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＊「Ⅲ. 研究成果の刊行物・別刷に収載」

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Ⅲ. 研究成果の刊行物・別刷

Kinetic characterization of methionine γ-lyases from the enteric protozoan parasite *Entamoeba histolytica* against physiological substrates and trifluoromethionine, a promising lead compound against amoebiasis

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Methionine γ-lyase (MGL) (EC 4.4.1.11), which is present in certain lineages of bacteria, plants, and protozoa but missing in mammals, catalyzes the single-step degradation of sulfur-containing amino acids (SAAs) to α-keto acids, ammonia, and thiol compounds. In contrast to other organisms possessing MGL, anaerobic parasitic protists, namely *Entamoeba histolytica* and *Trichomonas vaginalis*, harbor a pair of MGL isozymes. The enteric protozoon *En. histolytica* shows various unique aspects in its metabolism, particularly degradation of SAAs. Trifluoromethionine (TFM), a halogenated analog of Met, has been exploited as a therapeutic agent against cancer as well as against infections by protozoan organisms and periodontal bacteria. However, its mechanism of action remains poorly understood. In addition, the physiological significance of the presence of two MGL isozymes in these protists remains unclear. In this study, we compared kinetic parameters of the wild-type and mutants, engineered by site-directed mutagenesis, of the two MGL isotypes from *En. histolytica* (EhMGL1 and EhMGL2) for various potential substrates and TFM. Intracellular concentrations of L-Met and L-Cys suggested that these SAAs are predominantly metabolized by EhMGL1, not by EhMGL2. It is unlikely that *O*-acetyl-L-serine is decomposed by EhMGLs, given the kinetic parameters of cysteine synthase reported previously. Comparison of the wild-type and mutants revealed that the contributions of several amino acids implicated in catalysis differ between the two isozymes, and that the degradation of TFM is less sensitive to alterations of these residues than is the degradation of physiological substrates. These results support the use of TFM to target MGL.

Trans-sulfuration pathways are ubiquitous and play various roles, including in the formation of Met and Cys, transmethylation reactions, and the synthesis of polyamines, antioxidants, and cofactors [1]. As there are remarkable differences in trans-sulfuration pathways between organisms, these pathways, and in

Abbreviations
CS, cysteine synthase; EhMGL, *Entamoeba histolytica* methionine γ-lyase; Hcy, homocysteine; MGL, methionine γ-lyase; OAS, *O*-acetyl-L-serine; PG, L-propargylglycine; PLP, pyridoxal 5′-phosphate; SAA, sulfur-containing amino acid; TFM, trifluoromethionine (S-trifluoromethyl-L-homocysteine).

particular enzymes involved in the degradation of sulfur-containing amino acids (SAAs), have been exploited as a target for chemotherapeutic intervention in cases of cancer and infectious diseases [2,3]. Methionine γ-lyase (MGL) is one such enzyme, a member of the α-family of pyridoxal 5′-phosphate (PLP)-dependent enzymes [4]. MGL catalyzes the α,γ-elimination and γ-replacement of L-Met and homocysteine (Hcy), and α,β-elimination and β-replacement of L-Cys and S-substituted analogs, and produces ammonia, α-keto acids, and volatile thiols such as hydrogen sulfide and methanethiol [5]. MGL has been characterized in several bacteria, such as *Pseudomonas putida* [6], *Clostridium sporogenes* [7], *Aeromonas* sp. [6], *Citrobacter intermedius* [8], *Citrobacter freundii* [9], *Brevibacterium linens* [10], and *Porphyromonas gingivalis* [11], parasitic protozoa such as *Trichomonas vaginalis* [12] and *Entamoeba histolytica* [13], and the plant *Arabidopsis thaliana* [14].

MGL has been implicated in the degradation of toxic SAAs [15], and also in energy metabolism through the synthesis of pyruvate or 2-oxobutyrate in *En. histolytica* [16]. Volatile thiol compounds have also been implicated in the pathogenicity *in vivo* of the periodontal bacterium, *Po. gingivalis* [11]. It has been recently shown that in *Ar. thaliana*, α-ketobutyrate and methanethiol, generated by MGL, are utilized for isoleucine biosynthesis and the production of *S*-methylcysteine, the putative storage molecule for sulfide or methyl groups, which is formed by the transfer of the acetyl moiety of *O*-acetyl-L-serine (OAS) to methanethiol [14]. Unlike bacteria and plants, *T. vaginalis* and *En. histolytica* have two isozymes of MGLs that differ distinctly in substrate specificity [13,17]. However, the physiological roles of individual isotypes as well as the significance of their redundancy remain to be elucidated.

