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Active site inhibitor of aminoacyl-tRNA synthetase as a new antimicrobial agent

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Introduction

Specific recognition of amino acids and tRNAs by aminoacyl-tRNA synthetases is an essential step to maintain the fidelity of protein synthesis. Thus, the abortive activity of the tRNA synthetases may be detrimental for cell viability. Aminoacyl-tRNA synthetases catalyze the reaction in two steps, first by activating amino acids by adenylation and subsequently by transferring the adenylated amino acids to their cognate tRNAs. The tRNA synthetases are grouped into two classes depending on the features of conserved sequence and structure. The class I enzymes are defined by the conserved sequences such as HIGH and KMSKS in the N-terminal catalytic domain that contains Rossman fold (Webster *et al.*, 1984; Hountondji *et al.*, 1986; Ludmerer & Schimmel, 1987; Burbaum *et al.*, 1990). The class II enzymes show conserved motifs at three locations and the catalytic domains contain antiparallel β-sheets that are distinct from Rossman fold (Cusack *et al.*, 1990; Eriani *et al.*, 1990; Ruff *et al.*, 1991).

Recognition of cognate tRNA by aminoacyl RNA synthetases is idiosyncratic. Alanyl- and histidyl-tRNA synthetases recognize the features of the acceptor stem (Hou & Schimmel, 1988; McClain & Foss, 1988) while the methionyl- and glutamyl-tRNA synthetases make major interactions with the anticodon of their respective tRNA substrates (Rould *et al.*, 1989; Ghosh *et al.*, 1990). Individual tRNA synthetases are further classified depending on the taxonomic domains of the species although horizontal gene transfers have been reported in several cases (Shiba *et al.*, 1997). Species specificity of aminoacylation varies depending on tRNA synthetases. Glycyl- and tyrosyl-tRNA synthetases (Hipps *et al.*, 1995; Quinn *et al.*, 1995) did not show cross-reactivity between prokaryotic and eukaryotic systems while glutaminyl-tRNA synthetase reacted with the cognate tRNAs of different species (Lamour *et al.*, 1994; Nureki *et al*, 1995).

Species-specific reaction mechanisms have been also shown by differential sensitivity of tRNA synthetases to active site inhibitors. Pseudomonic acid specifically inhibited prokaryotic isoleucyl-tRNA synthetase (Yanagisawa *et al.*, 1994) and similar cases have been reported in arginyl (Walter & Kuhlow, 1985) and prolyl-tRNA synthetases (Heacock *et al.*, 1996). We were interested in an active site inhibitor of MetRS. The native MetRS of *E. coli* is a homodimer of 676 amino acid protomer and the removal of the C-terminal appendix results in the active monomer (Cassio & Waller, 1971). The X-ray structure of the monomeric enzyme was determined at the resolution of 2.5 Å (Brunie *et al.*, 1990). While the C-terminal domain is involved in the interaction with the anticodon of met-tRNA as a major recognition site (Ghosh *et al.* 1990; Perona *et al*, 1991; Kim, 1995), the N-terminal domain is involved in catalytic reaction (Hountondji *et al.* 1990).

The active site residues involved in binding to amino acid were investigated by alignment-guided mutagenesis (Schmitt *et al.*, 1997) and affinity labeling in prokaryotic MetRS proteins (Gillet *et al.*, 1997). In this work, such residues of the *E. coli* MetRS were identified by using its amino acid substrate analogue. We previously synthesized a series of methionine derivatives and tested their capability of inhibiting aminoacylation activity of the *E. coli* MetRS (*Lee et al.* 1998). Among the tested compounds, L-methionine hydroxamate was most potent in inhibition of the enzyme activity and cell growth. It was thus used to investigate the inhibition mechanism, species specificity and to identify residues important for the catalytic activity.

