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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 (*Mtb*LeuRS) 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 *Mtb*LeuRS 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 anticodon-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).

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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 $tRNA^{Leu}$, 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 aminoacylation and editing (16–20). The 3′-CCA_{76} end of $tRNA^{Leu}$ swings between the synthetic and editing active sites of LeuRS making specific interactions with LeuRS. Mutational studies at the tRNA 3′-CCA_{76} end revealed its role in orientating the CP1 domain relative to the LeuRS synthetic domain in aminoacylation. Furthermore, positioning 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 $tRNA^{Leu}$ ensuring generation of the correct product. The elbow region of L-shaped $tRNA^{Leu}$ is formed by the tertiary base pair interactions between the D- and TψC-loops. 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 $tRNA^{Leu}$ orientation and impacted upon aminoacylation and editing reactions (19). Based on available crystal structures of bacterial LeuRS, the G19:C56 tertiary base pair at $tRNA^{Leu}$ 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 (*Aa*LeuRS) showed that the CTD is involved in $tRNA^{Leu}$ binding *in vivo* and *in vitro* (23). Furthermore, deletion of the CTD of *Thermus thermophilus* LeuRS (*Tt*LeuRS) and *Escherichia coli* LeuRS (*Ec*LeuRS) abolished the enzymatic aminoacylation and post-transfer editing activities (21,24). Although some conserved residues in *Ec*LeuRS-CTD were mutated, no site-specific interactions between this domain and $tRNA^{Leu}$ were identified (22). The mechanism by which $tRNA^{Leu}$ is recognized by LeuRS-CTD in both aminoacylation and editing remains to be elucidated. Furthermore, the effect of the interactions between the CTD and $tRNA^{Leu}$ 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 antitrypanosomal agents (28,30). Therefore, the study on *M. tuberculosis* LeuRS (*Mtb*LeuRS) may improve the knowledge on the catalytic properties of LeuRSs originating from a different bacterial group, and promote the development of *Mtb*LeuRS inhibitors with an action mechanism that is different from that of currently used antitubercular agents.

Here we report the mutagenesis of several amino acid residues of the CTD of *Mtb*LeuRS in order to gain an improved understanding of the function of the CTD in LeuRS-$tRNA^{Leu}$ interaction. The consequences to $tRNA^{Leu}$ charging and $tRNA^{Leu}$-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 $tRNA^{Leu}$. The impact of these mutations on $tRNA^{Leu}$-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 $tRNA^{Leu}$ during quality control of leucyl-$tRNA^{Leu}$ formation.

MATERIALS AND METHODS

Materials

L-Leu, L-Nva, ATP, Tris–HCl buffer, $MgCl_2$ solution, dithiothreitol (DTT), activated charcoal and inorganic pyrophosphate (PPi) were purchased from Sigma (USA). [^{3}H] L-Leu, [^{3}H] L-Met, tetrasodium [γ-^{32}P] PPi and adenosine 5′-[α-^{32}P] triphosphate were obtained from PerkinElmer Life Sciences (USA). GF/C filters were from Whatman (Germany). PEI Cellulose F plates for thin layer chromatography (TLC) were purchased from Merck (Germany). Nickel-nitrilotriacetic acid (Ni^{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 obtained 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 *Mtb*LeuRS and its mutants and *Mtb*-$tRNA^{Leu}$

The gene encoding *Mtb*LeuRS 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′-ACTG*CATATG*ACCG AATCGCCAACC-3′ (NdeI site in italics) and reverse primer 5′-TA*GCGGCCGC*GATGACGAGATTGAC-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_6 tag at the C-terminus, thus generating the recombinant plasmid pET30a-mtb*lrs*. The genes encoding *Mtb*LeuRS mutants, including T341R, D450A, T909P, V910P, V910A, V910W, Asp mutants of Val914, Gln915, Lys919,

Val920, Arg921, Arg923, Leu949, Lys956, Ile958, Val960, Arg963, Leu964, Gln966 and Val968, as well as Ala and Lys mutants of Val914, Gln915, Arg921, Leu949 and Leu964 were constructed by KOD-plus mutagenesis kit using pET30a-mtb*lrs* 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 *Mtb*LeuRS and its mutants were induced in the presence of 200 μM IPTG at 22°C. The over-produced proteins with His_6 tags were purified by affinity chromatography using Ni^{2+}-NTA Superflow resin, followed by gel-filtration chromatography with Superose™ 12.

The gene encoding *Mtb*-$tRNA^{Leu}$ (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*-$tRNA^{Leu}$(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 $MgCl_2$, 4 mM ATP, 5 mM Leu, 2 mM tetrasodium [^{32}P] PPi (10 cpm/pmol) and 50 nM *Mtb*LeuRS 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% $HClO_4$ and 50 mM tetrasodium pyrophosphate). The total mixture was further filtered (Whatman GF/C filter) and washed with 20 ml of 10 mM tetrasodium pyrophosphate and 10 ml of 95% ethanol. ^{32}P-labeled ATP absorbed onto dried filters was counted by a scintillation counter (Beckman Coulter, USA).

