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The Deazapurine Biosynthetic Pathway Revealed: In Vitro Enzymatic Synthesis of $PreQ_0$ from Guanosine 5′-Triphosphate in Four Steps†

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ABSTRACT: Deazapurine-containing secondary metabolites comprise a broad range of structurally diverse nucleoside analogues 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 base $preQ_0$, 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 *Bacillus subtilis* QueE, a member of the radical SAM enzyme superfamily. The carboxylate moiety on CDG is converted subsequently to a nitrile to yield $preQ_0$ by either *B. subtilis* QueC or *Streptomyces 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 termed 7-deazapurines, are a structurally diverse class of nucleoside analogues 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 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 more than 40 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 C2 of the proferred purine was retained in the deazapurine product while C8 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, $preQ_0$[1] (see Figure 2), to the amino group found in 7-aminomethyl-7-deazaguanine, $preQ_1$ (*11*), which is subsequently incorporated into tRNA and modified to the hypermodified tRNA base, queuosine (*12*, *13*). BLAST analyses

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[1]Abbreviations: $preQ_0$, 7-cyano-7-deazaguanine; PTPS, 6-pyruvoyl-tetrahydropterin synthase; GCH I, GTP cyclohydrolase I; H_2NTP, 7,8-dihydroneopterin triphosphate; CPH_4, 6-carboxy-5,6,7,8-tetrahydropterin; PMSF, phenylmethanesulfonyl 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.

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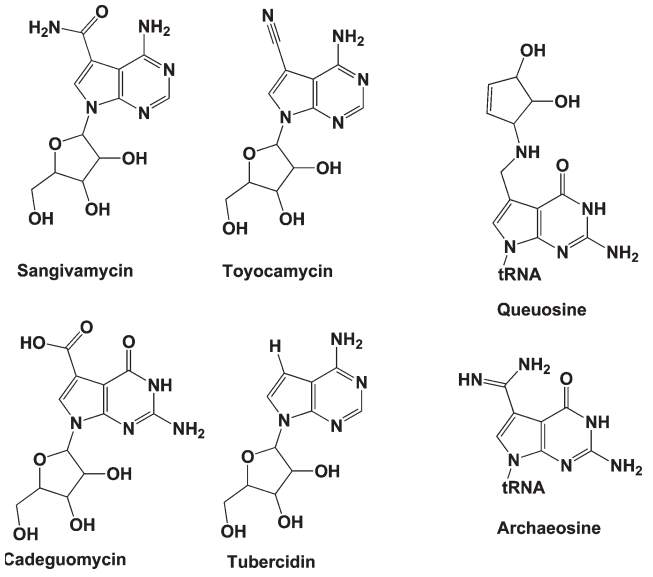

FIGURE 1: Representative examples of naturally occurring deazapurine-containing compounds. Sangivamycin, toyocamycin, cadeguomycin, and tubercidin are produced by various strains of *Streptomyces*. Archaeosine is a modified base found in archaebacterial tRNA. Queuosine is a hypermodified RNA base found in the wobble position of certain tRNA molecules in nearly all organisms.

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* homologue 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*, *toyB*, and *toyC*, which are homologous to *queC*, *queD*, and *queE*, 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 (H_2NTP). This is also the first step in the biosynthesis of folic acid (*16*). Indeed, the GCH I homologue of *E. coli*, FolE, is required for biosynthesis of queuosine and folic acid in that organism (*17*). A FolE homologue has also been implicated in the biosynthesis of archaeosine in *Haloferax volcanii* (*17*). In general, GCH I, QueD, QueC, and QueE homologues 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 H_2NTP to 6-carboxy-5,6,7,8-tetrahydropterin (CPH_4) (*18*). The formation of CPH_4 by QueD was unexpected in light of the significant amino acid sequence similarities between QueD and mammalian PTPS homologues that convert H_2NTP 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 reconstitution of in vitro biosynthesis of $preQ_0$.

