CHARACTERIZATION OF LEUCYL-TRNA SYNTHETASE FROM *HOMO SAPIENS* AND *ESCHERICHIA COLI* IN AMINOACYLATION, AMINO ACID EDITING AND INTERDOMAIN INTERACTIONS

BY

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DISSERTATION

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Abstract

Aminoacyl-tRNA synthetases (aaRSs) are ancient enzymes that charge tRNA with its cognate amino acid. In order to maintain fidelity during protein synthesis, editing mechanisms ensure that tRNAs are accurately charged. Leucyl-tRNA synthetase (LeuRS) has an editing active site that resides in a discrete domain called the connective polypeptide 1 domain (CP1). Post-transfer editing involves the translocation of mischarged tRNA from the aminoacylation to the editing active site where mischarged tRNA binds for hydrolysis of the noncognate amino acid to enhance fidelity. Based on crystal structure analysis, the CP1 domain rotates 30° relative to the canonical core where aminoacylation occurs during tRNA translocation and presumably facilitates the movement of tRNA from the core domain to the editing domain. Single molecule fluorescence resonance energy transfer (smFRET) techniques were employed to characterize this dynamic movement of tRNA from one domain of the enzyme to another.

Human cytoplasmic LeuRS (hscLeuRS) is typically found in a macromolecular complex containing at least eight other proteins. In order to study this enzyme, hscLeuRS was expressed independent of the complex in *Escherichia coli*. Enzymatic characterization of the isolated hscLeuRS suggested that it attaches a second leucine to Leu-tRNA^{Leu}. Liquid chromatography and mass spectrometry methods were used in an attempt to isolate this hypothesized "doubly charged" tRNA species and it is possible that hscLeuRS possesses a secondary function beyond aminoacylation reliant on a doubly charged Leu-Leu-tRNA^{Leu}. Further biochemical analysis of the hscLeuRS focused on its editing pocket. The editing site of hscLeuRS includes a highly conserved threonine discriminator and universally conserved aspartic acid that were mutationally characterized. Substitution of threonine to alanine uncoupled specificity similar to other LeuRSs. However, the introduction of bulky residues in the amino acid binding pocket

failed to block deacylation of tRNA, indicating that the architecture of the amino acid binding pocket is different compared to other characterized LeuRSs. In addition, mutation of the universally conserved aspartic acid abolished $tRNA^{Leu}$ deacylation. Surprisingly though, this editing-defective hscLeuRS maintained fidelity. This indicates that an alternate editing mechanism may have been activated upon failure of the post-transfer editing active site in order to maintain fidelity during protein synthesis.

To Mum and Dad for your love and encouragement

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Abbreviations

aaRS	aminoacyl-tRNA synthetase
ABD	anticodon binding domain
AIMP	aminoacyl-tRNA synthetase interacting multifunctional protein
AlaRS	alanyl-tRNA synthetase
AMP	adenosine 5′-monophosphate
Ap_4A	diadenosine 5′,5′′′-P1,P4-tetraphosphate
AspRS	aspartyl-tRNA synthetase
ArgRS	arginyl-tRNA synthetase
ATP	adenosine 5′-triphosphate
A76	adenosine at position 76 of tRNA
BME	β-mercaptoethanol
CAAD	cyanobacterial aaRS appended domain
cDNA	complementary DNA
CP1	connective polypeptide 1
cy3	cyanine-3
cy5	cyanine-5
ΔCP1	LeuRS lacking CP1 domain
D-arm	dihydrouridine arm
DHU	dihydrouridine
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol

E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EF-Tu	elongation factor Tu
EM	electron microscopy
ESI	electrospray ionization
gag	group specific antigen
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GDP	guanosine diphosphate
GFP	green fluorescent protein
GluRS	glutamyl-tRNA synthetase
GluProRS	glutamyl-prolyl tRNA synthetase
GlnRS	glutaminyl-tRNA synthetase
GTP	guanosine triphosphate
HA	hemagglutinin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-KOH
HIV	human immunodeficiency syndrome
HPLC	high performance liquid chromatographer
hscLeuRS	human cytoplasmic LeuRS
hscLysRS	human cytoplasmic LysRS
IleRS	isoleucyl-tRNA synthetase
IPTG	isopropyl β-$_D$-1-thiogalactopyranoside
k_{cat}	catalytic turnover number
k_{cat}/K_M	enzyme efficiency

K_M	Michaelis constant
LB	Luria broth
LC/MS	liquid chromatography/mass spectrometry
LeuRS	leucyl-tRNA synthetase
LSD	leucine specific domain
LysRS	lysyl-tRNA synthetase
MetRS	methionyl-tRNA synthetase
MIC	minimum inhibitory concentration
MITF	microphthalmia transcription factor
mRNA	messenger RNA
mTOR	mammalian Target of Rapamycin
N. crassa	*Neurospora crassa*
NHS	*N*-hydroxysuccinimide
N73	base at position 73 of tRNA
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEI cellulose	polyethyleneimine cellulose
PEG	polyethylene glycol
PGC	porous graphitic carbon
PP_i	pyrophosphate
P. horikoshii	*Pyrococcus horikoshii*
RNA	ribonucleic acid

SerRS	seryl-tRNA synthetase
smFRET	single molecule fluorescence resonance energy transfer
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
T-arm	TψC arm of tRNA
TBST	tris-buffered saline and tween 20
TCA	trichloroacetic acid
TCEP	*tris*(2-carboxyethyl)phosphine
ThrRS	threonyl-tRNA synthetase
TIR	total internal reflection
TLC	thin-layer chromatography
tmRNA	transfer-messenger RNA
TNF-α	tumor necrosis factor α
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer ribonucleic acid
TrpRS	tryptophanyl-tRNA synthetase
TyrRS	tyrosyl-tRNA synthetase
T4-PNK	T4-polynucleotide kinase
T. Thermophilus	*Thermus Thermophilus*
ValRS	valyl-tRNA synthetase
VMD	visual molecular dynamics

Chapter I: Introduction

I.1. General Features of Aminoacyl-tRNA Synthetases

Accurate protein synthesis is dependent upon a group of ancient enzymes: Aminoacyl-tRNA synthetases (aaRSs). Typically, each aaRS is responsible for activating one of the twenty standard amino acids, which provide diversity in protein structure that is important for protein function. Identity elements within the tRNAs serve as molecular determinants that confer specificity of the aaRS for tRNA (Giegé et al. 1998). This ensures precise interaction between the tRNA and synthetase, thus promoting accurate protein synthesis.

The aaRSs are divided into two classes based on their chemical properties, architecture of their catalytic domains as well as the presence of certain consensus sequences (Cusack et al. 1990; Eriani et al. 1990; Ribas de Pouplana and Schimmel 2001). Each class is then further separated into three subclasses a, b, and c based on their unique mechanistic properties, anticodon-binding domain characteristics as well as the organization of structural motifs unique to each of the two main classes (Table 1.1) (Moras 1992; Cusack 1995; Landes et al. 1995; Ribas de Pouplana and Schimmel 2001; Mascarenhas et al. 2008). A Rossmann dinucleotide binding fold defines the core of class I aaRSs that is also marked by two unique sequences, "KMSKS" (Lys-Met-Ser-Lys-Ser) (Hountondji et al. 1986) and "HIGH" (His-Ile-Gly-His) (Webster et al. 1984). The HIGH sequence plays a role in amino acid activation (Webster et al. 1984), while the KMSKS sequence stabilizes the 3′ end of tRNA and allows amino acid to be charged to it (Fersht et al. 1988; Perona et al. 1993; Arnez and Moras 1997).

Table I.1. Classification of aaRSs based on structural and chemical properties

Class I	Class II
Ia	IIa
LeuRS*	SerRS
IleRS*	ThrRS*
ValRS*	AlaRS*
MetRS*	GlyRS*
CysRS	ProRS*
ArgRS	HisRS
Ib	IIb
GlnRS	AspRS
GluRS	AsnRS
LysRS-I	LysRS-II*
Ic	IIc
TyrRS	PheRS*
TrpRS	
	SepRS
	PylRS

*These amino acids have a hydrolytic editing pathway to clear misactivated and mischarged amino acids

In contrast, class II aaRSs are usually found in dimeric or tetrameric form and possess a catalytic core that is comprised of seven antiparallel β-strands flanked by α-helices (Rossmann et al. 1974; Rould et al. 1989; Cusack et al. 1990). The canonical active site also harbors three conserved motifs. Motif 1 [gϕxxϕxxpϕϕ] is found at the interface of the dimer while motif 2 [fRxe-h/rxxxFxxx(d/e)] and motif 3 [gϕgϕgϕ(d/e)Rϕϕϕϕϕ] comprise part of the aminoacylation active site (ϕ represents a hydrophobic amino acid) (Cusack et al. 1990; Eriani et al. 1990; Ruff et al. 1991) (Figure I.1). Of note, in the past decade two new aaRSs that

activate O-phosphoserine and pyrrolysine have been added to the class II group (Polycarpo et al. 2004; Sethi et al. 2005).

Lysyl-tRNA synthetase (LysRS) has been historically associated with the class II aaRSs, but is unique because a few examples from archaea have been found to be represented in class I too (Ibba et al. 1997). *Methanococcus maripaludis* LysRS for example is a 62 kilodalton protein that resembles a class I synthetase, in contrast to other known examples of class II LysRSs that are bacterial and eukaryotic in origin (Ibba et al. 1997). Generally, class I and II LysRSs do not co-exist in organisms, with the exception of *Methanosarcineae*, where both the LysRSs are thought to be involved in the translation of pyrrolysine (Galagan et al. 2002; Ibba and Söll 2002; Polycarpo et al. 2003).

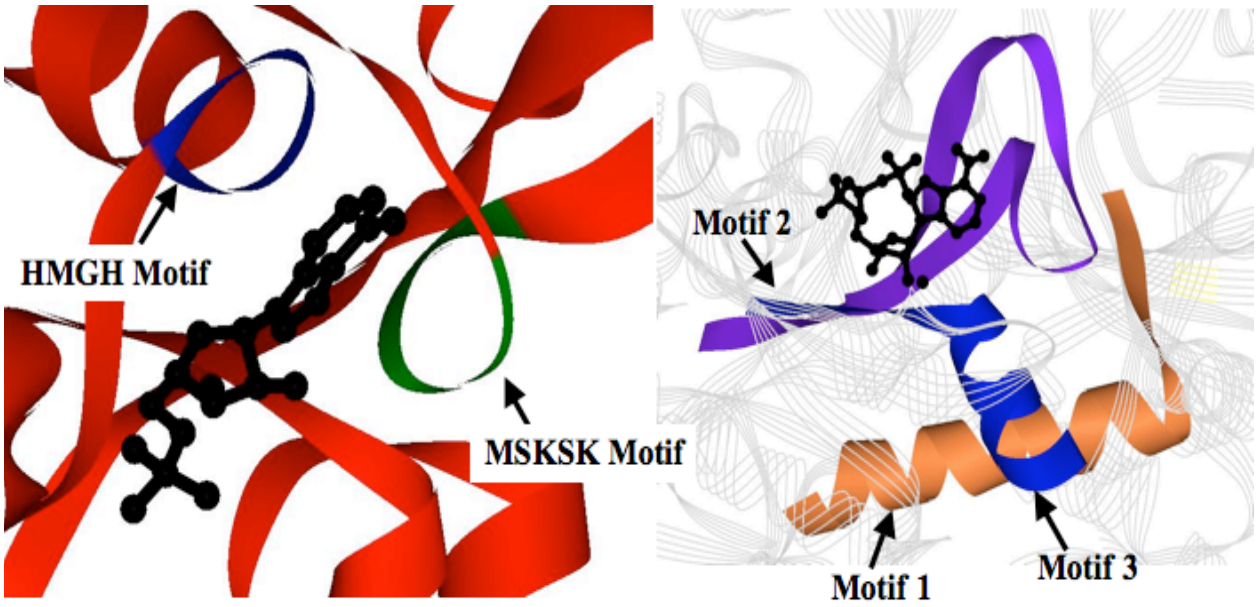


Figure I.1 Conserved sequences in class I and II aaRS active sites. The LeuRS enzyme from *Thermus thermophilus* (*T. thermophilus*) is shown on the left with a sulphamoyl analogue of leucyl-adenylate drawn in black within the synthetic active site. The conserved sequences HMGH and MSKSK are highlighted (PDB: 2VOC) (Rock et al. 2007). Glycyl-tRNA synthetase (GlyRS) is shown on the right with ATP drawn in black in the active site. The three conserved motifs characteristic of class II aaRSs are highlighted. Motif 1 is colored in orange, motif 2 in purple and motif 3 in blue. ATP is shown in black. (PDB: 1B76) (Arnez et al. 1999).

Some aaRSs have evolved to acquire insertions in addition to their catalytic canonical core. In many cases, these inserted domains enhance the enzyme's specificity and fidelity for the aminoacylation reaction (Schimmel and Ribas De Pouplana 2000). One example of this is the connective polypeptide 1 (CP1) hydrolytic editing domain (Starzyk et al. 1987; Lin et al. 1996) that is possessed by some aaRSs to maintain fidelity during the charging of tRNA with cognate amino acid. Another example of an inserted domain present in some aaRSs is the C-terminal anticodon binding domain (ABD). The ABD is essential for anticodon loop recognition of most aaRSs with the exception of leucyl-tRNA synthetase (LeuRS), seryl-tRNA synthetase (SerRS), and alanyl-tRNA synthetase (AlaRS) (Asahara et al. 1993; Asahara et al. 1993; Giegé et al. 1998). These ABDs are highly varied in their structure. For example, ABDs from glutamyl-tRNA synthetase (GluRS) (Nureki et al. 1995) and glutaminyl-tRNA synthetase (GlnRS) (Rould et al. 1989) are primarily made up of α-helices and β-strands, respectively.

I.2. Mechanism of Aminoacylation Reaction

During protein synthesis, aaRSs ensure that amino acid is charged to the 3′ end of its cognate tRNA with an error rate of less than 1 in 10^4 (Loftfield 1963). This charged tRNA is then bound by elongation factor Tu (EF-Tu) and transported to the ribosome for protein synthesis. Maintenance of fidelity during translation is crucial as mistakes can result in deceased viability of cells and disease (Karkhanis et al. 2006; Lee et al. 2006).

The aaRSs catalyze a two-step reaction mechanism. In the first step, amino acid is activated with ATP by the aaRS to form an aminoacyl-adenylate intermediate with the release of pyrophosphate (PP_i). In the second step, this activated amino acid then gets charged to the 3′ end of its cognate tRNA (Ibba and Söll 2004; Mascarenhas et al. 2008).

$$\text{ATP} + \text{Amino Acid (AA)} \leftrightarrows \text{AA-AMP} + PP_i \qquad \text{(I.1)}$$

$$\text{AA-AMP} + tRNA^{AA} \rightarrow \text{AA-}tRNA^{AA} + \text{AMP} \qquad \text{(I.2)}$$

Both classes of aaRSs catalyze the two-step aminoacylation reaction via different mechanisms. Class I aaRSs bind ATP in an extended conformation (Brick and Blow 1987; Fersht et al. 1988), while class II aaRSs bind ATP in a bent conformation (Rould et al. 1989; Cavarelli et al. 1994). In addition, the class I aaRSs bind the tRNA stem via the minor groove side (Rould et al. 1989), thus orienting the 2′-hydroxyl group of the A76 ribose for attachment of amino acid (Sprinzl and Cramer 1975). In contrast, class II aaRSs bind ATP in a bent conformation (Rould et al. 1989; Cavarelli et al. 1994) and bind the tRNA stem via the major groove side (Ruff et al. 1991), resulting in the 3′-hydroxyl group being more accessible for charging (Figure I.2) (Sprinzl and Cramer 1975).

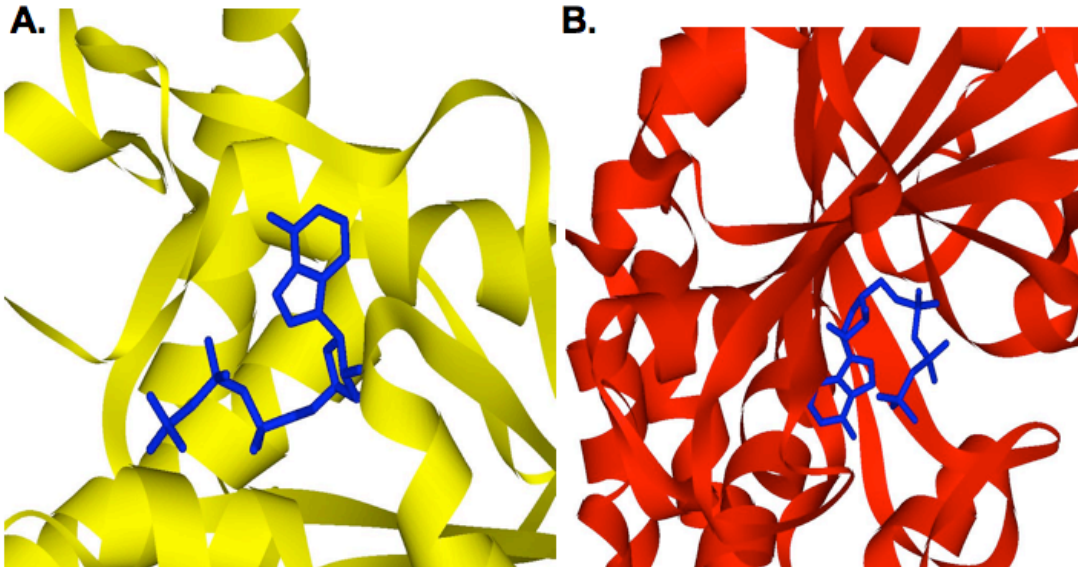
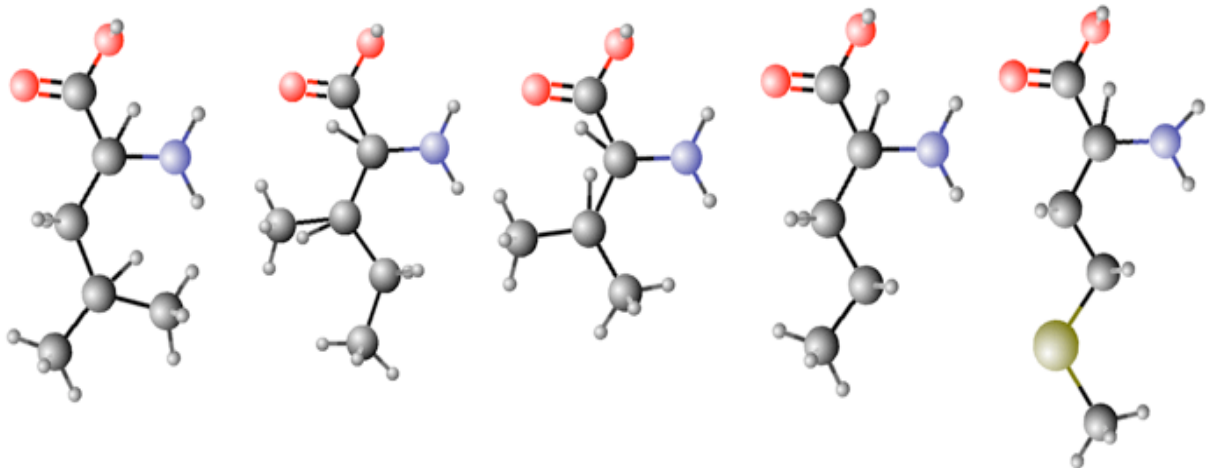


Figure I.2 Conformation of ATP during binding to aaRS. (A) The binding of ATP in an extended conformation to the active site of the class I tryptophanyl-tRNA synthetase (TrpRS) is shown on the left (PDB file: 1MAU) (Retailleau et al. 2003). The enzyme is highlighted in yellow and the ATP in blue. (B) The binding of ATP in a bent conformation to the active site of class II AlaRS is shown on the right (PDB file: 1YFR) (Swairjo and Schimmel 2005). The enzyme is highlighted in red and the ATP in blue.

I.3 Hydrolytic Editing of LeuRS

In order to ensure the highest level of fidelity during protein synthesis, some aaRSs have developed editing mechanisms to clear errors during the charging of tRNA (Hendrickson and Schimmel 2002; Geslain and Ribas de Pouplana 2004; Mascarenhas et al. 2008). Linus Pauling first predicted an error rate of 1 in 20 during protein synthesis when he projected a general inability of enzymes to distinguish fully between structurally similar amino acids that differed from each other by only a methyl group (Pauling 1958) (Figure I.3). However, Loftfield later measured the error rate for the misincorporation of valine for isoleucine in chicken ovalbumin to be much lower at an occurrence of less than 1 in 3000 (Loftfield 1963). In order to reconcile the projected error rate with high discrimination rate, Alan Fersht proposed that some aaRSs possess a secondary editing site where corrections can be made to confer a high specificity to the aaRS (Fersht 1977).

In order to protect against statistical errors that could be incorporated into proteins during translation, about half of the family of aaRSs have evolved proofreading mechanisms (Hendrickson and Schimmel 2002; Mascarenhas et al. 2008). These fidelity mechanisms are critical to the cell. Defects in aaRS editing result in cell death (Karkhanis et al. 2006; Nangle et al. 2006; Karkhanis et al. 2007) and have been shown to cause neurological disease in mammals (Lee et al. 2006).



Leucine Isoleucine Valine Norvaline Methionine

Figure I.3 Structurally similar amino acids. The red balls represent oxygen, the blue balls nitrogen, the yellow ball sulphur, the larger gray balls carbon, and the small gray balls hydrogen.

Alan Fersht hypothesized that some aaRSs, including isoleucyl-tRNA synthetase (IleRS) (Baldwin and Berg 1966), and valyl-tRNA synthetase (ValRS) (Fersht and Kaethner 1976) function via a double sieve mechanism in order to maintain fidelity (Fersht and Dingwall 1979). In this model, the aminoacylation active site serves as a coarse sieve for amino acid activation in the canonical core, for example, to exclude bulky amino acids, but allow smaller isosteric amino acids to pass through. The second hydrolytic active site operates as a fine sieve to bind to mischarged amino acid for hydrolysis, while correctly charged tRNAs are released to elongation factors (Figure I.4).

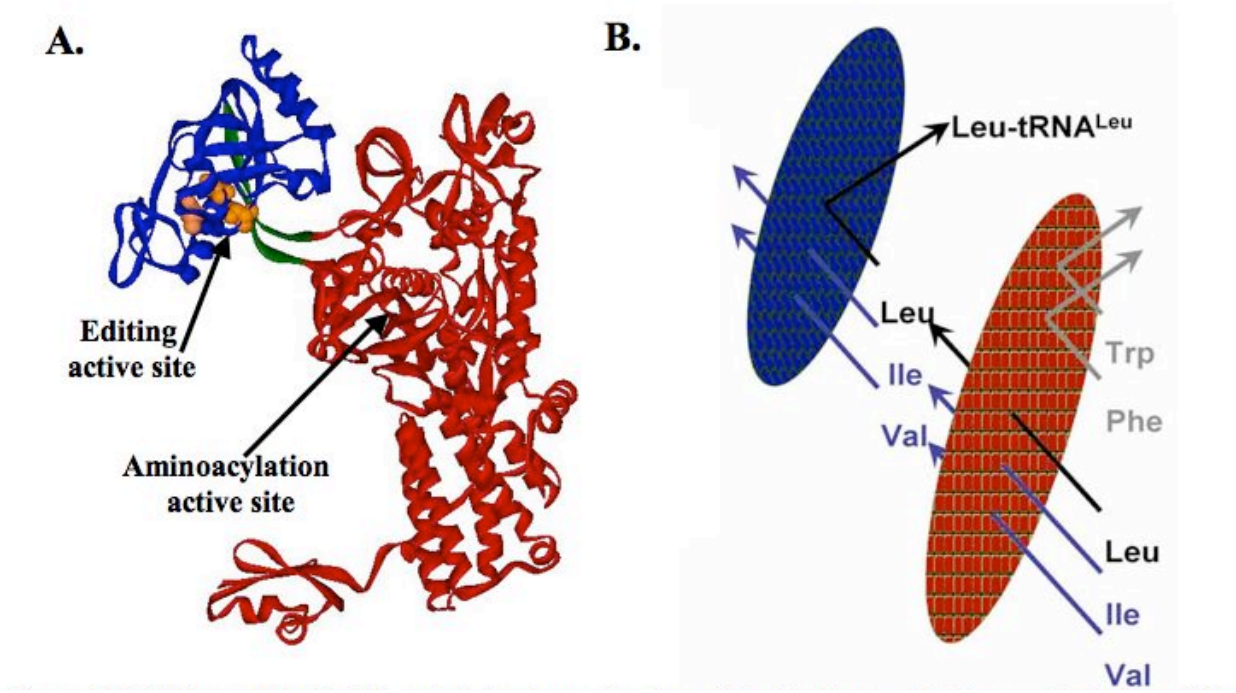


Figure I.4 *T. thermophilus* LeuRS crystal structure and cartoon of double sieve model. The overall structure of *T. thermophilus* LeuRS is shown on the left (A). Residues that impact editing function are shown in orange. The CP1 domain, β-strands, and the aminoacylation core domain are highlighted in blue, green, and red respectively. The double sieve model is shown on the right (B). LeuRS functions as a double sieve whereby the aminoacylation core domain serves as a "coarse sieve" that excludes larger amino acids and the editing active site acts as a "fine sieve" that blocks out cognate amino acid but allows non cognate amino acids to be hydrolyzed. The red region represents the coarse sieve while the blue region represents the fine sieve. Structure on left (PDB: 2BTE) adapted from (Tukalo et al. 2005) and cartoon on the right adapted from (Mursinna and Martinis 2002).

The aaRSs edit by different pathways: Pre-transfer and post-transfer of charging to the tRNA. In pre-transfer editing, misactivated amino acid is cleaved to release AMP and free amino acid. Post-transfer editing hydrolyzes mischarged tRNA to yield free tRNA and non-cognate amino acid (Figure I.5). It has been proposed that one editing pathway dominates over the other (Englisch et al. 1986; Williams and Martinis 2006). However, the exact relative contribution of each pathway toward fidelity is still largely unclear.

Some enzymes have been shown to activate a second editing pathway in the failure of the dominant editing pathway. For example, when the post-transfer editing active site of

Escherichia coli (*E. coli*) LeuRS is removed, the enzyme maintains overall fidelity through the activation of a pre-transfer editing pathway (Boniecki et al. 2008). Likewise, when post-transfer editing in the human cytoplasmic LeuRS (hscLeuRS) is mutationally knocked out, the enzyme fails to mischarge tRNA, indicating the activation of a second editing pathway to maintain overall accuracy (Chapter III) (Pang and Martinis 2009).

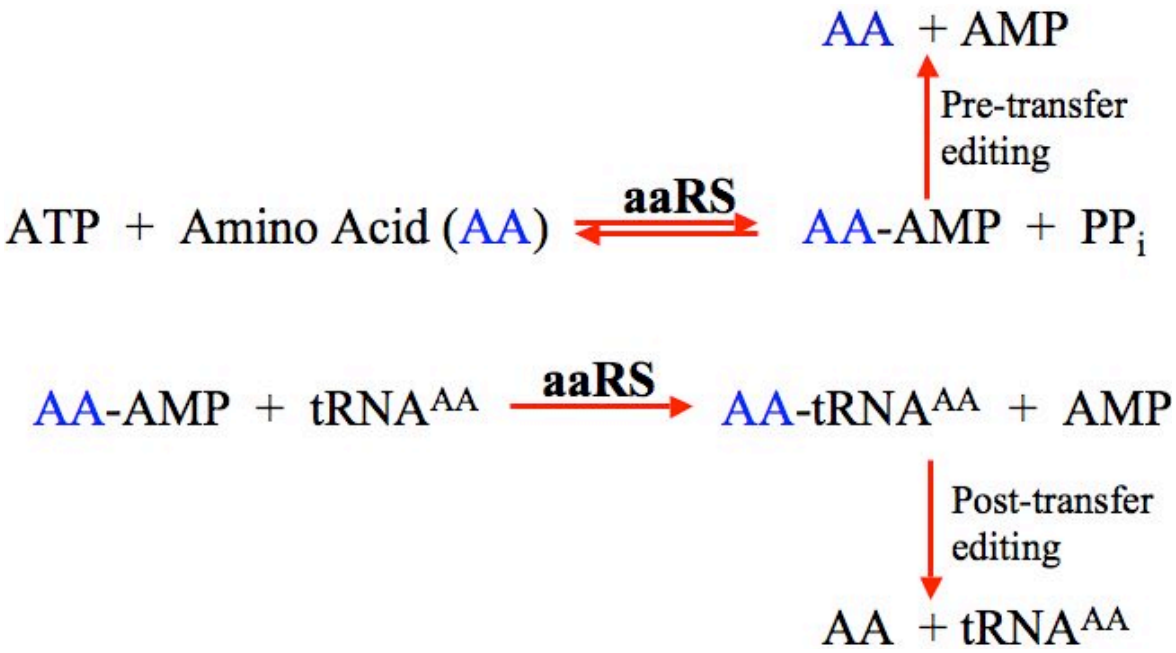


Fig I.5 Two editing mechanisms of aaRSs. Post-transfer editing occurs when the incorrectly charged tRNA gets hydrolyzed by the aaRS while pre-transfer editing occurs when the activated amino acid is cleaved to form free amino acid and AMP.

A number of aaRSs carry out proofreading functions in *cis* via a domain that possesses editing activity, which is inserted into the catalytic core (Hendrickson and Schimmel 2002). However, some archaea as well as bacterial aaRSs lack an editing domain that ensures fidelity during protein synthesis. In order to compensate for this, independent paralogs and homologues of editing domains possessing hydrolytic editing activity have been found to occur naturally. An example is the ProRS editing domain homologue YbaK that can edit mischarged Ala-tRNA^{Pro} and Cys-tRNA^{Pro} in *Haemophilus influenzae* (Wong et al. 2003;

An and Musier-Forsyth 2004). Additionally, ProX, a paralog of the ProRS editing domain from *Clostridium sticklandii* hydrolyzes Ala-tRNA^{Pro} (Ahel et al. 2003), while AlaX, an AlaRS homolog from *Methanosarcina barkeri* and *Sulfolobus solfataricus* cleaves mischarged Ser-tRNA^{Ala} and Gly-tRNA^{Ala} (Ahel et al. 2003; Chong et al. 2008).

In either the case of *cis* or *trans* editing, the mischarged 3′ end of the tRNA must be transferred from the aminoacylation to the editing active site. X-ray crystallography suggests that the 3′ end of tRNA must swing approximately 30 Å from the aminoacylation core domain to the CP1 domain of the enzyme via its β-strands (Figure I.6) (Tukalo et al. 2005). The flexibility of these β-strands connecting the aminoacylation core domain to the editing domain is essential for translocation and editing. Substitution of a specific glycine within the β-strands disrupted tRNA translocation (Mascarenhas and Martinis 2009). A "translocation peptide" within the CP1 domain of *E. coli* LeuRS also facilitates transfer of tRNA from the canonical core to the CP1 domain (Hellmann and Martinis 2009). Comparison of the crystal structures of LeuRS in various states of binding to tRNA suggested that the CP1 domain rotates through the different stages of aminoacylation and its movement is dependent on the flexibility of the β-strands (Mascarenhas and Martinis 2008).

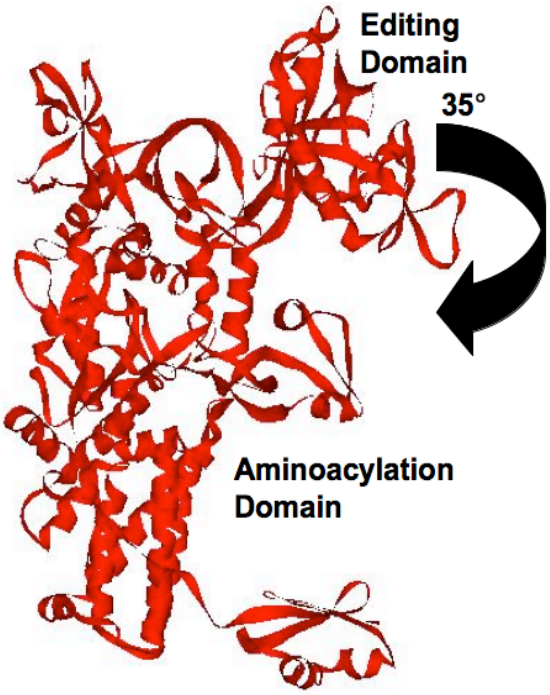


Figure I.6 Crystal structure of *T. thermophilus* LeuRS. The CP1 domain of LeuRS rotates 35° relative to the canonical core during the translocation of tRNA, PDB entry 2BTE. (Tukalo et al. 2005).

I.4. Aminoacyl-tRNA Synthetase Recognition of tRNA

In general, tRNAs have similar secondary as well as tertiary structure (Figure I.7) (Kim 1979). Thus, the aaRSs depend on embedded identity elements within the tRNA substrate to recognize and differentiate their cognate substrate for aminoacylation (Hou and Schimmel 1988; McClain and Foss 1988; Normanly et al. 1992). Certain structural features of the aaRS can also play an important role in ensuring specificity during tRNA binding. For example, the tRNA acceptor stem binds to the canonical core domain of a specific aaRS. In some class I aaRSs, a zinc binding domain is important for the binding of the acceptor stem of tRNA (Fourmy et al. 1995; Liu et al. 1995; Glasfeld and Schimmel 1997).

