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Alliin is a suicide substrate of *Citrobacter freundii* methionine γ-lyase: structural bases of inactivation of the enzyme

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The interaction of *Citrobacter freundii* methionine γ-lyase (MGL) and the mutant form in which Cys115 is replaced by Ala (MGL C115A) with the nonprotein amino acid (2*R*)-2-amino-3-[(*S*)-prop-2-enylsulfinyl]propanoic acid (alliin) was investigated. It was found that MGL catalyzes the β-elimination reaction of alliin to form 2-propenethiosulfinate (allicin), pyruvate and ammonia. The β-elimination reaction of alliin is followed by the inactivation and modification of SH groups of the wild-type and mutant enzymes. Three-dimensional structures of inactivated wild-type MGL (*i*MGL wild type) and a C115A mutant form (*i*MGL C115A) were determined at 1.85 and 1.45 Å resolution and allowed the identification of the SH groups that were oxidized by allicin. On this basis, the mechanism of the inactivation of MGL by alliin, a new suicide substrate of MGL, is proposed.

1. Introduction

Methionine γ-lyase (MGL; EC 4.4.1.11), a pyridoxal 5′-phosphate-dependent enzyme, catalyzes the γ-elimination reaction of L-methionine to produce methanethiol, ammonia and α-ketobutyrate. The enzyme also catalyzes the β-elimination reaction of L-cysteine and *S*-substituted L-cysteines (Tanaka *et al.*, 1985). It belongs to the family of PLP-dependent enzymes catalyzing transformations of sulfur-containing amino acids. Structural characteristics and peculiarities providing substrate and reaction specificities in this family have been considered by Messerschmidt *et al.* (2003). As in other members of this family, the homotetrameric molecule of *Citrobacter freundii* MGL is composed of two so-called 'catalytic dimers'. Two active sites in a catalytic dimer are formed by residues from both monomers (Nikulin *et al.*, 2008). MGL is biologically significant in the production of propionic acid for energy metabolism and in the degradation of toxic sulfur-containing amino acids (Nozaki *et al.*, 2005). The enzyme has been found in many microorganisms. Some of these are pathogens, including bacteria causing gangrenous emphysema (*Clostridium sporogenes*; Kreis & Hession, 1973; Revtovich *et al.*, 2012), tetanus (*C. tetani*; Revtovich *et al.*, 2012) and tooth decay (*Porphyromonas gingivalis*; Yoshimura *et al.*, 2000). MGL is considered to be an attractive target in pathogens for rational drug development because the enzyme is absent in mammalian hosts. Moreover, MGL is effective in cancer-cell treatment (Tan *et al.*, 2010; Morozova *et al.*, 2013). To date, two suicide substrates of MGL have been considered as possible new pharmaceuticals. The toxic properties of methylselenol, which is released from L-selenomethionine by cancer cells with the adenoviral-delivered MGL gene, have been used to inhibit tumour growth in rodents (Miki *et al.*, 2001). The therapy of breast tumours in mice with the methionine γ-lyase/annexin

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V/selenomethionine system caused a reduction in the size of the tumours (Van Rite *et al.*, 2013). The treatment of two MGL-containing eukaryotic pathogens *Trichomonas vaginalis* and *Entamoeba histolytica* with L-trifluoromethionine demonstrated a suppression of cell growth (Coombs & Mottram, 2001; Tokoro *et al.*, 2003) by the product of the enzymatic reaction, carbonothionic difluoride.

Allicin (allyl 2-propenethiosulfinate) is the best-known active compound of garlic (Cavallito & Bailey, 1944). It constitutes about 70% of the total thiosulfinates formed upon crushing the cloves (Han *et al.*, 1995) and is produced from the nonprotein amino acid alliin by the pyridoxal 5′-phosphate-dependent alliinase (EC 4.4.1.4; Stoll & Seebeck, 1951). Allicin is a highly reactive substance and possesses various biological activities such as antimicrobial, anticancer, antithrombotic *etc.* (Koch & Lawson, 1996; Lawson, 1998; Wills, 1956).

We have studied alliin as a substrate in the β-elimination reaction catalyzed by wild-type MGL and the mutant form MGL C115A. It was demonstrated that both enzymes catalyze the decomposition of alliin to allicin, ammonia and pyruvate. This reaction is followed by inactivation of the enzymes and modification of SH groups.

Here, we present three-dimensional structures of inactivated wild-type (*i*MGL) and mutant (*i*MGL C115A) enzymes determined at 1.85 and 1.45 Å resolution, respectively. It was shown that two cysteine residues of MGL C115A and three cysteine residues of the wild-type enzyme form mixed disulfides (*S*-allylmercaptocysteines) as a result of reaction with the activated disulfide bond [—S(O)—S—] of allicin.

2. Materials and methods

2.1. Materials

L-Methionine, *S*-ethyl-L-cysteine, 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), nicotinamide adenine dinucleotide reduced form (NADH), lactate dehydrogenase (LDH) from rabbit muscle, guanidine hydrochloride, Tris–HCl and (+)-L-alliin were purchased from Sigma–Aldrich. Pyridoxal 5′-phosphate (PLP) and D,L-dithiothreitol (DTT) were obtained from Serva. Capto DEAE and Superdex 200 prep grade were obtained from Amersham. Restriction enzymes, *Taq* DNA polymerase, calf intestine alkaline phosphatase, dNTP mixture, T4 DNA polymerase, pBluescript II SK(+/−), the rapid DNA ligation kit and the GenJet plasmid miniprep kit were purchased from Fermentas. 2-Nitro-5-thiobenzoic acid (NTB) was prepared according to Miron *et al.* (1998). The plasmid with the gene for D-2-hydroxyisocaproate dehydrogenase (HO-Hxo-DH) was a kind gift from K. Muratore.