Entamoeba histolytica, a causative agent of amoebiasis, affects an estimated 50 million people and results in 70 000 deaths per year worldwide [18]. The major clinical manifestations of amoebiasis are amoebic dysentery and extraintestinal abscesses, namely, hepatic, pulmonary and cerebral abscesses [19]. Although clinical resistance against metronidazole, the drug currently used most widely for invasive amoebiasis [3], has not yet been proven for clinical isolates, cases of treatment failure have been reported [3]. In addition, it was shown that metronidazole resistance was easily gained *in vitro* [20,21]. Moreover, metronidazole resistance is common in bacteria and the protozoan flagellates *Giardia intestinalis* and *T. vaginalis* [22]. Therefore, a novel amoebicidal drug is urgently needed.
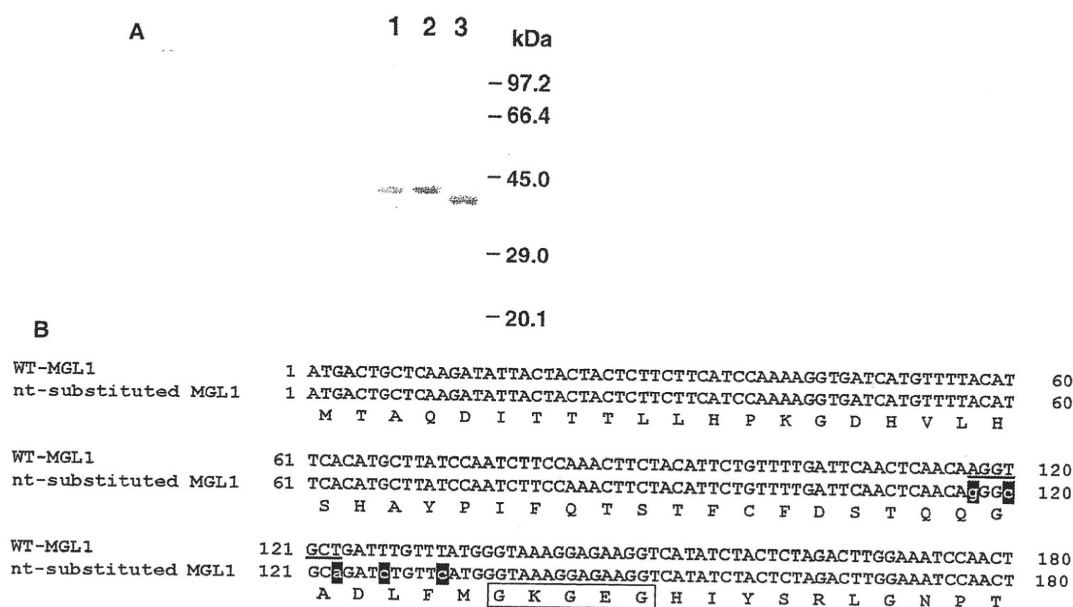
Trifluoromethionine [*S*-trifluoromethyl-L-homocysteine (TFM)], a halogenated Met analog in which a methyl moiety is replaced by a trifluoromethyl group [23], has been shown to be highly toxic to various bacteria [24], including *Po. gingivalis* [25], *T. vaginalis* [26], and *En. histolytica* [13] (Kobayashi and Nozaki, unpublished data). TFM affected the growth of *En. histolytica* and *T. vaginalis* trophozoites at micromolar levels *in vitro* [13,26], and also cured infections in mouse and hamster models [26] (Kobayashi and Nozaki, unpublished results). The limited presence of MGL among organisms, and the remarkable differences in the toxicity of TFM against amoeba and mammalian cells [IC_{50} for *En. histolytica* trophozoites or Chinese hamster ovary cells, 18 μM [13] or 865 μM (unpublished results)], give further support for TFM as a promising lead compound for the development of new chemotherapeutics against amoebiasis.

For the further development of antiamoebic agents based on TFM, elucidation of the underlying reaction mechanisms of MGLs and the interaction of TFM with the enzymes is required. In this study, we demonstrate differences in substrate specificity and kinetic parameters for four potential natural substrates and TFM of both the wild-type and mutants, created by site-directed mutagenesis of critical amino acid residues presumed to play an important role in catalysis, of the two isotypes of *En. histolytica* MGLs (EhMGL1 and EhMGL2). The results clearly demonstrate that EhMGL1, not EhMGL2, plays the predominant role in the degradation of Met and Cys in the amoeba trophozoites, whereas OAS seems to be decomposed by neither EhMGL1 nor EhMGL2. Our results also show that TFM is mainly degraded by EhMGL2, but not by EhMGL1. In addition, the contributions of the amino acids implicated in previous studies [17,27,28] to the catalysis of individual physiological and deleterious substrates differ greatly between the two EhMGL isotypes. The information provided by the present study should help in the further rational design of novel chemotherapeutic agents targeting MGL against amoebiasis.

Results and Discussion

Expression and purification of the genetically engineered wild-type of EhMGL1

We were unable to precisely determine kinetic constants for the reaction catalyzed by MGL isotypes from *En. histolytica*, due to the heterogeneity of EhMGL1 in the previous preparation [13] (approximately 20% of EhMGL1 was produced as a 35 kDa truncated form; Fig. 1A, lane 1). Our attempt to

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B

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WT-MGL1            1 ATGACTGCTCAAGATATTACTACTACTCTTCTTCATCCAAAAGGTGATCATGTTTTACAT   60
nt-substituted MGL1 1 ATGACTGCTCAAGATATTACTACTACTCTTCTTCATCCAAAAGGTGATCATGTTTTACAT   60
                     M  T  A  Q  D  I  T  T  T  L  L  H  P  K  G  D  H  V  L  H

WT-MGL1            61 TCACATGCTTATCCAATCTTCCAAACTTCTACATTCTGTTTTGATTCAACTCAACAAGGT  120
nt-substituted MGL1 61 TCACATGCTTATCCAATCTTCCAAACTTCTACATTCTGTTTTGATTCAACTCAACAGGGC  120
                     S  H  A  Y  P  I  F  Q  T  S  T  F  C  F  D  S  T  Q  Q  G

WT-MGL1           121 GCTGATTTGTTTATGGGTAAAGGAGAAGGTCATATCTACTCTAGACTTGGAAATCCAACT  180
nt-substituted MGL1 121 GCAGATCTGTTCATGGGTAAAGGAGAAGGTCATATCTACTCTAGACTTGGAAATCCAACT  180
                     A  D  L  F  M  G  K  G  E  G  H  I  Y  S  R  L  G  N  P  T
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Fig. 1. (A) Purified proteins (1.0 μg) were analyzed by 12% SDS/PAGE under reducing conditions, and stained with Coomassie brilliant blue. Lane 1: wild-type MGL1. Lane 2: nucleotide-substituted MGL1. Lane 3: MGL2. Molecular mass markers are indicated on the right. (B) Partial alignment of EhMGL1. Wild-type MGL1 (upper), nucleotide-substituted MGL1 (middle) and the deduced amino acids (lower) are aligned. The five substituted nucleotides are indicated in lower-case on a black background. A box represents the N-terminal end of the truncated sequence determined by Edmann degradation. The incidental Shine–Dalgarno-like sequence is underlined.

