A New Function of *S*-Adenosylmethionine: The Ribosyl Moiety of AdoMet Is the Precursor of the Cyclopentenediol Moiety of the tRNA Wobble Base Queuine†

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ABSTRACT: Queuosine (Q) [7-(((4,5-*cis*-dihydroxy-2-cyclopenten-1-yl)amino)methyl)-7-deazaguanosine] usually occurs in the first position of the anticodon of tRNAs specifying the amino acids asparagine, aspartate, histidine, and tyrosine. The hypermodified nucleoside is found in eubacteria and eucaryotes. Q is synthesized *de novo* exclusively in eubacteria; for eucaryotes the compound is a nutrient factor. In *Escherichia coli* the Q precursor (oQ), carrying a 2,3-epoxy-4,5-dihydroxycyclopentane ring, is formed from tRNA precursors containing 7-(aminomethyl)-7-deazaguanine ($preQ_1$) by the *queA* gene product. A genomic *queA* mutant accumulating $preQ_1$ tRNA was constructed. The QueA enzyme was overexpressed as a fusion protein with the glutathione *S*-transferase from *Schistosoma japonicum* and purified to homogeneity by affinity and anion-exchange chromatography. The enzyme QueA synthesizes oQ from $preQ_1$ in a single *S*-adenosylmethionine- (AdoMet-) requiring step, indicating that the ribosyl moiety of AdoMet is transferred and isomerized to the epoxycyclopentane residue of oQ. The identity of oQ was verified by HPLC and directly combined HPLC/mass spectrometry. The formation of oQ was reconstituted *in vitro*, applying a synthetic RNA. A 17-nucleotide microhelix (corresponding to the anticodon stem and loop of $tRNA^{Tyr}$ from *E. coli*) is sufficient to act as the RNA substrate for oQ synthesis. We propose that QueA is an *S*-adenosylmethionine:tRNA ribosyltransferase–isomerase.

Throughout all kingdoms of life a unique feature of tRNAs is their content of modified nucleosides. Up to now, 80 different modifications have been found in tRNA, but still, little is known about their biosynthesis and functions. An especially interesting modification is the deazaguanine derivative queuosine (Q)[1] [7-(((4,5-*cis*-dihydroxy-2-cyclopenten-1-yl)amino)methyl)-7-deazaguanosine]. Q is present in tRNAs of procaryotes and eucaryotes except archaebacteria and yeast. In tRNAs specific for Asn, Asp, His, and Tyr, Q replaces G_{34} in the first position of the anticodon. In mammals the Q content of these tRNAs is dependent on the developmental state of the respective cells. Embryonic tissues and regenerating rat liver and dedifferentiated tumor cells contain high amounts of the free base queuine (q) and of Q-undermodified tRNAs, whereas tRNAs in adult tissues and in differentiated cells are usually fully modified with Q (Nishimura, 1983). The extent of Q undermodification can be correlated with the morphological grading of solid tumors and is of value for the prognosis of neoplastic diseases (Shindo-Okada *et al.*, 1981; Emmerich *et al.*, 1985; Biing-Shiun *et al.*, 1992). Queuine alters the phosphorylation of specific proteins and enables cells to adapt their metabolism to hypoxic stress (Langgut *et al.*, 1990; Langgut & Kersten, 1990; Mahr *et al.*, 1990). These interesting properties prompted us to investigate the biosynthesis of queuosine.

Eucaryotes are unable to form Q and must obtain the compound as a nutrient factor or from their intestinal flora and insert the Q-base directly into the tRNA. Eubacteria synthesize Q *de novo* by a complicated biosynthetic pathway that was investigated in *Escherichia coli* (Figure 1). The biosynthesis commences with GTP, which is converted in an iron-requiring process to 7-(aminomethyl)-7-deazaguanine ($preQ_1$) by mechanisms and enzymes that are not yet characterized (Kersten & Kersten, 1990; Nishimura, 1983). $PreQ_1$ is then inserted into the first position of the respective tRNAs by exchange with guanine. The reaction is catalyzed by the tRNA–guanine transglycosylase (Tgt) (EC 2.4.2.29), the sole enzyme involved in Q biosynthesis so far purified and characterized (Okada & Nishimura, 1979). The $preQ_1$-containing tRNA is converted to epoxyqueuosine (oQ) [(((2,3-epoxy-4,5-*cis*-dihydroxycyclopent-1-yl)amino)methyl)-7-deazaguanosine] by attachment of an epoxycyclopentanediol moiety. *In vivo*, this reaction requires an intact *queA* gene (Reuter *et al.*, 1991) and methionine; however, no carbon atom of methionine is incorporated into oQ or Q (Katze *et al.*, 1977; Okada *et al.*, 1977). Consequently, the *queA* mutant strain JE10651 (Okada *et al.*, 1978) and methionine-starved *E. coli* are Q deficient and accumulate Q precursor molecules, mainly $preQ_1$. The last step in the Q biosynthesis is the reduction of oQ by an yet uncharacterized vitamin B12 dependent enzyme system (see Figure 1) (Frey *et al.*, 1988; Phillipson *et al.*, 1987).

The genes *tgt* and *queA* are cloned and sequenced. They are located at 9 min on the *E. coli* chromosome in one operon together with two genes participitating in protein secretion (*secD*, *secF*) and one open reading frame (ORF12) (Reuter *et al.*, 1991). The promoter of this operon is preceded by an upstream activator sequence containing a binding site for the *E. coli* factor of inversion stimulation (FIS). A similar promoter organization was found for many tRNA operons, suggesting a coordinate control of these modifying enzymes

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[1] Abbreviations: Q, queuosine; oQ, epoxyqueuosine; $preQ_1$, 7-(aminomethyl)-7-deazaguanine; Tgt, tRNA–guanine transglycosylase; GST, glutathione *S*-transferase; A_{260}, one absorption unit at 260 nm; DEAE, diethylaminoethyl; PMSF, phenylmethanesulfonyl fluoride; IPTG, isopropyl thiogalactoside; NTP, nucleoside triphosphate.

