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Crystallization and preliminary crystallographic characterization of recombinant L-methionine-α-deamino-γ-mercaptomethane lyase (methioninase)

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L-Methionine-α-deamino-γ-mercaptomethane lyase (rMETase) is involved in the α,γ-elimination of methionine to α-ketobutyrate, methanethiol and ammonia. The reaction catalyzed by rMETase reduces the methionine concentration of methionine-dependent tumor cells, arresting their growth. Towards the goal of developing rMETase into an effective antitumor therapeutic and also to understand the catalytic mechanism of this enzyme, rMETase from *Pseudomonas putida* has been expressed, purified and crystallized. The crystals belong to space group $P2_12_12$ and diffract X-rays to at least 2.68 Å resolution at 100 K using synchrotron radiation. The unit cell has parameters a = 152.8, b = 154.6, c = 80.8 Å and contains four molecules in the asymmetric unit.

1. Introduction

The chemotherapy of solid tumors, with a few exceptions, has had only limited efficacy (Devita *et al.*, 1993). Thus, the majority of solid cancers are generally not responsive to current chemotherapy regimens. For the most part, the existing drugs used for solid-tumor chemotherapy are not tumor selective and are therefore active against both tumor and normal cells, which gives the drugs limited efficacy and significant toxicity. Therefore, it is of critical importance to identify targets and agents that are tumor selective.

Asparagine dependence is one such target for the enzyme asparaginase. However, the asparagine-dependence target may be limited only to hematological tumors (Devita *et al.*, 1993). On the other hand, studies have shown that the amino acid methionine may be a tumor-specific target. Observations have indicated that a number of malignant cell lines have an absolute requirement for methionine, as they fail to grow on homocysteine (Tan *et al.*, 1996). Recent studies have demonstrated that *in situ* patient tumors are also frequently methionine dependent (Tan *et al.*, 1996). Normal cells and tissues, unlike methionine-dependent tumors, were found to use homocysteine in place of methionine for proliferation and are therefore methionine independent (Tan *et al.*, 1996). *In vitro* and *in vivo* studies have suggested that targeting the methionine dependence of tumors may exert tumor-selective efficacy *via* a tumor-specific cell-cycle block (Hoffman & Jacobson, 1980; Guo *et al.*, 1993). Under conditions of a limiting methionine source, methionine-dependent tumor cells arrest in the late-S/G_2 phase in the cell cycle (Guo *et al.*, 1993).

Recently, a number of investigators have attempted to exploit the methionine dependence of tumors for therapeutic effects *in vivo*. Breillout *et al.* (1987, 1990) observed that a methionine-depleted diet lowered the metastatic potential of methionine-dependent tumors without significant effects on local tumor growth in rats. Goseki *et al.* (1995) observed that a methionine-free total parental nutrition (TPN) mixture for rats bearing the Yashida sarcoma slowed tumor growth and extended the survival of the rats, especially with the use of doxorubicin. With the use of a methioninase, Kreis & Hession (1973) demonstrated an attenuation of growth of W-256 rat carcinosarcoma growing in rats. This clinical trial demonstrated that methionine depletion has clinical activity. However, dietary methionine starvation is insufficient in completely depleting the serum methionine and therefore does not completely arrest tumor growth.

A methonine-cleaving enzyme would lower methionine levels more than methionine starvation and thereby could have more therapeutic efficacy. For this purpose, Kreis & Hession (1973) attempted to purify a METase from *Clostridium sporogenes*. The enzyme reduced the growth of the Walker 256 carcinoma in rats more than a methionine-free diet. Subsequently, Ito *et al.* (1976) purified a METase from *P. putida*. This enzyme was composed of four identical subunits of molecular weight 43 kDa and catalyzed the α,γ-elimination of methionine to α-ketobutyrate, methanethiol and ammonia in the

presence of pyridoxal 5′-phosphate. The enzyme shares over 30% sequence identity with other pyridoxal phosphate containing enzymes such as cystathionine β-lyase and cystathionine γ-synthase. The crystal structures of *Escherichia coli* cystathionine β-lyase and *Nicotiana tabacum* cystathionine γ-synthase have been determined to 1.83 and 2.90 Å, respectively (Clausen *et al.*, 1996; Steegborn *et al.*, 1999). Tan *et al.* (1997) cloned METase from *P. putida* and obtained high-purity rMETase from *E. coli*. They found rMETase in combination with cisplatin produced synergistic anti-tumor efficacy on human colon cancers (Tan *et al.*, 1999). Yoshioka *et al.* (1998) also found that rMETase in combination with 5-fluorouracil prolonged the survival time of mice bearing Lewis lung cancer and inhibited the tumor growth.

