Structure of methionine γ -lyase from *Clostridium sporogenes*

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Methionine γ -lyase (MGL) is a pyridoxal 5'-phosphate-dependent enzyme that catalyzes the γ -elimination reaction of L-methionine. The enzyme is a promising target for therapeutic intervention in some anaerobic pathogens and has attracted interest as a potential cancer treatment. The crystal structure of MGL from *Clostridium sporogenes* has been determined at 2.37 Å resolution. The fold of the protein is similar to those of homologous enzymes from *Citrobacter freundii*, *Entamoeba histolytica*, *Pseudomonas putida* and *Trichomonas vaginalis*. A comparison of these structures revealed differences in the conformation of two flexible regions of the N- and C-terminal domains involved in the active-site architecture.

1. Introduction

Methionine γ -lyase (MGL; EC 4.4.1.11) is a pyridoxal 5'-phosphate (PLP)-dependent enzyme that catalyzes the γ -elimination reaction of L-methionine to give α -ketobutyric acid, methanethiol and ammonia (Supplementary Fig. S1; Tanaka *et al.*, 1985). Besides this physiological reaction, the enzyme catalyzes γ -replacement reactions of L-methionine and its analogues as well as β -elimination and β -replacement reactions of L-cysteine and S-substituted L-cysteines (Faleev *et al.*, 1994; Tanaka *et al.*, 1985). MGL has been found in a number of bacteria, including the human pathogens *Porphyromonas gingivalis* (Yoshimura *et al.*, 2000), *Clostridium sporogenes* (Kreis & Hession, 1973; Revtovich *et al.*, 2012) and *C. tetani* (Revtovich *et al.*, 2012). The enzyme has also been purified from the eukaryotic human pathogens *Entamoeba histolytica* (Tokoro *et al.*, 2003) and *Trichomonas vaginalis* (McKie *et al.*, 1998). The enzyme is also involved in the catabolism of methionine in the plant *Arabidopsis thaliana* (Rébeillé *et al.*, 2006).

MGL is being investigated as a potential biological anticancer agent, since malignant cells, unlike normal cells, are unable to synthesize methionine (Hoffman, 1982). Data on the anticancer activity of *P. putida* MGL have recently been reviewed (Hoffman, 2015).

The absence of MGL in mammals makes the enzyme a potential target for antimicrobial therapy. Suppression of growth of *T. vaginalis* (Coombs & Mottram, 2001), *P. gingivalis* (Yoshimura *et al.*, 2002) and *E. histolytica* (Sato *et al.*, 2010) cells by a suicide substrate of MGL, L-trifluoromethionine, has been demonstrated *in vitro* and *in vivo*. The complex of MGL with another suicide substrate, S-allyl-L-cysteine sulfoxide, revealed antimicrobial activity against *Staphylococcus aureus* and *Citrobacter freundii* (Anufrieva *et*

Table 1

Macromolecule-production information.

Source organism	<i>C. sporogenes</i>
DNA source	pET-28a::megL _s -HT
Forward primer	CGCGCGGCAGCCCCATGGAGAA
Reverse primer	CCGGATCTCAGTGGTGGTGGTG
Cloning vector	pET-28a
Expression vector	pET-28a
Expression host	<i>E. coli</i> strain BL21 (DE3)
UniProt identifier	J7TA22

al., 2015). Crystal structures of MGL holoenzymes from *P. putida* (PDB entry 2o7c; Kudou *et al.*, 2007), *C. freundii* (PDB entry 2rfv; Nikulin *et al.*, 2008), *E. histolytica* (PDB entry 3acz) and *T. vaginalis* (PDB entry 1e5f) have been determined.

The enzyme belongs to the evolutionary γ -family of PLP-dependent enzymes (Alexander *et al.*, 1994) involved in the metabolism of sulfur-containing amino acids. It is assigned to the aspartate aminotransferase (AspAT) family of PLP-dependent enzymes with a type I fold of polypeptide chains (Grishin *et al.*, 1995; Jansonius, 1998) and has features characteristic of enzymes of the cystathionine β -lyase subclass (Käck *et al.*, 1999). Like other enzymes of this subclass, MGL exists as a homotetramer with a molecular weight of about 200 kDa that possesses 222 symmetry. The tetrameric molecule is subdivided into two so-called catalytic dimers in which the two active sites contain residues from both subunits.

In this paper, we report the crystallization and crystal structure of the holoenzyme of MGL from *C. sporogenes* at 2.37 Å resolution together with a comparison of holoenzyme structures of the enzyme derived from different sources.

