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QueE: A radical SAM enzyme involved in the biosynthesis of 7-deazapurine containing natural products

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Abstract

7-Carboxy-7-deazaguanine (CDG) is a common intermediate in the biosynthesis of 7-deazapurine-containing natural products. The biosynthesis of CDG from GTP requires three enzymes: GTP cyclohydrolase I, 6-carboxy-5,6,7,8-tetrahydropterin (CPH₄) synthase, and CDG synthase (QueE). QueE is a member of the radical *S*-adenosyl-L-methionine (SAM) superfamily and catalyzes the SAM-dependent radical-mediated ring contraction of CPH₄ to generate CDG. This chapter focuses on methods to reconstitute the activity of QueE *in vitro*.

1. Introduction

Pyrrorolopyrimidine moieties are components of structurally diverse group of natural products that share a 7-deazapurine core. These compounds are found in all kingdoms of life and their function(s) range from secondary metabolites produced by *Actinomyces*, to the modified bases, queuosine and archaeosine, in tRNA [see (McCarty & Bandarian, 2012) for review]. The first example of a deazapurine, toyocamycin, was isolated on the basis of its biological activity in 1956 (Nishimura, Katagiri, Sato, Mayama, & Shimaoka, 1956). To date, >30 compounds with deazapurine cores have been observed in Nature (McCarty et al, 2012).

Prior to elucidation of the biosynthetic pathway to deazapurines, radiotracer isotope experiments had demonstrated that deazapurines were derived from purines in a pathway that retains some of the carbons of the sugar, and C-2 from the base, but removes C-8 (Buff & Dairman, 1975; Burg & Brown, 1968; Elstner & Suhadolnik, 1971; Elstner, Suhadolnik, Suh, 1975; Guroff & Strenkoski, 1966; Kuchino, Kasai, Nihei, & Nishimura, 1976; Reynolds & Brown, 1962; 1964; Smulson & Suhadolnik, 1967; Suhadolnik & Uematsu, 1970; Uematsu & Suhadolnik, 1970). Despite the structural diversity, it now appears the deazapurine moiety is biosynthesized in three steps from guanosine triphosphate (GTP) (McCarty et al, 2012; McCarty, Krebs, & Bandarian, 2012; McCarty, Somogyi, & Bandarian, 2009a; McCarty, Somogyi, Lin, Jacobsen, & Bandarian, 2009b; Miles, Roberts, McCarty, & Bandarian, 2014; Reader, Metzgar, Schimmel, & de Crécy-Lagard, 2004), and modified as appropriate by additional species-specific tailoring enzymes (McCarty et al, 2012; Bai, Fox, Lacy, Van Lanen, & Iwata-Reuyl, 2000; Dowling et al., 2016; Frey, McCloskey, Kersten, & Kersten, 1988; Lee, Van Lanen, & Iwata-Reuyl, 2007; Miles, McCarty, Molnar, & Bandarian, 2011; Miles, Myers, Kincannon, Britt, & Bandarian, 2015;

Nelp & Bandarian, 2015; Nelp, Astashkin, Breci, McCarty, & Bandarian, 2014; Nelp, Song, Wysocki, & Bandarian, 2016; Okada, Harada, & Nishimura, 1976; Okada et al., 1979; Phillips et al., 2008; Reuter, Slany, Ullrich, & Kersten, 1991; Shindo-Okada, Okada, Ohgi, Goto, & Nishimura, 1980; Song, Nelp, Bandarian, & Wysocki, 2015; Van Lanen et al., 2005; Watanabe et al., 1997) (Fig. 1). In the first step of the reaction GTP cyclohydrolase I (GCH I) converts GTP to 7,8-dihydroneopterin triphosphate (H_2NTP), which is subsequently modified to 6-carboxy-5,6,7,8-tetrahydropterin (CPH_4) by CPH_4 synthase (QueD), prior to undergoing a ring contraction to form 7-carboxy-7-deazaguanine (CDG) catalyzed by CDG synthase (McCarty et al, 2012; McCarty et al, 2009a; McCarty et al, 2009b; Phillips et al., 2008). CDG likely serves as the starting point for all known deazapurines.

QueE catalyzes the radical-mediated ring contraction of CPH_4 to form CDG (McCarty et al., 2009a). It is a member of the radical *S*-adenosyl-L-methionine (SAM) enzymes, which were initially identified on the basis of a conserved CxxxCxxC motif (Sofia, 2001; Frey, Hegeman, & Ruzicka, 2008). The Cys sidechains in the conserved motif coordinate three iron atoms of a site-differentiated 4Fe-4S cluster. The fourth iron to binds SAM through its amino and carboxylate moieties (Berkovitch et al., 2003; Walsby, Ortillo, Broderick, Broderick, & Hoffman, 2002; Walsby et al., 2005). In the +1 oxidation state, the iron-sulfur cluster reductively cleaves SAM to generate 5'-deoxyadenosyl radical (5'-dAdo•), which initiates chemistry by H-atom abstraction in most radical SAM enzymes (Moss & Frey, 2001). Recent bioinformatic studies suggest that radical SAM enzymes constitute a wide superfamily of >100,000 members (S. Brown & Babbitt, 2015).

In QueE, the reversible cleavage of SAM generates 5'-dAdo• which initiates ring contraction in CPH_4 is by abstracting a H-atom from C-6 of the substrate (Fig. 3) (McCarty et al., 2012). The substrate radical is thought to rearrange to a product radical that is quenched by H-atom abstraction from dAdo. Stereospecific abstraction of the C-7 *proR* hydrogen and elimination of the amino group complete the catalytic cycle and reforming SAM. In addition to detailed biochemical studies, QueE has been quite amenable to structural studies. Multiple high resolution crystal structures of the protein provide detailed snapshots of the active site of the protein in the $\pm$ various ligands, including the cofactor, substrate, and substrate analogs (Bruender et al., 2017; Bruender, Young, & Bandarian, 2015; McCarty et al., 2012; McCarty et al, 2009b; Wilcoxon, Bruender, Bandarian, & Britt, 2018) (Fig. 4).

Studies with QueE require access to a number of reagents. As with other radical SAM enzymes, QueE activity requires the presence of a means reducing the 4Fe-4S cluster to the catalytically active +1 state (Fig. 2 and Fig. 5). Dithionite is often used in the lab, but it has been demonstrated that higher activities are achieved with biological reducing systems, such as flavodoxin (Fld)/flavodoxin reductase (FPR)/NADPH (Bruender et al., 2015). However, it is also possible to bypass the FPR/NADPH and use dithionite-reduced Fld to achieve efficient reduction of the enzyme. In addition to a source of reductant, the substrate, CPH_4 , is not commercially available and must be obtained by enzymatic synthesis from GTP using the biosynthetic enzymes. This chapter will focus on the methods to obtain each of these components to reconstitute *in vitro* activity of recombinant QueE. We note that while the foregoing discussion will focus on the *B. subtilis* enzyme, the methods have also been

applied successfully to the *B. multivorans* protein as well (Dowling et al., 2013) and are therefore general to QueE proteins.

