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Bacterial Death Results from Mutations Made in the Translocation Peptide of Leucyl-tRNA Synthetase

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ABSTRACT

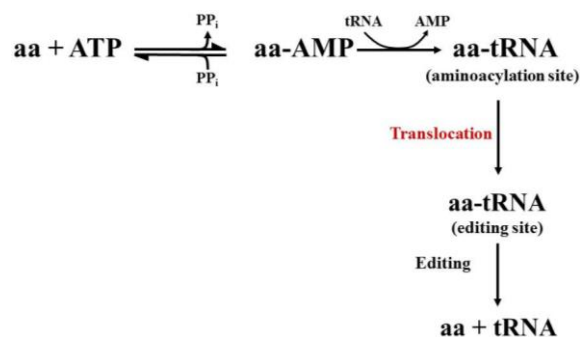
The family of aminoacyl-tRNA synthetases (aaRSs) ensures the fidelity of translation through providing a pool of correctly aminoacylated tRNA products that become incorporated by the ribosome. Leucyl-tRNA synthetase (LeuRS) has two functionally separate domains, one is the aminoacylation domain and the other is the CP1 editing domain. LeuRS can aminoacylate noncognate amino acids, therefore it relies on the CP1 editing domain to hydrolyze misaminoacylated tRNA products before they are released from the enzyme. The LeuRS enzyme must undergo a structural transition state in its reaction cycle in order to translocate the 3' acceptor stem of tRNA 30 Å from the aminoacylation active site to the CP1 domain hydrolytic active site. The translocation event is difficult to study, but we believe that we have generated mutations within LeuRS that alter the translocation event of tRNA. The mutations that we have generated lead to bacterial death in *Escherichia coli* (*E. coli*). Circular dichroism experiments indicate that our mutations do not significantly alter the secondary structure of LeuRS. *In vitro* biochemical studies demonstrate that these mutations reduce the rates of aminoacylation and hydrolysis, while also displaying misaminoacylation activity. We attribute these biochemical findings to the resulting bacterial death that is caused by these mutations.

Introduction

Polymerization of amino acids during canonical protein translation is an accurate process with a misincorporation rate of 1 per 2000 amino acids (Drummond & Wilke, 2008). During translation, cells employ many quality control mechanisms that maintain the precise production of proteins. The first quality control step is maintained by aminoacyl-tRNA synthetases (aaRSs) through the synthesis of aminoacylated tRNAs. All aaRSs rapidly bind their cognate amino acid and tRNA to turn-over an accurately aminoacylated tRNA product (Hendrickson & Schimmel, 2003). To ensure the fidelity of aminoacylation many aaRSs rely on a two-active site system termed “the double sieve model” for editing mistakes (Fersht, 1998; Fersht & Dingwall, 1979). Aminoacylation takes place in the synthetic active site termed “the coarse sieve,” which can exclude structurally dis-similar substrates. The second sieve is termed “the fine sieve,” and acts through a hydrolysis reaction to cleave incorrectly aminoacylated homologous products. When the editing process has been rendered defunct through mutational analysis, the results have been found to be deleterious for both prokaryotic and eukaryotic cells (Karkhanis, Boniecki, Poruri, & Martinis, 2006; Lee et al., 2006; Nangle, Zhang, Xie, Yang, & Schimmel, 2007)

The family of aaRSs are divided into two classes that are believed to have co-evolutionary pathways that have functionally merged from two different primitive biological processes (Woese, Olsen, Ibba, & Soll, 2000). Class I and class II aaRSs are distinct in both their structural features and their respective mechanisms of aminoacylation (Ibba & Soll,

2000; Woese et al., 2000). These structural features are known as protein folding motifs, which commonly occur in nature. One such structural motif is the Rossmann fold, which is known to bind to nucleotides and nucleotide derivatives such as nicotinamide adenine dinucleotide (NAD)(Rao & Rossmann, 1973). Class I aaRSs employ the Rossmann fold to house the aminoacylation active site, which structurally consists of parallel β - strands with the signature amino acid sequences KMSKS and HIGH (Rould, Perona, Soll, & Steitz, 1989; Schmitt, Meinnel, Blanquet, & Mechulam, 1994; Webster, Tsai, Kula, Mackie, & Schimmel, 1984). The active site within the Rossmann fold binds to ATP, the cognate amino acid and tRNA to carry out the two-step aminoacylation reaction (as can be seen below in the reaction schematic) (Arnez & Moras, 1997). For some class I aaRSs there is a second active site, which is housed within a separate domain. To generate this second domain, the Rossmann fold is split by a connective polypeptide domain (CP1) insertion sequence (Cusack, Yaremchuk, & Tukalo, 2000). The CP1 domain is only present in 4 of the 11 class I aaRSs. The CP1 domain houses the editing active site, which structurally excludes correctly aminoacylated products while hydrolyzing misaminoacylated amino acids from the tRNA (Mursinna, Lincecum, & Martinis, 2001; Swairjo & Schimmel, 2005). These two active sites work in conjunction to ensure that a pool of viable aminoacylated tRNAs are ready for ribosomal incorporation during protein translation, thereby reducing the misincorporation rate (Hendrickson & Schimmel, 2003).



