FLUORESCENCE SPECTROSCOPY AND
PRE-STEADY STATE KINETIC STUDIES
ON E. COLI METHIONYL TRANSFER RNA SYNTHETASE

BY

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I dedicate this thesis to my mom, who always pushes me to do better.
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LIST OF UNCOMMON ABBREVIATIONS

All amino acid abbreviations are the standard one letter and three letter codes.

Å (Angstrom = 10^{-10} m)

Bn (Benzyl)

Bzl (Carboxybenzyl)

CDI (N, N'- Carbonyl diimidazole)

DTT (Dithiothreitol)

FPLC (Fast Performance Liquid Chromatography)

IPTG (Isopropyl β-D-1-thiogalactopyranoside)

LBM (Lauria Bertani-Miller)

NEB (New England Biolabs)

NMR (Nuclear Magnetic Resonance)

OD_{600} (Optical Density at 600 nm)

PBS (Phosphate Buffer Saline)

PAGE (Polyacrylamide gel electrophoresis)

PMSF (Phenylmethanesulfonyl fluoride)

PPi (Inorganic pyrophosphate)

SOC (Super Optimal broth with Catabolite repression)

TBE (Tris Boric Acid EDTA)

TCA (Trichloroacetic acid)

THF (Tetrahydrofuran)
ABSTRACT

FLUORESCENCE SPECTROSCOPY AND PRE-STEADY STATE KINETIC STUDIES ON E. COLI METHIONYL TRANSFER RNA SYNTHErase

Our goal is to elucidate long range intramolecular signaling mechanisms in Escherichia coli methionyl tRNA synthetase (MetRS). In this thesis we utilize fluorescence spectroscopy and pre-steady state kinetics to unveil some features of MetRS catalysis that were previously unclear.

MetRS is a class I aminoacyl-tRNA synthetase that attaches methionine to tRNA Met in a two step reaction mechanism. In the first step, methionine is activated with ATP to form a methionyl adenylate. The second step is the transfer of methionine to the ribose 2'-O of A76. For the second step to occur efficiently, the enzyme needs to bind specifically to its cognate tRNA. This is accomplished by interaction of the anticodon binding domain of MetRS to the C34A35U36 anticodon triplet of tRNA Met. The binding of tRNA Met by MetRS involves a recognition event by MetRS Trp461 to the anticodon C34. The distance from Trp461 to the active site is ~ 50 Å, and we hypothesize there is a long-range communication between the two domains that signals the binding event for efficient aminoacylation.

The intrinsic tryptophan fluorescence of MetRS changes upon binding two of its substrates: enhancement is observed when methionine binds and quenching occurs when tRNA Met binds. Because tryptophan fluorescence is sensitive to protein environment, we exploit this property to probe structural changes in MetRS caused by substrate binding events.
By using molecular biology techniques, we determined that Trp253 in the active site is responsible for the increased fluorescence intensity. The reason for the increase is likely due to an electrostatic interaction between Trp253 and Tyr15 upon methionine binding which repositions the amino acid side chains. We also linked the tRNA\textsuperscript{Met}-induced fluorescence quench to Trp461.

Previous studies on the reaction mechanism of MetRS using steady state methods determined $K_M$ and $k_{cat}$ values for amino acid activation and for the overall aminoacylation reaction. Under single turnover reaction conditions, we determined the rate for the chemical step and for the actual amino acid transfer catalyzed by *E. coli* MetRS. We also confirmed that product release is not the rate limiting step in aminoacylation by the absence of a product formation burst under multiple turnover conditions.
CHAPTER ONE
INTRODUCTION

Genetic Code and RNA

Although DNA is the blueprint of life that encodes genetic information, in order for the information to be “alive”, organisms need RNA. Without messenger RNA (mRNA), ribosomal RNA (rRNA) and transfer RNA (tRNA), the genetic information in DNA will remain a meaningless sequence of four letter code. Conversion of genetic information to something functionally pertinent such as a protein is a long process. The information in a gene sequence first has to be transcribed into mRNA. The mRNA then interacts with rRNA and ribosomal proteins to become a cellular decoding machine. The code for the protein sequence in mRNA is translated at the ribosome by tRNA, which carries a specific amino acid for each trinucleotide codon. And indeed the catalytic activity for the coupling of the amino acids resides in the rRNA.\(^1\) Some even postulate that the first living organism utilized RNA to carry its metabolic function and only later evolved into storing its genetic information in a more stable form of nucleic acid (DNA).\(^2-4\)

The codon table catalogued by Nirenberg and coworkers in 1965 defines the rules for the 20 natural amino acids that make up proteins.\(^5\) There are 64 possible trinucleotide codons (Table 1), and the code is nearly universal for all organisms. With the discovery of selenocysteine and pyrrolysine utilizing UGA (opal) and UAG (amber) codons, the total number of genetically encoded amino acids has grown to 22.\(^6\) Recent developments in molecular biology have managed to expand the genetic code to insert unnatural amino
acids into proteins in various organisms such as *E. coli*,7, 8 yeast,9, 10 and mammalian
cells11 by manipulating components of the protein synthesis machinery.

**Table 1. Codon Table**

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**tRNA**

Robert W. Holley determined the complete nucleotide sequence of yeast tRNAAla in 196512 and shared the Nobel Prize in Physiology or Medicine in 1968 with H. Gobind Khorana and Marshall W. Nirenberg for the discovery of the genetic code (http://nobelprize.org/nobel_prizes/medicine/laureates/1968/). Almost a decade later, the L-shaped three-dimensional structure of yeast tRNA^Phe was determined by X-ray crystallography.13, 14 Each tRNA has the same L-shaped global structure (Figure 2). The number of nucleotides varies amongst tRNAs; by consensus, the acceptor adenine is numbered 76 and the anticodon is numbered 34 – 36.
In *E. coli* there are three tRNAs that accept methionine, two initiator (tRNA$^{\text{Met}}$) and one elongator (tRNA$^{\text{Met}}$). The methionine attached to the initiator tRNA is subsequently formylated by methionyl-tRNA$^{\text{Met}}$ formyltransferase (FMT). The FMT specifically recognizes the wobble pair C1•A72 in the tRNA$^{\text{Met}}$, which is G1•C72 in tRNA$^{\text{Met}}$.\(^{15}\)

![Figure 1. tRNA$^{\text{Met}}$ and tRNA$^{\text{Met}}$ cloverleafs. A. Endogenous tRNA$^{\text{Met}}$ type 1. B. Endogenous tRNA$^{\text{Met}}$. B. In vitro transcribed tRNA$^{\text{Met}}$. Modified nucleotides: 4-thiouridine (S), dihydrouridine (D), 2'-O-methylecytidine (Cm), 7-methylguanosine (m$^7$G), 5-methyluridine (T), pseudouridine (Ψ), 3-(3-amino-3-carboxypropyl) uridine (acp$^3$U).\(^{16, 17}\)](image)

Endogenous cellular tRNA undergoes post-transcriptional modification, primarily chemical modification of the nitrogenous bases; some examples of modifications are shown for *E. coli* tRNA$^{\text{Met}}$\(^{16}\) and tRNA$^{\text{Met}}$\(^{17}\) (Figure 1, A and B). In this thesis we use *in vitro* transcribed tRNA$^{\text{Met}}$ (Figure 1, C); although lacking nucleotide modifications, the tRNA$^{\text{Met}}$ is aminoacylated by MetRS just as efficiently as the native tRNA$^{\text{Met}}$.\(^{18, 19}\)
Aminoacyl-tRNA synthetases

Aminoacyl-tRNA synthetases (AARS) are a structurally diverse family of enzymes that attach amino acids to their cognate tRNAs as the first step of protein biosynthesis. Most organisms have one synthetase for each amino acid, although there may be several tRNA isoacceptors for an amino acid. Some organisms lack AsnRS and/or GlnRS and must use a transamidation pathway to synthesize a properly charged tRNA for Asn and/or Gln.

The synthetases are divided into two main classes based on mutually exclusive sequence motifs that reflect distinct active site topologies. Class I AARS active sites contain a Rossmann dinucleotide binding fold and an 11-residue signature sequence ending in His-Ile-Gly-His (HIGH) followed by the pentapeptide Lys-Met-Ser-Lys-Ser.
(KMSKS). Class II AARSs contain an antiparallel β-fold with three degenerate sequence motifs (Table 2). The two classes also differ in the way they bind tRNA: Class I enzymes approach the acceptor stem of tRNA from the minor groove side with the variable loop facing the solvent, whereas class II enzymes approach from the major groove side of the acceptor stem and the variable loop faces the synthetase.

AARSs synthesize aminoacyl-tRNA in a two-step mechanism. In the first step the enzyme catalyzes the condensation of its cognate amino acid with ATP, resulting in release of pyrophosphate. The activated amino acid is transferred to either the 3'-O or 2'-O of the cognate tRNA terminal adenosine by nucleophilic attack on the α-carbonyl of the AA-AMP by the A76 ribose hydroxyl group. Class I AARSs use the 2'-OH as the nucleophile, while class II AARSs use the 3'-OH. This difference is a consequence of which hydroxyl group is closer to the enzyme-bound adenylate.

Table 2. Aminoacyl tRNA synthetase (AARS) classification.

<table>
<thead>
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<th>Class I Subclass Ia</th>
<th>Subunit Composition</th>
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<td>IleRS α</td>
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<td>LeuRS α</td>
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<td>TyrRS α</td>
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<tr>
<td>PheRS (α,β), α</td>
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Motifs

(Φ=hydrophobic, x=any residue, X=absolutely conserved, x=very highly conserved)

| ΦhΦGh, | 1. gΦxxΦxΦΦΦΦ |
| kmsKS   | 2. fRxe-loop-h/rxxxFxx(d/e) |
|         | 3. gΦgΦgΦ(d/e)ΦΦΦΦΦΦ |

Catalytic Domain

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<td>2’ OH</td>
<td>3’ OH</td>
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**E. coli** MetRS

The endogenous methionyl-tRNA synthetase (MetRS) extracted from *E. coli* cells is a homodimer. Each monomer consists of 676 amino acids (76 kDa). MetRS can be turned into a functional monomer by selective protease digestion, reducing the MetRS to 547 amino acids (64 kDa) without significant loss of stability or activity. For experimental convenience a genetically engineered monomeric MetRS (MetRS547) is used in all the studies described here; our lab construct contains the N-terminal 547 amino acids of MetRS encoded in pET28a (Invitrogen) behind a His$_6$-affinity tag.

The *E. coli* MetRS547 has two major domains, the N-terminal catalytic domain (colored green in Figure 3) that has the HLGH (HIGH consensus motif) and KMSKS sequences responsible for the activation and transfer of methionine onto the acceptor stem of tRNA$^\text{Met}$. The catalytic domain is interrupted by a connective polypeptide region (Figure 3, red, CP Insertion) which contains a zinc(II) ion coordinated by four Cys residues (C145-PK-C148-KSPDQYGDN-C158-EV-C161) in a zinc knuckle structure. The C-terminal half or anticodon binding domain (Figure 3, blue) is a helical bundle domain containing a loop that makes contact with and recognizes the C$_{34}$A$_{35}$U$_{36}$ anticodon triplet of all methionine specifying tRNA isoacceptors. The two major
domains are connected by a stem contact fold motif (orange) which is observed in both class Ia and class Ib AARSs.

