Pyrroloquinoline Quinone Biogenesis: Demonstration That PqqE from *Klebsiella pneumoniae* Is a Radical S-Adenosyl-L-methionine Enzyme†

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**ABSTRACT:** Biogenesis of pyrroloquinoline quinone (PQQ) in *Klebsiella pneumoniae* requires the expression of six genes (pqqA–F). One of these genes (pqqE) encodes a 43 kDa protein (PqqE) that plays a role in the initial steps in PQQ formation [Velerop, J. S., et al. (1995) *J. Bacteriol.*, 177, 5088–5098]. PqqE contains two highly conserved cysteine motifs at the N- and C-termini, with the N-terminal motif comprised of a CXXC motif that is unique to a family of proteins known as radical S-adenosyl-L-methionine (SAM) enzymes [Sofia, H. J., et al. (2001) *Nucleic Acids Res.* 29, 1097–1106]. PqqE from *K. pneumoniae* was cloned into *Escherichia coli* and expressed as the native protein and with an N-terminal His tag. Anaerobic expression and purification of the His-tagged PqqE results in an enzyme with a brownish-red hue indicative of Fe–S cluster formation. Spectroscopic and physical analyses indicate that PqqE contains a mixture of Fe–S clusters, with the predominant form of the enzyme containing two [4Fe-4S] clusters. PqqE isolated anaerobically yields an active enzyme capable of cleaving SAM to methionine and 5′-deoxyadenosine in an uncoupled reaction (k<sub>obs</sub> = 0.011 ± 0.001 min<sup>−1</sup>). In this reaction, the 5′-deoxyadenosyl radical either abstracts a hydrogen atom from a solvent accessible position in the enzyme or obtains a proton and electron from buffer. The putative PQQ substrate PqqA has not yet been shown to be modified by PqqE, implying that PqqA must be modified before becoming the substrate for PqqE and/or that another protein in the biosynthetic pathway is critical for the initial steps in PQQ biogenesis.

The broad spectrum of chemical reactions catalyzed by enzymes frequently requires functional groups that are unavailable from the side chains of the 20 naturally occurring amino acids. In particular, there is a lack of electrophilic functional groups necessitating the involvement of exogenous metal ions or organic cofactors. The quino-cofactor family of enzymes contains intrinsic electrocatalytic centers that are derived from naturally occurring amino acids, thereby expanding the scope of amino acid side chains available within active sites of folded proteins. These cofactors arise via two fundamentally different pathways; in the first case, the pathway involves the addition of a redox active copper ion to a folded precursor protein followed by the “self-processing” of a tyrosine side chain to trihydroxyphenylalanine quinone (TPQ) or the cross-linked lylsyl tyrosine quinone (LTQ) (1). The second class of cofactors is derived from active site tryptophans and depends on an exogenous family of gene products to produce the active site tryptophanyl tryptophan quinone (TTQ) and cysteinyli tryptophan quinone (CTQ). Finally, there is the prokaryotic cofactor pyrroloquinoline quinone (PQQ), which is formed and then excised from a peptide to generate a “stand alone” cofactor. The CTQ-containing amine dehydrogenase and the PQQ biosynthetic operons contain uncharacterized enzymes with a conserved Cys–X–X–X–Cys–X–X–X–Cys motif that is specific to members of the radical S-adenosyl-L-methionine (SAM) enzyme family (2, 3). In this report, we document the first evidence of an active radical SAM enzyme in the biogenesis pathway leading to an amino acid-derived quino-cofactor.

PQQ is a noncovalently bound cofactor dominantly utilized by alcohol and sugar dehydrogenases localized in the periplasm of Gram-negative bacteria (4–6). The electrons obtained upon reduction of PQQ to PQH<sub>2</sub> are subsequently transferred to an electron transport chain responsible for ATP production (7). Certain bacteria, such as *Escherichia coli*, are unable to themselves produce PQQ, and PQQ has been designated as a prokaryotic vitamin in such cases (7). The role of PQQ in mammals is much more controversial. PQQ has been found in high concentrations in breast milk, shown to be an essential nutrient for proper growth and development in mice, and even suggested to be a novel B vitamin (8–10). These observations have stoked considerable interest in its physiological function and have highlighted the importance of understanding the biosynthetic pathway in vivo.

Insights into the PQ biosynthetic pathway have emerged piecemeal over the past two decades. A major breakthrough was...
the demonstration that PQQ is formed from the fusion of glutamate and tyrosine (Scheme 1) (11, 12). This observation paralleled the discovery of genes involved in PQQ biogenesis (13–15). In Klebsiella pneumoniae, there are six genes (designated pqqA–F) located in the pqq operon (15). pqqA encodes a 23-residue peptide (PqqA) with a strictly conserved glutamate and tyrosine that is believed to be the substrate for PQQ biogenesis (15). PqqC catalyzes the final step in PQQ biogenesis, which is the eight-electron oxidation and ring cyclization of 3a-(2-amino-2-carboxyethyl)-4,5-dioxo-4,5,6,7,8,9-hexahydroquinoline-7,9-dicarboxylic acid (AHQQ) (16, 17). Although no definitive functions for PqqB and PqqF have been experimentally demonstrated, sequence alignments suggest that both proteins may function as proteases. PqqD is a small 10 kDa protein that has no homology to other proteins in the Protein Data Bank, and PqqE contains a conserved cysteine motif that is present in proteins known as radical SAM enzymes (18).

The most detailed mechanistic information in the biosynthetic pathway describes the final step for PQQ formation catalyzed by PqqC (19), with little being known about the preceding chemical steps in biogenesis. An in vivo study of the genes from K. pneumoniae suggests that PqqA, PqqD, and PqqE are critical for the first steps in PQQ biogenesis (16). We now present the first in vitro characterization of one of these proteins, the radical SAM enzyme PqqE. As described herein, PqqE is most homologous to the radical SAM enzyme MoaA (18% identical and 38% similar to MoaA from Mycobacterium tuberculosis) (20). MoaA, which is involved in molybdenum cofactor biosynthesis, possesses two [4Fe-4S] clusters, one at its N-terminus and one at its C-terminus (21). The Fe–S cluster at the N-terminal domain provides a site for SAM binding and activation, whereas the second cluster at the C-terminus appears to play a role in anchoring and positioning of the 5′-GTP substrate. Spectroscopic and physical measurements of PqqE confirm two [4Fe-4S] clusters that display spectroscopic properties nearly identical to those found in other radical SAM enzymes. Importantly, these PqqE preparations are shown to reductively cleave SAM to methionine and 5′-deoxyadenosyl radical in an uncoupled reaction, and that the enzyme is capable of multiple turnovers. These studies raise many provocative questions regarding the coupling of a radical SAM enzyme to substrate oxidation and open up, for the first time, the full characterization of radical SAM enzymes in quino-cofactor biogenesis.

