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Structural and functional analysis of Vitamin K_2 synthesis protein MenD

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ABSTRACT

Here we describe in detail the crystal structures of the Vitamin K_2 synthesis protein MenD, from *Escherichia coli*, in complex with thiamine diphosphate (ThDP) and oxoglutarate, and the effects of cofactor and substrate on its structural stability. This is the first reported structure of MenD in complex with oxoglutarate. The residues Gly472 to Phe488 of the active site region are either disordered, or in an open conformation in the MenD oxoglutarate complex structure, but adopt a closed conformation in the MenD ThDP complex structure. Biospecific-interaction analysis using surface plasmon resonance (SPR) technology reveals an affinity for ThDP and oxoglutarate in the nanomolar range. Biochemical and structural analysis confirmed that MenD is highly dependent on ThDP for its structural stability. Our structural results combined with the biochemical assay reveal novel features of the enzyme that could be utilized in a program of rational structure-based drug design, as well as in helping to enhance our knowledge of the menaquinone synthesis pathway in greater detail.

Introduction

Vitamin K_2 (menaquinone), a naphthoquinone derivative, represents a group of lipophilic, hydrophobic vitamins that are required for the post-translational modification of certain proteins and are essential for blood coagulation [1,2]. Due to its absence in humans, menaquinone biosynthesis is an appealing target for the development of antibiotics against pathogenic microbes that depend on naphthoquinone for survival. The biosynthesis of menaquinone is best known in *Escherichia coli* for its involvement in aerobic respiration and menaquinone-mediated anaerobic respiration [3]. MenD (2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexadiene-1-carboxylate synthase) is the second of eight enzymes in the menaquinone biosynthesis process, which converts isochorismate and oxoglutarate to the intermediate product 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate (SEPHCHC), followed by a reaction with the MenH enzyme to produce SHCHC (2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate), pyruvate and carbon dioxide in a ThDP-dependent reaction [3–5]. Until now, very few attempts have been made to characterize the MenD protein using biochemical tools [6]. In addition, the protein's structure when associated with its substrate has not yet been determined due to the transient nature of the complex [7].

In this study, we describe the crystal structures of *Ec*MenD complex with ThDP and oxoglutarate with supporting biological assay using SPR.

Materials and methods

Protein purification, crystallization, and data collection. Detailed procedures for cloning and purification have been described elsewhere [8]. A crystal of the *Ec*MenD oxoglutarate complex was obtained from a solution using 0.5–0.6 M tri-Na citrate, 1 mM oxoglutarate, and 5 mM $MgCl_2$, whereas a crystal of the *Ec*MenD ThDP complex was obtained from 12% PEG 8K, 10% glycerol, 1 mM ThDP, 5 mM $MgCl_2$, and HEPES, pH 7.5. X-ray diffraction data were collected from the cooled crystals using an ADSC Quantum CCD 270 detector at beam line 4A, 6C (PLS, Korea) to 2.6 and 1.95 Å resolution for the ThDP and oxoglutarate complexes, respectively (Table 1). The data were integrated and scaled via the DENZO and SCALEPACK crystallographic data-reduction routines with the HKL-2000 program suite [9].

Structure determination. The structure was solved by molecular replacement using CNS [10] and MOLREP program [11] within CCP4 [12] simultaneously. Monomer of the apo *Ec*MenD structure (PDB:3FLM) was used as a model [8], two translated positions were found within the asymmetric unit for the *Ec*MenD oxoglutarate complex whereas eight molecules found in unit cell for

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Table 1
Data collection and refinement statistics for *Ec*MenD.

Crystal	*Ec*MenD + oxoglutarate	*Ec*MenD + ThDP
Space group	$P4_12_12$	$P1$
a, *b*, *c* (Å)	117.9, 117.9, 175.2	90.3, 90.4, 169.1
α, β, γ (°)	90.0, 90.0, 90.0	75.9, 82.9, 64.1
Resolution	50.0–1.95 (2.05–1.95)	50.0–2.40 (2.44–2.40)
Total reflection	85,185	141,599
Completeness (%)	94.2 (84.9)	84.1 (50.2)
R_{sym}[a] (%)	8 (46)	15 (26)
I/σ	19.0 (1.9)	6.22 (1.8)
Molecule per asymmetric unit	Two	Eight
R_{work}	0.20	0.19
R_{free}	0.25	0.25
r.m.s.d.		
Length (Å)	0.02	0.02
Angle (°)	2.3	2.1
Average *B*-factor	38.5	14.5
Ramachandran favorite (%)		
Most favored region	90	93
Additionally allowed region	9	6
Generously allowed region	1	1

[a] $R_{sym} = \sum(I - \langle I \rangle)/\sum\langle I \rangle$, where I is the intensity measurement for a given refraction and $\langle I \rangle$ is the average intensity for multiple measurements of this refraction.

the ThDP complex. The models underwent multiple cycles of editing, adjustment using the program Coot [13], restrained refinement with Refmac [14], and final validation using PROCHECK [15].

