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Crystallization and preliminary X-ray analysis of methylthioribose-1-phosphate isomerase from *Bacillus subtilis*

Haruka Tamura,[a] Hiroyoshi Matsumura,[a] Tsuyoshi Inoue,[a,b] Hiroki Ashida,[c] Yohtaro Saito,[c] Akiho Yokota[c] and Yasushi Kai[a]*

[a]Department of Materials Chemistry, Graduate School of Engineering, Osaka University, 2-1 Yamada-oka, Suita, Osaka 565-0871, Japan, [b]Structure and Function of Biomolecules Group, PRESTO, Japan Science and Technology Agency, 4-1-8 Honcho Kawaguchi, Saitama, Japan, and [c]Department of Molecular Biology, Graduate School of Biological Science, Nara Institute of Science and Technology (NAIST), 8916-5 Takayama, Ikoma, Nara 630-0101, Japan

Correspondence e-mail: kai@chem.eng.osaka-u.ac.jp

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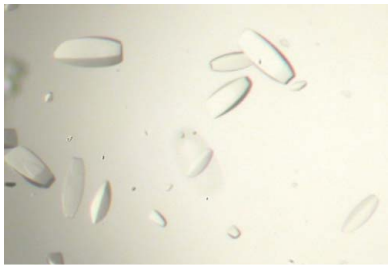


Methylthioribose-1-phosphate isomerase (MtnA) from *Bacillus subtilis*, the first enzyme in the downstream section of the methionine-salvage pathway, was crystallized using the sitting-drop vapour-diffusion method. Crystals grew using ammonium sulfate as the precipitant at 293 K. They diffracted to 2.5 Å at 100 K using synchrotron radiation and were found to belong to the tetragonal space group $P4_1$, with unit-cell parameters $a = b = 69.2$, $c = 154.7$ Å. The asymmetric unit contains two molecules of MtnA, with a V_M value of 2.4 Å^3 Da^{-1} and a solvent content of 48%.

1. Introduction

Methionine is an essential amino acid and plays an important role in a number of cellular functions, including the initiation of protein synthesis, biological methylation of DNA and rRNA and the biosynthesis of cysteine, phospholipids and polyamines. The biosynthesis of polyamines is involved in the control of many biological processes such as carcinogenesis, cell growth and differentiation. In the synthesis of the polymaines spermidine and spermine, methionine is consumed in a 1:1 stoichiometry with formation of the byproduct methylthioadenosine (MTA; Williams-Ashman & Canellakis, 1979). Since the amount of methionine in cells is limited and *de novo* synthesis of methionine is energetically costly, it is essential to salvage methionine and recycle the S atom from MTA (Trackman & Abeles, 1983). Moreover, it has long been known that MTA, once converted to adenine and methylthioribose-1-phosphate (MTR-1-P; Ferro *et al.*, 1978), is able to regenerate methionine (Williams-Ashman *et al.*, 1982; Winans & Bassler, 2002). This has been found in a wide variety of organisms, including mammals (Riscoe & Ferro, 1984; Riscoe *et al.*, 1988), *Trypanosoma brucei* (Riscoe *et al.*, 1988), *Saccharomyces cerevisiae* (Subhi *et al.*, 2003) and *Klebsiella pneumoniae* (Furfine & Abeles, 1988). The overall reaction scheme was determined in *K. pneumoniae* (Furfine & Abeles, 1988). However, the actual enzymes or genes involved in each step have not been unravelled.

A gene-inactivation study revealed that the S-box transcription-termination control system, first identified in *Bacillus subtilis*, was used for regulation of gene expression in response to methionine availability (Grundy & Henkin, 1998). The presence of the S-box motif provided the first indication that the *mtnKA* and *mtnWXBD* genes could play a role in recycling MTA (Murphy *et al.*, 2002). In 2003, we succeeded in showing the exact nature of five enzymes (MtnA, MtnW, MtnX, MtnB, MtnD) from *B. subtilis* in the six steps of the downstream section of the methionine-salvage pathway (Ashida *et al.*, 2003; Balakrishnan *et al.*, 1993; Sekowska *et al.*, 2004). The first step of the pathway in *B. subtilis* is catalyzed by MtnA, which converts MTR-1-P into methylthioribulose-1-phosphate (MTRu-1-P).

