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Three-Dimensional Structures of Noncovalent Complexes of *Citrobacter freundii* Methionine γ-Lyase with Substrates

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Abstract—Crystal structures of *Citrobacter freundii* methionine γ-lyase complexes with the substrates of γ- (L-1-amino-3-methylthiopropylphosphinic acid) and β- (S-ethyl-L-cysteine) elimination reactions and the competitive inhibitor L-norleucine have been determined at 1.45, 1.8, and 1.63 Å resolution, respectively. All three amino acids occupy the active site of the enzyme but do not form a covalent bond with pyridoxal 5′-phosphate. Hydrophobic interactions between the active site residues and the side groups of the substrates and the inhibitor are supposed to cause noncovalent binding. Arg374 and Ser339 are involved in the binding of carboxyl groups of the substrates and the inhibitor. The hydroxyl of Tyr113 is a potential acceptor of a proton from the amino groups of the amino acids.

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Key words: *Citrobacter freundii* methionine γ-lyase, pyridoxal 5′-phosphate, complexes with amino acids, three-dimensional structures

Methionine γ-lyase (EC 4.4.1.11, MGL) is a pyridoxal-5′-phosphate (PLP)-dependent enzyme catalyzing the reactions of γ-elimination and γ-replacement of L-methionine and its derivatives and the reactions of β-elimination and β-replacement of L-cysteine and S-substituted L-cysteines [1]. The enzyme also catalyzes the reactions of γ-elimination and γ-replacement of the phosphinic analog of methionine, Met-P_H [2]. Met-P_H has a high antibacterial activity [3], is an effective fungicide under field conditions [4], and inhibits the growth of tumor cells due to transformation into a metabolically stable phosphonic analog of S-adenosylmethionine [5].

The enzyme has been found in many bacteria [6] including the family Enterobacteriaceae (*Citrobacter freundii* [7]) and in pathogenic bacteria (*Bacteroides* ssp. [8], *Aeromonas* sp. [9], *Clostridium sporogenes* [10], *Porphyromonas gingivalis* [11]). MGL has also been found in pathogenic eukaryotes (the protozoa *Entamoeba histolytica* [12] and *Trichomonas vaginalis* [13]) and in the plant *Arabidopsis thaliana* [14].

The absence of the enzyme in mammals allows its consideration as a potential target for creation of medicinal preparations against pathogenic microorganisms. The efficiency of the MGL inhibitor 3-fluoro-L-methionine for suppression of the growth of *T. vaginalis* [15], *P. gingivalis* [16], and *E. histolytica* [17] has been shown *in vivo* and *in vitro*.

The prospects of application of the enzyme as an antitumor agent were also demonstrated *in vitro* and *in vivo* [18-21]. The possibility of using MLG for the therapy of Parkinson's disease, atherosclerosis, aging, and obesity is discussed in work [22].

MGL can be applied also in biotechnology. It is known that volatile mercaptans improve the quality and flavor of cheeses. Recently, the MGL gene has been expressed with this purpose from *Brevibacterium linen* BL2 into *Lactococcus lactis* [23].

However, in spite of the prospects of using the enzyme in medicine and biotechnology, the mechanism

Abbreviations: Met-P_H, L-1-amino-3-methylthiopropylphosphinic acid; MGL, methionine γ-lyase; PLP, pyridoxal-5′-phosphate.

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of its action has been studied insufficiently. The available data on the substrate and reaction specificity of MGL from *Pseudomonas putida* [24-28], *T. vaginalis* [13, 29], *E. histolytica* [12, 30], and *C. freundii* [2, 7], the structure of *P. putida* holoenzymes (PDB entry 2O7C [31]), *C. freundii* (PDB entry 2RFV [32]), and *T. vaginalis* (PDB entry 1E5F), and the structure of the *T. vaginalis* MGL complex with nonspecific irreversible inhibitor L-propargylglycine (PDB entry 1E5E) give no complete idea of the details and differences between the mechanisms of γ- and β-elimination reactions catalyzed by the enzyme.

