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Crystallization and preliminary X-ray analysis of 5′-methylthioribose kinase from *Bacillus subtilis* and *Arabidopsis thaliana*

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Recombinant *Bacillus subtilis* 5′-methylthioribose (MTR) kinase has been expressed, purified and subsequently crystallized using the hanging-drop vapor-diffusion technique. With PEG 2000MME as the precipitant, two different crystal forms have been grown in the absence and presence of the detergent CHAPS. These crystals belong to space groups $P2_12_12_1$ (unit-cell parameters a = 193.7, b = 83.2, c = 51.6 Å) and $P2_12_12$ (unit-cell parameters a = 213.8, b = 83.2, c = 51.5 Å), respectively. The crystals grown in the presence of CHAPS diffract to 2.2 Å resolution at Station X8C, National Synchrotron Light Source (NSLS). For both crystal forms, the presence of two monomers per asymmetric unit is predicted (Matthews coefficient V_M = 2.29 and 2.52 Å^3 Da^{-1}, respectively). Recombinant C-terminally histidine-tagged *Arabidopsis thaliana* MTR kinase has also been expressed, purified and refolded into its active form. Rod-shaped crystals of this protein were grown from PEG 8000 using the hanging-drop vapor-diffusion technique. These crystals exhibit the symmetry of space group $C2$ (unit-cell parameters a = 162.3, b = 83.3, c = 91.0 Å, β = 117.8°) and diffract to 1.9 Å resolution at Station X8C, NSLS. Two monomers are estimated to be present in the asymmetric unit (V_M = 2.82 Å^3 Da^{-1}).

1. Abbreviations

MTR, 5′-methylthioribose; MTA, 5′-methylthioadenosine; MTR 1-P, 5′-methylthioribose 1-phosphate; APH(3′)-IIIa, 3′,5″-aminoglycoside phosphotransferase type IIIa; PEG, polyethylene glycol; PEG MME, polyethylene glycol monomethyl ether; PEP, phosphoenolpyruvate; PK, pyruvate kinase; LDH, lactate dehydrogenase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propane sulfonate; NSLS, National Synchrotron Light Source.

2. Introduction

The essential amino acid methionine plays a critical role in a number of cellular functions including protein synthesis, biological methylation and polyamine biosynthesis. Since the *de novo* biosynthesis of methionine is energetically costly, pathways to salvage or recycle methionine have evolved in nearly all organisms (Sufrin *et al.*, 1995). In many microbes, plants and certain parasitic protozoa, 5′-methylthioribose kinase (EC 2.7.1.100; Ferro *et al.*, 1978) plays an essential role in methionine salvage, enabling these organisms to grow on non-methionine sulfur sources such as 5′-methylthioribose and 5′-methylthioadenosine (Riscoe *et al.*, 1988; Sekowska *et al.*, 2001). MTA is a byproduct and inhibitor of polyamine synthesis (Sekowska & Danchin, 2002) and is rapidly degraded in various microbes, plants and certain protozoa to MTR and adenine. MTR kinase catalyzes the phosphorylation of MTR to MTR 1-phosphate, which is subsequently converted to methionine *via* a series of intermediates (Murphy *et al.*, 2002; Sekowska & Danchin, 2002). In mammalian cells, however, the degradation of MTA is achieved in a single step by MTA phosphorylase, which converts MTA directly to MTR 1-P (Pegg & Williams-Ashman, 1969). This metabolic difference in the way MTA is removed has been explored and analogues of MTR have been synthesized and shown to be effective pro-drugs that selectively kill MTR kinase-containing organisms with little effect on mammalian cells (Gianotti *et al.*, 1990; Riscoe *et al.*, 1988). In the absence of a mammalian homolog, MTR kinase represents an ideal target for the design of novel antibiotics against a wide array of MTR kinase-containing pathogens (*Klebsiella pneumoniae*, *Bacillus anthracis etc.*) as well as selective herbicidal agents.