MATERIALS AND METHODS

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

Cloning of E. coli QueD, B. subtilis QueE and 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 His_6-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 an OD_{600} of ~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 an OD_{600} of ~0.5, at which point ferric chloride (final concentration of 50 μM) 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% H_2/95% N_2). 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 was pelleted by centrifugation at 26000*g* for 30 min at 4 °C. The cleared lysate was loaded onto two serially connected 1 mL HisTrapHP columns, which had been charged with $NiSO_4$ 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 a buffer A solution containing 0.3 M imidazole. The dark brown QueE protein eluted completely in a volume of ~4 mL. The protein was concentrated to ~3 mL in Microcon centrifugal concentrators (YM-10 membrane) in a tabletop 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 Na_2SO_4, and 10 mM DTT, and eluted via 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) cells 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 an OD_{600} of ~0.5, at which point $ZnSO_4$ 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 26000*g* and loaded on two serially connected 1 mL HiTrap Chelating HP columns, which had been charged with $NiSO_4$ and equilibrated with 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 cm × 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 N_2, and stored at −80 °C. The metal content was determined by inductively coupled plasma optical emission spectroscopy by Garratt-Callahan Co.

In Vitro Production of CPH4. CPH_4 was produced from GTP by the combined activities of *E. coli* FolE and CPH_4 synthase in situ. The reaction mixtures contained 50 mM PIPES (pH 7.4), 10 mM DTT, 10 mM $MgCl_2$, 0.5 mM GTP, 20 μM native recombinant *E. coli* FolE, and 20 μM native recombinant *E. coli* CPH_4 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 essentially to completion.

Activity Assays with B. subtilis QueE and QueC and S. rimosus ToyM. The reaction mixtures were prepared by adding *B. subtilis* QueE (200 μM) to a solution containing CPH_4 prepared enzymatically as described above. After we had accounted for carryover of buffer and reaction components from the CPH_4 synthesis and QueE addition, the reaction mixtures contained 50 mM PIPES (pH 7.4), 0.1 M NaCl, 20 mM Na_2SO_4, 7.7 mM $MgCl_2$, 10 mM DTT, and 0.4 mM CPH_4. 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, the appropriate protein (40 μM) was included. ATP (2 mM) was required for QueC and ToyM. In experiments probing the source of the nitrogen in $preQ_0$, 5 mM $(^{14}NH_4)_2SO_4$ or $(^{15}NH_4)_2SO_4$ was included. Reactions were allowed to proceed for 4 h in the anaerobic chamber and quenched by centrifugal filtration through Microcon centrifugal filtration devices (YM-10 membranes), and mixtures were flash-frozen in liquid nitrogen to prevent oxidative degradation of CPH_4. The quenched samples were kept at −80 °C until they were analyzed.

HPLC Assays for E. coli QueE, B. subtilis QueC, and S. rimosus ToyM Activity. Reaction mixtures were thawed, and an aliquot (40 μL) was injected directly on an Agilent Zorbax Eclipse C-18 column (4.6 mm × 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% acetonitirile (flow rate of 0.75 mL/min). The eluent was monitored (200−500 nm) using an Agilent 1100 photodiode array detector. Authentic $preQ_0$, synthesized as described previously (*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 semipreparative Agilent Eclipse C-18 column (9.4 mm × 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 to 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 conducted 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 a 1:1 H_2O/acetonitrile mixture (containing 0.1% 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 conducted in the quadrupole region with collision-induced dissociation (QCID, with N_2 as a collision gas) at laboratory collision energies of 10 eV. Accurate masses of fragments were determined, and they corresponded to the losses of CO_2 and NH_3 (negative ion mode) and NH_3 (positive ion mode).

Synthesis and Purification of Uniformly Labeled CDG for NMR. To confirm the identity of the product of QueE, uniformly labeled CDG was prepared using $[^{13}C_{10},^{15}N_5]$GTP. The reaction mixtures contained 50 mM PIPES·NaOH (pH 7.4), 10 mM $MgSO_4$, 10 mM DTT, 100 μM native recombinant *E. coli* FolE, 200 μM native recombinant *E. coli* CPH_4 synthase, and 6 mM labeled GTP in a total volume of 5 mL. This reaction was allowed to proceed for 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 for 8 h in the anaerobic chamber. All subsequent transformations were conducted 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 cm × 30 cm) which was pre-equilibrated in the same buffer, rinsed with 0.12 L of the same, and 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 redissolved 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 semipreparative C-18 column as described above. The CDG peak was collected manually and lyophilized.

NMR Spectroscopy of Enzymatically Produced Uniformly ^{13}C- and ^{15}N-labeled CDG. NMR spectra were acquired at 25 °C in DMSO-d_6 solvent on a Varian Inova-600 instrument operating at a ^1H frequency of 599.7 MHz, using a 5 mm cryogenic HCN single-axis gradient probe. The ^1H-decoupled ^{13}C 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 second-order polynomial.