Optimal coordination of the two active sites requires communication. The charged tRNA product initially within the aminoacylation active site, chemically communicates with the CP1 domain as it translocates into the editing hydrolytic active site (Hellmann & Martinis, 2009). In addition, there is structural communication that requires the CP1 domain to toggle in relation to the aminoacylation active site *via* flexible linker sequences that act as a molecular hinge (Mascarenhas & Martinis, 2008). These processes have been studied extensively for the class I aaRSs that have the CP1 domain insert such as: leucyl-tRNA synthetase (LeuRS) and the homologous isoleucyl-tRNA synthetase (IleRS) and valyl-tRNA synthetases (ValRS) (Starzyk, Burbaum, & Schimmel, 1989). Interestingly, though the two domains have many modes of communication, the CP1 domain is known to fold independently of the aminoacylation domain (Cusack et al., 2000; Fukai et al., 2000; Silvian, Wang, & Steitz, 1999). In addition, when the aminoacylation and CP1 domains have been expressed as separate independent enzymes, they can still carry out their respective reactions (Betha, Williams, & Martinis, 2007; Boniecki & Martinis, 2012; Lin, Hale, & Schimmel, 1996; Zhao et al., 2005).

X-ray crystallography data has recently shown that *E. coli* LeuRS's structure during the reaction cycle is highly dynamic (Palencia et al., 2012). Many of the domains undergo drastic structural and positional changes as the enzyme initiates aminoacylation followed by the hydrolytic reaction (Palencia et al., 2012). These structural and positional changes help

support the movement of tRNA between the aminoacylation and editing active sites, which are separated by about 30 Å (Palencia et al., 2012). It has been previously reported that the 3' acceptor stem of the tRNA must be translocated between the two active sites during LeuRS's reaction cycle (Lincecum et al., 2003; Mascarenhas & Martinis, 2008). Furthermore, the tRNA's translocation is believed to be specifically supported by interactions with the CP1 domain (Hellmann & Martinis, 2009; Mascarenhas & Martinis, 2008).

We hypothesize that the tRNA 3' acceptor stem has to first be translocated into the aminoacylation active site before the enzyme can begin its reaction cycle and that the CP1 domain structurally supports this initial translocation event, as has been previously suggested (Rock et al., 2007). Two computationally predicted hinge sites have been identified in the CP1 domain, which suggests that this region of the enzyme is highly dynamic and therefore capable of supporting this type of movement (Hellmann & Martinis, 2009; Mascarenhas & Martinis, 2008). The two molecular hinge sites have been previously described as the β -strand linker sequences and the translocation peptide (Hellmann & Martinis, 2009; Mascarenhas & Martinis, 2008). Additional mutations (D391A and F382A/N383A (FN1)) have been identified in the translocation peptide that has rendered the LeuRS enzyme non-functional in *E. coli* cells. Herein, we report our biochemical findings to support the claim that these additional sites within the translocation peptide may be integral in the pre-aminoacylation and post-aminoacylation movements of tRNA into and out of aminoacylation active site. These mutations have been found to reduce the rates of aminoacylation, hydrolysis, and to have misaminoacylation activity. Due to the reduced chemical reaction rates, we believe that the mutations may play a role in stabilizing the tRNA as it translocates between the two active sites.

Materials and Methods

Materials

T7 RNA polymerase (Invitrogen, Carlsbad, CA) was used to *in vitro* transcribe tRNA^{Leu} via run-off transcription (Sampson & Uhlenbeck, 1988). The *in vitro* transcription protocol is as follows: transcripts encoding the tRNA^{Leu} gene were obtained first by purifying 450 µg/mL of pTDNAleu14 (Normanly, Ogden, Horvath, & Abelson, 1986) using an alkaline lysis protocol. The pTDNAleu14 was restricted digested with *Bst*NI and incubated overnight at 60 °C. Digested plasmid was confirmed by agarose gel electrophoresis. The product resulting from the *Bst*NI digest was used as a template for *in vitro* transcription reactions containing 40 mM Tris-HCl, pH 8.0, 30 mM MgCl₂, 5 mM dithiothreitol (DTT), 0.01 % Triton X-100, 40 U RNase inhibitor (Eppendorf, Westbury, NY) 8 µg/ml pyrophosphatase (Sigma, St. Louis, MO), 5 mM spermidine, 50 µg/ml bovine serum albumin (BSA), 7.5 mM each of ATP, CTP, GTP, and UTP, 80 mg/ml polyethylene glycol (PEG) 8000, 60 µg/ml DNA plasmid template and 17 µM T7 RNA polymerase. The tRNA product was purified by urea-containing polyacrylamide gel electrophoresis (Sampson & Uhlenbeck, 1988) and quantitated based on absorbance at 260 nm using an extinction coefficient of 840,700 L/mol•cm (Puglisi & Tinoco, 1989). Purified tRNA^{Leu} was denatured at 80° C for 1 min, followed by an addition of 1 mM MgCl₂ and quick-cooling on ice for re-folding.

