Crucial role of the C-terminal domain of Mycobacterium tuberculosis leucyl-tRNA synthetase in aminoacylation and editing

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ABSTRACT

The C-terminal extension of prokaryotic leucyl-tRNA synthetase (LeuRS) has been shown to make contacts with the tertiary structure base pairs of tRNA^Leu as well as its long variable arm. However, the precise role of the flexibly linked LeuRS C-terminal domain (CTD) in aminoacylation and editing processes has not been clarified. In this study, we carried out aspartic acid scanning within the CTD of Mycobacterium tuberculosis LeuRS (MtLeuRS) and studied the effects on tRNA^Leu-binding capacity and enzymatic activity. Several critical residues were identified to impact upon the interactions between LeuRS and tRNA^Leu due to their contributions in the maintenance of structural stability or a neutral interaction interface between the CTD platform and tRNA^Leu elbow region. Moreover, we propose Arg921 as a crucial recognition site for the tRNA^Leu long variable arm in aminoacylation and tRNA-dependent pre-transfer editing. We also show here the CTD flexibility conferred by Val910 in regulation of LeuRS–tRNA^Leu interaction. Taken together, our results suggest the structural importance of the CTD in modulating precise interactions between LeuRS and tRNA^Leu during the quality control of leucyl-tRNA^Leu synthesis. This system for the investigation of the interactions between MtLeuRS and tRNA^Leu provides a platform for the development of novel antitubercular drugs.

INTRODUCTION

Aminoacyl-tRNA synthetases (aaRSs), which are a primitive and highly conserved protein family among all organisms, catalyze the formation of aminoacyl-tRNAs and provide materials for protein synthesis (1,2). Most reactions catalyzed by aaRSs are processed via two steps: first, amino acid is activated by ATP to form an aminoacyl-adenylate (aa-AMP) intermediate and second, the aminoacyl moiety is transferred to the 3′-terminus of the cognate tRNA to yield aminoacyl-tRNA (aa-tRNA) (2). Twenty aaRSs are classified into two groups based on their characteristic sequences and distinctive structural motifs of the synthetic active site (3–5). Leucyl-tRNA synthetase (LeuRS) belongs to class I aaRSs, which share a representative Rossmann fold in the synthetic domain (3,5). The characterized ancestral catalytic module combined with two other appended domains, the connective peptide 1 (CP1 domain) and the anticyodon-binding domain, constitute the overall architecture of LeuRS.

The overall error rate of aaRSs in translation is approximately 1 in 10,000 (6). This high fidelity in distinguishing cognate substrate from a large pool of analogs is mainly guaranteed by proofreading (editing) processes (7–10). Discrimination between cognate and non-cognate amino acids is important for the quality control of aaRSs because certain amino acids are in high degree of structural similarity. For instance, isoleucine (Ile) and leucine (Leu) differ only by a branched methyl group, and Leu and norvaline (Nva) are distinguished by the presence or absence of one methyl group. These Leu analogs as well as methionine (Met) and several non-standard amino acids can be misactivated by LeuRS in vitro (11,12). However, LeuRS and some other aaRSs have evolved editing functions to hydrolyze either misactivated aa-AMPs (pre-transfer editing) or mischarged tRNAs (post-transfer editing). The LeuRS CP1 domain is responsible for removal of non-cognate aa-tRNA^Leu (11). Furthermore, tRNA can significantly promote the hydrolytic reaction (13–15), partitioning editing pathways into tRNA-dependent and tRNA-independent ones. Multiple editing pathways collectively ensure the accuracy of products (9,10).
Compared with amino acid selection, recognition of tRNAs by aaRSs seems to be less complex but involves large areas of contacts between aaRSs and tRNAs. Precise interactions between tRNAs and aaRSs are critical for cognate aa-tRNA generation. For tRNA\textsubscript{Leu}, its amino acid acceptor stem and the elbow region at the corner of L-shaped tRNA make direct contacts with LeuRS and are recognized as two important sets for aminocaylation and editing (16–20). The 3'-CCA\textsubscript{76} end of tRNA\textsubscript{Leu} swings from the synthetic and editing active sites of LeuRS making specific interactions with LeuRS. Mutational studies at the tRNA 3'CCA\textsubscript{76} end revealed its role in orientating the CP1 domain relative to the LeuRS synthetic domain in aminocaylation. Furthermore, position- ing of the tRNA was suggested to be aided by the CP1 domain entrance pathway in post-transfer editing (20). These mutual interactions form a positive feedback mechanism between LeuRS and tRNA\textsubscript{Leu} ensuring generation of the correct product. The elbow region of L-shaped tRNA\textsubscript{Leu} is formed by the tertiary base pair interactions of the correct product. The elbow region is important for efficient leucylation because it maintains the stability of the overall conformation of tRNA. Recognition of the elbow region is important for efficient leucylation because nucleotide mutations in this domain distorted tRNA\textsubscript{Leu} orientation and impacted upon aminocaylation and editing reactions (19). Based on available crystal structures of bacterial LeuRS, the G19:G56 tertiary base pair at tRNA\textsubscript{Leu} elbow makes extensive interactions with the C-terminal domain (CTD) of LeuRS (21,22). A yeast three-hybrid selection and band-shift assays using the β-subunit of Aquifex aeolicus LeuRS (AaLeuRS) showed that the CTD is involved in tRNA\textsubscript{Leu} binding in vivo and in vitro (23). Furthermore, deletion of the CTD of Thermus thermophilus LeuRS (TtLeuRS) and Escherichia coli LeuRS (EcLeuRS) abolished the enzymatic aminocaylation and post-transfer editing activities (21,24). Although some conserved residues in EcLeuRS-CTD were mutated, no site-specific interactions between this domain and tRNA\textsubscript{Leu} were identified (22). The mechanism by which tRNA\textsubscript{Leu} is recognized by LeuRS-CTD in both aminocaylation and editing remains to be elucidated. Furthermore, the effect of the interactions between the CTD and tRNA\textsubscript{Leu} on pre-transfer editing has not yet been described.

In the present study, a system was established for the enzymological investigation of LeuRS from Mycobacterium tuberculosis, the leading pathogen of tuberculosis (TB) (25). Since its complete genome sequence was first published in 1998 (26), only few studies were performed on aaRSs of M. tuberculosis, while most of the understanding on bacterial aaRSs was obtained from E. coli or T. thermophilus. Recently, aaRSs have been identified as antibiotic targets (27–31). The focus on LeuRS as a drug target has been stimulated by the structural divergence between prokaryotic and eukaryotic LeuRSs, later validated by the emergence of the antifungal agent, AN2690, and the discovery of potent antitubercular agents. For MtbLeuRS-tRNALeu interaction. The consequences to tRNA\textsubscript{Leu} charging and tRNA\textsubscript{Leu}-dependent editing were investigated. Furthermore, proline (Pro) substitutions of residues proximal to the CTD were generated to examine the flexibility of the CTD in interactions with tRNA\textsubscript{Leu}. The impact of these mutations on tRNA\textsubscript{Leu}-binding affinity was analyzed by fluorescence quenching assays and yeast three-hybrid studies. Our data elucidate the specific role of the CTD in mediating interactions between LeuRS and tRNA\textsubscript{Leu} during quality control of leucyl-tRNA\textsubscript{Leu} formation.

