A Dissertation

Entitled

Biochemical Characterization of *Trypanosoma cruzi* Prolyl-tRNA Synthetase

by

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Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Doctor of Philosophy Degree in Chemistry

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A codon optimized gene of ProRS was constructed for expression in *E. coli* and the protein was purified for the purpose of crystal screening and analysis of the enzyme’s catalytic properties. To determine the oligomerization state of ProRS, analytical gel filtration and mass spectrometry techniques were applied and the results provide evidence
that the enzyme is a dimer in solution. Isothermal titration calorimetry has been used to show the binding of ATP to ProRS. The full length native ProRS was cleaved with trypsin in the presence or absence of substrates and the resultant fragments were analyzed by mass spectrometry. When tested by enzymatic assays, the cleaved enzyme shows substantial reduction in activity as compared to the unmodified ProRS enzyme. Results from the adenylate formation indicate that besides proline, ProRS activates other non-cognate amino acids; cysteine, threonine, glutamate, alanine, aspartate, glycine and the ketoacid α-ketoglutarate. This is partly in agreement with previous reports where bacterial ProRS enzymes are able to mischarge tRNA(Pro) with alanine or cysteine owing to their smaller or similar sizes relative to cognate proline.

The second part describes the recombinant human histidyl-tRNA synthetase (human HisRS) another class II aaRS which has been established to be a target antigen for anti-Jo-1. Anti-Jo-1 is a myositis specific autoantibody commonly found in patients with idiopathic inflammatory myopathies (IIM). The enzyme was studied using biochemical techniques; analytical gel filtration, dynamic light scattering and fluorescence spectroscopy. The data obtained show the evidence that human HisRS is a dimer in solution. Most assays available for studying aminoacylation activity rely on the use of either radiolabeled amino acids or radiolabeled tRNA. In an effort to develop a non-radiolabeling spectrophotometric assay for that could be used in detecting diverse aaRS activity is described. The *Archaeoglobus fulgidus* Elongation Factor Tu has been expressed and purified with an N-terminal histidine-tag and was used to the capture of aminoacylated tRNA in a ternary complex of EF-Tu: GTP: aa-tRNA. The detection of the resultant aminoacyl-tRNA was detected by fluorescence using RNA binding dye.
I dedicate this dissertation with love to my mom Marisela Akumu. Although you could neither read nor write you sacrificed all to set me on the path towards education. Thank you *(orio muno).*
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<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>% (v/v)</td>
<td>volume/volume</td>
</tr>
<tr>
<td>% (w/v)</td>
<td>% weight/volume</td>
</tr>
<tr>
<td>% poly</td>
<td>% Polydispersity</td>
</tr>
<tr>
<td>A-site</td>
<td>Aminoacyl site</td>
</tr>
<tr>
<td>aa-tRNA</td>
<td>Aminoacyl-tRNA</td>
</tr>
<tr>
<td>aaRS</td>
<td>Aminoacyl-tRNA synthetase</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>AEBSF</td>
<td>4-(2-Aminoethyl)-benzenesulfonylfluoride</td>
</tr>
<tr>
<td>Afu</td>
<td>Archaeoglobus fulgidus</td>
</tr>
<tr>
<td>Ala</td>
<td>Alanine</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>Asp</td>
<td>Aspartic acid</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATPγS</td>
<td>Adenosine 5′-O-(3-thio) triphosphate</td>
</tr>
<tr>
<td>BME</td>
<td>β-Mercaptoethanol</td>
</tr>
<tr>
<td>bp</td>
<td>base-pair</td>
</tr>
<tr>
<td>BAS</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CHCA</td>
<td>α-cyano-4-hydroxycinnamic acid</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton (unit)</td>
</tr>
<tr>
<td>DHB</td>
<td>2,5-dihydroxybenzoic acid</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic Light Scattering</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
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<td>Deoxyribonuclease</td>
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<tr>
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<td>Deoxyribonucleotide triphosphate</td>
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<td>Escherichia coli</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>EF-Ts</td>
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EF-Tu..........................Elongation Factor Tu
ExPASy.....................Expert Protein Analysis System

FPLC..........................Fast-Protein Liquid Chromatography

Glu .........................Glutamic acid
Gly .........................Glycine
GOI .........................Gene of Interest
GDP ..........................Guanosine diphosphate
GMP-PMP ..................Guanosine 5’-[β,γ-imido]triphosphate
GTP .........................Guanosine triphosphate

HEPES .....................2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HisRS ........................Histidyl-tRNA synthetase
HPA .........................3-hydroxypicolinic acid
HPLC .......................High Performance Liquid Chromatography
IPTG ........................Isopropyl-β-D-thiogalactopyranoside
ITC ..........................Isothermal titration calorimetry

α-KG ......................alpha Ketoglutarate
kDa ..........................kilodalton
KOD ..........................K. Kodakaraensis

LB ..........................Luria Broth medium
Lys ..........................Lysine

MALDI ........................Matrix-assisted laser desorption/ionization
MS ..........................Mass Spectrometry
MESG ........................2-amino-6-mercapto-7-methylpurine ribonucleoside
Mol. Wt. ...................Molecular Weight
m/z ..........................Mass/charge ratio
MWCO ........................Molecular Weight Cut-Off

NCBI .........................National Center for Biotechnology Information
NCBI-nr ...................National Center for Biotechnology Information non-redundance
Ni-NTA .....................Nickel-Nitriloacetic acid

ODx ..........................Optical Density, at x nm

PCR .........................Polymerase Chain Reaction
PDB ..........................Protein Data Bank
PEG ..........................Poly(ethylene glycol)
PEI ..........................Poly(ethylenimine)
PMF .........................Peptide Mass Fingerprinting
PMSF .........................Phenylmethylsulfonyl fluoride
PNP .........................Purine nucleoside phosphorylase
pI..............................Isoelectric point
PPI............................Inorganic pyrophosphate
Pro............................L-Proline
ProRS..........................Prolyl-tRNA synthetase

RNA.........................ribonucleic acid
rpm..........................rotations per minute
R_H...........................hydrodynamic radius

SA.........................Sinapinic acid
SDS-PAGE................Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SOC.........................Super Optimal Catabolite Repression Broth

TAE .........................Tris-acetate-EDTA
TBE..........................Tris-Borate-EDTA buffer
TCEP........................Tris(2-carboxyethyl)phosphine
*T. cruzi* ..............Trypanosoma cruzi
TFA..........................Trifluoroacetic acid
Thr..........................Threonine
TOF..........................Time of Flight
TOPO.........................Topoisomerase I
tRNA.............................transfer RNA
tRNA^Pro......................transfer RNA specific for Proline
Trp............................L-Tryptophan
Tris..........................Tris(hydroxymethyl)aminomethane

UV..........................Ultra Violet
Preface

The work presented in this dissertation is based on one of the two broad projects that I was involved in during my studies in the Department of Chemistry, University of Toledo. When I joined the laboratory of Dr Timothy Mueser co-advised by Dr Amanda Bryant-Friedrich in spring of 2009 my major project was to study how human flap endonuclease-1 recognizes its DNA substrates in the presence of divalent metal ions during DNA replication. Specifically, the project involved designing and synthesizing these DNA architectures that are recognized by FEN-1 using the automated DNA synthesizer that is available in our laboratory. The eventual goal was to solve the X-ray crystal structure of human flap endonucleases-1 in complex with DNA flap substrate and metal ion. I started off by cloning the codon optimized the human flap endonuclease-1 gene that was artificially synthesized then expressed and purified the protein followed by before carrying out crystal screening.

While crystal screening experiments were going on, the structure of human FEN-1 in complex with DNA substrate was published. This led to shift in focus of this project where I now concentrated on the design and synthesis of branched DNA architectures to which I used in studying the DNA-metal ion interactions. The purpose of the experiments was to determine if high affinity divalent metal ion binding sites exist in the DNA replication architectures. The initial experimental design for these binding studies was to
utilize isothermal titration calorimetry technique but due to unavailability of the
instrument at the time, alternative spectrophotometric measurements were utilized. Even
though the project did not go to completion, I learned several techniques including
automated DNA synthesis, DNA purification and analysis using spectroscopy and mass
spectrometry.

In the spring of 2012 through collaborative research work between Dr Mueser and
Dr David Dignam of the University of Toledo Health Science campus I was given the
opportunity to work on the project that involved characterizing two key aminoacyl-tRNA
synthetases. This second project set off with a goal to develop a novel non-isotopic assay
that could be applied in the detection of aminoacyl-tRNA synthetases. I was directly
involved in the molecular cloning, protein expression and purification of the three
aminoacyl-tRNA synthetases: human histidyl-tRNA synthetase and *Trypanosoma cruzi*
prolyl-tRNA synthetase with goal of obtaining crystals for solving high resolution
structures. The first goal of this second project was to solve the structure of the human
HisRS. I participated in the cloning, expression, purification and screening for crystals
for human HisRS in complex with substrates but the X-ray crystal structure got
published while crystal screens experiments were underway. Therefore, other
biochemical techniques including analytical gel filtration, dynamic light scattering and
mass spectrometry were utilized to characterize the human HisRS and the details are
discussed in Chapter 2.

After the human HisRS project, my research focused on trying to understand the
functional properties of the *Trypanosoma cruzi* prolyl-tRNA synthetase and part of this
work actually later came to develop and become the premise of my dissertation as
presented in chapter 4. The goal of this project was to understand the mechanism of the editing activity of *T. cruzi* prolyl-tRNA synthetase. I started off with cloning of a codon optimized gene that was expressed, purified before the screening for crystals was carried out. This was followed by biochemical characterization using isothermal titration calorimetry, analytical gel filtration, mass spectrometry and enzymatic assays as detailed in Chapter 4. The last part of the collaborative work involved the development of a non-isotopic assay that could be used in detecting aminoacyl-tRNA synthetases. During this project I was involved cloning, expression and purification of a histidine-tagged *Archaeoglobus fulgidus* Elongation Factor Tu, and purification of total tRNA from beef liver. The EF-Tu was used to capture aminoacyl-tRNA through the formation of a EF-Tu:GTP: aa-tRNA ternary complex using the human HisRS as described in Chapter 3.

While working in Dr Mueser’s laboratory I had an opportunity to train new members who joined in the lab for undergraduate research experience in molecular cloning, protein expression and purification techniques. This was very helpful since I gained a lot of team player and organizational skills through passing on the knowledge. Throughout this period besides improving my expertise in molecular biology, I have also learned other skills used in protein characterization like isothermal titration calorimetry, dynamic light scattering, MALDI-TOF mass spectrometry and enzymatic assays. All these skills will be very useful in my future research endeavors.
Chapter 1

Biochemical Characterization of Aminoacyl-tRNA Synthetases

1.1 Introduction

1.1.1 Aminoacyl-tRNA synthetases

Translation is a fundamental process in all living cells whereby proteins are synthesized from the information encoded by messenger ribonucleic acids (mRNAs). This process is carried out with the help of the ribosome, aminoacyl-tRNA synthetases, elongation factors and initiation factors. Aminoacyl-tRNA synthetases (aaRS) are a structurally and functionally heterogeneous group of enzymes that catalyze the attachment of amino acids to their cognate transfer RNAs (tRNAs) during protein synthesis in a two-step reaction in which both steps are catalyzed by aminoacyl-tRNA synthetase as shown in equations (1) and (2).

\[
\text{Amino acid} + \text{ATP} + \text{aaRS} \xrightarrow{} \text{aaRS-Amino acid-AMP} + \text{PPi} \quad (1)
\]

\[
\text{aaRS-Amino-acid-AMP} + \text{tRNA}^{AA} \xleftrightarrow{} \text{aaRS} + \text{Amino acid-tRNA}^{AA} + \text{AMP} \quad (2)
\]
In the first step, the amino acid is adenylated by reaction with ATP to yield aminoacyl adenylate and pyrophosphate. The aminoacyl adenylate remains tightly bound to aaRS until the AA-AMP-aaRS complex encounters a tRNA molecule. The activated amino acid is then transferred to the 3’adenosine of the tRNA molecule. Apart from Protein synthesis, aaRS are involved in other cellular functions like apoptosis, intron splicing and more others as detailed in Figure 1-1. This phenomenon of diversified roles of aaRS across all species has lead to their target for antimicrobial drugs. (Ibba et al., 2001; Waller et al., 2005)

During the translation process, each aminoacyl-tRNA synthetase must recognize its cognate tRNA and discriminate it from a pool of different tRNAs. Although all tRNAs have similar L-shaped tertiary structure each has a unique nucleotide sequence

Figure 1-1: Other cellular functions of aaRS. (adapted from Haussman & Ibba, 2008; Schimmel, 2008)
arrangement thus, each aaRS will recognize this sequence found in the acceptor stem and the anticodon stem to avoid misacylation. (Cavarelli & Moras, 1993)

1.1.2 Classification and cognate functions of aminoacyl-tRNA synthetases

Aminoacyl-tRNA synthetases from all species have been divided into two classes based on their characteristic conserved amino acid motifs in the active site. (Eriani et al., 1990) Each aaRS class contains ten synthetases one for each canonical amino acid. Enzymes in Class I use the Rossman fold made up of alternating beta-strands and alpha helices with a tetrapeptide signature sequence, His-hydrophobic-Gly-[His or Asp] commonly referred to as the HIGH and play a role in ATP binding. A second KMSKS signature sequence in which the second lysine is highly conserved across species exists in the loop region of the C-terminus of these Class I enzymes and it is believed to participate in amino acid activation. (Mechulam et al., 1991) Class II enzymes use an anti-parallel β-sheet similar to that found in biotin synthases which is characterized by three conserved motifs that participate in ATP binding although these motifs exist with very few invariant amino acids across species. (Cusack et al., 1990)

Class I aminoacyl-tRNA synthetases aminoacylate the 2′OH of the ribose of tRNA. Class II synthetases exhibit a wide diversity of oligomeric arrangements and exist as either dimeric or multimeric and are characterized by acylation that occurs on the 3′ hydroxyl of the last ribose of the tRNA. (Eriani et al., 1990) Based on how these enzymes recognize and bind to tRNA, they have been further classified into three subclasses: a, b, and c. This subdivision has been based on mechanistic and structural studies. (Moras, 1992) Table 1.1 summarizes the classification of the aaRS.
Two examples of aaRS, histidyl-tRNA synthetases and prolyl-tRNA synthetases, both belong to the class IIa subgroup and can be distinguished by having an N-terminal catalytic domain and also share a common C-terminal extension domain. (Arnez et al., 1997; Schimmel, 1987)

**Table 1.1:** Classification of aminoacyl-tRNA synthetases. (Eriani et al., 1990; Moras, 1992)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Class I</th>
<th>Class II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme sub-class</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>Ile</td>
<td>Glu</td>
<td>Ala</td>
</tr>
<tr>
<td>Leu</td>
<td>Gin</td>
<td>Ser</td>
</tr>
<tr>
<td>Val</td>
<td>Trp</td>
<td>Thr</td>
</tr>
<tr>
<td>Cys</td>
<td>Tyr</td>
<td>His</td>
</tr>
<tr>
<td>Met</td>
<td></td>
<td>Pro</td>
</tr>
<tr>
<td>Arg</td>
<td></td>
<td>Gly</td>
</tr>
<tr>
<td></td>
<td>a</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>Asn</td>
<td>Asp</td>
</tr>
<tr>
<td></td>
<td>Phe</td>
<td>Lys</td>
</tr>
<tr>
<td>Conserved characteristics</td>
<td>Active site structure motifs</td>
<td>Antiparallel β-sheet</td>
</tr>
<tr>
<td></td>
<td>Rossman fold</td>
<td>Motif 1, Motif 2, Motif 3</td>
</tr>
<tr>
<td></td>
<td>HIGH, KMSKS</td>
<td></td>
</tr>
<tr>
<td>ATP conformation</td>
<td>Extended</td>
<td>Bent</td>
</tr>
</tbody>
</table>

In addition to differences in quaternary structures, aaRS also differ in subunit molecular weights. For instance, enzymes from prokaryotes have lower molecular weight as compared to their homologs in eukaryotes because eukaryotic enzymes have N-terminal chain extensions and the presence of inter-domain sequence links. (Starzyk et al., 1987; Menguito et al., 1993)
1.1.3 Histidyl-tRNA synthetase

The histidyl-tRNA synthetase contains all the three characteristic motifs found in class II aaRS and like many other members in this class is a homodimeric enzyme as shown by the *E. coli* HisRS in Figure 1-2. (Menguito et al., 1993; Connolly et al., 2004) Domain arrangements of most histidyl-tRNA synthetases across species are characterized by a large catalytic domain extending from the N-terminus and an anticodon binding domain at the C-terminus (Figure 1-3). Others have extra extensions at the N-terminus whose functions have evolved to perform editing activity. (Merrit et al., 2010; Schimmel, 1987; Raben et al., 1992; Xu et al., 2012)

![Figure 1-2: Ribbon representation of the dimeric HisRS from *E. coli*. Monomers are colored green and blue with histidyl adenylate displayed in both active sites as red stick representation. Rendered in Pymol (PDB: 1KMN).](image-url)
The main distinguishing feature of HisRS catalytic mechanism from other class II aaRS is the histidine binding pocket located in the catalytic domain. The enzyme interacts with the imidazole moiety of the histidine substrate through hydrogen bonding and van der Waals interactions with residues in this binding pocket. (Schimmel, 1987; Arnez et al., 1997; Woese et al., 2000)

Figure 1-3: Domain arrangement of HisRS in different species. The domains (not drawn to scale) show a common arrangement in the catalytic region and anticodon binding regions. (adapted from information in the following references: Schimmel, 1987; Raben et al., 1992; Merrit et al., and 2010; Xu et al., 2012).

1.1.4 Prolyl-tRNA synthetases

Prolyl-tRNA synthetases (ProRS) are classified into two distinct groups: “prokaryotic-like” and “eukaryotic-like”. All possess the signature class II motif 1 responsible for dimer formation, a motif 2 and motif 3 that have functions in amino acid activation and anticodon binding domain as shown in Figure 1-4. (Eriani et al., 1990; Woese et al., 2000; Yaremchuk et al., 2000a; Yaremchuk et al., 2000b; Burke et al., 2001) These two groups of ProRS recognize the nucleotides in the tRNA acceptor stem differently in order to discriminate the cognate tRNA substrate. Prokaryotic-like ProRS
recognize the nucleotides at the bases in both the acceptor arm and anticodon whereas the eukaryotic-like ProRS recognize only the bases found in the anticodon binding domain. (Cusack et al., 1998; Beuning & Musier-Forsyth, 2001; Jakubowski, 2001)

In order to correct errors of misactivation of non-cognate tRNAs and amino acid misacylation that occur during protein synthesis most aaRS have evolved editing mechanisms. (Xue et al., 2003) During pre-transfer editing, the non-cognate aminoacyl-AMP is first hydrolyzed before the transfer to tRNA is done while post-transfer editing is achieved through the deacylation of the misactivated tRNA. (Jakubowski et al., 1978) ProRS is one of the class II aaRS that exhibit editing capability to edit errors arising from amino acid selection. This can occur by either pre-transfer before the amino acid is transferred to tRNA or by post-transfer where the mischarged amino acid is hydrolyzed. (Jakubowski, 1980; Moras, 1993)

**Figure 1-4:** Schematic diagram of “prokaryotic-like” and “eukaryotic-like” Prolyl-tRNA synthetases. The insertion domain between motif 2 and 3 in the editing domain found in prokaryotic ProRS (adapted and modified from Yaremchuk, et al., 2000a)
1.1.5 Transfer RNA recognition and amino acid discrimination

Structural studies have shown that each class of the aminoacyl-tRNA synthetase interacts with their cognate tRNA differently. (Beuning et al., 1997) This explanation was based on the fact that each tRNA has a unique nucleotide arrangement besides the common cloverleaf secondary structure and the L-shaped tertiary structure of all tRNAs. Majority of tRNA sequences contain recognition elements or sets of nucleotide sequences within the acceptor arm and the anticodon stem where aaRS bind. Members of class II aaRS with the exception of alanyl-RS bind tRNA in the major groove. (Sprinzl et al., 1996) Histidine tRNA contains a unique recognition feature, an extra nucleotide (G-1) found at the 5′ end of the acceptor stem. (Cooley et al., 1982)

In the genome of prokaryotes the G-1 extra nucleotide is found encoded within the tRNA gene while in the case of eukaryotes it is added after transcription. (L’Abbe et al., 1990; Cusack et al., 1997) HisRS interacts with histidine via a binding pocket that is lined with conserved residues. (Raben et al., 1992) This is facilitated by hydrogen bonding and van der Waals interactions of these side chains and the imidazole moieties of histidine substrate.

1.1.6 Aminoacyl-tRNA synthetases and their role in disease

The HisRS has received a lot of interest because of its being a target for autoantibodies in the human autoimmune diseases, polymyositis and dermatomyositis. (Arnett et al., 1981) The HisRS autoantigen (Jo-1) is the most commonly targeted in polymyositis, a rare human autoimmune muscle disease with about 5-25 % of patients producing anti-HisRS autoantibodies. (Miller et al., 1990; Hirakata et al., 1999; Targoff,
1992) Jo-1 syndrome is one of the autoimmune diseases called antisynthetase syndromes which are characterized by autoantibodies directed against aminoacyl-tRNA synthetases. (Miller et al., 1990) Polymyositis and dermatomyositis are both inflammation myopathies which result in muscle weakness and an elevation in muscle activity. (Francklyn et al., 1990) Characterizing the human HisRS, will provide information that will be helpful in studying its interaction with the anti-Jo-1 autoantibodies.