The first ubiquitous enzyme of the pathway, MtnA, belongs to a family of proteins related to the α-subunit of eukaryotic translation initiation factor 2B (eIF2B), which is a heteropentameric protein composed of α–ε subunits. Recently, the crystal structures of MtnA-homologous proteins [Ypr118w from *S. cerevisiae* and an archaeal regulatory subunit (aIF2Bα) from *Pyrococcus horikoshii* OT3] have been reported (Bumann *et al.*, 2004; Kakuta *et al.*, 2004). Despite

structural analyses and genetic studies, the active site remains unclear. The primary structure and molecular weight of MtnA from *B. subtilis* were very different from those of Ypr118w (38% identity, 45 kDa) and aIF2Bα (32% identity, 31 kDa). We have also established the generation of MTR-1-P, the substrate of MtnA, using recombinant *B. subtilis* MtnK. For the purpose of investigation into the detailed catalysis of MtnA, the crystallization of MtnA complexed with MTR-1-P is currently in progress.

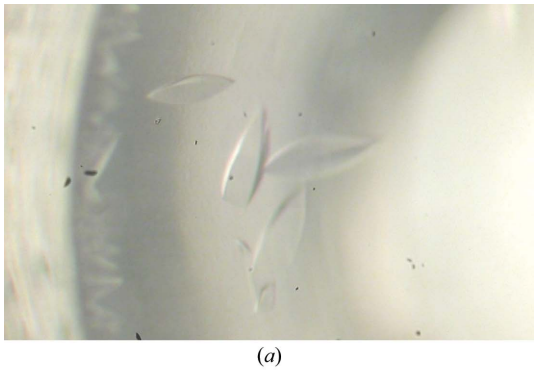
The methionine-salvage pathway is likely to be compartmentalized because of the extreme reactivity of the S atom towards dioxygen and radicals. Furthermore, the structure–function relationship of the series of *B. subtilis* enzymes remains unclear and their three-dimensional structures are unknown. Therefore, structural comparison between MtnA and its homologues will not only provide an evolutionary insight into two different catalytic mechanisms, but the three-dimensional structure of MtnA will also become an important first step in understanding the whole protein–protein network in the pathway. We report here the crystallization and the preliminary X-ray crystallographic study of MtnA from *B. subtilis*.

2. Materials and methods

2.1. Cloning and expression

The full-length *mtnA* gene was amplified from genomic DNA by PCR, which was performed with the following forward and reverse primers: 5′-GGAATTCCATATGACCCATTCATTTGCTG-3′ and 5′-CGGGATCCAAATGAGCAAAGTCC-3′. The products of PCR were digested with *Nde*I and *Bam*HI. The fragments were ligated at the *Nde*I/*Bam*HI site of pET15b (Novagen).

Escherichia coli strain BL21 (DE3) cells harbouring the *mtnA* 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 and 10 m*M* imidazole) and 1 m*M* PMSF and then disrupted using a French press.



(*a*)

(*b*)

Figure 1
Crystasls of MtnA from *B. subtilis*: (*a*) maximum dimensions 0.15 × 0.05 × 0.02 mm, (*b*) maximum dimensions 0.1 × 0.05 ×0.03 mm.