Preparation of mischarged $tRNA^{Leu}$

Residue Asp345 in *Ec*LeuRS is a conserved site crucial for post-transfer editing pathway among LeuRSs from different species. Substitution of Asp345 with alanine (Ala) abolished enzymatic activity of hydrolyzing mischarged $tRNA^{Leu}$ (33). Therefore, the homologous site in *Mtb*LeuRS (Asp450) was mutated, generating *Mtb*LeuRS-D450A mutant. It was used to mischarge *Mtb*-$tRNA^{Leu}$ with [^{3}H] Met which was available from PerkinElmer Life Sciences. Reactions were carried out at 30°C in a mixture containing 100 mM Tris–HCl (pH 8.2), 12 mM $MgCl_2$, 0.5 mM DTT, 4 mM ATP, 40 μM [^{3}H] Met, 10 μM purified *Mtb*-$tRNA^{Leu}$ and 1 μM *Mtb*LeuRS-D450A. The products were isolated by acid phenol–chloroform extraction (pH 4.5) and ethanol precipitation.

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 $MgCl_2$, 0.5 mM DTT, 4 mM ATP, 20 μM [^{3}H] Leu, 10 μM purified *Mtb*-$tRNA^{Leu}$ and 5 nM *Mtb*LeuRS 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 [^{3}H] leucyl-$tRNA^{Leu}$ were dried and radioactivity was quantified by a scintillation counter (Beckman Coulter). Kinetics of *Mtb*LeuRS and its mutants for *Mtb*-$tRNA^{Leu}$ in aminoacylation were determined in the presence of varying concentrations of $tRNA^{Leu}$ 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 $MgCl_2$, 0.5 mM DTT, 1 μM [^{3}H] Met-$tRNA^{Leu}$ and 5 nM *Mtb*LeuRS 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 *Mtb*LeuRS editing of Nva, reaction was initiated at 30°C by the addition of 0.5 μM *Mtb*LeuRS to a mixture containing 100 mM Tris–HCl (pH 8.2), 12 mM $MgCl_2$, 5 mM DTT, 3 mM ATP, 20 nM [α-^{32}P] ATP (3000 Ci/mmol) and 15 mM Nva with or without 5 μM purified *Mtb*-$tRNA^{Leu}$. Assays of the editing activity of *Mtb*LeuRS mutants were performed under the same conditions in the presence of 5 μM purified *Mtb*-$tRNA^{Leu}$. 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 [^{32}P] ATP, [^{32}P] AMP and aminoacyl-[^{32}P] 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 [^{32}P] AMP with a known [^{32}P] ATP concentration. The observed rate constants (k_{obs}) were obtained by linear regression of the graph of [^{32}P] AMP formation plotted against reaction time.

Determination of the $tRNA^{Leu}$ 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 $MgCl_2$, 0.5 mM DTT and 0.1 μM *Mtb*LeuRS was scanned at room temperature. The maximum emission was observed at 340 nm.

The fluorescence intensity of the enzyme titrated with *Mtb*-tRNA^{Leu} 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 *Mtb*LeuRS was amplified by PCR using primers: 5′-AGCT*CATATG*GCAGCAATGACCGAATCGCCAAC-3′ and 5′-CGAT*CATATG*CTAGATGACGAGATTGACCAG-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/*Mtb*LeuRS produced a hybrid protein of *Mtb*LeuRS with Gal4 activation domain. For the hybrid RNA, the gene encoding *Mtb*-tRNA^{Leu} was amplified by PCR from pTrc99B/*Mtb*-tRNA^{Leu}(CAG) plasmid using the following primers: 5′-CAGGAAACAGAC*CCCGGG*AATT-3′ and 5′-CAAAACAG*CCCGGG*TTGCATGCCT-3′ (SmaI sites indicated in italics) and inserted into the SmaI site of plasmids, pIIIA/MS2-1 and pIIIA/MS2-2, respectively. The resulting recombinant plasmids were designated pIIIA/MS2-*Mtb*tRNA^{Leu} and pIIIA/*Mtb*tRNA^{Leu}-MS2 according to the position of the gene of *Mtb*-tRNA^{Leu} relative to that of MS2 RNA. Subsequently, the two plasmids encoding hybrid protein and hybrid RNA were co-transformed into L40$_{coat}$ cells and transformants were selected by culture at 30°C on synthetic medium lacking uracil, Leu and histidine (SD/Ura-/Leu-/His-). Colonies were further restreaked onto medium supplemented with 160 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal); however, only a slight blue coloration could be observed after a week. Considering that the full length of *Mtb*LeuRS might be too large and consequently limit the expression level of its hybrid protein in yeast, resulting in very low activation of the lacZ gene, we attempted to truncate *Mtb*LeuRS without abolishing tRNA^{Leu}-binding capacity. A nonapeptide, KEEIDGKIT from *Mycoplasma mobile* LeuRS, designated the *Mm*Linker has previously been reported to compensate for the function of the LeuRS CP1 domain (35). Therefore, the *Mtb*LeuRS CP1 domain was replaced with the *Mm*Linker to produce the chimeric mutant designated *Mtb*LeuRS-*Mm*Linker and was selected as a hybrid protein partner. Plasmid pACTII/*Mtb*LeuRS-*Mm*Linker was constructed by KOD-plus mutagenesis kit based on pACTII/*Mtb*LeuRS and was used as the template for the construction of those encoding CTD mutants. Blue coloration was selected as described above. Activation of *lacZ* gene was quantified by measuring β-galactosidase activity using chlorophenol red β-D-galactopyranoside as a substrate as previously described (36). The activity of β-galactosidase was determined in 20 μg of crude extracts from three independent transformants. The co-transformants containing plasmids pACTII/IRP and pIIIA/IRE-MS2 or pACTII/IRP and pIIIA/MS2 were used as positive and negative controls, respectively.