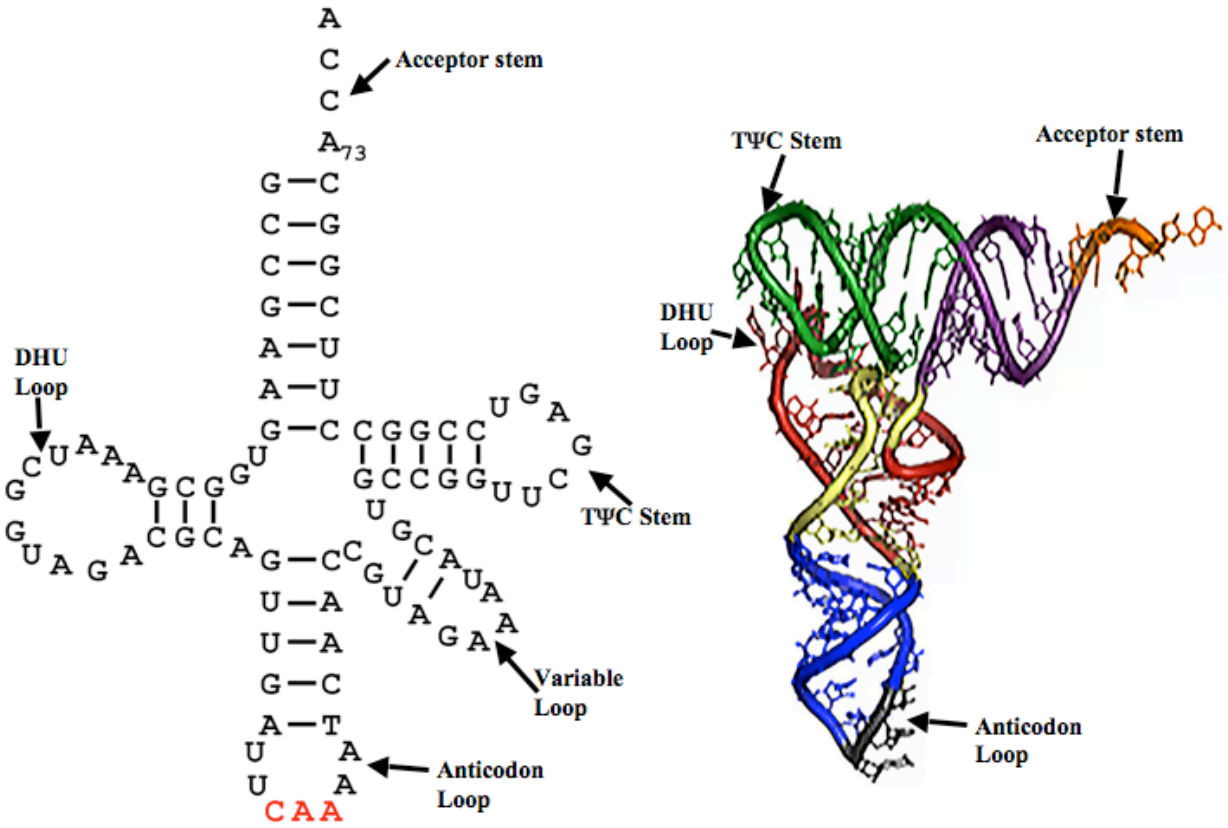


Figure I.7 Secondary and tertiary structure of tRNA. The secondary cloverleaf structure of tRNA is shown on the left with the anticodon-loop nucleotides shaded in red, while the tertiary structure of tRNA is shown on the right, where orange represents the 3′ acceptor end, purple the acceptor stem, green the TψC stem, red the dihydrouridine (DHU) stem, yellow the variable loop, blue the anticodon loop and black the anticodon nucleotides.

Identity elements on the tRNA enable a precise and complementary interaction between the aaRS and tRNA. The tRNA identity elements mostly cluster in the anticodon or acceptor stem region of the tRNA (Giegé et al. 1998). Some of these identity elements include single nucleotide variations, such as the discriminator base N73 in the acceptor stem of the tRNA that serves as a recognition factor to many aaRSs (Crothers et al. 1972). Also, unique base pairs in helices, particularly in the acceptor stem, ensure that tRNAs bind to their corresponding aaRS (Ibba and Söll 2004). Some other tRNAs, such as $tRNA^{Ser}$, rely on recognition elements in its D-stem and its long variable loop (Achsel and Gross 1993).

Anticodon nucleotides of tRNA are important for aaRS recognition as they ensure specific interaction between the aaRS and its cognate tRNA. The only exceptions are $tRNA^{Leu}$, $tRNA^{Ser}$, and $tRNA^{Ala}$ (Asahara et al. 1993; Asahara et al. 1993; Giegé et al. 1998). These tRNAs have many multiple isoacceptors with too different anticodon nucleotides to provide a unique anticodon-aaRS match. In the case of $tRNA^{Leu}$, the N73 discriminator base is primarily responsible for conferring specificity (Asahara et al. 1993; Breitschopf and Gross 1996; Soma et al. 1996). In addition, the *E. coli* $tRNA^{Leu}$ relies on the triple interaction that is formed between A_{15}:U_{48}, and A_{20a} at the D- and T-arms of tRNA, as well as the invariant $G_{18}G_{19}$ (Asahara et al. 1993; Asahara et al. 1998; Larkin et al. 2002) to form a unique structural motif for recognition by LeuRS.

Anti-determinants on the other hand prevent the productive interaction between aaRS and a non-cognate tRNA. The first anti-determinant was identified at position 34 of *E. coli* $tRNA^{Ile}$ which contains a lysidine, a modified cytosine, that hinders misaminoacylation by methionyl-tRNA synthetase (MetRS) (Muramatsu et al. 1988). Other examples of anti-determinants include modified nucleotide m^1G37 in yeast $tRNA^{Asp}$ that hinders aminoacylation by arginyl-tRNA synthetase (ArgRS) (Perret et al. 1990; Putz et al. 1994). In addition, the G37 nucleotide on $tRNA^{Ser}$ prevents charging by yeast LeuRS (Breitschopf et al. 1995). Finally, the A73 nucleotide on $tRNA^{Leu}$ inhibits aminoacylation by SerRS (Soma et al. 1996).

Some tRNA structural motifs can function as recognition elements for aaRSs. One instance involves $tRNA^{Ala}$ where the G3:U70 wobble pair causes a slight distortion in its acceptor helix causing it to complement the structure of AlaRS (McClain et al. 1988). Another example is the $tRNA^{Ser}$ whereby *T. thermophilus* SerRS recognizes $tRNA^{Ser}$ by its shape via backbone interactions (Biou et al. 1994).

I.5. Brief Overview of Eukaryotic LeuRS in its Macromolecular Complex

In higher eukaryotes, nine aaRSs are assembled into a multi-aaRS complex. This macromolecular complex consists of nine different enzymes including ArgRS, aspartyl-tRNA synthetase (AspRS), GlnRS, glutamyl-prolyl tRNA synthetase (Glu-ProRS), IleRS, LeuRS, LysRS, and methionyl-tRNA synthetase (MetRS). A 30 Å cryo-electron microscopy (EM) structure of the multi-aaRS complex is shaped like an asymmetric triangle with dimensions of approximately 19 nm x 16 nm x 10 nm. A central cleft that is 4 nm diameter deep extends about two-thirds of the length of the complex (Norcum and Warrington 1998; Norcum and Boisset 2002).

Three auxiliary non-synthetase proteins, p18, p38, and p43, now known as aminoacyl-tRNA synthetase interacting multifunctional protein (AIMP) 3, 2, and 1 respectively, are at the core that holds the subdomains of the macromolecule together. It is hypothesized that these auxiliary complexes stabilize the whole complex (Lee et al. 2004; Park et al. 2005; Kaminska et al. 2009). Auxiliary protein AIMP1 is located in the mid section of the complex (Norcum and Warrington 2000) and interacts with GlnRS and ArgRS (via its N-terminal region) to anchor them to the rest of the multi-synthetase complex (Park et al. 1999; Kaminska et al. 2009). The AIMP3 anchors the MetRS to the other components of the multi-synthetase complex (Kaminska et al. 2009). Based on tandem affinity purification from human cells, AIMP2, acts as a base for complex assembly (Quevillon et al. 1999; Robinson et al. 2000) and connects two sub-complexes, the first comprised of MetRS, AIMP3, Glu-ProRS, IleRS, and LeuRS and the second including AIMP1, GlnRS and ArgRS (Kaminska et al. 2009) (Figure 1.8). Auxiliary protein AIMP2 is critical for the assembly of the multi-synthetase

complex (Ahn et al. 2003) and links the two subcomplexes with AspRS and LysRS (Quevillon et al. 1999; Robinson et al. 2000; Kaminska et al. 2009).

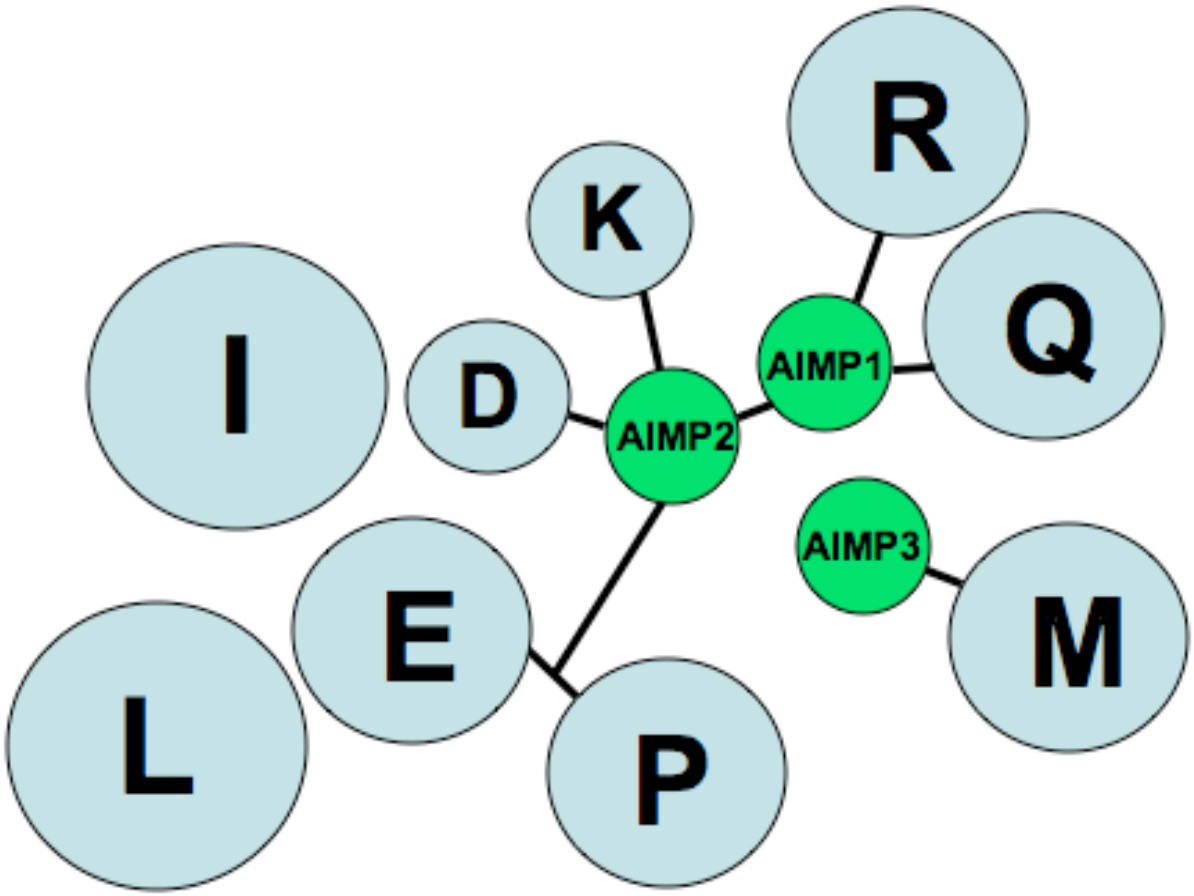


Figure I.8 Organization of the multi-synthetase complex. Schematic representation of the aaRS complex consisting of nine aaRSs and three auxiliary proteins in the core that stabilize the complex (Kaminska et al. 2009). Each aaRS is represented by its one-letter amino acid code within the circles shaded in blue. The three auxiliary proteins AIMP1, AIMP2 and AIMP3 are shaded in green. Connectivity between the various synthetases is denoted with black lines.

Certain events prompt individual aaRSs to be released from the multisynthetase complex, as in the case of LysRS (Yannay-Cohen et al. 2009). After the macromolecular complex comes apart, the three auxiliary proteins are then released to perform a myriad of cell signaling activities (Park et al. 2005; Ray et al. 2007; Kaminska et al. 2009). Additionally, many of the aaRSs that form this complex perform other roles in addition to aminoacylation, when they are released from the complex.

For example, Glu-ProRS can be switched from its classical role in translation by phosphorylation to become a gene-specific modulator of translation (Dever 2002; Mazumder et al. 2003). It forms a complex with ribosomal subunit L13a and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in response to interferon-γ to trigger translational silencing in ceruloplasmin. The Glu-ProRS complex binds to the 3′-untranslated region of ceruloplasmin to block mRNA translation (Sampath et al. 2004). Specific translational silencing of ceruloplasmin has been hypothesized to serve as a feedback mechanism to prevent over-accumulation of the protein, which might result in oxidative damage (Mukhopadhyay et al. 1997).

Of all the aaRSs within the complex, LysRS appears to be the most versatile and is associated with a myriad of activities. In an immunological function, LysRS can secrete Ap_4A in order to activate mast cells. Once Ap_4A is formed, it binds to Hint, which is a tumor suppressor gene. This causes reduced interaction between Hint and microphthalmia transcription factor (MITF), causing MITF to be released from the multicomplex to transactivate its responsive genes. (Lee et al. 2004). The LysRS can also act as a pro-inflammatory cytokine (Park et al. 2005) if its release is induced by tumor necrosis factor α (TNF-α). This secreted LysRS can bind macrophages in order to enhance TNF-α production and migration (Park et al. 2005; Nechushtan et al. 2009).

Assembly of the human immunodeficiency virus (HIV) with the viral Gag protein (Halwani et al. 2004) is dependent on LysRS that acts as a signal targeting $tRNA^{Lys}$ for incorporation into virions (Gabor et al. 2002; Javanbakht et al. 2002; Cen et al. 2004). The LysRS itself is also selectively packaged into HIV-1 separately from $tRNA^{Lys}$ (Cen et al. 2001). Once $tRNA^{Lys}$ is inside the virion, it anneals to the genomic DNA of the virus and can be used

to prime the reverse transcription of cDNA from the viral RNA genome (Mak and Kleiman 1997).

I.6. Alternate and Secondary Functions of aaRSs

As discussed briefly before, aaRSs may possess secondary functions that are not directly related to their aminoacylation role in protein synthesis (Figure 1.9) (Martinis et al. 1999). Many of these alternate functions have been implicated in disease states (Martinis et al. 1999). Additionally, aaRS-like proteins or paralogs of aaRSs that originate from complete or partial gene duplications of the aaRS gene also have adapted for diverse cellular functions (Schimmel and Ribas De Pouplana 2000).

Secondary activities of aaRSs in alternate functions include mitochondrial RNA splicing (Dujardin and Herbert 1997), translational regulation (Romby et al. 1996), and transcriptional regulation (Putney and Schimmel 1981; Condon et al. 1996). *Neurospora crassa* mitochondrial TyrRS as well as *Saccharomyces cerevisiae* and *Saccharomyces douglasii* mitochondrial LeuRS aid in group I intron splicing in the mitochondria (Herbert et al. 1988). It is proposed that they bind introns and promote folding of their cognate introns into an active conformation (Caprara et al. 1996; Rho et al. 2002; Boniecki et al. 2009). In the case of the *S. cerevisiae* mitochondrial LeuRS, the CP1 editing domain is important in splicing mitochondrial introns (Rho et al. 2002). LeuRSs from diverse origins can substitute for the mitochondrial LeuRS in the splicing reaction. In contrast, the *N. crassa* TyrRS is dependent on a small idiosyncratic N-terminal domain for splicing activity (Cherniack et al. 1990). The N-terminal domain is largely comprised of an α-helix (Paukstelis et al. 2005) and X-ray crystal structures indicate that TyrRS binds to the group I intron with a newly evolved surface that is different from where it binds $tRNA^{Tyr}$ during aminoacylation (Paukstelis et al. 2008). This N-terminal region, together

with the nucleotide-binding fold domain, and C-terminal RNA-binding domain of TyrRS are believed to promote splicing (Cherniack et al. 1990; Mohr et al. 2001).

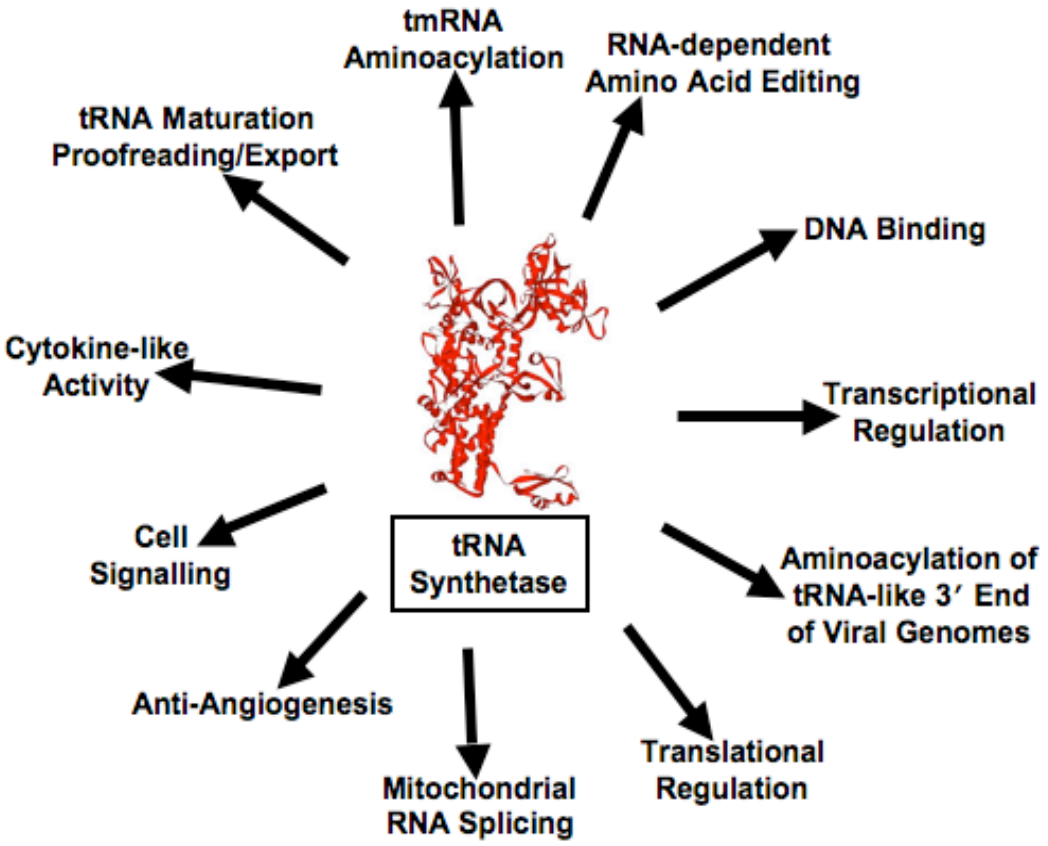


Figure I.9 Non-canonical activities of aaRSs. The aaRSs may be adapted for dual roles that coexist with their aminoacylation activity (Martinis and Pang 2007).

Several aaRSs charge non-canonical tRNA-like substrates. For example, *E. coli* AlaRS aminoacylates transfer-messenger RNA (tmRNA). A tmRNA is so named because of its tRNA- and messenger RNA (mRNA)-like properties that enable it to code and translate specific peptides (Atkins and Gesteland 1996; Jentsch 1996). The tmRNA facilitates release of stalled ribosomes from truncated mRNAs that lack a stop codon (Keiler et al. 1996). It enables ribosome recycling by switching the ribosome from the damaged mRNA to tmRNA to code for a C-terminal peptide tag, targeting the protein for degradation by proteases (Keiler et al. 1996) (Figure I.10).

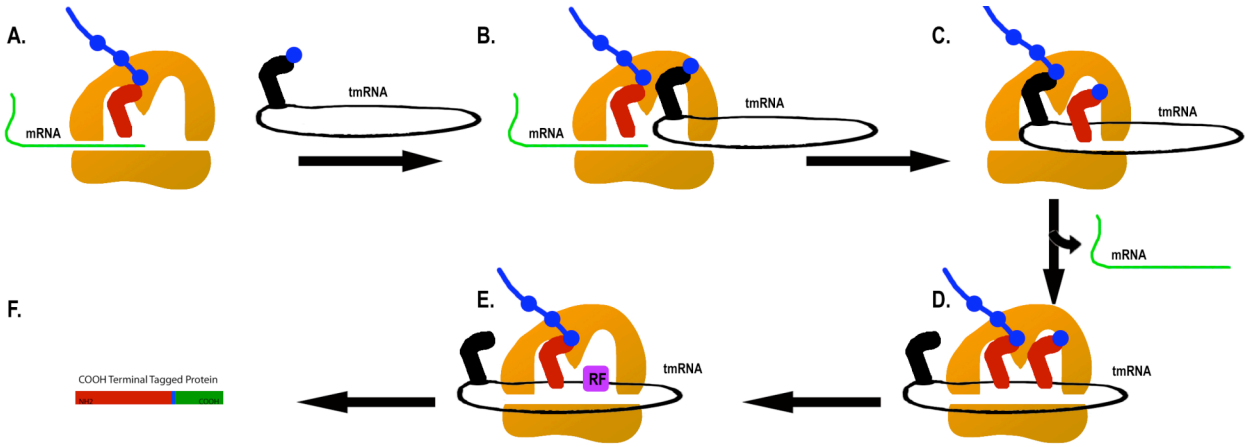


Figure I.10 Model of peptide tagging by tmRNA. (A) The tRNA in the ribosome reads mRNA. (B) The tmRNA is recognized by the ribosome (C) Damaged mRNA is expelled and tmRNA provides the mRNA template. (D) Translation of the tmRNA tag occurs. (E) Release factor releases the tagged protein. (F) The tagged protein is recognized by proteases and degraded. The mRNA is shaded in green, the tRNA in red, the tmRNA in black, the ribosome in orange, the protein and amino acids in blue, and the release factor is shaded in purple (Keiler et al. 1996).

The *E. coli* AlaRS acts as a transcriptional regulator of its own gene when the enzyme binds to a palindromic sequence that flanks its promoter site to repress transcription (Putney and Schimmel 1981). The *E. coli* threonyl-tRNA synthetase (ThrRS) regulates its production at the translational level by preventing ribosome binding for translation (Romby et al. 1996). It binds to two stem-loop structures upstream of the ribosome binding site, which mimics the anticodon arm of $tRNA^{Thr}$ (Romby et al. 1996).

Many of the aaRSs are also involved in various signaling activities, such as eukaryotic tyrosyl-tRNA synthetase (TyrRS) during apoptosis (Wakasugi and Schimmel 1999; Wakasugi and Schimmel 1999). TyrRS splits into two and the N- and C- terminal halves yield two distinct cytokines with the C-terminal end upregulating TNF-α and the N-terminal end mimicking interleukin-8 that is released by macrophages in an antigen response (Wakasugi and Schimmel 1999; Wakasugi and Schimmel 1999).

Several aaRS-like proteins have been identified that take part in diverse cellular activities (Figure I.11) (Schimmel and Ribas De Pouplana 2000). For example, *E. coli* YadB

protein resembles GluRS and attaches glutamate to a queuosine base (position 34) at the first anticodon position of $tRNA^{Asp}$. This modification expands and alters the ability of tRNA to read codons (Campanacci et al. 2004; Salazar et al. 2004). Finally, as mentioned before, a number of aaRSs that lack an editing domain such as ProRS (Wong et al. 2003) and AlaRS (Chong et al. 2008), have paralogs and homologues of editing domains in order to maintain overall fidelity (Ahel et al. 2003).

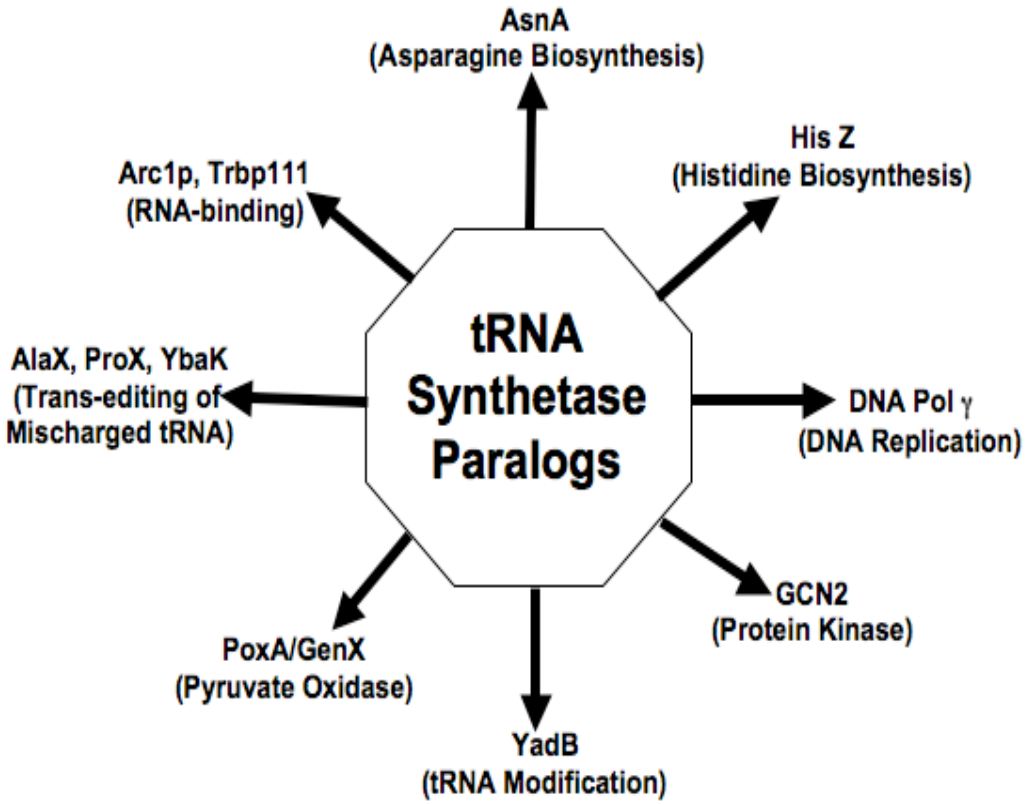


Figure I.11 Paralogs of aaRSs. Paralogs of aaRSs and their domains can provide important non-aminoacylation functions within the cell.

I.7. Antibiotics Targeting LeuRS

The dramatic rise in antibiotic resistant bacteria worldwide poses a serious threat to public health. This situation has fueled the search for antimicrobial agents which target essential cell processes but are not resisted by bacteria (Bush 2004). One of the best known

antibiotics that inhibit an aaRS is mupirocin (pseudomonic acid A), which selectively inhibits bacterial IleRS (Hughes and Mellows 1978; Hughes and Mellows 1980) and has been used as a topical antibiotic against methicillin-resistant *Staphylococcus aureus* (Sutherland et al. 1985).

More recently, a small molecule 5-fluoro-1,3-dihydroxy-2,1-benzoxaborole (AN2690) has been discovered that targets the editing active site of LeuRS (Figure I.12). It is an antifungal that contains the unusual atom: boron, and kills a range of organisms including yeasts, molds and dermatophytes. The minimum inhibitory concentration (MIC) needed for antifungal activity of AN2690 varies from 0.125 μg/mL to 2 μg/mL with dermatophytes having a generally higher MIC (Table 1.2) (Baker et al. 2006).

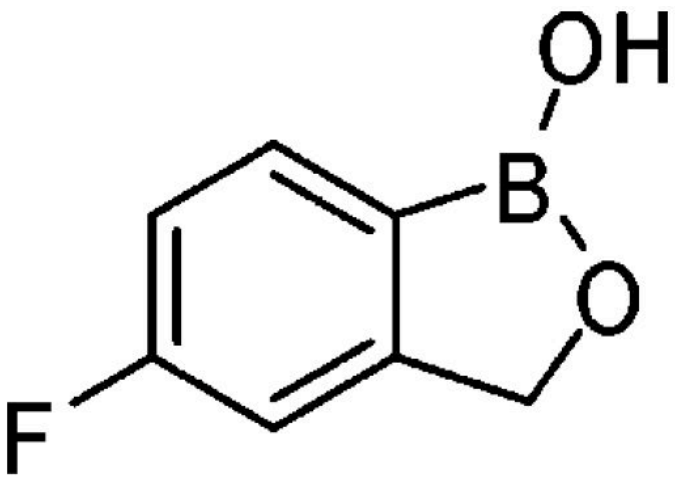


Figure I.12 Structure of boron-containing AN2690.

Table I.2 Table of broad-spectrum antifungal activity of AN2690

Fungi	**MIC (μg/mL)**
Yeasts	
Saccharomyces cerevisiae	0.125
Candida albicans	0.5
Candida krusei	1
Candida tropicalis	0.25
Cryptococcus neoformans	0.25
Malassezia furfur	1
Malassezia pachydermatis	1
Molds	
Aspergillus fumigatus	0.25
Rhizopus microsporus	2
Alternaria alternata	0.5
Penicillium chrysogenum	2
Cladosporium cladosporioides	0.5
Fusarium solani	2
Dermatophytes	
Trichophyton rubrum	2
Trichophyton mentagrophytes	2
Epidermophyton floccosum	1
Microsporum audouinii	2
Microsporum gypseum	2

The boron containing AN2690 traps $tRNA^{Leu}$ within the editing active site of LeuRS. The boron atom in AN2690 attacks one of the ribose *cis* diols of the 3′ adenosine in tRNA and then forms an oxaborole adduct with the ribose. Water molecules in the editing pocket stabilize the negatively charged boron atom (Rock et al. 2007) (Figure 1.13). The cross-linked editing complex impedes overall aminoacylation of $tRNA^{Leu}$. Thus protein synthesis within the cell ceases to occur. Therefore, the incorporation of boron atoms into rationally designed inhibitors represents a promising future for the development of new classes of therapeutic agents.

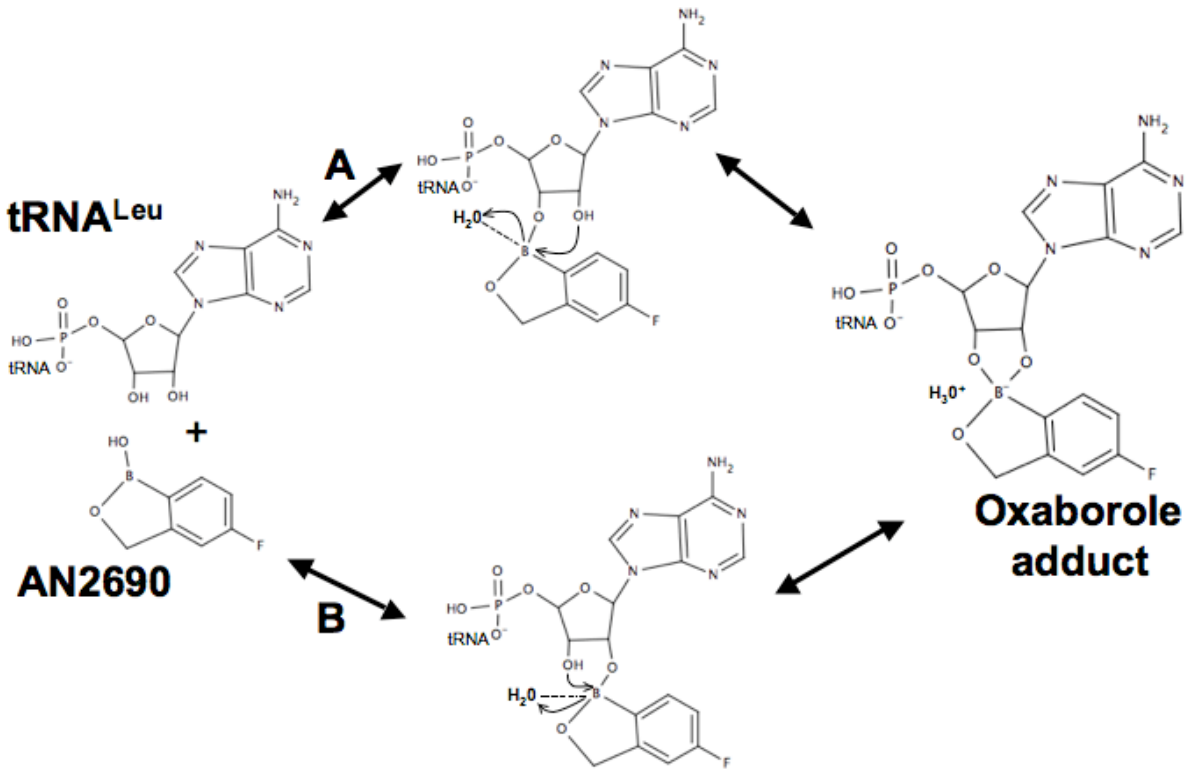


Figure I.13 Reaction mechanism of AN2690. Attack by the AN2690 boron on either the 3′ OH on the 3′ adenosine of tRNA (A) or the 2′ OH on the 3′ adenosine of tRNA (B). Water is present to help stabilize the negatively charged boron atom (Rock et al. 2007). Reproduced with permission.