2.2. Site-directed mutagenesis

The plasmid mglBlue was obtained by cloning the *mgl* gene into the pBluescript II SK(+/−) vector. Mutagenesis was performed by PCR. The fragment containing T/G (411) and G/C (412) replacements was amplified using mglBlu as the template by PCR with primers F1 (GATATCCATGGCTGACTGTCGTAC), R1 (TTCGGCATGCTGTGCGACAAAAACGCGTG) and R2 (TGCGACAAAAACGCGTGGGTGGCGCCGTA). The product was incubated with *Nco*I and *Sph*I and ligated *via* these sites into mglBlue. The fragment containing the Cys115Ala replacement was recloned from mglBlue into pET-28. The Cys115Ala substitution was confirmed by two-directional sequencing.

2.3. Preparation and assays of enzymes

Wild-type MGL and MGL C115A were purified from *Escherichia coli* cells bearing the genes of the enzymes in pET-28 plasmids (Manukhov *et al.*, 2005). The concentrations of homogeneous proteins were determined by the absorbance at 278 nm using an extinction coefficient ($A_{278}^{1\%}$) of 0.8 (Morozova *et al.*, 2010). The assays of the enzymes were determined by measuring the rates of the β- and γ-elimination reactions in 100 m*M* potassium buffer solution pH 8.0 containing 0.2 m*M* PLP, 1 m*M* DTT and either 2.5 m*M* *S*-ethyl-L-cysteine (β-elimination reaction) or 7 m*M* L-methionine (γ-elimination reaction) at 30°C. In the case of the β-elimination reaction a coupled assay with NADH and LDH was used to follow the accumulation of pyruvic acid as described previously (Morino & Snell, 1970). An assay with HO-Hxo-DH was utilized to determine the accumulation of α-ketobutyric acid in the γ-elimination reaction (Aitken *et al.*, 2003). The specific activities of wild-type MGL and MGL C115A were 9.2 and 1.6 U mg^{-1}, respectively, for the β-elimination reaction and 9.6 and 1.2 U mg^{-1}, respectively, for the γ-elimination reaction.

Allicin was determined by reaction with NTB. The standard reaction mixture consisted of 0.1 m*M* NTB, 0.2 m*M* PLP and 20 m*M* alliin in 100 m*M* potassium buffer solution pH 8.0 in a total volume of 1.0 ml. The reaction was started by adding the MGL and the initial rate was monitored by the decrease in absorbance at 412 nm at 30°C. The concentration of NTB consumed in the reaction with allicin was calculated based on ε_{412} = 14 150 M^{-1} cm^{-1} (Miron *et al.*, 1998).

2.4. Steady-state kinetics

Kinetic parameters for the β-elimination reaction of alliin were determined in reaction mixtures consisting of 100 m*M* potassium buffer pH 8.0, 0.2 m*M* PLP, 1 m*M* EDTA, 10 U LDH, 0.2 m*M* NADH, 5 m*M* DTT and a varied amount of alliin. To avoid inactivation of the enzymes during the course of the reaction, 5 m*M* DTT was added. The reaction was initiated by the addition of enzyme (wild-type MGL or MGL C115A) and the accumulation of pyruvate was determined as described above. Kinetic parameters were obtained by processing the data according to the Michaelis–Menten equation using the *EnzFitter* program. The molecular mass of a subunit was taken as 43 kDa.

2.5. Determination of SH groups

Ellman's reagent (DTNB) was used to determine the sulfhydryl groups. The enzyme solution was diluted with 0.1 *M* potassium buffer solution pH 8.0 (accessible SH groups) or

Table 1
Data-collection and refinement statistics.

Values in parentheses are for the highest resolution shell.

PDB code	4mkj	4mkk
Crystal	Cubic, yellow, 150 × 150 × 150 µm	Cubic, yellow, 150 × 150 × 150 µm
Space group	*I*222	*I*222
Unit-cell parameters (Å)	*a* = 56.44, *b* = 122.67, *c* = 128.28	*a* = 56.69, *b* = 122.86, *c* = 128.60
Wavelength (Å)	0.8943	0.8943
Measurements	300 frames, 0.5° each	300 frames, 0.4° each
Resolution (Å)	32.07–1.85 (1.95–1.85)	35.00–1.45 (1.63–1.45)
Completeness (%)	99.2 (95.5)	98.8 (92.5)
Multiplicity	6.10 (5.90)	4.63 (4.03)
$\langle I/\sigma(I)\rangle$	20.2 (3.37)	12.6 (1.48)
$R_{\rm merge}$†	0.067 (0.51)	0.051 (0.53)
Disordered protein residues	1, 50–57, 398	1, 50–57, 398
No. of protein atoms	3067	3128
No. of residues in alternative conformations	16	26
No. of water atoms	260	342
No. of unique reflections	38275	78999
$R/R_{\rm free}$	0.157/0.193	0.140/0.173
Mean temperature factor B (Å^2)	24.09	22.38
R.m.s. deviation from ideal values		
Bond lengths (Å)	0.007	0.006
Bond angles (°)	1.150	1.227
Chiral angles (°)	0.073	0.076
Planar angles (°)	0.006	0.005
Ramachandran plot (*MolProbity*)		
Favoured region (%)	97.18	97.69
Allowed region (%)	2.25	1.73
Outlier region (%)	0.56 [Ser190, LLP210]	0.58 [Ser190, LLP210]

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I(hkl)$ is the ith measured intensity of reflection hkl and $\langle I(hkl)\rangle$ is the intensity averaged from multiple observations of symmetry-related reflections.