RESULTS

*Mtb*LeuRS charged *Mtb*-tRNA^{Leu} with high activity

*Mtb*LeuRS consists of 969 amino acid residues with a molecular mass of 108 kDa. By sequence alignment with bacterial LeuRSs, *Mtb*LeuRS is 37.3% homologous to *Tt*LeuRS and 34.1% homologous to *Ec*LeuRS. In order to develop an effective system for *in vitro* investigation of *Mtb*LeuRS, *Mtb*LeuRS and *Mtb*-tRNA^{Leu}(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} isolated from *E. coli* exhibited high accepting activity with a plateau value of 1500 pmol/A_{260}. Approximately 17 mg of *Mtb*-tRNA^{Leu} were obtained from 5 g (wet weight) of cells. *In vitro* transcribed *Mtb*-tRNA^{Leu} was prepared with an accepting activity of 1600 pmol/A_{260}.

Optimal conditions for reactions catalyzed by *Mtb*LeuRS 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 *Mtb*LeuRS for *Mtb*-tRNA^{Leu} isolated from *E. coli* transformants were 7.80 ± 0.60 s^{-1} and 1.10 ± 0.20 μM. For transcribed *Mtb*-tRNA^{Leu}, 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} was a more competent substrate for *Mtb*LeuRS and used in subsequent study. In this way, an efficient system for the enzymological characterization of *Mtb*LeuRS was established which will be a useful platform for the development of novel antitubercular drugs.

Contributions of different editing pathways in *Mtb*LeuRS

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 *Mtb*LeuRS, a homologous mutation used to block post-transfer editing in LeuRSs from *E. coli* and *A. aeolicus* (T341R in *Mtb*LeuRS) was introduced (15). As expected, hydrolysis of mischarged tRNA^{Leu} by the mutant was completely abolished (Figure 1C), indicating a loss of the post-transfer editing activity of *Mtb*LeuRS-T341R. Editing of Nva in TLC assays was then examined. In the absence of tRNA^{Leu}, the mutant exhibited a k_{obs} value of 0.020 s^{-1} compared with that of the wild-type enzyme (0.025 s^{-1}) for AMP formation. However, following the addition of tRNA^{Leu}, the k_{obs} value of the mutant (0.83 s^{-1}) showed a little lower than that of the wild type (1.15 s^{-1}, Table 1, Figure 1A and B). Considering the lack of post-transfer editing activity in *Mtb*LeuRS-T341R mutant, it can be calculated that

