crystallization communications

Crystallization and preliminary X-ray diffraction analysis of mevalonate kinase from *Methanosarcina mazei*

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Mevalonate kinase (MVK), which plays an important role in catalysing the biosynthesis of isoprenoid compounds derived from the mevalonate pathway, transforms mevalonate to 5-phosphomevalonate using ATP as a cofactor. Mevalonate kinase from *Methanosarcina mazei* (*Mm*MVK) was expressed in *Escherichia coli*, purified and crystallized for structural analysis. Diffraction-quality crystals of *Mm*MVK were obtained by the vapour-diffusion method using 0.32 M MgCl$_2$, 0.08 M bis-tris pH 5.5, 16%(w/v) PEG 3350. The crystals belonged to space group $P2_12_12$, with unit-cell parameters a = 97.11, b = 135.92, c = 46.03 Å. Diffraction data were collected to 2.08 Å resolution.

1. Introduction

Isoprenoid compounds are ubiquitous in nature; they include hormones, insect pheromones, pigments, cell membranes, electron carriers and defensive agents and participate in important biological processes (Davis & Croteau, 2000; Sacchettini & Poulter, 1997; Liang *et al.*, 2002). The isoprenoids, which include phytol, retinol and ubiquinone, are derived from a common structural motif: isoprene (2-methyl-1,3-butadiene). In the chemical industry, about 95% of the isoprene produced is used to make *cis*-1,4-polyisoprene, a synthetic version of natural rubber that is used in a wide variety of applications (Williams, 1859–1860). Today, isoprene produced by the thermal cracking of naphtha or crude oil is readily available. For environmental and energy-saving reasons, attempts are now being made to produce isoprene using biological processes. The precursors of isoprene units in biological systems are the five-carbon building blocks isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) (Ogura & Koyama, 1998). IPP and DMAPP are synthesized in two different ways: the mevalonate (MVA) pathway and the methylerythritol phosphate (MEP) pathway (also called the non-mevalonate pathway) (Kuzuyama, 2002). The MVA pathway has evolved to provide a pool of the precursors in eukaryotes, archaea, eubacteria, plant cytoplasm and parasites of the genus *Trypanosoma* and *Leishmania* (Sgraja *et al.*, 2007). In contrast, the MEP pathway is mainly utilized in plant chloroplasts and eubacteria (Rohdich *et al.*, 2004).

Mevalonate kinase (MVK) catalyses the fourth step in the MVA pathway and is involved in the transfer of a phosphoryl group from ATP to mevalonate. Mevalonate kinase belongs to the distinct GHMP superfamily of ATP-dependent enzymes, which includes galactokinase (G), homoserine kinase (H), mevalonate kinase (M) and phosphomevalonate kinase (P) (Bork *et al.*, 1993; Andreassi *et al.*, 2009). These enzymes are involved in the biosynthesis of isoprenoids and amino acids as well as in carbohydrate metabolism and share several structural features. The ATP-binding site contains a conserved glycine-rich motif (PX_3GLGSSAA) in the N-terminal section that forms the phosphate-binding loop (Daugherty *et al.*, 2001; Krell *et al.*, 1998). As mevalonate kinase is an important regulatory point in the MVA pathway, intricate regulation is essential to ensure the production of isoprenoids in the proper amounts. Bacteria and
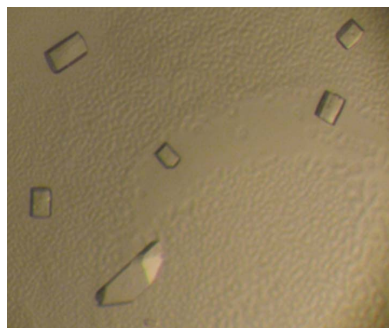
eukaryotes regulate MVKs by feedback inhibition, which can be divided into two classes (Dorsey & Porter, 1968). The first class are inhibited by metabolites downstream of the diphosphomevalonate decarboxylase reaction [IPP, DMAPP, geranyl diphosphate (GPP), farnesyl diphosphate (FPP) and longer-chain isoprenoids; Gray & Kekwick, 1972; Hinson *et al.*, 1997; Voynova *et al.*, 2004]. The MVK from the thermostable archaeon *Methanococcus jannaschii* was also inhibited by IPP, GPP and FPP metabolites (Huang *et al.*, 1999). The second class of MVKs are inhibited by diphosphomevalonate but not by metabolites downstream of the diphosphomevalonate decarbox-ylase (Andreassi *et al.*, 2004). The recently characterized MVK from the archaeon *Methanosarcina mazei* displays a feedback-resistant regulation profile that differs from those of previously characterized MVKs from both bacteria and eukaryotes (Primak *et al.*, 2011). Although it has been reported that *M. mazei* MVK has a much lower specific activity than *M. jannaschii* MVK, its different regulation profile without metabolite inhibition may be advantageous in opti-mizing the isoprenoid-biosynthesis pathway.