2. Expression and Purification of QueE

The 4Fe-4S cluster that is required in all RS enzymes is often oxygen sensitive, requiring the enzymes to be purified and studied under strictly anaerobic conditions. Since QueE is highly overexpressed in *E. coli* hosts, a single step purification under anoxic conditions, using the His₆ affinity tag step suffices to purify sufficient protein for biochemical, structural, and spectroscopic studies.

2.1. Expression of QueE

The gene of *queE* was amplified from genomic DNA of *B. subtilis* (ATCC 23857D) and ligated into pET28a using the *Nde*I and *Bam*HI restriction sites to make the plasmid pRM78 (McCarty et al., 2009b). The pET28a plasmid encodes kanamycin (Kan) resistance and expression is induced with isopropyl β-D-1-thiogalactopyranoside (IPTG). We find that it is necessary to express *queE* with pDB1282, which contains the genes *iscSUA-hscB-hscA-fdx-orf3* from *Azobacter vinelandii* that are involved in assembly of the iron-sulfur clusters in nitrogenase, to enhance cluster content (Frazzon, Fick, & Dean, 2009). The pDB1282 plasmid contains ampicillin (Amp) resistance and the expression of the *isc* operon is induced with arabinose.

Protocol for Expression of *queE* gene

1. Co-transform BL21(DE3) *E. coli* cells with plasmids pRM78 and pDB1282. Plate cells on LB agar plates containing 100 µg/mL Amp and 34 µg/mL Kan.
2. Prepare the LB media in 2.8 L Fernbach flasks by dissolving 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl in 1 L of deionized water. Autoclave 30 min and cool to room temperature before use.
3. Select a single colony from the transformation plate and inoculate 100 mL LB media containing both 100 µg/mL Amp and 34 µg/mL Kan (see step 1). Grow the starter culture at 37 °C overnight with shaking at 200 rpm.
4. Use the overnight culture to inoculate the expression cultures. Add 10 mL of overnight culture to the 1 L of fresh LB in 2.8 L Fernbach flasks supplemented with 100 µg/mL Amp and 34 µg/mL Kan. Allow the cells to grow at 37 °C with shaking at 175 rpm to an optical density at 600 nm (OD₆₀₀) of ~0.3 and induce expression of the *isc* genes from the pDB1282 plasmid by addition of 0.5 g/L of solid arabinose. Also add 50 µM (final concentration) FeCl₃ to ensure that enough iron is present for reconstitution of the cluster. Incubate the cultures with continued shaking at 37 °C to OD₆₀₀ of ~0.5 prior to inducing expression of QueE with IPTG (100 µM final concentration). Shake the cultures at 120 rpm for 6 h after inducing expression of QueE.
5. Harvest the cells at 5,000 *xg*, flash freeze in liquid nitrogen, and store at -80 °C until use.

2.2. Purification of QueE

Purification of QueE should be performed in an anaerobic chamber under a nitrogen atmosphere. We use a Coy chamber, which typically has an atmosphere composed of 97% nitrogen and 3% hydrogen. Anaerobicity is achieved by allowing H₂ to react with O₂ on the surface of the palladium catalyst within the chamber to generate water. Excess moisture in the chamber is removed by both dessicant packs and a Coy dehumidifier. The anaerobicity of the chamber is monitored by a Coy cam-12 detector. The chamber is in a laboratory which is maintained at 22 °C. Materials to be introduced into the chamber pass through an antechamber and made anaerobic by repeated cycling between vacuum and either N₂ or a 95% N₂/5% H₂ gas mix. We have modified the default cycle program for the antechamber so that it is vacuumed to 19.9 in Hg and refilled with N₂ four times prior to final round of evacuation and filling with mixed gas (95% N₂/5% H₂). To facilitate purifications steps, we have placed an ÄKTAprime plus inside the chamber. All tubes, tips, and other plasticware are placed in the glovebox at least 24 h prior to use. Water and concentrated buffer stocks are prepared aerobically and stirred in the glovebox for 12 h to exchange the gas. Cell disruption is carried out on ice and the centrifugation steps are at 4 °C. Cells are disrupted by a Branson sonifier as indicated below. We maintain the horn of the sonifier in the glove box and attach it to a controller outside the glove box.

Protocol for Purification of QueE

1. Place 5–10 g of frozen cell pellet in a stainless steel beaker with a stir bar and 1 mM phenylmethane sulfonyl fluoride (PMSF) in the middle of a crystallization dish filled with ice and cycle into the glove box.
2. Add deoxygenated lysis solution (4 mL/g of wet cell paste) containing 0.05 M piperazine N,N'-bis(2-ethanesulfonic acid) (PIPES-NaOH) (pH 7.4), 0.5 M KCl, 0.05 M imidazole, and 10 mM dithiothreitol (DTT).
3. While the suspension is stirred, lyse cells a total of 10 min, with 15 sec bursts at 50% power that are followed by 45 sec rests to allow the solution to chill in between.
4. Transfer the lysate into an Oakridge centrifuge tube with O-rings, tightly seal the tube inside the glove box, and remove from the chamber.
5. Centrifuge at 18,500 *xg* for 35 min at 4 °C to pellet cell debris.
6. Charge a 5 mL HisTrapHP column (GE Healthcare) with NiSO₄ according to manufacturer's directions and equilibrate with lysis solution (step 2).
7. Transfer the centrifuge tube into the anaerobic chamber and carefully decant the supernatant.
8. Using a 50 mL SuperLoop, load the protein onto the column. Wash with lysis solution (step 2) until the UV trace returns to baseline (~50 mL). Elute the protein with a 50 mL gradient from 0.05 M – 0.5 M imidazole in lysis buffer.

9. Pool the dark brown fractions containing QueE and exchange into a solution containing 0.05 M PIPES-NaOH (pH 7.4) and 10 mM DTT using a BioRad 10DG desalting column.
10. Concentrate to a volume of ~5–10 mL using an Amicon concentrator under N₂ with a YM-10 membrane (Millipore). N₂ can be brought into the glove box through a tube that is fed through a side nipple on the Coy chamber. Freeze the resulting protein in small aliquots in liquid nitrogen and store at –80 °C. A typical SDS-PAGE of purified QueE is shown in Fig. 6.
11. Quantify the QueE stock by a Bradford assay (BioRad) with BSA as standard. We have empirically determined a Bradford concentration correction factor of 0.67 for QueE, which is based on the absolute amino acid content (McCarty et al., 2012).