The worldwide increase in the danger of resistance of antibacterial agents and the need to come up with drug targets that do not affect the host organism has fueled a lot of research in trying to identify more drug targets. Aminoacyl-tRNA synthetases are being studied as promising drug targets for the development of cures against most diseases that are caused majorly by protozoans in humans. These include Plasmodium, Leishmania and other trypanosomal species. Several inhibitors have been shown to act on select aaRS enzymes. One such common compound is Mupirocin an isoleucyl-tRNA synthetase inhibitor developed for use as an antibacterial. (Ishiyama et al., 2001) Borrelidin, a threonyl-tRNA synthetase inhibitor, has been used to kill Plasmodium falciparum parasites. (Ishiyama et al., 2001; Otoguro et al., 2003)

1.2 Methods used in the in vitro analysis of aminoacylation

The principle technique utilized in studying aminoacylation is radiolabeling of either amino acids or $^{32}$P labeled tRNAs in which the rate of formation of radiolabeled aa-tRNA is measured. (Hoagland et al., 1957) To initiate the reaction, a radiolabeled amino acid is titrated into a mixture of aaRS, tRNA and ATP and the extent of aminoacylation monitored by scintillation counting. (Eigner & Loftfield, 1974; Wolfson
et al., 1998; Wolfson & Uhlenbeck, 2002) The newer technique that has been developed which involves the formation of a ternary complex of GTP bound EF-Tu captured aminoacylated labeled tRNA. Scintillation counting is used to measure the extent of aminoacylation based on the radiolabel used on the tRNA. (Dale & Uhlenbeck, 2005) More recently a spectrophotometric assay has been shown to measure aminoacylation without the use of radiolabeled amino acids or tRNAs. In this assay, an inorganic Pyrophosphatase enzyme was coupled to the aminoacylation reaction and the released pyrophosphate product (Pi) was monitored by the malachite green reaction. (Cestari & Stuart, 2013) In this study we apply the formation of an EF-Tu:aa-tRNA ternary complex and the use of spectrophotometric determination to study aminoacylation of key eukaryotic aminoacyl-tRNA synthetases. Of specific interest is to utilize archaeal Archaeoglobus fulgidus Elongation factor Tu, a thermally stable GTPase, which has a high affinity for aminoacylated tRNA in a fluorescence assay.

1.3 Project Goals

Aminoacyl-tRNA synthetases are key enzymes in the protein translation process that catalyze the aminoacylation of their cognate tRNAs. HisRS catalyzes the incorporation of L-Histidine to tRNA^{His} while ProRS catalyzes the incorporation of L-Proline to tRNA^{Pro} respectively during the initial step of protein biosynthesis. The Elongation Factor Tu plays the role of selecting the correct aa-tRNA to be delivered to the ribosome via hydrolysis of a GTP molecule bound to the EF-Tu: aa-tRNA complex in prokaryotes. The work of this thesis is focused on understanding the mechanisms of these three enzymes and their role in protein synthesis.
The first goal of this study was to obtain a high resolution structure for the human HisRS enzyme. After the structure was published, (Xu et al., 2012) while the study was going on, the change in approach of strategy led to utilizing available biochemical techniques to understand the functional properties of the enzyme especially how it interacts with its substrates. The HisRS has received a lot of interest because of it being a target of autoantibodies in the human autoimmune diseases, polymyositis and dermatomyositis. (Arnett et al., 1981; Hirakata & Mimori, 1999; Miller et al., 1990) The HisRS autoantigen (Jo-1) is the most commonly targeted in polymyositis a rare human autoimmune muscle disease with about 5-25% patients producing anti-HisRS autoantibodies. (Targoff, 1992)

The ProRS is one of the tRNA synthetases that have been discovered to have difficulty in discriminating against non-cognate amino acids during the aminoacylation reaction. Although there is a lot of information available on how ProRS editing activity in \textit{E. coli}, archaea and human, the editing mechanism in \textit{T. cruzi} ProRS has not been characterized before. The second goal of this study was to understand the editing mechanism of ProRS in \textit{T. cruzi}. The information gained form understanding the mechanism of this editing activity will be useful in designing inhibitors of ProRS.

Because of the central role the Elongation Factor Tu plays during protein synthesis in prokaryotes, there have been a lot of research findings from both structural and biochemical studies on how the enzyme binds the activated aminoacyl-tRNAs during the formation of the ternary complex with GTP and aa-tRNA. In the case of conventional aminoacylation assays in which EF-Tu is used to capture aa-tRNA, isotope labeled tRNA or amino acids are normally used. (Ribeiro et al., 1995) Therefore, there is need to
develop non-radiolabeling techniques to study aminoacylation in which EF-Tu is used in binding activated tRNA through the formation of a ternary complex. The third goal of the study was to develop a method that could be used to capture charged tRNA in vitro by utilizing the thermally stable Afu EF-Tu through the formation of a ternary complex without radiolabeling of either the amino acid or tRNA. The developed method will provide an alternative safer method that could be applied in studying the eukaryotic model of aminoacylation.

1.4 Organization of the Dissertation

The work presented in this dissertation focuses on two aminoacyl-tRNA synthetases (human HisRS and T. cruzi ProRS) and an Elongation Factor Tu from Archaeoglobus fulgidus. In chapter 2, the initial goal of the project was to solve the X-ray crystal structure of the human HisRS but while the study was going on, a high resolution structure of the enzyme was published. (Xu et al., 2012) Therefore, the experiments described address the interactions of human HisRS with its substrates using biochemical techniques. Chapter 3 describes the application of an N-terminal histidine-tagged Elongation Factor Tu (Afu EF-Tu) in generating ternary complex of EF-Tu: GTP: aa-tRNA that can be used in the detection of aminoacyl-tRNA synthetase activity without the use of radiolabeled amino acids or tRNAs. Also, the chapter describes the biochemical experiments that were carried out in investigating how Afu EF-Tu binds its substrates in addition to screening for crystals. Chapter 4 discusses the experiments that were carried out in order to obtain the functional properties of the T. cruzi ProRS. Besides the biochemical experiments which included analytical gel filtration, ITC and
screening for crystals, findings about the enzyme’s ability to activate non-cognate amino acids are described.
Chapter 2

Biochemical Characterization of the Human Histidyl-tRNA Synthetase

2.1 Introduction

2.1.1 Histidyl-tRNA synthetase

Histidyl-tRNA synthetase (HisRS) catalyzes the incorporation of L-Histidine to tRNA$^{\text{His}}$ during the initial step of protein biosynthesis in a two step reaction (equations 3 and 4).

\[
\begin{align*}
\text{HisRS} + \text{ATP} + \text{His} & \leftrightarrow \text{HisRS} \cdot \text{His-AMP} + \text{PPi} \\
\text{HisRS} \cdot \text{His-AMP} + \text{tRNA}^{\text{His}} & \leftrightarrow \text{HisRS} + \text{His-tRNA}^{\text{His}} + \text{AMP}
\end{align*}
\]

The HisRS protein has been expressed and purified from a variety of organisms to date. Several three-dimensional structures of HisRS have also been elucidated and more recently was the crystal structure of the human homolog. (Arnez et al., 1995; Aberg et al., 1997; Merrit, 2010, Xu et al., 2012) As mentioned earlier in the introduction (chapter 1), all the structures of HisRS show that the enzyme is dimeric in solution just like many class II aaRS enzymes with ATP binding in a similar class II aaRS manner and several
ATP analogs have also been shown to inhibit HisRS in *E. coli* and yeast. (Freist, 1981) The human HisRS is a dimeric, class IIa synthetase and it has a molecular weight 59 kDa and a pI of 6.01. The protein parameters are summarized in **Table 2.1**.

**Table 2.1:** Protein characteristics of human HisRS obtained using ProtParam program. (ExPASy proteomics server)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleotides</td>
<td>1551 bp</td>
</tr>
<tr>
<td>Amino acids</td>
<td>516</td>
</tr>
<tr>
<td>Molecular mass</td>
<td>58,262 Da</td>
</tr>
<tr>
<td>pI</td>
<td>5.62</td>
</tr>
<tr>
<td>Molar extinction coefficient</td>
<td>38,445 M⁻¹cm⁻¹</td>
</tr>
</tbody>
</table>

**2.1.2 Project Goal**

The human histidyl-tRNA synthetase (human HisRS) has attracted a lot of research since it is a target by autoantibodies in the human autoimmune diseases, polymyositis and dermatomyositis. (Mathews & Bernstein, 1983; Mathews *et al*., 1984) The focus of this study was to understand the enzyme’s structural function and how it interacts with substrates during aminoacylation. There was no solved crystal structure of the human HisRS at the beginning of this study and we set out to investigate both functional and structural aspects of the enzyme.

The first goal was to solve the X-ray crystal structure of the full length human HisRS. The second goal was to understand the biochemical properties of human HisRS. In order to achieve these goals, the following experiments were performed: 1.) Estimation
of the oligomeric state of human HisRS using analytical gel filtration and dynamic light scattering. 2.) Determination of binding properties of nucleotides to human HisRS in native and denatured state using fluorescence emission spectroscopy. 3.) Screening for crystals of human HisRS in complex with ligands. Information gained from this study will help in understanding the functional properties human HisRS and the knowledge could be applied in further understanding the enzyme’s role in the autoimmune diseases.

2.2 Materials and Methods

2.2.1 Reagents and Equipment

The sequence for the human HisRS, optimized for *E. coli* expression was synthesized by GenScript (Piscataway, NJ). Gateway® cloning reagents were purchased from Life Technologies (Carlsbad, CA). PCR and Plasmid DNA purification reagents, Minilute and Miniprep respectively were obtained from Qiagen (Valencia, CA). High molecular weight protein standards were purchased from Bio-Rad (Hercules, CA). All reagents were purchased from Fisher Scientific or Sigma-Aldrich® (St. Louis, MO).

2.2.2 Cloning, Expression and Purification of human HisRS

The cloning of the gene (sequence in Figure 2-1) was carried out using Gateway® Technology. Forward and reverse primers were designed for human HisRS gene to include a TOPO cloning site (CACC) and a TEV-protease site. The length of each primer was calculated so that the melting temperature was 54 °C. After amplification of the genes by PCR, the gene was inserted into the pENTR-D entry vector using TOPO assisted cloning. The next step was carried out through an LR clonase
multiple enzyme reaction in which the gene was inserted into pDEST-C1 destination vector before transformation into BL21 DE3* expression host cells.

2.2.2.1 Human HisRS gene amplification by PCR

The human HisRS PCR primers both forward (5′- CACC GAA AAC CTG TAT TTT CAG GGC ATG -3′ Tm = 54 °C) and reverse (5′-TTA GCA GAT ACA CAG CGG TTG G -3′ Tm =54 °C) were ordered in lyophilized form from Integrated DNA technology (IDT), (Coralville, IA). Working stock solutions of 250 µM concentration were made by dissolving the primers in Tris EDTA (TE) buffer (pH 8.0) to the primers. The primer mix solutions were made by diluting the stock primers to 10 µM final concentration for each primer. Gene amplification was achieved by KOD Hot Start DNA Polymerase (Millipore). For a complete PCR reaction the following components were needed: DNA template, KOD Hot Start polymerase enzyme, dNTPs, 10X PCR buffer containing magnesium ions. The PCR reaction was set up by mixing all the components

Figure 2-1: Amino acid sequence of the human HisRS codon optimized gene. NCBI gene ID is 3035. The N-terminal TEV protease cleavage site consisting of seven amino acid residues is underlined. The codon optimized gene was ordered from GenScript (Piscataway, NJ).
(Table 2.2) in a 0.2 mL PCR tube, mixed slightly and then vortexed briefly before they are placed in an Eppendorf Mastercycler Personal PCR machine. The PCR program used for the amplification of the human HisRS gene is detailed in Table 2.3. The PCR cycle was repeated 25 times with a final extension step of 2 minutes at 72 °C.

2.2.2.2 Human HisRS PCR Gel Purification

The PCR product was analyzed on 1 % (w/v) agarose gel prepared in 1X TAE (pH 8.0) (40 mM Tris-acetate, 1 mM EDTA). An equal volume of a gel-loading buffer (0.01 % (w/v) bromophenol blue, 40 % (v/v) glycerol, autoclaved Milli Q water) was added to each sample to assist in loading the samples into the wells. The gels were run at 90 V for 90 minutes in the 1X TAE (pH 8.0) buffer.

Table 2.2: PCR reaction set up for human HisRS.

<table>
<thead>
<tr>
<th>PCR component</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR grade water</td>
<td>21</td>
</tr>
<tr>
<td>Primer mix</td>
<td>3</td>
</tr>
<tr>
<td>DNA template</td>
<td>1</td>
</tr>
<tr>
<td>KOD start master mix</td>
<td>25</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>50</strong></td>
</tr>
</tbody>
</table>

Table 2.3: PCR program for the human HisRS gene amplification.

<table>
<thead>
<tr>
<th>Program</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activation</td>
<td>95</td>
<td>2 min</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>20 sec</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>54</td>
<td>10 sec</td>
<td>25</td>
</tr>
<tr>
<td>Extension</td>
<td>70</td>
<td>10 sec</td>
<td></td>
</tr>
</tbody>
</table>
The gel was stained with DNA gel stain 0.5X SYBR Gold dye (Invitrogen), before analysis and visualization on a Typhoon Phosphorimager (GE Healthcare). Bands corresponding to the size of human HisRS gene were cut out using a sterile blade before gel extraction following the protocol provided by the Qiaquick gel extraction reagents (Qiagen). The DNA samples were quantified by measuring UV absorbance at 260 nm on an Agilent Spectrophotometer and then stored at -20 °C for further cloning steps.

2.2.2.3 Gateway cloning of the human HisRS gene

Gateway cloning utilizes the activity of Lambda integrase. The insertion of the gene of interest into a cloning vector is a two step process via an entry vector as illustrated in Figure 2-2. First, the PCR products are inserted into an entry vector by topoisomerase-assisted cloning after which the gene is transferred into a destination vector using the transposase reaction that involves a λ integrase and excisionase enzymes.

The overall cloning process for the human HisRS gene was carried out using the Gateway cloning technology and the multiple steps are outlined in Figure 2-3. The PCR product of human HisRS was first inserted into pENTR-D which is the entry vector to generate an entry clone. This was achieved by using the CACC overhang which helps to maintain directionality of the gene during TOPO assisted cloning. The gene was swapped from the entry clone into a pDEST-C1 in a transposition reaction that utilizes an L-R clonase enzyme as represented in the scheme (Figure 2-2). The pENTR-D reaction was performed in a 0.2 mL PCR tube by mixing the components shown in Table 2.4 in the buffer containing 1.2 M NaCl and 0.06 M MgCl₂. The tube was then incubated at room temperature for 5 minutes for the reaction to go to completion.
Step 1: Insertion of gene of interest (GOI) into cloning vector

\[
\text{CACC} \quad \text{GOI} \quad + \quad \text{pENTR-D} \quad \xrightarrow{\text{Topoisomerase assisted cloning}} \quad \text{pENTR-D} \quad \text{CACC} \quad \text{GOI}
\]

Step 2: Switch gene of interest from cloning vector into expression (destination) vector

\[
\text{pENTR-D} \quad + \quad \text{pDEST-C1} \quad \xrightarrow{\text{LR clonase reaction}} \quad \text{pENTR-D} \quad + \quad \text{pDEST-C1}
\]

Figure 2-2: Gateway cloning overview. The gene of interest (GOI) is inserted into pENTR-D cloning vector via a topoisomerase assisted cloning reaction in step 1. This is made possible by addition of a CACC site on the gene of interest. In the second step, the GOI is switched into pDEST-C1 destination vector through a LR clonase reaction. The ccdB gene of is swapped with the GOI in a transposase manner.

Table 2.4: Reaction set up for the human HisRS insertion into pENTR-D vector. Reaction was done in a PCR tube after which the components were mixed and incubated at room temperature for 5 minutes.

<table>
<thead>
<tr>
<th>pENTR-D reaction mixture</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human HisRS PCR product</td>
<td>2</td>
</tr>
<tr>
<td>pENTR-D vector</td>
<td>1</td>
</tr>
<tr>
<td>Salt solution</td>
<td>1</td>
</tr>
<tr>
<td>Autoclaved water</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>6</strong></td>
</tr>
</tbody>
</table>
TOP10 cells were used to transform the pENTR-D reaction and plated on LB agar and incubated overnight at 37 °C. Several colonies were chosen and inoculated into fresh LB media containing Ampicillin 100 µg/mL and grown for overnight with shaking at 37 °C.

The cells were pelleted at 4,000 rpm for 10 min. The plasmid was isolated and purified using the Miniprep plasmid purification reagents (Qiagen). The purity of the pENTR-D plasmid was analyzed on a 1 % agarose gel electrophoresis to check the successful insertion of human HisRS gene into the entry vector with an expected size of 4.1 Kb.

![Diagram](image)

**Figure 2-3:** Scheme for the overall strategy for cloning and expression of human HisRS protein.

The LR-clonase enzyme mix was used to switch the human HisRS gene from pENTR-D to the destination vector pDEST-C1. The reaction was set up comprising the components shown in Table 2.5 in a 0.2 mL PCR tube. After the components are mixed
1X TE buffer is comprised of 20 mM Tris HCl (pH 8.0) and 1 mM EDTA, the tube was incubated at room temperature for 1 hour for the LR to allow the reaction to go to completion. After the 1 hour incubation proteinase K was then added to digest any traces of LR enzymes at 37 °C for 10 minutes. At this step, the human HisRS was now swapped from the entry vector into pDEST-C1 the destination vector made possible by direct cytotoxic gene (ccdB) which facilitates direct selection of positive clones containing gene of interest. The ccdB gene acts by trapping DNA gyrase, disrupting the DNA double strand hence killing the cell. (Bernard et al., 1993; Bernard et al., 1994)

Table 2.5: Reaction set up for human HisRS insertion into pDEST-C1 vector. Reaction was incubated at room temperature for 1 hour minutes. The 1X TE buffer is comprised of 20 mM Tris HCl pH 8.0 and 1 mM EDTA.

<table>
<thead>
<tr>
<th>pDEST-C1 reaction component</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDEST-C1 vector</td>
<td>2</td>
</tr>
<tr>
<td>Human HisRS /pENTR-D plasmid</td>
<td>2</td>
</tr>
<tr>
<td>LR Clonase enzyme mix</td>
<td>2</td>
</tr>
<tr>
<td>1X TE buffer to make</td>
<td>6</td>
</tr>
</tbody>
</table>

Transformation of human HisRS /pDEST-C1 into cloning host

The LR reaction mixture was transformed into E. coli cloning host cells Omnimax™ (Life Technologies, Grand Island, NY) that were freshly made in our laboratory using the following generalized procedure for making competent cells for both cloning and expression host by the CaCl₂ method. Briefly, A single colony was inoculated from a freshly transformed agar plate into 5 mL LB containing appropriate antibiotic and grown overnight at 37 °C. The overnight culture was transferred to 50 mL fresh LB and the culture grown until OD₆₀₀ reaches 0.4- 0.6 an indication of the log phase
of bacterial growth. The cells were then put on ice for 30 minutes before centrifugation at 2,000 rpm for 7 minutes. The cell pellet was then resuspended in a pre-chilled 60 mM CaCl₂ solution and further incubated on ice for 30 minutes before another centrifugation step at 1,600 rpm for 5 minutes. The cell pellet was resuspended in 10 mL of 60 mM CaCl₂ solution, chilled on ice for 30 minutes and centrifugation repeated. The final pellet was dissolved in 2 mL CaCl₂ solution and aliquots of 100 µL in Eppendorf tube and stored on ice until used or placed on dry ice before storage at -80 °C.

To carry out the transformation, 5 µL of the human HisRS /pDEST-C1 plasmid (50 ng/µL was added into an aliquot of 50 µL of Omnimax™ cloning host cells in an Eppendorf tube on ice. The tube was flicked gently in order to mix the contents after which the tube was incubated on ice for 30 minutes. The cells were heat shocked for 42 seconds at 42 °C followed by incubation on ice for a further 2 minutes. At this step 250 µL of room temperature SOC medium was added and the cells are shaken at 37 °C for 1 hr before plating on freshly made LB agar plate containing tetracycline. The plate was incubated overnight at 37 °C for colonies to grow. Single colonies were picked in the next day and inoculated into fresh LB medium and antibiotic for an overnight growth before the cells were harvested for plasmid isolation. The plasmid containing human HisRS gene was isolated and purified using the available plasmid purification Miniprep reagents (Qiagen) according to the manufacturer’s instructions. The isolated plasmid is analyzed by a 1 % (w/v) agarose gel electrophoresis to verify the correct size of pDEST-C1 plasmid containing the human HisRS gene.
2.2.2.4 Protein Expression of human HisRS

Small Scale Protein Expression

Once the pDEST-C1 plasmid containing the human HisRS gene was isolated from the cloning host, it was transformed into BL21 (DE3)* expression host cells following the transformation procedure described previously. The same protocol was applied to all the other proteins in this study. Typical protein expression studies were carried out on a small scale, using 5-10 mL LB culture and growing to an appropriate OD$_{600}$ before induction. Briefly, a single colony of freshly transformed human HisRS/pDEST-C1 LB gar + Streptomycin plate was inoculated into 3 mL LB medium containing antibiotic then grown overnight at 37 °C. The overnight culture was transferred to a 10 mL fresh LB medium and grown in a shaker at 37 °C until the OD$_{600}$ reached 0.6 before induction with 1 mM IPTG for 4 hours at 37 °C. The cells were harvested by centrifugation 4,000 rpm at 4 °C for 20 min using a Beckman Coulter™ TJ-25 centrifuge. Aliquots of cells at the time of induction (t= 0 hr) and at the end of induction (t= 4 hr) were taken out for running on an SDS-PAGE to check the expression of human HisRS protein. The samples were mixed with 2X SDS buffer, boiled for 5 minutes and run on 4-12 % SDS-PAGE for 35 minutes at 200 volts before staining with Coomassie blue protein stain. A protein marker was also run alongside to help determine the relative molecular weight of the human HisRS protein.