Three-dimensional structures of the complexes of *C. freundii* MGL with L-amino acids, Met-P_H and S-ethylcysteine (the substrates of the γ- and β-elimination reactions, respectively) and a competitive inhibitor of the enzyme, norleucine, are considered.

MATERIALS AND METHODS

Crystallization and data collection. The recombinant *C. freundii* MGL was isolated and purified according to the method described previously [7]. The holoenzyme was crystallized by the hanging drop vapor diffusion technique under the conditions described in [32]. Rhombic crystals were formed during a week and reached the size of 0.3-0.4 mm in two weeks.

The complexes with amino acids were obtained by soaking holoenzyme crystals in a cryoprotective solution (35% polyethylene glycol monomethyl ether 2000, 50 mM Tris-HCl, pH 8.5, 0.2 mM PLP, 25 mM DTT) with addition of the respective amino acid (6.8 mM S-Et-Cys, 40 mM Nle, or 48 mM Met-P_H) during different time intervals (5 to 120 min).

Diffraction data were collected using synchrotron radiation on line BW7B at the European Molecular Biology Laboratory (EMBL, Hamburg, Germany) using a MAR345 detector at 100K. The survey conditions were optimized with the BEST software [33]. The data were processed in the XDS software system [34].

Structure determination and refinement. The structures were solved by molecular replacement using a PHENIX software system [35]. The previously determined *C. freundii* MGL structure was used as an initial model at 1.35 Å (PDB entry 2RFV). The protocol of rigid body refinement was used at the first stage of structure refinement, followed by refinement of polypeptide chain position by the method of simulated annealing. For the model obtained, an electron density map was calculated and used as the initial experimental map. Later on, the refinement protocols were used with minimization of energy and optimization of model geometry, followed by manual correction in the COOT software [36]. The process of refinement was monitored by the model reliability factor R_{free} calculated by 5% of reflections excluded from refinement.

Location and orientation of the ligands were found using the COOT built-in function for determining the positions of small molecules in electron density maps. Unspecified electron density was assigned to protein-bound water molecules, and polyethylene glycol molecules were placed into the extensive regions of electron density. The structures of enzyme complexes with Met-P_H and Nle for cysteine in the fourth position of the polypeptide chain showed the excess electron density near the sulfur atom, leading to a conclusion about its oxidized state and substitution of 3-sulfenoalanine for this atom.

The structures were deposited to the Protein Data Bank. The characteristics of diffraction data and the statistics of structure refinements are given in the table.

RESULTS AND DISCUSSION

Citrobacter freundii methionine γ-lyase (PDB entry 1Y4I [37]) is assigned to the subclass of cystathionine β-lyase with type I folding of the polypeptide chain of PLP-dependent enzymes [38]. In a crystal, like in solution, the enzyme exists as a tetramer consisting of two dimers linked with a twofold symmetry axis (Fig. 1a) [39]. Each dimer contains two active centers formed by amino acid residues of both subunits of the dimer. The protein monomer consists of three domains: N-terminal, central PLP-binding, and C-terminal (Fig. 1b).

The binding of amino acids had no marked influence on the 3D structure of MGL (Fig. 2). The main differences between the structures are associated with positions of the mobile regions of the N- and C-terminal domains of the enzyme (amino acids residues 47-63 and 353-368; Fig. 2).

