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A Flexible Peptide Tether Controls Accessibility of a Unique C-terminal RNA Binding Domain in Leucyl-tRNA Synthetases

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Summary

A unique C-terminal domain extension is required by most leucyl-tRNA synthetases (LeuRS) for aminoacylation. In one exception, the enzymatic activity of yeast mitochondrial LeuRS is actually impeded by its own C-terminal domain. It was proposed that the yeast mitochondrial LeuRS has compromised its aminoacylation activity to some extent to adapt its C-terminus for a second role in RNA splicing, which is also essential. X-ray crystal structures of the LeuRS-tRNA complex show that the sixty-amino acid C-terminal domain is tethered to the main body of the enzyme via a flexible peptide linker and allows interactions with the $tRNA^{Leu}$ elbow. We hypothesized that this short peptide linker would facilitate rigid body movement of the C-terminal domain as LeuRS transitions between an aminoacylation and editing complex or in the case of yeast mitochondrial LeuRS, an RNA splicing complex. The roles of the C-terminal linker peptide for *Escherichia coli* and yeast mitochondrial LeuRS were investigated via deletion mutagenesis as well as by introducing chimeric swaps. Deletions within the C-terminal linker of *E. coli* LeuRS determined that its length, rather than sequence was critical to aminoacylation and editing activities. Although deletions in the yeast mitochondrial LeuRS peptide linker destabilized the protein in general, more stable chimeric enzymes that contained an *E. coli* LeuRS C-terminal domain showed that shortening its tether stimulated aminoacylation activity. This suggested that limiting C-terminal domain accessibility to $tRNA^{Leu}$ facilitates its role in protein synthesis and may be a unique adaptation for yeast mitochondrial LeuRS to accommodate its second function in RNA splicing.

Keywords

NAM2; tRNA; aminoacylation; amino acid editing; protein synthesis

Introduction

Leucyl-tRNA synthetases (LeuRS) are responsible for aminoacylation of leucine to $tRNA^{Leu}$ during protein synthesis.[1] In addition to its role in aminoacylation, LeuRS from yeast mitochondria is also vital to RNA splicing of two group I introns, bI4 and aI4α from the *cob* and *cox1 α* genes, respectively.[2–4] As a dual-function enzyme that is required for two essential

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activities in the cell, the yeast mitochondrial LeuRS must not only recognize its tRNA substrate during aminoacylation, but also the cognate introns that it assists in splicing.

LeuRSs are class IA aminoacyl-tRNA synthetases (aaRSs)[5,6] that contain a Rossmann nucleotide-binding fold,[7] which comprises its catalytic core, and an amino acid editing domain[8–11] called connective polypeptide 1 (CP112). The N-terminal canonical core of LeuRS resembles that of other class I aaRSs and is appended to a less conserved C-terminal anticodon binding domain.[13,14] Although many class I synthetases rely on this C-terminal domain for substrate selection via anticodon interactions, most LeuRSs appear to completely lack this interaction.[15–18]

Based on the co-crystal structure of *Thermus thermophilus* LeuRS, a unique C-terminal extension of about 60 amino acids folds into a separate domain and interacts with the elbow of the L-shaped tRNA.[19] Specifically, it appears to interact with a tertiary base pair formed between G19 and C56 of the tRNA.[19] In the absence of tRNA, the C-terminal domain extension is disordered in LeuRS crystal structures.[20]

Deletion analysis determined that the LeuRS C-terminal domain extension is important for aminoacylation[19,21–23] and amino acid editing.[23] Although the C-terminal domain deletion mutant of *Pyrococcus horikoshii* LeuRS failed to aminoacylate, it was previously shown that the its C-terminal domain is critical to prevent deacylation of correctly charged Ile-tRNA^{Ile} in the LeuRS editing active site.[24] Additionally, yeast complementation assays demonstrated that alterations at the C-terminus of the yeast mitochondrial LeuRS also impacted RNA splicing activity.[23,25] Surprisingly, deletion of this domain stimulated yeast mitochondrial LeuRS aminoacylation and amino acid editing activity,[23] yet abolished aminoacylation activities of other LeuRSs that were tested.[19,21–23] It is possible that yeast mitochondrial LeuRS C-terminal domain extension interactions with RNA have uniquely adapted for its role in RNA splicing.

The C-terminal domain extension is joined to the LeuRS by a short peptide linker (Figure 1), which we hypothesize facilitates movement of the small domain during aminoacylation and amino acid editing. We introduced a series of deletions within the peptide linker of *E. coli* and yeast mitochondrial LeuRSs. We also generated LeuRS chimeras that swapped the C-terminal domain extension and parts of the peptide linker. Our results suggest that restricting the range of rigid body movement of the C-terminal domain extension limits activity of *E. coli* LeuRS by hindering its accessibility for interactions with tRNA. In contrast, this restriction of the C-terminal domain extension for yeast mitochondrial LeuRS enhanced aminoacylation and editing activity. We hypothesize that the C-terminal domain extension-RNA interactions for yeast mitochondria have adapted for its role in RNA splicing, rendering a depreciated role for this unique domain's interactions with tRNA^{Leu} for protein synthesis.

Results

Deletions within the Peptide Linker to the E. coli LeuRS C-terminal Domain Extension Differentially Affect Leucylation and Hydrolytic Editing Activities

Previously, we showed that a small C-terminal domain extension of *E. coli* LeuRS abolished aminoacylation and tRNA deacylation activities.[23] Likewise, truncation of this LeuRS-specific C-terminal domain from *T. thermophilus*, *P. horikoshii* and *Aquifex aeolicus* also eliminated aminoacylation activity.[19,21,22] The *T. thermophilus* LeuRS-tRNA^{Leu} co-crystal structure shows that the C-terminal domain interacts at the backside of the tRNA elbow, near the G19:C56 base pair (Figure 1). Unlike *T. thermophilus* LeuRS, the *P. horikoshii* LeuRS C-terminal domain interacts with the long variable arm of its tRNA in the aminoacylation complex.[26] In the absence of the tRNA, the C-terminal domain is disordered in X-ray crystal

structures suggesting that it is mobile.[20] A short peptide links the unique C-terminal domain to the class I LeuRS canonical structure (Figure 1). We hypothesized that this flexible tether facilitates RNA-protein interactions of the C-terminal domain in the LeuRS aminoacylation complex and also translocation of the tRNA to the editing complex.

The C-terminal domain peptide linker is comprised of about eleven to twelve amino acids and is partially conserved (Figure 1). We hypothesized that shortening the peptide linker would restrict movement and hinder *E. coli* LeuRS-tRNA interactions to impair enzyme activity. We constructed a series of deletion mutations that successively shortened the C-terminal peptide linker (Figure 2A). The wild type and LeuRS deletion mutants were expressed in *E. coli* with an N-terminal six-histidine tag and purified via affinity chromatography. As the peptide linker length decreased, aminoacylation activity also decreased (Figure 2B). The largest deletion that eliminated 10 amino acids in the peptide linker nearly abolished aminoacylation activity. We propose that shortening the peptide linker constrained productive interactions of the essential *E. coli* LeuRS C-terminal domain with the corner of $tRNA^{Leu}$.