**EXPERIMENTAL PROCEDURES**

*Materials.* All chemicals and reagents were purchased from Sigma-Aldrich, Mallinckrodt, Acros, or Fisher. Chemicals and reagents were purchased at the highest purity available and used without further purification. pET24b, pET28b, and BugBuster were purchased from Novagen. *E. coli* BL21(DE3) and XL-1 Blue competent cells were purchased from Stratagene. All restriction enzymes (NdeI and BamHI) used for cloning reactions were purchased from New England BioLabs; calf intestinal alkaline phosphatase was from Invitrogen, and high-fidelity PFU polymerase and T7 DNA ligase were from Roche. Bradford Assay reagents were purchased from Bio-Rad. Bovine serum albumin was from Pierce. Ni²⁺-nitrilotriacetic acid (Ni-NTA) agarose resin was from Qiagen, and Q-Sepharose and Sephacryl S-300 were from Sigma.

Plasmids pPH149 and pPH151 containing the *E. coli* IscSUAHscBA-Fd and *E. coli* suf ABCDSE genes, respectively, were generously donated by P. Hänzelmann from the Institute for Structural Biology at the University of Würzburg (Würzburg, Germany). Plasmid pBCP-165 containing the pqq operon from *K. pneumoniae* was originally made in the laboratory of P. W. Postma (University of Amsterdam, Amsterdam, The Netherlands) and donated by R. Rucker from University of California, Davis. A clone containing the *sas2* gene from *E. coli* [TB1 (pUC18: *sas2*)] was generously donated by C. Roessner from the laboratory of I. Scott (Texas A&M University, College Station, TX).

**General Methods.** All inert atmosphere work was conducted in an 855-AC-controlled atmosphere chamber from Pias-Labs, Inc. (Lansing, MI). For inert atmosphere work, we made all buffer solutions anaerobic by purging solutions with argon for 1 mL/min. Reagents were either either prepared as anaerobic buffers or brought into the box in crystalline powder form and then reconstituted with anaerobic buffer. DNA sequencing was performed at the DNA sequencing facility at the University of California (Berkeley, CA). N-Terminal sequencing was conducted at the Stanford PAN facility. Iron and sulfide analyses were conducted using methods described by Beinert (22, 23). Protein concentrations were calculated using the Bradford assay. UV–vis spectra were recorded on either a Hewlett-Packard 8452 diode array spectrophotometer or a Cary 50 Bio spectrophotometer. UV–visible measurements were performed in long stem quartz cuvettes (Starna) sealed with a rubber septum. PCR’s were conducted on a PTC-200 Peltier thermal cycler (MJ Research).

**Cloning of pqqE from pBCP-165.** pBCP-165 was originally generated in the laboratory of P. W. Postma (15). The gene sequence for the pqq operon from *K. pneumoniae* has been deposited in the NCBI database and used as a reference for comparing DNA and protein sequencing information. The open reading frame for pqqE starts at base 3023 in the operon; however, this start codon is not the typical ATG codon found for most bacterial genes. Position 3023 in the deposited sequence is listed as a guanine base and was mutated to an adenosine to bring into the box in crystalline powder form and then reconstituted with anaerobic buffer. DNA sequencing was performed at the DNA sequencing facility at the University of California (Berkeley, CA). The gene for the pqq operon from *K. pneumoniae* has been deposited in the NCBI database and used as a reference for comparing DNA and protein sequencing information. The open reading frame for pqqE starts at base 3023 in the operon; however, this start codon is not the typical ATG codon found for most bacterial genes. Position 3023 in the deposited sequence is listed as a guanine base and was mutated to an adenosine to generate the ATG start site when appropriate primers were designed for the cloning of pqqE.

pqqE was cloned into the *NdeI* and *BamHI* restriction sites of pET28b (or pET24b) using standard cloning techniques. The following primers (obtained from Operon) were used to clone...
“ppqE out of pBCP-165: 5′-CGCATTACATAGCCAGGAGTAAACCCCATGCTACCTCG-3′ and 5′-CGCCGATCCTTACAGGTCCCGGTTTGAGATACGCTG-3′. Underlined bases show engineered restriction sites for Ndel and BamHI, respectively. The plasmids (named pET28b-ppqE and pET24b-ppqE) were isolated and sequenced using the T7 promoter and terminator primers, respectively.

After isolation, pET28b-ppqE was cotransformed into E. coli BL21(DE3) competent cells with either pPH149 or pPH151. The bacteria were plated onto LB agar plates containing both kanamycin (50 μg/mL) and chloramphenicol (50 μg/mL) and grown at 37 °C overnight. The following day, several colonies were picked and described in the Supporting Information for anaerobic growth and induced under identical anaerobic growth conditions as the wild-type that the medium also contained 50 μg/mL kanamycin (50 μg/mL) and grown in liquid LB medium at 37 °C for 18 h. Conditioned supernatants were pooled, reduced, and 50 μg/mL glyceral, immediately frozen in liquid nitrogen, and stored at −80 °C until further use.

Expression and Purification of PqqE. (i) Developmental Work. Initial efforts aimed at the expression and purification of PqqE were focused on either aerobic growth of the transformed E. coli cells, followed by purification of enzyme from inclusion bodies and chemical reconstitution, or expression of PqqE in E. coli grown anaerobically and subsequent isolation of the enzyme from soluble supernatants under aerobic conditions. These methods, which proved to be unsatisfactory for various reasons, are summarized in the Supporting Information.

(ii) N-Terminal HisTag-Containing PqqE. E. coli BL21(DE3) cells containing pET28b-ppqE and pPH151 were grown and induced under identical anaerobic growth conditions as described in the Supporting Information for anaerobic growth of E. coli BL21(DE3) containing pET28b-ppqE with the exception that the medium also contained 50 μg/mL of chloramphenicol.

For the purification of His8 tag-containing PqqE, all steps were performed under strictly anaerobic conditions in an inert atmosphere glovebox. Cell pellets (cell paste yields were approximately 4 g/10 L of LB medium) were brought into the glovebox and lysed for 30 min with Bugbuster in 50 mM Tris (pH 7.9), 1 mM DTT, 300 mM NaCl, 10 mM imidazole, and 5 μL of benzonase nuclease. The lysates were loaded into anaerobic centrifuge tubes (Beckman) and cleared via centrifugation at 15000g and 4 °C for 20 min. The lysates were then brought back into the glovebox and the reddish brown supernatants loaded onto a column containing 50 mL of Ni-NTA resin equilibrated with the same buffer (column size, 12 in. long and 2.5 in. wide). The bound protein was then washed with 100 mL of loading buffer followed by two, 100 mL stepwise gradient washes containing the same buffer but with 25 mM imidazole in the first and 50 mM imidazole in the second. PqqE was then eluted off the column with an increase in the imidazole concentration to 300 mM. The brownish red fractions were pooled and concentrated to approximately 5 mL using a 30 kDa Amicon membrane.