2. Materials and methods

2.1. Macromolecule production

The plasmid pET-28a-megL_s-sporog was constructed on the basis of the plasmid pET-28a and contained the *megL* gene from *C. sporogenes* with a His tag (Revtovich *et al.*, 2012). We obtained an amplicon containing the *megL* gene without a His tag by PCR. The gene was amplified using the primers 5'-CGCGCGGCAGCCCCATGGAGAA-3' and 5'-CCGGATCTCAGTGGTGGTGGTG-3' (the NcoI restriction site is underlined). To clone the obtained amplicons in pET-28a vector, the NcoI and EcoRI restriction sites were used. The resulting plasmid was used to transform *Escherichia coli* strain BL21 (DE3). For overexpression, the *E. coli* cells were grown at 37°C on an 'inducing' medium (Studier, 2005) with agitation (180 rev min⁻¹) for 24 h. Cells were collected by centrifugation and stored at -80°C. Cell disruption and release of nucleic acids were performed as described previously (Manukhov *et al.*, 2006). Further purification was performed using chromatography on a DEAE Sepharose column as described previously (Anufrieva *et al.*, 2015). The purity of the preparations was checked by electrophoresis under denaturing conditions (Laemmli, 1970). Crystallization was performed with preparations of about 95% homogeneity.

Table 2

Data collection and processing.

Diffraction source	Beamline 14.1, BESSY II
Wavelength (Å)	0.918409
Temperature (K)	100
Detector	Pilatus 6M
Crystal-to-detector distance (mm)	425.99
Rotation range per image (°)	0.1
Total rotation range (°)	100
Exposure time per image (s)	1.5
Space group	<i>P</i> ₄ ₃ ₂ ₁ ₂
<i>a</i> , <i>b</i> , <i>c</i> (Å)	91.09, 91.09, 175.93
α , β , γ (°)	90, 90, 90
Mosaicity (°)	0.263
Resolution range (Å)	45.55–2.37
Total No. of reflections	182391 (18619)
No. of unique reflections	30892 (3025)
Completeness (%)	99.48 (99.00)
Multiplicity	5.9 (6.2)
$\langle I/\sigma(I) \rangle$	16.97 (2.91)
<i>R</i> _{meas}	0.11 (0.68)
Overall <i>B</i> factor from Wilson plot (Å ²)	33.45

Macromolecule-production information is summarized in Table 1.

2.2. Crystallization

Crystals of MGL were obtained using the hanging-drop vapour-diffusion technique at 303 K. Drops were generated by mixing 2.0 µl of the enzyme in 50 mM Tris-HCl pH 8.5, 0.2 mM PLP with 2.0 µl precipitant solution on cover slips and were equilibrated against 1.0 ml of the same precipitant solution. MGL formed crystals in 35% polyethylene glycol monomethyl ether (PEG MME) 2000, 50 mM Tris-HCl pH 8.5, 0.2 mM PLP. The spindle-shaped crystals appeared after a week and attained dimensions of 0.15 × 0.4 mm within two weeks.

2.3. Data collection and processing

Data from a single crystal were collected on beamline 14.1 at BESSY II, Berlin, Germany equipped with a Pilatus 6M detector (Dectris) and were processed using *XDS* (Kabsch, 2010) with the *XDSAPP* GUI (Krug *et al.*, 2012). Detailed data-collection statistics are shown in Table 2. The crystals belonged to space group *P*₄₃₂₁₂, with unit-cell parameters *a* = 91.09, *b* = 91.09, *c* = 175.93 Å, and contained two monomers in the asymmetric unit. Prior to cryocooling in liquid nitrogen, the crystals were transferred into 35% PEG MME 2000, 50 mM Tris-HCl pH 8.5, 0.2 mM PLP.

2.4. Structure solution and refinement

The structure was solved by molecular replacement with the structure of *C. freundii* MGL (PDB entry 2rfv; 55% sequence identity) using *Phaser* (McCoy *et al.*, 2007) from the *CCP4* software suite (Winn *et al.*, 2011). The model was improved using manual rebuilding with *Coot* (Emsley & Cowtan, 2004) and maximum-likelihood refinement using *REFMAC5* (Murshudov *et al.*, 2011). Flexible loops of the protein and water molecules were removed from the initial model to exclude model bias during the first round of refinement. The

Table 3
Structure refinement.

