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Structural insights of the MenD from *Escherichia coli* reveal ThDP affinity

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ABSTRACT

MenD (2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexadiene-1-carboxylate) synthase belongs to the superfamily of thiamin diphosphate-dependent decarboxylases, which converts isochorismate and 2-oxoglutarate to SHCHC, pyruvate, and carbon dioxide. Here, we report the first crystal structure of apo-MenD from *Escherichia coli* determined in tetragonal crystal form. The subunit displays the typical three-domain structure observed for ThDP-dependent enzymes. Analytical gel filtration shows that *Ec*MenD behaves as a dimer as well as a tetramer. Circular dichroism and isothermal calorimetry results confirm *Ec*MenD dependency on ThDP, which concomitantly helps to stabilize with better configuration.

Introduction

Menaquinone (or vitamin K_2), a lipid-soluble molecule, plays a vital role in the electron transport chain in prokaryotes as well as being involved in the blood coagulating proteins in mammals [1]. Due to its absence in humans, menaquinone biosynthesis is recognized as an attractive target for the development of antibiotics for pathogenic microbes [2]. Menaquinone is derived from the common branch-point intermediate, chorismate, of the shikimate pathway in bacteria [3]. Eight proteins are involved in the biosynthesis of menaquinone, and have been extensively studied in gram negative *Escherichia coli*; among them four responsible genes (men BCDE) including MenD coding for menaquinone biosynthetic enzymes are linked at 49 min in the *E. coli* chromosome encodes α ketoglutarate decarboxylase and prearomatic compound 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate (SHCHC) synthase, respectively [4]. MenD (2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexadiene-1-carboxylate synthase; EC 2.5.1.64 or 2.2.1.9), belongs to the superfamily of thiamin diphosphate (ThDP) dependent decarboxylases [2]. This enzyme converts isochorismate and 2-oxoglutarate to SHCHC, pyruvate, and carbon dioxide. ThDP is a prosthetic group derived from vitamin B1, deprotonated at carbon 2 of the thiazolium ring, and this ylide then attacks the carbonyl carbon of 2-oxoglutarate (AKG) during enzymatic decarboxylation [1]. MenD is the only enzyme known to catalyze the addition of a ThDP intermediate to the β-carbon of a second substrate, a reaction that is similar to the Stetter reaction, which is a 1,4-addition, or conjugate addition, of an aldehyde to a β-unsaturated compound [5]. MenD is basically a dimer in solution with a molecular weight of 1.4–10^5 Da for the hexahistidine-tagged protein [4]. The protein is absolutely dependent on the presence of ThDP [6] and a divalent metal ion such as Mg^{2+} or Mn^{2+} for activity, with the maximum reaction rate observed in the presence of Mn^{2+} at pH 8.5 in the Tris–HCl buffer. A very low identity (approx. 20–30%) found for MenD sequences from different organisms reveals the diverse nature of this enzyme. At the early stages, MenD was assigned as the bifunctional enzyme SHCHC synthase, however, recently it assigned to SEPHCHC (2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexadiene-1-carboxylate) synthase [7,8].

In this study, we describe the crystal structure of apo *Ec*MenD (*E. coli* MenD) with supporting biological assay that reveals its affinity towards ThDP, FAD and oxoglutarate, providing further insight into its function and role as a bifunctional enzyme.

Materials and methods

Protein expression and purification. The full-length MenD gene (Accession No. NC_000913) was amplified via PCR using the *E. coli* K-12 genomic DNA as a template. The sequences of the forward and reverse oligonucleotide primers were designed as fol-

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lows: 5′-ggaattc**catatg**tcagtaagcgcatttaacc-3′ and 5′-ccg**ctcgag**tca taaatggcttacctgc-3′ (The bases in bold and underlined indicate the NdeI and XhoI digestion sites, respectively). DNA was then inserted into the NdeI/XhoI-digested expression vector, pET-28a (Novagen) to produce recombinant *Ec*MenD with a hexahistidine tag and a thrombin cleavage site at the N-terminus. Further, *Ec*MenD was then overexpressed in *E. coli* BL21 (DE3) cells. To accomplish this, the cells were grown at 310 K to an OD_{600} of 0.6 in a LB medium containing 50 μg ml^{-1} kanamycin. The protein expression was then induced via the addition of 0.7 mM IPTG, after which cell growth was continued at 295 K overnight. The cells were then harvested by 30 min of centrifugation at 4200g at 277 K. Next, the cell pellet was resuspended in an ice-cold lysis buffer [50 mM Tris–HCl pH 8.2, 200 mM NaCl, 5% (v/v) glycerol] and then disrupted using a sonicator (Sonics, USA). The crude cell extract was then centrifuged for 40 min at 12,000 rpm (Hanil Supra 21 K rotor) at 277 K. The recombinant protein in the supernatant fraction was purified via three chromatographic steps. The first step employed the N-terminal histidine tag by His-Trap chromatography (GE Healthcare). Second step, ion-exchange chromatography was conducted using a Q column (GE Healthcare), followed by chromatography on a prep-grade Superdex 200 26/60 (GE Healthcare) column that had previously been equilibrated with buffer A [25 mM Tris–HCl pH 8.2, 200 mM NaCl, 5 mM $MgCl_2$, 2 mM DTT]. The homogeneity of the purified protein was assessed by SDS–PAGE, after which the protein was concentrated to a final concentration of 25 mg ml^{-1} using a Centri-Prep centrifugal filter (Millipore) with buffer A.