2.3. Chemical Reconstitution of QueE

While as purified QueE has some intact 4Fe-4S cluster, it is necessary to carry out a chemical reconstitution of QueE under anaerobic conditions to obtain cofactor-replete enzyme. We have empirically determined that efficient reconstitution can be achieved when QueE is incubated with 8 molar excess of iron (FeCl₃) and sulfide (Na₂S) as described below.

1. Weigh out ~20 mg of Na₂S and FeCl₃ into separate glass scintillation vials. Cap, and cycle into the anaerobic chamber.
2. Dissolve the Na₂S in water and the FeCl₃ in saturated sodium bicarbonate to a final concentration of 100 mM.
3. Add 8 molar equivalents of FeCl₃ and Na₂S to the protein based on its corrected concentration determined by the Bradford assay (Section 2.2, step 11). Dropwise add the FeCl₃ and allow it to stir for about 3 min before adding the Na₂S.
4. Allow the reconstitution to proceed at room temperature for 4 h after Na₂S has been added.
5. At the end of the 4 h, the solution often has some precipitate. Remove the precipitate by centrifugation prior to desalting with a BioRad 10DG desalting column into solution of 0.05 M PIPES-NaOH (pH 7.4) containing 10 mM DTT.
6. Concentrate the desalted QueE using an Amicon concentrator under N₂ with a YM-10 membrane (Millipore) to a minimal volume (~1 mL).
7. Freeze the protein in liquid nitrogen as small aliquots and store at –80 °C. Typical yield from this procedure is ~150 mg of QueE from 5 g of wet cell paste.
8. As needed, QueE can be further purified by gel filtration on a HiPrep 16/60 Sephacryl S-200 high resolution column (GE Healthcare). Equilibrate the column with solution containing 0.05 M PIPES-NaOH (pH 7.4), 0.15 M NaCl and 10 mM DTT. We usually calibrate the column with chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), bovine serum albumin (BSA) (67 kDa), and aldolase (158 kDa).

9. Inject 1 mL of concentrated QueE onto the equilibrated column and collect fractions while washing with the loading buffer.
10. Analyze the fractions with an SDS-PAGE gel and compare to standards to determine the oligomeric state. We only elute fractions that correspond to dimeric QueE.
11. The fractions containing QueE are pooled and concentrated in the same manner as in step 6. The concentrated QueE is then aliquoted, flash frozen in liquid nitrogen, and stored at -80°C until use.

3. Expression and Purification of YkuN

The reduction of the FeS cluster in QueE to initiate the catalytic cycle can be accomplished by a number of methods (see Fig. 5). The simplest route is to use a strong reductant, such as sodium dithionite. However, we have shown that the biological reducing system, such as flavodoxin/flavodoxin reductase is a better choice for QueE, as significantly improves the activity (Bruender et al., 2015). We generally use the *B. subtilis* flavodoxin homolog, YkuN, in our assays to pass electrons to QueE either from dithionite, or flavodoxin reductase and NADPH.

3.1. Expression of *B. subtilis* flavodoxin YkuN

The gene *ykuN* was amplified by PCR from *B. subtilis* subsp. *subtilis* str. 168. The gene was A-tailed and ligated into the pGEM Easy-T vector (Promega) for blue and white screening. The gene was then moved to the pET29a expression vector to generate pNB466 (Bruender et al., 2015).

Protocol for expression of *B. subtilis* flavodoxin YkuN

1. BL-21 (DE3) *E. coli* cells were transformed with pNB466 and plated onto LB agar plates containing 34 $\mu\text{g/mL}$ Kan. Incubate at 37°C overnight.
2. Pick a colony from the LB agar plate to inoculate a 100 mL overnight starter culture containing 34 $\mu\text{g/mL}$ Kan. Incubate at 37°C overnight with shaking at 200 rpm.
3. Add 10 mL of the overnight culture to 1 L of fresh LB broth in 2.8 L Fernbach flasks containing 1 L LB each and supplemented with 34 $\mu\text{g/mL}$ Kan. Incubate at 37°C with shaking at 175 rpm until they reach $\text{OD}_{600}\sim 0.5$.
4. Induce expression of *ykuN* with 0.1 mM IPTG (final concentration) and add riboflavin to a final concentration of 0.1 mM at the time of induction to ensure that cofactor replete protein is obtained. Allow cells to express the protein at 37°C overnight while shaking at 175 rpm.
5. Collect the cells after ~ 18 h by centrifugation at 5,000 $\times g$ for 15 min at 4°C .
6. Flash freeze wet cell pastes in liquid nitrogen and store at -80°C until use.

3.2. Purification of YkuN

The expression vector for YkuN produces native protein, which is readily purified by successive anion exchange and hydrophobic interactions chromatographic steps as described below.

Protocol for the purification of YkuN

1. In a stainless steel beaker with a stir bar, add 40 g of frozen cell paste and solution (4 mL/g of wet cell paste) containing 0.05 M Tris-HCl (pH 7.2), 1 mM EDTA, 2 mM DTT, 1 mM PMSF. Place the beaker in the middle of a crystallization dish filled with ice.
2. While the suspension is stirred, lyse cells with a Branson digital sonifier for total of 10 min, with 15 sec bursts at 50% power that are followed by 45 sec rests to allow the solution to chill in between.
3. Centrifuge at 18,000 g for 35 min to remove cell debris.
4. Equilibrate a DEAE-Sepharose column (GE Healthcare) (11 $\times$ 3 cm) with 3 column volumes of the lysis solution (0.05 M Tris-HCl (pH 7.2), 1 mM EDTA, 2 mM DTT) on an ÄKTAprime FPLC.
5. Load the cleared lysate via a 50 mL SuperLoop onto the column. Wash the column with the loading buffer until the UV absorbance is at the baseline. Elute YkuN with a 0.85 L linear gradient 0 –1 M KCl in lysis solution.
6. Pool fractions containing YkuN on the basis of SDS-PAGE analysis of the yellow fractions (Flavodoxin (YkuN) binds FMN which is a yellow chromophore).
7. Slowly add an equal volume of 50 mM Tris-HCl (pH 7.0) containing 3 M ammonium sulfate and 2 mM DTT to the pooled YkuN fractions over 30 min.
8. Equilibrate a butyl-Sepharose column (GE Healthcare) (11 $\times$ 3 cm) with solution containing 0.05 M Tris-HCl (pH 7.0), 1.5 M ammonium sulfate, and 2 mM DTT on an ÄKTAprime FPLC.
9. Using a 50 mL SuperLoop load YkuN onto the column and wash with the loading buffer until the UV absorbance returns to baseline. Elute the protein with a 0.7 L linear gradient from the 1.5M –0 M ammonium sulfate in loading solution.
10. Identify YkuN-containing fractions by SDS-PAGE gel of yellow fractions.
11. Concentrate the protein to ~ 1 mL using Amicon concentrator under N_2 with a YM-10 membrane (Millipore). Freeze YkuN in small aliquots in liquid nitrogen and store at -80°C until use. See Fig. 6 for representative SDS-PAGE of purified YkuN.

