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In Vitro Reconstitution of the Radical *S*-Adenosylmethionine Enzyme MqnC Involved in the Biosynthesis of Futalosine-Derived Menaquinone

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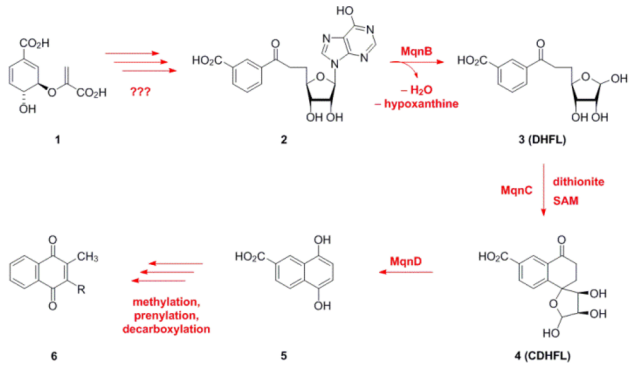
Supporting Information

ABSTRACT: The radical *S*-adenosylmethionine enzyme MqnC catalyzes conversion of dehypoxanthine futalosine (DHFL) to the unique spiro compound cyclic DHFL in the futalosine pathway for menaquinone biosynthesis. This study describes the *in vitro* reconstitution of [4Fe-4S] cluster-dependent MqnC activity and identifies the site of abstraction of a hydrogen atom from DHFL by the adenosyl radical.

Menaquinone (MK, vitamin K_2) is a lipid-soluble molecule that shuttles electrons between membrane-bound protein complexes in the respiratory chain. In the classic *Escherichia coli* pathway, it is biosynthesized from chorismate by eight enzymes (MenA–MenH).[1−3] Recently, an alternative route for menaquinone production was discovered, termed the futalosine pathway (Scheme 1).[4] Chorismate (**1**)-derived

Scheme 1. Menaquinone Biosynthesis via the Futalosine Pathway



futalosine (**2**) is converted to dehypoxanthine futalosine (DHFL, **3**) by futalosine hydrolase MqnB, and **3** is transformed into cyclic dehypoxanthine futalosine (CDHFL, **4**) by the radical *S*-adenosylmethionine (SAM) enzyme MqnC. MqnD then converts **4** to 1,4-dihydroxy-6-naphthoic acid (**5**). In the later steps of the pathway, on the basis of the annotation and clustering of the open reading frames in *Streptomyces coelicolor* A3(2), it is possible that SCO4491 (prenylation) and SCO4556 (methylation) could be involved in completing the biosynthetic pathway for production of menaquinone (**6**). The early and late steps in the pathway are currently unknown and under investigation. Humans and commensal intestinal bacteria lack this pathway; therefore, it represents an attractive target for the development of chemotherapeutic compounds.

Enzymes belonging to the radical SAM superfamily are typically characterized by the presence of a cysteine-rich CX_3CX_2C motif that ligates a [4Fe-4S] cluster.[5,6] The iron–sulfur cluster is responsible for the reductive cleavage of SAM, generating a 5′-deoxyadenosyl (5′-dA) radical and methionine. Amino acid sequence analysis revealed that MqnC contains this characteristic CX_3CX_2C iron–sulfur cluster motif. Therefore, a recombinant plasmid encoding *mqnC* from *Bacillus halodurans* C-125 was introduced into *E. coli* BL21(DE3)-T1R containing the *E. coli suf* operon for coexpression of His_6-MqnC with iron–sulfur cluster biogenesis proteins.[7] Anaerobic purification by nickel affinity chromatography yielded pure brown protein with three irons and two sulfides per monomer, suggesting incomplete reconstitution of the cluster. The UV–vis spectrum of MqnC had an absorbance maximum at approximately 410 nm that disappeared upon exposure of the protein to oxygen (Figure 1A).[8] Electron paramagnetic resonance (EPR) spectra and *g* values for MqnC treated with excess sodium dithionite were consistent with a protein-bound $[4Fe\text{-}4S]^+$ cluster (Figure 1B).

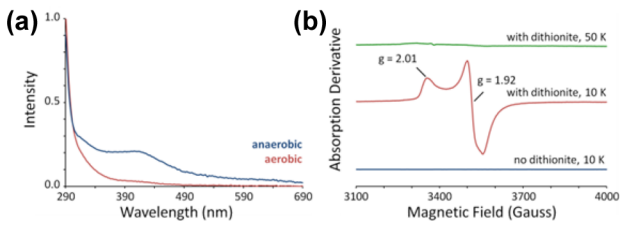


Figure 1. Spectroscopic properties of MqnC. (A) UV–vis absorbance of purified iron–sulfur cluster-reconstituted MqnC (160 μM) in 100 mM phosphate buffer (pH 7.5) before (blue) and after (red) exposure to oxygen. (B) EPR spectra of purified iron–sulfur cluster-reconstituted MqnC (280 μM) treated with (red and green) or without (blue) dithionite.

