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Spectroscopic, Steady-State Kinetic, and Mechanistic Characterization of the Radical SAM Enzyme QueE, Which Catalyzes a Complex Cyclization Reaction in the Biosynthesis of 7-Deazapurines

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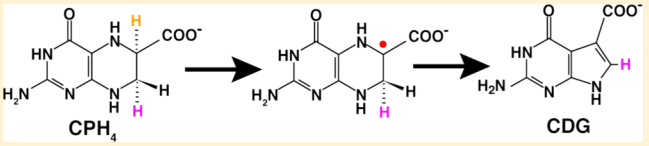
Supporting Information

ABSTRACT: 7-Carboxy-7-deazaguanine (CDG) synthase (QueE) catalyzes the complex heterocyclic radical-mediated conversion of 6-carboxy-5,6,7,8-tetrahydropterin (CPH_4) to CDG in the third step of the biosynthetic pathway to all 7-deazapurines. Here we present a detailed characterization of QueE from *Bacillus subtilis* to delineate the mechanism of conversion of CPH_4 to CDG. QueE is a member of the radical *S*-adenosyl-L-methionine (SAM) superfamily, all of which use a bound $[4Fe\text{-}4S]^+$ cluster to catalyze the reductive cleavage of the SAM cofactor to generate methionine and a 5′-deoxyadenosyl radical (5′-dAdo•), which initiates enzymatic transformations requiring hydrogen atom abstraction. The ultraviolet−visible, electron paramagnetic resonance, and Mössbauer spectroscopic features of the homodimeric QueE point to the presence of a single [4Fe-4S] cluster per monomer. Steady-state kinetic experiments indicate a K_m of 20 ± 7 μM for CPH_4 and a k_{cat} of 5.4 ± 1.2 min^{-1} for the overall transformation. The kinetically determined K_{app} for SAM is 45 ± 1 μM. QueE is also magnesium-dependent and exhibits a K_{app} for the divalent metal ion of 0.21 ± 0.03 mM. The SAM cofactor supports multiple turnovers, indicating that it is regenerated at the end of each catalytic cycle. The mechanism of rearrangement of QueE was probed with CPH_4 isotopologs containing deuterium at C-6 or the two prochiral positions at C-7. These studies implicate 5′-dAdo• as the initiator of the ring contraction reaction catalyzed by QueE by abstraction of the H atom from C-6 of CPH_4.



Compounds containing 7-deazapurine moieties are ubiquitous in nature and include structurally diverse nucleoside analogues produced by *Streptomyces* that have demonstrated antibiotic and antineoplastic activities (see refs 1 and 2 for reviews). The structural diversity in deazapurines arises from substitutions at C-7 and elsewhere in the molecule (see Figure 1a). In addition to secondary metabolites, 7-deazapurine moieties are also found in the hypermodified tRNA nucleosides queuosine[3] and archaeosine.[4] Queuosine is located in the wobble position of tRNAs bearing His, Tyr, Asp, and Asn with the 5′-GUN-3 anticodon;[5] the hypermodification is almost universally conserved in all kingdoms of life, but the precise physiological role of the modification remains to be established.[6,7] Archaeosine is located in the dihydrouridine loop of most archaeal tRNAs and is thought to play a role in stabilizing the tertiary RNA structure.[4] Eukaryotes do not have de novo pathways for biosynthesis of queuosine[8] but incorporate queuine (the free base of queuosine) from dietary sources into tRNA.[9,10]

The 7-deazapurine moiety that is at the core of all pyrrolopyrimidine nucleosides is derived from GTP in three steps (Figure 1b).[11−14] The first step is the conversion of GTP to 7,8-dihydroneopterin triphosphate (H_2NTP), which is catalyzed by GTP cyclohydrolase I (GCH I).[11] Interestingly, this step appears to have been co-opted from the primary metabolism as GCH I also catalyzes the first step in the biosynthesis of folic acid[15] and tetrahydrobiopterin.[16] The second step is the conversion of H_2NTP to 6-carboxy-5,6,7,8-tetrahydropterin (CPH_4) catalyzed by CPH_4 synthase (QueD or ToyB in Figure 1b).[13] Next, CDG synthase (QueE or ToyC in Figure 1b) catalyzes a dramatic and unprecedented heterocyclic rearrangement in the third step of the pathway to convert CPH_4 to 7-carboxy-7-deazaguanine (CDG).[14] CDG is the first 7-deazapurine in the biosynthetic pathway and likely the precursor to all naturally occurring 7-deazapurine-containing molecules.

CDG synthase is a member of the radical *S*-adenosyl-L-methionine (SAM) superfamily of enzymes.[17] Members of the radical SAM superfamily contain conserved cysteine residues that ligate iron present at three vertices of a [4Fe-4S] cluster. While the cysteine residues are typically arranged in a CX_3CX_2C motif,[17] exceptions have been noted.[18−20] In Dpn2, for example, the three Cys residues that coordinate the Fe−S cluster are distant in sequence.[20] The fourth iron is coordinated to the carboxylate and amino moieties of SAM.[21,22] In its +1 oxidation state, the cluster donates an electron to SAM, initiating the reductive cleavage of the C5′−S bond

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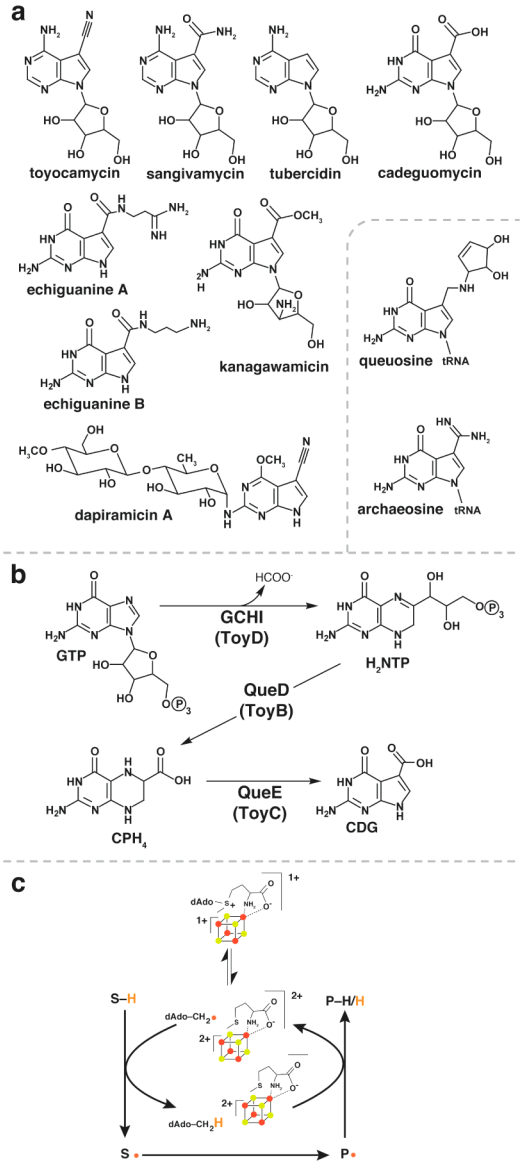

Figure 1. (a) Examples of 7-deazapurine nucleosides isolated from culture filtrates of soil bacteria. The hypermodified nucleosides queuosine and archaeosine are shown in the inset. (b) Pathway for the biosynthesis of the 7-deazapurine core of pyrrolopyrimidine nucleosides that involves three steps in which QueE catalyzes the key ring contraction step leading to the first 7-deazapurine intermediate. QueE is a member of the radical SAM superfamily of enzymes[17] and is the subject of this study. (c) General reaction scheme for radical SAM enzymes that use SAM catalytically.

affording methionine and a 5′-deoxyadenosyl radical (5′-dAdo•) (Figure 1c). 5′-dAdo• abstracts a hydrogen atom from either a site on the substrate molecule or the protein to initiate the catalytic cycle. SAM is re-formed at the end of the catalytic cycle in a subset of radical SAM proteins that include lysine 2,3-aminomutase[23] and spore photoproduct lyase;[24] however, in many others, cleavage of SAM is not reversed and 5′-dAdo is a product (see refs 25−28 for recent reviews).