MTR kinase has been classified *in silico* into the choline kinase family (Bateman *et al.*, 2002; Nitschke *et al.*, 1998; Sekowska *et al.*, 2001), despite the generally low sequence identity between the two proteins. The fold-recognition program *3D-PSSM* (Kelley *et al.*, 2000) also

predicts that MTR kinase is structurally similar to choline kinase as well as the antibiotic resistance protein 3′,5″-aminoglycoside phosphotransferase type IIIa [APH(3′)-IIIa]. The structures of APH(3′)-IIIa (Hon *et al.*, 1997) and choline kinase (Peisach *et al.*, 2003) reveal a similar protein fold and both resemble eukaryotic protein kinases. The lack of significant sequence similarity between MTR kinase, choline kinase and APH(3′)-IIIa prevents comparative or homology modeling (Baker & Sali, 2001). As a prelude to structure-based inhibitor-design studies, we have cloned *B. subtilis* and *Arabidopsis thaliana* MTR kinase genes. The recombinant proteins were expressed, purified and when necessary refolded prior to subsequent crystallization. These results and the preliminary X-ray analysis of the MTR kinase crystals are described.

3. Gene cloning, expression and purification

3.1. Initial cloning of *B. subtilis* and *A. thaliana* MTR kinase genes

The genes for *B. subtilis* and *A. thaliana* were identified by a BLAST search of GenBank (Altschul *et al.*, 1997) using the deduced amino-acid sequence from the *K. pneumoniae* gene (GenBank accession No. AF212863; Cornell *et al.*, 2003). The corresponding orthologs were amplified using gene-specific primers and subcloned into the pTrcHis2TOPO expression vector (Invitrogen). Briefly, *B. subtilis* chromosomal template DNA was purified from a 5 ml overnight culture of bacterial cells grown in BHI broth using a DNeasy kit (Qiagen). The MTR kinase gene was amplified using Ready-to-Go beads (Pharmacia), gene-specific primers (forward, ATG ATG ATG GGA GTC ACA; reverse, TTC CTT TAC AAG TAA CTT) and a standard thermocycling protocol (5 min at 368 K, followed by 35 cycles of 1 min at 368 K, 2 min at 328 K and 3 min at 345 K). The PCR product was ligated into the pTrcHis2TOPO expression vector and transformed into *Escherichia coli* TOP10 cells. The *A. thaliana* MTR kinase gene was similarly isolated and cloned, except that the PCR reaction contained a 2 µl sample of an *Arabidopsis* UniZap cDNA library (Stratagene) for template and gene-specific primers (forward, GAA TTC ATG TCT TTT GAG GAG TTT ACG; reverse, AGA TCT TTA ATG ATG ATG ATG ATG ATG ATG GCT TTG TTG TTG AAT TGC TGA AAC). In each case, plasmid clones isolated from positive transformants bearing the gene in the forward orientation were completely sequenced to ensure fidelity at the Veterans Affairs core sequencing facility. For initial expression analysis, plasmids were transformed into *E. coli* strain BL21(DE3)-pLysE.

3.2. *B. subtilis* MTR kinase expression and purification

The C-terminal His-tagged *B. subtilis* MTR kinase encoded in the pTRCHis2 vector did not produce crystals suitable for X-ray diffraction studies and therefore a non-tagged version of the *B. subtilis* MTR kinase gene was generated by engineering a stop codon right before the His-tag coding sequence using the QuickChange site-directed mutagenesis method (Stratagene). This engineered plasmid was transformed into *E. coli* BL21 CodonPlus (DE3) cells (genotype BF^{-} ompT hsdS($r_B^{-}m_B^{-}$) dcm+ Tet^r gal endA Hte[argU ileY leuW Cam^r]) (Stratagene). A 1 l culture of 2× yeast-tryptone (2YT) media with 100 µg ml^{-1} ampicillin was inoculated with 2 ml of overnight culture and grown at 310 K in an air shaker until the cell density reached an OD_{600} of 0.2. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture to a total concentration of 1 m*M* and the cells were incubated at 310 K for 3–4 h. The cells were harvested by centrifugation at 11 300*g* for 10 min at 277 K and lysed by sonication in 10 m*M* potassium phosphate pH 7.4 and 1 m*M* DTT (buffer *A*). After further centrifugation at 39 200*g* for 30 min at 277 K, the crude lysate was then loaded onto a 50 ml diethylaminoethyl cellulose (Whatman) anion-exchange column. The protein was eluted by applying a linear gradient from 0 to 0.5 *M* NaCl in buffer *A*. Fractions containing the partially purified protein were pooled and concentrated using a centrifuge concentrator (Amicon Ultra 10 kDa molecular-weight cutoff; Millipore) and further purified and desalted in buffer *A* by size-exclusion chromatography (Superdex 200 HR10/30; Pharmacia). Approximately 60 mg of non-tagged *B. subtilis* MTR kinase were produced per litre of cells.

