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Biochemical and Spectroscopic Studies of Epoxyqueuosine Reductase: A Novel Iron−Sulfur Cluster- and Cobalamin-Containing Protein Involved in the Biosynthesis of Queuosine

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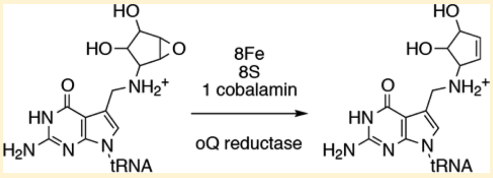
Supporting Information

ABSTRACT: Queuosine is a hypermodified nucleoside present in the wobble position of tRNAs with a 5′-GUN-3′ sequence in their anticodon (His, Asp, Asn, and Tyr). The 7-deazapurine core of the base is synthesized de novo in prokaryotes from guanosine 5′-triphosphate in a series of eight sequential enzymatic transformations, the final three occurring on tRNA. Epoxyqueuosine reductase (QueG) catalyzes the final step in the pathway, which entails the two-electron reduction of epoxyqueuosine to form queuosine. Biochemical analyses reveal that this enzyme requires cobalamin and two [4Fe-4S] clusters for catalysis. Spectroscopic studies show that the cobalamin appears to bind in a base-off conformation, whereby the dimethylbenzimidazole moiety of the cofactor is removed from the coordination sphere of the cobalt but not replaced by an imidazole side chain, which is a hallmark of many cobalamin-dependent enzymes. The bioinformatically identified residues are shown to have a role in modulating the primary coordination sphere of cobalamin. These studies provide the first demonstration of the cofactor requirements for QueG.



RNA is one of the most chemically diverse biological building blocks with >110 modifications reported to date.[1] Modifications can be as complex as the tricyclic ring structure observed in wybutosine,[2,3] or as simple as acetylation or thiolation of various positions on the purine or pyrimidine core. These modifications contribute to a myriad of functions, including, but not limited to, translational efficiency and accuracy, structural stabilization of the tRNA, enhanced recognition between the tRNA and its cognate aminoacyl-tRNA synthetase, and decoding of degenerate codons.[4,5] However, even simple modifications, such as methylation, are garnering attention as regulators of RNA processes. Only recently has the importance of such modifications come to light through identification of enzymes that reverse this methylation process, in a fashion analogous to that of DNA, adding a layer of regulation described as RNA epigenetics.[6,7]

Queuosine is one of the most highly decorated RNA modifications identified to date and has been observed in RNA across all domains of life.[8] The unique 7-deazapurine core of queuosine, which is also found in a variety of secondary metabolites,[9] is further adorned with a cyclopentenediol moiety derived from *S*-adenosyl-L-methionine (SAM).[10] Queuosine is present in the wobble position of tRNAs containing a 5′-GUN-3′ anticodon sequence that encodes Asp, Asn, His, and Tyr amino acids.[11] In bacteria, it is synthesized from the precursor GTP through an eight-step biosynthetic pathway (Figure 1),[10,12−22] which entail biosynthesis of 7-aminomethyl-7-deazaguanine, exchange into the wobble position, followed by two additional steps that occur on tRNA. Higher-order organisms obtain queuine, the free base of queuosine, from dietary sources and exchange this base into mature tRNA in place of guanine *en masse*.[23] While no discrete function is known for this modification, its absence has been correlated with numerous biological phenomena such as cancer pathology,[24−28] pathogenicity,[29,30] symbiosis,[31] and neurological disorders.[32]

Our understanding of the complete *de novo* biosynthetic pathway was completed recently with the discovery of epoxyqueuosine reductase (QueG), which catalyzes the final epoxide reduction, converting epoxyqueuosine to queuosine[22] (see Figure 1). QueG is homologous to reductive dehalogenase (RDH) enzymes that are essential to bacteria that utilize halogenated compounds, such as tetrachloroethylene, as their terminal electron acceptors.[33,34] RDHs are known to contain multiple iron−sulfur clusters and corrinoids as cofactors and require strong reductants for activity.[35−39] QueG retains the eight Cys residues that coordinate the two 4Fe-4S clusters in RDHs (Figure 2). Recent X-ray crystal structures of two RDHs provide views of the active sites of these enzymes.[40,41] The epoxide reduction reaction catalyzed by QueG is analogous to RDHs in that it is a two-electron reduction requiring a strong reductant. Prior to identification of oQ reductase, bacterial

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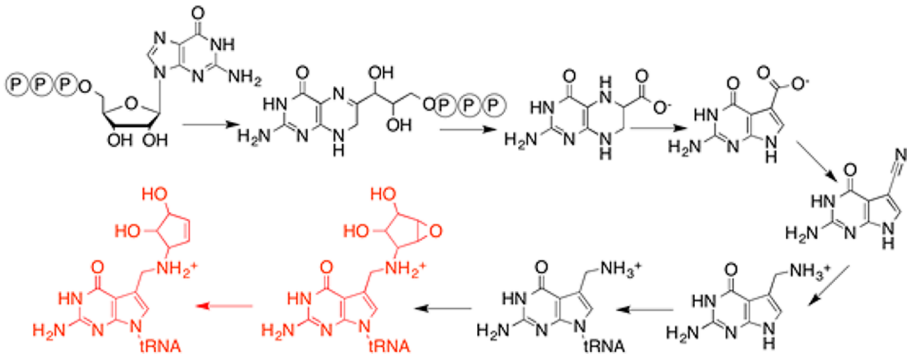

Figure 1. Biosynthesis of queuosine.

