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Crystallization and preliminary X-ray analysis of 2,3-diketo-5-methylthiopentyl-1-phosphate enolase from *Bacillus subtilis*

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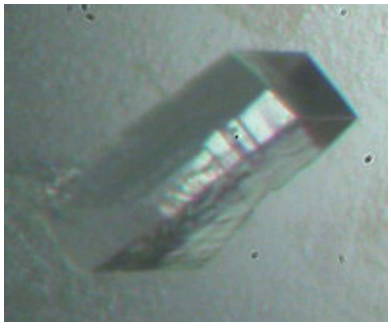

2,3-Diketo-5-methylthiopentyl-1-phosphate enolase (DK-MTP-1P enolase) from *Bacillus subtilis* was crystallized using the hanging-drop vapour-diffusion method. Crystals grew using PEG 3350 as the precipitant at 293 K. The crystals diffracted to 2.3 Å resolution at 100 K using synchrotron radiation and were found to belong to the monoclinic space group $P2_1$, with unit-cell parameters a = 79.3, b = 91.5, c = 107.0 Å, β = 90.8°. The asymmetric unit contained four molecules of DK-MTP-1P enolase, with a V_M value of 2.2 Å^3 Da^{-1} and a solvent content of 43%.

1. Introduction

Methionine is an essential amino acid for cellular functions such as protein synthesis (Cooper, 1983), transmethylation (Cantoni, 1951) and polyamine biosynthesis (Stipanuk, 1986; Tabor & Tabor, 1976). Bacteria and eukarya usually possess the methionine-salvage pathway (MSP; Sekowska *et al.*, 2004), which salvages methionine from methylthioadenosine, the end product of spermidine and spermine anabolism (Winans & Bassler, 2002). The MSP was first unravelled in *Bacillus subtilis* (Ashida *et al.*, 2003; Sekowska & Danchin, 2002) and functional MSPs have been experimentally demonstrated for various organisms (Pirkov *et al.*, 2008; Sekowska *et al.*, 2004; Sufrin *et al.*, 1995). At least seven enzymes are involved in the MSP of *B. subtilis*. Of these enzymes, 2,3-diketo-5-methylthiopentyl-1-phosphate enolase (DK-MTP-1P enolase) catalyzes the enolization of 2,3-diketo-5-methylthiopentyl-1-phosphate.

DK-MTP-1P enolase has attracted enormous attention owing to its homology to the large subunit of D-ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO). RuBisCO plays a key role in fixing CO_2 into a five-carbon sugar phosphate in the Calvin cycle (Lorimer, 1981). However, RuBisCO is an inefficient catalyst; therefore, the development of a super-RuBisCO is a potential avenue to improve crop productivity. Phylogenetic analyses have indicated that RuBisCOs can be divided into four forms (forms I–IV) and DK-MTP-1P enolase belongs to form IV (Tabita, 1999; Tabita *et al.*, 2007). Because the form IV proteins do not catalyze either carboxylation or oxygenation, they are called 'RuBisCO-like proteins' (RLPs). Based on their quaternary structures, different forms of RuBisCO can be also distinguished. In forms I and II a hexadecameric (L_8S_8) form is found that consists of eight 55 kDa large (L) subunits and eight 15 kDa small (S) subunits. A dimeric (L_2) enzyme, similar in structure to the L subunits of the hexadecameric enzymes, is restricted to some form III RuBisCOs and RLPs.

The activation and reaction of RuBisCO involve multiple discrete steps. In activation, RuBisCO is carbamylated by the addition of an activator CO_2 followed by binding of Mg^{2+}. In carboxylation, the substrate, D-ribulose-1,5-bisphosphate, binds to the carbamylated active site; this is followed by the binding of gaseous CO_2 to yield two molecules of 3-phosphoglycerate as products (Andrews & Lorimer, 1987; Hartman & Harpel, 1994). Structural analyses have clarified the RuBisCO structures in these multiple steps, which include apo decarbamylated (E), apo carbamylated (ECM), holo decarbamylated (E-ligand) and holo carbamylated (ECM-ligand) forms (Andersson & Backlund, 2008; Schneider *et al.*, 1992; Spreitzer & Salvucci, 2002).