Here we present spectroscopic and steady-state kinetic characterization of recombinantly expressed CDG synthase from *Bacillus subtilis* showing that it is indeed a member of the radical SAM superfamily. Furthermore, studies with site-specifically deuterated substrates afford detailed insights into the mechanism of the complex, radical-mediated ring contraction catalyzed by the enzyme.

EXPERIMENTAL PROCEDURES

Cloning, Expression, Purification, and Reconstitution of *B. subtilis* QueE. QueE from *B. subtilis* was cloned into pET28a for expression of His_6-tagged, recombinant protein, expressed in BL21(DE3) *Escherichia coli* cells (Novagen), and purified as described previously[14] with the following modifications. The eluent from the affinity chromatography step was loaded onto an Econo-Pac 10DG column (Bio-Rad), which had been pre-equilibrated in 0.05 M PIPES-NaOH (pH 7.4) buffer containing 10 mM DTT. QueE was eluted with 4 mL of the same buffer, and an aliquot (1 mL) was frozen at −80 °C for future use as nonreconstituted QueE. The remainder (∼3 mL) was reconstituted with iron and sulfide as follows. To a solution of QueE (∼1 mM) were rapidly added 15 μL of 1 M $FeCl_3$ and 15 μL of 1 M Na_2S with mixing following each addition. The reconstitution mixture was incubated at ambient temperature for 6 h, and then precipitated material was removed by brief centrifugation (1 min). The supernatant was loaded on an Econo-Pac 10DG column pre-equilibrated in buffer containing 0.05 M PIPES-NaOH (pH 7.4) and 10 mM DTT, and QueE was eluted with 4 mL of the same buffer. QueE reconstituted by this protocol was divided into aliquots, frozen in liquid nitrogen, and stored at −80 °C for future use.

Determination of QueE Concentration. The accurate amino acid content, which is required for determining the stoichiometry of the cofactor, was obtained by amino acid analysis. QueE was prepared for amino acid analysis as follows. QueE (0.1 mL, purified as described above) was passed over an illustra NICK column (GE Healthcare) pre-equilibrated in 10 mM NaOH. QueE was eluted from the column with 0.4 mL of the same, quantified with a Bradford assay with BSA as a standard, lyophilized, and submitted to the molecular structure facility at the University of California (Davis, CA) for amino acid analysis. A correction factor of 0.68 was calculated for the Bradford assay based on the amino acid analysis.

EPR Spectroscopy. EPR samples were prepared by combining QueE with $MgSO_4$, SAM, and sodium dithionite to final concentrations of ∼0.5, 10, 2, and 10 mM, respectively. The mixture was incubated for 5 min after the addition of sodium dithionite and frozen in an EPR tube. The sample was stored at −80 °C. Continuous wave (CW) X-band EPR experiments were performed on a Bruker ESP-300 spectrometer equipped with a standard rectangular resonator operating in TE102 mode and an ESR-900 flow cryostat (Oxford Instruments). Spectra were recorded at 10 K at a microwave frequency of 9.337 GHz. The power and modulation amplitude were 2 mW and 10 G, respectively.

Preparation of $^{57}FeSO_4$. Metallic ^{57}Fe was obtained from Isoflex USA (San Francisco, CA). The ^{57}Fe powder (0.1 g) was rinsed with 2 mL of $CHCl_3$ and dried under vacuum. The metal was combined with 2.63 mL of 1 M sulfuric acid (to give a ratio of 1.5 mol of acid/mol of iron) and incubated at 60 °C in the anaerobic chamber. The solution was neutralized after 90 min via the addition of solid sodium bicarbonate to a final concentration of 1 M.

Expression, Purification, and Reconstitution of ^{57}Fe-Labeled QueE for Mössbauer Spectroscopy. QueE containing ^{57}Fe for Mössbauer spectroscopy was expressed in the presence of the pDB1282 plasmid in *E. coli* BL21(DE3).

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Plasmid pDB1282 contains six genes (*iscS*, *iscU*, *iscA*, *hscA*, *hscB*, and *fdx*) from the *Azotobacter vinelandii* operon, which are important for biogenesis of FeS clusters.[29,30] The plasmid confers ampicillin resistance, and the genes are cloned behind an arabinose inducible promoter. Cells containing the QueE expression plasmid were grown in minimal medium based on a recipe from Fraenkel and Neinhardt[31] that contained 0.71% Na_2HPO_4, 1.35% KH_2PO_4, 0.000147% $CaCl_2{\cdot}H_2O$, 0.0246% $MgSO_4{\cdot}7H_2O$, 0.4% glucose, and 0.06% $(NH_4)_2SO_4$ along with 34 μg/mL kanamycin and 100 μg/mL ampicillin. When the cell density reached an OD_{600} of ~0.3, solid arabinose (0.5 g/L) was added to induce transcription of the genes in pDB1282 and $^{57}FeSO_4$ and cysteine were added to final concentrations 0.05 and 0.2 mM, respectively. At an OD_{600} of ~0.5, IPTG was added to a final concentration of 0.1 mM to induce expression of QueE. Cells were harvested by centrifugation 5 h after induction with IPTG and frozen in liquid N_2. Purification and reconstitution of ^{57}Fe-labeled QueE for Mössbauer spectroscopy were conducted as described above for the unlabeled protein except that $^{57}FeSO_4$ prepared as described above was included in the incubation mixture. Native or reconstituted [^{57}Fe]QueE was concentrated 2- or 3.5-fold, respectively, in Microcon centrifugal concentrators with YM-10 membranes (Millipore) and frozen in Mössbauer cups with liquid nitrogen.

Mössbauer Spectroscopy. Mössbauer spectra were recorded on a spectrometer from WEB research (Edina, MN) operating in the constant acceleration mode in transmission geometry. Spectra were recorded with the temperature maintained at 4.2 K. The sample was kept inside an SVT-400 dewar from Janis (Wilmington, MA), and a magnetic field of 53 mT was applied parallel to the α-beam. The reported isomer shift is relative to the centroid of the spectrum of a metallic foil of β-Fe at room temperature. Data analysis was performed using WMOSS from WEB research.

Determination of the Oligomerization State of QueE. All gel filtration steps were conducted using anaerobically prepared solutions in an anaerobic chamber. QueE (0.1 mL of 0.5 mM protein) was diluted to 0.5 mL with 0.05 M potassium phosphate buffer containing 0.15 M NaCl and loaded onto a HiPrep 16/60 Sephacryl S-200 high-resolution column (GE Healthcare), which was equilibrated in the same buffer. A mixture of molecular mass standards that included ~20 mg/mL chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), BSA (67 kDa), and aldolase (158 kDa) in a volume of 0.5 mL was injected separately to calibrate the column. Fractions of the eluent were collected; proteins were detected by SDS−PAGE, and the elution volume of QueE was compared to those of the standard to obtain the molecular mass of the complex.

Preparation and Purification of CPH_4. CPH_4 was prepared enzymatically by incubating GTP in the presence of recombinant GCH I and QueD, which were prepared as described previously.[13] The reaction mixture (10 mL) contained 0.02 M PIPES-NaOH (pH 7.4), 10 mM DTT, 5 mM GTP, 50 μM GCH I, and 15 μM QueD. The incubation was conducted in the anaerobic chamber to minimize oxidation of the air-sensitive CPH_4 product. After 20 h, the mixture was loaded onto a DEAE-Sepharose column (2.6 cm × 12.5 cm) that had been pre-equilibrated in 10 mM ammonium bicarbonate. The column was rinsed with the same buffer, and CPH_4 was obtained in the flow-through. CPH_4 was detected by HPLC analysis.[13] Fractions containing CPH_4 were pooled, lyophilized, and resuspended in ~1 mL of H_2O. The concentration of CPH_4 was quantified using the extinction coefficient for tetrahydrobiopterin[32] (ε_{297} = 8.71 × 10^3 M^{-1} cm^{-1} at pH 8.0).