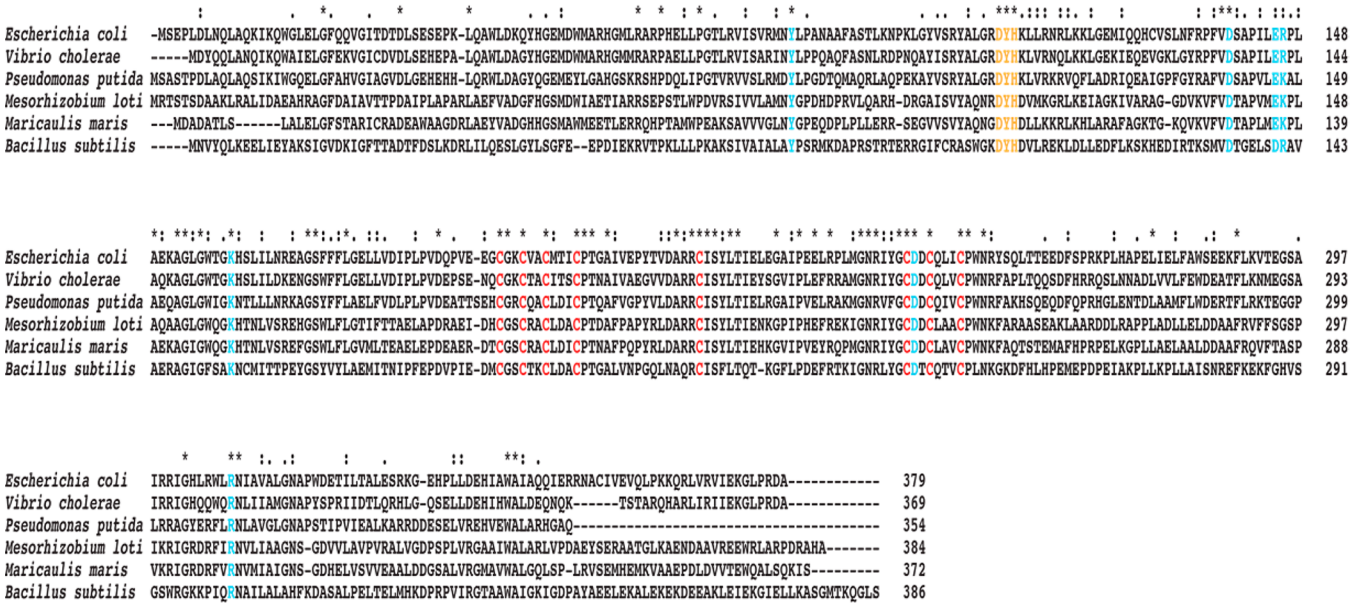

Figure 2. Representative multiple-sequence alignment of QueG homologues and *in vivo* alanine scanning of conserved residues of QueG. Conserved cysteine residues required for iron−sulfur cluster formation are colored red; the conserved DYH motif is colored orange, and other conserved residues mutated in the *in vivo* alanine scanning experiment are highlighted in blue. The accession numbers for the sequences are as follows: *Escherichia coli* (EGI43095), *Vibrio cholerae* (WP_000386321), *Pseudomonas putida* (WP_014754621), *Mesorhizobium loti* (WP_010912587), *Maricaulis maris* (WP_011642061), and *Bacillus subtilis* (NP_388772).

feeding experiments had demonstrated a cobalamin requirement for the conversion of oQ to Q.[42] *In vitro*, oQ reduction was shown to be stimulated by addition of exogenous cobalamin.[22] To date, however, there have been no systematic studies of the role(s) of the cofactors and conserved residues in catalysis in RDHs or QueG.

Herein, we report a biochemical and spectroscopic analysis of the cofactor requirements of QueG. We have established a method for obtaining cofactor replete, active recombinant protein in an anaerobic environment allowing the cofactor stoichiometry of the protein to be established unambiguously. The results demonstrate the presence of two iron−sulfur clusters and a cobalamin that are absolutely required for activity. In addition, an *in vivo* alanine scanning experiment has identified residues that are critical for catalysis. Analysis by electron paramagnetic resonance (EPR) spectroscopy of a subset of conserved residues that are essential for activity has revealed an interesting role for these residues in modulating the coordination environment of the cobalamin cofactor. Taken together, these analyses highlight the unique cofactor requirements necessary to facilitate the novel epoxide reduction catalyzed by QueG on RNA and show that despite the substantially different reaction catalyzed by oQ reductase, it utilizes similar cofactors, suggesting that reductive dehalogenation and epoxide reduction likely follow similar mechanistic pathways.

METHODS

Materials. All materials were purchased commercially (unless otherwise noted) and were of the highest purity. All assays and purification steps were conducted in a Coy anaerobic chamber in an atmosphere of 95% N_2 and 5% H_2. All buffers and materials were deoxygenated in the chamber several days prior to use and were made RNase-free when possible.