Resolution range (Å)	45.55–2.37 (2.42–2.37)
Completeness (%)	99.48 (99.00)
σ Cutoff	0
No. of reflections, working set	55150
No. of reflections, test set	2075
Final R_{cryst}	0.162 (0.234)
Final R_{free}	0.225 (0.285)
No. of non-H atoms	
Protein	6122
Ion	3
Water	194
Total	6319
R.m.s. deviations	
Bonds (Å)	0.008
Angles (°)	1.027
Average B factors (Å ²)	
Protein	34.4
Ion	47.1
Water	33.1
Ramachandran plot†	
Favoured regions (%)	96.6
Additionally allowed (%)	3.02
Outliers (%)	0.38

† The data were obtained using the MolProbity online service (Chen *et al.*, 2010)

final model, refined to 2.37 Å resolution by *PHENIX* (Adams *et al.*, 2002), contains two monomers in the asymmetric unit, two chloride ions, one sodium ion and 194 water molecules (Table 3). The structure has been submitted to the Protein Data Bank and assigned PDB entry 5dx5.

3. Results and discussion

3.1. Overall structure

C. sporogenes MGL is a homotetramer with a total molecular weight of about 170 kDa (Morozova *et al.*, 2013). Structures of the enzyme from *P. putida* (Motoshima *et al.*, 2000) and *C. freundii* (Mamaeva *et al.*, 2005) identify that it belongs to the cystathionine β -lyase structural subclass (Käck *et al.*, 1999). The tetrameric assembly can be subdivided into two so-called catalytic dimers in which two active sites contain residues from both subunits (Fig. 1*a*, green/blue or *A/B* and red/orange or *C/D* subunits). The monomer consists of three domains: N-terminal, central PLP-binding and C-terminal.

The N-terminal domain (residues 1–62) is composed of one short 3_{10} -helix and two α -helices, which are connected by a

Figure 1
Overall structure of *C. sporogenes* MGL. Catalytic dimers are shown in green/blue and red/orange. (*a*) Schematic model of a tetramer. PLP-binding sites are shown as yellow balls. (*b*) Stereoview of a catalytic dimer. (*c*) Solvent-accessible surface of the enzyme tetramer; 'paws' are shown in darker colours. (*d*) Antiparallel β -sheet-like structure organized by N-terminal β -strands of the two adjacent monomers (orange and green).

long loop containing 27 residues (Supplementary Fig. S2). The position of the loop is fixed in the tetramer by contacts with the neighbouring subunit (Supplementary Table S1).

The PLP-binding domain (residues 63–261) is typical of PLP-dependent enzymes with the type I fold (Jansonius, 1998). It includes a seven-stranded mainly parallel β -sheet (β 1– β 7) with $+ - + + + + +$ directions of the β -strands, seven α -helices (α 3– α 9), two 3_{10} -helices and one 5_{16} -helix, which are arranged on both sides of the β -sheet. Helices α 3, α 6 and α 7 and the second short 3_{10} -helix are located on one side of the β -sheet and shield it from solvent. Helices α 4, α 5 and α 8, the first short 3_{10} -helix and the 5_{16} -helix are located on the other side of the β -sheet and are included in the intermolecular interface. PLP is covalently bonded to the ϵ -amino group of

Lys212 (Supplementary Fig. S3) and is located near the N-terminus of the α 4 helix, the C-termini of the β 5 and β 6 strands and the N-terminus of the β 7 strand.

The C-terminal domain (residues 262–398) consists of a five-stranded antiparallel β -sheet, five α -helices (α 10– α 14) and one short 3_{10} -helix. The helices are located on both sides of the β -sheet.

The elements involved in contacts within a catalytic dimer belong mainly to the PLP-binding domain (loops α 2/ α 3, β 1/ α 4, α 4/ β 2, α 5/first 3_{10} -helix, β 6/ β 7, π -helix/ α 9 and helices α 4, α 5, α 9, the first short 3_{10} -helix and π -helix), having a centre of symmetry at Gly245/Ser246 in the π -helix/ α 9 loop. Another zone of the dimer contacts involves the α 1/ α 2 and α 2/ α 3 loops belonging to the N-terminal domain (Supplementary Table

Figure 2

(a) Superposition of the C^α traces of the two subunits of *C. sporogenes* MGL coloured according to increasing B -factor value from blue to yellow. The flexible regions of the N- and C-terminal domains are marked with ovals. (b) Superposition of the C^α traces of MGLs from various species. *C. sporogenes* MGL (PDB entry 5dx5) is in green, *C. freundii* MGL (PDB entry 2rfv) is in blue, *E. histolytica* MGL (PDB entry 3acz) is in cyan, *T. vaginalis* MGL (PDB entry 1e5f) is in orange and *P. putida* MGL (PDB entry 2o7c) is in magenta. The flexible N- and C-terminal domains are marked with ovals. (c) Stereoview of the superposition of the active sites coloured as in (b). Residues of monomer *B* are marked with asterisks. Hydrogen bonds are indicated by dotted lines.

