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The deazapurine biosynthetic pathway revealed: *In vitro* enzymatic synthesis of preQ₀ from guanosine-5'-triphosphate in four steps[†]

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Abstract

Deazapurine-containing secondary metabolites comprise a broad range of structurally diverse nucleoside analogs found throughout biology including various antibiotics produced by species of *Streptomyces* bacteria and the hypermodified tRNA bases queuosine and archaeosine. Despite early interest in deazapurines as antibiotic, antiviral, and antineoplastic agents, the biosynthetic route toward deazapurine production has remained largely elusive for more than 40 years. Here we present the first *in vitro* preparation of the deazapurine nucleoside, preQ₀, by the successive action of four enzymes. The pathway includes the conversion of the recently identified biosynthetic intermediate, 6-carboxy-5,6,7,8-tetrahydropterin, to a novel intermediate, 7-carboxy-7-deazaguanine (CDG), by an unusual transformation catalyzed by *B. subtilis* QueE, a member of the radical SAM enzyme superfamily. The carboxylate moiety on CDG is converted subsequently to a nitrile to yield preQ₀ by either *B. subtilis* QueC or *S. rimosus* ToyM in an ATP-dependent reaction, in which ammonia serves as the nitrogen source. The results presented here are consistent with early radiotracer studies on deazapurine biosynthesis and provide a unified pathway for the production of deazapurines in nature.

Compounds containing pyrrolopyrimidine functional groups, collectively referred to as 7-deazapurines, are a structurally diverse class of nucleoside analogs with demonstrated antibiotic, antineoplastic, and antiviral activities. Deazapurine containing compounds include the nucleoside antibiotics toyocamycin, sangivamycin, tubercidin, and cadeguomycin (Figure 1), which are produced by various species of *Streptomyces* (1,2). In addition, the hypermodified base, queuosine (Figure 1), which is located in the wobble position of 5'-GUN-3' anticodons in tRNA in a number of organisms (except yeast (3)) bearing tyrosine, histidine, asparagine and aspartate contains a deazapurine moiety (4). The occurrence of queuosine in tRNA is

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Supporting Information Available

Materials and detailed experimental procedures, controls for conversion of CPH₄ to CDG by QueE, controls showing conversion of CDG to preQ₀ by ToyM or QueC, tandem MS-MS fragmentation of preQ₀, and ¹H-decoupled ¹³C-NMR spectrum of CDG are shown in Supporting Information. This material is available free of charge on the internet at: <http://pubs.acs.org>.

almost universally conserved throughout biology. In archaea, a related deazapurine, archaeosine (Figure 1), is found in the D-loop of tRNA (5).

Since their first discovery over forty years ago, the biosynthetic steps required for deazapurine production have remained uncharacterized. Early studies on the biosynthesis of toyocamycin, in which radiolabeled purine-based precursors were fed to *Streptomyces rimosus*, revealed that carbon-2 of the preferred purine was retained in the deazapurine product, while carbon-8 was not (6,7). Similar results were obtained with radiotracer studies on queuosine biosynthesis in *Salmonella typhimurium* (8). Additional studies on the origin of the pyrrolo and cyano carbons of toyocamycin showed that they are derived from ribose present in the starting material (6). Collectively, these results suggest that substantial structural rearrangements occur during the course of conversion of a purine, such as guanosine, to a deazapurine-containing product.

In recent years, the availability of genome sequences has permitted comparative genomic analysis leading to identification of four genes (*queC*, *queD*, *queE*, and *queF*), which are involved in the queuosine biosynthetic pathway of *Bacillus subtilis* (9,10). Biochemical studies have shown that QueF catalyzes the NADPH dependent conversion of the nitrile moiety of 7-cyano-7-deazaguanine, preQ₀ (see Figure 2), to the amino group found in 7-aminomethyl-7-deazaguanine, preQ₁ (11), which is subsequently incorporated in tRNA and modified to the hypermodified tRNA base, queuosine (12,13). BLAST analysis of the protein sequences have permitted QueC, QueD and QueE to be tentatively annotated as an ATPase, a 6-pyruvoyltetrahydropterin synthase (PTPS), and a member of the radical SAM protein superfamily, respectively (9). A *queC* homolog was also found to be required for queuosine production in *Escherichia coli* (14). A cluster of 13 genes involved in the biosynthesis of toyocamycin and sangivamycin by *S. rimosus* contains three open reading frames, *toyM*, *B*, and *C*, which are homologous to *queC*, *D*, and *E*, respectively (15). This gene cluster also encodes ToyD, which has been shown experimentally to have GTP cyclohydrolase I (GCH I) activity, namely, the conversion of GTP to 7,8-dihydroneopterin triphosphate (H₂NTP). This is also the first step in the biosynthesis of folic acid (16). Indeed, the GCH I homolog of *E. coli*, FolE, is required for biosynthesis of queuosine and folic acid in that organism (17). A FolE homolog has also been implicated in the biosynthesis of archaeosine in *Haloferax volcanii* (17). In general, GCH I, QueD, QueC and QueE homologs have emerged as common enzymes in the biosynthetic pathways to deazapurine containing metabolites from GTP.