4. Expression and Purification of flavodoxin reductase (FPR)

FPR is used in tandem with YkuN to biologically reduce the [4Fe-4S] cluster of QueE by shuttling electrons that are ultimately derived from NADPH to QueE. This section describes expression and purification of FPR.

4.1. Expression of the *fpr* gene

The gene for *E. coli fpr* was synthesized in codon optimized form (Genscript), and cloned into *Hind*III and *Nde*I restriction sites of pET28a for expression of His₆ tagged protein. The sequence for the synthetic gene and the corresponding protein is shown in Fig. 6.

Protocol for *fpr* gene expression

1. Transform HMS174(DE3) *E. coli* cells with the *fpr* expression plasmid and plate onto an LB agar plate containing 34 µg/mL kanamycin. Put the LB agar plate at 37 °C overnight.
2. Select a colony from plate to inoculate a 0.1 L starter culture containing 34 µg/mL kanamycin and incubate at 37 °C overnight with shaking at 200 rpm.
3. Inoculate six 2.8 L Fernbach flasks containing 1 L LB each with 34 µg/mL Kan with 10 mL of overnight culture. Shake the cultures at 175 rpm at 37 °C.
4. At an OD₆₀₀~0.5 add 0.1 mM (final concentration) IPTG to induce expression. As with YkuN, add 0.1 mM riboflavin to ensure that cofactor replete protein is obtained.
5. Harvest the cells after ~ 6 h by centrifugation at 5,000 *xg* for 15 min at 4 °C.
6. Flash freeze the cell paste in liquid nitrogen and store at –80 °C until use.

4.2. Purification of FPR

FPR is produced as a His₆ tagged protein, which simplifies purification using Ni-NTA affinity chromatography. FPR is a flavoprotein and fractions containing the enzyme are readily identified by the bright yellow color due to the presence of the flavin chromophore and by SDS-PAGE.

Protocol for FPR purification

1. Place ~5 g of cell paste and a stir bar in a stainless steel beaker. Add lysis buffer containing 0.02 M KPi (pH 7.2) and 0.5 M NaCl at a ratio of ~4 mL buffer per g of cell paste. Add solid PMSF to obtain 1 mM final concentration. Put the beaker in the center of a recrystallization dish filled with ice.
2. Sonicate the cells with 48 bursts of 15 sec each at 50% power for 12 min with 45 sec rest to minimize heating of the sample.
3. Transfer the lysate to Oakridge tubes and centrifuge at 18,000 *xg* for 35 min.

4. Charge a 1 mL His trap column (GE Healthcare) with NiSO₄ according to manufacturer's protocol and equilibrate a 1 mL His-trap (GE Healthcare) with lysis buffer on an ÄKTAprime FPLC.
5. Load the clarified lysate onto the column using a 50 mL SuperLoop.
6. Wash the column with ~ 5 mL of lysis buffer until the UV absorbance is near baseline and stable.
7. Elute FPR with a 5 mL linear gradient in lysis buffer from 0.05 to 0.5 M imidazole. Pool the yellow fractions containing FPR.
8. Dialyze the pooled fraction against 4 L of 0.02 M HEPES-NaOH (pH 7.4) at 4 °C for ~16 h. Change the dialysis buffer twice.
9. Concentrate the protein with Amicon concentrator with a YM-10 membrane (Millipore) under N₂. Use liquid nitrogen to flash freeze the protein aliquots and store at -80 °C until use. See Fig. 6 for representative SDS-PAGE of purified YkuN.

5. Enzymatic synthesis of CPH₄ with GCH I and QueD from GTP

The substrate for QueE, CPH₄, is not commercially available but can be synthesized enzymatically using GCH I and QueD (see Fig. 1). In this section we describe protocols for preparation of GCH I and QueD, and the enzymatic preparation of CPH₄ from GTP. These methods can be adapted to synthesis of deuterated isotopomers of CPH₄ with label at *C7proR*, or *C7proS* (McCarty et al., 2012) (Fig. 8). The [7*R*-D] isotope of CPH₄ is generated by completing the first step of the reaction of GTP with GCH I in D₂O, followed by QueD in H₂O. The [7*S*-D] isotope of CPH₄ is made using 97% [U-D] GTP (Cambridge Isotope Laboratories, Inc.) as the starting material with the GCH I and QueD reactions run in H₂O. The C6 labeled isotope is obtained by reacting sepiapterin (Sigma), with QueD in D₂O. Fig. 8 summarizes the strategies for enzymatic synthesis of isotopologs of CPH₄.

5.1. Expression and purification of GCH I

The gene, for GCH I (*folE*) was amplified from the *E. coli* W3110 genomic DNA and cloned into the *Hind*III and *Nde*I site of pET28a to generate pJB163. GCH I is purified via anion exchange chromatography and hydrophobic interaction chromatography.

Protocol for expression of GCH I

1. Transform BL21 (DE3) *E. coli* cells with pJB163 and plate onto an LB agar plate containing 34 µg/mL Kan. Incubate the plate at 37 °C overnight.
2. Select a colony from the LB agar plate and use the colony to inoculate a 0.1 L starter culture containing 34 µg/mL Kan. Allow the culture to grow at 37 °C overnight.
3. Use 10 mL of starter culture to inoculate six 2.8 L Fernbach flasks, each having 1 L of LB with 34 µg/mL Kan and 100 µM ZnSO₄. Grow at 37 °C and 200 rpm.
4. At an OD₆₀₀~0.7 induce the cells with 100 µM IPTG.

5. Harvest the cells after 4 h of growth by centrifugation at 5,000 $\times g$ at 4 °C. Flash freeze the cell paste in liquid nitrogen and store at –80 °C until use.