We also tested each of the LeuRS peptide linker deletion mutants for deacylation of mischarged Ile-$tRNA^{Leu}$. In contrast to the aminoacylation activities, a majority of the deletion mutants retained significant deacylation activity against mischarged Ile-$tRNA^{Leu}$ compared to the wild-type enzyme. Only the largest deletions of 8 and 10 amino acids (Δ8 and Δ10, respectively) were decreased relative to the wild-type enzyme editing activity (Figure 2C). Thus, the LeuRS aminoacylation complex appeared to require greater flexibility of the linker region as compared to the amino acid editing complex.

We created a series of single-site deletion mutants at the more flexible C-terminal end of the peptide linker to scan for individual sites that might be important for activity (Figure 3A). We chose to delete the C-terminal linker starting at residue E797 (ΔE) in *E. coli* LeuRS. This site is analogous to K814 of *T. thermophilus* LeuRS (Figure 1A), which is the last amino acid at the C-terminal end of the *apo*-LeuRS structure that generated electron density during X-ray crystal structure analysis.[20] Each of the purified single amino acid deletion mutants showed similar aminoacylation activities compared to the wild-type enzyme (Figure 3B). We also introduced a series of two-amino acid deletions near the beginning of the folded C-terminal domain (Figure 3A). In contrast to the single amino acid deletion mutants, two of the double amino acid linker deletion mutants (ΔED and ΔVE) exhibited lower aminoacylation activity compare to the wild-type enzyme (Figure 3C). Since these two peptide linker deletion mutants shared a common E797 deletion, we substituted this site with three proline (E797PPP) or three glycine (E797GGG) residues in attempts to restrict or enhance the flexibility of the peptide tether to the C-terminal domain (Figure 3A). However, neither of these triple amino acid insertion mutants significantly affected aminoacylation (Figure 3D). When mischarged Ile-$tRNA^{Leu}$ substrates were introduced to all of the LeuRS deletion mutants (Figure 3A), we found that the mutants retained deacylation activities that were similar to wild type (data not shown). Based on the combined mutational analysis of the *E. coli* LeuRS peptide linker, we hypothesize that its length, rather than its specific sequence, is critical to conferring mobility to the unique C-terminal domain for interactions with $tRNA^{Leu}$ in the aminoacylation and editing complexes.

Restricting Access of the C-terminal Domain Extension of Yeast Mitochondrial LeuRS Enhances Aminoacylation and Editing Activities

In contrast to *E. coli*[23] and other LeuRSs,[19,21,22] deletion of the unique C-terminal domain of yeast mitochondrial LeuRS actually stimulated aminoacylation and editing activities.[23] We speculated whether deletions within the yeast mitochondrial LeuRS linker peptide that restrict access of the C-terminal domain might also enhance enzyme activity. We created a series of deletions within the C-terminal domain peptide linker of yeast mitochondrial LeuRS that

included [4, 6, 8] and [12] amino acids. With the exception of the shortest amino acid deletion mutant (Δ4; Figure 4A), the yeast mitochondrial LeuRS deletion mutants could not be stably expressed and purified. Only small changes in aminoacylation and editing activities were found for the yeast mitochondrial LeuRS (Δ4) deletion mutant (Figure 4B and C). The C-terminal domain deletion mutant, which enhanced aminoacylation and editing activities in previous experiments,[23] was normalized to the wild type activity and included for comparison to the Δ4 deletion mutant.

In order to further probe the peptide linker's influence on yeast mitochondrial LeuRS's protein synthesis activities, we employed a more stable chimera that contained a swapped C-terminal domain extension from *E. coli* LeuRS.[23] Previously, we showed that when the *E. coli* LeuRS C-terminal domain was fused to the yeast mitochondrial LeuRS, it also impeded activity. Thus, the activity is not necessarily specific to the molecular structure of the C-terminal domain *per se*, but its accessibility. To test this hypothesis, we used the chimeric enzyme to generate deletions and additional swaps within the C-terminal domain peptide tethers.

We created a series of deletions and chimeric variations in the peptide linker of the yeast mitochondrial LeuRS chimeric mutant that was fused to the *E. coli* LeuRS C-terminal domain extension (Figure 5A). A 4-amino acid deletion mutant of the yeast mitochondrial LeuRS chimera (Ym *Ec*CTD Δ4) significantly stimulated aminoacylation activity (Figure 5B) compare to the chimera enzyme without any deletions within the linker peptide. As found with the wild-type yeast mitochondrial LeuRS, larger deletions in the peptide linker were not stably expressed for purification. However, an eight-amino acid peptide linker deletion mutant that contained three (Ym *Ec*CTD Δ8/+3), six (Ym *Ec*CTD Δ8/+6), or nine (Ym *Ec*CTD Δ8/+9) amino acids from the *E. coli* LeuRS linker peptide restored protein stability and activity (Figure 5B). Within these three chimeric peptide linker swaps, successive increases in the length of the *E. coli* chimeric peptide linker progressively decreased aminoacylation activity. This is consistent with our previous hypothesis that restricting access of the yeast mitochondrial LeuRS C-terminal domain extension promotes aminoacylation activity.[23]

Hydrolytic editing activity of mischarged Ile-tRNA^{Leu} was also increased upon deletion of portions of the peptide linker of the yeast mitochondrial LeuRS chimeras that had an *E. coli* C-terminal domain (Figure 5C). The 4-amino acid deletion mutant (Ym *Ec*CTD Δ4) as well as the Ym *Ec*CTD Δ8/+3 and Ym *Ec*CTD Δ8/+6 mutants with variable peptide linker chain lengths had significantly enhanced deacylation activities against mischarged Ile-tRNA^{Leu} (Figure 5C). A complete swap of the peptide linker for Ym *Ec*CTD Δ8/+9 decreased editing, but was still stimulated over the chimeric yeast mitochondrial LeuRS that contained the *E. coli* C-terminal domain extension. Thus, these results suggest that decreasing the length of the C-terminal linker peptide and perhaps restricting its flexibility facilitated access of the tRNA to the enzyme's active site leading to an increase in overall enzymatic activity of yeast mitochondrial LeuRS.

Although we created several deletion mutants of yeast mitochondrial LeuRS, only the smallest 4-amino acid deletion mutant was expressed, suggesting that this linker region is important to stability of the tertiary structure. Previously, we showed that deletion of the C-terminal domain extension of yeast mitochondrial LeuRS increased the α-helical content of the folded protein.[23] We hypothesized that the presence of the C-terminal domain extension might influence enzyme activity by destabilizing the structure. We used circular dichroism (CD) to determine whether the increase in aminoacylation activities described above for two of the chimeric linker deletions, Ym *Ec*CTD Δ4 and Ym *Ec*CTD Δ8/+3 (Figure 5A), could also be attributed to increases in secondary structure. Both of the chimeric deletion mutants had more α-helical character than the wild-type enzyme (Figure 6). Thus, we hypothesize that the C-terminal linker

may impact structural interactions between the C-terminal domain and other regions of the LeuRS. It is also possible that these interactions are RNA-mediated.