further purify the full-length EhMGL1 with anion exchange and gel filtration chromatography failed (data not shown), suggesting that the truncated EhMGL1 probably forms a heterogeneous tetrameric complex with the full-length EhMGL1. We determined the N-terminus of the truncated EhMGL1 to be Gly46 (Fig. 1B, boxed) by Edmann degradation of the 35 kDa band excised from the SDS/PAGE gel, and postulated that the truncation was caused by a fortuitous initiation of translation at Met45 due to the similarity of the nucleotide sequence upstream of Met45 of EhMGL1 to the Shine–Dalgarno sequence (Fig 1B, underlined). The truncated enzyme lacking a glutathione *S*-transferase tag was purified by affinity chromatography, indicating that the full-length version and the truncated version form a tetramer. The truncation is potentially deleterious to the stability and activity of a tetramer, because this region is involved in a dimer–dimer interaction and catalytic reaction (e.g. *Ps. putida* MGL [28]). To eliminate the production of the truncated EhMGL1, we replaced five nucleotides within this region of the *EhMGL1* gene without causing amino acid substitutions (Fig. 1B, white lower-case on a black background), and applied the engineered EhMGL1 to protein expression. This genetically engineered EhMGL1 was purified to > 95% homogeneity

without traceable contamination of the truncated form (Fig. 1A, lane 2).

Comparison of the specific activity and kinetic parameters for potential substrates between the wild-type MGL isotypes

To understand the specific roles of the two MGL isotypes, which show 69% mutual identity [13], and also to demonstrate differences between them in reaction mechanisms towards physiological substrates and TFM, we determined the apparent specific activity (with a constant substrate concentration of 2 mM) and the kinetic parameters of recombinant EhMGL1 and EhMGL2 (Tables 1 and 2). Despite the heterogeneity of the EhMGL1 preparation used in the previous study [13], the kinetic constants of EhMGL1 in the present study largely agreed with the previous data, except that the relative activity towards Cys and OAS was underestimated by 4–5-fold previously (the relative specific activities of EhMGL1 towards Cys and OAS were 19.7% and 11.1% relative to that towards Met [13], and 116% or 42.4% in the present study). The discrepancy in the kinetic constants of EhMGL1 was probably attributable to the heterogeneity of the EhMGL1 preparation in the previous study. The V_{max}

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Table 1. Specific activities of wild-type and mutant EhMGL1 (A) and EhMGL2 (B). Apparent specific activity (mean ± SD in triplicate) is shown as µmol of α-keto acid produced·min^{-1}·mg^{-1} protein. ND, activity not detected (less than 0.05 µmol of product per min per mg of protein).

(A)

Substrate	Wild-type	Y108F	C110S	C110G	R55A
L-Methionine	1.39 ± 0.01	0.23 ± 0.02	1.11 ± 0.08	0.56 ± 0.04	ND
Trifluoromethionine	1.16 ± 0.10	2.54 ± 0.28	4.78 ± 0.19	1.61 ± 0.10	1.01 ± 0.15
DL-Homocysteine	1.83 ± 0.26	0.38 ± 0.05	1.18 ± 0.05	0.77 ± 0.02	ND
L-Cysteine	1.61 ± 0.35	0.52 ± 0.06	1.06 ± 0.26	1.12 ± 0.12	0.10 ± 0.01
O-Acetyl-L-serine	0.59 ± 0.12	0.67 ± 0.09	0.29 ± 0.04	0.82 ± 0.01	0.14 ± 0.02

(B)

Substrate	Wild-type	Y111F	C113S	C113G	R58A
L-Methionine	0.71 ± 0.02	ND	0.06 ± 0.0001	0.08 ± 0.002	ND
Trifluoromethionine	14.03 ± 2.03	7.76 ± 1.05	8.14 ± 0.70	14.67 ± 0.54	0.78 ± 0.05
DL-Homocysteine	7.42 ± 1.02	0.64 ± 0.17	1.90 ± 0.11	2.34 ± 0.06	ND
L-Cysteine	0.62 ± 0.02	0.15 ± 0.01	0.09 ± 0.01	0.75 ± 0.03	ND
O-Acetyl-L-serine	0.37 ± 0.04	0.26 ± 0.02	0.06 ± 0.01	0.90 ± 0.05	ND

(or the specific activity) of EhMGL2 against DL-Hcy previously reported (V_{max}, 1.31 µmol product·min^{-1}·mg^{-1} protein; relative specific activity compared to that against Met, 162%) was also underestimated (k_{cat}, 10.56 s^{-1}; relative specific activity 10.5-fold higher than that for Met, in the present study). In addition, the K_m of EhMGL2 for OAS in the previous study (0.89 mM) disagreed with that in the present study (52.33 mM). We assumed that these differences were attributable to the assay methods used; the α-keto acid assay was employed in the present study, whereas the nitrogen assay, which has less sensitivity, was used previously. Taken together, the specificities of the two isotypes are briefly summarized as follows. EhMGL1 showed comparable (within 1.1–3.1-fold differences) specific activities towards OAS and all SAAs tested in this study (0.59–1.83 µmol product·min^{-1}·mg^{-1} protein), whereas EhMGL2 showed 10–20-fold more activity with DL-Hcy than with other substrates (7.42 and 0.37–0.71 µmol product·min^{-1}·mg^{-1} protein, respectively).

The K_m of EhMGL2 for Met (3.58 mM) is six-fold higher than that of EhMGL1 (0.61 mM). In addition, the k_{cat} for Met of EhMGL1 is 1.6-fold higher than that of EhMGL2. The k_{cat}/K_m, which indicates the catalytic efficiency [29], of EhMGL1 is 10-fold higher than that of EhMGL2. Taking into account the intracellular Met concentrations, measured by NMR (2.1 ± 0.6 mM) or direct amino acid analysis (0.8 mM, [30]), we speculate that EhMGL1, but not EhMGL2, is involved in the degradation of Met under normal conditions. Similarly, the 2.0-fold higher k_{cat} and 2.7-fold lower K_m for Cys of EhMGL1 than of EhMGL2, together with the intracellular Cys concentration

(0.4 mM [30]), suggest that EhMGL1, but not EhMGL2, mainly catalyzes the degradation of Cys *in vivo*. Although Hcy is an essential component of the Met cycle [15], it is believed that Hcy must be maintained at low concentrations to avoid toxicity [31]. The intracellular Hcy concentration is unknown in amoebae, but is presumed to be several micromoles per liter, as shown for human plasma [32], a much lower concentration than the K_m of EhMGL1 and EhMGL2 for Hcy (1.5–3.0 mM). Thus, although the k_{cat}/K_m for Hcy of EhMGL2 was 5.5-fold higher than that of EhMGL1, the assumed Hcy concentrations suggest that neither EhMGL plays a significant role in the elimination of Hcy under physiological conditions.