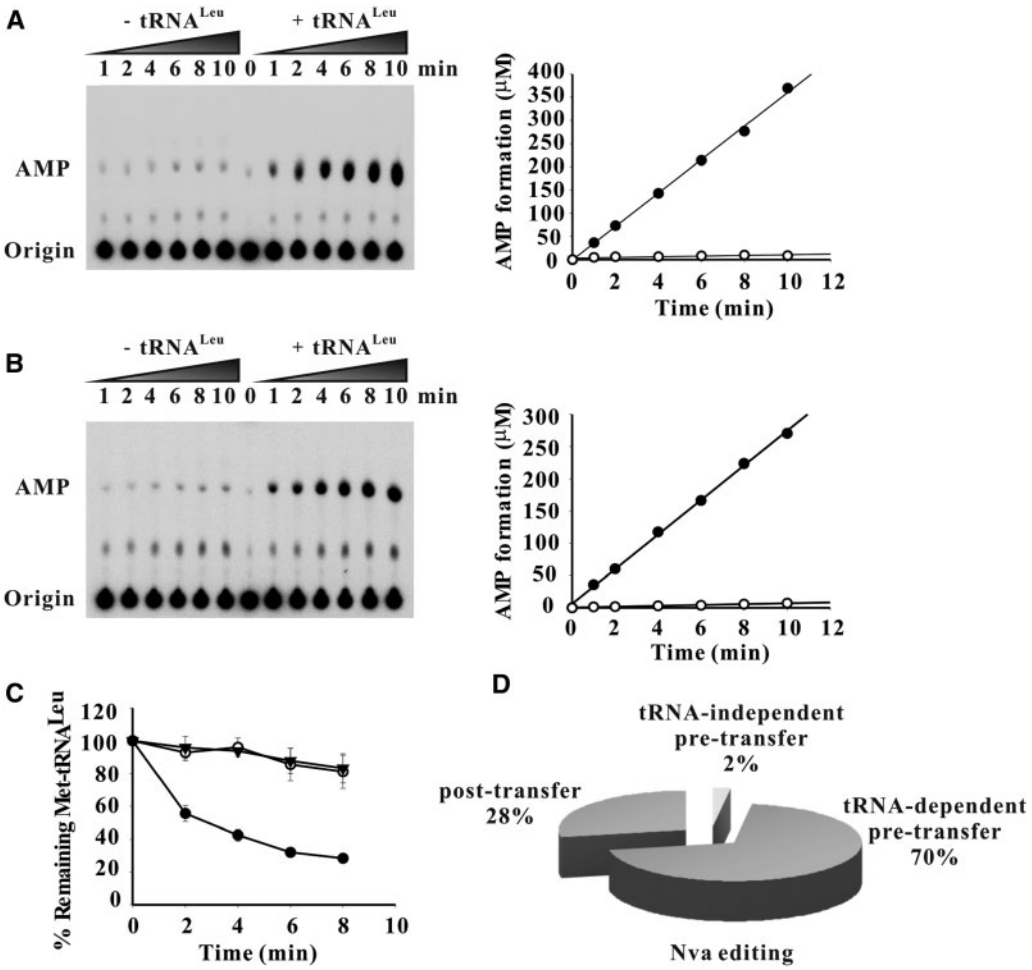

Figure 1. Editing property of *Mtb*LeuRS to Nva. AMP formation assay of 0.5 μM *Mtb*LeuRS (**A**) and the T341R mutant (**B**) based on TLC in the presence of 15 mM Nva with (filled circle) or without (open circle) 5 μM *Mtb*-tRNA^{Leu}. Linear regression graph of AMP formation plotted against reaction time was shown in the right. Slope of the graph was calculated as the observed rate of AMP formation summarized in Table 1. (**C**) Hydrolysis of 1 μM [^{3}H] Met-tRNA^{Leu} by 5 nM *Mtb*LeuRS (filled circle) and *Mtb*LeuRS-T341R (open circle). Spontaneous hydrolysis (filled inverted triangle) in the absence of enzyme was measured as the control. (**D**) Summary of pathways contributing to *Mtb*LeuRS editing. Proportion deduced from the rate of respective editing pathway to that of total editing was representative of percentage of each pathway shown in the pie chart.

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

Identification of important residues within *Mtb*LeuRS-CTD by aspartic acid scanning

Previous studies have shown that the CTD is important for tRNA^{Leu} binding and aminoacylation (21–23). According to the crystal structure of *Tt*LeuRS in complex with tRNA^{Leu}, the CTD (from Val^{817} to Val^{876}) directly contacts with the G19:C56 tertiary base pair of the L-shaped tRNA^{Leu} (Figure 2A and B). The corresponding *Mtb*LeuRS-CTD extends from Val^{910} to Ile^{969}. 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 *Mtb*LeuRS-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 *Mtb*LeuRS-CTD with tRNA^{Leu}. These residues were either located within β-sheets that form the interface between the CTD and the tRNA^{Leu} elbow or were in close proximity to the tRNA backbone based on the structure of the *Tt*LeuRS–tRNA^{Leu} complex (21). Among 14 Asp screening mutants, 5 mutants, including *Mtb*LeuRS-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), suggesting that the first step of aminoacylation was not affect by the CTD mutations.

Table 1. Observed rate constants of *Mtb*LeuRS and several mutants in AMP formation assays in the presence of Nva

Enzyme	$tRNA^{Leu}$	AMP formation k_{obs} (s^{-1})	Relative k_{obs}
W T	–	$(2.50 \pm 0.14) \times 10^{-2}$	0.02
	+	1.15 ± 0.07	1
−T341R	–	$(2.00 \pm 0.44) \times 10^{-2}$	0.02
	+	0.83 ± 0.098	0.72
−V910			
P	+	0.17 ± 0.023	0.15
A	+	1.00 ± 0.020	0.87
W	+	0.94 ± 0.020	0.82
−V914			
D	+	$(1.90 \pm 0.62) \times 10^{-2}$	0.02
A	+	$(9.50 \pm 0.98) \times 10^{-2}$	0.08
K	+	$(3.70 \pm 1.10) \times 10^{-2}$	0.03
−Q915			
D	+	$(4.60 \pm 1.28) \times 10^{-2}$	0.04
A	+	1.00 ± 0.066	0.87
K	+	0.65 ± 0.11	0.57
−R921			
D	+	$(2.70 \pm 0.46) \times 10^{-2}$	0.02
A	+	0.56 ± 0.034	0.49
K	+	1.02 ± 0.16	0.89
−L949			
D	+	$(3.90 \pm 0.84) \times 10^{-2}$	0.03
A	+	0.29 ± 0.053	0.25
K	+	0.11 ± 0.025	0.10
−L964			
D	+	$(4.90 \pm 0.80) \times 10^{-2}$	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 *Mtb*LeuRS 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, Gln915, Arg921, Leu949 and Leu964 within *Mtb*LeuRS-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 *Mtb*LeuRS-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 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_{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 *Mtb*LeuRS-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 *Tt*LeuRS–$tRNA^{Leu}$ complex has revealed that the amide group of residue Gln822 (homologous to Gln915 in *Mtb*LeuRS) 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_{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 *Ec*LeuRS caused a 2-fold decrease in catalytic efficiency (24). The resolution of *Ec*LeuRS–$tRNA^{Leu}$ complex has revealed a slight change in the orientation of the CTD compared with the structure of *Tt*LeuRS–$tRNA^{Leu}$ 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 *Mtb*LeuRS-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_{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