2.2.2.5 Confirmation of human HisRS protein expression by in-gel trypsin digestion

After running SDS-PAGE, the band thought to correspond to the human HisRS protein was excised using a sterile razor blade, cut into 1x 1 mm pieces and transferred
into a 0.6 mL low retention Eppendorf microcentrifuge tube. The in-gel trypsin digestion experiment was carried out following the manufacturer’s instructions (Sigma Aldrich). Briefly, the gel was destained to remove the Coomassie blue three times (30 minutes each cycle) in a destaining solution containing 5 % acetonitrile and ammonium bicarbonate at 37 °C before drying the samples in a Speed-vac (18,000 rpm, 25 °C). To initiate the trypsin digest, 20 µL of trypsin reaction buffer comprising of 5 % acetonitrile in 50 mM ammonium bicarbonate was added to the tube containing the dried gel before adding 1.0 µL of 1 µg/µL proteomics grade trypsin. The tube was placed in a water bath for digestion to proceed overnight at 37 °C. In the morning, the solution was placed in a Speed-vac to dry of the solvents and leave the peptides lyophilized before MALDI-TOF/TOF MS analysis.

Lyophilized peptides were dissolved in 10 µL of 0.1 % TFA and purified using the ZipTip C_{18} tips before MALDI MS analysis. To prepare the sample, 1 µL of the peptides after trypsin digestion was mixed with a saturated solution of α-cyano-4-hydroxycinnamic acid, the matrix for peptide analysis, in 1:2 ratio. The mixture was then placed onto a MALDI plate and allowed to air-dry for 15 minutes. Calibration was carried using BSA originally digested by trypsin as a standard. MALDI-TOF/TOF MS spectra were acquired using an UltraflExtreme MALDI-TOF/TOF mass spectrometer equipped with a smartbeam II laser (Bruker Daltonics) with the settings in the positive ion mode. The FlexAnalysis software (Bruker Daltonics) was used to analyze the data. Peak masses and intensities were detected by Flex analysis software. Peptides from human HisRS protein were examined using the MASCOT program with search parameters (Table 2.6).
Positive identification the protein was successful when the search hit random match probability values of less than 0.05. In order to generate the theoretical mass lists of the possible peptides from human HisRS digestion by trypsin, an *in silico* digest experiment with “MS digest” function was done using ProteinProspector, a bioinformatics tool available from the University of California, San Francisco. (http://www.prospector.ucsf.edu/prospector/mhhome.htm).

**2.2.2.6 Human HisRS large scale expression**

After the confirmation of the expressed human HisRS protein, a large scale protein expression for further protein purification using 2 liter shaker flasks in a benchtop shaker was done. Bacterial cells from a freshly transformed plate were inoculated into 250 mL erlenmeyer flasks containing 100 mL LB medium + Streptomycin and grown overnight at 37 °C. In the morning the overnight culture was transferred into a 2 liter flask containing 1 liter of LB medium+ Streptomycin (50 µg/mL). This was grown at 37 °C until the OD<sub>600</sub> reached 0.6 when the cells were induced with 1 mM IPTG and

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequence database</td>
<td>NCBI nr</td>
</tr>
<tr>
<td>Taxonomy</td>
<td>Eukaryotes <em>Homo sapiens</em></td>
</tr>
<tr>
<td>Enzyme</td>
<td>Trypsin</td>
</tr>
<tr>
<td>Allowed miscleavages</td>
<td>1</td>
</tr>
<tr>
<td>Fixed modifications</td>
<td>None</td>
</tr>
<tr>
<td>Variable modifications</td>
<td>Methionine oxidation</td>
</tr>
<tr>
<td>Peptide tolerance</td>
<td>0.3 Da</td>
</tr>
<tr>
<td>Peptide range</td>
<td>+1</td>
</tr>
<tr>
<td>Instrument</td>
<td>MALDI-TOF/TOF MS</td>
</tr>
</tbody>
</table>

*Table 2.6: MASCOT peptide search parameters for human HisRS.*
allowed to grow for 4 hours at 37 °C. The cells were harvested by centrifugation 4,000 rpm at 4 °C for 20 min using a Beckman Coulter™ TJ-25 centrifuge and frozen immediately at −80 °C for further lysis and subsequent purification steps.

**Human HisRS Cell lysis**

The cell pellet was thawed at on ice and resuspended in lysis buffer 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 % glycerol (v/v), 2 mM EDTA and shaken on ice for 60 minutes in the presence of 50 µg/mL of hen egg-white lysozyme. Other key components in the lysis buffer lysozyme used are and not limited to phenylmethylsulfonyl fluoride (PMSF) as the protease inhibitor, ß-mercaptoethanol (BME) or dithiothreitol (DTT) and 0.1 % Triton X-100. In order to break open the bacterial protoplasts, the lysate was then sonicated for 3 minutes 50 % duty cycle, 60 % power using the Branson™ Sonifier 250.

**2.2.2.7 Human HisRS Protein purification**

The purification of a crude extract of human HisRS was carried out using Bio-Rad BioLogic™ Duoflow FPLC system in three steps using a hydroxyapatite column as a first step and then a nickel metal affinity column. The FPLC buffers (*Table 2.7*) were made based on the column to be used at each purification step and also on how to remove the N-terminal 6His-tag during the last step.

The third purification step involved using TEV protease to cleave off the 6-histidine tag at the N-terminus following the detailed scheme (*Figure 2-4*) used to obtain pure protein. The lysate was dialyzed in the hydroxyapatite buffer A (50 mM KH$_2$PO$_4$/Na$_2$HPO$_4$ pH 7.0) overnight in order to match the conductivity to the buffer. The
lysate was loaded onto a hydroxyapatite column that was initially equilibrated with buffer A 50 mM KH$_2$PO$_4$/Na$_2$HPO$_4$ (pH 7.0).

**Table 2.7:** FPLC buffers for human HisRS purification.

<table>
<thead>
<tr>
<th>Column</th>
<th>Buffer A</th>
<th>Buffer B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxyapatite</td>
<td>50 mM KH$_2$PO$_4$/Na$_2$HPO$_4$ (pH 7.0)</td>
<td>500 mM KH$_2$PO$_4$/Na$_2$HPO$_4$ (pH 7.0)</td>
</tr>
<tr>
<td>Ni-NTA</td>
<td>50 mM NaH$_2$PO$_4$ (pH 7.4)</td>
<td>50 mM NaH$_2$PO$_4$ (pH 7.4)</td>
</tr>
<tr>
<td></td>
<td>500 mM NaCl</td>
<td>500 mM NaCl</td>
</tr>
<tr>
<td></td>
<td>5 mM imidazole</td>
<td>500 mM imidazole</td>
</tr>
</tbody>
</table>

The protein was eluted from the column using buffer B 0.5 M KH$_2$PO$_4$/Na$_2$HPO$_4$ (pH 7.0). Analysis of human HisRS from the hydroxyapatite column was done by running the eluted fractions on an SDS-PAGE. The fractions corresponding to human HisRS were identified, pooled together and concentrated using 15 mL Millipore™ centricon filter with a 10,000 MWCO and stored on ice for the next purification step. The yield after this step was ~200 mg from the A$_{280}$ estimation typically from a 4 liter grow up.

**Human HisRS Ni-NTA column purification**

The samples from the hydroxyapatite column were first dialyzed in the Ni-NTA buffer A 20 mM NaH$_2$PO$_4$ (pH 7.4), 500 mM NaCl, and 5 mM imidazole). The buffers used during the Ni-NTA column purification were based on the recommended by the resin supplier (GE Healthcare). The protein was loaded on the column that was pre-equilibrated with buffer A. Once all the lysate was on the column, 5 column volumes (30
mL) buffer A was run through to wash off any non-specific binding material. The protein was then eluted by a gradient of buffer B 20 mM NaH$_2$PO$_4$ (pH 7.4), 500 mM NaCl, 500 mM imidazole.

TEV Protease cleavage of N-terminal his-tag on human HisRS

The tobacco etch virus protease (TEV) recognizes a seven amino acid sequence (ENLYFQG/S) and cleaves between Q and G/S. It is used in the removal of the N-terminal his-tag during recombinant protein purification. The purified sample of human HisRS that was eluted from the hydroxyapatite column was placed in dialysis tubing before an aliquot of pure and freshly prepared TEV protease was added in a molar ratio of 20:1 protein to TEV and mixed gently. The dialysis tubing containing the human HisRS and TEV was then placed in a 2 liter beaker containing Ni-NTA buffer A and the

![Figure 2-4: Human HisRS purification scheme.](image)
reaction incubated at 4 °C for 12 hours. The contents were removed from the dialysis tubing then loaded onto the Ni-NTA affinity column that pre-equilibrated with buffer A. The desired human HisRS protein the first to elute from the column during buffer A run, was collected first while the bound 6His-tag and the TEV protease were washed off the column with buffer B which contained a higher amount of imidazole. The purity of the protein sample was analyzed by running an SDS-PAGE.

The purified protein was dialyzed against buffer A to reduce the imidazole concentrations that was associated with column purification. The purified protein was then concentrated using 15 mL Millipore™ centricon filter with a 10,000 MCWO before storing in small volume aliquots at -80 °C after adding 15 % glycerol. The yield of the protein after quantification by UV absorption A$_{280}$ nm was ~100 mg. For the preparation of human HisRS protein crystallization experiments an additional Superdex-200 size exclusion column equilibrated with buffer containing 25 mM Bis-Tris-HCl (pH 6.5), 100 mM NaCl was used. The final concentration of the purified human HisRS protein in this study was determined spectrophotometrically by applying the Beer-Lambert law (Equation 5).

$$ A = \varepsilon_{\lambda} c l $$

Where, $A$ is absorbance at wavelength $\lambda$, $\varepsilon_{\lambda}$ is the molar extinction coefficient at wavelength $\lambda$, $c$ is the concentration (mol per L) and $l$ is the cuvette path length. The extinction coefficient was estimated from the amino acid sequence using the ExPASy bioinformatics tool (expasy.org).
2.2.3 Biochemical Studies

2.2.3.1 Determination of human HisRS oligomeric state by Analytical Gel Filtration

Analytical gel filtration, also known as size exclusion chromatography (SEC), uses porous particles to separate molecules of different sizes. Molecules that are smaller than the pore size can enter the pores of the resin and therefore have a longer retardation than larger molecules that cannot enter the pores. Larger molecules remain un-retained in the resin and elute faster.

The oligomeric state of the human HisRS protein in this study was determined by analytical gel filtration on a Superose-12 size exclusion column. Prior to loading the samples onto the column it was first calibrated using high molecular weight gel filtration calibration protein standards (Bio-Rad) containing the following thyroglobulin (660 kDa), immunoglobulin G (150 kDa), ovalbumin (45 kDa), and bovine myoglobin (17 kDa). The column was equilibrated with a buffer containing 20 mM HEPES (pH 7.5), 100 mM KCl, 5 mM MgCl₂, 0.2 mM EDTA and 10 % glycerol. The purified human HisRS, a volume of 0.5 mL and a concentration of 1 mg/mL was injected at a flow rate of 0.5 mL/min at a chart speed of 2 cm/min. The elution was monitored by measuring the absorbance at 280 nm. The relative molecular weight of human HisRS was determined by comparing the relative mobility with that of the protein standards.

2.2.3.2 Determination of human HisRS percent polydispersity by Dynamic Light Scattering

Dynamic light scattering is one of the biophysical techniques used in determining the oligomerization of the proteins in solution. This technique applies the use of a beam
of monochromatic light which is directed through a buffer solution containing the protein of interest and the fluctuating intensity of the scattered light analyzed. The experiment is normally carried out with a pure protein solution in an appropriate buffer that is filtered prior to injection into the sample cell. The hydrodynamic radius ($R_H$) and apparent particle size of the enzyme is derived from $D$, (diffusion coefficient) using the Stokes–Einstein equation

$$R_H = \frac{k_B T}{6 \pi \eta D_t}$$  \hspace{1cm} (6)

Where, $k_B$ is the Boltzman constant, $T$ the temperature in Kelvin degrees and $\eta$ the viscosity of the solvent.

Before carrying out the dynamic light scattering experiment the human HisRS samples (1– 2 mg/mL) in buffer 25 mM Bis-Tris-Cl (pH 6.5), 100 mM KCl, 1 mM DTT was passed through a 0.1 $\mu$m membrane filter. The sample was then centrifuged briefly at 4 °C to eliminate air bubbles. The experiment was done on DynaPro Titan DLS (Wyatt Technology Corporation) instrument. A protein volume of 20 $\mu$L (1 mg/mL) was loaded into the DLS cuvette using a loading pipette tip and placed into the cuvette holder. After waiting for at least one minute for the sample to equilibrate to the desired temperature the data was collected. Data was acquired at 25 °C for 10 seconds, repeated 10 times and then averaged. The Dynamics 6.7.3 software was used to fit the data and calculate the hydrodynamic radius from the Stokes–Einstein equation.
2.2.3.3 Intrinsic Tryptophan Fluorescence Quenching of human HisRS

The intrinsic fluorescence of a protein is normally characterized by its emission intensity and emission maximum all which are influenced by the local environment of the tryptophan residues and can therefore, be altered when a ligand is introduced to the solution. A fluorescence spectrum of human HisRS protein was obtained at 25 °C on a PTI instrument Spectrophotometer (Photon Technologies International) with an excitation wavelength of tryptophan at 295 nm, emission was scanned from 300 to 400 nm. The slit band widths were set at 4.0 nm for both excitation and emission throughout the experiments. The protein concentration used was 0.5 mg/mL in buffer containing 5 μM in buffer 20 mM HEPES (pH 7.5), 100 mM KCl, 5 mM MgCl₂, 0.2 mM EDTA and 10 % glycerol. The binding of Mg²⁺-ATP and histidine to human HisRS were studied by measuring the quenching of tryptophan fluorescence at 25 °C. A 10 mm x 4 mm quartz cuvette was used to hold 400 μL of sample. The human HisRS protein (5 μM) in buffer 20 mM HEPES (pH 7.5), 100 mM KCl, 5 mM MgCl₂, 0.2 mM EDTA and 10 % glycerol, was titrated with either ATP or histidine in a concentration range of 0 to 250 μM, and the fluorescence spectra acquired in the same way as with the protein alone. ORIGIN software version 7 (Origin Lab. Corp., MA, USA) was for the analysis and display of data. All data were corrected for background fluorescence and dilution.

Acrylamide tryptophan quenching in of human HisRS in native or denatured state was also carried out. To denature the protein, Guanidinium-HCl added to 5 μM human HisRS to a final concentration of 4 M. The denaturation reaction was incubated on ice for 2 hours with aliquots taken out every 30 minutes. A control denaturation reaction involved incubating only Guanidinium-HCl (4 M) on ice in the same buffer without
human HisRS. After the denaturation, acrylamide quenching fluorescence measurements were carried out at a constant pH using by sequential addition of a freshly prepared 7 M (40 %) acrylamide stock solution. All data were corrected for background fluorescence and dilution. Fluorescence quenching using acrylamide was corrected for inner filter effect caused by the absorbance of acrylamide at 290 nm.

Quenching results were plotted as the ratio of the fluorescence intensity in the absence of quencher ($F_0$) to the intensity in the presence of quencher ($F$) against quencher concentration. The data was analyzed according to the Stern-Volmer equation. (Lakowicz, 1996)

$$F_0/F = 1 + K_{SV}[Q]$$

(7)

Where, $K_{SV}$ is the Stern-Volmer constant and $Q$ is the quencher.

2.2.3.4 Initial human HisRS crystallization experiments

Prior to protein crystallization set ups, the human HisRS was dialyzed against the buffer containing 25 mM Bis-Tris-HCl (pH 6.5), 100 mM NaCl and concentrating it to ~10 mg/mL. The hanging-drop vapor diffusion method (2+2 µL) droplets were set up in two Costar 24-well™ trays. In the first tray, a format gradient of two solutions was used as follows: To crystallize the apo HisRS A (10 % PEG4000) and B (40 % PEG4000) with the crystallization buffer as the well solution. In the second tray, the crystallization condition was the human HisRS complexed with two ligands (histidine and ATPγS). To prepare the protein sample, 10 mg/mL of human HisRS was added into a 0.2 mL Eppendorf microcentrifuge tube in the buffer. Then 10 mM ATPγS, 3 mM L-Histidine,
10 mM MgSO₄ and 10 mM TCEP were added to the protein before centrifuging briefly to reduce the contents. A gradient of solutions A (2 M (NH₄)₂SO₄) and solution B (1.6 M (NH₄)₂SO₄) were used in 100 mM NaPIPES (pH 6.5) as the well solution to set up the tray. All the droplets contained 1 µL of protein and 1 µL of the well solution. The trays were stored in a cold room at 4 °C.

Two crystallization screens were also set up in 96 well round bottom Corning™ trays for screening the apo and protein complex with protein concentration (12 mg/mL) using an automated Honeybee robot (Genomic Solutions). Sitting drop vapor diffusion method was used with the PEG/ion screen (Izaac et al., 2006). One hundred microliters dispensed automatically into the well from the deep block well block solutions. On the shelf of the tray, 1 µL each of protein and well solution was dispensed by the synQuad tips. The trays were sealed with a transparent tape and centrifuged using a Beckman Coulter™ TJ-25 centrifuge for 30 seconds at 1,000 rpm to mix the droplet contents. The trays were then stored at 4 °C. In order to monitor crystal growth of trays were frequently checked and observations done using a Nikon™ SMZ1500 microscope. Pictures of promising hits were taken using a Nikon™ CoolPix 990 digital camera.

2.3 Results and Discussion

2.3.1 Protein expression and purification of human HisRS

The gene carrying human HisRS was cloned into a pDEST-C1 vector. The first step was to amplify the gene using PCR. The PCR product was purified and run on a 1 % (w/v) agarose gel to verify the correct size. From Figure 2-5, the correct size of 1551 bp was compared against the 100 bp DNA ladder.
In a two step cloning process by the Gateway system, the gene was first inserted into the entry vector pENTR-D in TOPO assisted cloning reaction. The results are shown in Figure 2-6 with an expected size of 4.1 Kb in the left lane as compared to the 2-10 Kb supercoiled DNA ladder. The next cloning step using the LR clonase enzyme was used to insert the gene into cloning vector pDEST-C1 to generate the expression clone.

**Figure 2-5:** SYBR Gold™ stained 1% agarose gel of human HisRS PCR product. On the left: Lower molecular weight DNA ladder. On the right: human HisRS PCR product with the correct size (1551 bp).
The correct size of 5.2 Kb was verified by 1 % (w/v) agarose gel using a 2-10 Kb supercoiled DNA ladder shown in the lanes 2 and 3 of Figure 2-7. The expression clone in pDESCT-C1 was transformed into expression host *E. coli* BL21 (DE3)* competent cells in LB containing Streptomycin. Induction of protein expression was done by adding 1 mM IPTG at an OD$_{600}$ of 0.4 for 4 hours. Aliquots of cells at the time of induction (time 0 hr and at the end of induction (time 4 hr) were taken out and used for the confirmation of protein expression by running an SDS-PAGE. Once the protein expression conditions had been optimized, large scale expression was carried out under the same conditions to produce more material for FPLC purification. Harvested cells after each growth were centrifuged at 4,000 rpm and stored at -80 °C.

The pellet from large scale protein expression was lysed and purification was achieved using three steps. In the first step of human HisRS purification the lysate was
applied onto a hydrophobic interaction ceramic Hydroxyapatite shown in Figure 2-8. The fractions containing human HisRS protein as represented by the red box above the chromatogram in Figure 2-8 were pooled together and analyzed for purity by SDS-PAGE as shown in Figure 2-9). It can be seen from these results that though a considerable amount of human HisRS was eluted off the hydroxyapatite column, the material was still not pure as seen on the gel Lanes 3-7 in Figure 2-9.

![Image](image.png)

**Figure 2-7:** SYBR Gold™ stained 1 % agarose gel of human HisRS insertion into pDEST-C1 cloning vector. On the left: 2- 10 Kb supercoiled DNA ladder. On the right: pDEST-C1 plasmid with a band around 5.2 Kb ([((5335 bp- 1600 bp) + 1551 bp]. The sizes or pDEST-C1, cytotoxic ccdB gene and HisRS gene are 5335 bp, 1600 bp and 1551 bp respectively.

A next step involved a Ni-NTA metal affinity column. The gene was designed for expression with an N-Terminal 6His-tag and a TEV protease site. The tobacco etch virus protease (TEV) recognizes a seven amino acid sequence (ENLYFQG/S) and cleaves between Q and G/S. It is used in the removal of tags during recombinant protein purification. This is meant to make sure the 6His-tag does not interfere with downstream activities of the enzyme once it has been purified. So the next step involved two metal
affinity column purification steps. The lysate from the hydroxyapatite column was applied onto the Ni-NTA column following the Ni-NTA buffer 50 mM sodium phosphate, (pH 7.4), 300 mM NaCl and eluted with an imidazole gradient from 5 mM to 500 mM.