Protein synthesis is one of the most fundamental reactions in the cell that ensures its viability. It is evident that aaRSs require an editing function in order to ensure accurate protein synthesis and overall health of the cell. In view of this, I proposed to characterize the aminoacylation as well as editing function of hscLeuRS. As discussed earlier, hscLeuRS is part of a macromolecular complex in the cell, many components of which participate in alternate functions apart from translation. I characterized its editing activity and determined that there is a paradigm shift in the amino acid editing mechanism of hscLeuRS. I hypothesize that hscLeuRS carries out pre-transfer editing in the absence of post-transfer editing, indicating the re-emergence of a more redundant fidelity pathway.

Translocation of tRNA from the canonical core domain to the editing active site is critical for accurate charging to occur (Hellmann and Martinis 2009; Mascarenhas and Martinis 2009). The *E. coli* LeuRS was chosen as a model to study the protein-protein interactions that occur between the aminoacylation core domain and the editing domain in order to facilitate translocation of tRNA. I hypothesize that molecular crosstalk between the main body of the enzyme and CP1 domain takes place to ensure the successful translocation of tRNA. Immunoprecipitation pull-down assays as well as single molecule fluorescence resonance energy transfer (smFRET) experiments have been conducted to study the global interaction between the core and editing domain during the movement of the 3′ end of tRNA into the editing pocket of LeuRS.

Chapter II. Materials and Methods

II.1. Materials

Oligonucleotide primers were purchased from Integrated DNA Technologies (Coralville, IA). Restriction enzymes *Dpn*I and *Bst*NI were obtained from Promega (Madison, WI) and New England BioLabs Inc. (Beverly, MA) respectively. Cloned *Pfu* DNA polymerase and dNTP mix were acquired from Stratagene (La Jolla, CA). Crude calf liver tRNA was purchased from Novagen (San Diego, CA). Tritium-labeled amino acids, as well as [^{32}P]-ATP and [^{32}P]-PP$_i$, were obtained from Amersham Pharmacia Biotech (Piscataway, NJ).

II.2. Mutagenesis

Site directed mutagenesis was performed via the polymerase chain reaction (PCR). Each 50 μL PCR mixture contained 50 ng of DNA template, 100 ng each of forward and reverse primer, 200 μM dNTPs, and 2.5 U of *Pfu* DNA polymerase in commercial buffer. Each PCR reaction was carried out at 95°C for 30 sec; 55°C for 30 sec; and 68°C for 30 min for one cycle, and then 16 cycles at 95°C for 30 sec, 58°C for 30 sec and 68°C for 22 min. The reaction was completed with a final incubation at 68°C for 22 min. A restriction digestion of each PCR mixture with 20 units of *Dpn*I was carried out for 4 h at 37 °C and then used for transformation of *E. coli* DH5α. The mutant sequences of the LeuRS genes were confirmed by DNA sequencing (UIUC Core Sequencing Facility, Urbana, IL).

II.3. Protein Expression and Purification

Mutant and wild-type plasmids were used to transform *E. coli* strain BL21 (DE3) codon PLUS (Stratagene). A single colony was used to inoculate a 3 mL culture of LB with 100 μg/mL ampicillin and grown overnight at 37 °C. This 3 mL aliquot was then transferred to 1 L of LB containing 100 μg/mL ampicillin and grown at 37 °C until the OD_{600} was between 0.4

and 0.6. Protein expression was induced for 3 h at 37 °C by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG).

The cells were then harvested by centrifugation at 6000 rpm for 15 min in an Avanti J-E centrifuge (Beckman Coulter, Fullerton, CA). The cell pellet was resuspended in 7 mL of HA-1 buffer (20 mM NaP_i, 10 mM tris(hydroxymethyl)aminomethane (Tris), pH 8.0, 300 mM NaCl, 5 mM imidazole, and 5% glycerol) and then sonicated once at 50% power using a Vibra Cell sonicator (Sonic, Newtown, CT). The lysate was spun at 12,000 rpm for 30 min at 4 °C. The supernatant was collected and the pellet discarded.

The supernatant containing protein was combined with a HIS-Select HF Nickel Affinity (Sigma, St, Louis, MO) resin that had been pre-equilibrated with HA-1 buffer. The supernatant was rocked gently with the nickel resin for 2 h at 4 °C to allow for the N-terminal fused six-histidine tag on each protein to bind to the resin. The resin was then washed with a total of 100 mL HA-2 buffer (20 mM NaP_i, 10 mM Tris, pH 8.0, 500 mM NaCl, 5 mM imidazole, and 5% glycerol) and the protein was eluted with 10 mL 100 mM imidazole in HA-1 buffer. The protein solution was dialyzed against a 100 mM KP_i, pH 6.8, 10 mM β-mercaptoethanol buffer for 24 h with six changes of buffer. The protein was later stored at -20 °C and in 50% glycerol. The concentration of protein was determined on the basis of its absorbance at 280 nm and calculated using an estimated extinction coefficient specific for the protein as computed by the ExPASy Protparam tool (http://ca.expasy.org/tools/protparam.html).

II.4. Preparation and Purification of T7 RNA Polymerase Transcribed tRNA

The *E. coli* $tRNA^{Leu}_{UAA}$ ($tRNA^{Leu}$) was transcribed from plasmid ptDNAleu14 (Tocchini-Valentini et al. 2000) that encoded the gene for *E. coli* $tRNA^{Leu}$. A total of 450 μg of plasmid ptDNAleu14 was first digested overnight with 25 units of *Bst*NI at 60 °C in a total

volume of 1 mL and then used as a template for run-off transcription (Sampson and Uhlenbeck 1988). The T7 RNA polymerase was expressed from plasmid p6HRNAP (Gift from Dr. K. Musier-Forsyth, Ohio State University; Columbus, OH) for three hours at 37 °C with 1 mM IPTG and then purified over a nickel His-select™ column. Each transcription mixture contained 40 mM Tris, pH 8.0, 30 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 0.01% Triton X-100, 50 μg/mL bovine serum albumin (BSA), 4 mM of each NTP, 80 mg/mL PEG8000, 0.02 units/μL RNase inhibitor (Eppendorf, Hamburg, Germany), 2 mM spermidine, 0.01 mg/mL pyrophosphatase (Sigma, St. Louis, MO), and 0.8 μM T7 RNA polymerase. The reaction mixtures were incubated at 42 °C for 3 h followed by a second addition of 0.8 μM of T7 RNA polymerase, and then incubated for an additional 3 h period.

The reactions were precipitated with twice the volume of 100% ethanol and with 167 μg/mL glycogen as a carrier at -80 °C for at least 30 min. The RNA products were purified by electrophoresis on a 10% polyacrylamide gel that contained 8 M urea. The $tRNA^{Leu}$ band was visualized by UV shadowing and excised from the gel. The excised gel was crushed and soaked in 0.5 M NH_4OAC and 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0 and rocked gently at 37 °C to extract the tRNA. The supernatant was collected and the gel extracted two more times. Butanol extraction was used to concentrate the tRNA, which was then ethanol precipitated and resuspended in nuclease-free water (Ambion, Austin, TX). The concentration of the tRNA was calculated based on its extinction coefficient of 840,700 liters/mol·cm (Puglisi and Tinoco 1989).

II.5. Aminoacylation and Misaminoacylation Assays

Each aminoacylation reaction contained 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-KOH (HEPES), pH 7.6, 30 mM KCl, 30 mM $MgCl_2$, 0.02%

BSA, 1 mM DTT, 4 μM *in vitro* transcribed $tRNA^{Leu}$, 22 μM [^{3}H]-leucine (158 Ci/mmole) and 50 nM enzyme. Misaminoacylation assays were carried out in the same manner except with 1 μM enzyme, 4 μM *E. coli* transcribed $tRNA^{Leu}$ and 22 μM [^{3}H]-isoleucine (93 Ci/mmole). Reactions were initiated with 4 mM ATP and carried out in a 30 °C heat block. Aliquots of 10 μL were taken at different time points and quenched on Whatman filter pads that were pre-soaked with 5% trichloroacetic acid (TCA). The pads were washed three times for 10 min each with cold 5% TCA, once with cold 70% ethanol, and once with anhydrous ether. The washed pads were then dried under a heat lamp. Radioactivity was quantified in a Beckman LS 6000IC scintillation counter (Beckman Coulter, Fullerton, CA).

II.6. Isolation of Charged $tRNA^{Leu}$

Transcribed *E. coli* $tRNA^{Leu}$ at a final concentration of 8 μM was aminoacylated with 1 μM wild-type *E. coli* LeuRS or misaminoacylated with 1 μM editing-defective *E. coli* LeuRS in a reaction containing 60 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 22 μM [^{3}H]leucine or isoleucine, and 4 mM ATP. The reactions were carried out at room temperature for either 60 min (aminoacylation) or 180 min (misaminoacylation) and then quenched with 0.18% acetic acid (Schreier and Schimmel 1972). Protein was removed from the mixture by a phenol/chloroform/isoamyl alcohol mixture (125:24:1; Fisher Biotech, Fair Lawn, NJ) at pH 4.3. The charged tRNA was then ethanol precipitated and resuspended in 50 mM KP_i, pH 5.0.

II.7. Post-transfer Editing Assay

Deacylation reactions each contained 60 mM Tris, pH 7.5, 10 mM $MgCl_2$, 300 mM KCl, and approximately 4 μM of [^{3}H]-Ile-$tRNA^{Leu}$ or [^{3}H]-Leu-$tRNA^{Leu}$. Reactions were conducted in a 30 °C heat block and initiated with 50 nM enzyme. Aliquots of 10 μL were taken at

different time points and quenched on Whatman filter pads that were pre-soaked with 5% TCA. The pads were washed and analyzed as described above.

II.8. Inorganic Pyrophosphate (PP_i) Exchange Assay

Reactions containing 50 mM HEPES (pH 8.0), 10 mM $MgCl_2$, 300 mM KCl, 1 mM DTT, 1 mM [^{32}P]-PP_i (78 mCi/mL; Amersham Pharmacia Biotech), 1 mM leucine, and 1 μM enzyme were initiated with 1 mM ATP. Aliquots of 2 μL were taken at different time points and spotted on a cellulose polyethyleneimine (PEI) thin-layer chromatography (TLC) plate (Scientific Adsorbents Inc., Atlanta, GA). The reaction components were separated by TLC in 750 mM KH_2PO_4 (pH 3.5) and 4 mM urea (Hsu et al. 2006). The separated radiolabeled bands were visualized by phosphorimaging using a FUJIX BAS 1000 film (FUJI-FILM Medical Systems U.S.A., Stanford, CT) and quantified with a Storm 840 Molecular Dynamics imager (GE Healthcare, Piscataway, NJ).

II.9. ATPase Assay

Each reaction mixture contained 100 mM Tris, pH 7.5, 20 mM $MgCl_2$, 10 mM DTT, 5 mM amino acid, 36.2 μM α-[^{32}P]-ATP (3000 Ci/mmole), and 10 μM *E. coli* transcribed tRNA. The reactions were initiated with 1 μM enzyme. Aliquots of 2 μL were taken at different time points and spotted on a cellulose PEI TLC plate (Scientific Adsorbents Inc., Atlanta, GA). The reaction components were separated by TLC in 750 mM KH_2PO_4 (pH 3.5). The separated radiolabeled bands were visualized by phosphorimaging using a FUJIX BAS 1000 film (FUJI-FILM Medical Systems U.S.A., Stanford, CT) and quantified with a Storm 840 Molecular Dynamics imager (GE Healthcare, Piscataway, NJ).

Chapter III. Paradigm Shift in the Amino Acid Editing Mechanism of Human Cytoplasmic LeuRS

III.1. Introduction

The aaRSs establish the genetic code by accurately linking the correct tRNA to its cognate amino acid in the first step of translation (Ibba and Söll 2004; Mascarenhas et al. 2008). As discussed in the introduction, each aaRS ensures that the tRNA is correctly charged in a two-step reaction mechanism. The charged tRNAs are then shuttled to the ribosome via elongation factors.

The editing active site for the CP1 domains of IleRS, ValRS and LeuRS are comprised of conserved peptides that are only partially differentiated to confer specificity (Zhai et al. 2007). Each contains a universally conserved aspartic acid that is essential for hydrogen bonding to the amino group of the bound amino acid (Figure III.1). Hydrolytic editing activity is abolished when the aspartate is substituted by an alanine in LeuRS (Lincecum et al. 2003), ValRS (Fukunaga and Yokoyama 2005), and IleRS (Bishop et al. 2002).

In addition, a threonine-rich region is important for catalysis and substrate specificity. One of these threonines in LeuRS functions specifically as a specificity determinant. Substitution of this conserved threonine with an alanine abolishes editing specificity by removing a steric hindrance in the editing pocket to facilitate leucine binding (Mursinna et al. 2001). Thus, correctly charged Leu-tRNA^{Leu} is hydrolyzed in the second sieve before undergoing product release. Substitution of this conserved threonine with a bulky

residue in *E. coli* LeuRS blocks amino acid editing resulting in a mischarging phenotype (Mursinna and Martinis 2002; Tang and Tirrell 2002).

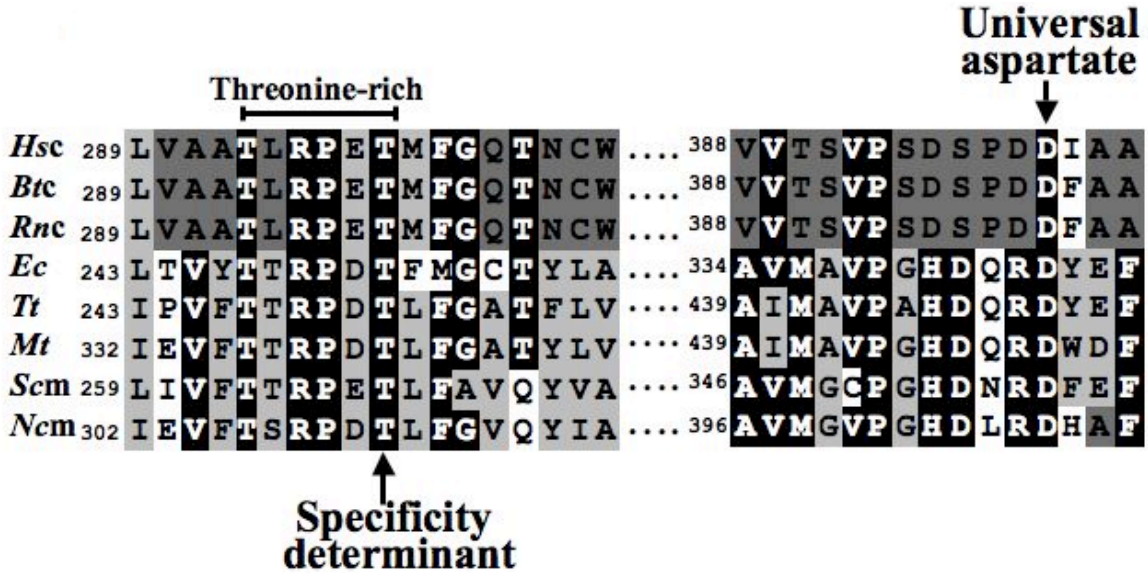


Figure III.1 Multiple sequence alignment of the LeuRS editing pocket. Sequence alignment of conserved regions within the LeuRS editing pocket. The threonine specificity determinant (Thr 298) and universally conserved aspartate (Asp 399) in hscLeuRS are indicated by arrows. Highly conserved residues are highlighted in black. Two shades of gray distinguish homologous amino acid sites. Abbreviations are as follows: *Hs, Homo sapiens; Bt, Bos taurus; Rn, Rattus norvegicus; Ec, E. coli; Tt, T. thermophilus; Mt, Mycobacterium tuberculosis; Sc, Saccharomyces cerevisiae; Nc, Neurospora crassa;* c, cytoplasmic; and m, mitochondrial.

In many higher organisms, and even some archaea and yeast, aaRS operates as a large or small complex. For example, in human cells, the aaRS macromolecular complex contains nine aaRSs including the Lys-, Asp-, Met-, Glu-Pro-, Ile-, Leu-, Arg-, and Glu-tRNA synthetases (Norcum and Boisset 2002). We wondered if viable editing mechanisms that typically reside in separate domains such as the CP1 editing module might be maintained in human aaRSs that are confined to the complex. The primary structure of hscLeuRS retains key amino acids in the editing active site that are important to bacterial LeuRS editing activities, although other sites within the region have diverged. For example, recent crystal structure information of the hscLeuRS editing domain shows that two residues have diverged to bulkier residues, leading to

a smaller hydrophobic pocket within the CP1 domain of hscLeuRS (Seiradake et al. 2009). Eukaryotic cytosolic LeuRS has also been found to contain an I4 insertion in the CP1 domain that was hypothesized to function as a lid by closing over the editing pocket. This would result in a capped tighter hydrophobic editing pocket for hscLeuRS compared to the more spacious bacterial LeuRS pocket that is open to the environment (Seiradake et al. 2009). We cloned the hscLeuRS gene and expressed it in *E. coli* independent of the multi-aaRS complex. Despite maintaining key features in the editing active site that are critical to its hydrolytic activity, mutational analysis of the human enzyme suggests that it has functionally differentiated to maintain fidelity.

III.2. Experimental Procedures

III.2.1 RNA Preparation

The CCA 3′ end of crude calf liver tRNA was added via a nucleotidyl transferase reaction. The plasmid pCCA encoding the nucleotide transferase enzyme was a gift from Dr. P. Schimmel (The Scripps Research Institute; La Jolla, CA). The enzyme was expressed in BL21 for 3 h at 37 °C with 1 mM IPTG and then purified over a nickel affinity column (Sigma, St. Louis, MO). Each nucleotidyl transferase reaction contained 100 mM glycine, pH 9.0, 10 mM $MgCl_2$, 1 mM DTT, 100 μM ATP, 100 μM CTP, 10 mg/mL crude calf liver tRNA, 5 μM *E. coli* CCA nucleotidyl transferase and was incubated in a 70 °C heat block for 15 min. Protein was removed from the mixture by extraction with phenol/chloroform/isoamyl alcohol (125:24:1; Fisher Biotech, Fair Lawn, NJ) that was pre-equilibrated at pH 4.3. The tRNA was then ethanol precipitated and resuspended in nuclease-free water (Ambion, Austin, TX).

The *E. coli* $tRNA^{Leu}$ was transcribed from the plasmid ptDNAleu14 (Tocchini-Valentini et al. 2000) that encoded the gene for *E. coli* $tRNA^{Leu}$. A total of 450 μg of

plasmid ptDNAleu14 was first digested overnight with 25 units *Bst*NI at 60 °C in a total volume of 1 mL and then used as a template for run-off transcription (Sampson and Uhlenbeck 1988) as described in Chapter II.

III.2.2 Mutagenesis

Site directed mutagenesis was performed via PCR using the template plasmid pHSLARS1 that encoded the wild-type hscLeuRS gene. The plasmid pHSLARS1 was a gift from Weimin Mao and MRK Alley (Anacor Pharmaceuticals; Palo Alto, CA) and was utilized to make mutants including T298A (pJPT298A) using primers T298A-Fwd (5'-CAG ACC TGA GGC CAT GTT TGG GC-3') and T298A-Rev (5'-GCC CAA ACA TGG CCT CAG GTC TG-3'), T298Y (pJPT298Y) using primers T298Y-Fwd (5'-CAG ACC TGA GTA CAT GTT TGG GC-3') and T298Y-Rev (5'-GCC CAA ACA TGT ACT CAG GTC TG-3'), and D399A (pJPD399A) using primers D399A-Fwd (5'-CTC CCC TGA TGC TAT TGC TGC CC-3') and D399A-Rev (5'-GGG CAG CAA TAG CAT CAG GGG AG-3'). Each 50 µL PCR mixture contained 50 ng of DNA template, 100 ng each of forward and reverse primer, 200 µM dNTPs, and 2.5 units of *Pfu* DNA polymerase in commercial buffer. Each PCR reaction was carried out as described before.

III.2.3 Expression and Purification of hscLeuRS

Mutant and wild-type plasmids were used to transform *E. coli* strain BL21 (DE3) codon PLUS (Stratagene). A single colony was used to inoculate a 3 mL culture of LB with 100 µg/mL ampicillin and grown overnight at 37 °C. This 3 mL aliquot was then transferred to 1 L of LB containing 100 µg/mL ampicillin and grown at 37 °C until the OD_{600} was between 0.6 and 1.0. Protein expression was induced overnight at room temperature by the addition of 1 mM IPTG and processed as described in Chapter II. The concentration of protein was

determined on the basis of its absorbance at 280 nm and calculated using an estimated extinction coefficient of 178,080 liters/mol·cm computed by the ExPASy Protparam tool (http://ca.expasy.org/tools/protparam.html).

III.3. Results

The editing active site within the CP1 domain of LeuRS is marked by conserved peptide motifs. A highly conserved threonine within a threonine-rich region acts as a specificity determinant (Figure III.1 and Figure III.2) and blocks leucine from binding for hydrolysis of correctly charged Leu-tRNA^{Leu}.

Substitution of this key threonine with a bulky residue such as tyrosine fills up the amino acid binding pocket in *E. coli* LeuRS to prevent editing (Mursinna and Martinis 2002; Tang and Tirrell 2002). In a separate motif, a universally conserved aspartic acid (Figure III.1) that is also found in IleRS and ValRS anchors the amino acid via a hydrogen bond with its amino moiety in the active site. Substitution of the aspartic acid by alanine abolishes editing activity in LeuRS and IleRS (Bishop et al. 2002; Lincecum et al. 2003; Fukunaga and Yokoyama 2005).

The threonine specificity determinant and universal aspartic acid are also found in hscLeuRS amongst many other conserved features of the editing active site. This suggests that LeuRS from a higher eukaryote maintains fidelity using an editing active site architecture that is common to lower organisms.

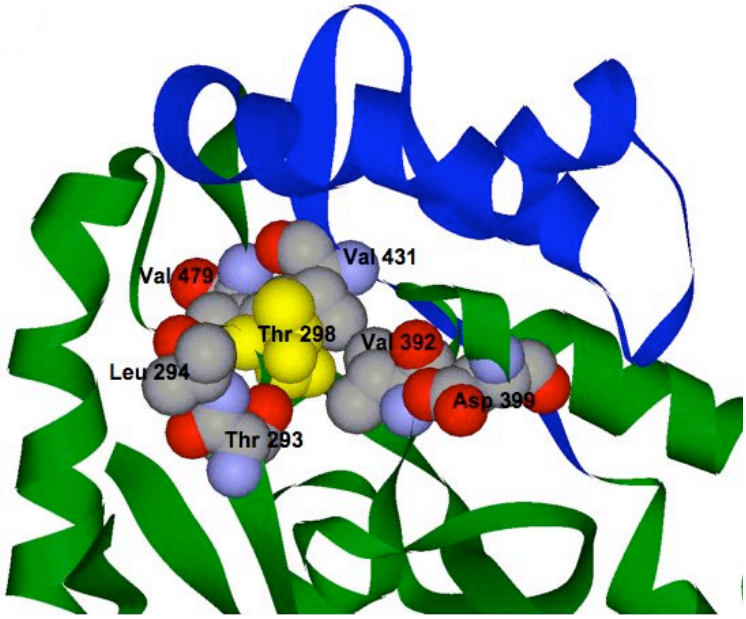


Figure III.2 Crystal structure of hscLeuRS CP1 domain. B. Crystal structure of the hscLeuRS CP1 domain (PDB entry 2WFD) showing the editing pocket containing the threonine-rich region and the universally conserved aspartate. Residues Thr 293, Leu 294, Thr 298, Val 392, Asp 399, Val 431 and Val 479 are shown in space-filling form. The atoms are colored as follows: oxygen, red; nitrogen, light blue; carbons, gray. Thr 298 is highlighted completely in yellow. The I4 insertion is highlighted in blue and the editing pocket in green.

We tested this hypothesis by substituting the conserved threonine at position 298 of hscLeuRS (Thr 252 in *E. coli* LeuRS) with an alanine (T298A) and also with a bulky tyrosine (T298Y). The universal aspartate at position 399 in hscLeuRS was also substituted with an alanine. The mutant and wild-type proteins were expressed in *E. coli* and purified by affinity chromatography with a six-histidine tag.

The enzymatic activities for each of the mutants were characterized and compared to wild-type hscLeuRS. Both the T298Y and D399A mutant hscLeuRSs exhibited robust aminoacylation of crude calf liver tRNA, albeit the latter activity was reduced compared to wild-type (Figure III.3A). As would be expected based on analysis of other LeuRSs (Mursinna et al. 2001), the T298A LeuRS mutant failed to accumulate Leu-tRNA^{Leu}

(Figure III.3A). The T298A hscLeuRS mutant also uncouples specificity in the editing active site and hydrolyzes Leu-tRNA^{Leu} (Figure III.3B).

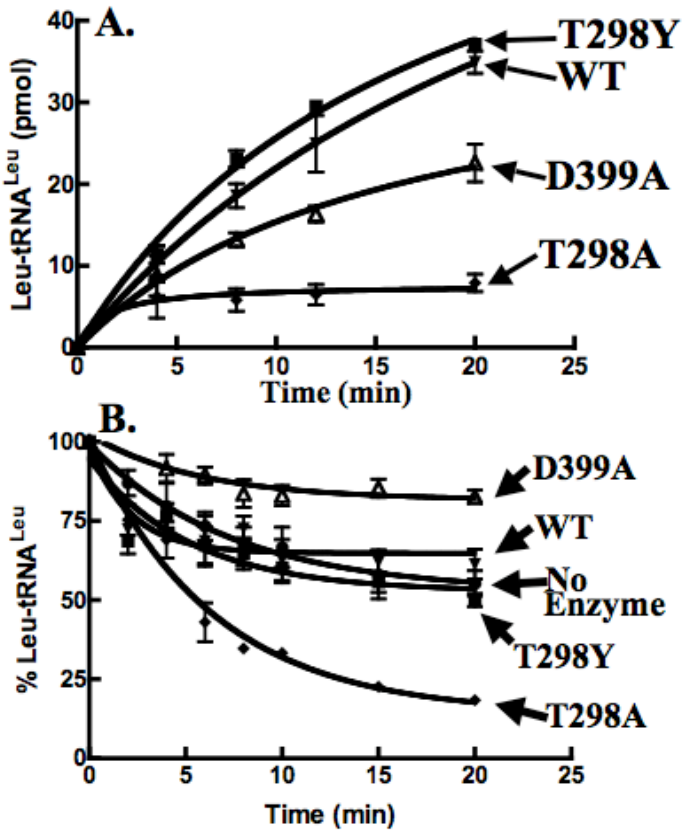


Figure III.3 Leucine aminoacylation and deacylation by wild-type and mutant hscLeuRS. A. Leucylation activity was measured with 500 nM enzyme and 4 mg/ml of crude calf liver tRNA (Novagen, Gibbstown, NJ). B. Post-transfer editing reactions were carried out with 500 nM enzyme and ~4 μM *E. coli* Leu-tRNA^{Leu}. All enzyme reactions were incubated at 30 °C. Wild type (WT) and mutant proteins are represented by the following symbols: WT, (▼); T298A, (♦); D399A, (△); T298Y, (■); and no enzyme, (●). Error bars are based on the results of three reactions each and indicated for each point.

We wondered if the relatively reduced aminoacylation activities of the D399A LeuRS mutant enzyme might be due in part to changes in the first step of amino acid activation step even though the mutation is located in the CP1 domain, which is completely separate from the canonical aminoacylation core. Pyrophosphate exchange assays determined that the D399A

LeuRS mutant activated leucine at reduced rates (Figure III.4). The decrease in leucine-dependent pyrophosphate exchange activity by the distal D399A mutation was largely due to a K_M effect (Table III.1). The K_M was about 40-fold higher for leucine activation by the D399A LeuRS mutant (0.9 $\pm$ 0.2 mM) as compared to the human wild-type enzyme (0.024 $\pm$ 0.009 mM). The k_{cat} for leucine activation of the wild type and mutant enzymes were 2.1 $\pm$ 0.4 s^{-1} and 5.6 $\pm$ 0.1 s^{-1}, respectively. This reduction in amino acid activation activity is consistent with the lower levels of leucylation activity for the D399A LeuRS mutant compared to the wild-type enzyme. These results suggest that a long-distance inter-domain communication mechanism for the protein exists between the aminoacylation and amino acid editing active site that is at least partially tRNA-independent.

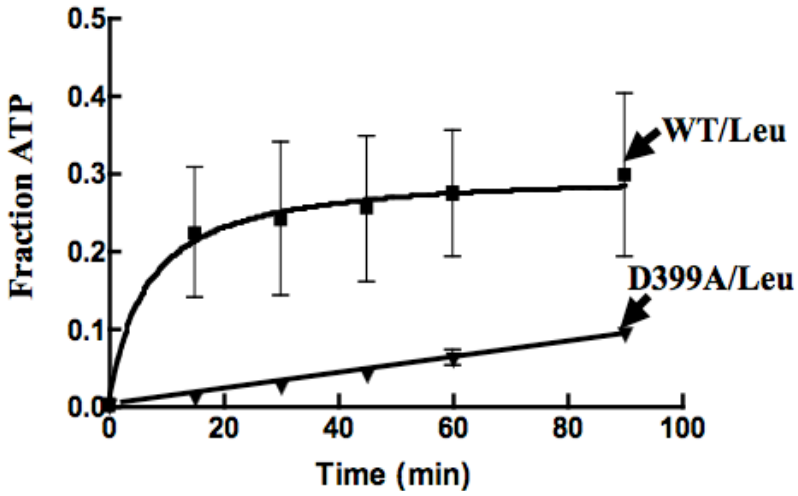


Figure III.4 Pyrophosphate (PP_i) exchange activity of wild type and D399A hscLeuRS. Leucine and isoleucine-dependent PP_i exchange assays were carried out with 1 µM hscLeuRS and 10 mM leucine. Symbols are as follows: WT with leucine, (■); and D399A with leucine, (▼). Error bars are based on the results of three reactions each and indicated for each point.

Table III.1 Apparent kinetic parameters for amino acid activation

	K_M (mM)		k_{cat} (s^{-1})		k_{cat}/K_M ($s^{-1}mM^{-1}$)	
Enzyme	**Leu**	**Ile**	**Leu**	**Ile**	**Leu**	**Ile**
Hsc WT	0.02 ± 0.0009	2.04 ± 0.80	2.1 ± 0.4	0.34 ± 0.2	94.3 ± 23.4	0.16 ± 0.04
Hsc D399A	0.90 ± 0.19	3.3 ± 1.2	5.6 ± 0.07	0.51 ± 0.04	5.6 ± 0.40	0.17 ± 0.04

Mischarged *E. coli* Ile-tRNA^{Leu} was isolated to test the wild-type and mutant LeuRSs directly for tRNA deacylation activity. The D399A mutant of LeuRS eliminated Ile-tRNA^{Leu} deacylation activity (Figure III.5), which is consistent with all other alanine substitutions at this key site in LeuRS (Lincecum et al. 2003), IleRS (Bishop et al. 2002) and ValRS (Fukunaga and Yokoyama 2005). In contrast though, substitution of the conserved threonine with a bulky tyrosine residue (T298Y) in the hscLeuRS maintained Ile-tRNA^{Leu} deacylation activity. This suggests that even though the threonine specificity determinant is maintained, the architecture around the amino acid binding pocket has changed for the human enzyme.

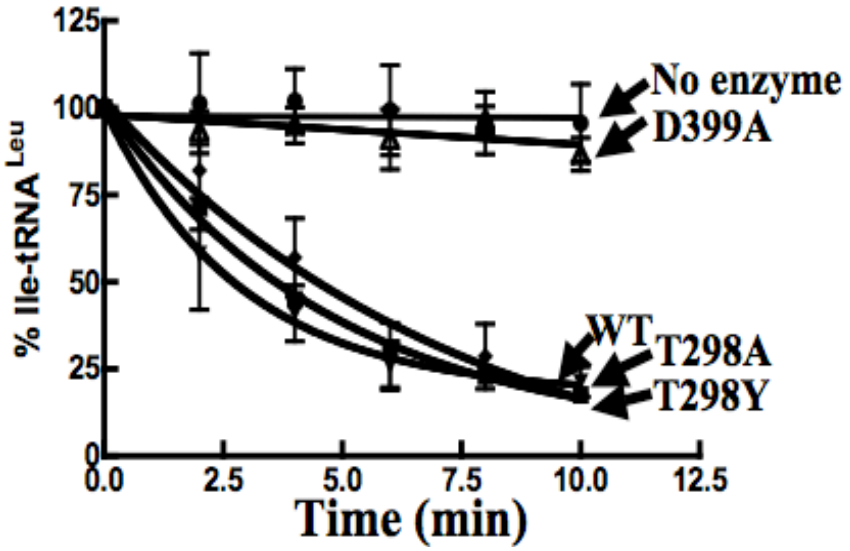


Figure III.5 Deacylation activity of wild-type and mutant hscLeuRS. Post-transfer editing assays were carried out with 500 nM enzyme and ~4 μM *E. coli* Leu-tRNA^{Leu} at 30 °C. Wild type (WT) and mutant proteins are represented by the following symbols: WT, (▼); T298A, (♦); D399A, (△); T298Y, (■); and no enzyme, (●). Error bars are based on the results of three reactions each and indicated for each point.

A recent crystal structure for the CP1 domain of hscLeuRS and the related yeast cytoplasmic enzyme showed that the eukaryotic LeuRS CP1 domain has acquired insertions (Seiradake et al. 2009). In particular, the I4 insertion (shown in blue on Figure III.2) is juxtaposed to the editing active site and has been hypothesized to act as a lid to sequester substrate in the hydrolytic pocket (Seiradake et al. 2009). It is possible that closing the I4 insertion lid might promote an induced fit for the mischarged $tRNA^{Leu}$ to be edited, even in the presence of a bulky residue substitution in the human LeuRS editing site.