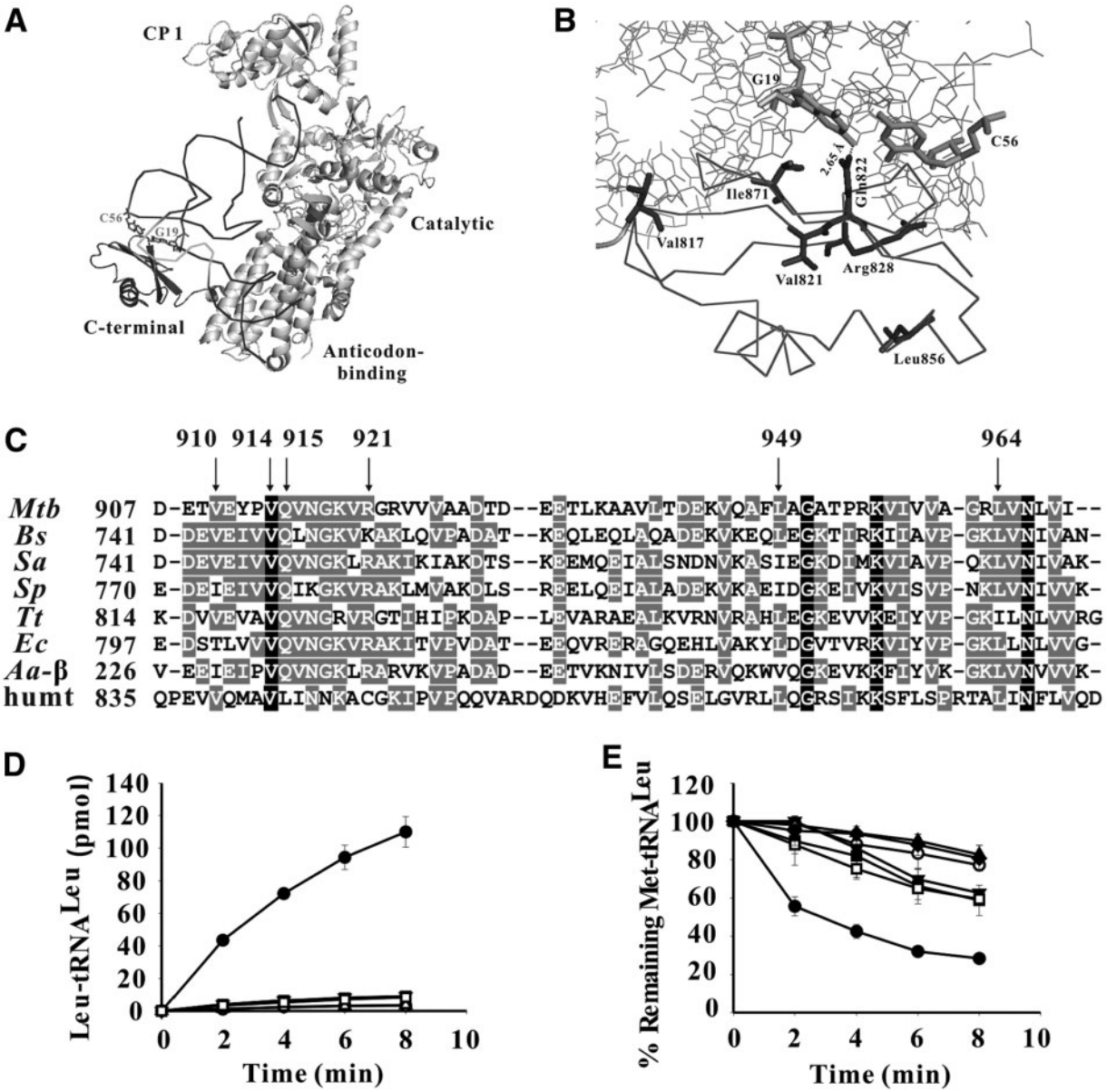

Figure 2. Location of the mutated residues within *Mtb*LeuRS-CTD in Asp scanning and the effects on enzymatic activities. (**A**) Overall representation of the crystal structure of *Tt*LeuRS bound with $tRNA^{Leu}$ (2BYT), showing the CTD in dark gray and the other parts of the enzyme including the CP1, catalytic and anticodon-binding domains in light gray. The main body of the tRNA is depicted in dark gray ribbon, and the long variable arm in light gray. The G19 and C56 nucleotides are illustrated as sticks. (**B**) Closer view of the orientation of the CTD (ribbon) relative to the tRNA (lines) elbow. Residues that depicted as sticks correspond to Val910, Val914, Gln915, Arg921, Leu949 and Leu964 of *Mtb*LeuRS mutated in the present work. The distance between residue Gln822 and nucleotide G19 is marked. (**C**) Primary sequence alignment of the CTD from representative bacteria and organellar LeuRS. Residues that are conserved or homologous are highlighted in black and gray, respectively. The residues of *Mtb*LeuRS mutated in this study are indicated by an arrow. *Mtb*, *Mycobacterium tuberculosis*; *Bs*, *Bacillus subtilis*; *Sa*, *Staphyloccocus aureus*; *Sp*, *Streptococcus pneumoniae*; *Tt*, *Thermus thermophilus*; *Ec*, *Escherichia coli*; *Aa*, *Aquifex aeolicus*; humt, human mitochondrial. (**D**) Aminoacylation catalyzed by 5 nM *Mtb*LeuRS and the Asp mutants. (**E**) Deacylation of 1 μM [^{3}H] Met-$tRNA^{Leu}$ was carried out by 5 nM *Mtb*LeuRS and the Asp mutants. *Mtb*LeuRS (filled circle), V914D (open circle), Q915D (filled inverted triangle), R921D (open triangle), L949D (filled square) and L964D (open square). Spontaneous hydrolysis (filled diamond) in the absence of enzyme was measured as the control.

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 *Mtb*LeuRS (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 *Mtb*LeuRS 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

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

Enzyme	k_d (μM)	Relative k_d
WT	1.17 ± 0.12	1
−V910P	5.86 ± 0.17	5
−V914		
D	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	6.33 ± 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.