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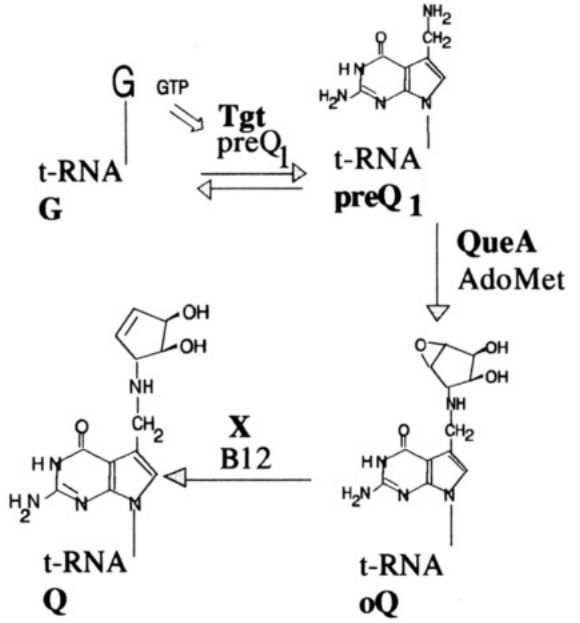

FIGURE 1: tRNA-dependent steps of queuosine biosynthesis [Tgt = tRNA–guanine transglycosylase; QueA = QueA enzyme (*S*-adenosylmethionine:tRNA ribosyltransferase–isomerase); X = unknown enzyme; $preQ_1$ = 7-(aminomethyl)-7-deazaguanine; AdoMet = *S*-adenosylmethionine; B12 = adenosylcobalamine; oQ = epoxyqueuosine; Q = queuosine].

with their substrate tRNAs (Slany & Kersten, 1992). Here we describe the overproduction, purification, and characterization of the QueA enzyme. Evidence is presented that QueA is an *S*-adenosylmethionine:tRNA ribosyltransferase–isomerase. During the enzyme reaction the ribosyl moiety of *S*-adenosylmethionine is isomerized to an epoxycyclopentanediol moiety that is transferred to tRNA-bound 7-(aminomethyl)-7-deazaguanine to yield epoxy-Q. This is the first example for the participation of the ribosyl moiety of AdoMet in a unique biosynthesis.

Furthermore, the tRNA-dependent steps of oQ formation were reconstituted using the enzymes Tgt and QueA and synthetic substrates. A completely unmodified 17-base oligoribonucleotide corresponding to the anticodon stem and loop of $tRNA^{Tyr}$ from *E. coli* serves as a substrate for the biosynthesis of epoxy-Q *in vitro*.

MATERIALS AND METHODS

Materials. Restriction endonucleases, nucleic acids, and DNA modifying enzymes were from Boehringer, Mannheim, Germany, Promega Corp., Madison, WI, Fermentas, Vilnius, Lithuania, or U.S. Biochemical Corp., Cleveland, OH. [α-^{32}P]-dATP, [γ-^{32}P]ATP and [^{3}H]guanine sulfate (custom synthesis, 7.7 Ci/mmol) were purchased from Amersham Corp., Little Chalfont, U.K. Culture media were from Difco Laboratories, Detroit, MI, and Merck GmbH, Darmstadt, Germany. Quiagen columns were obtained from Diagen, Hilden, Germany. Biochemicals and common reagents were from Sigma Chemical Co., St. Louis, MO, Serva AG, Heidelberg, Germany, Roth, Karlsruhe, Germany, or Fluka, Basel, Switzerland. Synthetic Q-base (designated queuine) and $preQ_1$ base [7-(aminomethyl)-7-deazaguanine] were kind gifts from S. Nishimura, Tokyo, Japan. The corresponding $preQ_1$ nucleoside was prepared by enzymatic insertion of $preQ_1$ into G-containing, Q-specific tRNA by the Tgt enzyme and subsequent hydrolysis and dephosphorylation. Columns and media for protein and tRNA purification were obtained either from Pharmacia, Uppsala, Sweden, or from Whatman, Maidstone, U.K.

A synthetic RNA oligonucleotide corresponding to the anticodon stem and loop of *E. coli* $tRNA^{Tyr}$ (5′-GCAGACU-GUAAAUCUGC-3′) was prepared by G. Ott, Bayreuth, with commercially available RNA synthesizer equipment from Diagen GmbH, Hilden, Germany.

Bacterial Strains and Plasmids. Bacterial strains used in this work included DH5α [endA1, hsdR17, supE44, thi-1, recA1, gyrA96, relA1, Δ(argF-lac)U169, ϕ*80dlacZ*ΔM15, λ^- (laboratory stock)], JM105 [Δ(lac-proAB), thi, StrA, endA, sbcB, hsdR, F′traD36 proAB $lacI^q$ZM15 (laboratory stock)], K12 wild type (German collection of microorganisms, DSM), JE10651 queA and various partially uncharacterized mutations [from S. Nishimura (Okada et al., 1978)], and K12queA (this work).