After concentration, the imidazole was removed using a gel filtration PD-10 column equilibrated with 50 mM Tris (pH 7.9), 1 mM DTT, and 300 mM NaCl. The protein was collected off the PD-10 column and concentrated to approximately 10 mg/mL with a 30 kDa Amicon membrane. Aliquots (200 μL) were loaded into glass vials (Agilent) equipped with rubber caps, brought out of the glovebox, and immediately frozen in liquid nitrogen. The protein was stored at −80 °C until further use.

(iii) Preparation of S-Adenosyl-1-methionine. The enzymatic synthesis, purification, and characterization of S-adenosyl-1-methionine (SAM) largely followed literature protocols (24–26). Details can be found in the Supporting Information.

(iv) Determination of Enzyme Activity for PqqE using LC–MS. LC–MS was performed on an Agilent LC 1100 series instrument equipped with an HP LC/MSD electrospray ion source and quadrupole mass spectrometer. Reversed-phase HPLC was performed on a Jupiter Proteo 4 μm, 90 Å, 250 mm × 4.60 mm C18 column (Phenomenex), and the following elution conditions were used for identifying the enzymatic products of the radical SAM reaction in PqqE: flow rate of 0.4 mL/min, 0.05% formic acid, 1.0% acetonitrile in water for 10 min, followed by a linear gradient from 1 to 30% acetonitrile over 30 min and ending with a linear gradient increase from 30 to 90% acetonitrile over the next 30 min. The quadrupole mass spectrometer was operated in the positive ion mode using the scan function from m/z 100 to 1000 (fragmentor, 70; gain, 1.0; threshold, 150; step size, 0.1). The elution profiles were recorded at 215 and 260 nm (10 nm bandwidth and referenced at 350 nm).

Samples for LC–MS were generated as follows. A stock solution of PqqE (~125 μM) in (pH 7.9) 50 mM Tris, 1 mM DTT, 300 mM NaCl, and 20% glycerol was prepared anaerobically and incubated with a 10-fold excess of sodium dithionite (DTT) for 10 min. Aliquots (100 μL) of reduced PqqE were diluted with an equal volume of buffer, and the reaction was initiated by a 10-fold addition of SAM (600 μM). The reaction mixture was left for 2 h and then the reaction quenched by the addition of neat formic acid to give a final concentration of 5% (v/v). The samples were pelleted via centrifugation and brought out of the glovebox, and 80 μL volumes were injected onto the LC–MS instrument.

(v) Quantification of 5′-Deoxyadenosine Formation. High-performance liquid chromatography (HPLC) was conducted on a Beckman system equipped with a diode array detector and operated by 32 Karat version 8.0. The software was also used for data collection and analysis. Reversed-phase HPLC was performed on a Jupiter Proteo 4 μm, 90 Å, 250 mm × 4.60 mm C18 column. The following elution conditions were used for quantifying the time-dependent formation of 5′-deoxyadenosine: flow rate of 1 mL/min, 0.05% formic acid, 1.0% acetonitrile in water for 30 min, followed by a linear gradient from 1 to 100% acetonitrile over 10 min. Spectra were recorded at 215 and 260 nm. Under these conditions, SAM and methionine elute in the dead time of the instrument (~5.0 min) and 5′-deoxyadenosine elutes at 23 min. The concentration of 5′-deoxyadenosine was determined via integration of the area under the peak and comparison to a linear calibration curve from a series of standards at known concentrations.

All assays were performed in an anaerobic chamber. For a typical assay, PqqE was prepared as a stock solution at 39 μM in 50 mM Tris (pH 7.9), 1 mM DTT, 300 mM NaCl, 20% glycerol, and 1 mM dithionite. The stock solution was left to incubate at room temperature for approximately 10 min, at which point SAM was added to the reaction mixture for a final concentration of 420 μM. The samples were left to incubate for various lengths of time and the reactions quenched with neat formic acid to a final concentration of 5%. The samples were pelleted via centrifugation and 100 μL aliquots injected into the HPLC instrument using the procedure outlined above.

(vi) EPR Characterization. Preparation of all samples was conducted in an anaerobic glovebox. In brief, a 1.5 mL stock solution of PqqE was prepared with 169 μM PqqE in 50 mM Tris (pH 7.9), 1 mM DTT, 300 mM NaCl, and 20% glycerol. The samples were cleared of precipitate at 14000 rpm for 10 min, diluted to a final concentration of 115 μM, loaded into EPR tubes
equipped with rubber septa, brought out of the glovebox, and immediately frozen in liquid nitrogen. To prepare the reduced PqqE sample, the stock solution was mixed with a 10-fold excess of sodium dithionite (1.15 mM) and left to incubate for approximately 10 min before being loaded into the EPR tubes. The reduced samples that contained SAM were prepared in a manner identical to that of the reduced PqqE sample, except a 10-fold excess of SAM (1.15 mM) was added after the 10 min incubation with dithionite. The total incubation time for the reduced PqqE with SAM was approximately 5 min.

EPR experiments were conducted at the CalEPR center at the University of California, Davis, using a Bruker ECS106 X-band spectrometer equipped with a TF100 cavity (ER4102ST) resonating at ~9.5 GHz and an Oxford ESR900 helium cryostat with an ITC503 temperature controller. The field modulation was 1.0 mT at 100 kHz. All spectra were background-corrected using a control without PqqE and SAM. Spin quantitation was achieved under nonsaturating conditions (40 K, 1 mW) with the double integrals of the EPR spectra using a series of Cu²⁺-EDTA solutions at concentrations between 22 and 177 μM for calibration.

(vii) Synthesis and Purification of PqqA. PqqA (WKKP-AFIDLRLGLEVTLYISNR) was synthesized on an ABI 431 synthesizer via standard Fmoc chemistry on a solid-phase support. The peptide was cleaved from the PEG resin under argon using reagent K (trifluoroacetic acid, phenol, water, thioanisole, and ethanedithiol), filtered through a fritted glass funnel, and precipitated into a bath of ice-cold diethyl ether. The precipitated peptide was centrifuged at 15000 g for 20 min at 4 °C and then washed several times with cold ether. The pellet was vacuum-dried overnight and then reconstituted with 50% acetonitrile in water and 0.1% trifluoroacetic acid. The reconstituted peptide was lyophilized to dryness and then stored at ~20 °C until it was purified.