Peptide Linker Deletions of Chimeric E. coli LeuRS with a Yeast Mitochondrial LeuRS C-terminal Domain Extension Differentially Affect Aminoacylation and Amino Acid Editing

Even though the unique C-terminal domain impacts *E. coli* and yeast mitochondrial LeuRS in surprisingly opposite ways, we showed that the C-terminal domains of *E. coli* and yeast mitochondrial LeuRS can be swapped and confer the respective wild-type like activities.[23] That is, if the *E. coli* LeuRS is fused to the yeast mitochondrial C-terminal domain extension, it maintains enzymatic activity. Likewise, as described above, the *E. coli* C-terminal domain extension hinders aminoacylation when attached to the yeast mitochondrial LeuRS.

The peptide linker of *E. coli* LeuRS fused to the yeast mitochondrial LeuRS C-terminal domain was targeted for deletion, followed by insertion of the yeast mitochondrial peptide linker sequences (Figure 7A). In the presence of the yeast mitochondrial C-terminal domain, deletions within the peptide linker of the *E. coli* LeuRS chimera had less impact than the wild type LeuRS deletion mutants that are described above. A six-amino acid (*Ec* YmCTD Δ6) and three-amino acid (*Ec* YmCTD Δ3) deletion mutant slightly decreased and increased aminoacylation activity, respectively (Figure 7B). Similar to the yeast mitochondrial LeuRS, a 9-amino acid deletion mutant from the C-terminal linker of the chimeric enzyme could not be stably expressed (data not shown). However, replacement of these nine amino acids with four amino acids from the yeast mitochondrial LeuRS peptide linker (*Ec* YmCTD Δ9/+4) restored stability and also significant aminoacylation activity. A complete swap of the peptide linker for *Ec* YmCTD Δ9/+8 yielded activities that were similar to wild-type *E. coli* LeuRS. Hydrolytic deacylation activities of Ile-tRNA^{Leu} were also tested and were largely unaffected by chimeric alterations in the C-terminal linker (Figure 7C). This is consistent with deletion analysis of the wild-type *E. coli* LeuRS peptide linker, which decreased aminoacylation activity, while maintaining amino acid editing activity.

Discussion

The C-terminal domain extension of LeuRS is essential to the enzymatic activities of most of the enzymes that have been tested.[19,21–23] Its interactions with tRNA^{Leu} in both the aminoacylation and editing complexes of LeuRS[19,26] are likely important to stabilizing RNA-protein interactions for catalysis. Even though specific mutations within the C-terminal domain failed to significantly impact aminoacylation,[23] we hypothesized that the C-terminal domain functioned by a shape-specific mechanism to bind to the tRNA^{Leu} elbow. This might be related to an indirect read-out of the tRNA backbone.[27] Alternatively, it could serve as a general RNA binding domain that enhances interactions with the tRNA substrate like the N-terminal appended domain of yeast cytoplasmic glutaminyl-tRNA synthetase (GlnRS)[28,29] and mammalian LysRS.[30] Similarly, an extra RNA-binding module at the C-terminus of human methionyl-tRNA synthetase (MetRS) was also shown to increase the enzyme's affinity for its tRNA substrate.[31]

Yeast mitochondrial LeuRS is unusual in that its aminoacylation and editing activities are impaired by the presence of the C-terminal domain.[23] Yet, when this C-terminal domain extension from the yeast mitochondrial LeuRS was fused to form a chimeric *E. coli* LeuRS, it failed to interfere with enzyme activity. These results further emphasize the lack of sequence-specificity that could underlie interactions of the C-terminal domain with tRNA^{Leu}.

It is possible that the C-terminal domain functions as an accessory RNA binding domain. Similar to the appended RNA binding domain of human MetRS[31] and LysRS[30] and yeast GlnRS,[29,32] the yeast mitochondrial LeuRS C-terminal domain is also lysine-rich with a

computed theoretical pI of 10.4.[33–35] This domain contains 12 lysine and 3 arginine residues (~23 % positively charged residues). This high level of basicity could confer substantial RNA binding interactions. Although the C-terminal domain of yeast mitochondrial LeuRS is inhibitory to its native enzyme, it is possible that this RNA binding module has adapted preferentially to accommodate its interaction with the group I introns that it assists in splicing. As a result, tRNA binding may be compromised, but sufficient to maintain protein synthesis activity in the cell.

We sought to restrict access of the C-terminal domain by introducing deletions into a flexible peptide tethered to the C-terminal domain extension. Our results clearly showed for the wild-type and *E. coli* LeuRS chimera that shortening the tether decreased aminoacylation activity. This is consistent with eliminating essential interactions with $tRNA^{Leu}$ by removal of the entire C-terminal domain extension. In contrast, editing activity was only minimally, if at all affected by the series of deletions. This suggests that the tRNA is in a more accessible orientation to the C-terminal domain extension in the LeuRS editing complex versus the aminoacylation complex.

Deletions of the peptide linker in the yeast mitochondrial wild-type and chimeric LeuRS tended to enhance its aminoacylation and editing activity. Circular dichroism in the absence of the tRNA substrate, showed that restricting accessibility of the C-terminal domain that is connected to yeast mitochondrial LeuRS, whether it originated from the yeast mitochondrial[23] or *E. coli* LeuRS increased secondary structure of the enzyme. It is possible that in the context of the yeast mitochondrial LeuRS, the C-terminal domain interacts or interferes with the structure and function of another important area of the enzyme that is required for tRNA binding or catalysis. Conversely, another region of the yeast mitochondrial LeuRS may stabilize the C-terminal domain when its peptide linker length is decreased. Although the C-terminal domain extension is disordered in X-ray crystal structures of *T. thermophilus* and *P. horikoshii* LeuRS in the absence of the tRNA, our biochemical results suggest that it could be more constrained in the yeast mitochondrial enzyme.

The yeast mitochondrial LeuRS is also a group I intron splicing factor and is essential to the production of electron transfer proteins that are required for respiration.[2–4] Although an *in vitro* splicing assay has not yet been developed to directly test the role of the C-terminal domain extension and its peptide tether in biochemical investigations, genetic[25] and three-hybrid studies[23] have indicated that it is important to its dual role in RNA splicing activity. We hypothesize that interactions with the C-terminal domain extension and its dependence on the peptide tether have co-adapted to maintain sufficient levels of charged Leu-$tRNA^{Leu}$ in the yeast mitochondria for protein synthesis, while facilitating excision of the bI4 and aI4α introns from the mRNAs encoding cytochrome b and the α-subunit of cytochrome oxidase.