Methods

Generating Mutations: The wild type *E. coli* LeuRS gene is encoded within the plasmid pRWecLeuRS, which was generated by extracting *E. coli* genomic DNA and utilizing PCR to

amplify and subsequently clone *leuS* (GeneBank Accession #EG10532) into pET15b (Fisher Scientific, Pittsburg, PA) as previously demonstrated by Härtlen, M. and Madern, D., 1987(Hartlein & Madern, 1987). To generate pRWecLeuRS we used synthesized oligonucleotides (Integrated DNA Technology (IDT), Coralville, IA). The forward primer 5'-CGCCATATGATGCAAGAGCAATACCGCCCG-3' and reverse primer 5'-CGCGGATCCCGGTTGCTGGTCTAACTCCTC-3', which contain the *NdeI* and *BamHI* restriction sites, respectively. The resulting pRWecLeuRS was used to insert mutations *via* PCR generating the plasmids: pRWFN1 (F382A/N383A), and pRHD391A (D391A). These mutations were constructed using synthesized complementary oligonucleotides, the FN1 mutation was generated with the forward primer 5'-GACTGAAAAAGGCGTGCTGGCTGCATCTGGCGAGTTCAACG-3' and the D391A mutation was generated with the forward primer 5'-CGAGTTCAACGGTCTTGCCCATGAAGCGGCCTTCAACG-3'. In addition, we generated the T252Y mutant to purify the mischarged product [³H] Ile-tRNA^{Leu} (as described below) with the following forward primer: 5'-CCCGCCGGACTACTTTATGGGTTGTACC-3'.(Mursinna & Martinis, 2002) DNA sequencing for all plasmids was carried out by Eurofins MWG Operon, Huntsville, AL.

Protein Expression: To express the LeuRS mutants FN1 and D391A, the pRHFN1 and pRHD391A plasmids were used to transform *E. coli* BL21 (Stratagene, La Jolla, CA). A single colony was used to inoculate 5 ml of Luria-Bertani medium containing 100 µg/ml ampicillin (LB-Amp) and grown overnight at 37 °C. The overnight culture was then transferred to 1 L of LB-Amp and grown at 37 °C to an OD₆₀₀ of 0.6-0.8. Expression of LeuRS was induced with 1 mM IPTG for 3 hrs at 37 °C. Harvested cells were lysed by sonication and each LeuRS, which contained an N-terminal six-histidine tag, was purified by affinity purification using HIS-Select HF nickel affinity resin as described before (Mascarenhas & Martinis, 2008). The purified proteins were concentrated *via* Pierce concentrators 50K MWCO (Thermo Scientific, Rockford, IL) and quantitated using the Bio-Rad protein assay according to the commercial protocol.

Growth Curve Analysis: Growth curves for selected *E. coli* KL231 transformants that contained plasmids pRWFN1 and pRWD391A were grown in 3 ml of minimal standard (MS) medium (Low, Gates, Goldstein, & Soll, 1971). The MS-media was supplemented with 100 µg/ml of each of the following amino acids: leucine, isoleucine, valine, arginine, histidine, lysine, methionine, phenylalanine, threonine, tryptophan, and tyrosine. It also included 40 µg/ml uracil, 40 µg/ml adenine, 200 µg/ml thymine, 100 µg/ml thiamine, and 2% glucose. Antibiotics including kanamycin (30 µg/ml), streptomycin (50 µg/ml), and ampicillin (100 µg/ml) were also added to generate MS-Amp/Kan/Str. Overnight cultures were transferred to 50 ml of MS-Amp/Kan/Str medium for growth curve analysis, which was conducted at 42 °C. Aliquots of 1 mL were taken every 2 hr until 20 hr of incubation were completed. At 30 hr and 48 hr another set of aliquots were recovered. Aliquots of the cell cultures were analyzed by taking the OD₆₀₀ using an ultraviolet-visible spectrophotometer (Thermo Scientific, Waltham, MA).