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 (Amersham Pharmacia Biotech) equilibrated with buffer *A*. The unbound proteins were flushed with buffer *A*. MtnA with an N-terminal histidine tag was eluted with a 150–225 m*M* imidazole gradient. Further purification to MtnA was carried out using a HiLoad 26/60 Superdex 75 prep-grade column (Amersham Pharmacia Biotech) equilibrated with 50 m*M* Na HEPES pH 7.4 containing 1 m*M* EDTA. Pooled fractions were concentrated to 30 mg ml^{-1} with a Vivaspin 20 ml (5000 MWCO PES, Vivascience) and used for crystallization without removal of the N-terminal histidine tag.

2.3. Crystallization

Crystallization screening using 30 mg ml^{-1} MtnA (in 50 m*M* Na HEPES pH 7.4 and 1 m*M* EDTA) was initially performed with Crystal Screen and Crystal Screen 2 (Hampton Research) in 96-well sitting-drop plates (Corning) at 293 K. The drop size was 2 µl, with a protein:reservoir ratio of 1:1. Oval-shaped crystals with maximum dimensions of 0.15 × 0.05 × 0.02 mm were obtained using Crystal Screen 2 condition No. 23 [100 m*M* MES pH 6.5, 1.6 *M* ammonium sulfate, 10%(*v*/*v*) 1,4-dioxane] within two weeks (Fig. 1*a*). However, the crystal diffracted with high mosaicity (1.6). Another small crystal appeared using Crystal Screen 2 condition No. 42 [100 m*M* Tris pH 8.5, 1.5 *M* ammonium sulfate, 12%(*v*/*v*) glycerol] after a month. To refine the conditions, we varied the pH, buffer and concentrations of protein or precipitant and attempted the hanging-drop vapour-diffusion method using a 2 µl drop containing equal volumes of protein and reservoir solution suspended over 400 µl reservoir solu-

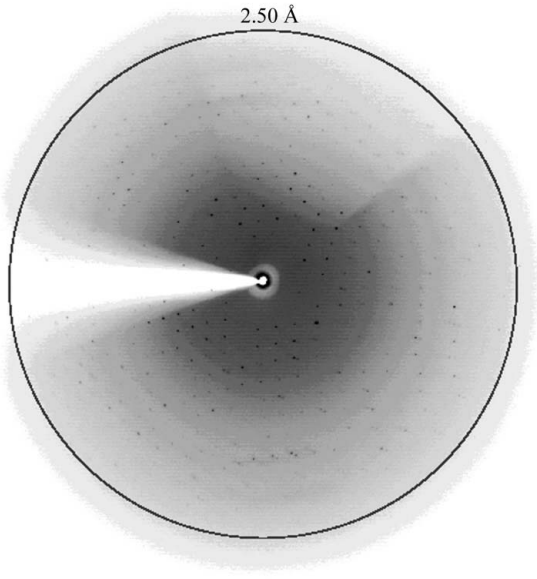


Figure 2
X-ray diffraction image from an MtnA crystal.

tion. Optimized crystallization was performed with the sitting-drop vapour-diffusion method at 293 K with a drop (2 µl) containing equal volumes of protein solution (10 mg ml^{-1} MtnA) and reservoir solution [50 m*M* MES, 50 m*M* Tris (final pH 7.4), 1.55 *M* ammonium sulfate, 5%(*v*/*v*) 1,4-dioxane, 6%(*v*/*v*) glycerol] suspended over 100 µl reservoir solution. Crystals suitable for diffraction with maximum dimensions 0.1 × 0.05 × 0.03 mm appeared after one week (Fig. 1*b*).