To construct a defined genomic *queA* mutation, the cloned *queA* gene was excised from plasmid pHH that contains the whole *tgt* operon (Reuter et al., 1991), as a *Cla*I fragment, and inserted into the *Hin*dII (isoschizomer of *Sal*I) site of pUC19, resulting in the plasmid pCC. A 263-bp deletion within the coding sequence of *queA* was created by cleavage of pCC with *Sal*I and subsequent religation (plasmid pCCΔS; see Figure 3. The insert of pCCΔS was transferred (*Hin*dIII/*Bam*HI) to the vector pMAK705 that was kindly provided by S. Kushner (Hamilton *et al.*, 1989). pMAK705 carries a temperature-sensitive pSC101 replicon and the gene for chloramphenicol acetyltransferase as a selection marker. The deletion was introduced into the genome of strain K12 by allelic replacement exactly as described by Hamilton *et al.*, (1989). For overexpression of the QueA enzyme the *queA* gene was inserted as a *Cla*I/*Dra*I fragment (fill in) into the i-*Sac* (*Ecl*136I) site of pUC19. The remaining upstream untranslated regions of *queA* were removed by treatment with exonuclease III and S1 nuclease (Henikoff, 1987). Suitable clones were identified by sequencing, and the corresponding insert was transferred to the expression vector pGEX-3X (Pharmacia) to yield an in-frame fusion of *queA* with the gene for glutathione *S*-transferase (*gst*) from *Schistosoma japonicum*. The construct is under control of the IPTG-inducible *tac* promoter and contains a $lacI^q$ gene for host independence. The genes were separated by an intervening factor Xa cleavage site and seven additional codons resulting from the cloning procedure (see Figure 2). The expression plasmid was designated pGEX-QA.

General Methods. DNA manipulations, restriction fragment isolations, and common methods for protein analysis were performed as described (Sambrook *et al.*, 1989). Protein concentrations were determined according to the method of Bradford (1976) using bovine γ-globulin as a standard. Native M_r determinations were performed by chromatography on a Superdex 200 column (Pharmacia) in 50 mM Tris-HCl pH 7.4, 100 mM KCl, and 2 mM DTT. Bulk tRNA was prepared from logarithmically growing cultures by phenol extraction and anion-exchange chromatography on DEAE-cellulose (Palatnik & Katze, 1977).

Purification of QueA. E. coli strain K12 was cotransformed with the expression plasmid pGEX-QA and the plasmid pLysS harboring the gene for T7 lysozyme (Studier *et al.*, 1990). The T7 lysozyme is inactive as long as it is located in the cytoplasma but facilitates cell lysis once it is released by cell disruption. A total of 2.5 L of YT medium was inoculated with 10 mL of an overnight culture of K12·pGEX-QA·pLysS. The cells were grown to an optical density (578 nm) of approximately 0.7, and protein expression was induced by addition of 0.2 mM IPTG. After an additional 5 h the cells were harvested by centrifugation and resuspended in 25 mL of buffer A [10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 100

mM NaCl, 6 mM DTT, 10% (v/v) glycerol] supplemented with 1% Triton X-100 and 0.2 mM PMSF. After cell disruption by sonication the mixture was spun at 100000*g* and 4 °C for 3 h. The clear supernatant was divided into aliquots that were applied to an affinity column (16 × 100 mm) filled with glutathione–Sepharose 4B (Pharmacia) that was preequilibrated with buffer A + 1% Triton X-100. The column was washed with buffer A + 100 mM NaCl until the absorbance (280 nm) of the eluate reached the basal level, and finally the bound fusion protein was eluted by applying buffer A + 20 mM glutathione (reduced form). The affinity purification was carried out at 4 °C at flow rates of approximately 0.3 cm/min. The eluted fusion protein was concentrated by ultrafiltration in Centricon 30 spin filter untis (Amicon) and cleaved by addition of factor Xa restriction protease (1 mg/400 mg of fusion protein) and $CaCl_2$ (final concentration 1 mM) overnight at room temperature. In a last purification step the cleavage products were separated on an FPLC MonoQ anion-exchange column (Pharmacia). Aliquots of the proteins were applied to the MonoQ column equilibrated with buffer B [10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM DDT, 10% (v/v) glycerol], and the column was developed by a gradient (1 mL/min flow) of buffer C (=buffer B +1 M NaCl) of the following shape (beginning time and ending time, linear increase of buffer C to the given percentage): 0–4 min, 0% C; 4–10 min, 16% C; 10–14 min, 16% C; 14–21 min, 35% C; 21–22 min, 100% C; 22–23 min, 100% C; 23–25 min, 0% C; 25–27 min, 0% C; end. The QueA protein eluted at a concentration of about 200 mM NaCl and was >97% pure as judged by SDS–PAGE.

Determination of Glutathione S-Transferase Activity. To monitor the purification process of the fusion protein, the enzymatic activity of the glutathione *S*-transferase (GST) part was measured in an optical test. GST conjugates glutathione to 1-chloro-2,4-dinitrobenzene, yielding a product with an absorbance maximum at 340 nm. The progress of the reaction can be conveniently monitored by reading the UV absorption of the reaction mixture. The reaction was carried out at 24 °C in glass cuvettes containing 2 mL of substrate solution (100 mM K_2HPO_4/KH_2PO_4, pH 6.5, 1 mM glutathione, 1 mM 1-chloro-2,4-dinitrobenzene). The reaction was started by the addition of an appropriate amount of test protein, and the initial increase in the absorbance at 340 nm was extrapolated. One unit was defined as the amount of protein necessary to increase the absorption 0.001 unit within 1 min; 1000 units (1 kilounit correspond to the formation of 0.2 μmol/min thioether product in a reaction volume of 2 mL assuming $\Delta\epsilon = 9.6\ mM^{-1}\ min^{-1}$ (Habig *et al.*, 1974).

tRNA–Guanine Transglycosylase (Tgt) Test. The Tgt enzyme was purified either according to Okada et al. (1979) or using a Tgt overproducing strain supplied by G. A. Garcia (University of Michigan, Ann Arbor, MI, personal communication). The Tgt test was performed as described (Okada *et al.*, 1979). The test allows distinction between Q-specific tRNAs that contain queuosine or epoxyqueuosine in the anticodon and tRNAs that are undermodified and contain G or the Q precursor $preQ_1$ in the respective position. Normally the Tgt enzyme exchanges the guanine residue in the anticodon with 7-(aminomethyl)-7-deazaguanine ($preQ_1$), but Tgt can also (i) catalyze the reverse reaction, that is, the exchange of tRNA-bound $preQ_1$ with guanine, and (ii) exchange guanine in undermodified Q-specific tRNAs with guanine (e.g., with [^{3}H]guanine). Once the synthesis of Q is completed, the tRNA is no longer a substrate for Tgt. These properties allow the specific labeling of Q-undermodified tRNAs with [^{3}H]-guanine. From the extent of [^{3}H]guanine incorporation into the respective tRNAs the amount of Q-undermodified tRNA can be estimated. In a typical experiment 10 μg of bulk tRNA was incubated with 2 μCi (2 μL) of [^{3}H]guanine sulfate (specific activity 7.7 Ci/mmol) and 0.75 μg of purified Tgt enzyme in a 20-μL reaction mixture (20 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 2 mM DTT) for 2 h at 37 °C. The tRNA was precipitated with ice-cold 10% trichloroacetic acid on filter papers, washed with 5% trichloroacetic acid and ethanol, and dried, and the incorporated activity was counted using a liquid scintillation counter.

