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# The Structure of a Biosynthetic Intermediate of Pyrroloquinoline Quinone (PQQ) and Elucidation of the Final Step of PQQ Biosynthesis

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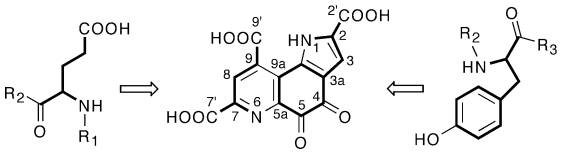
Pyrroloquinoline quinone (4,5-dihydro-4,5-dioxo-1-*H*-pyrrolo-[2,3-*f*]quinoline-2,7,9-tricarboxylic acid: PQQ) is an important redox-active cofactor used by a number of bacterial dehydrogenases.[1] PQQ has also received recent attention due to the claim that it serves as a novel B vitamin in mammals,[2] which underscores the importance of understanding the pathway for PQQ formation in vivo. This aromatic, heterocyclic, *o*-quinone (Scheme 1) is

***Scheme 1***



derived from a peptide precursor containing conserved glutamate and tyrosine residues,[3a] where all carbon and nitrogen atoms of these two amino acids are incorporated into PQQ.[3b,c] Not much is known about the chemical steps and therefore the function of the enzymes involved in PQQ biosynthesis, although gene clusters from several different organisms have been described.[4] In *Klebsiella pneumoniae* there are six genes (*pqqA-F*) in a transcriptional operon.[4a] Strains of *K. pneumoniae* and *Methylobacterium extorquens* deficient in *pqqC* produce a biosynthetic intermediate, which undergoes conversion to PQQ in the presence of PqqC.[4b,c,5] Identification of this compound has been hampered by the instability and low abundance of this species in bacterial cultures (65−90 nmol/L).[4c] Herein we describe the isolation and purification of the intermediate from a *pqqC* mutant strain of *M. extorquens* and its structural characterization using nuclear magnetic resonance (NMR) and UV−vis spectroscopies, mass spectrometry, and chemical derivatization. The intermediate, i.e., the substrate for PqqC, is shown to be a fully reduced derivative of PQQ, in which the five-membered ring has not been formed. We can therefore determine the reaction catalyzed by PqqC—the enzyme that catalyzes the last step in PQQ biosynthesis.

Partially isolated substrate from a *pqqC* mutant strain of *M. extorquens* was purified to homogeneity by a three-step procedure using high-performance liquid chromatography (HPLC) (see Supporting Information). The final yield of purified substrate from ∼50 g of cells was 0.60 $\mu$mol (∼0.2 mg). The substrate has a wine-red color and shows three distinct electronic transitions with absorption maxima at pH 7 at 222 nm ($\epsilon$ = 15 700 $M^{-1}$ $cm^{-1}$), 274 nm ($\epsilon$ = 8260 $M^{-1}$ $cm^{-1}$) and 532 nm ($\epsilon$ = 2010 $M^{-1}$ $cm^{-1}$) (Figure 1).

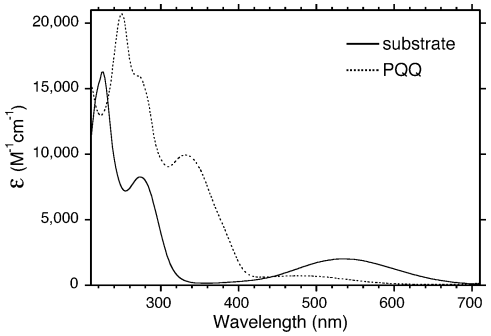
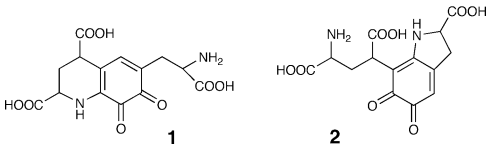