Protocol for purification of GCH I

1. GCH I is purified at 4 °C under aerobic conditions using an ÄKTA FPLC.
2. Weigh out ~10 g of cell paste and place in a stainless steel beaker with a stir bar. Dissolve in lysis buffer (4 mL/g) containing 0.02 M Tris-HCl (pH 8.0), 10 mM DTT and supplemented with 1 mM PMSF. Place beaker in the middle of a recrystallization dish packed with ice.
3. While stirring the cells, sonicate using a Branson digital sonifier with 40 bursts at 50% power for 15 sec, followed by 45 sec of rest to avoid heating the sample.
4. Pour the cell lysate into Oakridge tubes and centrifuge for 1 h at 18,000 $\times g$ and 4 °C.
5. Equilibrate a Q-Sepharose column (GE Healthcare) (2.6×14.5 cm) with ~ 3 column volumes of the lysis buffer.
6. Use a SuperLoop to inject the clarified lysate onto the column.
7. Wash the column with ~2 column volumes of loading buffer until the UV trace returns to near baseline.
8. Elute the protein with 0.85 L linear gradient from 0 –0.5 M KCl in loading buffer.
9. Identify the fractions containing GCH I by SDS-PAGE.
10. Pool the fractions containing GCH I and concentrate to ~50 mL using an Amicon concentrator with a YM-10 membrane (Millipore) under N₂.
11. Equilibrate a butyl-Sepharose column (GE Healthcare) (11 × 3 cm) with loading buffer containing 0.02 M Tris-HCl (pH 8.0), 1 M ammonium sulfate, and 10 mM DTT.
12. To the pooled fractions of GCH I from step 9 add an equal volume of buffer containing 20 mM Tris-HCl (pH 8.0), 2 M ammonium sulfate, and 10 mM DTT. This solution should be added slowly over a period of 30 min while the pooled fractions are stirring on a stir plate.
13. Use a SuperLoop to inject the fractions of GCH I onto the column. After loading, wash the column with the loading buffer until the UV trace has returned to baseline.
14. Elute the protein over a 0.7 L linear from 1 to 0 M ammonium sulfate in loading solution.
15. Identify and pool fractions containing GCH I by SDS-PAGE.

16. Dialyze the GCH I fractions against 4 L of buffer containing 20 mM Tris-HCl (pH 8.0) and 10 mM DTT at 4 °C for ~16 h. Dialyze against fresh buffer a second time.
17. Concentrate the GCH I to a minimal volume using an Amicon concentrator under N₂ gas with a YM-10 membrane (Millipore).
18. Aliquot the protein and flash freeze with liquid nitrogen. Store at –80°C until use.

5.2. Expression and purification of QueD

Native QueD from *E. coli* is expressed from a pET29 based plasmid with *ygcM* (b2765) cloned between *Hind*III and *Nde*I sites (McCarty et al, 2009a). The gene, *ygcM*, was PCR amplified from *E. coli* W3110 chromosomal DNA.

Protocol for expression of *queD* gene

1. Transform BL21(DE3) with vector containing *E. coli* QueD gene and incubate on a LB plate containing 34 µg/mL Kan at 37 °C overnight.
2. Inoculate 6 L of LB media containing 34 µg/mL Kan with 10 mL from a starter culture prepared from a single colony picked from the transformation plate.
3. Grow at 37 °C and 200 rpm to OD₆₀₀~0.5. Induce the cells with 0.1 mM IPTG. Since QueD requires Zn²⁺ for activity, supplement the cultures with 100 µL of 1 M ZnSO₄ (dissolved in water) to give a final concentration of 100 µM.
4. Continue to allow the cells to grow at 37 °C for 4 h after induction.
5. Collect cells by centrifugation at 5,000 *xg* at 4 °C. Use liquid nitrogen to flash freeze the cell paste and store at –80°C until use.

Purification of QueD—QueD is purified by a combination of anion exchange and hydrophobic interaction chromatographic steps as follows. All purification steps are carried out 4 °C under aerobic conditions.

1. In a stainless steel beaker containing a stir bar suspend ~10 g of cell paste in 40 mL of loading buffer containing 0.02 M Tris-HCl (pH 8) and 10 mM DTT, and supplemented with 1 mM PMSF. Place the beaker in a recrystallization dish packed with ice.
2. Lyse the cells by sonication using a Branson digital sonifier at with 48 bursts at 50% amplitude 45 sec rests to allow the mixture to remain cold.
3. Clear the lysate by centrifugation at 18,000 *xg* for 35 min at 4 °C.
4. Equilibrate a Q-Sepharose column (GE Healthcare) (2.6×14.5 cm) with loading buffer. Load the cleared lysate onto the column, wash with ~3 column volumes of the loading buffer and elute with a 0.8 L linear gradient from 0 –0.5 M NaCl in the loading buffer.

5. Identify fractions containing QueD by SDS-PAGE analysis. Pool the fractions and slowly add solid ammonium sulfate to 1 M saturation over 20 min while stirring.
6. Equilibrate a butyl sepharose CL-4B column (GE Healthcare) (2.6×14.5 cm) with a butyl loading buffer containing 0.02 M Tris•HCl (pH 8), 1 M ammonium sulfate, and 10 mM DTT.
7. Load the QueD onto the column, wash with ~2 column volumes of the loading buffer and elute with a 0.7 L linear gradient from 1M-0 ammonium sulfate in loading buffer.
8. Identify fractions containing QueD by SDS-PAGE analysis, pool, and concentrate in an Amicon pressure concentration device fitted with a YM-10 membrane (Millipore).
9. Dialyze the protein against 4 L of 0.02 M HEPES-NaOH (pH 7.5) at 4°C for ~16 h with one buffer change.
10. The protein can be concentrated as necessary by Amicon filtration as before.
11. Freeze the protein in small aliquots in liquid nitrogen and store at –80 °C.

5.4. Enzymatic synthesis of CPH₄

CPH₄ is synthesized by the combined action of GCH I and QueD from GTP. The resulting CPH₄ is readily purified by anion exchange chromatography on DEAE-Sephacrose. CPH₄ is light and oxygen sensitive. Therefore, the synthesis and purification of CPH₄ are carried out inside a Coy anaerobic chamber (5% H₂/95% N₂) and in the dark.

1. Set up a synthesis mixture that contains 0.05 M Tris-HCl (pH 8.0), 10 mM DTT, 0.5 mM GTP, 20 μM GCH I, and 20 μM QueD in a total volume of 20 mL. Stir the reaction 12 h under anaerobic conditions. The flask is kept dark by covering in foil.
2. Equilibrate a DEAE-Sephacrose (11 × 3 cm) column (GE Healthcare) with 10 mM ammonium bicarbonate. The column and the fractions are also kept dark by covering in foil.
3. Load the reaction mixture onto the column and elute the CPH₄ isocratically with 10 mM ammonium bicarbonate.
4. CPH₄ typically elutes ~0.45 L and can be detected by UV absorbance at 280 nm of the eluate.
5. Fractions containing CPH₄ are pooled and lyophilized. The lyophilization vessel is also kept dark by covering with foil.
6. The lyophilized CPH₄ is resuspended in a minimal volume of 0.05 M Tris-HCl (pH 8.0) and flash frozen small aliquots in liquid nitrogen and stored at –80°C until use.

7. CPH₄ is quantified using the extinction coefficient of tetrahydrobiopterin at 297 nm at pH 8.0 ($\epsilon_{297}=8.71\times10^3\text{ M}^{-1}\text{ cm}^{-1}$).