Materials and Methods

Materials

Tritium-labeled amino acids were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Oligonucleotide primers were obtained from MWG Biotech (High Point, NC) or Integrated DNA Technologies (Coralville, IA). RNA transcripts were synthesized *in vitro* and purified as described previously.[23]

Protein Mutagenesis and Purification

Deletion mutagenesis of wild-type *E. coli* and yeast mitochondrial LeuRS genes were carried out using plasmid p15ec3-1[36] or pYM3-17[37] respectively, as template for the polymerase chain reaction (PCR) with forward and reverse primers as described previously.[23] Plasmids,

pYMJLH63 encoding the yeast mitochondrial LeuRS main body fused to the *E. coli* LeuRS C-terminal domain and pECJLH71 encoding the *E. coli* LeuRS main body fused to the yeast mitochondrial LeuRS C-terminal domain,[23] were used as template for PCR to generate deletion and insertion chimeric linker mutants. Each mutant gene was confirmed by DNA sequencing (SeqWright, Houston, TX or UIUC Sequencing Facility, Urbana, IL). Mutant and wild-type plasmids expressing recombinant *E. coli* LeuRSs were used to transform *E. coli* BL21 (DE3) strain. Cell cultures were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37 °C for 2 hours for protein expression. The N-terminal six-histidine-tagged fusion protein was purified by affinity chromatography using His-Select™ Resin (Sigma-Aldrich; St. Louis, MO).[23]

Alternatively for yeast mitochondrial LeuRS expression, 500 ml cultures harboring the plasmid expressing the wild-type, deletion mutants, or chimeric yeast mitochondrial LeuRS were induced with 1 mM IPTG at 30 °C for 3 hours when the OD_{600} reached between 0.5 and 0.6. The cells were harvested and the expressed six-histidine tagged yeast mitochondrial proteins were affinity purified, [23] followed by desalting on a PD-10 column (GE Healthcare, Piscataway, NJ). Subsequently, anion exchange chromatography was carried out using an ÄKTA Purifier System (GE Healthcare, Piscataway, NJ). A MONO Q 5/50 GL column (GE Healthcare, Piscataway, NJ) was pre-equilibrated with Buffer A (50 mM NaP_i, pH 7.4 and 5 mM NaCl). After the affinity chromatography-purified proteins were bound, the column was washed with 10 volumes of Buffer A. A linear gradient of 20 volumes of Buffer B (50 mM NaP_i, pH 7.4 and 1 M NaCl) was used to elute the LeuRS. Fractions containing a single band of protein based on analysis via SDS-PAGE were pooled, concentrated, and equilibrated using a Centricon-50 (Amicon, Bedford, MA) in Buffer C (20 mM NaP_i, pH 7.5 and 100 mM NaCl), followed by storage in 50% glycerol at −20 °C. The protein's final concentration was determined by absorbance at A_{280} using a calculated extinction coefficient of 159,585 L/mol•cm.[35,38]

Enzyme Assays

The aminoacylation reaction of wild-type and mutant enzymes consisted of 60 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 8 μM folded $tRNA^{Leu}$ transcript, 21 μM [^{3}H]-L-leucine (150 μCi/ml), and 25 nM enzyme and was initiated by the addition of 4 mM ATP. Aminoacylation of *E. coli* and yeast mitochondrial LeuRS containing chimeric linker swaps were carried out using 50 nM enzyme. Hydrolytic editing assays were carried out at room temperature in 60 mM Tris, pH 7.5, 10 mM $MgCl_2$, and 0.4–0.8 μM [^{3}H]-Ile-$tRNA^{Leu}$ and the reactions were initiated with 100 nM enzyme. At specific time points, reaction aliquots of 10 μl for aminoacylation or 5 μl for editing were quenched by transferring to filter pads (Whatman, Clifton, NJ) that had been pre-soaked with 5% trichloroacetic acid. The pads were washed to remove free labeled amino acid and quantitated by scintillation counting as described previously.[23]

Circular Dichroism (CD)

CD measurements of wild-type and chimeric linker deletion mutants of yeast mitochondrial LeuRS were measured in the far-ultraviolet region using a Jasco J-720 spectropolarimeter. Each sample containing 0.8 μM protein in 5 mM KP_i, pH 7.5 was transferred to a cell with a 0.1 cm path length. Background signals for the cell and buffer were subtracted from each spectrum.

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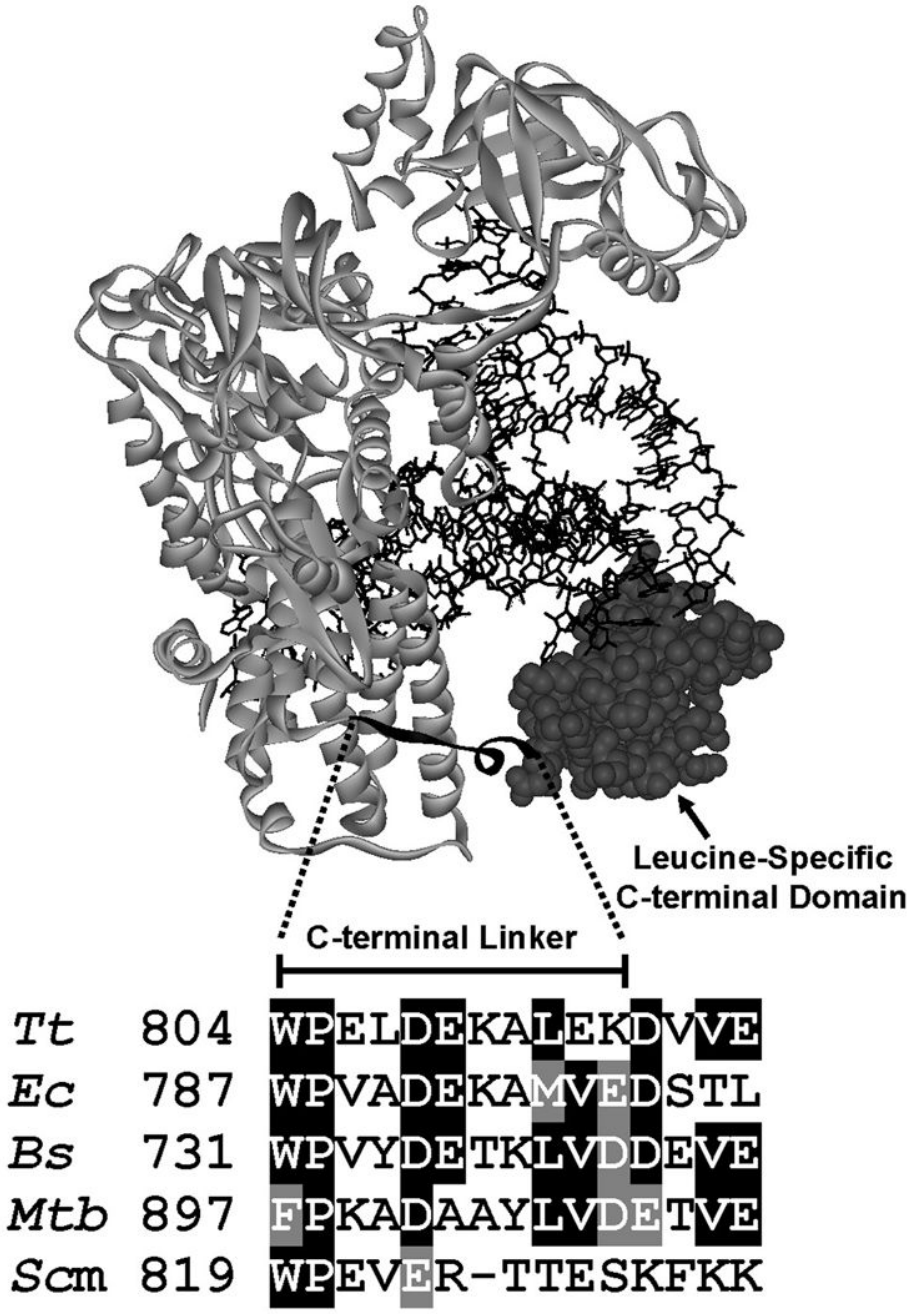