In the absence of deacylation activity, we expected that the mutant D399A hscLeuRS would mischarge $tRNA^{Leu}$ similar to other editing-defective LeuRSs (Bishop et al. 2002; Lincecum et al. 2003; Fukunaga and Yokoyama 2005). Crude calf liver tRNA was mischarged by the D399A LeuRS mutant, but only at low levels (data not shown) despite a robust aminoacylation activity (Figure III.3A). By comparison, crude *E. coli* tRNA was mischarged at higher levels (Figure III.6A) with a k_{cat} of 0.0018 ± 0.0005 s^{-1} by the D399A hscLeuRS mutant, albeit more weakly than LeuRS mutants from other origins that had ablated the tRNA deacylation activity. The D399A LeuRS mutant failed to charge isoleucine to *in vitro* transcribed *E. coli* $tRNA^{Leu}$, even at high concentrations of 1 μM enzyme (Figure III.6B). This suggests that the low levels of mischarging activity are dependent on tRNA modifications. It is possible that these RNA modifications stabilize tertiary structure to facilitate RNA-protein interactions for aminoacylation. As would be expected, wild-type hscLeuRS maintained fidelity and did not mischarge crude or transcribed *E. coli* tRNA.

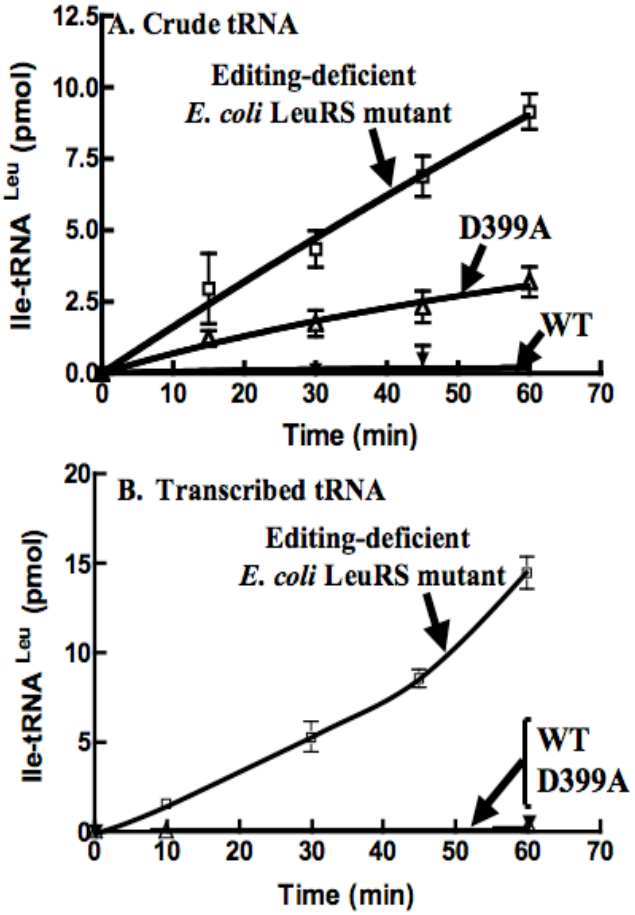


Figure III.6 Mischarging activity of wild type and mutant hscLeuRS with transcribed and crude *E. coli* tRNA. A. Misaminoacylation reactions were carried out with 1 μM enzyme and 20 μM of *in vitro* transcribed *E. coli* tRNA. B. Misaminoacylation reactions were carried out with 1 μM enzyme and 4 mg/ml of crude *E. coli* tRNA. Wild type (WT) and mutant proteins are represented by the following symbols: WT, (▼); D399A, (△) and an editing-deficient *E. coli* LeuRS mutant, (□). Error bars are based on the results of three reactions each and indicated for each point.

We hypothesized that, similar to the long-distance effect by the D399A mutation on leucine activation (Figure III.4), isoleucine activation might also be significantly reduced, resulting in only low mischarging activities for the editing-defective enzyme. However, the D399A mutation had a much less pronounced effect on the K_M and k_{cat} for isoleucine-dependent pyrophosphate with k_{cat} / K_M values that were nearly equivalent for the wild type and mutant enzymes (Table III.1).

Thus, these long distance inter-domain effects between the editing and aminoacylation active sites are sensitive to the cognate amino acid. It is also possible that low misaminoacylation activity by the D399A hscLeuRS could be due to a redundant fidelity mechanism that originates in the aminoacylation active site. In the absence of tRNA, isoleucine stimulated the D399A mutant hscLeuRS to form AMP with a k_{cat} of 0.06 $\pm$ 0.02 s^{-1} and K_M of 1.8 $\pm$ 0.5 mM in ATPase assays. If this represents a case of a putative pre-transfer editing pathway, then the mechanism would be independent of the D399A mutation in hscLeuRS since the activated isoleucyl-adenylates would be directly cleaved or expelled from the aminoacylation active site. As hypothesized, ATPase assays indicate higher levels of AMP production with the D399A mutant as compared to the wild type enzyme due to the hydrolysis of misactivated non-cognate amino acid adenylates in the presence of a pre-transfer editing pathway. In particular, pre-transfer edting appears to be tRNA dependent as there is a more robust production of AMP in the presence of *E. coli* transcribed tRNA (Figure III.7).

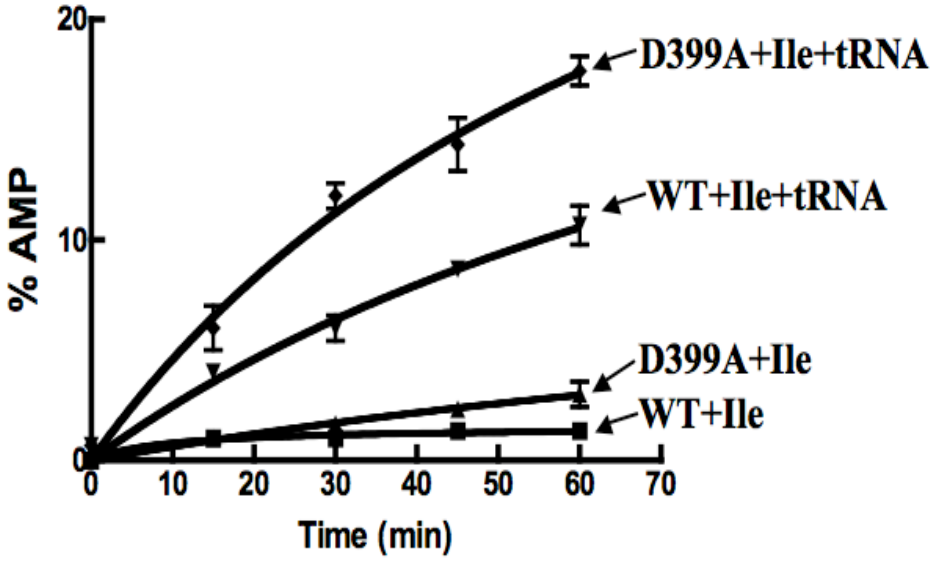


Figure III.7 ATPase activity assay with wild-type and mutant hscLeuRS. The ATPase assays were carried out with 1 µM enzyme and 5 mM of isoleucine in the absence or presence of 10 µM *E. coli* Leu-$tRNA^{Leu}$. Wild type (WT) and mutant proteins are represented by the following symbols: WT+Ile, (■); WT+Ile+tRNA, (▼); D399A+Ile, (▲); and D399A+Ile+tRNA, (♦). Error bars are based on the results of three reactions each and indicated for each point.

III.4 Discussion

Representative of all LeuRSs, the hscLeuRS contains specific active sites for aminoacylation and editing that are located in separate domains. X-ray crystal structures for LeuRSs from all three kingdoms (Fukunaga and Yokoyama 2005; Liu et al. 2006; Seiradake et al. 2009) have shown that within the CP1 editing domain, the LeuRS editing active site is marked by a threonine-rich peptide. In the human enzymes, it includes the highly conserved Thr 298 specificity determinant (Thr 252 in *E. coli* LeuRS) as well as Thr 293 (Thr 247 in *E. coli* LeuRS) that has been shown to be important in orientating the editing substrate in the editing active site for catalysis by LeuRS (Zhai and Martinis 2005). It also has the "GTG adenine-binding region" as well as a universally conserved aspartic acid that interacts with the amino group of the bound amino acid (Lincecum et al. 2003). However, four peripheral peptide insertions distinguish the hscLeuRS and other eukaryotic CP1 domains from their bacterial counterparts. X-ray crystallography structures for the hscLeuRS and yeast cytoplasmic LeuRS show that although the integrity of the editing core is clearly maintained, these peripheral insertions appear to induce some local structural rearrangements (Seiradake et al. 2009).

The I4 peptide insertion in the LeuRS CP1 domain is hypothesized to cap the editing active site when mischarged tRNA is bound (Seiradake et al. 2009). Another insertion, called I2, contacts the bound editing substrate analogue. In the yeast cytoplasmic LeuRS CP1 domain structure, the I2 insert also interacts with a network of water molecules. It is possible then that displacement of these water molecules could provide some plasticity for the editing active site to facilitate an induced fit mechanism. In the case of our results, water molecules could be displaced by the introduction of the bulky tyrosine residue at the Thr 298 specificity site in the hscLeuRS CP1 domain. Based on the structure of the hscLeuRS CP1 domain, we hypothesize

that the tyrosine residue could flip into vacant space over the neighboring Val 479 (Figure III.2) to allow binding of the editing substrate. Indeed, closure of the I4 lid might facilitate an induced fit mechanism for the hscLeuRS editing site that adequately includes the bulky Tyr 298 mutation.

The universal aspartic acid in the editing active site of the hscLeuRS is critical to tRNA deacylation as found for all other enzymes where it has been tested. Here, we demonstrated that an alanine substitution at the conserved site blocks deacylation activity *in vitro*. This is consistent with *in vivo* experiments showing that the introduction of a bulky positively charged lysine residue for the aspartic acid failed to protect yeast knock out cells from high concentrations of norvaline (Yao et al. 2008).

Surprisingly, the universally conserved aspartic acid in the CP1 domain was not only important to editing active site substrate interactions, but also interactions with the amino acid in the synthetic active site. Substitution of the aspartic acid resulted in a long distance K_M effect on the amino acid activation step, which would be expected to be governed primarily by the aminoacylation active site in the canonical core. Significantly, this long distance effect was selective for the cognate leucine amino acid suggesting that the CP1 domain might also distally influence amino acid discrimination within the aminoacylation core.

Since aminoacylation activity is also decreased for the D399A hscLeuRS mutant, it is possible that tRNA mediates this distal amino acid discrimination effect in the synthetic site. In this case, tRNA would be expected to bind in the so-called "exit/entry" position where its 3′ end is near the editing active site (Rock et al. 2007; Hellmann and Martinis 2009). Translocation of the tRNA could be slowed as it sweeps through the altered editing active site enroute to the aminoacylation active site. However, these long-distance effects were also detected in the

absence of tRNA during pyrophosphate exchange assays. Thus, subtle changes in the editing active site must be transmitted via nodes within the protein itself to influence amino acid activation (Sethi et al. 2009).

The D399A hscLeuRS mutant only exhibited weak mischarging activity relative to other corresponding LeuRS, IleRS, and ValRS mutants, where post-transfer editing activity was completely abolished. Since the kinetic parameters for isoleucine activation were similar to the wild type and mutant LeuRS, we hypothesize that the tRNA plays a significant role in maintaining fidelity. Indeed, mischarging activity seemed particularly sensitive to the presence of tRNA modifications and the origin of the tRNA. Ironically, *E. coli* crude tRNA yielded greater levels of mischarged tRNA. However, this could be because the *E. coli* tRNA was missing important elements that were embodied in the mammalian calf liver tRNA, which minimized mischarging of isoleucine by LeuRS. If this were the case, we hypothesize that the human enzyme relies, at least in part, on pre-transfer editing that originates from the aminoacylation active site. A redundant mechanism for amino acid editing that originates in the aminoacylation active site would allow fidelity to be rescued in the absence of post-transfer editing activity in the CP1 domain.

The hscLeuRS is part of a large macromolecular complex that includes nine aaRSs and three small auxiliary proteins (Han et al. 2003; Kaminska et al. 2009). A variety of approaches, such as electron microscopy, three-dimensional reconstruction (Wolfe et al. 2005), and tandem affinity purification (Kaminska et al. 2009) have been used to study the assembly of this macromolecular complex. Cryo-EM was used to elucidate the structure of the multi-aaRS complex at 30 Å. The imaging showed that this large complex is approximately 19 x 16 x 10 nm (Norcum and Warrington 1998; Norcum and Boisset 2002). Although the

3.25 Å X-ray crystal structure of the human cytoplasmic LeuRS CP1 domain has also been solved (Seiradake et al. 2009), it is unclear how this editing domain and the remainder of the human LeuRS fit into the macromolecular complex. In the context of the multi-aaRS complex, it is possible that domain motions such as the CP1 domain might be constrained. In this case, redundant fidelity pathways may also serve as a more efficient amino acid editing mechanism that doesn't require translocation of the mischarged tRNA to another editing domain. Recently, we showed that in the absence of the CP1 domain for *E. coli* and yeast mitochondrial LeuRS, the enzyme maintained fidelity by targeting the activated adenylate intermediate (Boniecki et al. 2008). In this case, isoleucyl-adenylate could be hydrolyzed within the aminoacylation active site (Gruic-Sovulj et al. 2005) or selectively released for hydrolysis in the cellular milieu (Hati et al. 2006). Redundant mechanisms in amino acid editing could be essential to maintain a level of protein synthesis fidelity, particularly in mammals that would guard against neurological disease (Lee et al. 2006).

We hypothesize that some of the architectural and mechanistic differences that are specific to hscLeuRS may have been acquired in part because of its association with the multi-aaRS complex. These differences have likely facilitated discovery of a novel class of benzoxaborole anti-fungal compounds, which are in development for the treatment of onchomycosis. In particular, AN3018 inhibits yeast cytoplasmic LeuRS by forming a covalent adduct with the 3′ adenosine of the $tRNA^{Leu}$ to trap it in the editing site (Rock et al. 2007). Significantly though, complementation experiments using yeast knock-out tester strains have indicated that the hscLeuRS is susceptible to AN2690 (Yao et al. 2008). Furthermore, the D399K hscLeuRS editing-deficient mutant is resistant to AN2690 in complemented yeast cells (Yao et al. 2008), indicating the importance of the Asp 399 residue in hscLeuRS for binding

within the editing site. The AN2690 inhibitor appears to bind to the human enzyme in these yeast complementation assays, in spite of crystal structure analysis of the hscLeuRS CP1 domain that suggests that the size of the hydrophobic editing pocket is smaller than that of the yeast enzyme (Seiradake et al. 2009). Nevertheless, a network of water molecules and an induced fit mechanism in the editing site could provide plasticity, especially in a version of hscLeuRS that is freed from its multi-aaRS macromolecular complex. It is also important to note that native residence within the multi-aaRS macromolecular complex might hinder inhibitor interactions with the hscLeuRS CP1 domain, particularly in a mechanism that requires cross-linking the large tRNA substrate that spans two protein domains.

Chapter IV. Formation of *bis*-Aminoacylated tRNA with Human Cytoplasmic LeuRS

IV.1. Introduction

The *E. coli* LeuRS charges tRNA with a single leucine via an aminoacylation reaction. Leucine is first activated with ATP by LeuRS to form an aminoacyl-adenylate intermediate with the release of PP_i. In the second step, this activated leucine is charged to the 2′ ribose hydroxyl of the 3′ end of its cognate tRNA (Ibba and Söll 2004; Mascarenhas et al. 2008), as is the case with class I aaRSs.

$$\text{ATP} + \text{Leucine} \leftrightarrows \text{Leucine-AMP} + PP_i \qquad \text{(IV.1)}$$

$$\text{Leucine-AMP} + tRNA^{Leu} \rightarrow \text{Leucine-}tRNA^{Leu} + \text{AMP} \qquad \text{(IV.2)}$$

This correctly charged tRNA is handed off to EF-Tu to be brought to the ribosome for participation in translation. Once the tRNA has successfully delivered the amino acid to the growing polypeptide chain in the ribosome, the tRNA is released and then binds LeuRS again for re-charging.

Interestingly, it has been shown that *T. thermophilus* PheRS can attach two molecules of phenylalanine to $tRNA^{Phe}$ to form a *bis*-aminoacylated species (Figure IV.1) (Stepanov et al. 1992). One amino acid is located on the 2′ hydroxyl and the other is on the 3′ hydroxyl group of the 3′ adenosine of tRNA. This *bis*-phenylalanylated-tRNA can actually participate in protein synthesis (Wang et al. 2006). It binds the aminoacyl-site of the ribosome and contributes an amino acid from its 3′ -OH group on the 3′ adenosine of tRNA. The donor tRNA can then dissociate from the ribosome as a monoacylated tRNA and continues on to participate in another round of translation in the ribosome (Wang et al. 2006).

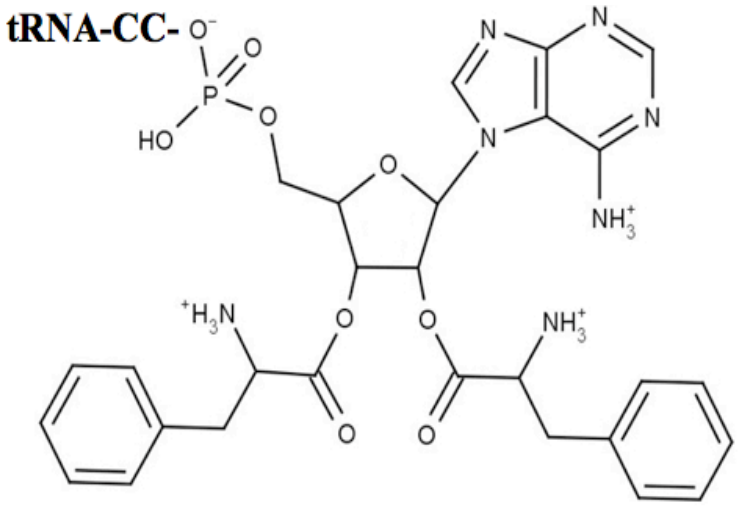


Figure IV.1 Structure of *bis*-aminoacylated adenosine at the 3′ end of tRNA. The 3′ adenosine of tRNA is *bis*-aminoacyated with two phenylalanines, one on the 2′ -OH and the other on the 3′ -OH.

The hscLeuRS enzyme displayed an interesting phenomenon during tRNA deacylation assays that I conducted to test post-transfer editing activity. I typically observe hydrolysis of charged and mischarged tRNA during deacylation assays (Pang and Martinis 2009). However, unlike the *E. coli* LeuRS, there was a subsequent re-accumulation of charged and mischarged tRNA with hscLeuRS. This recharging occurrence was unexpected since ATP was not one of the components of the hydrolysis assay. Great efforts were also made to ensure that there weren't any lingering ATP contaminants in the reactions. Based on previous findings regarding tandemly activated tRNAs (Stepanov et al. 1992; Wang et al. 2006), I wondered if the tRNA might be charged with two leucines at the terminal ribose of tRNA. I postulated that the additional amino acid that is freed during the initial hydrolysis of charged and mischarged tRNA might be re-attached to a new incoming tRNA. I then hypothesized that a dipeptide might be forming on the 3′ adenosine of tRNA.

In order to test our hypothesis, I carried out a series of experiments to confirm the presence of two leucines charged on the tRNA. Acidic gel electrophoresis to stably separate the

charged tRNA confirmed the initial decrease and subsequent regeneration of charged tRNA. Hydrolysis assays followed by nuclease digestion at various time points were carried out and I attempted to determine the chemical nature of the species charged to the tRNA via various techniques including TLC and liquid chromatography/mass spectrometry (LC/MS).

IV.2. Experimental Procedures

IV.2.1 Purification of hscLeuRS

The hscLeuRS was first purified via a HIS-Select HF Nickel affinity column as described in Chapter II. This was followed by anion exchange chromatography using a mono Q^{TM} 5/50 GL column (Amersham Pharmacia Biotech, Piscataway, NJ) on the ÄKTA Purifier Fast Protein Liquid Chromatography (FPLC) system (GE Healthcare, Piscataway, NJ) using a continuous NaCl gradient that was applied for 20 column volumes to elute hscLeuRS. The purified protein was dialyzed into 50% glycerol, 100 mM KP_i, pH 6.8, 10 mM β-mercaptoethanol buffer and stored at -20 °C.

IV.2.2 Isolation of mono- and *bis*-Aminoacylated tRNA Samples for Analysis

Radioactive charged tRNA was enzymatically synthesized as described in Chapter II. Non-radiolabeled tRNA was necessary as radioactive samples were prohibited from being purified over the LC/MS. The non-radioactive charged tRNA was also prepared as described in Chapter II, except 40 μM of transcribed *E. coli* tRNA and 80 μM of unlabeled leucine or isoleucine were substituted for 8 μM of transcribed *E. coli* tRNA and 22 μM [^{3}H]-leucine or isoleucine. A deacylation assay was conducted as described in Chapter II and aliquots were taken at 0, 5, and 10 min time points, followed by quenching in 400 mM sodium acetate, pH 5.0 and 0.1% sodium dodecyl sulfate (SDS).

Aliquots from deacylation assays were analyzed on an acidic gel (Varshney et al. 1991). The (mis)charged tRNA used for the deacylation assays was prepared as described in Chapter II except 1.2 mM of [^{14}C]-leucine or [^{14}C]-isoleucine (318 mCi/mmol) was used instead of 22 μM [^{3}H]-leucine or [^{3}H]-isoleucine. Quenched aliquots of 20 μL volume from deacylation assays were loaded onto a 10 % acidic polyacrylamide gel that had been pre-equilibrated in 25 mM NaOAc, pH 5.0 (Varshney et al. 1991). The gel was electrophoresed at 8 mA for approximately 12 h overnight. The gel was dried and phosphorimaged using a FUJI BAS 1000 phosphorimage screen (FUJI-FILM Medical Systems U.S.A., Stanford, CT) and quantified with a Storm 840 Molecular Dynamics Imager (GE Healthcare, Piscataway, NJ).

IV.2.3. Acetylation of Charged tRNA

The quenched aliquots from deacylation reactions were added to 50 μL of 5 mM sodium acetate pH 5.5, 10 μL of dimethyl sulfoxide, 20 μL of glacial acetic acid, and finally 20 μL of acetic anhydride. The reactions were incubated on ice for 1 h before being ethanol precipitated (De Pereda et al. 2004). Some samples were cleaved overnight with 5 units of P1 nuclease (Sigma, St. Louis, MO) at 37 ºC after acetylation and then ethanol precipitated the next day.

IV.2.4 Separation of tRNA Samples using TLC

Before separation by TLC, aliquots from deacylation reactions were subjected to either digestion with 0.1 μg/mL of RNase A or base hydrolysis with 0.5 M potassium hydroxide for 2 h at 37 ºC. The samples were spotted on a Whatman 60 Å silica gel TLC plate (GE Healthcare, Piscataway, NJ) and developed in a mobile phase containing chloroform/methanol/32 % acetic acid (5:3:1) (Larkin et al. 2002). After TLC separation, the plates were sprayed with ninhydrin and left to dry in an 80 ºC oven for 20 min in order to detect the leucine standards. These standards were then spotted with ^{14}C-leucine on the plate in order

to mark their positions. The plate was phosphorimaged using a FUJI BAS 1000 phosphorimage screen (FUJI-FILM Medical Systems U.S.A., Stanford, CT) and quantified with a Storm 840 molecular dynamics imager (GE Healthcare, Piscataway, NJ). In an effort to identify some of the spots on the plate, the spots were scrapped and soaked in methanol to remove the running buffer. They were then rocked in 4 M NH_4OH for 30 min at room temperature and left overnight at room temperature to evaporate to dryness.

IV.2.5 LC/MS of Aminoacylated Samples of tRNA

The pelleted tRNA samples that had been cleaved with P1 nuclease were dissolved in water and analyzed on a 6300 Series Ion Trap LC/MS (Agilent Technologies, Foster City, CA), using a 100 mm x 4.6 mm PGC Thermo Hypercarb column (Thermo Fischer, Waltham, MA). The sample was separated on the PGC Hypercarb column using 25 mM ammonium acetate as the aqueous phase (A) and acetonitrile as the organic mobile phase (B) on the HPLC. The sample was purified using a linear gradient that began at time zero with 5 % of B. The linear gradient increased from 5 % to 80 % B in 10 min, and then increased to 100 % B in the next 5 min. Finally, 100 % B was used to elute sample between 15-30 min. After HPLC purification, the sample was run through the 6300 Series Ion Trap MS equipped with an electrospray ionization (ESI) interface that was run in both the positive as well as negative mode. The experiments were conducted using a full-scan mode with a mass range *m/z* of 100-700. Alternatively, some samples were directly injected into a Micromas Q-TOF Ultima mass spectrometer (Waters Corporation, Milford, MA) to be analyzed.

IV.3. Results

IV.3.1. Initial Hydrolysis Followed by Re-charging of tRNA by hscLeuRS

The hscLeuRS enzyme was tested for post-transfer editing and displayed an unanticipated phenomenon. In the presence of hscLeuRS, I observed that after the usual decrease in charged and mischarged tRNA, there was a subsequent re-accumulation of charged and mischarged tRNA during the deacylation assays (Figure IV.2).

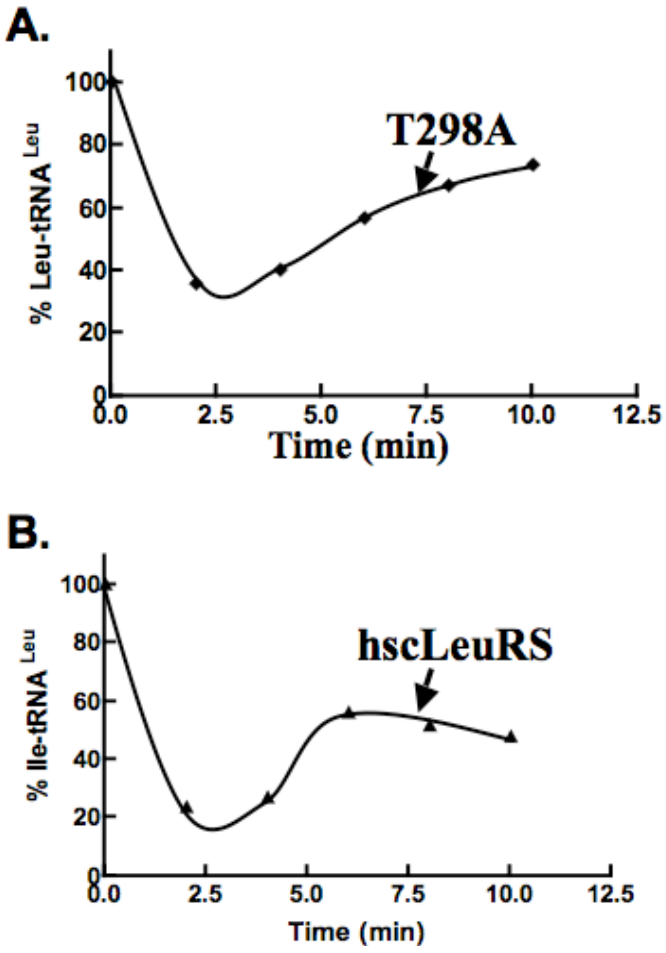


Figure IV.2 Recharging by wild-type and mutant hscLeuRS during deacylation reactions. Deacylation reactions were carried out with 1 µM enzyme and either A. ~4 µM *E. coli* Leu-tRNA^{Leu} or B. ~4 µM *E. coli* Ile-tRNA^{Leu}. All enzyme reactions were incubated at 30 °C. The hscLeuRS protein is represented by (▲) and mutant protein T298A by (♦).

Based on previous findings regarding tandemly activated tRNAs (Stepanov et al. 1992; Wang et al. 2006), I hypothesized that the tRNA might be charged with two leucines at the terminal ribose of tRNA. This recharging effect was surprising since ATP, which is typically required to charge tRNA, was not one of the components of the deacylation assays. I then hypothesized that the additional amino acid that is freed during the initial hydrolysis of charged and mischarged tRNA might be re-attached to a new incoming tRNA, possibly generating a dipeptide on the tRNA.

Steps were taken to ensure that the reaction was free of ATP. The hscLeuRS was purified as described in Chapter II in addition to a second round of purification over an anion exchange column using a FPLC system. This was done to ensure that hscLeuRS was free of any small molecule contaminants such as ATP. In addition, a control aminoacylation assay was carried out with purified hscLeuRS and leucine in the absence of externally added ATP. This control assay showed no charging activity, further confirming the absence of an ATP contaminant on hscLeuRS (Figure IV.3).

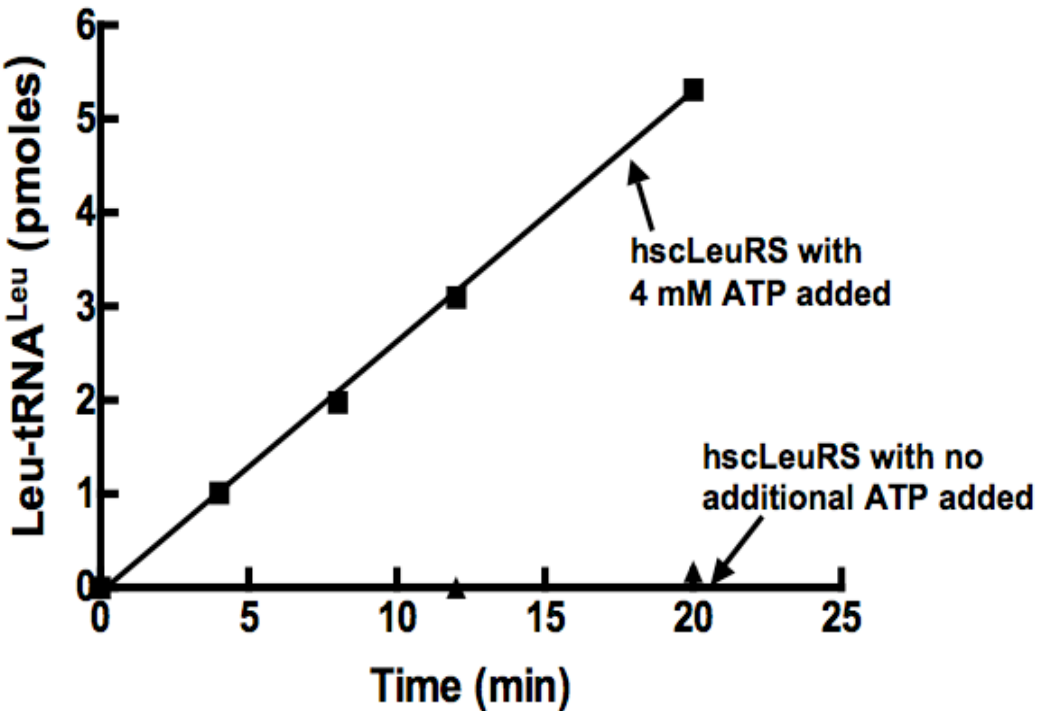


Figure IV.3 The hscLeuRS is unable to charge tRNA without the addition of ATP. Aminoacylation reactions contained 4 μM tRNA^{Leu}, 20 μM leucine, 500 nM enzyme, and either no ATP or 4 mM ATP. The hscLeuRS with 4 mM ATP and no additionally added ATP is represented by (■) and (▲) respectively.

In addition, acidic gel electrophoresis was employed to stably separate charged tRNAs in order to confirm the initial decrease and subsequent regeneration of charged tRNA. I focused mainly on using the hscT298A mutant that hydrolyzes correctly charged Leu-tRNA^{Leu} (Pang and Martinis 2009). Aliquots were taken at various time points during the course of the deacylation assay and separated on an acidic gel as described before. Analysis of the acidic gels revealed a reaccumulation of charged tRNA with T298A (Figure IV.4). Separating the charged tRNA samples on an acidic gel also helped confirm that free amino acid released during the deacylation wasn't sticking non-specifically to hscLeuRS, resulting in an erroneously high reading during deacylation assays.

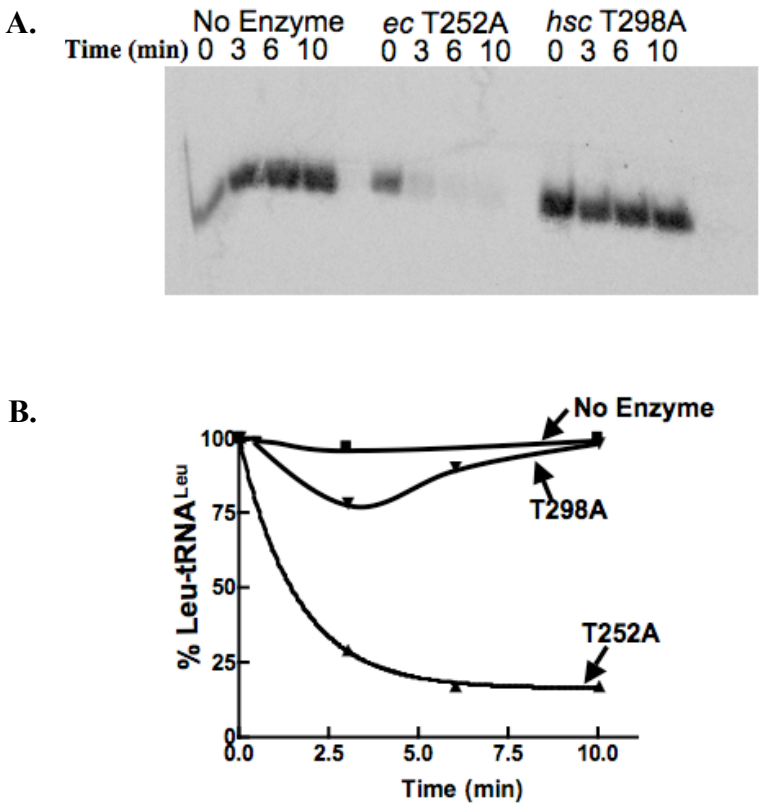


Figure IV.4 Reaccumulation of charged tRNA based on acid gel analysis. Deacylation reactions were carried out at 30 °C with 1 μM *E. coli* T252A or hscT298A, and ~4 μM *E. coli* Leu-tRNA^{Leu}. Aliquots taken at 0, 3, 6, and 10 min time points during deacylation were separated on a 10 % acid gel. The acid gel was electrophoresed dried and phosphorimaged using a FUJI BAS 1000 phosphorimage screen. The intensities of the bands appearing on the acid gel (A) were quantified with a Storm 840 Molecular Dynamics imager and plotted (B). The hscLeuRS T298A mutant is represented by (▼), *E. coli* T252A by (▲) and no enzyme by (■).