HPLC and Directly Combined HPLC/Mass Spectrometry (LC/MS) Analysis of tRNA. tRNA was hydrolyzed by P1 nuclease and dephosphorylated with alkaline phosphatase from *E. coli*. The resulting nucleosides were subjected to reversed-phase chromatography on a 4.6 × 250 mm Supelcosil (Supelco, Bellefonte, PA) LC-18 column using a 10 mM ammonium phosphate/methanol gradient as described (Frey et al., 1988). Single peaks were identified by retention time comparisons and cochromatography with synthetic reference nucleosides. 8-Bromouridine was used as an internal standard. The instrument, procedures, and interpretation of LC/MS data have been described (Pomerantz & McCloskey, 1990). tRNA digests (25 μg) were fractionated for LC/MS by reversed-phase chromatography (Supelco LC-18S column; 4.6 × 250 mm) using a 0.25 M ammonium acetate (pH 6)/40% (aqueous acetonitrile gradient (Pomerantz & McCloskey, 1990).

RESULTS

Purification and Physical Properties of QueA. An enzymatic test for QueA was not available; therefore, the enzyme was overexpressed as a fusion protein with the glutathione *S*-transferase (GST) from *S. japonicum*. This procedure permits the purification of the fusion protein by affinity chromatography on glutathione–Sepharose 4B. The *queA* gene was subcloned and its 5′ flanking regions deleted by exonuclease III. The insert was transferred to the expression vector pGEX-3X (Pharmacia). This resulted in an amino-terminal fusion of *queA* with the *gst* gene and a short intervening sequence coding for the cleavage site of the restriction protease factor Xa and seven additional amino acids (GIPSLHV) generated by the deletion and cloning procedure (Figure 2A). The overexpression was performed in *E. coli* strain K12 cotransformed with the cell lysis plasmid pLysS (see Materials and Methods). The construct was under control of the inducible *tac* promoter. After induction with 0.2 mM IPTG for 5 h, a soluble protein with an apparent M_r of 65 000 in SDS–PAGE accumulated within the cells to an amount corresponding to about 15–20% of the total cell protein. A protein extract from the induced cells displayed a high GST activity. The 65-kDa protein was selectively bound to glutathione–Sepharose 4B, indicating that it constituted the GST–QueA fusion protein (see Figure 2). Elution with 20 mM free glutathione yielded a protein fraction highly enriched with the GST–QueA fusion product. As judged by determination of the GST activity, this step resulted in a 4.4-fold purification of the fusion protein (see Table I). The eluate was concentrated and treated with factor Xa, and the reaction products were analyzed by SDS–PAGE. The 65-kDa protein band disappeared, and two new bands emerged, corresponding to the expected 39-kDa QueA and 26-kDa GST proteins. The GST component and residual impurities were removed in a final purification step by anion-exchange chromatography on a FPLC MonoQ column. Approximately >97% pure QueA as judged by SDS–PAGE was eluted from the column at a NaCl concentration of 200 mM (Figure 2 and Table I).

The native M_r of QueA was determined by size exclusion chromatography on a Superdex 200 column with Blue Dextran

