a final amount of 20 µg/assay) and 3 volumes of cold 10% TCA, and worked up as described above. Reactions with preQ$_1$-modified minihelix RNA were terminated and worked up as described above for assaying minihelix RNA substrates. Time course assays (up to 20 min) were performed in order to determine initial velocity conditions using nonlinear regression of DPM versus time. Michaelis-Menten parameters were calculated from the average of minimally four replicates by nonlinear regression analysis of the initial velocity versus substrate concentration using Kaleidagraph 3.0 (Synergy Software, Reading, PA).

**RESULTS**

**Preparation of Substrates.** To prepare AdoMet with specific radiochemical labeling in the ribosyl moiety ATP was first synthesized from glucose using the methodology of Schramm and coworkers (47), followed by the enzymatic conversion of ATP to AdoMet by MAT as described by Park et al. (49). Of the 8 enzymes required for the conversion of glucose to ATP, 6 are commercially available. PRPP synthetase and APRTase, the two enzymes not commercially available, were overproduced in *E. coli* and purified as described (41,42) for use in the synthesis. Thus, starting with [U-14C]glucose, [U-ribosyl-14C]ATP was synthesized enzymatically;
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purification of the ATP by reversed-phase HPLC (Fig. 4) provided [U-ribosyl-\(^{14}\)C]ATP in radiochemical yields of >85% after accounting for the loss of \(^{14}\)CO\(_2\) from C1 of glucose.

Recombinant \textit{E. coli} MAT was overproduced in \textit{E. coli} from pK8 and purified through the ammonium sulfate step as previously described (45). MAT catalyzed formation of [U-ribosyl-\(^{14}\)C]AdoMet from [U-ribosyl-\(^{14}\)C]ATP and methionine was carried out in reactions containing 20% acetonitrile, conditions that relieve the severe product inhibition observed with MAT (49,60), and the crude [U-ribosyl-\(^{14}\)C]AdoMet was subjected to reverse-phase HPLC (Fig. 4) to provide pure [U-ribosyl-\(^{14}\)C]AdoMet in radiochemical yields of > 90%. The purified AdoMet was subsequently stored at –90 °C with sulfate as the counter ion (pH < 1.5) to maximize chemical stability and minimize epimerization at the sulfur atom (61,62).

**FIGURE 4**

In order to prepare significant quantities of substrate tRNA an over-expression system for tRNA\(^{\text{Tyr}}\) was constructed by synthesizing the gene for \textit{E. coli} tRNA\(^{\text{Tyr}}\) (I) and ligating it into the pTrc99B vector, which had previously been shown to be an efficient vehicle for the over-expression of \textit{E. coli} tRNA\(^{\text{Asp}}\) (63), to give the expression plasmid pTrc-Tyr. After confirming the structure of the gene through sequencing, pTrc-Tyr was expressed in K12QueA, an \textit{E. coli} queA deletion mutant (37) that produces tRNA containing preQ\(_1\) at position 34 instead of queuosine. HPLC analysis of crude tRNA isolated from K12QueA/pTrc-Tyr cells at various post-induction times indicated that maximal tRNA\(^{\text{Tyr}}\) was present ~13 hrs post-induction (data not shown).

Crude tRNA was isolated using the acid guanidinium thiocyanate/phenol/chloroform protocol (52), and based on tyrosine-tRNA synthetase assays, ~25% of the crude tRNA was determined to be tRNA\(^{\text{Tyr}}\), whereas tRNA from the untransformed strain comprised only ~3% tRNA\(^{\text{Tyr}}\) (data not shown). The crude tRNA was fractionated first by mixed-mode chromatography on BND cellulose as previously described (54,64), with tRNA\(^{\text{Tyr}}\) eluting at 1 M NaCl and 10% ethanol
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(data not shown). The tRNA\textsuperscript{Tyr} enriched fractions were further purified on NACS resin (65), with tRNA\textsuperscript{Tyr} eluting at 800 mM NaCl (data not shown). Because we anticipated screening various metal ions for their effect on QueA activity, the purified preQ\textsubscript{1}-tRNA\textsuperscript{Tyr} was dialyzed at high temperature (80 °C) in citrate buffer containing EDTA to ensure complete removal of all bound metal ions.