Finally, I tested the viability of my hypothesis by conducting a PP_i exchange assay as described in Chapter II in order to determine if hscLeuRS activates dipeptidyl leucine-leucine. Interestingly, unlike *E. coli* LeuRS, hscLeuRS activates dipeptidyl leucine-leucine (Figure IV.5). The hscLeuRS was unable to activate tri-peptidyl leucine-leucine-leucine.

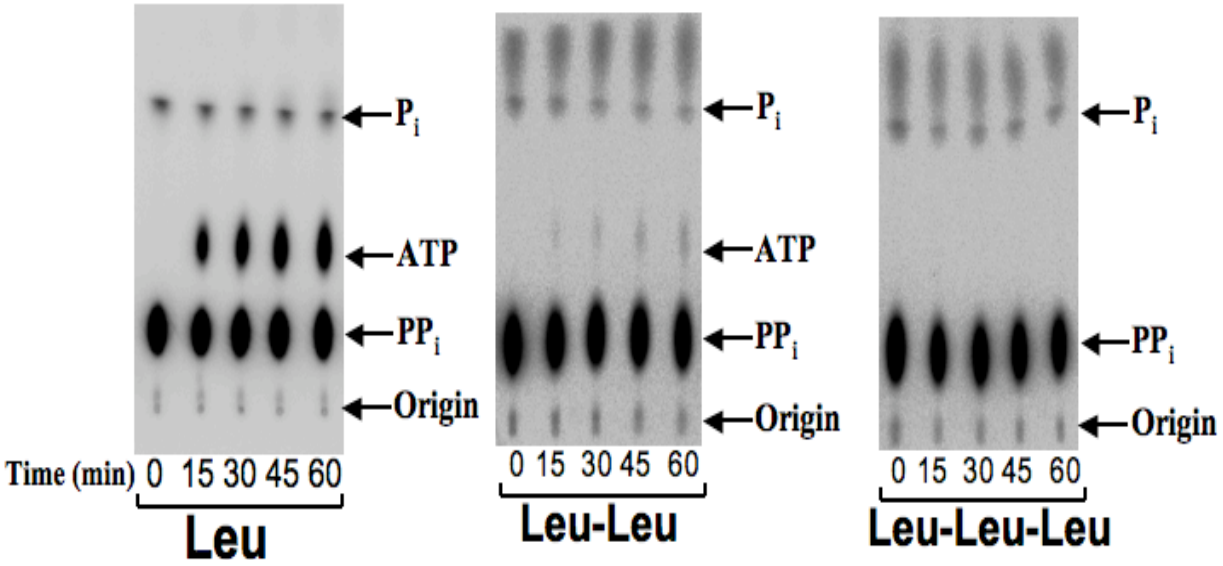


Figure IV.5 The hscLeuRS enzyme activates both leucine and dipeptidyl leucine-leucine. Spots representing ATP were visualized on TLC plates indicating activation of amino acid. Aliquots were quenched at 0, 15, 30, 45, and 60 minute time points. The presence of ATP spots on the first two plates indicates that Leu and Leu-Leu are activated by hscLeuRS. The absence of ATP spots on the right most plate indicates that tripeptidyl Leu-Leu-Leu is not activated in the first step of aminoacylation.

IV.3.2 Analysis by TLC Could Not Confirm Presence of *bis*-Aminoacylated tRNA Species

I reasoned that if a dipeptide was indeed forming on the 3′ adenosine of tRNA, then cleaving the tRNA into individual nucleotides would yield a modified adenosine corresponding to the 3′ adenosine with a dipeptide on it. I used RNase A to digest the quenched aliquots from deacylation assays in order to yield the free 3′ adenosine that had a radiolabeled amino acid charged on it. The digested samples were separated on a TLC plate as described above. I predicted that the modified adenosine would have a distinct shift on the TLC plate as compared to adenosine charged with just one leucine. In another set of experiments, I also treated aliquots from the deacylation assays with base in order to hydrolyze the amino acid from the 3′ adenosine. These samples were separated using TLC that also had free leucine and leucine-leucine dipeptides as standards. I observed a shift in spots with samples taken from the 4 min and 10 min time points that were digested with RNase A. These spots were scrapped and

quantified by ESI mass spectrometry. Unfortunately, the mass spectrometric data was inconclusive as I was unable to identify the origins of the spot that had shifted from the standards (Figure IV.6). I reasoned that the aminoacyl adenylate might be unstable and subject to hydrolysis within minutes as previously reported (Duca et al. 2008). Hence, I refined my strategy to stabilize the charged substrates by acetylating the aminoacylated tRNA samples in order to protect its free amine group. I then proceeded to use LC/MS to identify tRNA samples that were pre-acetylated (De Pereda et al. 2004).

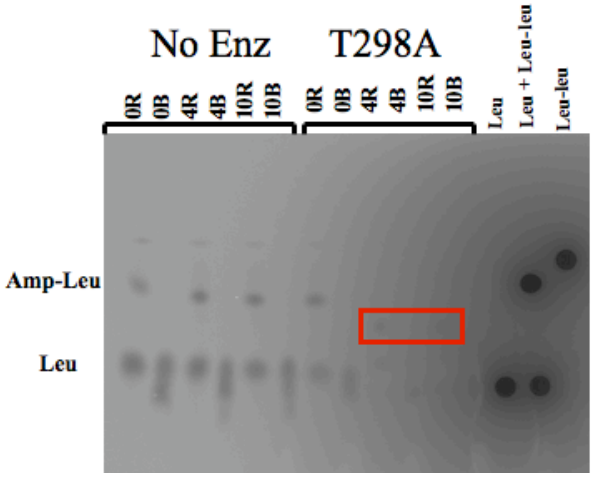


Figure IV.6 Samples separated via TLC did not reveal a dipeptidyl-adenosine species. Aliquots of tRNA samples obtained from various time points throughout the deacylation assay were subjected to either RNase A or base hydrolysis. Boxed in red are 2 unidentifiable spots appearing from RNase A digestions of aliquots obtained at time 4 and 10 min during deacylation of charged tRNA with hscT298A. "0R" represents RNase A digestion of aliquots taken at the 0 min time point during deacylation, "0B" represents base hydrolysis of aliquots taken at the 0 min time point during deacylation, "4R" represents RNase A digestion of aliquots taken at the 4 min time point during deacylation, "4B" represents base hydrolysis of aliquots taken at the 4 min time point during deacylation, and so forth.

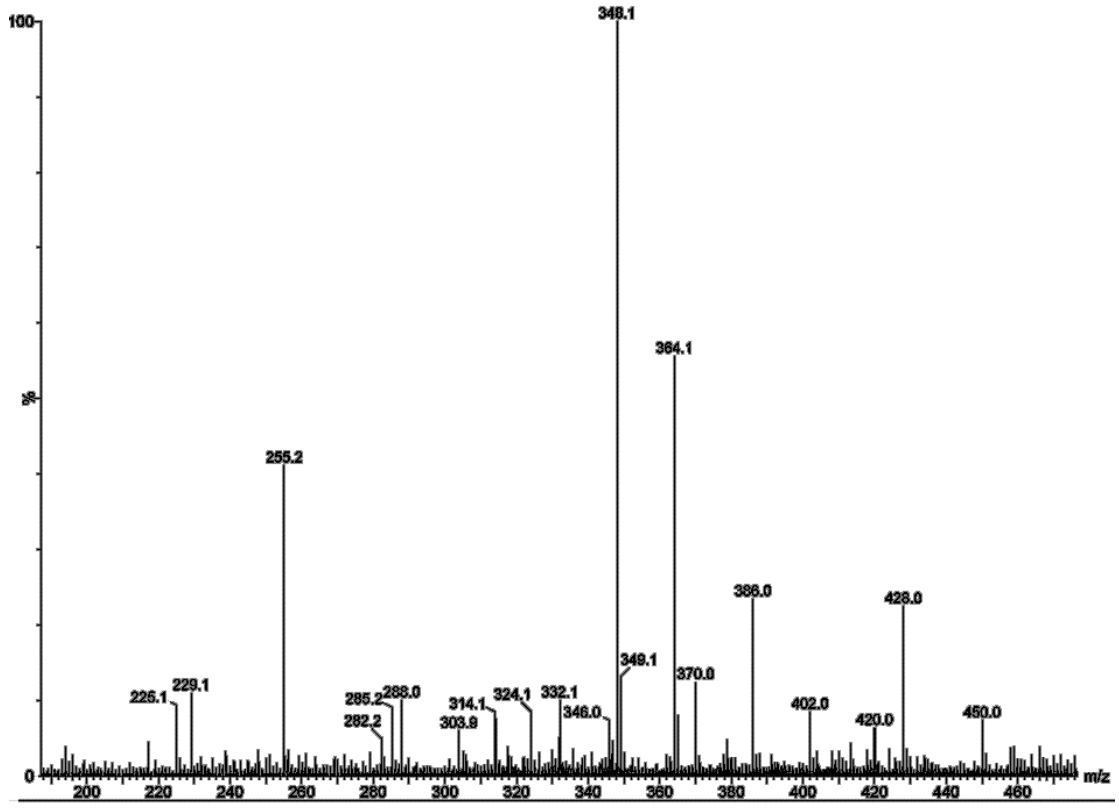
IV.3.3. Liquid Chromatographic and Mass Spectrometric Analysis Could Not Confirm Presence of *bis*-Aminoacylated tRNA Species

Since the separation of samples with TLC was inconclusive, I then directly purified the hypothesized dipeptidyl samples from the deacylation assays over a LC/MS. Deacylation assays were conducted and aliquots collected at 0, 5, and 10 min time points. These aliquots

were quenched and acetylated as described above. After acetylation, the samples were then digested overnight with 5 units of P1 nuclease at 37 °C before ethanol precipitation. Just before purification over the LC/MS, the pelleted samples were resuspended in water. I was very fortunate to have also received a chemically synthesized AMP-leucine-leucine compound from Dr. Sidney Hecht's group at Arizona State University that would function as a standard for the modified adenosine with two leucines on it.

Analysis via the 6300 Series Ion Trap LC/MS was unsuccessful as the instrument was not sensitive enough to detect samples present in picomole amounts. Subsequently, I injected the samples directly on a Micromas Q-TOF Ultima mass spectrometer (Waters Corporation, Milford, MA), which provided higher levels of sensitivity. The Q-TOF enabled me to conduct high resolution ESI MS on the samples. The AMP-leucine-leucine standard as well as a P1 nuclease cleaved tRNA sample were injected into the Q-TOF as controls. A good mass spectra was obtained with these controls in the Q-TOF and I attained a distinct peak indicating the presence of AMP (*m/z* 348.1) from my cleaved tRNA sample as well as one indicating the presence of AMP-leucine-leucine (*m/z* 574.2) (Figure IV.7).

A.



B.

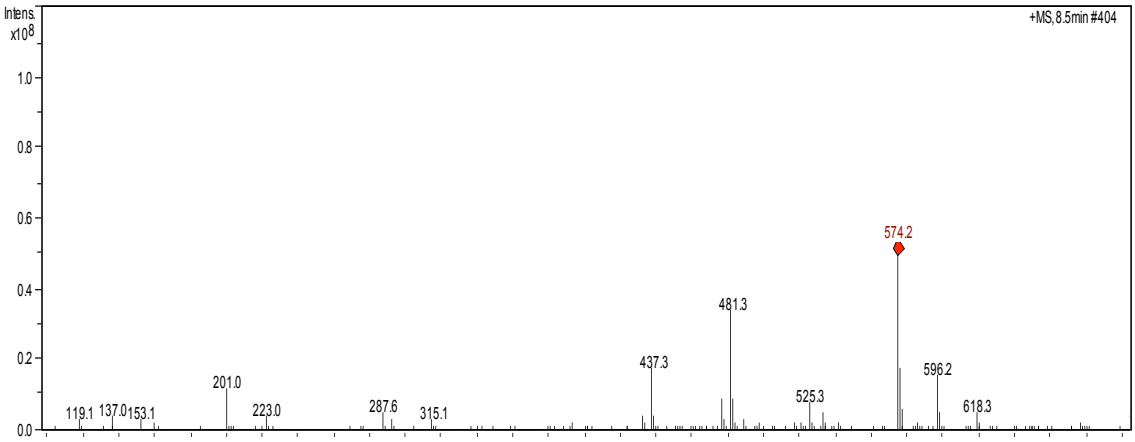


Figure IV.7 Spectra from Q-TOF indicating the presence of the controls. A. The peak at *m/z* 348.1 represents AMP from $tRNA^{Leu}$ liberated by P1 nuclease. B. Spectra from synthesized AMP-leucine-leucine standard. The peak at *m/z* 574.2 represents AMP-leucine-leucine. Samples were run using ESI in the positive mode.

Unfortunately, all other leucylated tRNA samples obtained from deacylation assays were not detected on both the LC/MS and Q-TOF, in spite of the extra stability conferred by acetylating the charged tRNA samples possibly due to their low concentrations.

IV.4. Discussion

The unanticipated phenomenon displayed by hscLeuRS during deacylation assays, whereby there was an initial dip followed by a reaccumulation of charged products (Figure IV.2), led me to hypothesize that the hscLeuRS might be charging two leucines on the 3′ adenosine of tRNA. It has been reported previously that *T. thermophilus* PheRS can form *bis*-aminoacylated $tRNA^{Phe}$ with 2 phenylalanines, one on the 2′ -OH and another on the 3′ -OH of the 3′ adenosine of tRNA (Stepanov et al. 1992) (Figure IV.2). However, it did not seem plausible that I was synthesizing a *bis*-leucylated species since there was no ATP in the reaction to facilitate the charging of amino acid on tRNA. The hscLeuRS was purified over a series of two columns to remove any small molecule contaminants and control aminoacylation experiments conducted with purified hscLeuRS and leucine in the absence of externally added ATP did not yield any charged product. Instead, I hypothesized that a dipeptide could be forming on the 3′ adenosine when a free amino acid forms a peptide bond with an amino acid on an existing charged tRNA species. The PP_i exchange assays that I conducted indicated that hscLeuRS, unlike the *E. coli* LeuRS, activates leucine-leucine, thus supporting my dipeptidyl tRNA formation hypothesis.

Paradoxically, Dr. Sidney Hecht's group has shown that in contrast to the *bis*-aminoacylated species, the dipeptidyl species is unable to participate in protein synthesis (Wang et al. 2006). Based on the many published articles on the alternate functions of aaRSs, it seemed plausible that the hscLeuRS was creating a dipeptidyl tRNA to help serve some of its

other purposes within the cell (Martinis and Pang 2007). Interestingly, some groups have shown that multiple peptide resistance virulence factors (*mprF*) can use Lys-tRNA^{Lys} to modify phosphatidylglycerol although it is still unclear as to how Lys-tRNA^{Lys} bound by EF-Tu is first sequestered by *mprF* (Peschel et al. 2001; Thedieck et al. 2006; Roy and Ibba 2008). This Lys-tRNA^{Lys} can then aminoacylate inner membrane lipids resulting in a change in cellular permeability to certain cationic antibiotics (Roy and Ibba 2008). Additionally, aminoacylated tRNAs can participate in a host of other functions including cell wall and porphyrin biosynthesis (Ibba and Söll 2004). It is thus possible that a dileucyl-tRNA^{Leu} might participate in similar functions, although it should be noted that new alternate functions of aaRSs are continuously being discovered, and it is difficult to say for sure what other functions a dileucyl-tRNA^{Leu} might serve.

Acidic gels were also used to confirm the increase in charged tRNA during the course of our deacylation assays. In order to isolate our hypothesized dipeptidyl-tRNA, a series of deacylation assays were conducted and aliquots were taken at various time points to be analyzed for possible dipeptidyl-tRNA. The isolated tRNA was cleaved with either RNase A or P1 nuclease and I attempted to identify a modified adenosine that would have two leucines on it. Various methods were employed, including the analysis of samples by TLC, LC/MS, as well as Q-TOF MS.

Separation via TLC proved inefficient as I did not observe a distinct upward shift in spots representing a di-charged species. The lack of a radiolabeled adenosine-leucine-leucine further complicated the experiment and TLC also failed to achieve high enough sensitivity in order to detect picomole quantities of product. I was further unsuccessful in trying to isolate the dipeptidyl species via MS from scrapped samples on the TLC plate. I later realized that an

adenosine aminoacylated with leucine(s) would be fairly unstable and that it would be necessary to acetylate the leucines attached to the adenosine in order to protect the free amino groups on leucine (Duca et al. 2008). However, in spite of acetylating the samples, LC/MS as well as Q-TOF MS continued to be unproductive in isolating the dipeptidyl species.

The elusiveness of this dipeptidyl-tRNA sample might be attributed to the very low quantities that it is produced during the reaction. We began with 4 μM of Leu-$tRNA^{Leu}$ during deacylation assays, resulting in a very low concentration of isolated tRNA sample. The initial concentration of charged tRNA species used in the deacylation assays was increased by 10-fold to 40 μM. However, attempts to isolate the dipeptidyl species continued to be unsuccessful. In addition, aminoacylated adenosyl sample is unstable and degrades over time, further lowering the detectable amount of dipeptidyl species left in the sample. One possibility of improving the yields of tRNA is to run full length acetylated tRNA products from the deacylation assays in order to reduce degradation and sample loss during the steps that are required to digest tRNA into individual nucleotides. This way, there will be a higher chance of isolating the elusive dipeptidyl species.

Chapter V. Characterization of Protein-Protein Interactions Between the CP1 Domain and Canonical Aminoacylation Core

V.1 Introduction

The aminoacylation site of aaRSs activates cognate amino acid as well as isosteric non-cognate amino acids (Hendrickson and Schimmel 2002; Mascarenhas et al. 2008). Misactivated amino acids could then be mistakenly charged on to tRNAs, resulting in mischarged tRNA. In order to maintain fidelity, some aaRSs such as LeuRS, IleRS and ValRS have an editing module termed the CP1 domain (Starzyk et al. 1987; O'Donoghue and Luthey-Schulten 2003) that binds incorrectly charged tRNAs and cleaves them (Lin et al. 1996). This prevents the mischarged species from being brought to the ribosome for participation in protein synthesis.

The CP1 domain is connected to the canonical core via two β-strands (Cusack et al. 2000) and its hydrolytic editing active site is separated from the aminoacylation site by approximately 30 Å. Based on the comparison of several crystal structures (Mascarenhas et al. 2008), the CP1 domain adopts different conformational states relative to the canonical core. This likely accommodates the various stages of tRNA binding, and is dependent on the rotational flexibility of the CP1 domain and also the dynamic nature of the β-strands (Mascarenhas and Martinis 2008; Mascarenhas and Martinis 2009).

When tRNA is bound to LeuRS, the CP1 domain rotates ~30° relative to its *apo* state during translocation (Tukalo et al. 2005). It has been hypothesized that this movement facilitates translocation of tRNA from the catalytic core domain to the CP1 domain for editing (Tukalo et al. 2005). During this process, the canonical core and CP1 domain have been proposed to interact via putative transient protein-protein interactions (Figure V.1) (Williams and Martinis 2006). Specifically, salt bridges were hypothesized to form between specific residues across the interface of the canonical core as well as CP1 domain in order to

allow crosstalk between both modules. These inter-domain interactions are thought to be especially essential when post-transfer editing is disrupted in *E. coli* LeuRS, and the cell is forced to rely on pre-transfer editing to ensure overall fidelity. It is possible this redundant or secondary pre-transfer editing mechanism relies on the formation of these transient salt bridges between the catalytic core domain and the CP1 domain. Upon activation, pre-transfer editing facilitates the hydrolysis of aminoacyl-adenylate intermediates (Williams and Martinis 2006).

In addition, more recent work has shown that a "translocation peptide" located within the vicinity of an Asp 391 hinge in *E. coli* LeuRS, is essential for the translocation of tRNA (Figure V.1) (Hellmann and Martinis 2009). The theoretically predicted Asp 391 hinge is hypothesized to confer flexibility that directs rotation of the CP1 domain relative to the main body. Mutations within this translocation peptide have resulted in amino acid toxicities owing to the bypassing of the editing domain by mischarged tRNA species (Hellmann and Martinis 2009). Translocation is essential for accurate charging of the tRNA. However structural analysis of this process has proven to be difficult due to its transient nature. Hence, we proposed to study the interactions between the canonical core and CP1 domain via biophysical techniques in order to elucidate the translocation process.

Herein, I characterized macromolecular protein-protein interactions between the CP1 editing domain and the aminoacylation core that might be important to translocation. A deletion mutant of *E. coli* LeuRS (LeuRS-ΔCP1) that lacks the entire CP1 domain (including both β-strands that link the CP1 domain to the core) (Boniecki et al. 2008) and constructs of the isolated LeuRS CP1 domain (Betha et al. 2007) (Figure V.1). Both these dissected domains are functional in their respective activities (Betha et al. 2007; Boniecki et al. 2008). To help us better decipher the translocation of editing substrates from the main body to the CP1 domain,

we tested these isolated active LeuRS domains for protein-protein interactions using immunoprecipitation pull-down assays as well as smFRET.

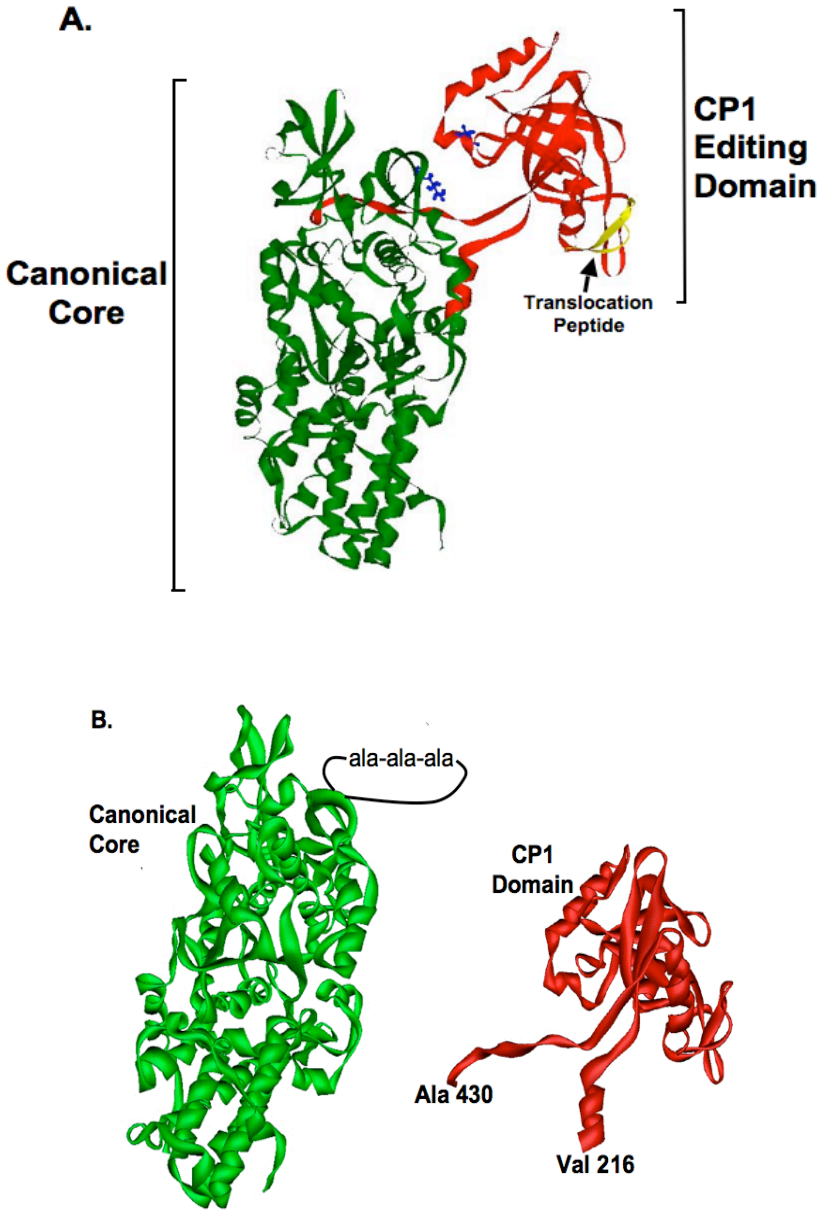


Figure V.1 Tertiary structures of *E. coli* LeuRS. (A) Homology model of wild-type *E. coli* LeuRS (Lee and Briggs 2004) with the translocation peptide highlighted in yellow. The residues Lys 186 and Ala 293 are depicted in ball and stick form in blue and are proposed to interact with each other (Williams and Martinis 2006). (B) Homology model of isolated ΔCP1 domain of *E. coli* LeuRS (Boniecki et al. 2008) on the left and isolated CP1 domain on the right (Betha et al. 2007). The canonical core domain is shaded in green while the CP1 domain is shaded in red.

V.2. Experimental Procedures

V.2.1 Immunoprecipitation Assays

Hemagglutinin (HA) and myc epitope tags were inserted on the his-tagged LeuRS-ΔCP1 (Boniecki et al. 2008) and CP1 domains (Betha et al. 2007) respectively on their N-terminal ends. Plasmid pBETeCP1-2-35 encodes the *E. coli* LeuRS CP1 domain from Val 216 to Ala 430. A HA tag was cloned in at the N-terminus between the *Xba*I and *Nco*I restriction sites using the primers HA-Fwd (5'-CTA GAA TGA CCT ACC CAT ACG ACG TCC CAG ACT ACG CTC A-3') and HA-Rev (5'-CAT GTG AGC GTA GTC TGG GAC GTC GTA TGG GTA GGT CAT T-3') to give pJPCP1HA. Subsequently, a myc tag was cloned in at the N-terminus of the canonical core domain between the *Xba*I and *Nco*I restriction sites using plasmid pMTV-ΔCP13a. Primers Myc-Fwd (5'-CTA GAA TGA CCG AAC AAA AAC TTA TTT CTG AAG AAG ATC TGC A -3') and Myc-Rev (5'-CAT GTG CAG ATC TTC TTC AGA AAT AAG TTT TTG TTC GGT CAT T-3') to give pJPmbmyc.

The LeuRS-ΔCP1-myc and LeuRS-CP1-HA were expressed and purified in a similar fashion as the untagged proteins. Varying concentrations of the lysate or purified form of LeuRS-ΔCP1-myc was incubated with 50 μl anti-myc antibody beads (Pierce, Rockford, IL) in binding buffer (50 mM HEPES, pH 7.6, 30 mM KCl, 20 μM leucine, and 30 mM $MgCl_2$) for 1 h at 4°C. The beads were washed three times with 1 ml of wash buffer (0.05% Triton X-100, 10% glycerol, 50 mM NaCl, 50 mM HEPES, pH 7.5). Varying concentrations of the lysates or purified forms of LeuRS-CP1-HA were then added for an overnight incubation at 4°C together with LeuRS-ΔCP1-myc immobilized to anti-myc antibody beads, in the absence or presence of *E. coli* transcribed tRNA. The next day, the beads were washed three times again with 1 ml of wash buffer at room temperature and eluted with the elution buffer from a ProFound Co-IP kit

(Pierce, Rockford, IL). Co-precipitation of LeuRS-ΔCP1 with CP1 was then detected by Western blotting using monoclonal anti-his antibodies (Sigma, St. Louis, MO).

V.2.2 Western Blots

Proteins were separated on a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. The gel was washed in Tris-Buffered Saline and Tween 20 (TBST) for 5 min. The proteins were then transferred by electrophoresis at 200 mA for 2 h in transfer buffer (25 mM Tris, pH 8.0, 250 mM glycine, 20% methanol) from the SDS-PAGE gel to a Protran BA85 nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) that had been presoaked in TBST for 5 min. The membrane was then removed and washed in TBST at room temperature for 10 min before blocking with 5% powdered milk at room temperature for 1 h. The membrane was incubated with a 1:1000 dilution of primary antibody in 5% milk TBST at room temperature for 1 h. Following that, it was washed three times in TBST for 10 min each time before incubation with 5% milk TBST solution containing a 1:5000 dilution of secondary antibody. Finally, the membrane was washed three times, 10 min each, at room temperature before being soaked in 0.56 mM 5-bromo-4-chloro-3-indolyl phosphate and 0.48 mM nitro blue tetrazolium (BCIP/NBT), 10 mM Tris-HCL, pH 9.2, and 59.3 mM $MgCl_2$ premixed liquid substrate solution (Sigma, St. Louis, MO) for 2 min until dark bands appeared on the filter. The reaction was quenched in water and air-dried.

V.2.3 Fluorescent Labeling of *E. coli* LeuRS Using an Orthogonal System

Plasmid pJPE317amber encodes the *E. coli* LeuRS E317amber mutant (harboring an amber stop codon at Glu 317) made using primers E317amber-Fwd (5'-CCA TTA ACG GGC GAA TAG ATT CCC GTT TGG GC-3') and E317amber-Rev (5'-GCC CAA ACG GGA ATC TAT TCG CCC GTT ATT GG-3'). The plasmid pSup_pAcPheRS_TRN

(Ryu and Schultz 2006) was a gift from Dr. P. Schultz (The Scripps Research Institute; La Jolla, CA) and is a single-plasmid system that encodes multiple copies of an amber suppressor tRNA from *Methanocaldococcus jannaschii* as well as an orthogonal PheRS harboring a D286R mutation that allows *p*-acetyl-L-phenylalanine to be substituted at an amber stop codon. *E. coli* BL21 (DE3) plysS (Novagen, Madison, WI) cells were transformed with pJPE317amber and pSup_pAcPheRS_TRN, and grown in LB containing 100 μg/ml of ampicillin, 34 μg/ml of chloramphenicol, and 1.0 mM *p*-acetyl-L-phenylalanine (RSP Amino Acid Analogues, Worchester, MA) at 37 °C, until the OD_{600} was between 0.4 and 0.6. Protein expression was induced overnight at room temperature by the addition of 0.5 mM IPTG and later purified as described in Chapter II.

V.2.4 Fluorescent Labeling of a Cysteine-knockout *E. coli* LeuRS Using Maleimide Chemistry

Plasmid pJP4cysko encodes the *E. coli* LeuRS with the mutations C37V, C128S, C505T, and C565T. I began with plasmid p15ec3-1 that encoded wild-type *E. coli* LeuRS and made base substitutions using PCR mutagenesis methods as described in Chapter II. The C37V mutation was made first using primers C37V-Fwd (5'-GAG AAG TAT TAC GTC CTG TCT ATG CTT-3') and C37V-Rev (5'-GAA GCA TAG ACA GGA CGT AAT ACT TCT C -3') to give plasmid pJPC37V. The plasmid pJPC37V was used to insert the C128S mutation using primers C128S-Fwd (5'-GAG CTG GCA ACC AGT ACG CCG GAA TAC-3') and C128S-Rev (5'-GTA TTC CGG CGT ACT GGT TGC CAG CTC-3') to give plasmid pJP2_cys. The plasmid pJP2_cys was used to insert the C505T mutation using primers C505S-Fwd (5'-GCG CGC TAC ACT AGC CCG CAG TAC AAA-3') and C505S-Rev (5'-TTT GTA CTG CGG GCT AGT GTA GCG CGC-3') to give plasmid pJP3_cys. Finally, the plasmid pJP3_cys was used to insert the C565T mutation using primers C565T-Fwd (5'-GAA ACA GTT GCT GAC

TCA GGG TAT GGT G-3') and C565T-Rev (5'-CAC CAT ACC CTG AGT CAG CAA CTG TTT C-3') to give plasmid pJP4cysko. The Glu 317 residue in pJP4cysko was substituted with a cysteine using primers E317C-Fwd (5'-CCA TTA ACG GGC GAA TGT ATT CCC GTT TGG-3') and E317C-Rev (5'-CCA AAC GGG AAT ACA TTC GCC CGT TAA TGG-3') to give pJP4cysko317C.

The protein was expressed from pJP4cysko317C in *E. coli* strain BL21 (DE3) codon PLUS cells (Stratagene, La Jolla, CA). This protein was purified over a HIS-Select HF Nickel Affinity (Sigma, St, Louis, MO) resin as described in Chapter II. The concentration of the protein calculated using an extinction coefficient of 166170 liters/mol·cm as computed by the ExPASy Protparam tool (http://ca.expasy.org/tools/protparam.html).

The protein was then diluted to 1 mg/ml in 500 μL phosphate buffered saline (PBS), flushed with nitrogen for 5 min and incubated at room temperature for 30 min. The reducing agent *tris*(2-carboxyethyl)phosphine (TCEP) (Thermo Scientific, Rockford, IL) was added at a final concentration of 1 mM to the protein and incubated further at room temperature for 20 min. The cyanine-5 (cy5) maleimide monoreactive dye (GE Healthcare, Piscataway, NJ) was dissolved in 50 μL of dimethylformamide (DMF), flushed with nitrogen for 5 min, and 25 μL was added to the 500 μL protein solution. The protein and dye solution together was again flushed with nitrogen and then incubated for 2 h in the dark. The reaction was later quenched into 0.5% β-mercaptoethanol (BME) and the labeled protein was then purified over a PD-10 desalt column (GE Healthcare, Piscataway, NJ).