In this study, the MVK from *M. mazei* (*Mm*MVK) was expressed in a soluble form in *Escherichia coli*, purified and crystallized for structural analysis. Structure determination of *Mm*MVK may provide clues for elucidation of the specific catalytic activity related to its regulation mechanism. It also offers an opportunity for the design of novel enzymes of the GHMP superfamily for activity enhancement.

2. Experimental

2.1. Protein expression and purification

*Mm*MVK (residues 1–301) was amplified from *M. mazei* genomic DNA by PCR with forward primer 5′-GCG **CAT ATG** GTT TCA TGT TCT GCG CCC-3′ and reverse primer 5′-GCG **GGA TCC** TCA ATC GAC CTT CAA CCC-3′ containing *Nde*I and *Bam*HI restric-tion sites (bold), respectively. The resulting purified 900 bp PCR product was ligated into the bacterial expression vector pET-28a (Novagen, Madison, Wisconsin, USA) to produce recombinant *Mm*MVK protein with a hexahistidine tag and thrombin cleavage site at the N-terminus (MGSSHHHHHHSSGLVPRGSH). The *Mm*MVK cloned in the vector was confirmed by DNA sequencing. The calcu-lated molecular mass of the tagged monomer was about 33.6 kDa and the theoretical isoelectric point was 6.6.
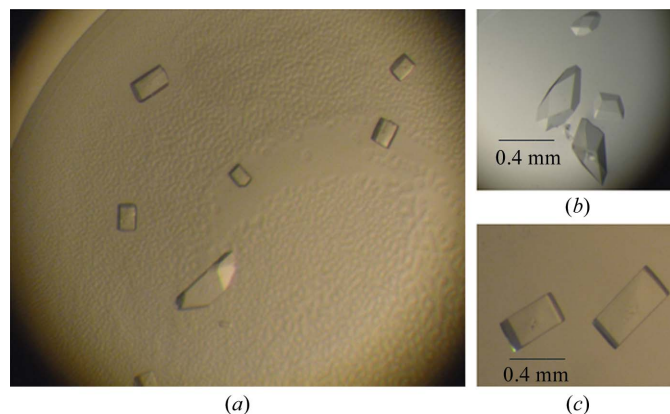

Figure 1
Crystals of *Mm*MVK obtained by the hanging-drop vapour-diffusion method. (*a*) Both orthogonal-shaped crystals and rhombohedral-shaped crystals formed in the same condition. (*b*) The rhombohedral-shaped crystals were finally used for data collection. (*c*) The orthogonal-shaped crystals in cryosolution were easily damaged during freezing and could not be used for data collection.

