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Purification, crystallization and preliminary X-ray diffraction analysis of human enolase-phosphatase E1

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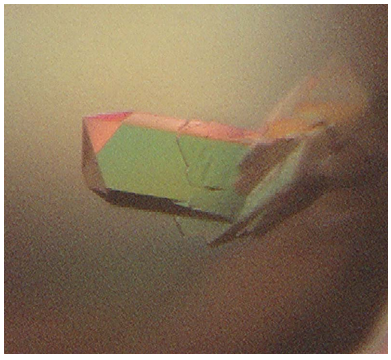

Enolase-phosphatase E1 (MASA) is a bifunctional enzyme in the ubiquitous methionine-salvage pathway and catalyzes the continuous reaction of 2,3-diketo-5-methylthio-1-phosphopentane to yield the acireductone metabolite. Recombinant human E1 enzyme has been crystallized using the hanging-drop vapour-diffusion method and diffraction-quality crystals were grown at 291 K using PEG 4000 as precipitant. Diffraction data were collected to 1.7 Å resolution from SeMet-derivative crystals at 100 K using synchrotron radiation. The crystals belong to space group $P2_12_12_1$, with unit-cell parameters a = 54.02, b = 57.55, c = 87.32 Å. The structure was subsequently solved by the multi-wavelength anomalous diffraction (MAD) phasing method.

1. Introduction

Enolase-phosphatase E1 (MASA) promotes the conversion of 2,3-diketo-5-methylthio-1-phosphopentane to 1,2-dihydroxy-3-keto-5-methylthiopentene anion (an acireductone) in the methionine-salvage pathway (Myers *et al.*, 1993). The pathway is a ubiquitous biochemical pathway that maintains methionine levels *in vivo* by recycling the thiomethyl moiety of methionine through a continuous degradation that leads from *S*-adenosyl methionine (SAM) to methylthioadenosine (MTA) in a wide variety of organisms, including bacteria (Shapiro & Schlenk, 1980; Marchitto & Ferro, 1985), protozoa (Sugimoto *et al.*, 1976), plants (Yung *et al.*, 1982) and mammals (Trackman & Abeles, 1983; Ghoda *et al.*, 1984). MASA was first purified and characterized in the methionine-salvage pathway of *Klebsiella pneumoniae*. The active bifunctional enzyme is a monomeric protein with MW 27 000 Da. The reaction is carried out by enolization and dephosphorylation of the substrate. The activity of MASA requires the presence of a magnesium ion as cofactor and no other prosthetic groups have been found to date (Myers *et al.*, 1993).

Preliminary sequence analysis indicates that MASA belongs to the L-2-haloacid dehalogenase (HAD) superfamily of hydrolases, which includes dehalogenases (C—Cl cleavage), phosphonatases (C—P cleavage), phosphotransferases and phosphatases (C—OP cleavage; Koonin & Tatusov, 1994; Baker *et al.*, 1998; Collet *et al.*, 1998; Ridder *et al.*, 1999; Stokes & Green, 2000; Hisano *et al.*, 1996). Despite having both low sequence identity and a diverse range of functions, the members of the superfamily share a similar tertiary structure, highly conserved sequence motifs in the active site and a common catalytic mechanism (Shin *et al.*, 2003). Members of this family whose structures are already known (haloacid dehalogenase, phosphonatase, Ca^{II} ATPase, phosphoserine phosphatase and β-phosphoglucomutase) consist of a typical Rossmann-fold core domain and a small α-helical cap domain. The active site is located at the domain–domain interface. The core domain responsible for catalysis has a highly conserved tertiary structure, while the cap domain has a diverse structure to accommodate the various substrates in the different reactions (Morais *et al.*, 2000). Furthermore, the catalytic mechanism of this superfamily involves the formation of a covalent aspartyl-ester enzyme intermediate which is supported by the three conserved motifs in the active-site scaffold. Motif I is D*X*D*X*(T/V), in which the absolutely conserved aspartate is responsible for formation of the enzyme–substrate intermediate (Collet *et al.*, 1998). Motif II contains a conserved serine or threonine that hydrogen bonds to the phos-

