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A Thiamine-Dependent Enzyme Utilizes an Active Tetrahedral Intermediate in Vitamin K Biosynthesis

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Supporting Information

ABSTRACT: Enamine is a well-known reactive intermediate mediating essential thiamine-dependent catalysis in central metabolic pathways. However, this intermediate is not found in the thiamine-dependent catalysis of the vitamin K biosynthetic enzyme MenD. Instead, an active tetrahedral post-decarboxylation intermediate is stably formed in the enzyme and was structurally determined at 1.34 Å resolution in crystal. This intermediate takes a unique conformation that allows only one proton between its tetrahedral reaction center and the exo-ring nitrogen atom of the aminopyrimidine moiety in the cofactor with a short distance of 3.0 Å. It is readily convertible to the final product of the enzymic reaction with a solvent-exchangeable proton at its reaction center. These results show that the thiamine-dependent enzyme utilizes a tetrahedral intermediate in a mechanism distinct from the enamine catalytic chemistry.

Thiamine diphosphate (ThDP)-dependent enzymes catalyze a broad range of reactions in diverse biological processes. Their catalysis involves activation of the ThDP cofactor as an ylide or carbene and formation of a planar enamine intermediate.[1] The reactive enamine intermediate has been successfully captured in crystal in several thiamine enzymes[2−5] and is generally accepted to be essential in the catalytic process. In the last two decades, although some atypical enamine tautomers have been suggested to play a role in catalysis of a few ThDP-dependent enzymes, the central role of enamine in thiamine catalysis has not changed.[6−9]

(1*R*,2*S*,5*S*,6*S*)-2-Succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate (SEPHCHC) synthase, or MenD, is a ThDP-dependent enzyme that catalyzes a distinctive Stetter-like 1,4-addition reaction in bacterial biosynthesis of vitamin K2 (Figures 1a and S1).[10,11] Previous structural studies showed that it is not significantly different from other ThDP-dependent enzymes in active-site architecture, cofactor binding, or overall three-dimensional structure, suggesting the use of the canonical enamine intermediate in its catalysis (Figure 1b).[12,13] However, the post-decarboxylation intermediate formed in situ presents a circular dichroism spectrum that is clearly different from that of the enamine intermediate of yeast transketolase (Figure 1c; also see the Supporting Information).[2]

To understand the reaction intermediate, we determined the crystal structures of ThDP-bound *Escherichia coli* MenD soaked

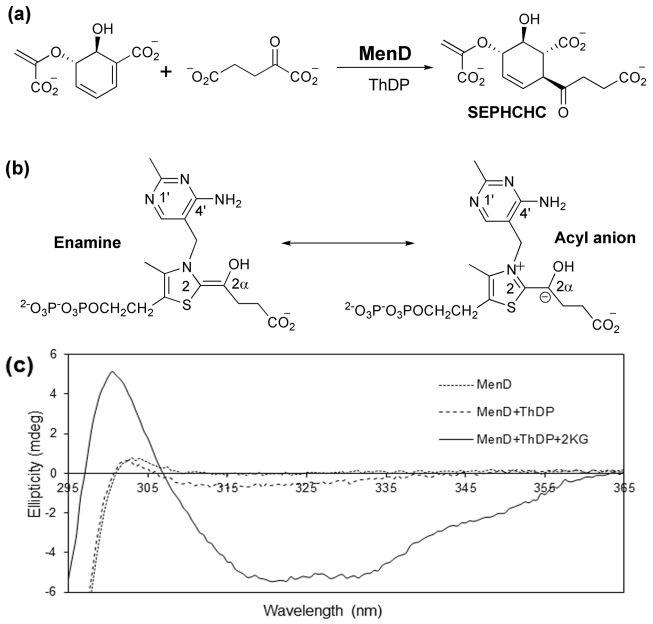

Figure 1. Thiamine disphophate-mediated reaction in vitamin K2 biosynthesis. (a) The MenD-catalyzed reaction. (b) The previously proposed enamine/acyl anion intermediate. (c) Circular dichroism spectrum of the intermediate. The solution contained 2 mM α-ketoglutarate (2KG), 33 μM MenD, 200 μM ThDP, and 2 mM $MgSO_4$ in 100 mM potassium phosphate buffer at pH 7.0, and the control solutions contained the enzyme with or without ThDP at the same concentration.