Preparation of [6-$^2H_1$]CPH_4. Figure 4 summarizes the strategies that were employed for synthesis of site-specifically deuterated CPH_4. Briefly, we have shown previously that *E. coli* QueD catalyzes the conversion of sepiapterin to CPH_4.[13] Therefore, this reaction was used to prepare CPH_4 containing deuterium at C-6 by conducting the reaction in D_2O. The reaction mixture (10 mL) contained 5 mM sepiapterin (Sigma), 0.02 M PIPES-NaOH (prepared from a 1 M stock solution in 99.9% D_2O), and 15 μM *E. coli* QueD that had been lyophilized to remove H_2O. The reaction was conducted in 99.9% D_2O (Sigma) and allowed to proceed for 20 h. The mixture was lyophilized and dissolved in H_2O, and CPH_4 was purified over a DEAE-Sepharose column as described above for the unlabeled compound.

Preparation of [7*R*-D]CPH_4. CPH_4 deuterated at the *pro-R* position of C-7 was prepared in two steps by the GCH I-dependent conversion of GTP to H_2NTP in D_2O, followed by the QueD-dependent conversion of the resulting H_2NTP to CPH_4 in H_2O. The GCH I reaction mixture (8 mL) contained 14.3 mM GTP, 0.05 M ammonium bicarbonate, and 0.13 mM GCH I that had been buffer exchanged into 99% D_2O (Sigma) containing 0.02 M Tris-HCl (pH 8.0). After 15 h in the anaerobic chamber, GCH I was removed when the solution was passed through centrifugal filter devices with a YM-10 membrane filter (Amicon) at 7000*g*. The flow-through was lyophilized to remove D_2O, redissolved in H_2O, and lyophilized a second time. The resulting H_2NTP was converted to CPH_4 in a reaction mixture (10 mL) that contained 0.02 M PIPES-NaOH (pH 7.4), 10 mM DTT, and 5 μM *E. coli* QueD. After 18 h, the reaction mixture was loaded onto a DEAE-Sepharose column and CPH_4 was purified as described above.

Preparation of [7*S*-D]CPH_4. CPH_4 deuterated at the *pro-S* position of C-7 was prepared enzymatically by incubating 97% [U-D]GTP (Cambridge Isotope Laboratories, Inc.) in the presence of GCH I and QueD in H_2O. The enzymatic production and subsequent purification of the deuterated CPH_4 were conducted as described for unlabeled CPH_4.

Determination of the Level of CPH_4 Deuteration by Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FT-ICR MS). CPH_4 samples were diluted 100-fold in a 1:1 H_2O/acetonitrile mixture containing 0.1% formic acid. FT-ICR MS was conducted in positive ion mode as previously described.[14] The level of deuteration was estimated by the ratio of $[M + H]^+$ ion peaks at m/z 212.1 and 213.2 for unlabeled and singly deuterated CPH_4, respectively.

Preparation of Flavodoxin (FldA) and Flavodoxin Reductase (FPR). *E. coli* strains containing the genes for *E. coli* FldA and FPR that had been cloned into intein-based expression vector pTYB1 (New England Biolabs, Ipswich, MA) were a generous gift from S. Booker (The Pennsylvania State University). FldA and FPR were expressed and purified by affinity chromatography to yield native proteins as previously described.[33] However, two experiments included in this work were conducted using His_6-tagged flavodoxin reductase. The gene encoding *E. coli* His_6-flavodoxin reductase was ordered from Genscript (see Figure S1 of the Supporting Information for the sequence), excised from the supplied pUC57 plasmid with *Nde*I and *Hin*dIII, and cloned into pET28a to give pET28:FPR. The recombinant protein was expressed in *E. coli* HMS174(DE3). *E. coli* HMS14(DE3) cells containing pET28:FPR were grown in LB at 37 °C with shaking, and

expression of His_6-FPR was induced with the addition of IPTG (0.1 mM) when the cell density had reached an OD_{600} of ~0.5. At that time, solid riboflavin was added to a final concentration of 0.1 mM. Cells were harvested by centrifugation 6 h after induction and frozen in liquid N_2. Frozen cells (~3 g) were resuspended in buffer containing 0.02 M potassium phosphate (pH 7.2), 0.5 M NaCl, 5 mM imidazole, and 1 mM PMSF and sonified at 60% amplitude using a Branson digital sonifier. The lysate was centrifuged for 30 min at 26500*g* and 4 °C. The cleared lysate was loaded onto a 1 mL HisTrapHP column (GE Healthcare) that had been pre-equilibrated in buffer containing 0.02 M potassium phosphate (pH 7.2), 0.5 M NaCl, and 5 mM imidazole (buffer A). The column was rinsed with 10 mL of buffer A, and the His_6-flavodoxin reductase was eluted with a 20 mL linear gradient to 100% buffer B that contained 0.5 M imidazole in buffer A. Fractions were analyzed by SDS−PAGE, and those containing the desired protein were combined and dialyzed twice against 4 L of 0.02 M HEPES-NaOH (pH 7.4). Flavodoxin reductase was quantified by the absorbance of flavin adenine dinucleotide[34] (ε_{456} = 7100 M^{-1} cm^{-1}).

Enzymatic Preparation of SAM. *E. coli* strain DM22-(pK8), which overexpresses *E. coli* SAM synthetase, was constructed by G. D. Markham (Fox Chase Cancer Center, Philadelphia, PA).[35,36] *E. coli* DM22(pK8) was grown in LB containing 30 μg/mL oxytetracycline at 37 °C while being shaken and harvested by centrifugation 12 h after inoculation. The cells (~12 g) were resuspended in 36 mL of 0.1 M Tris-HCl (pH 8.0) containing 1 mM EDTA and 50 μg/mL lysozyme and gently stirred at room temperature for 30 min. PMSF was added to a final concentration of 1 mM, and cells were sonified at 60% amplitude using a Branson digital sonifier. The cell lysate was centrifuged for 30 min at 26500*g* and 4 °C. The cleared lysate was divided into three portions and frozen in liquid nitrogen. SAM was generated enzymatically by incubating an aliquot of the lysate in a buffered solution (0.1 L) containing 0.1 M Tris-HCl (pH 8.0), 50 mM KCl, 1 mM EDTA, 20% acetonitrile, 26 mM $MgCl_2$, 13 mM ATP, and 10 mM methionine. After the mixture had been gently stirred for 5 h at room temperature, the reaction was quenched by adjusting the pH to 5.0 with HCl. The mixture was placed on ice for 15 min and centrifuged at 26500*g* for 30 min at 4 °C to remove precipitated material. The supernatant was diluted to 1 L with 1 mM sodium acetate (pH 5.0) and loaded on a CM-52 cation exchange resin (Whatman) (19.6 cm × 25 cm) that had been charged with 0.2 M sodium acetate (pH 5.0) and equilibrated in 1 mM sodium acetate (pH 5.0). The column was rinsed with 0.7 L of 1 mM sodium acetate, and SAM was eluted with a 0.9 L linear gradient from 0 to 0.2 M HCl. The presence and purity of SAM were confirmed by HPLC analysis, and fractions containing SAM were combined, lyophilized, resuspended in 4 mL of H_2O, and stored at −80 °C until they were used.

