ROLE OF PHENYLALANYL-TRNA SYNTHETASE IN AMINOACYLATION AND TRANSLATION QUALITY CONTROL

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By
Srujana Samhita Yadavalli, B.Tech.
Graduate Program in Microbiology

The Ohio State University
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Dissertation Committee:
Dr. Michael Ibba, Advisor
Dr. Irina Artsimovitch
Dr. Kurt Fredrick
Dr. Karin Musier-Forsyth
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ABSTRACT

Translational quality control occurs at several steps, including amino acid selection by aminoacyl-tRNA synthetases (aaRSs). Phenylalanyl-tRNA synthetase (PheRS) misactivates Tyr but is able to correct the mistake using a proofreading activity named editing. Using Escherichia coli PheRS as a model, we show that PheRS editing is the major proofreading step that prevents incorporation of Tyr at Phe codons during translation. In addition, mischarged aa-tRNAs released by PheRS can be resampled via a trans editing pathway that reduces the overall error rate of aminoacyl-tRNA synthesis, providing an additional quality control step prior to translation elongation.

While many aaRSs, such as PheRS, actively edit noncognate amino acids, editing mechanisms are not evolutionarily conserved and their physiological significance remains unclear. To address the connection between aaRSs and mistranslation, we used the evolutionary divergence of tyrosine editing by PheRS as a model system. Certain PheRSs such as those in Mycoplasma species are naturally error-prone and display a low level of specificity consistent with elevated mistranslation of the proteome. Mycoplasma mobile PheRS (MmPheRS) lacks canonical editing activity, relying instead on alternate low stringency quality control pathways. This mechanism of discrimination is inadequate for organisms where translation is usually more accurate. As a result, MmPheRS failed to support E. coli growth. However, minor changes in the defunct editing domain of the MmPheRS are sufficient to restore amino acid specificity and sustain E. coli growth,
indicating that translational accuracy is an evolutionarily adaptable trait. These findings indicate a mechanism by which aaRSs facilitate adaptation to changes in cellular physiology by altering the accuracy of translation of specific codons, which may prove advantageous for growth under different environmental conditions.

In addition to its role in cytoplasmic protein synthesis, PheRS is also essential for the proper functioning of organelles such as mitochondria. Structural and functional studies revealed that rearrangement of the RNA-binding and catalytic domains of human mitochondrial PheRS (mtPheRS) between closed and open states are required for completion of the aminoacylation reaction. These results combined with corroborating SAXS experiments indicate that conformational flexibility of the two functional modules in mtPheRS is essential for its phenylalanylation activity, consistent with the modular evolution of the aaRSs.

Defects in mitochondrial translation can lead to a number of mitochondrial diseases like diabetes, deafness, encephalopathy and other myopathies. Although mutations in mitochondria-encoded tRNAs are known to be pathogenic, it has recently become apparent that mutations in nuclear-encoded components of the mitochondrial translation machinery, such as the mtPheRS, could also lead to disease. Non-synonymous single nucleotide polymorphisms (ns-SNPs) occurring in regions distal to mtPheRS catalytic site, affect overall aminoacylation indirectly through refolding specific defects. Pathogenic mutations in mtPheRS associated with infant cardiomyopathy impact function
directly by impairing substrate binding and amino acid activation. Our work sheds light on the effects of pathogenic mutations in mtPheRS, which can provide a molecular basis for related mitochondrial diseases.
Dedicated to my family, especially to my father Nagaraj Yadavalli, my mother Nalini, my sister Sruti Samyama, my brother Abharan for their unconditional love, support and constant encouragement over the years.
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This work was supported by National Science Foundation grant to Dr. Ibba and the following people directly contributed to this dissertation work. Dr. Ibba helped me with research design and data analyses in all my projects. I am extremely thankful to Dr. Jiqiang Ling, who initially trained and helped me with the PheRS editing project. Dr. Ling designed the research and performed some crucial experiments that show resampling by *E. coli* PheRS (Chapter 2). Dr. Hervé Roy helped me optimize tRNA labeling protocol (Chapter 2). Dr. Shinichiro Shoji prepared the ribosomes and elongation factor-G (Chapter 2). Dr. Fredrick helped in the design and analysis of *in vitro* translation experiment (Chapter 2). Our collaborators, Dr. Mark Safro (Weizmann Institute, Israel), Dr. Dmitri Svergun (EMBL, Germany) and co-workers performed SAXS analyses
(Chapter 4). Dr. Rajat Banerjee helped me with tRNA binding assay (Chapter 4), designed the research, and performed biophysical analysis of mtPheRS nsSNPs (Chapter 5). Dr. Noah Reynolds performed yeast genetics and complementation and jointly performed the growth curve assays in liquid media (Chapter 5). Dr. Henna Tyynismaa and co-workers (University of Helsinki) analyzed patient data and discovered the infant cardiomyopathy-related mutations in mtPheRS (Chapter 5). Eric Caruso helped with active site titrations and aminoacylation assays (Chapter 5). Dr. Michael Ignatov and Dr. Musier-Forsyth helped me with time-resolved fluorescence spectroscopy (Appendix).

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VITA

March 10, 1985 ......................................................... Born - Manila, Philippines

2002-2006 ................................................................. B.Tech. Industrial Biotechnology, Anna University, India

2006-present ............................................................ Graduate Teaching and Research Associate, Department of Microbiology, The Ohio State University

PUBLICATIONS


**FIELDS OF STUDY**

Major Field: Microbiology
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<th>Symbol</th>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>2AP</td>
<td>2-aminopurine</td>
<td></td>
</tr>
<tr>
<td>4-hPro</td>
<td>4-hydroxyproline</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>adenosine or L-alanine</td>
<td></td>
</tr>
<tr>
<td>Å</td>
<td>angstrom (unit)</td>
<td></td>
</tr>
<tr>
<td>aa</td>
<td>amino acid</td>
<td></td>
</tr>
<tr>
<td>aaRS</td>
<td>aminoacyl-tRNA synthetase</td>
<td>(three letter amino acid code followed by suffix RS)</td>
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<tr>
<td>aa-tRNA</td>
<td>aminoacyl-tRNA</td>
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</tr>
<tr>
<td>Ala</td>
<td>L-alanine</td>
<td></td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine 5′-monophosphate</td>
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</tr>
<tr>
<td>ANS</td>
<td>1, 8-anilino naphthyl sulfonate</td>
<td></td>
</tr>
<tr>
<td>Arg</td>
<td>L-arginine</td>
<td></td>
</tr>
<tr>
<td>AS</td>
<td>active site</td>
<td></td>
</tr>
<tr>
<td>Asn</td>
<td>L-asparagine</td>
<td></td>
</tr>
<tr>
<td>Asp</td>
<td>L-aspartic acid</td>
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</tr>
<tr>
<td>ATP</td>
<td>adenosine 5′-triphosphate</td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>cytidine or L-cysteine</td>
<td></td>
</tr>
<tr>
<td>°C</td>
<td>degree Celsius (centigrade)</td>
<td></td>
</tr>
<tr>
<td>CCase</td>
<td>terminal tRNA nucleotidyl transferase</td>
<td></td>
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</table>
CP1          connective peptide 1
cpm          counts per minute
CTP          cytidine 5′-triphosphate
Cys          L-cysteine
d            deoxy
D            L-aspartic acid
Da           dalton (unit)
DEPC         diethylpyrocarbonate
DMSO         dimethyl sulfoxide
DNA          deoxyribonucleic acid
DNase        deoxyribonuclease
dNTP         deoxynucleotide 5′-triphosphate
DTD           D-tyrosyl-tRNA\textsuperscript{Tyr} deacylase
DTNB         5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman’s reagent)
DTT           dithiothreitol
E            enzyme or L-glutamic acid
Ec            \textit{Escherichia coli}
\textit{E. coli}  \textit{Escherichia coli}
EDTA         ethylenediaminetetraacetic acid
EF-G         elongation factor-G
EF-1A        elongation factor-alpha
EF-Tu        elongation factor-Tu
ES  editing site
F  L-phenylalanine
g  gram
G  guanosine or L-glycine
Gln  L-glutamine
Glu  L-glutamic acid
Gly  L-glycine
GMP  guanosine 5′-monophosphate
GTP  guanosine 5′-triphosphate
h  hour (unit)
H  L-histidine
Hcy  homocysteine
Hepes  N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]
His  L-histidine
Hse  homoserine
I  L-isoleucine
IF  initiation factor
Ile  L-isoleucine
INS  insertion domain
IPTG  isopropyl-β-D-thiogalactoside
K  L-lysine
$k_{\text{app}}$  apparent rate constant

xxi
$k_{\text{cat}}$  catalytic rate constant
$K_D$  binding constant
$k_{\text{obs}}$  observed rate constant
$k_{\text{off}}$  dissociation rate constant
$k_{\text{on}}$  association rate constant
$K_M$  Michaelis-Menten constant
$k_{\text{trans}}$  amino acid transfer rate constant
L  L-leucine
l  liter
LB  Luria-Bertani (growth medium)
Leu  L-leucine
Lys  L-lysine
m  milli
M  molar or L-methionine
MELAS  mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes
MERRF  myoclonic epilepsy with ragged red fibers
Met  L-methionine
$Mm$  *Mycoplasma mobile*
*M. mobile*  *Mycoplasma mobile*
min  minute
mRNA  messenger RNA
mt  mitochondrial
n  nano
N  L-asparagine
ND  not determined
nLeu  L-norleucine
ns  non-synonymous
nVal  L-valine
Orn  L-ornithine
p  pico
P  L-proline
PAGE  polyacrylamide gel electrophoresis
PCR  polymerase chain reaction
PEI  polyethyleneimine
PEP  phosphoenolpyruvate
Phe  L-phenylalanine
PK  pyruvate kinase
PPase  inorganic pyrophosphatase
PP_i  inorganic pyrophosphate
Pro  L-proline
PyC  pyrrolo-cytosine
Q  L-glutamine
R  L-arginine
<table>
<thead>
<tr>
<th>Acronym</th>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>S</td>
<td>substrate or L-serine</td>
</tr>
<tr>
<td>SAXS</td>
<td>small-angle X-ray scattering</td>
</tr>
<tr>
<td>Sc</td>
<td><em>Saccharomyces cerevisiae</em></td>
</tr>
<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>Sec</td>
<td>L-selenocysteine</td>
</tr>
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<td>L-serine</td>
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<td>SerHX</td>
<td>serine hydroxymate</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>T</td>
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</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
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<tr>
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</tr>
<tr>
<td>TLC</td>
<td>thin-layer chromatography</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>tris-(hydroxymethyl) aminomethane hydrochloride</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer RNA</td>
</tr>
<tr>
<td>Trp</td>
<td>L-tryptophan</td>
</tr>
<tr>
<td>Tyr</td>
<td>L-tyrosine</td>
</tr>
<tr>
<td>U</td>
<td>unit or uridine</td>
</tr>
<tr>
<td>UTP</td>
<td>uridine 5′-triphosphate</td>
</tr>
<tr>
<td>V</td>
<td>volume or L-valine</td>
</tr>
<tr>
<td>Val</td>
<td>L-valine</td>
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</tbody>
</table>

xxiv
W    water or L-tryptophan
WT   wild-type
Y    L-tyrosine
α-Abu α-aminobutyrate
γ-hLeu γ-hydroxyleucine
λ    wavelength
μ    micro
τ    fluorescence decay lifetime
τ_{avg} average fluorescence decay lifetime
CHAPTER 1

QUALITY CONTROL IN AMINOACYL-TRNA SYNTHESIS: ITS ROLE IN TRANSLATIONAL FIDELITY

1.1. Introduction

According to the central dogma, genetic information is processed from DNA to messenger RNA and then ultimately into the sequence of a protein. The translation of triplet nucleotide codons in mRNA to amino acid sequence is a high-fidelity process leading to functional protein synthesis and maintenance of cellular physiology. The overall error rate in translation is $\sim 10^{-4}$ (Ibba and Söll, 1999; Loftfield and Vanderjagt, 1972). During protein synthesis, aminoacyl-tRNAs produced by an aminoacyl-tRNA synthetase (aaRS) are bound by an elongation factor (EF-Tu in bacteria) and delivered to the ribosome for mRNA decoding (Ibba and Söll, 1999; Ling et al., 2009a). Two key steps that serve as quality control checkpoints in translation are: (i) accurate pairing of amino acids to tRNAs by aminoacyl-tRNA synthetases (aaRSs), and (ii) selection of correct aminoacyl-tRNA for decoding by a translating ribosome. The focus of this chapter will be the first checkpoint, describing the fidelity mechanisms during aa-tRNA synthesis employed by the aaRSs, a family of essential housekeeping enzymes.
Aminoacylation, or tRNA “charging”, is a two-step reaction (Fig. 1.1). In the first step the aaRS utilizes ATP to activate an amino acid and forms an aaRS-aminoacyl-adenylate complex, releasing inorganic pyrophosphate as a byproduct. In the second step the activated aminoacyl moiety is transferred to either the 2′- or 3′-hydroxyl group of the terminal adenine A76 of the acceptor end of tRNA forming an aminoacyl ester bond (Ibba and Söll, 2000; Mascarenhas et al., 2009). There are 20 aaRSs, one for each of the 20 standard amino acids. AaRSs can be divided into two mutually exclusive classes, I and II, based on their structural, functional and evolutionary relatedness (Burbaum and Schimmel, 1991; Cusack, 1993; Cusack et al., 1990; Eriani et al., 1990; Ibba and Söll, 2000). Each aaRS falls into either class I or class II, except for lysyl-tRNA synthetase (LysRS), which has a representative in both classes. Within each class there exist subclasses a, b and c on the basis of relative sequence and structural similarity. Although the two classes do not appear to share common ancestors, it is believed that the ancient aaRSs were constituted of just the canonical core aminoacylation sites (Schimmel and Ribas De Pouplana, 1995). During evolution aaRSs acquired several different appended domains including the editing domains (Ahel et al., 2003; Schimmel et al., 1993). In class I aaRSs, the aminoacylation active site has a Rossmann nucleotide binding fold defined by the two conserved motifs HIGH and KMSKS. The Rossmann fold binds ATP and amino acid and promotes catalysis (Eriani et al., 1990; Ibba and Söll, 2000). Class II aaRSs possess an antiparallel β-sheet active site architecture represented by the signature motifs 1, 2 and 3. Motifs 2 and 3 aid in binding ATP and amino acid, while motif 1 is
important for dimerization. Class I aaRSs except tyrosyl-tRNA synthetase (TyrRS) bind to the minor groove side of the tRNA acceptor stem and aminoacylate the tRNA at the 2′-OH (Carter et al., 1986; Yaremchuk et al., 2002). Class II aaRSs approach the tRNA acceptor stem from the major groove side, and with the exception of phenylalanyl tRNA synthetase (PheRS) aminoacylate the tRNA at the 3′-OH (Carter, 1993). Class I aaRSs exist as monomers with the exceptions of TyrRS, tryptophanyl tRNA synthetase (TrpRS) and methionyl tRNA synthetase (MetRS), whereas class II aaRSs are usually found to be dimeric or tetrameric. In multimeric aaRSs, tRNA is bound across all the subunits as seen in both class I TyrRS (Yaremchuk et al., 2002) as well as class II PheRS (Mosyak et al., 1995). Additionally in PheRS, tRNA binding is observed to be anticooperative between the two aminoacylation active sites (Holler, 1980). The impact of dimerization on catalysis of the aminoacylation reaction is well studied in TyrRS. The TyrRS dimer is asymmetric in solution with respect to substrate binding (Jakes and Fersht, 1975). The enzyme binds a single molecule of tRNA^{Tyr} or Tyr and forms only one molecule of tyrosyl adenylate per dimer, a feature referred to as “half-of-the-sites” reactivity. Similar asymmetry of the two synthetic active sites is observed in class I TrpRS (Trezeguet et al., 1986), class II glycyl tRNA synthetase (GlyRS) (Freist et al., 1996), PheRS (Baltzinger and Holler, 1982a) and histidyl tRNA synthetase (HisRS) (Guth et al., 2009). Recent studies suggest that the observed asymmetrical rates of amino acid activation at the two active sites are normalized to the overall rate of aminoacylation upon tRNA addition in some cases (Guth et al., 2009). Steady state and transient kinetic approaches reveal key differences between the two classes of aaRSs with respect to aminoacylation kinetics.
While product release is rate-limiting in monomeric class I aaRSs, it occurs readily in class II aaRSs (Zhang et al., 2006; Ling et al., 2009b) (Chapter 2). This difference in product release rates has important ramifications for the subsequent steps in protein synthesis as described in section 1.1.4.

1.1.2. Substrate selection by aaRSs

For accurate aminoacylation, an aaRS must recognize both a specific tRNA and the corresponding amino acid. Recognition of cognate tRNAs and their isoacceptors by an aaRS is facilitated by the presence of tRNA identity elements (Giegé et al., 1998). While some nucleotides act as positive elements (determinants) and promote productive interaction between the cognate tRNA and aaRS for charging, others serve as negative elements (antideterminants) that prevent mischarging of noncognate tRNA substrates. Systematic mutational analyses of tRNAs have identified numerous major and minor
identity elements, classified based on the degree to which they affect aminoacylation efficiency *in vitro* and/or *in vivo*. Major elements are usually well conserved in tRNAs across organisms and are located mainly in the acceptor stem including the discriminator base (N73) and the anticodon loop. Minor elements are specific to a particular organism and are distributed throughout the tRNA body. Some examples of identity determinants unique to a given tRNA species include the G3:U70 wobble base pair of tRNA$^{\text{Ala}}$ (Beuning et al., 1997; Gabriel et al., 1996; Hou and Schimmel, 1988; McClain et al., 1988), the Levitt pair G15:G48 of tRNA$^{\text{Cys}}$ (Hamann and Hou, 1997; Hou et al., 1993), G$^{-1}$ base of tRNA$^{\text{His}}$ (Francklyn and Schimmel, 1990; Francklyn et al., 1992; Himeno et al., 1989) and numerous modified nucleotides in many tRNAs (Giegé and Frugier, 2003; Giegé et al., 1998; Ibba and Söll, 2000). Other regions in tRNAs such as the elbow region of L-shaped tRNA, the variable arm, and the phosphate backbone contain major identity elements in some cases. Transplantation of critical identity elements can readily switch tRNA identity and allow aminoacylation by a noncognate aaRS. For example, *E. coli* tRNA$^{\text{Phe}}$ becomes an efficient substrate for charging by AlaRS upon substitution of the G3:U70 base pair (Hou and Schimmel, 1988; McClain and Foss, 1988). However, tRNA identity elements do not always work in isolation. These elements can coordinate with each other from distal sites in the tRNA and act synergistically to increase cognate aminoacylation efficiency. The ability of individual determinants to promote tRNA charging, together with the cooperativity among these elements, provides specificity for recognition of cognate tRNAs and their isoacceptors.
Binding the cognate amino acid is more challenging for many aaRSs (Fersht, 1979). While some amino acids have unique side chains, the similarity in chemical structures of related and/or isosteric amino acids creates a high probability of error in recognition leading to misactivation. For instance, the isosteric amino acid pair of isoleucine (Ile) and valine (Val) poses a difficulty for isoleucyl-tRNA synthetase (IleRS) (Fersht, 1977b) specificity whereas cysteine (Cys) is recognized via a very specific interaction with a Zn ion in the active site of cysteinyl-tRNA synthetase (CysRS) (Fersht and Dingwall, 1979b; Newberry et al., 2002; Zhang et al., 2003). Another example of structurally similar amino acids is the aromatic amino acid pair tyrosine (Tyr) and phenylalanine (Phe), which differ by a single hydroxyl group. While TyrRS has evolved a highly specific Tyr recognition active site cleft (Fersht et al., 1980), PheRS is known to occasionally misactivate Tyr instead of cognate Phe (Ibba et al., 1994).

1.1.3. Discovery of aaRS editing

The problem of accurate amino acid selection was noticed more than five decades ago by Linus Pauling, who predicted that there is a probability of 1 in 200 for noncognate Val binding by IleRS (Pauling, 1958). In 1966, Baldwin and Berg’s experiments with *E. coli* IleRS showed that although both Ile and Val were activated, only the valyl-adenylate was degraded upon tRNA^Ile^ addition (Baldwin and Berg, 1966). This observation, together with the subsequent measurement of a translation error rate of ~1 in 3000 (Loftfield and Vanderjagt, 1972), strongly suggested the existence of a proofreading function (termed “editing”) in aaRSs. In 1977, Alan Fersht proposed a “double-sieve” model to explain the
high fidelity in aminoacylation (Fersht, 1977a). According to this hypothesis, the synthetic active site acts as a first coarse sieve, which can bind and activate the cognate substrate as well as isosteres and smaller amino acids while rejecting larger amino acids. The editing site serves as a second fine sieve to selectively hydrolyze the isosteric amino acid but not the cognate amino acid based on size and chemical discrimination (Fersht, 1977b, 1999). In IleRS, entry of cognate Ile into the editing site is sterically hindered, whereas in the case of ValRS, cognate Val is discriminated on a chemical basis (Fersht, 1999; Fersht and Kaethner, 1976). Consistent with the double sieve mechanism, editing activities have been discovered in class I IleRS (Baldwin and Berg, 1966), ValRS (Fersht and Kaethner, 1976), LeuRS (Englisch et al., 1986) and in class II enzymes PheRS (Yarus, 1972), AlaRS (Tsui and Fersht, 1981), ThrRS (Dock-Bregeon et al., 2000) and ProRS (Beuning and Musier-Forsyth, 2000) (Table 1.1). Editing function is also found in aaRSs that do not possess a distinct editing domain. These include class I MetRS (Fersht and Dingwall, 1979a) and the class II aaRSs SerRS (Gruic-Sovulj et al., 2007) and LysRS (Jakubowski, 1999). The editing aaRSs employ rather unique strategies to proofread against noncognate substrates and the editing pathways utilized by class I and class II aaRSs are outlined in the following section.
Table 1.1. Editing by aa-tRNA synthetases: editing domains, amino acid substrates and mode of editing

<table>
<thead>
<tr>
<th>aaRS</th>
<th>Structural organization</th>
<th>Editing domain</th>
<th>Trans-editing factor</th>
<th>Amino acids edited Standard</th>
<th>Non-standard</th>
<th>References</th>
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<tbody>
<tr>
<td>IleRS α</td>
<td>CP1, AS⁴</td>
<td>-</td>
<td>Val</td>
<td>Hcy</td>
<td>Baldwin and Berg 1966, Eldred and Schimmel 1972, Lin et al., 1996, Nureki et al., 1998, Silvian et al., 1999, Dulic et al., 2010</td>
<td></td>
</tr>
<tr>
<td>LeuRS α</td>
<td>CP1, AS⁴</td>
<td>-</td>
<td>Val, Ille, Met</td>
<td>Hcy, g-hLeu, nLeu</td>
<td>Boniecki et al., 2008, Chen et al., 2000, Cusack et al., 2000, Englisch et al., 1986, Lincecum et al., 2003, Mursinna and Martinis 2002, Zhu et al., 2009, Dulic et al., 2010</td>
<td></td>
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<tr>
<td>MetRS α, α</td>
<td>AS⁵</td>
<td>-</td>
<td>Hcy</td>
<td>Fersht and Dingwall 1979, Jakubowski and Fersht 1981</td>
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<tr>
<th>aaRS</th>
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<th>Non-standard</th>
<th>References</th>
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<tbody>
<tr>
<td>ThrRS α₂</td>
<td>N2</td>
<td>ThrRS-ed</td>
<td>Ser</td>
<td>Dock-Bregeon et al., 2000, Dock-Bregeon et al., 2004, Korencic et al., 2004, Sankaranarayanan et al., 2000, Minaji and Francklyn 2010</td>
<td></td>
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<tr>
<td>PhεRS (αβ), α</td>
<td>B3/B4</td>
<td>-</td>
<td>Tyr, Ille</td>
<td>Yarus 1972, LIN et al., 1984, Roy et al., 2004, 2005, Kotik-Kogan et al., 2005</td>
<td></td>
<td></td>
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<tr>
<td>AlaRS α₁, α</td>
<td>C-terminal, AlaX-like</td>
<td>AlaXp</td>
<td>Gly, Ser</td>
<td>Ahel et al., 2003, Beebe et al., 2003, Tsui and Fersht 1981</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SerRS α₂</td>
<td>AS⁵</td>
<td>-</td>
<td>Thr, Cys</td>
<td>Ser-HX</td>
<td>Gnui-Sovulj et al., 2007</td>
<td></td>
</tr>
<tr>
<td>LysRS-I α₂</td>
<td>AS⁵</td>
<td>-</td>
<td>Hey, Hse, Orn</td>
<td>Jakubowski 1997, 1999</td>
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*AS refers to aminoaacylation active site
1.1.4. aaRS editing pathways

High-resolution structures of editing aaRSs in complex with non-hydrolyzable substrate analogs have provided invaluable insights into the molecular mechanisms of editing (Ling et al., 2009a; Mascarenhas et al., 2009). Proofreading of aminoacylation can occur either after activation prior to aminoacyl transfer (“pretransfer” editing) or after transfer (“posttransfer” editing) (Fig. 1.2).

Pretransfer editing involves hydrolysis of misactivated aminoacyl-adenylates (aa-AMPs) and may occur via one or more of the routes described below (Fig. 1.2A). (i) tRNA-independent, enzyme-catalyzed hydrolysis or cyclization of noncognate aa-AMP within the canonical aminoacylation active site (pathway 1), (ii) tRNA-independent selective release of noncognate aa-AMP, which then undergoes spontaneous hydrolysis in solution (pathway 2) or (iii) tRNA-dependent hydrolysis of misactivated aa-AMP either in the aminoacylation or the distal editing site (pathway 3). Examples of tRNA-independent enzymatic pretransfer hydrolysis are known for class II ProRS (Splan et al., 2008) and SerRS (Gruic-Sovulj et al., 2007). Class I MetRS cyclizes noncognate homocysteine (Hcy) into a non-productive thiolactone (Jakubowski and Fersht, 1981). This mechanism is also shared by class II LysRS against homoserine (Hse) (Jakubowski, 1997) and ornithine (Orn) (Jakubowski, 1999). IleRS utilizes tRNA-dependent pretransfer editing to hydrolyze misactivated Val-AMP (Baldwin and Berg, 1966). The LeuRS synthetic site can actively hydrolyze misactivated Ile-AMP in a tRNA-dependent fashion, which becomes evident upon inactivation of posttransfer editing (Boniecki et al., 2008; Yadavalli et al., 2008).
Posttransfer editing is well-studied for various aaRS systems where a distinct editing site ~35-40 Å away from the aminoacylation site deacylates the aminoacyl ester bond between noncognate amino acid and tRNA (Fig. 1.2B). Extensive structural and modeling studies led to the isolation and biochemical characterization of the posttransfer editing domains (Dock-Bregeon et al., 2000; Roy et al., 2004; Silvian et al., 1999; Tukalo et al., 2005). Post transfer editing activities are associated with both class I and II aaRSs including IleRS (Eldred and Schimmel, 1972), ValRS (Fersht and Kaethner, 1976), LeuRS (Englisch et al., 1986), PheRS (Yarus, 1972), ThrRS (Dock-Bregeon et al., 2000), ProRS (Beuning and Musier-Forsyth, 2000), and AlaRS (Beebe et al., 2003) to eliminate noncognate aminoacyl-tRNAs. In addition, freestanding editing domains are found to posttransfer edit noncognate aminoacyl-tRNAs (Fig. 1.2B, pathway 3; also see section 1.5). There are two modes in which posttransfer hydrolysis has been proposed to occur: (i) a direct translocation model (Fig. 1.2B, pathway 1), which involves movement of the 3'-CCA of mischarged tRNA from aminoacylation to editing site and/or (ii) a dissociation-reassociation model (Fig. 1.2B, pathway 2), where the mischarged tRNA is released and re-bound by the aaRS editing site where hydrolysis occurs (Chapter 2) (Ling et al., 2009a; Ling et al., 2009b). Support for the tRNA translocation model comes mainly from X-ray crystallography and structural studies in class I aaRSs, which show the conformations of aaRS and tRNA in posttransfer editing mode (Fukunaga and Yokoyama, 2005a; Tukalo et al., 2005) (Figs. 1.4, 1.5; also see section 1.2.2). The dissociation-reassociation model is favored by recent evidence showing resampling of mischarged aa-tRNA by the editing domain of an aaRS (Chapter 2) (Ling et al., 2009b).
This model is applicable to editing class II aaRSs and trans-editing factors due to class-specific differences in product release rates. Whereas the rate of product release is slow in class I aaRSs, class II aaRSs can rapidly release and re-bind aa-tRNAs. This difference affects not only the editing step but also aminoacylation and downstream steps in translation. For instance, the rate of aminoacylation is higher in the presence of elongation factor Tu (EF-Tu) for non-editing monomeric class I aaRSs such as cysteinyltRNA synthetase (CysRS) due to the sequestration of the aa-tRNA product by EF-Tu, resulting in an increase of enzyme turnover (Zhang et al., 2006). This observation is corroborated by structural modeling, which suggests that CysRS•aa-tRNA can form a ternary complex with EF-Tu. However, class I editing aaRSs and class II aaRSs cannot form ternary complexes with EF-Tu and aa-tRNA due to steric constraints (Ling et al., 2009b; Zhang et al., 2006). Since product release is not rate-limiting in class II aaRSs, any mischarged tRNA that escapes editing is bound and protected by EF-Tu. Therefore, EF-Tu works in competition with the editing domain of aaRSs (Chapter 2) (Ling et al., 2009b). This scenario provides a biological rationale for the existence of redundant quality control pathways involving pre-and/or posttransfer mechanisms. In addition, widespread freestanding editing domains such as AlaX appear to have evolved in order to resolve confounding problems in molecular recognition and prevent mistranslation (Guo et al., 2009b) (details in section 1.5).
Figure 1.2. Editing mechanisms in aaRSs. A, Pretransfer editing pathways in aaRSs. Misactivated aminoacyl adenylate (aa-AMP) can be hydrolyzed in a tRNA-independent manner either through the direct catalysis of aa-AMP hydrolysis by the aminoacylation active site (AS) (pathway 1) or through selective release of noncognate aa-AMP followed by spontaneous hydrolysis in solution (pathway 2). tRNA-dependent pretransfer hydrolysis (pathway 3) can occur within the aminoacylation active site (AS) in some aaRSs, or in the editing site (ES) presumably via translocation of aa-AMP through a channel between AS and ES. B, Posttransfer editing pathways in aaRSs. Mischarged aminoacyl-tRNA (aa-tRNA) can be hydrolyzed via translocation of the 3′-terminal CCA of tRNA from the aminoacylation active site (AS) to the distal editing site (ES) ~35Å away (pathway 1). Alternatively in class II aaRSs, the mischarged aa-tRNA may be released into solution, then either resampled by the aaRS (pathway 2) or hydrolyzed by an accessory trans-editing factor (pathway 3).
1.2. Class I aaRS editing mechanisms

