Biochemical and phylogenetic analyses of methionyl-tRNA synthetase isolated from a pathogenic microorganism, *Mycobacterium tuberculosis*

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Abstract ***Mycobacterium tuberculosis*** **methionyl-tRNA synthetase (MetRS) has been cloned and characterized. The protein contains class I signature sequences but lacks the Zn^{2+} binding motif and the C-terminal dimerization appendix that are found in MetRSs from several organisms including *E. coli* MetRS. Consistent with these features, the enzyme behaved as a monomer in a gel filtration chromatography and did not contain the bound Zn^{2+}. Nonetheless, it was active to the $tRNA^{Met}$ of *E. coli* as determined by in vivo genetic complementation and in vitro reaction. Phylogenetic analysis separated the *M. tuberculosis* and *E. coli* MetRSs into prokaryote and eukaryote-archaea group, respectively. This result is consistent with the taxonomic locations of the organism but is an interesting contrast to the case of its paralogous protein, isoleucyl-tRNA synthetase, and suggests that the two enzymes evolved in separate idiosyncratic pathways.**

Key words: Methionyl-tRNA synthetase; Zn^{2+} binding motif; Dimerization; Phylogeny; *Mycobacterium tuberculosis*

1. Introduction

The aminoacyl-tRNA synthetases (aaRSs) catalyze the first step in protein synthesis by covalently linking tRNA with its cognate amino acid. Comparison of sequences and structural information of these proteins from other organisms emphasizes the tremendous divergence of this family of enzymes despite their common functions [1–3]. Mechanistic distinctions among the tRNA synthetases [4] are further emphasized, for example, through species-specific RNA interactions which bar cross-aminoacylation between eukaryotic and prokaryotic tRNA and tRNA synthetase systems [5–7].

M. tuberculosis is an opportunistic agent that has re-emerged resistant to multiple prominent anti-tuberculosis treatments, promoting a search for new drugs [8]. The sequence and structural divergence of the tRNA synthetases between organisms highlight these essential enzymes as ideal targets to develop novel antibiotic agents. *M. tuberculosis* isoleucyl-tRNA synthetase (IleRS) was previously cloned, but surprisingly the protein sequence was determined to be eukaryotic-like [9] and, moreover, resistant to the prokaryotic IleRS-targeted antibiotic, pseudomonic acid [10,11]. IleRS is closely related to several paralogous tRNA synthetases including the methionine, valine, leucine, and cysteine enzymes [12] which have been suggested to have evolved by gene duplication before the separation of the three taxonomic domains [13]. Herein we report the cloning and characterization of *M. tuberculosis* MetRS. Comparative sequence and structural analyses of the protein revealed interesting features in its activity and evolutionary pathway.

2. Materials and methods

Two combinations of degenerate primers were designed from aligned MetRS sequences [14] to amplify a MetRS gene fragment from *M. kansasii* chromosomal DNA by polymerase chain reaction (PCR). The cloned PCR fragment was used to screen a λgt11 *M. tuberculosis* library. The cloned *M. tuberculosis* MetRS gene was engineered and cloned into pBluescript II SK^+ (Stratagene) using *Bam*HI and *Eco*RI. The resulting recombinant pSLM101 expressed the MetRS as an N-terminal β-galactosidase fusion protein. The protein was purified by $(NH_4)_2SO_4$ fractionation and DEAE-Sephadex chromatography [15]. The C-terminal truncated form of *E. coli* MetRS was purified from *E. coli* expressing pJB104 as described previously [16]. The kinetic parameters for both proteins were measured by in vitro aminoacylation of *E. coli* $tRNA^{fMet}$ (Sigma) [17]. In vivo aminoacylation activity of the *M. tuberculosis* MetRS was conducted using a *E. coli* strain MJR containing a defective MetRS that has a high K_m for methionine [18]. The recombinant plasmid, pSLM101, was introduced into MJR to test whether the *M. tuberculosis* MetRS rescues the methionine auxotrophy of the mutant strain. The sequence of the *M. tuberculosis* MetRS was aligned with MetRS and IleRS sequences from various organisms using the PILEUP program [19]. Gaps and less conserved sub-regions were deleted manually and the remaining 298 residues were used for construction of a phylogenetic tree. The maximum parsimony method was employed using the PROTPARS program that was provided in the PHYLIP program package [20]. A tree was also constructed by neighbor-joining using PROTDIST (by invoking the Dayhoff program option) and NEIGHBOR programs from the PHYLIP. Bootstrap analyses were performed with 500 replicates using SEQBOOT and CONSENSE programs.