6 *M* guanidine hydrochloride in the same buffer (total amount of SH groups) to a final enzyme concentration of 0.5 mg ml^{-1}. 5 m*M* DTNB was added to these samples (a final concentration of 0.8 m*M*). The absorbance at 412 nm was measured after 2 min (total amount of SH groups) or 15 min (accessible SH groups) at 25°C. Molar extinction coefficients for thionitrobenzoate of 14 150 M^{-1} cm^{-1} (Eyer *et al.*, 2003) and 13 700 M^{-1} cm^{-1} (Riddles *et al.*, 1983) were used to calculate the amount of accessible SH groups and the total amount of SH groups, respectively.

2.6. Preparation, crystallization and X-ray data collection

Wild-type MGL and MGL C115A inactivated by allicin were obtained by the reaction of these enzymes with alliin. A reaction mixture consisting of enzyme (5 mg ml^{-1}) and alliin (18 mg ml^{-1}) in 100 m*M* potassium buffer solution pH 8.0 containing 0.2 m*M* PLP was kept at 25°C for 10 min and was dialyzed against the same buffer to remove the products of the reaction. The samples were transferred into crystallization buffer (50 m*M* Tris–HCl pH 8.5, 0.5 m*M* PLP) and crystallized (Nikulin *et al.*, 2008). Rhombic-shaped crystals appeared after one week.

The crystals belonged to space group *I*222, with unit-cell parameters *a* = 56.44, *b* = 122.67, *c* = 128.28 Å for wild-type *i*MGL and *a* = 56.69, *b* = 122.86, *c* = 128.60 Å for *i*MGL C115A, and contained one subunit in the asymmetric unit. Diffraction data were collected on the MX BL14.2 beamline at BESSY II (Berlin, Germany) using a MAR CCD MX-225 detector and were processed using *XDS* (Kabsch, 2010). The detailed data-collection statistics are shown in Table 1.

2.7. Structure determination and refinement

The structures were solved by molecular replacement with the previously determined structure of *C. freundii* MGL (PDB entry 2rfv; Nikulin *et al.*, 2008) using the rigid-body procedure as implemented in *PHENIX* (Adams *et al.*, 2010). The model was improved using manual rebuilding with *Coot* (Emsley & Cowtan, 2004) and maximum-likelihood refinement using *PHENIX*. 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 final model of wild-type *i*MGL, which included PLP in the internal aldimine form, two polyethylene glycol molecules, three *S*-allylmercaptocysteine residues, a sodium ion and 259 water molecules, was refined to an $R_{\rm work}$ of 15.7% and an $R_{\rm free}$ of 19.3% for data between 32.07 and 1.85 Å resolution (Table 1). The structure has been deposited in the Protein Data Bank as PDB entry 4mkj. The final model of *i*MGL C115A, which included PLP in the internal aldimine form, two polyethylene glycol molecules, two *S*-allylmercaptocysteine residues, a chloride ion, a potassium ion, five sodium ions and 341 water molecules, was refined to an $R_{\rm work}$ of 14.0% and an $R_{\rm free}$ of 17.3% for data between 35.0 and 1.45 Å resolution (Table 1). The structure has been deposited in the Protein Data Bank as PDB entry 4mkk.

3. Results and discussion

3.1. Kinetic parameters of wild-type MGL and the C115A mutant form

Data on the steady-state parameters for the γ-elimination and β-elimination reactions catalyzed by MGL C115A are presented in Table 2. The mutant enzyme catalyzed both the γ- and β-elimination reactions with catalytic efficiencies lower that those for the wild-type enzyme by about one order of magnitude. This decrease is mainly owing to a decrease in the $k_{\rm cat}$ values for both reactions, as the $K_{\rm m}$ values proved to be comparable with those of wild-type MGL. In the case of a mutant form of *Pseudomonas putida* MGL, the homologous Cys116 proved to be more essential for catalysis of the γ-elimination reaction than for the β-elimination reaction (Kudou *et al.*, 2008).

Table 2
Kinetic parameters for the γ- and β-elimination reactions (for wild-type MGL and MGL C115A).

	Wild-type MGL			MGL C115A		
Substrate, reaction	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} M^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} M^{-1})
L-Met, γ-elimination reaction	6.2 ± 0.42†	0.7 ± 0.11†	8.86×10^3	0.22 ± 0.0011	0.301 ± 0.045	0.69×10^3
S-Ethyl-L-Cys, β-elimination reaction	5.03 ± 0.16†	0.17 ± 0.02†	29.6×10^3	0.57 ± 0.00086	0.25 ± 0.029	2.24×10^3
Alliin, β-elimination reaction	5.9 ± 0.0007	4.7 ± 0.56	1.26×10^3	0.41 ± 0.0017	1.75 ± 0.22	0.23×10^3

† Data from previous work (Manukhov *et al.*, 2006).

