Supplementary Material (ESI) for Chemical Communications

Interaction of PqqE and PqqD in the Pyrroloquinoline Quinone (PQQ) Biosynthetic Pathway links PqqD to the Radical SAM SuperFamily

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Supplementary Material

Experimental

Materials

All chemicals and reagents were purchased from Sigma-Aldrich, Acros, Cambridge Isotope Laboratories, or Fisher. Chemicals and reagents were used as is without further purification. *pET3a* was purchased from Novagen. *E. coli* BL21(DE3) and XL-1 Blue competent cells were purchased from Stratagene. *NdeI* and *HindIII* restriction enzymes were purchased from New England BioLabs, calf intenstinal alkaline phosphatase from Invitrogen, and high fidelity PFU polymerase and T7 DNA ligase from Roche. Bradford Assay reagents were purchased from BioRad, bovine serum albumin from Pierce, and Q-Sepharose and Sephacryl S-300 HR from Sigma.

Plasmid *pBCP-165* containing the *pqq* operon from *Klebsiella pneumoniae* was originally made in the laboratory of Peter W. Postma (University of Amsterdam, The Netherlands) and donated by Robert Rucker from University of California at Davis.

General Methods

Inert atmosphere experiments were carried out in an 855-AC-controlled atmosphere chamber from Plas-Labs, Inc. (Lansing, Michigan). For inert atmosphere

work, all buffer solutions were made anaerobic by purging solutions with argon for 1 mL/min. Reagents were either prepared as anaerobic buffers or brought into the box in powder form and then reconstituted with anaerobic buffer. DNA sequencing was performed at the DNA sequencing facility at the University of California at Berkeley. N-terminal sequencing was done at the Stanford PAN facility. Protein concentrations were calculated using the Bradford Assay. UV-vis spectra were recorded on a Hewlett-Packard 8452 diode array spectrophotometer. PCR reactions were carried out on a PTC-200 Peltier thermal cycler (MJ Research).

Cloning, Expression, Purification, and Characterization of PqqD.

Cloning of pqqD from pBCP-165.

pqqD was cloned into the *NdeI* and *HindIII* restriction sites of *pET3a* using standard cloning techniques. The following primers (obtained from Operon) were used to clone *pqqD* out of pBCP-165: 5'-GAGCACATATGCAGAAAACGTCCATC-3', 5'-GGACTAAGCTTTTACTCTGGCTCACG-3'. The underlined bases show the engineered restriction sites for *NdeI* and *HindIII*, respectively. The PCR reaction conditions were 95 °C melting, 55 °C annealing, and 72 °C elongation, each cycle carried out for 1 min for a total of 32 cycles. Ligation reaction mixtures were transformed into *E. coli* XL-1 Blue competent cells. The bacteria were plated onto LB agar plates containing ampicillin (amp, 50 μg/mL) and grown at 37 ºC overnight. The following day, several colonies were picked and grown in liquid LB media at 37 ºC containing 50 μg/mL amp. After several hours the bacteria had reached log phase and were quickly

mixed with 50 % glycerol, immediately frozen in liquid nitrogen and stored at -80 ºC until further use. The plasmid (named *pET3a-pqqD)* was isolated from these colonies and then sequenced using the T7 promoter primer, and the *pqqD* primer harboring the *HindIII* restriction site.

Expression and purification of PqqD.

pET3a-pqqD was transformed into *E. coli* BL21 (DE3) cells. The cells were plated onto LB agar containing 50 μg/mL of amp and grown overnight at 37 °C. The following day, several colonies were picked and grown in liquid LB media at 37 ºC containing 50 μg/mL amp. After several hours the bacteria had reached log phase and were quickly mixed with 50 % glycerol, immediately frozen in liquid nitrogen and stored at -80 ºC until further use.

100 mL starter cultures of *E. coli* BL21 (DE3) cells harboring plasmid *pET3a-pqqD* were grown overnight at 37 °C in LB media containing 50 μg/mL of amp. After approximately 16 h, 25 mL of the starter cultures were transferred to 8 L Erlenmeyer flasks containing 2 L of LB media with 50 μg/mL of amp. The flasks were left to shake aerobically at 30 ºC for approximately 24 h. Although *pET3a* has an IPTG inducible promoter, expression of PqqD was so leaky that induction was not necessary. After 24 h, the cells were harvested via centrifugation and frozen at -80 ºC until further use (cell paste yields were approximately 28 g per 8 L of LB).