A minihelix RNA corresponding to the anticodon stem-loop of \textit{E. coli} tRNA\textsuperscript{Asn} was synthesized via \textit{in vitro} transcription using recombinant T\textsubscript{7} RNA polymerase, and the minihelix RNA purified by continuous flow electrophoresis (data not shown) (57,66). Purification in this way provided the desired 17-mer in large amounts free of all other transcription products as determined from analytical PAGE (data not shown).

To quantify the amount of preQ\textsubscript{1} incorporated into the minihelix RNA by recombinant \textit{E. coli} TGT, [8-\textsuperscript{14}C]guanine was first incorporated into the RNA using TGT (67). After careful measurement of the specific radioactivity of the labeled RNA, the [8-\textsuperscript{14}C]guanine was subsequently eliminated by the TGT catalyzed incorporation of preQ\textsubscript{1}. Based on comparisons of specific radioactivity, greater than 99.8% of the [8-\textsuperscript{14}C]guanine present in the minihelix RNA was replaced by preQ\textsubscript{1}.

\textit{Effect of GST on the catalytic activity of QueA}. Previous investigations of QueA activity had employed only the GST-QueA fusion protein (37-39), but it was not known whether the GST domain compromised catalytic activity. To resolve this issue the activities of GST-QueA and the cleaved QueA were measured and compared under identical conditions; both QueA and GST-QueA had identical molar specific activities, 46.9 ± 7.0 and 46.7 ± 6.3 mmol min\textsuperscript{-1}mol\textsuperscript{-1} enzyme, respectively.

\textit{General Properties of Recombinant GST-QueA}. The pH profile of GST-QueA exhibited a bell-curve with activity between pH 7.5 and 10.5 (Fig. 5), suggesting the involvement of acid-
Characterization of Recombinant QueA

base catalysis where a minimum of two ionizable residues are required for protonation/deprotonation steps. Optimal activity was observed at pH 8.7, and thus was the pH used for subsequent characterization studies.

FIGURE 5

Monovalent ions gave only a slight change in activity, with the optimal concentration at 100 mM of KCl or NaCl (Table 1). Interestingly the presence of Mg$^{2+}$ or Mn$^{2+}$ in millimolar concentrations inhibited QueA activity, but had little or no effect in the submillimolar range (Fig. 6). In contrast, the presence of Ca$^{2+}$ in millimolar concentrations resulted in a modest stimulation of activity. Unexpectedly, the presence of several anions significantly increased activity (Table 1), with optimal activity occurring in the presence of the oxyanions EDTA (100 mM), acetate (750 mM), carbonate (25 mM), or phosphate (25 mM). This phenomenon was also observed with genomic DNA, where activity was highest in the presence of 200 µg/ml DNA. Furthermore, replacing Tris buffer with glygly increased activity another 2-fold (Table 1). Other anionic species, including fluoride and thiocyanate were more moderate activators, while sulfate exhibited comparably little activation of the enzyme. Remarkably, activation of QueA was completely eliminated in the presence of millimolar concentrations of magnesium (Fig. 6). Overall there was an approximate 10-fold difference in the specific activity of QueA when assaying activity in Tris buffer compared with glygly and one of the oxyanionic activators (Table 1).

FIGURE 6

Table 1

*Development of a new activity assay appropriate for small RNA substrates*. The failure of small RNAs to precipitate quantitatively with TCA required the development of an alternate assay for the analysis of small RNA substrates of QueA. A number of methods that utilized
Characterization of Recombinant QueA

selective binding of either AdoMet or RNA to chromatographic matrices were investigated, and good results were obtained with RNA binding to DEAE-cellulose. Since this was also an inexpensive matrix, protocols were optimized for this assay, and involved terminating reactions by decreasing the pH to 6.5 with the addition of acetic acid, briefly heating to 95 °C, and loading the assay solutions into Quik-Sep columns containing 250 µL of DEAE-cellulose equilibrated in 50 mM imidazole (pH 6.5). The unreacted AdoMet was collected by eluting the columns with 50 mM imidazole (pH 6.5), and the RNA subsequently collected by eluting with 50 mM imidazole (pH 6.5) and 1.0 M NaCl. The assay methodology was validated by carrying out replicate assays with preQ₁-tRNA^Tyr and processing one set with the TCA protocol and the other with the DEAE columns; initial velocity data from both series were indistinguishable (data not shown).