The protein was further purified over a series of three HiTrap™ desalting columns (GE Healthcare, Piscataway, NJ). The HiTrap™ columns were equilibrated in 70 mM Tris, pH 7.5, 350 mM NaCl, 1 mM DTT and the protein eluted in 20 mM Tris, 100 mM NaCl, and

1 mM DTT. The labeled protein was concentrated in a Centricon-50 (Amicon, Bedford, MA) to approximately 100 μL. The concentration of protein and dye was determined on the basis of its absorbance at 280 nm and 649 nm, and calculated using an extinction coefficient of 166170 liters/mol·cm and 250000 liters/mol·cm respectively as computed by the ExPASy Protparam tool (http://ca.expasy.org/tools/protparam.html). A correction factor of 0.05 for the cy5 dye was taken into consideration while reading the absorbance. The following equations were used to compute the [Dye/Protein] ratio.

$$[\text{cy5 dye}] = \text{Absorbance}_{649}/250000 \quad [V.1]$$

$$[\text{LeuRS}] = [\text{Absorbance}_{280} - (0.05 \times \text{Absorbance}_{649})] / 166170 \quad [V.2]$$

$$[\text{Dye/Protein}]_{final} = [\text{dye}] / [\text{protein}] \quad [V.3]$$

$$[\text{Dye/Protein}]_{final} = [0.66 \times \text{Absorbance}_{649}]/[\text{Absorbance}_{280} - (0.05 \times \text{Absorbance}_{649})] \quad [V.4]$$

V.2.5 Splint Ligation of tRNA with Modified Base

Initial efforts to label the tRNA with a cyanine-3 (cy3) dye involved the splint ligation of two RNA fragments using T4 RNA ligase (Fermentas, Glen Burnie, MD). The first RNA fragment (RNACGthy) consisted of a chemically synthesized fragment harboring a modified nucleotide 5-C6-Amino-2′-deoxythymidine (ChemGenes Corporation, Wilmington, MA). This fragment, RNACGthy (5'-GC(5-C6-Amino-2′-deoxythymidine) CGG AUG GUG GAA UCG-3'), was used to conjugate an *N*-hydroxysuccinimide (NHS) derivative of a cyanine dye later on (Edwards and Sigurdsson 2005). The second fragment (RNAtrans) consisted of a transcribed piece of tRNA (plasmid pJPtRNA3/4) that was ¾ of full length *E. coli* $tRNA^{Leu}$ and had the sequence RNAtrans (5'-GUA GAC ACA AGG GAU UUA AAA UCC CUC GGC GUU CGC GUC GUG CGG GUU CAA GUC CCG CUC CGG GUA CCA-3'). The underlined regions in both fragments represent the nucleotides flanking the splint. This ¾ length tRNA was

constructed via PCR deletion mutagenesis on ptDNALeu14 using primers pJPtRNA3/4-Fwd (5'-CTA ATA CGA CTC ACT ATA GTA GAC ACA AGG G-3') and pJPtRNA3/4-Rev (5'-CCC TTG TGT CTA CTA TAG TGA GTC GTA TTA G-3'). A DNA splint (DNAsp) with sequence DNAsp (5'- GTG TCT ACC GAT TCC -3') was used to hold the two fragments of tRNA together to be ligated. The ligations were carried out in a total volume of 10 μL with 10 μM each of the two RNA fragments as well as 20 μM of DNA splint in buffer containing 50 mM Tris(HCl), pH 7.4, 10 mM $MgCl_2$. 5 mM ATP, and 5 mM DTT. The RNAtrans was first phosphorylated at 30 °C with 15 units of T4-polynucleotide kinase (T4-PNK) (Fermentas, Glen Burnie, MD). Both RNA samples were heated at 80 °C for 2 min and then cooled at room temperature for 30 min. Ligation reactions were carried out for 3 h at 30 °C with 30 units of T4 RNA ligase. Finally, the DNA splint was digested with 5 units of DNase I (Sigma, St Louis, Mo) for 15 min at 30 °C. In order to analyze the ligation reactions, 2 μL of each ligation reaction was loaded on a 10% urea-PAGE gel that contained 8 M urea. The gel was analyzed for full length tRNA by visualizing with UV shadowing. A faint band corresponding to full length tRNA was excised from the gel and eluted as described in Chapter II. Unfortunately, less than 20 μM of full length tRNA in a 50 μL volume was extracted from the gel. I then resorted to purchasing full length $tRNA^{Leu}$ internally labeled with a cy3 dye from ChemGenes Corporation (Wilmington, MA) with the sequence RNAsynthesized (5'- GCC CGG AUG GUG GAA UCG GUA GAC ACA AG(cyanine-3)G GAU UUA AAA UCC CUC GGC GUU CGC GUC GUG CGG GUU CAA GUC CCG CUC CGG GUA CCA -3').

V.2.6 Preparation of PEGylated Quartz Slides

Silanized quartz slides were coated with polyethylene glycol (PEG) (Nektar Therapeutics, San Carlos, CA) and 1-2% (wt/wt) of biotin-PEG

(Laysan Bio. Inc., Arab, AL) and left to dry overnight. Neutravidin (Thermo Scientific, Rockford, IL) was then used to coat the PEGylated slide at a final concentration of 0.2 mg/ml (Ha 2001), followed by 20 nM of biotinylated histidine antibody (Qiagen, Valencia, CA), which was specific for the histidine tag on the labeled protein.

V.2.7 Single Molecule Data Acquisition and Analysis

All smFRET measurements were conducted with 10 nM cy5-LeuRS and 4 nM cy3-tRNA (ChemGenes, Wilmington, MA) at room temperature in a buffer containing 50 mM HEPES pH 7.6, 30 mM KCl, 30 mM $MgCl_2$, 1 μM ATP and an oxygen scavenger system (0.1 mg/ml glucose oxidase (Sigma, St. Louis, MO), 0.02 mg/ml catalase (Hoffmann-La Roche, Basel, Switzerland), 1% BME (Sigma, St. Louis, MO), 0.4% (wt/wt) β-D-glucose (Sigma, St. Louis, MO) and trolox (Sigma, St. Louis, MO)) to reduce photobleaching and photoblinking events (Rasnik et al. 2006). Data was collected using a wide field total internal reflection (TIR) fluorescence microscope. Both a prism and an oil-immersion objective (Olympus UplanSApo 100x numerical aperture 1.4) were used for TIR excitation. An electron multiplying charged coupled device (CCD) camera, iXon DV 887-BI (Andor Technology, South Windsor, CT) and an in-house C++ program were used to capture images at a 30 ms time resolution. The setup was corrected for filter leakage and all FRET values were calculated as the ratio of the acceptor intensity divided by the sum of the intensities of the donor and acceptor fluorophore (Ha 2001).

All image files obtained were analyzed using IDL (ITT Visual Information Solutions, Boulder, Co) software and Matlab (The MathWorks, Natick, MA) (Roy et al. 2008). Only time traces that contained a single photobleaching step for cy3 and cy5 were selected. TIRF movies were obtained using in-house software. The HaMMy program was used for Hidden Markov Modeling (HMM) analysis of the smFRET data (McKinney et al. 2006).

V.3. Results

V.3.1 Potential Non-covalent Protein-protein Interactions Detected from Co-Immunoprecipitation of ΔCP1 and CP1 Domains

A myc and HA tag was cloned to the *E. coli* LeuRS ΔCP1 and CP1 domains respectively at the N-terminus. The proteins were expressed with their respective tags and co-immunoprecipitation pull-down assays were conducted. Antibodies that recognize one or the other epitope tag were used to detect the presence of protein-protein interactions between the two domains (Figure V.2).

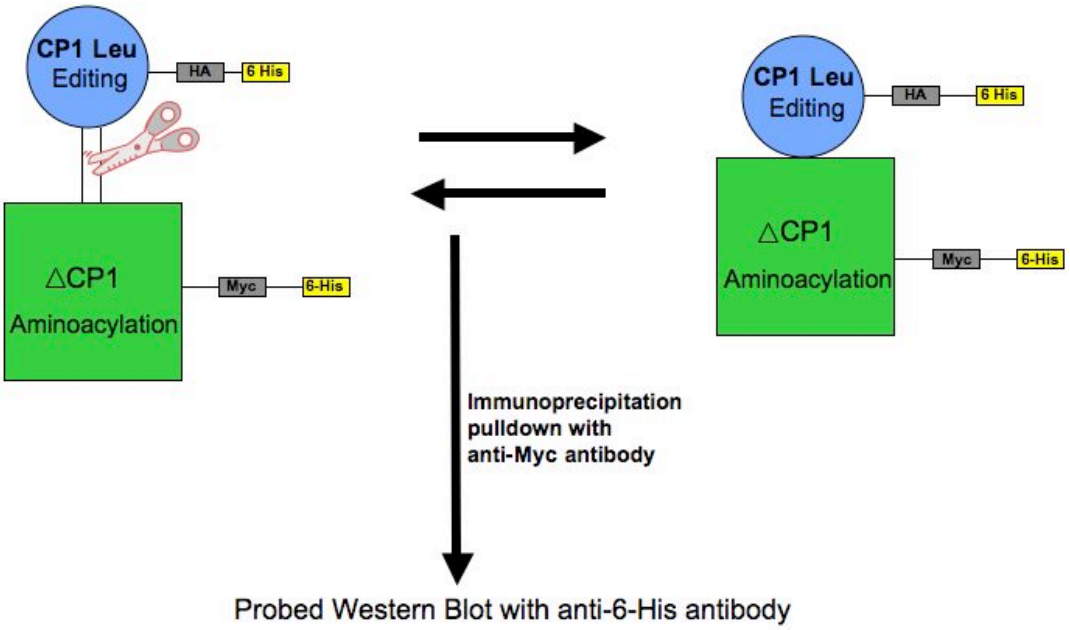


Figure V.2 Cartoon of immunoprecipitation pull-down assay. The LeuRS-ΔCP1 and CP1 domains were individually expressed and co-immunoprecipitation pull-down assays were conducted with them. The canonical core domain is represented by a green square, the CP1 domain by a blue circle, the myc- and HA-tags by gray rectangles, and the histidine-tag by a yellow rectangle.

Co-precipitation of LeuRS-ΔCP1 with CP1 was then probed by Western blotting. The pull-down assays were conducted with purified protein as well as protein in lysate form.

Interestingly, co-immunoprecipitation pull-downs with purified proteins did not yield bands that might have been indicative of interaction between the two domains. However, when lysates of the LeuRS-ΔCP1 with CP1 domain were used instead, the appearance of two bands representing both sub-domains suggested potential non-covalent protein-protein interactions (Figure V.3). We were intrigued by this and hypothesized that additional factors present in cell lysates were essential for interaction between the two domains to occur.

Mutations within the ΔCP1 and CP1 domains were also made based on previous data from our lab regarding two residues, Lys 186 (within the canonical core domain) and Ala 293 (within the CP1 domain). These two sites represented regions that are hypothesized to be responsible for molecular interactions between the two domains (Williams and Martinis 2006) (Figure V.1). However, the presence of these mutations did not enhance co-immunoprecipitation detection of protein-protein interaction between the two domains when used in either purified or lysate form.

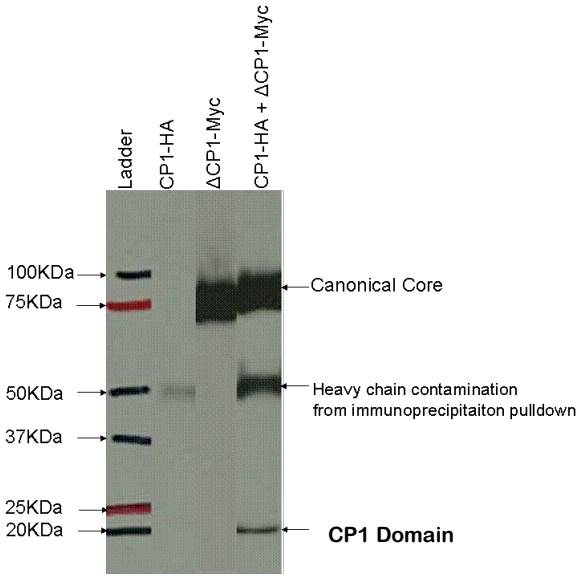


Figure V.3 Immunoprecipitation assays with ΔCP1-Myc and CP1-HA. Western blot showing results of co-immunoprecipitation pull-down of CP1-HA and ΔCP1-Myc in lysate form with antibody specific for the myc-tag. The protein ladder was obtained from Invitrogen Corporation (Carlsbad, CA).

V.3.2 Use of smFRET to Study Intramolecular Interactions of *E. coli* LeuRS during Translocation

Translocation is the movement of the 3′ end of tRNA from the main body to the CP1 domain of LeuRS. I hypothesized that in order for translocation to occur, the canonical core and CP1 domain interact with each other to facilitate the transfer of the 3′ end of tRNA from one domain to another. I used smFRET to probe for interactions of the aminoacylation core of LeuRS with its CP1 domain during translocation.

In short, FRET is a measure of the energy transfer between a donor and acceptor dye. Bulk FRET is a useful technique to examine coordinated motions in an enzyme, however, the molecular properties of the enzyme are complex and sometimes it is not possible to detect uncoordinated intermediate pathways via bulk FRET. Hence, a useful property of smFRET over bulk FRET is the detection of exact non-synchronized movements at specific regions of a protein that are potentially difficult to detect in bulk measurements (Lipman et al. 2003).

I carried out smFRET experiments with an *E. coli* LeuRS labeled on Cys 317 (mutated from Glu 317), which is on the surface of the CP1 domain (Figure V.4), and *E. coli* $tRNA^{Leu}$ with a dye in the anticodon stem. The efficiency of energy transfer, E, is represented by the equation:

$$E = (1 + (R/R_0)^6)^{-1} \qquad [V.5]$$

where R is the inter-dye distance, and R_0 is the Förster radius at which $E = 0.5$

Since the Förster distance for FRET is 50 Å and the distance between the synthetic active site and editing pocket is approximately 30 Å, the FRET dye pairs were places as far apart from each other to ensure the highest dynamic range for energy transfer to occur between both dyes. Given that the donor dye appended in the tRNA anticodon loop would bind in the canonical

core, I reasoned that the acceptor dye should be place as far back in the CP1 domain as possible. Thus, I chose to label Glu 317 located at the back of the CP1 domain (Figure V.4).

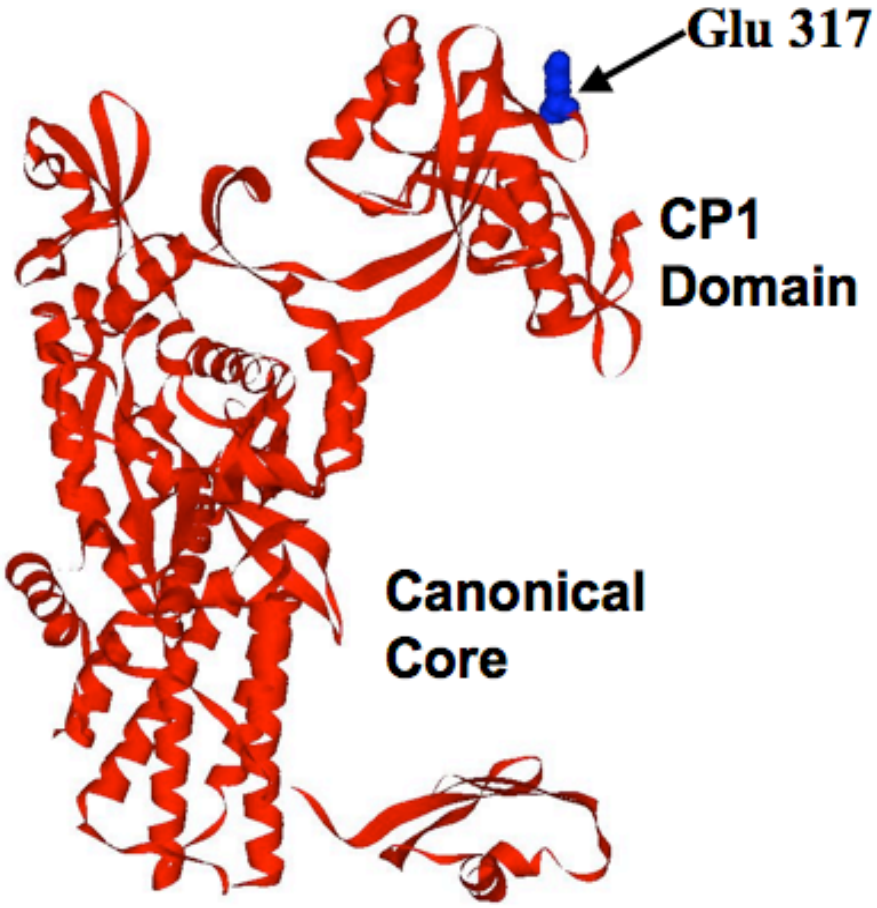


Figure V.4 Position of cy5 dye on LeuRS. The crystal structure of *T. thermophilus* showing residue Glu 317 in blue space-filling form on the CP1 domain. The Glu 317 was substituted with a cysteine and a cy5 dye was conjugated to the protein via that cysteine. PDB entry: 2BTE. (Tukalo et al. 2005).

In order to identify the best possible method of labeling protein with a cyanine-based dye, I attempted to label LeuRS via various methods, including the use of an orthogonal system (Wang et al. 2006) as well as via a cysteine using maleimide chemistry (Mujumdar et al. 1993). The orthogonal method allowed us to insert an unnatural amino acid at a stop codon, which could then be conjugated to a dye. The LeuRS was fluorescently labeled (Wang et al. 2003; Ryu and Schultz 2006) by incorporating *p*-acetyl-L-phenylalanine at Glu 317 of *E. coli* LeuRS.

The unique presence of a keto group in *p*-acetyl-L-phenylalanine facilitated the conjugation of a hydrazine-based cyanine dye to LeuRS. We were successful in expressing LeuRS with *p*-acetyl-L-phenylalanine, but at insufficiently high levels for us to label it efficiently. Insufficient amounts at less than 20 μM of protein in 50 μL volume were obtained. In addition, LeuRS with the *p*-acetyl-L-phenylalanine charged *E. coli* $tRNA^{Leu}$ poorly (Figure V.5).

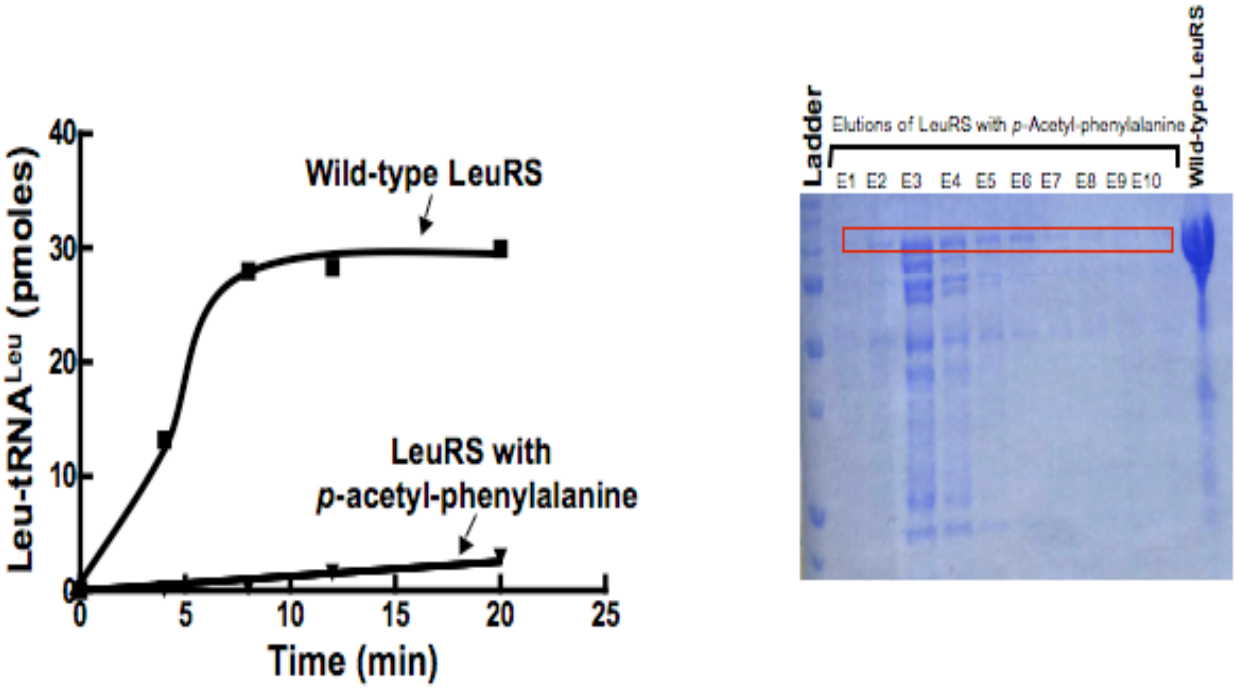


Figure V.5 Expression of LeuRS with *p*-acetyl-L-phenylalanine inserted at stop codon and aminoacylation with modified enzyme. On the left, aminoacylation reactions containing 4 μM tRNA, 4 mM ATP, 20 μM leucine or *p*-acetyl-L-phenylalanine, and 100 nM enzyme. The 10% SDS-PAGE gel of the elutions of LeuRS containing *p*-acetyl-L-phenylalanine is shown on the right. The protein of interest is boxed in red and E1 represents fraction 1 from the elutions; E2, fraction 2 from the elutions, and so forth.

In order to boost the labeling efficiency of protein, I then turned to labeling a partial cysteine knockout protein in which Cys 37, Cys 128, Cys 505, and Cys 565 had been mutated to valine, serine, serine, and threonine respectively. These cysteines were chosen based on the

analysis of the *T. thermophilus* crystal structure (PDB: 2BTE) with Visual Molecular Dynamics (VMD). These four cysteines were less than 3.5 Å from the surface, and were predicted to be accessible for labeling. The Glu 317 residue in the CP1 domain was mutated to a cysteine that was subsequently labeled with cy5 dye as described in the materials and methods section of this chapter. This method of labeling proved to be more successful as I was able to express approximately 130 μM of protein in a total volume of 100 μL. The labeling efficiency was calculated to be about 70%. Photobleaching analysis was carried out with the labeled LeuRS by subjecting the labeled species to continuous laser excitation. Approximately 50% of the LeuRS had just one photobleaching step, indicating that it was exactly labeled with one dye. I then carried out smFRET experiments with this labeled LeuRS.

The labeling of tRNA was initially carried out by the splint ligation of a tRNA comprising the modified nucleotide 5-C6-Amino-2′-deoxythymidine on which a NHS-cy3 could be conjugated. The first RNA fragment consisted of a chemically synthesized quarter length fragment of tRNA and harbored the modified nucleotide. The second fragment was a transcribed piece of tRNA that was ¾ of the full length *E. coli* $tRNA^{Leu}$. A DNA splint that was 15 nucleotides long was used to hold these two fragments together. The two RNA pieces were ligated together using T4 RNA ligase as described in the materials and methods section of this chapter. Unfortunately, this method was very inefficient and less than 20 μM of ligated full length tRNA in a 50 μL volume was obtained. The concentration of the modified full length tRNA was too low to be labeled. Eventually, labeled tRNA was obtained commercially from ChemGenes (Wilmington, MA) and this synthesized tRNA had a cy3 dye inserted between guanosines 28 and 29 in the anticodon stem (Figure V.6).

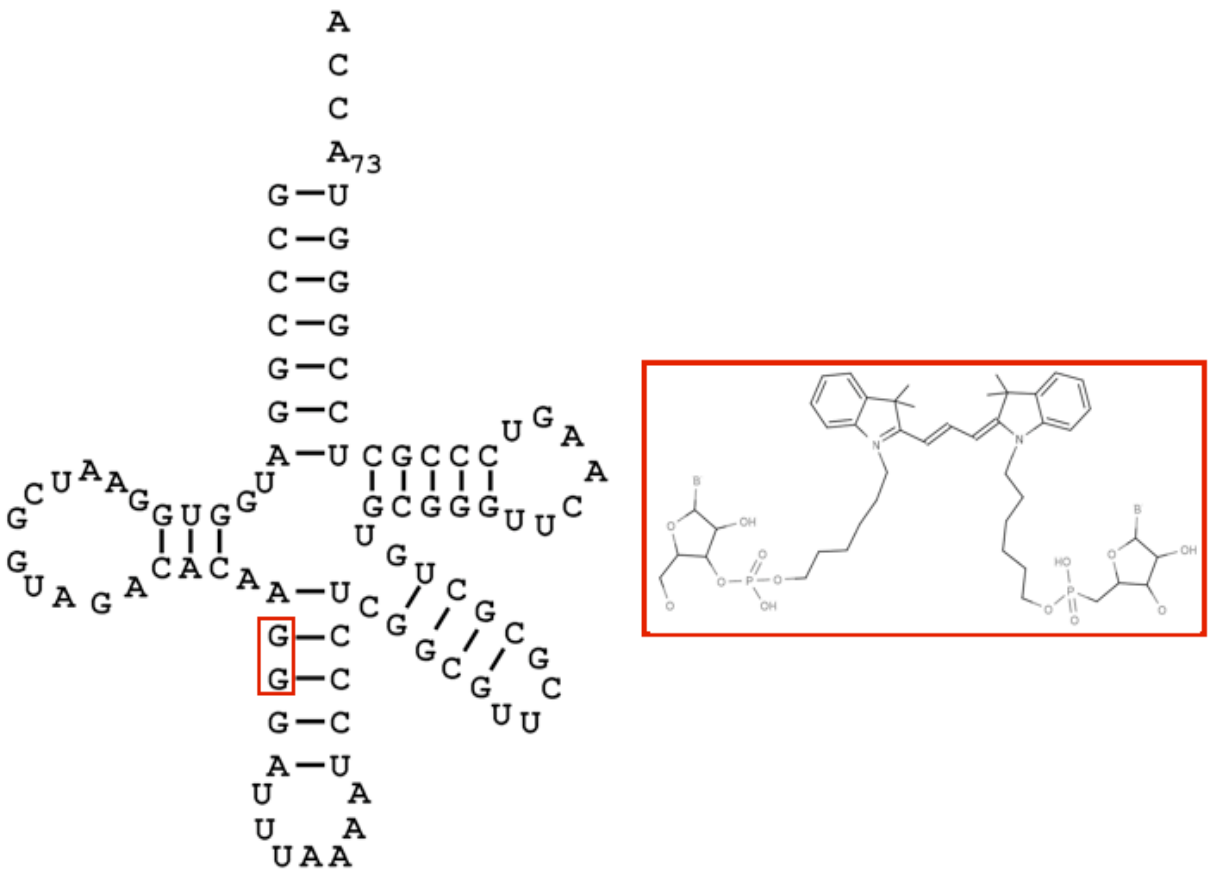


Figure V.6 Schematic of tRNA labeled with cy3. Labeled tRNA was chemically synthesized with a cy3 dye inserted between guanosines 28 and 29 in the anticodon stem boxed in red. The structure in the red box on the right shows the detailed structure of the dye between guanosine 28 and 29.

I carried out smFRET experiments with cy3 labeled tRNA as well as cy5 labeled LeuRS using Dr. Taekjip Ha's TIR microscope at the University of Illinois at Urbana-Champaign. The protein was efficiently immobilized on a quartz slide with approximately 500 fluorescently labeled single molecules conjugated to the slide using 20 nM histidine antibody specific for the his-tag on the protein (Figure V.7). Various concentrations of labeled LeuRS ranging from 2 nM to 20 nM and labeled tRNA ranging from 2 nM to 15 nM were tested. Finally, a concentration of 10 nM LeuRS and 4 nM tRNA was found to be optimal and used for all experiments.

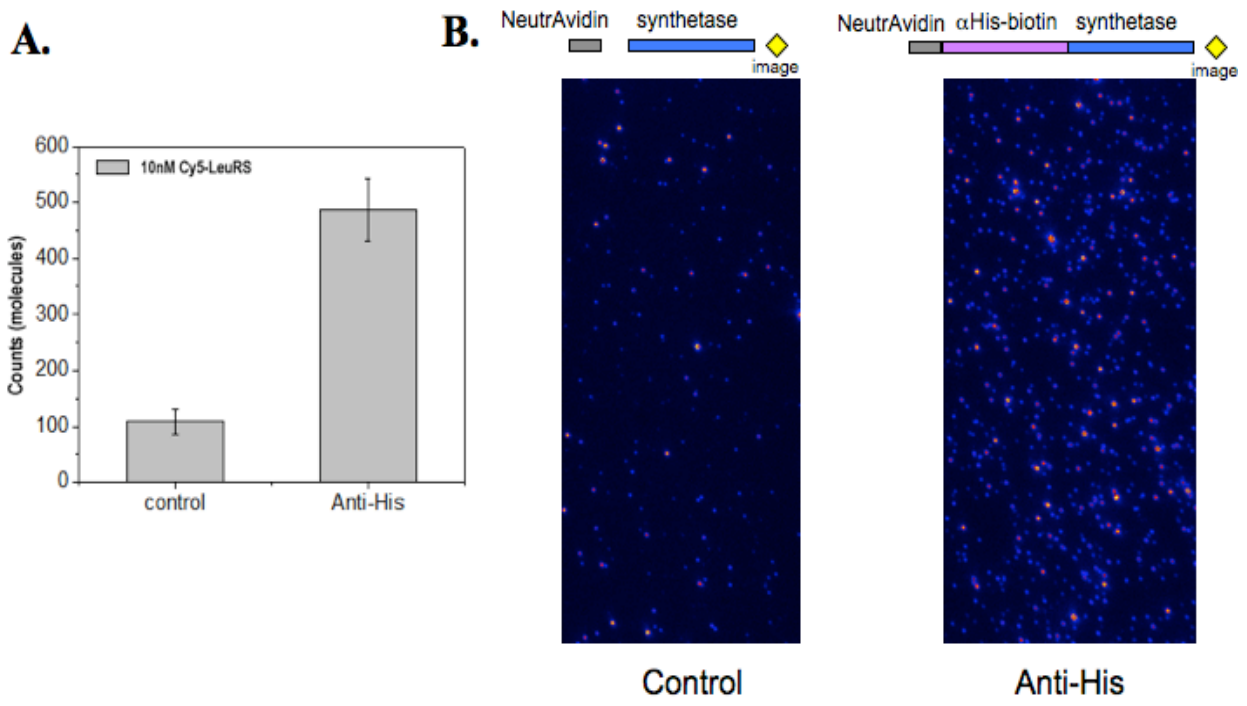


Figure V.7 Surface immobilization of histidine-tagged LeuRS on quartz slide. A. The histidine antibody is efficient in immobilizing labeled mutant his-tagged LeuRS (with 4 surface cysteines knocked out) on the surface of the slide. B. Picture of slide as seen from the TIR microscope. Each blue dot represents a single fluorescently labeled single molecule. Bars on top describe conjugation of LeuRS to the slide.

Control experiments were conducted with AN2690 to trap cy3-tRNA on cy5-LeuRS (Rock et al. 2007). As discussed previously in Chapter I.7, the boron containing compound AN2690 traps tRNA in the CP1 domain of LeuRS by forming an oxaborole adduct between the *cis* diols of the ribose (Rock et al. 2007). When this occurs, the donor dye on the tRNA and the acceptor dye on the LeuRS CP1 domain are close enough in proximity for FRET to occur, and I hypothesized there should be an instance of medium FRET. When the AN2690 comes off the complex, then tRNA will subsequently dissociate from the LeuRS and acceptor fluorescence will drop since the donor as well as acceptor dyes will be far apart. I incubated 10 mM of AN2690, 4 nM cy5-tRNA and 10 nM of cy3-LeuRS at 37 °C for 1 h. This complex was immobilized on the slide and a fairly strong acceptor signal was observed, indicative of energy

transfer between the donor and acceptor (Figure V.8). This suggested that both the LeuRS and tRNA were successfully labeled with fluorescent dye and that fluorescence from the donor dye on the tRNA was transferred to the acceptor dye on the LeuRS.

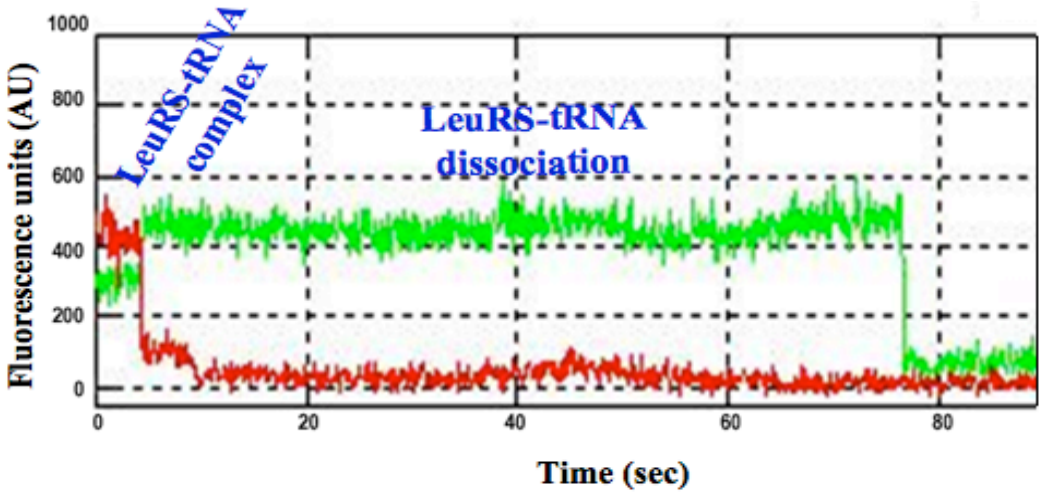


Figure V.8 The LeuRS-tRNA complex held together by AN2690. Here, smFRET experiments were carried out with 10 nM LeuRS, 4 nM tRNA and 10 mM AN2690. The red line represents acceptor intensity and the green line donor intensity. When the LeuRS and tRNA form a complex, acceptor fluorescence (red) is high but when the complex dissociates, acceptor fluorescence decreases.