Growth curves were also performed in *E. coli* BL21 cell (Stratagene, La Jolla, CA) transformants of pRWFN1 and pRWD391A. A single colony was used to inoculate 5 ml of LB-Amp and grown overnight at 37 °C. Overnight cultures were transferred to 50 ml of LB-Amp for growth curve analysis, which was conducted at 37 °C. Aliquots of 1 mL were taken every 2 hr until 20 hr of incubation were completed. At 30 hr and 48 hr another set of aliquots

were recovered. Aliquots of the cell cultures were analyzed by taking the OD₆₀₀ using an ultraviolet-visible spectrophotometer (Thermo Scientific, Waltham, MA).

Aminoacylation Assays: Leucylation reactions contained 60 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM DTT, 4 μM folded tRNA^{Leu}, 21 μM [³H]-L-leucine (150 μCi/ml) and 50 nM enzyme. Alternatively, isoleucylation reactions contained the same components, except 21 μM [³H]-L-isoleucine (radioactivity) was added instead of the tritiated leucine. Initial velocities for the leucylation reactions were obtained using 10 nM of enzyme. All reactions were initiated by the addition of 4 mM ATP and then quenched at specific time points by transfer to filter pads (Whatman, Clifton, NJ) that had been pre-soaked with 200 μL of 5% trichloroacetic acid (TCA). The pads were then washed three times with cold 5% TCA, followed by one wash with cold 70% ethanol at 10 min time durations for each washing. The pads were dried by soaking in ethyl ether and then under an ultra-red lamp. Each pad was transferred into a 20 ml scintillation vial with 3 mL of scintillation fluid (Fisher Scintisafe EconoF) and quantitated using a HIDEX Triathler Liquid Scintillation Counter.

Charged tRNA Preparation and Enzymatic Deacylation: The [³H]-Leu-tRNA^{leu} was generated in a one hour incubation of the leucylation reaction described above. The [³H]-Ile-tRNA^{leu} was prepared using an editing defective T252Y LeuRS mutant in an aminoacylation reaction that was incubated at 30 °C for 3 hr. Reactions were stopped using 0.18% acetic acid, and extracted using two equal volumes of phenol/chloroform/isoamyl alcohol, pH 4.3 (125:24:1) (Schreier & Schimmel, 1972). A one-half volume of 4.6 M NH₄OAc, pH 5.0, was added followed by an ethanol precipitation. The dried aminoacylated tRNA pellets were resuspended in 10 mM KH₂PO₄, pH 5.0. Hydrolytic editing assays were carried out at room temperature in 60 mM Tris, pH 7.5, 10 mM MgCl₂, and 0.8 μM [³H]-Leu-tRNA^{Leu} or 0.8 μM [³H]-Ile-tRNA^{leu} and was initiated with 100 nM enzyme (Mursinna et al., 2001). At specific time points, 5 μl aliquots were spotted onto filter pads, washed, and quantitated.

Structural Analysis of Wild type, FN1 and D391A mutants: Circular Dichroism was used to characterize the relative α-helix and β-sheet content of the wild type and mutant LeuRS enzymes. Each enzyme was diluted to 0.8 mg/mL in potassium phosphate buffer (10 mM KH₂PO₄ and 10 mM K₂HPO₄ pH 7.4). The enzymes were then inserted into a JASCO J-810 circular dichroism spectropolarimeter set at 25 °C using 50 nm/min scan speed, 0.5 sec time constant and a 1 nm bandwidth (Kelly, Jess, & Price, 2005).

Results

Examination of the Structural Impact of the FN1 and D391A mutations

The LeuRS mutations FN1 and D391A reside within the translocation peptide. The translocation peptide is approximately 12 Å away from bound tRNA (Hellmann & Martinis, 2009). Based on x-ray crystallography images of LeuRS in the aminoacylation and editing conformations, the translocation peptide is projected to move ± 12-15° with the rotational movement of the CP1 domain (Figure 1A) (Palencia et al., 2012).

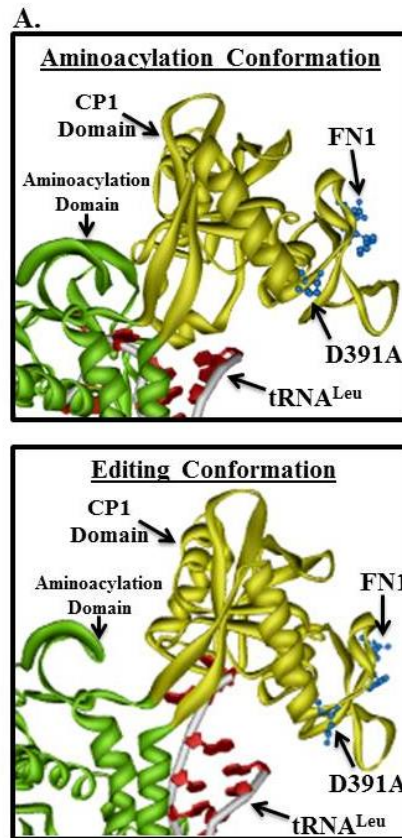


Figure 1A

Figure 1A. Secondary and Tertiary structure of the *E. coli* LeuRS and FN1 and D391A Mutants. *E. coli* LeuRS aminoacylation and editing X-ray crystal structures (Palencia et al., 2012). The CP1 domain (yellow) is inserted into the catalytic aminoacylation core (green) via two β -strand linkers. The mutants D391A and FN1 are depicted as ball and stick structures colored blue.