Kinetic parameters against OAS also revealed that the two EhMGLs have discernible catalytic properties (EhMGL1, 6.28 mM and 1.74 s^{-1}, and EhMGL2, 52.33 mM and 6.22 s^{-1}, for K_m and k_{cat}, respectively). Although the intracellular OAS concentration is unknown for amoebae, the presence of multiple isotypes of cysteine synthase (CS) makes it unlikely that EhMGLs are involved in the degradation of OAS. CS, which generates Cys from H_2S and OAS, has advantages (e.g. K_m and k_{cat} of EhCS1 are 1.27 mM and 395 s^{-1}, respectively) for OAS, as compared to EhMGLs [33]. Three isotypes of CS are constitutively expressed, as shown by immunoblotting [34] and a transcriptome analysis with a DNA microarray [35]. Thus, OAS is most likely utilized predominantly by CS. Taken together, these findings suggest that EhMGL1 is responsible for the decomposition and the maintenance of the cellular concentrations of Met and Cys, whereas the physiological substrates of EhMGL2 under normal growth conditions remain unknown.

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Table 2. Kinetic parameters of wild-type and mutant EhMGL1 (A) and EhMGL2 (B). Kinetic parameters were measured with at least five different concentrations. Values are means ± SD in triplicate. ND, not detectable; NT, not tested.

(A)

Substrate	Wild-type			Y108F			C110S			C110G			R55A		
	K_m (mM) ± SD	k_{cat} (s^{-1}) ± SD	k_{cat}/K_m	K_m (mM) ± SD	k_{cat} (s^{-1}) ± SD	k_{cat}/K_m	K_m (mM) ± SD	k_{cat} (s^{-1}) ± SD	k_{cat}/K_m	K_m (mM) ± SD	k_{cat} (s^{-1}) ± SD	k_{cat}/K_m	K_m (mM) ± SD	k_{cat} (s^{-1}) ± SD	k_{cat}/K_m
L-Methionine	0.61 ± 0.06	1.82 ± 0.11	2.99	NT	NT	NT	0.72 ± 0.02	0.93 ± 0.15	1.29	0.19 ± 0.01	0.36 ± 0.03	1.91	NT	NT	NT
Trifluoromethionine	0.10 ± 0.00	0.81 ± 0.08	8.02	0.57 ± 0.02	2.22 ± 0.08	3.88	NT	NT	NT	NT	NT	NT	0.83 ± 0.05	1.26 ± 0.06	1.52
DL-Homocysteine	3.03 ± 0.06	3.92 ± 0.15	1.30	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
L-Cysteine	0.64 ± 0.01	1.59 ± 0.14	2.48	1.01 ± 0.07	0.67 ± 0.06	0.66	0.46 ± 0.05	0.78 ± 0.03	1.69	0.34 ± 0.02	1.01 ± 0.02	3.00	NT	NT	NT
O-Acetyl-L-serine	6.28 ± 0.53	1.74 ± 0.12	0.28	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT

(B)

Substrate	Wild-type			Y111F			C113S			C113G			R58A		
	K_m (mM) ± SD	k_{cat} (s^{-1}) ± SD	k_{cat}/K_m	K_m (mM) ± SD	k_{cat} (s^{-1}) ± SD	k_{cat}/K_m	K_m (mM) ± SD	k_{cat} (s^{-1}) ± SD	k_{cat}/K_m	K_m (mM) ± SD	k_{cat} (s^{-1}) ± SD	k_{cat}/K_m	K_m (mM) ± SD	k_{cat} (s^{-1}) ± SD	k_{cat}/K_m
L-Methionine	3.58 ± 0.30	1.11 ± 0.13	0.31	NT	NT	NT	15.12 ± 0.24	0.47 ± 0.05	0.03	ND[a]	ND[a]	ND[a]	NT	NT	NT
Trifluoromethionine	0.92 ± 0.06	17.46 ± 1.21	19.05	0.29 ± 0.0003	5.80 ± 0.54	20.29	NT	NT	NT	NT	NT	NT	1.62 ± 0.15	1.19 ± 0.11	0.73
DL-Homocysteine	1.47 ± 0.12	10.56 ± 1.25	7.19	NT	NT	NT	NT	NT	NT	ND[a]	ND[a]	ND[a]	NT	NT	NT
L-Cysteine	1.70 ± 0.09	0.80 ± 0.08	0.47	ND	NT	NT	5.45 ± 0.09	0.24 ± 0.01	0.04	ND[a]	ND[a]	ND[a]	NT	NT	NT
O-Acetyl-L-serine	52.33 ± 1.52	6.22 ± 0.61	0.12	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT

[a] K_m is estimated to be less than 0.1 mM.

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Kinetic parameters of mutants of the two MGL isotypes

Among the several amino acid residues shown to interact with PLP, the importance of a few was evaluated in the amoebic MGL isotypes. Our preliminary crystallographic study suggests that Tyr111, Cys113 and Arg58 of EhMGL2 are oriented towards PLP in close proximity [36] (data not shown). Tyr114 of *Ps. putida* MGL (corresponding to Tyr108 and Tyr111 of EhMGL1 and EhMGL2, respectively) was implicated in γ-elimination, attacking the γ-position of a substrate as an acid catalyst [14]. Cys110 and Arg55 of EhMGL1, which correspond to Cys113 and Arg58 of EhMGL2, are also predicted to be located in similar positions.