Comparison of crystal structures of LeuRS suggests that the CP1 domain rotates during aminoacylation (Tukalo et al. 2005). I hypothesized that the CP1 domain moves towards the canonical core in order to facilitate the transfer of tRNA. A series of smFRET experiments were carried out with either labeled wild-type or T252A LeuRS in the presence of tRNA, ATP and either leucine or isoleucine to further elucidate the interaction of the CP1 domain with the canonical core during the translocation of tRNA. I first attempted to observe smFRET with cy5 labeled wild-type LeuRS and cy3-tRNA in the presence of ATP and leucine. I hypothesized that an instance of high FRET would be observed when the CP1 domain swings toward the canonical core in order to facilitate the transfer of the 3′ end of tRNA moving from the

canonical core to the CP1 domain. However, very rare instances of smFRET was observed, with only approximately 1 in 600 observed single molecules displaying high acceptor fluorescence. I attributed this to the high k_{cat} of 10 s^{-1} for the aminoacylation reaction, thus resulting in the inability to capture the transient nature of translocation. I then repeated the same experiment, but this time with cy5 labeled wild-type LeuRS and cy3-tRNA in the presence of ATP and isoleucine. I reasoned that mischarging with isoleucine would slow down the aminoacylation reaction and there would be a higher possibility of capturing the transient conformational change of the LeuRS during translocation. Unfortunately, this proved to be unsuccessful too and very rare instances of high acceptor fluorescence was observed.

Finally, I decided to insert the T252A mutation into the labeled wild-type LeuRS. The threonine has been shown to function as a specificity determinant and causes LeuRS to mistakenly cleave correctly charged tRNA when this threonine is mutated to an alanine (Mursinna et al. 2001). I hypothesized that the T252A mutant would result in a futile cycle of aminoacylation, whereby charged tRNA is constantly translocated to the CP1 domain for hydrolysis, thus availing uncharged tRNA for subsequent rounds of charging. I reasoned that this would increase the possibility of capturing the translocation event via smFRET. This also did not increase the observed occurrence of high FRET as compared to conditions tried previously with wild-type labeled LeuRS in the presence of leucine. Overall, the acceptor signal I obtained from the various smFRET experiments was not significantly higher than the noise reading, hence too low for further data processing (Figure V.9). In order to ensure the success of future smFRET experiments, further optimization is needed to obtain a stronger acceptor signal. Strategies to label the dye at different locations on the protein using improved labeling techniques can potentially increase the acceptor fluorescence signal.

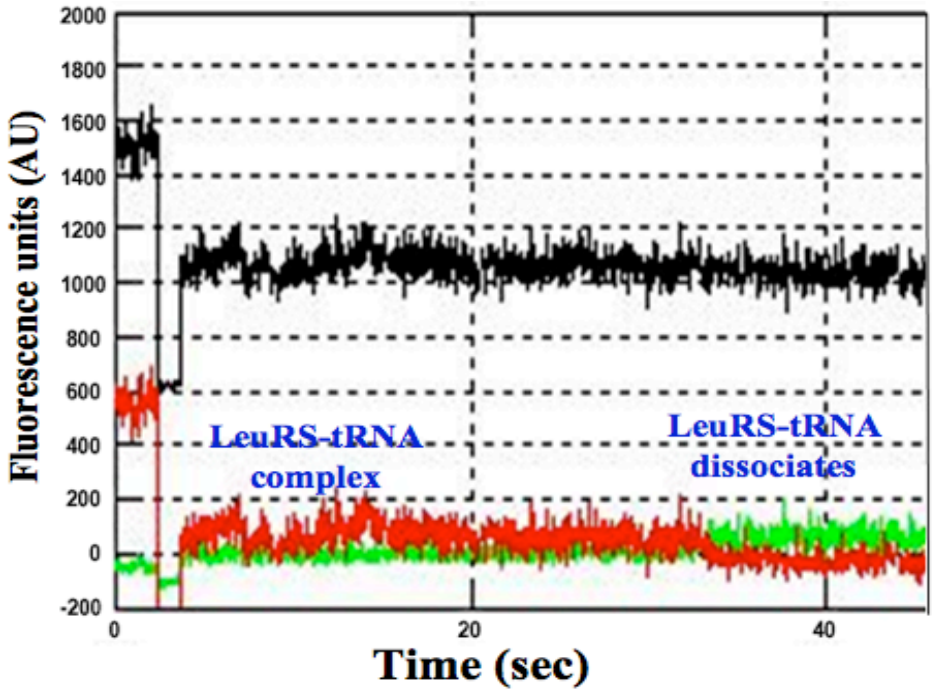


Figure V.9 Graph of fluorescence intensities with labeled LeuRS and tRNA. Here, smFRET experiments were carried out with 10 nM LeuRS, 4 nM tRNA and 1 μM ATP. The red line represents acceptor intensity and the green line donor intensity. When the LeuRS and tRNA are in complex, acceptor fluorescence is high but when the complex dissociates, acceptor fluorescence decreases. The black line represents total fluorescence.

V.4. Discussion

The aminoacylation and editing active site of LeuRS are separated by about 30 Å (Tukalo et al. 2005) requiring the 3′ end of tRNA to move a significant distance in order for editing to occur. I hypothesized that during this process, the canonical core and the CP1 domain are frequently interacting with each other at the molecular level in order to facilitate the transfer of tRNA. However, the translocation of tRNA is very transient and difficult to study via structural and biochemical analysis. Herein, I employed biophysical methods such as immunoprecipitation assays and smFRET techniques to elucidate the molecular crosstalk between the aminoacylation core and CP1 domain that facilitate the transfer of tRNA.

It was previously shown that two regions encompassing Lys 186 on the canonical core and Ala 293 on the CP1 domain *in E. coli* LeuRS, could stimulate pre-transfer editing via an unknown mechanism (Williams and Martinis 2006). This was further supported by data indicating that Lys 186 acts as a molecular hinge in IleRS to enable editing (Bishop et al. 2003). Immunoprecipitation pull-down assays were conducted to test if isolated constructs of the CP1 and canonical core could interact with each other. Due to potentially weak and transient interactions of the main body with the CP1 domain, I was unable to reproducibly show that these two domains were interacting. Interestingly, using the cell lysates of proteins for pull-down assays enhanced interaction between the separate domains, leading us to believe that an unknown component of the lysate was essential for inter-domain interaction to occur. In addition, K186E and A293D mutations were made in each of these constructs in order to promote interaction between both domains via the formation of salt bridges (Williams and Martinis 2006). However, this proved unsuccessful, again possibly due to weak and fleeting interactions between both domains.

Finally, smFRET techniques were employed to dynamically study the transient interactions between the main body and CP1 domain of *E. coli* LeuRS during the translocation of tRNA. These experiments were designed based on our hypothesis that the CP1 domain would swing towards the canonical core in order to facilitate the transfer of tRNA. A cy3 dye was inserted in the anticodon stem of tRNA while a cy5 dye was added at Cys 317 (previously Glu 317) of the *E. coli* LeuRS CP1 domain. During this event, I hypothesized there would be an occurrence of high FRET due to the close proximity of the donor dye on the tRNA anticodon stem and the acceptor dye on the CP1 domain. This approach also proved difficult because of the transient nature of translocation due to the high k_{cat} of 10 s^{-1} for aminoacylation. The high

K_D of 0.3 μM for tRNA binding to LeuRS also increased the difficulty of this experiment as only nanomolar concentrations of labeled LeuRS and tRNA could be used for smFRET experiments. In order to overcome the problem of the high k_{cat} of aminoacylation, an attempt was made to create a futile charging event, resulting in a continuous translocation cycle of tRNA from the aminoacylation core to the editing domain. This was carried out using the T252A LeuRS mutant, which hydrolytically cleaves correctly charged tRNA in the editing pocket, before releasing free tRNA to be charged again (Mursinna et al. 2001). In addition to this, we explored the possibility of lowering the k_{cat} of aminoacylation by substituting ATP in our smFRET experiments with ATPcPP, which is a non-hydrolysable ATP molecule with a carbon instead of oxygen linker between the α and β phosphate moieties. Finally, I attempted to carry out mischarging of tRNA with isoleucine in order to slow down aminoacylation enough to capture the translocation event with smFRET. All these optimized conditions failed to produce strong smFRET traces as acceptor fluorescence was not significantly higher than the background signal. On hindsight, one possibility to improve the acceptor signal in our smFRET experiments is to re-label the protein, perhaps at a different location. I can move the acceptor dye on the CP1 domain to the LSD (leucine specific domain), which has been shown by crystallographic structural studies to undergo larger movements relative to the CP1 domain during the aminoacylation process (Personal communication with Dr. Andrés Palencia).

In our current method of cysteine-maleimide labeling (Mujumdar et al. 1993), the protein still has to retain cysteines associated with the zinc binding domain in order for LeuRS to maintain activity. This has resulted in multiple labels being conjugated to a proportion of proteins due to the presence of more than one cysteine. With a more efficient labeling protocol in place, a more accurate and robust signal will be obtained from smFRET experiments. One

strategy of re-labeling the protein is to use recently developed snap-tag technology that can specifically label proteins with fluorophores (Keppler et al. 2003; Keppler et al. 2004). A snap-tag is two-thirds the size of a green fluorescent protein (GFP) molecule and originates from the O^6-guanine nucleotide alkyltransferase that covalently reacts with any fluorophore carried on the benzyl-group of benzyl-guanines. This snap-tag can be inserted at the backside of the CP1 domain in the vicinity of Glu 317. A second alternative for labeling a protein is via a lanthanide tag, which is a short peptide sequence consisting of 15-20 amino acids that can bind Tb(III) which has luminescent properties (Sculimbrene and Imperiali 2006). This approach will circumvent the problem of existing cysteines that are essential for the overall activity of the protein. Finally, the protein can be labeled via an orthogonal system developed by Peter Schultz's lab. This will allow for the insertion of a fluorescent amino acid such as L-(7hydroxycoumarin-4-yl)ethylglycine in the protein at an amber stop codon TAG (Wang et al. 2006). This is a more efficient orthogonal insertion method than the one I attempted before as it avoids the need to label an amino acid with dye as it already has fluorescent properties.

Another likelihood for the lack of success with smFRET is due to the protein losing its rotational flexibility while anchored to the quartz slide via its histidine tag. In order to overcome this, the LeuRS can be immobilized on nanodiscs, which are nano–sized phospholipids bilayers (Bayburt and Sligar 2009). Some cyanobacterial class I aaRSs have been found to associate with the cell membrane via a cyanobacterial aaRS appended domain (CAAD) (Luque et al. 2008). The CAAD contains transmembrane helices that mediate its association with the plasma membrane (Luque et al. 2008). It is possible that cloning a CAAD domain into *E. coli* LeuRS will first facilitate its localization to the phosphobilipids on the

nanodisc. The approach will enable the LeuRS to be tethered to a phosphobilipid layer, allowing it to remain in its natural conformation, hence retaining its rotational flexibility.

There is strong evidence for the interaction of the LeuRS canonical core and CP1 domain in order to facilitate translocation, which is important for the maintenance of fidelity and viability (Tukalo et al. 2005; Williams and Martinis 2006; Hellmann and Martinis 2009; Mascarenhas and Martinis 2009). I have conducted immunoprecipitation pull-down assays as well as smFRET experiments to study transient interdomain interactions during translocation, but was unable to clearly elucidate this process. Further development of the smFRET assay via techniques discussed above is needed to fully understand this transient event.

Chapter VI. Human Cytoplasmic LeuRS Involvement in Cell Signaling

VI.1. Introduction

The aaRSs are a versatile group of enzymes that are involved in aminoacylation as well as a host of signaling activities (Park et al. 2005). Aminoacylation itself is a two-part reaction mechanism that requires the aaRS to recognize nucleotide-based structures such as tRNA, as well as other small molecules such as amino acids and ATP (Mascarenhas et al. 2008). Thus, the aaRSs have evolved to interact with a diverse set of molecules allowing them great functional versatility. Sometimes, aaRSs even generate small molecules that go on to be involved in signaling activity. For example, the GlyRS, LysRS, and TrpRS are able to synthesize diadenosine polyphosphates (Ap_nA), which are involved in a host of signaling functions (Edgecombe et al. 1997; Nishimura et al. 1997).

As discussed in the previous chapters, aaRSs from higher eukaryotes such as humans form a macromolecular complex in the cell. It has been suggested that the aaRSs form a macromolecular complex in order to channel charged tRNA from the complex to the ribosome for translation to occur (Kyriacou and Deutscher 2008). Furthermore, the aaRS macromolecular complex is proposed to act as a depot for additional regulatory factors that the aaRSs may need to carry out alternate functions within the cell (Ray et al. 2007). Interestingly, many of the human aaRSs also have additional appended domains on them that can facilitate their interactions with other regulatory factors (Jia et al. 2008). Nevertheless, the aaRS can still be found in various subcellular locations either in its free form or in complex with other aaRSs, depending on the function it has to carry out (Wakasugi and Schimmel 1999; Park et al. 2005). Considering the expanded functionality of the aaRSs especially in terms of cell signaling

(Park et al. 2005), it is not surprising that they have been linked to many diseases (Park et al. 2008).

It has been shown that leucine is a critical factor for the activation of the mammalian target of rapamycin (mTOR) pathway (Lynch 2001). The mTOR pathway serves as a central regulator of cell metabolism, proliferation, growth, and ultimately survival by integrating both extracellular and intracellular signals (Laplante and Sabatini 2009). Although several factors mediate the leucine-induced mTOR activation (Bolster et al. 2004), they do not function as direct leucine sensors in the cell to monitor levels of free leucine available. Based on this, it is hypothesized that human cytoplasmic LeuRS functions as a leucine sensor in the cell to control mTOR activation (Personal communication with Dr Jung Ming Han, Seoul National University). Specifically, we hypothesized that hscLeuRS mediates mTOR directed translational control via hscLeuRS's non-catalytic activity. In collaboration with Dr. Sung Hoon Kim's lab at Seoul National University, mutations were made in the hscLeuRS to knock out its aminoacylation activity. In particular, we wanted to create mutants that would not bind and activate leucine efficiently, resulting in a lack of aminoacylation activity. Secondly, it has been shown previously that the *E. coli* LysRS generates Ap_4A during aminoacylation (Brevet et al. 1982), which could then go on to perform a host of signaling activities (Lee et al. 2004). Based on this prior information, we conducted experiments to confirm that hscLeuRS does not operate similarly via a mechanism that generates Ap_4A, that might then go on to affect mTOR signaling in the cell.

VI.2. Experimental Procedures

VI.2.1. Mutagenesis

A series of mutations were made within the editing pocket of hscLeuRS in order to severely disrupt leucine binding and activation. Site directed mutagenesis was performed via PCR using the template plasmid pHSLARS1 that encoded the wild-type hscLeuRS gene. The plasmid pHSLARS1 was a gift from Weimin Mao and MRK Alley (Anacor Pharmaceuticals; Palo Alto, CA). The F50A hscLeuRS mutant (pJPF50A) was generated using primers F50A-Fwd (5'-GTA TTT TGT AAC CGC CCC ATA TCC ATA TAT G-3') and F50A-Rev (5'-CAT ATA TGG ATA TGG GGC GGT TAC AAA ATA C-3'). The Y52A hscLeuRS mutant (pJPY52A) was generated using primers Y52A-Fwd (5'-GTA ACC TTC CCA GCT CCA TAT ATG AAT G-3') and Y52A-Rev (5'-CAT TCA TAT ATG GAG CTG GGA AGG TTA C-3'). The F50P hscLeuRS mutant (pJPF50P) was generated using primers F50P-Fwd (5'- GGG CAA GTA TTT TGT AAC CCC ACC ATA TCC ATA -3') and F50P-Rev (5'- TAT GGA TAT GGT GGG GTT ACA AAA TAC TTG CCC-3'). The F50H hscLeuRS mutant (pJPF50H) was generated using primers F50H-Fwd (5'-GGG CAA GTA TTT TGT AAC CCA CCC ATA TCC ATA TAT G-3') and F50H-Rev (5'-CAT ATA TGG ATA TGG GTG GGT TAC AAA ATA CTT GCC C-3'). The F50Y hscLeuRS mutant (pJPF50Y) was generated using primers F50Y-Fwd (5'-GGG CAA GTA TTT TGT AAC CTA CCC ATA TCC ATA TAT G-3') and F50Y-Rev (5'-CAT ATA TGG ATA TGG GTA GGT TAC AAA ATA CTT GCC C-3'). The F50W hscLeuRS mutant (pJPF50W) was generated using primers F50W-Fwd (5'-GGG CAA GTA TTT TGT AAC CTG GCC ATA TCC ATA TAT G-3') and F50W-Rev (5'-CAT ATA TGG ATA TGG CCA GGT TAC AAA ATA CTT GCC C-3'). Finally, the F50AY52A hscLeuRS mutant (pJPF50AY52A) was generated using primers F50AY52A-Fwd (5'-TTT GTA ACC

GCC CCA GCT CCA TAT ATG-3') and F50AY52A-Rev (5'-CAT ATA TGG AGC TGG GGC GGT TAC AAA-3'). Each 50 μL PCR mixture contained 50 ng of DNA template, 100 ng each of forward and reverse primer, 200 μM dNTPs, and 2.5 units of *Pfu* DNA polymerase in commercial buffer. Each PCR reaction was carried out using conditions as described in Chapter II.

VI.2.2. Detection of Ap_4A Formation during Aminoacylation

The synthesis of Ap_4A by *E. coli* LysRS was monitored at 37 °C in buffer containing 20 mM Tris, pH 7.8, 150 mM KCl, 0.1 mM lysine, 5 mM $MgCl_2$, 5 mM ATP, 1 mM DTT and 100 μM $ZnCl_2$. Reactions were initiated with 1 μM human cytoplasmic LysRS (hscLysRS). (The purified enzyme was a gift from Dr. Karin Musier-Forsyth, Ohio State University, Columbus, OH). A 20 μL aliquot was quenched into EDTA after 20 min (Brevet et al. 1982). The sample was then analyzed on a 6300 Series Ion Trap liquid chromatography/mass spectrometry (LC/MS) (Agilent Technologies, Foster City, CA). The column of choice was a 100 mm x 4.6 mm porous graphitic carbon (PGC) Thermo Hypercarb column (Thermo Fischer, Waltham, MA). The sample was separated on the PGC Hypercarb column using 50 mM ammonium acetate as the aqueous phase (A) and acetonitrile as the organic mobile phase (B) on the high performance liquid chromatographer (HPLC). The sample was purified using a linear gradient that increased from 10 % to 70 % B in 12 min. After HPLC purification, the sample was run through the 6300 Series Ion Trap equipped with an electrospray ionization (ESI) interface and was run in both the positive as well as negative mode. The experiments were conducted using a full-scan mode with a mass range *m/z* of 0-900 (Xing et al. 2004).

VI.3. Results

VI.3.1. Rationale for Mutations

Mutations were made in hscLeuRS with the goal of abolishing leucine binding and activation activity. We targeted the aminoacylation core in hscLeuRS, making mutants F50A, Y52A, F50P, F50H, F50Y, F50W, and F50AY52A. These residues were chosen because of their close proximity to the HMGH peptide in the enzyme, which plays an important role in amino acid activation (Figure VI.1) (Webster et al. 1984).

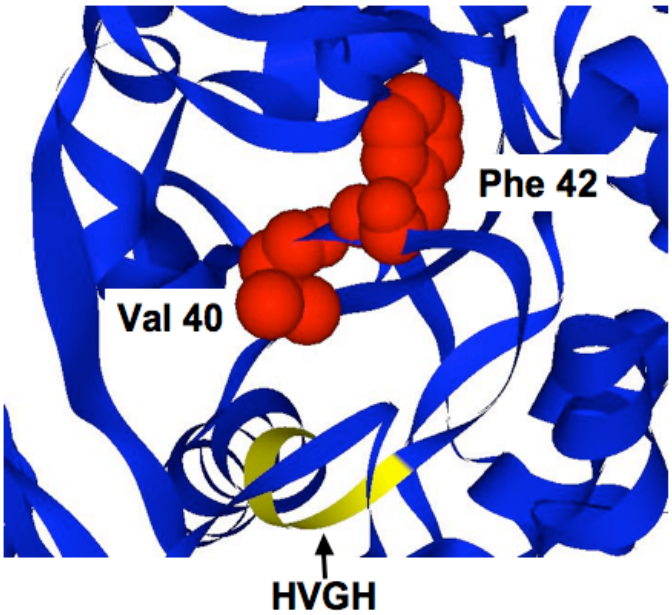


Figure VI.1 Crystal structure of the aminoacylation pocket of *Pyrococcus horikoshii* (*P. horikoshii*) LeuRS. The aminoacylation pocket of *P. horikoshii* is shaded in blue (PDB: 1WZ2). Highlighted in red are residues Val 40 and Phe 42 that are equivalent to Phe 50 and Tyr 52 in the hscLeuRS. The HVGH peptide is shaded in yellow.

I tested these mutants for aminoacylation activity as well as for amino acid activation activity. The mutants all had dramatically decreased aminoacylation activity as compared to the wild-type hscLeuRS (Figure VI.2). The kinetics for amino acid activation was determined by PP_i exchange assays as described in the experimental procedures section in Chapter III (Table VI.1).

The mutants mostly had a lower k_{cat} / K_M value compared to wild-type due to an increased K_M effect for amino acid activation. Interestingly, the F50H mutant had a 15-fold increase in the k_{cat} value for amino acid activation over wild-type hscLeuRS, possibly due to the positive charge on histidine, although the reason why is still largely unclear to us.

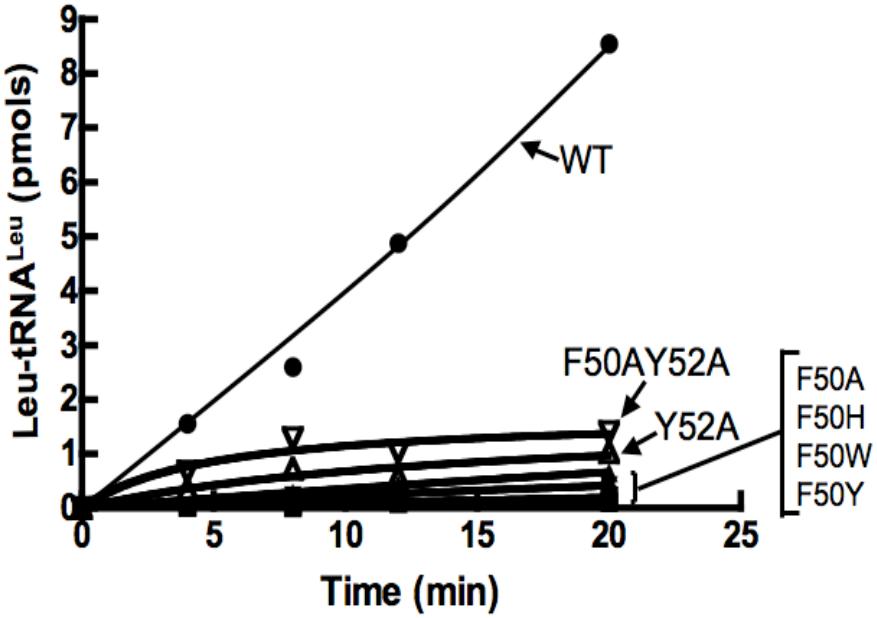


Figure VI.2 Mutants of hscLeuRS have diminished aminoacylation activity. Leucylation activity was measured with 500 nM enzyme and 4 μM of transcribed *E. coli* tRNA. All enzyme reactions were carried out at 30 °C. Wild type (WT) and mutant proteins are represented by the following symbols: WT, (●); F50AY52A, (▽); Y52A, (△); F50A, (▲); F50H, (▼); F50W, (◆); and F50Y, (■).

Table VI.1 Apparent kinetic parameters for leucine activation

Enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($s^{-1}mM^{-1}$)
WT	0.024 ± 0.0089	2.09 ± 0.80	94.3 ± 23.4
F50A	1.68 ± 0.47	0.017 ± 0.006	0.0056 ± 0.0002
Y52A	1.03 ± 0.11	3.11 ± 0.44	3.41 ± 0.37
F50AY52A	0.60 ± 0.09	0.30 ± 0.02	0.53 ± 0.05
F50P	1.36 ± 0.34	2.04 ± 0.17	1.29 ± 0.11
F50H	0.34 ± 0.01	32.3 ± 5.4	101.6 ± 16.9
F50Y	‡	‡	‡
F50W	0.25 ± 0.04	0.55 ± 0.03	2.83 ± 0.05

‡ Not determined because of low activity

VI.3.2. The hscLeuRS Does Not Generate Ap_4A

We carried out separate aminoacylation assays with hscLeuRS as well as hscLysRS to test for the generation of Ap_4A. The aminoacylation reactions were quenched as described in the experimental procedures section and quantitated based on LC/MS analysis. The LC/MS spectrum from the hscLysRS aminoacylation reaction yielded a strong peak corresponding to Ap_4A (Figure VI.3). A much smaller peak was found at a 200 times lower intensity in the spectrum from the hscLeuRS sample (Figure VI.3), which we assumed to be due to background noise. This indicated to us that the hscLeuRS, unlike the hscLysRS, could not generate Ap_4A during aminoacylation.

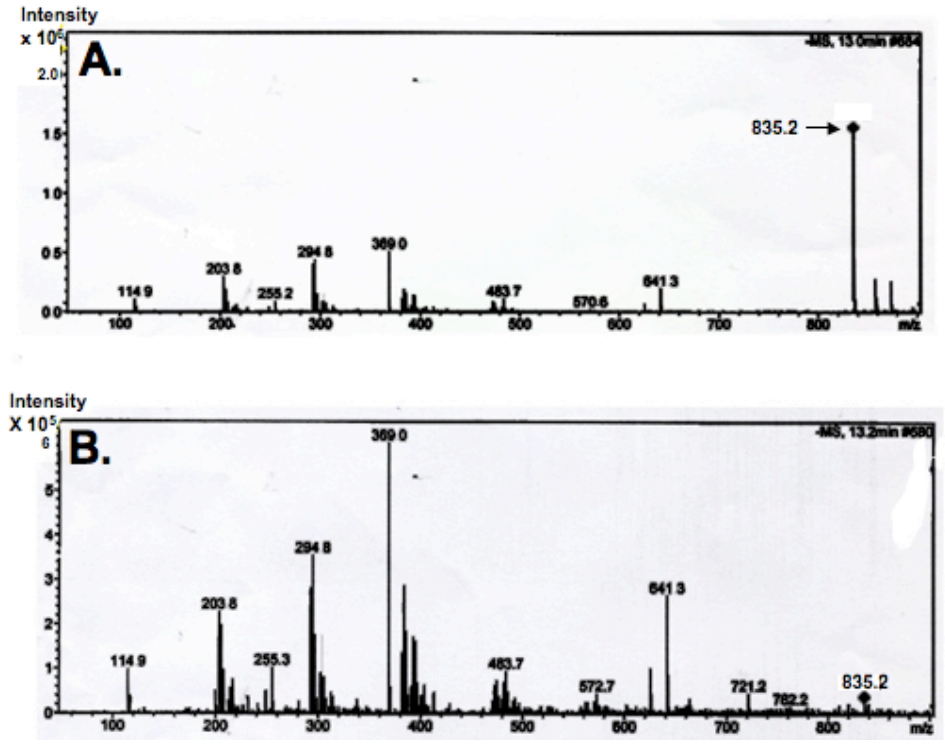


Figure VI.3 Mass spectrometric analysis reveals Ap_4A formation by hscLysRS but not hscLeuRS. A. The products from the aminoacylation reaction with LysRS run in the negative mode using ESI reveal a peak with *m/z* of 835.2 corresponding to Ap_4A. B. The products from the aminoacylation reaction with LeuRS run in the negative mode using ESI reveal a peak with *m/z* of 835.2 but at 200 times lower intensity than with hscLysRS.

VI.4. Discussion

The goal of our collaboration with the Kim lab at Seoul National University was to generate mutants in the hscLeuRS that would lack aminoacylation activity due to a deficiency in leucine binding and activation. I made seven mutants, mostly targeting a phenylalanine at position 50 in the aminoacylation pocket of hscLeuRS. This particular phenylalanine was chosen because it is located close to the HMGH peptide in the enzyme, which plays an important role in amino acid activation (Figure VI.1) (Webster et al. 1984). We reasoned that residues in proximity of the HMGH peptide would also be critical for amino acid activation to take place. As hypothesized, these mutants were found to have very low aminoacylation activity. They also had lowered k_{cat} / K_M values for leucine activation compared to the wild-type hscLeuRS. The F50H was an exception in that it had similar k_{cat} / K_M values for leucine activation compared to wild-type due to reasons that remain unclear. The Kim lab has continued to work with these mutants and will use them to further elucidate the role of hscLeuRS in mTOR signaling. Finally, I determined that unlike hscLysRS, the hscLeuRS does not generate Ap_4A during aminoacylation. This confirmation was helpful as it helped us eliminate the possibility of hscLeuRS generated Ap_4A signaling during the mTOR pathway.

Chapter VII. Protection of Charged tRNA by EF-Tu

VII.1 Introduction

Accurate translation of the genetic code is reliant on the fidelity of aaRSs, as well as the precision of EF-Tu in delivering correctly charged tRNAs to the ribosome for participation in protein synthesis. The EF-Tu has the responsibility of sampling a wide array of charged and mischarged tRNAs, before determining which aminoacylated-tRNAs to commit to ribosomal protein synthesis. Mischarged tRNAs bind EF-Tu with a wide range of affinities, however, correctly charged tRNAs bind with similar affinities (Dale and Uhlenbeck 2005; Sanderson and Uhlenbeck 2007; Sanderson and Uhlenbeck 2007). The overall binding affinity of EF-Tu for the aminoacylated tRNA is dependent on the sum of the individual thermodynamic contributions of the amino aid as well as the tRNA body (LaRiviere et al. 2001).

Some organisms have adapted to accommodate mischarged tRNAs in cases that involve indirect aminoacylation pathways (Ibba and Söll 2000). For example, many bacteria as well as archaea lack GlnRS and AsnRS, and generate a mischarged intermediate with GluRS and AspRS respectively that is later converted to correctly charged tRNA by transamidating enzymes (Wilcox and Nirenberg 1968; Ibba et al. 1997). In order to prevent the misreading of specific codons under these conditions, EF-Tu has evolved to have low binding affinity for certain essential misacylated tRNAs such as Glu-tRNA^{Gln}, Asp-tRNA^{Asn}, and Ser-tRNA^{Sec} that are part of important pathways (Stanzel et al. 1994; Becker and Kern 1998; Ambrogelly et al. 2007; Roy et al. 2007).

Nevertheless, EF-Tu is not error-proof and on occasion allows translation of mischarged tRNAs, as seen from the incorporation of noncognate amino acids *in vivo* during translation (Apostol et al. 1997; Döring et al. 2001; Tang and Tirrell 2002; Roy et al. 2004;

Wang et al. 2006). For example, *E. coli* LeuRS has been shown to misactivate multiple amino acids such as norvaline *in vivo* such that it is incorporated throughout the proteome (Apostol et al. 1997). Recent research has also shown that in order to increase the accuracy of protein synthesis, EF-Tu•GTP can dissociate from bound aminoacylated tRNA (Ling et al. 2009). If the tRNA is misaminoacylated, then this mischarged species can rebind to the aaRS and be resampled by its editing domain. This provides an additional proofreading step that can result in an increase in quality control (Ling et al. 2009).

It has been proposed that class I aaRSs are rate limited by product release and that EF-Tu forms a ternary complex with tRNA on the aaRS to facilitate its release (Zhang et al. 2006). In the context of *E. coli* LeuRS, I hypothesize that both charged and mischarged $tRNA^{Leu}$ are translocated from the aminoacylation active site to the CP1 domain before product release to EF-Tu. This is supported by data from our lab showing that a single T252A mutation in LeuRS uncouples specificity in the editing active site and allows hydrolysis of the correctly charged Leu-$tRNA^{Leu}$ (Mursinna et al. 2001) (Figure VII.1). I designed experiments with the aim of kinetically isolating the translocation of charged tRNA from the aminoacylation core to the editing active site.

I conducted *in vitro* aminoacylation competition assays with the T252A *E. coli* mutant LeuRS and EF-Tu. I hypothesized that the EF-Tu would compete with the editing site of T252A LeuRS to capture correctly charged Leu-$tRNA^{Leu}$ before translocation to the editing site, where product is released. As mentioned above, our lab has previously shown that a single T252A mutation in LeuRS uncouples specificity in the editing active site and allows hydrolysis of the correctly charged Leu-$tRNA^{Leu}$ (Mursinna et al. 2001). Hence, if the T252A LeuRS mutant releases Leu-$tRNA^{Leu}$ before binding to the CP1 domain for proofreading, then I would

expect EF-Tu to slow the apparent rate of hydrolysis of Leu-tRNA^{Leu} to accumulate correctly charged Leu-tRNA^{Leu}.