Cloning of *Bacillus subtilis* queG. The codon-optimized gene encoding *B. subtilis* QueG was obtained from Genscript (Piscataway, NJ). The sequence of the synthetic gene is available in the Supporting Information. The gene was excised from a supplied pUC57 vector by digestion with *Nde*I and *Hin*dIII and ligated into a similarly cut pET28a vector to obtain pZM419. The gene was then amplified from this vector using the primers 5′-AAAGGATCCATGAATGTTTACCAACTGA-

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Biochemistry 2015, 54, 4927−4935

AAGAAGAAC-3′ (forward) and 5′-AAAGGATCCGGACAG-ACCTTGTTTCGTCATACC-3′ (reverse) at an annealing temperature of 43 °C and included flanking *Bam*HI restriction sites. The amplified fragment was digested using *Bam*HI and cloned into a pASK-IBA43plus vector (IBA) that was similarly digested to yield pZM471 for expression of N-terminally His_6-tagged and C-terminally Strep-tag II QueG. Mutants of pZM419 and pZM471 were created using Stratagene Mutagenesis Kit according to the manufacturer's recommended protocol and using the primers listed in Table S1 of the Supporting Information.

Expression of *B. subtilis* QueG. *Escherichia coli* BL21-(DE3) cells containing pZM471 were grown in 12 L of LB containing 0.1 mg/mL ampicillin at 37 °C to an OD_{600} of approximately 0.3, at which time ferric chloride was added to a final concentration of 50 μM and the flasks were cooled to 18 °C. The cells were grown further to an OD_{600} of approximately 0.6, and protein expression was induced with 20 μL of 10 mg/mL anhydrotetracycline hydrochloride in dimethylformamide (Acros Organics) per liter of LB. Cells were grown overnight and harvested the next day by centrifugation (5000*g*), frozen in liquid N_2, and stored at −80 °C.

Purification of QueG. All steps were conducted in an anaerobic chamber as described in this section. Cells (~20 g) were suspended in buffer containing 100 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10 mM dithiothreitol (DTT), and 1 mM phenylmethanesulfonyl fluoride and sonified using a Branson digital sonifier (45% amplitude). The lysate was centrifuged for 30 min at 18000*g* (4 °C) to pellet insoluble material. The cleared lysate was loaded onto a 5 mL StrepTrap HP column (GE Healthcare) that had been equilibrated with buffer containing 100 mM Tris-HCl (pH 8.0) and 150 mM NaCl (buffer A). The column was washed with 25 mL of buffer A, and QueG was eluted with 25 mL of buffer A containing 2.5 mM *d*-desthiobiotin (Sigma-Aldrich). Fractions containing QueG were identified using sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and pooled. The concentration of the pooled protein was determined using the Bradford method with bovine serum albumin (ThermoScientific) as the standard.

Reconstitution of QueG. The reconstitution was conducted on ice, and the solution was stirred constantly. First, solid DTT was added to a final concentration of 10 mM, and the solution was allowed to mix for 15 min. Ten molar equivalents of ferric chloride dissolved in anaerobic water was added slowly over the course of 5 min, and the solution was allowed to mix for an additional 10 min. Ten equivalents of sodium sulfide dissolved in anaerobic water was added slowly over 5 min, and the solution was allowed to mix for an additional 3 h. The protein solution was buffer exchanged using a Bio-Rad DG-10 column into 20 mM Tris-HCl (pH 8.0), 100 mM KCl, and 2 mM DTT (buffer B). The protein was concentrated by centrifugation at 6000*g* (4 °C) using an Amicon Ultra-4 10 kDa cutoff concentrator to a final volume of <1 mL. To the resulting concentrated protein was added 100 μL of 20 mM hydroxocobalamin acetate salt (Sigma-Aldrich), and the solution was allowed to incubate at room temperature for 5 min. The protein was then loaded onto a Sephacryl S300 HR gel filtration column (16 cm × 60 cm, GE Healthcare) equilibrated in buffer B and eluted at a constant flow rate of 1 mL/min. Fractions containing QueG were identified using SDS−PAGE, pooled, and concentrated by centrifugation at 4000*g* (4 °C) using an Amicon Ultra-15 10 kDa cutoff concentrator. Aliquots were flash-frozen in liquid N_2 and stored at −80 °C. The protein concentration was determined using the Bradford method with bovine serum albumin (ThermoScientific) as the standard.

Amino Acid, Iron, Labile Sulfide, and Cobalamin Content Analyses. All subsequent analyses were completed on at least three independent protein preparations, and values are reported as averages.

Amino acid analysis was performed at the Molecular Structure Facility University of California—Davis. The protein was exchanged by gel filtration using a NICK Sephadex G-50 column (GE Healthcare) into 10 mM NaOH. A Bradford assay was performed on the eluate, and 0.3 mL of the remaining protein solution was utilized for the analysis. The amino acid correction factor was used to calculate the resulting stoichiometries during the cofactor content analyses.

Iron analysis was performed at the University of Arizona Department of Hydrology and Water Resources using a PerkinElmer Optima 5300 DV ICP-OES instrument. QueG was diluted into 1% (v/v) nitric acid to a concentration of 1 μM for analysis. The concentration of labile sulfide was determined using the Beinert method.[43]

The cobalamin content of QueG was determined by thermal denaturation of the protein and derivatization of cobalamin to dicyanocob(III)alamin by addition of potassium cyanide as follows. To a solution containing QueG at a known concentration was added potassium cyanide to a final concentration of 10 mM in a total reaction volume of 0.1 mL. The reaction mixture was allowed to incubate at room temperature in the dark for 30 min and held at 99 °C for 15 min. The mixture was placed directly on ice for 5 min and then centrifuged at 21000*g* for 5 min to pellet the precipitated protein. The concentration of dicyanocob(III)alamin in the supernant was determined by measuring the absorbance at 580 nm using an extinction coefficient of 10.13 mM^{-1} cm^{-1}.[44,45]

Size-Exclusion Chromatography To Determine Oligomerization State. Purified QueG (4 mg/mL) was loaded onto a Sephacryl S200 HR column (16 cm × 60 cm, GE Healthcare) equilibrated in 20 mM Tris-HCl (pH 8.0), 100 mM KCl, and 2 mM DTT. The column was run at a constant flow rate of 1 mL/min, and the fractions were analyzed by SDS−PAGE to observe the presence of the standards and QueG. The standards (Bio-Rad) used to determine the molecular weight of QueG were thyroglobulin (670 kDa), γ-globulin (150 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B_{12} (1.35 kDa).