Editing activities, both post- and/or pretransfer mechanisms, have been associated with the class Ia enzymes IleRS, LeuRS, ValRS and MetRS which share a high degree of homology (Burbaum and Schimmel, 1991; Eriani et al., 1990). IleRS is the first aaRS in which the existence of a distinct editing active site distal to the aminoacylation site was elucidated. Two specific mutations (Phe570Ser and Gly56Pro) in the synthetic active site of *E. coli* IleRS affected only amino acid activation but not posttransfer editing activity, clearly demonstrating that the aminoacylation and editing sites are functionally independent (Schmidt and Schimmel, 1994; Hendrickson and Schimmel, 2003) consistent with the double-sieve model. IleRS, LeuRS and ValRS contain a connective peptide 1 (CP1) domain insertion connected by β-strand linkers to the main body of the enzyme. This CP1 domain was shown to house the editing site by cross-linking experiments using chemically modified bromoacetyl-Val-tRNA$^{\text{Ile}}$ (Schmidt and Schimmel, 1995). Structural analyses revealed the location of the CP1 editing active site, ~35Å from the synthetic active site (Nureki et al., 1998; Silvian et al., 1999). All Ile/Leu/ValRSs have their CP1 domains inserted into a Zn-binding site with the exception of bacterial and mitochondrial LeuRSs. In these LeuRSs, the CP1 insertion occurs after the Zn-binding site (Cusack et al., 2000), which requires rotation of the editing domain by about 180° in order to reach the 3’end of mischarged tRNA. Isolated CP1 domains of Ile/Leu/ValRSs show robust editing activity against their misacylated tRNAs (Chen et al., 2000; Lin et al., 1996), however in some cases flexible β-linkers are necessary for the activity (Betha et al., 2007).
1.2.1. CP1-dependent editing by IleRS, LeuRS, and ValRS

Structural and mutational studies identified key residues in the CP1 editing site that interact with the side chain of the noncognate amino acid and the adenine (A76) of the tRNA. A critical Asp residue in the editing pocket of Ile/Leu/ValRSs interacts with the amino group of the noncognate amino acid in both pre- and posttransfer conformation via a non-covalent salt bridge (Fukunaga et al., 2004; Fukunaga and Yokoyama, 2005c; Lincecum et al., 2003). Disruption of this strictly conserved Asp residue leads to loss of editing, but not aminoacylation function (Bishop et al., 2002; Dock-Bregeon et al., 2004; Fukunaga and Yokoyama, 2005a, b, c; Lincecum et al., 2003). The three class Ia aaRSs contain a conserved threonine-rich sequence, which also interacts with the noncognate amino acid and contributes to editing specificity. One of the Thr residues in particular, Thr252 in *E. coli* LeuRS, is crucial for discrimination against cognate Leu through steric exclusion by the γ-methyl group of Thr (Mursinna et al., 2001). When Thr252 is replaced by Ala or Ser, Leu can enter the editing site leading to cognate Leu-tRNA\(^{\text{Leu}}\) hydrolysis. Conversely, introduction of a bulkier substitution such as Trp blocks the editing site to both the cognate and noncognate substrates (Mursinna and Martinis, 2002). Similarly, the conserved Thr230 in *T. thermophilus* IleRS (Fukunaga and Yokoyama, 2006), a semi-conserved His319 in *E. coli* IleRS (Hendrickson et al., 2002) (Fig. 1.3), and the conserved Lys277 in *E. coli* ValRS (Hountondji et al., 2002) are important for discrimination against the cognate substrate.

Although Ile/Leu/ValRSs are homologous both in terms of sequence and structure, the CP1 domain has evolved unique specificities required for editing in each
aaRS as discussed below (Cusack et al., 2000; Mascarenhas et al., 2009; Nureki et al., 1998; Schmidt and Schimmel, 1994; Schmidt and Schimmel, 1995; Silvian et al., 1999) (Table 1.1). In the *T. thermophilus* IleRS CP1 domain, noncognate Val is recognized specifically by residues His319 and Thr233 (Fig. 1.3), however they interact differently in the pretransfer and posttransfer modes (Fukunaga and Yokoyama, 2006). In the case of *E. coli* LeuRS, substrate specificity to the side chains of Met and Ile is conferred by a set of highly conserved CP1 residues Thr247, Met336 and Asp345 that form extensive hydrogen bonding interactions (Liu et al., 2006). In addition, the semiconserved residue Tyr330 aids in proper positioning of the terminal A76 of tRNA. The discrimination of Val versus isosteric Thr by the ValRS CP1 domain provided the first example of cognate amino acid discrimination by the editing site, which re-defined the originally conceived idea of the double sieve mechanism (Fersht and Kaethner, 1976). Rejection of Thr in the ValRS CP1 domain occurs on the basis of differences in the binding energies of the two amino acids and not by steric exclusion alone. In ValRS, noncognate Thr is held in the editing active site by Lys270, Thr272 and Asp279 (Fukunaga and Yokoyama, 2005c), and the strictly conserved GTG loop along with Phe264 provide conformational flexibility to accommodate mischarged Thr-tRNA\textsuperscript{Val}. The hydroxyl group of Thr interacts with conserved Asp residues through hydrogen bonds, whereas the methyl group of Val does not (Fukai et al., 2000).

Other regions that influence editing by class Ia aaRSs include a second insertion domain CP2 in LeuRS, which is important for both the aminoacylation as well as editing functions and appears to play a structural role in positioning the tRNA in the correct
orientation (Zhou et al., 2008). Also, the C-terminal domain of Ile/Leu/ValRSs, which is
distinct from the tRNA anticodon-binding domain, appears to be necessary for
aminoacylation and editing (Fukunaga and Yokoyama, 2007; Hsu et al., 2006). In the
case of P. horikoshii, truncation of the C-terminal domain in LeuRS leads to a loss in
specificity for the cognate tRNA\textsubscript{Leu}, resulting in the hydrolysis of mischarged Ile-
tRNA\textsubscript{Leu} as well as correctly charged Ile-tRNA\textsubscript{Ile}. This reveals the importance of the C-
terminal domain in P. horikoshii LeuRS in preventing misediting. In contrast, in the case
of yeast mitochondrial LeuRS, the C-terminal domain seems to have diverged from its
original function, probably owing to its dual role as a splicing factor (Hsu et al., 2006),
and deletion of this domain increases both the aminoacylation and editing activities.

\textbf{Figure 1.3. Recognition of Val by the IleRS CP1 editing domain} (reproduced with
permission from Fukunaga and Yokoyama 2006). (a) IleRS editing pocket showing the
conserved active site residues in the absence of Val. (b) Noncognate Val bound in the
IleRS CP1 editing active site. Ionic and hydrogen bond interactions are shown by dotted
lines. His319, a semi-conserved residue is crucial for steric exclusion of cognate Ile from
the editing site.
1.2.2. Mechanisms and partitioning of pre- versus posttransfer editing

The crystal structure of *T. thermophilus* IleRS bound to a nonhydrolyzable, noncognate substrate analog 5’-O-[N-(l-valyl)sulfamoyl]adenosine (Val-AMS) showed binding of Val in both the synthetic and editing active sites (Nureki et al., 1998). This suggests that IleRS can perform pre- and posttransfer editing of misformed Val-AMP and Val-tRNA\(^{\text{Ile}}\).

In the LeuRS system, detailed structural analyses reveal tRNA translocation during posttransfer editing. Cocystal structures of *P. horikoshii* and *T. thermophilus* LeuRS with tRNA\(^{\text{Leu}}\) show tRNA\(^{\text{Leu}}\) in different conformations attributed to the aminoacylation, intermediate, editing, and exit states (Fig. 1.4; Fukunaga and Yokoyama, 2005a; Tukalo et al., 2005). In addition to the movement of tRNA\(^{\text{Leu}}\), the *T. thermophilus* LeuRS itself undergoes specific domain rearrangements upon tRNA\(^{\text{Leu}}\) binding (Fig. 1.5), thus promoting the correct positioning of the 3’-CCA of tRNA for posttransfer hydrolysis (Tukalo et al., 2005). Biochemical and structural analyses implicate a role for the 3’-OH of tRNA via substrate-assisted catalysis (Nordin and Schimmel, 2002; Lincecum et al., 2003). With respect to pretransfer editing, despite detailed structural and biochemical analyses, the mechanism and site for pretransfer editing within the enzyme had remained unclear (Mascarenhas et al., 2009; Ling et al., 2009a). Experiments conducted using a selected DNA aptamer replacing a tRNA, combined with mutational analyses suggested pretransfer editing occurred within the CP1 domain (Nomanbhoy et al., 1999; Farrow and Schimmel, 2001; Hendrickson et al., 2002). Several theories were proposed to explain the translocation of misactivated adenylate from the synthetic active site to the CP1 editing site ~30–35Å away. Pretransfer editing has been proposed to occur in a tRNA-dependent
fashion and in this model, tRNA binding causes a conformational change in the IleRS leading to channel formation through which misactivated Val-AMP travels to the CP1 active site (Nomanbhoy et al., 1999; Silvian et al., 1999). It is hypothesized that one round of posttransfer editing is required to initiate multiple rounds of pretransfer editing (Nordin and Schimmel, 2003). This hypothesis awaits further verification as crystal structures of apo-enzymes have not been able to capture the channel. However, recent work has shed light on pretransfer mechanisms and the role of the editing site. Mutational analyses of specific residues on the surface of the CP1 domain as well as the synthetic core in the Thr252Tyr posttransfer editing-defective background unveiled a latent pretransfer editing activity in bacterial LeuRS (Williams and Martinis, 2006). Further studies performed by deleting the entire CP1 domain in LeuRS clearly illustrated a hidden tRNA-dependent pretransfer editing mechanism occurring within the aminoacylation active site (Boniecki et al., 2008; Yadavalli et al., 2008). Further kinetic studies support the idea that tRNA-dependent pretransfer editing in E. coli LeuRS occurs within the synthetic active site, thus obviating the need for a channeling mechanism (Zhu et al., 2009; Dulic et al., 2010). The contribution of pre- versus posttransfer editing pathways to overall editing is very specific in each of the class Ia aaRSs. MetRS (as described in detail in the next section) is solely dependent on pretransfer editing to remove misactivated adenylates (Fersht and Dingwall, 1979a; Jakubowski and Fersht, 1981). Although early work established that tRNA-dependent pretransfer editing is dominant in IleRS (Fersht, 1977b), there appears to be a significant contribution by the posttransfer editing component in hydrolysis of any mischarged Val-tRNA\textsuperscript{Ile} that has
escaped the pretransfer checkpoint (Dulic et al., 2010). Although *E. coli* LeuRS is thought to rely entirely on posttransfer editing, yeast cytoplasmic LeuRS, like IleRS, utilizes pretransfer editing as a major editing pathway in addition to a robust post-transfer editing activity (Englisch et al., 1986). In contrast, pre-steady state kinetics in ValRS implicate posttransfer editing to be the major pathway (Fersht and Kaethner, 1976). Recent biochemical studies confirmed that unlike IleRS and LeuRS, tRNA-dependent pretransfer editing is negligible in ValRS due to a rapid amino acid transfer step (Dulic et al., 2010).

![Figure 1.4. Translocation model for tRNA CCA-end movement toward the editing domain during posttransfer editing by LeuRS. Structural superposition of *T. thermophilus* tRNA\textsuperscript{Leu} in the posttransfer editing conformation (blue, PDB code 2BYT) onto *P. horikoshii* tRNA\textsuperscript{Leu} (red) and LeuRS in the aminoacylation state (PDB code 1WZ2). *P. horikoshii* LeuRS aminoacylation active site is shown in pale green and the CP1 editing domain in orange.](image)
With respect to pretransfer editing, despite detailed structural and biochemical analyses, the mechanism and site for pretransfer editing within the enzyme had remained unclear (Ko¨hrer et al., 2009; Ling et al., 2009a). Experiments conducted using a selected DNA aptamer replacing a tRNA, combined with mutational analyses suggested pretransfer editing occurred within the CP1 domain (Nomanbhoy et al., 1999; Farrow and Schimmel, 2001; 

Figure 1.5. Structure of T. thermophilus LeuRS in posttransfer editing conformation. Structural alignment of T. thermophilus LeuRS in the absence (gray, PDB code 1H3N) and presence (colored as following) of tRNA^{Leu} in the posttransfer editing conformation (PDB code 2BYT). In the editing conformation, LeuRS catalytic domain is shown in green, Zn-binding domain in orange, leucyl-specific insertion in blue, CP1 editing domain in magenta, anticodon-binding domain in red, and C-terminal domain in yellow. tRNA binding results in a 35° rigid-body rotation of the editing domain leading to interaction between the hinge region of the editing domain and the 3'-CCA-end of the tRNA. The leucyl-specific domain also rotates by ~19° with respect to the uncomplexed LeuRS.

1.2.3. Editing via intramolecular cyclization by MetRS

MetRS differs from other class Ia aaRSs in that it contains a truncated CP1 domain, which lacks editing activity. Instead the synthetic active site of MetRS doubles up as an editing site to clear misactivated noncognate adenylates via a tRNA-independent pretransfer editing pathway (Fersht and Dingwall, 1979a; Jakubowski, 1991). Homocysteine (Hcy), which is a smaller naturally occurring precursor in the methionine
biosynthetic pathway, differs from Met by a single methyl group and hence poses a challenge for accurate amino acid discrimination by MetRS. MetRS can bind smaller amino acids like Hcy, however following misactivation Hcy undergoes an intramolecular cyclization reaction forming the non-productive homocysteine thiolactone, which cannot participate in the second tRNA transfer step (Jakubowski and Fersht, 1981). Although the cyclization is a tRNA-independent process, the presence of tRNA accelerates the reaction by 2-fold. It is possible that the same cyclization reaction also occurs posttransfer (Hendrickson and Schimmel, 2003). Interestingly, cognate Met is also cyclized to form S-methyl homocysteine by the MetRS active site in vitro, but this reaction appears to be negligible in vivo (Jakubowski, 1993). Mutational analyses have identified several conserved residues in E. coli MetRS that play a crucial role in discrimination against Hcy and therefore appear to be important for editing. The backbone amino and carboxyl groups of Met form specific contacts with the strictly conserved Asp52 and Arg233 while the side chain methyl group and sulfur atom of Met form specific interactions with Trp305 and Tyr15 in the active site of MetRS (Ghosh et al., 1991; Kim et al., 1993). Structural studies provide further insight into specific recognition of Met in the active site (Serre et al., 2001). Met binding induces a series of conformational rearrangements leading to the formation of a protective hydrophobic cover around the side chain of Met. Hcy binding fails to induce any conformational change leaving the side chain accessible for cyclization by ATP. The MetRS active site appears to have evolved specifically to clear Hcy, the natural competitor of Met, since MetRS does not edit unnatural Met analogs such as selenomethionine, telluromethionine
or S-nitrosohomocysteine. This property has been exploited to incorporate unnatural amino acid analogs into proteins using Met auxotrophic strains (Besse et al., 1997; Jakubowski, 2000).

1.3. Class II aaRS editing mechanisms

Unlike most editing aaRSs of class I, which share a common CP1 editing domain, class II aaRSs differ greatly in their structural organization (Table 1.1), and employ distinct mechanisms to edit noncognate substrates.

1.3.1. ThrRS

ThrRS is the first class II enzyme whose editing site was well-characterized and mechanism best understood. ThrRS occasionally misactivates and misacylates Ser onto tRNA^{Thr}. In the active site of ThrRS, a Zn^{2+} ion is coordinated by 3 residues, Cys334, His385 and His511, as well as to a water molecule (Sankaranarayanan et al., 2000; Sankaranarayanan et al., 1999). The hydroxyl groups of cognate Thr and noncognate Ser can displace the water molecule and coordinate with the active site Zn^{2+}. Val, an isostere lacking the hydroxyl moiety is therefore not misactivated by ThrRS, but Ser is, and Ser-tRNA^{Thr} undergoes posttransfer hydrolysis (Dock-Bregeon et al., 2000). The N-terminal N2 domain of bacterial and eukaryal ThrRS is responsible for posttransfer editing activity. Deletion of the N-terminal editing domain did not affect aminoacylation activity but abolished posttransfer editing as this variant ThrRS can efficiently mischarge Ser-tRNA^{Thr}. High resolution X-ray crystal structures of *E. coli* ThrRS in complex with both
pre-and posttransfer substrate analogs were solved revealing a general base catalyzed editing mechanism involving two water molecules (Dock-Bregeon et al., 2004). His73, one of the histidines of the conserved HXXXH motif may deprotonate the first water molecule, which then performs a nucleophilic attack on the carboxyl carbon of the Ser. The second water molecule, stabilized by Lys156, acts as proton donor to the O3’ oxygen atom of the A76 leaving group. Substitution of His73 and Lys156 causes a drastic decrease in the catalytic rate corroborating this proposed hydrolytic mechanism (Waas and Schimmel, 2007). His77, Tyr104 and Asp180 are other molecular determinants that expel cognate Thr and mediate hydrolysis (Dock-Bregeon et al., 2004). Additionally the 2’-OH of A76 on tRNA^{Thr} is important for both aminoacylation and editing in *E. coli* ThrRS, where it is hypothesized to form a hydrogen bond with a catalytic water molecule (Minajigi and Francklyn, 2010). Recent biochemical and structural studies with the editing domain of ThrRS from *P. abyssi* (Pab-NTD) provided mechanistic insights into cognate amino acid discrimination at the editing active site (Hussain et al., 2010).

Binding studies by isothermal titration calorimetry revealed only a 10-fold difference in $K_D$ for noncognate Ser versus cognate Thr substrate analogs. Structural analyses implicated a critical role for the invariant Lys121, whose side chain is repositioned to remove the catalytic water molecule upon Thr binding, thereby preventing hydrolysis of correctly charged Thr-tRNA^{Thr} (Fig. 1.6). A series of small but important conformational changes involving editing site residues Tyr120 and Lys121 impair hydrolysis of cognate substrate. Hence, cognate Thr is not excluded from the editing site based on steric hindrance. This is analogous to the Thr rejection mechanism in ValRS (Fersht and Kaethner, 1976).
Detailed pre-steady state kinetic studies in *E. coli* ThrRS indicate that in the absence of tRNA\textsuperscript{Thr}, ThrRS also possesses a pretransfer editing function against misactivated Ser-AMP (Minajigi and Francklyn, 2010). In the presence of tRNA\textsuperscript{Thr} however, the aminoacyl transfer step is fast relative to the rate of pretransfer editing, and posttransfer hydrolysis becomes the dominant route (Fig. 1.7).

The N2 editing domain found to be conserved in bacterial and eukaryotic cytosolic ThrRSs is missing in most archaeal and mitochondrial versions of the enzyme (Beebe et al., 2004; Dock-Bregeon et al., 2000; Korencic et al., 2004). Instead many archaea harbor an unrelated N-terminal domain and in crenarchaea the ThrRS synthetic active site (Thr-cat) and editing domain (Thr-ed) are encoded by two separate genes, where Thr-ed acts in *trans* to hydrolyze misacylated Ser-tRNA\textsuperscript{Thr}. From an evolutionary standpoint, the lack of structural conservation with respect to the editing domains suggests that the ThrRS editing domain may have diverged early in a separate lineage. Alternatively the editing domain may have been acquired later in evolution in order to satisfy the need for fidelity (Ling et al., 2009a).
Figure 1.6. Superposition of posttransfer substrate analog complexes with *P. abyssi* ThrRS N-terminal domain (Pab-NTD) (adapted from Hussain et al., 2010). Pab-NTD-Ser3AA is represented in a darker shade and Pab-NTD-Thr-3AA in a lighter shade. Both complexes show similar positioning of Ser3AA and Thr3AA. Subtle repositioning of editing pocket residues Tyr120 and Lys121 can be observed. The positions of water molecules W1 and W2 are with respect to the Pab-NTD-Ser3AA complex, the catalytic water W1 is absent in the Pab-NTD-Thr3AA structure. The repositioning of Lys121 expels W1 through steric hindrance, which is crucial for discrimination against the cognate Thr in the editing domain.

Figure 1.7. Kinetic partitioning of quality control pathways in editing aaRSs. Pretransfer editing competes with the transfer of amino acid to the tRNA. A. If the aminoacyl transfer step is relatively slow, pretransfer hydrolysis of noncognate aa-AMP can occur while any mischarged aa-tRNA undergoes posttransfer editing. B. If the rate of transfer reaction is faster than the rate of pretransfer editing, most of the misactivated aa-AMP is rapidly transferred to the tRNA. Therefore, posttransfer hydrolysis becomes the dominant editing pathway. Scheme adapted from Dulic et al., 2010 and Minajigi and Francklyn 2010.
1.3.2. PheRS

PheRS has a heterotetrameric structure, \((\alpha\beta)_2\) wherein the \(\alpha\)-subunit contains the aminoacylation active site (Goldgur et al., 1997) and the B3/B4 domains of the \(\beta\)-subunit constitute a unique non-canonical editing site (Roy et al., 2004). Substrate specificity in the synthetic site of \(E.\ coli\) PheRS is determined by a conserved Ala294 residue of motif 3, which governs the size of the binding pocket (Ibba et al., 1994). Replacement of Ala294 with Gly reduces specificity to cognate Phe and this modified enzyme can efficiently misactivate several unnatural amino acids including \(p\)-Cl-Phe and noncognate Tyr (Ibba and Hennecke, 1995; Ibba et al., 1994; Kirshenbaum et al., 2002). However, Tyr appears to be the sole substrate for editing both \textit{in vitro} and \textit{in vivo} (Roy et al., 2004).

In early studies with PheRS, Yarus et al., (1972) observed that PheRS can hydrolyze mischarged Ile-tRNA\textsubscript{Phe} (Yarus, 1972), however further studies are required to assess the relevance of this activity \textit{in vivo}. Molecular modeling of the posttransfer editing site of \(E.\ coli\) PheRS based on the structure of \(T.\ thermophilus\) PheRS suggests that the editing site lies at the interface of the B3/B4 domains at a distance of \(~40\)Å away from the synthetic site (Kotik-Kogan et al., 2005; Roy et al., 2004). Amino acid discrimination at the editing site is dictated by a conserved Glu334, which forms a hydrogen bond with the \(p\)-hydroxyl group of Tyr (Ling et al., 2007a; Roy et al., 2004). Substitution of Glu334 with either Ala or Ile increases hydrophobicity at the editing site and reverses substrate specificity allowing for cognate Phe-tRNA\textsubscript{Phe} hydrolysis. In addition, mutagenesis studies showed that Arg244, His265, Gly318, Thr354 and Ala356 are all important for editing. Replacement of either Ala356 or Gly318 with the bulkier Trp blocks the editing site.
making it inaccessible to mischarged Tyr-tRNA\textsuperscript{Phe} (Ling et al., 2007a; Roy et al., 2004). All the important editing site residues identified in PheRS do not appear to play a catalytic role, but instead assist in efficient substrate binding. The 3'-OH group of A76 on tRNA\textsuperscript{Phe} may participate in catalysis by activating a nearby water molecule, which subsequently performs a nucleophilic attack leading to hydrolysis of the Tyr-tRNA\textsuperscript{Phe} ester bond (Ling et al., 2007a). PheRS can perform posttransfer hydrolysis either in \textit{cis} via the translocation of the 3'-end of mischarged Tyr-tRNA\textsuperscript{Phe} or in \textit{trans} where any mischarged Tyr-tRNA\textsuperscript{Phe} that is released into solution can re-bind to the PheRS editing site according to the “dissociation-reassociation” or “resampling” model (Chapter 2) (Ling et al., 2009b). The rate of release of mischarged Tyr-tRNA\textsuperscript{Phe} is comparable to that of cognate Phe-tRNA\textsuperscript{Phe}, which is consistent with the observation that product release is not rate-limiting in class II aaRSs, corroborating the resampling pathway (Baltzinger and Holler, 1982b; Ibba et al., 1995; Ling et al., 2009b; Zhang et al., 2006). Biochemical and kinetic studies also reveal that the PheRS editing site can compete effectively with EF-Tu to eliminate mischarged Tyr-tRNA\textsuperscript{Phe}, thereby preventing mistranslation of Phe codons (Chapter 2) (Ling et al., 2009b; Ling et al., 2007c). Posttransfer editing by PheRS appears to be the only proofreading step for translation of Phe codons since any mischarged Tyr-tRNA\textsuperscript{Phe} that evades editing is not later discriminated by either EF-Tu or the ribosome (Ling et al., 2007c; Roy et al., 2004) (Chapter 2). Whereas \textit{E. coli} PheRS has robust posttransfer editing function, the contribution of pretransfer editing to overall quality control in PheRS appears to be minimal (Roy et al., 2004).
The crystal structure of an N-terminal fragment of *P. horikoshii* PheRS suggests a different orientation for the editing site compared to that of *T. thermophilus* PheRS (Kotik-Kogan et al., 2005). Additionally, structure-based alignments show that the archaeal/eukaryal-type PheRSs have diverged from their bacterial counterparts and lack conservation of the critical residues found in bacterial PheRS (Sasaki et al., 2006). Ala-scanning mutagenesis of *P. horikoshii* PheRS identified key residues that may be involved in specific recognition of the *p*-OH group of Tyr and an Asn residue is suggested to play a role in catalysis.

Although eukaryotic cytosolic PheRSs share a similar structure to that of bacterial enzymes and possess posttransfer editing activity, their mitochondrial counterparts are monomers lacking the editing function (Igloi et al., 1978; Lin et al., 1984; Roy et al., 2005a) (See section 1.6 “Loss of editing function in aaRSs”). Also, unlike some of the other editing class II aaRSs, no *trans*-editing factors homologous to PheRS have been identified to date.