phoryl O atom or the carboxylate group of the substrate (Wang *et al.*, 2001). Motif III consists of a conserved lysine followed by several less conserved residues and a strictly conserved aspartate, which can help to coordinate the magnesium ion required for activity (Morais *et al.*, 2000; Wang *et al.*, 2001). Characteristic phosphoaspartate enzyme intermediates in this family have been proposed to function in signal transduction, phosphotransfer reactions (phosphatases and mutases) and the conversion of chemical energy to ion gradients (P-type ATPases; Welch *et al.*, 1998; Wang *et al.*, 2002; Toyoshima *et al.*, 2000).

In addition to the phosphatase, MASA also possesses enolase activity which cannot be classified as typical enzymatic enolization. This kind of enolization requires the presence of metal ions as cofactors, but the catalytic mechanism is still unclear. In order to further explore the mechanism of phosphoenzyme formation and hydrolysis and to elucidate how enolization occurs, it is important to determine the three-dimensional structure of human MASA. In addition, from this structure we expect to understand more about the methionine-salvage pathway in mammals and to shed light on the structure and function of other members of this family.

2. Materials and methods

2.1. Expression and purification of human MASA

A cDNA fragment encoding human MASA was amplified by RT-PCR (Lloyd *et al.*, 1998) and cloned into the bacterial expression vector pET28a (Novagen) with a His tag on the C-terminus. The recombinant plasmid was transformed into *Escherichia coli* strain BL21 (DE3) and overexpressed.

Cells were cultured at 310 K in 1 l LB medium containing 50 µg ml^{-1} kanamycin to an OD_{600} of 0.6–0.8. The cells were then induced with 1 m*M* isopropyl-β-D-thiogalactopyranoside (IPTG) and continuously incubated at 310 K for a further 4 h to an OD_{600} of 2.0. The cells were harvested by centrifugation, resuspended in lysis buffer (20 m*M* Tris–HCl pH 7.9, 500 m*M* NaCl, 5 m*M* imidazole, 10% glycerol) and homogenized by sonication. The soluble cell lysate obtained by centrifugation at 20 000*g* for 30 min was applied onto a Ni^{2+}-chelating affinity column (1.5 ml Ni^{2+}–NTA agarose) pre-equilibrated with lysis buffer. The contaminant protein was thoroughly washed off with wash buffer (20 m*M* Tris–HCl pH 7.9, 500 m*M* NaCl, 10–25 m*M* imidazole, 10% glycerol) for at least ten bed volumes and the target protein was eluted with 20 m*M* Tris–HCl pH 7.9, 500 m*M* NaCl, 60 m*M* imidazole, 10% glycerol for about 10 ml. The eluted sample was then concentrated using an Ultrafree 10 000 NMWL filter unit (Millipore) to 500 µl and further applied onto a Superdex-75 size-exclusion chromatography column with buffer *A* (20 m*M* HEPES pH 7.5, 150 m*M* NaCl). The target peak corresponding to a molecular weight of about 30 kDa (a monomer) was collected and loaded onto a MonoQ anion-exchange chromatography column (Pharmacia) equilibrated with buffer *B* (20 m*M* HEPES pH 7.5, 150 m*M* NaCl). After washing unbound protein with three bed volumes, a linear gradient of 0.2–0.6 *M* NaCl in the same buffer was applied. MASA was finally eluted with approximately 0.38 *M* NaCl. The purified sample was concentrated and transferred into a buffer containing 20 m*M* HEPES pH 7.5 and less than 50 m*M* NaCl. The purity of protein was then analyzed on SDS–PAGE (better than 90% purity) and was judged to be suitable for crystallization.