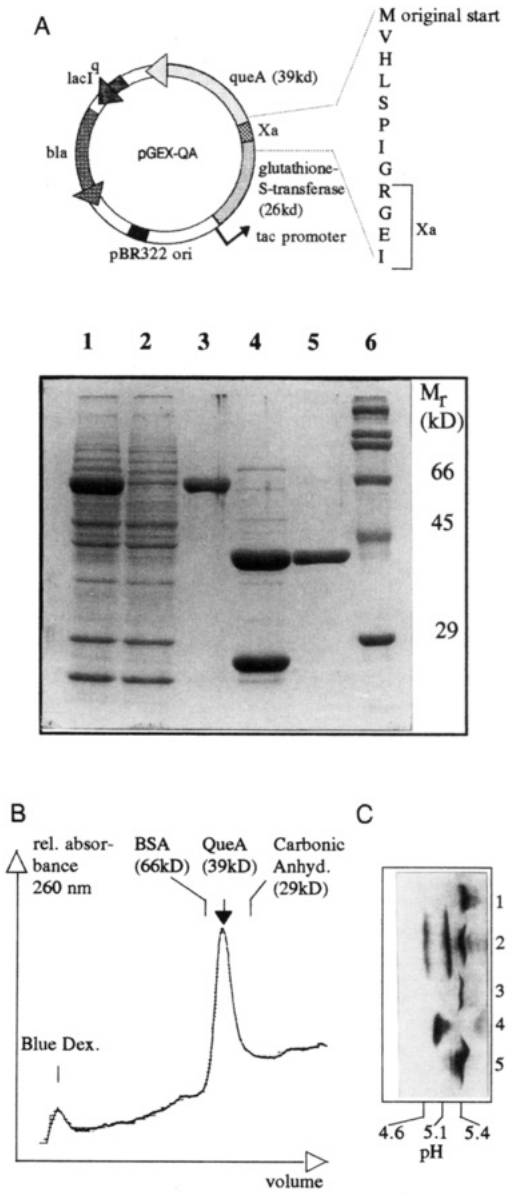

FIGURE 2: Purification and physical characterization of the QueA enzyme. (A) Schematic representation of the plasmid construct used to overexpress the fusion protein consisting of a glutathione *S*-transferase (*gst*) part, an intervening factor Xa cleavage site, and the *queA* gene. The amino acid sequence of the junction site is given in single-letter code. The picture shows a 10% SDS–polyacrylamide gel demonstrating the purification process. Lane 1, 30 μg of S100 protein extract from IPTG-induced cells; lane 2, 30 μg of the flow-through fraction from affinity chromatography on glutathione–Sepharose 4B; lane 3, 8 μg of eluted GST–QueA fusion protein; lane 4, 30 μg of fusion protein after factor Xa cleavage; lane 5, 8 μg of queA as eluted from MonoQ anion-exchange chromatography; lane 6, molecular weight standards. For further details see Materials and Methods. (B) Elution profile of QueA in size exclusion chromatography. QueA (1 mg) was subjected to chromatography on a Superdex 200 column together with 200 μg of Blue Dextran ($M_r > 2 \times 10^6$). The column was calibrated with bovine serum albumin (66 kDa) and bovine carbonic anhydrase (29 kDa). The elution times of the respective proteins are indicated. (C) Isoelectric focusing of the QueA protein. QueA protein (10 μg) was separated together with marker proteins on a 5% polyacrylamide gel in a gradient ranging from pH 3.5 to pH 10. Lane 1, QueA, p*I* = 5.4; lane 2, marker proteins (from left to right) soy trypsin inhibitor, p*I* = 4.6; β-lactoglobulin A, p*I* = 5.1; and bovine carbonic anhydrase, p*I* = 5.4; lane 3, tRNA–guanine transglycosylase, p*I* = 5.3; lane 4, bovine serum albumin, p*I* = 4.8; lane 5, bovine carbonic anhydrase, p*I* = 5.4.

(>2 × 10^6 kDa), bovine serum albumin (66 kDa, monomer), and carbonic anhydrase from bovine erythrocytes (29 kDa) as references. QueA eluted with a retention time corresponding to an M_r of 40 000, indicating that it is monomeric in solution (see Figure 2B). The p*I* of QueA is about pH 5.4 as judged by isoelectric focusing together with marker proteins. This is close to the theoretical value of pH 5.0 calculated from the amino acid composition (Figure 2C).

Construction of a Genomic queA Mutant. As a prerequesite for the elucidation of the QueA-catalyzed reaction, it was essential to obtain tRNA containing the Q precursor preQ$_1$, the substrate for the QueA enzyme. The *queA* strain JE10651 from S. Nishimura (Okada *et al.*, 1978) was derived by chemical mutagenesis and carries many additional mutations. Furthermore, the *queA* mutation is leaky, and tRNA from this strain contains not only Q precursors but also Q (not shown). Therefore, we constructed a defined *queA* mutant in a K12 wild-type background by allelic replacement according to Hamilton *et al.* (1989). A 263-bp *Sal*I fragment from *queA* (Figure 3A) was deleted and the mutation verified by Southern analysis (Figure 3B). tRNA from this K12queA strain was Q deficient and incorporated 55–60 pmol of [^{3}H]guanine per 1 A_{260} in the Tgt test (see Materials and Methods). tRNA from the parent strain K12 or a K12queA strain transformed with a plasmid copy (pCC) of the intact *queA* gene was fully modified with Q (Figure 3C). This confirms that the queuosine biosynthesis cannot further proceed in the constructed *queA* mutant. Furthermore, this mutant accumulates a Q precursor that was exchangeable with [^{3}H]guanine in the Tgt reaction. This precursor presumably constitutes the substrate for QueA. In HPLC analysis one additional nucleoside peak emerged in tRNA from K12queA that was not present in tRNA from K12 or K12queA transformed with pCC. The corresponding nucleoside was identified as preQ$_1$ nucleoside [7-(aminomethyl)-7-deazaguanosine] by retention time analysis compared to synthetic preQ$_1$ base and synthetic preQ$_1$ nucleoside (see Figure 5A and the next section).

Characterization of the QueA Reaction: A New Role for S-Adenosylmethionine. Purified QueA and preQ$_1$-containing tRNA from K12queA were used in a standard *in vitro* reaction assay to test various substances, especially coenzymes, for their ability to act as cosubstrate for QueA. On the basis of the *in vivo* results the enzymatic conversion of preQ$_1$ to oQ (or its precursor) by QueA concomitantly decreases the ability of the tRNA to be labeled in the Tgt assay. Because the incorporated amount of [^{3}H]guanine is directly proportional to the preQ$_1$ content, it is declined with the progress of the QueA-catalyzed reaction. Also, the preQ$_1$ nucleoside peak in the HPLC analysis was converted to a peak corresponding to the reaction product. In a typical reaction assay 100 μg of tRNA from K12queA was incubated with 10 μg of purified QueA protein in a 150-μL solution containing 20 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 2 mM DTT, and 1 mM respective test substance. The mixture was incubated for 3–5 h at 37 °C, and aliquots were withdrawn in regular intervals, heat inactivated (15 min at 65 °C), and analyzed by the Tgt test. After 5 h 50 μg of tRNA was ethanol precipitated, digested, dephosphorylated, and subjected to HPLC. From all possible precursor molecules tested [ribose- or ribitol-containing compounds: all dNTPs and rNTPs, NAD(H)$^+$, NADP(H)$^+$, FAD, adenosylcobalamin, riboflavin, ribose, ribitol, ribulose, AdoMet] only *S*-adenosylmethionine served as a substrate in the QueA reaction. The reaction rate as measured by the difference in [^{3}H]guanine incorporation before and after the reaction was directly proportional to the amount of AdoMet added (Figure 4). The [^{3}H]guanine incorporation dropped from approximately 60 pmol per A_{260} tRNA to a saturation level of 25 pmol per A_{260}, dependent on the concentration of AdoMet. Following incubation with *S*-adenosyl[*methyl*-^{14}C

Table I: Purification of the QueA Enzyme

fraction	vol (mL)	protein (mg)	GST act. (kilounits)[a]	sp act. (kilounits/mg)	purifn
S100	29	1247	2868	2.3	1
affinity eluate	43.5	160	1600	10.0	4.4
Xa cleavage	20	160 (+400 μg of Xa)	nd[b]	nd	4.4
MonoQ	45	77	nd	nd	>97% pure (SDS–PAGE)

[a] One kilounit (1000 units) corresponds to the formation of 0.2 μmol/min thioether reaction product (see Materials and Methods). [b] Not determined.