E. coli strain BL21 (DE3) transformed with the vector carrying *Mm*MVK was used for protein expression. A 10 ml aliquot of an overnight culture in Luria–Bertani (LB) medium was seeded into 1000 ml fresh M9 minimal medium containing 30 µg ml^{-1} kanamycin and was grown to an OD$_{600}$ of 0.6 at 310 K. 40 µg ml^{-1} seleno-methionine (Acros Organics, New Jersey, USA) was added together with 17 other solid L-amino acids (BioShop Canada Inc., Burlington, Canada). The protein was expressed at 303 K for 1 d with 0.4 m*M* isopropyl *β*-D-1-thiogalactopyranoside (IPTG). Cells were harvested by centrifugation (6000 rev min^{-1}, 6 min, 277 K), suspended in binding buffer (50 m*M* sodium phosphate pH 8.0, 300 m*M* NaCl, 5 m*M* *β*-mercaptoethanol, 1 m*M* MgCl$_2$) and disrupted by sonication. After centrifugation (12 000 rev min^{-1}, 1 h) at 277 K, the clear supernatant was filtered (qualitative filter paper, Advantec, Japan) and loaded onto an open column of nickel–NTA beads (Qiagen, Hilden, Germany) pre-equilibrated with binding buffer. The column was first washed with ten column volumes of binding buffer and then with 20 column volumes of washing buffer (50 m*M* sodium phosphate pH 8.0, 300 m*M* NaCl, 30 m*M* imidazole, 5 m*M* *β*-mercaptoethanol, 1 m*M* MgCl$_2$). The recombinant *Mm*MVK was eluted with elution buffer (50 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl, 300 m*M* imidazole, 5 m*M* *β*-mercaptoethanol, 1 m*M* MgCl$_2$). The fractions were exam-ined by SDS–PAGE. Fractions containing *Mm*MVK were pooled, concentrated and exchanged into 50 m*M* Tris–HCl pH 8.5, 1 m*M* DTT, 1 m*M* MgCl$_2$ by ultrafiltration with a Corning Spin-X UF concentrator with 30K molecular-weight cutoff (Corning Inc., New York, USA). The *Mm*MVK was further purified by anion-exchange chromatography on a Resource 15Q column (GE Healthcare, Piscataway, New Jersey, USA) with a linear gradient of 0–300 m*M* NaCl in 50 m*M* Tris–HCl pH 8.5, 1 m*M* DTT, 1 m*M* MgCl$_2$. The eluted fractions were examined by SDS–PAGE and the fractions containing *Mm*MVK were pooled and concentrated by ultrafiltration using a Corning Spin-X UF concentrator with 30K molecular-weight cutoff (Corning Inc.). The *Mm*MVK was finally purified by size-exclusion chromatography using a Superdex 200 column (GE Healthcare) in 20 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl, 1 m*M* DTT, 1 m*M* MgCl$_2$. The *Mm*MVK proteins separated by gel-filtration chromatography were concentrated to 10 mg ml^{-1} in 20 m*M* Tris pH 8.0, 1 m*M* DTT, 1 m*M* MgCl$_2$ for crystallization by ultrafiltration with the Corning Spin-X UF concentrator (Corning Inc.). Protein purity was examined by SDS–PAGE and native PAGE. All purification steps were carried out at 293 K with ice-cooled buffers, which we believe help to stabilize the sample. The protein concentration was determined by Bradford assay (Bradford, 1976). The N-terminal His tag was not removed for crystallization.

2.2. Crystallization

Crystallization of *Mm*MVK was initially carried out with Crystal Screen, Crystal Screen 2, Index (Hampton Research, California, USA), Wizard Screens I and II, Cryo Screens I and II (Emerald BioStructures, Bainbridge Island, Washington, USA) and laboratory-made solutions using a microbatch crystallization method at 291 K. Drops consisting of equal volumes (1 µl) of protein solution (10 mg ml^{-1} in 20 m*M* Tris–HCl pH 8.0, 1 m*M* DTT, 1 m*M* MgCl$_2$; buffer *A*) and screening solution were equilibrated under Al's oil in a 72-well microbatch plate (D'Arcy *et al.*, 2003). Crystals were initially produced in Index solution No. 82. Further screening to find optimal crystallization conditions was performed by hanging-drop vapour-diffusion trials, varying the salt and precipitant concentrations and the volume of the drop. The best crystals were grown at 291 K using 10 mg ml^{-1} *Mm*MVK protein in buffer *A* and reservoir solution
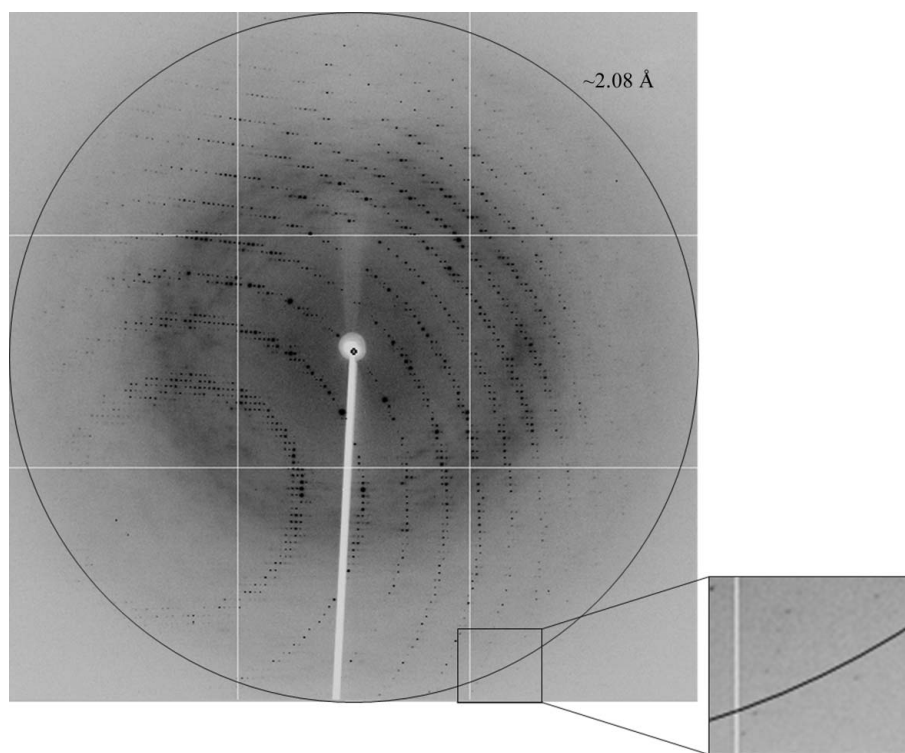
Figure 2
A diffraction image of *Mm*MVK. The circle corresponds to 2.08 Å resolution. The inset represents a magnified view of the area indicated by the square.