Steady-State Kinetics. QueA activity assays were performed under initial velocity conditions at variable substrate concentrations (with saturating co-substrate) to obtain steady-state kinetic parameters for the enzyme-catalyzed reaction. Initial velocities determined from incubations with saturating preQ₁-tRNA^Tyr (15 uM) and variable AdoMet gave a $K_m$ of 104 ± 8.6 µM for AdoMet and a $k_{cat}$ of 2.3 ± 0.1 min⁻¹ (Fig. 7), while incubations with saturating AdoMet (1 mM) and variable preQ₁-tRNA^Tyr provided a $K_m$ of 1.5 ± 0.1 µM for preQ₁-tRNA^Tyr (Fig. 7). When the preQ₁-modified minihelix RNA was the variable substrate, the $K_m$ was determined to be 37.6 ± 4.5 µM, with a $k_{cat}$ of 14.7 ± 0.7 min⁻¹. The kinetic parameters are collected in Table 2.

FIGURE 7

Table 2

DISCUSSION

The enzyme QueA catalyzes the penultimate step in the biosynthesis of the hypermodified tRNA nucleoside queuosine (Fig 3), a complex reaction involving the loss of adenine and
Characterization of Recombinant QueA methionine from the co-factor AdoMet, and the transfer and rearrangement of the ribosyl moiety to tRNA in the formation an epoxy-carbocycle. Although the enzyme has been known for almost a decade, the absence of a commercial source of AdoMet with specific radiochemical labeling in the ribosyl moiety rendered quantitative assays of QueA activity via the direct measurement of epoxyqueuosine problematic; previous studies utilized assays in which QueA activity was observed either indirectly by the inability of TGT to insert [³H]guanine into tRNA after reaction of QueA with AdoMet (37), or directly through the incorporation of tritium into tRNA from the in situ generation of [2,5',8-³H]AdoMet from [2,5',8-³H]ATP in a coupled MAT/QueA reaction (38). While the latter assay allowed for the confirmation of enzymatic activity from the direct observation of epoxyqueuosine formation, the assay was inefficient (~84% of the ³H is in a position not incorporated into epoxyqueuosine) and not amenable to the acquisition of accurate velocity data since the concentration of AdoMet remains unknown when it is generated in situ. 

The need to acquire quantitative initial velocity data therefore necessitated the synthesis of appropriately labeled AdoMet.

With the preparation of homogeneous substrate tRNA, preQ₁-modified minihelix RNA, and ribose-labeled AdoMet as described above it was possible to carry out quantitative measurements of QueA activity via the direct measurement of epoxyqueuosine formation. The observation of identical molar specific activities for the GST-QueA fusion protein and the cleaved QueA suggests that the GST domain does not compromise enzyme activity. However, because the QueA we isolate after Factor Xa cleavage still possesses an additional 7 N-terminal amino acids after cleavage due to the structure of the fusion protein (37), it can be argued that the cleaved QueA is inappropriate for measuring native enzyme activity. To partially address this we subcloned the E. coli queA gene into a pET expression vector and over-produced the wild-type QueA; based on the comparable levels of enzymatic activity and levels of recombinant enzyme
Characterization of Recombinant QueA measured in cell-free extracts containing GST-QueA or wild-type QueA, we conclude that the presence of the N-terminal fusion does not compromise enzyme activity.