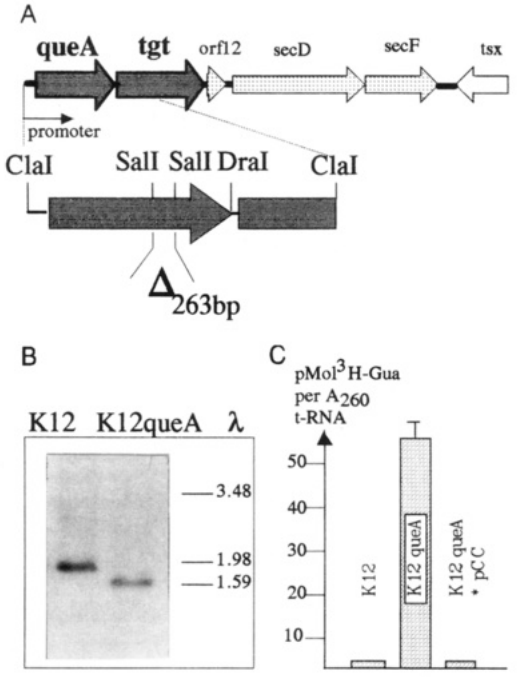

FIGURE 3: Construction of a genomic *queA* mutant. (A) Schematic representation of the *tgt* operon in *E. coli* and subcloning strategy. A 263-bp *Sal*I fragment was deleted from *queA* by allelic replacement (Hamilton *et al.*, 1989). (B) Southern analysis of genomic DNA from the mutant K12queA and a wild-type K12 strain. Genomic DNA (10 μg) from the indicated strains was digested with *Cla*I, separated on a 1% aragose gel, blotted, and hybridized against a *queA* probe. The fragment from K12queA shows the expected deletion. *Eco*RI/*Hin*dIII-digested λ DNA was used as a size standard. The numbers indicate the size of the respective fragments in kilobases. (C) tRNA–guanine transglycosylase test performed with tRNA extracted from *E. coli* K12, K12queA, and K12queA transformed with pCC carrying an intact copy of *queA*. The incorporation of [^{3}H]guanine into the tRNA of K12queA demonstrates that queuosine precursors are present in the respective Q-specific tRNAs instead of queuosine. For further details see text.

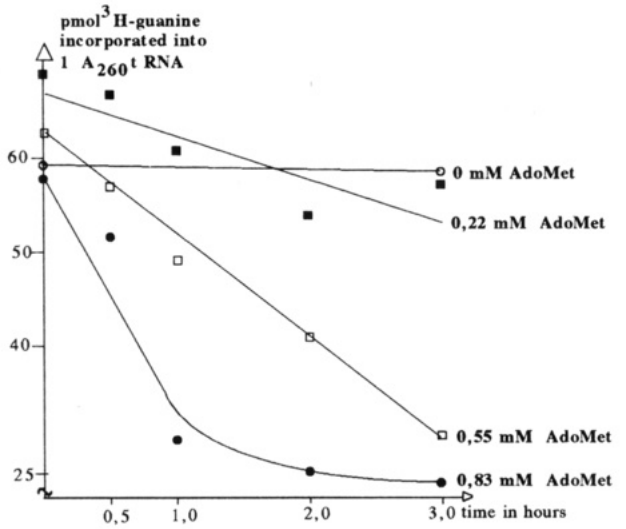

FIGURE 4: Time course of the QueA-catalyzed reaction. In a standard assay tRNA from the *E. coli* K12queA strain was incubated with purified QueA and varying concentrations of AdoMet (see text). At the indicated time points aliquots were removed and analyzed by the tRNA–guanine transglycosylase test (see Materials and Methods). The values of [^{3}H]guanine incorporation are directly proportional to the amount of Q-precursor left. Therefore, a decreasing ^{3}H incorporation indicates a proceeding QueA reaction that converts the Q precursor preQ$_1$ [7-(aminomethyl)-7-deazaguanine] to epoxyqueuosine.

or *carboxy*-^{14}C]methionine no radioactivity was incorporated into the tRNA (not shown), suggesting that neither the reactive methyl nor the equally activated 1-amino-1-carboxypropyl moiety of AdoMet is involved in the reaction. In HPLC analysis (Figure 5) the preQ$_1$ nucleoside peak disappeared, whereas a new peak appeared at a position normally occupied by epoxy-Q (Figure 5B). The identity of oQ as the reaction product of QueA and AdoMet with preQ$_1$ tRNA was confirmed by LC/MS of digested tRNA (Figure 6). The thermospray mass spectrum of oQ contains an ion (m/z 132) diagnostic for the 2,3-epoxycyclopentanylamino substitutent (Pomerantz & McCloskey, 1990). The reconstructed ion chromatogram of m/z 132 from digested preQ$_1$ tRNA (Figure 6A) shows no signal at the expected retention time of oQ. The reconstructed ion chromatogram from the digest of treated tRNA (Figure 6B), however, shows a peak for m/z 132 at the expected retention time for oQ (Pomerantz & McCloskey, 1990). The QueA reaction afforded no addition of another component. Neither ATP nor a redox acceptor/donor was necessary for the enzyme. Therefore, QueA must use a part of AdoMet to synthesize in one step epoxyqueuosine from preQ$_1$.

