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Methionine Analogue Probes Functionally Important Residues in Active Site of Methionyl-tRNA Synthetase

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Aminoacyl-tRNA synthetases are essential enzymes catalyzing the attachment of specific amino acids to cognate tRNAs. In the present work, the substrate analogue L-methionine hydroxamate was used to identify functional residues located in the active site of the *E. coli* methionyl-tRNA synthetase (MetRS). This compound inhibited bacteria, yeast, and human MetRS activities to a similar degree, suggesting a conserved active site structure and mechanism between MetRSs of different phylogenetic domains. Mutants of the *E. coli* MetRS resistant to methionine hydroxamate were also isolated. These mutants contained a substitution either at T10, Y15, or Y94. These residues are highly conserved among the different MetRSs and the mutants showed decreased aminoacylation activity, suggesting their functional and structural significances. The putative roles of these residues are discussed on a structural basis.

Keywords: Active site, Functional residues, L-Methionine hydroxamate, Methionyl-tRNA synthetase.

Introduction

Specific recognition of amino acids and tRNAs by aminoacyl-tRNA synthetases is an essential step in maintaining the fidelity of protein synthesis. Thus, the abortive activity of tRNA synthetases may be detrimental for cell viability. Aminoacyl-tRNA synthetases catalyze the reaction in two steps: first by activating amino acids through 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. Class I enzymes are defined by the conserved sequences such as HIGH and KMSKS in the N-terminal catalytic domain that contains a Rossman fold (Webster *et al.*, 1984; Hountondji *et al.*, 1986; Ludmerer and Schimmel, 1987; Burbaum *et al.*, 1990). Class II enzymes show conserved motifs at three locations and the catalytic domains contain antiparallel β-sheets that are distinct from the Rossman fold (Cusack *et al.*, 1990; Eriani *et al.*, 1990; Ruff *et al.*, 1991).

Recognition of cognate tRNA by aminoacyl-tRNA synthetases is idiosyncratic. Alanyl- and histidyl-tRNA synthetases recognize the features of the acceptor stem (Hou and Schimmel, 1988; McClain and 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) do not show cross-reactivity between prokaryotic and eukaryotic systems, while glutaminyl-tRNA synthetase may react with the cognate tRNAs of different species (Lamour *et al.*, 1994; Nureki *et al.*, 1995).

Species-specific reaction mechanisms have also been shown by differential sensitivity of tRNA synthetases to active-site inhibitors. Pseudomonic acid specifically inhibits prokaryotic isoleucyl-tRNA synthetase (Yanagisawa *et al.*, 1994) and similar cases have been reported with arginyl-(Walter and Kuhlow, 1985) and prolyl-tRNA synthetases (Heacock *et al.*, 1996). We have

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been interested in an active site inhibitor of MetRS. The native MetRS of *E. coli* is a homodimer of a 676 amino acid protomer, and the removal of the C-terminal appendix results in the active monomer (Cassio and Waller, 1971). The X-ray structure of the monomeric enzyme has been determined at a 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 the 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, active-site residues of *E. coli* MetRS were identified by using an amino acid substrate analogue. We previously synthesized a series of methionine derivatives and tested their capability of inhibiting aminoacylation activity of *E. coli* MetRS (Lee *et al.*, 1998a). Among the tested compounds, L-methionine hydroxamate was most potent in the inhibition of enzyme activity and cell growth. It was thus used to investigate the inhibition mechanism and species specificity, and to identify residues important for 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), stirred for 20 h at room temperature and concentrated *in vacuo*. The residue was dissolved in distilled water and washed with ethyl acetate several times. The aqueous solution was lyophilized to afford L-methionine hydroxamate as a sticky 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, $SC\underline{H}_2CH_2$), 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 the monomeric MetRS (N547mer) of *E. coli* and the native MetRS of *Mycobacterium tuberculosis*, respectively. Expression of the two proteins was induced by 1 mM IPTG, and the recombinant MetRS proteins were extracted from the harvested cells by ultrasonication. Proteins were then precipitated by 30–50% ammonium sulfate. The precipitate was redissolved in 50 mM potassium phosphate buffer, pH 7.35, and purified through a DEAE-Sephadex column using a NaCl gradient (0–0.5 M). The MetRS fractions were collected and concentrated using Centricon-30 (Amicon).