### 1.3.3. AlaRS

AlaRS is known to misactivate noncognate Ser and Gly at frequencies of 1/500 and 1/250, respectively, which is much higher than the overall error rate in aminoacylation of ~10^{-4} (Loftfield and Vanderjagt, 1972; Tsui and Fersht, 1981). This problem is circumvented by posttransfer hydrolysis of both Ser- and Gly-tRNA\textsubscript{Ala} by an internal editing domain of AlaRS, which is homologous to the N2 domain of bacterial and eukaryal ThrRSs (Beebe et al., 2004; Beebe et al., 2003). Co-crystal structures of
truncated AlaRS from *A. aeolicus* lacking the editing domain with cognate Ala, noncognate Ser, and Gly suggested an important role for Asn194 in the synthetic active site in accommodating Ser (Swairjo and Schimmel, 2005). Rigorous structural and mutational studies have identified a strictly conserved residue Asp235 in the active site of *E. coli* AlaRS as the major determinant for binding and misactivation of Ser (Guo et al., 2009b). While the carboxyl group of Asp235 interacts with the α-amino group of Ala, the same interaction helps bind the noncognate Gly and Ser. In the case of Ser, the γ-OH group makes two extra contacts in the AlaRS active site, with the carboxyl group of Asp235 and the backbone amino group of the neighboring Gly 237. Noncognate Ser is therefore naturally prone to misactivation by AlaRS. For this reason, several redundant posttransfer editing pathways in addition to the AlaRS editing site have evolved to ensure complete hydrolysis of mischarged Ser-tRNA\textsubscript{Ala}. Freestanding editing domains called AlaX proteins (AlaXps) are universally found in all organisms and help in solving the so-called “serine paradox” (Ahel et al., 2003; Guo et al., 2009b). In the AlaRS editing site, steric hindrance alone cannot explain the expulsion of cognate Ala while retaining the larger Ser moiety. Hence, the editing site must use chemical discrimination, maintaining specific hydrogen bonding interactions with Ser while repelling the hydrophobic side chain of Ala, similar to the case of Thr rejection by ValRS (Fersht, 1999; Tsui and Fersht, 1981). Mutational studies of *E. coli* AlaRS suggest that residues Cys666, Gln584 and Ile667 have important roles in determining editing site specificity (Beebe et al., 2003; Pasman et al., 2011). Cys666 is conserved in the signature motif HXXXH/CXXXH found in AlaRS and ThrRS sequences, and is involved in Zn\textsuperscript{2+} coordination. The
mutation of Cys666 to Ala switched editing specificity to cognate Ala-tRNA$^{\text{Ala}}$ and decreased Ser- and Gly-tRNA$^{\text{Ala}}$ hydrolysis. Cys666Ala AlaRS containing cells were highly sensitive to high concentrations of noncognate Ser and Gly in the growth medium. Cys666 was therefore identified as a critical editing determinant and the coordinated Zn$^{2+}$ ion has been proposed to provide Ser specificity. In $P$. horikoshii residue Thr30 is a critical determinant for binding Ser over Ala (Sokabe et al., 2005). Replacement of Gln584 in $E$. coli AlaRS, which is analogous to Thr30 in $P$. horikoshii AlaX, with Asn leads to greater deacylation of cognate Ala-tRNA$^{\text{Ala}}$, while hydrolysis of noncognate product is unaffected. Introduction of a Gln584His replacement into a Cys666Ala background leads to a robust mischarging activity for AlaRS and significant synthesis of both Ser- and Gly-tRNA$^{\text{Ala}}$. However, the Gln584His mutation alone has minimal effect on both misacylation and posttransfer editing (Beebe et al., 2003; Pasman et al., 2011). Ile667 appears to play a structural role in positioning the mischarged Ser-tRNA$^{\text{Ala}}$ in the correct conformation for hydrolysis. The Ile667Glu mutation is analogous to the Ala734Glu sticky mutation in mouse AlaRS, associated with neurodegeneration (Lee et al., 2006) and underscores the significance of the Ile667 residue in enhancing specificity and efficiency of Ser-tRNA$^{\text{Ala}}$ deacylation. Pretransfer editing has not been explicitly shown to occur in AlaRS and overall editing by AlaRS is known to be tRNA-dependent (Beebe et al., 2003).
1.3.4. ProRS

Pre- and posttransfer editing of Ala-AMP and Ala-tRNA$^{\text{Pro}}$, respectively, were first shown for *E. coli* ProRS (Beuning and Musier-Forsyth, 2000). ProRS in most bacteria contains a unique insertion domain “INS” (~180 residues) between the conserved class II motifs 2 and 3 (Cusack et al., 1998; Wong et al., 2002). This INS domain was shown to include the editing active site, which could fold into a functional unit when expressed independently (Wong et al., 2003). It is noteworthy that lower eukaryal and archaeal ProRSs contain N- and C-terminal extension domains, respectively (Wong et al., 2002). While *M. jannaschii* and *P. falciparum* ProRS can catalyze the posttransfer editing reaction, yeast and human ProRSs are deficient in this activity (SternJohn et al., 2007; Beuning and Musier-Forsyth, 2001; Ahel et al., 2003). Ala scanning mutagenesis of *E. coli* ProRS revealed key residues involved in posttransfer editing of Ala-tRNA$^{\text{Pro}}$ including the well-conserved Lys279 (Wong et al., 2002). Lys279Ala substitution leads to a dramatic reduction in the editing activity, while aminoacylation is not affected. Another residue critical for amino acid specificity is His369. The His369Ala mutation causes an increase in Pro-tRNA$^{\text{Pro}}$ deacylation while decreasing noncognate Ala-tRNA$^{\text{Pro}}$ hydrolysis by 80%. In addition to Ala, *in vitro* studies show that noncognate Cys is also misactivated and/or mischarged by ProRS (Ahel et al., 2002; Beuning and Musier-Forsyth, 2001). The ProRS INS domain, however, cannot deaclylate mischarged Cys-tRNA$^{\text{Pro}}$. This function is instead performed by a single domain *trans*-editing factor named YbaK, which is homologous to the INS editing domain (Wolf et al., 1999; Zhang et al., 2000) (discussed in detail in section 1.5). Recent biochemical data suggest that
Ybak deacylates Cys-tRNA$^{\text{Pro}}$ via a substrate-assisted catalytic mechanism, which relies on thiol-specific chemistry (So et al., 2011). Therefore, a triple sieve model is proposed for ProRS editing wherein the INS domain acts as the second sieve and Ybak serves as the third chemical sieve to clear mischarged Ala-tRNA$^{\text{Pro}}$ and Cys-tRNA$^{\text{Pro}}$, respectively (An and Musier-Forsyth, 2004, 2005; So et al., 2011).

Pretransfer editing is well-studied in the ProRS system. Although early studies show higher ATP consumption in the presence of Ala indicating pretransfer editing in *E. coli* ProRS (Beuning and Musier-Forsyth, 2000), the mechanism and site of pretransfer editing remained unclear for a long time. In recent biochemical studies, ProRS representatives from all three kingdoms have been shown to catalyze pretransfer hydrolysis of Ala-AMP (Hati et al., 2006; Splan et al., 2008). In the presence of tRNA, posttransfer editing is the dominant pathway, however in the absence of tRNA, significant pretransfer editing occurs. In addition, the INS domain was found to be dispensable for pretransfer editing activity, suggesting that hydrolysis of noncognate adenylates occurs within the synthetic active site. Kinetic studies using an *E. coli* ProRS ΔINS mutant, *M. jannaschii* and human ProRSs, all of which lack a separate posttransfer editing domain, show tRNA-independent pretransfer hydrolysis of Ala-AMP (Hati et al., 2006; Splan et al., 2008). About 80% of the noncognate adenylate is hydrolyzed actively by the *E. coli* ProRS and ≤20% of Ala-AMP is subject to spontaneous hydrolysis in solution via the selective release mechanism.
1.3.5. SerRS

Unlike most class II editing aaRSs, SerRS does not possess a distinct editing domain (Cusack et al., 1990). Kinetic analyses of misactivation of noncognate Thr, Cys and other unnatural amino acids by *E. coli* and *S. cerevisiae* SerRSs revealed the existence of a tRNA-independent pretransfer hydrolysis pathway (Gruic-Sovulj et al., 2007). Addition of tRNA did not stimulate the rate of AMP-formation. SerRS employs different pretransfer editing strategies: the noncognate aminoacyl-adenylates may either be hydrolyzed within the active site of SerRS or selectively released into solution leading to non-enzymatic hydrolysis. In the case of Thr, SerRS catalyzed Thr-AMP formation is 17-fold faster than the rate of spontaneous hydrolysis in solution indicating that misformed Thr-AMP is actively hydrolyzed by SerRS (Gruic-Sovulj et al., 2007). This study with SerRS, an aaRS that completely lacks a separate editing site, reaffirms that tRNA-independent pretransfer editing can easily occur within the confines of the synthetic active site as observed in the case of ProRS.

1.3.6. LysRS-II

LysRS is the only aaRS known to exist in both classes I and II. LysRS I and II are not present together in the same organism with only a few exceptions (Ibba et al., 1997b; Polycarpo et al., 2003). Biochemical and structural studies revealed that despite structurally divergent active site topologies, both LysRSs recognize the amino acid and tRNA substrates in a similar fashion (Ibba et al., 1999; Terada et al., 2002). While LysRS-I displays a high selectivity towards its cognate substrate and requires no editing
step, LysRS-II misactivates non-coded amino acids, homoserine and ornithine, and then cyclizes them to a lactone and lactam, respectively, via tRNA-independent pretransfer editing (Jakubowski, 1997, 1999; Levengood et al., 2004). Phe426 in the *E. coli* LysRS-II active site is implicated in amino acid specificity through hydrophobic interaction with the aliphatic side chain of Lys (Ataide and Ibba, 2004; Terada et al., 2002). Furthermore, a set of negatively charged residues Glu240, 278 and 428 contribute to effective binding of positively charged Lys. Introduction of a positively charged residue into this acidic pocket disrupts Lys binding and removal of the negative charge reduces the catalytic efficiency of lysylation (Ataide and Ibba, 2004).

### 1.4. Nucleotide determinants for editing

Recognition of unique identity elements on tRNA is important for aminoacylation (Giegé et al., 1998) as well as editing functions. Among class I aaRSs, the contribution of molecular determinants in tRNA to editing have been analyzed in detail for IleRS (Hale et al., 1997). The D-loop residues G16, D20 and D21 of tRNA^{ile} are critical for editing, but not for aminoacylation. Interestingly, posttransfer editing does not require these D-loop nucleotides (Farrow et al., 1999), which is consistent with the observation that the D-loop region is positioned away from IleRS in the posttransfer editing mode (Silvian et al., 1999). Hence the D-loop nucleotides of tRNA^{ile} may trigger a conformational change required for the translocation of misactivated and/or mischarged products from the synthetic to editing active site during pretransfer editing (Farrow et al., 1999). In contrast to the IleRS system, ValRS and LeuRS have common tRNA determinants for
aminoacylation and editing. Mutagenesis of the 3′-terminal A76 of tRNA<sub>Val</sub> critically impacted editing and also led to loss of aminoacylation specificity and a defect in posttransfer editing, highlighting the role of the terminal adenine in aminoacylation accuracy (Tardif and Horowitz, 2002; Tardif et al., 2001). Examination of several tRNA<sub>Leu</sub> deletion and substitution mutants indicated that the elbow region comprising the D/TΨC-loop is crucial for both posttransfer editing and aminoacylation (Du and Wang, 2003). However, structural and biochemical analyses of a C-terminal deletion mutant of LeuRS from <i>P. horikoshii</i> suggest that these identity elements may be recognized differently during aminoacylation and editing (Fukunaga and Yokoyama, 2005b).

The other important molecular element influencing editing is the position of the aminoacyl ester bond. Class I aaRSs aminoacylate at the 2′-OH of A76 on tRNAs, while all class II aaRSs (with the exception of PheRS) preferentially utilize the 3′-OH (Ibba and Söll, 2000; Sprinzl and Cramer, 1975). IleRS deacylates Val-2′-dA76 tRNA<sup>Ile</sup>, but not the 3′-dA76 version. This observation implies that IleRS requires transacylation of amino acid from the 2′-OH position to the 3′-OH of A76 on tRNA<sup>Ile</sup> for posttransfer hydrolysis to occur (Nordin and Schimmel, 2003). Indeed, rapid transacylation of amino acid between the 2′- and 3′- positions of terminal A76 is shown to occur in solution (Taiji et al., 1983). ValRS, on the contrary, hydrolyzes 2′-linked mischarged aminoacyl tRNAs (Nordin and Schimmel, 2003). Also, structural studies of LeuRS in the posttransfer editing mode demonstrate that only 2′-linked amino acids are substrates for editing (Lincecum et al., 2003).
In class II aaRSs, tRNA determinants for editing have been most extensively studied in AlaRS. AlaRS utilizes the same identity element, the conserved G3:U70 base pair, for aminoacylation and editing (Beebe et al., 2008). However, AlaRS utilizes two distinct motifs: the N-terminal catalytic domain and a separate motif in the editing domain to recognize the critical G:U base pair during aminoacylation and editing, respectively. Therefore, it is unlikely that misacylated tRNA undergoes direct translocation for posttransfer editing, where only the 3′ CCA-end of tRNA is believed to shuttle between the aminoacylation and editing sites. It is more plausible that the mischarged tRNA dissociates and re-binds to the editing site for proper recognition of the G:U base pair by the editing domain motif. In contrast to AlaRS, tRNA recognition by *E. coli* PheRS appears to be conserved between charging and editing (Ling et al., 2009b), which supports the *cis*-editing pathway (Chapter 2). In addition, substrates generated by modification and substitutions at the 3′-OH site of A76 in tRNA*Phe* are immune to posttransfer hydrolysis by PheRS, revealing a critical role for the 3′-OH in editing (Ling et al., 2007a; Roy et al., 2004).

### 1.5. Trans-editing factors and triple sieves

Posttransfer editing in *trans* has been observed for class II aaRSs, both PheRS and ProRS being able to resample noncognate mischarged products (Ling et al., 2009b) (Chapter 2). In addition to these posttransfer editing domains, several freestanding editing factors have been discovered that contribute to hydrolysis of misacylated aa-tRNAs (Table 1.1). These *trans*-acting factors are ubiquitous and diverse, suggesting divergent
evolution of these proofreading modules based on the selective pressure for their function (Ahel et al., 2003; Wydau et al., 2009). All the autonomous trans-acting factors found to date are homologous to class II editing domains, but not class I CP1 domains. This pattern is consistent with the differences in aminoacylation kinetics observed for class I versus class II aaRSs. Product release is rapid and not rate limiting in class II aaRSs unlike class I (Chapter 2) (Baltzinger and Holler, 1982; Ibba et al., 1995; Ling et al., 2009b; Zhang et al., 2006). Class I aaRSs do not readily release the mischarged aa-tRNAs and hence would not require proofreading by a trans editing factor. The first trans editing activity discovered was against D-Tyr by D-Tyr-tRNA_{Tyr} deacylase (DTD) (Calendar and Berg, 1967). Although only L-amino acids are employed in protein synthesis, TyrRS can mischarge D-Tyr. DTD can efficiently hydrolyze D-Tyr-tRNA_{Tyr}, and possibly other D-aa-tRNAs to a smaller extent, and this activity is essential for cell viability. DTDs are universal and three distinct classes are found across the three kingdoms of life: DTD1 in most bacteria and eukaryotes, DTD2 in archaea and plants, and DTD3 in cyanobacteria (Wydau et al., 2009). Sequence and structural similarities exist among DTDs and editing domains of some archaean ThrRSs (Dwivedi et al., 2005; Rigden, 2004). Specificity and enantioselectivity of E. coli DTD was switched to that of ThrRS when a single conserved Met129 residue was substituted by Lys, which is characteristic of ThrRS, indicating that the archaean ThrRS may have evolved from the freestanding DTD (Hussain et al., 2006).

Sequence homology studies identified several freestanding editing factors homologous to the editing domains of class II ProRS, AlaRS and ThrRS (Ahel et al., 2003; Wolf et al.,
1999; Zhang et al., 2000). The family of autonomous editing domains homologous to the ProRS INS domain (designated the Ybak superfamily) are further subdivided into at least 5 classes based on their sequence homology and substrate specificities (So et al., 2011; Kumar et al., 2012; Ahel et al., 2003). Although ProRS has an INS domain that can efficiently hydrolyze mischarged Ala-tRNA\textsuperscript{Pro}, it lacks editing activity against Cys-tRNA\textsuperscript{Pro} (Ahel et al., 2002; Beuning and Musier-Forsyth, 2001). This conundrum was solved with the discovery of \textit{H. influenzae} YbaK, a protein homologous to the INS domain (Wolf et al., 1999; Zhang et al., 2000; Wong et al., 2003). YbaK possesses Cys-tRNA\textsuperscript{Pro} deacylase activity both \textit{in vitro} and \textit{in vivo} (An and Musier-Forsyth, 2004; Ruan and Söll, 2005). In the absence of ProRS, YbaK deacylates any Cys-tRNA including Cys-tRNA\textsuperscript{Cys}. In the presence of ProRS, however, YbaK forms a ternary complex with ProRS and tRNA\textsuperscript{Pro}, thereby achieving the higher substrate specificity and efficiency required for Cys-tRNA\textsuperscript{Pro} hydrolysis (An and Musier-Forsyth, 2005). EF-Tu and YbaK act in competition to bind Cys-tRNA\textsuperscript{Pro}, suggesting that editing by YbaK is an important quality control step prior to Cys-tRNA\textsuperscript{Pro} release from ProRS and subsequent sequestration by EF-Tu. Altogether, a triple sieve model for proofreading has been proposed for the ProRS system where YbaK acts as a third “chemical” sieve (So et al., 2011). Another subclass of INS domain paralogs consists of the PrdX proteins. PrdX from \textit{C. sticklandii} can efficiently deacylate Ala-tRNA\textsuperscript{Pro} but not Cys-tRNA\textsuperscript{Pro}. The presence of PrdX likely compensates for the lack of INS domain in the ProRS of \textit{C. sticklandii} (Ahel et al., 2003).
Freestanding editing paralogs of AlaRS, termed the AlaX proteins (AlaXps) are conserved across the three kingdoms of life (Ahel et al., 2003; Guo et al., 2009a). AlaXps are classified into three types: Ia, Ib and II and harbor deacylation activity against tRNA$^{\text{Ala}}$ misacylated with Ser or Gly. Type II AlaXps contain a C-Ala domain homologous to the less conserved C-terminal domain in AlaRS (Guo et al., 2009a). Phylogenetic analyses suggest a common ancestry for all AlaXps and concurrent evolution of AlaXp-II and the editing domain of AlaRS. Indeed, structural and biochemical analyses have shown an important role for this C-Ala domain in linking the aminoacylation and editing sites, which in turn aids in accurate tRNA$^{\text{Ala}}$ recognition, thereby preventing mistranslation (Guo et al., 2009a). In addition, Ser misactivation seems to be a potential problem to AlaRS as shown by crystal structure analyses, wherein the $\gamma$-hydroxy group of Ser makes two extra contacts in the AlaRS synthetic active site. One of these interactions is the critical hydrogen bond with the highly conserved Asp235, which also plays an important role in transferring the activated aminoacyl moiety to the 3'-end of the tRNA (Guo et al., 2009b). The crystal structure of $P.$ horikoshii AlaX suggests an editing mechanism mediated by a network of hydrogen bonds formed between conserved Thr30, Asp20 and a water molecule (Sokabe et al., 2005). These structural studies along with mutagenesis data indicate that Thr30 is crucial for discrimination against Ala by AlaX. Thus, AlaXps play a prominent role acting as a third sieve in the AlaRS system to effectively clear any mischarged Ser-tRNA$^{\text{Ala}}$.

Trans-acting factors have also been associated with editing misacylated tRNA$^{\text{Thr}}$ (Korenčic et al., 2004). In crenarchaea, threonylation and editing functions are separately
carried out by ThrRS-cat and ThrRS-ed, respectively, each individually encoded by two different genes in the chromosome. ThrRS-ed acts in \textit{trans} to deacylate misformed Ser-tRNA$^\text{Thr}$, although it differs from other factors discussed above in that ThrRS-ed contains an additional tRNA anticodon-binding domain. Other hypothetical editing modules are being discovered in the genomes of different organisms, such as DTD in \textit{Plasmodium falciparum}, however it remains to be tested if any of them are catalytically active (Khan et al., 2011). AaRSs are believed to be modular enzymes composed of an ancient synthetic core to which other functional units including the editing domain and tRNA-binding domain may have been added later in evolution (Ahel et al., 2003; Schimmel and Ribas De Pouplana, 1995). Clearly, autonomous \textit{trans}-acting factors have been retained in many cases as an additional checkpoint beyond editing by the canonical editing domain in aaRSs. In some cases, \textit{trans} editing factors compensate for the loss of editing in the parental aaRS and in others the function is, so far, unknown.

\textbf{1.6. Loss of editing function in aaRS}

Some aaRSs have lost their editing function, including several mitochondrial enzymes such as the monomeric yeast and human mitochondrial PheRSs (Bullard et al., 1999; Roy et al., 2005a). These PheRSs lack a separate site for editing unlike their bacterial (Roy et al., 2004) and eukaryotic cytosolic counterparts (Roy et al., 2005a), which have heterotetrameric architectures. In the case of mitochondrial PheRS, biochemical, structural and kinetic analyses reveal high specificity of the enzyme towards both the amino acid and tRNA substrates (Reynolds et al., 2010b; Yadavalli et al., 2009)
Human mitochondrial ProRS also does not possess a posttransfer editing domain (Musier-Forsyth K, 1997). Archaeal and higher eukaryotic cytosolic ProRSs lack an intact editing domain, while ProRS in lower eukaryotes is defective in editing despite the presence of a bacterial-type INS editing domain (Beuning and Musier-Forsyth, 2001). Some of the eukaryotic cytosolic ProRSs display a higher initial amino acid selectivity to compensate for the loss of editing. Similarly, human mitochondrial LeuRS (Lue and Kelley, 2005) as well as Mycoplasma LeuRS (Li et al., 2011) and PheRS (Chapter 3) contain a defunct editing site that is defective in posttransfer editing. At least in the case of mitochondrial LeuRS, kinetic analyses suggest that the synthetic active site provides stringent selection against the noncognate substrate to maintain translational fidelity (Lue and Kelley, 2005). Nonetheless, yeast mitochondrial LeuRS has an intact editing site, which when disrupted has virtually no effect on cell viability (Karkhanis et al., 2006).

1.7. Mistranslation and role of editing in cell

The term mistranslation refers to a cumulative result of all the mistakes that occur during every step of protein synthesis ultimately leading to misincorporation of amino acids. As mentioned earlier, aaRSs provide the first checkpoint for quality control in translation. Following aminoacylation, EF-Tu binds and delivers the aa-tRNA to the ribosome. EF-Tu optimizes binding affinity for all cognate aa-tRNA pairs by thermodynamic compensation, while the noncognate aa-tRNAs have a wider range of affinities (LaRiviere et al., 2001). *In vitro* kinetic analyses showed that once the
noncognate Tyr-tRNA<sup>Phe</sup> evades posttransfer editing by PheRS, it is successfully bound and delivered by EF-Tu and utilized in protein synthesis by the ribosome resulting in mistranslation of Phe codons (Chapter 2) (Ling et al., 2007c). AaRSs exhibit a high level of fidelity during aminoacylation with an error rate of ~1 in 10,000 in vitro (Ibba and Söll, 1999), however the role of editing in vivo is yet to be resolved as quality control by aaRSs is often dispensable under normal laboratory conditions. Quality control mechanisms may become essential under different cellular stress conditions (Reynolds et al., 2010a). Firstly, nutrient limitation where the cognate to near cognate amino acid ratio is low may pose a major problem. Experimental evidence comes from mouse fibroblasts expressing an editing defective AlaRS (Lee et al., 2006) and an archaeal <i>S. sulfotaricus</i> ThrRS-ed deletion strain (Korencic et al., 2004) both of whose growth and viability are sensitive to exogenous addition of excess noncognate Ser. Studies with <i>E. coli</i> ValRS (Nangle et al., 2002) and LeuRS (Karkhanis et al., 2007) also reiterate the importance of aaRS editing activity in maintaining cell viability under conditions where noncognate amino acids are in excess. Secondly, lack of quality control can trigger heat shock or unfolded protein response (UPR) due to the accumulation of misfolded peptides. Mistranslation in cells expressing editing defective aaRSs can induce UPR causing upregulation of heat shock proteins as observed in editing deficient <i>E. coli</i> (Ruan et al., 2008) and mouse neuronal cells expressing an editing defective AlaRS (Lee et al., 2006). Thirdly, quality control is apparently more important under slow growth conditions. An editing defective IleRS renders <i>E. coli</i> more susceptible to antibiotics that inhibit DNA replication, ribosomal function or cell wall synthesis (Bacher et al., 2005). When these
IleRS editing deficient cells were aged, a statistically significant increase in mutation rate was observed, which was due to triggering of the bacterial SOS response involving error prone DNA repair (Bacher and Schimmel, 2007). Mutant mice expressing an Ala734Glu AlaRS variant that can misacylate tRNA^{Ala} with Ser suffer from hair loss and neurodegeneration (Lee et al., 2006). Misincorporation of Ser at Ala codons led to accretion of misfolded proteins specifically in Purkinje neural cells but not in other rapidly dividing cell types where misfolded proteins are diluted out. Lastly, mistranslation in mammalian cells expressing editing defective ValRS causes a decrease in cell viability and induces an apoptotic response (Nangle et al., 2006).

Overall, mistranslation due to aaRS editing defects seems to affect cell viability only when the wild-type (WT) allele is deleted and cells are grown in the presence of excess noncognate amino acid (Beebe et al., 2003; Nangle et al., 2002). In the yeast Candida albicans, codon ambiguity leads to a stress response wherein cellular growth rate is reduced by 50% upon mistranslation but does not cause cell death (Santos et al., 1999). In mammalian systems mistranslation has a more profound effect causing cell death, ataxia and neurodegenerative disease (Lee et al., 2006). On the other hand mitochondrial aaRS editing defects have little or no effect on cell viability. This can be explained either by the presence of other aaRS-independent quality control mechanisms, trans-acting factors, or an altered requirement for fidelity in mitochondria. Some obligate intracellular pathogenic Mycoplasma spp. have several aaRSs that lack editing activity leading to enhanced mistranslation, and appear to tolerate higher error rates (Li et al., 2011) (Chapter 3 and section 1.8). Recent studies using tRNA microarrays have revealed
misacylation of multiple tRNA species by MetRS in both mammalian as well as bacterial systems and increased misacylation was seen during innate immune response and under oxidative stress conditions (Jones et al., 2011; Netzer et al., 2009). Mistranslation is suggested to confer protection against reactive oxygen species (ROS) through addition of extra Met residues via misincorporation by MetRS (Levine et al., 1996). Mistranslation may therefore actually be advantageous to the cell under certain stress conditions, however it is yet to be tested if cells undergoing mistranslation and accumulating errors are as fit as their WT counterparts. Taken together these observations indicate that the selective forces exerted by cell-specific requirements and environmental conditions potentially shape quality control mechanisms.

1.8. Divergence of quality control mechanisms in bacterial translation

Mistranslation during protein synthesis can potentially follow two events: production of noncognate amino acid:tRNA pairs by aaRSs and inaccurate selection of aa-tRNAs by the ribosome (Reynolds et al., 2010a). Many aaRSs actively edit noncognate amino acids, but editing mechanisms are not evolutionarily conserved and their physiological significance remains unclear (Reynolds et al., 2010b; Yadavalli and Ibba, 2012). To address the connection between aaRSs and mistranslation, we use the evolutionary divergence of Tyr editing by PheRS as a model system. Certain PheRSs such as those in Mycoplasma species are naturally error-prone and display a low level of specificity consistent with elevated mistranslation of the proteome (Li et al., 2011) (Chapter 3). Mycoplasma mobile PheRS (MmPheRS) lacks canonical editing activity, relying instead
on discrimination against the noncognate amino acid during transfer step of aminoacylation for quality control. This mechanism of discrimination is inadequate for organisms where translation is usually more accurate. As a result, \textit{MmPheRS} failed to support \textit{E. coli} growth. However, minor changes in the defunct editing domain of the \textit{MmPheRS} were sufficient to enhance specificity to cognate amino acid and sustain \textit{E. coli} growth, indicating that translational accuracy is an evolutionarily adaptable trait. These findings indicate a mechanism by which aaRSs facilitate adaptation to changes in cellular physiology by altering the accuracy of translation of specific codons, which may prove advantageous for growth under different environmental conditions (Chapter 3).

1.9. Structure-function relationship in human mitochondrial phenylalanyl tRNA synthetase and its disease relevance

Structural studies suggest rearrangement of the RNA-binding and catalytic domains of human mitochondrial PheRS (mtPheRS) is required for aminoacylation (Klipcan et al., 2008). Crosslinking the catalytic and RNA-binding domains resulted in a “closed” form of mtPheRS that still catalyzed ATP-dependent Phe activation, but was no longer able to transfer Phe to tRNA and complete the aminoacylation reaction (Chapter 4). SAXS experiments indicated the presence of both the closed and open forms of mtPheRS in solution. Together, these results indicate that conformational flexibility of the two functional modules in mtPheRS is essential for its phenylalanylation activity (Yadavalli et al., 2009). This is consistent with the evolution of the aminoacyl-tRNA synthetases as modular enzymes consisting of separate domains that display independent activities.
Defects in mitochondrial translation are the underlying cause of a number of 
diseases including MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis, 
and stroke-like episodes), MERRF (myoclonic epilepsy with ragged red fibers), diabetes, 
deafness and other myopathies. Although the most common causes of these diseases are 
mutations in mitochondria-encoded tRNAs, it has recently become apparent that 
mutations in nuclear-encoded components of the mitochondrial translation machinery, 
such as the aminoacyl-tRNA synthetases (aaRSs), can also lead to disease. In some cases 
mutations can be directly impair enzymatic activity, but others may have indirect effects 
on function. Here we investigate the effects of two pairs of disease-related mutations on 
mtPheRS function. Firstly, we studied non-synonymous single nucleotide polymorphisms 
(ns-SNPs) that encode residues distal from the catalytic site of mtPheRS (Chapter 5). 
Both the ns-SNP variants, S57C, N280S and double mutant S57C/N80S mtPheRS 
displayed aminoacylation activity and stability comparable to WT mtPheRS, but were 
less stable at low pH. Nuclear-encoded mitochondrial proteins undergo partial unfolding/
refolding during their import. In vitro refolding assays showed that these variants retained 
less aminoacylation activity than WT mtPheRS upon refolding. Together, these data 
show that ns-SNPs can indirectly impact overall enzymatic activity of mtPheRS via 
refolding specific defects and thereby affect mitochondrial translation (Banerjee et al., 
2011).