6. Typical assay conditions for QueE

QueE, is assayed under anaerobic conditions using YkuN/Fpr/NADH to reduce the protein. The conversion of CPH₄ to CDG is followed by LC-MS analysis of the reaction. CDG is quantified by comparison of the peaks in the UV chromatograms to standards.

Protocol for assaying QueE *in vitro*

QueE is assayed in the anaerobic chamber using solutions that are prepared inside the chamber with anaerobic water. Assays are typically carried out in 0.05 M PIPES-KOH (pH 7.4) containing 10 mM DTT, 2 mM MgSO₄ and 2 mM SAM. The reduction of QueE is achieved by including 2 mM NADPH, 4 μM FPR and 10 μM YkuN. Alternatively, dithionite (10 mM) and YkuN can also be used to bypass FPR/NADPH. The reactions at various time points can be quenched with addition of 30% (w/v) trichloroacetic acid (TCA), to a final concentration of 3% (w/v) TCA. The reaction mixtures are then analyzed by LC-MS.

7. LC-MS analysis of turnover by CDG

CDG is readily detected and quantified by HPLC methods. We use the UV trace to quantify the product and the MS detector to ensure the identity of the peak. Reverse phase chromatography at 0.2 mL/min on a C18 column is carried out on a Vanquish UPLC that is interfaced with a LTQ Orbitrap XL instrument (ThermoFisher) operated in positive ion mode, scanning m/z 75–1000 at a resolution of 100,000.

Protocol for LC-MS detection of CDG

1. Centrifuge the quenched reaction mixtures to remove any precipitated protein.
2. Equilibrate a ThermoFisher Hypersil GOLD C18 column (2.1 $\times$ 150 mm, particle size of 1.9 μm) in Optima water (ThermoFisher) containing 0.1 % Optima TFA (ThermoFisher).
3. Inject an aliquot (60 μL) of the reaction mixture and elute with a gradient to 100% Optima acetonitrile (ThermoFisher) containing 0.1 % TFA with the following program: 0% acetonitrile from 0–3 min; 0–10% acetonitrile from 3–38 min; 100% acetonitrile from 38.1–40.1 min; and 0% acetonitrile from 40.1–43.1 min to re-equilibrate the column.
4. A representative chromatogram is shown in **Fig. 9**. Under these conditions, CDG elutes at 13 min. The UV-visible spectrum of the product has distinct features at 230 and 300 nm. Identity of the peak as CDG is confirmed by the extracted ion chromatogram at m/z 195.05. Note that the small difference in the retention times observed in the UV-visible and extracted ion chromatographic traces results from the short distance between the UV-visible detector and the electrospray.

5. Quantify the CDG by comparison to known standards using the area under the UV-visible trace corresponding to CDG.

11. Conclusions

The wealth of structural and biochemical data available on QueE makes this an ideal system for exploring structure/function relationships in a radical SAM enzyme. In addition to cofactor-replete enzyme, studies of QueE require access to a source of reductant and substrate. In this chapter we have described methods to all other components that are necessary for *in vitro* studies of the enzyme.

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Abbreviations:

AmSO₄	ammonium sulfate
Amp	ampicillin
CDG.	7-carboxy-7-deazaguanine
CPH₄	6-carboxy-5,6,7,8-tetrahydropterin
dAdo•	5'-deoxyadenosyl radical
DTT	dithiothrietol
IPTG	isopropyl β-D-1-thiogalactopyranoside
Kan	kanamycin
KPi	potassium phosphate
LB	lysogeny broth
PIPES	piperazine-N,N'-bis(2-ethanesulfonic acid)
PMSF	phenylmethane sulfonyl fluoride
SAM	<i>S</i> -adenosyl-L-methionine

References

- Bai Y, Fox DT, Lacy JA, Van Lanen SG, & Iwata-Reuyl D (2000). Hypermodification of tRNA in Thermophilic Archaea. *Journal of Biological Chemistry*, 275(37), 28731–28738. [PubMed: 10862614]
- Berkovitch F, Nicolet Y, Wan JT, Jarret JT, Drennan CL, Catherin. (2003). Crystal Structure of Biotin Synthase, an S-Adenosylmethionine-Dependent Radical Enzyme. *Science*, 303, 76–79.
- Brown S, & Babbitt P (2015). Using the Structure-Function Linkage Database to Characterize Functional Domains in Enzymes, 48, 2.10.1–2.10.16.