RESULTS AND DISCUSSION

Protein-BLAST reveals that *B. subtilis* QueE belongs to the family of *S*-adenosyl-L-methionine (SAM)-dependent organic

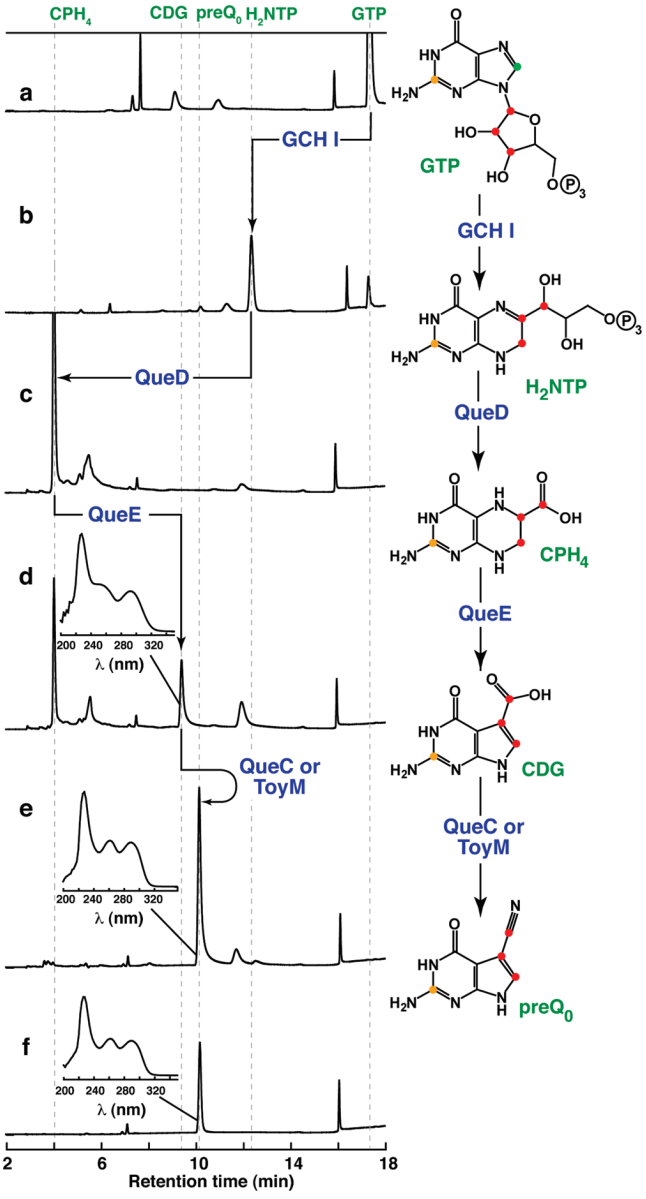

FIGURE 2: In vitro enzymatic synthesis of $preQ_0$ from GTP in four steps. GTP (a) is converted to $preQ_0$ in a reaction that included GTP cyclohydrolase I (GCH I), *E. coli* QueD homologue CPH_4 synthase, *B. subtilis* QueE (7-carboxy-7-deazaguanine synthase), and either *B. subtilis* QueC or *S. rimosus* ToyM (e). In control reactions where only (b) GCH I, (c) GCH I and QueD, or (d) GCH I, QueD, and QueE are included, H_2NTP, CPH_4, and CDG are observed, respectively. The reactions were monitored by HPLC [at (a) 260 nm or (b−f) 300 nm]; while a *y*-axis is not shown, all the chromatograms are to scale. The peak at ~6 min we ascribe to breakdown of CPH_4. Under conditions where all four enzymes were present (e), the reaction is driven to completion as evidenced by a lack of both breakdown product and the starting substrate (GTP). Insets show spectra corresponding to CDG and $preQ_0$. A trace with synthetic $preQ_0$ standard (f) is shown for reference. The coloring scheme of the carbon atoms in the biosynthetic pathway reflects the results of early radiotracer experiments.