3.3. *A. thaliana* MTR kinase expression and purification

To improve the initial expression, the C-terminal His-tagged *A. thaliana* MTR kinase gene was sub-cloned from pTRCHis2 into pET28a using the *Nco*I/*Sal*I restriction sites and transformed into *E. coli* BL21 CodonPlus(DE3) cells (Stratagene). The majority of the expressed protein was found in inclusion bodies as judged by SDS–PAGE of the lysate and the insoluble pellet (data not shown). Variation of the expression temperature or IPTG concentration did not improve the solubility of the overexpressed protein. Cells containing the inclusion bodies of the C-terminal His-tagged *A. thaliana* MTR kinase were therefore harvested by centrifugation at 11 300*g* for 10 min at 277 K and lysed by sonication in BugBuster (Novagen) protein-extraction reagent. After further centrifugation at 39 200*g* for 30 min at 277 K, the pellet was dissolved in 6 *M* guanidium hydrochloride (GnHCl), 0.7 *M* NaCl and 20 m*M* imidazole pH 8.0 (buffer *B*) and stirred constantly for 30 min at 277 K. To remove the insoluble debris, the sample was further centrifuged at 39 200*g* for 30 min and the soluble fraction filtered and loaded onto a 20 ml column of Ni–NTA Superdex (Qiagen) pre-equilibrated with buffer *B*. The unfolded protein was eluted with 6 *M* GnHCl, 0.5 *M* NaCl and 100 m*M* imidazole pH 8.0 at 277 K.

The unfolded *A. thaliana* MTR kinase was refolded by rapid pulse dilution using a peristaltic pump at 0.2 ml min^{-1} into a large volume of constantly stirring refolding buffer composed of 0.5 *M* arginine, 1 *M* D-glucose, 0.3%(*w*/*v*) CHAPS {3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate}, 1 m*M* EDTA, 20 m*M* DTT and 20 m*M* imidazole pH 8.0. The concentration of the unfolded protein before dilution was estimated by Coomassie Plus (Pierce; Bradford, 1976) and the final protein concentration was kept below 10 µg ml^{-1} to prevent irreversible aggregation. The dilute protein in refolding buffer was stirred at 277 K overnight and subsequently concentrated to 20–40 ml using a Stirred Ultra-Filtration Cell (Millipore) with a YM10 cellulose membrane (Millipore). The refolded protein was then dialyzed against 4 l of 20 m*M* imidazole pH 7.6 (buffer *C*) at 277 K overnight, concentrated and further purified in buffer *C* supplemented with 2 m*M* DTT using a Superdex 200 HR10/30 gel-filtration column (Pharmacia). Approximately 10–15 mg of the refolded active *A. thaliana* MTR kinase were produced per litre of cells.

4. Enzyme assay

Both the soluble non-tagged *B. subtilis* and the refolded C-terminal His-tagged *A. thaliana* MTR kinase were purified to homogeneity as judged by SDS–PAGE and native PAGE (data not shown). To ensure that both proteins were enzymatically active,