**MATERIALS AND METHODS**

**Materials**

\(L\text{-Leu, } L\text{-Nva, } ATP, \text{Tris–HCl buffer, } MgCl}_2 \text{ solution, di-thiothreitol (DTT), activated charcoal and inorganic pyrophosphate (PPI)} \) were purchased from Sigma (USA). \(^{[3}H\) \(L\text{-Leu, } ^{[3}H\) \(L\text{-Met, tetrasodium } [\gamma^{32}P] \text{PPi and adenosine } 5',\text{diphosphate (dATP)} \) were obtained from PerkinElmer Life Sciences (USA). GC/F filters were from Whatman (Germany). PEI Cellulose F plates for thin layer chromatography (TLC) were purchased from Merck (Germany). Nickel-nitrilotriacetic acid (Ni\textsuperscript{2+}-NTA) Superflow resin and gel extraction kits were from Qiagen (Germany). KOD-plus-mutagenesis kits were obtained from TOYOBO (Japan). T4 DNA ligase and other restriction endonucleases were from MBI Fermentas (Lithuania). DEAE-sepharose CL-6B was purchased from GE Healthcare (USA). Plasmid pET30a was purchased from Novagen (USA) and E. coli strain BL21 (DE3) was from Invitrogen (USA). The expression vector pTrc99B and E. coli strain MT102 were gifts from Dr. Gangloff of the Institut de Biologie Moléculaire et Cellulaire du CNRS, Strasbourg, France. Mycobacterium tuberculosis H37Rv strain genomic DNA was a gift from Prof. Y. F. Yao of Shanghai Jiao Tong University, School of Medicine, Shanghai, China.

**Gene cloning, expression and purification of MtbLeuRS and its mutants and Mtb-tRNA\textsubscript{Leu}**

The gene encoding MtbLeuRS was amplified from M. tuberculosis genomic DNA by PCR using primers designed on the basis of the NCBI-published sequence NC_000962.2: forward primer 5'-ACTGCAATAGACCGAATCGCCAACC-3' (NdeI site in italics) and reverse primer 5'-TACGGGCGCCGATGACGAGATTGAC-3' (NotI site in italics). The PCR products were then cleaved and inserted into the corresponding restriction sites of pET30a vector to include a His\textsubscript{6} tag at the C-terminus, thus generating the recombinant plasmid pET30a-mtb\textsubscript{lrs}. The genes encoding MtbLeuRS mutants, including T341R, D450A, T909P, V910P, V910A, V910W, Asp mutants of Val914, Gln915, Lys919,
Val920, Arg921, Arg923, Leu949, Lys956, Ile958, Val960, Arg963, Leu964, Glh966 and Val968, as well as Ala and Lys mutants of Val914, Glh915, Arg921, Leu949 and Leu964 were constructed by KOD-plus mutagenesis kit using pET30a-mtb/8 as a template. The identities of genes were confirmed by DNA sequencing (Biosune Bioscience, Shanghai, China). The recombinant plasmids were transformed into E. coli BL21 (DE3) and production of MtbLeuRS and its mutants were induced in the presence of 200 μM IPTG at 22°C. The over-produced proteins with His6 tags were purified by affinity chromatography using Ni2+-NTA Superflow resin, followed by gel-filtration chromatography with SuperoseTM 12.

The gene encoding Mtb-\text{tRNA}_{\text{Leu}} (CAG) isoacceptor in this study was chemically synthesized and inserted either between the EcoRI and PstI sites of the pTrc99B plasmid for expression in E. coli strain MT102 or between the EcoRI and HindIII sites of pUC19 with a T7 promoter upstream for in vitro transcription. Transformants containing pTrc99B/Mtb-\text{tRNA}_{\text{Leu}}(CAG) plasmids were grown at 37°C in the presence of 300 μM IPTG. Then the tRNAs were isolated from harvested cells by phenol extraction and DEAE-sepharose CL-6B anion-exchange chromatography as described previously (32). tRNA transcripts were synthesized by T7 RNA polymerase as described previously (20).

**ATP-PPI exchange assay**

Leu activation reaction was carried out at 30°C in a 50-μl mixture containing 100 mM Tris–HCl (pH 7.5), 10 mM KF, 10 mM MgCl2, 4 mM ATP, 5 mM Leu, 2 mM tetrasodium [32P] PPI (10 cpm/pmole) and 50 nM MtbLeuRS or its mutants. Aliquots (10 μl) were removed at 2-min intervals and immediately added to 200 μl quenching solution (2% activated charcoal, 3.5% HClO4 and 50 mM tetrasodium pyrophosphate). The total mixture was further filtered (Whatman GF/C filter) and washed with 20 μl of 10 mM tetrasodium pyrophosphate and 10 μl of 95% ethanol. 32P-labeled ATP absorbed onto dried filters was counted by a scintillation counter (Beckman Coulter). Kinetics of MtbLeuRS and its mutants for Mtb-\text{tRNA}^{\text{Leu}} in aminoacylation were determined in the presence of varying concentrations of tRNA\text{Leu} from 0.2 to 75 μM. Deacylation assays were performed at 30°C in 50-μl reaction volumes containing 100 mM Tris–HCl (pH 7.5), 12 mM MgCl2, 0.5 mM DTT, 1 μM [3H] Met-\text{tRNA}^{\text{Leu}} and 5 nM MtbLeuRS or its mutants. Aliquots (10 μl) were removed at 2-min intervals and processed as described for aminoacylation assays. Spontaneous hydrolysis in the absence of enzyme was measured as the control.

**Aminoacylation and deacylation assays**

Aminoacylation reactions were performed at 30°C in 50-μl volumes containing 100 mM Tris–HCl (pH 8.2), 12 mM MgCl2, 0.5 mM DTT, 4 mM ATP, 20 μM [3H] Leu, 5 μM purified Mtb-\text{tRNA}^{\text{Leu}} or the mutants. Aliquots (10 μl) were removed onto Whatman filter at 2-min intervals. After washing with 5% trichloroacetic acid three times and 95% ethanol twice, the filters precipitated with [3H] leucyl-\text{tRNA}^{\text{Leu}} were dried and radioactivity was quantified by a scintillation counter (Beckman Coulter). Kinetics of MtbLeuRS and its mutants for Mtb-\text{tRNA}^{\text{Leu}} in aminoacylation were determined in the presence of varying concentrations of tRNA\text{Leu} from 0.2 to 75 μM. Deacylation assays were performed at 30°C in 50-μl reaction volumes containing 100 mM Tris–HCl (pH 7.5), 12 mM MgCl2, 0.5 mM DTT, 1 μM [3H] Met-\text{tRNA}^{\text{Leu}} and 5 nM MtbLeuRS or its mutants. Aliquots (10 μl) were removed at 2-min intervals and processed as described for aminoacylation assays. Spontaneous hydrolysis in the absence of enzyme was measured as the control.