We have recently shown that *E. coli* QueD catalyzes the conversion of H₂NTP to 6-carboxy-5,6,7,8-tetrahydropterin (CPH₄) (18). The formation of CPH₄ by QueD was unexpected in light of the significant amino acid sequence similarities between QueD and mammalian PTPS homologs that convert H₂NTP to 6-pyruvoyltetrahydropterin. However, this observation provided the necessary framework within which to examine the role of the remaining two proteins, QueE and QueC leading to the successful re-constitution of *in vitro* biosynthesis of preQ₀.

MATERIAL AND METHODS

Materials

pGEM-T Easy system was obtained from Promega. Restriction endonucleases were from New England Biolabs. Oligonucleotides were synthesized by Operon Technologies. The vector pET28 and the expression strain BL21(DE3) were from Novagen. Sephacryl S-300 resin and Hitrap and HisTrap columns were from GE Healthcare. Proteins were quantified with BCA protein quantitation kit from Pierce with BSA as standard. All other chemicals were from Sigma-Aldrich or VWR Scientific.

Cloning of *E. coli* QueD, *B. subtilis* QueE, QueC and *S. rimosus* ToyM

The genes corresponding to *E. coli* QueD, *B. subtilis* QueE and QueC, and *S. rimosus* ToyM were cloned as documented in the Supporting Information.

Expression of *B. subtilis* His₆-QueE

E. coli BL21(DE3) containing pRM78 (for expression of QueE) and pBD1282 (for *in vivo* production of iron sulfur clusters (19)), was grown in 6 L of LB containing 34 µg/mL kanamycin and 100 µg/mL ampicillin at 37 °C to OD_{600nm}~0.3, at which point solid arabinose was added (to a final concentration of 0.05% (w/v)) to induce transcription of the genes in pBD1282. The cells were grown further to OD_{600nm}~0.5 at which point ferric chloride (50 µM final) was added and expression of QueE was induced by addition of IPTG (100 µM). Cells were harvested after 6 h by centrifugation (4000 × g), and frozen until use.

Purification of *B. subtilis* QueE

Purification of QueE was carried out in a Coy anaerobic chamber (5% H₂ / 95% N₂). Cells (~ 3 g) were suspended in 40 mL of buffer containing 20 mM potassium phosphate (pH 7.2), 0.5 M KCl, 5 mM imidazole, 1 mM dithiothreitol, and 1 mM PMSF and sonified using a Branson digital sonifier (60% amplitude). The lysate was placed in an Oakridge centrifuge tube and cell debris were pelleted by centrifugation at 26,000 ×g for 30 minutes at 4 °C. The cleared lysate was loaded onto two serially connected 1 mL HisTrapTMHP columns, which had been charged with NiSO₄ and equilibrated with a solution containing 20 mM potassium phosphate (pH 7.2), 0.5 M KCl, 1 mM DTT, and 5 mM imidazole (Buffer A). The column was rinsed with 20 mL of Buffer A solution of Buffer A containing 0.3 M imidazole. The dark brown QueE protein eluted completely in a volume of about 4 mL. The protein was concentrated to ~3 mL in Microcon centrifugal concentrators (YM-10 membrane) in a table top centrifuge inside the anaerobic chamber. The protein was loaded onto an Econo-Pac 10DG column, which had been pre-equilibrated with a 50 mM HEPES•NaOH (pH 7.5) buffer containing 0.2 M Na₂SO₄, and 10 mM DTT, and eluted with the addition of 4 mL of the same buffer. Sodium dithionite was added to the protein to a total concentration of 10 mM and the protein was concentrated through YM-10 Microcon centrifugal concentrators to ~2 mL.

Expression of *B. subtilis* QueC and *S. rimosus* ToyM

E. coli BL21(DE3) containing plasmids for overexpression of *B. subtilis* QueC or *S. rimosus* ToyM were grown in 2 L of LB containing 34 µg / mL kanamycin at 37 °C to OD_{600nm}~0.5, at which point ZnSO₄ was added to a final concentration of 100 µM and protein expression was induced by addition of IPTG (100 µM). Cells were harvested by centrifugation (4000 × g) 6 h after induction and frozen until use.