The pH at which optimal QueA activity was observed (pH 8.7), while high, is similar to the optimal pH reported for TGT activity (32), and is consistent with the need to deprotonate the 5′-carbon during the course of the reaction. Although there is no obvious need for Mg$^{2+}$ or Mn$^{2+}$ in the QueA catalyzed reaction, the inhibition observed with these metals was surprising given the ubiquity and importance of Mg$^{2+}$ to tRNA tertiary structure (68-70). Likewise, the activation observed in the presence of certain oxyanions was unexpected, particularly at the concentrations seen (Table 1).

We have obtained preliminary evidence that an enzyme-AdoMet complex can form in the presence of MgCl$_2$ (detected by native PAGE), while in the absence of MgCl$_2$ no significant binding of AdoMet to free enzyme occurs (data not shown). Given that we have recently determined that the enzyme follows an ordered-sequential kinetic mechanism in which tRNA binds first, followed by AdoMet (71), these observations are consistent with Mg$^{2+}$-dependent binding of AdoMet to free enzyme to form a dead-end complex. Although the basis for the activation of QueA remains unclear, the inhibition pattern exhibited by Mg$^{2+}$, unchanged in the presence or absence of activating anions, suggests that inhibition and activation are mechanistically distinct phenomena (Fig. 6). Further experiments will clearly be required to clarify the molecular basis of these phenomena; nevertheless, the characterization of these phenomena has allowed us to develop optimal conditions for analyzing enzyme activity.

Enzyme activity assays based on the precipitation of tRNA with TCA are widespread (72,73), but this protocol is not suitable when small RNAs are employed as substrates because they are refractory to TCA precipitation. While other methods have been developed for small RNAs (33,34), we sought a protocol that would give us the flexibility of measuring both product
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formation as well as substrate consumption, and so we investigated column based ion-exchange methods that allowed for the separate collection of product and unreacted substrate. The binding of RNA to DEAE-cellulose proved effective for resolving AdoMet from both small RNA and full-length tRNA, and provided a simple and efficient protocol for processing activity assays.

QueA exhibits classic hyperbolic Mechaelis-Menton kinetic behavior (Fig. 7), with both the tRNA and minihelix RNA serving as efficient substrates for the enzyme \( (k_{cat}/K_M = 1.7 \text{ and } 0.4 \mu\text{M}^{-1}\text{min}^{-1}, \text{ respectively}) \). While the \( K_M \) for minihelix RNA is 25-fold higher than for tRNA, this is almost completely offset by the larger \( k_{cat} \), such that \( k_{cat}/K_M \) for the two differ by < 3-fold. The similar values of \( k_{cat}/K_M \) indicate that all of the RNA structural elements required by QueA for recognition and catalysis are present in the anticodon stem-loop. Indeed, given that the sequence of the minihelix RNA differs from the anticodon stem-loop of tRNA\(_{\text{Tyr}}\) at 4 positions (including the presence of 2-methylthio-N6-isopentyl-adenosine at position 37 in tRNA\(_{\text{Tyr}}\) ), the differences in the kinetic parameters may be due to these sequence differences, and there may be no RNA contacts outside of the anticodon region at all. This is consistent with previous footprinting experiments (74), and is perhaps not surprising given that QueA catalysis is peripheral to the polynucleotide backbone, involving a preexisting modified base not present elsewhere in the tRNA. This can be contrasted with TGT catalysis, the prior step in queuosine biosynthesis, where replacement of the canonical base requires reaction directly with the sugar-phosphate backbone, and where RNA structural information is clearly essential to insure that reaction occurs only at the wobble position of the appropriate codons. Yet even in this case the decrease in \( k_{cat}/K_M \) was only 30-40 fold (33,34), demonstrating that the information content of the anticodon stem-loop is sufficient for achieving the requisite specificity. Thus, perhaps the more relevant question regarding QueA recognition and catalysis is not \textit{which} part of the tRNA is required, but whether \textit{any} part is required.
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Acknowledgement-We thank Mr. Olaf Happe and Ms. Gita Rabbani for technical assistance.
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71. Van Lanen, S. G., and Iwata-Reuyl, D. manuscript in preparation