The cell paste (14 g) was reconstituted on ice with 200 mL of 50 mM Tris (pH 7.9), 5 μL of Benzonase nuclease (Novagen), and lysed with Bugbuster for 30 min. After lysis, the cells were centrifuged at 15,000 x *g* at 4 ºC for 20 min. PqqD was then purified

from the supernatants via a two-step purification procedure consisting of strong anion exchange (Q-sepharose) followed by size exclusion chromatography.

The Q-sepharose was equilibrated with 50 mM Tris (pH 7.9), and a linear gradient from 0-500 mM KCl (800 mL) was used to elute PqqD from the column. Under these conditions, PqqD eluted at approximately 300 mM KCl. The protein fractions were analyzed via SDS-PAGE and fractions containing PqqD pooled, then dialyzed overnight at 4 °C into (3 x 2 L) 50 mM Tris (pH 7.9). After dialysis, the protein was concentrated to approximately 40 mg/mL using a 3.5 kDa Amicon membrane (Millipore). The protein was then further purified by size exclusion chromatography on a Sephacryl S-300 HR column equilibrated with 50 mM Tris (pH 7.9). The fractions were analyzed via SDS-PAGE and fractions containing PqqD pooled. The protein was finally concentrated down to approximately 18 mg/mL using a 3.5 kDa Amicon membrane, divided into 50 µL aliquots, frozen in liquid nitrogen and stored at -80 ºC until further use. Final yields of PqqD using this procedure were approximately 4.5 mg protein/g of cell paste.

PqqD Characterization

PqqD has been characterized by SDS gel electrophoresis, N-terminal sequencing, and high resolution mass spectrometry. An SDS-PAGE of PqqD before and after purification is shown in Supplementary Figure 1, demonstrating PqqD is obtained at greater than 95% purity. Confirmation of the correct protein sequence was also demonstrated via N-terminal sequence. N-terminal sequencing of the purified protein was determined by Edman degradation at the Stanford PAN facility (Found: MQKTSIVAFRRGYRLQWEAA, Predicted: MQKTSIVAFRRGYRLQWEAA).

High resolution mass spectrometry was performed (as described under LC-MS analyses of proteins) on an LTQ Orbitrap XL hybrid mass spectrometer equipped with an Ion Max electrospray ionization source (ESI; Thermo Fisher Scientific, Waltham, MA). Mass spectra were processed and deconvuluted using Xcalibur software (version 4.1, Thermo). The theoretical molecular weight (monoisotopic) for PqqD is 10435.96 daltons. The molecular weight determined via high-resolution mass spectrometry was 10435 daltons (see Supplementary Figure 1). Several peaks were also observed at larger molecular weights that could be attributed to oxidation presumably formed during aerobic purification.

Gel Permeation Chromatography

LC was performed on a Biorad FPLC running BioLogic software. Size exclusion chromatography was done on a Superdex 200 (GE healthcare) column equilibrated in 50 mM sodium phosphate buffer (pH 7.0), 150 mM NaCl running at 4°C at a flow rate of 0.25 mL/min and monitored at 280 nm. The column was standardized with Thyroglobulin, Apoferritin, beta amylase, YADH, Albumin, Carbonic anhydrase, Cytochrome C, Bovine Aprotonin, and NADH, which eluted at 35.8, 41.4, 47.0, 50.3, 55.8, 65.6, 70.8, 75.5 and 84.5 min (MW= 2,000, 667, 443, 200, 150, 66.0, 29.0, 12.4, 6.5, and 0.66 kDa), respectively. When PqqD was analyzed under these conditions, the protein eluted at 73 min., suggesting that PqqD exists as a monomer in solution.

Demonstration of an Interaction between PqqE and PqqD via Deuterium Exchange Mass Spectrometry.

Sample preparation for assays.

All assays were performed under strictly anaerobic conditions in an inert atmosphere glove box. PqqE was anaerobically grown and purified as previously described.[1] After purification, PqqE was concentrated to approximately 10 mg/mL using a 30 kDa amicon membrane (Millipore). PqqE was then buffer exchanged under anaerobic conditions in deuterated buffer (50 mM Tris (pH 7.9) in deuterium oxide, 1 mM DTT, 300 mM NaCl) via gel filtration over a Pd-10 column (GE healthcare). The brown red fractions were collected off the Pd-10 column and concentrated to approximately 2.5 mg/mL (57 μM) using a 30 kDa amicon membrane. The samples were distributed in 200 μL aliquots into glass agilent vials sealed with rubber septa, brought of the glove box and immediately frozen in liquid nitrogen. The samples were stored at – 80 °C until use.