**AMP formation assays**

Editing of non-cognate amino acids by aaRSs consumes ATP and releases AMP. The formation of AMP as a characteristic of editing reactions was measured by TLC as previously described (34). In assays of MtbLeuRS editing of Nva, reaction was initiated at 30°C by the addition of 0.5 μM MtbLeuRS to a mixture containing 100 mM Tris–HCl (pH 8.2), 12 mM MgCl2, 5 mM DTT, 3 mM ATP, 20 nM [α-32P] ATP (3000 Ci/mmol) and 15 nM Nva with or without 5 μM purified Mtb-\text{tRNA}^{\text{Leu}}. Assays of the editing activity of MtbLeuRS mutants were performed under the same conditions in the presence of 5 μM purified Mtb-\text{tRNA}^{\text{Leu}}. Aliquots (1.5 μl) were removed at the indicated times and quenched with 6 μl of 200 mM sodium acetate (pH 5.0). Quenched aliquots (1.5 μl) were spotted onto polyethyleneimine cellulose TLC plate and developed in a mobile phase containing 0.1 M ammonium acetate and 5% acetic acid to separate [32P] ATP, [32P] AMP and aminoacyl-[32P] AMP. The plates were visualized by phosphorimaging using Fluorescent Image Analyzer FLA-9000 (Fujifilm, Japan) and the results were analyzed using the Multi Gauge Version 3.0 software. The formation of AMP was quantified by gray densities based on comparison of [32P] AMP with a known [32P] ATP concentration. The observed rate constants (kobs) were obtained by linear regression of the graph of [32P] AMP formation plotted against reaction time.

**Determination of the tRNA\text{Leu} dissociation constant by fluorescence quenching assays**

For fluorescence quenching assays, the proteins were excited at 280 nm. The emission wavelength range of an equilibrium titration buffer containing 100 mM Tris–HCl (pH 8.2), 12 mM MgCl2, 0.5 mM DTT and 0.1 μM MtbLeuRS was scanned at room temperature. The maximum emission was observed at 340 nm.
The fluorescence intensity of the enzyme titrated with Mtb-tRNA$^{Leu}_{obs}$ was then measured at an emission wavelength of 340 nm. The dissociation constant ($k_d$) was determined by plotting changes in fluorescence intensity against final tRNA concentration using GraphPad Prism software. Bovine serum albumin or tyrosine as a control was performed using the same method.

**Yeast three-hybrid system construction**

In the first step to establish a functional yeast three-hybrid system (3HS), the gene encoding MtbLeuRS was amplified by PCR using primers: 5′-AGCTCTATAGGCAGCGAAT GACCGAATCGCCAAC-3′ and 5′-CGATCTATAGCT AGATGACGAGATTGACCAG-3′ (NdeI sites indicated in italics) and inserted into the NdeI site presented in the multiple cloning sites of the plasmid pACTII. The resulting recombinant plasmid pACTII/MtbLeuRS produced a hybrid protein of MtbLeuRS with Gal4 activation domain. For the hybrid RNA, the gene encoding Mtb-tRNA$^{Leu}_{obs}$ was amplified by PCR from pTrc99B/Mtb-tRNA$^{Leu}_{obs}$(CAG) plasmid using the following primers: 5′-CAGGAAACAGCCGCAGAAT-3′ and 5′-CAA AACGCCCCTGTTGATGCT-3′ (Smal sites indicated in italics) and inserted into the Smal site of plasmids, pIIIA/MS2-1 and pIIIA/MS2-2, respectively. The resulting recombinant plasmids were designated pIIIA/MS2-1 and pIIIA/MS2-2, respectively. The two plasmids encoding hybrid protein and hybrid RNA were co-transformed into L40coat cells and transformants were further restreaked onto medium supplemented with uracil, Leu and histidine (SD/Ura-/Leu-/His-). Colonies were selected according to the position of the gene of interest (indicated in italics) and inserted into the Smal site of the plasmid pACTII. The resulting recombinant plasmids were designated pACTII/MS2-1 and pACTII/MS2-2, respectively. The resulting recombinant plasmids were amplified for the hybrid RNA, the gene encoding Mtb-leuRS was amplified by PCR from pTrc99B/Mtb-leuRS isolated from E. coli and used as the template for the construction of the hybrid RNA.

**RESULTS**

*MtbLeuRS* charged *Mtb*-tRNA$^{Leu}_{obs}$ with high activity

*MtbLeuRS* consists of 969 amino acid residues with a molecular mass of 108 kDa. By sequence alignment with bacterial LeuRSs, *MtbLeuRS* is 37.3% homologous to *TtLeuRS* and 34.1% homologous to *EcLeuRS*. In order to develop an effective system for *in vitro* investigation of *MtbLeuRS*, *MtbLeuRS* and *Mtb*-tRNA$^{Leu}_{obs}$(CAG) were prepared by cloning and expression of their genes in *E. coli*. Both the enzyme and the tRNA were over-produced and purified to >90% homogeneity as detected by SDS–PAGE or denatured PAGE (Supplementary Figure S1). The *Mtb*-tRNA$^{Leu}_{obs}$ isolated from *E. coli* exhibited high accepting activity with a plateau value of 1500 pmol/A$^{260}$. Approximately 17 mg of *Mtb*-tRNA$^{Leu}_{obs}$ were obtained from 5 g (wet weight) of cells. *In vitro* transcribed *Mtb*-tRNA$^{Leu}_{obs}$ was prepared with an accepting activity of 1600 pmol/A$^{260}$.

Optimal conditions for reactions catalyzed by *MtbLeuRS* were assayed. The optimal pH, temperature and Mg$^{2+}$:ATP ratio for aminoacylation were 8.2, 30°C and 3:1, respectively. Under the optimized conditions, $k_{cat}$ and $K_m$ values of *MtbLeuRS* for *Mtb*-tRNA$^{Leu}_{obs}$ isolated from *E. coli* transformants were 7.80 ± 0.60 s$^{-1}$ and 1.10 ± 0.20 μM. For transcribed *Mtb*-tRNA$^{Leu}_{obs}$, the corresponding values were 2.04 ± 0.13 s$^{-1}$ and 2.74 ± 0.40 μM, respectively. Comparison of the different catalytic efficiencies showed that the over-expressed *Mtb*-tRNA$^{Leu}_{obs}$ was a more competent substrate for *MtbLeuRS* and used in subsequent study. In this way, an efficient system for the enzymological characterization of *MtbLeuRS* was established which will be a useful platform for the development of novel antitubercular drugs.