**Figure 2-8:** Hydroxyapatite column purification of human HisRS. Fractions 10-14 highlighted in red were pooled down for further concentration and analysis by SDS-PAGE electrophoresis. The lines are represented as follows: dotted line (baseline), disjointed line (conductivity) and diagonal wavy line (buffer B gradient) respectively on the chromatogram.
TEV protease was also purified using the same column since it had a non-cleavable 6His-tag at the N-terminal. Fractions containing human HisRS were pooled together and the concentration of the protein was determined. This was crucial because for a successful TEV site cleavage the ratio of TEV protease and protein should be about 1:20. Prior to the cleavage a buffer exchange was carried out on the sample before incubation with recombinant TEV protease in a ratio of 1:20 for an overnight duration at 4 °C while stirring. The final purification is carried out by elution from the Ni-NTA metal affinity column to get rid of TEV protease, 6His-tag and any un-cleaved components which remain bound. The chromatogram after the Ni-NTA metal affinity is represented in Figure 2-10. The fractions were concentrated and the purity of the protein from this step was analyzed by SDS-PAGE as seen in the results Figure 2-11.
One of the objectives of the study was to set up crystallization trays for human HisRS which therefore meant a very pure protein sample was needed. For the preparation of human HisRS protein crystallization experiments an additional Superdex-200 size exclusion column was used as seen in Figure 2-12. However some faint unwanted bands could be seen above the desired 58 kDa mark probably due to disulfide cross-linking since the protein had to be dialyzed against the crystallization buffer (25 mM Bis-Tris-HCl (pH 6.5), 100 mM NaCl) overnight prior to the size exclusion column.

Figure 2-10: Chromatogram of human HisRS purification by Ni-NTA column. Pure protein peak is shown by orange arrow. UV absorbance at 280 nm (green) and 260 (violet) curves are highlighted. The increasing imidazole gradient is indicated by the black line.
After concentration of the pure protein using 15 mL Millipore™ centicon filter with a 10,000 MCWO, the amount of the protein was determined by measuring UV A$_{280}$. Aliquots of 100 µL each containing about 10 mg/mL in 15 % (v/v) glycerol were flash-frozen on dry ice and stored at -80 °C until further use.

Figure 2-11: SDS-PAGE of human HisRS after Ni-NTA column purification. Lane 1: Protein Marker. Lane 2: lysate before Ni-NTA column. Lane 3: Pure human HisRS, the elution profile represented in Figure 10.
2.3.2 Human HisRS protein expression confirmation by in-gel trypsin digestion and MALDI-TOF/TOF MS

After trypsin digest of a gel sample the confirmation of the expressed protein was done by MALDI-TOF/TOF MS. The mass spectrum (Figure 2-13) was obtained in the positive ion mode. A total of  and peptides identified by searching the MASCOT search database for peptides corresponding to human HisRS and the results are represented in Table 2.8. A summary of all peptides identified shows sequence coverage of 38%.
**Figure 2-13:** MALDI-TOF/TOF MS of in-gel tryptic digest of human HisRS.
2.3.3 Characterization of human HisRS

2.3.3.1 Determination of human HisRS oligomeric state by Analytical Gel Filtration

The oligomeric state can be determined by analyzing the size of the protein in solution. Analytical gel filtration which separates molecules according to size was applied as a tool to determine whether human HisRS elutes as a monomer or dimer in solution. The experiment was carried out on a Superose-12 size exclusion column that was calibrated using protein molecular mass standards described in section 2.2.3.1. The results show that human HisRS is a dimer in solution with an observed apparent molecular weight of 114 kDa as represented in Figure 2-14. The calculated molecular weight is approximately 58 kDa for the monomer and 116 kDa for the dimer. This was a confirmation of the dimeric form of the enzyme since the crystal structure that was later

<table>
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<th>HisRS peptide</th>
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<th>m/z_{calc}</th>
<th>m/z_{observed}</th>
<th>ΔM</th>
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<td>136-144</td>
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<td>0.09</td>
</tr>
</tbody>
</table>
solved showed human HisRS being a dimer. (Xu et al., 2012) Another way to validate the presence of the functional dimeric human HisRS would be to perform an activity assay on the fractions eluted from the analytical gel filtration.

![Figure 2-14](image)

**Figure 2-14:** Determination of apparent molecular weight of human HisRS by analytical gel filtration. Protein standards are shown in blue diamonds while human HisRS is represented by red square.

### 2.3.3.2 Determination of human HisRS percent polydispersity by Dynamic Light Scattering

Dynamic light scattering is a technique used that is applied in determining the oligomerization of a protein in solution as a result of increased hydrodynamic radius. Polydispersity expressed as a percent is used as an indicator to predict formation of protein crystals. (Bernstein et al., 1998; Phillips et al., 1997) A low polydispersity of less than 15 % means the protein is monodispersed, with a higher conformational purity and
more likely to crystallize. About 1 mg/mL of protein was used in the dynamic light scattering experiment at 25 °C. From the results obtained, (Figure 2-15) the hydrodynamic radius of human HisRS in solution was 4.3 nm, polydispersity of 5.7 % and a relative molecular weight of 105 kDa. The relative molecular weight from the DLS results as presented in Figure 2-15 was about 10 kDa less than the expected dimer but well within the range required for crystallization based on the polydispersity below 15 %.

The results from DLS verified the dimeric form of human HisRS in solution just as with the analytical gel filtration experiment. DLS was also used to monitor the ligand-dependent changes in the oligomeric state of human HisRS. The presence of ATP in the
protein solution resulted into a large average mass and a significantly high % polydispersity (data not shown), well above the range recommended for good crystallizable results. Whether the latter observation was due to conformational changes upon ATP binding is it just because the DLS technique is very sensitive to the presence of aggregates in the protein sample will be probed further.

2.3.3.3 Intrinsic Tryptophan Fluorescence Quenching of human HisRS

Tryptophan as a chromophore is useful in probing protein structure and dynamics due to its sensitivity to chemical environment. (Burstein et al., 1973) Structural analysis of the human HisRS crystal structure shows that there are two tryptophan residues, one in the catalytic domain between motif 2 and motif 3 and the other in the C-term anticodon binding region. (Xu et al., 2012) The two residues Trp246 and Trp432 (Figure 2-16) can therefore be used to monitor the conformational changes in the human HisRS when ATP or ADP binds the active site since the tryptophan environment will be affected.

The protein was excited at the tryptophan excitation wavelength of 295 nm and emission spectra obtained between 300- 400 nm. The results show evidence of change in fluorescence intensity of a similar magnitude upon addition of 200 µM of either ATP or ADP to the protein solution (Figure 2-17 panels A and B). The trend however is not the same when 1 mM histidine is added to the protein in the presence of ATP or ADP. Addition of both histidine and ATP realized a greater decrease in fluorescence intensity (Figure 2-17 panel C) as compared to histidine and ADP (Figure 2-17 panel D). Addition of only histidine to the protein did not result into significant quenching in comparison with either ATP or ADP. According to the human HisRS structure, Trp246 is
~27 Å further away from the imidazole ligand that was used. (Xu et al., 2012) Observation from this experiment leads to a possible conclusion that the histidine binding pocket in human HisRS could not be located not so close to the position of the buried tryptophan in the protein’s catalytic domain.

Figure 2-16: Ribbon representation of human HisRS with the positions of the two tryptophan residues. The residues are shown in blue stick presentation, Trp246 located in the Catalytic domain, Trp432 in the anticodon binding domain. The N-terminal is shown in orange while the C-term in wheat. (Rendered in PyMOL, PDB 4G85)

Studies on ATP coordination in the E. coli HisRS nucleotide binding pocket that were carried out before the human HisRS crystal structure was published show that the binding pocket is filled with several arginine residues and that ATP binding is made possible by the change in conformation when histidine binds first before the nucleotide. (Merrit et al., 2010) This could explain why our fluorescence measurement results exhibit more tryptophan quenching especially in the case when both histidine and ATP are added.
to the protein prior to recording fluorescence measurements. Steady state fluorescence studies of ATP and histidine binding using the *E. coli* HisRS by Guth *et al.* showed a titration of ATP resulting in 5 % more tryptophan quenching than that of histidine in solution. (Guth *et al.*, 2009)

The specificity of ATP and ADP for the HisRS enzyme was also reported by Di Natale *et al.* and by comparing the two it was found that ADP behaved as a competitive inhibitor to ATP in the *Salmonella typhimurium* HisRS. (Di Natale, *et al.*, 1976) One important question however still needs to be addressed is what will be the effect of the presence of tRNA$^{\text{His}}$ to this conformational re-arrangement during the binding of ATP and histidine.

Solvent accessibility of the human HisRS tryptophan in native and denatured form was assessed using the non-ionic quencher acrylamide. Figure 2-18 panel A represents the fluorescence emission spectrum showing the decrease in fluorescence as the amount of acrylamide. When acrylamide was titrated into the denatured protein there was evidence of fluorescence quenching, which was proportional to the amount of acrylamide added. Up to 0.6 M acrylamide was used. Analysis of the Stern-Volmer plots of F$_0$/F vs. [acrylamide] show linear results as presented in Figure 2-18 panel B with a $K_{SV}$ value of 7.9 M$^{-1}$. Attempts to assess the acrylamide quenching of the native form in the presence and absence of ATP did was not successful because there was no significant difference in the F$_0$/F vs. acrylamide concentration plot (Figure 2-18 panel B). The $K_{sv}$ values obtained, 1.1 M$^{-1}$ (ATP) and 1.5 M$^{-1}$ (without ATP) do not present a significant difference in the extent of quenching for the two conditions.
In the tertiary structure, (Figure 2-16) Trp246 appears to be buried inside the catalytic domain in the native protein. Therefore, the denaturation with 4 M Guanidinium-HCl played a role in making the tryptophan residue accessible to the

Figure 2-17: Fluorescence emission spectra of intrinsic tryptophan quenching of human HisRS. A: Protein and ATP only. B: Protein and ADP only. C: Protein, histidine and ATP. D: Protein, histidine and ADP. The experiment was done at 22°C at pH 7.5. The volume of the sample in the cuvette was 400 µL while the concentrations of the protein, ATP (ADP) and histidine used were 0.5 µM, 200 µM and 1 mM histidine respectively. Protein sample was excited at 295 nm and the emission spectra collected from 300- 400 nm.
quencher resulting into a decrease in fluorescence. At this point however, without a known structure that fully describes the residues that are in the human HisRS ATP binding site it is difficult to determine how close the Trp246 is to ATP. To probe this subject further in determining if the residue has a characteristic polar environment or surface exposed, a mutant of Trp246 will be used and results compared with the wild type form.
Figure 2-18: Intrinsic tryptophan fluorescence quenching of human HisRS by acrylamide. A) Fluorescence emission spectra of Guanidinium-HCl denatured human HisRS recorded at 22 °C at pH 7.5 with increasing amounts of acrylamide (0 to 0.7 M). B) The Stern-Volmer plots F₀/F vs. acrylamide concentration. The lower line (black diamonds) represent the native protein in the presence of 200 μM ATP while the middle line (black squares) represents the native protein without ATP. The upper line (black triangles) represents the denatured protein without ATP.
2.3.3.4 Human HisRS Crystallization screens

Crystallization set ups were made under two different conditions. The first one was the human HisRS alone and the second condition where ligands were added (ATPγS and L-Histidine). There were no crystal hits in both conditions that were set up. Most of the drops had protein that was either clear or aggregated after several weeks. Further crystallization work of human HisRS was abandoned when the structure was published in favor of crystallizing the *Trypanosoma cruzi* prolyl-tRNA synthetase discussed in the coming chapters.

2.4 Conclusions

Human HisRS was successfully cloned, expressed with an N-terminal 6His-tag and a considerable amount of purified soluble protein was obtained using three purification steps. The TEV protease site was helpful in the removal of the 6His-tag during the last purification step. The identity of the expressed protein was confirmed by in-gel trypsin digestion and MALDI-TOF/TOF MS analysis.

The main study objective was to establish structural and functional information from the human HisRS by crystallizing the protein and obtaining X-ray crystal data to solve the structure. However, the publication of a paper by Xu and co-workers of native and mutants of HisRS while the study was ongoing (Xu *et al.*, 2012) led to the change of approach. The focus shifted to other biochemical experiments which included analytical gel filtration, dynamic light scattering and fluorescence spectroscopy which were carried out to characterize human HisRS. Both analytical gel filtration and dynamic light scattering were used to confirm that the human HisRS enzyme is a dimer in solution.
Percent polydispersity data obtained show that the enzyme is crystallizable in the presence of ATP and its analog.

Fluorescence emission spectroscopy was used to obtain data on how quenchers affect the solvent accessibility of the two intrinsic tryptophan residues in human HisRS. It should be noted that the fluorescence measurements were carried out at a single pH. It is not clear if the solvent accessibility of tryptophan in human HisRS will be pH dependent to conformational changes. Overall, this study provides some insights in the interaction of human HisRS with ATP and histidine in solution in a similar manner as the case with other homologs of the enzyme in *E. coli* and other species.

2.5 Future Perspectives

The most important aspect in fully characterizing this enzyme was the unavailability of pure His-tRNA\(^\text{His}\). With the availability of pure tRNA\(^\text{His}\) focus can shift to further studying how human HisRS binds its cognate tRNA. Therefore, part of the future experiments to be carried out will involve crystallization of a human HisRS complex with His-tRNA\(^\text{His}\).

The WHEP-TRS domain of the human HisRS enzyme (Figure 2-19) presents another area of exploring the enzyme. Although its functions remain fully understood, previous studies have indicated that the region could be an epitope for the Jo-1 antibodies. (Raben *et al.*, 1994; Xu *et al.*, 2012) Another possible strategy will be to make a construct of HisRS that does not contain the WHEP-TRS domain and study the functional and immunological properties in comparison with the full length. The possibility of crystallizing the short segment of the human HisRS enzyme containing only
the WHEP-TRS domain will be explored. Currently, only the solution NMR structure of the WHEP-TRS domain has been reported even though earlier immunological studies on a truncated version of human HisRS lacking this domain showed loss of antigenic activity and aaRS activity. (Raben et al., 1994)

**Figure 2-19:** Human HisRS WHEP-TRS domain. Inset: (A) Solution structure (green ribbon diagram) of the WHEP-TRS domain ranging from residues 1-73 at the N-Terminal. Picture was rendered in PyMOL PDB: 1X59. (B) Diagrammatic representation of the position of the WHEP-TRS domain at the N-terminus blue box residues 5-49. (Part B adapted from Xu et al., 2012)
Chapter 3

Exploring the Specificity of *Archaeoglobus fulgidus* Elongation Factor Tu for Eukaryotic tRNAs

3.0 Introduction

Several assays are used in the studying the aminoacylation activity of both prokaryotic and eukaryotic aminoacyl-tRNA synthetases. For a long time the major assay that has been conventionally used relied on the application of radioactive amino acids whereby the rate of formation of radiolabeled aa-tRNA is measured. Normally, for this type of assay $^3$H or $^{14}$C labeled amino acids are generally utilized. (Hoagland, 1957) To initiate the reaction, a radiolabeled amino acid is titrated into a mixture of aaRS, tRNA and ATP before the extent of aminoacylation is monitored by scintillation counting. (Eigner, 1974) The aminoacylation activity is determined by dividing the amount of radiolabeled amino acid bound to the scintillation filter by the total amount of tRNA in the reaction. (Eigner & Loftfield, 1974) However, this assay proceeds under saturating amino acid concentrations with $K_M$ ranges of 200- 400 $\mu$M. (Ibba *et al*., 1993; Hill & Schimmel, 1989; Eriani *et al*., 1993) The corresponding $K_M$ values for tRNA reported where this assay is used are also high in the range of 0.2- 2 $\mu$M. (Ibba *et al*., 1993; Hill &
Schimmel, 1989; Eriani et al., 1993) Therefore, large amounts of radioactivity is needed in order to detect aaRS activity at low tRNA concentrations. Another limitation when radiolabeled amino acids are used is the difficulty in obtaining the accurate aaRS activity since the fraction of activated tRNA has to be determined indirectly from the amount of bound amino acid. (Wolfson & Uhlenbeck, 2002)

A second isotope-based technique involves the use of radiolabeled tRNA (3′-[32P]-labeled tRNAs) together with unlabeled amino acids. Radiolabeled tRNA is incubated with the cognate amino acid and the aaRS of interest in the presence of ATP before monitoring the extent of aminoacylation by scintillation counting of aminoacylated tRNA. (Wolfson, 1998; Wolfson, 2002) The aminoacylation activity can also be determined by measuring the fraction of aminoacylated tRNA’s mobility on an acidic denaturing polyacrylamide gel. Using this assay detection ranges for the *E. coli* AlaRS as low as 50 nM have been achieved with the case of L-alanine concentrations of between 10 and 1 mM. (Wolfson et al., 1998) However, the assay also faces the same challenge associated with isotope labeling because it requires the tRNA to be radiolabeled before use.

More recently, spectrophotometric assays have been developed for use in the study of aaRS aminoacylation activity. One such example is where a multi enzyme coupling assay is applied especially by monitoring the first step of aminoacylation during the formation of the adenylate. The adenylate formed from reacting aaRS with its cognate amino acid is coupled to the oxidation of NADH. In this assay, three enzymes: adenylate kinase, pyruvate kinase and lactate dehydrogenase are utilized. First, the adenylate kinase in the presence of ATP and AMP produces two molecules of ADP. The ADP in the
presence of phosphoenolpyruvate is coupled to pyruvate kinase to produce pyruvate that is in turn coupled to lactate dehydrogenase. Monitoring of NADH oxidation is achieved by measuring absorbance at 340 nm when NAD\(^+\) is formed. The results from the *E. coli* AlaRS detection using this method showed that the formation of the activated tRNA\(_{\text{Ala}}\) is proportional to the amount of NADH that is oxidized with a detection limit of 13-270 nM. (Wu & Hill, 1993) Although this technique can achieve higher sensitivity, up to 4 fold in the case of AlaRS when compared to the conventional radiolabeled assays its major limitation is the number of coupling enzymes used. This could result into a higher reaction volume making the assay cumbersome and expensive to set up.

Another type of spectrophotometric based method utilizes the coupling of the inorganic pyrophosphatase enzyme but in this case the detection of aminoacylation is achieved by a malachite green assay. In this assay, the pyrophosphatase enzyme is coupled to the aminoacylation reaction and the released inorganic phosphate product (Pi) is monitored by the malachite green reaction. An example of this assay using *Trypanosoma brucei* IleRS was described by Cestari & Stuart. (Cestari & Stuart, 2013) The tRNA substrates used in this assay was prepared by *in vitro* transcription of *T. brucei* tRNA\(_{\text{Ile}}\) with T7 RNA polymerase. The aminoacylation assay reaction is stopped by addition of malachite green dye. To quantify the activity of aminoacylation, absorbance is measured at 620 nm. (Lloyd *et al.*, 1995; Dermyer *et al.*, 2007; Cestari & Stuart, 2013) The limitation of this assay is its dependence on the amount of amino acid used which could be as high as 50 mM. The efficiency for this technique is also very low especially when total tRNA or yeast tRNA are used. (Dermyer *et al.*, 2007)
The third technique that has been developed for assessing the activity of aaRS involves the formation of a ternary complex of GTP bound Elongation Factor Tu (EF-Tu) with aminoacylated labeled tRNA. Scintillation counting is used to measure the extent of aminoacylation based on the radiolabel used on the tRNA. (Ribeiro et al., 1995; Dale & Uhlenbeck, 2005) The majority of the assays described rely on the use isotope labeling. Either amino acids or tRNAs have to be radiolabeled during the aminoacylation reaction. Another important characteristic of the isotope dependent aminoacylation assays is the safety risks associated with isotope waste disposal and also the prohibitive cost of acquisition of radioalebeled amino acids makes these techniques unattractive for high throughput screening.

In this study we seek to apply the formation of the EF-Tu: aa-tRNA ternary complex and a spetrophotometric determination to study aminoacylation of eukaryotic aminoacyl-tRNA synthetases without radiolabeling. Of specific interest is to utilize the Archaeoglobus fulgidus Elongation Factor Tu (Afu EF-Tu) a thermally stable GTPase which has a high affinity for aminoacylated tRNA. In order to detect the activity of aaRS, the amount of tRNA bound to the EF-Tu in the ternary complex is measured by fluorescence with the help of a tRNA binding dye. The fluorescence intensity measured is assumed to be directly proportional to the amount of tRNA present in the reaction which represents the amount of aminoacylated tRNA.

3.1 Elongation Factor Tu

In bacterial ribosomal translation, the Elongation Factor Tu (EF-Tu) is the GTPase that delivers aminoacyl-tRNAs to the site A of the ribosome. This is made
possible by the ability of EF-Tu to bind tightly to aminoacyl-tRNA when in GTP-bound form, despite the differences in amino acid side chain properties and varied tRNA sequences. (Louie et al., 1984; Ott et al., 1990) Experimental studies using either *E. coli* or *T. thermophilus* EF-Tu with either GTP or its non-hydrolysable analogs have shown that EF-Tu and aminoacyl-tRNA form a ternary complex in the presence of GTP that binds to the ribosome before codon recognition takes place. (Rodnina et al., 1995) A representative of the ternary complex is shown in **Figure 3-1** in which the *T. aquaticus* EF-Tu is bound by yeast Phe-tRNA<sup>Phe</sup> in the presence of GDPNP. This activated EF-Tu in the ternary complex hydrolyzes GTP that causes it to inactive GDP-bound form. The GDP form of EF-Tu has lower affinity for aminoacyl-tRNA, thus the aa-tRNA is released from the complex at the A site to participate in the peptidyl transfer reaction as EF-Tu leaves the ribosome. (Rodnina et al., 1995; Rodnina et al., 1996; Dell et al., 1990; Polekhina et al., 1996) EF-Tu therefore plays a role in helping to discriminate between cognate and non-cognate aa-tRNA. (Thompson, 1988) It is not, however, clear how the GTP-bound form of EF-Tu differentiates between the diverse aa-tRNAs.