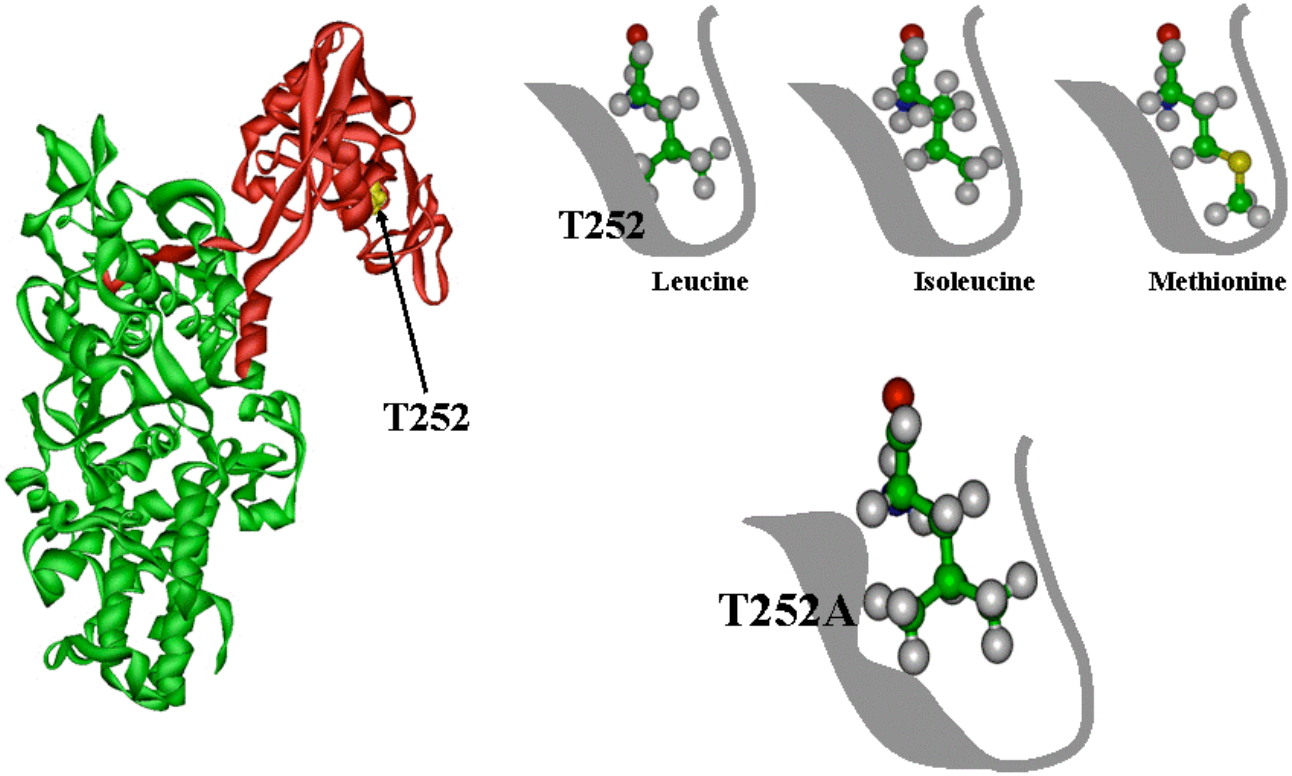


Figure VII.1 The Thr 252 residue in *E. coli* LeuRS. On the left is the homology model of wild-type LeuRS with conserved threonine at position 252 highlighted in yellow. On the right is a cartoon of the amino acid binding pocket within the editing active site, with Thr 252 ensuring that only non-cognate amino acids can bind within the hydrolytic editing pocket. Substitution of threonine with an alanine (T252A) allows for cognate leucine to bind within the pocket. This figure was partially adapted from reference (Mursinna and Martinis 2002). Reproduced with permission.

VII.2. Experimental Procedures

VII.2.1 Mutagenesis

The T252A/H537A mutant was generated using PCR mutagenesis that is described in Chapter II from plasmid pMURe10_T252A encoding the *E. coli* T252A mutant (Mursinna et al. 2001). The H537A mutant was inserted using primers H537A-Fwd (5'-CTA

ATA CGA CTC ACT ATA GTA GAC ACA AGG G-3') and H537A-Rev (5'-CCC TTG TGT CTA CTA TAG TGA GTC GTA TTA G-3') to give plasmid pJPT252A/H537A. This mutant was then used in rescue assays with charged tRNA.

VII.2.2 *E. coli* EF-Tu Expression, Activation and Use in Charged tRNA Rescue Assays

The *E. coli* EF-Tu was expressed from plasmid p20EfTu-25 (gift from Dr. Paul Schimmel, Scripps Research Institute, La Jolla, CA) using a protocol as described in Chapter II, except that the HA-I buffer was replaced with activation buffer S that contained 50 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 10 mM BME, 20 μM guanosine diphosphate (GDP), and 5% (v/v) glycerol. The EF-Tu was activated immediately before use in assays by incubating 40 μM EF-Tu•GDP with 3 mM phosphoenolpyruvate, 30 μg/mL pyruvate kinase, 10 mM DTT, 20 μM guanosine triphosphate (GTP), 20 mM $MgCl_2$, 50 mM HEPES, pH 7.0, and 0.05 M NH_4Cl at 37 °C for 3 h (Asahara and Uhlenbeck 2002).

Aminoacylation assays were carried out as described in Chapter II. The aminoacylation reactions each contained between 50 nM and 1 μM of *E. coli* LeuRS T252A/H537A mutant that had inactivated aminoacylation function as well as conferred editing for the correctly charged $tRNA^{Leu}$. In addition, 50 nM to 1 μM of *E. coli* EF-Tu was added to rescue charged tRNA from the T252A/H537A editing pocket.

VII.2.3 Pre-steady State Kinetics

Pre-steady state kinetics were measured using a rapid RQF-2 KinTek chemical quench flow apparatus (Kin-Tek, Austin, TX). Multiple turnover measurements were determined on a millisecond time scale. Each reaction contained 5 μM of enzyme in the presence or absence of 2 μM EF-Tu in buffer A containing 60 mM Tris, pH 7.5, 10 mM $MgCl_2$, and 1 mM DTT in one syringe. The other syringe contained 100 μM [^{14}C]-leucine (318 mCi/mmol), 5 mM ATP,

0.08U of PP_iase (Sigma, St. Louis, Mo), and 25 μM $tRNA^{Leu}$ also in buffer A. Aliquots of 25 μL were dispensed by the apparatus and quenched with 82 μL of 3 M ammonium acetate, pH 5.0. The collection tube of the apparatus held 10 μL of 10 % SDS to ensure the complete dissociation of enzyme from tRNA in order to quench the reaction. The total reaction volume was spotted on a filter pad that had been presoaked with 5% TCA. The pads were washed and quantitated as described in Chapter II.

The data was analyzed using GraphPad Prism 4 and the equation VII.1:

$$\text{Rate of product formation/[active site]} = A(1-e^{k_1 t}) + C(k_2 t) + B$$

where A is the amplitude of the exponential function, B is added to account for background radioactivity, C is the amplitude of the linear function, *k* is the observed rate, and t is time.

VII.3. Results

I hypothesized that EF-Tu competes with the editing site of LeuRS to capture correctly charged Leu-$tRNA^{Leu}$ before translocation to the editing site. In order to test this hypothesis, I chose to use the T252A LeuRS mutant as it was previously shown that T252A could bind correctly charged Leu-$tRNA^{Leu}$ and hydrolyze it (Mursinna et al. 2001). I conducted aminoacylation assays with T252A in the presence of EF-Tu, to assess if EF-Tu could out-compete the hydrolytic editing site and sequester correctly charged Leu-$tRNA^{Leu}$ before it bound to the T252A LeuRS editing pocket. Based on our experiments, the EF-Tu rescued Leu-$tRNA^{Leu}$ accumulation but not to wild-type levels (Figure VII.2). This indicated that the charged species was translocated to the editing domain before release to EF-Tu. Thus, I propose that EF-Tu was primarily facilitating the release of Leu-$tRNA^{Leu}$ from the editing active site.

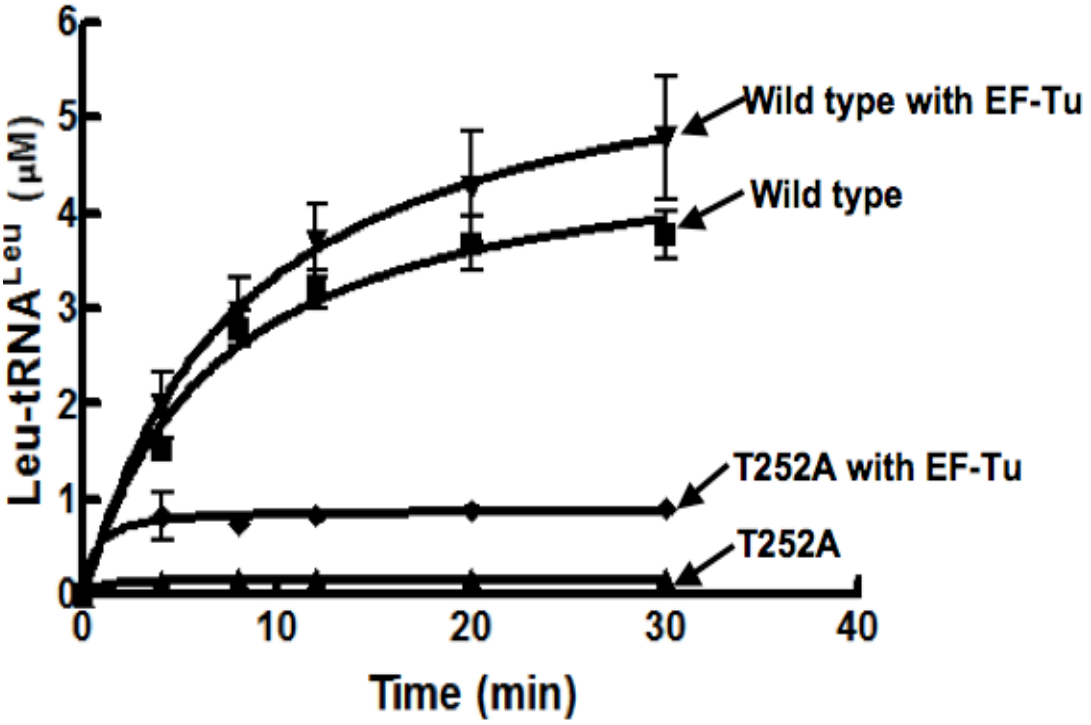


Figure VII.2 Aminoacylation competition assays with EF-Tu. Aminoacylation and rescue activity was measured with 50 nM enzyme, 4 μM transcribed *E. coli* tRNA, and 1 μM EF-Tu. Wild-type and mutant proteins are represented by the following symbols: Wild type, (■); wild type with EF-Tu, (▼); T252A, (▲); and T252A with EF-Tu, (♦). Error bars are based on the results of three reactions each and indicated for each point.

The use of the T252A/H537A mutant was chosen for this experiment because it does not aminoacylate tRNA, but binds correctly charged tRNA and hydrolyzes it within its editing site. The *E. coli* LeuRS was challenged with T252A/H537A LeuRS and EF-Tu, in order to test if EF-Tu was indeed facilitating the release of charged tRNA from LeuRS. In our assays, the EF-Tu competes with the editing site of the T252A/H537A LeuRS mutant for the correctly charged Leu-tRNA^{Leu}. If the T252A/H537A LeuRS mutant out-competes EF-Tu for Leu-tRNA^{Leu}, then the overall levels of Leu-tRNA^{Leu} would be expected to decrease. I hypothesized that EF-Tu would sequester Leu-tRNA^{Leu} from wild-type LeuRS before T252A/H537A, thus preventing a drop in Leu-tRNA^{Leu} levels. As predicted, my data showed that when wild-type LeuRS is challenged with T252A/H537A, EF-Tu rescues Leu-tRNA^{Leu}

from T252A/H537A to almost wild-type levels of Leu-tRNA^{Leu}, as in the absence of T252A/H537A LeuRS (Figure VII.3). This suggests that charged Leu-tRNA^{Leu} is indeed translocated to the CP1 editing domain first, where EF-Tu then binds correctly charged Leu-tRNA^{Leu}.

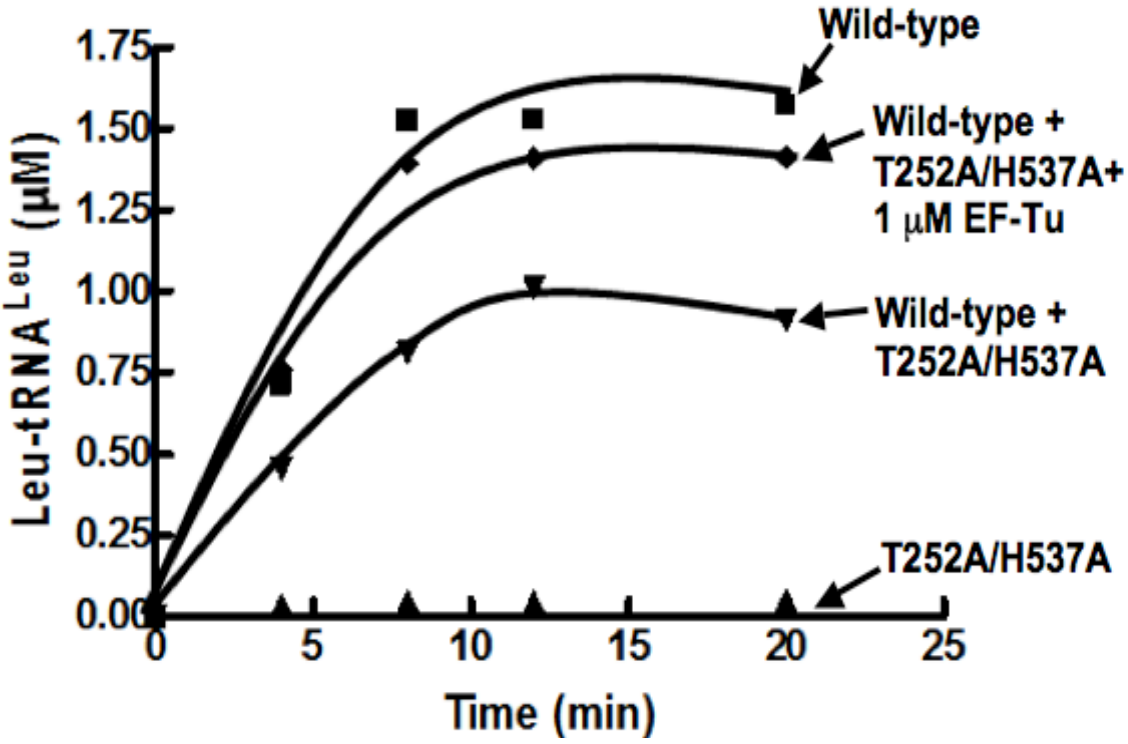


Figure VII.3 Aminoacylation competition assays with EF-Tu and T252A/H537A. Aminoacylation and rescue activity was measured with 50 nM enzyme and T252A/H537A, 4 μM transcribed *E. coli* tRNA, and 1 μM EF-Tu. Wild-type and mutant proteins are represented by the following symbols: Wild-type, (■); T252A/H537A, (▲); wild-type with T252A/H537A, (▼); and wild-type with T252A/H537A and 1 μM EF-Tu, (△).

It has been proposed that class I aaRSs are rate-limited by product release (Zhang et al. 2006). Thus, under multiple turnover conditions with limiting enzyme, a kinetic burst would be unmasked, which is when aminoacyl transfer occurs at much higher rates than steady-state kinetics (k_{cat}). Rapid-quench experiments (Fersht and Kaethner 1976) can be used

to isolate kinetic steps that represent transiently formed charged tRNA under multiple turnover conditions. This will determine if EF-Tu competing with wild-type LeuRS changes the initial kinetic burst of the enzyme.

Since rescue of aminoacylated product by EF-Tu was observed, I carried out rapid-quench kinetic experiments under multiple turnover conditions to measure changes in the rate constants. I reasoned that this would allow us to determine if LeuRS exhibits burst kinetics. If this is true, then I would expect EF-Tu to reduce this burst with wild-type LeuRS since class I aaRSs are rate-limited by product release (Zhang et al. 2006). Unfortunately, I did not observe a distinct display of burst kinetics during rapid-quench experiments with wild-type as well as T252A mutant. Our attempts to further optimize experimental conditions were impeded by difficulty in expressing *E. coli* EF-Tu at high enough concentrations for rapid-quench experiments.

VII.4. Discussion

Translation requires the specific attachment of amino acid to its cognate tRNA, before the charged tRNA is handed off to EF-Tu for delivery to the ribosome where protein synthesis occurs. In *E. coli* LeuRS, charging of tRNA occurs in the canonical core domain while editing occurs in the CP1 domain 30 Å away (Tukalo et al. 2005). I wondered if correctly charged tRNA was also translocated to the editing domain to be sampled, or if EF-Tu could directly pull the charged species off the canonical core domain. Additionally, it was reported that charged tRNAs could be released from EF-Tu to be resampled by the aaRS (Ling et al. 2009). Thus, it seemed plausible that the tRNA would skip sampling by its editing domain, since there was another chance whereby it could return from the EF-Tu and be edited.

I carried out a series of experiments with *E. coli* LeuRS to determine if correctly charged Leu-tRNA^{Leu} is translocated to the CP1 domain before being handed off to the EF-Tu. The T252A mutant was chosen because of its unique ability to cleave correctly charged tRNA within its editing site (Mursinna et al. 2001). When the T252A LeuRS mutant was challenged with an EF-Tu in an aminoacylation assay, there continued to be a low accumulation of Leu-tRNA^{Leu}, suggesting that the EF-Tu was ineffective in sequestering correctly charged tRNA before transfer to the editing pocket for hydrolysis. I also concluded that both correctly charged and mischarged tRNAs are translocated to the editing pocket to be sampled before release from the aaRS. In order to further confirm this, a large excess of EF-Tu over tRNA should be used in future experiments in order to ensure that EF-Tu is not limiting in the reaction.

It has been reported that EF-Tu binds to charged and mischarged tRNA species with different affinities (LaRiviere et al. 2001). Thus, I speculated that EF-Tu would bind correctly charged tRNAs tightly, ensuring their quick release from the editing pocket. Conversely, when mischarged tRNA is present, EF-Tu has a low affinity for it and the tRNA lingers within the CP1 domain for editing to occur. Interestingly, previous investigations have showed that class I aaRSs are rate limited by product release (Zhang et al. 2006), suggesting that EF-Tu is needed to bind correctly charged tRNA at the CP1 domain to facilitate its release from the enzyme.

I carried out competition assays with wild-type LeuRS and the T252A/H537A LeuRS mutant, in the absence and presence of EF-Tu. The EF-Tu successfully competed with the T252A/H537A for the binding of charged tRNA and I noticed that EF-Tu competed with the T252A/H537A mutant for Leu-tRNA^{Leu}. This suggested that EF-Tu tightly binds to Leu-tRNA^{Leu} to facilitate its release from LeuRS. I then conducted a series of multiple turnover quench flow experiments in order to kinetically show EF-Tu facilitating the release of charged

tRNA as described in the results section. I was however unsuccessful in this as I was unable to express EF-Tu to sufficiently high levels.

Thus, a new method of expressing EF-Tu should be developed in order for subsequent experiments to be successful. Since EF-Tu is made in large amounts within the cell (Jacobson and Rosenbusch 1976), it is possible to purify EF-Tu directly from *E. coli* cells via a matrix-bound GDP derivative (Jacobson and Rosenbusch 1977). With greater amounts of purified EF-Tu, quench flow experiments can then be conducted with greater accuracy. In addition, the ratio of EF-Tu to tRNA in rescue experiments described above should be optimized to ensure that tRNA is the limiting substrate. The higher concentrations of purified EF-Tu will allow rescue experiments to be conducted with a large excess of EF-Tu over tRNA.

Chapter VIII. Conclusion

The aaRSs ensure fidelity during protein synthesis. Each tRNA is accurately charged by its corresponding aaRS with cognate amino acid before being brought to the ribosome by EF-Tu for participation in protein synthesis (Hendrickson and Schimmel 2002; Mascarenhas et al. 2008). Based on its interaction with tRNA as well as ATP, the aaRSs have evolved to interact with a diverse set of molecules allowing them great functional flexibility. Many of these aaRSs have acquired alternate functions such as cell signaling activities on top of their involvement in protein synthesis (Martinis and Pang 2007).

My thesis research consists of two distinct themes, the first being the characterization of hscLeuRS. Considering the expanded functionality of the aaRSs especially in terms of cell signaling (Park et al. 2005), it is not surprising that they have been linked to many diseases (Park et al. 2008). Hence, a deeper understanding of the hscLeuRS, in particular its editing function, is beneficial for advancements in pharmaceutical research that focus on aaRSs as targets for antibiotics (Rock et al. 2007). The second theme of my thesis research centers on the interactions between the canonical core domain as well as the CP1 domain of *E. coli* LeuRS in order to facilitate the transfer of the 3′ end of tRNA into the editing pocket for it to be sampled. Previous research has shown that the CP1 domain rotates ~30° when tRNA is bound, relative to its *apo* state during translocation (Tukalo et al. 2005) and it has been hypothesized that this movement facilitates translocation of tRNA from the catalytic core domain to the CP1 domain for editing (Tukalo et al. 2005).

I obtained the plasmid expressing hscLeuRS from Anacor Pharmaceuticals. I focused mainly on characterizing the editing functions of hscLeuRS and mutations were made primarily in the editing pocket of the enzyme, including the Thr 298 and Asp 399 residues. The

hscLeuRS is distinct from its bacterial counterparts as it contains four peripheral insertions not found in the bacterial enzymes (Seiradake et al. 2009). These peripheral insertions have resulted in an architectural change in the CP1 domain although the integrity of the editing core is still maintained (Seiradake et al. 2009). In my case, the mutation of Thr 298 to a bulky tyrosine did not have any effect on the editing function of hscLeuRS unlike in the *E. coli* enzyme, which knocked out post-transfer editing (Mursinna and Martinis 2002; Pang and Martinis 2009). I hypothesized that this tyrosine residue could flip into vacant space over the neighboring Val 479 to allow continued binding of the editing substrate. Closure of one of the insertions (I4) in a cap-like function over the editing pocket might facilitate an induced fit that accommodates the bulky Tyr 298 mutation in the hscLeuRS editing pocket.

I also demonstrated how a universally conserved aspartic acid in the CP1 domain was important for post-transfer editing as well as for interactions with the amino acid in the synthetic active site (Pang and Martinis 2009). Substitution of this aspartic acid with an alanine led to a loss of post transfer editing as well as an increase in the K_M for leucine activation (Pang and Martinis 2009). In addition, the overall aminoacylation activity was decreased for the D399A mutant compared to wild-type, consistent with the increase in K_M for leucine activation with the D399A mutant.

The D399A mutant lacked mischarging activity with transcribed *E. coli* tRNA even in the absence of post-transfer editing (Pang and Martinis 2009). This led me to believe that in the absence of a post-transfer editing mechanism, a more ancient pre-transfer editing pathway is activated in order to maintain overall fidelity in aminoacylation. Interestingly, the D399A mutant was sensitive to the presence of tRNA modifications as well as the origin of tRNA.

Unlike the weak mischarging observed with crude *E. coli* tRNA, no mischarging by D399A was observed with transcribed *E. coli* tRNA (Pang and Martinis 2009).

The hscLeuRS is part of a macromolecular complex in the cell (Kaminska et al. 2009) although it is still unclear how the hscLeuRS with its CP1 domain fits into the macromolecular complex. It is possible that the movement of the CP1 domain is impeded within the complex. Hence, a more redundant fidelity pathway is required to serve as the primary editing mechanism, as it does not require the translocation of mischarged tRNA from one domain to another. The hscLeuRS CP1 domain in turn could be present to perform an alternate function or interact with other molecules that together with hscLeuRS serve another purpose in the cell.

In view of the many other alternate functions that aaRSs can potentially serve, I collaborated with Dr. Sung Hoon Kim's lab at Seoul National University. The Kim lab is interested in studying hscLeuRS as a leucine sensor within the mTOR pathway. I worked with them to generate mutations in the hscLeuRS that would lack aminoacylation activity. I focused on making mutants that would be deficient in leucine binding and activation. I made mutations primarily at Phe 50 in the aminoacylation pocket of hscLeuRS. These mutants were found to have very low aminoacylation activity and had lowered k_{cat} / K_M values for leucine activation compared to the wild-type hscLeuRS. The F50H mutation was an exception and had similar k_{cat} / K_M values for leucine activation compared to wild-type due to reasons that are still unclear. The Kim lab has continued to work with these mutants and will use them to further elucidate the role of hscLeuRS in mTOR signaling.

The second theme of my thesis was to study the interactions between the canonical core as well as the CP1 domain of LeuRS. The *E. coli* LeuRS, unlike the hscLeuRS, is a monomer that relies on post-transfer editing in order to ensure fidelity. In order for post-transfer editing

to occur, I hypothesized based on previous crystal structure data (Tukalo et al. 2005) that interaction between the canonical core and CP1 domain is essential. Co-immunoprecipitation assays were used to capture this interaction. However, due to potentially weak and transient interactions of the main body with the CP1 domain, I was unable to reproducibly show that these two domains were interacting. Interestingly, cell lysates of proteins used for pull-down assays enhanced interaction between the separate domains, leading us to believe that an unknown component of the lysate was essential for inter-domain interaction to occur. I initially hypothesized that the presence of EF-Tu and/or tRNA was important for the interactions of both domains, however, the addition of purified EF-Tu and tRNA to the individually purified ΔCP1 as well as CP1 domain did not enhance interactions between the two sub-domains during pull-down assays. It is possible that other factors such as concentrations of substrates as well as environmental conditions similar to those in cell lysates play a part in enhancing interaction between the two domains.

In collaboration with Dr. Taekjip Ha's lab, smFRET experiments were conducted in order to dynamically study the transient interactions between the main body and CP1 domain of *E. coli* LeuRS during the translocation of tRNA. This approach also proved challenging because of the transient nature of translocation due to the high k_{cat} of 10 s^{-1} for aminoacylation. The high K_D of 0.3 μM for tRNA binding to LeuRS also increased the difficulty of this experiment as only nanomolar concentrations of labeled LeuRS and tRNA could be used for smFRET experiments. I was thus unable to observe strong smFRET signals from our experiments due to many technical challenges. However, a more efficient re-labeling strategy is one possibility of improving the acceptor signal in our smFRET experiments. With an efficiently working smFRET set-up, I will be able to dynamically observe the detailed

conformational changes that LeuRS undergoes in order to bring about the translocation of tRNA. In fact, smFRET technology can even be harnessed to obtain in depth kinetic information of the transition steps between each conformational change of LeuRS during the translocation of tRNA. This would ultimately deepen our understanding of the tRNA translocation process in LeuRS that is fundamental for accurate charging of tRNA to occur.

Finally, I carried out a series of experiments with *E. coli* LeuRS to determine if the translocation of tRNA could be omitted and correctly charged Leu-tRNA^{Leu} handed off directly to the EF-Tu at the canonical core. I found *E. coli* EF-Tu to be ineffective in sequestering correctly charged tRNA before transfer to the editing pocket for hydrolysis. I also concluded that both correctly charged and mischarged tRNAs are translocated to the editing pocket to be sampled before release from the aaRS.

The competition assays with wild-type LeuRS and the T252A/H537A LeuRS mutant, in the absence and presence of EF-Tu suggested that EF-Tu binds to Leu-tRNA^{Leu} tightly in order to facilitate its release from LeuRS. The *E. coli* LeuRS is a class I enzyme that displays burst kinetics (Zhang et al. 2006). Multiple turnover quench flow experiments to check for a release in burst kinetics with EF-Tu were unsuccessful as I was unable to express EF-Tu to sufficiently high levels.

In summary, the interactions between the canonical core and editing domain of *E. coli* LeuRS are very transient and have been difficult to trap. In order to maintain a high level of accuracy during charging, the enzyme resorts to a high catalytic turnover in order to ensure that the editing domain samples all the charged tRNA before being released to EF-Tu. The hscLeuRS on the other hand carries out pre-transfer editing that does not require the translocation of tRNA from one domain to another. It is possible that hscLeuRS has evolved to

depend on pre-transfer editing because the dynamics of the CP1 domain are impeded when it is in the macromolecular complex.

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Appendix. Plasmid Constructions

Plasmid Name	Parent Plasmid	Description
pJPCP1HA	pBETeCP1-2-35	*E. coli* CP1 (V216-A430) domain with HA tag
pJPmbHA	pMTV-ΔCP13a	*E. coli* ΔCP1 domain with myc tag
pJPCP1myc	pBETeCP1-2-35	*E. coli* CP1 (V216-A430) domain with myc tag
pJPmbmyc	pMTV-ΔCP13a	*E. coli* ΔCP1 domain with myc tag
pJPT252A/H537A	pMURe10_T252A	*E. coli* T252A/H537A
PJPmmK186E	pJPmbmyc	*E. coli* Main body with myc-tag and K186E mutation
PJPCHA293D2	pJPmbHA	*E. coli* CP1 with HA tag and A293D mutation
pJPE317amber	p15ec3	Amber stop codon replaces codon GAA (Glu 317)
pJPN275amber	p15ec3	Amber stop codon replaces codon AAT (Asn 275)
pJPE367amber	p15ec3	Amber stop codon replaces codon GAA (Glu 367)
pJPE329amber	p15ec3	Amber stop codon replaces codon GAA (Glu 329)
pJPE317amberChis	pJPE317amber	Amber stop codon replaces codon GAA (Glu 317) with his-tag at C-terminus in pET21
pJPN275amberChis	pJPN275amber	Amber stop codon replaces codon AAT (Asn 275) with his-tag at C-terminus in pET21
pJPE367amberChis	pJPE367amber	Amber stop codon replaces codon GAA (Glu 367) with his-tag at C-terminus in pET21
pJPE329amberChis	pJPE329amber	Amber stop codon replaces codon GAA (Glu 329) with his-tag at C-terminus in pET21
pJPC37V	p15ec3-1	C37V in *E. coli* LeuRS
pJP2_cys	pJPC37V	C37V/C128S in *E. coli* LeuRS
pJP3_cys	pJP2_cys	C37V/C128S/C505S in *E. coli* LeuRS
pJP4cysko	pJP3_cys	C37V/C128S/C505S/C565T in *E. coli* LeuRS
pJP4cysko317C	pJP4cysko	C37V/C128S/C505S/C565T/E317C in *E. coli* LeuRS
pJP4cysko329C	pJP4cysko	C37V/C128S/C505S/C565T/E329C in *E. coli* LeuRS
pJP4cysko275C	pJP4cysko	C37V/C128S/C505S/C565T/N275C in *E. coli* LeuRS
pJPA109C	pJPEFTu3cysko	*E. coli* EF-Tu C82A/C138A/C256A/A109C
pJPV171C	pJPEFTu3cysko	*E. coli* EF-Tu C82A/C138A/C256A/V171C
pJP4cyskoT252A	pJP4cysko	C37V/C128S/C505S/C565T/E317C/T252A in *E. coli* LeuRS
pJP5cysko317C	pJP4cysko	C37V/C128S/C505S/C565T/E317C/C179S in *E. coli* LeuRS
pJP6cysko317C	pJP5cysko317C	C37V/C128S/C505S/C565T/E317C/C179S/C175GC176R in *E. coli* LeuRS
pJP8cysko317C	pJP6cysko317C	C37V/C128S/C505S/C565T/E317C/C179S/C175GC176R/C285G in *E. coli* LeuRS
pJP9cysko317C	pJP8cysko317C	C37V/C128S/C505S/C565T/E317C/C179S/C175GC176R/C285G/C769A in *E. coli* LeuRS
pJPEFTuC82A	p20EfTu-25	*E. coli* EF-Tu C82A
pJPEFTu2_cys	pJPEFTuC82A	*E. coli* EF-Tu C82A/C138A
pJPEFTu3cysko	pJPEFTu2_cys	*E. coli* EF-Tu C82A/C138A/C256A
pJPtRNA3/4	ptDNAleu14	3/4 of tRNA (ptLeu14). 1/4 of tRNA deleted, starting from 5' end. GCCCGGAUGGUGGAAUCG deleted. *E. coli* $tRNA^{Leu}$
pJPC2U	ptDNAleu14	C2U mutation in *E. coli* UAA $tRNA^{Leu}$
pJPC3U	ptDNAleu14	C3U mutation in *E. coli* UAA $tRNA^{Leu}$
pJPC4U	ptDNAleu14	C4U mutation in *E. coli* UAA $tRNA^{Leu}$
pJPC2UG71A	pJPC2U	C2U and G71A mutation in *E. coli* UAA $tRNA^{Leu}$
pJPC3UG70A	pJPC3U	C3U and G70A mutation in *E. coli* UAA $tRNA^{Leu}$
pJPC4UG69A	pJPC4U	C4U and G69A mutation in *E. coli* UAA $tRNA^{Leu}$
pJPD399A	pHSLARS1	HscD399A
pJPT298A	pHSLARS1	HscT298A
pJPT298Y	pHSLARS1	HscT298Y
pHSLARS1	pHSLARS1	HscWT from Anacor Pharmaceuticals
pJPDATY2	pJPD399A	HscD399A/T298Y
pJPTYTV	pJPT298Y	HscT293V/T298Y
pJPDATV	pJPD399A	HscD399A/T293V
pJPDATYTV	pJPDATV	HscD399A/T293V/T298Y
pJPT298W	pHSLARS1	HscT298W
pJPF50A	pHSLARS1	HscF50A
pJPY52A	pHSLARS1	HscY52A
pJPY54A	pHSLARS1	HscY54A
pJPQ629A	pHSLARS1	HscQ629A
pJPF50AY52A	pJPF50A	HscF50A/Y52A
pJPF50Y	pHSLARS1	HscF50Y
pJPF50P	pHSLARS1	HscF50P
pJPF50H	pHSLARS1	HscF50H
pJPF50W	pHSLARS1	HscF50W

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