***In Vitro* Activity Assays To Determine Requirements of Iron−Sulfur Clusters and Cobalamin.** The conversion of oQ to Q by QueG was assayed using a synthetic 17-mer oQ-containing stem loop substrate corresponding to the anticodon loop of Tyr-tRNA and prepared as previously described.[22] The assays contained 20 mM PIPES-NaOH (pH 7.4), 10 mM sodium dithionite, 0.75 mM methyl viologen, 0.75 mM hydroxocobalamin (if present in assay), and 5 μL of ~0.5 mM oQ stem loop. The reactions were initiated by addition of 5 μM QueG enzyme in a total volume of 0.1 mL. The reaction was allowed to proceed in the dark at room temperature for 5 h and quenched using QIAzol reagent (Qiagen), which contains phenol. RNA from the assay was purified, digested to nucleosides, and analyzed by liquid chromatography and mass spectrometry (LC/MS) as previously described.[22]

***In Vivo* Alanine Scanning Complementation Experiment.** The Δ*yjeS E. coli* strain from the Keio Collection[46] was

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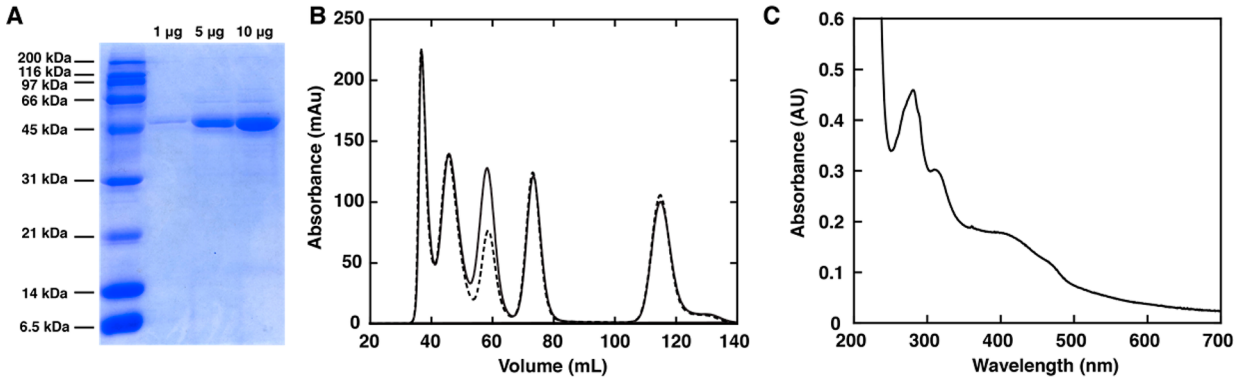

Figure 3. Purification, UV−visible spectrum, and quaternary state determination of QueG. (A) An SDS−PAGE gel of purified QueG. On the basis of the gel, QueG is >95% pure with a molecular weight consistent with the predicted weight of 48.7 kDa. (B) Size-exclusion chromatogram of protein standards (---) superimposed with a chromatogram of protein standards, including purified QueG enzyme (—). On the basis of the elution profiles, only the peak pertaining to the ∼44 kDa standard changes with inclusion of QueG in the solution mixture. The molecular weight of QueG is ∼48.7 kDa, leading to the increase in only the absorbance of the concurrent standard peak. The standard peaks (from left to right) are thyroglobulin (670 kDa), γ-globulin (150 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B_{12} (1350 Da). (C) UV−visible spectrum of the purified enzyme that contains features consistent with inclusion of both iron−sulfur clusters (∼420 nm) and cob(II)alamin (∼475 nm) as cofactors.