Purification of *B. subtilis* QueC and *S. rimosus* ToyM

Cells (~2 g) containing either *B. subtilis* QueC or *S. rimosus* ToyM were lysed by sonication in 20 mM potassium phosphate (pH 7.2) containing 0.5 M KCl, 40 mM imidazole, and 1 mM PMSF using a Branson digital sonifier (60% amplitude). Cleared lysates were obtained by centrifugation at 26,000 ×g and loaded on two serially connected 1 mL HiTrap Chelating HP columns, which had been charged with NiSO₄ and equilibrated with in 20 mM potassium phosphate (pH 7.2) containing 0.5 M KCl, and 40 mM imidazole. Proteins were eluted with a linear gradient from 40 mM to 0.5 M imidazole (pH 7.2) in 20 mM potassium phosphate containing 0.5 M KCl over 25 mL at a flow rate of 0.5 mL/min. Fractions containing the desired protein were identified by SDS-PAGE, combined, and concentrated to ~1.5 mL. The protein samples were loaded (0.75 mL/min) onto a Sephacryl S-300 size exclusion column (2.6 × 60 cm), which had been pre-equilibrated in 20 mM HEPES•NaOH (pH 7.5) and eluted with the same buffer. Fractions containing the desired protein were identified by SDS-PAGE,

combined, and concentrated using Amicon pressure concentrators and centrifugal devices containing YM-10 membranes. Protein was aliquoted, frozen in N₂ and stored at -80°C. Metal content was determined by inductively coupled plasma optical emission spectroscopy by Garratt-Callahan Company.

In vitro production of CPH₄

CPH₄ was produced from GTP by the combined activities of *E. coli* FolE and CPH₄ synthase *in situ*. The reaction mixtures contained 50 mM PIPES (pH 7.4), 10 mM DTT, and 10 mM MgCl₂ 0.5 mM GTP, 20 μM native, recombinant *E. coli* FolE, and 20 μM native recombinant *E. coli* CPH₄ synthase (18). Reactions were allowed to proceed at ambient temperature in the dark for 3 h in a Coy anaerobic chamber. HPLC analysis indicated that the reactions went to essentially to completion.

Activity assays with *B. subtilis* QueE, QueC and *S. rimosus* ToyM

The reaction mixtures were prepared by adding *B. subtilis* QueE (200 μM) to a solution containing CPH₄ prepared enzymatically as described above. After accounting for carry-over of buffer and reaction components from the CPH₄ synthesis and QueE addition, the reaction mixtures contained 50 mM PIPES (pH 7.4), 0.1 M NaCl, 20 mM Na₂SO₄, 7.7 mM MgCl₂, 10 mM DTT and 0.4 mM CPH₄. Sodium dithionite (10 mM) and S-adenosyl-L-methionine (2 mM) were added as required. For the experiments probing the reaction catalyzed by QueC or ToyM, 40 μM of the appropriate protein was included. ATP (2 mM) was required for the QueC and ToyM. In experiments probing the source of the nitrogen in preQ₀, 5 mM (¹⁴NH₄)₂SO₄ or (¹⁵NH₄)₂SO₄ were included. Reactions were allowed to proceed 4 h in the anaerobic chamber, quenched by centrifugal filtration through Microcon centrifugal filtration devices (YM-10 membranes), and flash frozen in liquid nitrogen to prevent oxidative degradation of CPH₄. The quenched samples were kept at -80 °C until analyzed.

HPLC assays for *E. coli* QueE, *B. subtilis* QueC, and *S. rimosus* ToyM activity

Reactions were thawed and an aliquot (40 μL) injected directly on an Agilent Zorbax Eclipse C-18 column (4.6 × 250 mm), which had been pre-equilibrated in 10 mM tetrabutylammonium bromide and 50 mM potassium phosphate (pH 6.8). The reaction components were resolved with a 20 min gradient from 0 to 30% acetonitrile (flow rate of 0.75 mL/min). The eluent was monitored (200-500 nm) using an Agilent 1100 photodiode array detector. Authentic preQ₀, synthesized as described (20) was injected as a standard.

Purification of QueE and QueD (ToyM) products for mass spectrometry

Samples of 7-Cyano-7-deazaguanine (CDG) prepared as described above for the HPLC assays were purified using a semi-preparative Agilent Eclipse C-18 column (9.4 × 250 mm) equilibrated in water and run at a flow rate of 2.5 mL/min. CDG was eluted with a linear gradient from 0-7.5% acetonitrile in water over 10 min. CDG eluting from the column was collected in a glass vial and lyophilized.