Table 3
Inactivation of wild-type MGL and MGL C115A and modification of SH groups of the enzymes upon incubation with alliin.

	Wild-type MGL	MGL C115A
Incubation with alliin, 10 min		
Inactivation (%)		
γ-Elimination reaction of L-Met	74	63
β-Elimination reaction of *S*-ethyl-L-Cys	31	13
Modified SH groups		
Accessible SH groups	2	1
Buried SH groups	1	1
Incubation with alliin, 60 min		
Inactivation (%)		
γ-Elimination reaction with L-Met	100	85
β-Elimination reaction with *S*-ethyl-L-Cys	100	32
Modified SH groups		
Accessible SH groups	2	1
Buried SH groups	4	2

Both wild-type MGL and MGL C115A catalyzed the β-elimination reaction of alliin to yeld allicin, ammonia and pyruvate (Fig. 1).

The conventional method for the assay of the β-elimination reaction catalyzed by PLP-dependent enzymes is based on pyruvic acid quantification. The rates of formation of both pyruvic acid and allicin in the β-elimination reaction of alliin were determined. According to the data for alliinase, both methods gave identical values for the specific activity of the enzyme (Miron *et al.*, 1998). The consumption of 1 mol NTB is equivalent to the appearance of half a mole of allicin, which corresponds to the formation of 1 mol of pyruvic acid. In the cases of wild-type MGL and the mutant enzyme this ratio was not satisfied. The specific activity obtained using the LDH method was twice that obtained using the NTB method. This can occur because of the reaction of the thiol groups with allicin. In the following, the LDH method was used to determine the steady-state parameters for the β-elimination reaction of alliin. Kinetic parameters of the β-elimination reaction of alliin catalyzed by wild-type MGL and MGL C115A are presented in Table 2. Both enzymes catalyzed the β-elimination reaction of alliin with k_{cat} values comparable to the k_{cat} values for the β-elimination reaction of *S*-ethyl-L-cysteine, while the affinity of wild-type MGL and the mutant enzyme for aliin proved to be poorer than that for *S*-ethyl-L-cysteine (Table 2).

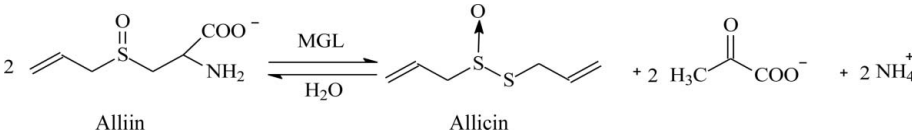

Figure 1
The β-elimination reaction of alliin catalyzed by MGL.

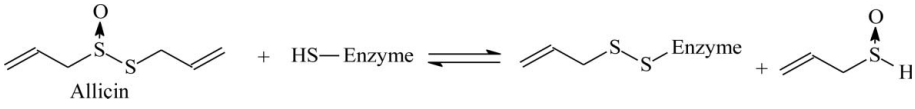

Figure 2
The inactivation of an enzyme owing to the oxidation of SH groups by allicin in the course of the β-elimination reaction of alliin.

3.2. Inactivation of wild-type MGL and MGL C155A in the course of the β-elimination reaction of alliin

Inactivation of the enzymes was observed when the β-elimination reaction of alliin was performed without DTT present. The enzymes lost about 70% of activity in the γ-elimination reaction during first 10 min, followed by total inactivation after 60 min incubation. The inactivation of both the wild-type and mutant enzymes in the β-elimination reaction proved to be less substantial (Table 3). It may be assumed that the inactivation of the enzymes in the course of the β-elimination reaction of alliin is owing to the oxidation of SH groups by allicin (Cavallito & Bailey, 1944; Rabinkov *et al.*, 1998; Fig. 2).

According to the amino-acid sequence (Manukhov *et al.*, 2006) there are seven cysteine residues in the polypeptide chain of MGL. Their locations in a catalytic dimer are shown in Fig. 3. We determined that two cysteine residues in the native wild-type enzyme react with DTNB and seven SH groups react with the reagent in 6 *M* guanidine hydrochloride. One cysteine residue was determined in MGL C115A and six residues were determined in the denaturated enzyme. Cys4 is located on the surface of a subunit (Fig. 3). Cys115, which is highly conserved in the heterologous MGLs (Kudou *et al.*, 2008), is situated in the active site of the enzyme (Nikulin *et al.*, 2008). The side chains of Cys4 and Cys115 should be considered as accessible to modification by DTNB in native wild-type MGL and the side chain of Cys4 as accessible to the reagent in the native mutant enzyme.

We determined that the three SH groups are not titrable in the inactivated

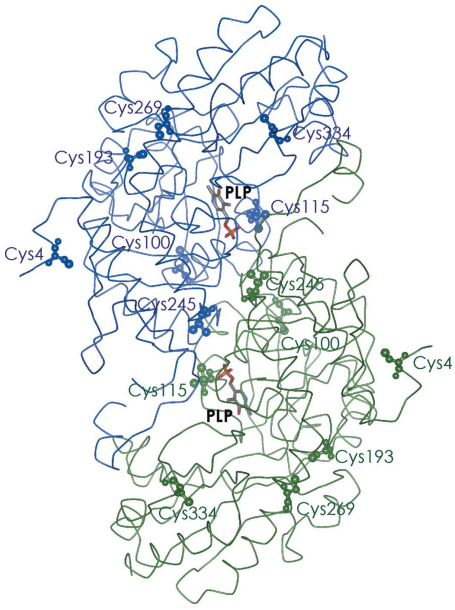

Figure 3
Polypeptide-chain fold of a catalytic dimer and the locations of cysteine residues. Subunits are shown in blue and green, and PLP molecules are shown in red.