The enzyme bound Zn^{2+} ion was determined using the sulfhydryl-specific reagent *p*-hydroxylmercuryphenylsulfonate (PMPS, Sigma), which reacts with cysteines and forms a 250-nm complex, simultaneously releasing Zn^{2+} into solution [21]. The free Zn^{2+} is trapped with 4-(2-pyridylazo)resorcinol (PAR, Sigma) that may be quantitatively detected at 500 nm [22,23]. The quaternary structure of the protein was determined by high-performance liquid chromatography (HPLC) analysis (Gynkotek) using size exclusion column (TSK3000SW).

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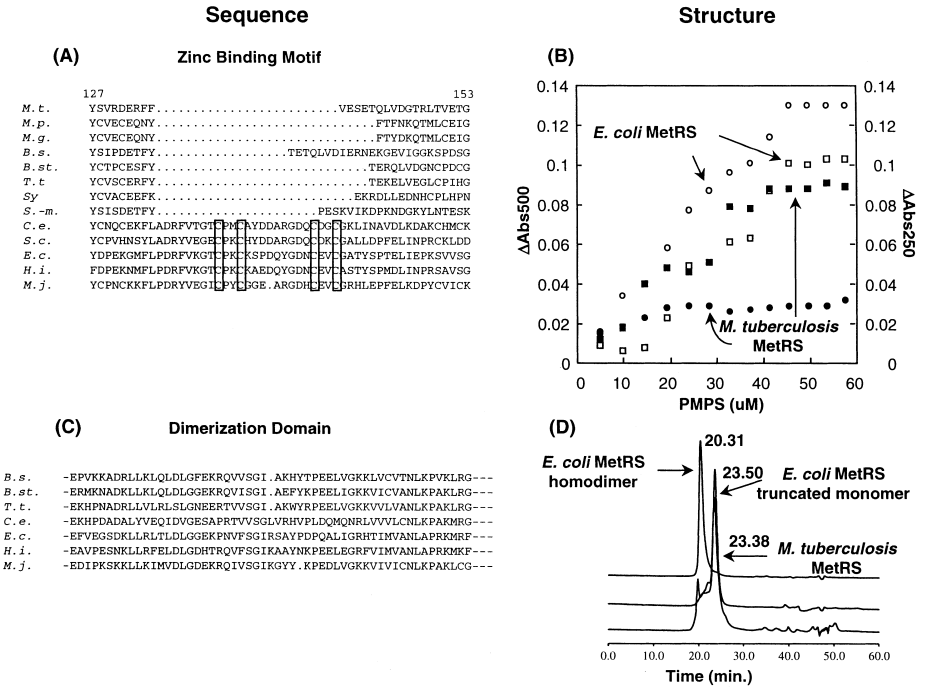

Fig. 1. Sequence and structure of *M. tuberculosis* MetRS in the regions of Zn^{2+} binding motif and C-terminal appendix. The *M. tuberculosis* MetRS lacks (A) the Zn^{2+} binding motif and (C) the C-terminal dimerization appendix. The sequence alignment of MetRSs from various organisms was made by the PILEUP program. The sequence numbers correspond to the amino acids of the *M. tuberculosis* MetRS. Cysteines in the Zn^{2+} binding motif were highlighted by boxes. B: The enzyme bound Zn^{2+} was dissociated by chemical modification of cysteine using PMPS which was monitored by the absorbance change at 250 nm (squares). The released Zn^{2+} was measured by the absorbance change at 500 nm (circles). The Zn^{2+} release was detected from the *E. coli* MetRS according to the amount of PMPS but not from the *M. tuberculosis* MetRS. D: The quaternary structure of *M. tuberculosis* was determined by high pressure liquid chromatography using sizing column. The *M. tuberculosis* MetRS contains 32 extra amino acids at its N-terminal end originating from β-galactosidase, resulting in a 551 amino acid polypeptide. The native homodimer (676-mer protomer) and truncated monomer (547-mer) of the *E. coli* MetRS were used as size references. The elution profile of the *M. tuberculosis* MetRS suggests that it is a monomer.