Figure 3. Crystal structure of *E. coli* MetRS (PDB ID: 1QQT)\(^{32}\) with colors as indicated. Residues for HLGH, KMSKS, Trp461, and zinc binding motifs are shown in ball and stick conformation.

**Domain-domain communication and long-range interaction**

The anticodon binding domain of MetRS has a highly conserved tryptophan residue (Trp461) that is essential for recognition of the tRNA\(^{\text{Met}}\) CAU anticodon. Upon mutation of Trp461 to Phe, the *K*_M for tRNA is increased by 50-fold, while mutation of Trp461 to Ala results in reduced aminoacylation activity by at least 10^6^-fold.\(^{27}\) It has therefore been predicted that MetRS utilizes Trp461 to recognize the anticodon by making aromatic interaction with C\(_{34}\). The anticodon recognition loop of MetRS that
contains Trp461 is approximately 50 Å away from the active site, but somehow
information regarding binding of the CAU anticodon gets transferred to the active site,
resulting in efficient tRNA aminoacylation. The CAU anticodon of tRNA^{Met} is the
dominant identity element for \textit{E. coli} MetRS to recognize its cognate tRNA, and can
confer methionylation in noncognate tRNAs. For example, the anticodon of tRNA^{Ile} is
GUA, but substitution of G_{34} with C enables efficient methionylation by \textit{E. coli} MetRS.\textsuperscript{33}

Split proteins of \textit{E. coli} MetRS have been constructed, and some of these are
active in tRNA methionylation. Depending on the site of the stop-restart sequence, some
constructs can complement a chromosomal MetRS defect in \textit{E. coli}, indicating that they
retain activity \textit{in vivo}. The split MetRS is almost as active \textit{in vitro} as the unsplit protein as
long as the split proteins are expressed in the same cell. If expressed in different cells, the
N-terminal part is not soluble and activity is regained if it is refolded in the presence of
the C-terminal fragment.\textsuperscript{34} The ability of these split proteins to aminoacylate tRNA^{Met} \textit{in vivo}
and \textit{in vitro} suggests that covalent connectivity of the active site and anticodon
binding domain is not necessary for activity.

Efficient aminoacylation of tRNA^{Met} by MetRS does however require covalent
connectivity between the two arms of the L-shaped tRNA. Synthetic RNA mimicking the
acceptor stem of tRNA^{f_{Met}} (Figure 4, microhelix^{f_{Met}}) is a poor substrate for MetRS with \sim \textit{10}^{6}-fold reduced activity.\textsuperscript{19, 35, 36} A small RNA mimic of the tRNA^{f_{Met}} anticodon stem
loop (ACSL^{f_{Met}}) acts as an inhibitor in the tRNA^{f_{Met}} aminoacylation assay. This
inhibition effect is abolished when the CAU sequence is changed to GAU, indicating that
the hairpin binds in the same mode as the anticodon of full-length tRNA^{f_{Met}}. However,
this anticodon binding alone is not enough to enhance the aminoacylation of the microhelix$^{\text{Met}}$.19

Together, these biochemical experiments indicate that binding of tRNA$^{\text{Met}}$ C$_{34}$ by Trp461 contributes to efficient tRNA aminoacylation ~ 50Å away, and a structurally intact, although not necessarily contiguous MetRS, is essential for this communication.

![Figure 4. Sequences of tRNA$^{\text{fMet}}$ in cloverleaf representation and its corresponding L-shape conformation. Microhelix$^{\text{fMet}}$ is the acceptor stem of the tRNA capped by a loop similar to the TΨC loop. Anticodon stem-loop$^{\text{fMet}}$ microhelix is the equivalent of the anticodon stem loop of the tRNA$^{\text{fMet}}$ with extra G-C pair for stability.19](image)

**Fluorescence spectroscopy**

Luminescence is a phenomenon that occurs when a molecule emits light from an electronically excited state.$^{37}$ Fluorescence and phosphorescence are the two types of luminescence, differentiated by their excited state. Fluorescence occurs when the excited electron maintains its spin and is paired to the electron in the ground state. The return of the excited electron to the ground state is spin-allowed and rapid. The energy when the
excited electron returns to the ground state is released as a photon; consequently the wavelength of the photon is dependent upon the energy gap of the excited and ground state. As seen in Jablonski’s diagram, when an electron gets excited by absorbing light it can move to several higher energy states (Figure 5). The excited electron then undergoes vibrational equilibrium and moves to a specific energy state (S1, S2) before emitting a photon and returning to the ground state. Hence, the wavelength of emitted fluorescence is always longer than the wavelength of excitation light. The number of photons emitted is not always the same as the number absorbed; the ratio of emitted photon to absorbed photon is referred to as quantum yield.

Figure 5. Jablonski's diagram for luminescence phenomenon.
Molecules that exhibit fluorescence are mostly aromatic, and in proteins the three natural amino acids that fluoresce are phenylalanine, tyrosine and tryptophan. Phenylalanine has a maximum emission near 285 nm and tyrosine emits at 303 nm; the fluorescence of both amino acids is relatively insensitive to solvent polarity. Tryptophan has a fluorescence maximum around 350 nm and is very sensitive to its local environment, making it suitable for probing structural changes in proteins.\textsuperscript{38}

*E. coli* MetRS is amenable to a variety of biophysical and spectroscopic techniques. In particular, fluorescence spectroscopy is a very suitable method for this protein, as it has 11 tryptophan residues. Changes in wavelength and intensity of tryptophan emission spectra can be related to structural changes in the protein due to denaturation, ligand/substrate binding, conformational transitions and subunit association or dissociation. Intrinsic protein fluorescence is usually observed by excitation at 295 - 305 nm (avoiding the excitation of tyrosine residues at 280 nm) with emission monitoring at 340 – 350 nm.\textsuperscript{37}

**Unnatural Amino Acid Fluorescence**

By exploiting engineered orthogonal synthetase-tRNA pairs, many synthetic amino acids with tailored characteristics have been incorporated into proteins.

Currently there are two methods for incorporation of unnatural amino acids. One method by Kwon and Tirrell\textsuperscript{8} utilizes yeast PheRS (yPheRS) and yeast tRNA\textsuperscript{Phe}_{CUA} (ytRNA\textsuperscript{Phe}_{CUA}). Substitution of Thr416 with glycine relaxes the active site specificity of yPheRS, enabling it to activate tryptophan analogues (Figure 6). These are then incorporated into proteins using an *E. coli* expression system. The drawback of this
method is that the expression has to be carried out using a Phe/Trp auxotroph *E. coli* strain in minimal media and the incorporation only reaches 98%. The rest of the 2% at the site of the target protein is occupied by phenylalanine and tryptophan due to the mutant yPheRS’s promiscuity or by lysine due to cross-reactivity of the ytRNA\textsuperscript{Phe} with *E. coli* LysRS.

![Figure 6. Tryptophan analogues 6ClW (6-chorotryptophan), 6BrW (6-bromotryptophan) and BT (benzothienylalanine).\textsuperscript{8}](image)

A second method, using an *M. janaschii* TyrRS (mjTyrRS) and mjtRNA\textsuperscript{Tyr\textsubscript{CUA}} orthogonal pair was developed by Peter Schultz.\textsuperscript{7} Many unnatural amino acids have been successfully incorporated into target proteins at specific sites. Examples of unnatural amino acids with designed functionality are shown in Figure 7: *p*-propargyloxyphenylalanine \textbf{1} and *p*-azidophenylalanine \textbf{2} can be selectively modified by copper(I) catalyzed [3+2] cycloadditions with an azide or alkyne derivative respectively; the heavy atom in *p*-iodophenylalanine \textbf{3} is used to help phasing in X-ray crystallography; \textsuperscript{15}N-labeled *O*-methyltyrosine \textbf{4} is an NMR probe, photoactive *p*-benzoyl-phenylalanine \textbf{5} is a cross-linking agent to study protein-protein or protein-
nucleic acid interactions and amino acid dansyl side chain 6 is fluorescent. Unnatural amino acid incorporation by this method is virtually 100%, but for every unnatural amino acid a specific synthetase has to be generated by mutations and genetic selection.

![Chemical structures](image)

**Figure 7.** Examples of unnatural amino acids that have been incorporated into proteins.

**Enzyme kinetics of *E. coli* MetRS**

The reaction catalyzed by MetRS can be divided into two steps, shown below:

\[
\text{Met + ATP + MetRS} \rightleftharpoons \text{MetRS-Met-AMP + PPi} \quad (1)
\]

\[
\text{MetRS-Met-AMP + tRNA}^{\text{Met/fMet}} \rightleftharpoons \text{MetRS + Met-tRNA}^{\text{Met/fMet}} + \text{AMP} \quad (2)
\]

Aminoacyl adenylate formation (1) is typically monitored as an enzyme-catalyzed pyrophosphate exchange reaction using \(^{32}\text{P-ATP}\) as a substrate and quantifying \(\gamma^{32}\text{P-ATP}\) production. The aminoacylation of tRNA is monitored by a trichloroacetic acid (TCA) precipitation assay, where cold 5% TCA precipitates both the enzyme and tRNA. With
the use of $^{35}$S-methionine, product formation is monitored in a discontinuous radioassay. The TCA precipitation assay is able to allow determination of steady state kinetics parameters ($K_M$ and $k_{cat}$) of MetRS but does not allow measurement of the actual transfer step (2).

Pre-steady state kinetic assays are able to measure the aminoacyl transfer rate separate from the binding/recognition step and are also useful in determining if there is a third step in the reaction (i.e. product release) as the rate limiting step. Pre-steady state kinetic experiments are commonly done with a rapid chemical quench assay.

**Goal of Research**

The long-term objective of research in the Alexander lab is to use *E. coli* MetRS as a model system for anticodon-stimulated aminoacylation of tRNA (long-range intramolecular communication).

The projects described in this thesis use fluorescence spectroscopy, unnatural amino acid incorporation and pre-steady state kinetic analyses to probe conformational changes and mechanistic features of *E. coli* MetRS.
CHAPTER TWO
MATERIALS AND METHODS

Buffers and Chemicals

All buffers are made in RNase free Milli-Q water.

Charging assay buffer: 20 mM Tris-HCl pH 7.5, 10 mM MgCl2, 150 mM NH₄Cl

Fluorescence buffer: 20 mM Tris-HCl pH 7.5, 10 mM MgCl2

HisTrap Buffer A: 20 mM Tris-HCl pH 8, 300 mM NaCl

HisTrap Buffer B: 20 mM Tris-HCl pH 8, 300 mM NaCl, 500 mM Imidazole

Luria Bertani Broth Miller (LBM): 10 g tryptone, 5 g yeast extract, 10 g NaCl in 1 L

MetRS storage buffer: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM MgCl2

NEB Buffer 2: 10 mM Tris-HCl pH 7.9, 50 mM NaCl, 10 mM MgCl2, 1 mM DTT

Phosphate Buffer Saline (PBS): 8.1 mM Na₂HPO₄, 1.76 mM KH₂PO₄, 2.7 mM KCl, 137 mM NaCl pH 7.4

Super Optimal broth with Catabolite repression (SOC): 2 % w/v tryptone, 0.5 % w/v yeast extract, 8.6 mM NaCl, 2.5 mM KCl, 20 mM MgSO₄, 20 mM Glucose.