Crystal structures of EF-Tu in complex with aa-tRNA and also with the Elongation Factor Ts (EF-Ts) have provided information on how EF-Tu interacts with RNA and other proteins at the molecular level. (Niessen et al., 1995; Kawashima et al., 1996) For instance, the crystal structures of *Thermus aquaticus* EF-Tu complexed with yeast Phe-tRNA<sup>Phe</sup> or Cys-tRNA<sup>Cys</sup> from *E. coli* show the protein interacting with both tRNA bodies via the helices of the acceptor (A) and TΨC (T) helices although not with the same hydrogen bonding patterns for the two tRNA substrates. (Nissen et al., 1995,
Nissen *et al.*, 1999) Aminoacyl-tRNAs form a ternary complex with EF-Tu: GTP as shown in equation 8 during the aminoacylation reaction.

\[
\text{aa-tRNA} + \text{EF-Tu: GTP} \leftrightarrow \text{aa-tRNA} + \text{EF-Tu: GTP}
\]  

(8)

It has been shown that the abundance of EF-Tu inside an *E. coli* cell exceeds that of EF-Ts and the ribosome almost ten times making it one of the most abundant proteins. (Furano, 1976) This underscores the importance of the formation the ternary complex for its importance in making sure the uncharged tRNAs do not compete for cognate aminoacyl-tRNA synthetases as these latter species have similar affinities for both

**Figure 3-1:** Ternary complex of EF-Tu bound to tRNA^{Phe}. Three monomers of *Thermus thermophilus* EF-Tu in ribbon representation (green, cyan and red interact with yeast Phe-tRNA^{Phe} polymer in orange). GDPNP is shown as a stick representation in magenta while the magnesium ion is shown as a blue sphere. (Rendered in PyMOL. PDB ID: 1TTT).
charged and uncharged tRNA species. (Pingoud et al., 1973) This observation and other studies make EF-Tu an interesting GTPase whose association with the ribosome during the translation process is under investigation. Based on these findings, a lot of research has been going on in trying to develop antibiotics that target bacterial EF-Tu. (Wolf et al., 1974)

The archaeal organism *Archaeoglobus fulgidus* is a sulfur reducing hyperthermophile that is found living in hydrothermal vents, hot springs and also in oil deposits. It is known to survive under extreme temperatures ranging from 60-90 °C. (Slesarev et al., 1993; Klenk et al., 1997)

*Archaeoglobus fulgidus* EF-Tu is a monomer, similar to its homologs in eukaryotes and it contains the three domains typical of GTPases. The protein parameters for *Afu* EF-Tu (Table 3.1) were obtained from the ExPASy website using ProtParam tool. The choice for an archaeal protein was because of two major reasons. First, archaeal proteins represent simplified versions of their eukaryotic counterparts and two, because of their thermal stability they are easier to work with especially under varied temperature conditions.
3.1.2 Project Goals

The major goal of this project was to utilize the thermally stable *Afu* EF-Tu to capture activated eukaryotic tRNAs through the formation of the ternary complex without radiolabeling of either the amino acid or tRNA. In the case of conventional aminoacylation assays in which EF-Tu is used to capture aaRS, isotope labeled tRNA or amino acids are normally used. (Ribeiro *et al.*, 1995) Therefore, there is need to develop non-radiolabeling techniques to study aminoacylation in which EF-Tu is used in binding activated tRNA through the formation of a ternary complex.

The generation of a ternary complex of *Afu* ET-Tu using eukaryotic tRNA without having to use radioisotopes will provide an alternative safer method that could be applied in studying the detection of the activity of various eukaryotic aminoacyl-tRNA synthetases. Another goal of the project was to obtain a high resolution crystal structure of *Afu* EF-Tu in complex with its substrates (GDP, GTP or non-hydrolyzable analogs of GTP).

**Table 3.1:** Protein characteristics of *Afu* EF-Tu obtained using ProtParam program. (ExPASy proteomics server)

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</tr>
<tr>
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</tr>
<tr>
<td>pI</td>
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</tr>
<tr>
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</tbody>
</table>
First, the *Afu* EF-Tu gene was cloned and expressed with an N-terminal his-tag not only to aid in the purification using metal affinity chromatography but also to enable it to bind the nickel coated surface that will be used in the generation of the ternary complex with aa-tRNA. In-gel trypsin digestion and mass spectrometry analysis were used to identify the expressed protein. Screening for crystals has been carried out under different conditions. Also, in order to gain insights into the functional properties of *Afu* EF-Tu, several biochemical techniques were utilized in this study. Analytical gel filtration and dynamic light scattering experiments were applied to determine the oligomeric state of the enzyme in solution while ITC and fluorescence emission spectroscopy were applied in investigating the interactions of *Afu* EF-Tu with GTP and other substrates. Information obtained from structural and binding studies as well as the mechanism of GTP hydrolysis and tRNA binding during the ternary complex formation will be helpful in understanding how the enzyme functions in archaea and other prokaryotes.

### 3.2 Materials and Methods

#### 3.2.1 Reagents and Equipment

The gene coding for *Afu* EF-Tu was obtained from the American Type Culture Collection (amino acid sequence in Figure 3-2). Gateway® cloning reagents were purchased from Life Technologies (Carlsbad, CA). PCR and Plasmid DNA purification reagents, MiniElute and Miniprep, respectively were obtained from Qiagen (Valencia, CA). All other reagents were purchased from Fisher Scientific or Sigma-Aldrich® (St. Louis, MO).
3.2.2 EF-Tu cloning

3.2.2.1 PCR amplification of EF-Tu gene

The cloning of the *Afu* EF-Tu gene was carried out using Gateway® Technology. Forward and reverse primers were designed to include a TOPO cloning site (CACC). After amplification of the genes by PCR, the gene is inserted into the pENTR-D entry vector using TOPO assisted cloning. The next step was carried out through an LR clonase multiple enzyme reaction in which the gene is inserted into a pDEST-C1 destination vector before transforming into BL21 DE3* expression host cells.

**Figure 3-2:** Amino acid sequence of the *Afu* EF-Tu. The gene sequence was obtained from the NCBI website, Gene ID: AA90301.

PCR primers both forward (5′–CACC GCT AAG GAA AAG GAG CAC ATT AAT G–3′ Tm 54 °C) and reverse (3′–TTA TTT TCT TGG GGT AAG GTC GAG -3′ Tm 54 °C) were ordered in lyophilized form from Integrated DNA technology (IDT), (Coralville, IA). Working stock solutions of 250 µM concentration were made by dissolving the primers in Tris EDTA (TE) buffer (pH 8.0) to the primers. The primer mix solutions were made by diluting the stock primers to 10 µM final concentration for each primer. Gene amplification was achieved by KOD Hot Start DNA Polymerase (Millipore). For a complete PCR reaction the following components were needed: DNA
template, KOD DNA polymerase enzyme, dNTPs, and 10X PCR buffer containing magnesium ions.

The PCR reaction was set up by mixing all the components (Table 3.2) in a 0.2 mL PCR tube, mixed slightly and then vortexed briefly before they are placed in an Eppendorf Mastercycler Personal PCR machine.

**Table 3.2:** PCR reaction set up for *Afu* EF-Tu.

<table>
<thead>
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<th>PCR component</th>
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</tr>
</thead>
<tbody>
<tr>
<td>PCR grade water</td>
<td>21</td>
</tr>
<tr>
<td>Primer mix</td>
<td>3</td>
</tr>
<tr>
<td>DNA template</td>
<td>1</td>
</tr>
<tr>
<td>KOD start master mix</td>
<td>25</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>50</strong></td>
</tr>
</tbody>
</table>

**Table 3.3:** PCR program for *Afu* EF-Tu.

<table>
<thead>
<tr>
<th>Program</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activation</td>
<td>95</td>
<td>2 min</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>20 sec</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>53</td>
<td>10 sec</td>
<td>18</td>
</tr>
<tr>
<td>Extension</td>
<td>70</td>
<td>10 sec</td>
<td></td>
</tr>
<tr>
<td>Additional extension</td>
<td>70</td>
<td>2 min</td>
<td></td>
</tr>
</tbody>
</table>

The PCR product was analyzed on a 1 % (w/v) agarose gel prepared in 1X TAE (pH 8.0), 40 mM Tris-acetate, and 1 mM EDTA. An equal volume of a gel-loading
buffer (0.01 % (w/v) bromophenol blue, 40 % (v/v) glycerol and autoclaved Milli Q water were added to each sample to assist in loading the samples into the wells. The gels were run at 90 V for 80 minutes in the 1X TAE (pH 8.0) buffer. The gel was stained with DNA gel stain 0.5X SYBR Gold (Invitrogen), before visualization on a Typhoon Phosphorimager (GE Healthcare). Bands corresponding to the size of the Afu EF-Tu gene were cut out using a sterile blade before gel extraction following the protocol provided by the QiaQuick gel extraction reagents (Qiagen). The DNA samples were quantified by UV absorbance at 260 nm on an Agilent Spectrophotometer and then stored at -20 °C for further cloning steps.

3.2.2.2 Gateway Cloning of Afu EF-Tu

The overall cloning process for the Afu EF-Tu gene was carried out using the Gateway cloning technology. This multiple step process is outlined in Figure 2-3. The PCR product of Afu EF-Tu was first inserted into a pENTR-D with the help of a TOPO assisted CACC cloning site. The gene was swapped from the entry clone into a pDEST-C1 in a transposition reaction that utilizes an L-R clonase enzyme mix as outlined in Figure 2-3. The pENTR-D reaction was performed in a 0.2 mL PCR tube by mixing the components in Table 3.4 in buffer containing 1.2 M NaCl and 0.06 M MgCl₂. The tube was then incubated at room temperature for 5 minutes for the insertion reaction to proceed to completion.

The pENTR-D reaction was transformed in TOP10 cloning host cells and plated on agar + Ampicillin (100 µg/mL) plates for overnight growth of colonies. Several colonies were subsequently identified and inoculated into fresh LB media containing
Ampicillin (100 µg/mL and grown overnight. The plasmid was purified using the CTAB Miniprep protocol as described by Wilson, 1990 with modifications. (Wilson, 1990) Cells from an overnight culture were harvested by centrifugation at 4,000 rpm. Typically, 20 mL of cells were used. After centrifugation, the pellet was dissolved in 200 µL Lysis-STET-buffer containing 8 % Sucrose, 5 % Triton X-100, 50 mM EDTA, 50 mM Tris (pH 8.0) and lysozyme to a final concentration of 50 µg/mL lysozyme and incubated on ice for 30 min. The mixture was boiled for 90 seconds at 95 °C then, centrifuged for 10 min using a Beckman Coulter™ TJ-25 centrifuge at 15,000 rpm. A pipette loading tip was used to remove the pellet containing cell debris, protein, and genomic DNA from the supernatant. To the supernatant, 100 µL 5 % (w/v) Cetyltrimethylammonium bromide (CTAB) / 0.2 M NaCl was added and incubated for 3 min to precipitate RNA and plasmid DNA before centrifugation at 1,000 rpm for 15 min. At this step the pellet was resuspended in 300 µL 1.2 M NaCl containing 5 µg/mL RNase A, before incubation at 37 °C for 30 min. In order to precipitate RNA, 750 µL of 95 % ethanol was added to the mixture and incubated for 1 h at 30 °C before another centrifugation step at 10, 000 rpm. The pellet containing the plasmid was dried at 37 °C then resuspended in 50 µL 1X TE buffer. The purity of the pENTR-D plasmid was analyzed on a 1 % agarose gel to check the successful insertion of *Aflu* EF-Tu gene into the entry vector.
After a successful pENTR-D reaction, the LR-clonase enzyme mix was carried out to switch the *Afu* EF-Tu gene from pENTR-D to the destination vector pDEST-C1. The reaction was set up comprising the components shown in Table 3.5 in a 0.2 mL PCR tube. After the components were mixed in 1X TE buffer, comprised of 20 mM Tris HCl (pH 8.0) and 1 mM EDTA, the tube was incubated at room temperature for 1 hour for the LR for the reaction to go to completion. After the 1 hour incubation, Proteinase K was then added to digest any traces of LR enzymes at 37 °C for 10 minutes. At this step, the *Afu* EF-Tu was swapped from the entry vector into pDEST-C1, the destination vector.

**Table 3.4:** *Afu* EF-Tu pENTR-D reaction set up. Reaction was done in a PCR tube after which the components were mixed and incubated at room temperature for 5 minutes.

<table>
<thead>
<tr>
<th>pENTR-D reaction mixture</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Afu</em> EF-Tu PCR product</td>
<td>2</td>
</tr>
<tr>
<td>pENTR-D vector</td>
<td>1</td>
</tr>
<tr>
<td>Salt solution</td>
<td>1</td>
</tr>
<tr>
<td>Autoclaved water</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>6</strong></td>
</tr>
</tbody>
</table>

After a successful pENTR-D reaction, the LR-clonase enzyme mix was carried out to switch the *Afu* EF-Tu gene from pENTR-D to the destination vector pDEST-C1. The reaction was set up comprising the components shown in Table 3.5 in a 0.2 mL PCR tube. After the components were mixed in 1X TE buffer, comprised of 20 mM Tris HCl (pH 8.0) and 1 mM EDTA, the tube was incubated at room temperature for 1 hour for the LR for the reaction to go to completion. After the 1 hour incubation, Proteinase K was then added to digest any traces of LR enzymes at 37 °C for 10 minutes. At this step, the *Afu* EF-Tu was swapped from the entry vector into pDEST-C1, the destination vector.

**Table 3.5:** Set up for the *Afu* EF-Tu pDEST-C1 reaction. Reaction is incubated at room temperature for 1 hour minutes. The 1X TE buffer is comprised of 20 mM Tris HCl (pH 8.0) and 1 mM EDTA.

<table>
<thead>
<tr>
<th>pDEST-C1 reaction component</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDEST-C1 vector</td>
<td>1</td>
</tr>
<tr>
<td><em>Afu</em> EF-Tu /pENTR-D plasmid</td>
<td>3</td>
</tr>
<tr>
<td>LR Clonase enzyme mix</td>
<td>2</td>
</tr>
<tr>
<td>1X TE buffer to make</td>
<td>8</td>
</tr>
</tbody>
</table>
Transformation of *Afu EF-Tu /pDEST-C1* into cloning host

The LR reaction mixture was then transformed into *E. coli* cloning host cells Omnimax™ (Life Technologies, Grand Island, NY) following the CaCl$_2$ method as described in section 2.2.2.3. Briefly, the *Afu EF-Tu /pDEST-C1* plasmid was added to an aliquot of 50 µL of Omnimax™ (Life Technologies) cells in an Eppendorf tube on ice. The tube was flicked gently in order to mix the contents after which the tube was incubated on ice for 30 minutes. The cells were heat shocked for 42 seconds at 42 °C followed by incubation on ice for a further 2 minutes. At this step 250 µL of room temperature SOC medium was added and the cells shaken at 37 °C for 1 hr before plating on freshly made LB agar plates containing tetracycline (15 µg/mL). The plate was incubated overnight at 37 °C for colonies to grow. Single colonies were picked in the next day and inoculated into fresh LB medium and antibiotic for overnight growth before the cells were harvested for plasmid isolation. The plasmid containing *Afu EF-Tu* gene was isolated and purified using plasmid purification Miniprep reagents (Qiagen) according to the manufacturer’s instructions as described in section 2.2.2.2. The isolated plasmid was analyzed on a 1 % (w/v) agarose gel to verify the correct size of the pDEST-C1 plasmid containing the *Afu EF-Tu* gene.

3.2.2.3 Protein Expression of *Afu EF-Tu*

Small Scale Protein Expression

Once the pDEST-C1 plasmid containing the *Afu EF-Tu* gene was isolated from the cloning host, it was transformed into Tuner (DE3) expression host cells following the transformation procedure described in section 2.2.2.3. Protein expression studies were
carried out on a small scale, using 5-10 mL LB culture and growing to an appropriate 
OD\textsubscript{600} before induction. Briefly, a single colony of freshly transformed \textit{Afu} EF-Tu /pDEST-C1 LB gar + Streptomycin (50 µg/mL) plate was inoculated into 3 mL of LB medium containing antibiotic then grown overnight at 37 °C. The overnight culture was transferred to 10 mL of fresh LB medium+ Streptomycin and grown in a shaker at 37 °C until the OD\textsubscript{600} reached 0.6 before induction with 0.8 mM IPTG for 6 hours at 37 °C.

The cells were harvested by centrifugation at 4,000 rpm. Aliquots of cells at the time of induction (t= 0 hr) and at the end of induction (t= 6 hr) were taken out for analysis on an SDS-PAGE to check the expression of \textit{Afu} EF-Tu protein. The samples were mixed with 2X SDS buffer, boiled for 5 minutes and run on 4-12 % SDS-PAGE for 35 minutes at 200 volts before staining with Coomassie blue protein stain. A protein marker was also run alongside to help determine the relative molecular weight of the \textit{Afu} EF-Tu protein. The expression tests were carried out using two different cell lines: Tuner DE3 and BL21 (DE3)*. Sorbitol, betaine and M9 salts as expression additives were included in the medium to optimize protein expression. The LB medium with M9 salts combination was the best growth condition when Tuner DE3 cells were used. Therefore, all subsequent expression experiments using shaker flasks were done under these conditions.

\textbf{3.2.2.4 Large scale Protein expression of \textit{Afu} EF-Tu}

Two methods were utilized in growing the \textit{Afu} culture for protein expression. The first method involved the use of 2 L shaker flasks to grow the cultures while shaking. The
second method a batch reactor fermentor was utilized whereby oxygen control, pH, nutrients and temperature were maintained throughout the growth.

The LB medium enriched with M9 salts was prepared and autoclaved before use. Briefly, 10 mL of overnight cultures were prepared containing Streptomycin (50 µg/mL) 0.5 % glucose (w/v) and inoculated with freshly transformed bacterial colonies of *Afu EF-Tu* in Tuner (DE3) competent *E. coli* cells and incubated overnight at 37 °C. The overnight growth was transferred to 2 L shaker flasks with fresh LB + Streptomycin (50 µg/mL enriched with 200 mM sorbitol. The large scale culture was incubated at 37 °C while shaking at 220 rpm until OD$_{600}$ reached 0.4 before inducing with IPTG to a final concentration of 0.8 mM for 6 hours at 37 °C. The cells were harvested by centrifugation 4,000 rpm at 4 °C for 20 min using a Beckman Coulter™ TJ-25 centrifuge, frozen on dry ice and kept at -80 °C for further cell lysis experiments.

**Large scale production of *Afu EF-Tu* using BioFlo III batch fermentor**

Two liter batch fermentations were conducted in a New Brunswick BioFlo III fermentor (New Brunswick Scientific) with a 2 L working volume at 37 °C (**Figure 3-2**).
For each batch, an overnight inoculum a freshly transformed colony of *Afu* EF-Tu was transferred into the fresh culture of the batch reactor in the morning. Typically the cells were grown in autoclaved 1.8 L LB media + Streptomycin. The media was also enriched by adding 200 mL of 20 % lactose and trace elements which were sterilized separately. The pH of the culture was sustained at 7.0 by adding potassium phosphate buffer (50 mM KH$_2$PO$_4$/K$_2$HPO$_4$, pH 7.5) whenever it dropped below 6.5 while agitation was maintained at 270 rpm. Oxygen was supplied from a connected tank to maintain the aerobic conditions for cell growth while the growth of the cells was monitored by taking out 0.5 mL culture for UV measurements at OD$_{600}$. When the OD$_{600}$ reached 12, (typically the culture started becoming more turbid after 4 to 6 hours after inoculation) the culture was induced by 1 mM IPTG for 3 hours at 37 °C before harvesting the cells. The harvested cells were centrifuged using a Beckman Coulter™ TJ-25 centrifuge for 20 minutes kept at -80 °C until the lysis step.

**Figure 3-3:** BioFlo III benchtop 2 L batch reactor for overexpression of *Afu* EF-Tu. Expression conditions were controlled; temperature, oxygen and pH.
3.2.2.5 Confirmation of Afu EF-Tu protein expression

After running SDS-PAGE, the band corresponding to the Afu EF-Tu protein size was cut excised out in order to carry out the in-gel trypsin digestion and confirmation of the protein identity by MALDI-TOF/TOF MS as described earlier in section 2.2.2.5. Peptides from Afu EF-Tu protein by the MASCOT program with search parameters outline in Table 3.6. Theoretical mass lists of the possible peptides from Afu EF-Tu were generated by an in silico trypsin digest experiment using the “MS digest” function of ProteinProspector (University of California, San Francisco).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequence database</td>
<td>NCBI nr</td>
</tr>
<tr>
<td>Taxonomy</td>
<td>Archaea</td>
</tr>
<tr>
<td>Enzyme</td>
<td>Trypsin</td>
</tr>
<tr>
<td>Allowed miscleavages</td>
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</tr>
<tr>
<td>Fixed modifications</td>
<td>None</td>
</tr>
<tr>
<td>Variable modifications</td>
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</tr>
<tr>
<td>Peptide tolerance</td>
<td>0.3 Da</td>
</tr>
<tr>
<td>Peptide range</td>
<td>+1</td>
</tr>
<tr>
<td>Instrument</td>
<td>MALDI-TOF/TOF MS</td>
</tr>
</tbody>
</table>

3.2.3.6 EF-Tu Protein purification

Cell lysis

The pellet cells was thawed on ice and resuspended in lysis buffer 50 mM Tris-HCl (pH 7.5), 300 mM NaCl, 1 mM DTT, 1 mM PMSF and 50 μg/mL of hen egg-white lysozyme before shaking on ice for 1 hour. The lysate was then sonicated at 50 % duty cycle, 60 % power for minutes using the Branson™ Sonifier 250. The crude lysate was
centrifuged using a Beckman Coulter™ TJ-25 centrifuge at 18,000 rpm for 30 minutes. At this stage the protein of interest was in the supernatant.