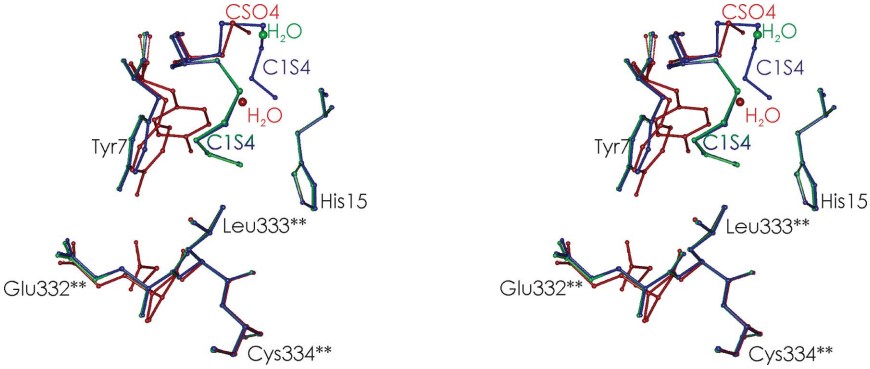

Figure 4
Superposition of *S*-allylmercaptocysteine 4 (denoted C1S4) and its environment in wild-type *i*MGL (PDB entry 4mkj, blue), *i*MGL C115A (PDB entry 4mkk, green) and wild-type MGL (PDB entry 2rfv, red). Residues from the monomer of another catalytic dimer are marked **. The oxidized 3-sulfenolamine 4 is denoted CSO4.

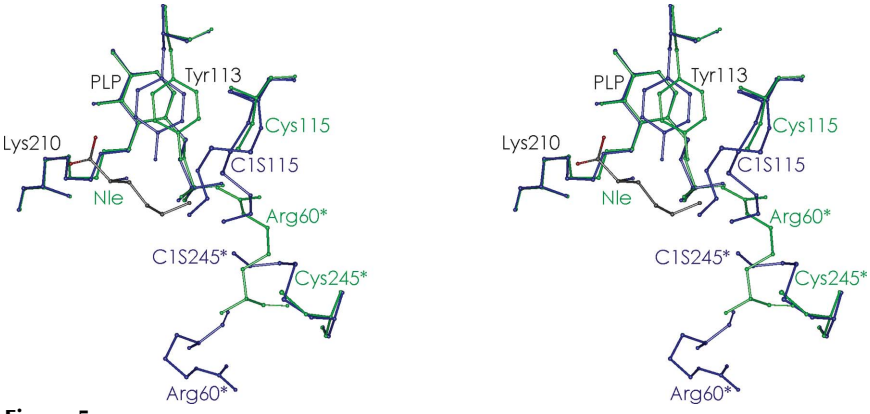

Figure 5
Superposition of active-site residues in the Michaelis complex of wild-type MGL with L-norleucine (PDB entry 3jwb, green) and in wild-type *i*MGL (PDB entry 4mkj, blue). The oxidized Cys115 and Cys245 are labelled C1S115 and C1S245, respectively. Residues from another monomer of the catalytic dimer are marked *.

wild-type enzyme and the two SH groups are absent in *i*MGL C115A. Consequently, inactivation of the enzymes is accompanied by a loss of accessible SH groups (Cys4 and Cys115 in the case of wild-type MGL and Cys4 in the case of MGL C115A) and one buried SH group (Table 3).

As the inactivation of MGL C115A in the γ-elimination reaction proved to be almost the same as that of wild-type MGL, we believe that modification of a buried SH group rather than of the accessible Cys4 is mostly responsible for the inactivation of both enzymes in the γ-elimination reaction.

Incubation of wild-type MGL with alliin for 60 min led to fully inactivated enzyme and to a total loss of thiol groups (Table 3). In the case of the mutant form, 85% inactivation was accompanied by the loss of one accessible and two buried SH groups. This difference could be a result of the slow rate of the β-elimination reaction of alliin and therefore a lower concentration of allicin in the reaction mixture compared with the concentration of allicin accumulating in the β-elimination reaction of alliin catalyzed by the wild-type enzyme.

Several thiol-containing compounds were used to reduce the disulfide bonds of proteins modified by allicin. Mixed disulfides formed by the modification by allicin of the SH groups of papain, $NADP^+$-dependent alcohol dehydrogenase from *Thermoanaerobium brockii* and NAD^+-dependent alcohol dehydrogenase from horse liver could be restored by glutathione, β-mercaptoethanol or DTT (Rabinkov *et al.*, 1998). Incubation (after 60 min incubation with alliin) of wild-type *i*MGL and *i*MGL C115A with DTT (50-fold excess with respect to the concentration of the enzyme, 30 min) leads to full restoration of the γ- and β-elimination activities of the enzymes. This clearly demonstrates that inactivation of both enzymes is caused by the oxidation of SH groups by allicin.