Reconstitution of the QueA Reaction with a Synthetic RNA Substrate. To determine the requirements of the QueA enzyme interaction with the tRNA, *in vitro* experiments were performed using a synthetic RNA oligonucleotide as a substrate. A commercially synthesized 17-base oligoribonucleotide with the sequence of the acceptor stem and loop of the *E. coli* tRNA^{Tyr} (5′-GCAGACUGUAAAUCUGC-3′) was applied. This short RNA is capable of forming a 5-base-pair stem connected by a 7-base-pair loop. HPLC analysis showed that this oligoribonucleotide contained the four common nucleosides, A, C, G, and U, and some minor impurities probably derived from incomplete deprotection during synthesis (Figure 7A). Preliminary experiments revealed that this oligonucleotide constitutes a substrate for the Tgt enzyme (data not shown). Therefore, it was possible to insert synthetic preQ$_1$ into the anticodon loop with the Tgt enzyme. The reaction was carried out at 30 °C to stabilize the short stem under conditions otherwise identical with the Tgt test. The reaction products were analyzed by HPLC, and the RNA was found to contain a single additional nucleoside corresponding to preQ$_1$ nucleoside (Figure 7B). A total of 20 μg of this modified oligonucleotide was further incubated with 5 μg of QueA enzyme and 1 mM AdoMet for 5 h at 30 °C. This resulted in an almost complete conversion of the preQ$_1$ nucleoside to oQ, indicating that the Tgt as well as the QueA enzyme does not need the complete three-dimensional tRNA structure or any modifications to accept an RNA as substrate (Figure 7C).

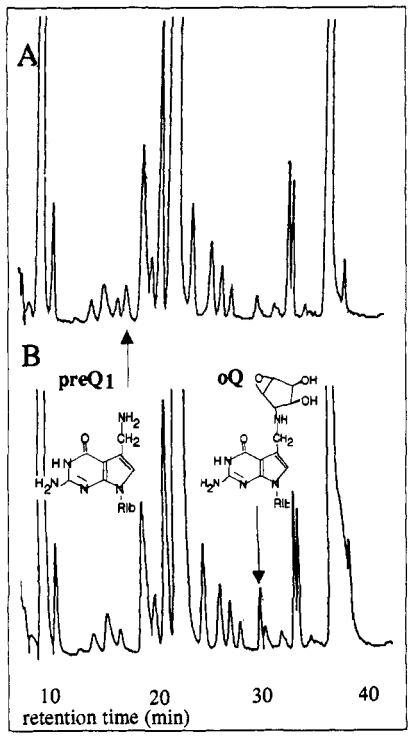

FIGURE 5: Reversed-phase HPLC analysis of tRNA nucleosides; detection by UV absorbance at 254 nm (for technical details see Materials and Methods). (A) Analysis of tRNA from *E. coli* K12queA. The arrow designates an additional peak (corresponding to $preQ_1$ nucleoside) that is not present in tRNA from *E. coli* K12. (B) The same tRNA as in (A) was analyzed after incubation with purified QueA enzyme and 1 mM AdoMet. The $preQ_1$ nucleoside peak disappears, and a new peak appears (arrow) corresponding to epoxyqueuosine.

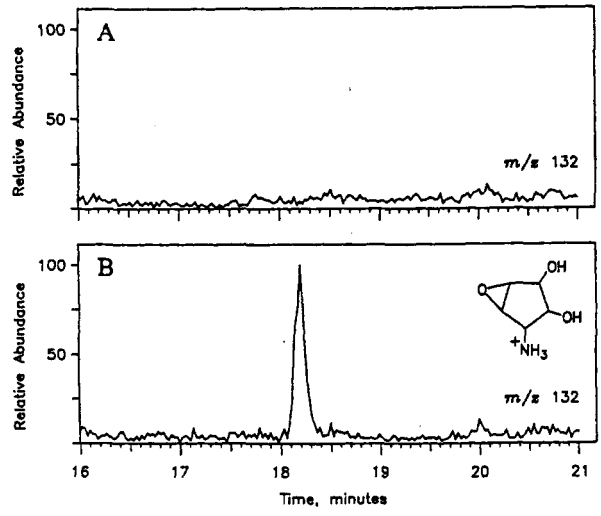

FIGURE 6: Reconstructed ion chromatograms of the m/z 132 ion, diagnostic for oQ, from LC/MS analysis of nucleosides from digests of $preQ_1$ tRNA (A) and $preQ_1$ tRNA treated with QueA and *S*-adenosylmethionine (B). Note that the separation protocol of the tRNA digest for LC/MS differs from the standard procedure (see Materials and Methods). Therefore, the retention times of the m/z 132 ion and of oQ are different in Figures 5–7.

DISCUSSION

The multistep biosynthesis of queuosine consists of many extraordinary reactions. In the primary Fe ion dependent step the deazaguanine derivative $preQ_1$ [7-(aminomethyl)-7-deazaguanine] is synthesized presumably from GTP. From the purine ring sytem N-7 and C-8 are excised, and it was suggested that this reaction may proceed by the same mechanism essential for the biosynthesis of pteridine or