S1), which interact with the $\alpha 12$ – $\beta 10$ region of the C-terminal domain of an adjacent subunit. As a result, both monomers in the catalytic dimer form the large and planar interaction surface (Fig. 1*b*).

Two catalytic dimers form a tetrameric structure with 222 symmetry that is stabilized by a network of intermolecular interactions (Supplementary Table S1). It is noteworthy that most of these interactions are provided by the N-terminal domains. Helix $\alpha 1$ of subunit *A* interacts with a monomer (subunit *C*) from the second catalytic dimer, and the $\alpha 1/\alpha 2$ loop interacts with another monomer (subunit *D*) from the second catalytic dimer. These residues are organized as a two-stranded antiparallel β -sheet-like structure with the centre of symmetry at Ile31. The isoleucines from the two different subunits form typical β -sheet hydrogen bonds between N and O atoms of the main chain (Fig. 1*d*). Thus, each dimer has two N-terminal regions (residues 22–37) which form two β -sheet-like structures during interaction with the same regions of another dimer and stabilize the whole tetramer structure like ‘paws’ (Fig. 1*c*). Upon tetrameric structure formation about 30% of the monomer surface becomes inaccessible to solvent.

The tetramer has only two flexible regions: the $\alpha 2/\alpha 3$ loop (residues 51–54 of the N-terminal domain) and the $\alpha 13$ helix with adjacent loops (residues 357–373 of the C-terminal domain) (shown in ovals in Fig. 2*a*). Helix $\alpha 13$ is located between the PLP-binding and C-terminal domains and is involved in formation of the PLP-binding site, but this helix makes no direct contacts with the atoms of the PLP-binding domain. The positions of these regions vary in MGLs from different sources and notable differences are observed in the N-terminal domain (shown with ovals in Fig. 2*b*).

3.2. The PLP-binding site

The cofactor-binding pocket is formed by amino acids from the PLP-binding domain of one subunit and the N-terminal domain of the neighbouring subunit of the catalytic dimer (Fig. 2*c*). The phosphate ‘handle’ of PLP is fixed by seven

hydrogen bonds, two of which are bifurcated. The hydroxyl group of Ser209 and the amide group of Gly88 form bifurcated hydrogen bonds to O1P and O4P and to O1P and O3P, respectively (Supplementary Fig. S1). The O1P atom accepts a hydrogen bond from the hydroxyl group of Thr211 and the O3P atom interacts with the amino group of Met89. On the other side of PLP, the phosphate handle forms three hydrogen bonds to amino acids of the adjacent subunit of a catalytic dimer (indicated by asterisks). The hydroxyl group of Tyr58* is involved in a short hydrogen bond to O2P, and the side chain of Arg60* interacts with the PLP phosphate handle using two N atoms: NE contacts the O3P atom of the phosphate moiety and NH2 contacts the O2P atom.

The cofactor is bound to the apoenzyme *via* the aldimine bond between the C4' atom of PLP and the ϵ -amino group of Lys212, forming a so-called internal aldimine. The position of the PLP pyridine ring is stabilized by a hydrogen bond between the N1 atom of the ring and the OD1 atom of Asp187, which is a residue that is strictly conserved in the aspartate aminotransferase family.

The position of the PLP pyridine ring is confined by Thr189 and Ser209 on one side and by Tyr113 on the other side. The phenol ring of Tyr113 is coplanar with the pyridine ring of PLP and placed at a distance of 3.5 Å from it. Stacking interactions between the two rings provide additional stabilization of the PLP position. Sideways movement of the PLP ring is limited by Ile92 on the one side and Phe190 on the other.

Besides the interaction with the PLP phosphate handle, the NH2 atom of the Arg60* guanidine makes a hydrogen bond to the hydroxyl of Tyr113.