**Contributions of different editing pathways in *MtbLeuRS***

Previous studies have shown that LeuRSs from different species employ several editing pathways for the removal of incorrect products (15,37,38). The contribution of each editing pathway is reflected by the rate of AMP formation assayed by TLC (34). To investigate the editing properties of *MtbLeuRS*, a homologous mutation used to block post-transfer editing in LeuRSs from *E. coli* and *A. aeolicus* (T341R in *A. aeolicus*) was introduced (15).

The fluorescence intensity of the enzyme titrated with *Mtb*-tRNA$^{Leu}_{obs}$ was then measured at an emission wavelength of 340 nm. The dissociation constant ($k_d$) was determined by plotting changes in fluorescence intensity against final tRNA concentration using GraphPad Prism software. Bovine serum albumin or tyrosine as a control was performed using the same method.

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tRNA-dependent pre-transfer editing accounts for ~70% of the total editing activity of MtbLeuRS. This proportion is similar to that of AaLeuRS (65%) but greater than that of EcLeuRS (35%) and human cytoplasmic LeuRS (9%) (15,38), suggesting a significant role for tRNA Leu in MtbLeuRS pre-transfer editing pathway. Overall, tRNA-dependent editing pathways contributed significantly to the editing function of MtbLeuRS as summarized in Figure 1D.

Identification of important residues within MtbLeuRS-CTD by aspartic acid scanning

Previous studies have shown that the CTD is important for tRNALeu binding and aminoacylation (21–23). According to the crystal structure of TtLeuRS in complex with tRNALeu, the CTD (from Val817 to Val876) directly contacts with the G19:C56 tertiary base pair of the L-shaped tRNALeu (Figure 2A and B). The corresponding MtbLeuRS-CTD extends from Val910 to Ile969. Primary sequence alignment of LeuRSs from several prokaryotes (Figure 2C) showed that the CTD is highly conserved and universally consists of up to 50% hydrophobic residues. To identify critical residues for the function of MtbLeuRS-CTD, several conserved or semi-conserved residues, including hydrophobic and charged ones, were mutated to Asp, with the hypothesis that the introduction of a negative charge may disrupt the interaction of MtbLeuRS-CTD with tRNALeu. Among 14 Asp screening mutants, 5 mutants, including MtbLeuRS-V914D, -Q915D, -R921D, -L949D and -L964D, exhibited a complete loss of aminoacylation activity (Figure 2D and Supplementary Figure S2A), although their Leu activation was retained (Supplementary Figure S2B).
Lys residue (V914K) resulted in abolition of aminoacylation activity and markedly decreased post-transfer editing activity (Figure 3A and B). In the presence of tRNA Leu, the $k_{\text{obs}}$ values for AMP formation of the two mutants were as low as 0.095 s$^{-1}$ and 0.037 s$^{-1}$, respectively (Table 1), suggesting a loss of tRNA-stimulated editing. Moreover, the binding affinity between the mutants and tRNA Leu was dramatically reduced as indicated by a 3- to 4-fold increase in $k_d$ values (3.37 μM for MtbLeuRS-V914A and 4.18 μM for -V914K, Table 2). Therefore, both the hydrophobic property and the length of the side chain of Val914 are highly crucial for tRNA Leu recognition during aminoacylation and editing processes, as observed in the V914L mutant which partially restored enzymatic activities (Supplementary Figure S3). Substituting Leu949 with Ala or Lys greatly impacted upon enzymatic aminoacylation and editing activities (Figure 3G, H, Tables 1 and 3), similar to the effects observed by substitution at Val914.

Gln915 is conserved among prokaryotic LeuRSs, with the exception of a Leu substitution in human mitochondrial LeuRS (Figure 2C). The crystal structure of the TtLeuRS–tRNA Leu complex has revealed that the amide group of residue Gln822 (homologous to Gln915 in MtbLeuRS) stretches toward the elbow of L-shaped tRNA Leu and forms a hydrogen bond with the purine ring of nucleotide G19 (Figure 2B) (21). To determine whether this interaction is specifically required for tRNA Leu recognition, Gln915 was mutated to Ala (Q915A) to disrupt the hydrogen bond. However, this mutation did not affect leucylation of tRNA Leu and deacylation of mischarged tRNA Leu (Figure 3C and D). The catalytic efficiency ($k_{\text{cat}}/K_m$) of the mutant remained similar to that of the wild-type enzyme as did the rate of AMP formation (1.00 s$^{-1}$, Tables 1 and 3). Previous studies have shown that the homologous mutation in EcLeuRS caused a 2-fold decrease in catalytic efficiency (24). The resolution of EcLeuRS–tRNA Leu complex has revealed a slight change in the orientation of the CTD compared with the structure of TtLeuRS–tRNA Leu complex, therefore it is possible that some subtle structural differences present around the interaction interfaces between tRNA Leu and LeuRSs from E. coli and M. tuberculosis. The results suggested that the hydrogen bond between MtbLeuRS-Gln915 and tRNA Leu is not important. However, substitution of Gln915 with Lys (Q915K) affected tRNA Leu leucylation and resulted in an ~2-fold decrease in catalytic efficiency (Figure 3C and Table 3). Although this mutation did not impact upon hydrolysis of mischarged tRNA Leu, the $k_{\text{obs}}$ of the mutant for AMP formation was reduced (0.65 s$^{-1}$, Figure 3D and Table 1), implying that tRNA-dependent pre-transfer editing pathway was disrupted. The data showed that substitution of Gln915 with any charged residue affects the fidelity of leucyl-tRNA Leu formation.

Although no interaction was observed between tRNA Leu elbow and residue Leu964 located at the entry of the last β-sheet of the CTD, substitution of Leu964 with negatively charged Asp or positively charged Lys also resulted in markedly reduced enzymatic catalytic efficiency.