radical-generating enzymes (*21*). Members of the radical SAM superfamily contain a CX_3CX_2C 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 CPH_4 and $preQ_0$ (see Figure 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 CPH_4, produced enzymatically from GTP by GCH I and *E. coli* QueD (CPH_4 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 CPH_4 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 of 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 CPH_4. Reaction products were separated by HPLC and detected by UV spectroscopy (see Figure 2). To generate the starting CPH_4 substrate, GTP [retention time of 17.3 min (Figure 2a)] was converted to H_2NTP by *E. coli* FolE [retention time of 12.4 min (Figure 2b)], which was subsequently converted to CPH_4 in the presence of *E. coli* CPH_4 synthase [retention time of 4.0 min (Figure 2c)] as we have shown previously (*18*). Upon addition of reductively activated *B. subtilis* QueE, a significant portion of the CPH_4 is converted to a new product that elutes at 9.4 min (compare panels c and d of Figure 2) and exhibits a UV−visible spectrum that is different from that of CPH_4 (*18*). The conversion of CPH_4 to this new product occurred only in the presence of SAM and dithionite (see Figure S1 of the Supporting Information). The product was purified with a semipreparative HPLC column, lyophilized, and analyzed by Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS). To simplify the assignment, the reactions were also conducted with [$^{15}N_5$]GTP or [$^{13}C_{10}$,$^{15}N_5$]GTP and the products of these reactions were also analyzed. An $[M - H]^-$ ion at m/z 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 at m/z 197.0259 and 204.0497 were observed in the [$^{15}N_5$]GTP and [$^{13}C_{10}$,$^{15}N_5$]GTP samples, respectively (Figure 3a). The comparison of $[M - H]^-$ ions observed in these samples shows that the product contains one fewer nitrogen atom than the starting CPH_4. These 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 *Streptomyces hygroscopicus* (*23*); it is tempting to suggest that CDG produced by a QueE homologue in that organism is the source of the CDG base of the pyrrolopyrimidine nucleoside. Indeed, the ^{13}C NMR chemical shifts of uniformly ^{13}C- and ^{15}N-labeled CDG are very similar to the corresponding shifts published for the base of cadeguomycin (see Figure S4 of the Supporting Information) (*24*).

QueC or ToyM Catalyzes Conversion of 7-Deaza-7-carboxyguanine to $PreQ_0$. We next examined whether CDG, produced by QueE, could serve as a substrate for QueC and ToyM. Interestingly, a conserved SXGXDS motif found in members of 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 $preQ_0$, with externally derived nitrogen to yield the nitrile group. An X-ray crystal structure of QueC from *B. subtilis* has been determined revealing, in addition to the PP_i loop, a structural zinc metal ion (*25*). Inductively

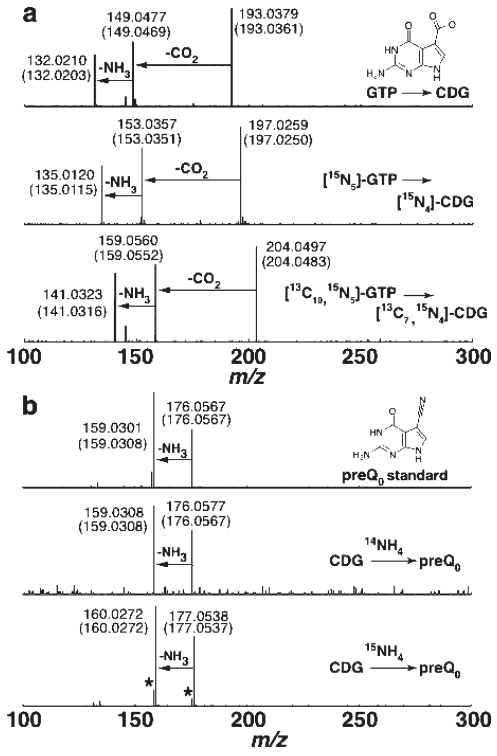

FIGURE 3: Tandem MS/MS (QCID) analysis of products of (a) *B. subtilis* QueE (7-carboxy-7-deazaguanine synthase) and (b) *S. rimosus* ToyM (7-cyano-7-deazaguanine synthetase). Calculated (in parentheses) and observed masses are shown. In each case, enzymatically generated products were purified by semipreparative HPLC and subjected to high-resolution MS/MS analysis. In panel a, CDG was produced in reactions that included unlabeled GTP, [$^{15}N_5$]GTP, or [$^{13}C_{10}$,$^{15}N_5$]GTP. MS/MS spectra were obtained by isolating and fragmenting the $[M - H]^-$ ion at *m*/*z* 193, 197, or 204, corresponding to CDG obtained with unlabeled GTP, [$^{15}N_5$]GTP, or [$^{13}C_{10}$,$^{15}N_5$]GTP, respectively. The fragmentation corresponds to loss of CO_2 and further loss of NH_3 from CDG. In panel b, $^{14}NH_4^+$ or $^{15}NH_4^+$ was included to demonstrate that the cyano nitrogen of $preQ_0$ derives from exogenous ammonia. MS/MS analysis was conducted in the positive ion mode by isolating and fragmenting the $[M + H]^+$ ion at *m*/*z* 176 or 177, corresponding to synthetic $preQ_0$, $preQ_0$ generated enzymatically in the presence of $^{14}NH_4^+$ or in the presence of $^{15}NH_4^+$. The fragmentaion corresponds to loss of NH_3 from $preQ_0$. Asterisks denote a small amount of $preQ_0$ with ^{14}N in the cyano moiety. The same results were obtained with $preQ_0$ generated by *B. subtilis* QueC.