- Bruender NA, Grell TAJ, Dowling DP, McCarty RM, Drennan CL, & Bandarian V (2017). 7-Carboxy-7-deazaguanine Synthase: A Radical S-Adenosyl-l-methionine Enzyme with Polar Tendencies. *Journal of the American Chemical Society*, 139(5), 1912–1920. [PubMed: 28045519]
- Bruender NA, Young AP, & Bandarian V (2015). Chemical and Biological Reduction of the Radical SAM Enzyme CPH4 Synthase. *Biochemistry*, 54(18), 2903–2910. [PubMed: 25933252]
- Buff K, & Dairman W (1975). Biosynthesis of Biopterin by Two Clones of Mouse Neuroblastoma. *Molecular Pharmacology*, 11, 87–93. [PubMed: 1167928]
- Burg AW, & Brown GM (1968). Biosynthesis of Folic Acid 8. Purification and properties of the enzyme that catalyzes the production of formate from carbon atom 8 of guanosine triphosphate. *Journal of Biological Chemistry*, 243(9), 2349–2358. [PubMed: 4296838]
- Dowling DP, Bruender NA, Young AP, McCarty RM, Bandarian V, & Drennan CL (2013). Radical SAM enzyme QueE defines a new minimal core fold and metal-dependent mechanism. *Nature Chemical Biology*, 10(2), 106–112. [PubMed: 24362703]
- Dowling DP, Miles ZD, Köhrer C, Maiocco SJ, Elliott SJ, Bandarian V, & Drennan CL (2016). Molecular basis of cobalamin-dependent RNA modification. *Nucleic Acids Research*, 44, gkw806–12.
- Elstner EF, & Suhadolnik RJ (1971). The Biosynthesis of the Nucleoside Antibiotics 9. Purification and properties of guanosine triphosphate 8-formylhydrolyase that catalyzes productino of formic acid from the ureido carbon of guanosine triphosphate. *Journal of Biological Chemistry*, 246(22), 6973–6981. [PubMed: 4331201]
- Elstner EF, Suhadolnik RJ, Suh. (1975). Guanosine Triphosphate-8-formylhydrolyase. *Methods in Enzymology*, 43, 515–520. [PubMed: 1134369]
- Frey B, McCloskey J, Kersten W, & Kersten H (1988). New Function of Vitamin B12: Cobamide-Dependent Reduction of Epoxyqueuosine to Queuosine in tRNAs of *Escherichia coli* and *Salmonella typhimurium*. *Journal of Bacteriology*, 170(5), 2078–2082. [PubMed: 3129401]
- Frey PA, Hegeman AD, & Ruzicka FJ (2008). The Radical SAM Superfamily, 43(1), 63–88.
- Guroff G, & Strenkoski CA (1966). Biosynthesis of Pteridines and of Phenylalanine Hydroxylase Cofactor in Cell-free Extracts of *Pseudomonas* Species (ATCC 11299a). *Journal of Biological Chemistry*, 241(20), 2220–2227. [PubMed: 5911609]
- Frazzon J, Fick JR, & Dean DR (2009). Biosynthesis of iron-sulphur clusters is a complex and highly conserved process, 1–6.
- Kuchino Y, Kasai H, Nihei K, & Nishimura S (1976). Biosynthesis of the modified nucleoside Q in transfer RNA. *Nucleic Acids Research*, 3(2), 393–398. [PubMed: 1257053]
- Lee BWK, Van Lanen SG, & Iwata-Reuyl D (2007). Mechanistic Studies of *Bacillus subtilis* QueF, the Nitrile Oxidoreductase Involved in Queuosine Biosynthesis †. *Biochemistry*, 46(44), 12844–12854. [PubMed: 17929836]
- McCarty RM, & Bandarian V (2012). Biosynthesis of pyrrolopyrimidines. *Bioorganic Chemistry*, 43(C), 15–25. [PubMed: 22382038]
- McCarty RM, Krebs C, & Bandarian V (2012). 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. *Biochemistry*, 52(1), 188–198. [PubMed: 23194065]
- McCarty RM, Somogyi A, & Bandarian V (2009a). *Escherichia coli* QueD Is a 6-Carboxy-5,6,7,8-tetrahydropterin Synthase. *Biochemistry*, 48(11), 2301–2303. [PubMed: 19231875]
- McCarty RM, Somogyi A, Lin G, Jacobsen NE, & Bandarian V (2009b). The Deazapurine Biosynthetic Pathway Revealed: In Vitro Enzymatic Synthesis of PreQ 0 from Guanosine 5'-Triphosphate in Four Steps. *Biochemistry*, 48(18), 3847–3852. [PubMed: 19354300]
- Miles ZD, McCarty RM, Molnar G, & Bandarian V (2011). Discovery of epoxyqueuosine (oQ) reductase reveals parallels between halorespiration and tRNA modification. *Pnas*, 108(18), 7368–7372. [PubMed: 21502530]
- Miles ZD, Myers WK, Kincannon WM, Britt RD, & Bandarian V (2015). Biochemical and Spectroscopic Studies of Epoxyqueuosine Reductase: A Novel Iron–Sulfur Cluster-and Cobalamin-Containing Protein Involved in the Biosynthesis of Queuosine. *Biochemistry*, 54(31), 4927–4935. [PubMed: 26230193]

- Miles ZD, Roberts SA, McCarty RM, & Bandarian V (2014). Biochemical and Structural Studies of 6-Carboxy-5,6,7,8-tetrahydropterin Synthase Reveal the Molecular Basis of Catalytic Promiscuity within the Tunnel-fold Superfamily. *Journal of Biological Chemistry*, 289(34), 23641–23652. [PubMed: 24990950]
- Moss M, & Frey PA (2001). The Role of S-Adenosylmethionine in the Lysine 2,3-Aminomutase Reaction. *Journal of Biological Chemistry*, 262(31), 14959–14862.
- Nelp MT, & Bandarian V (2015). A Single Enzyme Transforms a Carboxylic Acid into a Nitrile through an Amide Intermediate. *Angewandte Chemie International Edition*, 54(36), 10627–10629. [PubMed: 26228534]
- Nelp MT, Astashkin AV, Breci LA, McCarty RM, & Bandarian V (2014). The Alpha Subunit of Nitrile Hydratase Is Sufficient for Catalytic Activity and Post-Translational Modification. *Biochemistry*, 53(24), 3990–3994. [PubMed: 24914472]
- Nelp MT, Song Y, Wysocki VH, & Bandarian V (2016). A Protein-derived Oxygen Is the Source of the Amide Oxygen of Nitrile Hydratases. *The Journal of Biological Chemistry*, 291(15), 7822–7829. [PubMed: 26865634]
- Nishimura H, Katagiri K, Sato K, Mayama M, & Shimaoka N (1956). Toyocamycin, a new anti-candida antibiotics. *Journal of Antibiotics*, 9, 60–62. [PubMed: 13345725]
- Okada N, Harada F, & Nishimura S (1976). Specific replacement of Q base in the anticodon of tRNA by guanine catalyzed by a cell-free extract of rabbit reticulocytes. *Nucleic Acids Research*, 3(10), 2593–2603. [PubMed: 792816]
- Okada N, Noguchi S, Kasai H, Shindo-Okada N, Ohgi T, Goto T, & Nishimura S (1979). Novel Mechanism of Post-translation Modification of tRNA. *Journal of Biological Chemistry*, 254(8), 3067–3073. [PubMed: 372186]
- Phillips G, Yacoubi El, B., Lyons B, Alvarez S, Iwata-Reuyl D, & de Crécy-Lagard V (2008). Biosynthesis of 7-Deazaguanosine-Modified tRNA Nucleosides: a New Role for GTP Cyclohydrolase I. *Journal of Bacteriology*, 190(24), 7876–7884. [PubMed: 18931107]
- Reader JS, Metzgar D, Schimmel P, & de Crécy-Lagard V (2004). Identification of Four Genes Necessary for Biosynthesis of the Modified Nucleoside Queuosine. *Journal of Biological Chemistry*, 279(8), 6280–6285. [PubMed: 14660578]
- Reuter K, Slany R, Ullrich F, & Kersten H (1991). Structure and Organization of Escherichia coli Genes Involved in Biosynthesis of the Deazaguanine Derivative Queuine. *Journal of Bacteriology*, 173(7), 2256–2264. [PubMed: 1706703]
- Reynolds JJ, & Brown GM (1962). Enzymatic Formation of the Pteridine Moiety of Folic Acid from Guanine Compounds. *Journal of Biological Chemistry*, 237, 2713–2715. [PubMed: 14491714]
- Reynolds JJ, & Brown GM (1964). The Biosynthesis of Folic Acid IV. Enzymatic synthesis of dihydrofolic acid from guanine and ribose compounds. *Journal of Biological Chemistry*, 239(1), 317–325. [PubMed: 14114859]
- Shindo-Okada N, Okada N, Ohgi T, Goto T, & Nishimura S (1980). Transfer ribonucleic acid guanine transglycosylase isolated from rat liver. *Biochemistry*, 19(2), 395–400. [PubMed: 6986171]
- Smulson ME, & Suhadolnik RJ (1967). The Biosynthesis of the 7-Deazaadenine Ribonucleoside, Tubercidin, by Streptomyces tubercidicus. *Journal of Biological Chemistry*, 242(12), 2872–2876. [PubMed: 6027250]
- Sofia HJ (2001). 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 Research*, 29(5), 1097–1106. [PubMed: 11222759]
- Song Y, Nelp MT, Bandarian V, & Wysocki VH (2015). Refining the Structural Model of a Heterohexameric Protein Complex: Surface Induced Dissociation and Ion Mobility Provide Key Connectivity and Topology Information. *ACS Central Science*, 1(9), 477–487. [PubMed: 26744735]
- Suhadolnik RJ, & Uematsu T (1970). Biosynthesis of the Pyrrolopyrimidine Nucleoside Antibiotic, Toyocamycin. *Journal of Biological Chemistry*, 245(17), 4365–4371. [PubMed: 5498424]