The crude peptide was purified via reversed-phase HPLC using a semipreparative Luna 5 μm, 100 Å, 250 mm × 10.00 mm C₁₈ column (Phenomenex). The following elution conditions were used for the purification of PqqA: flow rate of 2 mL/min, 0.05% formic acid, 1.0% acetonitrile in water for 5 min, followed by a linear gradient from 1 to 15% acetonitrile over 5 min, followed by a linear gradient from 15 to 65% acetonitrile over 50 min, and finishing with a wash from 65 to 100% acetonitrile over 10 min. Spectra were recorded at 215 and 280 nm. Under these conditions, PqqA elutes at approximately 38% acetonitrile. The fractions were analyzed via mass spectrometry and appropriate fractions pooled and lyophilized to dryness. The final yield for purified peptide was approximately 60%. A detailed description of the methods and instrumentation used for obtaining high-resolution mass spectra of the purified peptide and the peptide subject to reaction conditions is given in the Supporting Information.

MALDI-TOF Mass Spectrometry Assay Used To Characterize Enzyme-Induced Modifications of PqqA. Purified PqqA was brought into the glovebox and anaerobically reconstituted with 50 mM Tris (pH 7.9) and 300 mM NaCl. After purification, the peptide had extremely poor solubility in water or buffer, and therefore, the concentration of the soluble peptide in solution was determined via an estimated extinction coefficient at 280 nm of 6970 M⁻¹ cm⁻¹ (estimation based on a linear combination of known extinction coefficients for aromatic residues). Saturated solutions of PqqA gave a final concentration of ~200 μM.

All assays were performed in an anaerobic chamber. For a typical assay, PqqE was prepared as a stock solution at 90 μM in 50 mM Tris (pH 7.9), 1 mM DTT, 300 mM NaCl, 20% glycerol, and 1 mM dithionite. The stock solution was left to incubate at room temperature for approximately 10 min, at which point 50 μL aliquots were taken out and added to an equal volume of buffer containing a 10-fold excess of SAM (450 μM). The samples were then mixed with stoichiometric amounts of PqqA (45 μM), left to incubate for various lengths of time, and quenched with neat formic acid to a final concentration of 5% (v/v). The reaction mixtures were pelleted via centrifugation, and approximately 40 μL of the sample solution was loaded onto a C₈ zip tip (Millipore) and washed several times with water. The peptide/protein mixture was then eluted onto the MALDI plate with 80% acetonitrile, 0.05% formic acid, and ~1 mM sinapinic acid.

MALDI-TOF mass spectrometry was performed on a Voyager DE Pro instrument (Applied Biosystems). The samples were analyzed in positive ion reflectron mode (accelerating voltage, 20000 V; grid, 75; guide wire, 0; delay time, 130 ns; number of shots per spectrum, 100; mass range, 500–5000; low mass gate, 500). Calibration curves were generated using the mass spectrometry standards (Sigma), ProteoMass ACTH fragment 18–39 (2464.1989), and insulin-oxidized B chain (3494.6513).

RESULTS

Initial Efforts Aimed at Aerobic Expression of PqqE. Other investigators have reported the successful expression of radical SAM enzymes under aerobic conditions (27). However, when E. coli cells containing the pqqE expression vector [pET24b (see Experimental Procedures)] were grown and induced under aerobic conditions, PqqE was found solely in insoluble inclusion bodies. A purification scheme consisting of protein refolding followed by anion exchange and gel filtration resulted in an enzyme that was >95% pure [according to SDS–PAGE analysis (data not shown)]. These preparations of PqqE were purged with argon, brought into an inert atmosphere glovebox, and anaerobically reconstituted in the presence of DTT with a 10-fold excess of Fe²⁺/Fe³⁺ and S²⁻ ions. After the protein had been desalted through a PD-10 column, the enzyme exhibited a red-brown hue indicative of Fe–S cluster formation.

The UV–vis spectrum of reconstituted PqqE had absorbance maxima at 390 and 420 nm and labile iron and sulfide contents of 8.5 ± 0.8 and 6.7 ± 0.6 mol/monomer of protein, respectively. The EPR spectrum (data not shown) of reconstituted PqqE appeared to be consistent with a [4Fe-4S]⁺ cluster (g = 2.05 and 1.94); we had expected this form of the enzyme to be EPR silent because of the typical diamagnetic [4Fe-4S]³⁺ oxidation state found in other radical SAM enzymes (28, 29). Reduction of the enzyme with a 10-fold excess of dithionite had little effect on the EPR or UV–vis spectrum, even in the presence of SAM. The anaerobic reconstitution of apo-PqqE with iron and sulfide ions was performed in the presence of 1 mM DTT, and we had originally suspected that the redox potential of the cluster(s) might have permitted reduction to the [4Fe-4S]⁻ state by the excess dithiothreitol (g² = −0.33 V at pH 7). This would be consistent with the fact that no change in the spectrum is observed upon addition of a 10-fold excess of dithionite. It is clear from the spectral shape and g values that this species is not a [3Fe-4S]⁻ cluster (30), but whether the species is a [4Fe-4S]⁻ or [4Fe-4S]³⁻ cluster is still unknown. Regardless, these spectral characteristics are not consistent with those found for other radical SAM enzymes and, thus, dictated a new approach to obtaining active enzyme.
A soluble expression system was developed for PqqE by growing and inducing the cells under anaerobic conditions, eliminating the need to refold the protein. However, aerobic purification of these preparations resulted in physical and spectroscopic properties (after anaerobic chemical reconstitution) similar to that of refolded PqqE. Further, despite extensive efforts, these forms of the protein failed to exhibit activity toward SAM cleavage. Therefore, it seemed mandatory to use a strictly anaerobic expression and purification protocol, which has been shown to enhance the activity for other radical SAM enzymes (27, 28, 31).

Cloning, Expression, and Anaerobic Purification of N-Terminal His₆ Tag-Containing PqqE. In the context of maintaining strictly anaerobic conditions during cell growth and purification, a His₆ tag was appended to the N-terminus of PqqE (see Experimental Procedures). Additionally, *E. coli* BL21(DE3) cells were cotransfected with vectors expressing the *E. coli suf* ABCDSE (*pPH151*) genes as well as *pqqE*, with the goal of facilitating Fe–S cluster repair and assembly (32, 33). In a previously published report, *pPH151* was cotransformed with a gene encoding the radical SAM protein MOCS1A into *E. coli* BL21(DE3) cells (27), resulting in a 1.7–2.2-fold increase in the amount of soluble MOCS1A; a similar 2-fold increase for soluble PqqE was obtained in our case.