Figure 1.

Co-crystal structure of *T. thermophilus* LeuRS and primary sequence alignment of the LeuRS C-terminal linker. The X-ray co-crystal structure of *T. thermophilus* LeuRS bound to $tRNA^{Leu}$ in the editing complex[19] has the unique C-terminal domain of LeuRS which is depicted in dark colors as a space-filling model. The main body of the enzyme and the bound tRNA are highlighted in gray and black, respectively. The C-terminal domain that is tethered to the main body of the enzyme via a flexible linker peptide is illustrated as a black ribbon. A sequence alignment of the C-terminal linker peptide is shown below with amino acid residues that are conserved and homologous highlighted in black and gray, respectively. A portion of the unique C-terminal domain is included in the alignment. Abbreviations are as follows: *Tt*,

Thermus thermophilus; Ec, Escherichia coli; Bs, Bacillus subtilis; Mtb, Mycobacterium tuberculosis, and *Sc*m, mitochondrial *Saccharomyces cerevisiae*.

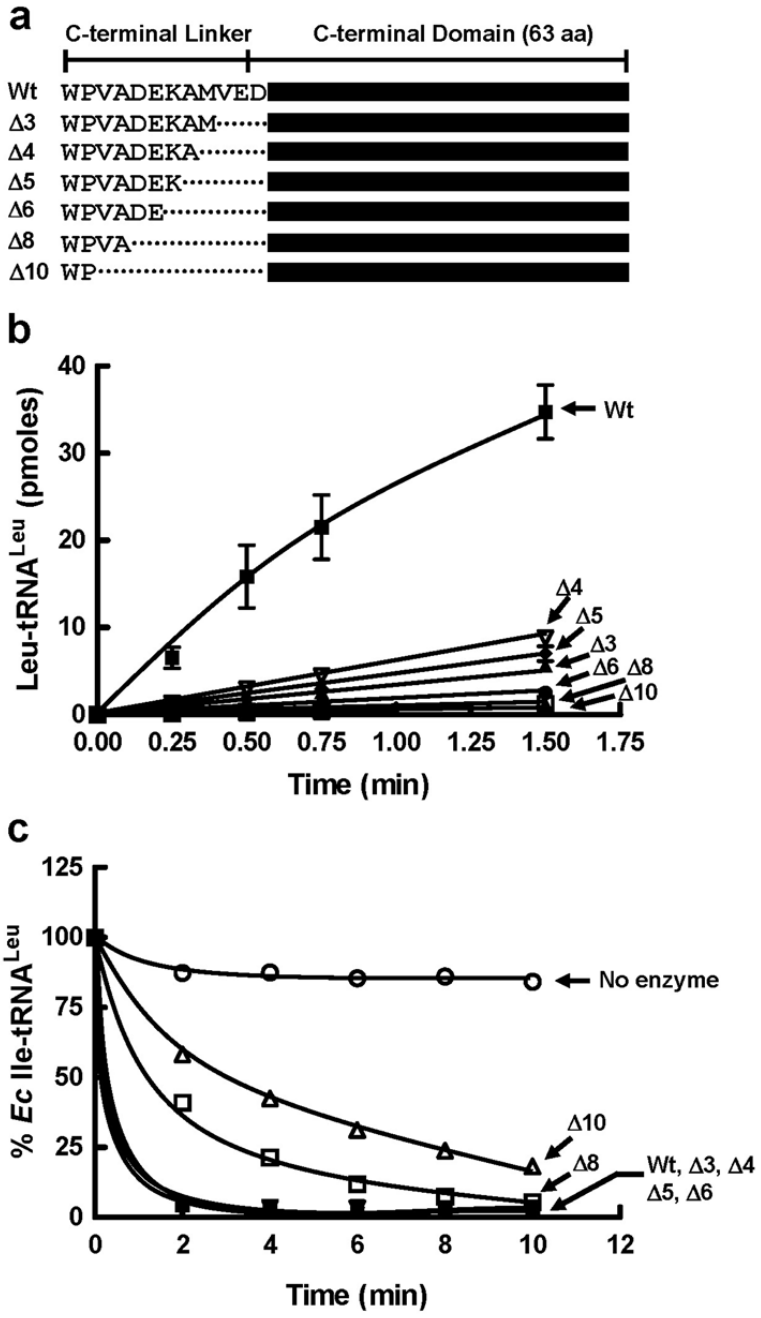

Figure 2.

Enzymatic activities of *E. coli* LeuRS wild-type and C-terminal linker deletion mutants. (A) Schematic of C-terminal linker sequence and amino acid deletion sites. The solid bars represent the C-terminal domain of 63 amino acids (aa). Amino acids in the linker peptide that were deleted are indicated by dotted lines. (B) Leucylation reactions were carried out using 21 μM [^{3}H]-L-leucine (150 μCi/ml), 4 μM *in vitro* transcribed *E. coli* tRNA^{Leu}, and 25 nM LeuRS. (C) Hydrolytic editing activities were measured using 100 nM enzyme and approximately 0.7 μM *E. coli* (*Ec*) [^{3}H]-Ile-tRNA^{Leu}. Error bars are based on reactions that were repeated at least in triplicate and are present for each point, but nominal in some cases. Abbreviations for the wild-type and C-terminal linker deletion mutants are as follows: wild-type LeuRS (Wt), ■; 3-

amino acid deletion mutant (Δ3), ▲; 4-amino acid deletion mutant (Δ4), ▽; 5-amino acid deletion mutant (Δ5), ◆; 6-amino acid deletion mutant (Δ6), ●; 8-amino acid deletion mutant (Δ8), □; 10-amino acid deletion mutant (Δ10), △; and no enzyme, ○.