Materials and Methods

Chemical synthesis of L-methionine hydroxamate A solution of N-Boc-L-methionine (1 g, 4 mmol) and N-methylmorpholine (1 ml, 9 mmol) in CH_2Cl_2 (10 ml) was cooled to -15 °C and slowly treated with isobutyl chloroformate (0.57 ml, 4.4 mmol). After stirring for 1 h at -15 °C, the reaction mixture was treated with hydroxylamine hydrochloride (0.235 g, 4.4 mol), and stirred at -15 °C for 1 h and then at room temperature for 16 h. The mixture was quenched with H_2O (30 ml) and extracted three times with CH_2Cl_2 (30 ml). The combined organic phase was washed with H_2O, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel with CH_2Cl_2:CH_3OH (9:1) to afford N-Boc-L-methionine hydroxamate as a white solid (0.423 g, 40%). Trifluoroacetic acid (0.5 ml) was added to a solution of N-Boc-L-methionine hydroxamate (264 mg, 1 mmol) in CH_2Cl_2 (2 ml). A solution was stirred for 20 h at room temperature and concentrated *in vacuo*. A residue was dissolved in distilled water and washed with ethylacetate several times. The aqueous solution was lyophilized to afford L-methionine hydroxamate as a stick yellow solid (140 mg, 50%). ^1H NMR (CD_3OD, 300 Hz) δ 3.83 (t, 1 H, J = 6.8 Hz, CHC=O), 2.5-2.6 (m, 2 H, $SC\underline{H_2}CH_2$), 2.0-2.2 (m, 5 H, CH_3S and $SCH_2C\underline{H_2}$), IR (KBr) 3420, 1675, $[\alpha]_D = +10.2$ (CH_3OH, c 1.03), MS (EI) m/z 165 (M^++1)

Preparation of MetRS proteins Phagemids pJB104 (Mellot *et al.*, 1989) and pSLM101 (Kim *et al.*, 1998) were used to express a monomeric MetRS (N547mer) of *E. coli* and the native form MetRS of *Mycobacterium tuberculosis*, respectively. The two proteins were induced by 1 mM IPTG and the recombinant MetRS proteins were extracted from the harvested cells by ultrasonication. The proteins were then precipitated by 30-50% ammonium sulfate. The precipitate was then redissolved in 50 mM potassium phosphate buffer, pH 7.35, and purified through DEAE-Sephadex column using NaCl gradient (0-0.5 M). The MetRS fractions were collected and concentrated by Centricon-30 (Amicon).

To isolate a gene encoding cytoplasmic MetRS of *Saccharomyces cerevisiae*, genomic DNA was prepared by the method previously described (Fasiolo *et al.*, 1981). The MetRS gene of *S. cerevisiae* was then isolated by PCR using the specific primers (5'CCGGATCCAATGTCTTTCCTCATTTCC/5'CCCAAGCTTGGGTCTTTTACTTT TTCTTCTTCC). The isolated gene was cloned into pET28a (Novagen) using *Bal*I and *Eco*RI to express the protein as His fusion protein. The recombinant cDNA clone encoding human cytoplasmic MetRS was provided by Dr. K. Shiba (Cancer Institute, Tokyo, Japan). The cDNAs for N-terminal truncated (N-terminal 267 amino acid truncated) form of human MetRS was isolated by PCR. The 1902 base pair PCR fragment was then subcloned into pET28a using *Eco*RI and *Hin*dIII and the protein were expressed as His fusion proteins. The recombinant His-tagged MetRSs of human and yeast were purified by nickel affinity column chromatography following the method described by manufacturer (Invitrogen).

Kinetic analysis Aminoadenylation activity of *E. coli* MetRS was determined by ATP-PPi exchange assay using [^{32}P]-labeled pyrophosphate. The reaction contained 100 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 10 mM KF, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.1 mg/ml bovine serum albumin (BSA), 10 mM methionine, 2 mM ATP and 2 mM [^{32}P] PPi. The reaction was initiated by addition of 1 nM MetRS proteins and quenched by mixing aliquots with 1 % activated charcoal suspended in ice cold 15 % $HClO_4$ containing 0.4 M PPi. The quenched reaction mixtures were filtered on glassfiber filter pads (Schleicher and Schuell, 2.4 cm) using vacuum manifold. The partially dried filters were added to the Hydrofluor (Packard) and the enzyme-bound radioactive methionine adenylate was quantitated by liquid scintillation.