### Table 1. Observed rate constants of MtbLeuRS and several mutants in AMP formation assays in the presence of Nva

| Enzyme | tRNA Leu | AMP formation $k_{\text{obs}}$ (s$^{-1}$) | Relative $k_{\text{obs}}$ |
|--------|----------|----------------------------------------|------------------------|
| W T    | –        | (2.50 ± 0.14) × 10$^{-2}$              | 0.02                   |
| –T341R | +        | 1.15 ± 0.07                           | 1                      |
| –V910  | –        | (2.00 ± 0.44) × 10$^{-2}$              | 0.02                   |
| P      | +        | 0.17 ± 0.023                          | 0.15                   |
| A      | +        | 1.00 ± 0.020                          | 0.87                   |
| W      | +        | 0.94 ± 0.020                          | 0.82                   |
| D      | +        | (1.90 ± 0.62) × 10$^{-2}$              | 0.02                   |
| A      | +        | (9.50 ± 0.98) × 10$^{-2}$              | 0.08                   |
| K      | +        | (3.70 ± 1.10) × 10$^{-2}$              | 0.03                   |
| –Q915  | D        | (4.60 ± 1.28) × 10$^{-2}$              | 0.04                   |
| A      | +        | 1.00 ± 0.066                          | 0.87                   |
| K      | +        | 0.65 ± 0.11                           | 0.57                   |
| –R921  | D        | (2.70 ± 0.46) × 10$^{-2}$              | 0.02                   |
| A      | +        | 0.56 ± 0.034                          | 0.49                   |
| K      | +        | 1.02 ± 0.16                           | 0.89                   |
| –L949  | D        | (3.90 ± 0.84) × 10$^{-2}$              | 0.03                   |
| A      | +        | 0.29 ± 0.053                          | 0.25                   |
| K      | +        | 0.11 ± 0.025                          | 0.10                   |
| –L964  | D        | (4.90 ± 0.80) × 10$^{-2}$              | 0.04                   |
| A      | +        | 0.93 ± 0.13                           | 0.81                   |
| K      | +        | 0.28 ± 0.036                          | 0.24                   |

All the data in the table are the averages from three independent AMP formation assays with the standard deviations indicated.

Moreover, these five mutants were impacted greatly upon Met-tRNA Leu hydrolysis (Figure 2E). Editing of Nva by further TLC assays showed that tRNA Leu addition did not increase the rates of these mutants for AMP formation (Table 1) compared with that of the wild-type MtbLeuRS in the absence of tRNA Leu, suggesting a severe reduction in tRNA-dependent pre- and post-transfer editing activity. The $k_d$ values of these Asp mutants with tRNA Leu determined in fluorescence quenching assays showed a 4- to 9-fold increase compared with that of the wild type (Table 2), indicating an impairment upon LeuRS-binding affinity for tRNA Leu. These results suggest that residues Val914, Glu915, Arg921, Leu949 and Leu964 within MtbLeuRS-CTD are important for the recognition of tRNA Leu in aminoacylation and editing.

### Further investigation of critical residues

To elucidate the action modes of these critical residues in the interaction between MtbLeuRS-CTD and tRNA Leu, further mutated forms were generated containing substitutions with smaller Ala or positively charged lysine (Lys) residues.

Val914 is absolutely conserved among prokaryotic LeuRSs and is located within the first β-sheet of the CTD. Substitution of this residue with either a small, non-polar Ala residue (V914A) or a positively charged residue (V914K) resulted in abolition of aminoacylation activity and markedly decreased post-transfer editing activity (Figure 3A and B). In the presence of tRNA Leu, the $k_{\text{obs}}$ values for AMP formation of the two mutants were as low as 0.095 s$^{-1}$ and 0.037 s$^{-1}$, respectively (Table 1), suggesting a loss of tRNA-stimulated editing. Moreover, the binding affinity between the mutants and tRNA Leu was dramatically reduced as indicated by a 3- to 4-fold increase in $k_d$ values (3.37 μM for MtbLeuRS-V914A and 4.18 μM for -V914K, Table 2). Therefore, both the hydrophobic property and the length of the side chain of Val914 are highly crucial for tRNA Leu recognition during aminoacylation and editing processes, as observed in the V914L mutant which partially restored enzymatic activities (Supplementary Figure S3). Substituting Leu949 with Ala or Lys greatly impacted upon enzymatic aminoacylation and editing activities (Figure 3G, H, Tables 1 and 3), similar to the effects observed by substitution at Val914.

Glu915 is conserved among prokaryotic LeuRSs, with the exception of a Leu substitution in human mitochondrial LeuRS (Figure 2C). The crystal structure of the TtLeuRS–tRNA Leu complex has revealed that the amide group of residue Gln822 (homologous to Gln915 in MtbLeuRS) stretches toward the elbow of L-shaped tRNA Leu and forms a hydrogen bond with the purine ring of nucleotide G19 (Figure 2B) (21). To determine whether this interaction is specifically required for tRNA Leu recognition, Glu915 was mutated to Ala (Q915A) to disrupt the hydrogen bond. However, this mutation did not affect leucylation of tRNA Leu and deacylation of mischarged tRNA Leu (Figure 3C and D). The catalytic efficiency ($k_{\text{cat}}/K_m$) of the mutant remained similar to that of the wild-type enzyme as did the rate of AMP formation (1.00 s$^{-1}$, Tables 1 and 3). Previous studies have shown that the homologous mutation in EcLeuRS caused a 2-fold decrease in catalytic efficiency (24). The resolution of EcLeuRS–tRNA Leu complex has revealed a slight change in the orientation of the CTD compared with the structure of TtLeuRS–tRNA Leu complex, therefore it is possible that some subtle structural differences present around the interaction interfaces between tRNA Leu and LeuRSs from E. coli and M. tuberculosis. The results suggested that the hydrogen bond between MtbLeuRS-Glu915 and tRNA Leu is not important. However, substitution of Glu915 with Lys (Q915K) affected tRNA Leu leucylation and resulted in an ~2-fold decrease in catalytic efficiency (Figure 3C and Table 3). Although this mutation did not impact upon hydrolysis of mischarged tRNA Leu, the $k_{\text{obs}}$ of the mutant for AMP formation was reduced (0.65 s$^{-1}$, Figure 3D and Table 1), implying that tRNA-dependent pre-transfer editing pathway was disrupted. The data showed that substitution of Glu915 with any charged residue affects the fidelity of leucyl-tRNA Leu formation.

Although no interaction was observed between tRNA Leu elbow and residue Leu964 located at the entry of the last β-sheet of the CTD, substitution of Leu964 with negatively charged Asp or positively charged Lys also resulted in markedly reduced enzymatic catalytic efficiency.
in aminoacylation and AMP formation rate in editing (Tables 1 and 3), whereas hydrolysis of mischarged tRNA$^{Leu}$ was moderately influenced. However, L964A mutation did not greatly influence aminoacylation and editing activities of MtbLeuRS (Figure 3I, J, Tables 1 and 3), suggesting that non-charged residue is favorable at position 964 for the maintenance of the CTD function in the interaction of MtbLeuRS with tRNA$^{Leu}$.