Crystallization and data-collection. Initial crystallization was conducted at 295 K via 96 well sitting-drop Intelli-plates using the Hydra II Plus One system (Matrix Technology™) with approximately 800 conditions and a ratio of 200 nl of precipitants to 200 nl of protein solution over 70 μl volume of well solution. Crystallization trials were conducted using screening kits obtained from Hampton Research and Emerald BioStructures. After 2–3 days, micro crystals were observed from Emerald BioStructures Wizard III condition No. 15 (1.6 M Na-citrate) and from Hampton Research's Salt RX1 condition No. 21 (1.2 M tri-Na citrate, 0.1 M Bis–Tris propane, pH 7.0). These micro crystals were diffracted poorly to 7 Å and the space group was undecided. These crystallization conditions were further optimized via the hanging-drop vapour-diffusion method using reservoir solution (0.5–0.6 M tri-Na citrate). Tetragonal shaped crystal ($0.4 \times 0.3 \times 0.2$ mm) were obtained after one month at 295 K. The crystals of the *Ec*MenD were cryo-protected with 0.8 M tri-Na citrate and 25% (v/v) ethylene glycol. The X-ray diffraction data were collected from the cooled crystal using an ADSC Quantum CCD 270 detector at beam line BL-17A (Photon factory, Japan). The crystal was oscillated by 1.0° per frame over a total range of 100° at a wavelength of 1.0000 Å. X-ray diffraction data to 2.7 Å was collected. The data were integrated and scaled via DENZO and SCALEPACK crystallographic data-reduction routines with *HKL*-2000 program suite [9].

UV circular dichroism analyses. The *Ec*MenD protein was structurally characterized to see the effect of ThDP, FAD and AKG on secondary structure in terms of ellipticity, as well as thermal stability, using a circular dichroism (CD) spectropolarimeter. The experiments were performed on a Jasco J-715 spectropolarimeter (JASCO, Japan) equipped with a temperature-controlling unit, using a 0.1 cm path length cell with a 1 nm bandwidth and 4 s response time. The standard far-UV CD spectra were collected at room temperature at a scan speed of 20 nm/min with a 0.1 nm step resolution. Six accumulations taken from 260 to 190 nm were added and averaged, followed by the subtraction of the solvent CD signal. Thermal denaturation was monitored at 222 nm, increasing the temperature by 1 °C/min from 5 to 100 °C using the CD spectrometer, and the data was converted into fraction unfolded. The concentration of *Ec*MenD was 0.2 mg/ml in 25 mM Tris buffer, pH 8.0. The thermal mid point (Tm) was calculated as described previously [10,11].

Isothermal calorimetry (ITC). ITC experiments were performed at 298 K using a high-precision VP-ITC system (Microcal Inc.). Protein and cofactors (ThDP) were dialysed into 25 mM Tris–HCl pH 8.2, 200 mM NaCl, 5 mM $MgCl_2$, 2 mM DTT. The concentrations of the protein (cell) and cofactor (injector) were kept at 30 and 300 μM, respectively. The heat evolved following each 2 μl injection was obtained from the integral of the calorimetric signal. The heat arising from the binding reaction was obtained as the difference between the heat of reaction and the corresponding heat of dilution. Analysis of the data was performed using Origin software (Microcal 1998).

Structure determination. The structure was solved by molecular replacement using MOLREP program within CCP4 [12], where a monomer of the holoenzyme *E. coli* MenD structure (PDB ID: 2JLC) [13] was used as a model, two translated positions were found within the asymmetric unit. Multiple cycles of editing, adjustment of the model-using coot [14], restrained refinement and final refinement were carried out with refmac program within CCP4 [12]. The final models were validated with PROCHECK [15].

Results and discussion

Protein purification, crystallization and X-ray diffraction analysis

*Ec*MenD (62 kDa) was overexpressed in *E. coli* in a soluble form with a yield of ~80 mg of homogeneous protein per liter of culture. Based on the results of size exclusion the molecular weight of this protein was calculated approximately 120 and 240 kDa as a dimer and tetramer, respectively. Approximately 80% of the total protein eluted as a dimer and 20% as a tetramer (data not shown). These results support previous reports that MenD from various organisms behave as a dimer or a tetramer, or both [4,13]. In contrast to previous *Ec*MenD crystallization conditions [13,16], we obtained first time the apoenzyme crystal with tetragonal form having dissimilar space groups and unit cell parameters. Initially, very irregular micro crystals were observed from the dimer protein; however, after optimization, tetragonal shaped crystals were observed when a reservoir solution containing 0.5–0.6 M tri-Na citrate was used over a period of one month. Flash-cooled *Ec*MenD crystal using 25% (v/v) ethylene glycol as a cryo-protectant was diffracted to 2.7 Å. Auto-indexing was conducted using DENZO, and the results indicated that the crystal was a member of the tetragonal space group $P4_12_12$ on the basis of systematic absences and with following unit cell parameters: $a = b = 118.0$, $c = 176.4$ Å, and $\alpha = \beta = \gamma = 90°$. These results indicate that two monomers are likely present in the asymmetric unit, which has a corresponding calculated Matthews's coefficient (V_M) of 2.42 $Å^3\ Da^{-1}$ and a solvent content of 49.5% [17]. Data-collection and refinement statistics are provided in Table 1.

Structure determination

We have determined the crystal structure of apo *Ec*MenD forming dimeric structure. Two independent subunits of an *Ec*MenD homodimer in the asymmetric unit adopt similar conformations. When we superposed the A and B chains for 530 C^{α} (except 175–177 and 471–493) within the dimer *Ec*MenD structure, the overlay with a root-mean-square deviation (r.m.s.d.) of 0.30 Å. However, a higher r.m.s.d. 0.61 Å was observed when compared with the recently reported holoenzyme *E. coli* MenD [13], suggesting some sort of conformational change among them. Monomer structure of *Ec*MenD comprised of 23 α-helices and 17 β-stranded unequally divided within 3 domains (Fig. 1A). Continuous electron density was observed for almost the entire polypeptide chains in the apo