MGL1(Y108F) and MGL2(Y111F) showed a 79–100% reduction in the α,γ-elimination of both Met and Hcy as compared to the wild-type MGLs, whereas these mutations only slightly affected the α,β-elimination of OAS (a 1.14-fold increase or only a 28% reduction as compared to wild-type MGL1 or MGL2, respectively). These results were similar to the Tyr114 mutant of *Ps. putida* MGL [27]. Unlike the case of OAS, MGL1(Y108F) and MGL2(Y111F) showed reduced α,β-elimination for Cys (68% or 76% reduction); for example, MGL1(Y108F) showed a 1.6-fold increase in the K_m and a 58% decrease in the k_{cat} for Cys. This implies that the hydroxyl group of Tyr108 of EhMGL1 is actively involved in the β-elimination and γ-elimination of the C–S bond, but not the β-elimination of the C–O bond, of OAS.

The Cys near the active site was shown to be important for activity by chemical modification with 2-nitrothiocyanobenzoic acid and labeling with a PLP analog, *N*-(bromoacetyl)pyridoxamine phosphate, in *Ps. putida* MGL [37,38]. Cys116 was shown to be located in close proximity to Tyr114 [28]. This Cys is not conserved in other PLP α-family enzymes; Cys is substituted by Gly or Pro in cystathionine γ-lyase, cystathionine β-lyase, and cystathionine γ-synthase [27,28]. In *B. linens* MGL, Gly is substituted for Cys at this position. *B. linens* MGL degrades neither Cys nor cystathionine [10], whereas *Ar. thaliana* MGL decomposes Cys but degrades cystathionine only marginally, in spite of the presence of Gly at this position [39]. The Cys to Ser or Thr mutations of *Ps. putida* MGL caused a reduction in activity [28]. Neither *En. histolytica* MGL nor *T. vaginalis* wild-type MGL degrades cystathionine [13,17]. The Cys → Gly mutation of *T. vaginalis* MGLs reduced γ-elimination activity towards Met and Hcy 5–13-fold, but only slightly changed β-elimination activity for Cys and

OAS (0.38–2.5-fold) [17]. Thus, it was proposed that this Cys plays an important role in substrate specificity, i.e. the preference of substrates for γ-elimination in *T. vaginalis* MGLs.

Amoebic MGL2(C113S) showed reduced activities towards Met, Cys, and Hcy (9–26% of that of the wild-type), whereas MGL1(C110S) showed only a marginal reduction (65–80% of that of the wild-type). MGL1(C110S) and MGL2(C113S) showed reduced k_{cat} values for Met or Cys (49–51% or 29–42% of that of wild-type MGL1 or MGL2, respectively), whereas the K_m values remained unchanged for MGL1(C110S) (72–118% of that of the wild-type) or increased 3.2–4.2-fold for MGL2(C113S). In contrast to the Cys → Ser mutation, the Cys → Gly mutation caused 2.5-fold and 1.4-fold increases in activity towards OAS for MGL1(C110G) and MGL2(C113G), respectively. MGL2(C113G) also showed a 20% higher level of activity towards Cys than wild-type MGL2. Interestingly, the K_m values of MGL1(C110G) for Met and Cys were reduced by 70% and 48%, respectively. In contrast, the k_{cat} values of MGL1(C110G) for Met and Cys decreased by 80% and 34%, respectively. Additionally, MGL1(C110G)-catalyzed reactions of Met or Cys showed saturation with 0.125 M substrate, suggesting the K_m to be < 0.1 mM (Table 1, indicated by asterisks). Taken together, these findings show that the contribution of this Cys to the catalytic reaction clearly differs between EhMGL1 and EhMGL2; Cys113 of MGL2 is heavily involved in substrate specificity, whereas Cys110 of MGL1 is not so essential for catalysis. However, as mutations of Cys110 of MGL1 produced 56% and 32% reductions in the specificity constants with Met and Cys, respectively, this residue might be also important for catalysis.

Arg55 of EhMGL1 and Arg58 of EhMGL2 are located near the PLP of the neighboring subunit of the catalytic dimer, as revealed by X-ray crystallography (unpublished data), similar to what is found for MGLs from *Ps. putida* [28] and *Ci. freundii* [40]. The mutation of this Arg to Ala was shown to abolish the activity for Met of *Ps. putida* MGL [28]. Similarly, the R58A mutation of MGL2 completely abolished activity towards Met, Cys, Hcy, and OAS, whereas residual activity remained for MGL1(R55A) towards Cys and OAS, but not Met and Hcy. We confirmed by gel filtration that the apparent molecular mass of MGL1(R55A) and MGL2(R58A) was approximately 175 kDa, similar to that of wild-type MGLs (data not shown). Thus, interference with dimerization was not a reason for the observed loss of activity. It was also shown that a mutant containing the corresponding Arg mutation formed a tetramer in *Ps. putida* MGL

[28]. It is worth considering the utilization of MGL1(R55A) and MGL2(R58A) mutants for dominant negative effects, because these EhMGL mutants were shown to be associated with endogenous EhMGL in a heterotetrameric complex (data not shown).

Kinetic parameters of MGL wild-type and mutants towards TFM

The specific activity of EhMGL2 against TFM was 12-fold higher than that of EhMGL1. This increase is mostly attributable to a large difference in k_{cat}; the k_{cat} of EhMGL2 was 21-fold higher than that of EhMGL1 (17.5 s^{-1} and 0.81 s^{-1}). By contrast, the K_m of EhMGL2 was nine-fold higher than that of EhMGL1 (0.92 and 0.10 mM, respectively). Thus, the catalytic efficiency, expressed as k_{cat}/K_m, of EhMGL2 is only 2.4-fold higher than that of EhMGL1 (19.05 and 8.02, respectively; Table 2). It is remarkable that the k_{cat} of EhMGL1 for TFM was comparable to that for Met and Cys, whereas the k_{cat} of EhMGL2 for TFM was 16–22-fold higher than that for these physiological substrates. The K_m of EhMGLs for TFM was 52–470-fold lower than that of the closest mammalian counterpart (rat liver cystathionine γ-lyase, $K_m = 48$ mM) [41].