In addition to sufficient activity and the absence of toxicity, an important requirement for a therapeutically useful rMETase would be a longer half-life of the enzyme and the absence of side effects. Towards the goal of developing rMETase into an effective antitumor therapeutic and also to understand the catalytic mechanism of α,γ-elimination of methionine by this class of pyroxidal phosphate containing lyases, structural studies on rMETase have been undertaken. Here, we report the crystallization and preliminary X-ray crystallographic characterization of the recombinant rMETase from *P. putida*.

Figure 1
Orthorhombic crystal of native recombinant methioninase.

2. Materials and methods

2.1. Purification and crystallization

Native recombinant *P. putida* METase was expressed in *E. coli* BL21 (DE3) strain and purified as described previously (Tan *et al.*, 1997). The purified rMETase was at a concentration of 10–20 mg ml^{-1} in a solution consisting of 120 m*M* sodium chloride, 10 m*M* sodium phosphate pH 7.2. rMETase in solution was frozen on dry ice and acetone and then lyophilized at 193 K under a vacuum of 10 kPa for 72 h. For crystallization experiments, lyophilized rMETase was dissolved in water and dialyzed overnight against water. Following the dialysis, the sample was dialyzed further in a solution containing 150 m*M* sodium chloride and 10 m*M* sodium phosphate buffer pH 7.2. The dialyzed sample was then concentrated to a final concentration of 10 mg ml^{-1} for crystallization experiments.

Initial screening for crystallization conditions of rMETase was performed using the vapor-diffusion technique at room temperature (295 K) using a standard screening protocol Wizard-96 (Emerald Biostructures). Subsequent refinement of the pH, precipitant and protein concentrations resulted in rectangular rod-shaped crystals (Fig. 1). Diffraction-quality crystals were grown reproducibly using 1.25 *M* ammonium sulfate in 100 m*M* MES–HCl pH 6.0 as the reservoir solution. The sitting-drop experiments consisted of 3 µl of protein solution (8 mg ml^{-1}) and 3 µl of reservoir solution. Crystals required two weeks to grow to approximate dimensions of 0.05 × 0.05 × 0.8 mm.

2.2. X-ray data collection

X-ray diffraction data sets were collected at a wavelength of 1.08 Å using the synchrotron source at SSRL beamline 7-1. Prior to data collection, the crystals were briefly transferred to a cryoprotectant of 25% glycerol in mother liquor. Data to 2.68 Å were collected at 100 K using a 345 mm MAR Research imaging-plate detector with a crystal-to-detector distance of 300 mm and 0.50° oscillation per frame.

The space group and unit-cell parameters were determined by careful application of the autoindex option of *MOSFLM* (Leslie, 1992); the space group was subsequently confirmed to be $P2_12_12$ by the systematic absences of the axial reflections $h00, h = 2n + 1$ and $0k0, k = 2n + 1$. The data were processed and integrated using *MOSFLM* (Leslie, 1992; Steller *et al.*, 1997) and scaled using *SCALA* from the *CCP*4 (Collaborative Computational Project, Number 4, 1994) suite of programs. The self-rotation functions were calculated using *POLARRFN* (Collaborative Computational Project, Number 4, 1994); *AMoRe* (Navaza, 1994) was used for cross-rotation and translation searches.

Table 1
Unit-cell parameters and data-collection statistics of rMETase.

Unit-cell parameters (Å)	
a	152.83
b	154.64
c	80.748
Space group	$P2_12_12$
No. of subunits per asymmetric unit	4
Matthews coefficient (Å^3 Da^{-1})	2.8
Solvent content (%)	55
Resolution [$I/\sigma(I) \geq 2.0$] (Å)	2.68
$R_{\rm merge}$ (%)	8.9
Total No. of observations	281502
No. of unique observations	75496
Completeness (%)	99.8
$I/\sigma(I)$	6.8
Last shell	
Resolution range (Å)	2.87–2.68
$R_{\rm merge}$ (%)	26.9
Completeness (%)	99.9
$I/\sigma(I)$	2.4