Fourier transform ion-cyclotron resonance mass spectrometry

Mass spectrometry experiments were carried out on a Bruker Apex Qh ultrahigh resolution 9.4 T Fourier transform ion-cyclotron resonance (FT-ICR) instrument (Bruker Daltonics, Billerica, MA). The lyophilized samples prepared as described above were dissolved in H₂O:acetonitrile 1:1 (containing 0.1% of formic acid). Positively and negatively charged ions were generated by electrospray ionization (ESI) by direct infusion with a flow rate of 2.5 μL/min. The ion optics of the instrument were tuned to optimize for ions in the *m/z* range of 100-300. The instrument was externally calibrated with conventional standards (Agilent mix and trifluoroacetic acid solutions), which allowed us to determine accurate masses with a mass

accuracy of < 2 ppm. Tandem MS/MS fragmentation experiments were carried out in the quadrupole region with collision-induced dissociation (QCID, with N₂ as a collision gas) at 10 eV laboratory collision energies. Accurate masses of fragments were determined and they corresponded to the losses of CO₂ and NH₃ (negative ion mode) and NH₃ (positive ion mode) losses.

Synthesis and Purification of Uniformly labeled CDG for NMR

To confirm the identity of the product of QueE, uniformly labeled CDG was prepared using [¹³C₁₀, ¹⁵N₅]-GTP. The reactions contained 50 mM PIPES•NaOH (pH 7.4), 10 mM MgSO₄, 10 mM DTT, 100 μM native recombinant *E. coli* FolE, 200 μM native recombinant *E. coli* CPH₄ synthase and 6 mM labeled GTP in a total volume of 5 mL. This reaction was allowed to proceed 4 h at ambient temperatures under anaerobic conditions and in the dark. Sodium dithionite was added to a final concentration of 10 mM followed by S-adenosyl-L-methionine (2 mM). QueE, which was freshly prepared as described above, was added to a final concentration of 200 μM. The reaction was allowed to proceed 8 h in the anaerobic chamber. All subsequent transformations were carried out outside of the anaerobic chamber. Protein was removed by filtration through Amicon centrifugal devices and the resulting filtrate was diluted to 40 mL with 0.35 M ammonium bicarbonate (pH 8.0). The material was then loaded on a Q-Sepharose column (1.6 × 30 cm) which was pre-equilibrated in the same buffer, rinsed with 0.12 L of the same then eluted with a linear gradient to 0.5 M ammonium bicarbonate (pH 8.0) over 0.5 L. Fractions containing CDG were identified by HPLC, pooled and lyophilized. The resulting solid was re-dissolved in 40 mL of water and lyophilized to remove remaining ammonium bicarbonate. Solid residue from the second lyophilization was dissolved in 0.5 mL of water and CDG was purified further on a semi-preparative C-18 column as described above. The peak of CDG peak was collected manually and lyophilized.

NMR Spectroscopy of enzymatically produced uniformly ¹³C/¹⁵N-labeled CDG

NMR spectra were acquired at 25 °C in DMSO-d₆ solvent on a Varian Inova-600 operating at a ¹H frequency of 599.7 MHz, using a 5 mm cryogenic HCN single-axis gradient probe. The ¹H-decoupled ¹³C spectrum was acquired with a spectral width of 33167 Hz using 32k complex data points with a relaxation delay of 1.5 s and 10314 transients. Backward linear prediction was used to replace the first 16 data points, and an exponential multiplier was applied with a line broadening of 5 Hz before Fourier transformation. Baseline correction was performed using a 2nd order polynomial.

RESULTS AND DISCUSSION

Protein-BLAST reveals that *B. subtilis* QueE belongs to the S-adenosyl-L-methionine (SAM)-dependent organic radical generating enzymes (21). Members of the radical SAM superfamily contain a CX₃CX₂C-motif for binding a [4Fe-4S] cluster, which when reductively activated, cleaves SAM to 5'-deoxyadenosyl radical, which in turn initiates radical mediated transformations of the substrate (22). cursory examination of the structures of CPH₄ and preQ₀ (see Fig. 2) clearly highlights the fact that a substantial and unprecedented rearrangement of the carbon skeleton is required. We hypothesized that QueE may indeed be involved in this transformation, though the order by which QueE and QueC act remained unknown. Preliminary experiments showed that when CPH₄, produced enzymatically from GTP by GCH I and *E. coli* QueD (CPH₄ synthase), is incubated with either QueE or QueC, a new product is observed with QueE only. Therefore, we pursued identification of the product of the reaction catalyzed by QueE.