in 10 mM α-ketoglutarate solution at room temperature for 21 s, 2 min, 15 min, and 90 min to a resolution of 1.34−2.3 Å by molecular replacement (Table S1). The resulting structures (Figure S2) overlap very well, with a root-mean-square deviation (rmsd) of less than 0.2 Å over all comparable backbone carbon atoms, and each protein subunit contains an essentially identical cofactor-bound intermediate with well-defined electron density (Figures 2a and S3). This stable intermediate contains a planar thiazolium ring with a C_2 appendage derived from succinic semialdehyde (Figure 2a). Its $C_{2\alpha}$ atom is clearly tetrahedral, and its terminal carboxyl group is firmly fixed in the active site by salt bridges and

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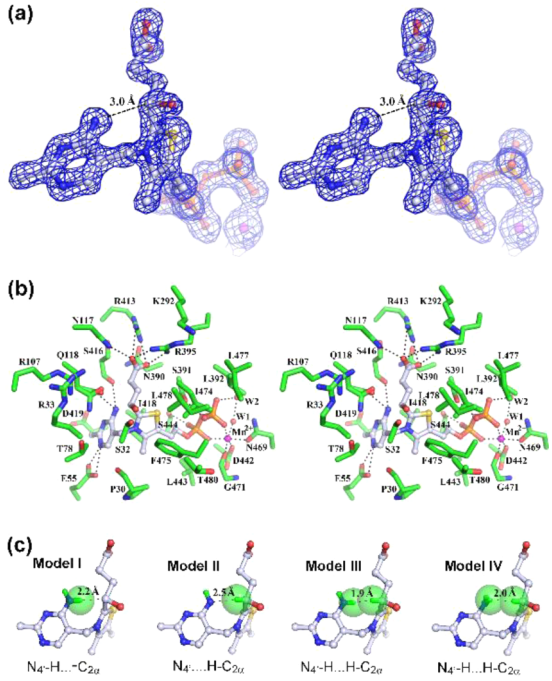

Figure 2. Crystal structure of the tetrahedral intermediate complexed with MenD at 1.34 Å. (a) Stereo diagram of the $2mF_o - DF_c$ electron density map of the tetrahedral intermediate contoured at 2.0σ in blue mesh. (b) Stereo diagram of the tetrahedral intermediate bound to the active site. (c) Four possible models of the tetrahedral intermediate with different protonation states at $C_{2\alpha}$ and $N_{4'}$. The intermediate is represented in sticks with gray carbon atoms, while the conserved active-site residues are represented in sticks with green carbon atoms and the Mn(II) ion is denoted with a magenta sphere in (b). The broken lines denote hydrogen bonds or metal-chelating bonds with a distance ≤3.6 Å in (b), and the green spheres denote the van der Waals surfaces of protons between $C_{2\alpha}$ and $N_{4'}$ in (c). Only the hydrogen atoms on either $C_{2\alpha}$ or $N_{4'}$ are indicated by green sticks in (c), with the dotted lines indicating the shortest interatomic distances; those attached to other atoms have been omitted for simplicity.

hydrogen bonds with the conserved residues Arg395 and Arg413 (Figure 2b).

In the covalent intermediate, all of the bond lengths and angles of its planar thiazolium ring are comparable to those of a similar enzyme-free ThDP adduct (Figure S4).[14] Its $C_2-C_{2\alpha}$ bond is a typical σ bond with a bond length of 1.49 Å (Table S2), which is comparable to that of the enzyme-bound ThDP or 3-deaza-ThDP adducts with a tetrahedral and protonated $C_{2\alpha}$ atom.[5,15−17] Importantly, the $C_{2\alpha}$ to $N_{4'}$ distance is short (3.03 Å), and the $C_{2\alpha}$ atom takes an *S* configuration to position one of its bonds in the direction of the $N_{4'}$ atom. In comparison, the $C_{2\alpha}$ atom of other enzyme-bound adducts always takes a configuration to allow the $C_{2\alpha}-OH$ to form a hydrogen bond with $N_{4'}$ and thus prevent any of the $C_{2\alpha}$ bonds from spatially colliding with it (Table S2), despite the fact that the $C_{2\alpha}$ to $N_{4'}$ distance is also short (3.3−3.8 Å).[5,15−17]

