Crystallization and preliminary X-ray analysis of L-methionine γ-lyase 1 from *Entamoeba histolytica*

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L-Methionine γ-lyase (MGL) is a pyridoxal phosphate-dependent enzyme that is involved in the degradation of sulfur-containing amino acids. MGL is an attractive drug target against amoebiasis because the mammalian host of its causative agent *Entamoeba histolytica* lacks MGL. For the development of anti-amoebic agents based on the structure of MGL, one of two MGL isoenzymes (EhMGL1) was crystallized in the monoclinic space group $P2_1$, with unit-cell parameters $a = 99.12$, $b = 85.38$, $c = 115.37$ Å, $\beta = 101.82°$. The crystals diffract to beyond 2.0 Å resolution. The presence of a tetramer in the asymmetric unit (4 × 42.4 kDa) gives a Matthews coefficient of 2.8 $Å^3$ Da^{-1} and a solvent content of 56%. The structure was solved by the molecular-replacement method and structure refinement is now in progress.

1. Introduction

Amoebiasis, which is caused by infection with the enteric protist *Entamoeba histolytica*, is the second largest parasitic disease after malaria; it affects over 50 million people and results in 70 000 deaths annually (World Health Organization, 1998). Its major clinical manifestations are amoebic dysentery and extraintestinal abscesses (Stanley, 2003).

The sulfur-containing amino-acid metabolism of *E. histolytica* has a number of unique features (Nozaki *et al.*, 2005; Ali & Nozaki, 2007). For example, *E. histolytica* lacks the transsulfuration pathway and thus is unable to convert methionine to cysteine and *vice versa*. Instead, it possesses L-methionine γ-lyase (MGL; EC 4.4.1.11), which is involved in the degradation of sulfur-containing amino acids (Tokoro *et al.*, 2003; Sato *et al.*, 2008). MGL contains pyridoxal 5′-phosphate (PLP) as a cofactor and has been categorized as belonging to the γ-family of PLP-dependent enzymes (Alexander *et al.*, 1994). MGL catalyzes α,γ- or α,β-elimination of sulfur-containing amino acids and produces ammonia, α-keto acids and volatile thiols such as hydrogen sulfide or methanethiol (Tanaka *et al.*, 1985). It also catalyzes β- or γ-replacement of cysteine, *S*-substituted cysteine, methionine and related compounds (Tanaka *et al.*, 1985). MGL is only present in a limited range of bacteria, protozoa and plants (Nakayama *et al.*, 1984; Kreis & Hession, 1973; Yoshimura *et al.*, 2000; Dias & Weimer, 1998; Manukhov *et al.*, 2005; Goyer *et al.*, 2007; Coombs & Mottram, 2001; Nozaki *et al.*, 2005; Rebeille *et al.*, 2006). Crystal structures have been reported for MGLs from *Pseudomonas putida* (Motoshima *et al.*, 2000; Sridhar *et al.*, 2000), *Trichomonas vaginalis* (PDB code 1e5f; G. Goodall, J. C. Mottram, G. H. Coombs & A. J. Lapthorn, unpublished work) and *Citrobacter freundii* (Mamaeva *et al.*, 2005; Nikulin *et al.*, 2008).

MGLs from parasitic protozoa are unique in that they exist as two isoenzymes with distinct substrate specificities (*e.g.* EhMGL1 and EhMGL12 in *E. histolytica*; 69% amino-acid sequence identity; Tokoro *et al.*, 2003; Sato *et al.*, 2008). A previous study demonstrated that EhMGL1, but not EhMGL2, predominantly degrades methionine and cysteine (Sato *et al.*, 2008). In contrast, trifluoromethionine (TFM), the halogenated analogue of methionine, which exhibits toxicity by being degraded by MGL in *Porphyromonas gingivalis*,

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T. vaginalis and *E. histolytica* (Yoshimura *et al.*, 2002; Coombs & Mottram, 2001; Tokoro *et al.*, 2003), was almost exclusively degraded by EhMGL2. We also showed that the K_m of EhMGL1 towards TFM was ninefold lower than that of EhMGL2, while the k_{cat} of EhMGL2 was 22-fold higher than that of EhMGL1 (Sato *et al.*, 2008), indicating that the catalytic action of MGLs towards TFM might be different. For structure-based design of TFM derivatives, comparison of the tertiary structures of the two EhMGL isotypes should be helpful. We have previously crystallized ligand-free EhMGL2 (Sato *et al.*, 2006) and determined its X-ray structure as well as those of structures of complexes with substrates and inhibitors (unpublished results). In the present study, we describe the bacterial expression, purification, crystallization and preliminary X-ray diffraction studies of EhMGL1.