Aminoacylation reaction was determined by adding 5 nM of various MetRS proteins to 20 mM HEPES, pH 7.5, 100 μM EDTA, 150 mM NH_4Cl, 1 mg/ml BSA, 2 mM ATP, 4 μM $tRNA^{fMet}$, 4 mM $MgCl_2$, 20 μM methionine, 0.2 μM [^{35}S] methionine. Aliquots were taken from the reaction and mixed with 10 % trichloroacetic acid containing 2 mM methionine on Whatman 3 MM filter pad (2.3 cm) to quench the reaction. The aminoacylated $tRNA^{Met}$ was quantitated by liquid scintillation counter in 5 ml Betafluor. Kinetic analysis for the effect of L-methonine

hydroxamate on aminoacylation were carried out by varying concentrations of methonine (5-160 μM) and methionine hydroxamate (3.75-240 μM).

Results

MetRS catalyzes the reaction between methionine and ATP to form the reaction intermediate, methionine adenylate. This intermediate is subsequently transferred to the acceptor end of the cognate tRNA. In theory, compounds structurally mimicking one of the substrates or reaction intermediate have a potential to interfere with the reaction process. We previously synthesized methionine derivatives and tested whether they affect the aminoacylation activity of *E. coli* MetRS (Lee *et al.* 1998). Among the tested compounds, L-methonine hydroxamate (Fig. 1) showed the most significant inhibition effect.

To understand the molecular mechanism for the inhibition effect of L-methonine hydroxamate, kinetic analysis was carried out (Fig. 2B). We thought that L-methonine hydroxamate would inhibit aminoacyl adenylation reaction because it contains methionine residue that would compete with methionine substrate for the binding to MetRS. To our surprise, the aminoacyl adenylation reaction was not affected by L- methonine hydroxamate (Fig. 2A). However, L-methonine hydroxamate showed a competitive inhibition to the aminoacylation of $tRNA^{Met}$ (Fig. 2B). The estimated K_i of L-methonine hydroxamate was 19.6 μM. These results suggest that L-methonine hydroxamate interferes with the reaction process after the formation of methionyl adenylate.

We previously showed that L- but not D-form of methionine hydroxamate showed inhibition effect on the cell growth of a few microorganisms (Lee *et al.*, 1998). Here, we tested whether the growth inhibition effect of L-methionine hydroxamate could be compensated by the competition by methionine or by high dosage of the *E. coli* MetRS. LD_{50} of L-methonine hydroxamate on the *E. coli* cells in M9 minimal medium was 0.1 μg/ml. However, LD_{50} was increased to 16.7 and 17.3 μg/ml, respectively when the cells were grown in the M9 minimal medium containing 400 μg/ml methionine or in LB rich medium. LD_{50} of L-methionine hydroxamate to the cells overexpressing the recombinant *E. coli* MetRS was also increased to 19 μg/ml in M9 minimal medium. These results confirm that the lethal effect of L-methionine hydroxamate is related to methionine substrate and MetRS.

We then investigated whether the reaction mechanism of MetRS is conserved between MetRSs of different species by comparing the effect of L-methonine hydroxamate on the aminoacylation activities. The purified MetRS proteins of *Mycobacterium tuberculosis*, *Saccharomyces cerevisiae* and human (cytoplasmic forms) showed the similar degree of sensitivity to L-methonine hydroxamate as the *E. coli* MetRS (Fig. 3), suggesting that the reaction mechanism and the active site structures are conserved.

Discussion

Analogues of enzyme substrates or reaction intermediates can be a useful probe to investigate the molecular mechanism of the reaction as well as to identify functionally important residues. In the present work, a methionine derivative, L-methionine hydroxamate, was used to determine whether the reaction mechanism was conserved among MetRSs of different species and to identify the amino acid residues important for the catalysis of *E. coli* MetRS

The overall sequence homology of different MetRS proteins is in the range of 21-26%. The yeast (Walter *et al.*, 1989) and human (Lage & Dietel, 1996, *Rho* et al, 1999) cytoplasmic MetRS proteins contain unique N-terminal extensions that are not found in other prokaryotic MetRS proteins. Nevertheless, these enzymes still contain the conserved sequence elements. They were also active to the *E. coli* $tRNA^{Met}$ and sensitive to L-methionine hydroxamate to the similar level as the *E. coli* MetRS (Fig. 3), suggesting that the reaction mechanism is well conserved among different MetRSs.