The unique $C_{2\alpha}$ stereochemistry and short $C_{2\alpha}$ to $N_{4'}$ distance significantly affect the protonation states of the $C_{2\alpha}$ and $N_{4'}$ atoms in the covalent intermediate. Out of the four possible models with different protonation patterns at these two atoms (Figure 2c), the first two (I and II) are strongly favored by the absence of steric repulsion resulting from having only one proton between $C_{2\alpha}$ and $N_{4'}$, whereas the other models are disfavored by the overlap of the van der Waals surfaces of the two hydrogen atoms between these two atoms. In models I−III, $N_{4'}$ is constrained to have all of its associated protons in the same plane of the pyrimidine ring because of a heavy energy penalty for breaking this coplanarity.[18] In model IV, this constraint is removed, which reduces but is unable to eliminate the steric repulsion. Both intermediates III and IV are similar to the off-pathway protonation products of the enamine/acyl anion intermediate in catalysis of many other ThDP-dependent enzymes and are expected to be dissociated at the $C_2-C_{2\alpha}$ bond to release succinic semialdehyde, like the latter in aqueous solution.[1] However, Palmer and co-workers showed that no succinic semialdehyde was released for at least 30 min from the intermediate formed between ThDP-bound MenD and α-ketoglutarate,[19] further negating the likelihood of the protonated model III or IV.

Although the crystal structure is unable to determine the protonation state at $C_{2\alpha}$ and $N_{4'}$ at the current resolution (1.34 Å), the four possible models can be differentiated by their chemical reactivity and proton exchangeability. Model I is the typical acyl anion tautomer with a potential amine-to-carbanion hydrogen bond, while model II could be considered to take a near attack position to allow deprotonation of $C_{2\alpha}$ by the neutral imine, particularly when primed by isochorismate, the second MenD substrate. These two different forms of the intermediate may interconvert and exist in equilibrium with the proton shuttling between $C_{2\alpha}$ and $N_{4'}$. Thus, both models I and II are active, on-pathway intermediates with an exchangeable $C_{2\alpha}$ proton. In contrast, both III and IV are off-pathway intermediates with neither catalytic activity nor an exchangeable $C_{2\alpha}$ proton because they are variants of the protonated acyl anion tautomer.

To directly determine the solvent exchangeability of the proton associated with $C_{2\alpha}$, the putative enzyme-bound intermediate was freshly prepared and mixed with one equal volume of deuterated water. The exchange reaction was quenched after 30 min of incubation at room temperature, and the succinic semialdehyde−ThDP adduct was isolated by HPLC. The resulting adduct was found to produce two molecular ions at m/z 527 and 528 with an intensity ratio of 100:96.6, compared with 100:22.5 for the non-deuterated control (Figures 3a and S5), indicating 39.8% deuterium incorporation. In its NMR spectrum (Figure 3b), the chemical shift of its $C_{2\alpha}-H$ was determined at 5.31 ppm by comparison to those of the $C_{2\alpha}$ protons in similar ThDP adducts such as 2-(1-hydroxyethyl)-ThDP.[20] The intensity of this $C_{2\alpha}-H$ signal is decreased by 32.8% compared with the non-deuterated adduct after normalization with the intensity of the $C_{6'}$ proton on the aminopyrimidine ring at 7.30 ppm,[21] while those of other protons are not significantly changed (Figure S6). The mass spectrometry and NMR results exhibit differences due to uncertainties in NMR intensity integration but are roughly consistent, demonstrating that deuteration occurs at $C_{2\alpha}$ with an exchange rate in the range of 65.8% (NMR result) to 79.6% (MS result) under the given conditions (maximum deuterium incorporation = 50%). These experiments clearly show that $C_{2\alpha}$ is associated with an exchangeable proton in the post-decarboxylation intermediate, supporting its identity as either model I or model II.

To test its activity, the putative intermediate was first prepared in solution and allowed to react with excess isochorismate in the presence of the menaquinone biosynthetic enzyme MenH,[22] which converted the SEPHCHC product to stable 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate

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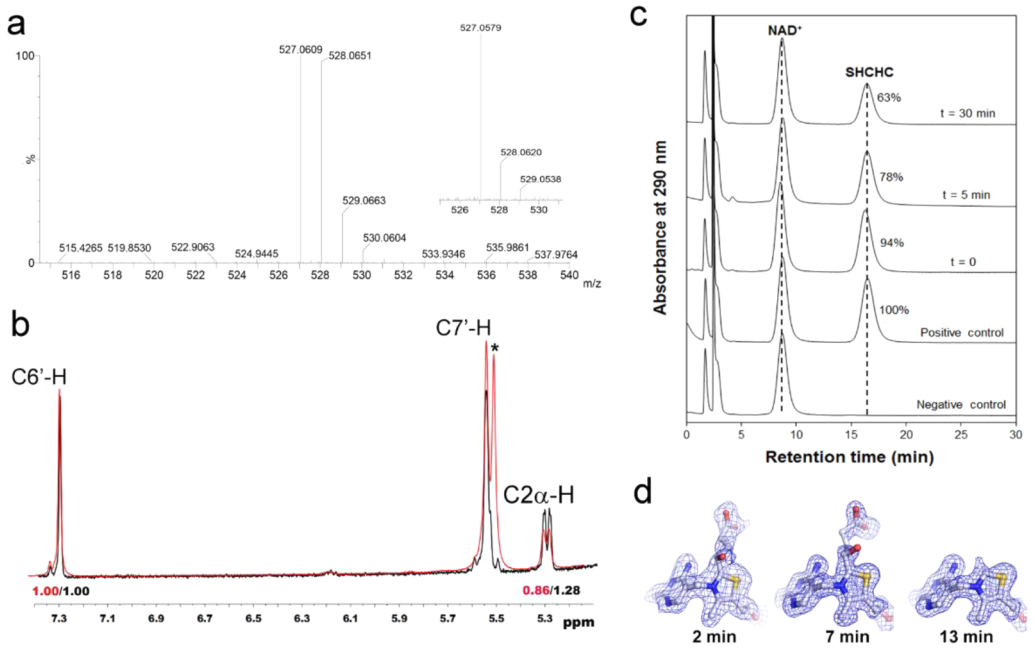

Figure 3. $C_{2\alpha}$–H exchangeability and activity of the putative intermediate. (a) ESI-MS molecular ion of the isolated succinic semialdehyde–ThDP adduct after 30 min of deuterium exchange at room temperature. The inset shows the molecular ion of the non-deuterated adduct from the control reaction. (b) Partial ^{1}H NMR spectra of succinic semialdehyde–ThDP purified from the deuterium-exchange reaction (red line) and the control reaction (black line). The intensity of $C_{2\alpha}$–H at 5.31 ppm is normalized to the intensity of $C_{6'}$–H at 7.30 ppm and provided below the corresponding peaks. The peak with an asterisk at 5.51 ppm belongs to $C_{7'}$–H of free ThDP. (c) Conversion of the putative intermediate to SEPHCHC in solution. SEPHCHC was converted to SHCHC by MenH for HPLC detection using NAD^+ as an internal control. The percent yields after normalization to the positive control are indicated. (d) Gradual disappearance of the succinic semialdehyde group of the intermediate in crystal. Shown is the $2mF_o$–DF_c electron density map contoured at 1.0σ in blue mesh after the crystal was soaked in 250 μM isochorismate at room temperature for the indicated time periods.

(SHCHC) for quantitative measurement by HPLC. Relative to the control reaction, the single-turnover experiment produced 94%, 78%, and 62% of SHCHC when the intermediate was freshly prepared (t = 0) or stored at room temperature for 5 or 30 min before reaction with isochorismate, respectively (Figure 3c). The lower product levels relative to the control may be due to inactivation of the putative intermediate by side reactions such as oxidation,[23] but nonetheless, these results unequivocally demonstrate that the intermediate is active in solution. Subsequently, the putative intermediate was prepared in crystal by soaking and subjected to a second soaking in isochorismate solution at room temperature. In the resulting crystal structures with a resolution from 1.60 to 2.24 Å, the electron density of the C_2 appendage gradually decreases in quality with increasing soaking time, ranging from essentially unchanged at 2 min to complete disappearance at 13 min (Figure 3d), while other parts of the intermediate and the polypeptide chains remain unchanged. In all cases, SEPHCHC was detected in the soaking solution with a coupled assay[22] but was not found in the solved structures, indicating that it quickly exited the active site after formation. These observations unambiguously demonstrate that the putative intermediate is also active in crystal.