TCA Wash Solution: 5% TCA and 1 mM L-Methionine
Protein Expression

Plasmid pSW101 is a vector in which the portion of the *E. coli* MetG gene encoding the N-terminal 547 amino acids was inserted into expression vector pET28a (Novagen). The expression vector encodes a hexahistidine tag on the N-terminal side of the cloned protein. Restriction sites used are *Nde*I on the 5'-end of the insert and *Xho*I on the 3'-end. This places the gene behind an IPTG inducible lac promoter.

Transformation of chemically competent *E. coli* cells Rosetta 2 (DE3) (Novagen) is done by incubating a mixture of ~ 100 ng plasmid and 50 µL cells on ice for 5 minutes, and in a water bath at 42°C for 45 seconds; the mixture is quickly cooled on ice. After 5 minutes on ice, 300 µL of SOC media is added and the culture is left to grow in a shaker incubator at 37°C (225 rpm). After the culture has grown for at least one hour, 50 – 200 µL of the culture is then plated on LB agar with kanamycin (50 µg/mL) and incubated at 37°C for 16 – 18 hours.

*E. coli* cells from the plate are transferred to liquid media (LB supplemented with kanamycin at 50 mg/L). Cell growth is monitored at 600 nm. After OD$_{600}$ reaches ~ 0.6, protein expression is induced by adding IPTG (final concentration 1 mM). Three hours after induction, the culture is centrifuged at 6000 × g for 15 minutes and cells are washed with PBS and spun down in 50 mL conical tubes for storage at -80°C.

Protein purification and quantification

Typically 5 grams of cell pellet from 1 L culture are suspended in HisTrap Buffer A with addition of 1 mM PMSF (protease inhibitor). The cell suspension is lysed by subjecting it to 10,000 psi internal pressure with a French Press cell disruptor. The lysate
is centrifuged twice for 15 minutes each under 15,000 × g. The supernatant is filtered using a 0.45 µM syringe filter before applying it to a nickel affinity column (HisTrap FF 5 mL, GE Lifesciences) pre-equilibrated with HisTrap Buffer A. The column is washed with 10 column volumes of HisTrap Buffer A, followed by a step gradient of 10 columns volume 4% HisTrap Buffer B (equivalent to buffer A with 20 mM imidazole). Protein is eluted from the column with 50% B (250 mM imidazole).

Fractions containing *E. coli* MetRS (as determined by UV absorbance at 280 nm and SDS PAGE) are pooled and dialyzed overnight in 2X MetRS storage buffer. After the dialyzed proteins are concentrated, glycerol, water and DTT are added to a final buffer concentration of 1X MetRS storage buffer, 40% glycerol and 1 mM DTT. Protein concentration is determined by absorbance at 280 nm. Protein is serially diluted in Milli-Q water with dilution factors ranging from 200 to 1000. The extinction coefficient 94770 M⁻¹cm⁻¹ for MetRS547 is calculated by the ProtParam tool on the ExPAsy proteomics server (http://ca.expasy.org/tools/protparam.html). The first 21 amino acids (underlined) include the six histidine tag and flanking sequence encoded by vector pET28a (Novagen) making a total of 568 amino acids and molecular mass of 64,694.2 g/mol. The sequence for *E. coli* MetRS MetRS457 expressed from pSW101 is as follow:

MGSSHHHHHHSSGLVPRGSMTQVAKILVTCALFYANGSIHLGHMLEHIQADVWVRYQRMRGHEVNIFCA
DDAHGTPIMLKAQQLGITPEQMIGEMSQEHOQDFAPGNIYSYNTHDEQNRQLEILYKENGFIKN
RTISQYDPEKGMFLPDRFVKGTCPKSCPQDQYGDNCVATGYSPTHELIEPKSVSAGTFVMDHSHFFFF
DLPSFSEMLQAWTRSGALQEQVANKMQEFVESGLQQWDISRDAPYFGFEIPNAAPKYFYWLDAPIGYMGS
FKNLCDKRGDSVSFIXWVKDSTAEYHFIGKDIVYHFSLFWMLEGNSFRPSNLFVHGYVTVNGAKMS
KSRGTIFKAATWLNHFADSFRLYTTYTAKLSSTRIDDLNLEDVQRVNAIVNKLASRAGFINKRFD
The gene MetG for *E. coli* dimeric MetRS (MetRS676) was amplified from *E. coli* (K12 strain) genomic DNA and cloned into pET28a (Novagen) using the *Nde*I and *Xho*I (5' and 3' respectively) restriction sites. The plasmid for expression of MetRS676 is named pMetG111. Expression, purification and quantification of MetRS676 are the same as the MetRS547 described above. The sequence for *E. coli* MetRS676 with extinction coefficient 96,260 M⁻¹cm⁻¹ and molecular weight of 78,418.0 g/mol is:

MGSSHHHHHHSSGLVPRGSHMTQVAVKILVTCALFYANGSIHLGHMLEHIQADVWVRYQRMRGHEVNFC
DDAHGTPIMLKAQQLGITPEQMIGEMSQEHTDQFAGFNISYDNHYTHSEENNQLSELIYSRLKENGFIKN
RTISQLYDPEKMFLPDRFKGTKCPKSPDQYGDNCEVCGATYSPTLEIEFKSVSGAFPVMDSEHFF
DLPSFSEMLQAWTRSGALQEQQKMQEFESGLQWISSDAFYPGEFPNAPGKYFYVWLDAPIGYMGS
FKNLCDKRGDSVSFDEYWKKDSTAELYHFIGKDIVFHSLSFW/APLEGSNFRKPSNL/FVHYVTVNAGAMS
KSRGTKFIASTWHFDASLRYYATAKLSRIDDDLLNLEDVQRVNADIVNKVNLASRNAGFINKRF
GVLAELADPQLYKTFTDAEVI/GEAWESREFGKAVREIMALADLANVDEQAPWVVAQRGGRADLDQAI
CSMGINLFRVLMYLKPVLPKLEAEAFLNELTWIGQQLPGHVKVPFKALYNRIMQVEA/LEASK
EEVKAAPA/VTGPLLADDPIQETITTFDDFAKVDLRVALEN/EAEEV/GSDKK/RLLTDLGLGEEKRNVFSGIR
YPDPQALIRH/TIMVANLAPRKMRFGISEGMVMAAGPGGKDIFLLSPDAGAKPGHQVK

**Synthesis and purification of L-(7-hydroxycoumarin-4-yl) ethylglycine**

L-(7-hydroxycoumarin-4-yl) ethylglycine 1 was synthesized according to the published procedure.⁴⁰ Synthesis proceeded by converting N-α-Cbz-L-glutamic acid α-
benzyl ester into (2S)-2-benzyloxycarbonylamino-5-oxo-heptanedioic acid 1-benzyl ester 7-ethyl ester 2 followed by silica gel flash chromatography purification. Compound 2 is converted into coumarinyl amino acid 1 by the von Pechmann reaction. Excess resorcinol acts as a scavenger for the benzyl cations from the starting material protecting group.41

Figure 8. Synthesis of L-(7-hydroxy coumarin-4-yl) ethylglycine 1. (a) \(N,N'\)-Carbonyl diimidazole, rt, 2 hours; (b) ethyl magnesium malonate, rt, overnight; (c) resorcinol, methanesulfonic acid, rt, 2 hours.

Z-Glu-OBzl was dissolved in anhydrous THF (6 mL per mmoles of Z-Glu-OBzl) at room temperature under argon. Carbonyl diimidazol (1.1 eq) was added slowly and the mixture was stirred for two hours at room temperature. The formation of carbon dioxide was visible as the amino acid became activated. Magnesium ethyl malonate (0.55 eq) was added and the reaction was stirred overnight at room temperature. The magnesium ethyl malonate was previously prepared by reacting equimolar magnesium ethoxide (0.572 g) and ethyl malonate (1.468 g) in 10 mL anhydrous ethanol.42 The ethanol was removed by rotary evaporation and subject to vacuum evaporation overnight. The product was extracted with ether and washed with 10 % NaHCO₃, water and brine. The residue was purified by silica gel flash chromatography (ethyl acetate:hexane 50:50). The resulting compound 2 was concentrated by rotary evaporation and dried under vacuum overnight.
Compound 2 was slowly added to resorcinol (5 molar excess) in methane sulfonic acid (5 mL per mmole of 2) and stirred for one hour at room temperature. Five volumes of diethyl ether was then added to the mixture and cooled to -30°C. The precipitate was washed with cold diethyl ether, resolubilized in water, and lyophilized. L-(7-hydroxycoumarin-4-yl) ethylglycine was purified using reversed phase liquid chromatography on Biologic Duoflow FPLC (BioRad) with a Single StEP 16.5 g C18 Flash Column (Thomson Instrument Company). The structure of purified L-(7-hydroxycoumarin-4-yl) ethylglycine was confirmed by mass spectrometry and NMR spectroscopy and by incorporation into myoglobin (encoded in the plasmid pBADMyo provided by Schultz Lab).

**Incorporation of L-(7-hydroxycoumarin-4-yl) ethylglycine into MetRS**

Plasmids for incorporating L-(7-hydroxycoumarin-4-yl) ethylglycine into *E. coli* MetRS547 were provided by Peter Schultz’s lab. The system utilizes two plasmids; the plasmid called pBKCou which supplies mutant *M. janaschii* TyrRS and the second plasmid (pBADMyo) which encodes for the orthogonal tRNA and target protein (myoglobin).