3.3. Crystal structures of wild-type *i*MGL and *i*MGL C115A

3.3.1. Modification of SH groups of the enzymes. In the crystal structures of the inactivated enzymes, the residues Cys4, Cys115 and Cys245 in wild-type MGL and Cys4 and Cys245 in the mutant form contained *S*-allylmercapto 'tails' (Fig. 2). This confirms the mechanism of the reaction of allicin with thiols (Cavallito & Bailey, 1944; Rabinkov *et al.*, 1998, 2000; Miron *et al.*, 1998).

Cys4 is on the surface of the MGL molecule (Fig. 3) and one may assume that Cys4 is not important for the catalysis of the γ- and β-elimination reactions. In the three-dimensional structure of the holoenzyme (PDB entry 2rfv) Cys4 was found to be

oxidized to 3-sulfenoalanine (Fig. 4) and its side chain is placed parallel to the main-chain residues 16–17 and is stabilized by a hydrogen bond to the main-chain N atom of Gly17. Preparations of MGL with oxidized Cys4 possessed the same activities in the γ- and β-elimination reactions as preparations with a free SH group. The three-dimensional structures of both modified enzymes revealed a rotation of the side chain of *S*-allylmercaptocysteine 4 by 180° into the inter-subunit area between two catalytic dimers (Fig. 4). As a result, the *S*-allylmercapto fragment of the modified Cys4 is situated close to the C-terminal part of the adjacent subunit of another catalytic dimer (residues 331**–334**). This location does not influence the contacts between two catalytic dimers. Nevertheless, owing to steric hindrance the side chain of Tyr7 has only one position from the two alternatives found in wild-type MGL (Fig. 4).

3.3.2. Active site of wild-type *i*MGL. Two alternative positions of *S*-allylmercaptocysteine 115 were found in the crystal structure of the modified wild-type enzyme. These positions do not influence the positions of the other active-site residues, but the long side chain of the oxidized Cys115 decreases the accessibility of the active sites to substrates and inhibitors. It protrudes into the area where the side chains of substrates and inhibitors are situated in the three-dimensional structures of the Michaelis complexes (Revtovich *et al.*, 2011). Superposition of the active-site residues of the Michaelis complex of wild-type MGL with L-norleucine and the active-site residues of wild-type *i*MGL reveals that the C^{δ} and C^{ε}

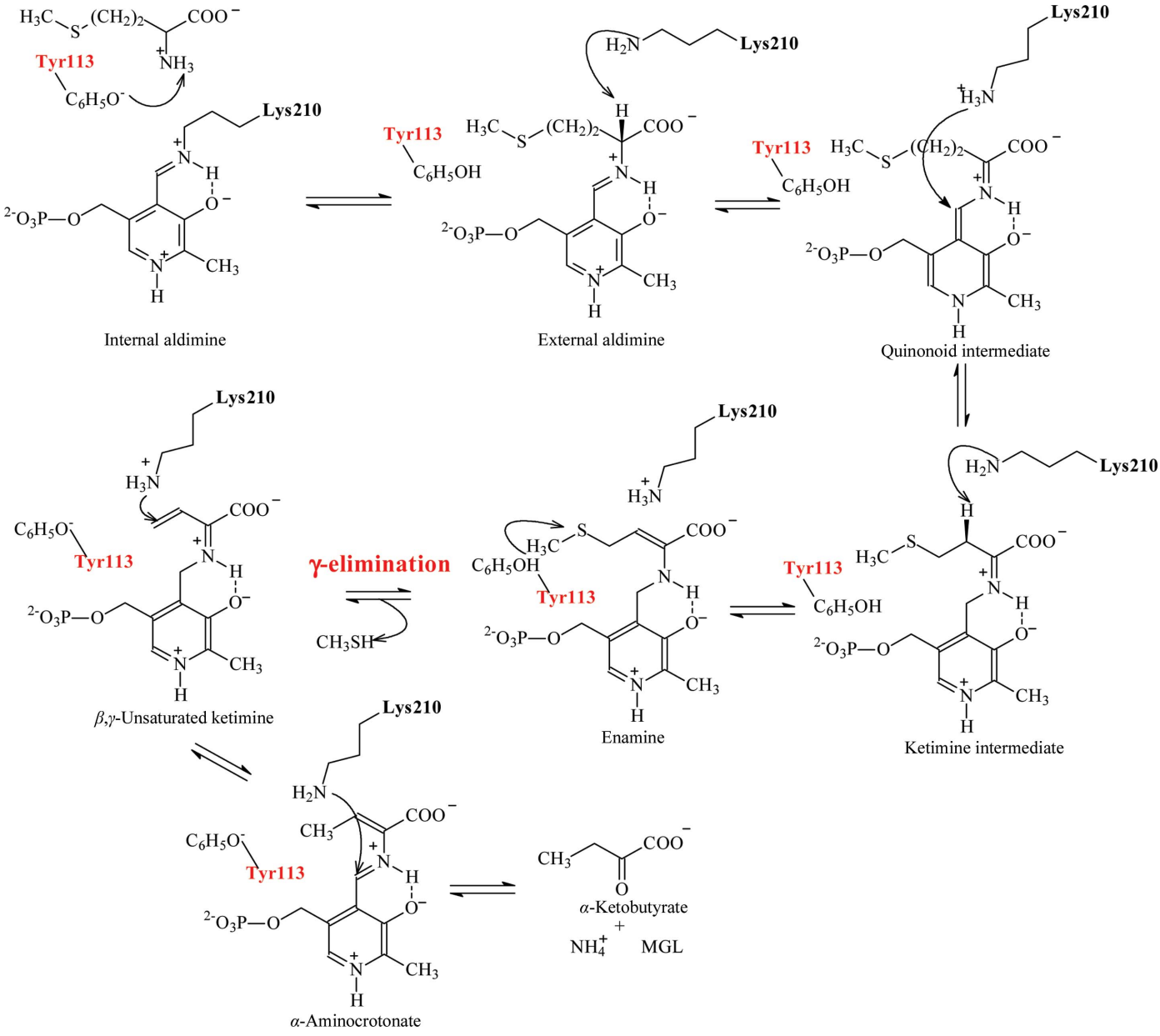

Figure 6
Mechanism of the γ-elimination reaction of L-Met catalyzed by MGL. Adapted from Brzović *et al.* (1990).