Since aminoacyl-tRNA synthetases are essential for cellular protein synthesis, inhibition of

any single tRNA synthetase would give a detrimental effect on cell viability. As expected, L-methionine hydroxamate inhibited the growth of *E. coli* cells. This result also suggests that tRNA synthetases can be a molecular target to develop a growth controlling agent and structural diversity of tRNA synthetases can be explored to enhance specificity to a target enzyme.

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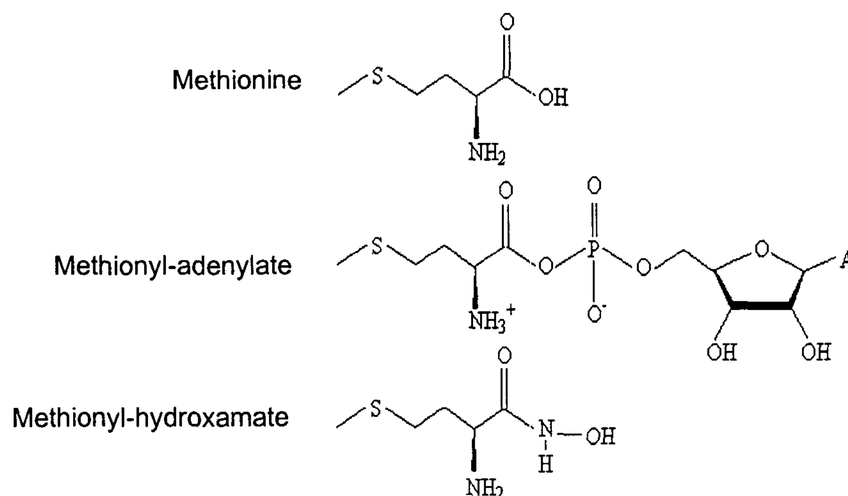

Fig. 1. Chemical structure of methionine hydroxamate. “A” in the methionyl adenylate indicates adenine base connected to ribose.