A stock solution of PqqE in deuterated buffer was prepared by mixing the enzyme under anaerobic conditions with sodium dithionite, dithiothreitol (DTT), and glycerol. PqqD was made anaerobic by several evacuation/argon purge cycles on a Schlenk Line. The final concentration of each component in the stock solution was 49 μM (PqqE), 500 μM (dithionite), 1 mM (DTT), and 20 % v/v (glycerol). A 25 μL aliquot of the stock solution was removed and mixed with 7.3 μL of PqqD (1.71 mM) and 17.7 μL of deuterated buffer to give a final concentration of PqqD and PqqE of 25 μM in solution.

For the samples containing SAM, a ten-fold excess of SAM to protein was added to these reaction mixtures. The samples were left to incubate for exactly two hours and then quenched with neat formic acid to a final concentration of 5% v/v. 10 μL aliquots of the assay mixtures were injected onto the LC-MS and analyzed as described under LC-MS methods.

For the preparation of controls containing bovine serum albumin, BSA was brought into the glove box as a lypholized powder and anaerobically reconstituted with deuterated buffer. Assays were initiated and analyzed under identical conditions to those described for assays containing PqqE. Samples were run in triplicate. Controls were also performed that demonstrated all of the deuteriums exchanged into PqqD could be back exchanged out of the protein, indicating deuterium exchange into PqqD is a reversible process.

LC-MS methods.

Protein samples were analyzed using an Agilent 1200 series liquid chromatograph (LC; Santa Clara, CA) that was connected in-line with an LTQ Orbitrap XL hybrid mass spectrometer equipped with an Ion Max electrospray ionization source (ESI; Thermo Fisher Scientific, Waltham, MA).

The LC was equipped with C_8 guard (Poroshell 300SB-C8, 5 μm, 12.5 × 2.1 mm, Agilent) and analytical (75 × 0.5 mm) columns and a 100 μL sample loop. Solvent A was 0.1% formic acid/99.9% water and solvent B was 0.1% formic acid/99.9% acetonitrile (v/v). Sample solutions contained in 0.3 mL polypropylene snap-top vials

sealed with rubber septa caps (Wheaton Science, Millville, NJ) were loaded into the Agilent 1200 autosampler compartment prior to analysis. For each sample, approximately 50 to 100 picomoles of analyte was injected onto the column. Following sample injection, analyte trapping was performed for 5 min with 99.5% A at a flow rate of 90 μL/min. The elution program consisted of a linear gradient from 35% to 95% B over 34 min, isocratic conditions at 95% B for 5 min, a linear gradient to 0.5% B over 1 min, and then isocratic conditions at 0.5% B for 14 min, at a flow rate of 90 μL/min. The column and sample compartment were maintained at 35 ºC and 10 ºC, respectively. Solvent (Milli-Q water) blanks were run between samples, and the autosampler injection needle was rinsed with Milli-Q water after each sample injection, to avoid cross-contamination between samples.

The connections between the LC column exit and the mass spectrometer ion source were made using PEEK tubing (0.005" i.d. × 1/16" o.d., Western Analytical, Lake Elsinore, CA). External mass calibration was performed prior to analysis using the standard LTQ calibration mixture containing caffeine, the peptide MRFA, and Ultramark 1621 dissolved in 51% acetonitrile/25% methanol/23% water/1% acetic acid solution (v/v).[2] The voltages applied to the ion source optics were adjusted automatically for optimum desolvation and transmission of protein ions using Tune Plus software (version 2.4, Thermo), while directly infusing 50% water/50% acetonitrile (v/v) solutions of horse myoglobin (10^{-5} M, Sigma, St. Louis, MO) into the ESI source at a flow rate of 5 μL/min using a syringe pump. The ESI source parameters were as follows: ion transfer capillary temperature 275 ºC, normalized sheath gas (nitrogen) flow rate 25%, ESI voltage 2.0 kV, ion transfer capillary voltage 33 V, and tube lens voltage 125 V. Mass spectra were

recorded in the positive ion mode over the range m/z = 500 to 2000 using the Orbitrap mass analyzer, in profile format, with full MS automatic gain control target settings of 3×10^4 and 5×10^5 charges for the linear ion trap and the Orbitrap, respectively, and an Orbitrap resolution setting of 6×10^4 (at m/z = 400, FWHM). The mass resolution was sufficient to resolve the isotopic distributions of the multiply charged protein ions, formed by ESI, that were measured in this study. Thus, an ion's mass and charge could be determined independently, i.e., the charge is determined from the reciprocal of the spacing between adjacent isotope peaks in the m/z spectrum).[3] Mass spectra were processed using Xcalibur software (version 4.1, Thermo).