Previously published results have shown that the residues H392 and E393 specifically interact with the tRNA molecule to promote its translocation between the two active sites of LeuRS. This claim was supported by biochemical and computational evidence (Hellmann & Martinis, 2009). The residues D391, H392 and E393 are all part of a computationally predicted hinge region that has been projected to be a very dynamic region of the CP1 domain. As can be seen in figure 1A, the molecular hinge divides the translocation peptide into two halves, with both ends forming α -helical structures. D391 sits in the center of translocation peptide. Being that D391 is at the center of the molecular hinge site, it may contribute to crucial conformational changes the translocation peptide may experience during the transient translocation step. In addition, because of the position of D391, this residue could form electrostatic interactions with the bound tRNA. The F382 and N383 residues are outside of the molecular hinge region, and are at the distal end of the translocation peptide in relation to tRNA (Figure 1A). Therefore, it would be unlikely that these residues have direct interactions with tRNA. However, based on the structures of LeuRS and the positions of F382 and N383, these residues most likely play vital secondary structural roles in helping to provide the correct orientation of the translocation peptide so that it may interact with the tRNA. F382 and N383 are proximal to other aromatic residues in the CP1 domain, which could allow for base-stacking interactions thus stabilizing the translocation peptide.

As will be addressed, these two mutations have been characterized as non-functional in *E. coli* cells. To rule out the possibility that the bacterial death caused by these mutations result

from regional or global denaturation of the secondary structural features of LeuRS, we conducted circular dichroism analysis of the mutant enzymes in relation to the wild type enzyme (Figure 1B). As indicated by figure 1B, the circular dichroism spectrum indicates that the mutants have similar secondary structural features to the wild type enzyme.

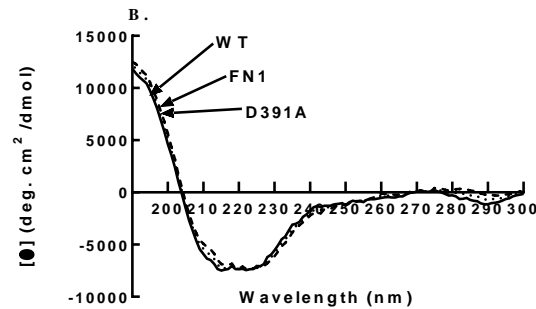


Figure 1B

Figure 1B. Secondary and Tertiary structure of the *E. coli* LeuRS and FN1 and D391A Mutants. Circular Dichroism spectrum of WT (wild type), (—) FN1, (---) and D391A, (· · ·). Sample concentration was 0.8 mg/mL in potassium phosphate buffer (10 mM KH_2PO_4 and 10 mM K_2HPO_4 pH 7.4). Each enzyme was scanned in triplicate using a JASCO J-810 circular dichroism spectropolarimeter set at 25 °C using 50 nm/min scan speed, 0.5 sec time constant and a 1 nm bandwidth (Kelly et al., 2005).

Growth curve analysis indicates FN1 and D391A are non-functional in E. coli cells

The pRWFN1 and pRWD391 plasmids were used to transform *E. coli* BL21 cells and were then grown in liquid LB-Amp media over a period of 48 hrs. The growth of the cells was monitored spectroscopically to determine their intracellular behavior. The FN1 and D391A mutants grew in a similar manner to the wild type enzyme (Figure 2A).

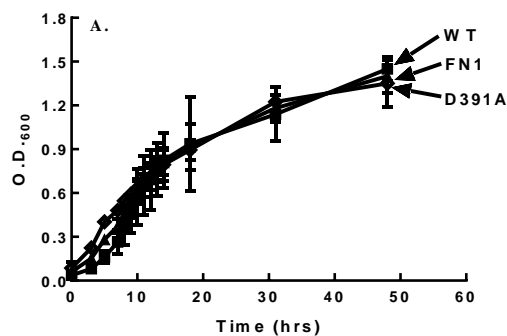


Figure 2A

Figure 2A. Growth curve of wild type LeuRS, and the mutants FN1, and D391A, using *E. coli* BL21 strain and the temperature sensitive *E. coli* KL231 strain. Growth Curve of WT, FN1 and D391A in *E. coli* BL21. Cell growth was carried out at 37 °C. Symbols represent the following: WT (wild type), (■); FN1, (▲); and D391A, (◇). Error bars are present for all points and represent growth curves that were repeated in triplicate.

