

Interaction of PqqE and PqqD in the pyrroloquinoline quinone (PQQ) biosynthetic pathway links PqqD to the radical SAM superfamily†‡

Stephen R. Wecksler,^a Stefan Stoll,^b Anthony T. Iavarone,^a Erin M. Imsand,^a Ha Tran,^a R. David Britt^b and Judith P. Klinman*^a

Received 15th April 2010, Accepted 16th July 2010

DOI: 10.1039/c0cc00968g

pqqD is one of six genes required for PQQ production in *Klebsiella pneumoniae*. Herein, we demonstrate that PqqD interacts specifically with the radical SAM enzyme PqqE, causing a perturbation in the electronic environment around the [4Fe-4S]⁺ clusters. This interaction redirects the role for PqqD in PQQ biosynthesis.

PQQ is the essential cofactor for glucose and methanol dehydrogenases found predominantly in gram-negative bacteria.¹ The biosynthetic operon for this prokaryotic cofactor is found in many pathogenic organisms, making the pathway an attractive target for new antibiotics. PQQ has also been found in high concentrations in human breast milk and is necessary for proper growth and development in mice, though its participation as a cofactor for mammalian enzymes remains to be proven.²

The biosynthetic pathway for this important cofactor is still poorly understood. It is known that PQQ is formed from the fusion of glutamate (Glu) and tyrosine (Tyr) (Scheme 1).³ The genes in the *pqq* operon from many organisms have been described and compared.⁴ In *Klebsiella pneumoniae*, there are six such genes designated *pqqA-F*.⁵ PqqA is a peptide with conserved Glu and Tyr that has been implicated as the substrate for biosynthesis.^{4,5} PqqB shares homology to the β -lactamase family, and PqqF is likely to function as a protease. PqqC catalyzes the ring closure and 8-electron oxidation of 3a-(2-amino-2-carboxyethyl)-4,5,-dioxo-4,5,6,7,8,9,-hexahydroquinoline-7,9-dicarboxylic acid (AHQQ) in the final step of biosynthesis.⁶ PqqE contains a conserved

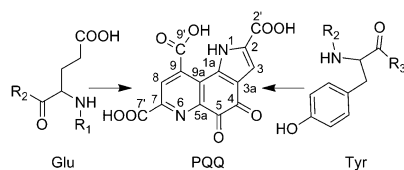
cysteine triad that is found in the radical *S*-adenosyl-L-methionine (SAM) family,⁷ and has recently been shown to cleave SAM to methionine and 5'-deoxyadenosine in an uncoupled reaction.⁸ Efforts to link the activity of PqqE to the cross-linking of the conserved Glu and Tyr residues within the putative substrate PqqA have, thus far, been unsuccessful.⁸

One of the least understood proteins within the biosynthesis pathway is PqqD. A BLAST search of this protein in the NCBI database yielded little information, other than revealing homology to PqqD proteins within the *pqq* operon. Further complicating the assignment of PqqD is the fact that in approximately 4% of PQQ-producing organisms, the genes for *pqqC* and *pqqD* are fused together to encode a single polypeptide chain. Due to this fact, several groups have hypothesized that PqqD may function to dissociate tightly bound PQQ from PqqC.^{1,4} The recent crystal structure for PqqD from *Xanthomonas campestris*, in which a putative PQQ binding site was identified at the dimer interface, appeared to support this role.⁹ Worth noting is that the PQQ binding site was identified by a docking study, and attempts to crystallize PqqD with PQQ have not been successful.⁹ In fact, there is little experimental evidence to support the role of PqqD as a “dissociase” or PQQ carrier protein, and Toyama *et al.* have shown that removal of the *pqqD* gene from the *pqqC/D* construct does not alter the function of PqqC.¹⁰ Furthermore, a *pqqD* knockout study demonstrated that this gene is critical for PQQ formation, arguing for a more prominent role in the biosynthetic pathway.⁵

Recently, we performed a refined BLAST search of PqqD uncovering a possible link between PqqE and PqqD. In a search for a “conserved domain” architecture of PqqD, a series of unknown proteins arose that contain a radical SAM domain fused to a PqqD domain (Fig. 1). While the open-reading frame from *Dehalococcoides* sp. *CBDB1* (entry (6) in Fig. 1) has no known function, *albA* (entries (4) and (5) in Fig. 1) is involved in the biosynthesis of subtilisin A, with a possible role in the cross-linking of amino acid side chains.¹¹

In this paper, we present the first evidence for a direct interaction of PqqE with PqqD. These data redirect the investigation of the role for PqqD within the PQQ biosynthetic pathway, and likely have important functional implications for the sub-family of radical SAM/PqqD domain containing enzymes.