The *E. coli* MetRS547 gene was subcloned from pSW101 with an *Nco*I restriction site on the 5' side of the gene (to include the six histidine tag) and a *Kpn*I site at the 3'-end (added to pSW101 using QuikChange™ protocols) into pBADMyo, replacing the myoglobin gene (this plasmid is now called pBADMetRS). The same mutation method was used to insert the TGA (stop/amber) codon into MetRS at positions replacing either Trp204 or Trp461.
Top10 cells (Invitrogen) were transformed with pBKCou and pBADMetRS mutants under kanamycin (50 µg/mL) and tetracycline (15 µg/mL) selection. The cells with the two plasmids were grown in LBM liquid media supplemented with kanamycin (50 µg/mL) and tetracycline (15 µg/mL) and 1 mM L-(7-hydroxycoumarin-4-yl) ethylglycine. When the culture reached OD<sub>600</sub> ~ 0.6, protein induction was initiated with the addition of L-arabinose at a final concentration of 0.2% w/v and the induction was allowed to proceed overnight. Protein extraction and purification was achieved following the standard His<sub>6</sub>-Tag affinity column protocol described above for MetRS547. Protein concentration determination of L-(7-hydroxycoumarin-4-yl) ethylglycine incorporated MetRS was accomplished using the modified Bradford protein assay.<sup>43</sup>

**Fluorescence spectroscopy**

A MetRS547 solution of 500 µL was made up in fluorescence buffer with protein concentration between 1 - 2 µM. Titration of methionine was carried out by adding 0.5 – 5 µL of solution containing the same protein concentration with varying amounts of methionine (2 – 50 mM) into the 500 µL MetRS547 sample in a Quartz SUPRASIL Macro/Semi-micro Cell (PerkinElmer) equipped with a small magnetic stir bar. The excitation wavelength was set at 295 nm with a 5 nm slit width, and the fluorescence signal was measured at 340 nm with a 5 nm slit width. The same procedure also applied for tRNA<sup>Met</sup> titration using annealed tRNA<sup>Met</sup> (10 – 180 µM). For tRNA<sup>Met</sup> titration experiments of L-(7-hydroxycoumarin-4-yl) ethylglycine incorporated MetRS547, the excitation wavelength was 360 nm and the emission was monitored at 450 nm.
The fluorescence emission signal was collected at 0.5 second intervals on a Perkin-Elmer LS50B Luminescence Spectrometer using a time drive method at 25ºC. The solution of MetRS547 was stirred continuously and each addition of ligand was equilibrated for 100 – 200 seconds.

**tRNA\textsuperscript{Met} in vitro transcription and purification**

*E. coli* tRNA\textsuperscript{Met} is transcribed using a 2’-O methylated primer according to the method developed by Perona with minor modifications.\textsuperscript{44, 45} The template for tRNA\textsuperscript{Met} in vitro transcription is two 60-nucleotide DNA primers with 15 nucleotides overlapping (underlined on the sequences below) on the 3’-end. The sequence for the tRNA\textsuperscript{Met} 5’-end primer, which contains T7 RNA polymerase promoter (in bold type) is as follows:

5’-AAT TCC TGC AGT AAT ACG ACT CAC TAT AGG CTA CGT AGC TCA GTT GGT TAG AGC ACA TCA

The 5’ two nucleotides for the tRNA\textsuperscript{Met} 3’-end transcription primer are 2’-O methyl modified ribonucleotides; the sequence is as follows:

5’-mUmGG TGG CTA CGA CGG GAT TCG AAC CTG TGA CCC CAT CAT TAT GAG TGA TGT GCT CTA ACC

Oligonucleotides were purified by 16% denaturing PAGE in TBE buffer. Bands were visualized by UV shadowing, cut from the gel, electroeluted, and ethanol precipitated. Precipitated oligonucleotides were washed with 70% ethanol, dried overnight in a Savant DNA SpeedVac Concentrator (Thermo Fisher Scientific), resolubilized with Milli-Q water and quantified by absorbance at 260 nm. The extinction
coefficient for the 5'-primer is 589,600 M\(^{-1}\) cm\(^{-1}\) and 569,900 M\(^{-1}\) cm\(^{-1}\) for the 3'-primer (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/).

Gel-purified oligonucleotides were mixed at 4 µM each in 1X NEB Buffer 2 with 400 µM dNTPs and 50 U/mL Klenow Fragment (3’→5’ exo⁻) (New England Biolabs, Inc). The mixture was cycled in a thermocycler between 4°C and 37°C at 30 second intervals for 20 cycles.

*In vitro* transcription of tRNA\(^{\text{Met}}\) was accomplished by mixing the Klenow-extended primer (template final concentration 1 µM), 250 mM Hepes-KOH (pH 7.5), 30 mM MgCl\(_2\), 2 mM spermidine, 40 mM DTT, 0.1 mg/mL bovine serum albumin, 4 mM NTPs and 95 µg/mL T7 RNA polymerase (purified in house). The reaction proceeds at 37ºC for 6 hours with constant rotation on a tube rotator. The tRNA\(^{\text{Met}}\) is precipitated at -20ºC overnight with one reaction volume of 7.5 M ammonium acetate and two reaction volumes of 2-propanol. The precipitate is further purified by 12% denaturing PAGE in TBE buffer. The tRNA\(^{\text{Met}}\) band is visualized by UV shadowing, isolated and extracted from the gel by electroelution using an Elutrap System (Schleicher and Schuell) in 0.5X TBE Buffer at 150 V for 3 hours twice. Extracted tRNA\(^{\text{Met}}\) was precipitated by adding one tenth volume of 3 M sodium acetate (pH 5.2) and one volume of 2-propanol overnight at -20ºC. The tRNA\(^{\text{Met}}\) is pelleted by centrifugation at 16,000 \(\times\) g for 15 minutes, washed with 70% ethanol and dried by Savant DNA SpeedVac Concentrator for several hours at room temperature. Dried tRNA\(^{\text{Met}}\) pellets were resolubilized in 20 mM Hepes-NaOH (pH 7.5) and quantified by UV spectrophotometry at 260 nm. The extinction coefficient 762,900 M\(^{-1}\) cm\(^{-1}\) used in quantification is obtained by entering the
tRNA\textsuperscript{Met} transcript sequence (below) into the IDT sequence analyzer tool mentioned above.

5'- /5Phos/rGrGrC rUrArC rGrUrA rGrCrU rCrArG rUrUrG rGrUrU rArGrA rGrCrA rCrArU rCrArC rUrCrA rUrArA rUrGrA rUrGrG rGrGrU rCrArC rArGrG rUrUrC rGrArA rUrCrC rCrGrU rCrGrU rArGrC rCrArC rCrA -3'

The tRNA\textsuperscript{Met} is refolded before use in every experiment in 20 mM Hepes-NaOH (pH 7.5) and 1 mM MgCl\textsubscript{2}. Refolding is carried out by heating the tRNA\textsuperscript{Met} solution (without magnesium chloride) in a water bath at 80°C followed by slow cooling to room temperature; when the water temperature reaches 60°C, one tenth volume of 10 mM MgCl\textsubscript{2} is added to make the final concentration of magnesium 1 mM.

**Aminoacylation Assay**

The aminoacylation activity of MetRS is assayed using L-[\textsuperscript{35}S]-Methionine (37.0 TBq/mmol) purchased from PerkinElmer. The typical reaction condition is 100 μM methionine, 4 mM ATP and 4 μM tRNA\textsuperscript{Met} in charging assay buffer. The concentration range of MetRS assayed is 20 nM – 1 μM, depending on the activity of the MetRS variants. For a steady state kinetics experiment, a minimum of five time points are taken in the range of 1 – 2 minutes reaction time by spotting a 5 μL aliquot of the reaction onto a 2.3 mm diameter Whatman filter paper impregnated with 200 μL of 5 % TCA and 1 mM methionine. The filter paper is left to stand for 15 seconds before dropping it into 250 mL of ice cold 5% TCA, 1 mM methionine. The filter papers are washed 5 times at 10 minutes each with 250 mL 5% TCA, 1 mM methionine and then washed with 95 %
ethanol before being dried under infrared lamp. Dried filter papers are mixed with 6 mL of Ecolite(+) LC Fluid (MP Biomedicals) and radioactivity is counted using a Beckman Coulter LS-6500 liquid scintillation counter.

Single turnover kinetic analyses

Single turnover reactions of tRNA\textsuperscript{Met} aminoacylation are carried out using chemical quench method with a RQF-3 Rapid Quench Flow Instrument (KinTek Corporation). The assay uses an excess of enzyme over tRNA to prevent multiple catalytic cycles.

Reactions are performed in charging assay buffer with final concentrations of 2 µM tRNA\textsuperscript{Met}, 40 µM MetRS, 100 µM methionine and 4 mM ATP. Each time point is quenched with 3 M sodium acetate (pH 4.5), collected in a 1.5 mL eppendorf tube containing 150 µL phenol:chloroform (1:1) pH 4.5, vortexed for 10 seconds and centrifuged for 1 minute at 16000 \( \times \) g. The aqueous layer is then spotted onto two pieces of TCA impregnated Whatman filter paper. Washing, drying and radioactivity counting methods are the same as the steady state experiment described above.

There are two types of single turnover reactions. The first type is where the adenylate is formed \textit{in situ}; the rate resulting from this analysis is the chemical rate \( (k_{chem}) \) which is the rate of amino acid activation and transfer combined. Reaction components are separated in two syringes, the first syringe contains 4 µM tRNA\textsuperscript{Met} and 80 µM MetRS and the second syringe contains 200 µM Methionine, 8 mM ATP and 3 µL of L-[\textsuperscript{35}S]-methionine (37.0TBq/mmol, PerkinElmer).
The second type of single turnover experiment is where the adenylate is pre-formed in the second syringe (by mixing MetRS, methionine and ATP), while the other syringe holds the tRNA^{Met}. The rate value resulting from this analysis is the actual transfer rate \( k_{\text{trans}} \) of the amino acid to the tRNA^{Met}.

Data are fitted using Origin 8 (OriginLab Corporation) with non-linear curve fitting to a first order exponential equation:

\[
y = y_0 + A(1 - e^{-kt})
\]

where \( A \) is the scaling constant, \( y_0 \) is the y axis intercept, \( k \) is the observed rate constant and \( t \) is time in seconds.

**Multiple turnover kinetic analyses**

The principle multiple turnover kinetic analyses are similar to the steady state aminoacylation reaction previously described, except the time points taken are much faster (0.01 – 3 seconds). The experimental technique is similar to the single turnover reaction to obtain \( k_{\text{chem}} \). Typical reaction conditions are 10 µM tRNA^{Met}, 0.5 µM MetRS, 100 µM methionine and 4 mM ATP. These conditions allow the enzyme to perform multiple catalytic cycles. Experiments are carried out as for the first type of single turnover except the first syringe contains 20 µM tRNA^{Met} and 1 µM MetRS.
Chemical quench setup

The drive syringes (A and B) are filled with charging assay buffer and C is filled with quench buffer (3 M sodium acetate pH 4.5). Reaction mixtures are loaded into 1 mL syringes and attached to the sample ports. The instrument is programmed to a constant quench volume with a zero second delay quench time.