To isolate the gene encoding cytoplasmic MetRS of *Saccharomyces cerevisiae*, genomic DNA was prepared by a method previously described (Fasiolo *et al.*, 1981). The MetRS gene of *S. cerevisiae* was then isolated by PCR using specific primers (5′-CCGGATCCAATGTCTTTCCTCATTTCC-3′/5′-CCCAAGCTTGGGTCTTTTACTTT TTCTTCTTCC-3′). The isolated gene was cloned into pET28a (Novagen) using *Bam*HI and *Eco*RI to express the protein as a His fusion protein. The recombinant cDNA clone encoding human cytoplasmic MetRS was provided by Dr. K. Shiba (Cancer Institute, Tokyo, Japan). The cDNA for the N-terminal truncated (N-terminal 267 amino acids truncated) form of human MetRS was isolated by PCR. The 1902 bp PCR fragment was then subcloned into pET28a using *Eco*RI and *Hind*III and the proteins 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 the manufacturer (Invitrogen).

Kinetic analysis Aminoadenylation activity of *E. coli* MetRS was determined by ATP-PPi exchange assay using [^{32}P]-labeled pyrophosphate (PPi). 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 the 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 glass fiber filter pads (Schleicher and Schuell, 2.4 cm) using a vacuum manifold. The partially-dried filters were added to Hydrofluor (Packard) and the enzyme-bound radioactive methionine adenylate was quantitated by liquid scintillation.

Aminoacylation reactions were performed 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, and 0.2 μM [^{35}S] methionine. Aliquots were taken from the reaction and mixed with 10% trichloroacetic acid containing 2 mM methionine on a Whatman 3 MM filter pad (2.3 cm) to quench the reaction. The aminoacylated $tRNA^{Met}$ was quantitated by liquid scintillation counting in 5 ml Betafluor. Kinetic analysis for the effects of L-methionine hydroxamate on aminoacylation was carried out by varying concentrations of methionine (5–160 μM) and methionine hydroxamate (3.75–240 μM).

Isolation of resistant mutants *E. coli* strain JM109 was inoculated in LB broth containing 40 μg/ml L-methionine hydroxamate and cultivated at 37°C until the cells reached the stationary growth phase. The mature cells were then transferred into fresh LB broth containing L-methionine hydroxamate. The cells resistant to L-methionine hydroxamate were enriched by serial transfer of the mature cells to fresh broth containing L-methionine hydroxamate for three times. Cells were harvested

after the repeated enrichment. The chromosomal *metG* genes were isolated by PCR from the L-methionine hydroxamate-resistant cells and cloned into pBluescript KS+ (Stratagene) using *Eco*RI and *Xba*I restriction sites. Mutations were confirmed by sequencing of the *metG* genes.

Results

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

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

We previously showed that the L- but not the D-form of methionine hydroxamate inhibited the cell growth of a few microorganisms (Lee *et al.*, 1998a). Here, we tested whether the growth inhibition effect of L-methionine hydroxamate could be compensated by competition with methionine or by high dosage of *E. coli* MetRS. The LD_{50} of L-methionine hydroxamate for *E. coli* cells in M9 minimal medium was 0.1 μg/ml. However, the LD_{50} was

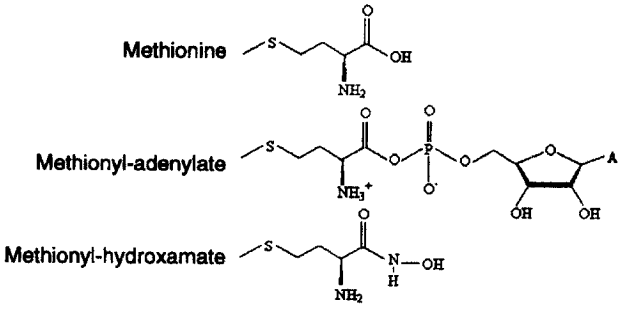

Fig. 1. Chemical structure of methionine hydroxamate. "A" in the methionyl adenylate indicates the adenine base connected to ribose.