In all of the complexes, the amino acids occupy positions close to the typical position for the formation of the external aldimine with PLP but do not form a covalent

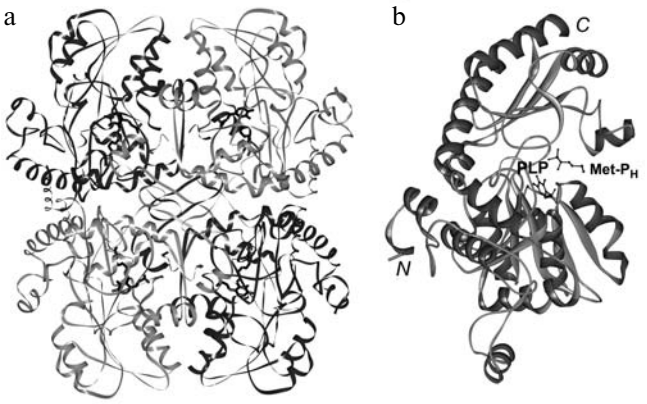

Fig. 1. a) Tetrameric organization of MGL. The position of PLP in active centers is shown. b) Monomer ribbon model. Positions of PLP and Met-P_H are shown.

Data collection and refinement statistics structures of MGL complexes with amino acids

PDB ID	3JWA	3JW9	3JWB
Amino acid	Met-P_H	S-Et-Cys	Nle
Amino acid soaking time in solution, min	5	40	120
Space group		*I222*	
Unit cell parameters (at $\alpha = \beta = \gamma = 90°$), Å	$a = 56.42$ $b = 123.01$ $c = 127.01$	$a = 56.49$ $b = 122.87$ $c = 127.21$	$a = 56.76$ $b = 123.15$ $c = 127.62$
Wavelength, Å		0.843	
Resolution, Å*	20.00-1.45 (1.47-1.45)	20.00-1.80 (1.84-1.80)	20.00-1.63 (1.65-1.63)
Completeness, %*	98.20 (98.60)	96.30 (92.40)	92.50 (81.60)
$I/\sigma(I)$*	18.93 (4.95)	18.83 (3.63)	11.35 (3.40)
Redundancy*	4.20 (4.10)	3.60 (3.35)	5.20 (4.10)
R_{merge}, %*	4.00 (28.30)	5.20 (34.20)	7.10 (33.90)
Disordered protein residues		1, 398	
Number of non-hydrogen atoms of the protein	3102	3049	3076
Number of water molecules	298	330	320
Number of polyethylene glycol molecules	5	1	3
Number of unique reflections*	77926(2697)	40626(2696)	54913(2356)
R/R_{free} of final model, %*	14.1/18.2(27.2/30.1)	16.7/19.9(23.1/29.3)	16.0/21.7(21.8/28.8)
Mean temperature factor B, $Å^2$	19.4	23.2	26.8
R.m.s.d. from ideal values			
bond length, Å	0.005	0.005	0.004
bond angles, deg	1.004	0.987	0.919
Positions of amino acid residues on Ramachandran plot, %			
most favored	98.7	98.7	98.7
additionally allowed	0.8	1.0	1.0
permissible	0.5	0.3	0.3
R.m.s.d. of positions of C_α atoms of the complex and holoenzyme of MGL, except for regions 47-63 and 353-368, Å	0.28	0.17	0.19

* Values for the high-resolution layer are given in parenthesis.

Fig. 2. Superposition of structures of MGL complexes with amino acids on the holoenzyme structure (PDB entry 2RFV) at C_α atoms. Mobile regions of the MGL polypeptide chain, 47-63 and 353-368, are enclosed in ovals.

bond with it. In all of the structures the coenzyme is bound to the apoenzyme via the aldimine bond between the C4′ atom of PLP and the ε-amino group of Lys210, forming the so-called internal aldimine. Amino acid positions are stabilized by electrostatic and H-bonds, which they form with amino acid residues of the enzyme active center and the coenzyme and by contacts of their side chains with the amino acids of a hydrophobic pocket formed by side groups of the residues of both monomers of the catalytic dimer: Tyr113, Cys115, Ala118, Phe188, Thr209, Val338, and Leu340 of the first monomer and Phe49*, Leu57*, Tyr58*, Leu61*, and Phe235* of the second monomer. The distance from the nitrogen atom of amino groups of the bound amino acids to the C4′ atom of PLP is 3.30 Å for Met-P_H, 2.65 Å for S-Et-Cys, and 3.10 Å for Nle. In spite of the similarity of amino acid positions in the enzyme active center, positions of their functional groups and the formed H-bonds are different.