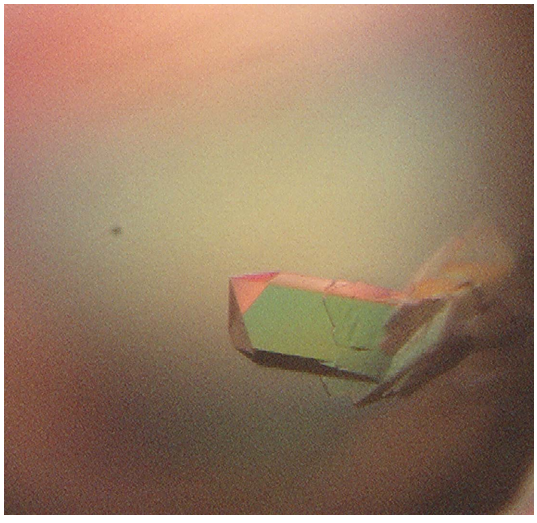

Figure 1
An orthorhombic crystal of SeMet-labelled MASA protein. The crystals have dimensions of 0.5 × 0.2 × 0.3 mm.

The SeMet MASA protein was also expressed in *E. coli* strain BL21 (DE3). After incubation overnight in LB medium containing 50 µg ml^{-1} kanamycin, the cells were diluted with adaptive medium (20% LB medium, 80% M9 medium) and grown at 310 K to an OD_{600} of 0.6–0.8. The cells were harvested and resuspended in M9 medium, transferred into restrictive medium (3% glucose) and then grown to an OD_{600} of 0.6–0.8 before induction. L-SeMet at 60 mg l^{-1}, lysine, threonine and phenylalanine at 100 mg l^{-1}, leucine, isoleucine and valine at 50 mg l^{-1} and 0.5 m*M* IPTG were added and incubation continued at 289 K for about 20 h. The cells were harvested and the L-SeMet-labelled MASA protein was purified and crystallized by the same method as for the native protein.

2.2. Crystallization

The purified protein was concentrated using an Ultrafree 10 000 NMWL filter unit (Millipore) to 25 mg ml^{-1} in a solution containing 20 m*M* HEPES pH 7.5, 30 m*M* NaCl. The preliminary crystallization conditions were obtained by the sparse-matrix sampling technique (Jancarik & Kim, 1991) with the hanging-drop vapour-diffusion method using Crystal Screen I and Crystal Screen II reagent kits (Hampton Research). Crystals suitable for X-ray diffraction were obtained from 30% PEG 4000, 0.1*M* Tris–HCl pH 8.5, 0.2 *M* magnesium chloride hexahydrate. 1 µl protein solution was mixed with 1 µl reservoir solution and equilibrated against 300 µl reservoir solution at 291 K. Crystals grew in 4–5 d.

2.3. X-ray crystallographic studies

The three-wavelength MAD X-ray diffraction data set for L-SeMet-labelled MASA protein was collected at 291 K using a MAR CCD detector and synchrotron-radiation beamlines 3W and 1A at BSRF (Beijing Synchrotron Radiation Facility). The crystal was flash-cooled for data collection and no cryoprotectant was used. The program *AUTOMAR* was used for data indexing, integration and scaling, and determination of the unit-cell parameters (Bartels & Klein, 2003).

3. Results

The initial native protein crystallization trials were carried out using Crystal Screen reagent kits from Hampton Research with different protein concentrations. Bundles of needle-like crystals were obtained from a condition containing 30% PEG 8000, 0.1 *M* sodium cacodylate pH 6.5, 0.2 *M* ammonium sulfate. Although these crystals diffracted X-rays, they were too brittle and showed apparent twinning and were