consisting of 0.32 *M* MgCl$_2$, 0.08 *M* bis-tris pH 5.5, 16%(*w*/*v*) PEG 3350. The drop consisted of 3 µl protein solution and 3 µl reservoir solution. Orthogonal-shaped and rhombohedral-shaped crystals appeared after two weeks and grew to maximum dimensions of 0.4 × 0.3 × 0.1 and 0.4 × 0.3 × 0.2 mm, respectively (Fig. 1).

2.3. X-ray data collection and processing

Before data collection, crystals were quickly transferred into a cryosolution consisting of 0.32 *M* MgCl$_2$, 0.08 *M* bis-tris pH 5.5, 17%(*w*/*v*) PEG 3350, 10% glycerol for less than 10 s. Only the rhombohedral-shaped crystals were stable during the quick soaking; the orthogonal-shaped crystals were always damaged in cryosolutions supplemented with different cryoprotectants such as glycerol, ethylene glycol, PEG 400, glucose and MPD. The rhombohedral-shaped crystals were flash-cooled in liquid nitrogen. X-ray diffraction data were collected on beamline 5C at the Pohang Accelerator Laboratory, Republic of Korea using an ADSC Quantum 210 CCD detector. For multiple-wavelength anomalous dispersion (MAD), three data sets were collected at wavelengths of 0.97947 Å (peak), 0.97954 Å (edge) and 0.97179 Å (remote) at 100 K. 180 images of data were collected with an oscillation angle of 1° and an exposure time of 1 s at each wavelength. All diffraction images were indexed, integrated and scaled using the *HKL*-2000 suite (Otwinowski & Minor, 1997). Data statistics are shown in Table 1. The intensities were converted to structure-factor amplitudes using *TRUNCATE* from the *CCP*4 suite (French & Wilson, 1978; Winn *et al.*, 2011).

3. Results

*Mm*MVK (residues 1–301) was expressed in *E. coli* and sufficiently purified for crystallization. The apparent mass of the protein was estimated to be about 33 kDa from SDS–PAGE, which is similar to

Table 1
Data statistics for the SeMet derivative.

Values in parentheses are for the highest resolution shell.