2. Materials and methods

2.1. Overexpression and purification of recombinant MGL1

When we attempted to express the *EhMGL1* gene (AB094499) in a bacterial expression system (pGEX-6P-1 vector; GE Healthcare Biosciences) as an amino-terminal glutathione *S*-transferase (GST) fusion protein, a fraction of protein (~20%) was produced as a truncated form starting from Gly46 (Sato *et al.*, 2008). This truncation was caused by an inadvertent translation initiation at Met45 which was likely to be a consequence of the similarity of the nucleotide sequence upstream of Met45 to the Shine–Dalgarno sequence. We therefore mutated five nucleotides in this region, which did not cause amino-acid substitution (Sato *et al.*, 2008). This genetically engineered EhMGL1 construct allowed us to successfully express and purify EhMGL1 protein without truncation. For large-scale preparation, this plasmid was introduced into the *Escherichia coli* BL21 Star (DE3) strain (Invitrogen) and the transformant was grown in 2 l M9 minimal medium (Sambrook *et al.*, 1989) supplemented with 1 m*M* MgSO₄, 0.1 m*M* CaCl₂, 3.3 µ*M* FeCl₃, 59 µ*M* thiamine–HCl, 82 µ*M* biotin, 20 µ*M* PLP and 50 µg ml⁻¹ ampicillin at 310 K. The medium was prepared with commercially available mineral water to supply trace elements. The induction of expression, affinity purification and on-column digestion of the GST-EhMGL1 fusion protein were carried out as described previously for EhMGL2 (Sato *et al.*, 2006). The fraction containing GST-removed EhMGL1 was eluted from a GSTrap HP column (1.6 × 2.5 cm; GE Healthcare Bio-

sciences) with 50 m*M* HEPES–NaOH pH 7.5, 150 m*M* NaCl and 20 µ*M* PLP and concentrated to 1–2 ml with Amicon Ultra-4 Ultracel-10K. EhMGL1 was further purified by size-exclusion chromatography on a HiPrep 16/60 Sephacryl S-300 column (16 × 60 cm; GE Healthcare Biosciences) equilibrated with 50 m*M* sodium phosphate pH 7.2 containing 150 m*M* NaCl and 20 µ*M* PLP. EhMGL1 was eluted as a tetramer as previously described (Tokoro *et al.*, 2003). The purified EhMGL1 contained ten extra amino-acid residues (GPL-GSPEFPG) at the amino-terminus derived from the linker peptide between GST and EhMGL1. The fractions containing the recombinant EhMGL1 were dialyzed against 10 m*M* HEPES–NaOH pH 7.4 and concentrated using an Amicon Ultra-4 Ultracel-50K to ~20 mg ml⁻¹. About 10 mg purified EhMGL1 was produced from 2 l culture. The purity was estimated to be >95% pure by densitometric quantitation of the corresponding band on SDS–PAGE (Fig. 1, lane 4) and the retained activity was comparable to the previous study (Sato *et al.*, 2008).

2.2. Crystallization and X-ray diffraction data collection

Crystallization conditions were screened at 277 and 293 K by the sitting-drop vapour-diffusion method in 96-well CrystalClear Strips (Hampton Research). A 0.5 µl droplet containing about 20 mg ml⁻¹ EhMGL1 dissolved in 10 m*M* HEPES–NaOH pH 7.4 was mixed with an equal volume of reservoir solution and the droplet was allowed to equilibrate against 100 µl reservoir solution. In the initial screening experiment, Crystal Screen (Jancarik & Kim, 1991), Crystal Screen II (Hampton Research) and Wizard Screens I and II (Emerald Biostructures) were used as reservoir solutions. Of the 194 conditions used, reagents containing ammonium sulfate as a precipitant gave thin plate-shaped crystals at 277 K. Conditions were further optimized at 277 K by varying the concentration of ammonium sulfate and the buffer pH. The effects of additives were also examined using Additive Screen kits from Hampton Research. The best crystals grew at 277 K from reservoir solution containing 1.8 *M* ammonium sulfate, 0.1 *M* cacodylate buffer pH 6.2, 0.1 *M* lithium citrate and 0.01 *M* betaine.