Normal growth was observed because the genomic copy of wild type LeuRS was still active in the *E. coli* BL21 cells, thus rescuing growth. Interestingly, we observed a similar result when the pRWFN1 and pRWD391 plasmids were used to transform the temperature sensitive *E. coli* KL231 cells, which were grown at 30 °C. Again, this result is due to expression of genomic LeuRS within *E. coli* KL231 cells at 30 °C (Low et al., 1971). The non-functional behavior of these mutants became evident when the genomic copy of LeuRS was rendered functionally inactive within *E. coli* KL231 cells grown at 42 °C (Figure 2B) (Low et al., 1971).

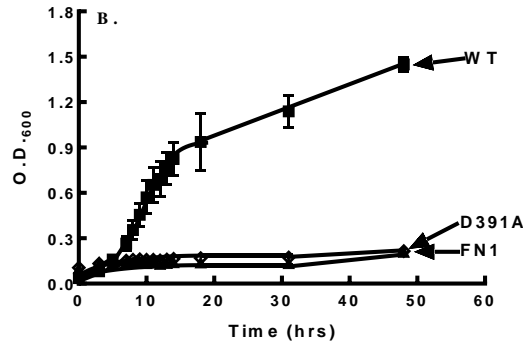


Figure 2B

Figure 2B. Growth curve of wild type LeuRS, and the mutants FN1, and D391A, using *E. coli* BL21 strain and the temperature sensitive *E. coli* KL231 strain. Growth Curve of WT, FN1 and D391A in *E. coli* KL231. Cell growth was carried out at 42 °C. Symbols represent the following: WT (wild type), (■); FN1, (▲); and D391A, (◇). Error bars are present for all points and represent growth curves that were repeated in triplicate.

At this elevated temperature, both mutants were unable to complement *E. coli* KL231 as shown in Figure 2B and therefore failed to significantly support cellular growth. Therefore, from the growth curve analysis, we have shown that FN1 and D391A reduce the growth of *E. coli* cells by approximately 80% and that this finding indicates that these mutations are non-functional. Thus, these mutations must significantly impact the biochemical behavior of bacterial LeuRS.

Biochemical evidence supports the role of FN1 and D391A in tRNA movement

In order to determine how these enzymes impact the biochemical behavior of LeuRS, the FN1 and D391A mutants were purified through ion-exchange chromatography as previously described in the *Methods* section. The purified enzymes were initially tested for their ability to leucylate tRNA (Figure 3A).

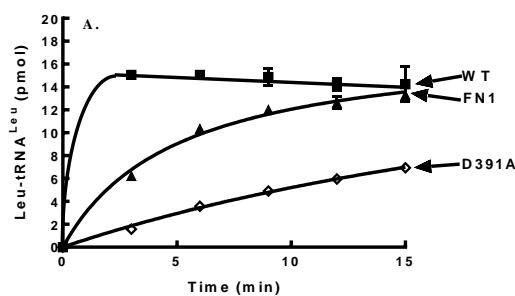


Figure 3A

Figure 3A. Biochemical Analysis of WT, FN1 and D391A LeuRS Enzymes. Leucylation activity of wild type and mutant LeuRSs. The reaction was carried out with 50 nM LeuRS, 4 μM tRNA^{Leu} transcript, and 22 μM [³H]-leucine (167 μCi/ml). Symbols represent: WT (wild type), (■); FN1, (▲); D391A, (◇); and No Enzyme (x). Error bars are present for all points and represent reactions that were repeated in triplicate.

As indicated in figure 3A, these mutants have reduced leucylation activity when compared to the wild type enzyme. This finding correlates with the results of bacterial death by suggesting that the mutant enzymes cannot turn-over leucylated tRNA fast enough for ribosomal incorporation. This would then lead to leucine-deficient proteins and thereby increase the amount of aberrant proteins being produced in the cell, thus cellular death.

Additionally, it can be seen in figure 3A that both FN1 and D391A fail to reach a maximal reaction velocity, which is represented by the plateau of leucylation activity (as seen in the wild type results). This plateau value represents an equilibrium between the acylation event and the deacylation events (Bonnet & Ebel, 1972). The deacylation events can occur through spontaneous hydrolysis of the ester linkage between the amino acid and tRNA or through enzyme-mediated deacylation, which also results in the hydrolysis of the acylated product (Bonnet & Ebel, 1972). For the wild-type enzyme these processes are in near exact equilibrium, but for FN1 and D391A equilibrium is never reached (figure 3A). The results indicate that the mutant enzymes may reduce the rates of translocation and therefore fail to produce enough acylated product to be in equilibrium with the deacylated products. Based on our data, FN1 and D391A seem to influence the translocation of the 3' acceptor stem of tRNA into the leucylation active site and then into the CP1 editing active site. We performed deacylation assays with the misacylated substrate Ile-tRNA^{Leu} as previously described. In relation to wild type LeuRS, FN1 and D391A had very slow deacylation activity and failed to reach an equilibrium state (Figure 3B).