Depending on the activation and ligand-binding states, two flexible catalytic loops, called loop-6 and the 60s loop, are either 'open' or 'closed' to adjust the degree of solvent accessibility to the active site (Schreuder *et al.*, 1993).

DK-MTP-1P enolase is considered to partially share the same activation mechanism as RuBisCO. On the basis of structural and biochemical studies (Imker *et al.*, 2007), the enzyme from *Geobacillus kaustophilus* is activated *via* carbamylation of Lys173 at the active site and subsequent coordination by Mg^{2+}. Therefore, the structure of DK-MTP-1P enolase must exhibit multiple states including E, ECM, E-ligand and ECM-ligand forms, as demonstrated by structures of RuBisCO. At present, crystal structures of DK-MTP-1P enolase are not available in all forms. The structure of the apo decarbamylated form (E) remains unknown, although crystal structures of DK-MTP-1P enolase from *G. kaustophilus* (60% sequence identity to the *B. subtilis* enzyme) have been reported in four forms (Imker *et al.*, 2007): a PO_4^{3-}-bound decarbamylated (unactivated) form (E-PO_4^{3-}), a carbamylated (activated) form complexed with Mg^{2+} (ECM), a carbamylated form complexed with Mg^{2+} and bicarbonate (ECM-HCO_3^-) and a carbamylated form complexed with Mg^{2+} and the alternate substrate 2,3-diketohexane-1-phosphate (ECM-DK H-1P). If the structure of the E form of DK-MTP-1P enolase were available, the dynamic events that occur in the discrete catalytic steps of DK-MTP-1P enolase could be elucidated. It is also possible to compare the structures with those of RuBisCO in the discrete catalytic steps, which might provide clues about the functional and evolutionary relationships between RLP and RuBisCO. Furthermore, we can compare the E-form structures of DK-MTP-1P enolase and other RLPs from *Chlorobium tepidum* (26% sequence identity to the *B. subtilis* enzyme; Li *et al.*, 2005) and *Rhodopseudomonas palustris* (25% sequence identity to the *B. subtilis* enzyme; Tabita *et al.*, 2007), the molecular functions of which remain unknown. Such comparisons may provide further insights into the molecular mechanisms of RLPs of currently unknown function.

In order to understand the structure–function relationship, crystallization of the apo decarbamylated form (E) of DK-MTP-1P enolase from *B. subtilis* (molecular weight 45.1 kDa) has been attempted. Here, we report the purification, crystallization and preliminary X-ray crystallographic study of *B. subtilis* DK-MTP-1P enolase.

2. Materials and methods

2.1. Cloning and expression

The full-length DK-MTP-1P enolase (*mtnW*) gene was amplified from genomic DNA by PCR, which was performed with forward and reverse primers 5′-GAGCTCTCATATGAGTGAGTGAGTTA-TTAG-3′ and 5′-GCGGATCCTCATACGGCTTC-3′, respectively. Because of the presence of an *Nde*I site in *mtnW*, full-length *mtnW* was subcloned in pBC (Stratagene) and then partially digested with *Nde*I before digestion with *Bam*HI. The fragments were ligated into the *Nde*I/*Bam*HI site of pET15b (Novagen). The resulting recombinant protein consists of the DK-MTP-1P enolase sequence with 20 extra residues (MGSSHHHHHHSSGLVPRGSH) at the N-terminus containing the 6×His tag and a thrombin cleavage site. *Escherichia coli* strain BL21 (DE3) cells harbouring the DK-MTP-1P enolase expression plasmid were grown for 8 h in LB medium containing 50 µg ml^{-1} ampicillin at 310 K. The cells were grown for a further 16 h at 303 K. The cells were then harvested by centrifugation at 6000*g* for 30 min. The cell pellet was resuspended in buffer *A* (50 m*M* Na HEPES pH 7.4, 500 m*M* NaCl, 10 m*M* imidazole and 10 m*M* 2-mercaptoethanol) containing 1 m*M* PMSF and disrupted using a French press.