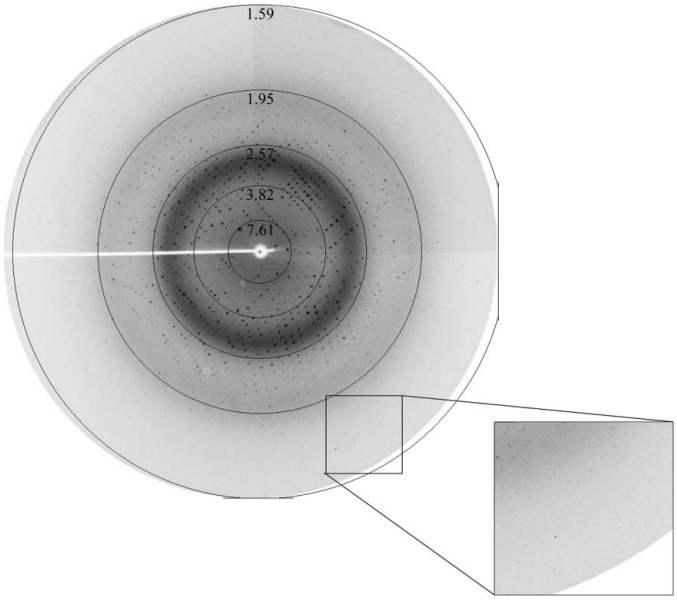

Figure 2
Typical diffraction of SeMet-labelled MASA crystals using synchrotron radiation at the BSRF. The resolution at the edge of the plate is 1.58 Å.

thus unsuitable for good diffraction. By adding 1–5% PEG 400 to the reservoir solution as an additive, the crystals grew thicker but were still in bundles. The diffraction data was so poor that the space group and the unit-cell parameters could not be easily determined. The best crystal of native MASA diffracted to a resolution of 3.5 Å.

When preparing for crystallization of the SeMet-labelled MASA protein, we used the same conditions as used for the native protein while screening again using the kits from Hampton Research. Surprisingly, better crystals were obtained from a totally different condition containing 30% PEG 4000, 0.1 *M* Tris–HCl pH 8.5, 0.2 *M* magnesium chloride hexahydrate. Drops containing 1 µl protein solution and 1 µl reservoir solution were equilibrated against 300 µl reservoir solution at 291 K. Crystals grew in 4–5 d and reached their full size in about 10 d (Fig. 1). Crystals from this optimum reservoir solution condition were compact and stable and diffracted X-rays to a resolution of at least 2 Å in-house. The native protein also crystallized well using this condition and diffracted to 1.9 Å resolution. To determine the structure of MASA, crystals of the SeMet-labelled protein were used at the synchrotron-radiation source at BSRF. A three-wavelength MAD X-ray diffraction data set for L-SeMet-labelled MASA protein was collected to a resolution of 1.7 Å (Fig. 2). The crystals belong to space group $P2_12_12_1$, with unit-cell parameters $a = 54.02$, $b = 57.55$, $c = 87.32$ Å. The value of the Matthews coefficient (Matthews, 1968) is 2.19 Å^3 Da^{-1} for one molecule in the asymmetric unit and the estimated solvent content is 45.0%. Table 1 shows the relevant statistics for the three SeMet data sets.

Phases for the structure of human MASA were determined by the multi-wavelength anomalous diffraction (MAD) method and the structure has since been solved. The details of the structure are currently being studied. In addition, this study should elucidate the molecular mechanisms for the phosphatase and enolase activity of MASA and provide a clue for understanding the functional switch of MASA.

Table 1
Data-collection and processing statistics for the SeMet MASA crystals.

Values for the outermost shell are given in parentheses. The resolution limit for the highest resolution shell is 1.7 Å.

	SeMet λ_1 (edge)	SeMet λ_2 (peak)	SeMet λ_3 (remote)
Space group	$P2_12_12_1$		
Unit-cell parameters (Å)	$a = 54.02$, $b = 57.55$, $c = 87.32$		
Wavelength (Å)	0.9800	0.9798	0.9000
Resolution (Å)	30–1.7	30–1.7	30–1.7
Total reflections	185221	236864	185438
Unique reflections	30414	30337	30550
$R_{\rm merge}$† (%)	9.03 (45.43)	9.11 (36.33)	9.34 (37.68)
Completeness (%)	99.9 (98.3)	99.7 (97.5)	100 (99.8)
Average $I/\sigma(I)$	3.5 (0.9)	4.1 (1.1)	3.6 (1.4)

† $R_{\rm merge} = \sum_i |I_i - \langle I\rangle| / \sum \langle I\rangle$, where I_i is an individual intensity measurement and $\langle I\rangle$ is the average intensity for all reflections.

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