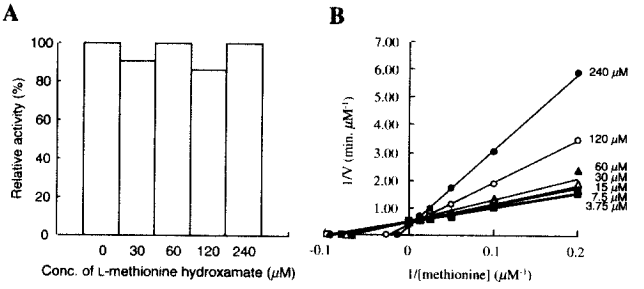

Fig. 2. The effect of L-methionine hydroxamate on aminoadenylation and aminoacylation reactions of the *E. coli* MetRS. **A.** Aminoadenylation reaction catalyzed by the *E. coli* MetRS was monitored by ATP-PPi assay as described in Materials and Methods. **B.** Lineweaver-Burk plot of aminoacylation reactions of *E. coli* MetRS in the presence of varying concentrations of methionine hydroxamate, *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 μM (●).

increased to 16.7 and 17.3 μg/ml, respectively, when cells were grown in M9 minimal medium containing 400 μg/ml methionine or in LB rich medium. The LD_{50} of L-methionine hydroxamate for 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 the 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-methionine hydroxamate on the aminoacylation activities. The purified MetRS proteins of *Mycobacterium tuberculosis*, *Saccharomyces cerevisiae*, and human (cytoplasmic forms) showed a similar degree of sensitivity to L-methionine hydroxamate as *E. coli* MetRS (Fig. 3), suggesting that the reaction mechanism and the active site structures are conserved.

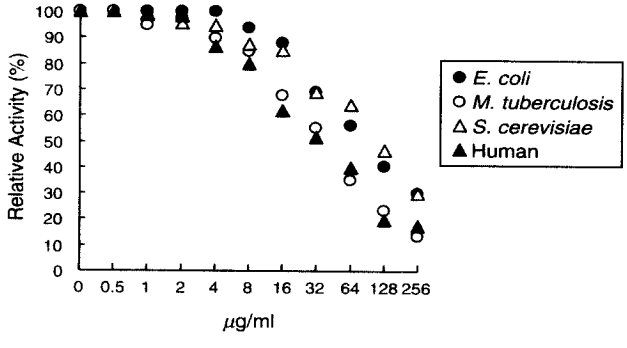

Fig. 3. L-Methionine hydroxamate inhibits MetRSs isolated from different species. Different concentrations of L-methionine hydroxamate were added to the aminoacylation reaction carried out by the recombinant human, *S. cerevisiae* cytoplasmic, *M. tuberculosis*, and *E. coli* MetRS proteins.

To determine the amino acid residues involved in methionine adenylation, the mutants resistant to L-methionine hydroxamate were selected by a sequential enrichment of cells growing in the presence of L-methionine hydroxamate. The selected mutants contained substitutions of T10M, Y15A, or Y94H. These three residues are well conserved among different MetRSs (Fig. 4). The activity and stability of these mutants were tested by genetic complementation and immunoblotting, respectively. The *E. coli* host MJR mutants contain a defective MetRS that poorly binds to methionine and thus cannot grow on methionine-depleted minimal media (Starzyk and Schimmel, 1989). If the mutant is active in aminoacylation, it will complement and support the cell growth on minimal media. Mutants T10M and Y15A, but not Y94H, retained the activity in genetic complementation. The stability test determined by immunoblotting showed that the mutant Y94H was unstable, suggesting that Y94 is important in maintaining the native MetRS conformation (Fig. 5A). However, mutants T10M and Y15A showed wild-type stability, indicating that these residues are not important for the MetRS structure. Mutants T10M and Y15A were purified and compared for aminoacylation activity and resistance to L-methionine hydroxamate treatment. The activity of the Y15A mutant was barely detectable and the T10M mutant showed about 5% of wild-type activity, suggesting that these residues are critical for the enzyme activity (Fig. 5B).