Preparation of 7-Carboxy-7-deazaguanine (CDG). CDG was prepared by refluxing 50 mg of $preQ_0$ (prepared as described in ref 37) in 3.5 mL of 6 M sodium hydroxide for 3 h. The reaction mixture was diluted to 110 mL of 0.35 M ammonium acetate and loaded onto a Q-Sepharose column (1.6 cm × 20 cm) pre-equilibrated in the same buffer. The column was rinsed with 40 mL of 0.35 M ammonium acetate, and CDG was eluted with a gradient to 0.5 M ammonium acetate over 0.4 L. The presence and purity of CDG were confirmed by HPLC analysis, and fractions containing CDG were combined, lyophilized, and resuspended in 1 mL of H_2O. The concentration of CDG ultimately obtained was measured by UV−visible absorbance and quantified using the extinction coefficient for the 7-carboxy-7-deazaguanine nucleoside cadeguomycin (ε_{298} = 7607 M^{-1} cm^{-1}).[38]

Steady-State Kinetic Analysis of QueE. Steady-state kinetic experiments were conducted to measure the initial rate of QueE activity as a function of CPH_4, Mg^{2+}, and SAM concentration. The reaction mixtures contained 0.05 M PIPES-NaOH (pH 7.4), 10 mM DTT, 1.2 μM QueE, 24 μM FldA, and 2.2 μM FPR. Reaction mixtures for measuring the dependence of initial activity on CPH_4 concentration and Mg^{2+} concentration contained 1.6 mM NADPH, but reaction mixtures for measuring the dependence of initial activity on SAM concentration contained 2 mM NADPH. Reaction mixtures also contained 1.6 mM SAM (Sigma), 1 mM $MgSO_4$, and 1 mM CPH_4 unless the concentration of one of these ingredients was varied. In all cases, reactions (65 μL) were initiated by addition of CPH_4 quenched after 2 min by addition of 6.5 μL of 30% (w/v) TCA. An aliquot (25 μL) was injected directly on an Agilent Zorbax Eclipse C-18 column (4.6 mm × 250 mm) pre-equilibrated in water. A 30 min gradient from 0 to 30% acetonitrile was used to elute analyte components at a flow rate of 0.75 mL/min. The elution profile was monitored by UV absorbance spectroscopy from 200 to 500 nm using an Agilent 1100 photodiode array detector, and the peak area on the UV chromatogram corresponding to CDG was noted.

Dependence of Activity on QueE Concentration. A linear relationship between the formation of CDG and the concentration of QueE was established by reaction mixtures that contained 0.05 M PIPES-NaOH (pH 7.4), 10 mM DTT, 1 mM $MgSO_4$, 2 mM SAM (Sigma), 2 mM NADPH, 24 μM FldA, 5 μM FPR, 1 mM CPH_4, and 0.7, 1.4, 2.0, or 2.7 μM QueE in a total volume of 0.2 mL. The reactions were initiated by addition of substrate, and time points were taken by withdrawing 30 μL at 2.5, 5, and 10 min and adding them to 3 μL of 30% (w/v) TCA to quench activity. An aliquot (25 μL) was analyzed by HPLC as described above, and the peak area on the UV chromatogram corresponding to CDG was noted.

Stoichiometry of CDG Produced to SAM in the Reaction Catalyzed by QueE. The reaction mixtures contained 0.05 M PIPES-NaOH (pH 7.4), 10 mM DTT, 1 mM $MgSO_4$, 10 μM SAM (Sigma), 2 mM NADPH, 2.4 μM QueE, 12 μM FldA, 2 μM FPR, and 1 mM CPH_4 in a total reaction volume of 0.15 mL. The reaction was initiated by addition of substrate, and time points were taken by withdrawing 30 μL at 30, 60, 90, and 120 min and combining it with 3 μL of 30% (w/v) TCA to quench the reaction. A quantity of the quenched reaction mixtures (25 μL) was analyzed by HPLC as described above, and the peak area on the UV chromatogram corresponding to CDG was noted.

Analysis of the Deuterium Content of 5′-dAdo from Site-Specifically Deuterated CPH_4. QueE reactions were conducted using unlabeled or C-6-, C-7-*R*-, or C-7-*S*-deuterated CPH_4 and analyzed by LC−MS to examine incorporation of substrate deuterium into 5′-dAdo and CDG. The reaction mixtures contained 0.05 M PIPES-NaOH (pH 7.4), 10 mM DTT, 2 mM $MgSO_4$, 4 mM SAM (produced enzymatically as described above), 2 mM NADPH, 19 μM QueE, 20 μM FldA, 20 μM His_6-FPR, and 2 mM CPH_4 (either unlabeled or labeled as 6-D, 7*R*-D, or 7*S*-D) in a total reaction volume of 0.1 mL. An additional reaction mixture containing 60% D_2O and unlabeled CPH_4 was used as well to assess the extent to which solvent-derived deuterium can label 5′-dAdo and CDG pools.

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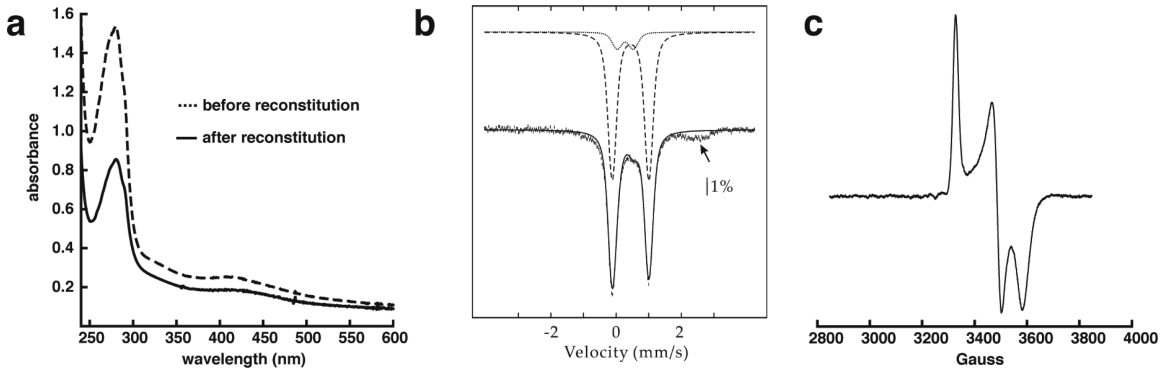

Figure 2. (a) Representative UV−visible spectra of as-isolated (20 μM) and reconstituted (10 μM) QueE. (b) 4.2 K, 53 mT Mössbauer spectrum of reconstituted QueE (0.89 mM). The solid line overlaid with the experimental data is a simulation assuming two quadrupole doublets with the following parameters: (1) $\delta(1)$ = 0.44 mm/s, $\Delta E_Q(1)$ = 1.13 mm/s (80% of total intensity), (2) $\delta(2)$ = 0.30 mm/s, $\Delta E_Q(2)$ = 0.50 mm/s (11% of total intensity). The individual contributions are shown as dashed and dotted lines, respectively. (c) EPR spectrum of 0.68 mM QueE containing 2 mM SAM, 10 mM sodium dithionite, and 10 mM $MgSO_4$. EPR spectra were measured at 10 K with the following settings: microwave frequency, 9.337 GHz; microwave power, 2 mW; modulation amplitude, 10 G. $MgSO_4$ is not required to observe the EPR signal.

Reactions were quenched after 20 min with the addition of 10 μL of 30% (w/v) TCA, and 0.1 mL of the sample was analyzed by LC−MS.

RESULTS AND DISCUSSION

Purification, Determination of the Quaternary Structure, Metal Ion Content, and Spectroscopic Characterization. Recombinant QueE from *B. subtilis* was expressed in *E. coli* as a His_6 fusion protein together with pDB1282, which contains the *isc* operon encoding genes involved in the biosynthesis of Fe−S clusters in *A. vinelandii*.[29,30] The pDB1282 construct is commonly included during expression of radical SAM proteins to improve the yield of holoproteins. QueE was purified in an anaerobic chamber by Ni^{2+}-affinity chromatography and emerged from the column in dark brown fractions. The purity was estimated to be at least 95% by SDS−PAGE (Figure S2 of the Supporting Information). Analytical gel filtration reveals a molecular mass between that of ovalbumin (46 kDa) and BSA (67 kDa), consistent with dimerization of the 29 kDa monomers to form a homodimer.