The use of strictly anaerobic conditions (for growth, induction, and protein purification, see Experimental Procedures) resulted in a protein that was >90% pure (Figure 1) at an average yield of 0.5–1.5 mg/L of LB medium. The mobility of PqqE on SDS gel electrophoresis is consistent with a calculated molecular mass for His₆-PqqE of 44.9 kDa. There is a small impurity still present after anaerobic purification that has a molecular mass of approximately 30 kDa and has been tentatively assigned to a FK506 binding protein (FKBP)-type peptidyl-prolyl cis–trans isomerase. N-Terminal sequencing of the purified protein preparation unveiled an impurity with a calculated molecular mass for His₆-PqqE of 44.9 kDa. There is a small impurity still present after anaerobic purification that has a molecular mass of approximately 30 kDa and has been tentatively assigned to a FK506 binding protein (FKBP)-type peptidyl-prolyl cis–trans isomerase. N-Terminal sequencing of the purified protein preparation unveiled an impurity with a calculated molecular mass for His₆-PqqE of 44.9 kDa. There is a small impurity still present after anaerobic purification that has a molecular mass of approximately 30 kDa and has been tentatively assigned to a FK506 binding protein (FKBP)-type peptidyl-prolyl cis–trans isomerase. N-Terminal sequencing of the purified protein preparation unveiled an impurity with a calculated molecular mass for His₆-PqqE of 44.9 kDa. There is a small impurity still present after anaerobic purification that has a molecular mass of approximately 30 kDa and has been tentatively assigned to a FK506 binding protein (FKBP)-type peptidyl-prolyl cis–trans isomerase. N-Terminal sequencing of the purified protein preparation unveiled an impurity with a calculated molecular mass for His₆-PqqE of 44.9 kDa. There is a small impurity still present after anaerobic purification that has a molecular mass of approximately 30 kDa and has been tentatively assigned to a FK506 binding protein (FKBP)-type peptidyl-prolyl cis–trans isomerase. N-Terminal sequencing of the purified protein preparation unveiled an impurity with a calculated molecular mass for His₆-PqqE of 44.9 kDa.

Anaerobically purified PqqE has a brown color indicating the presence of Fe–S clusters. Although the enzyme is stable for days under anaerobic conditions and in the presence of DTT, it rapidly decomposes and eventually precipitates in the presence of oxygen. This form of enzyme is not amenable to anaerobic reconstitution with an excess of ferrous or sulfide ions as these treatments resulted in fast precipitation of the enzyme. The iron and sulfide contents found in the anaerobic preparations are 10.4 ± 0.9 and 7.0 ± 1.0 mol/monomer of protein, respectively.

Spectroscopic Characterization of PqqE. PqqE has an optical absorption spectrum (Figure 2) similar to that found for other radical SAM enzymes with absorbance maxima at 390 and 420 nm and a shoulder at 550 nm (27, 28, 31). The broad absorption spectrum undergoes complete and immediate bleaching in the presence of a 10-fold excess of sodium dithionite and then slowly re-forms upon being exposed to air. The UV–vis spectrum, extinction coefficients, and iron and sulfide analyses indicate that PqqE grown and isolated anaerobically contains multiple Fe–S clusters, the nature of which was further probed via EPR.

The continuous wave X-band EPR spectra of as-isolated PqqE, PqqE (with and without glycerol) reduced with dithionite (DT), and reduced PqqE with a 10-fold excess of SAM (with and without glycerol) are shown in Figure 3. As-isolated PqqE is nearly EPR silent in the g = 2 region, consistent with what is expected for a diamagnetic [4Fe-4S]²⁺ cluster (30). The spectrum does contain a small nearly isotropic S = 1/2 resonance at g = 2.01, similar to that found for a [3Fe-4S]⁺ cluster (20, 21). At 40 K, this signal saturates easily with a half-saturation power (P₁/₂) of 3 mW. Quantitation of this signal gives approximately 0.01 spin/monomer of protein, showing that this signal is due to only a minor component of the enzyme. There is also a very weak g = 4.3 signal observed (data not shown) that is characteristic of adventitiously bound ferric ions (30).

When PqqE is reduced with a 10-fold excess of dithionite, new features appear in the EPR spectrum. There is a strong rhombic signal with g values of 2.06, 1.96, and 1.91 (Figure 3) that have been attributed to the reduced [4Fe-4S]⁺ species (29, 30). The
shape and position of these signals are nearly identical to those observed in the radical SAM enzyme pyruvate-formate lyase-activating enzyme (PFL-AE) (34) and very similar to those found for MOCSIA (27). Spin quantitation gives ~0.17 spin/monomer of protein. Unlike the oxidized enzyme, this species does not saturate at 40 K ($P_{1/2} > 200$ mW).

Interestingly, there is only a moderate change in the EPR spectrum upon addition of SAM (Figure 3). Also, the spin quantitation shows that the number of spins is unchanged. This behavior is very different from that found for PFL-AE, where it has been demonstrated that the presence of SAM significantly changes the EPR spectrum of the reduced $[4\text{Fe}-4\text{S}]^+$ cluster (34). There has also been a report on anaerobic ribonucleotide reductase that shows a change of the axial $g$ = 1.96 signal to a more rhombic one in the presence of SAM (29). The effect of SAM on the EPR spectrum of the reduced $[4\text{Fe}-4\text{S}]^-$ cluster was further probed by measuring EPR samples without glycerol. Glycerol is known to be able to coordinate to the $[4\text{Fe}-4\text{S}]^-$ cluster, inducing spectroscopic changes similar to that for SAM coordination (29). When glycerol was omitted from the samples, the EPR spectrum of the reduced enzyme looked nearly identical to that which contained glycerol (Figure 3). However, the reduced enzyme with SAM (without glycerol) looked slightly different, with the $g$ = 2.00 feature more pronounced than when both SAM and glycerol were present in the sample. There were also small changes in the signal shape at $g$ = 1.96.

**Demonstration of Enzymatic Activity for PqqE.** PqqE was incubated anaerobically at room temperature with a 10-fold excess of dithionite and SAM and left for 2 h. The reaction was then quenched with formic acid and the mixture analyzed by LC−MS. A second reaction (without PqqE) was run under identical conditions as a control reaction. In the control reaction (Figure 4A), the major species elutes immediately after the solvent front at approximately 8 min and has been assigned to S-adenosylmethionine. Standards of SAM elute at an identical time and give the expected mass-to-charge ratio for the singly charged positive ion at mass/charge = 299.5 ($C_{15}H_{23}N_{2}O_{3}S^+$). Two other peaks are observed in the control reactions at approximately 10 min (mass/charge = 293.5) and 33.5 min (mass/charge = 298.5), which are attributable to the singly charged ions of adenine ($C_{5}H_{5}N_{5}$ + $H^+$) and methylthioadenosine ($C_{11}H_{14}N_{5}O_{3}S^+$), respectively. These compounds are typical thermal decomposition products of SAM and have been observed by others (35).