(Figure 3E and F). However, the R921A mutant displayed a decrease of ~3-fold in catalytic efficiency (Table 3), similar to that observed in the homologous R811A mutation of *Ec*LeuRS (24). The K_m value for $tRNA^{Leu}$ was 5.2 μM, which was ~5-fold of that of the wild type, suggesting a reduction in affinity between the mutant and tRNA. Consistently, tRNA-stimulated editing of Nva was weakened as indicated by a 2-fold decrease in k_{obs} value for AMP formation (0.56 s^{-1}, Table 1). Moreover, the post-transfer editing activity was not affected (Figure 3F), thus it can be deduced that the R921A mutation primarily impacted upon tRNA-dependent pre-transfer editing activity of which ~30% was retained compared with that of the wild type. Together, these data suggest that the electrostatic property of Arg921 contributes crucially to aminoacylation and tRNA-dependent pre-transfer editing activities of *Mtb*LeuRS.

The role of residue Val910 in flexibility of the *Mtb*LeuRS-CTD

Structurally, the CTD is compacted in *Tt*LeuRS only in the presence of $tRNA^{Leu}$, implying a structural rearrangement within the CTD induced by $tRNA^{Leu}$ binding (21). Recent resolution of the structures of *Ec*LeuRS–$tRNA^{Leu}$ 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^{787} to Asp^{798} (corresponding to Phe^{897} to Glu^{908} in *Mtb*LeuRS) indicated that the length of the linker controls the movement range of the CTD and the accessibility of the CTD to $tRNA^{Leu}$ 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 Thr909 and Val910 (Val816 and Val817 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 $tRNA^{Leu}$ 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 of the V910P mutant (9.2 μM) for $tRNA^{Leu}$ increased 8.5-fold, while the k_{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 $tRNA^{Leu}$ by the mutant was severely disrupted too (Figure 4B). Moreover, its k_{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 $tRNA^{Leu}$-binding affinity as indicated by a 5-fold increase of the k_d value of the V910P mutant for $tRNA^{Leu}$ (5.86 μM, 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 can be substituted by other residues, which is Ile in *Streptococcus pneumonia* LeuRS and *Aa*LeuRS 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–$tRNA^{Leu}$ interaction during both aminoacylation and editing processes.

Confirmation of $tRNA^{Leu}$-binding capacity using the yeast 3HS

To test the effects of *Mtb*LeuRS-CTD mutations on $tRNA^{Leu}$-binding capacity *in vivo*, a functional yeast 3HS was developed for assay of *Mtb*LeuRS–$tRNA^{Leu}$ interactions. In determining protein and RNA partners capable of interacting as hybrids to activate the expression of reporter genes, a yeast clone with moderate blue color was observed in $L40_{coat}$ co-transformants containing plasmids pACTII/*Mtb*LeuRS-*Mm*Linker and pIIIA/*Mtb*$tRNA^{Leu}$-MS2 (data not shown), revealing that *Mtb*LeuRS-*Mm*Linker and *Mtb*-$tRNA^{Leu}$ interact in $L40_{coat}$ transformants. Consistently, the *Mtb*LeuRS-*Mm*Linker retained $tRNA^{Leu}$-binding capacity as it exhibited ~50% of the aminoacylation activity of the wild type (Supplementary Figure S4). However, transformants containing the combinations of pACTII/*Mtb*LeuRS-*Mm*Linker and pIIIA/MS2 or pIIIA/*Mtb*$tRNA^{Leu}$-MS2 and pACTII failed to produce a blue coloration, demonstrating that this interaction is specific. To quantify the binding affinity between *Mtb*-$tRNA^{Leu}$ and