None of the mutations examined in this study, except for MGL2(R58A), greatly affected the activity towards TFM, suggesting that the mechanism of the MGL-catalyzed reaction of TFM is relatively independent of these amino acids, unlike the case for physiological substrates. The activity of MGL2(R58A) towards TFM was similar to that of wild-type MGL for the physiological substrates. Moreover, the effects of the Y108F substitution on the K_m and k_{cat} of EhMGL1 for TFM are opposite to those of Y111F of EhMGL2; the K_m and k_{cat} of EhMGL1(Y108F) increased 5.6-fold and 2.7-fold, respectively, as compared to those of wild-type EhMGL1, whereas the K_m and k_{cat} of EhMGL2(Y111F) decreased threefold. The Tyr → Phe mutation caused only a 2.1-fold reduction in the catalytic efficiency (k_{cat}/K_m) of EhMGL1 (8.02 to 3.88), whereas the corresponding mutation of EhMGL2 did not have a significant effect (19.05 to 20.29). The degradation of TFM probably proceeds without interaction with Tyr111, Cys113, and Arg58 (in the case of EhMGL2), possibly due to the electronegativity of the trifluoromethyl group of TFM. It is also worth noting that the role of Tyr108 (or Tyr111) in the degradation of TFM significantly differs between EhMGL1 and EhMGL2.

As indicated, EhMGL2, but not EhMGL1, displayed a remarkable preference for TFM. Although

elucidation of the mechanisms responsible for this observation await further study, we speculate that the preference is associated with the functional group bound to the γ-carbon: the trifluoromethyl moiety. EhMGL2 also showed a remarkable preference for Hcy, similar to TFM. Three fluorides on the methyl carbon of TFM and a sulfur atom of the thiol group of Hcy may participate in the formation of additional hydrogen bonds in the catalytic pocket. Although we previously reported X-ray crystallography of EhMGL2 [36], EhMGL2 cocrystallized with either TFM or Hcy has not yet been obtained.

Crosslinking of EhMGLs and a scavenger protein by TFM

It was previously proposed that a thiol derived from the degradation of TFM by MGL, carbonothionic difluoride, crosslinks the primary amino group of proteins, which results in toxicity [41]. This model was supported by the detection of released fluoride, a byproduct of crosslinking with carbonothionic difluoride [41]. We attempted to directly demonstrate that TFM-derived product(s) causes protein modification. We investigated whether the recombinant EhMGL was modified after the incubation with TFM by examining the mobility of the proteins by SDS/PAGE. When recombinant EhMGL1 or EhMGL2 was incubated with TFM, at least three additional bands were found (Fig. 2A, lane 1, open arrowheads). Incubation of EhMGLs with Met or without substrates did not result in the appearance of these bands (Fig. 2A, lanes 2 and 3). Preincubation of EhMGLs with L-propargylglycine (PG), a suicide substrate of PLP–enzyme, prior to the mixing with TFM, abolished these extra bands (Fig. 2A, lane 4). Immunoblot assay with antibody to EhMGL2 (Fig. 2C) showed that when EhMGL1 was reacted with TFM, but not with Met, or pretreated with PG, EhMGL1 was no longer recognized by the antibody (the equal loading of proteins was verified by silver staining; Fig. 2A), suggesting that EhMGL1 was chemically altered by unknown modifications caused by the decomposition of TFM catalyzed by MGL. Suppression of the antibody's reactivity by the treatment with TFM was also observed for EhMGL2, but not for EhMGL1. The additional bands described above (open arrowheads) were not recognized by the antibody, suggesting that these bands were also chemically modified. Alternatively, these bands were not derived from EhMGLs, but were minor contaminants in the recombinant protein preparations. To examine whether irrelevant proteins can also serve as scavengers of carbonothionic difluoride produced from TFM by

–73–
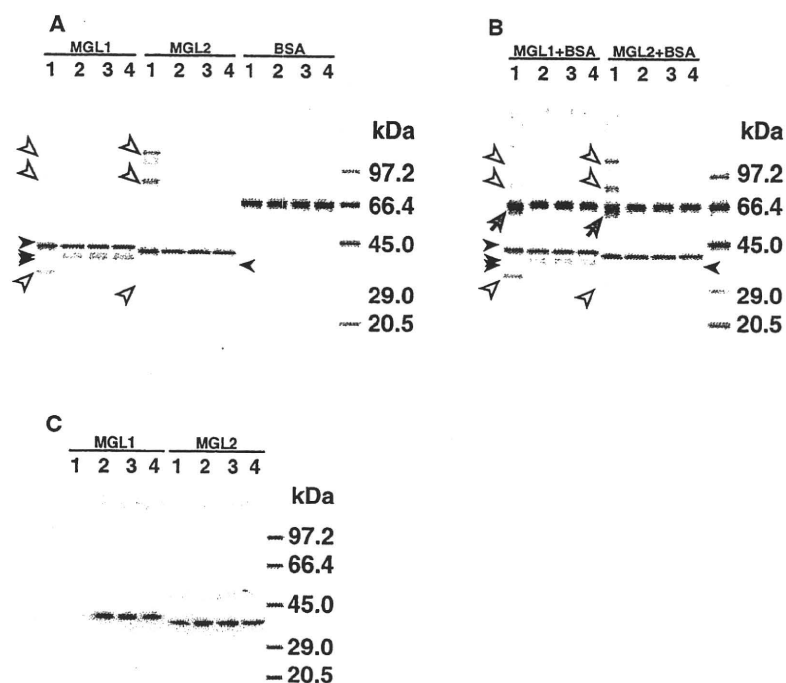
Fig. 2. *In vitro* crosslinking by TFM produced by recombinant MGLs. (A) The recombinant EhMGL1 or EhMGL2 or BSA was preincubated with 4 mM PG (lane 4) or without PG (lanes 1–3) for 30 min at 37 °C, and incubated with 4 mM TFM (lanes 1 and 4), Met (lane 2) or 2.5% dimethylsulfoxide (control, lane 3) in 100 mM sodium phosphate (pH 7.0) containing 20 μM PLP and 1 mM dithiothreitol for 1 h at 37 °C. The reaction mixtures containing 50 ng of EhMGL or 100 ng of BSA were electrophoresed on a 5–20% SDS/PAGE gel under reducing conditions, and subjected to silver staining. (B) The same reactions were performed with the mixtures of EhMGL and BSA. (C) The reaction mixtures of (A) were subjected to immunoblot analysis with antibody to EhMGL1 (left) or EhMGL2 (right). One-fourth of the volume of each reaction mixture (corresponding to 25 ng of EhMGL) was analyzed. Open arrowheads, filled arrowheads and gray arrows depict the bands that appeared upon incubation with TFM, contaminants of MGL preparations, and a smeared band probably corresponding to crosslinked BSA, respectively. Molecular mass markers are indicated on the right.