Before and after each aliquot is collected, the sample loops, mixer chamber and delay line are washed with Milli-Q water and methanol. Washes are performed by attaching the exit line to a vacuum pump and inserting the flush line into water then into a

Figure 9. Schematic of the KinTek RQF-3 Rapid-Quench Flow Instrument. The apparatus is used to perform the rapid chemical quench assay. The diagram is from the KinTek Corporation website (http://www.kintek-corp.com/).
methanol reservoir (20 and 3 seconds respectively). The system is dried for 30 seconds by letting air enter the flush line while the exit line is still attached to the vacuum.
CHAPTER THREE

FLUORESCENCE STUDIES OF *E. coli* MetRS547

Background

The fluorescence phenomenon occurs when light is absorbed by a fluorophore and the electrons are excited from the ground state. When the excited electrons return to the ground state, they release energy in the form of fluorescence. The emitted wavelength is independent to the absorbed light but is always longer than the wavelength of the absorbed light due to vibrational equilibrium of the excited state. Molecules that are fluorophores are usually aromatic compounds. In proteins there are three naturally occurring fluorescent amino acids, phenylalanine, tyrosine and tryptophan. Among these three, tryptophan is the most sensitive to environmental change and can be selectively excited at 295 nm. This characteristic makes intrinsic tryptophan fluorescence suitable for the study of protein-ligand interactions and conformational changes in proteins.37

When either trypsin-modified MetRS or genetically engineered MetRS547 is titrated with methionine, the intrinsic fluorescence intensity increases by 40 %.46 In contrast, titration of dimeric MetRS or monomeric MetRS (trypsin modified) with tRNA\textsuperscript{Met} results in quenching of fluorescence by 18%.47 These results suggest that when MetRS binds methionine or tRNA\textsuperscript{Met}, the environment of one or more tryptophan residues is affected. We sought to determine whether these fluorescence changes are the result of global conformational changes in the protein or are from interaction of a specific ligand (methionine and tRNA\textsuperscript{Met}) with one or more tryptophan residues.
Construction of MetRS457 variants

MetRS457 variants in this chapter were generated by applying QuikChange™ (Stratagene) protocols. In pSW101, the mutant Trp253 → Phe is generated by changing the codon from TGG to TTT, Tyr15→Phe from TAC to TTC, Phe300→A from TTC to GGC and Trp461→Phe from TGG to TTT. The codon change from TGG (tryptophan) to TAG (stop/amber) is performed on pBADMetRS to generate Trp204→Cou and Trp461→Cou for the incorporation of L-(7-hydroxycoumarin-4-yl) ethylglycine into MetRS547. The codon substitutions and integrity of the variants were confirmed by DNA sequencing.

The plasmid pSW101 with individual amino acid substitutions for W253F, Y15F, F300A and W461F were expressed in Rosetta 2(DE3) E. coli cells (Novagen). The variant proteins are soluble and expressed at the same level as the wild type MetRS547. Purification by nickel affinity column (HisTrap, GE Lifesciences) was achieved as described in Chapter 2. The concentrated MetRS457 mutants were quantified by UV spectroscopy with extinction coefficients of 89,270 M⁻¹cm⁻¹ for W253F and W461F, 93,280 M⁻¹cm⁻¹ for Y15F and 94,770 M⁻¹cm⁻¹ for W300A.

For L-(7-hydroxycoumarin-4-yl) ethylglycine incorporation, W204Cou and W461Cou were expressed in Top10 chemically competent E. coli cells (Invitrogen) and purified as described in Chapter 2.

Fluorescence study on methionine titration to MetRS547

We are interested in using fluorescence to monitor conformational change(s) upon substrate binding and catalysis. Prior studies by Sylvan Blanquet⁴⁶ using endogenous
dimeric MetRS demonstrated that the binding constant for methionine can be determined by intrinsic fluorescence spectroscopy. By saturating a MetRS solution with methionyl adenylate or methionine, fluorescence intensity increases by 40% and 26%, respectively. The only tryptophan in the MetRS active site is Trp253, which is conserved in all organisms. Comparing the crystal structure of MetRS with and without with methionine reveals three residues that change their orientation: Trp253, Tyr15 and Phe300 (Figure 10).

![Figure 10](image)

**Figure 10.** Superimposed crystal structures of MetRS547 with and without methionine (PDB ID: 1PG2 and 1QQT respectively). The blue residues are in the absence of methionine. Gray color indicates residue positions with methionine.

Trp253 changes its orientation upon methionine binding, suggesting that the observed fluorescence increase comes from Trp253. However, as there is no direct interaction between methionine and the indole side chain of Trp253, other structural
changes occurring in the active site may result in the fluorescence enhancement. Tyr15 flips towards Trp253, placing the phenol oxygen within electrostatic interaction range of the Trp253 indole N-hydrogen. Phe300 also flips toward Trp253 and the phenyl ring stacks with the indole six-membered ring (Figure 11). To investigate which of these interactions may result in the increase in fluorescence intensity upon methionine binding, three amino acid substitutions were made to MetRS547: W253F, Y15F and F300A.

Figure 11. Three active site residues from E. coli MetRS (Trp253, Phe300, Tyr15) and methionine from PDB ID: 1PG2.48 Superimposed residues (blue) are the amino acid positions from the MetRS crystal structure without methionine (PDB ID: 1QQT).32 The distances are shown in Angstroms.48

Two main hypotheses arise from previous fluorescence studies and from the crystal structures: either the increase in fluorescence on methionine binding is caused by global structural changes in MetRS or the fluorescence enhancement is due only to the
change in Trp253 environment. If the latter were true, fluorescence enhancement could arise from an electrostatic interaction between the Tyr15 hydroxyl and the hydrogen of Trp253 indole nitrogen, and/or from the effect of aromatic stacking between Phe300 and Trp253.

An *E. coli* MetRS547 variant with Trp253 to Phe substitution was made to test whether the fluorescence increase is caused by the change in the environment of Trp253 instead of a structural change; this variant should no longer exhibit increased fluorescence upon methionine binding. Substitution of Tyr15 with Phe is used to test the idea that fluorescence increases because of an electrostatic interaction between the tyrosine hydroxyl group to tryptophan indole N-hydrogen. Finally, substituting Phe300 with Ala will test the effect of aromatic interaction on the fluorescence enhancement.

**Methionine titration study results and discussion**

MetRS547 variants were constructed, expressed and purified as described in Chapter 2. Aminoacylation assays at 4 μM tRNA\textsuperscript{Met}, 100 μM methionine and 4 mM ATP were used to test whether the mutants are still active. The MetRS547 variants W253F, Y15F and F300A are still able to aminoacylate tRNA\textsuperscript{Met}, although with 7 – 50 fold reduced initial rates compared to wild type MetRS547 (Figure 12). The reduced initial rates at 100 μM methionine are not surprising, as these active site residues are all conserved amino and will likely impair the ability of MetRS to bind methionine.
Figure 12. Aminoacylation of tRNA\textsuperscript{Met} by MetRS547, W253F, Y15F and F300A. Reactions were performed at 4 µM tRNA\textsuperscript{Met}, 100 µM methionine and 4 mM ATP. Initial rates for WT MetRS547 is 0.328 s\(^{-1}\), W253F 0.007 s\(^{-1}\), Y15F 0.043 s\(^{-1}\), and F300A 0.046 s\(^{-1}\). \([^{35}\text{S}]\)Met-tRNA\textsuperscript{Met} is quantified by precipitation with TCA followed by liquid scintillation counting of the radioisotope.

Substitution of Trp253 to Phe produces a MetRS547 variant that no longer exhibits increased fluorescence intensity upon titration with methionine (Figure 13). This is a clear indication that Trp253 is the fluorophore that senses and signals methionine binding. Substitution of Tyr15 to Phe maintains the hydrophobicity of the side chain but eliminates the electrostatic interaction. Titrating methionine into a solution of Y15F resulted in no fluorescence enhancement (Figure 13). This result supports the hypothesis that the fluorescence increase is triggered by interaction of the Tyr15 hydroxyl group with the Trp253 indole N-hydrogen. Methionine titration of the Phe300A variant still results in fluorescence intensity enhancement, although the intensity increase is only 20 % compared to 40 % for the wild type MetRS547 (Figure 13). This suggests that the
stacking of Phe300 with the indole group is not crucial for the fluorescence activity of Trp253 but may help provide an optimal hydrophobic environment.

**Figure 13.** Representation plot of methionine titration of MetRS547 and its variants. The relative fluorescence intensity difference is plotted against methionine concentration. Fluorescence intensity of wild type MetRS547 is increased by 40 %. W253F and Y15F substitutions eliminates fluorescence enhancement. W300F fluorescence intensity enhancement is reduced to 20 %.

**Methionine titration conclusion**

The loss of methionine-induced fluorescence enhancement of W253F is a clear indication that Trp253 is the residue most directly affected by methionine binding to MetRS. The environmental change that results in the increased fluorescence of Trp253 is caused by side chain orientation rearrangement of Tyr15 and Phe300 when methionine binds to MetRS.

Our results support a model in which Tyr15 swings in towards Trp253 upon methionine binding and positions the hydroxyl group in close proximity to the Trp253
indole nitrogen. The interaction is possibly an electrostatic interaction between the indole $N$-hydrogen and the phenolic oxygen of Tyr15. This idea is supported by the absence of fluorescence enhancement with substitution from Tyr to Phe at position 15.

The reduced fluorescence enhancement from 40% to 20% for the Phe to Ala substitution that was made at position 300 suggests that the aromatic interaction between the phenyl group and the Trp253 indole helps to position Trp253 (for the interaction with Tyr15) and provides the hydrophobic environment for optimal fluorescence.

**Fluorescence study on tRNA$^{\text{Met}}$ titration to MetRS547**

Just as binding of methionine results in altered MetRS fluorescence, so does binding of tRNA$^{\text{Met}}$. Sylvan Blanquet and coworkers \(^{47, 49}\) have studied tryptophan fluorescence quenching of both native and trypsin-modified *E. coli* MetRS with native *E. coli* tRNA$^{\text{fMet}}$ and tRNA$^{\text{Met}}$. His work also includes titration of the MetRS-tRNA$^{\text{fMet}}$ complex with methionyl adenylate and the effects of KCl and magnesium ion on the quenching of fluorescence; he also demonstrated that tRNA$^{\text{Met}}$-induced fluorescence quenching can be used to determine the dissociation constant of tRNA$^{\text{Met}}$.