Binding assay using SPR. Biospecific-interaction analysis was performed using a BIAcore 2000 biosensor system (Amersham, Sweden). The immobilization of the *Ec*MenD protein (0.1 mg/ml) in 10 mM sodium acetate buffer (pH 4.5) on a CM5 (carboxymethylated)-certified grade sensor chip coupled to 3000 resonance units (RUs). All measurements were carried out in 10 mM HEPES, pH 7.5/8.0, 150 mM NaCl. In order to determine the association rate constant, ThDP, oxoglutarate and FAD were passed over the chip surfaces at various concentrations at 25 °C and a flow rate of 10 μl/min.

Results and discussion

Overall structure

The monomer structure of *Ec*MenD comprised of 23 α-helices and 17 β-strands (Fig. 1A) unequally divided between three domains and capable of forming a dimer structure. The *Ec*MenD subunit has 64% of its residues in secondary structure elements and displays the three-domain architecture typical of ThDP-dependent enzymes. The N-terminal domain I has 195 residues and shows similar topology to domain III (Val347 to Leu556), each domain consisting of a central six-stranded parallel β-sheet sandwiched between several α-helices. In contrast, the central domain II (Asp213 to His337) contains five β-sheets and seven α-helices. The electrostatic surface map of *Ec*MenD reveals a patchy surface charge, but domain II shows greater hydrophilicity compared to the other domains. The average *B*-factor of the *Ec*MenD ThDP complex is 14.5 Å^2, but a higher *B*-factor of 38.5 Å^2 was observed for the *Ec*MenD oxoglutarate complex, with a *B*-factor of almost double (~70 Å^2) for the regions Ala29 to Leu36 (β1 to α2), Ala171 to Asp180 (β6 to α7), and Asn469 to Gly471 (β15 to α21), which correspond to the highly flexible loop region. Analysis of the dimer interface indicated that 13% (2600 Å^2) of the surface area of the monomer (20,160 Å^2) is occluded on formation of the dimer.

Flexible loop region

The most significant difference between the oxoglutarate complex and the ThDP complex structure, presumably caused by the binding of ThDP, is the movement of the loop (Tyr489 to Asn494) and the disorder region Gly472 to Phe488 (Fig. 1B and C). The substrate-binding loop (Tyr489 to Asn494) in the apo form [8] as well as in the oxoglutarate complex structure was shifted by 4 Å and moves towards the region Gly182 to Asp195 (α7) of another asymmetric unit (lie within ~3.5 Å). This movement, along with a rotation in the side chain of Asn470, Gln493, and Asn494 (Fig. 1C), causes the substrate-binding cavity to widen, allowing it to accommodate the bound substrate, and thus demonstrating the flexibility of this loop in accommodating substrates of various chain lengths. The crystal structure of the *Ec*MenD ThDP complex revealed a similar binding

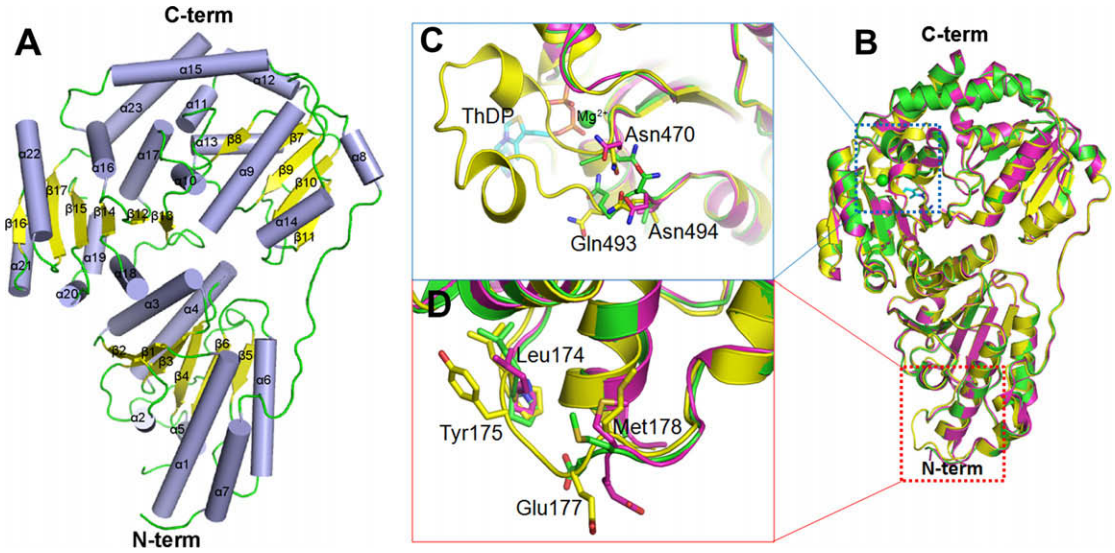

Fig. 1. Monomer structure of *Ec*MenD. (A) Ribbon diagram of *Ec*MenD monomer composed of 17 β-sheets and 23 α-helices unequally divided into three domains. (B) Superposition of various *Ec*MenD structures. Oxoglutarate complex (pink), apo (green) and ThDP complex (yellow). (C) Active site pocket of *Ec*MenD anchoring ThDP and Mg^{2+} shows a disordered region in the apo and oxoglutarate complex structures, and an ordered region in the ThDP complex structure. (D) Flexible region (Leu174-Met178).