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FOOTNOTES

1Abbreviations: Q, queuosine; TGT, tRNA-guanine transglycosylase; QueA, S-adenosylmethionine:tRNA ribosyltransferase-isomerase; MAT, S-adenosylmethionine synthetase; APRTase, adenine phosphoribosyl transferase; PRPP synthetase, ; DTT, dithiothreitol; IPTG, isopropyl β-D-thiogalactopyranoside; glygly, glycylglycine; PCR, polymerase chain reaction; HPLC, high pressure liquid chromatography; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid.
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FIGURE LEGENDS

Fig. 1. The structure of queuosine and its location in tRNA.

Fig. 2. The *de novo* biosynthesis of queuosine in Bacteria.

Fig. 3. The reaction catalyzed by QueA.

Fig. 4. A) Purification of [U-ribosyl-\(^{14}\)C]ATP. Purification was carried out by mixed-mode HPLC using a semi-preparative Hypersil 5 C18 column (250 x 10 mm, 5 µm, Phenomenex). The ATP eluted under isocratic conditions of 83.3 mM triethylammonium acetate (pH 6.0) and 6% methanol at 4 mL/min. The peak at ~10 min is due to adenine, the peak at ~12 min is NADPH, and ATP is at ~18 min. B) Purification of [U-ribosyl-\(^{14}\)C]AdoMet. Purification was carried out by reverse-phase HPLC using the above column with elution under isocratic conditions of 20 mM ammonium acetate (pH 6.0) and 2% methanol. [\(^{14}\)C]AdoMet elutes at ~13 min, and the small amount of residual [\(^{14}\)C]ATP elutes at ~5 min.

Fig. 5. pH profile. QueA Activity was assayed with 100 mM buffer, 50 mM KCl, 20 mM MgCl\(_2\), 0.5 mM DTT, 2 µM preQ\(_1\)-tRNA\(_{\text{Tyr}}\), 200 µM AdoMet, and 250 nM QueA under initial velocity conditions. Buffers used are represented as the following: ■ MES (pK\(_a\) 6.1), □ MOPS (pK\(_a\) 7.2), ◆ Tris-HCl (pK\(_a\) 8.1), CHES (pK\(_a\) 9.3), and ▲ CAPS (pK\(_a\) 10.4). Data are the average of at least 4 independent replicates. Standard error was < 10% in all cases.

Fig. 6. Magnesium inhibition. The reactions were run in 100 mM gly-gly (pH 8.7), 0.5 mM DTT, 1 µM preQ\(_1\)-tRNA\(_{\text{Tyr}}\), 100 µM AdoMet, and 100 nM QueA at 37°C under initial velocity conditions. The species present are as follows: ○ Mg(CH\(_3\)COO)_2, X MgCl\(_2\), and ◆ Na(CH\(_3\)COO). Data are the average of at least 4 independent replicates. Standard error was < 10% in all cases.

Fig. 7. Steady-state kinetic analysis. Assays were carried out in 100 mM gly-gly pH 8.7, 100 mM EDTA pH 8.7, 100 mM KCl, 0.5 mM DTT, and variable substrate concentrations. A) Assays contained 0.75 µM preQ\(_1\)-tRNA\(_{\text{Tyr}}\), 200 nM QueA, with [AdoMet] varied from 1 to 250 µM. B) Assays contained 500 µM AdoMet, 80 nM QueA, with [tRNA\(_{\text{Tyr}}\)] varied from 0.38 to 7.5 µM. C) Assays contained 500 µM AdoMet, 80 nM QueA, with [RNA] varied from 1.0 to 200 µM. Data are the average of at least 4 independent replicates. Standard error was < 10% in all cases.
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**TABLES**