The oxygens of the Met-P_H phosphinic group are at the distance of H-bonds with the nitrogen atom of Arg374 guanidinium group and the nitrogen atom of the Ser339 main chain, and the nitrogen atom of the Met-P_H amino group is at the distance of an H-bond with the oxygen atom of the Tyr113 side chain (Fig. 3). The water molecule present in the enzyme active center forms a network of H-bonds between the nitrogen atom of the Asn160 side chain, the second nitrogen atom of the Arg374 side chain, the oxygen atom of the Met-P_H phosphinic group, and the 3′O-atom of PLP.

Like in the case of Met-P_H, the carboxyl and amino groups of Nle form H-bonds with atoms of the Arg374 and Tyr113 side chains and with the nitrogen atom of the Ser339 main chain (Fig. 4). In this complex, position of the Nle carboxyl group much more corresponds to position of the carboxyl group typical of the external aldimine of PLP-dependent enzymes. It is located opposite the Arg374 bifurcated side chain and, in contrast to Met-P_H, forms H-bonds not with one but with both nitrogen atoms of the Arg374 side chain.

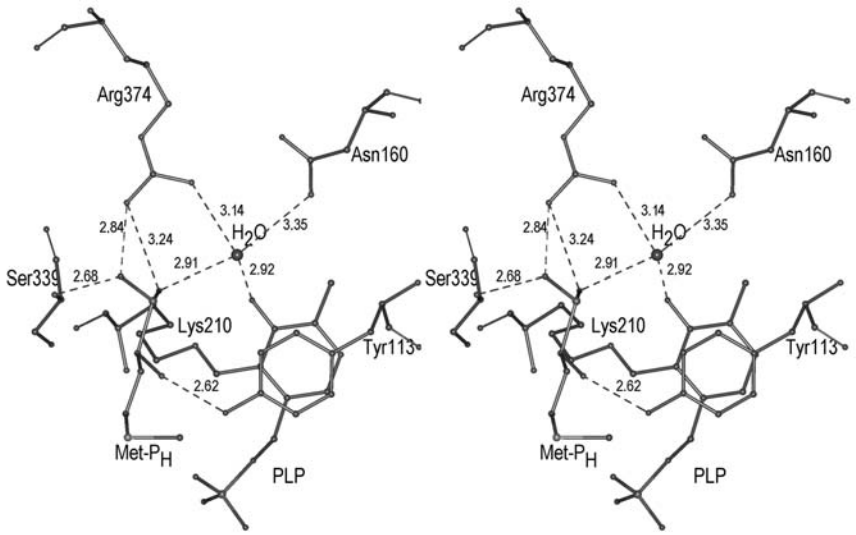

Fig. 3. H-bonds formed by Met-P_H in complex with MGL (stereo pair).

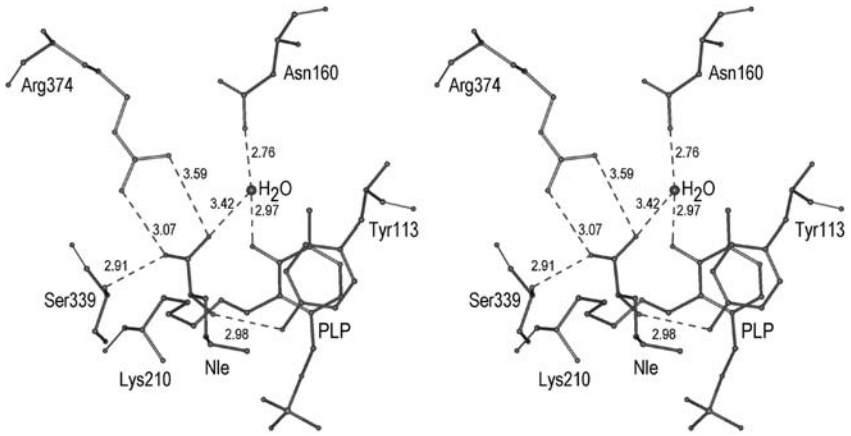

Fig. 4. H-bonds formed by Nle in complex with MGL (stereo pair).