EhMGLs, MGL was incubated with TFM in the presence or absence of BSA, electrophoresed, and silver-stained or immunoblotted with antibody to EhMGL2 (Fig. 2B). Although we did not observe BSA-derived extra bands, the band corresponding to BSA on a silver-stained gel was smeared only when BSA was incubated with TFM and EhMGLs (gray arrows), suggesting that unknown modifications or degradation of BSA probably occurred.

As we observed differences in reactivity with the TFM-derived product between the two EhMGLs, we examined whether the sensitivities of the two EhMGLs to inactivation by TFM differ. We preincubated EhMGLs with TFM at a molecular ratio of 1 : 1000, and further tested for the Met-degrading activity on the basis of the detection of α-keto acid after the addition of 2 mM Met (Fig. 3). Approximately 85% of EhMGL2 activity remained after 1 h, whereas 75% of EhMGL1 activity was lost. MGL activity following preincubation with Met was indistinguishable from

that without preincubation, confirming that the decrease was not due to inactivation of MGL during the preincubation. These results clearly showed that significant differences in sensitivity to TFM exist between the two EhMGLs. Although we did not identify specific proteins that were crosslinked and inactivated *in vivo* by the MGL-mediated degradation of TFM, except for the amoebic MGL itself, we speculate that carbonothionic difluoride generates crosslinks surrounding proteins in the cytosol of the parasite, leading to the observed toxicity to the cell.

The fact that EhMGL2, which is more active in the degradation of TFM, is less sensitive than EhMGL1 seems to contradict the notion that the product of the degradation is the enzyme inactivator. However, we speculate that the distribution of possible primary amines, which are target of the TFM adducts (carbonothionic difluoride), in close proximity to the catalytic pocket differs between MGL1 and MGL2, and that this difference may influence the sensitivity to the

-74-
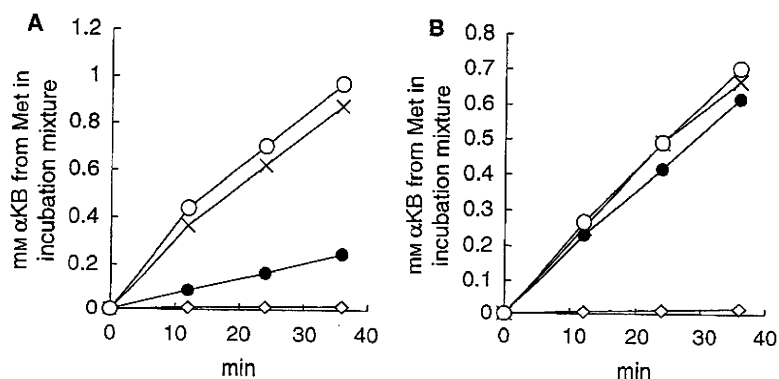
Fig. 3. Inactivation of MGL by incubation with TFM. The recombinant EhMGL1 [(A) 15 ng·μL^{-1}] or EhMGL2 [(B) 30 ng·μL^{-1}] was preincubated with 0.35 mM or 0.7 mM TFM respectively (filled circles), PG (diamonds), Met (crosses) or control (0.625% dimethylsulfoxide) (open circles) at 37 °C for 1 h. After preincubation, the mixtures were further incubated with 2 mM Met for 0, 12, 24 or 36 min, and the amount of α-keto acid was measured. The means for the triplicate measurements of the amount of α-keto acids produced after the addition of 2 mM Met are plotted. Error bars are omitted for clarity (standard errors < 0.03).

inactivation by the TFM adducts. A comparison of primary structures indicated that 28 basic amino acids (i.e. Lys and Arg) were conserved, whereas eight and 12 are unique to MGL1 and MGL2, respectively [13]. Thus, these eight MGL1-specific Lys and Arg residues may be involved in the inactivation by TFM adducts.

Roles of two MGL isotypes in *En. histolytica*

The kinetic parameters of the two MGL isotypes suggest that EhMGL1 is the primary isotype involved in the degradation of Met and Cys. Both an immunoblot study [13] and a transcriptome analysis (supplemental data of [35]) showed that EhMGL1 and EhMGL2 were expressed at comparable levels. To directly confirm the *in vivo* activity of the two isozymes in the parasite, we measured specific activities of MGL in the amoebic extracts using two representative physiological substrates, i.e. Met and Hcy. The specific activities with Met and Hcy in the parasite lysate (the 15 000 *g* supernatant fraction) were 0.456 and 2.28 nmol of product·min^{-1}·mg^{-1} of lysate, respectively. Assuming that the substrate specificity is similar between native and recombinant EhMGLs and that recombinant EhMGLs are fully active, the EhMGL1/EhMGL2 ratio was determined to be 1 : 1.38 (data not shown). This ratio agreed well with the data from the immunoblot and transcriptome analyses. The constitutive expression of EhMGL1 and EhMGL2 *in vitro* ([13] and this study) and *in vivo* [35] strongly suggests that both isotypes play indispensable and nonoverlapping roles during proliferation and intestinal infection. As the K_m of EhMGL2 for most naturally occurring SAAs and related compounds was significantly higher