Table 1. Salt Effects on QueA Activity

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Maximal Activity Concentration (mM)</th>
<th>Relative Activity&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>100</td>
<td>1.00</td>
</tr>
<tr>
<td>K&lt;sub&gt;2&lt;/sub&gt;CO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>25</td>
<td>1.00</td>
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<tr>
<td>NaOAc</td>
<td>750</td>
<td>0.93</td>
</tr>
<tr>
<td>DNA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>----</td>
<td>0.90</td>
</tr>
<tr>
<td>Na&lt;sub&gt;2&lt;/sub&gt;PO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>25</td>
<td>0.80</td>
</tr>
<tr>
<td>KF</td>
<td>500</td>
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</tr>
<tr>
<td>NaSCN</td>
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<td>0.60</td>
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<td>CaCl&lt;sub&gt;2&lt;/sub&gt;</td>
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</tr>
<tr>
<td>Na&lt;sub&gt;2&lt;/sub&gt;SO&lt;sub&gt;4&lt;/sub&gt;</td>
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<tr>
<td>KCl</td>
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<td>0.28</td>
</tr>
<tr>
<td>NaCl</td>
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<td>0.25</td>
</tr>
<tr>
<td>none&lt;sup&gt;c&lt;/sup&gt;</td>
<td>----</td>
<td>0.22</td>
</tr>
<tr>
<td>Tris-HCl&lt;sup&gt;d&lt;/sup&gt;</td>
<td>----</td>
<td>0.11</td>
</tr>
</tbody>
</table>

<sup>a</sup> Reactions were carried out in 100 mM gly-gly (pH 8.7), 0.5 mM DTT, 100 µM adomet, 1 µM tRNA<sup>Tyr</sup>, and 100 nM GST-QueA plus added chemical.

<sup>b</sup> Isolated from salmon sperm; 10 µg/ 50 µL reaction (concentration by UV-Vis spectroscopy with ε=50 ug/A<sub>260</sub>).

<sup>c</sup> Reaction was carried out in 100 mM gly-gly (pH 8.7), 0.5 mM DTT, 100 µM adomet, 1 µM tRNA<sup>Tyr</sup>, and 100 nM GST-QueA.

<sup>d</sup> Reaction was carried out in 100 mM Tris-HCl (pH 8.7), 0.5 mM DTT, 100 µM adomet, 1 µM tRNA<sup>Tyr</sup>, and 100 nM GST-QueA.
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Table 2. Michaelis constants for QueA

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_M$ (µM)</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$k_{cat}/K_m$ (µM$^{-1}$min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adomet</td>
<td>104.4 ± 8.6</td>
<td>2.3 ± 0.1</td>
<td>-----</td>
</tr>
<tr>
<td>tRNA$^{\text{Ty}}$</td>
<td>1.5 ± 0.2</td>
<td>2.5 ± 0.1</td>
<td>1.7</td>
</tr>
<tr>
<td>Minihelix RNA</td>
<td>37.7 ± 4.5</td>
<td>14.7 ± 0.7</td>
<td>0.4</td>
</tr>
</tbody>
</table>
Queuosine (Q) $R = H$
Mann Q $R = \beta$-D-mannose
Gal Q $R = \beta$-D-galactose
The diagram illustrates the conversion of GTP to Q-tRNA through the following steps:

1. **GTP** is converted to **preQ₁** using an unknown reaction.
2. **preQ₁** is then modified by TGT-tRNA to form **preQ₁-tRNA**.
3. **preQ₁-tRNA** is further modified by QueA and AdoMet to form **oQ-tRNA**.
4. **oQ-tRNA** is then modified by **B₁₂** to form the final product, **Q-tRNA**.
Figure 4
Figure 5
Figure 6

- Activity (nM/min) vs. Concentration (mM)

- The graph shows a significant increase in activity with concentration.
Figure 7

A

Velocity (nM/min) vs. [Adomet] (uM)

B

Velocity (nM/min) vs. [tRNA] (uM)

C

Velocity (uM/min) vs. [17-mer] (uM)
tRNA modification by S-adenosylmethionine: tRNA ribosyltransferase-isomerase (QueA): Assay Development and Characterization of the Recombinant Enzyme
Steven G. Van Lanen, Sylvia Daoud Kinzie, Sharlene Matthieu, Todd Link, Jeff Culp and Dirk Iwata-Reuyl

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