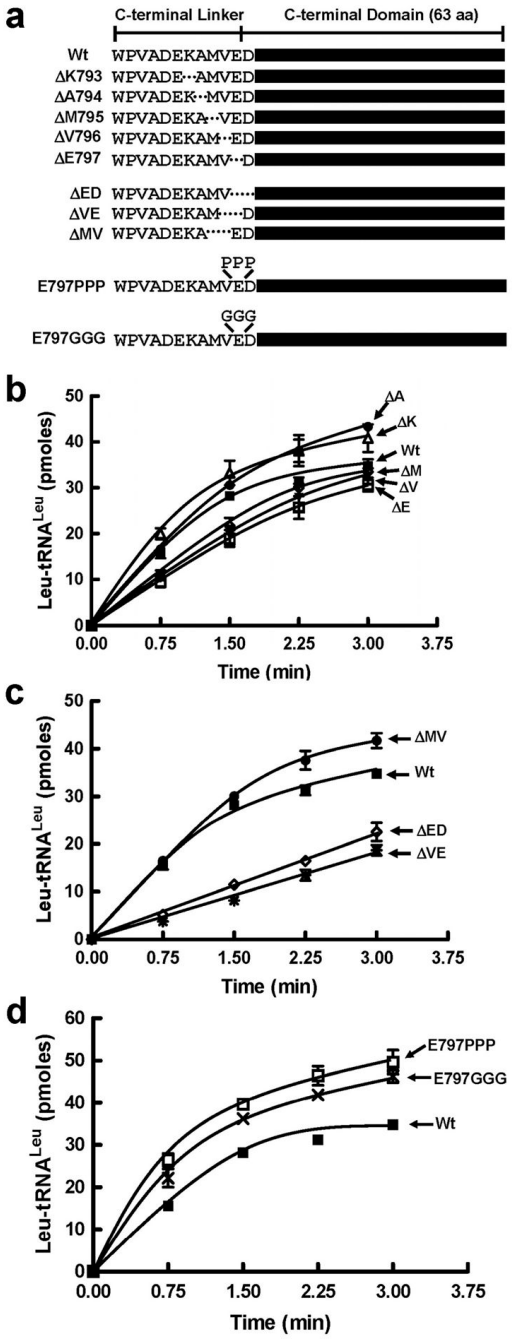

Figure 3.

Enzymatic activities of *E. coli* LeuRS wild-type and C-terminal linker deletion and insertion mutants. (A) Schematic of C-terminal linker sequence and amino acid deletion and insertion sites. The solid bars represent the C-terminal domain of 63 amino acids (aa). Specific single or double amino acid deletions are indicated by the dotted line. The triple proline (PPP) and glycine (GGG) substitutions are shown above the glutamic acid residue at position 797. (B) Leucylation reactions were carried out using 21 μM [^{3}H]-L-leucine (150 μCi/ml), 4 μM *in vitro* transcribed *E. coli* tRNA^{Leu}, and 25 nM LeuRS. (C) Hydrolytic editing activities were measured using 100 nM enzyme and approximately 0.7 μM *E. coli* (*Ec*) [^{3}H]-Ile-tRNA^{Leu}. Error bars are based on reactions that were repeated at least in triplicate and are present for

each point, but nominal in some cases. Abbreviations for the wild-type and C-terminal linker deletion mutants are as follows: wild-type LeuRS (Wt), ■; ΔK793, △; ΔA794, ●; ΔM795, ◇; ΔV796, ▼; ΔE797, □; ΔM795/V796, ○; ΔV796/E797, ✶; ΔE797/D798, ▲; E797GGG, ×; and E797PPP, ▽.

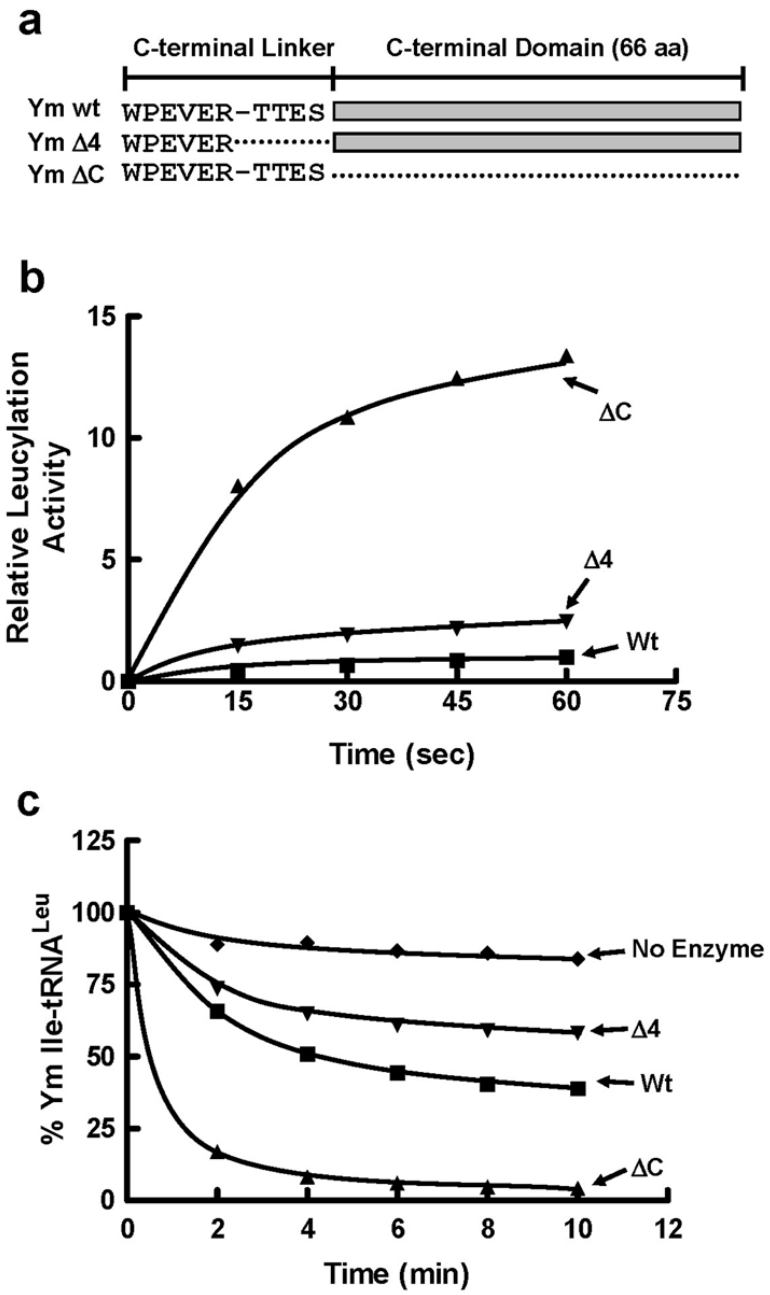

Figure 4.