The arrangement of the residues at the PLP is almost identical in all known MGL structures (Fig. 2*c*) and they can be superimposed with root-mean-square deviations of 0.55 Å (*C. freundii* versus *C. sporogenes*), 0.44 Å (*E. histolytica* versus *C. sporogenes*), 0.56 Å (*P. putida* versus *C. sporogenes*) and 0.23 Å (*T. vaginalis* versus *C. sporogenes*). A significant difference is observed in the position of Arg60 compared with *C. freundii* MGL. Nevertheless, the position of Arg60 in the

Figure 3

The catalytic dimer interface. The position of L-norleucine inside the active site was modelled *via* superposition of the structure of *C. freundii* MGL complexed with L-norleucine (PDB entry 3jwb). (a) Stereoview of the solvent-accessible surface. Subunits are shown in green and in blue. (b) Profile of hydrophobicity from blue to brown with increasing hydrophobicity. Hydrophobic amino acids are in grey, neutral hydrophilic amino acids are in green and charged hydrophilic amino acids are in blue.

structures of complexes of *C. freundii* MGL with substrates and inhibitors (Revtovich *et al.*, 2014) is the same as in the reported structure.

There are also minor differences in some residues involved in formation of the PLP-binding site. In MGL from *C. freundii* isoleucine is present instead of Met89, in MGL from *E. histolytica* serine is present instead of Thr211 and in MGL from *P. putida* tyrosine is present instead of Phe190. The enzyme from *T. vaginalis* has no differences in the residues forming the hydrogen-bond network with PLP compared with MGL from *C. sporogenes*.

The conserved residues Tyr113, Arg60, Cys115, Lys241, Tyr58 and Asp242 are proposed to be important for catalysis of the γ -elimination reaction (Kudou *et al.*, 2007). It was postulated that the hydrogen bond between Arg60 NH₂ and the hydroxyl of Tyr113 decreases the p*K*_a value of Tyr113 and thus acts as a base activating the incoming substrate (Messerschmidt *et al.*, 2003; Revtovich *et al.*, 2011). Tyr113 has been proposed to be a general acid catalyst in the stage of the γ -elimination of methylmercaptan from methionine (Messerschmidt *et al.*, 2003). Apparently, the acid–base chemistry of the Tyr113 hydroxyl group is provided by a positive charge on the Arg60* side chain and a hydrogen bond between it and Cys115. The network of hydrogen bonds between Tyr113, Cys115, Asp242* and Lys241* may provide ‘storage’ for a proton abstracted from the amino group of methionine at Lys241* and its return to the Tyr113 hydroxyl group to perform acidic catalysis. Some evidence confirming these ideas has been presented in Morozova *et al.* (2014).

3.3. Substrate-binding pocket

Since the structure does not contain any ligand in the active site, we modelled its position in the substrate-binding pocket using the structure of *C. freundii* MGL complexed with L-norleucine (PDB entry 3jwb) described previously (Revtovich *et al.*, 2011).

The substrate-binding pocket is organized by residues from both monomers of the catalytic dimer (Fig. 3*a*) and apparently can be divided into two functional areas. The first area is generally represented by residues from monomer *B* of the catalytic dimer and forms a hydrophobic contact area for the methyl group of the methionine substrate (Messerschmidt *et al.*, 2003). The residues involved are Phe49*, Ile57*, Leu61* and Leu237* from monomer *B* and Val340 from monomer *A*. A second ‘charged’ area of the binding pocket is predominantly formed by residues from monomer *A* of the catalytic dimer. The residues include Tyr360, Thr356, Asn160 and Arg376 from one side and Tyr113, Gly114, Cys115, Tyr58*, Arg60* and Lys241* from other side (Fig. 3*b*). These residues are able to form hydrogen bonds to carboxyl or amino groups of a substrate, have positive/negative charges and may be responsible for substrate delivery to the cofactor and for the correct substrate orientation in the active site of the enzyme.

Most residues forming the substrate-binding pocket are strongly conserved among MGLs. These include Phe49, Tyr58, Arg60, the region Tyr113–Cys115, Asn160, Lys241, Val340,

Thr356 and Arg374; Leu61 is substituted by Ile in some primary sequences. Ile57 and Leu237 are not conserved in MGL from different sources (Manukhov *et al.*, 2006).

Recently, we have shown (Anufrieva *et al.*, 2015) that mixtures of *C. sporogenes* MGL with a number of sulfoxides, analogues of L-methionine and S-ethyl-L-cysteine, possess antibacterial activity. It has also been demonstrated (Morozova *et al.*, 2013) that *C. sporogenes* MGL has cytotoxic activity compared with *P. putida* MGL. The development of studies to apply the enzyme in pharmacology requires detailed knowledge of its structural and functional characteristics. To this end, it is necessary to obtain structures modelling the intermediates of β - and γ -elimination reactions based on the structure of *C. sporogenes* MGL at a resolution below 2.0 Å.

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