lysogenized using the λDE3 Lysogenization Kit (Novagen) following the manufacturer's recommended protocol. The kanamycin resistance cassette, which was inserted in place of the *yjeS* gene in the Keio Collection, was removed by transformation with the pCP20 plasmid[47] following a published method.[48] Briefly, 30 μL of stationary phase overnight culture of the lysogenized knockout strain was used to inoculate 3 mL of LB containing 34 μg/mL kanamycin. The culture was then left to grow at 37 °C and 200 rpm to an OD_{600} of ∼0.6−0.7. The cells were then made electrocompetent by placing 1 mL of the culture in a prechilled, sterile 1.5 mL tube and spun at 13000*g* (4 °C) for 1 min to pellet the cells. The pellet was resuspended in 1 mL of prechilled sterile water and was again spun at 13000*g* (4 °C) for 1 min to pellet the cells. This process was repeated. The pellet from the final spin was resuspended in 50 μL of the residual supernatant and transformed with the pCP20 plasmid, resuspended in 1 mL of room-temperature SOC medium, and left to shake at 30 °C and 200 rpm for 1 h. The culture was then added to 100 mL of LB containing 100 μg/mL ampicillin and 34 μg/mL chloramphenicol and allowed to shake at 30 °C and 200 rpm overnight. An aliquot (30 μL) of the overnight growth was used to inoculate a 3 mL LB culture, and it was allowed to shake at 30 °C and 200 rpm until the OD_{600} reached ∼0.1, at which time the temperature was shifted to 42 °C and the culture was allowed to grow to an OD_{600} of ∼0.8−1.0. The culture was then streaked onto an LB/agar plate and left overnight at 37 °C. Colonies were selected that could no longer grow on either kanamycin or ampicillin and verified to be the correct Δ*yjeS E. coli* strain by colony polymerase chain reaction. Strains verified to be Kan^{-r} and Δ*yjeS* were made competent and transformed with a pET28a plasmid containing wild-type QueG or QueG variants. Site-directed variants were prepared using the primers listed in Table S1 of the Supporting Information. A single colony was used to inoculate 50 mL of LB containing 34 μg/mL kanamycin and was grown at 37 °C and 200 rpm to an OD_{600} of ∼0.5−0.7. Then, the culture was induced by addition of 5 μL of 1 M IPTG and left to continue growing for 16 h total. The cells were harvested the next day, and total RNA was extracted and analyzed on the LC/MS instrument as previously described.[22] Each variant was analyzed in this manner at least in triplicate.

Electron Paramagnetic Resonance Spectroscopy of Wild-Type QueG and Its Variants. EPR samples were prepared under anaerobic conditions by mixing concentrated, purified protein with a final glycerol concentration of 15% (v/v) to a total volume of ∼0.15 mL and frozen in liquid nitrogen in an EPR tube. Continuous wave (CW) X-band EPR experiments were preformed on a Bruker Biospin EleXsys E500 spectrometer equipped with a cylindrical TE_{011}-mode resonator (SHQE-W), an ESR-900 liquid helium cryostat, and an ITC-5 temperature controller (Oxford Instruments). Spectra were recorded at 40 K at microwave frequencies of 9.323 GHz [wild-type (WT)], 9.333 GHz (D104A), 9.321 GHz (Y105A), 9.373 GHz (H106A), and 9.376 GHz (D134A). The power and modulation amplitude were 0.2 mW and 2 G, respectively. Simulations were conducted in MatLab using the EasySpin suite of programs.[49]

RESULTS AND DISCUSSION

Purification of QueG. Previous studies of purified QueG confirmed that the enzyme was sufficient for conversion of oQ to Q and demonstrated that addition of exogenous cobalamin stimulated activity.[22] However, the protein used in those studies was recalcitrant to expression and purification to a more homogeneous state, and the observation that cobalamin stimulated activity was presumably due to the lack of cofactor replete protein. Therefore, we obtained a codon-optimized gene encoding the *B. subtilis* homologue and developed a method of expression in *E. coli* and purification to obtain >95% pure protein (Figure 3A). QueG is a monomer as judged by size-exclusion chromatography (Figure 3B). On the basis of the sequence homology to reductive dehalogenases, we hypothesized that the enzyme would contain iron−sulfur clusters and cobalamin as cofactors. Indeed, the UV−visible spectrum of QueG (Figure 3C) includes features common to both [4Fe-4S] clusters at ∼420 nm[50] and cob(II)alamin at ∼475 nm.[51]

Cofactor Stoichiometry. After establishing the protein purification and cofactor incorporation scheme, we turned our attention to determining whether the iron−sulfur clusters and cobalamin are required for activity. The basic strategy for obtaining replete protein entailed reconstitution of the purified protein in two stages with iron and sulfide first, followed by

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Biochemistry 2015, 54, 4927−4935

cobalamin. Excess cofactors were removed by gel filtration prior to activity and stoichiometry measurements.

To determine the cofactor requirements, an aliquot of the reconsitution mixture was assayed for activity following the initial affinity purification steps, after reconstitution with iron and sulfide, and after addition of cobalamin. As shown in Figure 4, the as-isolated protein has no activity whereas protein that has been subjected to additions of iron, sulfur, and cobalamin is active. However, reconstitution with iron and sulfide is not sufficient for activity; therefore, oQ reductase activity requires FeS clusters and cobalamin. To determine the stoichiometry of the clusters and cobalamin present, amino acid analysis was conducted on reconstituted QueG after the final size-exclusion step to obtain an accurate protein concentration. On the basis of the amino acid analysis, the protein concentration obtained using the Bradford method overestimates the QueG concentration by ~1.7-fold. QueG was subjected to ICP-OES to determine the amount of iron, and the Beinert method[43] was used to determine the amount of labile sulfide. Finally, a cyanolysis was conducted to quantify cobalamin content.[44,45] The iron, sulfide, and cobalamin stoichiometries derived from the analyses described above are summarized in Table 1. Purified and reconstituted QueG contains 6.5 mol of iron, 8 mol of labile sulfide, and 1 mol of cobalamin per monomer.