2.2. Protein purification

The disrupted cells were centrifuged at 400 000*g* at 277 K for 30 min. The supernatant was loaded onto a 5 ml HiTrap Chelating column (GE Healthcare Biosciences) equilibrated with buffer *A*. The unbound proteins were flushed with buffer *A*. DK-MTP-1P enolase with an N-terminal histidine tag was eluted with a 215–300 m*M* imidazole gradient. Further purification of DK-MTP-1P enolase was carried out using a gel-filtration chromatography column (HiLoad 26/60 Superdex 200 prep grade, GE Healthcare Biosciences) equilibrated with buffer *B* (50 m*M* Na HEPES pH 7.4, 500 m*M* NaCl, 10 m*M* DTT and 1 m*M* EDTA). Pooled fractions were concentrated to 10 mg ml^{-1} using a Vivaspin 20 ml (10 kDa cutoff, Vivascience) and used for crystallization without removal of the N-terminal histidine tag. The protein concentration was determined by measuring the absorbance at 280 nm with a calculated molar absorption coefficient of 23 080 M^{-1} cm^{-1} (Gill & von Hippel, 1989). The purity was checked by SDS–PAGE analysis (Fig. 1).

2.3. Crystallization

Crystallization screening of 10 mg ml^{-1} DK-MTP-1P enolase in buffer *B* was initially performed with PEG/Ion Screen (Hampton Research) using the hanging-drop vapour-diffusion method at 293 K. The drop size was 2 µl, with a protein:reservoir solution ratio of 1:1. Crystals with maximum dimensions of 0.1 × 0.05 × 0.05 mm were obtained using PEG/Ion Screen condition No. 13 [200 m*M* sodium thiocyanate, 20%(*w*/*v*) PEG 3350] within a week. To refine the conditions, we varied the pH and the concentration of protein, salt and precipitant. Optimized crystallization was performed using the hanging-drop vapour-diffusion method at 293 K with a 2 µl drop containing equal volumes of protein (10 mg ml^{-1} DK-MTP-1P enolase) and reservoir solution [140 m*M* sodium thiocyanate, 21%(*w*/*v*) PEG 3350] suspended over 300 µl reservoir solution.

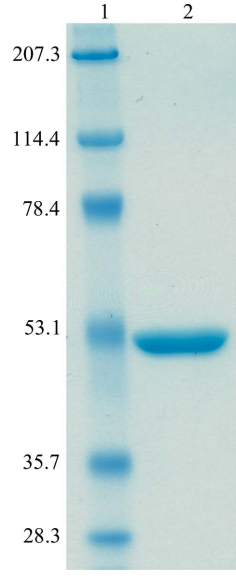

Figure 1
SDS–PAGE analysis of purified *B. subtilis* DK-MTP-1P enolase. Lane 1, molecular-weight markers (kDa). Lane 2, purified *B. subtilis* DK-MTP-1P enolase for crystallization.

Table 1
Statistics of crystal parameters and X-ray diffraction data collection.

Values in parentheses are for the highest resolution shell (2.42–2.30 Å).

Synchrotron beamline	SPring-8 BL44XU
Wavelength (Å)	1.0
Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 79.3, b = 91.5, c = 107.0, β = 90.8
Resolution range (Å)	30.5–2.30
No. of molecules per ASU	4
V_M (Å^3 Da^{-1})	2.2
V_{solv} (%)	43
No. of measured reflections	259484
No. of unique reflections	64719
R_{merge}† (%)	8.4 (27.3)
Completeness (%)	95.0 (95.3)
$I/\sigma(I)$	7.2 (2.4)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the value of the ith measurement of the intensity of a reflection, $\langle I(hkl)\rangle$ is the mean value of the intensity of that reflection and the summation is the over all measurements.

2.4. Data collection

The crystals were soaked for a few seconds in reservoir solution containing 10%(*v*/*v*) glycerol. Flash-cooled crystals were then mounted in a nitrogen stream at 100 K. X-ray diffraction data were collected to 2.3 Å resolution on a DIP-6040 detector (Bruker-AXS) using synchrotron radiation of wavelength 1.0 Å on the BL44XU beamline at SPring-8. Data collection was performed with a total oscillation range of 220°, a step size of 1.0° and an exposure time of 10 s per frame. All diffraction data were processed using the *MOSFLM* program (Leslie, 2006) and the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