QueE catalyzes conversion of CPH₄ to 7-deaza-7-carboxyguanine

Recombinant QueE was purified under strictly anaerobic conditions and used within a few hours of purification to preserve the [4Fe-4S] cluster. To promote assembly of metal cofactor, the overproducing strain also contained the pBD1282 plasmid which has been shown to support incorporation iron and sulfide into the [4Fe-4S] clusters of similar proteins (19). The anaerobic conditions, under which all reactions were conducted, also minimized degradation of oxygen labile intermediate CPH₄. Reaction products were separated by HPLC and detected by UV spectroscopy (see Fig. 2). To generate the starting CPH₄ substrate, GTP (retention time: 17.3 min, Fig. 2a) was converted to H₂NTP by *E. coli* FolE (retention time: 12.4 min, Fig. 2b), which was subsequently converted to CPH₄ in the presence of *E. coli* CPH₄ synthase (retention time: 4.0 min, Fig. 2c) as we have shown previously (18). Upon addition of reductively activated *B. subtilis* QueE, a significant portion of the CPH₄ is converted to a new product that elutes at 9.4 min (compare Fig. 2c and 2d) and exhibits a UV-visible spectrum that is different from CPH₄ (18). The conversion of CPH₄ to this new product occurred only in the presence of SAM and dithionite (see Fig. S1). The product was purified with a semi-preparative HPLC column, lyophilized, and analyzed by Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS). To simplify the assignment, the reactions were also carried out with [¹⁵N₅]-GTP or [¹³C₁₀, ¹⁵N₅]-GTP and the products of these reactions were also analyzed. A [M-H]⁻ ion with a *m/z* of 193.0379 was identified in the unlabeled samples, which was absent from the spectra of product derived from singly or doubly labeled GTP. Instead, unique [M-H]⁻ ions with *m/z* of 197.0259 or 204.0497 were observed in the [¹⁵N₅]-GTP and [¹³C₁₀, ¹⁵N₅]-GTP samples, respectively (Fig. 3a). The comparison of [M-H]⁻ ions observed in these samples shows that the product contains one fewer nitrogen atom than the starting CPH₄. The above observations are consistent with the formation of 7-carboxy-7-deazaguanine (CDG) as the product of QueE. Cadeguomycin, in which CDG is appended to a ribose, has been isolated from *S. hygroscopicus* (23); it is tempting to suggest that CDG produced by a QueE homolog in that organism is the source of the CDG base of the pyrrolopyrimidine nucleoside. Indeed, the ¹³C-NMR chemical shifts of uniformly ¹³C/¹⁵N-labeled CDG are very similar to the corresponding shifts published for the base of cadeguomycin (see Fig. S4) (24).

QueC or ToyM catalyze conversion of 7-deaza-7-carboxyguanine to preQ₀

We next examined whether CDG, produced by QueE, could serve as substrate for QueC and ToyM. Interestingly, a conserved SXGXDS motif found in the PP_i-loop ATPase superfamily, is present in QueC (14). One can envision that QueC and ToyM catalyze an ATP-dependent conversion of CDG to preQ₀, with externally derived nitrogen to yield the nitrile group. An X-ray crystal structure of QueC from *B. subtilis* has been solved revealing, in addition to the PP_i loop, a structural zinc metal ion (25). Inductively coupled plasma optical emission spectroscopy of recombinant QueC from *B. subtilis* and its *S. rimosus* homolog, ToyM, revealed the presence of 0.88 and 0.80 equivalents of zinc metal ion per monomer, respectively, for the proteins used in our studies.

When CPH₄ generated as described above by successive actions of GCH I and QueD, is incubated with QueE and either *B. subtilis* QueC or *S. rimosus* ToyM, a peak with retention time of 10.3 min, identical to synthetic preQ₀, is observed in the chromatogram (compare Fig. 2e and 2f). In addition, the spectral properties of this molecule are identical to those of synthetic preQ₀ (see insets). While the formation of enzymatically produced preQ₀ was ATP-dependent, it occurred in the *absence* of an exogenously supplied nitrogen source. (Fig. S2). As will be discussed shortly, however, enough ammonia was present in the reaction mixture to effect the conversion. FT-ICR MS analysis of the product generated by QueC or ToyM showed a [M+H]⁺ ion with *m/z* of 176.0567, confirming that both of these proteins convert CDG to preQ₀ (calculated *m/z* 176.0567). When the reaction was carried out with [¹³C₁₀, ¹⁵N₅]-GTP as starting material, [M+H]⁺ ions with *m/z* of 187.0684 or 187.0683 were observed with ToyM

or QueC, respectively (calculated m/z 187.0683, see Fig. S3). The molecular weight difference between preQ₀ derived from unlabeled and labeled precursor GTP reveals the loss of one ¹⁵N from the starting material, as with CDG, and incorporation of one ¹⁴N.