**Heating step**

A heating step included after cell lysis of the *Afu* EF-Tu protein was used as a first purification step. The supernatant was heated in a hot bath at 70 °C for 30 minutes in order to precipitate out non-heat stable *E. coli* proteins present in the lysate. After the heat treatment, the lysate was kept on ice for 30 minutes before another centrifugation step at 18,000 rpm for 30 minutes. The supernatant was saved for the column purification.

**Ni-NTA metal affinity column purification of *Afu* EF-Tu**

The supernatant was filtered using a syringe filter before loading onto a 10 mL nickel affinity column filled with an Ni-NTA superflow resin (Qiagen) equilibrated with buffer A 20 mM NaH$_2$PO$_4$ (pH 7.4), 5 mM imidazole using Bio-Rad duoflow FPLC system at 3 mL/min. The column was washed with 5 column volumes of wash buffer B 20 mM NaH$_2$PO$_4$ (pH 7.4), 20 mM imidazole) followed by elution of EF-Tu with buffer C containing (20 mM NaH$_2$PO$_4$ (pH 7.4), 500 mM imidazole). Three column loadings were carried out per each 4 liter large scale preparation. Fractions collected were analyzed for purity using SDS-PAGE along with chromatograms. Peaks from the Ni-NTA column purifications were identified and pooled together. The fractions were concentrated to ~1 mL using the Amicon Ultra-15 centricon filter units with a 10,000 MWCO membrane (Millipore, USA). The concentration of the protein was obtained by UV spectroscopy at an OD$_{280}$.
Intact mass of *Afu* EF-Tu by MALDI-TOF/TOF MS

The protein was desalted in 10 mM ammonium acetate to thoroughly remove salts. To obtain MALDI data, 1 µL (0.5 mg/mL) of protein sample was mixed with 1 µL of saturated sinapinic acid dissolved in aqueous 0.1 % TFA. The sample was spotted on the MALDI target plate and data acquired as described in section 2.2.2.5. Protein calibration Standard II (Bruker) was used to calibrate the instrument prior to acquiring data.

3.2.3 Biochemical Studies

3.2.3.1 Determination of *Afu* EF-Tu oligomeric state by Analytical gel filtration

Analytical size-exclusion chromatography was done using a Superose-12 size exclusion column, equilibrated with a buffer containing 20 mM HEPES (pH 7.5), 100 mM KCl, 5 mM MgCl₂, 0.2 mM EDTA and 10 % glycerol. The purified *Afu* EF-Tu (V= 0.5 mL; [2 mg/mL]) was injected at a flow rate of 0.5 mL/min: The molecular weight standards were obtained from high molecular weight gel filtration calibration protein standards (Bio-Rad) containing the following thyroglobulin (660 kDa), immunoglobulin G (150 kDa), ovalbumin (45 kDa), and bovine myoglobin (17 kDa).

3.2.3.2 Determination of *Afu* EF-Tu percent polydispersity by Dynamic light scattering

The experiment to determine polydispersity of *Afu* EF-Tu followed the steps described in section 2.2.3.2. Briefly, protein samples (1– 2 mg/mL) were filtered using a 0.1 µM membrane filter and centrifuged briefly at 4 °C to eliminate bubbles. The experiment was carried out on a DynaPro Titan DLS (Wyatt Technology Corporation) instrument. Data were acquired at 25 °C for 10 seconds, repeated 20 times and then
averaged. Samples of the EF-Tu protein in the presence of (GTP or GDP) were also treated the same way as the protein alone. Dynamics 6.7.3 software was used to fit the calculated diffusion coefficient and hydrodynamic radius from the Stokes–Einstein equation.

3.2.3.3 Afu EF-Tu Tryptophan Fluorescence Quenching

A fluorescence spectrum of Afu EF-Tu protein was obtained at 25 °C using a PTI Spectrophotometer (Photon Technologies International). The protein was excited at 295 nm. The excitation and emission bandwidths were set at 4.0 nm and the emission spectra collected starting from 300- 400 nm. The protein concentration used was 5 μM in buffer containing 20 mM HEPES (pH 7.5), 100 mM KCl, 5 mM MgCl₂, 0.2 mM EDTA. Correction of fluorescence values was done based on the blank. Fluorescence spectra were also acquired in the same way to study the effects of GTP and GMP-PNP binding on Afu EF-Tu. Origin software version 7 (Origin Lab. Corp., MA, USA) was used to analyze the data. All data were corrected for background fluorescence and dilution effects.

3.2.3.4 Determination of nucleotide binding to Afu EF-Tu by Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) was used to study the interaction between Afu EF-Tu and GDP or GMP-PNP. Protein samples were dialyzed into the ITC buffer containing 20 mM Na/KPO₄ (pH 7.4), 100 mM KCl, 2 mM MgCl₂ while GDP and GMP-PNP were directly dissolved in the dialysis buffer. All solutions were degassed for 5
minutes. Titrations were carried out using a MicroCal ITC 2000 Microcalorimeter (Northampton, MA).

In a typical experiment, GDP or GMP-PNP (1 mM) was titrated into 20 μM Afu EF-Tu protein while the reference cell contained 1.5 mL of the corresponding buffer. Experiments were performed at 25 °C and 35 °C. The ITC experiments were set up as follows: 30 injections of 5 μL and 10 s except for the first injection that only contained 1 μL. The rotation speed of the syringe was 270 rpm, and the temperature of the cell 25 °C. Blank titrations as controls were first carried out using buffer-buffer run to give heat of injection. The GDP or GMP-PNP into buffer titrations were used to generate the heat of dilutions associated with ligand binding.

Another set of titrations were carried out using an alternative buffer 10 mM HEPES (pH 7.5), 20 mM MgCl₂ using under similar conditions and instrument parameters. The data was fitted to a single-site binding model by a non-linear regression analysis using Origin 7.0 to determine the binding parameters; dissociation constant (K_D), enthalpy of binding (ΔH) and the binding stoichiometry (N).

3.2.3.5 Initial crystallization experiments of Afu EF-Tu

_Afu_ EF-Tu alone and in complex with GTP the presence of (GTP or GMP-PNP) was screened for crystal growth at room temperature and at 4 °C against two commercial screens (Hampton Research). The Honeybee crystallization robot was used for tray set up. Crystallization screens were also set up as described in section 2.2.3.4 in 96 well round bottom Corning™ trays for screening the apo and ligand complex with protein concentration of 60 mg/mL of Afu EF-Tu. The nucleotide concentration was made to a
final concentration of 6 mM in the complex. The Honeybee robot (Genomic Solutions) was used to dispense both the 100 µL well solution as well as the 1 µL droplets. One hundred microliters were dispensed automatically into the well from the deep block well block solutions. On the shelf of the tray, 1 µL each of protein and well solution were dispensed by the synQuad tips. The tray was sealed using transparent tape and centrifuged for 30 seconds at 1000 rpm to mix the droplet contents. The trays were kept at room temperature and monitoring of crystal growth was checked frequently. Observations in the trays were done using a Nikon™ SMZ1500 microscope. Pictures of promising hits were taken using a Nikon™ CoolPix 990 digital camera.

3.2.4 Aminoacylation Assay development

3.2.4.1 Eukaryotic tRNA purification from beef liver

Purification of total beef liver tRNA was carried out at 4 °C following the procedure of Petrissant et al. (Petrissant, et al., 1971) Briefly, an aliquot of 100 g of frozen beef liver was homogenized in a blender for 180 sec in 200 mL of buffer containing 0.15 M sodium acetate (pH 4.5). A concentrated aqueous solution of phenol (140 mL) was added and homogenized for a further 180 sec. The homogenate was centrifuged for 40 min at 700 rpm. The aqueous layer was collected and re-extracted with 140 mL of phenol after which it was stirred vigorously on ice for 30 minutes. The homogenate was centrifuged for 40 minutes at 6000 rpm and the supernatant collected for further purification on a DEAE-Sephacel column. The supernatant was loaded onto a DEAE column that was pre-equilibrated with buffer containing 0.15 M sodium acetate, (pH 4.5). Once the tRNA sample was loaded the column was washed with 0.15 M
sodium acetate, (pH 4.5) containing 0.3 M sodium chloride. The tRNA was eluted with a high salt buffer (1 M sodium chloride). The fractions were collected (35 mL each) and the tRNA UV absorbance monitored at 260 nm. All fractions containing high tRNA concentrations were pooled together and precipitated with 95 % ethanol. The pure tRNA was quantified by UV absorbance at 260 nm and the aliquots were stored in 95 % ethanol at -80 °C until use.

3.2.4.2 Attempts to form Afu EF-Tu: GTP: aa-tRNA ternary complex

The four step assay that was utilized is summarized in Figure 3-4. The first step was the aminoacylation reaction done to generate His-tRNA\textsuperscript{His}. This was carried out in a reaction buffer containing 20 mM HEPES (pH 7.5), 1 mM MgCl\textsubscript{2} and 100 mM KCl.

\begin{center}
\includegraphics[width=0.5\textwidth]{figure3-4.png}
\end{center}

Figure 3-4: Scheme for the formation of EF-Tu: GTP: aa-tRNA ternary complex formation.
In a microcentrifuge tube 2.5 µM human HisRS, 0.05 µM tRNA, 200 µM histidine, 5 mM ATP and reaction buffer to make a total volume of 100 µL were mixed together and incubated at 30 °C for 20 minutes. Control reactions were: 1) without ATP and 2) human HisRS as the aaRS representative omitted. The reaction was stopped by adding 3 volumes (150 µL) of 90 % ethanol in 0.1M sodium acetate (pH 4.5) and incubating the reaction on ice for 30-60 minutes. The reaction tubes were centrifuged at 4 °C for 15 minutes and the supernatant discarded. The resulting white pellet was dried in a desiccator for 1 hr, after which the pellet was stored at -20 °C. Step two involved the formation of the EF-Tu GTP complex. This was carried out by mixing 20 µM purified *Afu* EF-Tu, 200 µM GTP, 1 mM phosphoenol pyruvate and 0.02 mg/mL pyruvate kinase in the reaction buffer 20 mM HEPES (pH 7.5), 1 mM MgCl₂, 100 mM KCl. The contents were mixed together and incubated on ice at 37 °C for 10 minutes shaking gently.

Once the EF-Tu GTP binary complex was generated, the next step was to generate the ternary complex of EF-Tu GTP: His-tRNA^{His} ternary complex in the third step. The white pellet of His-tRNA^{His} from step 1 was dissolved in 25 µL deionized water in a microcentrifuge tube. To the tube, 10 µL 10X binding buffer 200 mM HEPES (pH 7.5), 10 mM MgCl₂, 1 M KCl and 40 µL of the EF-Tu::GDP complex from step 2 were added and the reaction mixture incubated at 30 °C for 10 minutes to generate the ternary complex. Then 30 µL of 25 % nickel resin suspension was added to the tube. The reaction tube was shaken at room temperature for 10 minutes before placing on ice. The complex was washed with 1 mL 10X binding buffer 20 mM HEPES (pH 7.5), 1 mM MgCl₂, and 100 mM KCl. This was done by centrifugation at 4 °C for 1 minute discarding the supernatant after each wash step. The washing step was done three
additional times. The ternary complex was eluted with 50 µL of buffer containing 0.5 M imidazole (pH 6.5), 0.1 M NaCl, 1 mM MgCl₂ by centrifugation for 1 minute at 4 °C. The ternary complex was after this step assumed to be in the supernatant.

In order to detect the formation of the ternary complex, about 50 µL of supernatant containing the ternary complex were pipetted into a 384 microtiter plate and SYBR Gold nucleic acid dye was added to a final concentration of 0.3X (180 nM). Another sample was put in a plastic transparent cuvette and SYBR Gold added to the same final concentration of 0.3X (180 nM). Fluorescence was measured on a BioTek™ Synergy™ H4 Hybrid Microplate Reader at an excitation wavelength of 495 nm, emission 537 nm. The background fluorescence resulting from the dye alone was compared to the fluorescence from the ternary complex in the presence of dye. The concentration of SYBR Gold was estimated from the molar extinction coefficient of 50,000 M⁻¹cm⁻¹ based on the literature values of cyanine dyes. (Haugland, 1996) After measuring its absorbance the assumption was made where a 1X dye concentration is equivalent to 0.6 µM. The sensitivity of the SYBR Gold dye binding to tRNA crude extract was determined by measuring the fluorescence at different dye concentrations.

3.3 Results and Discussion

3.3.1 Expression and purification of Afu EF-Tu

The gene carrying EF-Tu was cloned into a pDEST-C1 vector and amplified using PCR. The PCR product was purified and analyzed on a 1 % (w/v) agarose gel to verify the size. From Figure 3-5, it can be seen that the correct size of 1271 bp is apparent when compared to the 100 bp DNA ladder.
In a two step cloning process using Gateway technology, the gene was inserted into the entry vector pENTR-D via TOPO assisted cloning. The results are shown in Figure 3-6 panel A. A band corresponding to the expected size of 3.85 Kb product is indicated in lane 2 when compared to the 2-10 Kb supercoiled DNA ladder. The LR clonase reaction was used to insert the gene into the cloning vector pDEST-C1 to generate the expression clone. The 5.0 Kb product from the LR reaction was verified by analysis on a 1 % (w/v) agarose gel using a 2-10 Kb supercoiled DNA ladder as shown in lanes 2 and 3 of Figure 3-6 panel B. After successful entry into the expression vector, EF-Tu expression studies were carried out.

Figure 3-5: SYBR Gold™ stained 1 % agarose gel of the PCR product of *Afu* EF-Tu. Lane 1: 100 kb ladder. Lanes 2-3: *Afu* EF Tu PCR.
The expression clone in pDESCT-C1 was transformed into expression host *E. coli* BL21 (DE3)* competent cells in LB containing streptomycin. Induction of protein expression was done by adding 1 mM IPTG at an OD$_{600}$ of 0.4 for 4 hours at 37 °C. The confirmation of protein expression was carried out by running an SDS-PAGE. Once the protein expression conditions were optimized, large scale expression was carried out under the same conditions to produce more material for FPLC purification. After centrifugation, lysis and heat treatment, the protein was purified in one step on a Ni-NTA metal affinity FPLC column. The eluted protein was concentrated and quantified while the purity was checked by SDS-PAGE. The results of the purification are represented in Figure 3-7 and Figure 3-8.

**Figure 3-6:** SYBR Gold™ stained 1 % agarose gel of cloning of *Afu* EF-Tu. (A) pENTR-D entry vector insertion reaction. Lane 1: 2-10 Kb supercoiled DNA ladder. Lane 2: EF Tu *Afu* in pENTR-D plasmid with the correct expected size of 3.85 Kb in Lane 2. (B) EF-Tu insertion into pDEST-C1 destination vector. Lane 2: DNA Ladder. Lane 3-4: EF-Tu/pDEST-C1 with the expected size 5.0 Kb. The vector pDEST-C1 size is 5335 bp; ccdB dead gene is 1600 bp swapped out with the EF-Tu gene that is 1272 bp.
From the SDS-PAGE (Figure 3-7) the purification of EF-Tu was achieved on the Ni-NTA column. It should be noted that the protein was expressed with a 6His-tag at the N-terminus which played a role in the metal affinity purification making it possible to get pure and soluble material with one column purification. The heating step after cell lysis was also very important in achieving the initial separation from unwanted proteins. This is normally applied to the majority of proteins whose source is from thermophiles.

Figure 3-7: Ni-NTA metal affinity purification chromatogram for *Afu* EF-Tu. The arrow in red shows the peak corresponding to pure eluted *Afu* EF-Tu protein with green and purple traces corresponding to absorbance at 280 and 260, respectively. The red arrow represents the peak for EF-Tu and the yellow arrow represents the impurities (other proteins and nucleic acids). EF-Tu fractions represented by the red rectangle were pooled, concentrated down and analyzed on SDS-PAGE (Figure 3-8).
3.3.2 Characterization of Afu EF-Tu by MALDI-TOF/TOF MS

The next step was to confirm the identity of the protein. The band corresponding to the size of EF-Tu was excised from the gel. The EF-Tu peptides were generated by in-gel tryptic digestion followed by identification using MALDI-TOF/TOF MS. The mass spectrum of the EF-Tu tryptic digest (Figure 3-9) was obtained in the positive ion mode and peptides (Table 3-7) were identified by comparison to data from the MASCOT search database following the protocol described earlier in section 2.2.2.5. In order to obtain the intact mass of Afu EF-Tu by MALDI-TOF/TOF MS, the protein was desalted.

Figure 3-8: SDS-PAGE of the purification of Afu EF-Tu from Ni-NTA affinity column. Lane1: t= 0 hr. Lane 2: t= 4 hr. Lane 3: crude extract. Lane 4: lysate before heat. Lane 5: lysate after heat. Lane 6: Protein Marker. Lane 7: flow through. Lane 8-15: Fractions 6-13 from the Ni-NTA column purification (Figure 3-7).
in 10 mM ammonium acetate thoroughly using Amicon™ filters (MILLIPORE) to remove salts. MALDI-TOF/TOF MS data was obtained when 1 µL of the protein sample was mixed with 1 µL of a saturated solution of sinapinic acid dissolved in aqueous 0.1 % TFA. The sample was spotted onto the MALDI target plate and data acquired as described in section 2.2.2.5. Protein calibration Standard II (trypsinogen, 23 kDa; Protein A, 44 kDa and Bovine serum albumin, 66 kDa (Bruker)) was used to calibrate the instrument prior to acquiring data and the spectrum is presented in Figure 3-10. In the results there is a peak at $m/z$ 46,976.681. This peak differs in mass from the calculated mass of the intact protein (Table 3.1) probably this may be attributed to the presence of post-translational modifications of the expressed protein. A second peak at $m/z$ 23,532.888 was also seen and since it is much lower than the $(M+2H)^{2+}$ it could be a product of proteolysis or disulfide linkage in the protein.
Figure 3-9: MALDI-TOF/TOF MS of in-gel tryptic digest of *Afu* EF-Tu. A total of twenty peptides were positively identified with 60% sequence coverage as shown in the Appendix B.
Figure 3-10: MALDI-TOF/TOF MS results showing the intact mass of EF-Tu.
3.3.2.2 Determination of *Afu* EF-Tu oligomeric state by Analytical Gel Filtration

Analytical gel filtration was performed to determine the oligomeric state of EF-Tu using a Superose-12 size exclusion column. Calibration was achieved using protein molecular mass standards described in section 2.2.3.1. The results show that EF-Tu is a monomer in solution with an observed molecular weight of 48.9 kDa as represented in *Figure 3-11*. The calculated molecular weight is of EF-Tu 47.3 kDa. This result lies within the monomeric size range for EF-Tu. This confirmed the monomeric form of the enzyme in agreement with the crystal structures of EF-Tu enzymes from other species.

### Table 3.7: Peptides identified from trypsin digestion of *Afu* EF-Tu by MALDI-TOF/TOF MS. The peptide sequences with their respective observed mass values in Da are shown against the theoretical mass values.

<table>
<thead>
<tr>
<th><em>Afu</em> EF-Tu peptide</th>
<th>Sequence position</th>
<th>m/z&lt;sub&gt;calc&lt;/sub&gt;</th>
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<th>ΔM</th>
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<td>227-245</td>
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<tr>
<td>SIEMHHEPIQEAYPDDNIGFNVR</td>
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<td>2653.246</td>
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<td>RGDVAGHPDNPPTVVK</td>
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<td>1517.403</td>
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<tr>
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<td>382-392</td>
<td>1340.735</td>
<td>1340.353</td>
<td>-0.382</td>
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</table>
3.3.2.3 Determination of Afu EF-Tu percent polydispersity by Dynamic Light Scattering

About 1 mg/mL of protein was used in the dynamic light scattering experiment at 25 °C. The EF-Tu alone showed a hydrodynamic radius of 4.1 nm a polydispersity of 12.2 % with a relative molecular weight of 90 kDa. When the experiment was done in the presence of GTP, a hydrodynamic radius of 4.4 nm was obtained with a relative molecular weight of 108 kDa.

Figure 3-11: Determination of apparent molecular weight of Afu EF-Tu by analytical gel filtration. Protein standards are represented by blue diamonds while EF-Tu is represented by red square.
The DLS results show a percent polydispersity of less than 15 which implies that EF-Tu can crystallize in solution, however, the molecular weight results are inconsistent with the monomer results obtained from the gel filtration experiment. It is not clear if the dimeric size obtained is due to the aggregation of EF-Tu or indeed is a dimer when in solution since the relative molecular weight was 2 times higher than the expected weight of ~47 kDa. Similar results were also obtained when GTP was added in the protein solution giving a dimer instead of a monomer size.