The UV−visible spectrum of the "as-isolated" protein measured in the anaerobic chamber immediately after purification displays a broad shoulder at ∼410 nm, which is characteristic of proteins containing [4Fe-4S] clusters (see Figure 2a). The three conserved cysteine residues in QueE are in a CX_3CX_2C motif, which is present in radical SAM proteins.[17]

The iron and labile sulfide contents of the protein were determined by ICP-OES and the Beinert method,[39] respectively. The "as-purified" protein contains 1.5 ± 0.4 equiv of Fe and 2.4 ± 0.1 equiv of sulfide. To improve the content of [4Fe-4S] clusters, the protein was reconstituted at room temperature in the presence of iron and inorganic sulfide. The A_{410}/A_{280} ratio, which reports on Fe−S content, improved from 0.12 to 0.15 upon reconstitution (Figure 2a). Moreover, the reconstitution results in an increase in Fe and sulfide content to 4.2 ± 0.8 and 7.1 ± 1.0, respectively. These observations are consistent with presence of a single [4Fe-4S] cluster in QueE.

To determine the types and quantity of Fe−S clusters associated with QueE more rigorously, we have used a combination of Mössbauer and EPR spectroscopy on samples enriched with ^{57}Fe. As-isolated, ^{57}Fe-labeled QueE contains 1.4 Fe atoms and 1.9 sulfides per monomer. The combination of EPR and Mössbauer spectroscopies (Figure S3 of the Supporting Information) reveals that as-isolated QueE harbors ∼0.2 equiv of $[4Fe\text{-}4S]^{2+}$ clusters and ∼0.2 equiv of $[2Fe\text{-}2S]^{2+}$ clusters, in addition to a small amount (<0.01 equiv) of $[3Fe\text{-}4S]^{+}$ clusters. The presence of multiple Fe−S cluster types has been observed previously in other Fe−S cluster-containing enzymes, including radical SAM enzymes, e.g., pyruvate formate lyase activase.[24] Reconstitution of as-isolated QueE as described in Experimental Procedures leads to a significant uptake of ^{57}Fe and sulfide to 2.8 Fe atoms and 3.9 sulfides per QueE. The 4.2 K, 53 mT Mössbauer spectrum of reconstituted QueE is dominated by a quadrupole doublet with an isomer shift (δ) of 0.44 mm/s and a quadrupole splitting parameter (ΔE_Q) of 1.13 mm/s, which are typical of $[4Fe\text{-}4S]^{2+}$ clusters (dashed line in Figure 2b). This quadrupole doublet accounts for ∼80% of the total intensity, which after taking into account the Fe and amino acid analyses reveals that the ^{57}Fe-reconstituted QueE contains ∼0.6 equiv of $[4Fe\text{-}4S]^{2+}$ clusters. In addition, the spectrum reveals a distinct shoulder at ∼0.6 mm/s. This position is typical of the high-energy line of a quadrupole doublet associated with $[2Fe\text{-}2S]^{2+}$ clusters. This feature can be modeled by a quadrupole doublet (δ = 0.30 mm/s, ΔE_Q = 0.50 mm/s, 11% of total intensity, dotted line in Figure 2b) and suggests the presence of ∼0.15 $[2Fe\text{-}2S]^{2+}$ cluster per QueE. The added contribution of these two quadrupole doublets is shown as a solid line in Figure 2b. The missing absorption (e.g., the broad features at approximately −1 and 2−2.5 mm/s) could (at least in part) emanate from Fe−S clusters with the $S = {}^1/_2$ ground state. However, their presence can be confidently ruled out on the basis of the fact that an identical sample is EPR silent (data not shown). Most likely, these features emanate from high-spin Fe(II) complex(es). The broadness of the features suggests the presence of multiple Fe(II) complexes, e.g., Fe(II) coordinated by four thiolate ligands and Fe(II) coordinated by five or six N/O ligands. Although the stoichiometry of [4Fe-4S] clusters (∼0.6 equiv) and total Fe−S clusters (∼0.75 equiv) is less than one in the ^{57}Fe-reconstituted protein, the results suggest that QueE harbors one [4Fe-4S] cluster, which is assigned to the essential radical SAM [4Fe-4S] cluster coordinated by the canonical CX_3CX_2C motif. The small amount of [2Fe-2S] cluster is presumably due to oxidative degradation.

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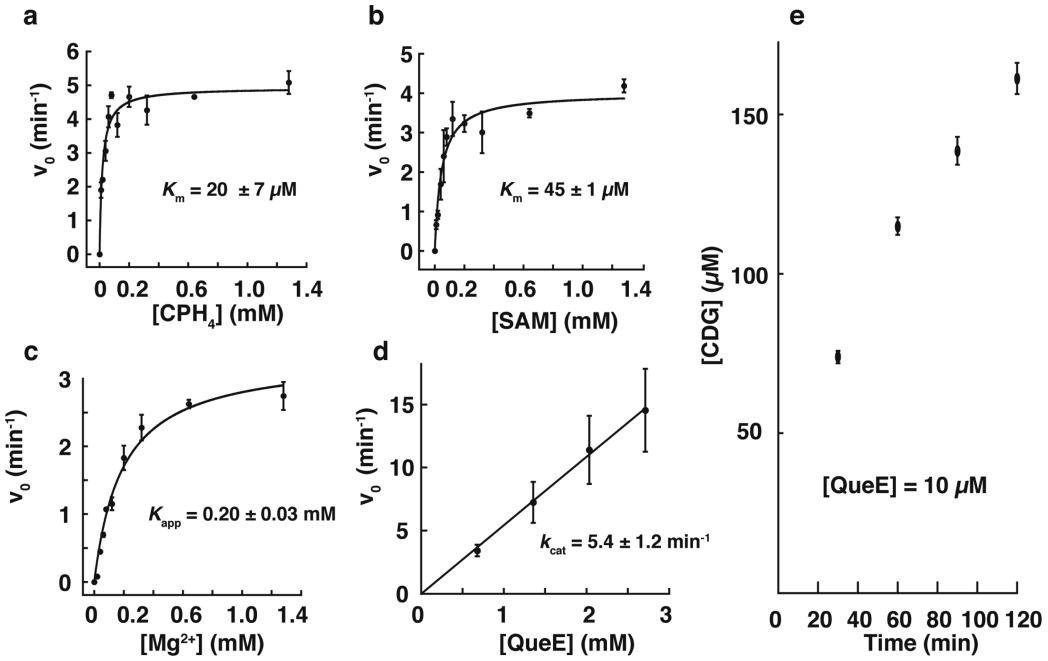

Figure 3. Initial rates (v_0) of CDG produced by QueE as a function of CPH_4 (a), SAM (b), or Mg^{2+} (c) concentration. Data are averages of three experiments, and curves were fit to the average values to yield K_m and K_{app}. (d) Initial rate (v_0) of CDG production as a function of QueE concentration. The results are an average of two experiments. (e) CDG concentrations observed in reaction mixtures that contained 10 μM QueE showing SAM is utilized catalytically. Data shown in panel e are an average of three experiments.