The cyano nitrogen of preQ₀ is derived from ammonia

To examine whether ammonia was the source of the nitrogen in the cyano group of preQ₀, a series of reactions was carried out in which CPH₄ (produced enzymatically from GTP as described above) was incubated with QueE and QueC (or ToyM) and either ¹⁵NH₄SO₄ or ¹⁴NH₄SO₄. FT-ICR MS analysis of the preQ₀ produced in these reactions shows incorporation of ¹⁵N into the product when a source of labeled ammonia is included (Fig 3b). In tandem MS/MS analysis of the products generated with either QueC (or ToyM) in the presence of ¹⁴NH₄⁺, a [M+H]⁺ ion with m/z identical to synthetic preQ₀ is observed (m/z 176.0577). By contrast, when ¹⁵NH₄⁺ is added, the peak shifts to a [M+H]⁺ ion at m/z 177.0538, consistent with incorporation of the exogenous labeled ammonia into preQ₀. In the samples where ¹⁵NH₄⁺ was supplied, the [M+H]⁺ ion with m/z 176.0538, which is ≤20% as large as that of ¹⁵N-labeled molecule, is also observed; this presumably derives from ammonia produced in conversion of CPH₄ to CDG. These results confirm that the nitrogen atom found appended to the substituent at the 7-position of deazapurines is derived from ammonia.

Paradigm for biosynthesis of deazapurine-containing compounds

Based on our results we propose a general paradigm for biosynthesis of deazapurine containing compounds in nature, which incorporates H₂NTP, CPH₄ and CDG as common intermediates (see Fig. 2). We hypothesize that CDG may be the central precursor to all deazapurines, and that the presence of a QueC homolog in a genome may signal the capacity of the organism to produce compounds containing a nitrogen at the carbon-8 moiety of the molecule. This pathway is interesting for several reasons. First, it contains a novel PTPS homolog, QueD, which has evolved a function distinct from mammalian PTPS enzymes despite few changes in amino acid sequence. Second, the pathway includes QueE, a previously uncharacterized member of the diverse and growing radical SAM enzyme superfamily, which appears to carry out a complex transformation (CPH₄→CDG). Third, the pathway involves a novel ATP-dependent conversion of a carboxylic acid to a nitrile. Fourth, the cyano nitrogen of preQ₀ is derived from ammonia and not N-7 from the base of the proffered GTP. The pathway proposed here also accounts for the existing body of radiotracer experiments on the biosynthesis of 7-deazapurine containing metabolites (6-8). While we cannot rule out that there may be species-specific variation in some of the steps of the pathway, the successful *in vitro* reconstitution of the pathway from GTP to preQ₀ provides the framework for future studies of the chemical transformations that underlie the biosynthesis of 7-deazapurine containing compounds.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ABBREVIATIONS

preQ₀, 7-cyano-7-deazaguanine
PTPS, 6-pyruvoyltetrahydropterin synthase
GCH I, GTP cyclohydrolase I
H₂NTP, 7,8-dihydroneopterin triphosphate
CPH₄, 6-carboxy-5,6,7,8-tetrahydropterin
PMSF, phenylmethylsulfonyl fluoride
DTT, dithiothreitol
SAM, S-adenosyl-L-methionine

HPLC, high performance liquid chromatography
 ESI, electrospray ionization
 CDG, 7-carboxy-7-deazaguanine
 PIPES, 1,4-piperazinediethanesulfonic acid
 FT-ICR MS, Fourier transform ion cyclotron resonance mass spectrometry
 QCID, quadrupole collision-induced dissociation