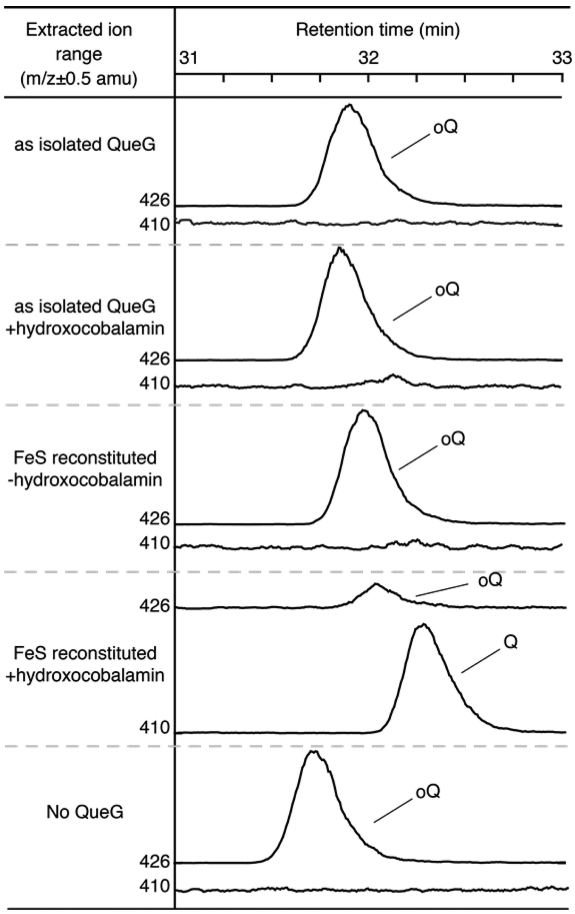

Figure 4. Iron–sulfur clusters and cobalamin are required for QueG activity *in vitro*. Extracted ion chromatograms of digested nucleosides of oQ (m/z 426) and Q (m/z 410) from the oQ stem loop used as the substrate in an *in vitro* activity assay of QueG. Protein was assayed for activity before and after reconstitution in the presence or absence of exogenous hydroxocobalamin. The appearance of turnover in only enzyme with reconstituted iron–sulfur clusters and added hydroxocobalamin demonstrates the necessity of both for activity.

Table 1. Analysis of the Cofactor Content of QueG

cofactor	stoichiometry (mol/monomer of QueG)[a]
iron	6.54 ± 1.61
labile sulfide	8.01 ± 1.08
cobalamin	1.01 ± 0.11

[a]The values are consistent with the presence of two [4Fe-4S] clusters and one cobalamin cofactor per monomer of QueG. The concentration of QueG protein used to obtain the stoichiometric values was calibrated with amino acid analyses as described in Methods.

Recently, two structures of reductive dehalogenase proteins have been reported that are relevant in this discussion.[40,41] Both structures show that RDHs have two [4Fe-4S] clusters and a cobalamin. While there is limited sequence similarity between QueG and RDHs, the Cys residues that coordinate the two iron–sulfur clusters are conserved in QueG (shown in red in Figure 2). Intriguingly, oQ reductase has the same complement of cofactors as RDHs, despite the differences in the overall reactions catalyzed by these enzymes.

***In Vivo* Screening for Essential Residues.** The initial difficulties in the expression and purification of QueG and the complicated reconstitution methods that are required to obtain cofactor replete active protein made it necessary to develop an alternative method to rapidly assess the importance of residues in catalysis. In these experiments, we utilized the Δ*queG* strain of *E. coli* from the Keio collection[46] and examined the oQ/Q content of the cells upon expression of wild-type and site-directed variants of QueG on a plasmid. Potential catalytic residues were identified from a multiple-sequence alignment that was constructed with ~1100 sequences obtained through a protein BLAST search[52,53] of the nonredundant database with the *E. coli* QueG homologue (YjeS; b4166) as the initial search sequence. The candidates obtained from the BLAST search had 30–80% sequence identity. Analysis of the extensive alignment identified multiple absolutely conserved motifs (Figure 2). Two of the conserved motifs, $CX_2CX_2CX_3C$ and CX_2CX_3C with another distally conserved Cys, are likely involved in forming the iron–sulfur clusters as both of these motifs are also the sequence signatures associated with enzymes involved in reductive dehalogenation, where they had been proposed to coordinate two [4Fe-4S] clusters that are necessary for electron transfer and reduction of the cobalamin cofactor.[35,54−56] Another absolutely conserved sequence was a DYH motif. This DYH motif was of great interest as it was reminiscent of the DxHxxG motif commonly found in cobalamin-dependent enzymes, specifically, enzymes that bind cobalamin in its base-off/His-on conformation, such as methionine synthase[57] and glutamate mutase.[58,59] In this conformation, a histidine side chain displaces the dimethylbenzimidazole moiety of cobalamin, which in solution coordinates the cobalt atom through the N3 atom in the lower axial position. On the basis of the conserved residues observed in the multiple-sequence alignment, mutations were made in a pET28a plasmid harboring the *B. subtilis* QueG enzyme (pZM419). In all, 18 single-mutation variants in which the residue of interest was mutated to an alanine were generated. The Δ*yjeS E. coli* Keio Collection strain was then lysogenized with λDE3 and made electrocompetent to allow for expression of the wild-type or variant *queG* gene using

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Biochemistry 2015, 54, 4927–4935