Secondly, we analyzed mutations I293T and D355V in hmtPheRS, found in 
association with infant cardiomyopathy (Chapter 5). The residues I293 lies in the linker 
region close to the ATP binding pocket in the active site, whereas D355 is located in the
anticodon binding domain. As expected, I293T substitution in mtPheRS led to a defect in ATP binding. The D355V replacement led to a slight decrease in affinity for Phe binding. Additionally, the I293T, D355V single and double mutant mtPheRSs displayed lower stability upon refolding compared to WT mtPheRS (Chapter 5). These data shed light on the molecular impact of these mutations on infant cardiomyopathy. Taken together our mutational analyses of mtPheRS suggest that mutations occurring in functional domains of mtPheRS not only affect aminoacylation directly but may also affect stability. Mutations outside the functional domains may affect overall aminoacylation function via refolding specific defects as seen in the case of ns-SNP variants of mtPheRS.
CHAPTER 2

PHENYLALANYL-TRNA SYNTHETASE EDITING IS A MAJOR TRANSLATIONAL QUALITY CONTROL CHECKPOINT IN BACTERIA

2.1. Introduction

Faithful translation of genetic information from mRNA to protein is critical for normal cellular functions. The protein synthesis machinery utilizes aminoacyl-tRNAs (aa-tRNAs), which are formed by aminoacyl-tRNA synthetases (aaRSs) and delivered to the ribosome by elongation factors (EF-Tu in bacteria and EF-1A in archaea and eukaryotes) (Ibba and Söll, 2000; Ogle and Ramakrishnan, 2005). The overall translation error rate of $10^{-4}$ is a net accumulation from several steps, including transcription ($\sim 10^{-5}$), aa-tRNA synthesis ($\sim 10^{-4}$), and ribosomal decoding ($\sim 10^{-4}$) (Loftfield and Vanderjagt, 1972; Rosenberger and Foskett, 1981; Ibba and Söll, 1999; Ogle and Ramakrishnan, 2005). As the error rates from all the above steps are similar and additive, elevated mistakes during any step, such as aminoacylation, may limit the overall accuracy of protein synthesis. As one of the first checkpoints for translational fidelity, aaRSs selectively pair the correct amino acids with their cognate tRNAs in a two-step aminoacylation reaction. AaRSs first activate the amino acid with ATP to form an aminoacyl-adenylate intermediate, and then catalyze the esterification of the activated amino acid to the 2’ or 3’ hydroxyl at the 3’ end of tRNA. AaRSs are extremely selective for their cognate tRNAs due to highly specific
binding and kinetic proofreading (Ibba and Söll, 1999; Guth and Francklyn, 2007). In contrast, several aaRSs lack sufficient discrimination against structurally similar near-cognate amino acids during activation. For example, phenylalanyl-tRNA synthetase (PheRS) misactivates Tyr at a level higher than the overall translation error rate (Lin et al., 1984; Roy et al., 2005a). However, such mistakes by aaRSs are not directed to protein synthesis, due to a proofreading step (Baldwin and Berg, 1966). This step, called editing, occurs through hydrolysis of misactivated amino acids (pretransfer editing) or misacylated aminoacyl-tRNAs (posttransfer editing), while the correct products are excluded from the hydrolytic reaction. Editing activities have been shown to play important roles in vivo (Döring et al., 2001; Roy et al., 2004; Lee et al., 2006), and are found in both aaRS structural classes (I and II) (Ibba and Söll, 2000; Hendrickson and Schimmel, 2003). Mischarged aa-tRNAs that escape aaRS editing may dissociate from an aaRS and potentially enter translation via binding to EF-Tu.

EF-Tu was long considered to bind all aa-tRNAs with roughly equal efficiencies, the only exceptions being fMet-tRNA\(^{\text{fMet}}\), Ser/Sec-tRNA\(^{\text{Sec}}\), Glu-tRNA\(^{\text{Gln}}\) and Asp-tRNA\(^{\text{Asn}}\) (Stanzel et al., 1994; Becker and Kern, 1998; Dale and Uhlenbeck, 2005a; Ambrogelly et al., 2007; Roy et al., 2007). The weak affinity of EF-Tu for Glu-tRNA\(^{\text{Gln}}\) and Asp-tRNA\(^{\text{Asn}}\) prevents genetic ambiguity that would result from misincorporating Glu and Asp at Gln and Asn codons, respectively, while fMet-tRNA\(^{\text{fMet}}\) and Sec-tRNA\(^{\text{Sec}}\) instead bind specialized translation factors (Ambrogelly et al., 2007). Uhlenbeck and colleagues found that EF-Tu displays selectivity for both the amino acid and the tRNA body (LaRiviere et al., 2001; Asahara and Uhlenbeck, 2002; Dale et al., 2004; Asahara and Uhlenbeck, 2005). It was proposed that since EF-Tu binds amino acids with various
affinities, their corresponding tRNAs have evolved to compensate for the differences in affinity thermodynamically, so that EF-Tu binds all cognate aa-tRNAs uniformly (Dale and Uhlenbeck, 2005a). In contrast to cognate aa-tRNAs, EF-Tu binds their noncognate counterparts with a wide range of affinities, which may lead to reduced incorporation efficiencies for some mischarged amino acids.

The last step at which translational fidelity can be monitored is ribosomal decoding. Proper matching of codons and anticodons is a prerequisite for efficient decoding, and the ribosome utilizes both thermodynamic and kinetic discrimination mechanisms to reject aa-tRNAs with near-cognate or noncognate anticodons (Gromadski and Rodnina, 2004a; Cochella and Green, 2005). It is less clear whether ribosomes are able to discriminate against misacylated tRNAs with cognate codons.

We investigated the effect on binding by EF-Tu and ribosomal selection of misacylated tRNAs, to better understand the quality control mechanisms that prevent misincorporation of Tyr at Phe codons. We found that neither EF-Tu nor the ribosome can effectively discriminate noncognate Tyr-tRNA^{Phe} from the cognate Phe-tRNA^{Phe}, as revealed by the aa-tRNA dissociation rate constants and poly(U)-directed in vitro translation assays. Our data suggest that PheRS editing is the major proofreading step that prevents infiltration of Tyr into Phe codons during translation. In addition, using Tyr-tRNA^{Phe} editing by PheRS as a model, we show here that a fraction of mischarged tRNA dissociates from the aaRS prior to entering the editing site. Interestingly, rather than being sequestered directly by EF-Tu for protein synthesis, released mischarged tRNAs can rebind to the aaRS leading to resampling by the editing site and a reduction in the
aminoacylation error rate. The net effect of this resampling is to provide an additional quality control step before translation elongation.

2.2. Materials and methods

2.2.1. Strains, plasmids, site-directed mutagenesis and general methods

*E. coli* JM109/pKECA-Tu producing His6-tagged *E. coli* EF-Tu was a gift from B. Kraal (Leiden University, Leiden, the Netherlands). *E. coli* strain XL1-Blue/pQE31-FRS expressing the WT *E. coli* PheRS was a gift from DA Tirrell (California Institute of Technology). PheRS variants were previously obtained by site-directed mutagenesis (Roy et al., 2004). *E. coli* tRNA<sub>Phe</sub> and mRNA transcripts were prepared using *in vitro* T7 RNA polymerase run-off transcription as described (Roy et al., 2004). Native *E. coli* tRNA<sub>Phe</sub>, tRNA<sub>Met</sub> and poly(U) were purchased from Sigma-Aldrich.

2.2.2. Aminoacylation

Aminoacylation by PheRS was performed at 2 °C or 37 °C in the presence of 0.1 M Na-HEPES pH 7.2, 30 mM KCl, 10 mM MgCl₂, 2 mM ATP, 1-30 μM *E. coli* tRNA<sub>Phe</sub> transcript, 20 μM [¹⁴C] Phe (252 cpm/pmole) or 50 μM [³H] Tyr (464 cpm/pmole), 5-750 nM PheRS, with or without 10 μM activated *E. coli* EF-Tu. Tyrosylation experiments were performed at 37 °C as described (Roy et al., 2004) using 5 μM *E. coli* tRNA<sub>Phe</sub> transcript, 50 μM [³H]Tyr (464 cpm/pmole), 0.5 μM PheRS, with or without 5 μM *E. coli* EF-Tu. EF-Tu was activated in 50 mM Tris-HCl, 1 mM DTT, 68 mM KCl, 6.7 mM MgCl₂, 2.5 mM phosphoenolpyruvate (PEP), 0.5 mM GTP, and 30 μg/ml pyruvate kinase (PK) at 37 °C for 30 minutes before use.
2.2.3. Deacylation

Deacylation assays were performed as in (Ling et al., 2007a). For competition experiments, 0.75 μM PheRS WT or protein storage buffer was pre-mixed with 10 μM activated EF-Tu or BSA for 3 min at 37 °C. A final concentration of 1 μM Tyr-tRNA\(^{\text{Phe}}\) was added to the mixture and the remaining Tyr-tRNA\(^{\text{Phe}}\) concentration determined at different time points.

2.2.4. Determination of \(k_{\text{off}}\) values for EF-Tu

*E. coli* EF-Tu was activated in 50 mM Na-Hepes pH 7.2, 5 mM DTT, 150 mM NH\(_4\)Cl, 20 mM MgCl\(_2\), 3 mM PEP, 2 mM GTP, and 30 μg/ml PK at 37 °C for 20 minutes. A final concentration of 0.5 μM \([^{14}\text{C}]\) Phe-tRNA\(^{\text{Phe}}\) or \([^{3}\text{H}]\) Tyr-tRNA\(^{\text{Phe}}\) was added to 10 μM activated EF-Tu and incubated on ice for 5 min. RNase A was added to the reaction mixture to a final concentration of 100 μg/ml and aliquots were taken at each time point, spotted on 3 MM discs pre-soaked with 5 % trichloroacetic acid (TCA), washed, dried and scintillation counted.

2.2.5. 2AP-tRNA\(^{\text{Phe}}\) synthesis and aminoacylation

A blunt tRNA\(^{\text{Phe}}\) gene lacking the last four nucleotides at the 3’-end was cloned into pUC-18 vector and *E. coli* XL1-Blue cells were transformed with this plasmid. The blunt tRNA\(^{\text{Phe}}\) was *in vitro* transcribed and gel purified as described previously (Roy et al., 2004). A tetranucleotide oligo 5’p ACC-2AP p 3’ where A76 on the tRNA 3’-end was replaced with 2-aminopurine was obtained commercially (TriLink BioTechnologies) and ligated to the blunt tRNA\(^{\text{Phe}}\) to form an intact tRNA\(^{\text{Phe}}\) using T4 RNA ligase (NEB). The
ligated tRNA was phenol-chloroform extracted and gel filtered using sephadex G-25 (GEC). This tRNA was then dephosphorylated using antarctic phosphatase enzyme (NEB). A second phenol chloroform extraction was performed followed by gel filtration using sephadex G-50 (Pharmacia biotech). aa-tRNA\textsuperscript{Phe} complexes were prepared as described previously (Ling et al., 2007a). In addition, gel filtration using sephadex G-25 was performed to remove any excess \([^3]\text{H}\)Tyr or \([^{14}\text{C}]\text{Phe}\). The charging level and concentration of aa-tRNA\textsuperscript{Phe} was measured by scintillation counting.

2.2.6. Determination of binding rate constants by stopped-flow fluorimetry

Binding rates for 2APtRNA\textsuperscript{Phe}, Phe-2APtRNA\textsuperscript{Phe} and Tyr-2APtRNA\textsuperscript{Phe} were obtained by monitoring changes in 2-aminopurine fluorescence in a stopped-flow spectrophotometer (SX.18MV, Applied Photophysics). An excitation wavelength of 315 nm was used and emission cut-off was 360 nm. The concentration of 2AP-tRNA\textsuperscript{Phe} or aa-tRNA\textsuperscript{Phe} in one syringe was maintained constant at 0.1 µM while varying the PheRS concentration in the second syringe (0.5-10 µM). \(\alpha\text{A294G}\) PheRS was used in case of 2AP-tRNA\textsuperscript{Phe} and \(\alpha\text{A294G}\ \beta\text{G318W}\) PheRS (editing defective mutant) was used with both aa-tRNA\textsuperscript{Phe} species. The rate constants \((k_{\text{obs}})\) were obtained by fitting the observed increase in fluorescence to a single exponential model using the in-built SX.18MV software. The observed rates were then plotted against varying PheRS concentration and fitted to a linear equation to obtain the association \((k_{\text{on}})\) and dissociation \((k_{\text{off}})\) rate constants.
2.2.7. Poly(U)-directed poly-Phe and poly-Tyr synthesis

5 µM *E. coli* EF-Tu was activated by incubation in a buffer containing 50 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 20 mM NH₄Cl, 1 mM ATP, 30 µM GTP, 30 mM 2-mercaptoethanol, 1 mM DTT, 5 mM PEP and 20 µg/ml pyruvate kinase for 30 min at 37 °C. *In vitro* translation was carried out by reconstituting 200 nM 70S ribosomes, 1 µM *E. coli* EF-G, 0.3 µg/µl poly(U) mRNA and activated EF-Tu. 1 µM [¹⁴C]Phe-tRNA²Phe and 1 µM [³H]Tyr-tRNA²Phe synthesized with native *E. coli* tRNA²Phe were added to initiate the reaction at 37 °C. For competition assays, varying concentrations of *E. coli* αA294G PheRS (0 nM, 5 nM, 50 nM or 500 nM) were added to the pre-mixed buffer containing activated EF-Tu. Aliquots were spotted on 3MM Whatman paper pre-soaked with 10 % TCA, incubated in 10 % TCA at room temperature for 20 min, washed twice in 5 % TCA at 90 °C for 10 min each, dried and scintillation counted.
2.3. Results

2.3.1. Misacylation of tRNA\textsuperscript{Phe} by editing-defective PheRSs and substrate dissociation during editing

EF-Tu binds aa-tRNAs, including Tyr-tRNA\textsuperscript{Phe}, with high affinity at low temperature and provides protection from both spontaneous and enzymatic hydrolysis (at 4 °C, $K_D = 5$-50 nM) (LaRiviere et al., 2001; Ling et al., 2007c). It has been shown that EF-Tu significantly increases the steady-state levels of cognate and misacylated tRNAs (Wolfson and Uhlenbeck, 2002; Lee et al., 2006), which prompted us to further investigate the mischarging activities of PheRS variants in the presence and absence of EF-Tu. The WT PheRS did not produce accumulated Tyr-tRNA\textsuperscript{Phe} irrespective of EF-Tu addition (Fig. 2.1A). As the WT and editing defective PheRSs display similar aminoacylation activities (Ling et al., 2007c), it is likely that the WT PheRS hydrolyzes Tyr-tRNA\textsuperscript{Phe} before it is trapped by EF-Tu. In contrast, editing-defective PheRS variants displayed significantly increased tyrosylation levels in the presence of EF-Tu, likely through EF-Tu protection of synthesized Tyr-tRNA\textsuperscript{Phe} from hydrolysis. Superimposition of the EF-Tu:Phe-tRNA\textsuperscript{Phe} structure onto the PheRS:tRNA\textsuperscript{Phe} complex by overlaying the tRNA backbones revealed extensive steric clashes (Nissen et al., 1995; Goldgur et al., 1997). These structural constraints suggest that EF-Tu will not be able to protect Tyr-tRNA\textsuperscript{Phe} from hydrolysis until after it is released by PheRS. This allowed us to use EF-Tu to trap and stabilize any Tyr-tRNA\textsuperscript{Phe} that dissociates from PheRS before entering the editing site. An \textit{E. coli} PheRS active site variant (αA294G) with enhanced Tyr activation activity was first tested (Ibba et al., 1994; Roy et al., 2004). This PheRS variant, which has WT editing activity, did not accumulate Tyr-tRNA\textsuperscript{Phe} at 2 °C in the absence of EF-Tu.
Addition of EF-Tu led to accumulation of Tyr-tRNA\textsubscript{Phe} under the same conditions, indicating that at least a fraction of Tyr-tRNA\textsubscript{Phe} dissociates from PheRS before entering the editing site. It is possible, however, that dissociation of noncognate Tyr-tRNA\textsubscript{Phe} is slower than cognate Phe-tRNA\textsubscript{Phe}. To investigate if PheRS discriminates against noncognate Tyr-tRNA\textsubscript{Phe} at the product release step, the kinetic parameters of Phe-tRNA\textsubscript{Phe} and Tyr-tRNA\textsubscript{Phe} binding to PheRS were determined. To prevent hydrolysis of Tyr-tRNA\textsubscript{Phe}, an editing defective PheRS variant (αA294G βG318W) was employed. Phe-tRNA\textsubscript{Phe} and Tyr-tRNA\textsubscript{Phe} showed no significant differences in association and dissociation rate constants measured (Table 2.1), and the values were comparable to those previously measured for \textit{E. coli} PheRS binding of Phe-tRNA\textsubscript{Phe} (Baltzinger and Holler, 1982b). This suggests that mischarged Tyr-tRNA\textsubscript{Phe} can readily dissociate and re-bind PheRS. In other words, PheRS may also compete with EF-Tu for mischarged tRNAs, which would constitute an additional quality control step during aminoacylation.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>(k_{\text{on}}) ((\mu\text{M}^{-1}\text{s}^{-1}))</th>
<th>(k_{\text{off}}) ((\text{s}^{-1}))</th>
<th>(K_d) ((\mu\text{M}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>tRNA\textsubscript{Phe}</td>
<td>59±4</td>
<td>12±2</td>
<td>0.21±0.04</td>
</tr>
<tr>
<td>Phe-tRNA\textsubscript{Phe}</td>
<td>80±11</td>
<td>12±2</td>
<td>0.16±0.03</td>
</tr>
<tr>
<td>Tyr-tRNA\textsubscript{Phe}</td>
<td>57±8</td>
<td>14±5</td>
<td>0.24±0.09</td>
</tr>
</tbody>
</table>

\textbf{Table 2.1. Binding rate constants for Phe-tRNA\textsuperscript{Phe} and Tyr-tRNA\textsuperscript{Phe} to αA294G βG318W PheRS.} Parameters were determined by stopped-flow fluorometry and data analysis was performed as described in materials and methods. Errors correspond to the standard deviation from three independent experiments.
Figure 2.1. Tyrosylation of *E. coli* tRNA\textsubscript{Phe} by PheRS variants. **A**, Tyrosylation in the presence (filled bars) and absence (open bars) of EF-Tu (5 μM). The Y-axis represents the levels of Tyr-tRNA\textsubscript{Phe} synthesized after 6 minutes. PheRS variants contain either a wild-type editing site or mutations in the β subunit that reduce the editing activity. Relative posttransfer editing activities determined previously are shown in the figure. Data sets are the average of three independent experiments. This experiment was performed by Jiqiang Ling and Srujana S Yadavalli. **B**, Tyrosylation by 0.75 μM *E. coli* PheRS (αA294G WT β), at 2 °C +/- 10 μM activated *E. coli* EF-Tu. This experiment was performed by Jiqiang Ling.
2.3.2. EF-Tu and the ribosome do not discriminate Tyr-tRNA\textsuperscript{Phe} from Phe-tRNA\textsuperscript{Phe}

in vitro

EF-Tu increased the tyrosylation level of tRNA\textsuperscript{Phe} by PheRS, suggesting that Tyr-tRNA\textsuperscript{Phe} might be protected by EF-Tu. To directly address this possibility, we tested the dissociation rates of Tyr-tRNA\textsuperscript{Phe} and Phe-tRNA\textsuperscript{Phe} from E. coli EF-Tu using an RNase A protection assay as previously described (LaRiviere et al., 2001; Asahara and Uhlenbeck, 2002). The $k_{off}$ rates of Tyr-tRNA\textsuperscript{Phe} and Phe-tRNA\textsuperscript{Phe} were very similar at 2 °C (data not shown), comparable to values for cognate aa-tRNAs determined using T. thermophilus EF-Tu (LaRiviere et al., 2001; Dale et al., 2004). The $k_{off}$ values of both Tyr-tRNA\textsuperscript{Phe} and Phe-tRNA\textsuperscript{Phe} remained similar, ~7 min\textsuperscript{-1} (Fig. 2.2A) when the reaction temperature was raised to 37 °C, but increased by approximately 30-fold, consistent with previous observations (Vorstenbosch et al., 2000). It has been predicted that some misacylated tRNAs bind EF-Tu either too tightly or too weakly to allow efficient delivery to the ribosome (LaRiviere et al., 2001; Asahara and Uhlenbeck, 2005). However, many noncognate aa-tRNAs bind to EF-Tu with affinities similar to their cognate counterparts (Asahara and Uhlenbeck, 2005). The data collected here show that E. coli EF-Tu does not discriminate Tyr-tRNA\textsuperscript{Phe} from Phe-tRNA\textsuperscript{Phe}.

In a previously established in vivo assay using an amber suppressor tRNA\textsubscript{Phe\ CUA}, we observed that Tyr was misincorporated into UAG amber codons upon expression of an editing-defective PheRS (Roy et al., 2004), suggesting that the ribosome is able to use Tyr-tRNA\textsubscript{Phe\ CUA} as a substrate. What remained unknown, however, was how efficiently the ribosome utilizes the mischarged Tyr-tRNA\textsuperscript{Phe}. To test decoding efficiency, we performed poly(U)-directed poly-Tyr and poly-Phe synthesis. The synthesis rates of poly-
Tyr and poly-Phe were almost identical (Fig. 2.2B), confirming that the ribosome lacks discrimination between Tyr and Phe side chains in the context of tRNA\textsuperscript{Phe}. Together, our experiments demonstrate that Tyr-tRNA\textsuperscript{Phe} is not discriminated by either the EF-Tu or the ribosome. Hence, any Tyr-tRNA\textsuperscript{Phe} that escapes PheRS editing can potentially contribute to mistranslation of Phe codons.
Figure 2.2. EF-Tu and ribosome do not discriminate Tyr-tRNA<sub>Phe</sub> from Phe-tRNA<sub>Phe</sub>. A, Determination of $k_{\text{off}}$ rates of Phe- and Tyr-tRNAs<sub>Phe</sub> from EF-Tu. The dissociation rates are $7.7 \pm 0.2\ \text{min}^{-1}$ (Phe-tRNA<sub>Phe</sub>) and $6.8 \pm 1.2\ \text{min}^{-1}$ (Tyr-tRNA<sub>Phe</sub>) at 37 °C. Data sets are the average of three independent experiments. B, Decoding of Phe codons by Tyr- and Phe-tRNAs<sub>Phe</sub> using poly(U)-directed poly-Phe and poly-Tyr synthesis. The reaction mixture contains 200 nM 70 S ribosomes, 1 µM EF-G, 5 µM EF-Tu, 0.3 µg/µl poly(U) mRNA, and 1 µM $[^{14}\text{C}]$ Phe-tRNA<sub>Phe</sub> or $[^{3}\text{H}]$ Tyr-tRNA<sub>Phe</sub> synthesized with native <i>E. coli</i> tRNA<sub>Phe</sub>. As controls, 1 µM $[^{14}\text{C}]$ Phe or $[^{3}\text{H}]$ Tyr are added instead of $[^{14}\text{C}]$ Phe-tRNA<sub>Phe</sub> or $[^{3}\text{H}]$ Tyr-tRNA<sub>Phe</sub>. Data sets are the average of three independent experiments. This experiment was performed by Jiqiang Ling. Srujana S Yadavalli helped with reagent preparation and experimental set-up.
2.3.3. PheRS competes effectively with EF-Tu for Tyr-tRNA\textsuperscript{Phe}

EF-Tu readily binds Tyr-tRNA\textsuperscript{Phe}, and can deliver it to the ribosome for translation elongation just as efficiently as it does Phe-tRNA\textsuperscript{Phe} (Ling et al., 2007c). This observation, and the fact that EF-Tu is one of the most abundant proteins in \textit{E. coli}, would seem to suggest that any mischarged tRNAs that escape PheRS will be sequestered by EF-Tu. However, PheRS with a WT editing site did not produce detectable levels of Tyr-tRNA\textsuperscript{Phe} at 37 °C even in the presence of excess EF-Tu, suggesting that at physiological temperatures, little EF-Tu bound Tyr-tRNA\textsuperscript{Phe} is available for the ribosome. To directly test the competition, purified Tyr-tRNA\textsuperscript{Phe} was added to a mixture of activated EF-Tu and PheRS. Tyr-tRNA\textsuperscript{Phe} was completely hydrolyzed by PheRS even though EF-Tu was present in excess (Fig. 2.3), confirming that the PheRS editing site effectively competes with EF-Tu for free Tyr-tRNA\textsuperscript{Phe} under physiological conditions.

\textbf{Figure 2.3.} PheRS editing site competes with EF-Tu for Tyr-tRNA\textsuperscript{Phe}. Hydrolysis of Tyr-tRNA\textsuperscript{Phe}. 0.75 µM WT β subunit EcPheRS and 10 µM activated \textit{E. coli} EF-Tu were mixed and pre-incubated at 37 °C for 3 min before the addition of 1 µM Tyr-tRNA\textsuperscript{Phe}. This experiment was performed by Jiqiang Ling.
2.3.4. Tyr-tRNA\textsuperscript{Phe} is resampled prior to translation elongation

While our data showed that PheRS could compete with EF-Tu to edit mischarged tRNA, it remained unclear how effective this competition would be compared to Tyr-tRNA\textsuperscript{Phe}•EF-Tu•GTP binding by the ribosome. The effect of translating ribosomes on resampling and editing of mischarged tRNAs was investigated using poly(U)-directed ribosomal polypeptide synthesis. Previous studies showed that ribosomes utilize Tyr-tRNA\textsuperscript{Phe} and Phe-tRNA\textsuperscript{Phe} with equal efficiencies in the presence of poly(U) and purified EF-Tu and EF-G, but in the absence of PheRS (Ling et al., 2007c). Addition of increasing amounts of PheRS to these experiments can help us directly test the competition between the PheRS editing site and activated EF-Tu during ribosomal translation (Fig. 2.4). Upon addition of purified Phe-tRNA\textsuperscript{Phe}, poly-Phe was rapidly synthesized with approximately 100 % of the radiolabeled Phe finally incorporated into the polypeptide. When Tyr-tRNA\textsuperscript{Phe} was used in the absence of PheRS, yields of poly-Tyr were comparable to those obtained for poly-Phe. If, instead, PheRS was included at 0.1 % the level of EF-Tu, poly-Tyr synthesis was reduced by more than half, and at higher concentrations of PheRS poly-Tyr synthesis was virtually eliminated. These data indicate that the majority of free Tyr-tRNA\textsuperscript{Phe} is hydrolyzed before its utilization in translation despite the presence of both EF-Tu and ribosomes, a step that provides a substantial reduction in the error rate of protein synthesis.
Figure 2.4. Resampling and editing of misacylated tRNA. Poly(U)-directed poly-Phe and poly-Tyr synthesis at 37 °C. As controls, 1 μM [¹⁴C] Phe or [³H] Tyr were added instead of [¹⁴C] Phe-tRNA^Phe or [³H] Tyr-tRNA^Phe. For poly-Tyr synthesis 0, 5, 50 or 500 nM PheRS was included as indicated. PheRS was also added to poly-Phe synthesis reactions at the same concentrations but no change in poly-Phe synthesis was observed (data not shown).

2.4. Discussion

2.4.1. The editing activity of PheRS is essential for translational quality control

Our data demonstrate that once Tyr-tRNA^Phe evades editing by PheRS, incorporation of Tyr at Phe codons is inevitable. EF-Tu and the ribosome, which are downstream to aa-tRNA synthesis, do not provide further proofreading mechanisms to avoid Tyr misincorporation. This is distinct from some naturally misacylated tRNAs, such as Glu-tRNA^Gln and Asp-tRNA^Asn, which are discriminated by EF-Tu (Stanzel et al., 1994; Becker and Kern, 1998). Uhlenbeck and coworkers proposed that since EF-Tu and possibly the ribosome display selectivity for different amino acids, their cognate tRNAs might have evolved to compensate for the binding affinities thermodynamically, so that all cognate aa-tRNAs can be translated uniformly (Dale and Uhlenbeck, 2005a). Our
findings support this hypothesis, as Tyr-tRNA\textsuperscript{Phe} accumulation is normally prevented by the PheRS editing activity and as a result there is no selective pressure to drive further evolution of tRNA\textsuperscript{Phe}. This hypothesis also explains why unnatural amino acids are usually efficiently incorporated into proteins \textit{in vivo}, as the necessary orthogonal aaRSs do not exist in nature.

2.4.2. Resampling and editing of mischarged tRNA

\textit{In vitro} poly-Tyr synthesis using Tyr-tRNA\textsuperscript{Phe} was virtually eliminated upon addition of sufficient PheRS, suggesting that the majority of the mischarged tRNA was accessed and edited by the synthetase before it could be used for translation elongation (Fig. 2.4). While this contradicts the notion that aa-tRNAs are almost irreversibly bound to EF-Tu until GTP hydrolysis occurs on the ribosome, the data are consistent with substrate binding kinetics. EF-Tu binds aa-tRNAs very slowly, with an association rate constant ($k_3$ in Fig. 2.5A) of approximately 0.1 $\mu$M$^{-1}$ s$^{-1}$ at physiological temperatures (LaRiviere et al., 2001; Asahara and Uhlenbeck, 2002; Roy and Ibba, 2008). In contrast, the association rate of \textit{E. coli} PheRS for Tyr-tRNA\textsuperscript{Phe} is $\sim$60 $\mu$M$^{-1}$ s$^{-1}$. The 500-fold difference in association rate constants ensures that PheRS can effectively compete with EF-Tu for Tyr-tRNA\textsuperscript{Phe} even though EF-Tu is in about 100-fold excess over PheRS in \textit{E. coli} (Furano, 1975; Neidhardt et al., 1977; Jakubowski and Goldman, 1984). Once bound by PheRS, Tyr-tRNA\textsuperscript{Phe} is likely to be hydrolyzed rapidly.

The dissociation rate of Tyr-tRNA\textsuperscript{Phe} from \textit{E. coli} PheRS is about 14 s$^{-1}$, so $k_2$ in Figure 2.5A is estimated to be greater than 200 s$^{-1}$ from the equation $K_M = (k_{1}+k_{2})/k_{1}$ ($K_M$ of PheRS for Tyr-tRNA\textsuperscript{Phe} hydrolysis exceeds 5 $\mu$M (Ling et al., 2007a)). This indicates
that the association of Tyr-tRNA$^{\text{Phe}}$ to the PheRS editing site is nearly unidirectional. Conversely, the binding of aa-tRNAs by EF-Tu is predicted to be readily reversible, consistent with the recent descriptions of non-ribosomal processes that rely on canonical elongator aa-tRNAs as substrates (Villet et al., 2007; Watanabe et al., 2007; Lloyd et al., 2008; Roy and Ibba, 2008).