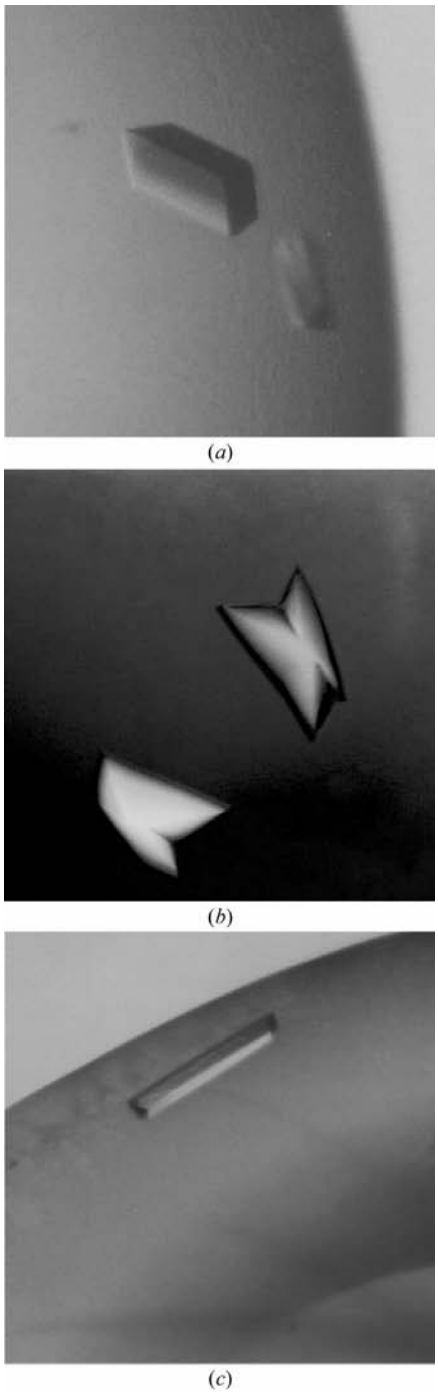

Figure 1
MTR kinase crystals. (*a*) Crystals of *B. subtilis* MTR kinase in complex with ATP-γ-S and MTR (~0.1 × 0.05 × 0.05 mm). (*b*) Crystals of *B. subtilis* MTR kinase grown in the presence of CHAPS (~0.4 × 0.4 × 0.05 mm). (*c*) Crystals of refolded *A. thaliana* MTR kinase (~0.3 × 0.05 × 0.05 mm) observed with heavy precipitate.

their kinase activity was assayed by an end-point ^{14}C-MTR-based radioactive assay as described previously (Cornell *et al.*, 1996; Gianotti *et al.*, 1990) and by a continuous colorimetric assay that enables direct spectroscopic measurement of native substrate phosphorylation (Barker *et al.*, 1995). This general kinase assay responds to the production of ADP during kinase catalysis. ADP is converted back to ATP by pyruvate kinase in the presence of phosphoenolpyruvate. Pyruvate kinase converts phosphoenolpyruvate to pyruvate, which is rapidly removed by lactate dehydrogenase (LDH). During the production of lactate by LDH, NADH is oxidized to NAD^+. MTR kinase is the rate-limiting enzyme and the rate of NADH consumption, measurable by absorbance at 340 nm (ε = 6290 M^{-1} cm^{-1}), therefore corresponds to the rate of MTR kinase catalysis. In brief, a 500 µl reaction mixture containing 1 m*M* PEP, 5 m*M* DTT, 1 m*M* ATP, 2 m*M* $MgCl_2$, 0.3 m*M* NADH, 10 U PK and 20 U LDH in 20 m*M* imidazole pH 8.0 with varied MTR kinase or substrate concentrations was incubated at 298 K for 10 min and the rate of decrease in absorbance at 340 nm was measured on a Ultrospec 2100 Pro UV–visible spectrophotometer (Biochrom). Typically, after 10 min incubation the decrease in absorbance was linear until all NADH was consumed. The maximum specific activities of the *B. subtilis* and the refolded *A. thaliana* enzymes were found to be approximately 1.0 and 1.5 µmol min^{-1} mg^{-1}, respectively.

Table 1
Diffraction data statistics.

Values in parentheses correspond to the highest resolution shell.