***Figure 1.*** UV−vis spectra of the substrate and PQQ measured in 50 mM potassium phosphate buffer, pH 7.0, at 25 °C.

The UV−vis spectrum is clearly indicative of a quinone species and displays the greatest similarity in terms of peak positions and intensities to model compounds for the lysyltyrosine *o*-quinone (LTQ) cofactor of lysyl oxidase.[6] Electrospray ionization (ESI) mass spectra of both substrate and PQQ were obtained as described elsewhere.[7] The substrate spectrum has major peaks at $m/z$ = 339 ($[M + H]^+$) and $m/z$ = 337 ($[M - H]^-$) (data not shown). PQQ yields the predicted $m/z$ = 329 ($[M - H]^-$), which shows that the PqqC substrate is 8 amu greater in mass. These results indicate that the substrate is a highly reduced form of PQQ or a derivative thereof. Further characterization was obtained by reaction of the substrate with fluorescamine and 2,4,6-trinitrobenzene sulfonic acid (TNBS)—reagents specific for primary amines.[8] The results demonstrated the presence of a primary amine in the substrate, although an accurate quantification was not obtained nor was the location of the amine.[9] Taken together, these data can be accommodated by two alternate structures, **1** and **2** (Chart 1), which lack either a final five-membered or six-membered ring.

***Chart 1***



**1** **2**

The $^1$H NMR spectrum of the substrate in 5 mM DCl/$D_2O$ shows eight distinct protons.[10] *a*: $\delta$ 2.09, ddd, $J$ = 5.9, 8.5, 13.6 Hz; *b*: $\delta$ 2.32, ddd, $J$ = 4.5, 5.9, 13.6 Hz; *c*: $\delta$ 2.83, dd, $J$ = 7.4, 14.2 Hz; *d*: $\delta$ 3.00, dd, $J$ = 6.1, 14.2 Hz; *e*: $\delta$ 3.56, t, $J$ = 5.9 Hz; *f*: $\delta$ 4.08, dd, $J$ = 6.1, 7.4 Hz; *g*: $\delta$ 4.17, dd, $J$ = 4.5, 8.5 Hz; *h*: $\delta$ 6.55, s. However, since the amine protons exchange with solvent ($D_2O$), the location of the primary amino group could not be

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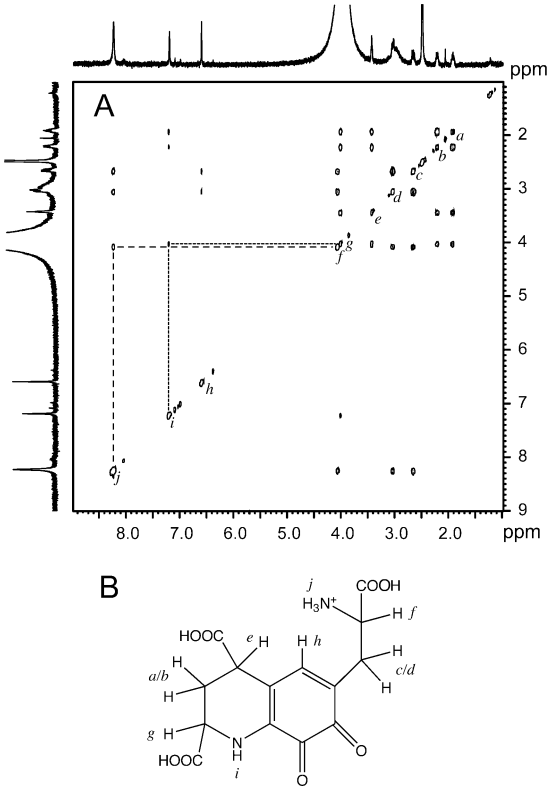


***Figure 2.*** (A) 2D TOCSY spectrum measured in DMSO-$d_6$, 0.1% TFA, using a 70-ms mixing time. *a*: $\delta$ 1.91; *b*: $\delta$ 2.19; *c*: $\delta$ 2.65; *d*: $\delta$ 3.02; *e*: $\delta$ 3.43; *g*: $\delta$ 4.03; *f*: $\delta$ 4.08; *h*: $\delta$ 6.59; *i*: $\delta$ 7.19; *j*: $\delta$ 8.23. The dashed lines show cross-peaks due to coupling from amine protons to the α-protons on the glutamate and tyrosine moieties, respectively. (B) Chemical structure of the substrate for PqqC. Labels refer to resonances in the spectra above. Note that proton labeled *f* has moved slightly downfield of proton labeled *g* by switching from $D_2O$ to DMSO-$d_6$ as solvent.