than that of EhMGL1, the physiological substrates of EhMGL2 and precise biological role of MGL2 *in vivo* under normal growth conditions are still not well understood. However, it is conceivable that EhMGL1 plays a central role in the control of SAA concentrations in the cell under normal conditions, whereas EhMGL2 is involved in the control of SAA homeostasis in cases where intracellular SAA concentrations are elevated to toxic levels, e.g. on exposure to high concentrations of Cys precursors, including Ser, or the engulfment of excessive amounts of bacteria or host cells. This can be interpreted as follows to explain the regulatory mechanism of the intracellular Met concentration. Under physiological Met or Cys concentrations, EhMGL1 is fully active, whereas EhMGL2 is only partially active, due to its higher K_m and lower k_{cat}/K_m. However, at higher Met concentrations, EhMGL2 plays a supplementary role in reducing the concentration of this toxic amino acid. In addition, EhMGL2 may be present specifically to degrade Hcy. Gilchrist *et al.* [35] reported that EhMGL1 was overexpressed 15-fold at the mRNA level 1 day after amoebae were inoculated into the mouse cecum, but not a month later, when they colonized the intestine (only 1.3-fold increase), whereas EhMGL2 mRNA was repressed 1.8–4.2-fold during this period [35], suggesting that the expression of EhMGL1 is induced under stress conditions. We also speculate that EhMGL2 may prefer substrates other than those used in this study, e.g. *S*-adenosylmethionine, *S*-adenosylhomocysteine, and *S*-methylmethionine. The reaction catalyzed by MGLs is considered to be unidirectional, because one of the products from Met, methanethiol, is highly volatile and immediately evaporates extracellularly [25].

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However, as it is not reasonable to speculate that *En. histolytica* discharges methanethiol, while it incorporates sulfide, we propose that *En. histolytica* salvages methanethiol. This is plausible if *En. histolytica* possesses a pathway to produce Cys from Met in which MGL is used to provide reactive thiol molecules such as sulfide and methanethiol, which are in turn utilized as substrates to form Cys and *S*-methylcysteine as proposed for *Ar. thaliana* [14]. One of the major thiols produced by amoebic MGLs, hydrogen sulfide, is probably assimilated to form Cys in a reaction also catalyzed by CS [34]. This organism has three isozymes of CS [42], which convert OAS and hydrogen sulfide to Cys [15]; one of these may utilize methanethiol instead of hydrogen sulfide as an alanyl acceptor. Genes encoding enzymes that utilize methanethiol as a substrate, such as *O*-acetylhomoserine sulfhydrylase (EC 2.5.1.49) and methanethiol oxidase (EC 1.8.3.4), are not present in the *En. histolytica* database. Metabolomics or fluxomics using amoebic transformants overexpressing EhMGL1 or EhMGL2 should elucidate the physiological substrates and functions of these enzymes.

The excellent reactivity of TFM, a promising lead to target MGL

We demonstrated in this study that TFM is an ideal lead compound as a prodrug targeting MGL, from an enzymological point of view. The excellent ability of TFM to act as a prodrug is primarily attributable to the high k_{cat} and low K_m of MGL2 against TFM. It is considered that both EhMGL1 and EhMGL2 are, despite their clear differences in K_m and k_{cat}, probably responsible for the decomposition of TFM, because the concentration that is effective against the amoebae is two orders of magnitude lower than the K_m values. It was reported that the incorporation of TFM into proteins and recycling via the Met cycle are extremely poor [43,44], which reinforces the notion that TFM and its derivatives are not very toxic to mammalian cells (data not shown). Finally, the elucidation of reaction mechanisms against both physiological substrates and prodrugs such as TFM should provide a rationale for the further design of TFM derivatives.

Experimental procedures

Chemicals

All chemicals of analytical grade were purchased from Wako Pure Chemical Industries (Osaka, Japan) or Sigma-Aldrich

(St Louis, MO, USA) unless otherwise stated. PG was purchased from PepTech Corp. (Burlington, MA, USA). TFM was a gift from T. Toru and N. Shibata (Graduate School of Engineering, Nagoya Institute of Technology, Nagoya, Japan).

Mutagenesis, expression and purification of recombinant enzymes

To eliminate the production of a truncated EhMGL1 in *Escherichia coli*, due to the fortuitous translation initiation at the second Met (Met45) within the coding region, five synonymous nucleotide changes were introduced into EhMGL1 (accession number AB094499). Nested PCR was performed with appropriate oligonucleotide primers (supplementary Table S1) and pGEX6P1–EhMGL1 [13] as template, and subsequently with primers having *Bam*HI and *Xba*I sites for 'nested PCR', using the first PCR product as template. To make use of the *Bam*HI site in the vector and the *Xba*I site in the EhMGL1 gene, the product of the nested PCR was replaced with the corresponding region in pGEX–EhMGL1 [13] to produce pGEX–EhMGL1fl. The following mutations were introduced into EhMGL1 and EhMGL2 (AB094500), using the GeneTailor site-directed mutagenesis system (Invitrogen, Carlsbad, CA, USA): Y108F, C110S, C110G and R55A in EhMGL1; and Y111F, C113S, C113G and R58A in MGL2. PCRs were performed with the corresponding oligonucleotide primers (supplementary Table S1) and methylated pGEX–EhMGL1fl and pGEX–EhMGL2 [13] as templates. The transformation and selection of mutated plasmids were performed according to the instructions of the manufacturer. Both wild-type and mutated proteins were expressed and purified as described previously [13,36].

Activity assay and measurement of kinetic parameters

The MGL activity was measured on the basis of the production of α-keto acids [45]. Assays were carried out in 60 μL of 100 mM sodium phosphate (pH 7.0) containing 1 mM dithiothreitol and 20 μM PLP, and 3–60 μg·mL⁻¹ of the recombinant enzymes, at 37 °C for 10 min. The ranges of substrate concentrations for the measurement of kinetic parameters were 0.125–20 mM for Met, Cys, OAS, and TFM, and 0.125–2 mM for Hcy. Specific activity with 2 mM substrates was also measured. These measurements were performed independently. To assay the activity of MGLs in the parasite, amoeba cells were lysed with 100 mM sodium phosphate (pH 7.0) containing 20 μM PLP and 0.1% Triton X-100. The insoluble materials were eliminated by centrifugation at 15 000 *g* for 10 min, and subsequently 12.4 and 1.24 mg·mL⁻¹ of the supernatant was incubated with 2 mM Met and Hcy, respectively. After the reaction

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