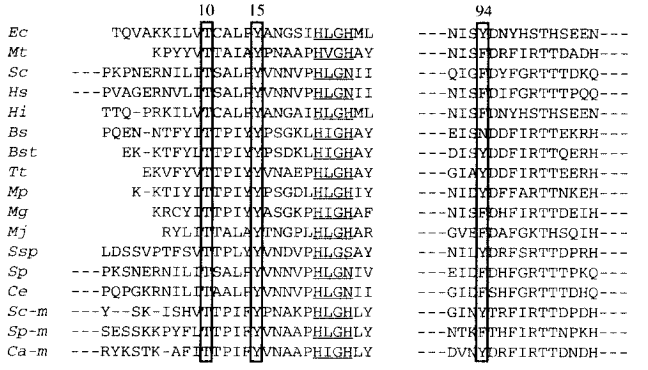

Fig. 4. Alignment of MetRS proteins at the mutation sites. The sequences of MetRS proteins from various species were aligned using the MACAW program (Karlin and Altschul, 1990) and the aligned sequences including the mutated residues are shown. Ec (*E. coli*, D10483), Mt [*M. tuberculosis*, (Kim *et al.*, 1998)], Sc (*S. cerevisiae* cytoplasmic, J01339), Hs (*H. sapiens* cytoplasmic, X94754), Hi (*H. influenza*, HI1276), Bs (*B. subtilis*, D26185), Bst (*B. stearothermophilus*, X57925), Tt (*T. thermophilus*, M64273), Mp (*M. pneumoniae*, U00089), Mg (*M. genitalium*, B64238), Mj (*M. jannaschii*, Q58357), Ssp (*Synechocystis sp.*, D90907), Sp (*S. pombe* cytoplasmic, Z98978), Ce (*C. elegans* mitochondrial, Z81038), Sc-m (*S. cerevisiae* mitochondrial, GI135147), Sp-m (*S. pombe* mitochondrial, D1022268), Ca-m [*C. albicans* mitochondrial, (Lee *et al.*, 1998b)].

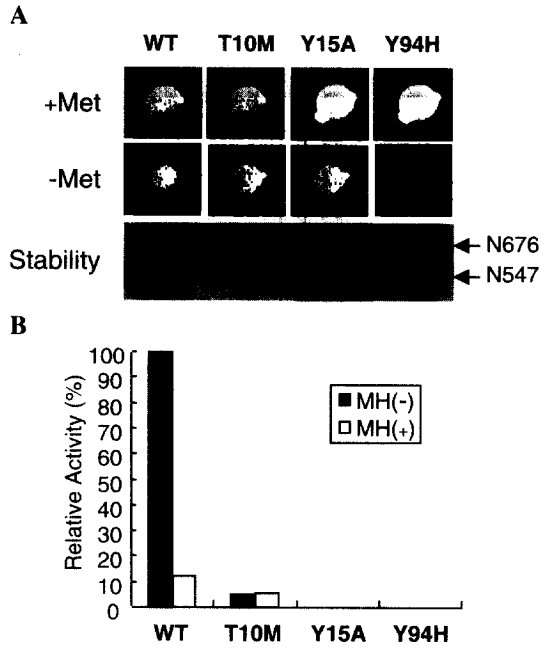

Fig. 5. Complementation of the *E. coli* MetRS mutants and effect of L-methionine hydroxamate on wild-type and mutant MetRSs. **A.** *E. coli* tester strain MJR containing plasmids encoding wild-type, T10M, and Y15A mutants were inoculated on M9 minimal media with or without methionine. The MJR strain is a methionine auxotroph because its MetRS is defective in binding to methionine. The cell growth on M9 minimal media without methionine indicates that the exogenous MetRS is active in aminoacylation. **B.** The relative aminoacylation activities of wild-type and mutant MetRSs are shown in the absence (open bar) and presence (solid bar) of L-methionine hydroxamate (200 μg/ml).

The activity of the T10M mutant was not affected by L-methionine hydroxamate as expected.