The same peaks are also observed in the reaction mixture containing PqqE, but two new features emerge from the chromatogram. The peak eluting at 8 min (assigned to SAM) has decreased in intensity, and there is a new species that elutes at 28 min (see Figure 4B). This ion has an $m/z$ of 252.4 ($C_{10}H_{13}N_{5}O_{3}$ + $H^+$) and is due to 5'-deoxyadenosine, as standards of 5'-deoxyadenosine elute at an identical time and give an identical mass-to-charge ratio and isotope pattern. The other product of the radical SAM reaction is methionine. Although methionine absorbs weakly at 260 nm, it can be observed when the LC−MS spectrum is monitored at 215 nm. In this case (data not shown), a large peak was observed that eluted at approximately 12 min and had an $m/z$ of 150.2. This peak elutes at a time identical to those of standards of methionine and gives the expected mass-to-charge ratio and isotope pattern for the singly charged, protonated species ($C_{5}H_{11}NO_3S^+$ + $H^+$).

**PqqE Is Capable of Undergoing Multiple Turnovers.** Anaerobic incubation of PqqE with dithionite and SAM results in the production of methionine and 5'-deoxyadenosine. The amount of 5'-deoxyadenosine formed varied with time, and experiments were performed to determine the pseudo-first-order rate constant for the reaction. In these experiments, PqqE (39 $\mu$M) was reacted with an approximate 10-fold excess of both sodium dithionite (420 $\mu$M) and SAM (420 $\mu$M) at 23 ºC for various amounts of time, and then the reaction was quenched by the addition of formic acid and the mixture analyzed via RP-HPLC (protocols outlined in Experimental Procedures).

The time-dependent formation of 5'-deoxyadenosine is shown in Figure 5. The reaction is linear over 45 min, during which time PqqE undergoes approximately one turnover. The rate measured for the linear portion of this reaction was 0.417 ± 0.019 $\mu$M/min. When the enzyme concentration was decreased 2-fold (19 $\mu$M), the rate of 5'-deoxyadenosine was measured at 0.236 ± 0.0011 $\mu$M/min. These experiments clearly show an enzyme dependence of 5'-deoxyadenosine formation and give a pseudo-first-order rate constant for the uncoupled reaction ($k_{obs}$) of 0.011 ± 0.001 min$^{-1}$. The amount of 5'-deoxyadenosine formation was also found to be dependent on dithionite concentration, with multiple turnovers observed only when in excess of PqqE (i.e., greater than stoichiometric amounts).

**Incorporation of Deuterium into 5'-Deoxyadenosine during Turnover.** PqqE was exchanged into D$_2$O by gel filtration over a PD-10 column, and reactions were initiated and analyzed in a procedure identical to that described above. The chromatograms from the reactions in D$_2$O are nearly identical to those shown in Figure 4B. Peaks are observed at 8, 12, and 28 min, corresponding to SAM, methionine, and 5'-deoxyadenosine, respectively. Although the retention time for the products is identical to those observed in H$_2$O, the mass-to-charge ratio and isotope patterns are significantly different. The mass spectrum of 5'-deoxyadenosine formed from the radical SAM reaction of
PqqE in H\textsubscript{2}O is shown in Figure 6A. The expected \( m/z \) for the singly charged, protonated form of 5'-deoxyadenosine is 252.3 (C\textsubscript{10}H\textsubscript{12}N\textsubscript{5}O\textsubscript{3} + H\textsuperscript{+}). The species that elutes at 28 min has an \( m/z \) of 252.4, and its isotope pattern and elution time are identical to those of standards containing 5'-deoxyadenosine. When the reaction was conducted in D\textsubscript{2}O, the isotope distribution for the peak eluting at 28 min (Figure 6B) shifts with the largest abundance centered at 253.4. This change in isotopic distribution indicates the incorporation of a deuterium in 5'-deoxyadenosine (C\textsubscript{10}H\textsubscript{12}D\textsubscript{5}N\textsubscript{5}O\textsubscript{3} + H\textsuperscript{+}). Controls containing 5'-deoxyadenosine were incubated in D\textsubscript{2}O under identical conditions in the absence of PqqE and showed no evidence of deuterium incorporation in the mass spectra.

Analyses of Reaction Mixtures Containing Reduced PqqE, SAM, and PqqA. In vivo experiments conducted by others strongly suggest that PqqA is the substrate for PQQ biogenesis and that both PqqA and PqqE are required for the initial steps in PQQ formation (\textsuperscript{27}). PqqE isolated anaerobically contains multiple iron–sulfur clusters and yields an active enzyme capable of reductive cleavage of SAM. The next goal was to test whether the radical SAM activity in PqqE is coupled to the putative substrate, PqqA.

In these experiments, PqqE was anaerobically incubated with a 10-fold excess of dithionite for approximately 10 min. The protein was then mixed with a 10-fold excess of SAM, followed by the addition of stoichiometric amounts of PqqA. The addition of a large excess of peptide to protein was not possible as this caused fast precipitation of the protein. Controls containing reduced PqqE and PqqA without SAM were also run. The reaction mixtures were left for several hours, quenched by the addition of formic acid, and analyzed by mass spectrometry.

Confirmation of the intact peptide after HPLC purification was achieved via high-resolution mass spectrometry [calculated \( m/z \) of 2631.49, found (M + H\textsuperscript{+}) \( m/z \) of 2632.52]. When PqqA was incubated with reduced PqqE and SAM, the mass-to-charge ratio found via MALDI-TOF mass spectrometry after reaction (data not shown) was determined to be \( m/z \) 2632.4 (M + H\textsuperscript{+}). The spectrum revealed no modifications of PqqA and no differences between the control peptides and that for PqqA subjected to reaction conditions. Varying amounts of reducing agent, SAM, PqqE, PqqA, and time also resulted in nearly identical mass spectra. The reactions were analyzed via EPR, LC–MS and SDS–PAGE, and no new peaks were observed in the EPR spectra, chromatograms or gels. Finally, high-resolution mass spectrometry confirmed the identity of the intact peptide after reaction and failed to show any change in the mass-to-charge ratio before and after reaction. Although these reaction mixtures failed to show any modifications of PqqA, the products of the radical SAM reaction were still observed. The amount of 5'-deoxyadenosine formed in these reactions was also quantified and was similar to that measured without the peptide present.

DISCUSSION

The UV–vis spectrum, labile iron and sulfide analyses, and EPR spectra have provided a good framework for characterizing the nature of the Fe–S clusters present in PqqE. PqqE has strong absorption bands in the visible region with absorbance maxima at 390 and 420 nm and a shoulder at 350 nm, which rapidly and completely bleach in the presence of dithionite. The extinction coefficient at 420 nm is \( \sim 33000 \) M\textsuperscript{-1} cm\textsuperscript{-1}, more similar to what could be expected for two [4Fe-4S]\textsuperscript{2+} clusters (\([4Fe-4S]^{2+} + e_{410} \sim 15000\) M\textsuperscript{-1} cm\textsuperscript{-1}) as opposed to a [4Fe-4S]\textsuperscript{2+} cluster and a [2Fe-2S]\textsuperscript{2+} cluster ([2Fe-2S]\textsuperscript{2+} + e_{410} \sim 8000–10000 M\textsuperscript{-1} cm\textsuperscript{-1}) (26). The iron and sulfide analyses from these preparations were determined to be 10.4 ± 0.9 and 7.0 ± 1.0 mol/monomer of protein, respectively. These results implicate the presence of two [4Fe-4S] centers as has been reported for MOCS1 (27). Alignment of the sequence for PqqE from \textit{K. pneumoniae} with gene sequences for \textit{pqqE} and \textit{pqqF} from other sources indicates that the presence of two [4Fe-4S]\textsuperscript{2+} clusters is to be expected in all cases (Figure 7). The origin of the excess labile iron remains unresolved and may be a consequence of a weak binding of iron to the N-terminal His\textsubscript{6} domain. However, the possibility that the excess iron is part of a mononuclear iron site or plays some type of structural role in PqqE cannot be ruled out by our data.