QueE is active only in the presence of SAM and sodium dithionite.[14] SAM is required as a source of the 5′-dAdo• radical, and sodium dithionite is required to reduce the [4Fe-4S] cluster to the +1 oxidation state (Figure 1c). The EPR spectrum of reconstituted QueE obtained in the presence of sodium dithionite and SAM revealed a paramagnetic species with g values of 2.003, 1.912, and 1.861 (Figure 2c). Interestingly, QueE is only EPR active in the presence of both sodium dithionite and SAM (Figure S4 of the Supporting Information). A similar result was observed with lysine 2,3-aminomutase,[40] and it was subsequently shown that the binding of SAM to the [4Fe-4S] cluster in that enzyme increases the reduction potential of the cluster from −479 ± 5 to −430 ± 2 mV.[41]

Steady-State Kinetic Analysis of QueE. Steady-state kinetic analysis of QueE was conducted to determine the steady-state kinetic parameters for the optimal concentrations of components to be included in the activity assays. In these experiments, *E. coli* FldA, FPR, and NADPH were utilized to reductively activate QueE. FldA and FPR are bacterial proteins that contain flavin mononucleotide and flavin adenine dinucleotide, respectively, and catalyze the in vivo transfer of electrons from NADPH to activate a number of enzymes, including anaerobic ribonucleotide reductase,[42] B_{12}-dependent methionine synthase,[34] pyruvate formate lyase activase,[43] biotin synthase,[44] lipoyl synthase,[33] lysine 2,3-aminomutase,[45] and AtsB.[46] Control experiments showed that dithionite at various concentrations [1−10 mM (data not shown)] also activated the protein; however, excess FldA, FPR, and NADPH:QueE consistently resulted in higher activities. Therefore, the biological reducing system was used in all subsequent experiments.

The steady-state kinetic data obtained with QueE showing the dependence of activity on either CPH_4, SAM, or $MgSO_4$ concentration while the other two components were kept at nearly saturating levels are shown in Figure 3a−c. These experiments reveal a K_m of 20 ± 7 μM for CPH_4 (Figure 3a). A K_{app} of 45 ± 1 μM was observed for SAM (Figure 3b). We note that the SAM used in this experiment was commercially obtained and contains a mixture of the biologically active (*S*,*S*) along with inactive (*R*,*S*) diastereomer. In addition, various additional contaminants, including methylthioadenosine and (*S*)-adenosylhomocysteine, may be present. Therefore, the actual K_{app} for the biologically relevant enantiomer may be even lower.

QueE requires magnesium metal ion for activity. Control experiments show that ∼11-fold more product is formed when $MgCl_2$ or $MgSO_4$ (1 mM each) was included in the reaction mixture. Steady-state measurements at saturating concentrations of the substrate and SAM indicate a K_{app} of ∼0.2 mM for magnesium (Figure 3c). To the best of our knowledge, while electron density consistent with Zn^{2+} was found in ThiC,[18] a magnesium divalent metal ion requirement has not been demonstrated in other radical SAM proteins.

The turnover number of the enzyme can be estimated under nearly saturating conditions of all assay components. Figure 3d shows the rate of formation of CDG as a function of QueE concentration. The data shown in Figure 3d are representative of the data generally obtained with this enzyme and show a k_{cat} of ∼5.4 ± 1.2 min^{-1}. Turnover numbers for QueE across various preparations generally range from 2.4 to 6.1 min^{-1}. This range of values is in line with turnover numbers observed with other radical SAM enzymes such as BtrN (2.3 ± 0.22 min^{-1}),[47] lipoate synthase (0.175 ± 0.10 min^{-1}),[33] pyruvate formate lyase activase (5 min^{-1}),[48] and DesII (1.0 ± 0.1 min^{-1}),[49] but lower than that observed with lysine 2,3-aminomutase (∼27 s^{-1}).[50]

QueE Utilizes SAM Catalytically. To determine if QueE utilizes SAM in a catalytic capacity or as a reactant, QueE was assayed in a reaction mixture containing a 100-fold excess of CPH_4 (1 mM) over SAM (10 μM), in the presence of 2.4 μM

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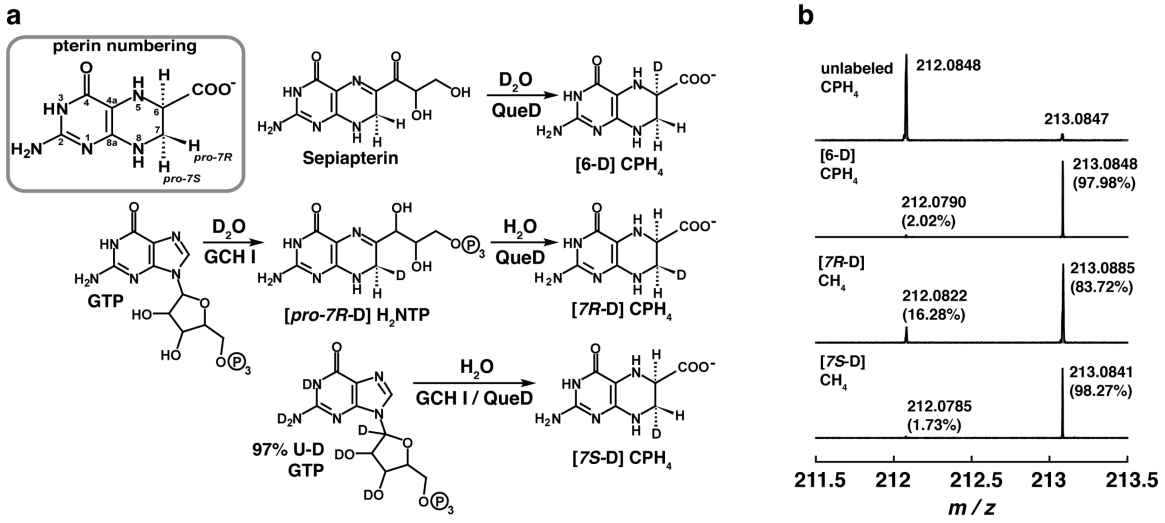

Figure 4. (a) Scheme for site-selective deuteration of CPH_4. (b) FTICR-MS spectra showing the relative amounts of deuterated vs nondeuterated CPH_4 obtained in the preparation of pure, site-specifically deuterated CPH_4.

QueE. As shown in Figure 3e, 150 μM CDG is produced, which is a 15-fold excess over the level of SAM present in the reaction mixture. Therefore, we conclude that SAM is regenerated at the end of each catalytic cycle and that QueE belongs to class I of radical SAM enzymes, which regenerate the cofactor at the end of each catalytic cycle.[25] This result, however, does not rule out the involvement of an enzyme-based radical that propagates the radical chain from 5′-dAdo$^\bullet$ to the substrate and back.

QueE Assays with Site-Specifically Deuterated CPH_4. Abstraction of a hydrogen atom from CPH_4 to initiate the catalytic cycle can occur at either C-6 or C-7, as has been previously proposed.[51] To probe the site of hydrogen atom abstraction on the substrate and gain insights into the mechanism of the reaction, isotope transfer experiments were conducted with three monodeuterated CPH_4 isotopomers that contain deuterium atoms at C-6 or C-7. The stereospecific deuteration reactions were accomplished enzymatically as described in Experimental Procedures and illustrated in Figure 4a. The starting compound for synthesis of deuterated analogues of CPH_4 with GCH I and QueD was GTP. Previous studies have shown that the conversion of GTP to H_2NTP by GCH I introduces a solvent-derived proton into the *pro*-7*R* position.[52] The stereoselectivity of the QueD reaction is not known; however, we presumed that it would be similar to its 6-pyruvoyltetrahydropterin synthase (PTPS) that places a solvent-derived proton on the *si* face of the substrate at C-6.[52] QueD is homologous to PTPS and retains the catalytic residues that have been shown to be required for PTPS activity, including the residues that coordinate a catalytic zinc metal ion.[53] Moreover, H_2NTP is an alternate substrate for QueD, which catalyzes its conversion to CPH_4. Additional alternate substrates for QueD include sepiapterin and 6-pyruvoyltetrahydropterin, all which are turned over to CPH_4.[13] Therefore, commercially available sepiapterin was used as the starting point for the enzymatic synthesis of [6-D]CPH_4. The three stereoselectively labeled CPH_4 isotopologs were prepared by using a combination of deuterated or unlabeled GTP in enzymatic transformations that were conducted in either H_2O or D_2O (see Figure 4a). The deuteration levels of resulting CPH_4 were determined by FT-ICR MS (Figure 4b) to be 97.98% for [6-D]CPH_4, 83.72% for [7*R*-D]CPH_4, and 98.27% for [7*S*-D]CPH_4.