EPR

Preparation of all samples was done in an anaerobic glove box. A 1.5 mL stock solution of PqqE was prepared with 169 μM PqqE in 50 mM Tris (pH 7.9), 1 mM DTT, 1.7 mM dithionite and 300 mM NaCl. The samples were diluted to a final concentration of 115 μM and a stoichiometric amount of PqqD was added to the solutions. The reduced samples that contained SAM were prepared in an identical manner, except a ten-fold excess of SAM (1.15 mM) was added to the reaction mixture. The samples were loaded into EPR tubes equipped with rubber septa, brought out of the glove box and immediately frozen in liquid nitrogen. The total incubation time for the samples was approximately 5 min.

EPR experiments were carried out at the CalEPR center at the University of California at Davis, using a Bruker ECS106 continuous-wave X-band spectrometer equipped with a TE_{102} cavity (ER4102ST) resonating at about 9.5 GHz and an Oxford

ESR900 helium cryostat with an ITC503 temperature controller. The microwave power was 6.3 mW, and the field modulation was 1.0 mT at 100 kHz. All spectra were background corrected using a control without PqqE, PqqD, and SAM.

The EPR spectra were modeled as a three-component system, each component being a spin system of one spin $S = 1/2$ (as typical for $[Fe_4S_4]^+$) with a rhombic g tensor, with three distinct principal values. The spectral broadening was modeled as g-strain, where each principal value of the g tensor is assumed to be independently distributed over a range with a Gaussian probability distribution. The spectra were simulated and fitted with EasySpin[4] (www.easyspin.org), using matrix diagonalization of the spin hamiltonian. In the least-squares fitting procedure, the nine principal g values, the nine g strain linewidths (Gaussian FWHM, full width at half maximum), and the relative populations of the three components were varied until the sum-of-squares of the residuals between simulated and experimental spectra was minimal.

The spectral components are shown in Supplementary Figure 1 and the fitted parameters are listed in Supplementary Table 1. For both conditions with and without SAM, the addition of PqqD to PqqE results in a drastic decrease in component A and an increase in component B and C.

Far UV CD

All samples for CD were prepared in an anaerobic glove box. Samples of PqqE were prepared using a Pd-10 column equilibrated with 50 mM Tris (7.9) and 1 mM spectroscopic grade tris(2-carboxyethyl)phosphine (TCEP). Samples of PqqD in 50 mM Tris (pH 7.9) were made anaerobic by several evacuation/argon purge cycles on a

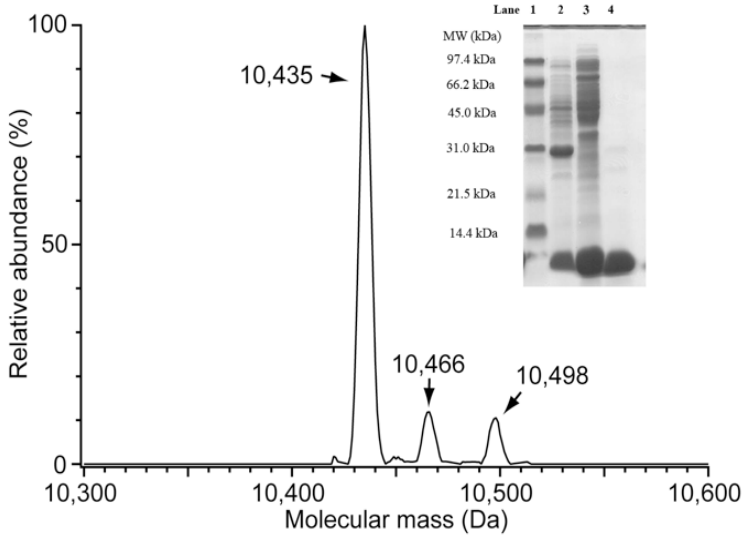
Schlenk Line. Spectra were collected in a 1 cm split-cell, anaerobic cuvette containing a final concentration of 2.5 μM PqqE or PqqD in separate chambers. Dilution was performed with water to decrease the signal to noise ratio resulting from buffering components, and the final concentrations resulting from dilution were 600 μM Tris and 12 μM TCEP. Controls were performed to ensure that dilution with water did not affect the isolated spectrum of either PqqE or PqqD. The spectrum of PqqE and PqqD prior to mixing was obtained, and then the two chambers were exposed to each other for approximately 5 minutes before the spectrum of the combined proteins was obtained. Additionally, the spectra of either PqqE or PqqD alone were obtained in the same split-cell cuvette with water occupying the unused chamber, and these spectra suggested that the beta sheet contribution to the spectrum of the combined proteins largely was provided by PqqE. The additive spectra of PqqE and PqqD was equal to the spectrum of the two proteins when isolated prior to mixing in the split-cell cuvette. Control spectra containing BSA also used 2.5 μM protein, and the sample was made anaerobic by bringing the protein into the anaerobic box as dry powder and dissolving with anaerobic water. All spectra were obtained at 25 °C using an AVIV Model 410 circular dichroism spectrometer (Lakewood, NJ) at an acquisition rate of 1 nm/s as the sum of three accumulations.