3. Results and discussion

3.1. Sequence and structural characteristics

An open reading frame of 1557 base pairs encoding 519 amino acids was isolated from the genomic library of *M. tuberculosis*. The N-terminal sequence which folds into the Rossmann nucleotide binding pocket contains the conserved class I signature sequence motifs as HVGH and KMSKS and the C-terminal sequence contains proline and tryptophan residues which are conserved across most of the MetRS proteins (data not shown). However, the *M. tuberculosis* MetRS showed contrasting characteristics compared to that of *E. coli*. The *M. tuberculosis* MetRS as well as other parallel enzymes from Gram-positive bacteria and cyanobacteria lack an inserted peptide within the CP1 (connective polypeptide 1) region [24] that is found in those from eukaryote, archaea, *E. coli* and *H. influenzae* (Fig. 1A). This CP1 region contains four cysteines involved in binding to Zn^{2+} that was shown to be important to maintain the active conformation of the catalytic site [21,23,25]. We predicted that the *M. tuberculosis* lacked a bound Zn^{2+} and thus tested for its presence using the sulfhydryl-specific reagent, PMPS, which reacts with cysteines to form a 250-nm complex, while releasing free Zn^{2+} into solution. The released Zn^{2+} was trapped with PAR that may be quantitatively detected at 500 nm [22,23]. While Zn^{2+} [22,23] was released from the *E. coli* MetRS by the treatment of PMPS, it was not observed for the *M. tuberculosis* MetRS (Fig. 1B), supporting the absence of a Zn^{2+} binding pocket.

The C-terminal region, which contains the oligomerization domain of the protein, is extremely divergent among all of the MetRSs. The *T. thermophilus* and *B. stearothermophilus* enzymes are the only MetRSs that exhibit any significant homology in their dimerization regions. Although the *M. tuberculosis* MetRS is most closely related to these enzymes, it lacks the entire dimerization domain (Fig. 1C). Specifically, the polypeptide contains only 519 amino acids, and thus is significantly shorter than the other bacterial MetRS proteins which range from 616 to 677 amino acids. The quaternary structure of the protein was determined by chromatography using a gel filtration column. The native homodimer (676 amino acid protomer) [26] and genetically truncated monomer (547 amino acids) [27] of the *E. coli* MetRS were used as a reference. The dimeric and monomeric *E. coli* proteins were eluted from the column at 20.31 and 23.50 min, respectively.

Table 1
Kinetic parameters for aminoacylation of *E. coli* $tRNA^{fMet}$

MetRS	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m	Relative k_{cat}/K_m
E. coli	1.0	6.85	6.85	1
M. tuberculosis	3.2	2.83	0.88	0.13

Each reaction contained 5 nM of MetRS and 0.1–80 μM *E. coli* $tRNA^{fMet}$ and was carried out at 37°C as described previously [17].

M. tuberculosis MetRS was eluted at 23.38 min, suggesting a monomeric protein (Fig. 1D).

3.2. In vivo and in vitro aminoacylation activity

The Zn^{2+} binding to MetRS was previously shown to be important for the enzyme activity [21,23,25] and the C-terminal dimerization appendix is also involved in the interaction with the acceptor stem of the bound tRNA [28]. We thus investigated whether the *M. tuberculosis* MetRS lacking these two peptide motifs is active in aminoacylating initiator tRNA of *E. coli*. As shown in Table 1, the K_m and k_{cat} for the recombinant *M. tuberculosis* MetRS were, respectively, about 3-fold higher and 2.5-fold lower compared to the *E. coli* MetRS. In vitro aminoacylation experiments clearly showed that *M. tuberculosis* MetRS recognizes *E. coli* $tRNA^{fMet}$. This result suggests that there should be alternative mechanisms to yield a stable and active MetRS enzyme although the Zn^{2+} binding motif within the CP1 domain is conserved among a number of MetRS. Clearly, since a subset of the MetRSs (Fig. 1A) lacks the Zn^{2+} binding motif, these enzymes have adopted a different conformation, perhaps because of distinct evolutionary pressures, to maintain an active catalytic domain.

Because *E. coli* uses an initiator and elongator tRNA for a single methionine codon, the in vitro data did not conclude whether the enzyme specifically recognizes both types of $tRNA^{Met}$. We thus performed in vivo complementation tests using a methionine auxotroph *E. coli* strain, MJR. The auxotrophy of this strain is due to the increased K_m of its MetRS for methionine. The introduction of pSLM101 expressing the recombinant *M. tuberculosis* MetRS rescued the methionine-dependent *E. coli* strain, indicating the *M. tuberculosis* MetRS can specifically charge both the initiator and elongator *E. coli* $tRNA^{Met}$ (Fig. 2).

3.3. Phylogenetic relationship

While the *M. tuberculosis* MetRS contains the class I-defining signature sequences, it lacks a Zn^{2+}-binding motif and the C-terminal dimerization appendix (Fig. 1). An evolutionary path of MetRS was deduced through a phylogenetic tree based on 13 MetRS and 17 IleRS protein sequences. Because MetRS and IleRS have significant N-terminal sequence similarities in the catalytic domain, an initial alignment was easily generated by the PILEUP program. A phylogenetic tree was constructed from the alignment using a parsimony method described in Section 2.