2.3. Molecular replacement

The crystal structure of rMETase was determined by molecular-replacement methods using *AMoRe* (Navaza, 1994). The starting search model for molecular replacement was obtained with no prior knowledge of the sequence identity or biochemical properties of the enzyme. The primary sequence of *P. putida* rMETase was used as a starting point to obtain the search model for molecular replacement. Using the primary sequence of rMETase, a search was performed in *BLAST* to obtain the sequence of homologous proteins. This search resulted in the primary sequences of 15 proteins from different classes, primarily belonging to cystathionine β-lyases, cystathionine γ-lyases and cystathionine γ-synthases. The top two hits, with a sequence identity of nearly 35%, were cystathionine β-lyase from *E. coli* (Clausen *et al.*, 1996) and cystathionine γ-synthase from *N. tabacum* (Steegborn *et al.*, 1999). A subsequent search of the Protein Data Bank (Berman *et al.*, 2000) yielded coordinates for these two enzymes. The crystal structures have been determined to 1.83 and 2.90 Å resolution, respectively, and both are tetramers. The *E. coli* cystathionine β-lyase tetramer was used as the starting search model for molecular replacement.

A cross-rotation function was calculated in the resolution range 20.0–5.0 Å with a sampling step of 2.5° and an integration radius of 35 Å using the program *AMoRe* (Navaza, 1994). The cross-rotation functions calculated using the tetramer as the search model resulted in four peaks of equal height and equal correlation coefficients (CC). These four peaks could be grouped into two sets. The two peaks in the first set had values of $\alpha = 82.3$, $\beta = 78.0$, $\gamma = 103.1°$ and $\alpha = 82.0$, $\beta = 78.2$, $\gamma = 283.8°$; the two peaks in the second set had values of $\alpha = 98.3$, $\beta = 78.1$, $\gamma = 77.0°$, and $\alpha = 97.7$, $\beta = 78.2$ and $\gamma = 257.0°$. The four best solutions with equal CC were used in translation-function searches in the same resolution range. All the four rotation functions resulted in the same translation function with comparable CC and *R* factors.

3. Results and discussion

Crystals of *P. putida* rMETase belong to orthorhombic space group $P2_12_12$ and diffract to at least 2.68 Å resolution. The unit-cell parameters and data-collection statistics for the native data set are given in Table 1. Based on the unit-cell volume, it is assumed that the asymmetric unit contains one tetramer (172 kDa). Self-rotation calculations revealed two strong peaks in the $\kappa = 180°$ section in addition to the peaks arising from the 222 point-group symmetry (Fig. 2). The two peaks correspond to rotations of $\omega = 48.0$, $\varphi = 90$, $\kappa = 180°$, and $\omega = 47.6$, $\varphi = -90$ and $\kappa = 180°$ and are consistent with the cross-rotation function results (§2). The top four solutions of the cross-rotation function can be grouped in two sets of two, each set having the same α and β values but differing by 180° in the γ value. Consequently, the cross- and self-rotation function values together suggest that four monomers of a tetramer in the asymmetric unit are packed as two independent dimers, with each monomer in the dimer being related by a twofold non-crystallographic axis. This arrangement of the subunits of the rMETase tetramer in the asymmetric unit is similar to that observed in the crystal structure of *E. coli* cystathionine β-lyase and is defined as the $\alpha 2$ dimer (Clausen *et al.*, 1996). Based on this model, the CC was 39.2% and the *R* factor was 51.3%. Rigid-body refinement improved the CC to 45.5% and lowered the *R* factor to 49.9%. Complete structure determination and refinement of rMETase is in progress.

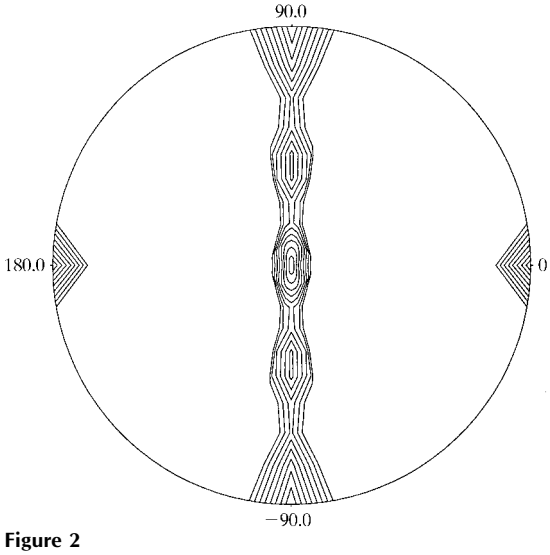

Figure 2
The $\kappa = 180°$ section of the self-rotation function of recombinant methioninase. The integration radius is 25.0 Å and was calculated using the data in the resolution range 30.0–3.0 Å.

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