The DNA for *pqqD* from *K. pneumoniae* was cloned into *E. coli* and expressed as a native protein in high yield (see ESI† for details on cloning, purification, and characterization). N-terminal sequencing and high-resolution mass spectrometry confirmed the identity of the protein. The enzyme has no



Scheme 1 Fusion of Glu and Tyr to make PQQ.

^a Department of Chemistry, Department of Molecular and Cell Biology, and the California Institute for Quantitative Biosciences, University of California, Berkeley, CA 94720, USA. E-mail: klinman@berkeley.edu; Fax: +1 510 643 6232; Tel: +1 510 642 2668

^b Department of Chemistry, University of California, Davis, CA 95616, USA. E-mail: rdbrutt@ucdavis.edu; Fax: +1 530 752 8995; Tel: +1 530 752 6377

† This article is part of the ‘Enzymes and Proteins’ web-theme issue for ChemComm.

‡ Electronic supplementary information (ESI) available: Experimental protocols. See DOI: 10.1039/c0cc00968g



Fig. 1 PqqD and radical SAM domain sequence alignments. Separate PqqD and PqqE domains from (1) *K. pneumoniae*, (2) *Kluyvera intermedia* and (3) *Xanthobacter autotrophicus* Py2. Entries (4)–(6) denote genes in which the PqqD and radical SAM domains are fused: antilisterial bacteriocin (subtilosin) biosynthesis gene *albA* from (4) *Bacillus subtilis*, (5) *Bacillus cereus* *Rock4-2*; (6) unknown protein (NCBI Reference Sequence: YP_308556.1, *Dehalococcoides* sp. *CBDB1*). Red-colored amino acids denote highly conserved residues and * denotes completely conserved residues in PqqD, PqqE, and fused genes.

noticeable color or detectable cofactors after purification. Gel filtration experiments demonstrated that PqqD exists as a monomer in solution (see ESI†). Although these data contrast with the dimer identified by crystallography, those authors also noted that only the monomer was observed in solution.⁹

Radical SAM enzymes contain an extremely oxygen-sensitive [4Fe–4S] cluster, precluding many standard biochemical and biophysical techniques to examine the binding of PqqD to PqqE. In light of this limitation, a simple affinity tag assay was devised (see ESI†) to investigate whether column-immobilized PqqE would bind PqqD (the PqqE that was previously characterized contained an N-terminal His-tag to aid in purification).⁸ The absence of evidence for a tight and specific binding of PqqE to PqqD implied either no binding or a relatively weak interaction between these proteins. The latter was probed using hydrogen/deuterium (H/D) exchange into the backbone of PqqD under anaerobic conditions.¹² In these experiments, PqqE (25 μ M) was first exchanged into deuterated buffer and then incubated with PqqD (25 μ M) for 2 h. Exchange was quenched by the addition of formic acid and the samples were analyzed by high-resolution mass spectrometry. The mass spectrum for the 10+ charge state of PqqD is shown in Fig. 2. When PqqD was incubated anaerobically in deuterated buffer for two hours, the protein gained approximately 22 daltons corresponding to the incorporation of 22 deuteriums (approximately 21% of amide protons). When this experiment was performed in the presence of PqqE, PqqD only incorporates 20 deuteriums. The extent of deuterium incorporation was unaffected by the addition of SAM. When a control was run with bovine serum albumin (BSA), the same amount of deuterium was incorporated as with PqqD alone. Although there is not a large difference in deuterium incorporation between the controls and PqqD in the presence of PqqE, this initial screening experiment was reproducible ($n = 4$), indicating interaction between PqqE and PqqD that produces partial protection of PqqD from H/D exchange.

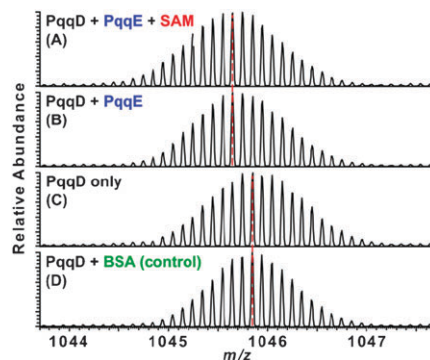


Fig. 2 Isotopically resolved ESI mass spectra of the protein, PqqD, showing detail for the 10+ ion.