For X-ray diffraction experiments at 100 K, a crystal mounted in a nylon loop was transferred into and briefly soaked in a solution containing 2.2 *M* ammonium sulfate, 0.1 *M* cacodylate buffer pH 6.2, 0.1 *M* lithium citrate, 0.01 *M* betaine and 20%(*w/v*) glycerol and then frozen by rapidly submerging it in liquid nitrogen. X-ray diffraction data were collected using the rotation method on the BL5A beamline of Photon Factory (Tsukuba, Japan) at a wavelength of 1.000 Å using
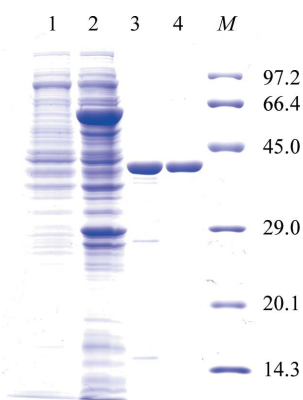

Figure 1
A 12% SDS–PAGE gel stained with Coomassie Brilliant Blue showing the apparent homogeneity of the purified EhMGL1. Lane 1, *E. coli* soluble extract before IPTG induction; lane 2, *E. coli* soluble extract after IPTG induction; lane 3, an eluate from a GSTrap HP column after digestion with PreScission Protease; lane 4, the purified EhMGL1 after size-exclusion chromatography using a HiPrep16/60 Sephacryl S-300 column; lane *M*, molecular-weight markers (kDa).
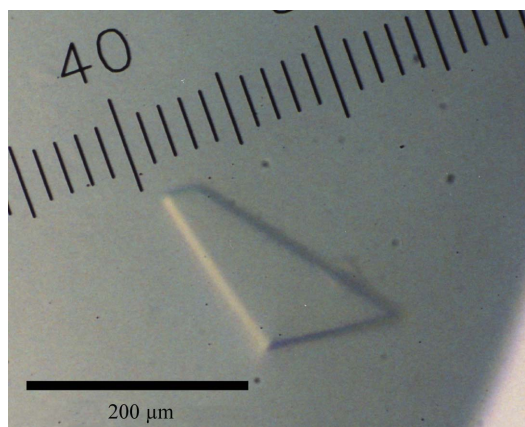

Figure 2
A typical crystal of EhMGL1.

Table 1
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	1.000
Space group	$P2_1$
Unit-cell parameters	
a (Å)	99.12
b (Å)	85.38
c (Å)	115.37
β (°)	101.82
Solvent content† (%)	56
Resolution range (Å)	50.0–1.93 (2.00–1.93)
No. of reflections	491182
Unique reflections	140502
Completeness (%)	96.3 (87.0)
R_{merge}‡ (%)	7.2 (39.1)
$I/\sigma(I)$	8.4 (2.57)

† Assuming the presence of four molecules in the asymmetric unit. ‡ $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$.

a CCD ADSC Quantum 315 detector. A total of 180 frames were recorded with an oscillation angle of $1.0°$, an exposure time of 5.0 s per frame and a crystal-to-detector distance of 213 mm. Data were processed and scaled with *HKL*-2000 and *SCALEPACK* (Otwinowski & Minor, 1997).

3. Results and discussion

Of the 194 crystallization conditions screened using commercially available screening kits, reagents containing ammonium sulfate gave thin plate-shaped crystals. After optimization of the pH, the concentration of ammonium sulfate and additives, crystals grew to typical dimensions of $0.02 \times 0.3 \times 0.4$ mm (Fig. 2) in 5 d under the conditions 1.8 *M* ammonium sulfate, 0.1 *M* cacodylate buffer pH 6.2, 0.1 *M* lithium citrate and 0.01 *M* betaine at 277 K. Diffraction patterns were recorded as 180 frames at 100 K using one crystal. Analysis of the symmetry and systematic absences in the recorded diffraction patterns indicated that the crystals belonged to the monoclinic space group $P2_1$, with unit-cell parameters a = 99.12, b = 85.38, c = 115.37 Å, β = 101.82°. Assuming the presence of four EhMGL1 molecules (4×42.4 kDa) in the asymmetric unit, the V_M value was calculated to be 2.8 Å^3 Da^{-1} with an estimated solvent content of 56%; these values are within the range commonly observed for protein crystals (Matthews, 1968). A total of 491 182 observed reflections were merged to 140 502 unique reflections in the 50.0–1.93 Å resolution range. The data-collection and processing statistics are summarized in Table 1.

An attempt to solve the structure using the molecular-replacement method with the *MOLREP* program (Navaza, 1994) as implemented within the *CCP*4 package (Collaborative Computational Project, Number 4, 1994) was carried out using the refined coordinates of EhMGL2 (unpublished results; 69% sequence identity to EhMGL1). A promising solution with a homotetramer structure was obtained (correlation coefficient and *R* factor of 0.557 and 51.3%, respectively) and the model was subsequently subjected to rigid-body refinement, giving an *R* factor of 46.8%. Amino-acid residues of the initial homotetramer model that differ from those of the search model were replaced with Ala. Using the molecular-replacement solution,

refinement of the tetramer structure of EhMGL1 is in progress. In parallel with the refinement, we are now trying to prepare crystals of EhMGL1 complexed with substrate analogues and inhibitors.

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