	B. subtilis	*B. subtilis* (CHAPS)	*A. thaliana* (refolded)
Space group	$P2_12_12_1$	$P2_12_12$	$C2$
Unit-cell parameters			
a (Å)	193.72	213.80	162.46
b (Å)	83.22	83.20	83.24
c (Å)	51.59	51.46	91.08
β (°)			117.83
Wavelength (Å)	1.54	1.0	1.0
No. measured reflections	237370	456570	278393
No. unique reflections	17397	47711	82470
Average redundancy	13.64	9.57	3.38
Resolution (Å)	3.0 (3.11–3.00)	2.2 (2.28–2.20)	1.9 (1.97–1.90)
$R_{\rm merge}$† (%)	9.7 (27.9)	7.2 (29.2)	4.5 (17.5)
Completeness (%)	99.7 (100)	100.0 (100.0)	98.7 (90.8)
Average $I/\sigma(I)$	19.0 (9.2)	14.9 (5.8)	16.9 (4.8)

† $R_{\rm merge} = \sum\sum|I(k) - \langle I(k)\rangle|/\sum I(k)$, where $I(k)$ and $\langle I\rangle$ represent the diffraction intensity values of the individual measurements and the corresponding mean values. The summation is over all unique measurements.

5. Crystallization and X-ray analysis

5.1. *B. subtilis* MTR kinase

The non-tagged *B. subtilis* MTR kinase was concentrated to 7.5–15 mg ml^{-1} in 10 m*M* potassium phosphate pH 7.4, 2 m*M* DTT, 1 m*M* MTR and 1 m*M* ATP-γ-S. The initial crystallization hit was obtained from the Clear Strategy Screen I (Molecular Dimensions Ltd) sparse-matrix screen. The protein was crystallized by the hanging-drop vapor-diffusion technique from a drop consisting of 2–3 µl protein solution mixed with an equal volume of 22%(*w*/*v*) PEG 2000MME, 0.1 *M* Tris–HCl pH 7.5 and 0.3 *M* sodium acetate suspended over a 0.5 ml reservoir of the same precipitating agent. Small rod-shaped crystals grew at room temperature in 3 d (Fig. 1*a*). The crystals were subsequently soaked in a cryoprotectant consisting of 25%(*w*/*v*) PEG 2000MME, 0.1 *M* Tris–HCl pH 7.5, 0.3 *M* sodium acetate and 25%(*v*/*v*) ethylene glycol for 10 s and flash-frozen prior to data collection. The crystals diffracted to 3.0 Å resolution on an R-AXIS IV^{++} image-plate detector using Cu $K\alpha$ X-rays from an RU-H3R rotating-anode generator. A complete set of data was collected and processed using *d*TREK* v.8.0 (Pflugrath, 1999). The crystals belong to space group $P2_12_12_1$ and are estimated to contain two monomers per asymmetric unit ($V_{\rm M}$ = 2.29 Å^3 Da^{-1}; Matthews, 1968). The diffraction and data-reduction statistics for all MTR kinase crystals obtained to date are summarized in Table 1.

To improve the size and diffraction quality of *B. subtilis* MTR kinase crystals, we explored selective detergents as crystallization aids. In the presence of 8 m*M* {3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate} or {3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propane sulfonate}, the non-tagged *B. subtilis* MTR kinase crystallized as two-dimensional thin plates (Fig. 1*b*) from the same condition described above. Interestingly, in the presence of CHAPS the crystals can be grown in the presence or absence of MTR and ATP-γ-S. These thin plates diffract to 2.2 Å resolution at Station X8C, NSLS, Brookhaven National Laboratory and have improved data-reduction statistics (Table1). The presence of CHAPS changes the symmetry and increases the *a* axis by 20 Å.

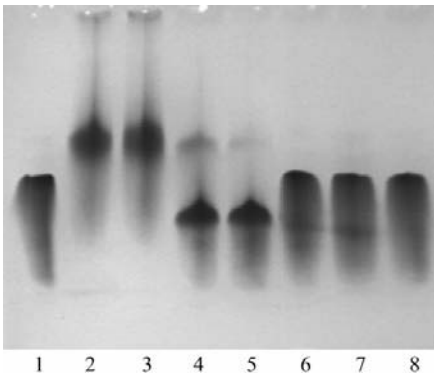

Figure 2
Coomassie-stained native polyacrylamide gel of non-tagged *B. subtilis* MTR kinase pre-treated with various heavy-atom compounds (lanes 2–8). Lane 1, native protein (control); lane 2, $HgCl_2$; lane 3, $Hg(CH_3COO)_2$; lane 4, PCMB; lane 5, PCMBS; lane 6, K_2PtCl_4; lane 7, TMLA; lane 8, $KAu(CN)_4$.