Arg921 is the only charged amino acid among the five crucial residues. Based on the effect of the R921D mutation on enzymatic synthetic and editing activities (Figure 2D and E), we proposed the importance of the positive charge at position 921. To investigate it, Lys and Ala mutants of Arg921 were generated. The R921K mutant showed similar aminoacylation and editing activities compared with the wild type.
Structurally, the CTD is compacted in \( Tt \) LeuRS only in the presence of tRNA\(^{\text{Leu}} \), implying a structural rearrangement within the CTD induced by tRNA\(^{\text{Leu}} \) binding (21). Recent resolution of the structures of \( Ec \) LeuRS–tRNA\(^{\text{Leu}} \) complex revealed a rotation of the CTD during tRNA translocation between the synthetic and editing active sites of LeuRS (22), suggesting the dynamic nature of the CTD. This nature correlates with the flexible peptide linker that connects the CTD to the main body of LeuRS. Previous deletion analysis within the C-terminal linker of \( Ec \) LeuRS extending from Trp\(^{597} \) to Asp\(^{790} \) (corresponding to Phe\(^{897} \) to Glu\(^{988} \) in Mtb LeuRS) indicated that the length of the linker controls the movement range of the CTD and the accessibility of the CTD to tRNA\(^{\text{Leu}} \) elbow (39). However, deletion mutagenesis failed to identify specific sites important for the flexibility of the CTD and may alter the structure of the enzyme. Therefore, single-point mutations were made in residues Thr\(^{909} \) and Val\(^{910} \) (Val\(^{816} \) and Val\(^{817} \) in \( Tt \) LeuRS) proximal to the N-terminus of the Mtb LeuRS-CTD. These two residues were substituted by Pro which is suggested to provide a rigid conformation to proteins, and the effect on tRNA\(^{\text{Leu}} \) recognition was studied. Compared with the aminoacylation activity of the T909P mutant (data not shown), that of the V910P mutant was dramatically reduced (Figure 4A), implying a more severe effect caused by the Pro mutation of Val910. Further kinetics analysis showed that the \( K_m \) value for the V910P mutant (9.2 \( \mu \)M) for tRNA\(^{\text{Leu}} \) increased 8.5-fold, while the \( k_{\text{cat}} \) (2.4 s\(^{-1} \), Table 3) decreased 3.3-fold compared with that of the wild type, resulting in a sharp decline in catalytic efficiency. The hydrolysis of mischarged tRNA\(^{\text{Leu}} \) by the mutant was severely disrupted too (Figure 4B). Moreover, its \( k_{\text{obs}} \) for AMP formation declined to 0.17 s\(^{-1} \) in the presence of tRNA (Table 1), indicating a great impact upon Mtb LeuRS editing by the Pro mutation. Circular dichroism spectra of the wild-type Mtb LeuRS and the V910P mutant were almost the same (data not shown), suggesting that this effect is not derived from the changes in the secondary structure. It could be attributed to impaired tRNA\(^{\text{Leu}} \)-binding affinity as indicated by a 5-fold increase of the \( k_d \) value of the V910P mutant for tRNA\(^{\text{Leu}} \) (5.86 \( \mu \)M, Table 2). To exclude the effects of interference to the intrinsic specificity of the residue or steric hindrance caused by Pro substitution, Val910 was further mutated to a smaller Ala or tryptophan (Trp) residue. The mutations had little effect on enzymatic aminoacylation or editing activities (Figure 4, Tables 1 and 3), indicating that the conformational plasticity of the CTD might have been impacted by the Pro mutation, since the enzyme could accommodate smaller or broader side chain at that level. Primary sequences alignment (Figure 2C) confirmed that Val910 is Ile in \( Streptococcus pneumoniae \) LeuRS and AaLeuRS and threonine (Thr) in \( Ec \) LeuRS. These results suggest that the position of Val910 contributed to the flexibility of the Mtb LeuRS-CTD in the accommodation of LeuRS–tRNA\(^{\text{Leu}} \) interaction during both aminoacylation and editing processes.

**Table 2.** \( k_d \) values between tRNA\(^{\text{Leu}} \) and Mtb LeuRS or its mutants determined by fluorescence titration at 280-nm excitation and 340-nm emission wavelengths

| Enzyme     | \( k_d \) (\( \mu \)M) | Relative \( k_d \) |
|------------|-------------------------|-----------------|
| WT         | 1.17 ± 0.12             | 1               |
| -V910P     | 5.86 ± 0.17             | 5               |
| -V914D     | 5.50 ± 0.15             | 4.7             |
| A          | 3.37 ± 0.31             | 2.9             |
| K          | 4.18 ± 0.24             | 3.6             |
| -Q915D     | 10.17 ± 0.84            | 8.7             |
| -R921D     | 7.37 ± 0.57             | 6.3             |
| -L949D     | 6.35 ± 0.30             | 5.4             |
| -L964D     | 5.57 ± 0.32             | 4.8             |

All the data in the table represent the average values from three independent experiments with the standard deviations indicated.

凝集素在Unleu的识别中的作用

凝集素-CTD的结构

凝集素的CTD在LeuRS中是紧凑的，除了在Unleu的条件下，引起结构上的重排。通过tRNA\(^{\text{Leu}} \)的结合（21）。最近的结构解析揭示了\( Ec \) LeuRS–tRNA\(^{\text{Leu}} \)复合物中CTD的旋转，发生在合成和编辑活性位点之间（22），表明了CTD的动态性。这一性质与可柔性的肽链相连，该链连接CTD到主体LeuRS。之前的删除分析位于C末端的LeuRS的肽链从Trp\(^{597} \)到Asp\(^{790} \)（对应于Phe\(^{897} \)到Glu\(^{988} \)在Mtb LeuRS）表明长度的肽链控制了CTD的移动范围和CTD的可接近性。CTD与tRNA\(^{\text{Leu}} \)肘部的相互作用。然而，删除突变体未能识别出特定的位点对CTD的灵活性很重要，并可能改变酶的结构。因此，单点突变在Thr\(^{909} \)和Val\(^{910} \)（Thr\(^{816} \)和Val\(^{817} \)在\( Tt \) LeuRS）的N-端接近Mtb LeuRS-CTD。这两个残基被替换为Pro，这是因为Pro被建议提供一个刚性的蛋白质结构，并在tRNA\(^{\text{Leu}} \)和
Figure 3. Effects of mutagenesis at Val914, Gln915, Arg921, Leu949 and Leu964 on aminoacylation and post-transfer editing activities. Aminoacylation catalyzed by 5nM MtbLeuRS (filled circle) and the mutants of Val914 (A), Gln915 (C), Arg921 (E), Leu949 (G) and Leu964 (I). Hydrolysis of 1μM [3H] Met-tRNA\textsubscript{Leu} by these enzymes is shown in (B), (D), (F), (H) and (J), respectively. The Ala mutants are represented by the symbol (open circle) and Lys mutants by (filled inverted triangle), respectively. Spontaneous hydrolysis (open triangle) in the absence of enzyme was measured as the control.
Table 3. Steady-state kinetics of MtbLeuRS and its mutants for tRNA^Leu in aminoacylation reaction at 30°C