	Peak	Edge	Remote
Space group	$P2_12_12$		
Unit-cell parameters (Å)	$a = 97.11$, $b = 135.92$, $c = 46.03$		
No. of chains in the asymmetric unit	2		
V_M (Å^3 Da^{-1})	2.24		
Solvent content (%)	45.19		
Wavelength (Å)	0.97947	0.97954	0.97179
Resolution (Å)	50–2.08 (2.12–2.08)	50–2.12 (2.15–2.12)	50–2.23 (2.28–2.23)
Unique reflections	37136 (1420)	36224 (1843)	30700 (1889)
Completeness (%)	98.5 (76.8)	99.9 (100)	99.7 (100)
R_{merge}† (%)	8.5 (63.3)	7.7 (82.1)	7.4 (76.4)
Multiplicity	7.1 (6.2)	7.2 (6.9)	7.2 (7.3)
$\langle I/\sigma(I) \rangle$	13.7	12.4	10.8

† $R_{merge} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $\langle I(hkl) \rangle$ is the average intensity of the *i*th observations.

the theoretical molecular mass of 33.6 kDa including the histidine tag. On gel-filtration chromatography the protein separated with an effective mass of 64.6 kDa, suggesting that the protein exists as a dimer in solution, which is consistent with previously reported results (Primak *et al.*, 2011). We collected a 2.08 Å resolution MAD data set (Fig. 2) at three wavelengths, which was processed in the primitive orthorhombic space group $P2_12_12$, with unit-cell parameters $a = 97.11$, $b = 135.92$, $c = 46.03$ Å (Table 1). The asymmetric unit contained two *Mm*MVK molecules with a Matthews coefficient V_M of 2.24 Å^3 Da^{-1} and a solvent content of 45.19% (Matthews, 1968). A detailed discussion of the structure determination will be published elsewhere.

References

Andreassi, J. L. II, Dabovic, K. & Leyh, T. S. (2004). *Biochemistry*, **43**, 16461–16466.

Andreassi, J. L. II, Vetting, M. W., Bilder, P. W., Roderick, S. L. & Leyh, T. S. (2009). *Biochemistry*, **48**, 6461–6468.

Bork, P., Sander, C. & Valencia, A. (1993). *Protein Sci.* **2**, 31–40.

Bradford, M. M. (1976). *Anal. Biochem.* **72**, 248–254.

D'Arcy, A., Mac Sweeney, A., Stihle, M. & Haber, A. (2003). *Acta Cryst.* D**59**, 396–399.

Daugherty, M., Vonstein, V., Overbeek, R. & Osterman, A. (2001). *J. Bacteriol.* **183**, 292–300.

Davis, E. M. & Croteau, R. (2000). *Top. Curr. Chem.* **209**, 53–95.

Dorsey, J. K. & Porter, J. W. (1968). *J. Biol. Chem.* **243**, 4667–4670.

French, S. & Wilson, K. (1978). *Acta Cryst.* A**34**, 517–525.

Gray, J. C. & Kekwick, R. G. (1972). *Biochim. Biophys. Acta*, **279**, 290–296.

Hinson, D. D., Chambliss, K. L., Toth, M. J., Tanaka, R. D. & Gibson, K. M. (1997). *J. Lipid Res.* **38**, 2216–2223.

Huang, K.-X., Scott, A. I. & Bennett, G. N. (1999). *Protein Expr. Purif.* **17**, 33–40.

Krell, T., Coggins, J. R. & Lapthorn, A. J. (1998). *J. Mol. Biol.* **278**, 983–997.

Kuzuyama, T. (2002). *Biosci. Biotechnol. Biochem.* **66**, 1619–1627.

Liang, P.-H., Ko, T.-P. & Wang, A. H.-J. (2002). *Eur. J. Biochem.* **269**, 3339–3354.

Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.

Ogura, K. & Koyama, T. (1998). *Chem. Rev.* **98**, 1263–1276.

Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.

Primak, Y. A., Du, M., Miller, M. C., Wells, D. H., Nielsen, A. T., Weyler, W. & Beck, Z. Q. (2011). *Appl. Environ. Microbiol.* **77**, 7772–7778.

Rohdich, F., Bacher, A. & Eisenreich, W. (2004). *Bioorg. Chem.* **32**, 292–308.

Sacchettini, J. C. & Poulter, C. D. (1997). *Science*, **277**, 1788–1789.

Sgraja, T., Smith, T. K. & Hunter, W. N. (2007). *BMC Struct. Biol.* **7**, 20.

Voynova, N. E., Rios, S. E. & Miziorko, H. M. (2004). *J. Bacteriol.* **186**, 61–67.

Williams, C. G. (1859–1860). *Proc. R. Soc. Lond.* **10**, 516–519.

Winn, M. D. *et al.* (2011). *Acta Cryst.* D**67**, 235–242.