obtained from this spectrum. The substrate was, thus, repurifed by HPLC and resuspended in DMSO-$d_6$ that contained 0.1% trifluoroacetic acid, pH $\approx$ 2, to stabilize the compound.[9] The resulting NMR spectrum (Figure 2A) shows the appearance of two new peaks at 7.19 and 8.23 ppm, which are assigned to a secondary amine and a protonated primary amine, respectively, based on the integration of the peaks. Although *J*-coupling could not be discerned in the 1D spectrum and residual water is overlapping two protons at ~4 ppm, a 2D TOCSY (**to**tal **c**orrelated **s**pectroscop**y**) experiment provides information about the connectivities and allows for a complete structural characterization.

Analysis of the 2D TOCSY spectrum leads to identification of the substrate as 3a-(2-amino-2-carboxyethyl)-4,5-dioxo-4,5,6,7,8,9-hexahydroquinoline-7,9-dicarboxylic acid (Figure 2B). The TOCSY spectrum shows that the $-NH_3^+$ protons (*j*) are in a spin system with protons derived from the tyrosine moiety of the molecule (*f*, *c*, and *d*), allowing us to locate the primary amine in the position shown. This peak integrates to three protons, consistent with amine protonation under the conditions of data acquisition (pH $\approx$ 2). Conversely, the less basic secondary amine is neutral under these conditions as determined by the peak integral, and the −NH proton (*i*) is in a spin system with protons derived from the glutamic acid moiety of the molecule (*g*, *a*, and *b*). This secondary amine is expected to be very acidic ($pK_a$ < 2) due to the neighboring *o*-quinone, which causes electron delocalization from the nitrogen lone pair into the ring; this explains the relatively high chemical shift of the NH proton. Similarly low $pK_a$ values and downfield-shifted NMR resonances have been observed for −NH protons of quinone model compounds of LTQ.[6] Other interesting cross-peaks in the spectrum include a weak, four-bond allylic coupling between the lone proton on the tyrosine ring (*h*) to protons *c* and *d*. We postulate that spontaneous addition of the primary amine into the quinone system is hampered by the reduced electrophilicity of the quinone due to electron donation of the secondary amine. However, at pH 8, the compound is rather labile ($t_{1/2}$ = 135 min),[9] where addition of the primary amine is presumably the first step in the decomposition of the compound.

The elucidation of the substrate structure of PqqC provides us with the necessary information to deduce the nature of the final step in PQQ biosynthesis. The results show that PqqC facilitates ring closure and an unprecedented eight-electron oxidation of the substrate, which raises a number of important questions regarding the mechanism of this enzyme. This study also lays the foundation for further work concerning PQQ biosynthesis, including the roles of other enzymes in the pathway, the functions of which are currently unknown.

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**Supporting Information Available:** Description of substrate purification, including chromatograms for each step, and $^1$H NMR spectrum of the substrate. This material is available free of charge via the Internet at http://pubs.acs.org.

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(9) Fluorescamine assays were done in 50 mM Na-borate buffer, pH 9.0 as described.[8a] TNBS reactions were done in 0.1 M $NaHCO_3$ buffer, pH 8.5 and incubated for 1 h at 40 °C before quenching with HCl as described.[8b] Both fluorescamine and TNBS assays yielded 0.4−0.6 mol amine per mol substrate based on tyrosine as a standard. The low yield can in part be explained by the instability of the substrate under mildly alkaline conditions. The substrate was shown to have a half-life of 135 min in 0.1 M Na-phosphate buffer, pH 8.0, at 25 °C in a pseudo-first-order reaction, by monitoring its disappearance by HPLC (data not shown).
(10) The $^1$H NMR spectrum along with complete assignments is available in the Supporting Information. All NMR experiments were performed on a Bruker DRX-500 instrument at 25 °C. Samples (60 μL) were in a 1.5-mm capillary that was kept nonspinning inside a 5-mm NMR tube.

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