Binding of the tRNA$^{\text{fMet}}$ does not seem to be affected by the functional state of the acceptor stem. Ferguson et al.\(^{50}\) carried out quenching studies on MetRS with 3'-labeled tRNA$^{\text{fMet}}$, where tRNA$^{\text{fMet}}$ was oxidized with periodate prior to coupling with dansyl hydrazine. This labeling method attached the fluorophore to the 3'-O ribose of A$_{76}$, thus inactivating tRNA$^{\text{fMet}}$ for aminoacylation. The tRNA$^{\text{fMet}}$ dissociation constant by MetRS intrinsic fluorescence quenching is still attained even though the modified tRNA$^{\text{fMet}}$ is unable to accept methionine.
The CAU anticodon of tRNA\textsuperscript{\textit{fMet}} is sufficient for MetRS to recognize it from all other cellular tRNAs. The role of tRNA\textsuperscript{\textit{Met}} C\textsubscript{34} as a strong determinant for tRNA recognition by MetRS was shown 20 years ago by Schulman et al.\textsuperscript{51} The CAU anticodon is transplantable into tRNA\textsuperscript{\textit{Ile}}, such that the resulting tRNA\textsuperscript{\textit{Ile}}\textsubscript{CUA} is aminoacylated by \textit{E. coli} MetRS with comparable efficiency.\textsuperscript{27} Later studies also showed that the anticodon stem loop (a fragment of tRNA\textsuperscript{\textit{fMet}}) acts as a competitive inhibitor of tRNA\textsuperscript{\textit{fMet}} in the aminoacylation reaction.\textsuperscript{35}

Cross-linking studies identified Lys465 in MetRS as one residue in close proximity to where the tRNA\textsuperscript{\textit{Met}} interacts with MetRS.\textsuperscript{52} Substitution of Trp461 with Phe reduced the relative aminoacylation efficiency 50-fold,\textsuperscript{18} and substitution to Ala rendered the mutant virtually unable to aminoacylate tRNA\textsuperscript{\textit{Met}}.\textsuperscript{27} Trp461 is conserved in MetRS from almost all organisms except in yeast cytoplasmic MetRS (mitochondrial yeast MetRS has the equivalent Trp461). Interestingly, yeast cytoplasmic MetRS lacking the conserved Trp461 is able to aminoacylate \textit{E. coli} tRNA\textsuperscript{\textit{Met}} efficiently.\textsuperscript{53}

The distance from MetRS Trp461 to its active site is approximately 50 Å, but the interaction between C\textsubscript{34} and Trp461 is sufficient to trigger efficient aminoacylation.\textsuperscript{33} It has been hypothesized that the recognition event is conveyed by a structural change in the enzyme, the tRNA or both (by an induced fit or mutually induced fit mechanism). The crystal structure of \textit{Aquifex aeolicus} MetRS complexed with tRNA\textsuperscript{\textit{Met}} showed an aromatic stacking interaction of Trp422 (the equivalent Trp461 in \textit{E. coli}) and C\textsubscript{34} of the tRNA\textsuperscript{\textit{Met}} anticodon.\textsuperscript{54} A molecular dynamics study by Budiman\textsuperscript{55} showed that there are highly correlated movements between Trp461 and amino acids in the active site and CP domains of the enzyme.
Previous biochemical and structural studies strongly suggest direct interaction between Trp461 and C_{34} of the tRNA^{Met} anticodon, but the connection between this recognition and tRNA-induced fluorescence quenching has not been directly tested. We sought to link the fluorescence quenching to tRNA^{Met} binding and explore the possibility of studying structural changes in the MetRS-tRNA^{Met} complex by fluorescence spectroscopy.

**tRNA^{Met} titration of MetRS547**

The MetRS547 variant W461F (Trp to Phe substitution) was constructed to test whether the fluorescence quenching seen upon tRNA^{Met} binding is due to the quenching of single tryptophan (Trp461) or to a global conformational change affecting other tryptophan residues. Phenylalanine is chosen to eliminate the fluorescence signal at position 461 yet maintain the aromatic nature at that position. A previous study by Ghosh^{18} demonstrated that the W461F $K_M$ for tRNA^{Met} binding is increased by 50-fold compared to the wild-type enzyme (75 and 1.2 µM, respectively). The aminoacylation rate is essentially the same ($k_{cat}$ 4.3 and 3.4 s$^{-1}$ respectively), suggesting that Phe461 is able bind C_{34} (with reduced affinity) but the binding signal is still transferred to the active site with the same efficiency as Trp461 in the case of wild-type protein (hence the same aminoacylation rate). To determine whether tRNA-induced fluorescence quenching is a result of anticodon binding at Trp461, tRNA^{Met} was titrated into a solution of either wild-type or W461F MetRS547.

The inner filter effect caused by tRNA^{Met} absorbance at 295 nm is corrected experimentally. A plot of $I/I_0$ vs. tRNA^{Met} concentration (where $I$ is the fluorescence
intensity of MetRS in the presence of methionine and I₀ is the fluorescence intensity without methionine) is obtain by titrating tRNA^{Met} into a tryptophan solution in fluorescence buffer. The reduced fluorescence intensity caused by partial absorption of the excitation light is then used to correct the titration of MetRS.

Figure 14. tRNA^{Met} titration of MetRS WT and W461F. The inner filter effect of tRNA^{Met} has been corrected by adjusting the fluorescence signal to the curve obtained by titrating RNA^{Met} into a tryptophan solution.

As previously demonstrated, tRNA binding resulted in a quench of the protein intrinsic fluorescence. The calculated Kₐ for tRNA^{Met} is 0.75 ± 0.06 µM. Mutation of Trp461 to Phe did not completely diminish the quenching phenomenon, but did reduce the quench to 5 % compared to 20 % for wild-type MetRS547. This indicates that the fluorescence signal involved in the tRNA^{Met} binding associated quenching belongs primarily to Trp461. The reduced quenching level suggests that at least one other tryptophan in MetRS is affected by tRNA^{Met} binding. Since the amino acid residue at position 461 is the only tryptophan that has been shown to interact directly with tRNA^{Met},
there is a high probability that the remaining 5% quench is caused by a structural change in W461F which in turn changes the environment of one or more tryptophans.

Locating other tryptophan(s) affected by tRNA\textsuperscript{Met} binding to W461F is not possible by simply making substitutions at other tryptophan residues, because the solubility of the protein decreases as more tryptophan substitutions are introduced.

**tRNA\textsuperscript{Met} titration conclusion**

The reduced fluorescence quenching from 20% in wild-type MetRS547 to 5% in W461F when the protein was titrated with tRNA\textsuperscript{Met} is clear evidence that the major fluorescence quenching phenomenon comes from Trp461. The 5% quenching may have come from structural change(s) in other part(s) of the protein that affect the electronic environment of one or more tryptophan residues.

**Unnatural amino acids incorporation**

With eleven tryptophan residues, MetRS547 has a very high intrinsic fluorescence intensity. This provides an advantage in sensitivity but is also a drawback since it is difficult to pinpoint where the changes in fluorescence originate. It is not possible to mutate all but one tryptophan to identify which one is affected by structural change, because some tryptophans are integral to the protein structure (as evidenced by decreased solubility). Labeling the protein with a fluorescent dye is another method to probe protein-ligand interactions, but this method requires reactive chemical groups such as cysteine thiols. In order for this method to work, one needs a single cysteine accessible to coupling, which either exists in the protein in the first place or is added by a point
mutation. MetRS has eight cysteine residues; all but four of these cysteines are complexed with the zinc ion in the CP insertion knuckle and are unreactive to coupling. Previous attempts to mutate the other four residues to serine have been carried out, but the introduction of more than one substitution causes the expressed protein to become insoluble, thus fluorescent dye labeling is deemed unsuitable for MetRS.

Recent developments in manipulating the genetic code have enabled researchers to introduce unnatural amino acids in the protein synthesis repertoire. Utilizing an orthogonal tRNA and synthetase pair, amino acids with tailored characteristics such as fluorescence, $^{15}$N nuclear spin labeled, or “click” reactivity have been incorporated into protein in various biological systems.

A fluorophore can be introduced in the protein by incorporating a fluorescent unnatural amino acid (UAA). The advantage of this method is that the UAA has different excitation and emission spectra from tryptophan so there is no resonance energy transfer problem that occurs in proteins with multiple tryptophans. Unlike fluorescent dyes, the UAA does not have to be solvent exposed and can be incorporated almost anywhere in the protein provided that the protein can tolerate the bulkier UAA.

The method of incorporating UAA that we use here is developed by Peter Schultz’s lab at UC Berkeley by using a two vector tandem system. One vector (pBK-CouRS) carries the mutant M. janaschii TyrRS that has been selected to accept the UAA and the second vector (pBAD/JYAMB) is the protein expression vector which also expresses an M. janaschii tyrosyl amber suppressor tRNA ($Mj$tRNA$_{\text{Tyr}}^{\text{CUA}}$). This two-vector system was a generous gift from the Schultz lab.
In order to apply this method, we subcloned *E. coli* MetRS547 gene from our pSW101 plasmid into pBAD/JYAMB with *Nco*I and *Kpn*I restriction sites, thus the MetRS547 in pBAD/JYAMB will then have an N-terminal His<sub>6</sub>-tag. The unnatural amino acid L-(7-hydroxycoumarin-4-yl) ethylglycine (Cou) is not commercially available so it was synthesized as described by Wang and Brun.<sup>40, 41</sup>

This method allowed us to insert Cou, which has a different fluorescence spectrum from tryptophan, into MetRS547 at specific sites. Cou absorbs light at 350 nm and emits fluorescence light at 450 nm. By inserting Cou into MetRS457 at specific sites we intend to monitor structural changes when MetRS547 binds tRNA<sup>Met</sup>. This method is employed based on the result of our previous study (above) that W461F quenching is not completely diminished, suggesting that there other tryptophans affected by tRNA<sup>Met</sup> binding.

![Figure 15. L-(7-hydroxycoumarin-4-yl) ethylglycine (Cou). This fluorescent unnatural amino acid absorbs at $\lambda_{ex} = 360$ nm and fluoresces at $\lambda_{em} = 450$ nm](image)

Trp204 (Figure 16) is chosen for Cou incorporation based on previous molecular dynamics study by Budiman<sup>55</sup> showing a correlated movement of Trp461 and the CP
insertion domain; furthermore the location of Trp204 is away from the surface where tRNA\textsuperscript{Met} binds. We also incorporated Cou at position 461, replacing the conserved tryptophan, as a control for non-specific tRNA interactions leading to structural perturbation. Tryptophan is selected because it is the largest natural amino acid in proteins and the size of the amino acid replaced has to be big enough to accommodate L-(7-hydroxycoumarin-4-yl) ethylglycine.

Figure 16. Crystal structure of MetRS showing Trp204 and Trp461. This view is rotated compared to Figure 3 to show the “back” side of the protein. Coordinates are from PDB ID: 1PFV.\textsuperscript{48}
L-(7-hydroxycoumarin-4-yl) ethylglycine incorporation results

L-(7-hydroxycoumarin-4-yl) ethylglycine was synthesized, purified and characterized following the method described in Chapter 2 with 53% overall yield. Excitation and emission spectra of Cou in fluorescence buffer shows two excitation peaks (340 nm, 367 nm) and a single emission maximum at 464 nm (Figure 17).

**Figure 17.** Emission and excitation spectrum of L-(7-hydroxycoumarin-4-yl) ethylglycine. Excitation spectrum is collected by monitoring the fluorescence signal at 460 nm, and the emission spectrum is generated by exciting at 350 nm.

Incorporation of Cou into MetRS457 (W204Cou and W461Cou) was successful although with a lower protein yield than for wild-type MetRS547 due to the nature of the arabinose expression system used. The purity of the isolated W204Cou and W461Cou is also less than wild-type MetRS547, because the lower expression level leads to nonspecific binding of *E. coli* proteins to the nickel affinity column (Figure 18).
Figure 18. SDS-PAGE of L-(7-hydroxycoumarin-4-yl) ethylglycine incorporated into MetRS547. Left panel, Coomasie blue stained gel. Right panel, the same gel visualized under UV light. Lane 1, High Range Rainbow molecular weight marker (GE Lifesciences); lane 2, W204Cou; lane 3, W461Cou; lane 4, wild-type MetRS547.