| Enzyme | $K_m$ (μM) | $k_{cat}$ (s⁻¹) | $k_{cat}/K_m$ (s⁻¹·μM⁻¹) | Relative $k_{cat}/K_m$ |
|--------|------------|-----------------|--------------------------|----------------------|
| W T    | 1.1 ± 0.2  | 7.8 ± 0.6       | 7090.9                   | 1.0                  |
| −V910  | 9.2 ± 1.0  | 2.4 ± 0.1       | 260.9                    | 0.037                |
| P      | 1.1 ± 0.2  | 7.0 ± 0.6       | 6363.6                   | 0.9                  |
| W      | 1.4 ± 0.3  | 9.2 ± 0.5       | 6571.4                   | 0.93                 |
| −V914  | D nd       | nd              | –                        | –                    |
| A      | 1.9 ± 0.3  | 13.3 ± 1.0      | 7000                     | 0.99                 |
| K      | 1.3 ± 0.1  | 5.0 ± 0.3       | 3846.2                   | 0.54                 |
| −Q915  | D nd       | nd              | –                        | –                    |
| A      | 5.2 ± 0.7  | 13.6 ± 1.3      | 2615.4                   | 0.37                 |
| K      | 1.8 ± 0.3  | 10.6 ± 0.9      | 5888.9                   | 0.83                 |
| −L949  | D nd       | nd              | –                        | –                    |
| A      | 8.3 ± 1.0  | 5.6 ± 0.6       | 674.7                    | 0.095                |
| K      | 27.3 ± 1.9 | 5.0 ± 0.5       | 183.2                    | 0.026                |
| −L964  | D nd       | nd              | –                        | –                    |
| A      | 1.2 ± 0.1  | 7.2 ± 0.5       | 6000                     | 0.85                 |
| K      | 17.4 ± 2.3 | 10.6 ± 0.8      | 609.2                    | 0.086                |

nd: not determined.

All the data in the table are the average values from three independent experiments with the standard deviations indicated.

MtbLeuRS-MmLinker or the corresponding CTD mutants, the activity of β-galactosidase indicative of protein–RNA interactions in the co-transformants was assayed. The transformants expressing hybrid proteins of the CTD truncation mutant exhibited a low β-galactosidase activity which was ~30% of that of the wild-type (Figure 5), implying a disruption of the interaction of MtbLeuRS-MmLinker with tRNA^Leu. The Asp substitutions of Val914, Gln915, Arg921, Leu949 and Leu964 impacted upon tRNA^Leu-binding capacity in vivo either, as indicated by a decrease of the β-galactosidase activity. Similar effect was observed with the V910P mutation which was shown to be obstructive to the flexibility of the CTD. The interaction between MtbLeuRS-MmLinker and tRNA^Leu was restored by the R921K mutant for which transformants exhibited β-galactosidase activity comparable to that of the wild type, and this was consistent with its enzymatic results in vitro. The functional yeast 3HS verified the importance of these residues within the MtbLeuRS-CTD in tRNA^Leu recognition in vivo.

DISCUSSION

Outside the ancestral synthetic site of aaRSs, numerous extension regions have evolved and appended to the main enzymatic architecture, causing wide divergence in the communication between aaRSs and tRNAs or conferring new properties and functions on these enzymes (24,40–43). The C-terminal extensions in prokaryotic aaRSs are structurally diversified from primary sequence to tertiary conformation and functionally homologous for tRNA binding (21,27,44,45). However, their specific functional mechanisms vary among aaRSs. For example, the class II histidyl-tRNA synthetase from E. coli recognizes anticodon triplet of the tRNA via its C-terminal extension which thus plays an important role in tRNA selection (45). Among class I prokaryotic LeuRSs, this specific extension compacts into a β-domain with the four-stranded β-sheet extending as a platform for the elbow region of L-shaped tRNA^Leu based on their direct contacts in co-crystal structure of TrLeuRS–tRNA^Leu complex (21). Although the CTD is implicated in the interaction with tRNA^Leu, the functional mechanism of the small domain has not been clarified. Our study revealed several critical residues within the MtbLeuRS-CTD that played different roles in the quality control of leucyl-tRNA^Leu formation. Hydrophobic residues Val914 and Leu949 were shown to be important for the tRNA^Leu elbow-binding platform in aminoacylation and editing processes. Structurally, these two residues kept against from the tRNA^Leu body (Figure 2B), and thus did not contact with tRNA^Leu. However, the corresponding Asp mutants were impaired in tRNA binding as indicated by fluorescence quenching assays and yeast three-hybrid studies. We suggest that residues Val914 and Leu949 contribute to the conformational stability of the CTD by maintaining the internal hydrophobic environment in which hydrophobic residues constitute almost half of the domain. Alternatively, the overall architecture of the CTD is required for the orientation of tRNA^Leu as tRNA^Leu recognition by LeuRS is revealed to be dependent on the tertiary structure of tRNA^Leu and conformable architecture of LeuRS (21). Different from Val914 and Leu949, the side chains of the equivalent residues of Gln915 and Leu964 directed toward nucleotide G19 of tRNA elbow within 4 Å distance based on the structure of TrLeuRS bound with tRNA^Leu (Figure 2B) (21). Introduction of charged amino acids, Asp or Lys, into these positions decreased enzymatic aminoacylation and editing activities, while Ala replacement did not cause such effects. Based on the location of these residues relative to tRNA^Leu, it could be speculated that the introduced charges influence the proper orientation of the G19:C56 base pair of tRNA^Leu, such impacted upon the precise interactions between LeuRS and tRNA^Leu in reactions. Further analysis of the β-sheet platform of the CTD revealed that residues that face toward tRNA^Leu elbow are rare charged. Therefore, we suggest that the orientation of the tertiary base pair of tRNA^Leu is positioned and maintained through a neutral platform presented within the CTD that provide adjustable interactions with tRNA^Leu during tRNA^Leu translocation.