![Figure 3-12](image)

**Figure 3-12:** Dynamic light scattering of *Afu* EF-Tu alone. In solution EF-Tu shows a reasonable crystallizable polydispersity range of less than 15 % with a molecular weight of 90 kDa.
After homology modeling and amino acid analysis of EF-Tu using available EF-Tu crystal structures from other species, it was established that *Afu* EF-Tu has two tryptophan residues, one in the catalytic domain (Trp58) and the Trp201 in the nucleotide binding region. The model in Figure 3-14 is based on the *Thermus thermophilus* EF-Tu structure (PDB ID: 1EXM).

The protein was therefore excited at 295 nm and emission spectra obtained between 300- 400 nm. To determine the extent of quenching of the two intrinsic

<table>
<thead>
<tr>
<th>R (nm)</th>
<th>%Pd</th>
<th>MW-R (kDa)</th>
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</thead>
<tbody>
<tr>
<td>4.4</td>
<td>11.3</td>
<td>108</td>
</tr>
</tbody>
</table>

**Figure 3-13:** Dynamic light scattering of *Afu* EF-Tu: GTP complex. The complex in solution shows a reasonable crystallizable polydispersity range of less than 15%.

**3.3.2.4 *Afu* EF-Tu Tryptophan Fluorescence Quenching**

The complex in solution shows a reasonable crystallizable polydispersity range of less than 15%.
tryptophan residues of EF-Tu in native state the EF-Tu fluorescence spectrum was obtained in the presence of GTP and its non-hydrolysable analog GMP-PNP.

Results presented in Figure 3-15 show that quenching was evident when GTP was bound. Figure 3-15 panel B represents the GTP dependent concentration versus the change in fluorescence up to 500 μM GTP. Since the *Afu* EF-Tu structure is not available structure from *Thermus thermophilus* (Figure 3-14) was used as the model to show the distance between the tryptophan residues and the GTP binding site. Trp195 is 9.4 Å the closet to the nucleotide site and it is assumed to be the one that resulted in the significant quenching that was observed. The second residue, Trp200 appears far away about 20 Å
from the GTP binding site. As expected, the quenching is as a result of the residue that lies close to the active site, Trp195. It is unclear whether both tryptophans in *Afu* EF-Tu Trp58 and Trp201 contributed to the fluorescence quenching equally or one if one completely more buried inside than the other. Another observation that can be made is on the amount of GTP used during the experiment shows that saturation was not reached since the plot of change in fluorescence versus GTP concentration was still linear at 400 µM GTP.

Figure 3-15: *Afu* EF-Tu GTP binding measured as change in fluorescence versus GTP concentration. (A) Fluorescence emission spectra of EF-Tu increasing amount of GTP. (B) Linear plot of change in fluorescence vs. GTP concentration.

It has been previously demonstrated in *T. thermophilus* and *T. aquaticus* that EF-Tu binds GMP-PNP nearly as well as it binds GTP. (Wagner et al, 1995) Studies of EF-Tu properties *in vitro* have shown that the enzyme’s affinity for GDP is high (nM range) almost two orders of magnitudes more than GTP because of the low dissociation rate. (Fasano, *et al.*, 1978) However, on the other hand, the binding affinity of charged tRNA to the GTP-bound EF-Tu varies almost ten times, and this is mainly dependent on the
nature of the amino acid involved. (Louie et al., 1984) The reason for these affinity differences from the structural perspective however, is still not understood. Mutational analysis experiments of the residues involved in the nucleotide binding pocket will be helpful in understanding the structure function relationship of EF-Tu and its substrates.

3.3.2.5 Determination of nucleotide binding to Afu EF-Tu by Isothermal Titration Calorimetry

The ITC experiment was performed to determine the binding of GDP and GMP-PNP to EF-Tu. The titrations of GDP or GMP-PNP into the EF-Tu protein solutions elicited exothermic heats ranging from 0-0.6 µcal/sec for all the 30 injections. Each injection comprising of 5 µL of ligand, however, in all the attempted titrations with either GDP or GMP-PNP saturation could not be reached. Therefore, the results from the ITC experiment for EF-Tu binding to both nucleotides (GDP or GMP-PNP) could not give interpretable data (not shown). Attempts to carry out the titrations at a higher temperature condition (35 °C) also resulted in similar data that could not be successfully analyzed for binding interpretation.

3.3.2.6 Afu EF-Tu crystallization screens

Crystallization attempts for EF-Tu were carried out using two different conditions. In the first set up EF-Tu alone was screened for crystal growth while additional set ups involved the screening of EF-Tu in complex with GMP-PNP (the non-hydrolysable analog of GTP). There were no crystal hits under both sets of conditions. Most of the droplets indicated signs of protein aggregation which could not develop into crystals even after several weeks.
3.3.3 *Afu* EF-Tu: GTP: aa-tRNA Ternary complex formation

The first step in exploring the possibility of the ternary complex formation involved testing the binding of the purified tRNA to SYBR Gold™ sensitivity. This was evaluated by measuring fluorescence emission of dye in the presence of tRNA. Figure 3-14 represents the results of fluorescence emission measurements with varying amounts of dye ranging from 0.05X to 0.8X which corresponds to 30–480 nM. From the results, it is shown that the fluorometer measurements using a quartz cuvette (Figure 3-16 panel A and Figure 3-17) gave a higher intensity of up to ~5000 after subtracting the buffer measurements. On the other hand, the measurements using a self-masking microtiter well plate, the fluorescence intensity was much lower (Figure 3-16 panel B). The SYBR Gold™ concentration was calculated based on the manufacturer’s supplied stock concentration of 10,000X using a molar extinction coefficient of 50,000 M⁻¹cm⁻¹ as reported by Haugland. (Haugland, 1996)

After establishing that the dye can be detected on a fluorometer in the 0.05-0.1X which corresponds to 30-60 nM range based on the assumed theoretical molar extinction coefficient of 50,000 M⁻¹cm⁻¹ the next step was to set up the reaction for generating the EF-Tu GTP: aa-tRNA ternary complex. This was done by adding the crude tRNA to the GTP bound EF-Tu as described in the methods section 3.2.4.2.

**Principle of the experiment:**

After forming the ternary complex using EF-Tu, GTP, aaRS, amino acid and tRNA, it was envisaged that the aaRS in the presence of a cognate amino acid and tRNA will generate an activated aa-tRNA molecule. Since GTP-bound EF-Tu has a high
affinity for aa-tRNA, it will bind to it hence forming the ternary complex. The *Afu* EF-Tu was expressed with a 6His-tag at the N-terminus which was to be utilized in binding to the nickel functionalized resin. After the EF-Tu was bound to the resin all other non-specific components in the reaction were washed off including the unbound tRNA from the reaction. The complex was eluted off by washing with 0.5 M imidazole. The detection was achieved by adding SYBR Gold and measuring fluorescence (Table 3.8). The fluorescence intensity measured was assumed to be directly proportional to the amount of activated tRNA bound to the SYBR Gold dye.

**Figure 3-16:** Fluorescence measurement to determine sensitivity of tRNA to SYBR Gold™ dye. (A) 50 nM crude tRNA and SYBR Gold™ dye (60-400 nM) mixture was placed in a quartz cuvette and fluorescence measured. (B) 50 nM crude tRNA and dye sample mixed in a 384 well microtiter plate and fluorescence measured using a plate reader at an excitation of 495 nm and emission of 537 nm. (1X of dye = 600 nM)
Figure 3-17: Fluorescence emission spectra of SYBR Gold™. The dye alone in buffer solution at concentrations starting from 0.05 to 0.25X (30–150 nM) was excited at 495 nm and emission spectra acquired at 537 nm in order to assess the sensitivity of tRNA dye (SYBR Gold™).
To detect the amount of tRNA in the ternary complex after elution, the reaction samples were taken out and applied onto a TBE gel for analysis. Results are shown in Figure 3-18. In control experiments either EF-Tu or tRNA or aaRS were omitted. Attempts to detect the aminoacyl-tRNA from both set ups (microcentrifuge or spin column) after isolating the ternary complex resulted into very low sensitivity. Fluorescence values obtained (Table 3.8) were not significant enough to make a distinction between the full assay and that resulting from the control assays. Therefore, at

**Table 3.8:** Fluorescence emission of aa-tRNA in the EF-Tu: GTP: aa-tRNA ternary complex. The fluorescence intensity (arbitrary units) was carried out by applying 50 µL of the eluted sample into a 384 corning microtiter plate. (A) The experiment was done in a 0.5 L microcentrifuge tube. (B) The experiment was done with in 0.5 mL Red dot Ultrafree spin column. B13- C14 represents the microplate well position.
this stage it will not be possible to conclude that the formation of EF-Tu ternary complex was successful based on the results that were obtained. The challenge for the assay arose from the difficulty to form aminoacyl-tRNA that is required to bind to the EF-Tu during the aminoacylation step. The partitioning of total tRNA and the activated tRNA still remains the most crucial step for the success of this set up especially since total crude tRNA was used instead of pure tRNA$^{His}$.

More attempts will be made in order to isolate the ternary complex using properly charged tRNA and the aminoacylation step could be verified by the conventional isotope techniques. After a successful formation of the ternary complex, the next step will be to obtain the complex binding constants by considering the three reactions represented in equations 9-11 based on the amounts of EF-Tu and tRNA used.

\[
\text{aa-tRNA} \xrightarrow{K_{\text{free}}} \text{amino acid + tRNA} \quad (9)
\]

\[
\text{aa-tRNA: EF-Tu GTP} \xrightarrow{K_{\text{bound}}} \text{amino acid + tRNA + EF-Tu GTP} \quad (10)
\]

\[
\text{aa-tRNA + EF-Tu GTP} \xleftrightarrow{K_{\text{assoc}}} \text{aa-tRNA: EF-Tu GTP} \quad (11)
\]

Pingoud et al., have shown that the equilibrium between the activated GTP-bound EF-Tu and aminoacyl-tRNA is fast when compared that of the deacylation of aminoacyl-tRNA. (Pingoud et al., 1973) The observation forms the basis as to why EF-Tu effectively prevents charged aminoacyl-tRNA from competing with uncharged tRNAs for their cognate aminoacyl-tRNA synthetases which are known to bind both.
3.4 Conclusions

The main goal was to explore the application of *Afu* EF-Tu in developing a non-radiolabeling assay that can be used to study aminoacylation by utilizing the formation of the EF-Tu: GTP: aa-tRNA ternary complex. *Afu* EF-Tu protein was successfully cloned, expressed with an N-terminal 6His-tag and a considerable amount of purified soluble protein was obtained using one purification step by Ni-NTA metal affinity column chromatography. The identity of the expressed protein was confirmed by MALDI-TOF/TOF MS analysis after in-gel trypsin digestion. Initial results have shown that 6His-tagged EF-Tu in the presence of GTP is able bind to the nickel resin. This is important
because the GTP-bound EF-Tu will be utilized to capture activated tRNA thus forming the desired ternary complex. However, in an attempt to form the ternary complex in the presence of aaRS and purified beef liver tRNA the experiment was unsuccessful. The quantification of tRNA from the ternary complex that was generated could not be achieved owing to the very low fluorescence measured even though up to 50 nM TRNA was used as compared to ~100 nM SYBR dye.

Several biochemical experiments including analytical gel filtration, dynamic light scattering and fluorescence spectroscopy were carried out to characterize the *Afu* EF-Tu enzyme. Analytical gel filtration confirmed that the enzyme is a monomer in solution. Percent polydispersity data obtained from dynamic light scattering show that the enzyme is crystallizable. Fluorescence spectroscopy was used to obtain data on how quenchers affect the solvent accessibility of the two intrinsic tryptophan residues. It should be noted that the fluorescence measurements were also carried out in the presence of non hydrolysable analog of GTP, GMP-PNP as a control. Studies on the binding of GDP to EF-Tu using ITC did not yield the desired results since all the titrations carried out were only able to yield isotherms without reaching saturation of ligand binding. Titrations were carried out with two different temperature conditions (25 °C and 35 °C) and under a variety of buffer conditions. It was established that the HEPES and sodium phosphate buffers are able to give stable ligand into buffer titrations at pH 7.4 and 7.5 thus being the only promising condition under which future experiments could be tried.
3.5 Future Perspectives

For the development of the aminoacylation detection assay, the main challenge has been to obtain activated tRNA. Therefore, the formation of the EF-Tu: GTP: aa-tRNA experiments have not been concluded. Initial experiments in this study have shown the possibility of using SYBR Gold to detect tRNA recovered from the ternary complex up to 50 nM. The next step will be to address the formation of aa-tRNA that can be complexed with EF-Tu in the presence of GTP. Formation of the ternary complex using pure tRNA will be applied to validate the performance the proposed assay (Figure 3-19). Additionally, the stability of the ternary complex will be determined by carrying out the experiment under different ionic strengths.

To validate the potential of this technique a similar experiment using radiolabeled amino acids will be carried out and a comparison of how much tRNA is bound to the EF-Tu complex done since detection with radioisotopes can go as low as picomolar tRNA concentration. This method once established and validated will be helpful, since it can be applied as a non-radiolabeling assay for screening aminoacyl-tRNA synthetase activity and potentially developed for high throughput screening of aaRS inhibitors.
Figure 3-19: Proposed scheme for the formation of the EF-Tu: GTP: aa-tRNA ternary complex using nickel-affinity coated micro-plate assay.

1. Apply GDPPNP bound EF-Tu to plate nickel coated surface
2. Wash off unbound EF-Tu
3. Aminoacyl-tRNA synthetase + cognate amino acid + ATP to generate aa-tRNA\textsuperscript{aa}
4. Bind aa-tRNA to EF-Tu to form ternary complex
   \((\text{EF-Tu} \bullet \text{GDPPNP} \bullet \text{aa-tRNA}^{\text{aa}})\)
5. Wash off unbound tRNA
6. Add tRNA dye, visualize aa-tRNA\textsuperscript{aa} bound on the EF-Tu
Chapter 4

Biochemical Characterization of *Trypanosoma cruzi* Prolyl-tRNA Synthetase

4.1 Introduction

4.1.1 Prolyl-tRNA synthetase

Prolyl-tRNA synthetase is a member of the class II aminoacyl-tRNA synthetase family. It activates proline in the presence of ATP through the formation of Proline-AMP and transfers the activated proline to the 3’OH of pro-tRNA$^{Pro}$, as shown in equations 12 and 13. Elongation factor Tu binds aminoacyl-tRNA and delivers it to the ribosome for the protein synthesis.

\[ \text{Pro} + \text{ATP} \rightarrow \text{Pro-AMP} + \text{PPi} \quad (12) \]

\[ \text{Pro-AMP} + \text{tRNA}^{Pro} \rightarrow \text{AMP} + \text{Pro-tRNA}^{Pro} \quad (13) \]

Part of the accuracy in aminoacylation is achieved by the ability of the aaRS to discriminate between cognate and non-cognate amino acids and cognate tRNAs. The accuracy of amino acid selection is 1 in $10^4$ to $10^5$ and for the cognate tRNA, 1 in $10^6$ (Fersht, 1981; Jakubowski & Goldman, 1992). To correct errors in aminoacylation, aaRS
have developed pre-transfer editing, where non-cognate aminoacyl-adenylates are hydrolyzed and post-transfer editing mechanisms, in which the incorrectly aminoacylated tRNA is hydrolyzed (Jakubowski & Fersht, 1981). Editing activities in aaRS were first reported in class I and class II aaRS including isoleucyl-tRNA synthetase, leucyl-tRNA synthetase, threonyl-tRNA synthetase, (Dock-Bregion et al., 2000), prolyl-tRNA synthetase (Beuning & Musier-Forsyth, 2000; Beuning & Musier-Forsyth, 2001) and phenylalanyl-tRNA synthetase (Roy et al., 2004). ProRS possesses pre-transfer and post-transfer editing activities and this has so far been reported for alanyl-adenylate in all three kingdoms of life (Beuning & Musier-Forsyth, 2001). Crystal structures solved for prolyl-tRNA synthetases are summarized in Table 4.1. This information provides a basis for understanding the structural and biochemical function of ProRS.
Trypanosoma cruzi, a flagellated protozoal parasite, is the etiological agent of Chagas disease. It is transmitted into the human host by Triatomine insects popularly known as “Kissing bugs” during a blood meal. The disease is endemic in Central and South America and it is estimated that over 20 million people are affected. (Clayton, 2010) As important components of the protein translation machinery, aaRS have become targets for designing drugs that target parasites and bacteria.

The amino acid sequence identities of most aaRS enzymes from trypanosomal organisms and their human counterparts are in the range of 30 % and below making these
enzymes structurally diverse among species. (Merrit et al., 2010) Analysis of different ProRS across species (Figure 1-4) reveals diverse structural arrangements of the domains including the variable presence of an insertion domain. These differences in domain arrangement and diverse residues in the nucleotide binding site could be utilized in designing inhibitors of ProRS as potential antibacterial and antiprotozoal drugs. For example, Mupirocin, which targets bacterial Isoleucyl-tRNA synthetase, is used as a topical antibiotic to treat skin infections caused by gram positive organisms, including MRSA (Hughes & Mellows, 1978).

4.1.1 Project Goal

Aminoacylation requires a high level of accuracy to ensure incorporation of the correct amino acid into the growing polypeptide. *E. coli*, archael and human ProRS do not distinguish easily between cysteine, proline and alanine during amino acid activation and in some cases, aminoacylation. (Ahel et al., 2002; Ahel et al., 2003; Beuning & Musier-Forsyth, 2000; Beuning & Musier-Forsyth, 2001) The enzyme requires an editing mechanism to remove incorrectly activated amino acids. In protozoa, such as *Trypanosoma*, ProRS contains an N-terminal extension that may participate in editing.

The goal of this project is to understand the mechanism of the editing activity of *T. cruzi* ProRS. The significance of understanding how this enzyme carries out its editing activity in *T. cruzi* will widen the scope knowledge about the functional properties of ProRS. The information gained will be helpful in developing drugs that inhibit *T. cruzi* ProRS.

To achieve the objective of this study, a codon optimized gene of *T. cruzi* was constructed by GenScript (Piscataway, NJ) for expression in *E. coli*. A summary of *T.
*cruzi* ProRS gene and protein parameters obtained from the ExPASy proteomics server using the ProtParam program as presented in Table 4.2. The full length protein is 726 amino acid residues with a slightly acidic pI. After the ProRS protein was expressed and purified, a series of experiments were carried out. Analytical gel filtration, isothermal titration calorimetry (ITC), partial digestion of the native enzyme with trypsin and studies of the protein’s enzymatic activity were performed as part of the proteins physical and enzymatic characterization. MALDI-TOF/TOF mass spectrometry and a pyrophosphate release assay were also carried out in order to obtain functional properties of ProRS.

Table 4.2: Properties of *T. cruzi* ProRS.

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</tr>
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4.2 Materials and Methods

4.2.1 Reagents and Equipment

A codon optimized synthetic gene encoding for *Trypanosoma cruzi* Prolyl-tRNA synthetase (ProRS) was constructed for expression in *E. coli* by GenScript (Piscataway, NJ). Reagent for PCR product and plasmid DNA purification (MiniElute and Miniprep) were obtained from Qiagen (Valencia, CA). EnzChek® pyrophosphate assay reagents
were from Life Technologies (Carlsbad, CA). Other reagents were from Fisher Scientific or Sigma-Aldrich® (St. Louis, MO).

### 4.2.2 ProRS cloning into expression vector

The codon optimized gene coding for *T. cruzi* ProRS was initially cloned into pDEST-C1 vector to be expressed as a fusion protein with a polyhistidine element at the N-terminus using the Gateway® cloning in *E. coli*. Although this fusion protein was expressed in large quantities, it was insoluble under a number of conditions of bacterial growth and extraction. However, a plasmid construct in pET12a encoding a form of the protein without any additional elements fused to it was readily expressed in *E. coli* (Tuner strain); growing the bacteria at temperature of 17 °C proved to a critical requirement for solubility.