The excitation spectrum of W204Cou (Figure 19) surprisingly showed a maximum at 280 nm not present for Cou alone (Figure 17). This 280 nm excitation peak is due the resonance energy transfer\textsuperscript{37, 56} from tryptophan and tyrosine residues in the protein to the coumaryl amino acid due to spectral overlap of tryptophan and tyrosine emission with Cou excitation.
Figure 19. W204Cou emission and excitation spectra. The excitation spectrum shows maxima at 280 nm and 330 nm. The emission spectrum shows maximum intensity at 440 nm. Excitation spectrum is collected by monitoring the fluorescence signal at 460 nm, and the emission spectrum is generated by exciting at 350 nm.

The activity of these UAA-containing enzymes to catalyze tRNA^{Met} aminoacylation was analyzed. The W204Cou variant could still aminoacylate tRNA^{Met}, although with 30% reduced activity (Figure 20). As might be expected given the replacement of Trp461, W461Cou exhibited no detectable activity.

Titration of W461Cou with tRNA^{Met} does not produce any effects on the coumaryl amino acid fluorescence, which is not surprising because the larger size and different polarity from tryptophan leads to loss interaction with the C_{34} of the anticodon. However, while W204Cou was active in aminoacylation (Figure 21), no change in fluorescence was observed on tRNA binding (data not shown).

The absence of fluorescence intensity change in W204Cou when titrated with tRNA^{Met} could come from two reasons; either the position 204 does not have a structural
change upon tRNA$^{\text{Met}}$ binding or the change is too small to impact the coumaryl amino acid environment.

**Figure 20.** Aminoacylation activity of MetRS547. Initial rate of wild-type MetRS aminoacylation is 0.293 s$^{-1}$, W204Cou is 0.097 s$^{-1}$ and W461Cou is not measureable. W204Cou is 33 % active relative to the wild-type enzyme.

Unnatural amino acid incorporation conclusion

L-(7-hydroxycoumarin-4-yl) ethylglycine was successfully incorporated into MetRS547. Titration of W204Cou and W461Cou with tRNA$^{\text{Met}}$ did not show any change in the coumaryl amino acid fluorescence intensity. No fluorescence change in W461Cou is predicted since the bulkier and more polar coumaryl amino acid could not interact with C$_{34}$ of the tRNA$^{\text{Met}}$ anticodon.
The absence of coumaryl fluorescence change in W204Cou when titrated with tRNA\textsuperscript{Met} could be interpreted that the position 204 did not undergo structural change upon tRNA\textsuperscript{Met} binding or that the change is too subtle to affect the coumaryl amino acid.
CHAPTER FOUR

PRE-STEADY STATE KINETIC ANALYSES OF E.coli MetRS

Background

Aminoacylation of tRNA\textsuperscript{Met} by MetRS is a two step reaction. First, methionine is activated with ATP to form methionyl adenylate, then the methionine is transferred to the A\textsubscript{76} 2'-O ribose of tRNA\textsuperscript{Met}, as shown below

\begin{equation}
\text{Met} + \text{ATP} + \text{MetRS} \rightleftharpoons \text{MetRS}\cdot\text{Met-AMP} + \text{PPi}
\end{equation}

\begin{equation}
\text{MetRS}\cdot\text{Met-AMP} + \text{tRNA}\textsuperscript{Met} \rightleftharpoons \text{MetRS} + \text{Met-tRNA}\textsuperscript{Met} + \text{AMP}
\end{equation}

With steady state kinetic methods we can determine the $K_M$ and $k_{cat}$ ($K_M$ reflects the productive substrate-enzyme complex stability, $k_{cat}$ is the reaction rate) for reaction (1) and the overall reaction (1+2), but using steady state kinetics alone we are not able to obtain the amino acid transfer rate. Although steady state kinetic methods coupled with mutagenesis techniques have been useful to reveal the relationship between amino acid sequence and MetRS activity,\textsuperscript{19, 57, 58} two features of the MetRS mechanism remain elusive, the amino acid transfer rate and the rate limiting step. Pre-steady state kinetic methods can be used to address these questions.

There are two types of pre-steady state experiments, single turnover and multiple turnover reaction. In a single turnover experiment, the concentration of substrate is much lower than the enzyme so the product formed results only from a single turnover reaction. Depending on the reaction setup, two rates can be obtained, the chemical rate ($k_{chem}$) and the transfer rate ($k_{trans}$). In the case of MetRS, when the methionyl adenylate is formed \textit{in situ}, the chemical rate is obtained as the combined rate of adenylate formation, tRNA\textsuperscript{Met}
binding and methionine transfer. If the methionyl adenylate is pre-formed before the addition of tRNA^{Met}, the “uncoupled” methionine transfer rate can be measured.

A multiple turnover reaction is in principle the same as a steady state kinetics experiment, except aliquots are removed at shorter time points so the product formed is quantified before the substrate-enzyme complex reaches equilibrium. This method is designed to allow us to see whether product release is the rate limiting step in methionylation of tRNA^{Met}, such that a burst of product formation in the earlier portion of the reaction is observed followed by a linear steady state rate.

Pre-steady state kinetic studies on class II synthetases *E. coli* HisRS\(^{59}\) and *E. coli* ThrRS\(^{60}\) revealed that the transfer rate is faster than the steady state rate and that amino acid activation is the rate limiting step. On the other hand, the study by Zhang et al\(^{61}\) on *E. coli* CysRS (a class I synthetase) revealed a burst of product formation on multiple turnover kinetics, suggesting product release as the limiting rate.

\[
\text{Met} + \text{ATP} + \text{MetRS} \overset{k_1}{\longrightarrow} \text{Met}\cdot\text{ATP}\cdot\text{MetRS} \overset{k_{\text{act}}}{\longrightarrow} \text{PPi} + \text{Met-AMP}\cdot\text{MetRS}
\]

\[
\text{Met-tRNA}^{\text{Met}} + \text{MetRS} \overset{k_2}{\longrightarrow} \text{AMP} + \text{Met-tRNA}^{\text{Met}}\cdot\text{MetRS} \overset{k_{\text{trans}}}{\longrightarrow} \text{tRNA}^{\text{Met}}\cdot\text{Met-AMP}\cdot\text{MetRS}
\]

**Figure 21.** MetRS aminoacylation diagram. \(k_1\) is the rate of methionine and ATP binding, \(k_2\) is the rate of tRNA^{Met} binding and \(k_3\) is the rate of product release; \(k_{\text{act}}\) is the rate of amino acid activation and \(k_{\text{trans}}\) is the rate of amino acid transfer. In rapid equilibrium system, the chemical rate (\(k_{\text{chem}}\)) is the sum of \(k_{\text{act}}\) and \(k_{\text{trans}}\).

The objective of this study is two-fold: 1) to determine whether the *E. coli* MetRS catalytic mechanism is the same as the other class I synthetases and 2) to determine whether mechanistic differences between monomeric and dimeric forms of the enzyme
can be identified. By using pre-steady state kinetics we aim to determine $k_{\text{chem}}$, $k_{\text{trans}}$ and the rate limiting step of tRNA$^{\text{Met}}$ aminoacylation by MetRS (Figure 21). In this chapter we analyze both the monomeric MetRS (MetRS547) and dimeric MetRS (MetRS676) to investigate whether there is any difference in their mechanisms.

**Pre-steady state single turnover results**

We performed pre-steady state kinetic analyses following the methods described by Francklyn et al.\textsuperscript{45} with some modifications. Unlike described in the published method, adding 20 µL of 20 % SDS to the quench reaction aliquots is not sufficient to loosen the MetRS active site to allow the $^{35}$S-methionine to be washed from the precipitate, so we used 150 µL phenol:chloroform (1:1) to extract the MetRS from each aliquot prior to TCA precipitation. This reduced the radioactivity count from the wash, giving higher signal-to-noise ratios at the shortest time intervals. Our effort to isolate the enzyme-bound methionyl-adenylate with G-25 Sephadex Medium (GE Healthcare) was unsuccessful, so the adenylate for the $k_{\text{trans}}$ experiment is pre-formed in the syringe sample. We also found that since the 150 µL of each aliquot of the chemical quench flow exceeded the capacity of one filter, we used two filter papers to precipitate each aliquot; this way we lessened the radioactivity count variations of the replicate experiment.

The rates for both the chemical step ($k_{\text{chem}}$) and methionine transfer ($k_{\text{trans}}$) are obtained by fitting the data to a single exponential equation using Origin 8 (OriginLab Corp.). The equation is shown below:

$$y = y_0 + A(1 - e^{-kt})$$
where $y$ is the product concentration, $y_0$ is the $y$ axis intercept, $A$ is the scaling constant, $k$ is the observed rate constant (in $s^{-1}$) and $t$ is the time in seconds.

The fitting converged well ($R^2$ value $\sim 0.99$) for both types of single turnover experimental data on the global scale. The data for the $k_{\text{chem}}$ determination fits less well ($R^2$ value $\sim 0.98$) for times less than 0.3 seconds, while the $k_{\text{trans}}$ determination fit just as well over the full data set.

Using optimized experimental conditions, we determined that the $k_{\text{chem}}$ and $k_{\text{trans}}$ values for MetRS547 and MetRS676 are the same (Table 3), in agreement with the steady state result that the monomeric form behaves similar to the dimer. The rate of the chemical step for *E. coli* MetRS is consistently lower than its transfer rate, unlike in CysRS where both rates are virtually the same at $15 \, s^{-1}$.61 These results suggest that the adenylation rate for MetRS is slower than for CysRS but not slower than the transfer rate. Thus, the adenylation step is not rate limiting as in the case of the class II synthetases (*E. coli* HisRS62 and ThrRS60).

**Table 3.** The rates of the chemical step ($k_{\text{chem}}$) and the amino transfer step ($k_{\text{trans}}$) for monomeric (MetRS547) and dimeric *E. coli* MetRS (MetRS676). Each value represents three independent trials.

<table>
<thead>
<tr>
<th></th>
<th>$k_{\text{chem}}, , s^{-1}$</th>
<th>$k_{\text{trans}}, , s^{-1}$</th>
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<tbody>
<tr>
<td>MetRS547</td>
<td>$12.61 \pm 0.05$</td>
<td>$15.19 \pm 0.81$</td>
</tr>
<tr>
<td>MetRS676</td>
<td>$12.84 \pm 0.15$</td>
<td>$14.69 \pm 1.14$</td>
</tr>
</tbody>
</table>
Figure 22. Representative plots of single turnover aminoacylation of tRNA$^{Met}$ by MetRS547. A. Determination of $k_{chem}$ where the adenylate is formed \textit{in situ}. B. Determination of $k_{trans}$ where the adenylate is pre-formed. Each curve was fitted to a single exponential equation. Reactions contained 2 µM tRNA$^{Met}$ and 40 µM MetRS547.

Figure 23. Replot of single turnover aminoacylation reaction of MetRS547. The data on $k_{chem}$ and $k_{trans}$ determination under 0.2 seconds revealed different kinetics for the two reactions.
When we compared the data between 0.01 – 0.2 seconds (Figure 23), we can see a lag on the \( k_{chem} \) experiment, and the data points merged at 0.2 second. The lag is seen in all the \( k_{chem} \) experiments performed, no matter how we incubated MetRS with the substrates (Table 4). From this we hypothesize that the lag is due to a slow pre-steady state adenylate reaction, not because of slow binding of tRNA, methionine or ATP.