Ni-NTA affinity immobilization assay for determining binding of PqqD to N-terminal His_6-tagged PqqE.

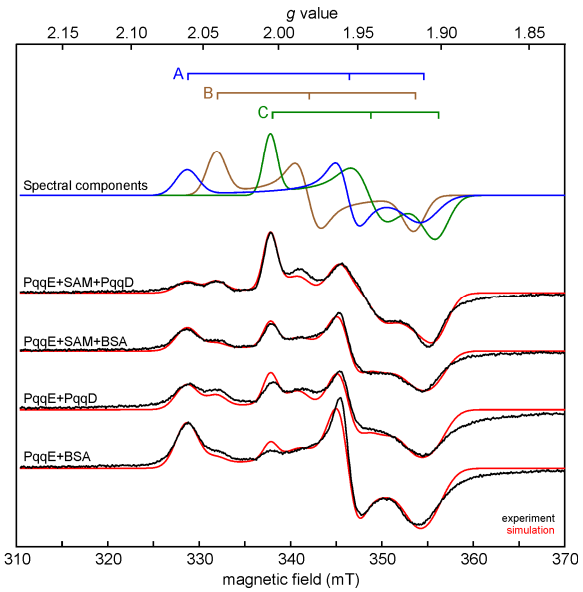
400 μg of PqqE in 50 mM Tris (pH 7.9), 1 mM DTT, was added to 1.5 mL eppendorf tubes containing approximately 200 μL of Ni-NTA agarose resin (Qiagen) equilibrated in 50 mM Tris (pH 7.9). An equal molar amount of PqqD was added to the assay mixtures. The samples were thoroughly mixed and left to bind for approximately 10 minutes, then centrifuged at 2,000 x g for 3 minutes. The supernatants were removed from the solutions and the samples washed several times with buffer. 200 μL of 50 mM Tris (pH 7.9) containing 300 mM Imidazole was added to the tubes. The samples were thoroughly mixed and then centrifuged at 2,000 x g for another 3 minutes. The supernatants were removed and analyzed via SDS-PAGE.

Supplementary Figures

Supplementary Figure 1. SDS-PAGE and deconvoluted mass spectrum of purified PqqD from *Klebsiella pneumoniae*. Low molecular weight marker (lane 1), crude PqqD (lane 2), PqqD after Q-Sepharose anon exchange (lane 3), PqqD after Sephacryl S-300 HR (lane 4).



Supplementary Figure 2. Spectral fits to the EPR spectra



Supplementary Table 1. Spectral analysis of PqqE/PqqD spectra

Magnetic parameters of the three components

	Component A	Component B	Component C
g values	2.060(2) 1.955(8) 1.910(8)	2.040(2) 1.980(8) 1.915(8)	2.004(2) 1.942(8) 1.902(8)
g strain broadening (FWHM)	0.0227 0.0135 0.0180	0.0150 0.0150 0.0150	0.0173 0.0206 0.0105

Microwave frequency 9.479 GHz

Fractional compositions of the four samples

	Component A	Component B	Component C
PqqE+PqqD +SAM	28%	15%	56%
PqqE+BSA +SAM	63%	9%	27%
PqqE+PqqD -SAM	57%	16%	27%
PqqE+BSA -SAM	81%	7%	12%

References

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