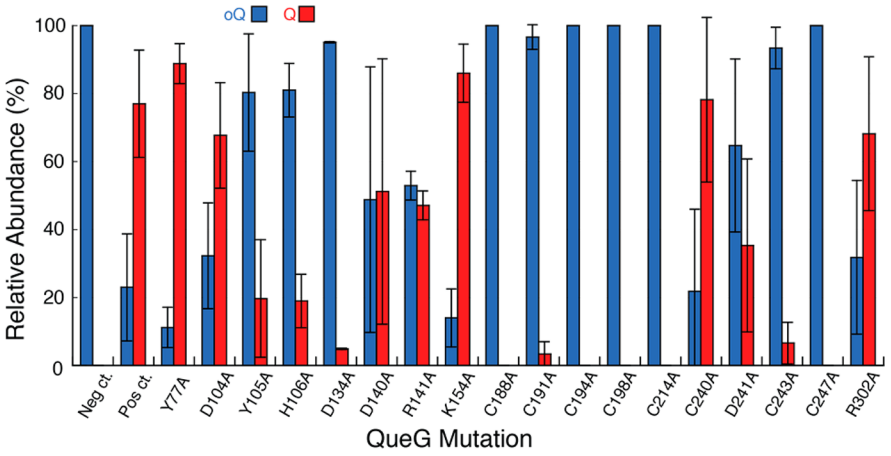

Figure 5. *In vivo* QueG mutagenesis. The relative abundances of Q and oQ were calculated from the extracted ion chromatograms of oQ (blue) and Q (red) present in total RNA nucleosides from overnight growths of Δ*queG E. coli* overexpressing the indicated variant form of QueG (*B. subtilis* numbering). All values are normalized to the sum of both oQ and Q and given as a percentage of the total.

T7 polymerase after transformation with the corresponding pET28a plasmid. Cells were induced in the log phase of growth and allowed to grow overnight. Total RNA was isolated, digested to nucleosides, and analyzed for the presence of oQ versus Q via LC/MS. The effects of the mutations on the production of both oQ and Q *in vivo* are shown in Figure 5. Mutation of almost all of the cysteine residues thought to be involved in cluster formation completely negated the ability to convert oQ to Q. Curiously, the C240A point mutation did not seem to have any effect on activity in comparison to that of the wild-type protein. However, there have been examples of iron−sulfur cluster-containing enzymes in which a single mutation in a cysteine residue comprising an iron−sulfur cluster is not sufficient to abolish cluster formation and activity.[60] Mutations in residues comprising the DYH motif showed differing results. The introduction of Y105A and H106A variants of QueG into the knockout strain reduced the extent of cellular Q relative to oQ. By contrast, the D104A mutation did not alter the oQ/Q ratio relative to that of the wild-type.

Interestingly, the D134A and R141A variants significantly reduced the Q content of the strain relative to that of the wild-type. The importance of the Cys residues to catalysis in QueG is easy to justify because of the observation that analogous Cys residues in RDH proteins coordinate the two [4Fe-4S] clusters in the protein. The significance of D104, Y105, H106, and D134, however, was not immediately obvious.

EPR Spectroscopy of QueG and Its Variants. To further probe the role of residues that appeared to be essential as determined by the *in vivo* experiments, we conducted electron paramagnetic resonance studies to investigate whether these residues are in the vicinity of the cobalamin cofactor. Cobalamin can exist in three oxidation states with distinct UV−visible spectral signatures. However, in the +2 oxidation state, the cofactor is paramagnetic and may be studied by EPR.

Reconstituted wild-type, D104A, Y105A, H106A, and D134A proteins were analyzed by EPR spectroscopy. The EPR spectrum of the wild-type protein (Figure 6A) is best simulated by a rhombic **g** and **A** tensor (Table 2). Interaction of the unpaired spin with cobalt ($I = {}^{7}/_{2}$) introduces extensive fine structure, which is best fit with a rhombic **A** tensor (A_x = 186 MHz, A_y = 133 MHz, and A_z = 340 MHz). We observe no additional strongly coupled nuclei. If a nitrogen (I = 1) from the dimethylbenzimidazole or His imidazole side chain had been present, we would have observed triplet splittings of the g_z features, as is observed in other cobalamin-dependent enzymes.[57,61,62] Therefore, the data clearly show that upon binding of cobalamin to QueG, the dimethylbenzimidazole ligand that coordinates the cobalt in solution is dissociated, but not replaced with a His side chain. This observation is similar to what has been observed in the structures of the RDH proteins.[40,41]

To determine if the DYH motif is in the vicinity of the cofactor, the residues in this motif were mutated to Ala, the purified proteins were reconstituted with cobalamin, iron, and sulfide, and EPR spectra of each variant were recorded (Figure 6C,E,G). The cobalamin content of the variants was quantified by cyanolysis to be 0.85−1.2 per monomer. The spectra of the wild-type and H106A variant are nearly identical and can be simulated with very similar sets of parameters (Table 2). The spectrum of the D104A variant, however, shows subtle differences from that of the wild-type that are best captured in simulations where nearly axial **g** and **A** tensors are included. By contrast, there is a very dramatic change in the spectrum of the Y105A variant relative to that of the wild-type, whereby there is significantly less **g** tensor anisotropy and fewer **A** tensors. The differences between the wild-type, D104A, H106A, and Y105A variants are reminiscent of the observations with the H759G variant of cobalamin-dependent methionine synthase (MetH). In MetH, binding of the cobalamin leads to an interchange of the dimethylbenzimidazole for the imidazole side chain of the His759 residue.[57] Mutation of the His residue reveals two sets of EPR cobalamin spectra, one of which corresponds to a cob(II)alamin interacting with a water molecule (five-coordinate), and another of which is essentially four-coordinate. The EPR spectra of wild-type QueG and all the variants reported here are consistent with five-coordinate geometry around the cobalamin, but the differences in the **g** and **A** tensors suggest that in all but the Y105A variant, the interaction between the putative water ligand and the cobalamin is weakened.[63]