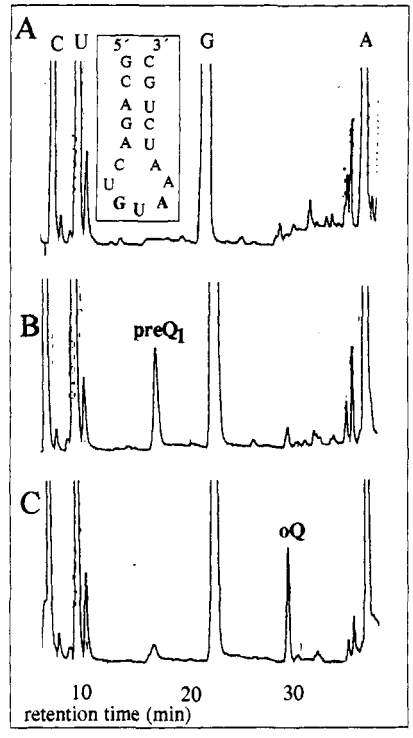

FIGURE 7: *In vitro* reconstitution of epoxyqueuosine formation using completely synthetic substrates. (A) A 17-base-pair oligoribonucleotide corresponding to the anticodon stem and loop of the Q-specific $tRNA^{Tyr}$ from *E. coli* (inset) was synthesized commercially and subjected to HPLC analysis (see Materials and Methods). Besides the four nucleosides A, C, G, and U, a small amount of undefined byproducts of the reaction can be seen. (B) Preparation of a synthetic QueA substrate. An aliquot of the oligoribonucleotide shown in (A) was incubated with Tgt enzyme and synthetic $preQ_1$ base. A new nucleoside peak appears corresponding to $preQ_1$ nucleoside. (C) Incubation of the synthetic $preQ_1$ RNA substrate with QueA enzyme and AdoMet results in an almost complete conversion of the $preQ_1$ nucleoside to epoxyqueuosine as demonstrated by HPLC analysis. A small amount of $preQ_1$ is still detectable, indicating the original elution point of this nucleoside.

riboflavin (Nishimura, 1983). Yet nothing is known about the enzymes catalyzing the formation of $preQ_1$. Similar deazanucleosides are very rare and are found mainly as secondary metabolites, e.g., the antibiotic tubercidin (7-deazaadensoine) from *Streptomyces tubercidicus*. $PreQ_1$ base is inserted into the tRNA by the Tgt enzyme in exchange for guanine, and the resulting $preQ_1$–containing tRNA is further modified by the attachment of a 1,2-epoxycyclopentane-4,5-*cis*-diol moiety to yield oQ. It has been demonstrated that this reaction occurs in members of the family *Enterobacteriaceae* under aerobic and strictly anaerobic conditions, excluding the participation of molecular oxygen in this process. This fact and the structural similarity of the vicinal *cis*-hydroxyl group-containing epoxycyclopentane moiety with pentoses lead to the proposal that a ribose- or ribitol-carrying molecule is a likely precursor of oQ (Frey *et al.*, 1988).

It has been found that tRNAs with $preQ_1$ accumulate if methionine-requiring strains of *E. coli* were cultured in methionine-limiting medium. However, no radioactivity derived from labeled methionine added to the culture appeared in the respective Q nucleosides (Katze *et al.*, 1977; Okada *et al.*, 1977). Although it was suggested that *S*-adenosylmethionine could be involved in this reaction (Nishimura, 1983), so far no coenzyme- or ribose-containing compound was definitely identified as the donor of the epoxycyclopentanediol moiety of oQ. The presented results demonstrate that *S*-adenosylmethionine serves this purpose. Purified QueA was prepared by a fusion protein system resulting in a slightly

modified QueA protein with seven additional amino acids at the NH_2 terminus. This enzyme preparation synthesized oQ from $preQ_1$-containing tRNAs in one step with AdoMet as the sole cofactor. Additional intermediary products were not detectable. The reaction rate was dependent on the concentration of AdoMet that had to be present in stoichiometric amounts. Addition of AdoMet, labeled at the methyl or carboxyl group, did not result in any incorporation of radioactivity into the reaction product. Consequently, the cyclopentane moiety must be derived from the ribose residue of AdoMet. It is difficult to imagine that the adenine molecule is completely rearranged and all four nitrogen atoms are eliminated to form the C_5 cyclopentanediol ring.

Since AdoMet, labeled at the ribose moiety, is not commercially available, the reaction rate was followed indirectly. This was done by measuring the proportion of remaining $preQ_1$ substrate with the Tgt test.

Interestingly, all activated groups that can be transferred by AdoMet are used for tRNA modification: the most prominent methylation is the formation of ribothymidine, the 1-amino-1-carboxypropyl moiety can be found as a modification of uridine (Nishimura *et al.*, 1974), and the ribosyl group emerges in queuosine. Q biosynthesis is to our knowledge the first reaction at all that utilizes this group of AdoMet. Considering the vitamin B12 dependent reduction of epoxy-Q to Q that constitutes one of three known reactions requiring cobalamin in *E. coli*, it is very striking that these two important coenzymes participate together in the biosynthesis of a modified nucleoside. Possibly there might exist an additional function of Q besides its effect in protein biosynthesis, namely, an influence on methionine or cobalamin metabolism.

The requirements of the QueA enzyme to recognize an RNA as cognate substrate were tested using synthetic substrates. Whereas it was clear that QueA needs an RNA bound 7-(aminomethyl)-7-deazaguanine, it is unknown whether the whole three-dimensional tRNA structure is essential for the enzyme reaction. It has been demonstrated that the eucaryotic tRNA–guanine transglycosylase, which inserts nutritional Q-base into eucaryotic Q-specific tRNAs, acts regardless of the specificity of the tRNA, depending only on the sequence of positions 33, 34, and 35 in the tRNA anticodon loop (Carbon *et al.*, 1983). These positions are occupied by U, G, and U in the respective Q tRNAs. A synthetic oligonucleotide consisting of 17 bases corresponding to the sequence of the anticodon stem and loop of the natural Q-specific $tRNA^{Tyr}$ from *E. coli* proved to be a suitable substrate for QueA, prerequisite that it was modified with $preQ_1$ nucleoside by the Tgt enzyme. Therefore, none of the two enzymes necessarily interacts with the entire tRNA or any previously introduced modifications therein. It even might be possible that the specificity of the QueA enzyme is solely determined by the Tgt enzyme and that the unique feature of a $preQ_1$ molecule inserted into an RNA loop is sufficient for the QueA enzyme to synthesize oQ. In contrast to aminoacylation, that is also possible with microhelix substrates, Q modification does not require any free RNA 3′ terminus. Therefore, it might be imagined also that other RNA substrates besides tRNAs become modified with Q as long as they are capable of forming a stem and loop resembling a Q-specific tRNA anticodon arm.

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