Our hypothesis is that abstraction of a hydrogen atom from the substrate by 5′-dAdo$^\bullet$ initiates the conversion of CPH_4 to CDG. Therefore, QueE was incubated with unlabeled and deuterated substrates, and the deuterium contents of 5′-deoxyadenosine and the extent of deuterium remaining in the product were analyzed. In these experiments, QueE was incubated in the presence of excess unlabeled or deuterated CPH_4 (2 mM) for 20 min prior to the reactions being quenched and the mixtures analyzed by LC−MS for incorporation of deuterium into 5′-dAdo or CDG, respectively. While 5′-dAdo is not a product of the QueE reaction, some 5′-dAdo is present during turnover. Occasional abortive cleavage of SAM is another source of 5′-dAdo. Analysis of the extracted ion chromatograms from LC−MS runs shows that 5′-dAdo is clearly observable (m/z 252 in Figure S5 of the Supporting Information). Comparison of the mass envelopes for 5′-dAdo when unlabeled CPH_4 or [6-D]CPH_4 is incubated with QueE reveals multiple deuteriums in 5′-dAdo with the latter (see Figure 5). By contrast, deuterium from the 7*S* or 7*R* position of CPH_4 is never found in 5′-dAdo (compare MS traces for 5′-dAdo in Figure 5). Interestingly, however, deuterium is present in CDG (m/z 195) only when [7*S*-D]CPH_4 is used as the substrate (compare MS traces for CDG in Figure 5). Deuterium from [C-6-D]- or [7*R*-*D*]CPH_4 is never retained in the product. The retention of deuterium derived from [7*S*-D]CPH_4 is consistent with previous radiotracer feeding experiments showing that when [C-1-^{3}H]ribose was present in the growth medium, tritium was retained in toyocamycin.[54] In these experiments, the tritiated ribose would have been converted to [C-1′-^{3}H]GTP and subsequently to [7*S*-^{3}H]CPH_4 by the combined actions of GCH I and QueD.

The MS spectra of 5′-dAdo from the reaction mixtures containing [6-D]CPH_4 show clearly that 5′-dAdo is multiply deuterated. Under the conditions employed, 41% of the observed 5′-dAdo was monodeuterated, 37% was dideuterated, 14% was trideuterated, and 8% of the 5′-dAdo was not deuterated at all. The presence of multiply deuterated 5′-dAdo is consistent with SAM being used catalytically (Figure 3e). One would imagine that if the initial hydrogen atom abstraction is reversible and/or the productlike radical abstracts a hydrogen

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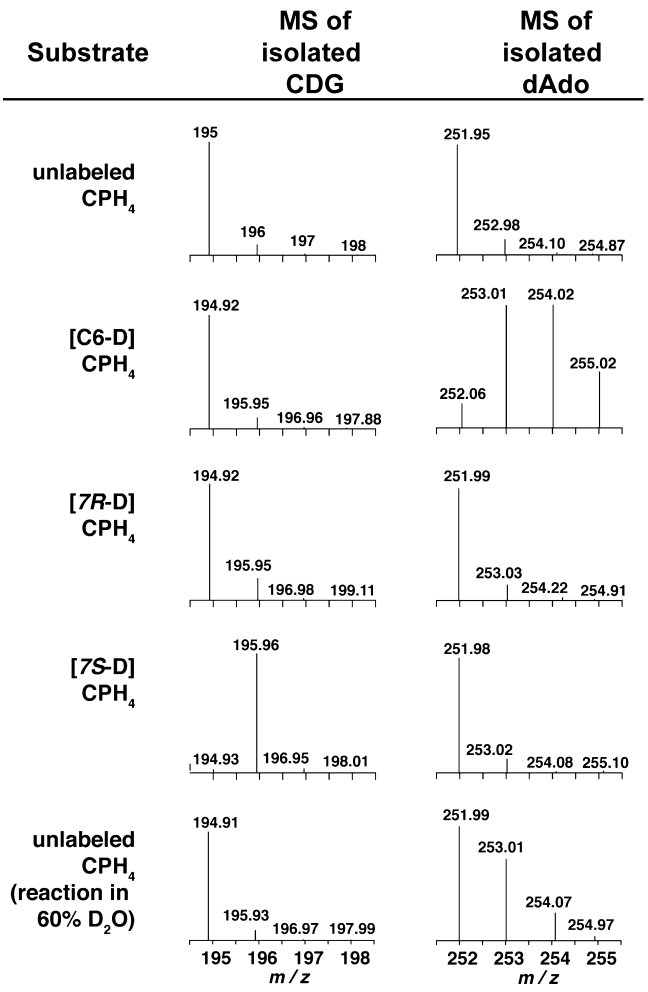

Figure 5. Mass spectra of CDG (m/z 195.0) and dAdo (m/z 252.1) isolated from turnover of QueE with unlabeled or various site-specifically deuterated CPH_4 substrates. An additional experiment was conducted with an unlabeled substrate in D_2O to determine if reductively cleaved SAM can be quenched by solvent or a solvent exchangeable site (see the text).

atom from the 5′-dAdo, a deuterium isotope effect for the transfer of the heavy deuterium isotope would discriminate in favor of protium transfer. At this time, it is not possible to confirm a steady-state kinetic isotope effect, as there is no measurable difference between the rates for conversion of saturating levels of unlabeled CPH_4 and [6-D]CPH_4 to CDG, suggesting that hydrogen atom abstraction may not be rate-limiting under V_{max} conditions (Figure S6 of the Supporting Information).

We were surprised to observe a pool of unlabeled 5′-dAdo in the reactions with the [6-D]CPH_4 because on the basis of MS analysis, unlabeled CPH_4 comprises at most 2% of the total substrate pool (Figure 4b). The 8% hydrogen incorporation may reflect in part an isotope effect that favors turnover with unlabeled substrate; in other words, the deuterated substrate is discriminated against. A second possibility, however, is that the protiated pool results from abortive reductive cleavage of the cofactor: 5′-dAdo$^{\bullet}$ formed under some conditions may abstract a hydrogen atom from a solvent exchangeable site. Klinman and co-workers, for example, have observed the incorporation of deuterium into 5′-dAdo in an abortive SAM cleavage reaction catalyzed by PqqE, a radical SAM enzyme involved in the biosynthesis of pyrroloquinoline quinone, when the reaction was conducted in D_2O.[55] To determine the contribution of such abortive cleavage reactions to the unlabeled 5′-dAdo pool, an additional QueE reaction was conducted with unlabeled CPH_4 in ∼60% D_2O (Figure 5). LC−MS analysis revealed that 33% of the 5′-dAdo was monodeuterated and 58% of the 5′-dAdo remained undeuterated. We also observed a small quantity of dideuterated species (∼8%), the source of which is not clear at present, but it may be from abortive reactions where there are back hydrogen atom transfers to a solvent exchangeable position. These results, nevertheless, are consistent with the notion that at least some (if not most) of the 8% unlabeled 5′-dAdo that we observe in the presence of [6-D]CPH_4 results from abstraction of a hydrogen atom from a solvent exchangeable pool.

Mechanism of QueE. A working model for the reaction catalyzed by QueE that is consistent with all biochemical and spectroscopic data to date is shown in Figure 6. The deuterium transfer experiments collectively support the model in which radical-mediated rearrangement of CPH_4 to CDG is initiated by direct abstraction of a hydrogen atom from C-6 of the substrate by 5′-dAdo$^{\bullet}$, which is formed by reductive cleavage of the SAM cofactor by the +1 oxidation state of the [4Fe-4S] cluster. The proposed C-6-centered radical is reminiscent of the α-Lys radical intermediate observed in the reaction catalyzed by LAM.[56,57] The substrate radical in QueE may be stabilized by delocalization of unpaired spin density onto the adjacent carboxylate group; in LAM, simulations of the EPR spectra of the radical indicated that 20% of the spin density is delocalized into the carboxylate moiety.[56] The CPH_4 radical can be stabilized further by captodative mechanisms. The glycyl radical in pyruvate formate lyase has been proposed to be stabilized by delocalization of the spin density at the α-carbon by combined electron withdrawing and donating effects of the its carbonyl group and the amide nitrogen, respectively.[58] The C-6-based radical is sandwiched between a carboxylate moiety (electron-withdrawing) and a nitrogen atom (electron-donating).