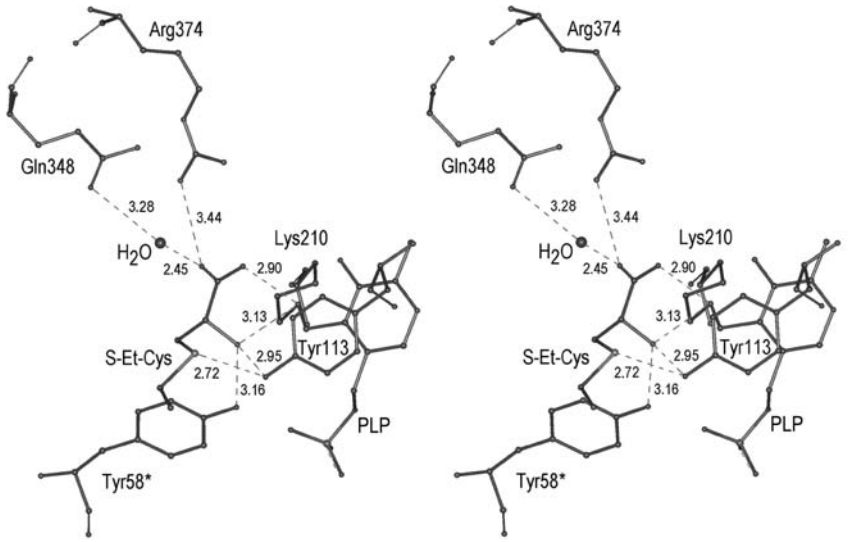

Fig. 5. H-bonds formed by S-Et-Cys in complex with MGL (stereo pair).

The water molecule in the active center of the complex occupies a position analogous to its position in the structure of the complex with Met-P_H and forms a network of H-bonds between the nitrogen atom of the N160 side chain, the oxygen atom of the Nle carboxyl group, and the 3′O-atom of PLP.

The network of H-bonds formed by S-Et-Cys significantly differs from the H-bonds present in the MGL complexes with Met-P_H and Nle (Fig. 5). The oxygen of the S-Et-Cys carboxyl group lacks an H-bond with the nitrogen atom of the Ser399 main chain, and one of the oxygen atoms of the carboxyl group is at the distance of an H-bond from the nitrogen atom of the Arg374 guanidinium group. This oxygen atom is also bound to the Gln348 side chain through a water molecule different from the water molecule in the two structures discussed above. The second oxygen atom of the carboxyl group is at the distance of an H-bond from the aldimine nitrogen atom. The nitrogen atom of the S-Et-Cys amino group is at the distance of an H-bond from the Tyr113 hydroxyl group, from the oxygen atom of the Tyr58* side chain of the second monomer of the catalytic dimer, and from the aldimine nitrogen atom. Since, as is shown for the holoenzyme [40], the nitrogen atom of the internal aldimine is charged positively, mutual position of the nitrogen atoms of the aldimine bond and the S-Et-Cys amino group nitrogen observed in crystal is possible only in the absence of charge on the amino group.

In PLP-dependent catalysis, the transaldimination reaction resulting in the substitution of external for internal aldimine occurs by way of nucleophilic attack of the α-amino group of the substrate at the C4′-atom of the

coenzyme, which needs deprotonation of the α-amino group for most of the natural amino acids. Analysis of 3D structures of the enzymes of the cystathionine β-lyase subclass suggested [41, 42] that this role is played by a dissociated hydroxyl group of the conservative tyrosine residue of the active center (Tyr113 of *C. freundii* MGL).