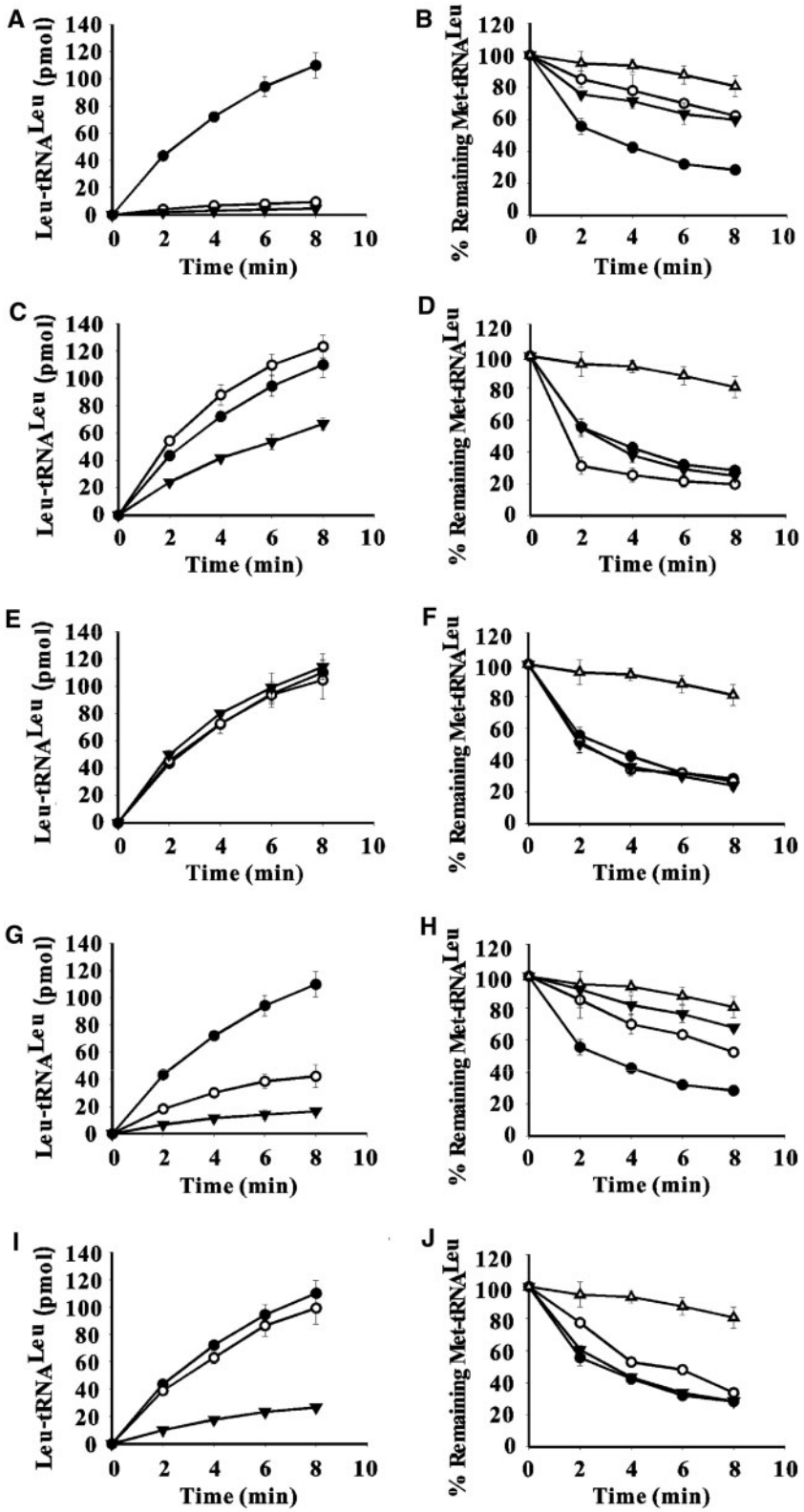

Figure 3. Effects of mutagenesis at Val914, Gln915, Arg921, Leu949 and Leu964 on aminoacylation and post-transfer editing activities. Aminoacylation catalyzed by 5 nM *Mtb*LeuRS (filled circle) and the mutants of Val914 (**A**), Gln915 (**C**), Arg921 (**E**), Leu949 (**G**) and Leu964 (**I**). Hydrolysis of 1 μM [^{3}H] Met-tRNA^{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 *Mtb*LeuRS and its mutants for $tRNA^{Leu}$ in aminoacylation reaction at 30°C

Enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}mM^{-1}$)	Relative k_{cat}/K_m
W T	1.1 ± 0.2	7.8 ± 0.6	7090.9	1.0
−V910				
P	9.2 ± 1.0	2.4 ± 0.1	260.9	0.037
A	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	nd	nd	–	–
K	nd	nd	–	–
−Q915				
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
−R921				
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.

*Mtb*LeuRS-*Mm*Linker 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 *Mtb*LeuRS-*Mm*Linker 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 *Mtb*LeuRS-*Mm*Linker 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 *Mtb*LeuRS-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 α 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 *Tt*LeuRS–$tRNA^{Leu}$ 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 *Mtb*LeuRS-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 *Tt*LeuRS 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 $tRNA^{Leu}$, thus 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 *Mtb*LeuRS, enabling system of *Mtb*LeuRS suitable for investigation of the function of the CTD in this pathway. Our data showed that mutations of Val914,

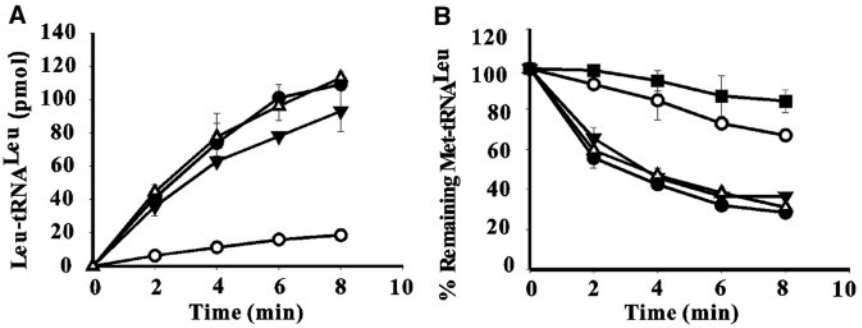

Figure 4. Effects of Val910 mutations on aminoacylation and editing activities. (**A**) Aminoacylation catalyzed by 5 nM *Mtb*LeuRS and the Val910 mutants with 10 μM *Mtb*-tRNA^{Leu}. (**B**) Hydrolysis of 1 μM [^{3}H] Met-tRNA^{Leu} by 5 nM *Mtb*LeuRS and the Val910 mutants. Spontaneous hydrolysis (filled square) in the absence of enzyme was measured as the control. Symbols are *Mtb*LeuRS (filled circle), V910P (open circle), V910A (open triangle) and V910W (filled inverted triangle).