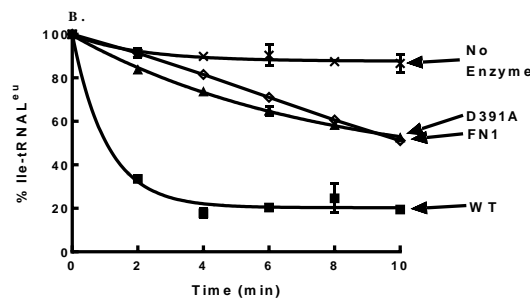


Figure 3B

Figure 3B. Biochemical Analysis of WT, FN1 and D391A LeuRS Enzymes. Deacylation of mischarged *E. coli* Ile-tRNA^{Leu} by wild type and mutant LeuRSs. Reaction conditions include 1.5 μ M [³H]-Ile-tRNA^{Leu} and 5 nM enzyme. Symbols represent: WT (wild type), (■); FN1, (▲); D391A, (◇); and No Enzyme (x). Error bars are present for all points and represent reactions that were repeated in triplicate.

FN1 and D391A deacylated approximately 50% of the available Ile-tRNA^{Leu} substrate. In general, deacylation activity is represented by three events that cannot be separated in this assay. First, there is spontaneous deacylation occurring in solution (Bonnet & Ebel, 1972). Second, there is enzymatic deacylation which involves the translocation of tRNA into the editing active site (Hellmann & Martinis, 2009). Third, is the deacylation of tRNA when it binds directly in the editing active site without undergoing a translocation event (Betha et al., 2007). Therefore, from our data it appears that FN1 and D391A both have significantly reduced translocation activity of the 3' acceptor stem of the tRNA into the editing active site.

Lastly, we conducted a misaminoacylation biochemical assay to assess whether FN1 and D391A would allow for the misaminoacylation of tRNA in the presence of noncognate amino acid. We have likened the aminoacylation active site to a “coarse sieve” whereby all structurally homologous amino acids would be charged to the 3' acceptor stem of tRNA. The FN1 and D391A mutants are believed to inhibit efficient tRNA translocation between the active sites. Figure 3C indicates that FN1 and D391A have misaminoacylation activity in the presence of noncognate amino acid. From the graph, FN1 and D391A have 2.5-5 times higher misaminoacylation activity than the wild type enzyme. These mischarging activities are directly correlated to a defunct translocation pathway resulting from these two mutations.

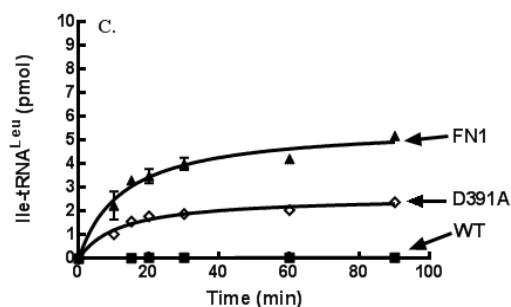


Figure 3C

Figure 3C. Biochemical Analysis of WT, FN1 and D391A LeuRS Enzymes. Misaminoacylation activity of wild type and mutant enzymes. Isoleucine mischarging reactions were carried out with 1 μ M LeuRS enzyme, 4 μ M tRNA^{Leu} transcript, and 22 μ M [³H]-isoleucine (167 μ Ci/ml). Symbols represent: WT (wild type), (■); FN1, (▲); D391A, (◇); and No Enzyme (x). Error bars are present for all points and represent reactions that were repeated in triplicate.

Discussion and Conclusions

From the structural and biochemical data we have gathered, FN1 and D391A do not appear to significantly impact the structural or functional characteristics of either active site of LeuRS. Structural data obtained through circular dichroism suggests that these mutants do not significantly alter the regional or global structure of LeuRS when compared to the spectra of the wild type enzyme. Furthermore, based on recently published x-ray crystallography data (Palencia et al., 2012), we believe these sites of mutations are too far removed from either active to significantly impact their functionality. Since we can rule out the possibility that these mutants structurally alter LeuRS, we believe that these mutants influence the biochemistry of LeuRS by altering the chemical communication between the active sites.

We hypothesize that FN1 and D391A affect the chemical communication between the two active sites by inhibiting efficient movement of the tRNA. Based on our findings, we have demonstrated that both of these mutations reduce the rate of leucylation as well skew the equilibrium of product formation. It has been previously reported that the leucylation reaction is a two-step reaction which can be broken down into a synthesis step followed by a product-release step (Francklyn, First, Perona, & Hou, 2008; Hellmann & Martinis, 2009). Mutations made within the translocation peptide were reported to alter the rate of the product-release step (Hellmann & Martinis, 2009). By extension, we hypothesize that D391A (which is within the molecular hinge region) and FN1 alter the rate of leucylation as was demonstrated in figure 3A, by reducing the efficiency of the tRNA translocation.