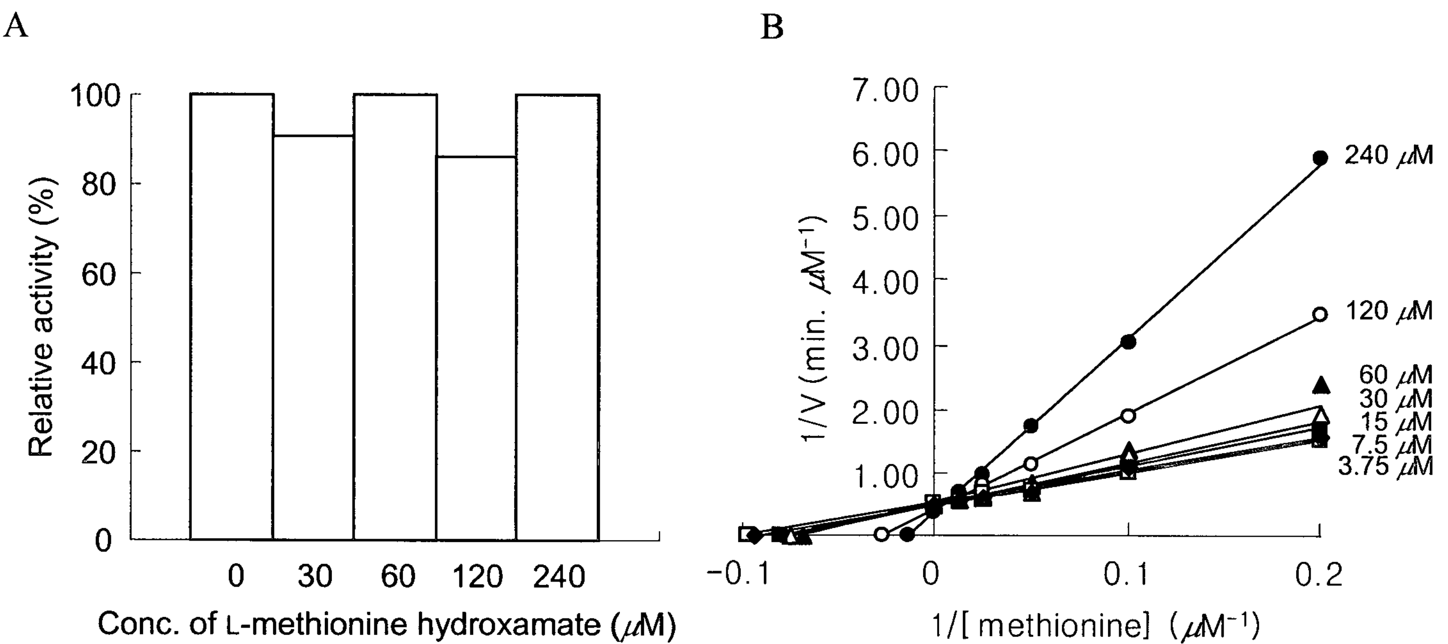

Fig. 2. The effect of L-methionine hydroxamate on aminoadenylation and aminoacylation reactions of the *E. coli* MetRS. A. Aminoadenylation reaction catalyzed by the *E. coli* MetRS was monitored by ATP-PPi assay as described in Materials and Methods. B. Lineweaver-Burk plot of aminoacylation reaction of the *E. coli* MetRS in the presence of varying concentrations of the *E. coli* MetRS (5 nM) was used in the aminoacylation reaction in the presence of [^{35}S]-labeled methionine. The concentrations of the added L-methionine hydroxamate were 3.75 (□), 7.5 (◆), 15 (■), 30 (△), 60 (▲), 120 (○), and 240 *m*M (●).

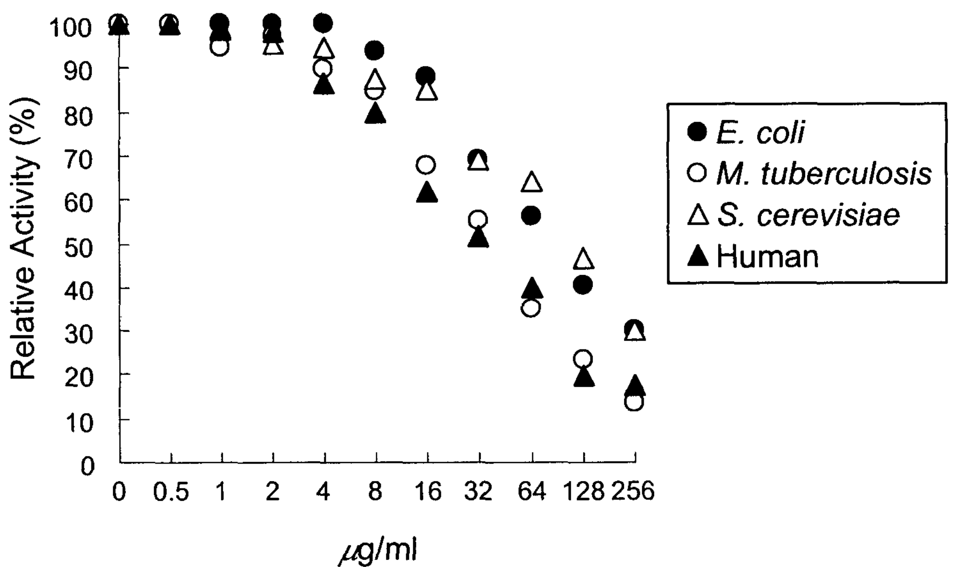

Fig. 3. L-Methionine hydroxamate inhibits MetRSs isolated from different species. Different concentrations of L-methionine hydroxamate were added to the amino- acylation reaction carried out by the recombinant human, *S. cerevisiae* cytoplasmic, *M. tuberculosis* and *E. coli* MetRS proteins and the inhibition effects of L-methionine hydroxamate were compared.