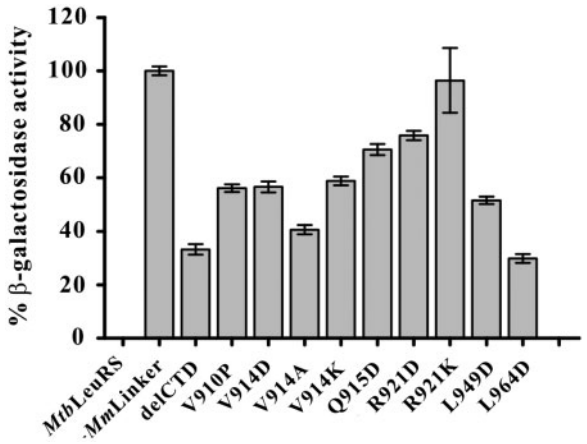

Figure 5. Measurement of β-galactosidase activity of yeast transformants. Plasmids pACTII/*Mtb*LeuRS-*Mm*Linker or its CTD mutants as indicated were transformed into L40$_{coat}$ cells containing plasmid pIIIA/*Mtb*tRNA^{Leu}-MS2. Every 20 μg of crude extracts were used in the assay. The percentages showed in the vertical axis were measured β-galactosidase activity compared with that of the wild-type, which was set as 100%. The values represented the averages from three independent transformants. Error bars represent the standard deviation.

Gln915, Leu949 and Leu964 impaired tRNA^{Leu} stimulated pre-transfer editing activity to varying degrees, indicating that the proper orientation of tRNA^{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 *Ec*LeuRS–tRNA^{Leu} 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 tRNA^{Leu} 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 *Mtb*-tRNA^{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^{Leu} from different species, the pairing should be conserved. It has been suggested previously that the orientation of the long variable arm of tRNA^{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^{Leu} variable arm in aminoacylation and editing reactions. Checking of the long variable arm of tRNA has been found in ancestral LeuRS from *Haloferax volcanii* and SerRS as well as TyrRS (46–48), although it has not been reported in bacterial LeuRSs. Our results in *Mtb*LeuRS suggest that the specific recognition of the long variable arm of tRNA^{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 *Ec*LeuRS–tRNA^{Leu} showed that the translocation of tRNA^{Leu} 3′-CCA$_{76}$ 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^{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 *Mtb*LeuRS and tRNA^{Leu} during tRNA^{Leu} translocation. Compared with previous studies in *Ec*LeuRS (39), we hypothesized that the potential for movement of the small CTD decreases with increased rigidity in the conformation of

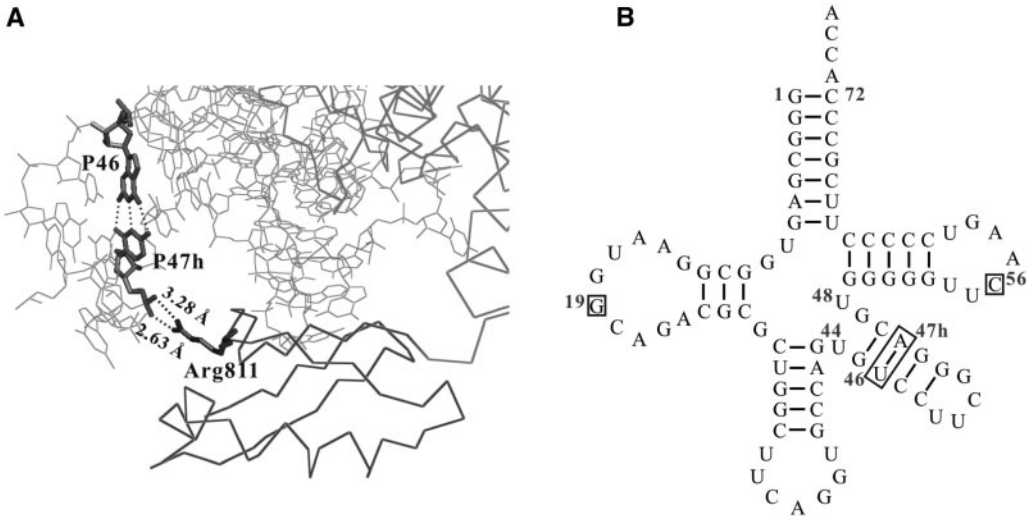

Figure 6. Orientation of residue Arg921 relative to nucleotide 47 h of $tRNA^{Leu}$. (**A**) View focused on the interaction between residue Arg811 (Arg921 in *Mtb*LeuRS) within the CTD and the nucleotide C47h (highlighted as sticks) of $tRNA^{Leu}$ long variable arm in crystal structure of *Ec*LeuRS–$tRNA^{Leu}$ complex (4AQ7). Distances between them are indicated, as well as hydrogen bonds between nucleotides 47 h and 46. For clarity, the main body of LeuRS and tRNA are omitted. (**B**) Cloverleaf structure of *M. tuberculosis* $tRNA^{Leu}$(CAG). Nucleotides involved in this study are boxed.

the peptide near the CTD, as showed by the position of Val910 in the plasticity of *Mtb*LeuRS-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-$tRNA^{Leu}$ 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 *Mtb*LeuRS using *Mtb*-$tRNA^{Leu}$ 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*-$tRNA^{Leu}$ 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, *Mtb*LeuRS and *Mtb*-$tRNA^{Leu}$, respectively. Based on this efficient system, a search for *Mtb*LeuRS 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 *Tt*LeuRS–$tRNA^{Leu}$ 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 *Mtb*LeuRS 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 *Mtb*LeuRS in the development of novel antitubercular drugs.

SUPPLEMENTARY DATA

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

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