The CHAPS crystals belong to space group $P2_12_12$, with unit-cell parameters a = 213.8, b = 83.2, c = 51.5 Å (Table 1). Two monomers are estimated to be present in the asymmetric unit (V_M = 2.52 Å^3 Da^{-1}; Matthews, 1968).

5.2. *A. thaliana* MTR kinase

The refolded *A. thaliana* MTR kinase was concentrated to 10 mg ml^{-1} in 20 m*M* imidazole pH 7.6, 2 m*M* DTT, 1 m*M* MTR and 1 m*M* ATP-γ-S. Heavy precipitate was observed almost ubiquitously during crystallization screening. The initial crystallization hit was obtained from the Crystal Screen Lite (Hampton) sparse-matrix screen. The protein was crystallized from a drop consisting of 2 µl protein solution mixed with an equal volume of 10%(*v*/*v*) PEG 8000, 0.1 *M* sodium cacodylate pH 6.5 and 0.2 *M* magnesium acetate suspended over a 0.5 ml reservoir of the same precipitating agent. Long rod-shaped crystals grew from the precipitate at room temperature in 3 d (Fig. 1*c*). The crystals were soaked in a cryoprotectant solution consisting of 10%(*v*/*v*) PEG 8000, 0.1 *M* sodium cacodylate pH 6.5, 0.2 *M* magnesium acetate and 25%(*v*/*v*) ethylene glycol for 10 s and flash-frozen prior to data collection. A complete set of data was collected to 1.9 Å resolution at Station X8C, NSLS and processed using *d*TREK* v.8.0 (Pflugrath, 1999). The crystals belong to space group *C*2, with unit-cell parameters a = 162.3, b = 82.3, c = 91.0 Å, β = 117.8° (Table 1). Two monomers are estimated to be present in the asymmetric unit (V_M = 2.82 Å^3 Da^{-1}; Matthews, 1968).

6. Searching for heavy-atom derivatives

Given that MTR kinase is part of the methionine-recycling pathway, perhaps not too surprisingly it appears that in most organisms the enzyme has low methionine content. This prevents us from using a selenomethionine protein to determine the crystallographic phases. The *A. thaliana* enzyme is of interest to us because unlike most other MTR kinases, this protein has 13 methionines in 420 residues, making selenomethionine MAD phasing feasible. The expression, purification, refolding and crystallization of selenomethionine-substituted *A. thaliana* MTR kinase is in progress. The sequence of *A. thaliana* MTR kinase is 35% identical to that of the *B. subtilis* enzyme and could therefore potentially be used as a model for the molecular-replacement solution of the *B. subtilis* enzyme.

B. subtilis MTR kinase, however, has only five methionines in 399 residues. Three of these methionines are found at the N-terminus (residues 1–3) and are therefore likely to be disordered. The low content of ordered methionine would make selenomethionine MAD phasing difficult. *B. subtilis* MTR kinase aggregates into oligomers and shows a series of discrete bands on a native polyacrylamide gel (data not shown). These aggregates can be removed with the addition of DTT. This phenomenon of thio-dependent aggregation suggests accessible cysteine residues that may react with mercury compounds. Using native PAGE, we have screened a number of heavy-atom compounds as potential derivatives (Boggon & Shapiro, 2000). The protein was incubated with various heavy-atom compounds at room temperature for 5 min and then loaded onto a native gel. Fig. 2 shows that mercury chloride, mercury acetate, *p*-chloromercuric benzene sulfonate (PCMBS) and *p*-chloromercuric benzoate (PCMB) all altered the mobility of the protein, whilst potassium tetrachloroplatinate, trimethyllead acetate (TMLA) and potassium dicyanoaurate (Fig. 2) did not produce a gel shift. We are currently optimizing the soaking conditions to find a suitable heavy-atom derivative to phase the data and determine the structure.

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