Several possible mechanisms can be proposed from the CPH_4 radical intermediate en route to CDG. One possibility is homolytic cleavage of the C−N bond, which would be followed by ring opening to form an imine in the proximity of the unpaired spin at the C4a position of the starting substrate. Such a homolytic cleavage would set up the molecule for a favorable Baldwin 5-*exo*-trig radical-mediated ring closure;[59] addition of C-centered radicals to imines is precedented.[60] An alternative mechanism involving an azacyclopropylcarbinyl radical can also be proposed; such an intermediate would be strictly analogous to the central radical intermediate in the reaction catalyzed by lysine 2,3-aminomutase.[61] In LAM, the intermediate can be stabilized by delocalization of the unpaired spin into the pyridoxal 5′-phosphate cofactor. In QueE, the unpaired spin may be further stabilized by delocalization. In either mechanism, a nitrogen-centered radical is formally shown to result. However, it is expected that it will be quickly quenched by the transfer of a hydrogen atom from 5′-dAdo to form 5′-dAdo$^{\bullet}$ and the initial 7-carboxy-7-amino product. 5′-dAdo$^{\bullet}$ would subsequently recombine with Met to re-form SAM and regenerate the +1 oxidation state of the cluster.

The conversion of the 7-carboxy-7-amino intermediate to CDG requires the loss of the amino group and aromatization of the five-membered ring. Loss of an unactivated amino group on the surface would seem to be difficult. However, we note that QueE activity is magnesium-dependent and that the metal ion

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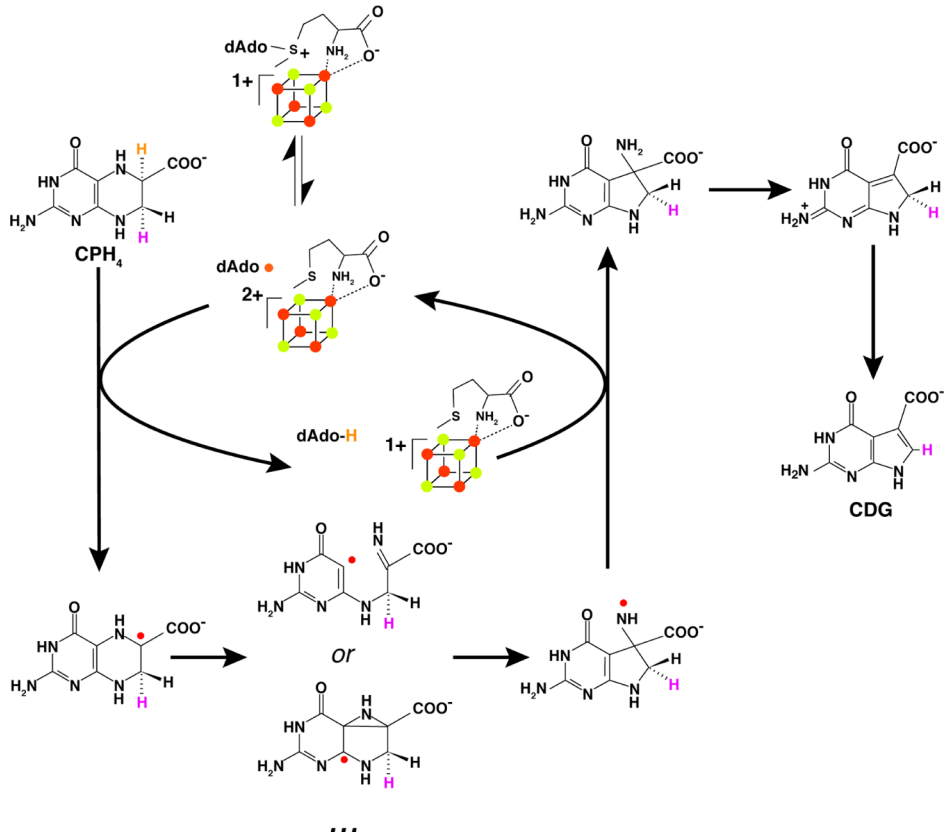

Figure 6. Reaction mechanism proposed for conversion of CPH_4 to CDG by QueE. Hydrogen bound to C-6 of CPH_4 (orange) is abstracted by 5′-dAdo$^{\bullet}$ to initiate the reaction. Homolytic cleavage of the C−N bond of the substrate leads to an imine intermediate, which is proposed to undergo 5-*exo*-trig ring closure to the new five-membered ring. Alternatively, the ring rearrangement may proceed via an azacyclopropylcarbinyl intermediate. The proposed nitrogen-centered radical is subsequently quenched by 5′-dAdo to generate 5′-dAdo, which subsequently combines with Met to re-form the cofactor. The exocyclic amino group is shown to facilitate the elimination and rearomatization reactions that are required to form CDG.

may have role in catalyzing the elimination. In addition, the loss of ammonia may be facilitated by the donation of an electron by the exocyclic amino group and/or the ring nitrogen atoms. For example, Figure 6 shows the use of the exocyclic amino group to eliminate ammonia followed by the general base-assisted abstraction of the *pro-R* proton from C-7 of the substrate to generate CDG.

The magnesium dependence observed for the QueE reaction was unexpected, and several roles may be envisioned for the divalent cation in the reaction. Mg^{2+} may coordinate the substrate carboxylate group leading to activation of the C-6 proton by inductive effects akin to the mechanisms proposed for exchange of acetate protons of EDTA in the presence of divalent metal ions.[62] Alternatively, as proposed above, it can interact with N-5 of the substrate to facilitate the ring contraction chemistry. At this stage, we cannot distinguish between these and other role(s) for the metal ion.

Summary. We have demonstrated that QueE is a member of the radical SAM family and that it catalyzes a complex radical-mediated ring contraction reaction. The isotope transfer experiments implicate direct abstraction of a hydrogen atom from the substrate by 5′-dAdo$^{\bullet}$ as setting the stage for the complex radical ring contraction chemistry that follows.

■ ASSOCIATED CONTENT

Supporting Information

Sequence of codon-optimized FPR (Figure S1), SDS−PAGE gel of purified QueE (Figure S2), spectroscopic characterization of ^{57}Fe-labeled QueE (Figure S3), EPR spectra of QueE obtained in the presence and absence of SAM and dithionite (Figure S4), extracted ion chromatograms of 5′-dAdo observed in QueE reactions (Figure S5), and comparison of CDG production in the presence of unlabeled and [6-D]CPH_4 (Figure S6). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

BSA, bovine serum albumin; CPH_4, 6-carboxy-5,6,7,8-tetrahydropterin; CDG, 7-carboxy-7-deazaguanine; DEAE, diethylaminoethyl; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; FT-ICR MS, Fourier transform ion cyclotron resonance mass spectrometry; FldA, flavodoxin; FPR, ferredoxin:NADP oxidoreductase; GCH I, GTP cyclohydrolase I; GTP, guanosine 5′-triphosphate; H_2NTP, 7,8-dihydroneopterin triphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; ICP-OES, inductively coupled plasma optical emission spectroscopy; IPTG, isopropyl *β*-1-thiogalactopyranoside; LC−MS, liquid chromatography−mass spectrometry; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; PIPES, 1,4-piperazinediethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; PTPS, 6-pyruvoyltetrahydropterin synthase; QueE, CDG synthase; QueD, CPH_4 synthase; SAM, *S*-adenosyl-L-methionine; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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