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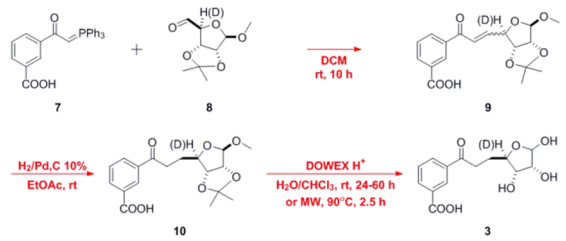
On the basis of gene disruption studies in *S. coelicolor*, MqnC is predicted to catalyze the conversion of **3** to **4**.[4] To test the activity of MqnC, DHFL was synthesized as illustrated in Scheme 2 (experimental methods are described in detail in the Supporting Information). When MqnC was incubated under anaerobic conditions with sodium dithionite, DHFL, and SAM (**11**) in phosphate buffer (pH 7.5), the production of CDHFL and 5′-deoxyadenosine (5′-dAd) was observed by high-performance liquid chromatography (HPLC) as confirmed by co-elution with authentic standards (Figure 2A). CDHFL was not observed in control reaction mixtures from which sodium dithionite, DHFL, SAM, or MqnC had been omitted. Assay and control samples were also analyzed by liquid chromatography and mass spectrometry (LC–MS) using an Agilent 1200 liquid chromatograph (ChemStation) with a Bruker Daltonics micrOTOF-Q II ESI-Qq-TOF mass spectrometer (HyStar). The observed mass for cyclic DHFL in negative ion mode was m/z 293.1 as compared to the theoretical exact mass of m/z 293.1 [$[M - H]^-$ (Figure 2C)]. The authentic standard for CDHFL was purified from the previously described *S. coelicolor* SCO4326-disruptant strain (observed mass of m/z 293.1).[4]

Scheme 2. Synthesis of DHFL and [4-^{2}H]-DHFL



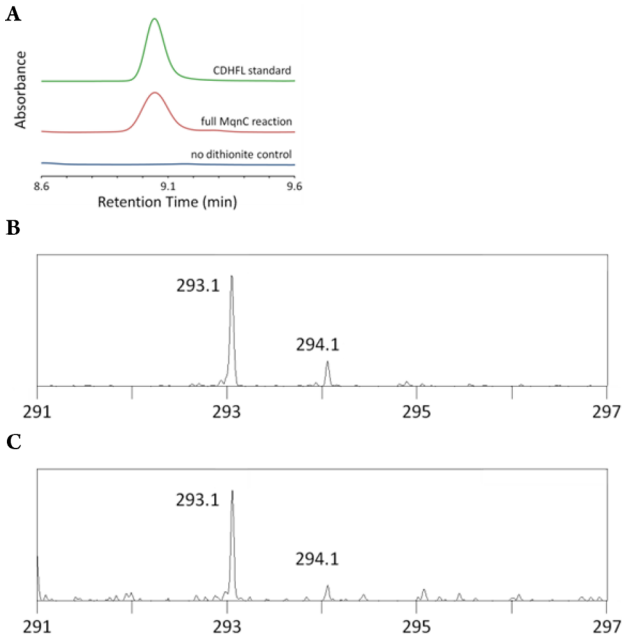


Figure 2. MqnC activity *in vitro*. (A) HPLC chromatograms monitoring absorbance at 250 nm. (B) Mass spectrum for CDHFL purified from the *S. coelicolor mqnD*-disruptant strain. (C) Mass spectrum for CDHFL from the full MqnC reaction. Calculated exact mass of CDHFL of m/z 293.1 ($[M - H]^-$).

These results confirm the predicted role of MqnC as the DHFL cyclase.

Deuterium labeling of DHFL was used to elucidate the site of hydrogen atom abstraction by the 5′-dA radical (**13**) during MqnC catalysis. DHFL that was site-specifically deuterated at C4′ was synthesized and used as a substrate for MqnC. The transfer of deuterium from [4-^{2}H]-DHFL to **13** was monitored by LC–MS (Figure 3). The data revealed an increase of a single mass unit for 5′-dAd, thus demonstrating that the hydrogen from C4′ of DHFL is abstracted by the 5′-dA radical. The low efficiency of label transfer is most likely due to the substantial levels of uncoupled 5′-dAd formation [10:1 (Figure S5 of the Supporting Information)] as well as a primary isotope effect on the initial hydrogen atom abstraction, which would favor consumption of [4-^{1}H]-DHFL over [4-^{2}H]-DHFL.