We, thus, considered whether PqqD could couple the radical SAM activity of PqqE to PqqA by forming a protein radical intermediate, similar to that found during the interaction of pyruvate formate lyase with pyruvate formate lyase-activating enzyme.¹³ To probe this hypothesis, we incubated PqqD (115 μ M) anaerobically with reduced PqqE (115 μ M) and SAM (1.15 mM). The absence of detectable fragmentation of PqqD after the addition of air to PqqD/PqqE/SAM/dithionite samples ruled out the generation of a protein radical at the conserved Gly (Fig. 1) in PqqD.¹³

The same samples were analyzed by continuous-wave X-band EPR spectroscopy at cryogenic temperatures. The EPR spectra also do not give any indication of protein-based radicals. We have shown previously that reduced PqqE gives an EPR spectrum from [4Fe–4S]⁺ similar to that seen for other radical SAM enzymes.⁸ Upon addition of SAM, the spectrum is perturbed slightly (Fig. 3), perhaps due to binding of SAM near the reduced [4Fe–4S]⁺ cluster. However, when the reduced PqqE (115 μ M) samples were incubated with a stoichiometric amount of PqqD, the EPR spectra changed significantly (Fig. 3), both in the presence and in the absence of SAM (1.15 mM). The spectra indicate that the features are much more pronounced in the presence of both SAM and PqqD. These features are absent from samples containing reduced PqqE with or without SAM. Controls of reduced PqqE with BSA (115 μ M) also failed to reveal any new features. PqqD itself is EPR silent.

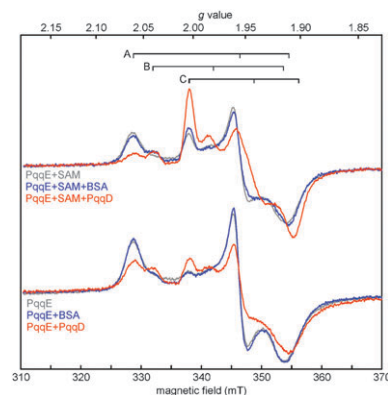


Fig. 3 Continuous-wave EPR spectra (40 K, 9.479 GHz, 6.3 mW) of PqqE anaerobically reduced with dithionite (gray trace), in the presence of either BSA (blue trace) or PqqD (red trace), with a ten-fold excess of SAM (top) and without SAM (bottom).

We have been able to model the spectra as mixtures of three components A, B, and C (see Fig. 3 and ESI†). The addition of PqqD induces the appearance of component C with g factors 1.902, 1.942, and 2.004. The new component observed in the EPR spectra is indicative of a perturbation at one or both of the two $[4\text{Fe-4S}]^+$ centers of PqqE in the presence of PqqD. Although further work is needed to clarify the structural details of the interaction, the data in Fig. 3 provide clear evidence for complex formation between PqqD and PqqE.

Further support for this interaction was obtained by analyzing the overall gross secondary structure of PqqE and PqqD by far UV circular dichroism (CD) spectroscopy before and after incubation. Experiments were performed using 2.5 μM each of PqqD and PqqE in a 1 cm pathlength, anaerobic, split-cell cuvette containing a mixing window. The three-dimensional structure of PqqD and a homology model of PqqE developed using the closest structural relative of the enzyme (MoaA) indicate that PqqD and PqqE are predominantly α -helical proteins containing some β -sheets and regions of random coiling.^{9,14} Consistent with these structures, the spectrum of PqqD and PqqE prior to mixing gave a predominantly α -helical signal (Fig. 4, blue trace). Subsequent to mixing, molar ellipticity of the PqqD/PqqE complex increased 4-fold (Fig. 4, red trace), suggesting that formation of the complex results in an increase of the helical content of PqqD and/or PqqE. Incubation of either protein with BSA did not result in altered CD spectra, thus reinforcing the specificity of the observed results for the interaction of PqqD and PqqE.

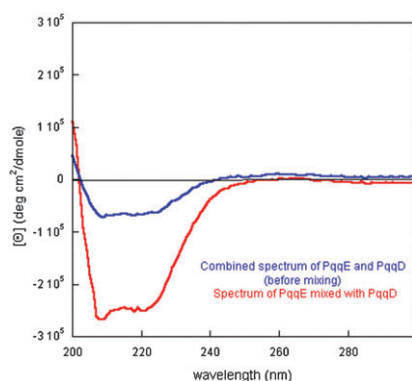


Fig. 4 Far UV CD spectra of PqqE and PqqD before (blue trace) and after (red trace) mixing.

Experiments were then performed to ascertain whether PqqD could link the radical SAM activity of PqqE to a chemical reaction in PqqA (*i.e.* the cross-linking of the Glu and Tyr, Scheme 1). Anaerobic incubation of PqqD with varying amounts of PqqE, PqqA, SAM and dithionite resulted in no detectable spectral changes or modifications (by UV-vis, LC-MS, SDS PAGE, EPR, and high-resolution MS) of PqqA, PqqD or PqqE, even though the radical SAM products methionine and 5'-deoxyadenosine were still detected. The rate and amount of 5'-deoxyadenosine formed in this uncoupled reaction was also quantified, and showed no difference from those reactions in which PqqD was omitted.⁸

The pronounced changes in the EPR spectrum of the $[4\text{Fe-4S}]^+$ centers in PqqE in the presence of PqqD, the perturbation of the PqqD CD spectra in the presence of PqqE,

the modest protection of PqqD to deuterium exchange, and the identification of several proteins where a “PqqD” domain is fused to a “radical SAM” domain, provide strong support for a specific interaction between these two proteins. Although the precise functional role of this interaction remains to be elucidated, these data completely refocus studies on the biosynthesis of PQQ toward a novel structural/catalytic role for the PqqD/PqqE pair.