**Table 4.** Single turnover reaction setup.

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<thead>
<tr>
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<th>Sample Syringe 2</th>
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<tbody>
<tr>
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<td>MetRS</td>
<td>Met, ATP, tRNA</td>
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<td>Met, ATP</td>
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<tr>
<td>( k_{trans} )</td>
<td>tRNA</td>
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</tbody>
</table>
Pre-steady state kinetics multiple turnover results

Figure 24. Multiple turnover aminoacylation by MetRS547. The data is linear over the course of the reaction with a calculated slope of 0.433 s\(^{-1}\). Reactions contained 0.5 µM MetRS547, 10 µM tRNA\(^{\text{Met}}\), 100 µM methionine and 4 mM ATP.

We observed a linear progress curve and no apparent “burst” in pre-steady state aminoacylation by \textit{E. coli} MetRS (Figure 24), as was also seen previously by Mulvey in 1978\(^{63}\) for \textit{B. stearotheophilus} MetRS. When product release is rate limiting in the case of \textit{E. coli} CysRS\(^{61}\) and GlnRS\(^{64}\), a higher rate of product formation under one turnover is observed, followed by a slower linear rate. This higher rate (burst) follows a single exponential equation which corresponds to the chemical rate (\(k_{\text{chem}}\)). The absence of a burst in MetRS multiple turnover experiments suggests that product release is not the rate limiting step.
The conditions used to perform the multiple turnover reaction are not at saturating substrate levels due to two limitations: first, 0.5 μM MetRS is our lowest allowable concentration due to limits of detection; second, annealing high concentrations of tRNA\textsuperscript{Met} reduced the tRNA\textsuperscript{Met} amino acid acceptance due to improper folding. Even though the substrate concentration is not saturating, we should have seen the burst at time points under 0.2 seconds, a time when the enzyme performed a single turnover reaction (compare to Figure 23), if product release was rate limiting.

![Graph](image)

**Figure 25.** Multiple turnover aminoacylation of tRNA\textsuperscript{Met} by MetRS547 under 0.2 seconds. The curve deviates from linearity at times below 0.1 seconds.

While the multiple turnover data is found to be linear from 0.1 seconds onward, it deviates from linearity below 0.1 seconds (Figure 25). This lag is seen in all experiments, whether the protein is incubated with methionine and tRNA\textsuperscript{Met}, ATP and tRNA\textsuperscript{Met}, methionine alone or ATP alone. This suggests that the order of addition does
not matter, and the lag is not caused by slow substrate binding. This phenomenon is unexpected and has not been seen before in other class I synthetases studied by pre-steady state methods (E. coli CysRS$^{61}$, ValRS$^{61}$ and GlnRS$^{64, 65}$). For these three synthetases, a rapid burst in aminoacylation rate is observed, indicating that product release is the rate limiting step. Because we also observe the lag in single turnover reactions for chemical rate ($k_{chem}$) determination (Figure 23), we conclude that the lag is most likely caused by slow pre-steady state adenylate formation.

**Pre-steady state kinetics conclusions**

By using pre-steady kinetic methods we obtained the $k_{chem}$ and $k_{cat}$ for tRNA$^{Met}$ aminoacylation by E. coli MetRS (Table 3). There are no observable differences in the single turnover and multiple turnover aminoacylation reaction of tRNA$^{Met}$ for the monomeric MetRS (MetRS547) and dimeric MetRS (MetRS676), which agrees with previous steady state kinetics studies.$^{66}$ The absence of a burst of product formation seen in the tRNA$^{Met}$ aminoacylation by MetRS is clear indication that product release is not the rate limiting step which is unlike the other class I synthetases investigated to date.

The lag in aminoacylation in the earliest time intervals seen in the single turnover experiment for $k_{chem}$ determination and in multiple turnover experiments is very likely due to pre-steady state amino acid activation. In order to conclusively explain this lag, we need to determine the pre-steady state rates of adenylate formation with and without tRNA$^{Met}$.

The product concentrations are lower than what we expected even after taking into account the amino acid acceptance ability of our annealed tRNA$^{Met}$ transcript. This
may be due to fractionation of tRNA$^{\text{Met}}$ into the aqueous phase in the phenol-chloroform extraction. In the single turnover experiment, the calculated rates ($k_{\text{chem}}$ and $k_{\text{trans}}$) are independent of the absolute concentration of the product. This was not the case in multiple turnover experiments, where the rate is dependent on the product concentration, thus inefficiency in the product recovery will underestimate the obtained rate.

One question that remains unanswered is the cause of a lower $k_{\text{cat}}$ value (~3 s$^{-1}$) in steady state analyses. We are certain that amino acid transfer ($k_{\text{trans}}$ ~14 s$^{-1}$) is not the rate limiting step. Since the chemical step ($k_{\text{chem}}$ ~12 s$^{-1}$) is the combined rate of adenylate formation and amino acid transfer, we deduce that the adenylate formation is also not a rate limiting step. We can eliminate the possibility that the substrate binding is rate limiting because the $k_{\text{chem}}$ value is the same no matter what substrate is pre-incubated with MetRS. The absence of burst in multiple turnover experiments is an indication that product release is not the rate limiting step. Further work must be done to clarify the source of the low steady-state aminoacylation rate ($k_{\text{cat}}$).
CHAPTER FIVE
CONCLUSIONS AND FUTURE DIRECTIONS

Fluorescence spectroscopy conclusion

As shown in the work presented herein, the fluorescence intensity increase of MetRS upon methionine binding seen previously by Blanquet et. al\textsuperscript{46} is caused by the change of Trp253 environment. The conformational change is caused by repositioning of three amino acids: Trp253, Tyr15 and F300.\textsuperscript{48} Furthermore, the increased fluorescence of Trp253 when methionine binds to MetRS is likely also due to an electrostatic interaction between the \textit{N}-indole of Trp253 and the tyrosine hydroxyl group. The amino acid substitution from tyrosine to phenylalanine at position 15 supports this conclusion. Additionally, the substitution from phenylalanine to alanine at position 300, resulting in lower fluorescence enhancement, indicates the role of Phe300 is to help provide a hydrophobic environment for Trp253 in methionine binding.

We managed to link the tRNA\textsuperscript{Met} binding to MetRS and its tRNA\textsuperscript{Met}-induced fluorescence quenching. The substitution of Trp to Phe at position 461 made the MetRS\textsubscript{547 W461F} lose most of the fluorescence quenching; this result is consistent with the previous biochemical studies\textsuperscript{18} and X-ray crystallography\textsuperscript{54} which show that Trp461 interacts with C\textsubscript{34} of the tRNA\textsuperscript{Met} anticodon. Although C\textsubscript{34} is a strong determinant for recognition by MetRS, we also have to consider the interaction of A\textsubscript{35} and U\textsubscript{36} with MetRS. While most of the fluorescence quenching comes from the Trp461 interaction with C\textsubscript{34}, the 5 \% quench seen in W461F might be due structural changes in the protein caused by the interaction with the rest of the anticodon. In addition to the anticodon, base
A$_{73}$ in the acceptor stem is also shown to be a major identity element for tRNA$^{\text{Met}}$ aminoacylation, indicating the interaction of A$_{73}$ with the CP catalytic domain might have also caused a global structural change.

L-(7-hydroxycoumarin-4-yl) ethylglycine was successfully incorporated into MetRS547 at position 204 (W204Cou) and position 461 (W461Cou) using the methods developed by Peter Schultz. The purity of both proteins is lower than the wild-type due to a low expression level that leads to co-purification of *E. coli* proteins with the nickel affinity column. The relative activity of W204Cou is only 33 % compared to the wild-type MetRS547; this decrease might be caused by structural perturbations due to steric effects of the large coumaryl amino acid or the purity of W204Cou.

Although W204Cou is still active in aminoacylation, no change is observed in coumaryl fluorescence intensity upon titrating the protein with tRNA$^{\text{Met}}$. The reasons for this may be rationalized if there is no structural change at position 204 when MetRS binds to tRNA$^{\text{Met}}$ or any global structural change is too subtle to affect the coumaryl amino acid.

**Future direction for fluorescence spectroscopy**

In order to more completely dissect conformational changes that may be observed by fluorescence spectroscopy, the rates of methionine binding and tRNA$^{\text{Met}}$ should be determined by stopped-flow fluorescence spectroscopy. Now that synthesis and incorporation of the coumaryl-amino acid has been demonstrated, there may be other positions in MetRS where this UAA could be incorporated such as Trp305, because this position is at the opposite site of where tRNA$^{\text{Met}}$ binds and variant W305F activity is the
same as the wild-type MetRS547 (data not shown). In combination with more standard mutagenesis studies, incorporation of this UAA should prove a useful tool in structure-function analysis of *E. coli* MetRS.

The discriminator base at position 73 is a common feature in all tRNAs\textsuperscript{68} and is an important identity element of tRNA\textsuperscript{Met}.\textsuperscript{67} The residues on MetRS that interact with A$_{73}$ have yet to be identified. This aspect of the tRNA:MetRS interaction should be further clarified to undertake more complete analysis of the long-range communication that occurs between the active site and anticodon binding domains.

Fluorescence resonance energy transfer can be used to measure distance in a molecule (a spectroscopic ruler). This method has a potential to elucidate structural changes in *E. coli* MetRS upon tRNA\textsuperscript{Met} binding, possibly the distance change in the CP insertion and the anticodon binding domain. A major challenge of attaching a donor and acceptor to the same protein is the heterogeneity of the labeled protein. This problem can be solved by using an incorporated UAA as a donor; we then would only need to label the protein with an acceptor.

**Pre-steady state kinetics**

The pre-steady state kinetic experiments reported here were performed according to methods by Francklyn and coworkers\textsuperscript{45} with some modifications to fit the characteristics of *E. coli* MetRS. Both the monomeric (MetRS547) and dimeric (MetRS676) forms of *E. coli* MetRS have the same $k_{\text{chem}}$ and $k_{\text{trans}}$ value (as shown in **Table 3**). The chemical step ($k_{\text{chem}}$) is lower than the amino acid transfer rate ($k_{\text{trans}}$). There is a lag below 0.2 seconds in the $k_{\text{chem}}$ experiment that persists no matter what
substrate is incubated with MetRS. This leads us to conclude that the lag is not caused by slow substrate binding but is due to a slow pre-steady state adenylate formation.

The multiple turnover experiment shows linear progress with no observable burst of product formation. In contrast there is a lag under 0.1 seconds. This leads us to conclude that the rate limiting step is not product release as it is for the other class I synthetases studied to date.

**Future direction for pre-steady state kinetics**

In order to fully characterize the individual rates that make up the full catalytic cycle of MetRS, the pre-steady state adenylation rate for MetRS with and without tRNA should be determined. This may also necessitate determination of the rate of methionine binding and tRNA\textsuperscript{Met} by stopped-flow fluorescence.

It should be investigated whether the different tRNA substrates (elongator vs. initiator) show any difference in pre-steady state kinetics.
REFERENCES


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