### 2.4.3. A concerted translocation model for class II-type editing

In class II aaRS enzymes such as PheRS, ProRS and ThrRS, mischarged tRNA is synthesized at the active site and hydrolyzed at the distinct editing site ~40 Å away. It had been believed that direct translocation of mischarged tRNA between these two sites on the aaRS was an absolute requirement for editing (Roy et al., 2004). Our data now show that while this may hold true for class-I aaRSs, class-II type editing includes an additional resampling pathway (Fig. 2.5B). Following synthesis at the active site, a portion of Tyr-tRNA$^{\text{Phe}}$ directly translocates to the editing site through the movement of the 3′ end and is hydrolyzed in cis, while the rest of the mischarged Tyr-tRNA$^{\text{Phe}}$ either partially (Yang et al., 2006) or completely dissociates from PheRS. At physiological temperatures, PheRS efficiently competes with EF-Tu to rebind released Tyr-tRNA$^{\text{Phe}}$, with the Tyr moiety entering the editing site to be rapidly hydrolyzed in trans. In contrast to the reversible binding by EF-Tu, the binding and hydrolysis of Tyr-tRNA$^{\text{Phe}}$ by the PheRS editing site is unidirectional, promoting the equilibrium of EF-Tu binding to shift towards releasing Tyr-tRNA$^{\text{Phe}}$. This maintains a very low level of EF-Tu bound Tyr-tRNA$^{\text{Phe}}$ and restricts its use in protein synthesis. Nevertheless, the cis-editing pathway should not be excluded. Even in the presence of EF-Tu at 2 °C, where aa-tRNAs are more...
tightly bound (Roy and Ibba, 2008), WT EcPheRS produces Tyr-tRNA\textsubscript{Phe} much more slowly than the editing-defective variant (data not shown), suggesting that a significant portion of the editing activity can not be sequestered by EF-Tu. Previously it was shown that in minimal medium enriched with Tyr and depleted for Phe, co-expressing WT and editing-defective PheRS variants resulted in Tyr misincorporation at Phe codons (Roy et al., 2004), indicating that Tyr-tRNA\textsubscript{Phe} overproduced by the editing-defective PheRS could not be completely hydrolyzed in \textit{trans}. Although the relative contributions of \textit{cis-} and \textit{trans}-editing remain to be characterized, it is likely that PheRS \textit{trans}-editing complements \textit{cis}-editing by efficiently hydrolyzing misacylated Tyr-tRNA\textsubscript{Phe} that escapes from the enzyme, mimicking the function of autonomous \textit{trans}-editing factors (Ahel et al., 2003; Wong et al., 2003; Korencic et al., 2004).

2.4.4. \textit{Trans}-editing pathways

Several autonomous \textit{trans}-editing factors have been identified that hydrolyze mischarged tRNAs (Ahel et al., 2003; Wong et al., 2003; An and Musier-Forsyth, 2004; Korencic et al., 2004). One such protein, YbaK, was shown not to compete with EF-Tu for mischarged tRNA when free, but may compete more effectively when in complex with ProRS (An and Musier-Forsyth, 2005). The same may also be true for the \textit{trans}-editing factor AlaX and AlaRS that compete with EF-1A to clear Ser-tRNA\textsubscript{Ala}, accumulation of which can cause severe neurodegeneration (Lee et al., 2006). Like PheRS, AlaRS is a class II aaRS, and is not likely to form a complex with EF-Tu and Ser-tRNA\textsubscript{Ala}, indicating that Ser-tRNA\textsubscript{Ala} must be released from AlaRS to be protected by EF-Tu. The notion that Ser-tRNA\textsubscript{Ala} dissociates from WT AlaRS is also supported by studies of
tRNA recognition during editing. The G3:U70 pair of tRNA$^{\text{Ala}}$ is critical for both aminoacylation and posttransfer editing (Musier-Forsyth et al., 1991; Beebe et al., 2008), but is recognized by distinct domains during the two reactions (Beebe et al., 2008). The significant rearrangement of G3:U70 recognition during aminoacylation and editing correlates well with a trans-editing pathway, but not with cis-editing alone.

Aminoacylation by class I aaRSs is suggested to be rate-limited by product release for both editing (IleRS and ValRS) and non-editing (CysRS) enzymes (Eldred and Schimmel, 1972; Zhang et al., 2006). The dissociation rates of cognate aa-tRNAs from ValRS are estimated to be 3-4 s$^{-1}$, comparable to the overall editing rate of ValRS (Nomanbhoy and Schimmel, 2000; Zhang et al., 2006). Structural modeling revealed that in class I editing aaRSs, the CP1 domain sterically excludes EF-Tu from forming a complex with the aaRS and aa-tRNA (Zhang et al., 2006), consistent with the observation that bacterial EF-Tu does not increase aminoacylation or stimulate misacylation by LeuRS (Hausmann et al., 2007; Hausmann and Ibba, 2008). Such steric clashes may prevent EF-Tu from enhancing dissociation of misacylated aa-tRNAs, allowing editing to occur through the dominant cis-pathway in class I aaRSs. All known autonomous trans-editing factors have evolved from class II aaRSs, consistent with the notion that class I enzymes do not release misacylated aa-tRNAs and so would not need to utilize trans-editing for proofreading. These class-specific differences in proofreading may reflect early evolutionary constraints on accuracy that facilitated the introduction of particular amino acids into the genetic code.
Figure 2.5. Concerted translocation model for PheRS editing. A, Scheme of competition for Tyr-tRNA\textsuperscript{Phe} between PheRS and EF-Tu. As $k_1[\text{PheRS}] > k_3[\text{EF-Tu}]$ and $k_2 >> k_1$, the majority of free-standing Tyr-tRNA\textsuperscript{Phe} is bound by PheRS and hydrolyzed, whereas only a small fraction is utilized by the ribosome in protein synthesis. B, model for concerted editing pathways. Top, cis-editing pathway. Upon synthesis at the active site (AS), a fraction of Tyr-tRNA\textsuperscript{Phe} directly translocates to the editing site for hydrolysis, which is not accessible to EF-Tu. Bottom, trans-editing pathway. Tyr-tRNA\textsuperscript{Phe} dissociates from PheRS and is competed for by EF-Tu and PheRS. Tyr-tRNA\textsuperscript{Phe} bound to the editing site is rapidly hydrolyzed to yield a very low level of EF-Tu bound Tyr-tRNA\textsuperscript{Phe} in vivo.
CHAPTER 3

DIVERGENT EVOLUTION OF QUALITY CONTROL MECHANISMS IN MICROBIAL TRANSLATION

3.1. Introduction

Accurate transfer of genetic information is critical for cellular maintenance and integrity. Accordingly, different stages in gene expression ensure an optimal level of fidelity (Johansson et al., 2008). DNA replication is an extremely accurate process with an error rate of 1 in $10^8$ (Kunkel, 2004), whereas mRNA transcription and translation are relatively less accurate with misincorporation rates of $10^{-5}$ (Blank et al., 1986) and $10^{-4}$ (Loftfield and Vanderjagt, 1972; Kramer and Farabaugh, 2007), respectively. Fidelity in translation is dependent upon several steps including synthesis of cognate aminoacyl tRNAs (aa-tRNAs) by aminoacyl tRNA synthetases (aaRSs), binding of aa-tRNAs by elongation factor Tu (EF-Tu) and accurate selection of aa-tRNAs by the ribosome (Ibba and Söll, 1999; Ling et al., 2009a).

tRNA charging by aaRSs is a two-step reaction, (i) activation of a specific amino acid and (ii) transfer or ligation of amino acid to the 3’ end of tRNA forming an aminoacyl ester bond (Ibba and Söll, 2000). Roughly half of the aaRSs display a high level of specificity of ~3000:1 or greater for the cognate amino acid over noncognate...
substrate, while the other half utilize an additional proofreading function called “editing” to maintain accuracy (Mascarenhas et al., 2009; Yadavalli and Ibba, 2012). Quality control by editing aaRSs occurs via a double sieve mechanism (Fersht, 1977a). The first sieve is the specificity for cognate substrate conferred by the synthetic active site of the aaRS. The second sieve is an editing or proofreading activity to clear either misactivated amino acids or mischarged tRNAs (Fersht, 1977b, 1999). Hence, editing can be classified as pre- or posttransfer depending on the stage of hydrolysis of misformed products during aminoacylation (Mascarenhas et al 2009; Ling et al., 2009a; Jakubowski 2012; Yadavalli and Ibba 2012). AaRS editing has been shown to be important in vivo and defects in editing lead to mistranslation and can be detrimental to cells (Roy et al., 2004; Lee et al., 2006). In bacteria, expression of editing-defective aaRSs can trigger stress responses (heat shock or unfolded protein response) due to the accumulation of misfolded proteins (Ruan et al., 2008). Loss of editing can increase bacterial susceptibility to antibiotics that affect key cellular processes (Bacher et al., 2005), and induce mutations that trigger DNA repair via the SOS mechanism in aged cells (Bacher and Schimmel, 2007). In mammalian cells, mistranslation resulting from expression of editing-defective aaRSs can lead to cell death and neurodegeneration (Lee et al., 2006, Nangle et al., 2006). However, proofreading pathways are not universal and different organisms appear to tolerate different levels of translation error rates (Reynolds et al., 2010a). The limits on translational fidelity in a particular organism reflect the fine balance between the cost and benefit of errors, which may vary depending on the cellular physiology. For example, codon ambiguity in the pathogenic yeast Candida albicans leads to around 100-fold higher errors than typically observed in translation, and induces a general stress response
that provides a selective advantage to the organism (Santos et al., 1999; Miranda et al., 2006). Recent studies reveal misacylation of multiple tRNA species by methionyl-tRNA synthetase (MetRS) in both bacteria (Jones et al., 2011) and mammalian cells (Netzer et al., 2009). An increase in the level of mismethionylation is observed in mammalian cells during innate immune response and oxidative stress conditions, which could serve to protect cellular proteins against damage from reactive oxygen species (Levine et al., 1996).

Phenylalanyl-tRNA synthetase (PheRS) is a well-studied example of an aaRS that utilizes different quality control mechanisms in bacteria, eukaryotic cytosol and in mitochondria (Roy et al., 2004; Reynolds et al., 2010b). Bacterial and eukaryotic cytosolic PheRSs have an (αβ)$_2$ heterotetrameric structure, where the $\alpha$ subunit harbors the synthetic active site and the $\beta$ subunit contains a distinct editing site (Goldgur et al., 1997; Roy et al., 2004). *E. coli* PheRS maintains high specificity for cognate amino acid in the synthetic active site and also employs editing for additional accuracy (Reynolds et al., 2010b). Yeast *Saccharomyces cerevisiae* cytosolic PheRS (ctPheRS) lacks high specificity and relies on editing activity to clear any mistakes. In contrast to ctPheRS, mitochondrial PheRS (mtPheRS) achieves accuracy solely through high substrate specificity, and does not have editing activity. Recently, some obligate intracellular pathogenic *Mycoplasma spp.* have been shown to harbor proteomes with significant ambiguities at specific codons (Li et al., 2011). In particular, misincorporation rates at Leu and Phe codons were estimated to be as high as 1 in 200. Investigation of the editing activity of leucyl-tRNA synthetase (LeuRS) from *Mycoplasma mobile* suggests that mistranslation mainly stems from aaRS editing defects. Similarly PheRSs from
Mycoplasma and related species display remarkable sequence divergence, with extensive substitution of editing site residues. Whereas E. coli PheRS utilizes multiple pathways to maintain fidelity in aminoaacylation, Mycoplasma mobile PheRS (MmPheRS) appears to have lost at least one major quality control pathway. Here we show that MmPheRS is highly error prone in amino acid selection with an error rate greater than 1 in 3000. Although MmPheRS lacks robust posttransfer editing against mischarged tRNA, it has acquired alternative, albeit less efficient, quality control pathways. We also show that point mutations can effectively modulate the ability of PheRS to discriminate against noncognate amino acid. Thus, changes in the editing mechanisms of MmPheRS may reflect divergent selection pressure on the translation quality control machinery across bacteria during evolution.

3.2. Materials and methods

3.2.1. General methods

Site-directed mutagenesis was performed by PCR using primers obtained from Sigma. Aminoacylation, ATP consumption, deacylation and steady state kinetic assays were performed as described previously (Roy et al., 2004, 2005a).

3.2.2. Preparation of M. mobile PheRS

GeneIDs for M. mobile pheS encoding the α subunit and pheT encoding the β subunit are MMOB3170 and MMOB5160 respectively. MmPheRS gene sequence was codon-optimized for expression in E. coli, specifically TGA codons were substituted with TGG codons and the modified operon was synthesized (GenScript). The intergenic sequence
between *pheS* and *pheT* genes was replaced with the sequence from that in *E. coli* in order to ensure efficient transcription and translation of the two subunits in *E. coli*. The *MmPheRS* gene was subcloned into pQE31 vector (Qiagen) at SacI and HindIII restriction sites. The resulting plasmid pQE31-His<sub>6</sub>-*MmPheRS* was used to transform BL21(DE3)pLysS cells. The cells were grown to an OD<sub>600</sub> = 0.4 at 37 °C, 250 rpm, then grown until an OD<sub>600</sub> = 0.7 at 22 °C, 250 rpm. IPTG was then added to a final concentration of 0.5 mM and cells were grown overnight at 22 °C, 250 rpm. Cells were harvested, the pellet resuspended in a buffer containing 25 mM Tris-HCl (pH 8.0), 300 mM NaCl, 10 % glycerol and 5 mM imidazole, and flash-frozen using liquid N<sub>2</sub> and left at -80 °C. The frozen cells were thawed at 37 °C for 10 min, sonicated and supernatant was collected after centrifugation at 50,000 × g for 1 h. The supernatant was applied to a TALON® metal affinity resin column (Clontech), followed by washing and the protein was eluted with Tris-HCl, pH 8.0, 300 mM NaCl, 250 mM imidazole and 10 % glycerol. Fractions containing *MmPheRS* were checked for electrophoretic purity by SDS-PAGE, pooled and dialyzed overnight at 4 °C in buffer containing 10 mM Tris-HCl (pH 8.0), 200 mM NaCl, 5 mM NaH<sub>2</sub>PO<sub>4</sub> and 10 % glycerol, following which protein was aliquoted and stored in the same buffer with 50 % glycerol. *MmPheRS* variants were similarly purified using the above-mentioned protocol.

### 3.2.3. Cloning and *in vitro* transcription of *M. mobile* tRNA<sub>Phe</sub>

The gene for *M. mobile* tRNA<sub>Phe</sub><sup>GAA</sup> (GeneID: MMOB9150) was synthesized using synthetic DNA oligomers according to standard procedures (Sampson and Uhlenbeck, 1988) and cloned into pUC19 vector using BamHI and HindIII restriction sites to yield
pUC19-\textit{MmtRNA}^{\text{Phe}}. This plasmid (200 µg) was digested with BstNI to generate 3’ CCA and used as a template for run-off transcription using T7 RNA polymerase. The tRNA transcript was purified on denaturing 12% polyacrylamide gel and extracted by electrodialysis in 90 mM Tris-borate/2 mM EDTA, pH 8.0. tRNA was phenol and chloroform extracted, ethanol precipitated and resuspended in DEPC-treated ddH$_2$O. Refolding was carried out by heating the tRNA at 70 °C for 2 min, followed by addition of MgCl$_2$ to a concentration of 2 mM and subsequent slow cooling to room temperature.

3.2.4. Tyr-tRNA$^{\text{Phe}}$ preparation and hydrolysis assays

1 µM wild-type (WT) \textit{MmPheRS} was used to aminoacylate 5 µM tRNA$^{\text{Phe}}$ to produce Tyr-tRNA$^{\text{Phe}}$, which was then purified and reactions were performed as described previously (Roy et al., 2004). Reaction mixture contained 1 µM Tyr-tRNA$^{\text{Phe}}$ and 100-500 nM WT or mutant PheRS. BSA was used as a negative control and 0.1M NaOH as a positive control.

3.2.5. Steady state kinetics

Steady state kinetics were carried out at 37 °C according to Roy et al., 2004 and 2005a. For ATP-PP$_i$ exchange kinetics for amino acid activation, concentrations of substrates were varied from 0.5-200 µM for Phe, 0.1-9 mM for Tyr and 0.5-20 mM for ATP. Enzymes were added to a final concentration of 100-150 nM. For steady state aminoacylation kinetics, concentrations of substrates were varied from 0.5-100 µM for Phe, 20-400 µM for Tyr and 0.5-40 µM for tRNA$^{\text{Phe}}$. For cognate and noncognate
charging reactions final PheRS concentrations used were 100 nM and 500 nM, respectively.

3.2.6. Single turnover kinetics

Experiments were performed at 22 °C on a quench flow RQF-3 KinTek instrument, using the constant quench option. The buffer for PheRS contained 100 mM Na-Hepes (pH 7.2), 30 mM KCl, 10 mM MgCl$_2$ and 10 mM DTT, while the quench solution contained 3M sodium acetate (pH 4.5). In syringe A, enzyme•aminoacyl-adenylate complex was formed in situ by incubating 6-8 μM PheRS, 40 μM $[^{14}$C]-Phe (215 cpm/pmol) or 80 μM $[^{14}$C]-Tyr (360 cpm/pmol), 4 mM ATP and 2 units/ml of inorganic pyrophosphatase. Syringe B contained 2-4 μM tRNA$^{\text{Phe}}$. Reaction aliquots of 36 μl (18 μl each syringe) were quenched and precipitated in 5 % trichloroacetic acid. The radiolabeled aa-tRNA products were quantified by scintillation counting. Amount of aa-tRNA formed was plotted versus time and fit to single exponential equation, $y = C + A*(1 - \exp(-k_{\text{trans}}*t))$, where $C$ is the $y$ intercept, $A$ is the amplitude, $k_{\text{trans}}$ is the rate of aminoacyl transfer and $t$ is time in seconds.

3.2.7. E. coli NP37 pheS$^{ts}$ complementation

*E. coli* NP37 strain containing pheS$^{ts}$ mutation (Kast et al., 1992) was co-transformed with plasmids pQE31-MmPheRS (amp$^\text{r}$) and pREP4 (kan$^\text{r}$, Qiagen) to ensure tight transcriptional regulation of plasmid-encoded PheRS. The transformants were plated on LB supplemented with 100 μg/ml ampicillin, 50 μg/ml kanamycin and incubated at 30 °C. Individual colonies were then streaked and grown on the same media as above at both
30 °C and 42 °C for 24-48 h. To ensure the colonies appearing at 42 °C were not revertants, the presence of the pheS<sup>ts</sup> mutation in <i>E. coli</i> NP37 was confirmed by sequencing. Mutant <i>MmPheRS</i> constructs were screened for complementation similarly.

3.3. Results

3.3.1. <i>MmPheRS</i> lacks canonical posttransfer editing activity

In order to determine if the high error rates observed in the <i>M. mobile</i> proteome are in part due to PheRS-specific editing defects, we analyzed the editing activity and aminoacylation specificity of <i>MmPheRS</i>. Comparison of sequences of editing domains of bacterial PheRSs with that of <i>Mycoplasma</i> showed a high degree of divergence among <i>Mycoplasma</i> spp., including signature motif regions known to be important for PheRS editing (Fig. 3.1). Structural and biochemical investigations of the <i>E. coli</i> PheRS (<i>EcPheRS</i>) editing site revealed that conserved residues βR244, βH265, βG318, βE334, βT354, and βA356 are all involved in editing (Ling et al., 2007a). R244, which interacts with the tRNA backbone particularly at C75, is replaced by Lys in most <i>Mycoplasma</i> spp. <i>MmPheRS</i> retains the well conserved His in the signature motif QPxHxFD, which is proposed to play a structural role in stacking the substrate in the editing site (Ling et al., 2007a). Other key residues important for editing in <i>EcPheRS</i> are not well conserved in <i>MmPheRS</i> including the second signature motif, GVMGGxxS/T, which is difficult to align correctly due to extensive sequence changes. Based on these significant differences in the editing site we predicted that <i>MmPheRS</i> would lack posttransfer editing activity as a result of its divergent editing domain. As expected, <i>MmPheRS</i> failed to enzymatically deacylate mischarged Tyr-<i>tRNA<sub>Phe</sub></i> instead showing a profile comparable to that of
spontaneous hydrolysis (Fig. 3.2). To ascertain if Tyr-tRNA\textsuperscript{Phe} hydrolysis by \textit{Mm}PheRS is negligible, we compared its editing activity to that of an \textit{Ec}PheRS editing-deficient mutant (\(\alpha A294G \beta G318W\)), which has \(\sim80\)-fold lower trans editing activity than WT \textit{Ec}PheRS (Ling et al., 2007a). \(\alpha A294G \beta G318W\) \textit{Ec}PheRS hydrolyzed Tyr-tRNA\textsuperscript{Phe} slower than WT \textit{Ec}PheRS but much more rapidly in comparison to \textit{Mm}PheRS, confirming the loss of trans editing activity in the latter.

**Figure 3.1. Alignment of B3/B4 domain editing site residues in bacterial PheRSs.**

Key residues for editing are highlighted in black and indicated by arrows. Organism abbreviations are as follows: \textit{Mycoplasma mycoides} subsp. \textit{mycoides} (\textit{Mmy}), \textit{Mycoplasma capricolum} (\textit{Mca}), \textit{Mesoplasma florum} (\textit{Mfl}), \textit{Spiroplasma citri} (\textit{Sci}), \textit{Mycoplasma pneumoniae} (\textit{Mpn}), \textit{Mycoplasma genitalium} (\textit{Mge}), \textit{Mycoplasma gallisepticum} (\textit{Mga}), \textit{Mycoplasma penetrans} (\textit{Mpe}), \textit{Ureaplasma parvum} (\textit{Upa}), \textit{Mycoplasma synoviae} (\textit{Msy}), \textit{Mycoplasma agalactiae} (\textit{Mag}), \textit{Mycoplasma pulmonis} (\textit{Mpu}), \textit{Mycoplasma mobile} (\textit{Mmo}), \textit{Mycoplasma arthritidis} (\textit{Mar}), \textit{Mycoplasma hyopneumoniae} (\textit{Mhy}), \textit{Acholeplasma laidlawii} (\textit{Ala}), \textit{Phytoplasma australiense} (\textit{Pau}), \textit{Clostridium acetobutylicum} (\textit{Cac}), \textit{Bacillus subtilis} (\textit{Bst}), \textit{Staphylococcus aureus} (\textit{Sau}), \textit{Escherichia coli} (\textit{Eco}) and \textit{Thermus thermophilus} (\textit{Tth}).

Figure adapted from Li et al., 2011.
Figure 3.2. *Mm*PheRS lacks canonical posttransfer editing activity. Posttransfer hydrolysis of preformed Tyr-tRNA\(^{\text{Phe}}\) by *Mm*PheRS. WT EcPheRS and 0.1M NaOH serve as positive controls. αA294G βG318W *E. coli* PheRS is an editing-deficient mutant, which serves as an additional negative control.

3.3.2. *Mm*PheRS is inherently error prone

Tyr-tRNA\(^{\text{Phe}}\) hydrolysis assays suggest that the editing active site of *Mm*PheRS is unable to hydrolyze mischarged tRNA\(^{\text{Phe}}\). It is possible that the synthetic active site of *Mm*PheRS may have evolved high specificity to Phe to compensate for this loss and therefore may not misacylate noncognate Tyr. Whereas it is not surprising for *Mm*PheRS to aminoacylate cognate Phe (Fig. 3.3A), it is interesting that this enzyme can misacylate Tyr remarkably well (Fig. 3.3B). The level of mischarging by *Mm*PheRS was comparable to that of the αA294G βG318W EcPheRS editing-deficient mutant. The ability of *Mm*PheRS to bind and mischarge Tyr is in stark contrast to WT EcPheRS, which does not produce any mischarged Tyr-tRNA\(^{\text{Phe}}\). Our results suggest that the *Mm*PheRS synthetic active site has low specificity for Tyr and therefore is inherently error-prone.
Figure 3.3. *MmPheRS* is error-prone in aminoacylation. A, Phenylalanylation of *MmtRNA<sub>Phe</sub>* by *MmPheRS* and *EcPheRS*. B, Tyrosylation of *MmtRNA<sub>Phe</sub>* by *MmPheRS*, *EcPheRS* and the αA294G βG318W *E. coli* PheRS editing-deficient mutant.

3.3.3. Amino acid specificity of *MmPheRS*

Steady state kinetic analyses were performed to determine the relative amino acid specificities of *MmPheRS* during amino acid activation and overall charging. Steady state charging kinetics revealed a Phe:Tyr specificity ratio during aminoacylation of about 440:1 (Table 3.1). Thus, *MmPheRS* misacylates tRNA<sub>Phe</sub> with Tyr to levels greater than the typical threshold of ~1 in 3000 commonly associated with accuracy in amino acid selection (Loftfield and Vanderjagt, 1972; Beuning and Musier-Forsyth, 2000; Ling et al., 2009a).
Table 3.1. Steady state kinetic parameters for aminoacylation by \textit{MmPheRS}. Amino acid concentrations were varied in the following ranges: 0.5-100 µM for Phe, 20-400 µM for Tyr. Kinetic parameters were determined as averages from three independent reactions and their standard errors are shown.

\[\text{Specificity} (\text{Phe/Tyr}) = \frac{\frac{k_{\text{cat}}}{K_M}}{\text{Phe}} / \frac{\frac{k_{\text{cat}}}{K_M}}{\text{Tyr}}\]

Next, we examined in more detail the amino acid specificity during the first activation step. Previous work has shown that the \textit{EcPheRS} synthetic active site is highly specific for Phe over Tyr (~8000:1, Reynolds et al., 2010b). The \(\alpha\)A294 residue is the major specificity determinant and a glycine mutation at this position enlarges the active site pocket allowing it to bind Phe analogs such as \(p\)-Cl Phe and Tyr (Ibba et al., 1994). The \(\alpha\)A294G variant \textit{EcPheRS} exhibits a 100-fold loss in specificity for Phe over Tyr (Reynolds et al., 2010b). Consistently, yeast \textit{S. cerevisiae} cytosolic PheRS (ctPheRS) which contains a Gly (G458) at the position homologous to \(\alpha\)A294 in \textit{EcPheRS} also exhibits a low Phe:Tyr specificity ratio (~500:1) (Reynolds et al., 2010b). Conversely an \(\alpha\)G458A replacement in \textit{Sc} ctPheRS restores high specificity for Phe over Tyr. Steady state amino acid activation kinetics indicate that the \textit{MmPheRS} active site displays a low specificity for Phe over Tyr of ~500:1 (Tables 3.2 and 3.3). This ratio is comparable to that of \textit{Sc} ctPheRS, however, it is interesting to note that \textit{MmPheRS} retains an alanine residue at the \(\alpha\)A294 position. This suggests an alternative mechanism for loss of active site specificity in \textit{MmPheRS} compared to \textit{Ec} and \textit{Sc} ctPheRS, where \(\alpha\)A294 acts as a
major determinant for specificity. Taken together, our data show that *Mm*PheRS lacks amino acid specificity during aminoacylation in addition to loss of editing activity.

\[
\text{Specificity} = \frac{k_{\text{cat}}/K_M}{(\text{mutant}/\text{WT})}
\]

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<th></th>
<th>(k_{\text{cat}}) (s(^{-1}))</th>
<th>(K_M) (µM)</th>
<th>(k_{\text{cat}}/K_M) (s(^{-1}) µM(^{-1}))</th>
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| WT (ATP) | 23±0.7\(^a\) | 600±70\(^a\) | 0.04±0.002
| WT        | 10±0.2                  | 10±1.3          | 1.0±0.2                                      |
| βT305A    | 28                   | 10              | 2.8                                           |
| βQ306A    | 15                   | 5               | 3                                             |
| βT305A/Q306A | 19                   | 9               | 2.1                                           |

Table 3.2. ATP-PPi exchange kinetics for Phe activation by *Mm*PheRS and mutants. Substrate concentrations were varied as follows: 0.5-200 µM for Phe and 0.5-20 mM for ATP. \(^a\)Data represent \(k_{\text{cat}}\) and \(K_M\) for ATP; Other parameters represent \(k_{\text{cat}}\) and \(K_M\) for Phe.

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<th>(k_{\text{cat}}) (s(^{-1}))</th>
<th>(K_M) (µM)</th>
<th>(k_{\text{cat}}/K_M) (s(^{-1}) µM(^{-1}))</th>
<th>Specificity(^a) (Phe/Tyr)</th>
<th>Relative specificity(^b) (Mutant/WT)</th>
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<tbody>
<tr>
<td>WT</td>
<td>8±0.4</td>
<td>3900±540</td>
<td>0.0021±0.0003</td>
<td>~500</td>
<td>1</td>
</tr>
<tr>
<td>βT305A</td>
<td>ND</td>
<td>ND</td>
<td>0.0018</td>
<td>~1400</td>
<td>2.8</td>
</tr>
<tr>
<td>βQ306A</td>
<td>ND</td>
<td>ND</td>
<td>0.0015</td>
<td>~1500</td>
<td>3</td>
</tr>
<tr>
<td>βT305A/Q306A</td>
<td>ND</td>
<td>ND</td>
<td>0.0007</td>
<td>~3000</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 3.3. ATP-PPi exchange kinetics for Tyr activation by *Mm*PheRS and mutants. Tyrosine concentrations were varied in the range 0.1-9 mM. \(^a\)Specificity (Phe/Tyr) = \((k_{\text{cat}}/K_M)\)Phe/ \((k_{\text{cat}}/K_M)\)Tyr
\(^b\)Relative specificity = (specificity)\(_{\text{Mutant}}\)/ (specificity)\(_{\text{WT}}\)
\(^c\)The \((k_{\text{cat}}/K_M)\) is estimated using sub-saturating Tyr concentrations from the slope of the equation, \(V = k_{\text{cat}} [E][S]/K_M\). ND, not determined.