In addition to the residues in the conserved DYH motif, we also examined the effect of mutating the absolutely conserved D134 to A on the EPR spectrum of the variant. Interestingly, the EPR spectrum of this variant (Figure 6I) exhibits features of both wild-type and Y105A variants. We did not attempt to simulate the complex pattern; however, the spectrum of the

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Biochemistry 2015, 54, 4927−4935

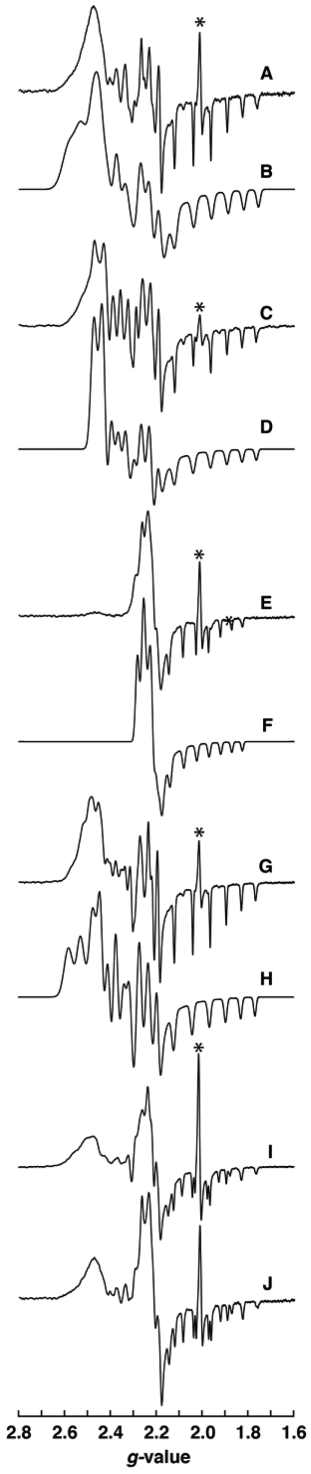

Figure 6. EPR of wild-type QueG and its variants. The EPR spectra and simulations of (A) the wild-type and variants (C) D104A, (E) Y105A, (G) H106A, and (I) D134A of QueG in the cob(II)alamin form. Simulations are shown in resonances B, D, F, H, and J for the wild-type and variants in the same order, respectively. The asterisk denotes a signal that results from degradation of the iron−sulfur clusters during purification and reconstitution.

protein is reasonably reproduced (Figure 6J) by mathematically adding experimental spectra of the wild-type and Y105A proteins, suggesting that the cobalamin in this variant exists as a 50:50 mixture of the forms that are present in the wild-type and Y105A proteins.

Table 2. EPR Simulation Parameters for Cob(II)alamin in Wild-Type QueG and Its Site-Directed Variants

protein	g_x	g_y	g_z	A_x (MHz)	A_y (MHz)	A_z (MHz)
wild-type[a]	2.437	2.355	2.061	186	133	340
H106A[b]	2.435	2.354	2.064	186	130	330
D104A	2.396	2.349	2.065	125	125	333
Y105A	2.282	2.250	2.063	9	11	235

[a]The simulation of the wild-type spectrum better matched the experimental spectrum when an **A** tensor strain of [48.6 17 0] (MHz) was included. [b]The simulation of the H106A variant was best fit with an **A** tensor strain of [30 0 0] (MHz).

The molecular mechanisms by which these conserved residues modulate the environment of the cobalamin remain to be established by structural methods, and we cannot exclude the possibility that they are distant from the active site. However, taken in the context of the *in vivo* analysis of oQ:Q ratios in a Δ*queG* knockout strain, it seems likely that these residues are in the active site of the protein.

CONCLUSIONS

The identity of QueG, which catalyzes the final step in the biosynthesis of queuosine, was surprising in that it revealed the protein to be homologous to reductive dehalogenases. In this paper, we demonstrate that despite the drastic differences in the reactions catalyzed by these enzymes, QueG and RDHs utilize the same set of cofactors to accomplish their respective chemical transformations. Reconstitution experiments with QueG demonstrate that iron−sulfur clusters and cobalamin are required for function. These data establish a direct link between cobalamin and RNA modification for the first time since the feeding experiments noted a role for cobalamin in oQ reduction *in vivo*.[42] On the basis of the similarity of the EPR spectroscopic parameters to those of MetH,[61−63] the cobalamin cofactor in QueG is bound in a base-off conformation but is five-coordinate, presumably by interactions with a ligand, such as water, that does not have a nuclear spin. Intriguingly, the conserved DYH motif does not provide an imidazole side chain to coordinate the cofactor. However, the significant perturbations of the electronic environment of the cobalamin observed in these variants suggest that these residues are in the active site of the protein. Therefore, our findings place QueG in the class of enzymes that displace the dimethylbenzimidazole of the cobalamin cofactor upon binding to the protein. Understanding the mechanism of QueG, the role of the conserved residues in activity, and the correlations between the reactions catalyzed by QueG and RDHs must await high-resolution structures of QueG.

ASSOCIATED CONTENT

Supporting Information

The sequence of the codon-optimized gene and sequences of the primers used for constructing the site-directed variants. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00335.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

EPR, electron paramagnetic resonance; oQ, epoxyqueuosine; Q, queuosine; QueG, epoxyqueuosine reductase; RDH, reductive dehalogenase.

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