The resulting phylogenetic tree of these proteins placed the *M. tuberculosis* MetRS and IleRS into different lines of evolution (Fig. 3, boxed). While the *M. tuberculosis* IleRS was more eukaryotic-like as previously described [9,29], the MetRS was closely related to the eubacterial type. The grouping of *M. tuberculosis* MetRS to the eubacterial MetRSs is convincing since the branch between eubacteria and eukaryote-archaea group exhibits a 100% bootstrap value. We also obtained a similar tree by neighbor-joining method (data not shown). The tree placed the *M. tuberculosis* MetRS in the clade consisting of *S. cerevisiae* mitochondrial, *M. genitalium*, *M. pneumoniae*, *B. subtilis*, *B. stearothermophilus*, *Synechocystis* sp. and *T. thermophilus* MetRSs. Thus, *M. tuberculosis* MetRS and IleRS have evolved in different pathways, although they may have originated from a common ancestor by gene duplication before the diversification of eubacteria, archaea and eukarya.

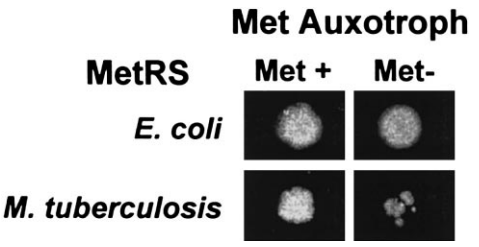

Fig. 2. Complementation of *M. tuberculosis* MetRS in *E. coli*. Recombinant plasmids expressing the *M. tuberculosis* MetRS (pSLM101) and the active monomeric *E. coli* MetRS (pJB104) were introduced into the methionine auxotroph, *E. coli* MJR strain. The in vivo activity of the plasmid-encoded proteins was assessed by the ability to rescue the auxotrophy of the MJR strain.

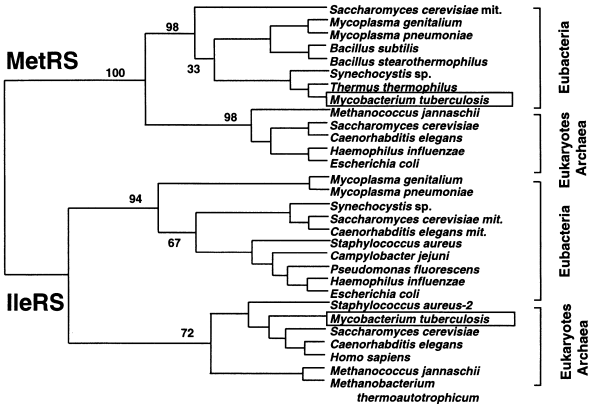

Fig. 3. Phylogenetic relationship of *M. tuberculosis* MetRS and IleRS. Numbers indicate the percent bootstrap value. The MetRS and IleRS of *M. tuberculosis* are highlighted by boxes. The accession numbers for MetRS and IleRS proteins are shown below. MetRS: *S. cerevisiae* mitochondrial (X14629); *M. genitalium* (U39680); *M. pneumoniae* (P75091); *B. subtilis* (D26185); *B. stearothermophilus* (X57925); *Synechocystis* sp. (D64002); *T. thermophilus* (M64273); *M. tuberculosis* (this study); *M. jannaschii* (U67567); *S. cerevisiae* (J01339); *C. elegans* (Z73427); *H. influenzae* (HI1276); *E. coli* (J01649). IleRS: *M. genitalium* (B64238); *M. pneumoniae* (U00089); *Synechocystis* sp. (D90907); *S. cerevisiae* mitochondrial (L38957); *C. elegans* mitochondrial (Z81038); *S. aureus* (X74219); *C. jejuni* (U15295); *P. fluorescens* (P18330); *H. influenzae* (P43824); *E. coli* (D10483); *S. aureus* episomal (X75439); *M. tuberculosis* ([9]); *S. cerevisiae* (X07886); *C. elegans* (Z70310); *H. sapiens* ([5]); *M. jannaschii* (Q58357); *M. thermoautotrophicum* (M59245).

The phylogeny of the *M. tuberculosis* MetRS sequence is more consistent with the other mycobacterial tRNA synthetase sequences including seryl- and leucyl- (S. Martinis, unpublished data) and tyrosyl-tRNA synthetases [30], which are also prokaryotic-like. IleRS (and GlyRS also) in this organism show archaea-eukarya features and may be the result of horizontal gene transfer from the archaea-eukarya domain into the bacteria domain [29]. Unlike the more eukaryotic IleRS which is resistant to the antibiotic, pseudomonic acid [9], *M. tuberculosis* MetRS thus offers more optimal molecular target to screen for inhibitors which can distinguish the human counterpart.