The kinetic characteristics of this mutant were further analyzed. The values of K_m for methionine and k_{cat} for aminoacylation of the wild-type MetRS were determined to be 7.9 μM and 5.6 s^{-1}, respectively. The aminoacylation activity of the wild-type MetRS was decreased to 12.2% by treatment with L-methonine hydroxamate (200 μg/ml). The T10M mutant showed a K_m increase to 58 μM for methionine and a k_{cat} decrease to 3.4 s^{-1} (Table 1), implying that T10 is involved in amino acid binding.

Table 1. Kinetic parameters for aminoacylation of the wild-type and T10M mutant of *E. coli* MetRS.

	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m	Relative k_{cat}/K_m
Wild-type	7.9	5.6	0.71	1.00
T10M Mutant	58.0	3.4	0.06	0.08

Aminoacylation reactions were carried out by adding 5 nM of the purified *E. coli* MetRS at 37°C as described previously (Kim and Schimmel, 1992).

Discussion

Analogues of enzyme substrates or reaction intermediates can be useful probes to investigate the molecular mechanism of a 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.

Since L-methionine hydroxamate contains a methionine residue, its binding sites were expected to be near to or overlap with those involved in methionine binding. The mutants isolated by their resistance to L-methionine hydroxamate showed substitutions at T10 and Y15 that are well conserved among different MetRSs (Fig. 4) and located in the first β-sheet of the Rossman fold (Fig. 6, labeled red). The artificial cleavage of this peptide region at Y15 inactivated the *E. coli* MetRS (Kim and Kim, 1997), implying its functional importance. Residues P14, Y15, R233, V298, H301, and W305 are known to be involved in binding to methionine (Fourmy *et al.*, 1991; Ghosh *et al.*, 1991; Schmitt *et al.*, 1997) and are clustered within 20 Å along the first β-sheet (Fig. 6). In particular, it was suggested that W305 and Y15 are responsible for recognition of the methionine residue. Interestingly, the aromatic ring of Y15 needs to be moved to link its hydroxyl group to the sulfur atom of methionine *via* a hydrogen bond (Kim *et al.*, 1993). Although L- methionine hydroxamate does not bind to Y15 of the naked MetRS due to its hydroxylamine moiety, it can bind to Y15 and T10 when the active site of MetRS is opened by binding to methionine and ATP. This interpretation is consistent with the experimental results that L-methionine hydroxamate did not affect the aminoacyl adenylation reaction but rather inhibited the subsequent aminoacylation reaction (Fig. 2A). Y94 is tightly connected to residues in other peptide regions such as Y91, A203, and R206. Thus, mutation at this residue would affect the stability of the protein as observed in this work (Fig. 5A).

The overall sequence homology of different MetRS proteins is in the range of 21–26%. Yeast (Walter *et al.*, 1989) and human (Lage and 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 at similar levels 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 serve as molecular targets to develop growth controlling agents. The structural diversity of tRNA synthetases can also be explored to enhance specificity to a target enzyme.

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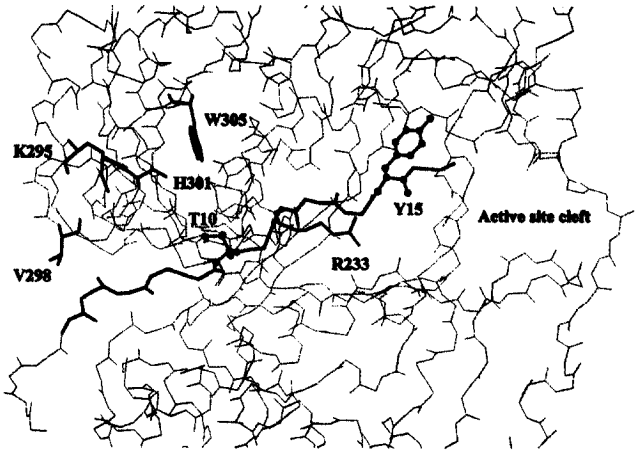

Fig. 6. Locations of T10 and Y15 in the *E. coli* MetRS structure. Residues of T10 and Y15 (marked red) are located in the first β-strand (marked red) of the Rossman fold. P14, R233, V298, H301, and W305 (marked blue) and K295 (marked yellow) were reported to be involved in the interaction with methionine and in the transfer of methionine adenylate to the acceptor of the bound tRNA, respectively.

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