REFERENCES

1. Suhadolnik, RJ. Nucleoside antibiotics. Wiley-Interscience; New York: 1970. Pyrrolopyrimidine nucleosides; p. 298-353.
2. Wu RT, Okabe T, Namikoshi M, Okuda S, Nishimura T, Tanaka N. Cadeguomycin, a novel nucleoside analog antibiotic. II. Improved purification, physicochemical properties and structure assessment. *J Antibiot (Tokyo)* 1982;35:279-284. [PubMed: 7076576]
3. Kasai H, Kuchino Y, Niehei K, Nishimura S. Distribution of modified nucleoside Q and its derivatives in animal and plant RNA's. *Nucleic Acids Res* 1975;2:1931-1939. [PubMed: 1187350]
4. Harada F, Nishimura S. Possible anticodon sequences of tRNA His, tRNA Asn, and tRNA Asp from *Escherichia coli* B. Universal presence of nucleoside Q in the first position of the anticodons of these transfer ribonucleic acids. *Biochemistry* 1972;11:301-308. [PubMed: 4550561]
5. Kilpatrick MW, Walker RT. *Zentralbl. Bakteriell. Mikrobiol. Hyg. 1 Abt. Orig. C* 1982;3:78-89.
6. Suhadolnik RJ, Uematsu T. Biosynthesis of the pyrrolopyrimidine nucleoside antibiotic, toyocamycin. VII. Origin of the pyrrole carbons and the cyano carbon. *J. Biol. Chem* 1970;245:4365-4371. [PubMed: 5498424]
7. Uematsu T, Suhadolnik RJ. Nucleoside antibiotics. VI. Biosynthesis of the pyrrolopyrimidine nucleoside antibiotic toyocamycin by *Streptomyces rimosus*. *Biochemistry* 1970;9:1260-1266. [PubMed: 5418715]
8. Kuchino Y, Kasai H, Nihei K, Nishimura S. Biosynthesis of the Modified Nucleoside Q in Transfer RNA. *Nuc. Acids. Res* 1976;3:393-398.
9. Reader JS, Metzgar D, Schimmel P, de Crecy-Lagard V. Identification of four genes necessary for biosynthesis of the modified nucleoside queuosine. *J. Biol. Chem* 2004;279:6280-6285. [PubMed: 14660578]
10. de Crecy-Lagard V. Identification of genes encoding tRNA modification enzymes by comparative genomics. *Methods Enzymol* 2007;425:153-183. [PubMed: 17673083]
11. Van Lanen SG, Reader JS, Swairjo MA, de Crecy-Lagard V, Lee B, Iwata-Reuyl D. From cyclohydrolase to oxidoreductase: discovery of nitrile reductase activity in a common fold. *Proc Natl Acad Sci U S A* 2005;102:4264-4269. [PubMed: 15767583]
12. Okada N, Harada F, Nishimura S. Specific Replacement of Q Base in the Anticodon of tRNA by Guanine Catalyzed by a Cell-free Extract of Rabbit Reticulocytes. *Nuc. Acids. Res* 1976;3:2593-2603.
13. Okada N, Nishimura S. Isolation and Characterization of a Guanine Insertion Enzyme, a Specific tRNA Transglycosylase, from *Escherichia coli*. *J. Biol. Chem* 1979;254:3061-3066. [PubMed: 107167]
14. Gaur R, Varshney U. Genetic analysis identifies a function for the queC (ybaX) gene product at an initial step in the queuosine biosynthetic pathway in *Escherichia coli*. *J. Bacteriol* 2005;187:6893-6901. [PubMed: 16199558]
15. McCarty RM, Bandarian V. Deciphering deazapurine biosynthesis: pathway for pyrrolopyrimidine nucleosides toyocamycin and sangivamycin. *Chem Biol* 2008;15:790-798. [PubMed: 18721750]
16. Burg AW, Brown GM. The biosynthesis of folic acid. 8. Purification and properties of the enzyme that catalyzes the production of formate from carbon atom 8 of guanosine triphosphate. *J. Biol. Chem* 1968;243:2349-2358. [PubMed: 4296838]
17. Phillips G, El Yacoubi B, Lyons B, Alvarez S, Iwata-Reuyl D, de Crecy-Lagard V. The biosynthesis of the 7-deazaguanosine modified tRNA nucleosides: a new role for GTP cyclohydrolase-I. *J. Bacteriol* 2008;190:7876-7884. [PubMed: 18931107]