Recombinant DK-MTP-1P enolase from *B. subtilis* was successfully expressed and purified to homogeneity. Crystals suitable for diffraction with maximum dimensions of 0.1 × 0.15 × 0.3 mm appeared after 5 d (Fig. 2). The crystals diffracted to 2.3 Å resolution at 100 K using the BL44XU beamline at SPring-8 (Fig. 3). The intensities were indexed in the monoclinic space group $P2_1$ based on the systematic absences, with unit-cell parameters a = 79.3, b = 91.5, c = 107.0 Å, β = 90.8°. The value of the Matthews coefficient is 2.2 Å^3 Da^{-1} for four monomers in the asymmetric unit, corresponding to a solvent content of 43%, a typical value for protein crystals (Matthews, 1968). The self-rotation function at χ = 180° was calculated for data in the 15–3.5 Å resolution range with an integration radius of 40 Å (*POLARRFN*; Collaborative Computational Project, Number 4, 1994). The result indicated the existence of pseudo-orthorhombic symmetry at (θ, φ, χ) = (90.3, 180, 180°) and (0.3, 180, 180°). The peak intensity with respect to the crystallographic axis was 64.4%. Details of the data processing and statistics are shown in Table 1. Structure determination was performed by molecular replacement using *MOLREP* v.7.3 (Vagin & Teplyakov, 1997) from the *CCP*4 v.6.0 software suite (Collaborative Computational Project, Number 4, 1994). A dimer of RuBisCO-like protein from *C. tepidum* (26% sequence identity; PDB code 1ykw; Li *et al.*, 2005) was used as a search model. The results of molecular replacement suggested that these crystals contain two independent dimers per asymmetric unit. Refinement of the structure is in progress.

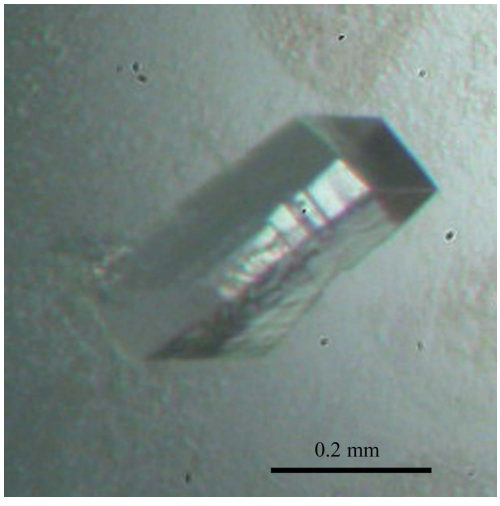

Figure 2
Crystal of *B. subtilis* DK-MTP-1P enolase (maximum dimensions 0.1 × 0.15 × 0.3 mm).

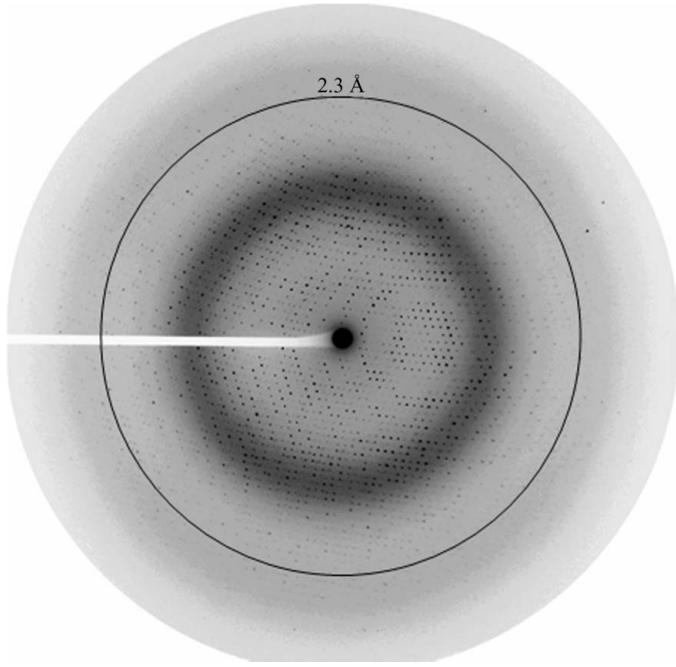

Figure 3
X-ray diffraction image from a *B. subtilis* DK-MTP-1P enolase crystal.

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