3.3.4. *Mm*PheRS displays weak tRNA-dependent pre-transfer editing activity

Although we did not detect any Tyr-tRNA\(^{\text{Phe}}\) hydrolysis activity in *trans*, it is possible that *Mm*PheRS may *cis*-edit Tyr-tRNA\(^{\text{Phe}}\) or pretransfer edit noncognate Tyr-AMP. To investigate these possibilities, ATP consumption assays were performed using either cognate Phe or noncognate Tyr, in the presence or absence of *Mt*RNA\(^{\text{Phe}}\). Background
rates of ATP hydrolysis in the absence of enzyme were subtracted during each individual experiment. The level of ATP consumption in the presence of noncognate Tyr was similar to that in the presence of cognate Phe. However ATP consumption was higher in the presence of tRNA and the noncognate Tyr, suggesting that there is weak tRNA-dependent editing activity (Fig. 3.4A). To distinguish between cis-posttransfer editing and tRNA-dependent pretransfer editing, we utilized a nonchargeable substrate, 2′-deoxy EctRNA\textsuperscript{Phe} (Roy et al., 2004). In the presence of noncognate Tyr and 2′-deoxy EctRNA\textsuperscript{Phe}, the ATP consumption rate was similar to that found in the presence of Tyr and chargeable tRNA (Fig. 3.4B). These data indicate that MmPheRS exhibits a weak tRNA-dependent pretransfer hydrolysis activity.

\textbf{Figure 3.4. ATP hydrolysis by MmPheRS.} A, ATP hydrolysis activity in the presence of cognate Phe (1 mM) or noncognate Tyr (5 mM), with or without Mm\textit{tRNA}_{\text{Phe}} (10 \mu M). B, tRNA-dependent ATP hydrolysis in the presence of noncognate Tyr (5 mM) with \textit{MmtRNA}_{\text{Phe}} (5 \mu M), 2′-deoxy EctRNA\textsuperscript{Phe} (5 \mu M) or no tRNA. ATP hydrolysis was monitored in the absence of enzyme during each experiment and subtracted from data points.
3.3.5. Amino acid transfer rates

Amino acid activation kinetics showed similar rates of activation ($k_{cat}$) for both Phe and Tyr, while tRNA charging kinetics revealed that the overall rate ($k_{cat}$) for noncognate Tyr is about 10-fold lower than that of cognate Phe. This suggests MmPheRS may kinetically discriminate against noncognate Tyr at a step subsequent to activation, either aminoacyl transfer or product release. Using αA294G and αA294S EcPheRS variants, which can accommodate unnatural Phe analogs such as p-chlorophenylalanine (p-Cl Phe), amino acid transfer rate constants have been measured for cognate Phe and p-Cl Phe (Ibba et al., 1995). It was shown that p-Cl Phe is transferred at a faster rate to tRNA$^{Phe}$ compared to cognate Phe. It was proposed that decreased stability of the PheRS•aminoacyl-AMP complex leads to increased rates of tRNA transfer and charging. Accordingly we expected that EcPheRS would form an unstable activation complex with noncognate Tyr, which would then be transferred at a higher rate than that of cognate Phe. Using rapid quench assays under single turnover conditions, we measured $k_{trans}$ for Tyr as well as Phe. An EcPheRS editing deficient mutant (αA294G βG318W) was employed to prevent hydrolysis of mischarged Tyr-tRNA$^{Phe}$ during the time-scale of the reaction. The PheRS•aminoacyl-AMP complex is not stable following purification, so the complex was formed in situ in the first syringe as described previously (Zhang et al., 2006). The sample was then mixed rapidly with tRNA$^{Phe}$ in the second syringe and quenched at various time intervals. The observed $k_{trans}$ for Tyr is ~1.5 fold higher (30 s$^{-1}$) than that of Phe (19 s$^{-1}$) (Figs. 3.5A and B). The Phe transfer rate constant is consistent with the previously published value determined using αA294G EcPheRS. We then measured cognate and noncognate amino acid transfer rates for MmPheRS. The rate of Phe transfer,
\( k_{\text{trans}} = 10 \text{s}^{-1} \) (Fig. 3.5C) was faster than the rate of Tyr transfer, which is \( \sim 2.5 \) fold slower (Fig. 3.5D). In summary, our data suggest a distinct mechanism of discrimination against noncognate Tyr by \( MmPheRS \) during aminoacylation, in contrast to \( EcPheRS \) where Tyr is favored during the transfer step.

![Graphs showing amino acid transfer by bacterial PheRSs.](image)

**Figure 3.5. Amino acid transfer by bacterial PheRSs.** Transfer of Phe (A, C) and Tyr (B, D) by \( EcPheRS \) and \( MmPheRS \) respectively. Transfer rate constants \( \left( k_{\text{trans}}, \text{s}^{-1} \right) \) were measured under single turnover conditions using 6-8 \( \mu \text{M} \) PheRS and 2-4 \( \mu \text{M} \) tRNA\(^{\text{Phe}}\) as described in section 3.2.6 (Materials and methods).
3.3.6. Restoring proofreading in *MmPheRS*

*MmPheRS* lacks the robust posttransfer editing pathway found in *EcPheRS*. *MmPheRS* displays weak tRNA-dependent pretransfer editing activity and has acquired a less stringent discrimination against Tyr during the transfer step. Using *E. coli* NP37 strain containing a temperature-sensitive (ts) *pheS* allele, we tested if this error-prone PheRS can rescue growth at restrictive temperature. WT *EcPheRS* displayed robust complementation, while *MmPheRS* was unable to complement *E. coli* NP37 at 42 °C. The *EctRNA* is homologous to *MmtRNA* and both *EcPheRS* and *MmPheRS* can cross-aminoacylate either tRNA (data not shown), suggesting the lack of complementation by *MmPheRS* does not result from a tRNA recognition defect. We attempted to restore posttransfer editing in *MmPheRS* by replacing editing site residues at key positions known to be important for editing function, using *EcPheRS* as a model. The first signature motif QPxHxFD in *MmPheRS* showed conservative replacements and retained the invariant His residue (see above). However, the sequences in the second motif GVMGGxxS/T are less conserved. The conserved glycine residues βG315, βG318 and βG319 found in *EcPheRS* and many bacterial PheRSs are substituted with relatively bulkier corresponding residues βY302, βT305 and βQ306 in *MmPheRS*. We hypothesized that these naturally occurring substitutions in place of smaller glycine residues may occlude Tyr from the editing pocket. Therefore we mutated these residues to alanines to obtain βY302A, βT305A and βQ306A variants of *MmPheRS*. We screened these *pheS* genes with Ala mutations for their ability to rescue the ts phenotype of *E. coli* NP37. Genes encoding βT305A and βQ306A *MmPheRS* rescued growth at 42 °C as well.
as the double mutant βT305A/Q306A MmPheRS (Fig. 3.6), which also complements, albeit poorly. We further tested if these point mutations conferred an editing activity against mischarged Tyr-tRNA\(^{\text{Phe}}\) \textit{in vitro}. \textit{Trans}-editing assays showed that the Ala substitutions at T305A and Q306A do not restore posttransfer editing. In addition, ATP consumption assays did not show an increased rate of AMP production confirming that these mutants did not acquire editing function (data not shown). The single mutants βT305A and βQ306A MmPheRS and the double mutant did show reduced mischarging activity with Tyr compared to WT (Fig. 3.7). Amino acid activation kinetics revealed that the mutations βT305A and βQ306A conferred higher specificity towards the cognate substrate, Phe versus Tyr (Tables 3.2 and 3.3). Hence, point mutations in the editing domain of MmPheRS, near the α/β domain interface, increase discrimination against noncognate Tyr. This suggests communication between the editing and synthetic active sites, consistent with the recent observation that fusion of the CP1 editing domain to MmLeuRS leads to enhancement of amino acid discrimination during the activation step in addition to conferring posttransfer hydrolysis activity (Boniecki and Martinis, 2012).
Figure 3.6. Rescue of growth phenotype of *E. coli* NP37 (*pheS*<sup>ts</sup>). *E. coli* NP37 was transformed with WT *Mm*PheRS, mutant *Mm*PheRSs βT305A, βQ306A, βT305A/Q306A, WT *Ec*PheRS as positive and empty vector as negative controls.

Figure 3.7. Aminoacylation by *Mm*PheRS mutants. A, Phenylalanylation and B, tyrosylation by WT *Mm*PheRS and mutants βT305A, βQ306A, βT305A/Q306A.
3.4. Discussion

Despite four decades of research in the field of aaRS editing the \textit{in vivo} significance of editing function remains unclear. Although aaRSs are essential to cells, there is often no growth defect upon loss of aaRS editing (Ruan et al., 2008). Editing mechanisms may become necessary under stress conditions such as nutrient limitation, or in aging cells, which accumulate erroneous proteins that form misfolded aggregates. However, the fact that some organisms display greater tolerance to errors compared to others suggests that proofreading by aaRSs has evolved to maintain a level of fidelity suited to the physiology of particular organisms.

3.4.1. Divergence of bacterial PheRS

Sequence comparisons of several aaRS families in \textit{Mycoplasma} reveal a high degree of divergence of their editing domains compared to other bacterial aaRSs (Li et al., 2011). An extreme example is that of \textit{MmLeuRS} that lacks the entire CP1 editing domain, which is substituted by a 9-amino acid linker region. In the case of PheRS, none of the \textit{Mycoplasma} spp. examined to date have completely lost their editing domains, which may in part be explained by the requirement of the \(\beta\)-subunit C-terminus for tRNA anticodon recognition. Nevertheless, the amino acid composition of PheRS from \textit{Mycoplasma} is highly diverged, which may be partly explained by their AT-rich genomes (Dybvig and Voelker, 1996). Variation in AT/GC content of bacterial genomes has been shown to influence the amino acid composition of the proteome (Lobry, 1997). Bioinformatic analyses have shown that organisms containing AT-rich genomes likely possess a greater bias to AT-rich codons encoding the amino acids Phe, Tyr, Met, Ile,
Asn and Lys, and a lower number of GC-rich codons coding for Gly, Ala, Arg and Pro (Singer and Hickey, 2000). This nucleotide bias is believed to be the driving force for the codon reassignment for Trp, from UGG to UGA. We compared the sequence of MmPheRS to that of EcPheRS and found a correlation of amino acid composition as predicted by the nucleotide bias (Fig. 3.8). This could provide at least in part an explanation for the substitutions of various synthetic and editing site residues observed in MmPheRS. Therefore, the effect of nucleotide bias appears to be one of the non-specific factors causing a shift in amino acid composition, and functional properties, of the Mycoplasma proteome including PheRS.

![Figure 3.8. Amino acid composition of PheRS.](image)

**Figure 3.8. Amino acid composition of PheRS.** Blue bars represent *M. mobile* PheRS and green bars represent *E. coli* PheRS.
3.4.2. *M. mobile* PheRS has evolved to decrease accuracy

Our biochemical and kinetic investigation of *Mm*PheRS reveals a loss of the high stringency posttransfer editing pathway and acquisition of low stringency discrimination during transfer. This discrimination against noncognate Tyr during transfer is clearly insufficient to eliminate errors in aminoacylation and subsequent translation (Li et al., 2011). The low fidelity of *Mm*PheRS is mainly due to the relatively low specificity for cognate versus the noncognate amino acid. The selectivity for the cognate amino acid takes into account the cellular concentrations of amino acids in addition to aaRS specificity (Reynolds et al., 2010b). It is noteworthy that *de novo* amino acid biosynthesis pathways are missing in *M. mobile* (Dybvig and Voelker, 1996). This obligate parasite instead derives its amino acids from the host. Hence, it is possible that the level of fidelity maintained by PheRS is fine-tuned based on the availability of individual amino acids in cells. *M. mobile* PheRS has evolved to be less accurate as per the physiological demands of the organism and probably does not require stringent proofreading mechanisms utilized by other bacteria such as *E. coli*.

3.4.3. AaRS-editing defects allow for translational ambiguity at specific codons

The *Mycoplasma* proteome has clearly adapted to accumulate higher levels of ambiguous decoding. Translational ambiguity can arise *via* different mechanisms, for instance ribosomal ambiguity (*ram*) mutations cause miscoding by ribosomes most likely by increasing the affinity of noncognate aa-tRNA in a non-specific, codon-independent manner (Karimi and Ehrenberg, 1994). Antibiotic-induced ribosomal miscoding is believed to occur in a similar fashion (Moazed and Noller, 1987). Another mechanism of
generic mistranslation involves mismethionylation of several tRNA species by MetRS in bacteria as well as mammalian cells, where it is hypothesized to protect against oxidative stress (Netzer et al., 2009; Jones et al., 2011). *M. mobile* has a variable proteome with misincorporations often confined to specific codons (Li et al., 2011). In particular, decoding of Leu and Phe codons is highly erroneous with rates of approximately 1 in 200 versus a low error rate $\leq$1 in 3000 across other codons. Data presented here, together with recent studies on *Mm*LeuRS (Li et al., 2011), suggest that aaRS-editing defects are in part responsible for the high error rates observed in Mycoplasma protein synthesis. We propose that aaRSs allow for error modulation by restricting the errors to a subset of codons rather than causing a global defect. This hypothesis is supported by the observation that editing defects are not seen in all aaRSs but only in particular cases such as PheRS and LeuRS in *M. mobile*. Our results show that *Mm*PheRS can alter its specificity to favor the cognate amino acid simply through point mutations. In conclusion, aaRSs can facilitate adaptation to changes in cellular physiology by regulating codon ambiguity at specific sites during translation.
CHAPTER 4

LARGE-SCALE MOVEMENT OF FUNCTIONAL DOMAINS FACILITATES AMINOACYLATION BY HUMAN MITOCHONDRIAL PHENYLALANYL-TRNA SYNTHETASE

4.1. Introduction

Aminoacyl-tRNA synthetases (aaRSs) play a key role in maintaining translational fidelity by synthesizing correctly paired aminoacyl-tRNAs (aa-tRNAs) (Ataide and Ibba, 2006; Ling et al., 2009a). Aminoacyl-tRNA synthesis occurs in two steps: (i) amino acid activation forming aminoacyl adenylate (aa-AMP) and (ii) transfer of amino acid to the 3' end of cognate tRNA. While some aaRSs are highly selective for their substrate amino acids, others require a correction mechanism called “editing” to eliminate the incorrect products (Jakubowski and Goldman, 1992; Hendrickson and Schimmel, 2003). AaRSs are divided into two mutually exclusive groups, class I and class II, based on their structure and function (Cusack et al., 1990; Eriani et al., 1990; Ribas De Pouplana and Schimmel, 2001). Class I aaRSs are usually monomeric, whereas class II aaRSs mostly function as dimers or tetramers. A common feature of all aaRS enzymes is the modular
arrangement of functional units along the sequence (Alexander and Schimmel, 2001). A catalytic domain in the N-terminal half is often responsible for aminoacyl adenylate synthesis, and RNA recognition elements are usually present in the C-terminal region. It is believed that the catalytic domain constitutes the ancestral core enzyme to which several accessory domains including sequences important for RNA recognition, oligomerization, assembly and complex formation have been appended later in evolution (Ibba et al., 1997a).

Phenylalanyl-tRNA synthetase (PheRS), a class II aaRS, exists as an (αβ)_2 heterotetramer in the bacterial (Goldgur et al., 1997) and eukaryotic cytosolic forms (Moor et al., 2002), where the α subunit constitutes the catalytic module and the B3/B4 domain of the β subunit forms the editing site (Roy et al., 2004; Kotik-Kogan et al., 2005). PheRS is instead active as a monomer in the organelles of eukaryotes (Sanni et al., 1991; Bullard et al., 1999) and lacks an editing function (Roy et al., 2005a). Human mitochondrial PheRS (mtPheRS) is highly homologous to the corresponding domains of bacterial PheRS, consisting of an N-terminal catalytic α domain fused to a C-terminal B8-like anticodon binding domain (ABD) from the β subunit (Klipcan et al., 2008). The crystal structure of mtPheRS revealed a fully closed conformation of the enzyme in which the anticodon binding domain located at the C-terminus overlaps with the acceptor stem of tRNA^{Phe} if the substrate is modeled in a position similar to that seen in the bacterial heterotetrameric PheRS-tRNA^{Phe} complex. To bind tRNA^{Phe} correctly, the ABD must undergo an ~160° hinge-type rotation upon tRNA binding (Klipcan et al., 2008).
MtPheRS is therefore proposed to exist in two conformations, a “closed” state when it is not bound to the tRNA and an “open” state upon tRNA binding. To test this hypothesis, we restrained the movement of the ABD relative to the catalytic domain, which led to a loss in aminoacylation but not Phe activation. SAXS analysis confirmed the existence of the open and closed states in solution, underscoring the importance of conformational mobility in mtPheRS for maintaining the function of this essential housekeeping enzyme.

4.2. Materials and methods

4.2.1. Protein expression, purification and characterization

*E. coli* strains BL21 (pArgU218)/pET21c-PheRS expressing C-terminal His$_6$-tagged mtPheRS was a gift from Dr. L. Spremulli (University of North Carolina, Chapel Hill, NC). Residues K33 and T351 of mtPheRS were replaced by cysteines through PCR mutagenesis. These residues are separated by a ~5 Å distance, suitable for disulfide bond formation. Primers were obtained from IDT (Integrated DNA Technologies). Rosetta (DE3) cells containing pRARE plasmids encoding tRNAs for rare codons were transformed with the mutant mtPheRS plasmid construct and selected on medium containing both ampicillin and chloramphenicol. The cells were grown at 37 °C to OD = 0.8 and induced with 0.5 mM IPTG for 4 h. Cells were harvested, sonicated and supernatant was collected after centrifugation at 25,000×g for 1 h. The supernatant was applied to a TALON® metal affinity resin column (Clontech), followed by washing and the protein was eluted with Tris-HCl, pH 8.0, 300 mM NaCl, 250 mM imidazole and 10
% glycerol. Fractions containing mtPheRS were checked for electrophoretic purity by SDS-PAGE, pooled and dialyzed overnight at 4 °C in 50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂ and 100 mM KCl. The purified enzyme was concentrated, adjusted to 50 % (v/v) glycerol, and aliquots were stored at -80 °C. The presence of disulfide bonds in K33C / T351C mtPheRS was determined by non-reducing SDS-PAGE. Samples were mixed with a loading buffer containing 100 mM Tris-HCl, pH 6.8, 20 % (v/v) glycerol, 4 % (w/v) SDS, 0.05 % (w/v) bromophenol blue and 10 mM DTT (for reduced samples only) before electrophoresis. A 4-15 % pre-cast polyacrylamide gel (Biorad) was used to resolve the proteins and visualized using Coomassie blue staining. Aminoacylation and active site titration assays were performed as described before (Ibba et al., 1994; Ling et al., 2007b).

4.2.2. Fluorescence intensity measurements and tRNA binding

2AP-labeled E. coli tRNA\textsuperscript{Phe} was synthesized as described previously (Chapter 2). Different concentrations of WT and mutant mtPheRS were allowed to equilibrate with 0.1 µM 2AP-tRNA\textsuperscript{Phe} in 0.1 M Tris, pH 7.5, for 15 mins. Fluorescence intensities of protein-tRNA complexes were measured using a Fluorolog-3 spectrofluorimeter (Horiba Jobin Yvon). From the fluorescence intensity curves, area under the curve (AUC) was determined. The percentage increase in AUC with increase in protein concentration was then compared.
4.2.3. Small angle X-ray scattering (SAXS)

Synchrotron small angle X-ray scattering data were collected by the Svergun lab, at the bending magnet beamline X33 (DORIS III storage ring, DESY) of the EMBL Hamburg Outstation using a pixel detector PILATUS 1M (DECTRIS, Switzerland). To monitor for radiation damage, four successive 30 second exposures of the same sample were done and no changes were detected. The sample-to-detector distance was 2.7 m providing the range of scattering vectors $0.08 < s < 6 \text{ nm}^{-1}$ ($s=4\pi \sin \theta / \lambda$, where $2\theta$ is the scattering angle and $\lambda=0.15 \text{ nm}$ is the X-ray wavelength). Solutions of mtPheRS alone and its complex with tRNA were measured at three different concentrations of mtPheRS in the range from 1 to 5 mg/ml (sample temperature 10 ºC). The data were processed following standard procedures using PRIMUS (Konarev et al., 2003) and GNOM (Svergun 1992), and calibrated against a reference solution of bovine serum albumin (MM = 66 kDa) to determine the overall parameters (molecular mass, radius of gyration $R_g$, and maximal diameter $D_{\text{max}}$). The estimation of excluded volume $V_{\text{ex}}$ and low-resolution \textit{ab initio} models of mtPheRS solutes were obtained using DAMMIF (Svergun 1999; Franke and Svergun, 2009). This program builds a compact interconnected bead model fitting the experimental data $I_{\text{exp}}(s)$ to minimize discrepancy:

\[
\chi^2 = \frac{1}{N-1} \sum_j \left[ \frac{I_{\text{exp}}(s_j) - cI_{\text{calc}}(s_j)}{\sigma(s_j)} \right]^2,
\]
where N is the number of experimental points, c is a scaling factor and \( I_{\text{calc}}(s) \) and \( \sigma(s_j) \) are the calculated intensity and the experimental error at the momentum transfer \( s_j \), respectively. The scattering patterns from the atomic models were computed by CRYSOL (Svergun et al., 1995). The volume fractions of mtPheRS-tRNA complex and free tRNA as well as fitting to the measured SAXS data were computed using OLIGOMER (Konarev et al., 2003).

4.3. Results

4.3.1. Catalytic activity of open and closed mtPheRS

Wild-type (WT) and K33C/T351C mtPheRS samples were subjected to electrophoresis under reducing and non-reducing conditions (Fig. 4.1). Both the samples migrated to a similar extent when 10 mM DTT was included in the sample loading buffer (lanes 1, 2; monomeric mtPheRS, ~48 kDa) while under non-reducing conditions K33C/T351C mtPheRS migrated differently (lane 4) compared to WT (lane 3). The faster migration of the mutant is consistent with the formation of an intramolecular disulfide bond between the two cysteines at positions 33 and 351. In addition, intermolecular disulfide bond formation was observed in both the WT as well as double cysteine mutant mtPheRSs, forming dimers of ~96 kDa. This is expected considering the presence of six cysteines in WT mtPheRS. None of these six cysteines are in proximity to each other or to the newly introduced cysteine residues at positions 33 and 351, ruling out the possibility of formation of other intramolecular disulfide bonds. Additionally, titration of the free thiols
using Ellman’s reagent, 5, 5′-dithiobis-2-nitrobenzoic acid (DTNB) or 4, 4′-dithiopyridine (DTDP) confirmed the presence of equal numbers of reactive thiol groups in the WT and mutant mtPheRSs under non-reducing conditions (data not shown).

**Figure 4.1. SDS-PAGE analysis of purified human mtPheRS.** WT and mutant mtPheRS samples were analyzed on a 4-15 % (w/v) polyacrylamide gradient gel and the proteins were visualized by staining with Coomassie blue. Lanes 1 and 2 show WT and K33C / T351C mtPheRSs respectively, in the presence of 10 mM DTT. Lanes 3 and 4 represent WT and K33C / T351C mtPheRSs under non-reducing conditions. M stands for molecular weight marker.
WT and mutant mtPheRSs were tested for their ability to catalyze the first step in aminoacylation, formation of an enzyme-bound aminoacyl adenylate, in the absence of reducing agents. Time courses for PheRS-Phe-AMP formation were monitored using the active site titration protocol. The closed form of the K33C/T351C mutant was able to form stable enzyme phenylalanyl adenylate complexes at approximately 60% of the level shown by WT mtPheRS under the same conditions (Fig. 4.2). These data indicate that the first step of the Phe-tRNA synthesis pathway, Phe-AMP formation, is not dependent on the conformational state of mtPheRS. Next we investigated the ability of open and closed mtPheRS to catalyze the second step of the aminoacylation reaction by monitoring Phe-tRNA accumulation. Aminoacylation assays performed under non-reducing conditions suggest that K33C/T351C mtPheRS has negligible activity compared to WT when it is restrained in the closed conformation (Fig. 4.3A). Addition of 10 mM DTT to the aminoacylation reaction, which would allow K33C/T351C mtPheRS to adopt an open conformation, increased Phe-tRNA synthesis by the mutant to approximately 90% of WT level (Fig. 4.3B), indicating that conformational flexibility of functional domains is essential for the aminoacylation activity of mtPheRS.
Figure 4.2. Formation of PheRS:Phe-AMP complex. Phenylalanyl-adenylate complex formation by WT (●) and K33C/T351C (■) mtPheRSs.

Figure 4.3. Aminoacylation by mtPheRS. Phenylalanylation by WT (●) and K33C/T351C (■) mtPheRS, in the absence of any reducing agents (A), and in the presence of 10 mM DTT (B).
4.3.2. tRNA binding by mtPheRS

It is possible that either the closed form of K33C/T351C mtPheRS is unable to bind tRNA\textsuperscript{Phe} or that binding does not result in the correct conformational change necessary for tRNA\textsuperscript{Phe} aminoacylation. In order to test these two possibilities we attempted to measure binding constants for tRNA\textsuperscript{Phe} using fluorescence techniques. It has been estimated previously that mtPheRS has a $K_M$ for tRNA\textsuperscript{Phe} of $\sim 18 \mu M$ (Bullard et al., 1999) which, given the low yields of active mitochondrial tRNA\textsuperscript{Phe} transcribed \textit{in vitro}, precludes accurate determination of binding constants. Comparison of binding at sub-saturating concentrations suggests that WT mtPheRS binds tRNA\textsuperscript{Phe} better than the closed mutant form (Fig. 4.4), consistent with the proposal that conformational flexibility is crucial for maintaining the aminoacylation function. The different possible conformations in solution of mtPheRS were further investigated by small angle X-ray scattering (SAXS).
**Figure 4.4. tRNA^{Phe} binding by mtPheRS.** Binding of 2AP-\textit{EctRNA}^{Phe} to mtPheRS. WT (●) and K33C/T351C (■) mtPheRS. Experiment was performed by Srujana S. Yadavalli and Dr. Rajat Banerjee.
4.3.3. Modeling of mtPheRS structure by SAXS

The conformations of mtPheRS both alone and in complex with tRNA were probed by SAXS. The overall parameters of mtPheRS and the mtPheRS-tRNA\textsuperscript{Phe} complex are summarized in Table 4.1. The experimental scattering curve from native mtPheRS (Fig. 4.5, curve 1, dots) cannot be adequately fitted by the calculated scattering curve from the crystal structure of mtPheRS (PDB code 3cmq.pdb, (Klipcan et al., 2008)) showing the discrepancy $\chi= 3.04$ (Fig. 4.5, curve 1, blue line). At the same time, the fit calculated from the proposed open conformation of mtPheRS provides much better agreement with the experimental data with the discrepancy $\chi= 1.38$ (Fig. 4.5, curve 1, red line). However, smearing of the scattering profile suggests that the structure in solution may be flexible. Such a conformational mobility would not be surprising due to the extended linker region (residues 290-322 in mature mtPheRS), connecting the catalytic module of the enzyme to the anticodon binding domain. These results are indicative of the conformational flexibility of mtPheRS that exists in solution, and demonstrate that both the “crystal” and “solution forms” are two functionally relevant states.
Table 4.1. Overall structural parameters evaluated from SAXS data. $R_g$, $MM_{exp}$, $D_{max}$, and $V_{ex}$ are the radius of gyration, molecular mass (MM), maximum size, and excluded volume derived from scattering data. $MM_{calc}$ is the MM calculated from primary sequence. $\chi$ values are the discrepancies between experimental data and calculated scattering curves. Data generated by Drs. Svergun, Safro and their co-workers.