Continuous wave X-band EPR of PqqE isolated anaerobically is consistent with the hypothesis that the as-isolated enzyme primarily contains two diamagnetic [4Fe-4S]\textsuperscript{2+} clusters. The presence of a small axial g = 2.01 signal (0.01 spin per monomer) has been attributed in other radical SAM enzymes to a [4Fe-4S]\textsuperscript{2+} cluster and a [2Fe-2S]\textsuperscript{2+} cluster ([2Fe-2S]\textsuperscript{2+} + e\textsubscript{410} \sim 8000–10000 M\textsuperscript{-1} cm\textsuperscript{-1}) (26). The iron and sulfide analyses from these preparations were determined to be 10.4 ± 0.9 and 7.0 ± 1.0 mol/monomer of protein, respectively. These results implicate the presence of two [4Fe-4S]\textsuperscript{2+} clusters as has been reported for MOCS1 (27). Alignment of the sequence for PqqE from \textit{K. pneumoniae} with gene sequences for \textit{pqqE} and \textit{pqqF} from other sources indicates that the presence of two [4Fe-4S]\textsuperscript{2+} clusters is to be expected in all cases (Figure 7). The origin of the excess labile iron remains unresolved and may be a consequence of a weak binding of iron to the N-terminal His\textsubscript{6} domain. However, the possibility that the excess iron is part of a mononuclear iron site or plays some type of structural role in PqqE cannot be ruled out by our data.

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cluster, followed by further reduction to a [4Fe-4S]$^+$ cluster. Although we propose that the loss of the $g = 4.3$ and 2.01 spectral changes upon reduction is due to the conversion of small amounts of the [3Fe-4S]$^+$ cluster to the final [4Fe-4S]$^+$ cluster, we cannot rule out the conversion of some unspecified paramagnetic species to a diamagnetic form.

EPR has also been used as a valuable tool to probe SAM coordination to the reduced cluster (25). The binding of SAM was investigated in the absence and presence of glycerol, as glycerol has been previously shown to bind to the reduced [4Fe-4S]$^+$ cluster and induce spectral changes similar to that of the binding of SAM (29). When glycerol was omitted, there are small perturbations in the EPR spectrum in the presence of SAM with the $g = 2.00$ feature becoming more pronounced and the $g = 1.96$ feature changing shape. This indicates that the $g = 2.00$ signal might be part of a rhombic minority species with $g$ values of 2.00, ~1.95, and 1.92. When SAM was added to reduced PqqE in the presence of glycerol, only small perturbations in the EPR spectrum were observed with the shape and $g$ values nearly identical to those without SAM (see Figure 3). These results suggest that either SAM is not coordinated to the [4Fe-4S]$^+$ cluster or it is coordinated only weakly. Whether this species is chemically relevant is unclear.

The spectroscopic and physical measurements described above indicate that anaerobically purified PqqE contains multiple Fe-S clusters, with the predominant form of the isolated enzyme containing two [4Fe-4S]$^{2+}$ clusters. The EPR and UV–vis spectra suggested that at least one of the clusters would be redox active with characteristics similar to those found for other radical SAM enzymes. The role of the second cluster is still unclear, but in analogy to that for MOCS1A, it may play a role in positioning the substrate for H-atom abstraction.


<table>
<thead>
<tr>
<th>Cluster</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>K. pneumonia</td>
<td>WMSDFCRSDEKEKDFSCRCACFMTCSADNADVEWGSKSP</td>
</tr>
<tr>
<td>M. petroleiphilum</td>
<td>WMSDFCRSDEKEKDFSCRCACFMTCSADNADVEWGSKSP</td>
</tr>
<tr>
<td>C. psychrerythraea</td>
<td>WMSDFCRSDEKEKDFSCRCACFMTCSADNADVEWGSKSP</td>
</tr>
<tr>
<td>M. capsulatus</td>
<td>WMSDFCRSDEKEKDFSCRCACFMTCSADNADVEWGSKSP</td>
</tr>
<tr>
<td>N. oceani</td>
<td>WMSDFCRSDEKEKDFSCRCACFMTCSADNADVEWGSKSP</td>
</tr>
<tr>
<td>P. fluorescens</td>
<td>WMSDFCRSDEKEKDFSCRCACFMTCSADNADVEWGSKSP</td>
</tr>
<tr>
<td>P. putida</td>
<td>WMSDFCRSDEKEKDFSCRCACFMTCSADNADVEWGSKSP</td>
</tr>
<tr>
<td>P. syringae</td>
<td>WMSDFCRSDEKEKDFSCRCACFMTCSADNADVEWGSKSP</td>
</tr>
<tr>
<td>E. coli AT1b</td>
<td>WMSDFCRSDEKEKDFSCRCACFMTCSADNADVEWGSKSP</td>
</tr>
</tbody>
</table>
When PqqE was anaerobically incubated with an excess of SAM and dithionite, methionine and 5′-deoxyadenosine were formed as products of the enzymatic reaction (Figure 4). The cleavage of SAM to methionine and 5′-deoxyadenosine radical is energetically unfavorable (bond dissociation energy for the C–S bond of 60 kcal/mol) and is possible only via enzymatic activation (Scheme 2) (38, 39). This radical is highly reactive and generally initiates the next step in catalysis via abstraction of a H-atom from the protein followed by (1) abstraction of a H-atom from the protein followed by (2) reduction of the 5′-deoxyadenosyl radical by dithionite to form 5′-deoxyadenosyl radical. When the reaction was performed in D2O and the reaction mixtures were analyzed via LC–MS, an increase of one mass unit in the mass-to-charge ratio of 5′-deoxyadenosine (Figure 6) indicates that solvent-derived deuterium has been incorporated into the product.