The data on pH-dependence of the kinetic parameters of the methionine γ-elimination reaction catalyzed by *C. freundii* MGL [43] also suggested that the Tyr113 hydroxyl group with pK_a 7.2 is a base that accepts the proton from the methionine amino group. In the structure under discussion, the Tyr113 hydroxyl group is at the distance of an H-bond from the sulfur atom of the S-Et-Cys side group, obviously being a proton donor in the H-bond. In the two structures considered above, the Tyr113 side residue occupies a position also suggesting that the hydroxyl group is a potential proton acceptor from the amino group of amino acids. Thus, the structural characteristics of three complexes demonstrate that one of the functions of *C. freundii* MGL Tyr113 residue is the function of a base accepting a proton from the α-amino group of the substrate.

The study of catalytic parameters of the *P. putida* mutant MGL form with substitution of a homologous Tyr114 residue has shown that later on, in the course of enzymatic reaction, this residue can perform the function of an acid catalyst at the stage of elimination of the side group of the natural substrate [27]. Analysis of the data on pH-dependence of the catalytic parameters of reactions of decomposition of the natural substrate, methionine, its analogs Met-P_H and S-Met-Cys, and the modeling of the MGL external aldimine with S-Met-Cys suggest that the Tyr58* side group can participate as an acid catalyst in catalysis of the stage of elimination of the Met-P_H and S-Met-Cys side groups [43]. However, in the structure of the MGL complex with S-Et-Cys, the Tyr58* hydroxyl group is distant from the sulfur atom of the substrate. Probably, during the formation of external aldimine and subsequent stages of the enzymatic reaction, conformational changes in the active center enable the Tyr58* hydroxyl group to occupy a position favorable for protonation of the leaving group.

The characteristic feature of the structures of the complexes is the turn of the carbonyl group of the peptide bond between the Thr338 and Ser339 residues by 180° relative to its position in the holoenzyme, resulting in local reconstruction of the protein main chain. The position of the C=O group observed in the holoenzyme constricts the cavity of the enzyme active center, rendering impossible the correct orientation of substrate relative to the coenzyme, which is necessary for the formation of a covalent bond with the latter. Such turn of the carbonyl group of the homologous serine residue is observed also in the structures of covalent (*T. vaginalis* MGL, PDB entry 1E5E) and noncovalent (*Homo sapiens* cystathionine γ-lyase, PDB entry 3COG) complexes with L-propargylglycine.

Previously we have shown that the competitive inhibitors Gly, L-Ala, and L-Nle form covalent complexes with the enzyme in solution [44]. The parameters of steady state kinetic were defined for the substrates L-Met, L-Gly(vinyl) [45], and L-Met-P_H [2]. Microspectrophotometry shows that the spectral characteristics of MGL complexes with L-Met and L-Gly(vinyl) in crystals are analogous to the spectra of complexes in solution [40], i.e. MGL crystals are catalytically competent. At the same time, the K_d values determined for L-Met and L-Gly(vinyl) in crystal (2.3 and 26 mM, respectively) proved to be close to the values obtained in solution (0.7 and 6.7 mM). In the present work, the amino acid concentrations used for soaking the crystals (48 mM Met-P_H, 6.8 mM S-Et-Cys, and 40 mM Nle) considerably exceeded the corresponding K_m values for Met-P_H and S-Et-Cys (1.2 and 0.17 mM, respectively) and the K_i value (0.6 mM) for Nle. However, the two substrates and the inhibitor are located in the enzyme active center close to PLP but not bound to it covalently. Such location is probably caused by interaction between the amino acid side chains and hydrophobic residues of the active center, which in crystals may result in slowdown of their movement to the active center and appearance of the observed intermediate position in the enzyme active center.

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