We believe the role for PqqD may be to orient the active site of PqqE, effectively positioning the 5'-deoxyadenosyl radical to abstract a hydrogen atom from PqqA. The fact that no modifications of PqqA have been observed so far suggests that PqqA itself may not be the substrate for the PqqD/PqqE pair, and that PqqA may need to be hydroxylated by another enzyme in the biosynthetic pathway before reaction with PqqD/PqqE can occur.⁸ Further studies to examine this unexpected and new interaction in the PQQ biosynthetic pathway are underway.

This work was supported by research grants from the National Institutes of Health (GM039296 to JPK, GM073789 to RDB and F32GM080795 to SRW). SRW thanks Z. Nagel for help with the gel filtration experiments. EMI thanks the Marqusee lab (University of California, Berkeley) for the use of their CD instrument.

Notes and references

- 1 P. M. Goodwin and C. Anthony, in *Advances in Microbial Physiology*, ed. R. K. Poole, Elsevier, Amsterdam, 1998, vol. 40, pp. 1–80.
- 2 A. E. Mitchell, A. D. Jones, R. S. Mercer and R. B. Rucker, *Anal. Biochem.*, 1999, **269**, 317; J. Killgore, C. Smidt, L. Duich, N. Romero-Chapman, D. Tinker, K. Reiser, M. Melko, D. Hyde and R. B. Rucker, *Science*, 1989, **245**, 850; T. Kasahara and T. Kato, *Nature*, 2003, **422**, 832.
- 3 D. R. Houck, J. L. Hanners and C. J. Unkefer, *J. Am. Chem. Soc.*, 1988, **110**, 6920; D. R. Houck, J. L. Hanners and C. J. Unkefer, *J. Am. Chem. Soc.*, 1991, **113**, 3162.
- 4 J. J. M. Meulenber, E. Sellink, N. H. Riegman, M. van Kleef and P. W. Postma, *Mol. Gen. Genet.*, 1992, **232**, 284; H. Toyama, L. Chistoserdova and M. E. Lidstrom, *Microbiology*, 1997, **143**, 595; N. Goosen, H. P. A. Horsman, R. G. M. Huinen and V. D. P. Putte, *J. Bacteriol.*, 1989, **171**, 447.
- 5 J. S. Velterop, E. Sellink, J. J. M. Meulenber, D. S. Bulder and P. W. Postma, *J. Bacteriol.*, 1995, **177**, 5088.
- 6 O. T. Magnusson, H. Toyama, M. Saeki, R. Schwarzenbacher and J. P. Klinman, *J. Am. Chem. Soc.*, 2004, **126**, 5342; O. T. Magnusson, H. Toyama, M. Saeki, A. Rojas, J. C. Reed, R. Liddington, J. P. Klinman and R. Schwarzenbacher, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 7913.
- 7 H. J. Sofia, G. Chen, B. G. Hetzler, J. F. Reyes-Spindola and N. E. Miller, *Nucleic Acids Res.*, 2001, **29**, 1097.
- 8 S. R. Weckler, S. Stoll, H. Tran, O. T. Magnusson, S.-P. Wu, D. King, R. D. Britt and J. P. Klinman, *Biochemistry*, 2009, **48**, 10151.
- 9 T.-Y. Tsai, C.-Y. Yang, H.-L. Shih, A. H.-J. Wang and S.-H. Chou, *Proteins: Struct., Funct., Bioinf.*, 2009, **76**, 1042.
- 10 H. Toyama, J. Fukumoto, M. Saeki, K. Matsushita, O. Adachi and M. E. Lidstrom, *Biochem. Biophys. Res. Commun.*, 2002, **299**, 268.
- 11 K. E. Kawulka, T. Sprules, C. M. Diaper, R. M. Whittall, R. T. McKay, P. Mercier, P. Zuber and J. C. Vederas, *Biochemistry*, 2004, **43**, 3385.
- 12 J. G. Mandell, A. M. Falick and E. A. Komives, *Proc. Natl. Acad. Sci. U. S. A.*, 1998, **95**, 14705.
- 13 A. F. V. Wagner, M. Frey, F. A. Neugebauer, W. Schäfer and J. Knappe, *Proc. Natl. Acad. Sci. U. S. A.*, 1992, **89**, 996.
- 14 P. Hänzelmann and H. Schindelin, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 12870.