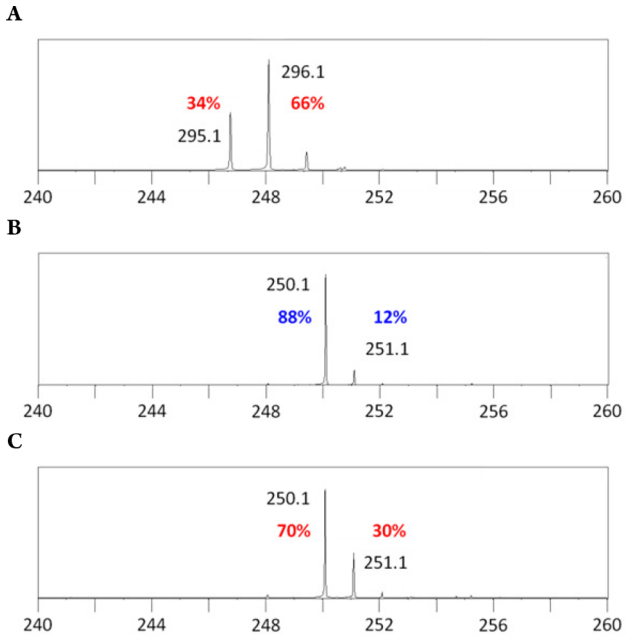


Figure 3. MqnC-mediated hydrogen atom abstraction by the 5′-dA radical occurs at C4′ of DHFL. (A) Mass spectrum demonstrating 66% deuterated [4-^{2}H]-DHFL. (B) 5′-dAd derived from the MqnC reaction using DHFL as the substrate. (C) 5′-dAd derived from the MqnC reaction using [4-^{2}H]-DHFL as the substrate. Calculated exact masses ($[M - H]^-$) of DHFL, [4-^{2}H]-DHFL, and 5′-dAd: m/z 295.1, 296.1, and 250.1, respectively.

A mechanistic proposal for the MqnC-catalyzed reaction is outlined in Scheme 3. Reductive cleavage of SAM generates adenosyl radical **13**, which then abstracts a hydrogen atom from DHFL **3** to give radical **15**. Cyclization gives **16**, which after deprotonation transfers an electron back to the $[4Fe\text{-}4S]^{2+}$ cluster to give CDHFL **4**.

In summary, the activity of the radical SAM enzyme MqnC was successfully reconstituted. EPR studies confirmed that MqnC contains a [4Fe-4S] cluster, and isotope labeling studies demonstrated that the C4′ hydrogen atom of DHFL **3** is abstracted by the 5′-deoxyadenosyl radical. A mechanistic proposal for the MqnC-catalyzed reaction is described. Experiments are in progress to test this proposal.

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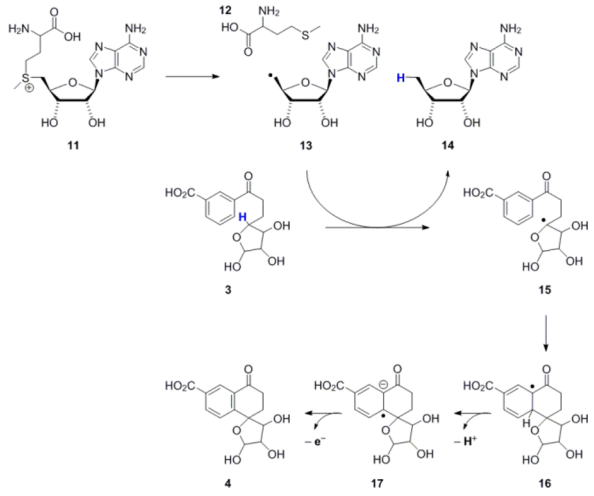
Scheme 3. Proposed MqnC Reaction Mechanism



■ ASSOCIATED CONTENT

Supporting Information

Detailed procedures for cloning the *mqnC* expression construct, MqnC expression and purification, DHFL synthesis, CDHFL isolation, and HPLC and LC−MS methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

L.E.C. was responsible for the biochemical studies. D.F., S.H.A., and S.-H.K. synthesized DHFL. L.E.C. and T.P.B. designed the experiments and prepared the manuscript.

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Notes

The authors declare no competing financial interest.

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