- Uematsu T, & Suhadolnik RJ (1970). Nucleoside antibiotics. VI. Biosynthesis of the pyrrolopyrimidine nucleoside antibiotic toyocamycin by *Streptomyces rimosus*. *Biochemistry*, 9(5), 1260–1266. [PubMed: 5418715]
- Van Lanen SG, Reader JS, Swairjo MA, de Crécy-Lagard V, Lee B, & Iwata-Reuyl D (2005). From cyclohydrolase to oxidoreductase: Discovery of nitrile reductase activity in a common fold. *PNAS*, 102(12), 4264–4269. [PubMed: 15767583]
- Walsby CJ, Ortillo D, Broderick WE, Broderick JB, & Hoffman BM (2002). An Anchoring Role for FeS Clusters: Chelation of the Amino Acid Moiety of S-Adenosylmethionine to the Unique Iron Site of the [4Fe–4S] Cluster of Pyruvate Formate-Lyase Activating Enzyme. *Journal of the American Chemical Society*, 124(38), 11270–11271. [PubMed: 12236732]
- Walsby CJ, Ortillo D, Yang J, Nnyepi MR, Broderick WE, Hoffman BM, & Broderick JB (2005). Spectroscopic Approaches to Elucidating Novel Iron–Sulfur Chemistry in the “Radical-SAM” Protein Superfamily. *Inorganic Chemistry*, 44(4), 727–741. [PubMed: 15859242]
- Watanabe M, Matsuo M, Tanaka S, Akimoto H, Asahi S, Nishimura S, et al. (1997). Biosynthesis of Archaeosine, a Novel Derivative of 7-Deazaguanosine Specific to Archaeal tRNA, Proceeds via a Pathway Involving Base Replacement on the tRNA Polynucleotide Chain. *Journal of Biological Chemistry*, 272(32), 20146–20151. [PubMed: 9242689]
- Wilcoxon J, Bruender NA, Bandarian V, & Britt RD (2018). A Radical Intermediate in *Bacillus subtilis* QueE during Turnover with the Substrate Analogue 6-Carboxypterin. *Journal of the American Chemical Society*, 140(5), 1753–1759. [PubMed: 29303575]

Figure 1. Biosynthesis of the 7-deazapurine core.

The 7-deazapurine core is obtained in three steps from GTP in Nature.

Figure 2. Reduction cleavage of SAM.

The [4Fe-4S] cluster in RS enzymes reductively cleaves SAM to 5'-dAdo•, which initiates the catalytic cycle.

Figure 3. Radical mediated ring contraction mechanism of QueE.

The dAdo• abstracts a hydrogen atom from the C-6 position of CPH₄ which is thought to undergo rearrangement to a *gem*-amino carboxylate product. The product maintains the C-7 *proS* proton of the substrate.

Figure 4. Crystal Structure of QueE.

The overall structure of QueE with SAM, CPH₄, and 4Fe-4S cluster (A), and close up of the active site (B). SAM is bound to the cluster through its α-amino and α-carboxylate moieties.

Figure 5. Biological Reduction Pathways.

The biological reduction pathway from NADPH or the chemical reduction via dithionite.

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Figure 6. SDS-PAGE of purified proteins.

The gel shows (1) ladder, (2) QueE, (3) FPR, and (4) YkuN.

ATGGGCAGCAGCCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCC**CATATG**
GCTGATTGGGTAACAGGCCAAAGTCACTAAAGTGCAGAACTGGACCGACGCCCTGTTTAGTCTCA
CCGTTACGCCCCCGTGCTTCCGTTTACCGCCGGGCAATTTACCAAACCTTGGCCTTGAAATCGAC
GGCGAACGCGTCCAGCGCGCCTACTCCTATGTAAACTCGCCCGATAATCCCGATCTGGAGTTTTA
CCTGGTCACCGTCCCCGATGGCAAATTAAGCCCACGACTGGCGGCACTGAAACCAGGCGATGAA
GTGCAGGTGGTTAGCGAAGCGGCAGGATTCTTTGTGCTCGATGAAGTGCCGCACTGCGAAACGC
TATGGATGCTGGCAACCGGTACAGCGATTGGCCCTTATTTATCGATTCTGCAACTAGGTAAAGATT
TAGATCGCTTCAAAAATCTGGTCCTGGTGCACGCCGCACGTTATGCCGCCGACTTAAGCTATTTGC
CACTGATGCAGGAACGGAAAAACGCTACGAAGGAAAACGCGCATTCAGACGGTGGTCAGTCG
GGAAACGGCAGCGGGGTCGCTCACCGGACGGATACCGGCATTAATTGAAAGTGGGGAACCTGGA
AAGCACGATTGGCCTGCCGATGAATAAAGAAACAGCCATGTGATGCTGTGCGGCAATCCACAG
ATGGTGCGCGATACACAACAGTTGCTGAAAGAGACCCGGCAGATGACGAAACATTTACGTCGCC
GACCGGGCCACATGACAGCGGAGCATTACTGGTAAT**AAGCTT**

Figure 7. Sequence of codon optimized FPR.

The cloning sites are shown in italic.

Figure 8. Enzymatic synthesis of isotopologs of CPH_4 .

(a) Isotopologs of CPH_4 can be prepared enzymatically by carrying out the GCH I and QueD reactions in H_2O or D_2O . (b) Mass spectra of the synthetic isotopologs. Reprinted with permission from American Chemical Society (McCarty et al, 2012)

Figure 9. LC-MS analysis of turnover by QueE.

Production of QueE is monitored at 254 nm using a diode array detector (**A**). CDG has characteristic spectral features at 229 and 299 nm (**B**). The in-line mass spectrometer allows confirmation of the mass of the CDG (**C**).