As a tRNA-binding domain, the role of the CTD in tRNA-dependent pre-transfer editing has not yet been examined. This pathway contributes 70% of the editing activity of MtbLeuRS, enabling system of MtbLeuRS suitable for investigation of the function of the CTD in this pathway. Our data showed that mutations of Val914,
Gln915, Leu949 and Leu964 impaired tRNA\textsubscript{Leu} stimulated pre-transfer editing activity to varying degrees, indicating that the proper orientation of tRNA\textsubscript{Leu} maintained by the CTD is dispensable for the function of tRNA in pre-transfer editing. Furthermore, Arg921 was shown to be specifically crucial for tRNA-dependent pre-transfer editing due to its electrostatic property. Based on the recently solved co-crystal structure of \textit{Ec} LeuRS–tRNALeu in which the long variable stem of tRNA is intact (22), the side chain of the equivalent residue Arg811 forms an electrostatic interaction with the phosphate group of nucleotide C47h of tRNALeu which is within 3.3 Å distance (Figure 6A). Elimination of the electrostatic interaction by Ala substitution severely decreased enzymatic catalytic efficiency and tRNA-dependent pre-transfer editing activity, whereas Lys mutation had little effect, suggesting its importance in aminoacylation and editing. In the 3D structure of \textit{Mtb}-tRNA\textsubscript{Leu}(CAG) (Figure 6B), nucleotide A47h forms hydrogen bonds with U46, constituting the second base pair of the variable stem. Although nucleotides at those positions differ among tRNA\textsubscript{Leu} from different species, the pairing should be conserved. It has been suggested previously that the orientation of the long variable arm of tRNA\textsubscript{Leu} is a specific structural element for recognition, which is determined mainly by the single unpaired base at the 3'-base of the arm (21). Therefore, we propose that the electrostatic interaction between residue Arg921 and nucleotide A47h may confer stability on the orientation of the tRNA\textsubscript{Leu} variable arm in aminoacylation and editing reactions. Checking of the long variable arm of tRNA has been found in ancestral LeuRS from \textit{Halofexx volcanii} and SerRS as well as TyrRS (46–48), although it has not been reported in bacterial LeuRSs. Our results in \textit{Mtb} LeuRS suggest that the specific recognition of the long variable arm of tRNA\textsubscript{Leu} is critical for aminoacylation and tRNA-dependent pre-transfer editing.

Proteins possess intrinsic plasticity (49). This dynamic structure provides the foundation for the conformational changes that occur during interactions with other molecules as does in the family of aaRSs. The recent co-crystal structures of \textit{Ec} LeuRS–tRNA\textsubscript{Leu} showed that the translocation of tRNA\textsubscript{Leu} 3'–CCA76 end correlates with rotation of four independently folded domains of LeuRS, including the CP1 domain, zinc fingers, Leu-specific domain and the CTD (22). All four domains are flexibly linked to the canonical structure of LeuRS. In this study, the relationship between the flexibility of the CTD and its function was investigated. A rigid Pro substitution of Val910 proximal to the CTD dramatically decreased enzymatic binding affinity for tRNA\textsubscript{Leu} which was consistent with the observed impairment in aminoacylation and editing activities. In contrast, Ala or Trp substitutions had little effect on these functions. These results suggest that the introduction of Pro with rigid conformation prevents the rotation of the CTD and therefore, hinder the maintenance of the interactions between \textit{Mtb}LeuRS and tRNA\textsubscript{Leu} during tRNA\textsubscript{Leu} translocation. Compared with previous studies in \textit{Ec}LeuRS (39), we hypothesized that the potential for movement of the small CTD decreases with increased rigidity in the conformation of...
the peptide near the CTD, as showed by the position of Val910 in the plasticity of MtbLeuRS-CTD.

A C-terminal extension region can as well be found in archaeal/eukaryotic cytosolic LeuRSs, but no homology is shared in the primary sequence among LeuRSs from three kingdoms. Furthermore, this region has been shown to be functionally divergent. This region is essential for tRNA leucylation as negligible leucyl-tRNA<sup>Leu</sup> synthesis was observed in the C-terminal deletion mutant of Pyrococcus horikoshii LeuRS (41). However, the deletion of the C-terminus of hcLeuRS did not affect the aminoacylation activity, but affected its interaction with arginyl-tRNA synthetase in the mammalian macromolecular complex (42). The present work revealed a crucial role of the bacterial LeuRS-CTD in tRNA binding and its recognition in both aminoacylation and editing. Collectively, these data provide a basis for the understanding of the acquisition of the C-terminal module, which may have occurred after the divergence of the LeuRSs. This could have either been driven by evolutionary pressures on the interaction between LeuRS and tRNA or as a result of the expansion of the LeuRS in terms of its function and organization.

TB has become a great threat to human health since the first pathogenic strain, H37Rv, was discovered more than a century ago (50). It has been estimated that approximately one-third of the world’s population comprises latent carriers of M. tuberculosis (http://www.who.int/gtb). The use of antibiotics for the treatment of this disease for half a century has failed to curtail the spread of TB; what is more, the four-drug combination regimen has led to the appearance and spread of multi-drug resistant strains (MDR-TB) on a global scale (25). To identify new targets for drug discovery in TB therapy, we examined the enzymatic properties of MtbLeuRS using Mtb-tRNA<sup>Leu</sup> isolated from E. coli, which exhibited higher catalytic efficiency compared with the corresponding transcripts synthesized in vitro. It has been reported in three kingdoms that post-transcriptional modifications of tRNAs are necessary for its maturation and function, including folding, structural stability and accurate decoding (51). Some modified nucleosides have been described as identity determinants or anti-determinants for aaRS recognition (52). Therefore, we suggest that the modifications obtained during the expression of Mtb-tRNA<sup>Leu</sup> in E. coli may have improved the catalytic constants, resulting in high charging capacity. It contributes to two efficient partners that exhibited high catalytic and accepting activities, MtbLeuRS and Mtb-tRNA<sup>Leu</sup>, respectively. Based on this efficient system, a search for MtbLeuRS inhibitors will be performed by screening a focused library that comprises small molecular compounds, which will be designed to be directed toward either the synthetic or editing active sites of bacterial LeuRS using the crystal structure of TtLeuRS–tRNA<sup>Leu</sup> as a template. The selectivity of the inhibitors, which is of great importance for a drug, can be achieved as some structural variations are present between the active sites of the prokaryotic and eukaryotic LeuRSs, especially between the editing domains as revealed by the structural analysis (21,22,53). Otherwise, although human mitochondrial LeuRS shares a degree of homology with bacterial-type LeuRSs, it displays divergence in the editing domain that disrupts proofreading activity (54). This makes it possible to identify inhibitors that specifically target MtbLeuRS but would not affect the human mitochondrial LeuRS. The bioavailability of the inhibitors is another key issue to be considered. It has been reported that compounds that mimic reaction intermediates always exhibit low inhibitory activities against pathogen growth (31). These compounds are often polar, which may prevent their diffusion through hydrophobic membrane layers. However, the discovery of the so-called Trojan horse antibiotics (31), which can be efficiently taken up using a
peptide transporter and processed as an active compound following the hydrolysis of the transporter in the cytoplasm, provides clues to the problem of active uptake. Due to the essential role of aaRSs for cell function, the inhibition of aminoacylation will prevent protein synthesis and arrest microorganism growth. Therefore, our work provides a potential platform for the application of MtbLeuRS in the development of novel antitubercular drugs.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online: Supplementary Figures 1–4.

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