Collectively, all of the biochemical and crystallographic results support the conclusion that the trapped ThDP-bound intermediate is an active, on-pathway intermediate in MenD catalysis and takes a structure as either the acyl anion (model I) or its near-attack variant (model II). More likely, the intermediate exists in both structural forms in an equilibrium that is dominated by model II because of its much higher stability (Figure 4). In this structural model of the intermediate (Figure 2c), the protonated $C_{2\alpha}$ is activated by the neutral

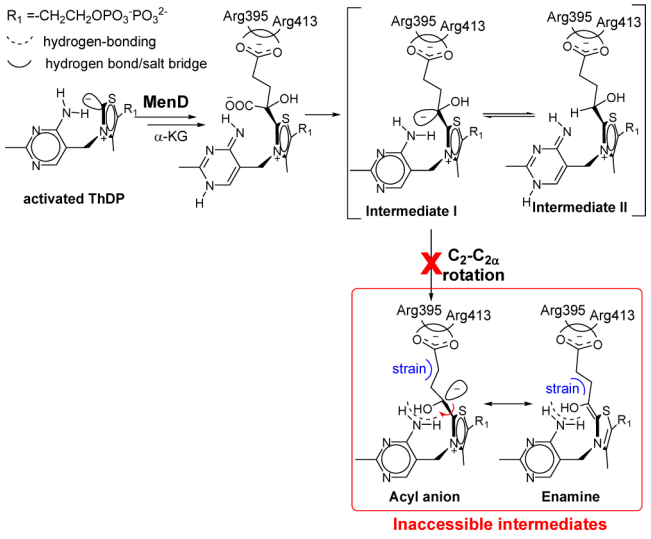

Figure 4. Proposed mechanism for formation of the tetrahedral intermediate in MenD catalysis. The strong interaction at the terminal carboxylate is proposed to restrict the rotation around C_2–$C_{2\alpha}$ so that no hydrogen bond is formed between $C_{2\alpha}$–OH and $N_{4'}$ of the cofactor throughout the reaction process. This disables the formation of the enamine intermediate and enables the formation of the tetrahedral intermediate. Intermediates I and II are models I and II in Figure 2c, respectively.

iminopyrimidine of the cofactor, similar to the activation of C_2–H of the ThDP cofactor.[24] In both cases, $N_{4'}$ of the cofactor and the reaction center at either C_2 or $C_{2\alpha}$ are coerced into a short distance to allow only one proton between them

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and to force the cofactor to take the iminopyrimidine form. In connection to this activation mechanism, it is interesting to note that $C_{2\alpha}$–H and C_2–H are comparable in pK_a.[25]

As noticed earlier, the MenD intermediate is formed as a result of the special stereochemical environment at $C_{2\alpha}$ that leads to collision between $C_{2\alpha}$ and $N_{4'}$, whereas a hydrogen bond between $C_{2\alpha}$–OH and $N_{4'}$ is formed to avoid unfavorable interactions in known enzyme-bound tetrahedral adducts of ThDP or its 3-deaza analogue.[5,15−17] From this difference, prevention of the $C_{2\alpha}$–OH to $N_{4'}$ hydrogen bond is suggested to be the key to forming the tetrahedral intermediate in MenD. This is apparently achieved by conformational control of the C_2 appendage via strong interactions of its terminal carboxylate with the conserved Arg395 and Arg413 (Figure 2b). This conformational control is thus believed to be the point where MenD diverges from other ThDP-dependent enzymes (Figure 4). In its absence, the ThDP-bound intermediate naturally falls to an energy minimum after decarboxylation by taking the energetically favorable enamine structure and simultaneously forming the $C_{2\alpha}$–OH to $N_{4'}$ hydrogen bond in most known ThDP-dependent enzymes.[2−5] In its presence in MenD, the intermediate is forced to retain the tetrahedral structure after decarboxylation, which is stabilized by protonation but remains catalytically active because of the short distance between $C_{2\alpha}$–H and $N_{4'}$ of the neutral iminopyrimidine (Figure 4). The role of this conformational control is supported by the more than 100-fold activity decrease in *Bacillus subtilis* MenD mutated at the equivalent residue of *E. coli* MenD Arg395.[13]

Besides being different from the enamine intermediate, the tetrahedral MenD intermediate is also different from the previously identified noncanonical enamine intermediates in both structure and catalytic mode.[6−9] It is strained and may be required for the catalysis of the unique 1,4-addition reaction. Further studies are needed to better understand its differences from the canonical enamine intermediate in reactivity and catalytic mechanism.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b03437.

Experimental procedures and additional results in Figures S1−S6 and Tables S1 and S2 (PDF)
Crystallographic data (CIF)

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Notes

The authors declare no competing financial interest.

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