Enzymatic Activities of yeast mitochondrial LeuRS wild type and deletion mutants. (A) Schematic of wild-type, C-terminal domain deletion and 4-amino acid C-terminal linker deletion mutants. The solid bars represent the C-terminal domain of 66 amino acids (aa). (B) Leucylation reactions were carried out using 21 μM [^{3}H]-L-leucine (150 μCi/ml), 4 μM *in vitro* transcribed ymtRNA^{Leu}, and 50 nM LeuRS. Each reaction was repeated at least three times and normalized relative to the activity of the wild-type enzyme. (C) Post-transfer editing activity was measured in triplicate using 100 nM of LeuRS and approximately 0.4 μM of yeast mitochondrial (Ym) [^{3}H]-Ile-tRNA^{Leu}. The C-terminal domain deletion mutant was included for comparison. Symbols and abbreviations for the LeuRSs are as follows: wild-type (Wt), ■;

C-terminal domain deletion mutant (ΔC), ▲; 4-amino acid linker deletion mutant (Δ4), ▼; and no enzyme, ◆. Error bars are based on reactions that were repeated at least in triplicate and are present, but nominal for each point.

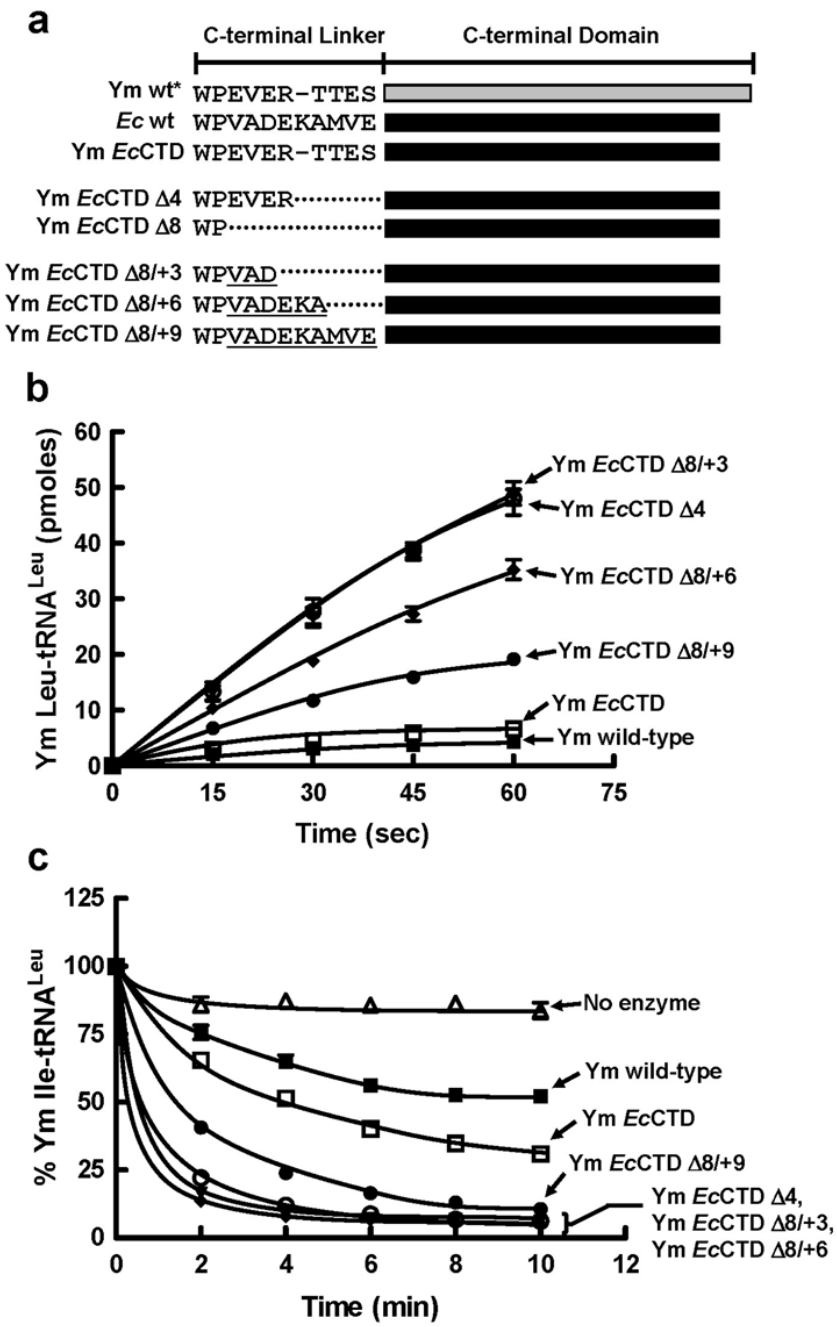

Figure 5.

Enzymatic activities of C-terminal linker mutations of yeast mitochondrial LeuRS chimeras that have an *E. coli* LeuRS C-terminal domain. (A) Schematic of *E. coli* LeuRS C-terminal linker deletions and insertions. The bars in black and gray represent the C-terminal domains of *E. coli* (63 amino acids) and yeast mitochondrial (66 amino acids) LeuRS, respectively. An asterisk marks the wild type yeast mitochondrial LeuRS protein. The dash in the linker sequence of wild-type yeast mitochondrial LeuRS originated from the sequence alignment and represents a gap generated by the program[39] and the dotted lines represent missing amino acid residues. Chimeric peptide linker swaps were constructed by inserting unique *E. coli* LeuRS C-terminal linker sequences (underlined) into deletion mutants. (B) Leucylation reactions were

carried out using 21 μM [^{3}H]-L-leucine (150 μCi/ml), 4 μM *in vitro* transcribed yeast mitochondrial tRNA^{Leu}, and 50 nM LeuRS. (C) Hydrolytic editing activities were measured using 100 nM enzyme and approximately 0.4 μM yeast mitochondrial (Ym) [^{3}H]-Ile-tRNA^{Leu}. Error bars are based on reactions that were repeated at least in triplicate and are present for each point, but nominal in some cases. Abbreviations for the wild-type and C-terminal linker deletion and insertion mutants are as follows: wild-type LeuRS (Wt), ■; Ym *Ec*CTD, □; Ym *Ec*CTD Δ4, ○; Ym *Ec*CTD Δ8/+3, ▼; Ym *Ec*CTD Δ8/+6, ◆; Ym *Ec*CTD Δ8/+9, ●; and no enzyme, △.