Based on the biochemical evidence presented in figure 3A, we hypothesize that the reduction in leucylation activity is two-fold. First, the initial rates of leucylation for the mutant enzymes are reduced by 50% or more. It has been previously proposed that tRNA translocation consists of the initial movement of the tRNA 3' acceptor stem into the leucylation active site and then a second translocation event which places the 3' acceptor stem in the hydrolytic active site (Hellmann & Martinis, 2009; Rock et al., 2007). If FN1 and D391A play a structurally supportive role in the translocation peptide's interaction with tRNA, then the initial rates of leucylation would naturally be reduced as the CP1 domain is less effective at initiating the movement of tRNA into the leucylation active site. Thus, this biochemical data suggests that FN1 and D391A both may cause the early release of tRNA before it is translocated to the CP1 domain for editing. The early release of the tRNA may happen before

the tRNA is charged with amino acid during the initial translocation into the aminoacylation active site.

As a result of this hypothesis, we propose that these mutants may initiate a leaky futile reaction cycle. The futile reaction cycle may consist of the following steps: 1. LeuRS binds tRNA, 2. enzyme fails to rapidly aminoacylate the tRNA, 3. LeuRS pre-maturely releases the tRNA, 4. the tRNA re-binds to the enzyme. This futile reaction mechanism would significantly reduce the population of leucylated tRNA in the reaction tube, thus increasing the concentration of deacylated tRNA. Therefore, the equilibrium of the leucylation reaction is skewed toward the deacylated product, which can be seen in figure 3A as both mutants fail to reach the maximal reaction velocity of wild type LeuRS.

We have hypothesized that FN1 and D391A affect the pre-aminoacylation translocation step, but we also believe these mutants may affect the post-aminoacylation translocation step. Our biochemical data supports this finding. In figure 3B, the rates of hydrolysis for both mutants are reduced, which we hypothesize is the result of a defunct translocation pathway into the editing active site. The hydrolysis activity that was observed is mainly attributed to the direct binding of the mischarged tRNA into the editing active site without undergoing translocation. We have hypothesized that the hydrolysis reaction encompasses non-enzymatic hydrolysis, hydrolysis *via* direct binding to the editing active site and hydrolysis through a translocation pathway that originates at the leucylation active site and ends at the editing active site. If all modes of editing were occurring we would see the rapid hydrolysis of misacylated tRNA until equilibrium was reached, as is the case with wild type LeuRS. However, for FN1 and D391A we see a reduced rate of hydrolysis as well as a failure to reach equilibrium. We believe that this result stems from a lack of translocation of tRNA between the two active sites, therefore the only operating modes of hydrolysis are non-enzymatic and the direct binding of tRNA to the CP1 domain.

Additionally, this finding is supported by the misaminoacylation activity of both FN1 and D391A. We have shown (Figure 3C) that these mutants produce approximately 2-5 picomoles of Ile-tRNA^{Leu} in a 90 minute reaction. This product is less than half of the Leu-tRNA^{Leu} product that is generated by these mutants in a 15 minute reaction. Though these mutants do not have robust mischarging rates, we believe that this finding is significant. We attribute the formation of aberrant product to the lack of movement of tRNA into the CP1 domain from the aminoacylation active site. Just like with previous biochemical findings, we feel that the initial rates of translocation into the active site are reduced, thus dropping the overall production of product. Once mischarged product is generated, it is released from the enzyme without undergoing translocation, which allows mischarged product to be detected. Non-enzymatic deacylation and deacylation *via* direct binding to the CP1 domain also occur, which further reduces the concentration of mischarged tRNA product.

From these *in vitro* studies, we have advocated that our mutants have defunct translocation pathways for both the pre-aminoacylation and post-aminoacylation translocation events. We have also supported our findings with *in vivo* experiments which demonstrate that FN1 and D391A are non-functional in *E. coli* cells. We hypothesize that these mutants reduce the rates of the canonical biochemical reactions of LeuRS and thus render the mutant enzymes unable to produce sufficient product to meet the synthesis demands of the bacterial cell. When expressed in bacterial cells, these mutant enzymes may result in a reduced occurrence of ribosomal leucine incorporation during translocation.

In conclusion, we hypothesize that these mutations have not significantly altered the structure of LeuRS. We further believe that these mutations do not directly alter the functionality of the active sites. Based on our data, we believe that these residues in the wild type enzyme may help promote the conformational changes that the translocation peptide may undergo throughout LeuRS's reaction cycle. Therefore, FN1 and D391A may reduce the rates of translocation, which impairs the biochemical processes of LeuRS that ultimately leads to bacterial death.

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