coupled plasma optical emission spectroscopy of recombinant QueC from *B. subtilis* and its *S. rimosus* homologue, ToyM, revealed the presence of 0.88 and 0.80 equiv of zinc metal ion per monomer, respectively, for the proteins used in our studies.

When CPH_4 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 a retention time of 10.3 min, identical to synthetic $preQ_0$, is observed in the chromatogram (compare panels e and f of Figure 2). In addition, the spectral properties of this molecule are identical to those of synthetic $preQ_0$ (see insets). While the formation of enzymatically produced $preQ_0$ was ATP-dependent, it occurred in the absence of an exogenously supplied nitrogen source (Figure S2 of the Supporting Information). 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 an $[M + H]^+$ ion at *m*/*z* 176.0567, confirming that both of these proteins convert CDG to $preQ_0$ (calculated *m*/*z* 176.0567). When the reaction was carried out with [$^{13}C_{10}$,$^{15}N_5$]GTP as the starting material, $[M + H]^+$ ions at *m*/*z* 187.0684 and 187.0683 were observed with ToyM and QueC, respectively [calculated *m*/*z* 187.0683 (see Figure S3 of the Supporting Information)]. The molecular weight difference between $preQ_0$ derived from unlabeled and labeled precursor GTP reveals the loss of one ^{15}N from the starting material, as with CDG, and incorporation of one ^{14}N.

The Cyano Nitrogen of $PreQ_0$ Is Derived from Ammonia. To examine whether ammonia was the source of the nitrogen in the cyano group of $preQ_0$, a series of reactions was carried out in which CPH_4 (produced enzymatically from GTP as described above) was incubated with QueE and QueC (or ToyM) and either $^{15}NH_4SO_4$ or $^{14}NH_4SO_4$ FT-ICR MS analysis of the $preQ_0$ produced in these reactions shows incorporation of ^{15}N into the product when a source of labeled ammonia is included (Figure 3b). In tandem MS/MS analysis of the products generated with either QueC (or ToyM) in the presence of $^{14}NH_4^+$, an $[M + H]^+$ ion with an *m*/*z* value identical to that of synthetic $preQ_0$ is observed (*m*/*z* 176.0577). By contrast, when $^{15}NH_4^+$ is added, the peak shifts to an $[M + H]^+$ ion at *m*/*z* 177.0538, consistent with incorporation of the exogenous labeled ammonia into $preQ_0$. In the samples where $^{15}NH_4^+$ was supplied, the $[M+H]^+$ ion at *m*/*z* 176.0538, which is ≤20% as large as that of the ^{15}N-labeled molecule, is also observed; this presumably derives from ammonia produced in conversion of CPH_4 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. On the basis of our results, we propose a general paradigm for biosynthesis of deazapurine-containing compounds in nature, which incorporates H_2NTP, CPH_4, and CDG as common intermediates (see Figure 2). We hypothesize that CDG may be the central precursor to all deazapurines, and that the presence of a QueC homologue in a genome may signal the capacity of the organism to produce compounds containing a nitrogen at the C8 moiety of the molecule. This pathway is interesting for several reasons. First, it contains a novel PTPS homologue, QueD, which has evolved a function distinct from that of 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 ($CPH_4 \rightarrow$ CDG). Third, the pathway involves a novel ATP-dependent conversion of a carboxylic acid to a nitrile. Fourth, the cyano nitrogen of $preQ_0$ is derived from ammonia and not N7 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 the possibility 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 $preQ_0$ provides the framework for future studies of the chemical transformations that underlie the biosynthesis of 7-deazapurine-containing compounds.

SUPPORTING INFORMATION AVAILABLE

Materials and detailed experimental procedures, controls for conversion of CPH_4 to CDG by QueE, controls showing conversion of CDG to $preQ_0$ by ToyM or QueC, tandem MS/MS fragmentation of $preQ_0$, and ^1H-decoupled ^{13}C NMR spectrum

of CDG. This material is available free of charge via the Internet at http://pubs.acs.org.

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