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Menaquinone Biosynthesis: Biochemical and Structural Studies of Chorismate Dehydratase

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Supporting Information

ABSTRACT: Menaquinone (MK, vitamin K) is a lipid-soluble quinone that participates in the bacterial electron transport chain. In mammalian cells, vitamin K functions as an essential vitamin for the activation of several proteins involved in blood clotting and bone metabolism. MqnA is the first enzyme on the futalosine-dependent pathway to menaquinone and catalyzes the aromatization of chorismate by water loss. Here we report biochemical and structural studies of MqnA. These studies suggest that the dehydration reaction proceeds by a variant of the E1cb mechanism in which deprotonation is slower than water loss and that the enol carboxylate of the substrate is serving as the base.

Menaquinone (**9**, vitamin K) is a lipid-soluble cofactor that shuttles electrons between membrane-bound redox enzymes in the bacterial respiratory chain.[1] It is also an important vitamin in mammalian cells where it participates as a cosubstrate in the carboxylation of glutamic acid residues in various proteins involved in blood coagulation[2] and bone morphogenesis.[3] In addition to the classical pathway in *Escherichia coli*,[4−6] an alternative pathway for menaquinone biosynthesis was reported in *Streptomyces coelicolor* A3(2).[7−10] In this pathway, MqnA is a chorismate dehydratase catalyzing the conversion of chorismate **1** to 3-[(1-carboxyvinyl)oxy]-benzoic acid (**2**) (Figure 1).[11] In the next step, the radical SAM enzyme MqnE (SCO4494) catalyzes the transformation of **2** into aminofutalosine **3**.[11,12] Aminofutalosine **3** is then converted to compound **4** by aminofutalosine hydrolase (MqnB, SCO4327) or by a combination of aminofutalosine deaminase (SCO5662) and MqnB.[13−17] Cyclization of **4**, catalyzed by another radical SAM enzyme, MqnC (SCO4550), gives compound **5**,[18] which is then converted to 5,8-dihydroxy-2-naphthoic acid **6** by MqnD (SCO4326).[7] The genes likely to be involved in the conversion of **6** to menaquinone **9** have been identified, on the basis of frequent clustering with other Mqn genes and sequence similarity to proteins of closely related function in ubiquinone biosynthesis.[4,6] SCO4491 (MqnP) is likely to catalyze the prenylation reaction to give **7**;[19] SCO4490 (MqnL) and SCO4492 (MqnM) are likely to catalyze the decarboxylation, and SCO4556 (MqnG) is likely to catalyze the methylation reaction to give menaquinone **9**.[7]

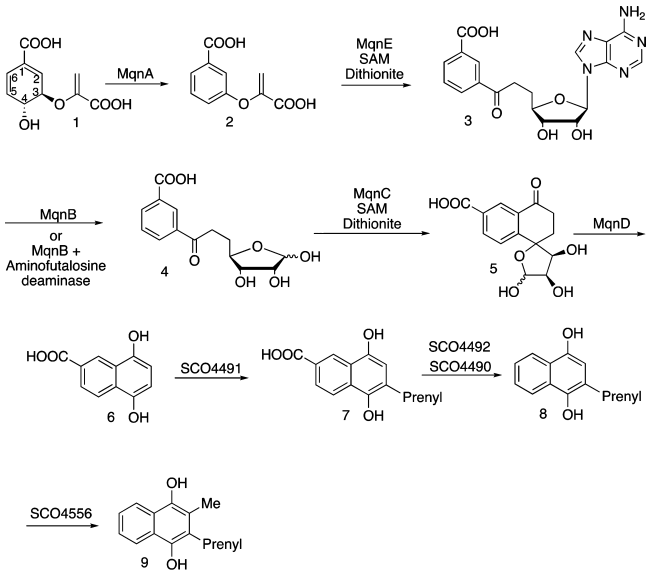

Figure 1. Aminofutalosine-dependent pathway to menaquinone **9**.

In this paper, we report H/D exchange experiments, a pH−rate profile, and structural studies of the MqnA/3−hydroxybenzoic acid complex. These studies are consistent with a variant E1cb mechanism[20] for MqnA in which substrate-mediated deprotonation is slower than water loss.