18. McCarty RM, Somogyi A, Bandarian V. *Escherichia coli* QueD Is a 6-Carboxy-5,6,7,8-tetrahydropterin Synthase. *Biochemistry* 2009;48:2301–2303. [PubMed: 19231875]
19. Frazzon J, Fick JR, Dean DR. Biosynthesis of iron-sulphur clusters is a complex and highly conserved process. *Biochem. Soc. Trans* 2002;30:680–685. [PubMed: 12196163]
20. Quaranta D, McCarty R, Bandarian V, Rensing C. The copper-inducible *cin* operon encodes an unusual methionine-rich azurin-like protein and a preQ₀ reductase in *Pseudomonas putida* KT2440. *J. Bacteriol* 2007;189:5361–5371. [PubMed: 17483220]
21. Sofia HJ, Chen G, Hetzler BG, Reyes-Spindola JF, Miller NE. Radical SAM, a novel protein superfamily linking unresolved steps in familiar biosynthetic pathways with radical mechanisms: functional characterization using new analysis and information visualization methods. *Nucleic Acids Res* 2001;29:1097–1106. [PubMed: 11222759]
22. Frey PA, Hegeman AD, Ruzicka FJ. The Radical SAM Superfamily. *Crit. Rev. Biochem. Mol. Biol* 2008;43:63–88. [PubMed: 18307109]
23. Tanaka N, Wu RT, Okabe T, Yamashita H, Shimazu A, Nishimura T. Cadeguomycin, a novel nucleoside analog antibiotic. I. The producing organism, production and isolation of cadeguomycin. *J Antibiot (Tokyo)* 1982;35:272–278. [PubMed: 7076575]
24. Wu RT, Okabe T, Namikoshi M, Okuda S, Nishimura T, Tanaka N. Cadeguomycin, A novel nucleoside analog antibiotic. *J Antibiot (Tokyo)* 1982:279–284. [PubMed: 7076576]
25. Cicmil N, Huang RH. Crystal structure of QueC from *Bacillus subtilis*: an enzyme involved in preQ₁ biosynthesis. *Proteins* 2008;72:1084–1088. [PubMed: 18491386]

Sangivamycin

Toyocamycin

Queuosine

Cadeguomycin

Tubercidin

Archaeosine

Figure 1.

Representative examples of naturally occurring deazapurine-containing compounds. Sangivamycin, toyocamycin, cadeguomycin and tubercidin are produced by various strains of *Streptomyces*. Archaeosine is a modified base found in archaeobacterial tRNA. Queuosine is a hypermodified RNA base found in the wobble position of certain tRNA molecules in nearly all organisms.

Figure 2.

In vitro enzymatic synthesis of preQ₀ from GTP in four steps. GTP (a) is converted to preQ₀ in a reaction that contained GTP cyclohydrolase I (GCH I), *E. coli* QueD homolog CPH₄ synthase, *B. subtilis* QueE (7-carboxy-7-deazaguanine synthase) and either *B. subtilis* QueC or *S. rimosus* ToyM (e). In control reactions where only (b) GCH I, (c) GCH I and QueD, (d) GCH I, QueD and QueE, are included, H₂NTP, CPH₄ and CDG are observed, respectively. The reactions were monitored by HPLC (at (a) 260 or (b)-(f) 300 nm); while a y-axis is not shown, all the chromatograms are to scale. The peak ~6 min we ascribe to breakdown of CPH₄. Under conditions where all four enzymes were present (e) the reaction is driven to completion as evidenced by lack of both breakdown product and the starting substrate (GTP).

Insets show spectra of corresponding to CDG and preQ₀. A trace with synthetic preQ₀ standard (**f**) is shown for reference. The coloring scheme of the carbon atoms in the biosynthetic pathway reflects the results of early radiotracer experiments.

Figure 3. Tandem MS-MS (QCID) analysis of products of (a) *B. subtilis* QueE (7-carboxy-7-deazaguanine synthase) and (b) *S. rimosus* ToyM (7-cyano-7-deazaguanine synthetase). Calculated (in parentheses) and observed masses are shown. In each case, enzymatically generated products were purified by semi-preparative HPLC and subjected to high resolution MS-MS analysis. In (a), CDG was produced in reactions that contained unlabeled, [¹⁵N₅] or [¹³C₁₀, ¹⁵N₅]-GTP. MS/MS spectra were obtained by isolating and fragmenting the [M-H]⁻ ion with *m/z* 193, 197 or 204, corresponding to CDG obtained with unlabeled, [¹⁵N₅] or [¹³C₁₀, ¹⁵N₅]-GTP, respectively. The fragmentation corresponds to loss of CO₂ and further loss of NH₃ from CDG. In (b), ¹⁴NH₄⁺ or ¹⁵NH₄⁺ was included to demonstrate that the cyano

nitrogen of preQ₀ derives from exogenous ammonia. MS/MS analysis was carried out in the positive ion mode by isolating and fragmenting the [M+H]⁺ ion with *m/z* 176 or 177, corresponding to synthetic preQ₀, preQ₀ generated enzymatically in the presence of ¹⁴NH₄⁺, or the presence of ¹⁵NH₄⁺. The fragmentation corresponds to loss of NH₃ from preQ₀. Asterisks denote a small amount of preQ₀ with ¹⁴N in the cyano moiety. The same results were obtained with preQ₀ generated by *B. subtilis* QueC.