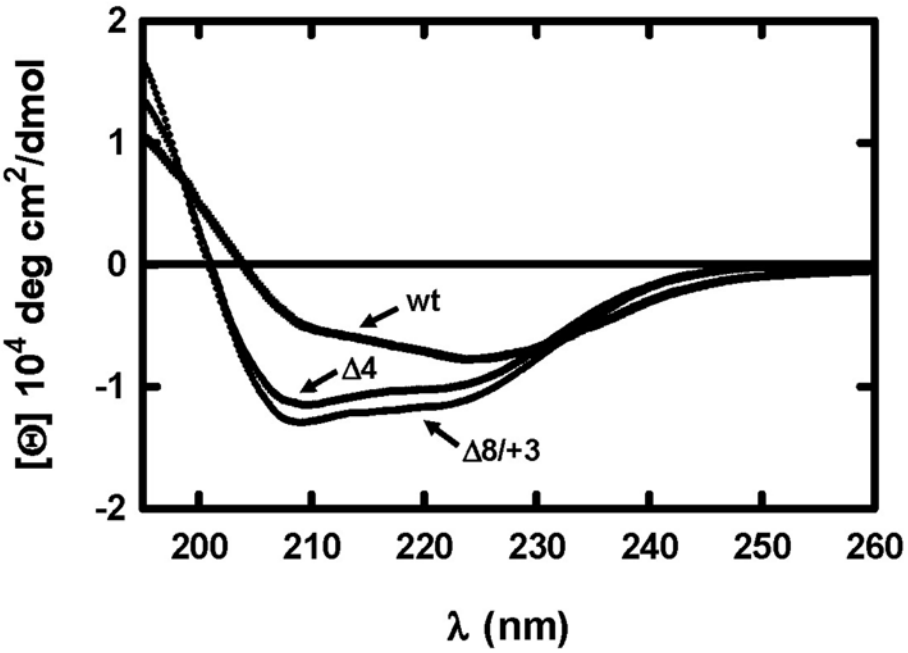

Figure 6.
CD spectra of the yeast mitochondrial LeuRS C-terminal linker chimeric swaps. Protein samples were prepared in 5 mM KPi, pH 7.5. Abbreviations for the wild-type and deletion mutants are as follows: wild-type, wt; ym *Ec*CTD chimera with a 4-amino acid deletion, Δ4; ym *Ec*CTD chimera with a 3-amino acid insertion, Δ8/+3.

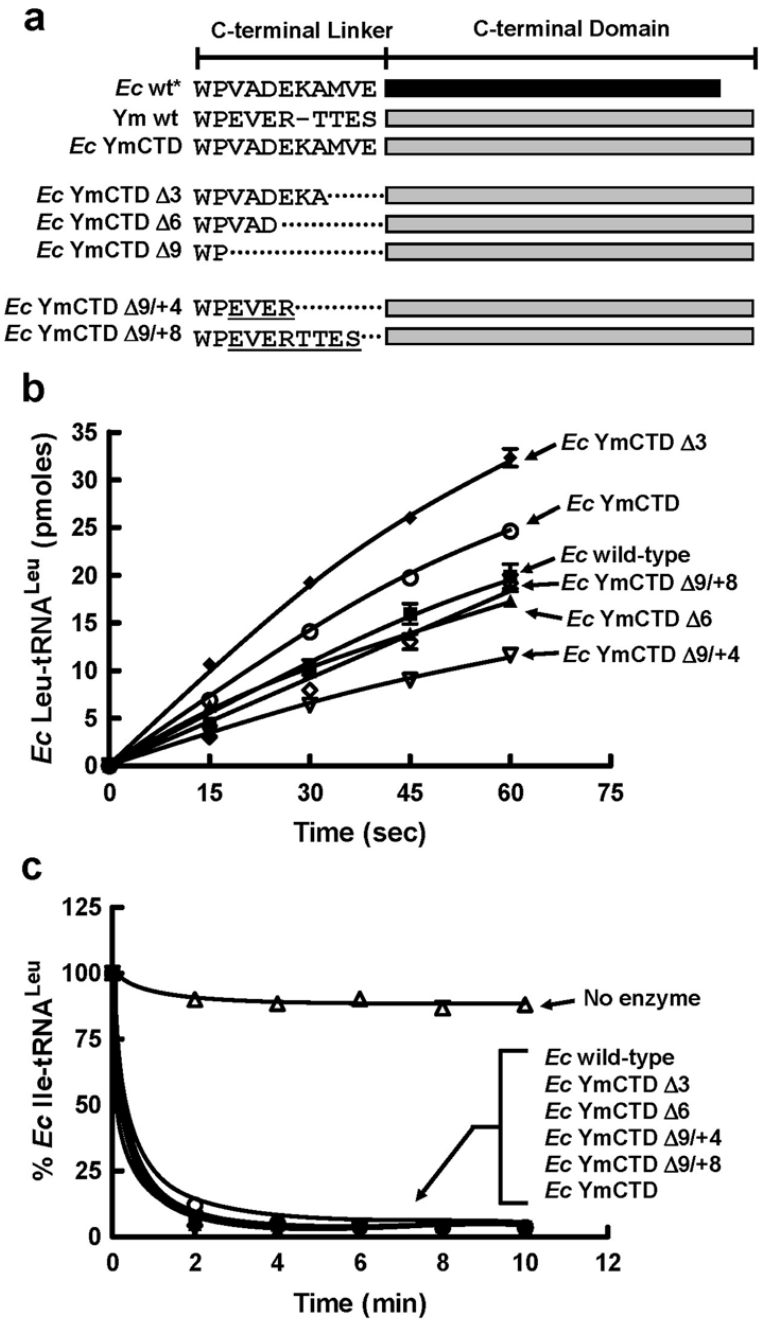

Figure 7.

Enzymatic activities of C-terminal linker mutations of *E. coli* LeuRS chimeras that have a yeast mitochondrial LeuRS C-terminal domain. (A) Schematic of *E. coli* LeuRS C-terminal linker deletions and insertions. The bars in black and gray represent the C-terminal domains of *E. coli* (63 amino acids) and yeast mitochondrial LeuRS (66 amino acids), respectively. An asterisk marks the wild type *E. coli* LeuRS protein. The dash in the linker sequence of wild-type yeast mitochondrial LeuRS originated from the sequence alignment and represents a gap generated by the program[39] and the dotted lines represent missing amino acid residues. Chimeric peptide linker swaps were constructed by inserting unique yeast mitochondrial LeuRS C-terminal linker amino acids (underlined) into deletion mutants. (B) Leucylation

reactions were carried out using 21 μM [^{3}H]-L-leucine (150 μCi/ml), 4 μM *in vitro* transcribed *E. coli* tRNA^{Leu}, and 50 nM LeuRS. (C) Hydrolytic editing activities were measured using 100 nM enzyme and approximately 0.7 μM *E. coli* [^{3}H]-Ile-tRNA^{Leu}. Error bars are based on reactions that were repeated at least in triplicate and are present, but nominal for each point. Abbreviations for the wild-type and C-terminal linker deletion and insertion mutants are as follows: wild-type LeuRS (Wt), ■; *Ec* YmCTD, ○; *Ec* YmCTD Δ3, ◆; *Ec* YmCTD Δ6, ▲; *Ec* YmCTD Δ9/+4, ▽; *Ec* YmCTD Δ9/+8, ◇; and no enzyme, △.