The mechanistic analysis of the metal ion-independent MqnA-catalyzed reaction is shown in Figure 2. Because the chorismate structure prevents the trans periplanar arrangement of the C3 proton and the C4 hydroxyl groups, an E2 elimination mechanism is unlikely and our experiments have focused on differentiating between an E1 and an E1cb mechanism. The E1 mechanism involves rate-limiting carbocation formation followed by fast deprotonation. Catalysis of this reaction would require an active site acid to activate the hydroxide as a leaving group to form carbocation **10**. This suggests that the enzyme should function optimally under acidic to neutral conditions and that the enzyme would be inactive under basic conditions. This prediction is not in

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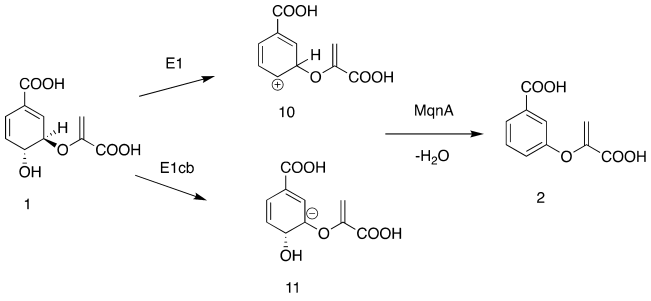

Figure 2. Mechanistic proposals for the MqnA-catalyzed chorismate dehydration reaction.

agreement with the experimental pH−rate profile for MqnA, which shows that the enzyme retains 47% of its maximal pH 6.5 activity at pH 9 (Figure S4). This suggests that MqnA does not proceed via an E1 mechanism.

The E1cb mechanism for the formation of **2** involves initial deprotonation, by a base, to form carbanion **11** followed by loss of water. There are two variants of the E1cb mechanism. In the first version, the deprotonation reaction is fast and reversible and the departure of the leaving group is slow. This mechanism is widely used for the enzymatic elimination of water.[20] A standard method for experimentally probing for this mechanism is to look for the incorporation of deuterium into unreacted starting material when the reaction is run, to partial conversion, in deuterated buffer. For the MqnA-catalyzed reaction, liquid chromatography/mass spectrometry (LC/MS) analysis of a reaction mixture containing chorismate (10 mM) and MqnA (5 μM) in 100% deuterated buffer (potassium phosphate, pH 7.5) run for 25 min (95% conversion) demonstrated that no H/D exchange of the chorismate C3H occurred (Figure S5). As the LC−MS sensitivity for chorismate detection is low, the unreacted chorismate was converted to phenyl pyruvate by heating the mixture at 70 °C prior to MS analysis.[21] This experiment argues against version 1 of the E1cb mechanism for MqnA.

In the second version of the E1cb mechanism ($E1cb_I$), the deprotonation reaction is slow relative to departure of the leaving group and the H/D exchange reaction is not observed. While we are not aware of an enzymatic dehydration reaction that proceeds via an $E1cb_I$ mechanism, this mechanism has been found in the glycoside hydrolases[22−24] and in non-enzymatic systems.[25−28] In contrast to previously studied E1cb dehydrations, the loss of water from carbanion **11** is likely to be fast because of the energy gained by product aromatization. These considerations, as well as the pH−rate profile and the absence of H/D exchange at C3 of unreacted chorismate, suggest that MqnA may follow this mechanism.

It was not possible to identify the MqnA catalytic residues from the previously published unliganded structure of the *Deinococcus radiodurans* MqnA (Protein Data Bank entry 2I6E). Our attempts to obtain crystals with **1** or **2** bound at the active site were unsuccessful. However, we were able to obtain a structure of MqnA complexed with 3-hydroxybenzoic acid (3HBA), a truncated analogue of the reaction product (Protein Data Bank entry 6O9A). The data collection and refinement statistics are listed in Tables S2 and S3. This structure demonstrated that there are no acidic or basic residues in a 6 Å zone surrounding the bound ligand (Figure 3). This suggested that the enol ether carboxylate of the substrate functions as the base. The amide NH of Ser65 is located 3.9 Å from C4 of the