<table>
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<th>Sample</th>
<th>$R_g$, nm</th>
<th>$MM_{exp}$, kDa</th>
<th>$MM_{calc}$, kDa</th>
<th>$D_{max}$, nm</th>
<th>$V_{ex}$, nm$^3$</th>
<th>$\chi$</th>
</tr>
</thead>
<tbody>
<tr>
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<td>46.4</td>
<td>48.4</td>
<td>10.5</td>
<td>98</td>
<td>1.38</td>
</tr>
<tr>
<td>MtPheRS+tRNA</td>
<td>3.12</td>
<td>68.7</td>
<td>71</td>
<td>12.5</td>
<td>80</td>
<td>1.60</td>
</tr>
<tr>
<td>Ratio</td>
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<td>1.48</td>
<td>1.47</td>
<td>1.2</td>
<td>0.82</td>
<td>-</td>
</tr>
</tbody>
</table>

Figure 4.5. Scattering profiles of mtPheRS alone and its complex with tRNA. Experimental SAXS data of mtPheRS alone (curve 1) and the complex (curve 2) are represented in logarithmic scale by dots with error bars. The fitting curves are shown by solid lines. Fits obtained from the crystal structures of “closed” and “opened” conformations of mtPheRS are shown in (1) as blue and red lines respectively. Fit obtained by OLIGOMER for the model of the complex is shown in (2) as red line. For better visualization the scattering curve of the protein was multiplied by a factor of 20. Data generated by Drs. Svergun, Safro and their co-workers.
Given that the solution of the complex contained excess tRNA, no direct modeling of its structure was possible. A set of tentative models of the complex, which obey the contact conditions, was generated interactively by MASSHA (Konarev et al., 2001). The scattering patterns of these models were calculated and taken in a linear combination with the computed scattering from the tRNA molecule (PDB code 1eiy) to fit the experimental pattern from the complex using OLIGOMER. The overall best model (Fig. 4.6) provides the fit with $\chi^2 = 1.60$ (Fig.4.5, curve 2) at the volume fractions of 53 % and 47 % for the 1:1 mitPheRS-tRNA complex and free tRNA, respectively. This model further suggests that the formation of the mtPheRS-tRNA complex is accompanied by significant rearrangement of the anticodon binding domain.
4.4. Discussion

4.4.1. The open conformation of mtPheRS aminoacylates tRNA$^{Phe}$

Biochemical data indicate that while the open form of mtPheRS only binds tRNA$^{Phe}$ moderately more strongly than the closed form, only the open form is competent in aminoacylation. This is consistent with models of the open form derived from the crystal structure of mtPheRS, which suggested that accurate binding of tRNA recognition elements would necessitate a large conformational rearrangement in the C-terminal
anticodon-binding domain. The use of SAXS to augment existing crystallographic data confirmed the existence of the open form of mtPheRS, and provided a model for tRNA_{Phe} recognition. As seen for other class II aaRSs, mtPheRS recognizes the major groove side of the acceptor stem (Fig. 4.6) but does so via an unusually small contact region with tRNA_{Phe} (74 and 17 protein residues for bacterial and mitochondrial PheRSs, respectively). The majority of the interactions with tRNA_{Phe} are concentrated around the anticodon loop and 3’-CCA-end, and the corresponding amino acid sequences are strictly conserved throughout bacterial and mitochondrial PheRSs, and also occupy similar positions in space. These data indicate that mtPheRS has only retained the common, evolutionarily conserved, tRNA_{Phe} recognition domains while losing idiosyncratic structures such as the B2 RNA binding domain of bacterial PheRS (Roy and Ibba, 2006). This minimal recognition strategy is consistent with the ability of monomeric mtPheRS to efficiently cross-aminoacylate tRNA_{Phe} from E. coli, T. thermophilus, and yeast cytoplasm, a property absent from (αβ)_{2}-type PheRSs (H. Roy and M. Ibba, personal communication).

4.4.2. Evolution of mtPheRS and the modularity of aaRSs

The protein-RNA recognition interface of mtPheRS is considerably smaller than that of its cytoplasmic counterparts, reflecting the loss of several accessory domains during evolution of the organellar enzyme from its bacterial ancestor (Brown, 2001; Roy et al., 2005a). This reduction in the size and complexity of organellar PheRS explains both the
relaxed specificity of tRNA$_{Phe}$ recognition (above) and the reliance on a large conformational change to complete aminoacylation. ($\alpha\beta)_2$-type PheRSs bind tRNA across more than one subunit (Goldgur et al., 1997), while the monomeric mtPheRS instead ensures accurate recognition and aminoacylation by co-ordinating the movement of the N- and C-terminal domains, which bind the 3’-CCA and anticodon of tRNA, respectively. These data reveal how conserved modules can maintain the canonical role of PheRS, Phe-tRNA$_{Phe}$ synthesis, in different structural contexts despite the presence or absence of idiosyncratic modules with roles ranging from proofreading to DNA-binding (Dou et al., 2001; Roy et al., 2005a). AaRSs are believed to have evolved in a piece-wise fashion where different modules are arranged along the protein sequence (Alexander and Schimmel, 2001). Our data now show that the functional independence of such modules ensures canonical aaRS activity is retained despite the frequent addition or loss of other secondary activities during evolution. This structural and evolutionary versatility allows aaRSs to acquire functions outside protein synthesis, where they have recently been shown to play numerous key roles (Park et al., 2005; Ray et al., 2007).
CHAPTER 5

PATHOGENIC MUTATIONS IN HUMAN MITOCHONDRIAL
PHENYLALANYL-TRNA SYNTHETASE

5.1. Introduction

In eukaryotes ATP synthesis by oxidative phosphorylation occurs in mitochondria. Mitochondria maintain a small genome separate from that found in the nucleus, as exemplified by the human mitochondrial genome that encodes 13 proteins, 2 rRNAs and 22 tRNAs. Polypeptides encoded by the human mitochondrial genome are subunits of respiratory chain complexes, and are essential for mitochondrial function. To maintain viability, mitochondria must import numerous components of their translational machinery from the cytosol, including proteins and some RNAs (Bonnefond et al., 2005; Alfonzo and Söll, 2009; Frechin et al., 2010). Numerous proteins are imported into mitochondria including nuclear-encoded organelle-specific aminoacyl-tRNA synthetases (aaRSs), whose role is to correctly pair amino acids with their cognate tRNAs during translation (Duchene et al., 2005).

Mitochondria are responsible for ATP synthesis during aerobic respiration, and different mutations that compromise this essential function have been linked to a wide range of human diseases including mitochondrial myopathy, encephalopathy, lactic
acidosis and stroke-like episodes (MELAS), myoclonic epilepsy with ragged red fibers (MERRF), diabetes and deafness (Montoya et al., 2009). The most commonly reported class of mitochondrial genome mutations associated with diseases are those found in genes encoding tRNAs (Putz et al., 2007). Mutations in tRNAs can compromise mitochondrial translation in a number of ways (Florentz and Sissler, 2001; Florentz et al., 2003), either by directly impairing protein synthesis (Sissler et al., 2004; Kirino et al., 2005; Ling et al., 2007b; Montoya et al., 2009; Li and Guan, 2010) or by disrupting biogenesis or folding of tRNA (Wittenhagen et al., 2003; Roy et al., 2005b; Mollers et al., 2005; Maniura-Weber et al., 2006). Recent reports have shown that mitochondrial aminoacylation of tRNA may also be impaired in some diseases as a result of mutations in nuclear genes that encode mitochondrially-targeted aaRSs. For mitochondrial aspartyl-tRNA synthetase (encoded by DARS2) and arginyl-tRNA synthetase (RARS2), loss of exons, premature termination and missense mutations lead to aaRS variants with severe reductions in aminoacylation activity, leading to diseases such as leukoencephalopathy (Scheper et al., 2007) and hypoplasia (Edwardson et al., 2007). Another class of DARS2 pathogenic mutations linked to leukoencephalopathy was also recently reported, where a single amino acid substitution does not impair aminoacylation but instead prevents mitochondrial import of aspartyl-tRNA synthetase (Messmer et al., 2011). In other instances, the molecular basis by which changes to an organelle aaRS lead to mitochondrial dysfunction are unclear, as for example in the case of a mutation in the LARS2 gene (encoding leucyl-tRNA synthetase) which may play a role in susceptibility to type 2 diabetes (t Hart et al., 2005; Reiling et al., 2010).
Most mitochondrial matrix proteins, such as the aaRSs, are synthesized in the cytosol with an N-terminal targeting sequence that facilitates import. During their subsequent import across the mitochondrial membranes, nuclear-encoded matrix proteins are either unfolded or partially unfolded so that they adopt a molten globule conformation (Bychkova et al., 1988; van der Goot et al., 1991; Huang et al., 2002; Wilcox et al., 2005). Following import, mitochondrial proteins such as aaRSs must then be refolded correctly within the organelle matrix to ensure proper function. Defects in refolding of matrix proteins could be expected to lead to mitochondrial dysfunction, perhaps by triggering an unfolded protein response or by limiting levels of stable folded active protein (Longley et al., 2010). One example of the latter has been reported for a common mutant of mitochondrial medium-chain acyl-CoA dehydrogenase (MCAD) found in MCAD deficiency patients, where a single amino acid substitution decreases protein folding and assembly in mitochondria (Bross et al., 1995).

Despite the growing number of disease-related non-synonymous single nucleotide polymorphisms (ns-SNPs) and other mutations in both cytosolic and mitochondrial aaRS-encoding genes, in most cases the molecular basis underlying loss of activity is still unknown (Antonellis and Green, 2008). To investigate the possible effects of mutations on folding and function of mitochondrial aaRSs, we studied two sets of mutations in mtPheRS. mtPheRS is a monomer for which a high-resolution crystal structure is available (Klipcan et al., 2008). The first pair constitutes the ns-SNPs of the mtPheRS gene, FARS2 (S57C and N280S), listed in the NCBI single nucleotide polymorphism database (dbSNP). Both ns-SNPs correspond to amino acid changes distal to the catalytic
or tRNA binding sites of mtPheRS. The mtPheRS ns-SNP variants retained similar aminoacylation activity as the wild-type (WT) but displayed reduced stability and refolding defects, suggesting a mechanism by which aaRSs mutations that do not directly impact enzymatic activity can still lead to loss of function. The second set of mutations, I293T and D355V, are found to be associated with infant cardiomyopathy, discovered during a recent screening and clinical exome sequencing of patients with the disease. Mutation I293T is located near the ATP binding site of the catalytic domain and D355V lies in the tRNA anticodon binding domain, respectively. As expected, these mutations affect aminoacylation function directly due to substrate-binding defects. In addition to their direct impact on function, the mutations I293T and D355V also appear to reduce stability of mtPheRS, further affecting the overall aminoacylation efficiency.

5.2. Materials and Methods

5.2.1. Protein expression, purification and characterization

Point mutations were introduced by site-directed mutagenesis using the Quikchange procedure (Stratagene). Primers and chemicals were obtained from Sigma. WT mtPheRS and variants were purified according to previously published procedures (Chapter 4) (Yadavalli et al., 2009). *Escherichia coli* strain BL21 (pArgU218)/pET21c-PheRS expressing C-terminal His$_6$-tagged mtPheRS was a gift from Prof. Linda Spremulli (University of North Carolina, Chapel Hill, NC). Rosetta (DE3) cells containing pRARE plasmids encoding tRNAs for rare codons were transformed with the mutant mtPheRS plasmid construct and grown on LB medium containing ampicillin (100 µg/ml) and chloramphenicol (30 µg/ml). Purification of mtPheRS S57C/N280S was performed using
the auto-induction method (Tyler et al., 2005). Briefly, cells were grown overnight in LB medium containing ampicillin and chloramphenicol, re-inoculated into fresh media and then grown to OD\textsubscript{600} = 0.6. 10 ml of this culture was transferred to 1 L auto-induction medium and grown overnight at 25 °C, and cells were then harvested and protein purification performed as described above for mtPheRS. Human mitochondrial or \textit{E. coli} tRNA\textsuperscript{Phe} transcripts were prepared by run-off transcription using T7 RNA polymerase and gel purified as described in Chapter 3. Plasmid DNA containing either the human mitochondrial or \textit{E. coli} tRNA\textsuperscript{Phe} gene construct was used as template.

5.2.2. tRNA aminoacylation

Aminoacylation was performed at 37 °C in aminoacylation buffer (100 mM Na-Hepes (pH 7.2), 30 mM KCl, 2 mM ATP, 10 mM MgCl\textsubscript{2}) containing 25-40 µM L-\textsuperscript{[14C]}Phe (215 cpm/pmol, Perkin Elmer Lifesciences), 3 µM native \textit{E. coli} tRNA\textsuperscript{Phe}, and 100-200 nM mtPheRS. 9 µl aliquots were removed and spotted on 3MM filter disks (Whatman), washed three times in 10 % trichloroacetic acid and dried. The amount of radioactivity retained was determined by liquid scintillation counting. One unit of mtPheRS activity corresponded to the amount of enzyme necessary to catalyze the formation of 1 nmol of Phe-tRNA\textsuperscript{Phe} min\textsuperscript{-1} mg\textsuperscript{-1} protein at 37 °C.

5.2.3. Active site titration

Active site titration was done in a 50 µl reaction mixture containing 100 mM Na-Hepes pH 7.2, 30 mM KCl, 10 mM MgCl\textsubscript{2}, 2 mM ATP, 25 µM L-\textsuperscript{[14C]}Phe (214 cpm/pmol), 5
mM β-mercaptoethanol and 2 units/ml of inorganic pyrophosphatase. The reaction was initiated by the addition of mtPheRS to reaction mixture pre-incubated at 37 °C for 5 min. After adding the enzyme, the reaction was performed for 10 min at 37 °C and then filtered through a nitrocellulose membrane (Whatman PROTRAN BA85) pre-washed with cold 0.5X aminoacylation buffer. The filters were then washed with 3 ml cold 0.5X aminoacylation buffer and dried at 80 °C for 15 min. The amount of radioactivity retained was quantified by liquid scintillation counting.

5.2.4. Steady state kinetics

Steady state kinetic assays were carried out at 37 °C according to Roy et al., 2004 and 2005. For ATP-PP$_i$ exchange kinetics for amino acid activation, concentrations of substrates were varied from 5-800 µM for Phe and 1-25 mM for ATP. For steady state aminoacylation kinetics, concentrations of mitochondrial tRNA$^{Phe}$ varied between 0.05-4 µM. Enzymes were added to a final concentration of 100-150 nM.

5.2.5. Fluorescence spectroscopy

Fluorescence was measured using a Fluorolog-3 spectrofluorimeter (Horiba Jobin Yvon) equipped with a constant-temperature cell holder with integration time of 1 s. Protein concentrations were 2.5 µM for all fluorescence measurements. Tryptophan was selectively excited at 295 nm and the emission was recorded from 310 to 450 nm. Both excitation and emission slit widths were set at 5 nm for all experiments unless mentioned
otherwise. The $\lambda$ maxima values were obtained by taking the first derivative of the corresponding emission spectra. All measurements were carried out at least three times.

5.2.6. Urea-induced unfolding

Urea-induced denaturation of WT mtPheRS and variants was performed with increasing concentrations of the denaturant. Protein samples were incubated at a desired urea concentration for 18-24 h at 25 °C to attain thermodynamic equilibrium (no further change in fluorescence intensity). Thermodynamic equilibrium was generally reached after 5 h of incubation. The final concentrations of the protein and denaturant in each sample were determined by spectrophotometry and refractive index measurements, respectively. Data are expressed in terms of the fraction unfolded ($F_{un}$) calculated from the equation:

$$ F_{un} = \frac{(F_{obs} - F_N)}{(F_N - F_U)} $$  \hspace{1cm} (1)

where $F_{obs}$ was the observed value of the tryptophan emission maxima at a given denaturant concentration and $F_N$ and $F_U$ are the values of native and unfolded protein, respectively. By assuming a simple two-state model, transitions were fitted to the following equation:

$$ F_{un} = \exp\left(-\Delta G_{U-N}^{H_2O} + m[D]\right)/RT / \left[1 + \exp\left(-\Delta G_{U-N}^{H_2O} + m[D]\right)/RT\right] $$  \hspace{1cm} (2)

where $\Delta G_{U-N}^{H_2O}$ is the free energy difference in the absence of denaturant, $m$ reflects the co-operativity of the reaction, $D$ is the denaturant concentration, $R$ is universal gas constant and $T$ is temperature in Kelvin (Finn et al., 1992). Kyplot (version 2.0 beta 15...
(32 bit), Koichi Yoshioka, 1997-2001) was used to obtain the parameters using the non-linear square fit method.

5.2.7. Acid denaturation

Acid denaturation of mtPheRS was investigated as a function of pH using KCl-HCl (pH 0.5-1.5), Gly-HCl (pH 2.0-3.5), sodium acetate (pH 4.0-5.5) and sodium phosphate (pH 6.0-8.0) buffers (Dubey and Jagannadham, 2003). Analytical grade chemicals were used for buffer preparation (all 50 mM), which were then filtered and stored at -20 °C, and thawed at room temperature immediately prior to use. Protein samples were prepared individually at each different pH in the appropriate buffer to a final concentration of 2.5 µM. The samples were incubated for 18-24 h at 25 °C to ensure thermodynamic equilibrium. The final pH and concentration of the protein in each sample were then re-measured.

5.2.8. Refolding assay

mtPheRS (10 µM) was incubated in aminoacylation buffer either at a different pH (pH 1-6 at intervals of 0.5) or until complete loss of activity in active site titration assays was attained (usually 5-6 h). The reactions were then diluted 10 fold in aminoacylation buffer and activity was again followed by active site titration. Two separate control experiments were performed, in one case the dilution was done only in 100 mM Na-Hepes buffer pH 7.2, and in the second case dilutions were done in aminoacylation buffer corresponding to
the same pH (only Na-Hepes was replaced by suitable buffer at that pH as mentioned below). All the experiments were repeated three times and averages calculated.

5.2.9. ANS binding assay

The extent of exposure of hydrophobic surfaces in the enzyme was measured by the ability to bind to the fluorescent dye ANS (Semisotnov et al., 1991). A stock solution of ANS was prepared in methanol, and the dye concentration was determined using an extinction coefficient of 5000 M$^{-1}$ cm$^{-1}$ at 350 nm (Khurana and Udgaonkar, 1994). Protein (2.5 µM) was incubated with a 25-fold molar excess of ANS for more than 30 min at room temperature in the dark, and the ANS fluorescence then measured. The excitation wavelength was set to 420 nm to avoid inner filter effect, and emission spectra were collected between 430 and 550 nm. The intensities at 482 nm were recorded and plotted as a function of pH.

5.2.10. Yeast complementation

*S. cerevisiae* W303 *msf1Δ* was created through the replacement of the *MSF1* open reading frame with a KanMX4 cassette by homologous recombination in a W303 *MSF1* homozygous diploid. W303 *msf1::KanMX4* was then obtained by sporulation and dissection. The mtPheRS was inserted into the low copy-number centromeric plasmid pFL36, resulting in the plasmid pFL36-*FARS2*. To ensure that the *FARS2* protein was targeted to the *S. cerevisiae* mitochondria, the human mitochondrial targeting signal sequence in the *FARS2* gene was substituted with the *MSF1* mitochondrial targeting
sequence and start codon (66 bp). To allow for native MSF1 regulation, the hybrid FARS2 with MSF1 mitochondrial targeting sequence was cloned with flanking regions consisting of an additional 414 bp of sequence upstream of the MSF1 start codon and 151 bp of sequence downstream of the MSF1 stop codon. The FARS2 S57C, N280S, and S57C/N280S mutations were introduced by site-directed mutagenesis. The haploid W303 msf1::KanMX4 strain was separately transformed with plasmids pFL36-FARS2, pFL36-S57C, pFL36-N280S, or pFL36-S57C/N280S, crossed with W303 MSF1, sporulated, and dissected onto YPDA. Resulting W303 msf1::KanMX4 haploids carrying the appropriate plasmid were selected and utilized in *S. cerevisiae* growth assays. Growth curve assays were conducted in triplicate at 30 °C in 100 mL YPGA (peptone, glycerol, adenine), cultures shaken at 300 rpm.

### 5.3. Results

#### 5.3.1. Free energy of unfolding of two naturally occurring variants of mtPheRS

While many disease-causing ns-SNPs have been described that lead to changes in the active-site, editing-site, oligomer-interface or subcellular localization of aaRSs, it is not known if ns-SNPs that do not target these properties could also impact function. Several ns-SNPs of mtPheRS have been documented (Fig. 5.1A): three correspond to amino acid replacements in the mitochondrial targeting sequence (G3S, S4P, L6P), two are in the tRNA anticodon binding domain (H408Q, T410P), and one is in the catalytic domain (T246M), while two others are located outside any of these key functional regions of the protein (S57C, N280S). Neither S57C nor N280S would be expected to impact
mitochondrial targeting or enzymatic activity, making them suitable candidates to investigate the potential effect of ns-SNPs that map outside known functional regions. While the clinical association with these ns-SNPs is still unknown, both SIFT (Ng and Henikoff, 2003) and Polyphen (Ramensky et al., 2002) software predict that S57C might be deleterious to function while the N280S substitution is expected to be tolerated. Several recent reports indicated that multiple mutations, particularly double ns-SNP mutants in a single gene, can cause severe disease phenotypes (Leung et al., 2009; Thummer et al., 2010; Tejedor et al., 2010; Shetty et al., 2011), prompting us to investigate the S57C/N280S double mutant in parallel with the corresponding single mutants. Ser57 and Asn280 map to positions away from the catalytic center and anticodon-binding domain of mtPheRS, and neither variant showed significant loss in secondary structure or aminoacylation activity in vitro compared to WT (Fig. 5.1B). To ascertain if these amino acid replacements lead to changes in stability, urea denaturation profiles were determined for each variant and for the corresponding double mutant, and compared to WT mtPheRS. All the protein samples were incubated for 18-24 h at room temperature to ensure complete thermodynamic equilibrium. The changes in emission maxima of tryptophan fluorescence progressively shifted towards longer wavelengths indicating solvent exposure of tryptophans due to unfolding of native protein structure (Fig. 5.2A). Calculation of the free energies of unfolding based on urea denaturation revealed only marginal differences for the ns-SNP variants compared to WT, while the double mutant was significantly more stable (Table 5.1). Urea denaturation profiles were also measured in the presence of 1,8-anilino naphthyl sulfonate (ANS), which exhibits
enhanced fluorescence upon binding to exposed hydrophobic patches, to investigate if any stable intermediates accumulated during protein folding/ unfolding (Semisotnov et al., 1991). A dramatic increase in ANS fluorescence was observed at around 3 M urea and remained high up to 5 M urea and then leveled off completely around 9 M urea indicating complete unfolding (Fig. 5.2B). The ANS fluorescence increase around 3 M urea indicated the potential formation of a “molten globule” like intermediate. In addition to the observed retention of secondary structure during unfolding, molten globule states are characterized by loss of tertiary interactions, which was confirmed by near UV-CD (data not shown). The S57C, N280S and S57C/N280S variant mtPheRSs all displayed nearly identical behavior to WT during unfolding, indicating that WT mtPheRS and its variants all form stable molten globule like intermediates at around 3 M urea (data not shown).
Figure 5.1. Structure and activity of mtPheRS. **A**, Crystal structure of mtPheRS (Klipcan et al., 2008). The phenylalanyl-adenylate bound in the active site of the enzyme and the amino acid substitutions associated with human ns-SNPs are shown. **B**, Aminoacylation of tRNA by mtPheRS. The reactions were performed using 3 µM *in vitro* transcribed *E. coli* tRNA\(^\text{Phe}\) and 200 nM mtPheRS variants. Data points are an average of three replicates, with error bars representing one standard deviation.
Figure 5.2. Unfolding of mtPheRS. A, Equilibrium urea unfolding of mtPheRS variants. The tryptophan emission maxima data obtained after taking the first derivative of tryptophan emission spectra using a Fluorolog-3 spectrofluorimeter (Horiba Jobin Yvon) with integration time of 1 s equipped with a constant-temperature cell holder. The excitation wavelength was 295 nm and the emission was recorded from 310 to 450 nm. Both excitation and emission slit widths were 5 nm. The protein concentration was 2.5 µM. All the data were repeated three times and standard deviations were measured. B, ANS binding of mtPheRS variants as a function of urea concentration. The protein concentration was 2.5 µM. The protein samples were incubated overnight and then 25-molar excess of ANS was added. The protein samples were kept at 25 °C in the dark for 1 h before measurements were taken. Data were the average of three measurements. Experiments were performed by Dr. Rajat Banerjee and Srujana S. Yadavalli.
Table 5.1. Unfolding parameters of mtPheRS and its variants. Urea-induced chemical unfolding was performed with increasing concentration of the denaturant. The tryptophan emission maxima value as a function of urea concentration was recorded. Data were normalized and analyzed according to equation 1 and 2 (section 5.2.6). Data analyzed by Dr. Rajat Banerjee.

### 5.3.2. Human mitochondrial ns-SNP variants with reduced refolding capability

Aminoacylation activity measurements (Fig. 5.1B) showed that mtPheRS ns-SNP variants had similar charging capacity for cognate tRNA\(^{\text{Phe}}\) as WT, indicative of native-like catalytic cores. Mitochondrial import requires proteins to cross the acidic intermembrane space, which can result in partial pH-dependent denaturation and unfolding due to protonation of acidic residues (Bychkova et al., 1988; van der Goot et al., 1991). The stability of mtPheRS variants was further investigated by measuring aminoacylation activity following extended incubation at room temperature. WT mtPheRS retained over 90% activity following incubation at room temperature for 24 h, whereas the S57C and N280S variants retained less than 30% of their initial activity (Fig. 5.3A). To investigate if this loss in activity was indicative of a reduced ability to refold from a partially denatured state, the number of active sites was determined after low pH denaturation and refolding. WT mtPheRS retained ~80-95% of the active sites present prior to denaturation, while the S57C and N280S variants retained ~40-65% and...
30-60 %, respectively, suggesting that both were defective in refolding compared to WT (Fig. 5.3B).

**Figure 5.3. Effect of refolding on mtPheRS activity.** A, Aminoacylation activity after incubation at room temperature. Active fractions of 10 µM of all the protein samples were kept in aminoacylation buffer. After 24 h, the protein samples were diluted to 100 nM in aminoacylation buffer and aminoacylation assays were performed in triplicate. The average values along with standard deviations were calculated and plotted. B, Refolding of mtPheRS variants. The protein samples were incubated 5 h at room temperature at a concentration of 10 µM active fraction in aminoacylation buffer. The protein samples were then diluted to 1 µM and active site titration performed. Dilution with the same pH buffer instead of aminoacylation buffer was used as a control. The data were taken from pH 1-6.5, but below pH 5.5, no activity was observed after refolding. All activities are based on aminoacylation plateaus. Data generated by Dr. Rajat Banerjee.
5.3.3. *In vivo* activity of ns-SNP variants of mtPheRS

To investigate the possible effects on growth of the ns-SNP-encoded mtPheRS variants, we complemented a *Saccharomyces cerevisiae* strain lacking its endogenous mitochondrial PheRS (*MSF1*). The mtPheRS genes encoding the ns-SNP variants and the corresponding double mutant were inserted into the low copy-number plasmid pFL36, resulting in the plasmids pFL36-FARS2, pFL36-S57C, pFL36-N280S, and pFL36-S57C/N280S, which were then used to complement *S. cerevisiae msf1Δ* cells. To test the growth of mtPheRS WT and ns-SNP variants, growth was monitored in liquid media (Fig. 5.4). In liquid respiratory media a modest, but significant, growth difference between the WT and S57C/N280S double mutant was observed (Fig. 5.4). While the S57C/N280S double mutant mtPheRS is able to support growth of *S. cerevisiae* on respiratory media, the negative impact on growth that is observed suggests that the mitochondria in this strain have some defect in their ability to respire.
5.3.4. Effect of infant cardiomyopathy related mutations on aminoacylation

Dr. Tyynismaa and co-workers at the University of Helsinki screened patients who suffered from infant cardiomyopathy and performed clinical exome sequencing. Interestingly, two mutations were found to be associated with the disease and were mapped to the FARS2 gene encoding mtPheRS. One of the mutations, I293T occurs in the linker region, close to the ATP-binding motif in the catalytic site and the other, D355V, belongs to the tRNA binding domain of mtPheRS (Fig. 5.5).
Figure 5.5. Mutations in mtPheRS associated with infant cardiomyopathy. Structure of mtPheRS (Klipcan et al., 2008) highlighting the two mutations found in relation to infant cardiomyopathy. I293 is located close to the ATP binding region of the catalytic domain and D355 lies within the tRNA anticodon binding region.

To test the functional effects of the three FARS2 identified mutations, we expressed and purified the mutants as recombinant proteins in E. coli. In vitro aminoacylation assays showed that the charging activities of the mtPheRS single mutants I293T and D355V were comparable to WT (Fig. 5.6A). We also tested the activity of the double mutant I293T/D355V, which showed significant reduction in steady state aminoacylation capacity. In order to probe for potential differences in substrate binding by mutant mtPheRSs compared to WT, we determined steady state kinetic constants for amino acid activation (Tables 5.2 and 5.3). ATP-PPi exchange kinetic assays for amino acid activation revealed a significant difference in ATP binding between the WT and I293T mtPheRS. The I293T replacement led to an ~4X decrease in the catalytic efficiency of
amino acid activation due to a 2.5X increase in $K_M$ and a small decrease in $k_{cat}$ (Table 5.2). The D355V variant was unaffected whereas I293T/D355V mtPheRS showed a 5-fold loss in catalytic efficiency resulting from reduction in $k_{cat}$. Phe binding is essentially unaffected in I293T and D355V variants but the corresponding double mutant displayed a 10-fold loss in Phe binding (Table 5.3). Interestingly, we also observed that the D355V replacement, which is distal from the active site led to an increase in $K_M$ for Phe. Therefore in vivo, under limited substrate availability, the D355V mutation may significantly decrease mtPheRS aminoacylation activity. Asp 355 is in involved in a close network of interactions with the conserved residue Arg 294 near motif 3 (which is right next to Ile 293), Tyr 152 in motif 2 and Arg 37. The D355V replacement may cause Arg 294 and other neighboring residues to adopt different conformations, leading to perturbation of the Phe binding site. Steady state charging assays were also performed to determine the effect of mutations on tRNA$^{Phe}$ binding. Both the I293T and D355V mtPheRS variants showed only modest changes in their catalytic efficiencies for mt tRNA$^{Phe}$ charging (Table 5.4).

In addition to the direct impact on function, we analyzed the effect of these amino acid replacements on the stability of mtPheRS. We first determined the active fraction of the mutant and WT mtPheRSs by active site titration, followed by incubation at room temperature for 24 h and re-measured activity to quantify the properly folded fraction (Fig. 5.6B). Our results show that the single mutants I293T, D355V and double mutant hmtPheRSs refold only to about 35-45 % of the original active fraction whereas the WT retains almost 80-90 % of its active folded form. Therefore, the I293T and D355V
substitutions in mtPheRS not only impair aminoacylation function directly but also affect stability of mtPheRS leading to a decrease in overall charging capacity.