2.4. Data collection

The crystals were soaked for a few seconds in reservoir solution containing 20%(*v*/*v*) glycerol and 2.0 *M* ammonium sulfate. Flash-cooled crystals were then mounted in the nitrogen stream at 100 K. X-ray diffraction data were collected to 2.5 Å on a Rigaku R-AXIS V detector using synchrotron radiation of wavelength 1.0 Å at the BL38B1 beamline at SPring-8 (Fig. 2). Data collection was performed with a total oscillation range of 180°, a step size of 1.0° and an exposure time of 100 s. All diffraction data were processed using the *HKL*2000 program package (Otwinowski & Minor, 1997) and the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

Form II crystals diffracted well to 2.5 Å at 100 K using the BL38B1 beamline at SPring-8 and were found to belong to the tetragonal space group $P4_1$ (or $P4_3$), with unit-cell parameters $a = b = 69.2$, $c = 154.7$ Å. The value of the Matthews coefficient is 2.4 Å^3 Da^{-1} for a dimer in the asymmetric unit, corresponding to a solvent content of 48%, a typical value for protein crystals (Matthews, 1968). This is corroborated by analysis of the self-rotation function at $\chi = 180°$ (*POLARRFN*; Collaborative Computational Project, Number 4, 1994; Fig. 3). The result indicates the existence of a local twofold axis at $(\theta, \varphi, \chi) = (90, 30, 180°)$. The peak intensity with respect to the crystallographic axis was 41.8%. Details of 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.4.2 software suite (Collaborative Computational Project, Number 4, 1994), using the coordinates of Ypr118w from *S. cerevisiae* (PDB code 1w2w) as a search model. The results of molecular replacement suggest that the crystals contain two monomers per asymmetric unit and belong to space group $P4_1$. Refinement of structural details is now in progress.

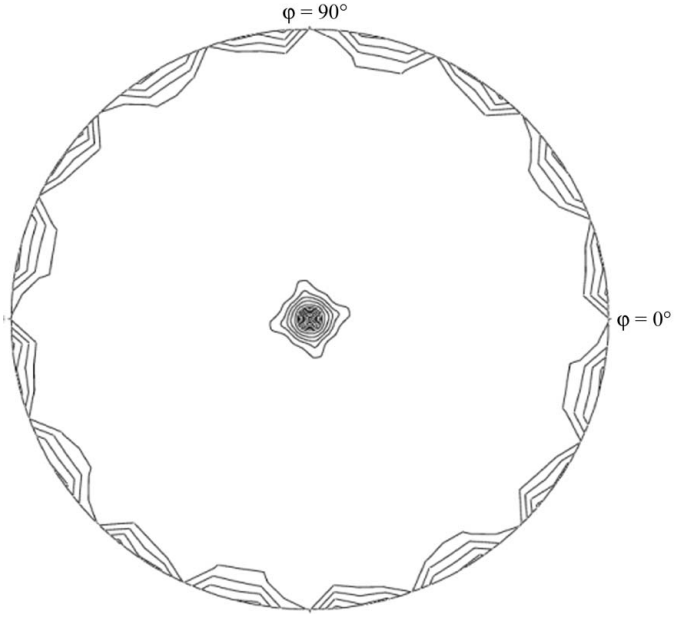


Figure 3
Stereographic projections of the self-rotation function calculated at $\chi = 180°$ with crystal data in the 20–7 Å resolution range with an integration radius of 40 Å. The result indicates the existence of a non-crystallographic twofold axis at $(\theta, \varphi, \chi) = (90, 30, 180°)$.

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

Values in parentheses are for the highest resolution shell (2.54–2.50 Å).

Crystal system	Tetragonal
Wavelength (Å)	1.0
Space group	$P4_1$
Unit-cell parameters (Å)	$a = b = 69.2$, $c = 154.7$
Resolution range (Å)	46.7–2.50
No. of molecules per AU	2
V_M (Å^3 Da^{-1})	2.4
V_{solv} (%)	48
No. of measured reflections	97701
No. of unique reflections	25293
R_{merge}† (%)	6.6 (30.6)
Completeness (%)	96.6 (83.9)
Average $I/\sigma(I)$	24.5 (3.2)

† $R_{merge} = \sum |I(k) - I| / \sum I(k)$, where $I(k)$ is the value of the kth measurement of the intensity of a reflection, I is the mean value of the intensity of that reflection and the summation is over all measurements.

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