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References

[1] Francklyn, C., Musier-Forsyth, K. and Martins, S.A. (1997) RNA 3, 954–960.
[2] Martinis, S.A. (1996) *Escherichia coli* and *Salmonella typhimurium* Cellular and Molecular Biology, 2nd edn. (Neidhardt, F.C., Ed.) pp. 887–901, ASM Press, Washington, DC.
[3] Schimmel, P. (1987) Annu. Rev. Biochem. 56, 125–158.
[4] Martinis, S. and Schimmel, P. (1995) in: tRNA-Structure, Biosynthesis and Function (Soll, D. and RajBhandary, U.L., Eds.) pp. 349–370, ASM Press, Washington, DC.
[5] Shiba, K., Schimmel, P., Motegi, H. and Noda, T. (1994) J. Biol. Chem. 269, 1–7.
[6] Hipps, D., Shiba, K., Henderson, B. and Schimmel, P. (1995) Proc. Natl. Acad. Sci. USA 92, 5550–5552.
[7] Quinn, C.L., Tao, N. and Schimmel, P. (1995) Biochemistry 34, 12489–12495.
[8] Bloom, B.R. and Murray, C.J. (1992) Science 257, 1055–1064.
[9] Sassanfar, M., Kranz, J.E., Gallant, P., Schimmel, P. and Shiba, K. (1996) Biochemistry 35, 9995–10003.
[10] Sutherland, R., Boon, R.J., Griffin, K.E., Masters, P.J., Slocombe, B. and White, A.R. (1985) Antimicrob. Agents Chemother. 27, 495–498.
[11] Fuller, A.T., Banks, G.T., Mellows, G., Barrow, K.D., Woolford, M. and Chain, E.B. (1971) Nature 234, 416–417.
[12] Hou, Y.-M., Shiba, K., Mottes, C. and Schimmel, P. (1991) Proc. Natl. Acad. Sci. USA 88, 976–980.
[13] Brown, J.R. and Doolittle, W.F. (1995) Proc. Natl. Acad. Sci. USA 92, 2441–2445.
[14] Shiba, K., Suzuki, N., Shigesada, K., Namba, Y., Schimmel, P. and Noda, T. (1994) Proc. Natl. Acad. Sci. USA 91, 7435–7439.
[15] Mellot, P., Mechulam, Y., Le Corre, D., Blanquet, S. and Fayat, G. (1989) J. Mol. Biol. 208, 429–443.
[16] Burbaum, J.J. and Schimmel, P. (1991) Biochemistry 30, 319–324.
[17] Kim, S. and Schimmel, P. (1992) J. Biol. Chem. 267, 15563–15567.
[18] Starzyk, R.M. and Schimmel, P. (1989) J. Biomol. Struct. Dyn. 7, 225–234.
[19] Needleman, S.B. and Wunsch, C.D. (1970) J. Mol. Biol. 48, 443–453.
[20] Felsenstein, J. (1993) Department of Genetics, The University of Washington, Seattle, WA.
[21] Landro, J.A. and Schimmel, P. (1993) Proc. Natl. Acad. Sci. USA 90, 2261–2265.
[22] Hunt, J.B., Neece, S.H., Schachman, H.K. and Ann, G. (1984) J. Biol. Chem. 259, 14793–14803.
[23] Nureki, O., Kohno, T., Sakamoto, K., Miyazawa, T. and Yokoyama, S. (1993) J. Biol. Chem. 268, 15368–15373.
[24] Starzyk, R.M., Webster, T.A. and Schimmel, P. (1987) Science 237, 1614–1618.
[25] Schmitt, E., Panvert, M., Mechulam, Y. and Blanquet, S. (1997) Eur. J. Biochem. 246, 539–547.
[26] Barker, D.G., Ebel, J.-P., Jakes, R. and Bruton, C.J. (1982) Eur. J. Biochem. 127, 449–457.
[27] Cassio, D. and Waller, J.-P. (1971) Eur. J. Biochem. 20, 283–300.
[28] Kim, S., Landro, J.A., Gale, A.J. and Schimmel, P. (1993) Biochemistry 32, 13026–13031.
[29] Shiba, K., Motegi, H. and Schimmel, P. (1997) Trends Biochem. Sci. 22, 453–457.
[30] Nair, S., de Pouplana, L.R., Houman, F., Avruch, A., Shen, X. and Schimmel, P. (1997) J. Mol. Biol. 269, 1–9.