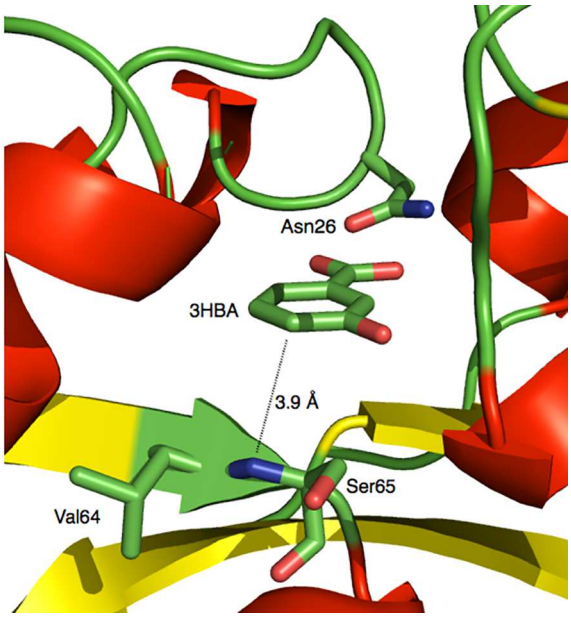

Figure 3. Structure of the MqnA active site with bound 3-hydroxybenzoic acid (3-HBA) viewed looking into the active site. This structure shows that there are no protein-derived acidic or basic residues available to catalyze the dehydration reaction in a 6 Å zone around 3HBA. The ligand carboxylate is hydrogen-bonded to Asn26, and the water at C4 of chorismate may hydrogen bond to the amide NH group of Ser65 (model shown in Figure S7).

ligand, suggesting that hydrogen bonding of this to the C4 alcohol of chorismate may activate it as a leaving group. The structure also shows that the carboxylate base is positioned on the solvent-exposed side of the active site. This suggests that the absence of H/D exchange in unreacted chorismate is unlikely due to slow exchange of the carboxylic acid with buffer.

On the basis of these experiments, we propose the mechanism for MqnA shown in Figure 4. In this mechanism,

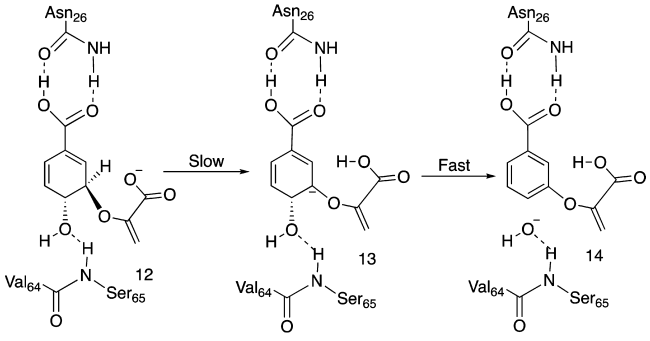

Figure 4. Proposed mechanism for MqnA involving an $E1cb_I$ mechanism showing the substrate-mediated deprotonation and the hypothetical protonation of the C4 hydroxylation by the amide NH group of Ser65.

the enol ether carboxylate removes the C3 proton of chorismate. The resulting carbanion is stabilized by delocalization in the two ring C−C double bonds as well as the C1 carboxylate of chorismate. This carboxylate is further polarized by hydrogen bonding to the highly conserved Asn26. Rapid loss of water from carbanion **13** may be facilitated by hydrogen bonding to the NH group of Ser65 (located 3.9 Å from C4 of

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the ligand) and by the aromatization of the benzene ring. It is interesting to note that the chemical decomposition of chorismate also gives **2** under basic, but not acidic, conditions (Figure S6).[29]

While chorismate is an intermediate in the biosynthesis of many different substituted benzene rings, the simple dehydration reaction catalyzed by MqnA has not been previously reported. A survey of natural product structures suggests that the biosynthesis of NP25301 (Figure S6) in *Streptomyces* UMA-044 (not sequenced) is likely to use an MqnA ortholog.[30] In addition, the MqnA genome neighborhood network[31,32] reveals several uncharacterized MqnA-containing gene clusters. It is therefore likely that MqnA is not restricted to menaquinone biosynthesis and that other examples of this facile chorismate dehydration reaction will be found in natural products biosynthesis.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.9b00105.

Detailed procedures for overexpression and purification of MqnA, enzymatic assays, kinetics, H/D exchange, crystallization, and structure determination (PDF)

Accession Codes

UniProtKB/TrEMBL entry Q9RXE3, Protein Data Bank entry 6O9A.

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Notes

The authors declare no competing financial interest.

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