**Figure 5.6. Aminoacylation activity and stability of mtPheRS variants.**

**A,** Aminoacylation of tRNA$_{\text{Phe}}$ by mtPheRS. The reactions were performed using 2.5 µM in vitro transcribed *E. coli* tRNA$_{\text{Phe}}$ and 100 nM mtPheRS variants. Data points are an average of three replicates, with error bars representing one standard deviation. **B,** Amino acid activation after incubation at room temperature. Active fractions of 1-5 µM of all the protein samples were incubated in aminoacylation buffer. After 24 h, amino acid activation was performed. Data from at least three independent experiments were averaged and the resulting standard errors are shown.
### Table 5.2. Steady state PPi exchange kinetic constants for ATP with wild-type and mutant mtPheRSs. ATP was varied in the range of 1-25 mM. Enzyme concentrations were normalized by active site titration. Data represent averages and standard errors from three independent trials.

<table>
<thead>
<tr>
<th>mtPheRS</th>
<th>$k_{\text{cat}}^{\text{ATP}}$ (s$^{-1}$)</th>
<th>$K_M^{\text{ATP}}$ (mM)</th>
<th>$k_{\text{cat}}/K_M$ (s$^{-1}$ mM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>7.4±0.3</td>
<td>2.9±0.3</td>
<td>2.5±0.3</td>
</tr>
<tr>
<td>I293T</td>
<td>5±0.1</td>
<td>7.3±0.9</td>
<td>0.7±0.1</td>
</tr>
<tr>
<td>D355V</td>
<td>5.6±0.1</td>
<td>2.5±0.04</td>
<td>2.2±0.02</td>
</tr>
<tr>
<td>I293T/D355V</td>
<td>1.5±0.02</td>
<td>2.9±0.05</td>
<td>0.5±0.01</td>
</tr>
</tbody>
</table>

### Table 5.3. Steady state PPi exchange kinetic constants for Phe with wild-type and mutant mtPheRSs. Phe was varied in the range of 5-800 µM. Enzyme concentrations were normalized by active site titration. Data represent averages and standard errors from three independent trials.

<table>
<thead>
<tr>
<th>mtPheRS</th>
<th>$k_{\text{cat}}^{\text{Phe}}$ (s$^{-1}$)</th>
<th>$K_M^{\text{Phe}}$ (µM)</th>
<th>$k_{\text{cat}}/K_M$ (s$^{-1}$ µM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>1.7±0.1</td>
<td>7.3±0.5</td>
<td>0.2±0.01</td>
</tr>
<tr>
<td>I293T</td>
<td>1.6±0.2</td>
<td>8.6±1.7</td>
<td>0.2±0.02</td>
</tr>
<tr>
<td>D355V</td>
<td>2.3±0.02</td>
<td>20.9±1.2</td>
<td>0.1±0.01</td>
</tr>
<tr>
<td>I293T/D355V</td>
<td>0.2±0.001</td>
<td>10.1±5</td>
<td>0.02±0.01</td>
</tr>
</tbody>
</table>

### Table 5.4. Steady state aminoacylation kinetic constants for tRNA$^{\text{Phe}}$ with wild-type and mutant mtPheRSs. Human mitochondrial tRNA$^{\text{Phe}}$ was varied in the range of 0.05-4 µM. Kinetic parameters were estimated using sub-saturating concentrations of tRNA$^{\text{Phe}}$. Enzyme concentrations were normalized by active site titration. Data represent averages and standard errors from at least three independent trials. $^a$k_{cat}/K_M was estimated directly from the slope of the equation, $V = k_{\text{cat}}[E][S]/K_M$. ND, not determined.

<table>
<thead>
<tr>
<th>mtPheRS</th>
<th>$k_{\text{cat}}^{\text{tRNA}}$ (s$^{-1}$)</th>
<th>$K_M^{\text{tRNA}}$ (µM)</th>
<th>$k_{\text{cat}}/K_M$ (s$^{-1}$ µM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.09±0.01</td>
<td>1.2±0.2</td>
<td>0.07±0.01</td>
</tr>
<tr>
<td>I293T</td>
<td>0.05±0.005</td>
<td>1.2±0.4</td>
<td>0.05±0.01</td>
</tr>
<tr>
<td>D355V</td>
<td>0.07±0.01</td>
<td>1.4±0.2</td>
<td>0.05±0.01</td>
</tr>
<tr>
<td>I293T/D355V</td>
<td>ND</td>
<td>ND</td>
<td>0.04±0.001 $^a$</td>
</tr>
</tbody>
</table>

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5.4. Discussion

5.4.1. Formation of stable molten globule states by human mitochondrial aaRSs

Nuclear-encoded proteins are imported into mitochondria either unfolded or in a partially unfolded extended conformation such as a molten globule state (Schwartz et al., 1999). Unfolding can be driven by different factors, including components of the protein import machinery itself and the mitochondrial membrane potential (Huang et al., 2002; Wilcox et al., 2005; Schleiff and Becker, 2011). The data presented here show that for mtPheRS partial unfolding to form a molten globule like intermediate can readily occur, and that this requires moderately acidic pH conditions similar to those associated with the generation of local pH gradients across the mitochondrial membrane (Khalifat et al., 2008). An acidic pH environment at the mitochondria outer membrane matrix has been shown to help the steroidogenic acute regulatory protein (StAR) adopt a functional molten globule structure (Bose et al., 1999) and several other examples have also been described where either a lower pH or specific membrane composition allows a protein to adopt predominantly a molten globule form (van der Goot et al., 1991; Shin et al., 1997). The ability of mtPheRS to form remarkably stable molten globule like intermediates at an acidic pH suggests that import into the mitochondrial matrix of these and perhaps other aaRSs might be facilitated by adopting such a structure. Our data also suggest that the presence of the N-terminal mitochondrial targeting sequence of mtPheRS does not alter the protein’s folding. mtPheRS lacking a targeting sequence produced heterologously in E. coli retained full activity in vivo, and also refolded into its native active state in vitro without the aid of any chaperones (data not shown).
5.4.2. Ns-SNPs of mitochondrial aaRSs encode refolding-specific defects that reduce overall aminoacylation activity

In addition to the well-characterized examples that have been linked directly to specific diseases, considerably more mutations in aaRS-encoding genes have been uncovered through sequencing of ns-SNPs. In some instances the extensive knowledge that already exists on the structure and function of aaRSs allows reasonably confident predictions to be made as to the possible effects of such mutations. In other examples, such as the ns-SNPs of FARS2 studied here, how amino acid substitutions might impact function is unclear, making it difficult to discern whether or not a particular mutation may be deleterious. The ns-SNP variants of mtPheRS showed comparable cognate tRNA charging activity to WT. However, both ns-SNP variants of mtPheRS refolded less efficiently than the WT \textit{in vitro}, resulting in a net reduction in aminoacylation activity.

Whether this change in aaRS folding and aminoacylation activity compared to WT is significant for mitochondrial biogenesis is unclear. Although more severe or long-term effects in human cells cannot be excluded, the ability of the ns-SNP variants to function in yeast nevertheless indicates that these mutants do not critically limit mitochondrial import or translation as reported for other ns-SNPs (Ueki et al., 2006; Rotig 2010; Messmer et al., 2011). The only exception was the S57C/N280S double mutant, which was less active \textit{in vivo} than either of the respective single mutants, despite the fact that all three mtPheRS showed comparable losses in aminoacylation activity \textit{in vitro}. One notable feature of the S57C/N280S protein is that it unfolds more slowly than either of
the single mutants or WT, which could potentially lead to defects in protein import and supports the assertion that unfolding is an important step during translocation of mtPheRS.

These findings reveal the existence of a new class of ns-SNPs in the aaRS family that have no obvious effect on the maturation or activity of newly synthesized proteins, but instead perturb optimal unfolding and refolding. While these mutations are initially less detrimental than those that, for example, target the active site they nevertheless have the potential to significantly limit aaRS activity. Mitochondrial aaRSs are the most vulnerable to such mutations as their activity is dependent on proper refolding following translocation into the organelle. What is still unclear is to what extent unfolding and refolding defects might also effect cytoplasmic aaRSs, perhaps by making them less stable and reducing their half lives, or by acting synergistically with other class of mutations. Further studies are now warranted to investigate the extent to which other uncharacterized ns-SNPs affect both cytoplasmic and mitochondrial aaRS refolding, and how this contributes to diseases resulting from such mutations.

5.4.3. Mutations in mtPheRS associated with infant cardiomyopathy directly impair function

Mutations that lead to mitochondrial dysfunction and disease are commonly found in genes encoding tRNAs (Putz et al., 2007). Mutations in tRNAs can directly impair mitochondrial protein synthesis (Florentz et al., 2003; Sissler et al., 2004; Kirino et al., 2005; Ling et al., 2007b; Li and Guan 2010) or affect tRNA biogenesis or folding.
(Wittenhagen and Kelley, 2003; Roy et al., 2005b; Mollers et al., 2005; Maniura-Weber et al., 2006). In recent years mutations in human mitochondrial aaRS-encoding genes have been identified that lead to defects in gene expression, enzymatic activity and subcellular localization (Scheper et al., 2007; Edvardson et al., 2007; Riley et al., 2010; Messmer et al., 2011; Belostotsky et al., 2011). Mutations in functional domains such as those examined here, I293T and D355V in mtPheRS can directly affect aminoacylation function. We show that the I293T mutation in the linker region near the active site influences ATP binding. The D355V mutation affects Phe binding and may affect the flexibility of the tRNA anticodon binding domain of mtPheRS, a key feature required for proper tRNA recognition and aminoacylation (Chapter 4) (Yadavalli et al., 2009). Combining the two mutations in the double mutant mtPheRS led to a loss in binding of both substrates, Phe and ATP, which can explain the aminoacylation defect. Refolding assays indicated that the mutations I293T and D355V in mtPheRS also affect activity indirectly by altering stability resulting in lower levels of active folded fraction of enzyme. These studies are one of the first to elucidate the direct and indirect effects of disease-related mutations on mtPheRS function. Together our work sheds light on the molecular impact of mutations in mtPheRS associated with mitochondrial disorder and cardiomyopathy in infants.
CHAPTER 6

CONCLUSIONS AND PERSPECTIVES

Accurate translation of mRNA into protein is vital for maintenance of cellular integrity. Translational fidelity is achieved by two key events: (i) synthesis of correctly paired aminoacyl-tRNAs by aminoacyl-tRNA synthetases (aaRSs) and (ii) stringent selection of aminoacyl-tRNAs (aa-tRNAs) by the ribosome (Ibba and Söll, 1999). AaRSs define the genetic code by catalyzing the precise formation of aminoacyl ester-linked tRNAs via a two-step reaction (Ibba and Söll, 2000). AaRSs ensure faithful aa-tRNA synthesis via high substrate selectivity and/or by proof-reading (editing) of noncognate products (Mascarenhas et al., 2009; Ling et al., 2009a; Yadavalli and Ibba 2012). About half of the aaRSs rely on proofreading mechanisms to achieve high levels of accuracy in aminoacylation. Editing functions in aaRSs contribute to the overall low error rate in protein synthesis. Defects in aaRS editing can be detrimental to cells and can cause neurodegeneration in mammals (Bacher et al., 2005; Lee et al., 2006; Reynolds et al., 2010a).

PheRS maintains editing activity against noncognate Tyr and loss of editing leads to mistranslation in vivo in E. coli (Roy et al., 2004). In Chapter 2, using E. coli PheRS as our model we illustrated the significance of PheRS editing in preventing miscoding of
Phe codons as Tyr. In vitro binding and translation assays showed that EF-Tu and ribosome bind and utilize Phe-tRNA$^{\text{Phe}}$ and Tyr-tRNA$^{\text{Phe}}$ similarly. Our study showed that PheRS editing is a major quality control checkpoint in preventing mistranslation of Phe codons, as downstream steps do not effectively discriminate against noncognate substrate (Ling et al., 2007c). Accordingly it is expected that any mischarged tRNA that escapes aaRS editing would be sequestered by EF-Tu and delivered to ribosomes. However, our in vitro competition assays revealed that the PheRS editing site competes efficiently against EF-Tu for any mischarged Tyr-tRNA$^{\text{Phe}}$ and hydrolyzes it via trans editing (“resampling”). These studies revealed that efficient resampling of mischarged tRNAs serves as an additional, and previously unknown, proofreading step prior to translation elongation (Ling et al., 2009b). In addition, the finding that binding of aa-tRNA to EF-Tu does not constitute a committed step in protein synthesis also has important implications for the trafficking of aa-tRNA to pathways outside protein synthesis (Banerjee et al., 2010).

As many aaRSs actively edit noncognate amino acids, it was believed that editing mechanisms, and the cellular requirements for translation fidelity, are evolutionarily conserved. Recent findings indicate that certain aaRSs such as those in *Mycoplasma* species are naturally error-prone and display a low level of specificity consistent with elevated mistranslation of the proteome (Li et al., 2011). This observation implies divergence of translation quality control across bacteria, the physiological significance of which remains unclear. In Chapter 3, we utilized the divergence of Tyr editing by PheRS to understand the correlation between aaRSs and mistranslation. *Mm*PheRS has evolved to be less accurate in amino acid selection and utilizes less stringent quality control
pathways. *Mm*PheRS failed to support *Escherichia coli* growth, suggesting that the level of discrimination against noncognate amino acid may be inadequate for organisms where translation is usually more accurate. However, point mutations in the defunct editing domain of *Mm*PheRS are sufficient to restore specificity for Phe over Tyr, and sustain *E. coli* growth. Structural studies are now required to provide further insights into amino acid selection in *Mm*PheRS and how these point mutations in the editing site modulate substrate binding. More broadly, our findings shed light on the divergence of quality control mechanisms in bacterial translation. We propose that aaRSs may facilitate adaptation to changes in physiology by altering the translational accuracy of specific codons, which may be advantageous for growth under different environmental conditions.

In Chapter 4, we investigated the structure-function relationship in human mtPheRS. Structural studies in mtPheRS suggested a massive rearrangement of the tRNA anticodon binding domain with respect to the catalytic domain (Klipcanc et al., 2008). Using biochemical crosslinking experiments, we showed that both the closed and open forms of mtPheRS are functionally relevant. While both forms are capable of amino acid activation, only the open form recognizes tRNA\(^{Phe}\) efficiently (Yadavalli et al., 2009). SAXS analyses and modeling confirmed the existence of a dynamic equilibrium between the closed and open forms of mtPheRS in solution, and stabilization of the open form upon tRNA\(^{Phe}\) binding. This minimal tRNA\(^{Phe}\) recognition strategy is unique to monomeric mtPheRS. Our results show that the functional independence of aaRS modules ensures canonical aminoacylation function is retained despite the frequent addition or loss of other secondary activities during evolution (de Pouplana and
Schimmel, 2001). This structural and evolutionary versatility allows aaRSs to readily acquire roles outside protein synthesis (Park et al., 2005).

We extended our studies of in human mtPheRS to investigate the effects of pathogenic mutations as discussed in Chapter 5. Although several mutations in mitochondria-encoded tRNA species have been shown to cause mitochondrial dysfunction and disease (Putz et al., 2007), little is known about pathogenicity of mutations in nuclear-encoded components of the translation machinery such as the aaRSs. MtPheRS is a nuclear-encoded protein whose import into mitochondria requires partial unfolding and refolding in the mitochondrial matrix for function (Wilcox et al., 2005). We first analyzed the effects of two disease-related non-synonymous single nucleotide polymorphisms (ns-SNPs), S57C and N280S, occurring in sites distal to the catalytic domain of mtPheRS, on the aminoacylation activity. MtPheRS can form a molten globule intermediate during folding/unfolding, which is proposed to be an important structural intermediate during mitochondrial protein import (van der Goot et al., 1992). The mtPheRS variants resulting from the ns-SNPs retained full charging activity, but showed reductions in activity after unfolding and refolding compared to WT. Our data show that these ns-SNPs induce specific functional defects upon refolding leading to an overall decrease in charging capacity. In addition to the ns-SNPs, we examined the effects on mtPheRS function of two mutations I293T and D355V associated with infant cardiomyopathy. These residues, I293 and D355, occur near the ATP binding site and tRNA anticodon binding region, respectively. As predicted, the I293T replacement affects ATP binding and the D355V substitution may disrupt the conformational flexibility of mtPheRS, which is necessary for tRNA recognition and
aminoacylation (Chapter 4). In addition to direct effects on charging function, these amino acid replacements also showed reduced amino acid activation upon refolding similar to the ns-SNP variants. Together, our work provides insights to the molecular mechanisms of pathogenicity of these disease-related mutations in mtPheRS.

**Perspectives**

In Chapter 2, we proposed a concerted model for posttransfer editing based on our studies with *E. coli* PheRS. If Tyr-tRNA$^{Phe}$ is produced during aminoacylation, it would be expected to partition between the *cis*- and *trans*-editing pathways. The extent of partitioning between each pathway, and how this globally effects translation quality control, are unknown. We will examine the distribution of mischarged Tyr-tRNA$^{Phe}$ between the two pathways, using quenched-flow kinetic approaches. We will monitor the formation and deacylation of Tyr-tRNA$^{Phe}$ in the presence or absence of factors that affect the *trans*-editing pathway. EF-Tu competes with the PheRS editing site for Tyr-tRNA$^{Phe}$ after it is released from PheRS. Using a combination of PheRS and EF-Tu variants, we will assess the relative contributions of *cis*- and *trans*-editing pathways during editing by *E. coli* PheRS.

In Chapter 3, our detailed biochemical analyses of *Mm*PheRS suggest divergent evolution of this enzyme across bacteria. Both the aminoacylation and editing active sites of *Mm*PheRS fail to discriminate against noncognate Tyr efficiently. We observed that point mutations in the defunct editing domain enhance specificity for Phe over Tyr, and sustain *E. coli* growth. To gain structural insights into the evolution of this error-prone PheRS, we will utilize X-ray crystallography and resolve its structure. One major
challenge is the low yield of *Mm*PheRS, which appears to be toxic to *E. coli*. To obtain large amounts of purified protein, we will culture *Mm*PheRS strains using large-scale fermentation systems. Protein crystallization, diffraction and structural analysis will be carried out in collaboration with the X-ray crystallography facility at OSU (Research Core Facilities).

In addition to *Mycoplasma* spp., sequence analysis of other pathogenic organisms revealed defunct editing domain motifs in *Helicobacter pylori*. We predict that *H. pylori* PheRS is error-prone similar to *Mm*PheRS. Using recombinantly expressed *H. pylori* PheRS, we can study the biochemical activity of the enzyme. The *in vitro* transcript of *H. pylori* tRNA<sup>Phe</sup> is efficiently chargeable by *E. coli* PheRS. Additionally, *in vivo* experiments can be performed as *H. pylori* is genetically tractable unlike *Mycoplasmas*. It remains to be seen what phenotypic or evolutionary advantages are conferred upon *H. pylori* by an error-prone aaRS.

In Chapter 5, our studies are one of the first to characterize the biochemical effects of mtaaRS mutations *in vitro*. We will analyze more aaRS mutations that are associated with genetic disorders. Knowledge of pathogenic mutations is important for genetic counseling and/or early screening for disease susceptibility by genetic testing. Detailed functional analyses are necessary to help identify the potentially deleterious mutations among the numerous mutations/variants now listed in disease databases.
APPENDIX A

TIME-RESOLVED FLUORESCENCE OF PYRROLO-CYTOSINE AS A PROBE TO STUDY tRNA DYNAMICS IN PHERS EDITING

As discussed in Chapter 2, we proposed a concerted translocation model for posttransfer editing by PheRS using *E. coli* PheRS as a model. According to the model (Fig. 2.5), mischarged Tyr-tRNA\textsuperscript{Phe} is edited via the following pathways: (i) in *cis* by direct translocation of the 3′ CCA end of enzyme-bound Tyr-tRNA\textsuperscript{Phe} and/or (ii) in *trans*, where Tyr-tRNA\textsuperscript{Phe} dissociates and then re-binds to PheRS. To gain insight into tRNA dynamics during posttransfer editing, we attempted to utilize a fluorescence resonance energy transfer (FRET) method employing labeled tRNA\textsuperscript{Phe} and PheRS. Site-specific labeling of PheRS without significantly affecting the aminoacylation function using currently available probes that are suitable for FRET proved to be challenging. However, site-specific labeling of the 3′ end of tRNA\textsuperscript{Phe} was achieved using fluorescent nucleotide analogs such as 2AP (Chapter 2) and pyrrolo-cytosine (PyC). Fluorescence intensities of 2AP and PyC are highly sensitive to local conformational changes and are quenched in double stranded DNA or RNA most likely via base stacking interactions (Jean and Hall, 2001; Berry et al., 2004). 2AP and PyC were introduced at positions 76 and 75 respectively, according to the protocol described in Chapter 2. Both 2AP- and PyC-
tRNA\textsuperscript{Phe} were chargeable with cognate Phe and noncognate Tyr (Fig. A.1). Although PyC-tRNA\textsuperscript{Phe} showed reduced charging levels with both Phe and Tyr (Fig. A.1C and D) these values are comparable to those obtained previously for charging of PyC-labeled \textit{E. coli} tRNA\textsuperscript{Cys} with Cys (Zhang et al., 2008).

\textbf{Figure A.1. Aminoacylation of 2AP- and PyC-labeled \textit{E. coli} tRNA\textsuperscript{Phe}.} Charging of 2AP-tRNA\textsuperscript{Phe} with A, Phe and B, Tyr. Charging of PyC-tRNAPhe with C, Phe and D, Tyr.
2AP displays a single exponential decay profile and therefore 2AP-tRNA\textsubscript{Phe} was first used to check if there is any corresponding enhancement in fluorescence upon protein binding. 2AP was excited at 315 nm and emission was monitored at 370 nm. Upon addition of PheRS, an enhancement in fluorescence intensity was observed, which increased further when the other substrates ATP and Phe were included (Fig. A.2). Time-resolved fluorescence studies were carried out with 2AP-tRNA\textsubscript{Phe} using an excitation wavelength of 300 nm instead of the ideal wavelength of 315 nm, due to inherent instrument limitations. Approximately 70-75% of 2AP-tRNA\textsubscript{Phe} displayed a lifetime, $\tau_1=11$ ns, corresponding to the lifetime of free 2AP (Jean and Hall, 2001) and the remaining fraction showed a shorter lifetime, $\tau_2 \sim 3$-4 ns (data not shown), indicating that 2AP-tRNA\textsubscript{Phe} exists in two different conformational states in the tRNA-bound form. Excitation at 300 nm, however, led to a high level of interference from the indirect excitation of the numerous tryptophan residues in PheRS. To address this problem we employed PyC-tRNA\textsubscript{Phe} to conduct time-resolved fluorescence assays. PyC can be excited at 350 nm, away from the absorption wavelengths of nucleotides and of aromatic amino acid residues, and has an emission maximum at 460 nm. PyC-tRNA\textsubscript{Phe} by itself displayed a triple exponential decay with 60-80% of the population with a longer lifetime $\tau_1=9$-10 ns, 20-30% with $\tau_2=2.5$ ns and 10-15% with the shortest lifetime, $\tau_3=0.1$ ns, with an average lifetime, $\tau_{\text{avg}} \sim 6$ ns.
Figure A.2. Fluorescence of 2AP-labeled *E. coli* tRNA\(^{\text{Phe}}\). 0.1 µM 2AP-tRNA\(^{\text{Phe}}\), 0.5 µM *Ec*PheRS WT, 30 µM Phe and 2 mM ATP were used. \(\lambda_{\text{Ex}}=315\) nm and \(\lambda_{\text{Em}}=370\) nm.

Figure A.3. Typical trace for PyC-tRNA\(^{\text{Phe}}\) fluorescence intensity decay. Sample contains 300 nM PyC-tRNA\(^{\text{Phe}}\) in phosphate buffer pH 7.5. The phase angle (open triangles) and modulation ratio (open circles) are shown. Lines represent triple exponential fit for the decay data points. Measurements were made using a K2 frequency-domain time-resolved spectrophotometer (ISS).
We monitored the fluorescence intensity decay of PyC-tRNA\textsuperscript{Phe} in the presence of \textit{E. coli} PheRS $\alpha$A294G variant, which retains WT editing activity. Addition of 600 nM PheRS led to a decrease in $\tau_{\text{avg}}$ from 6.4 ns to 5.6 ns (Fig. A.4). The observed decrease in the average lifetime was due to the decrease in the fractional population of the longer lifetime species (~10 ns), which led to a corresponding increase in the shorter lifetime species (~2 ns). Titration of PyC-tRNA\textsuperscript{Phe} using increasing concentrations of PheRS further shortened the $\tau_{\text{avg}}$ of PyC-tRNA\textsuperscript{Phe} (Fig A.4). This data suggests that binding of PheRS to PyC-tRNA\textsuperscript{Phe} alters the environment of PyC such that the tRNA 3’ end may adopt alternate conformational states, leading to fluorescence quenching and correspondingly shorter lifetimes.

\textbf{Figure. A.4. Average lifetimes of PyC-tRNA\textsuperscript{Phe} in the presence of PheRS.} \textit{E. coli} PheRS $\alpha$A294G was added in varying concentrations of 600 nM, 2 $\mu$M, 6 $\mu$M, and 12 $\mu$M to 300 nM PyC-tRNA\textsuperscript{Phe}. 
Next, fluorescence intensity decay curves were obtained using editing deficient variants of *E. coli* PheRS, including $\alpha$A294G $\beta$A356W and $\alpha$A294G $\beta$G318W, which have ~50X and 78X reduction in editing compared to WT, respectively (Ling et al., 2007a). Both the replacements, A356W and G318W appear to block access to the editing site, thereby causing a loss of editing activity. We titrated PyC-tRNA$^{\text{Phe}}$ with varying concentrations (2 $\mu$M, 6 $\mu$M, and 12 $\mu$M) of the PheRS editing deficient variants and measured the fluorescence lifetimes. The average lifetimes were then compared to those in the presence of PheRS $\alpha$A294G, which contains a WT editing site.

![Figure. A.5. Average lifetimes of PyC-tRNA$^{\text{Phe}}$ in the presence of editing deficient PheRS.](Image)

300 nM PyC-tRNA$^{\text{Phe}}$ was mixed with 2 $\mu$M, 6 $\mu$M, 12 $\mu$M of the indicated PheRS variant. At each concentration of PheRS, average lifetimes of PyC-tRNA$^{\text{Phe}}$ were calculated and plotted against PheRS concentration.
PyC-tRNA$^{Phe}$ displayed a decrease in $\tau_{avg}$ with increasing concentrations of $\alpha A294G \beta A356W$ PheRS, similar to that of $\alpha A294G$ PheRS. In the presence of 2 $\mu$M $\alpha A294G$ PheRS, PyC-tRNA$^{Phe}$ displayed three lifetimes, $\tau_1=10.2$ ns (35-40% of population), $\tau_2=2.5$ ns (35-40%) and $\tau_3=0.2$ ns (15-20%). Increasing concentrations of $\alpha A294G$ PheRS altered the relative populations with $\tau_1$ and $\tau_2$, leading to an overall decrease in $\tau_{avg}$. Analysis of individual lifetimes, $\tau_1$, $\tau_2$ and $\tau_3$ in the case of $\alpha A294G \beta A356W$ PheRS, showed a decrease in $\tau_{avg}$ with increasing PheRS mainly due to significant lowering of the longer lifetime component, $\tau_1$. For example, as the concentration of $\alpha A294G \beta A356W$ PheRS was increased to 12 $\mu$M, the distribution of lifetimes was as follows: $\tau_1=6.41$ ns (40-45% species), $\tau_2=2$ ns (40%) and $\tau_3=0.2$ ns (15-20%). These data suggest that PyC-tRNA$^{Phe}$ adopts a different conformation in the presence of $\alpha A294G \beta A356W$ PheRS when compared to $\alpha A294G$ PheRS. It is possible that the PyC is involved in a stacking interaction with $\beta W356$ or neighboring residues such that it causes a decrease in its lifetime $\tau_1$ and $\tau_{avg}$. Addition of $\alpha A294G \beta G318W$ PheRS led to an increase in $\tau_{avg}$ of PyC-tRNA$^{Phe}$ with increasing PheRS. It is likely that this mutant, which has an ~80X loss of editing activity, completely blocks entry of the tRNA 3’ end to the editing site. At 2 $\mu$M $\alpha A294G \beta G318W$ PheRS, the distribution of lifetimes was as follows: $\tau_1=10.8$ ns (50-55% species), $\tau_2=3$ ns (30%) and $\tau_3=0.2$ ns (15%), leading to an overall increase in $\tau_{avg}$ relative to PheRS $\alpha A294G$ and $\alpha A294G \beta A356W$ variants. Clearly, the 3’ end of PyC-tRNA$^{Phe}$ is less stacked in the context of the $\alpha A294G \beta G318W$ PheRS editing deficient mutant.
Together the time-resolved fluorescence studies show that the 3’ end of tRNA\textsuperscript{Phe} adopts different conformations in the environments of the WT editing site and of the editing site variants that restrict tRNA mobility. Single molecule FRET experiments can further help to resolve the populations of tRNA species bound to either the active site, editing site or unbound in solution as well as determine the rate of translocation of mischarged tRNA to the editing site. Site-specific labeling of PheRS with less bulky probes, or improved technology for incorporation of fluorescent amino acid analogs, together with labeled tRNA\textsuperscript{Phe} such as PyC-tRNA\textsuperscript{Phe} may provide a useful way to perform FRET assays to measure tRNA dynamics in the future.
REFERENCES