The quenching of the 5′-deoxyadenosyl radical to form 5′-deoxyadenosine could occur through two different mechanisms: (1) abstraction of a H-atom from the protein followed by fast outer-sphere electron transfer to the protein-derived radical or (2) reduction of the 5′-deoxyadenosyl radical by dithionite to the corresponding carbanion followed by rapid abstraction of a proton from the solvent. Although no organic radicals were observed in the EPR, the first mechanism cannot be ruled out because outer-sphere electron transfer to the protein-derived radical may be so fast that it is undetectable via EPR under our assay conditions; further, abstraction of a H-atom from a solvent exchangeable position on PqqE would lead to the same deuterated product as direct transfer of a proton from solvent. The “uncoupled” production of 5′-deoxyadenosine in several other radical SAM enzymes has been observed previously (40, 41), but to the best of our knowledge, this is the first example of an uncoupled radical SAM reaction that leads to the incorporation of a solvent deuterium into 5′-deoxyadenosine. This may be suggestive of a solvent-accessible amino acid residue in the active site of PqqE that is not shared with other radical SAM enzymes. At this juncture, we are not sure how the radical is quenched, but the aggregate experiments show that a 5′-deoxyadenosyl intermediate is formed from reductive cleavage of SAM by PqqE and are consistent with the proposed mechanism for radical SAM enzymes (38, 39). The fact that deuterium incorporation is only approximately 50% can be attributed to incomplete buffer exchange in PqqE (see Experimental Procedures) and dilution of D2O with protiated glycerol added at a mole ratio of 8% to reaction mixtures.

The amount of 5′-deoxyadenosine formed from the uncoupled radical SAM reaction in PqqE varied with time, undergoing multiple turnovers over the course of several hours. Furthermore, decreasing the enzyme concentration 2-fold caused the rate of 5′-deoxyadenosine formation to decrease by a factor of 2, clearly indicating the dependence of 5′-deoxyadenosine formation on enzyme. The rate for the production of 5′-deoxyadenosine was linear for approximately 45 min, and during this time period,

\[ k_{\text{obs}} = 0.011 \pm 0.001 \text{ min}^{-1}. \]

Although this rate constant seems quite slow for an enzymatic reaction, the uncoupled rate is only approximately 6 times slower than single-turnover rate constants of biotin synthase (0.07 min\(^{-1}\)) (42), 10 times slower than that for lipoyl synthase (0.175 min\(^{-1}\)) (43), and ~30 times slower than that for AtsB (0.38 min\(^{-1}\)) (31). This rate is only ~7 times slower when compared to the rate-limiting step for TPQ biogenesis (\(k_{\text{bio}} = 0.08 \text{ min}^{-1}\)) (44), indicating that the turnover rate is at least comparable with those found for the formation of other quinone cofactors.

We have proceeded on the premise that PqqE catalyzes the first step in PQQ biogenesis, thought to be the linking of the amino acid side chains of glutamate and tyrosine in PqqA. Previous in vivo labeling experiments demonstrated PQQ was formed from the fusion of glutamate and tyrosine, also providing evidence for how these amino acids are linked together (see Scheme 1) (11, 12). A carbon–carbon bond is formed between C9 from glutamate and C9a from tyrosine. It seems likely that the radical SAM enzyme could catalyze this step since the large activation energy needed to break the aromaticity of the tyrosine ring could be overcome by formation of a glutamate radical generated via H-atom abstraction by the 5′-deoxyadenosyl radical.

We tested PqqA as a substrate for PqqE by incubating the enzyme with varying amounts of SAM, dithionite, and PqqA. For these reactions, the enzyme needed to be stabilized with glycerol and PqqA slowly titrated into the reaction mixtures due to significant protein precipitation problems. Reactions were also attempted with a small amount of ethanol or denaturant to circumvent potential peptide aggregation. The reaction mixtures were left for various periods of time and then analyzed via MALDI-TOF mass spectrometry, LC–MS, SDS–PAGE, EPR, and high-resolution mass spectrometry. Under all conditions, no modifications of the peptide or (radical) intermediates were observed, even though the uncoupled reaction products (methionine and 5′-deoxyadenosine) were still detected. Furthermore, there were no new peaks observed in the EPR, LC–MS traces, or bands in the SDS–PAGE analyses. The reaction mixtures were also exposed to molecular oxygen after anaerobic incubation, on the premise that O2 may play a role in trapping radical intermediates and driving the reaction toward products. However, these experiments failed to show any new products or modifications of PqqA. Experiments performed with glutamate and tyrosine as potential substrates for PqqE also failed to show any new products.

The fact that no modifications of PqqA have been observed may be due to a lack of sensitivity of detection (for instance, if only a very small amount of product were formed), or perhaps a reversible cleavage of SAM with an equilibrium that favors the reactants. Experiments that aim to test these hypotheses using spin-trapping agents and also utilizing radiolabeled [2,3,5\(^{-3}\)H]SAM to look for tritium scrambling into the peptide are under way.

Alternatively, other proteins in the PQQ biosynthetic pathway may be necessary for the initial steps in PQQ biogenesis. One possible candidate is PqqD, a gene product with no known
homology to any annotated protein. PqqA may also need to be modified by another gene product in the pqq operon prior to reaction with PqqE. One important question that we would like to resolve is that of which chemical step happens first, the cross-linking of PqqA or the hydroxylation of the conserved tyrosine. Veletrop et al. have demonstrated that molecular oxygen is critical for PQQ biogenesis (15), and it is reasonable to suggest that the hydroxylation of the tyrosine in PqqA occurs via activation of molecular oxygen. We had initially thought that PqqE may be responsible for both of these steps; however, we have yet to observe this type of unprecedented radical SAM chemistry.

It has been recently demonstrated in TTQ biogenesis that mutation of critical residues in the mauG gene results in tryptophan cross-linking and either hydrogen peroxide or oxygen with reducing equiva-

lents results in tryptophan cross-linking via a radical mechanism. Ongoing experiments occur before cross-linking of PqqA, after which PqqE catalyzes the cross-linking via a radical mechanism. Ongoing experiments are aimed at oxidatively modifying the conserved tyrosine in PqqA, with the goal of assessing whether such derivations of PqqA are capable of entering into reaction with PqqE.

ACKNOWLEDGMENT

We thank Prof. Robert Rucker for the donation of plasmid pBCP-165 containing the pqq operon, Dr. Petra Hänzelmann for the donation of plasmids pPH149 and pPH151 containing the E. coli IscSUA-HscBA-Fd and E. coli suf ABCDSE genes, respectively, and Dr. Charles Roessner for a clone containing the pqqE gene from T. thermophilus. We thank Dr. Tony Iavarone for obtaining high-resolution mass spectra of peptides, and Ms. Mae Tulfo for help preparing the manuscript.

SUPPORTING INFORMATION AVAILABLE

Methods for the aerobic and anaerobic growth and induction of E. coli BL21(DE3) cells harboring the pET24b-pqqE plasmid, aerobic purification of PqqE, anaerobic reconstitution of PqqE with iron and sulfide ions, synthesis and purification of SAM, and a detailed description of the high-resolution mass spectrometry experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES