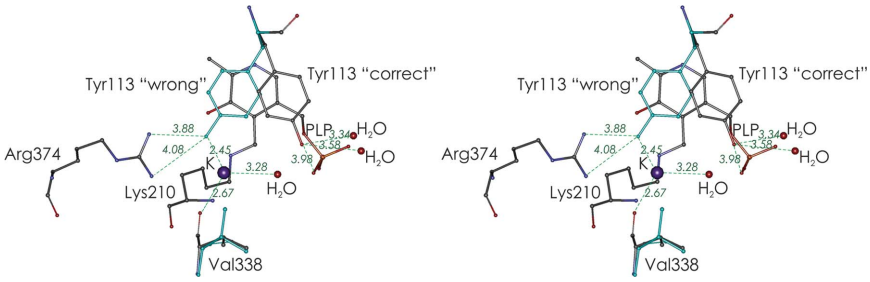

Figure 7
The 'wrong' and 'correct' positions of Tyr113 in the active site of *i*MGL C115A.

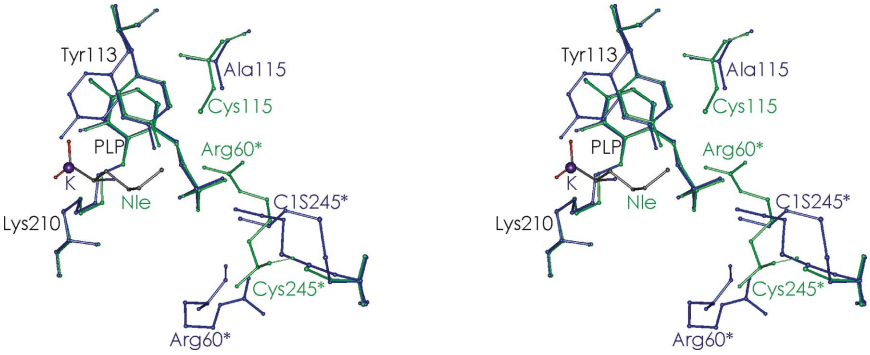

Figure 8
Superposition of the active-site residues in the Michaelis complex of wild-type MGL with L-norleucine (PDB entry 3jwb, green) and *i*MGL C115A (PDB entry 4mkk, blue). The oxidized Cys245 is labelled C1S245. Residues from another monomer of a catalytic dimer are marked *.

atoms of L-norleucine and the C^{η} atoms of *S*-allylmercaptocysteine 115 in both positions almost overlap (Fig. 5). Such positions of the allylmercapto 'tail' do not forbid accommodation of amino acids in the active site but may require some conformational changes to accept them.

It was proposed above that the modification of a buried SH group by allicin may be responsible for inactivation of the wild-type and mutant enzymes in the γ-elimination reaction. Thus, oxidation of Cys245 may be the main cause of inactivation.

The main difference in the overall structures of wild-type *i*MGL and *i*MGL C115A compared with the structures of the holoenzyme (Nikulin *et al.*, 2008), Michaelis complexes (Revtovich *et al.*, 2011) and the external aldimine with glycine (Revtovich *et al.*, 2014) proved to be a change of the positions of the N-terminal residues 51–60. In the structures of the inactivated enzymes, Cys245 is located close to the cofactor-binding Arg60 and the lengthening of its side chain leads to a displacement of residues 51–60. As a result, hydrogen-bonding interactions of the Arg60* and Tyr58* side chains with the O2P and O3P atoms of the PLP phosphate group are lost. This does not influence the conformation of the phosphate handle compared with that in the structure of the holoenzyme. The displacement leads to a loss of the positive charge of the Arg60 side chain near to the side chain of the active-site Tyr113 observed in the structures of the Michaelis complexes (Revtovich *et al.*, 2011) and the external aldimine with glycine (Revtovich *et al.*, 2014).

A dual role has been proposed for the conserved active-site tyrosine residue (Tyr113 in *C. freundii* MGL) in the family of PLP-dependent enzymes involved in the metabolism of sulfur-containing amino acids. It was supposed that it acts as a base, abstracting a proton from an amino group of the incoming substrate, and as a general acid catalyst at the stage of γ-substituent elimination (Fig. 6; Messerschmidt *et al.*, 2003, Clausen *et al.*, 1996, 1998). In the β-elimination reaction, the active-site lysine was proposed to be a general acid catalyst to eliminate the β-substituent (Clausen *et al.*, 1996). The replacement of Tyr114 of *P. putida* MGL by Phe provided evidence that this residue is a general acid catalyst (Inoue *et al.*, 2000). The data on the pH dependence of the kinetic parameters of the γ-elimination reaction of L-methionine catalyzed by *C. freundii* MGL (Faleev *et al.*, 2009) suggested that the Tyr113 hydroxyl group is a base that accepts a proton from the amino group of L-methionine, which is necessary for the transaldimination stage to proceed.

We postulate that the acid–base chemistry of the Tyr113 hydroxyl group is provided by the positive charge of the Arg60 side chain, the hydrogen bond between it and Cys115, and the network of hydrogen bonds between Cys115, Asp240* and Lys239* present in the spatial structures of the *C. freundii* (Nikulin *et al.*, 2008) and *P. putida* (Motoshima *et al.*, 2000) holoenzymes. These residues are highly conserved in MGLs from different sources (Kudou *et al.*, 2007). The network of hydrogen bonds between them has been proposed to be important for L-methionine recognition in the active site of *P. putida* MGL (Fukumoto *et al.*, 2012). The distance between Cys115 and Tyr113 becomes shorter by 0.82 Å on going from the structure of the holoenzyme (3.98 Å) to the structure of the external aldimine with glycine (3.16 Å) owing to reciprocal tilts of the PLP and Tyr113 rings. A simultaneous shortening of the distance between Lys239* and Cys115 by about 1 Å is observed. In the spatial structure of the external aldimine with glycine the side chain of the hydroxyl group of Tyr113 is ionized (Revtovich *et al.*, 2014). One may assume that the proton abstracted from the amino groups of substrates by Tyr113 is 'stored' on the side chain of Asp240* or the SH group of Cys115 and may be returned to the hydroxyl group of Tyr113 in one of the subsequent stages of the γ-elimination reaction to perform catalysis of methanethiol elimination. The possible discussed interactions provide optimal position(s) of Tyr113 for catalysis of the transaldimination stage of the β- and γ-elimination reactions and also the γ-substituent elimination stage.

In wild-type *i*MGL, the side chain of Tyr113 and an electron pair on the S^{δ} atom of *S*-allylmercaptocysteine 115 in two positions are at distances of 3.52 and 4.33 Å. A loss of the positive charge of the Arg60 side chain near to the Tyr113 hydroxyl group and hydrogen-bond interactions with the S^{δ} atom of *S*-allylmercaptocysteine 115 might increase the pK_a value of the Tyr113 hydroxyl group.

We speculate that in the physiological reaction the change in interactions brought about by the oxidation of Cys245 and Cys115 may lead to breakage of the external aldimine formation and methanethiol elimination stages. In the β-elimination reaction it may result in retardation of the transaldimination stage.

3.3.3. Active site of *i*MGL C115A. The lack of a hydrogen-bonding interaction of Tyr113 with Cys115 results in two positions of the Tyr113 side chain in the structure of the modified mutant enzyme (Fig. 7). The 'correct' position of Tyr113 corresponds to its position in wild-type *i*MGL.

In the 'wrong' position of Tyr113, the aromatic ring moves toward the other side of the PLP ring relative to its N1–C4 axis (Fig. 7). In the experimental electron density at Tyr113 a large positive peak was identified which was assigned as a potassium cation. It is retained in the active site of the enzyme despite the potassium buffer used in the β-elimination reaction of alliin having been changed to Tris–HCl buffer during the crystallization procedure. The cation is coordinated by the main-chain carbonyl O atom of Val338, the hydroxyl group of Tyr113 in the 'wrong' position and a water molecule (Fig. 7). In this position, the hydroxyl group of Tyr113 occupies a position close to those of the carboxylic groups of the substrates and inhibitors in the three-dimensional structures of Michaelis complexes with amino acids (Fig. 8).

To accommodate a substrate, the side chain of Tyr113 should be in the 'correct' position and the main-chain carbonyl group between residues Val338 and Ser339 must turn by 180°, as demonstrated in the three-dimensional structures of the Michaelis complexes (Revtovich *et al.*, 2011) and the external aldimine with glycine (Revtovich *et al.*, 2014). The turn must disturb the interaction between the potassium ion and the main-chain O atom of Val338, leading to dissociation of potassium ion from the active site and thus enabling a return of the Tyr113 ring to the 'correct' position.

The oxidation of Cys4 and Cys245 practically did not influence the β-elimination activity of the mutant form compared with that of the wild-type enzyme (Table 3). This demonstrates that the possible increase of the pK_a value of the Tyr113 side chain while the residue is in the 'correct' position and the steric hindrance existing in the 'wrong' position do not retard the transaldimination stage for the mutant enzyme. Meantime, the slowdown of the β-elimination reaction for the oxidized wild-type enzyme was found to be 31% (time of incubation 10 min). The hydrophobic *S*-allylmercapto 'tail' of Cys115 and the hydrogen-bonding interaction of its S^{δ} atom with Tyr113 may increase the pK_a value of Tyr113 more significantly than the replacement of Cys115 by Ala. This may account for the retardation of the transaldimination stage in the case of wild-type *i*MGL.

4. Conclusion

MGL is considered to be a target enzyme for the design of new antibacterial agents. Alliin was found to be a suicide substrate of MGL. The structural bases of the inactivation of the enzyme have been determined. The results demonstrate the important role of interactions of the Cys115/Tyr113/Arg60 triad in the catalysis of the γ-elimination reaction of methionine. Research on other sulfoxides, analogues of methionine and cysteine, as possible suicide substrates of the enzyme will provide new knowledge for the rational design of effective inhibitors of the enzyme.

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