Large scale protein expression was performed in a 10 L Virti-Culture Laboratory Fermentor equipped with a refrigerated circulating water birth and the culture was purged with air. Cells were grown in LB medium with M9 salts, 1 % glucose (w/v) and Ampicillin (100 µg/mL). A 100 mL of overnight culture was inoculated with freshly transformed bacterial colonies of ProRS in Tuner (DE3) competent *E. coli* and incubated for overnight at 37 °C. The overnight culture growth was used to inoculate a 10 L culture. Temperature was maintained at 37 °C until OD$_{600}$ reached 0.4- 0.6. The temperature was lowered to 17 °C and IPTG was added to 0.8 mM followed by growth for 12 hours. Cells were harvested by centrifugation at 4 °C for 20 min., the pellets washed in phosphate buffer saline and stored at -80 °C.
4.2.3 ProRS Protein purification

Preliminary studies were performed to identify the best conditions for solubility of ProRS. All steps were performed at 0-4 °C. Partially thawed cells (81 grams) were suspended at 10 mL buffer per gram of cells with a Dounce homogenizer in 50 mM Tris-HCl (pH 7.5), 10 % glycerol (v/v), 2 mM DTT, 1 mM PMSF, and 0.1 % Triton X-100 (v/v) and 50 µg/mL of hen egg-white lysozyme per volume. (NH₄)₂SO₄ was added to 0.25 M and MgCl₂ was added to 15 mM. After 30 minutes, the suspension was centrifuged 15,000 rpm for 30 minutes and the supernatant was used for polyethylene glycol fractionation. Polyethylene glycol 8000 (PEG8000) was added as a 50 % (v/v) to the extract to 7.5 % (v/v); precipitated material was removed by centrifugation. Polyethylene glycol was added to the supernatant to 17.5 % (v/v) and the precipitate containing ProRS was collected by centrifugation. The precipitate was dissolved in Poros HQ anion exchange buffer A35 (mM Tris-HCl, (pH 7.5), 10 % glycerol (w/v), 35 mM (NH₄)₂SO₄, 0.2 mM EDTA, 1 mM DTT) and loaded onto the 30 mL column equilibrated with buffer A. The flow rate was 2 mL/min. After applying the sample, the column was washed with five column volumes of buffer A. ProRS eluted in the material that did not bind to the column. Fractions containing ProRS were combined and concentrated fourfold using an Amicon® Ultra-15 Centricon filtration device (Millipore) with 10,000 MWCO filters by centrifugation at 4,000 rpm at 4 °C. The concentrated material was dialyzed against 20 mM HEPES (pH 7.5), 10 % glycerol (w/v), 0.2 mM EDTA, and 1 mM DTT for 12 hrs. This material was applied to a 20 mL column of SP-Sepharose equilibrated in the same buffers as used for dialysis. The column was washed with two column volumes of starting buffer and eluted with a ten column volume linear
gradient of \((\text{NH}_4)_2\text{SO}_4\) from 0 to 0.25 M in 10 column volumes. ProRS eluted early in the gradient at \(~0.08\text{ M (NH}_4)_2\text{SO}_4\). Fractions containing ProRS were concentrated, quick frozen on dry ice and stored at \(-70^\circ\text{C}\). Figure 4-1 summarizes the steps for protein purification.

\[\text{Cell lysis} \downarrow \]
\[\text{Polyethylene glycol fractionation} \downarrow \]
\[\text{Poros HQ} \downarrow \text{Analysis by SDS-PAGE} \]
\[\text{SP- Sepharose} \downarrow \text{Analysis by SDS-PAGE} \]
\[\text{Superose-12 size exclusion column} \downarrow \text{Analysis by SDS-PAGE} \]
\[\text{Pool fractions from Superose-12 and concentrate}\]

**Figure 4-1:** Purification scheme that was followed in obtaining soluble ProRS.

**4.2.4 Confirmation of ProRS protein expression**

After SDS-PAGE, the species corresponding to the ProRS protein was excised for in-gel trypsin digestion as described in section 2.2.2.5. Samples were prepared by mixing 1 \(\mu\text{L}\) of the peptides after trypsin digestion mixed with the matrix, \(\alpha\)-cyano-4-hydroxycinnamic acid in the ratio of 1:2. The mixture was placed on a MALDI plate and air-dried for 15 minutes. MALDI-TOF/TOF spectra were acquired using a
UltrafleXtreme MALDI-TOF/TOF mass spectrometer equipped with a smartbeam II laser (Bruker Daltonics, Billerica, MA) as described in section 2.2.2.5.

Peptides from ProRS protein were examined using the MASCOT program with search parameters in section 2.2.2.5 (Table 2.6) with taxonomy set at “other alveolata” and peptide 0.3. Theoretical mass lists of peptides from ProRS digestion by trypsin were generated by using the “MS digest” function of ProteinProspector (University of California, San Francisco).

4.2.5 Biochemical Studies

4.2.5.1 Determination of ProRS oligomeric state by Analytical Gel Filtration

Analytical gel filtration was performed using a Superose-12 column, equilibrated with 20 mM HEPES (pH 7.5), 100 mM KCl, 5 mM MgCl₂, 0.2 mM EDTA and 10 % glycerol (v/v). Molecular weight standards (Bio-Rad) were thyroglobulin (660 kDa), immunoglobulin G (150 kDa), ovalbumin (45 kDa), and bovine myoglobin (17 kDa).

4.2.5.2 Determination of ATP binding to ProRS by Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) was used to study interactions between ProRS and ATP. ProRS samples were passed through Sephadex G-50 medium (GE Healthcare) equilibrated in 20 mM HEPES-NaOH (pH 7.5), 100 mM KCl, 5 mM MgCl₂, and 0.2 mM EDTA immediately before the experiment. Mg-ATP was prepared in the same buffer. Protein and ATP solutions were degassed for 5 minutes using reduced pressure.

The titrations were performed using a MicroCal VPITC Microcalorimeter (Northampton, MA) at 25 °C. The instrument and experimental parameters were: an
initial injection of 1 μL of 600 μM Mg-ATP over 2 seconds; subsequent injections were 5 μL over 10 seconds; stir rate was 270 rpm; the temperature of the cell 25 °C; ProRS was 20. The heat of dilution was determined from a separate bland titration of ATP into buffer. Data was analyzed using Origin 7.0 with a one site binding model.

4.2.6 Studies of trypsin cleaved ProRS

4.2.6.1 Trypsin digestion of ProRS

Digestion of ProRS was performed on ice in 20 mM HEPES-NaOH (pH 7.5), 100 mM KCl, 5 mM MgCl₂, 0.2 mM EDTA, 5% glycerol (w/v) 0.5 mM DTT. One set of reactions was performed in the absence of ligands and a second in presence 5 mM Mg-ATP and 1 mM proline, for 7 hours and 20 hours. Trypsin concentration ranging from 0-2 µg/mL was used. Times of incubation and trypsin concentration are indicated in the respective figures. After the reaction the aliquots were analyzed by SDS-PAGE. The cleaved enzyme was loaded onto a Superose-12 size exclusion column (24 mL bed volume) equilibrated with 20 mM HEPES-NaOH (pH 7.5), 100 mM KCl, 5 mM MgCl₂, 0.2 mM EDTA, 10% glycerol (w/v) 0.5 mM DTT run at a flow rate of 0.5 mL/min. Fractions of 0.5 mL were collected and analyzed by SDS-PAGE. Fractions containing the major fragments were concentrated for subsequent analysis using MALDI-TOF MS. Adenylate formation assay was performed using intact and trypsin cleaved ProRS.

4.2.6.2 MALDI-TOF/TOF MS analysis of intact and fragments after cleavage

ProRS that had been subjected to limited Trypsin cleavages and Superose-12 size exclusion chromatography was used for MALDI-TOF/TOF MS analysis. After dialysis against 10 mM ammonium acetate, 1 μL of protein sample (~5 nM) was mixed with 1 μL
of a saturated solution of sinapinic acid dissolved in 0.1 % TFA and spotted on the MALDI plate. The MALDI-TOF/TOF MS spectra were acquired as described in section 3.2.3.6.

4.2.6.3 ProRS pyrophosphate assay

EnzChek Pyrophosphate assay reagents (Life Technologies, Carlsbad, CA) were used to monitor the activity by measuring amount of inorganic pyrophosphate released in the adenylate forming step catalyzed by amioacyl-tRNA synthetases in the forward direction (Equation 1). PPi was converted to Pi by inorganic phosphatase (Figure 4-2) and phosphate was detected by the conversion of amino-6-mercaptop-7-methylpurine ribonucleoside (MESG) to 2-amino-6-mercaptop-7-methylpurine catalyzed by purine nucleotide phosphorylase (Webb, 1992).
Assays were performed using a Varian Cary 50 Bio UV/Visible Spectrophotometer (McKinley Scientific Sparta, NJ). A one 1 mL ProRS Pyrophosphate assay reaction in a 1 mL quartz cuvette was set up by adding the components as follows: amino acid (1 mM), inorganic pyrophosphatase (0.03 U/mL), ATP (250 µM), DTT (1 mM), purine nucleoside phosphorylase (1 U/mL), MESG (0.2 mM) and ProRS (2 µM). The reaction buffer comprised of 50 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, and 0.1 mM NaN₃. PPI formation was monitored by measuring the absorption at 360 nm at 30 °C. Measurements were done in triplicate and the readings from the blank reaction (one without amino acid) were subtracted as background absorption. Activity measurements

Figure 4-2: Adenylate formation assay. The enzymatic conversion of MESG to ribose 1-phosphate and 2-amino-6-mecapto-7-methylpurine results in a shift in UV-absorption maximum from 330 nm to 360 nm. (Modified from Webb, 1992)
were performed at different concentrations ATP and proline until the dynamic range for this assay was determined. To examine the specificity of purified intact and trypsin cleaved ProRS, the assay was performed with seven non-cognate amino acids cysteine, threonine, lysine, glutamic acid, aspartic acid, alanine and glycine and one ketoacid (α-ketoglutarate).

4.3 Results and Discussion

4.3.1 ProRS protein expression and purification

The gene carrying ProRS was initially amplified and cloned into a pDEST-C1 vector which would allow for expression of ProRS as a fusion protein with a poly histidine tag on the N-terminus that can be removed proteolytically. Although the plasmid was correctly constructed for expression, the protein was insoluble under all growth and solution conditions tested. A second plasmid was constructed in pET12a that directed the synthesis of the ProRS without the fusion tag. This plasmid directed the synthesis of active, soluble enzyme; high levels of expression depended on growth temperature (17 °C) and the appropriate bacterial expression strain (Tuner DE3). The protein was purified by column chromatography and the results of the Superose-12 purification results shown in Figure 4-3. Confirmation of the expressed protein was done by trypsin in-gel digestion and MALDI-TOF/TOF MS data was obtained in the positive ion mode; peptides were identified by MASCOT search database and as shown in Figure 4-4 and Table 4.3.
Figure 4-3: SDS-PAGE of ProRS expression after cloning into pET12a. The protein was expressed in Tuner DE3 *E. coli* cells before purification by Q-Sepharose and Superose-12 columns. Lane 1: Protein Marker. Lane 2: Flow through. Lane 3-6 fractions collected from Superose-12 column. Lane 7: lysate from Q-Sepharose after concentration.
Figure 4-4: MALDI-TOF/TOF MS of in-gel tryptic digest of ProRS. A complete list of the peptides identified and the sequence coverage provided in Table 4.5 while the sequence coverage is shown in Appendix B.
4.3.2. Determination of ProRS oligomeric state by Analytical Gel Filtration

Analytical gel filtration to determine the oligomeric state of ProRS was performed on a Superose-12 size exclusion column. The calculated dimer molecular mass calculated based on the sequence is 162 kDa; in accord with this value, the molecular mass obtained from analytical gel filtration is 168 kDa. This result is in accord with the classification of this enzyme in dimeric class II aaRS.
Figure 4-5: Determination of apparent molecular weight of ProRS by analytical gel filtration. ProRS (V= 0.5 mL; [2 mg/mL]) was injected at a flow rate of 0.5 mL/min and eluted at 22 °C. Protein standards are represented by blue diamonds, intact ProRS (red square), large trypsin cleaved fragment (black triangle) and small trypsin cleaved fragment (black square). Inset is the SDS-PAGE of purified ProRS.
4.3.3 Determination of ATP binding to ProRS by Isothermal Titration Calorimetry

To study ATP binding to ProRS by Isothermal titration calorimetry, Mg-ATP was titrated into ProRS at 25 °C as shown in Figure 4-6. ATP binding to ProRS is exothermic at 25 °C. The data was fit with a one-site binding model to obtain the thermodynamic parameters, \( N = 0.54 \pm 0.1, K = 2.21 \times 10^4 \pm 3.9 \times 10^3 \text{ M}^{-1}, \Delta H = -9,000 \pm 20 \text{ kcal mol}^{-1}, \Delta S = -10.3 \text{ cal deg}^{-1} \). From the molar ration value obtained it can be postulated that the ATP binding observed equals to one molecule per dimer since the ~0.54 binds a single subunit in the ProRS dimeric enzyme.

![Figure 4-6: Isothermal titration calorimetry of ProRS with Mg•ATP. Mg•ATP (600 µM) was titrated into ProRS (20 µM) at 25 °C as described in Experimental Procedures. The upper panel represents the instrumental response and the lower panel represents integrated data and the fit (solid line) to a one site binding model. Inset: is the summary of the thermodynamic parameters obtained.](image-url)
Some of the class II aaRS including ProRS although being symmetric homodimers, they exhibit a negative cooperativity during substrate binding. (Fersht, 1975) While studying the asymmetry of dimeric aaRS enzymes Lazdunski et al. described a “flip flop” type of subunit interdependence during adenylation reaction that results in a negative cooperativity mechanism of binding. (Lazdunski, 1971) In the mechanism, Lazdunski et al. describes alternating functions for each of the two nucleotide binding sites whereby when one binds ATP, it inactivates the second site’s inability to binding a second ATP molecule. Fersht while studying the tyrosyl-AMP formation on the other hand described a different mechanism for the “half-of-the sites” model in the tyrosyl-tRNA synthetase from Bacillus stearothermophilus. (Fersht, 1975)

In this case it is postulated that during the binding of ATP and tyrosine to the each site in the dimeric enzyme, a symmetrical complex of aaRS-(aa-AMP₂) is formed at the first site that results into a sterically hindrance to binding of the second site. Based on the ITC results in which ~0.5 molecule of ATP binds per monomer of ProRS this could imply that the two molecules are binding only to the first site, although this possibility will have to be confirmed by carrying out more kinetic experiments. It can therefore be postulated that the kind of binding exhibited in T. cruzi ProRS could be the case in which binding of ATP to site one is inhibiting the second site in a negative cooperativity manner. This still remains a possible explanation for the ITC results.

However, this is not conclusive since there could be a possibility of just one half of the enzyme being inactive in solution during the titrations. If so, this could be attributed to the inactivation of the protein; some aggregated material was being observed at the end of the each ITC titration experiment. More information from kinetic
experiments and the availability of a crystal structure depicting ATP/proline binding in the active site of *T. cruzi* ProRS will shed light on this phenomenon.

### 4.3.4 Modification of ProRS by proteolytic trypsin cleavage

#### 4.3.4.1 Trypsin Proteolysis

Limited proteolysis using trypsin was applied to show the changes in the pattern and fragmentation of ProRS due to conformational changes of the exposed amide bonds or flexible regions within the protein upon ligand binding. MALDI TOF-TOF MS was applied to analyze the mass sizes of the proteolytic fragments. The effect of substrate protection of native ProRS from trypsin cleavage was carried out in the presence of ATP (5 mM) and proline (2 mM). The control reaction was one in which no substrate (neither ATP nor proline) was added during the digestion. The first reaction was done by carrying out the digestion on ice for 7 hrs before stopping the reaction. The intact protein and the fragments were analyzed by SDS-PAGE (*Figure 4-7*). Lanes 2-7 represent the reactions in which there was no ligand added prior to the digestion. Lanes 8-12 are the reactions in which ATP and proline were added. The same experiment was repeated on ice but digestion done for a longer time for at least 20 hours (*Figure 4-8*).

From the SDS-PAGE, there is evidence of trypsin being protected from completely cleaving ProRS in reactions that contained ATP and proline. There is a continuous decrease in the band size for the original ProRS and an increase in band size for the proteolytic products. Another observation was that the extent of cleavage is proportional to the amount of trypsin used in the reaction. The amount of trypsin used highest (2 µg/mL) resulted into a higher extent of cleavage as shown in lanes 7 and 12, *Figure 4-7*. 

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The trypsin modified form was separated on a Superose-12 size exclusion column (Figure 4-9) and the eluted fractions were analyzed on an SDS-PAGE (Figure 4-10). Based on the results from the size exclusion column, there are two major fragments. The main proteolytic product is the fragment that elutes near the apparent molecular weight of IgG (150 kDa) and it is possibly a dimer. The second product most likely a monomeric fragment eluted close to myoglobin (17 kDa) as seen on the SDS-PAGE gel Figure 4-10.
Figure 4-7: SDS-PAGE after trypsin cleavage of ProRS in the absence or presence of ATP and proline for 7 hours. Reactions were performed in 20 mM HEPES-NaOH (pH 7.5), 100 mM KCl, 5 mM MgCl$_2$, 0.2 mM EDTA, 5 % glycerol (w/v) 0.5 mM DTT on ice.

Figure 4-8: SDS-PAGE after trypsin cleavage of ProRS in the absence or presence ATP and proline for 20.5 hours. Reactions were performed in 20 mM HEPES-NaOH (pH 7.5), 100 mM KCl, 5 mM MgCl$_2$, 0.2 mM EDTA, 5 % glycerol (w/v) 0.5 mM DTT on ice.
Figure 4-9: Elution profile of trypsin cleaved ProRS. The fragments separated on a Superose-12 size exclusion column equilibrated in 20 mM HEPES-NaOH (pH 7.5), 100 mM KCl, 5 mM MgCl₂, 0.2 mM EDTA, 5 % glycerol (w/v) 0.5 mM DTT and analyzed by SDS-PAGE. The elution position of protein standards and the intact ProRS are indicated by red arrows on the figure. The elution of cleaved ProRS is shown by fractions 1-11 on the figure.
The trypsin cleavage fragments were analyzed by MALDI-TOF/TOF MS to determine their molecular mass; the MALDI-TOF/TOF mass spectra are shown in Figure 4-11, Figure 4-12 and Figure 4-13. The results are in agreement with those obtained from the gel filtration experiment. The larger fragment produced two peaks one at \( m/z \) 59,854.837 and another at \( m/z \) 29,922.764. Analysis of the smaller fragment yielded a peak at \( m/z \) 19,985.854 although it was not observed on the elution profile but surprisingly, a band corresponding to its molecular weight appears on the SDS-PAGE of ProRS trypsin cleaved fragments. The fragments were separated on Superose-12 size exclusion column (Figure 4-9) and fractions 1-10 were analyzed by SDS-PAGE. Lane I represents the intact ProRS and lane T is the trypsin cleaved ProRS before loading on the column.

4.3.4.2 Characterization of ProRS trypsin cleaved products by MALDI-TOF/TOF MS

The trypsin cleavage fragments were analyzed by MALDI-TOF/TOF MS to determine their molecular mass; the MALDI-TOF/TOF mass spectra are shown in Figure 4-11, Figure 4-12 and Figure 4-13. The results are in agreement with those obtained from the gel filtration experiment. The larger fragment produced two peaks one at \( m/z \) 59,854.837 and another at \( m/z \) 29,922.764. Analysis of the smaller fragment yielded a peak at \( m/z \) 19,985.854 although it was not observed on the elution profile but surprisingly, a band corresponding to its molecular weight appears on the SDS-PAGE.
PAGE gel. Additionally, other minor fragments appeared at the bottom of the SD-PAGE gel but also could not be eluted on the column. These mass value of intact ProRS obtained was 81,406 Da.

**Figure 4-11**: MALDI-TOF/TOF MS spectrum of the intact ProRS.
Figure 4-12: MALDI-TOF/TOF MS spectrum of the large fragment of trypsin cleaved ProRS.
Trypsin was used in modifying the ProRS enzyme. Trypsin cleaves peptide bonds from the carboxyl terminus before lysine or arginine except when either is preceded by proline. The amino acid sequence of ProRS has 55 lysines and 50 arginine residues and all of them are evenly distributed in the entire sequence. Residues that are more solvent exposed will tend to be more accessible for the proteolytic cuts. More analysis of the fragments is required to establish if the main tryptic sites are located at the N-terminal or C-terminal regions and how if they are solvent accessible during substrate binding. Trypsin digestion in the presence of ATP or proline alone will be carried out to probe

**Figure 4-13:** MALDI-TOF/TOF MS showing the smaller fragment of the trypsin cleaved ProRS.
further their effects individually on the solvent accessibility of the active site residues of ProRS.

**4.3.5 Pyrophosphate assay using intact and trypsin cleaved ProRS**

Despite there being a lot of information on the aminoacylation studies on prolyl-tRNA synthetases, there are no studies on the *T. cruzi* ProRS. In this experiment, the objective was to investigate the enzyme’s ability to form adenylate with non-cognate amino acids and a ketoacid besides the cognate amino acid proline by applying a spectrophotometric method. The pyrophosphate assay was originally described by Webb in 1992 for measuring phosphate release kinetics in biological systems (Webb, 1992). When coupled to an inorganic pyrophosphatase enzyme the sensitivity of this reaction is amplified to enable quantification of inorganic phosphate. Upson and coworkers previously applied this spectrophotometric assay to study the release of ATP during Acetyl-CoA synthetase and Luciferase reactions whereby ATP released was measured to fempto level (Upson *et al.*, 1996).

In the assay, adenylate formation generates pro-AMP and PPi with the PPi being converted to 2Pi by inorganic pyrophosphatase. The Pi is then used by purine nucleotide phosphorylase to convert amino-6-mercapto-7-methylpurine ribonucleoside (MESG) to 2-amino-6-mercapto-7-methylpurine. PPi released is estimated from the amount of 2-amino-6-mercapto-7-methylpurine (11,000 M⁻¹cm⁻¹ at 360 nm) formed (Webb, 1992). The amount of PPi released is one half of the Pi formed since one molecule of PPi is converted to two Pi by inorganic phosphatase. The assay was used to test the release of PPi during the Pro-AMP formation at 30 °C and pH 7.4 by monitoring product formation through absorbance at 360 nm. The initial pyrophosphatase assay focused on the
basal absorbance of the ProRS reaction in the absence of any amino acid and there was a substantial low absorbance measurement. The results indicated represent the different reactions that were carried out by varying the amount of amino acid and ProRS (Figure 4-14). Different conditions were assayed besides using proline which is the cognate amino acid for ProRS other different non-cognate amino acids were used in the study as shown in Figure 4-15 and Figure 4-16. The trypsin cleaved and intact of ProRS activity was also determined.

Figure 4-14: Time-course for pyrophosphate release. The 2 µM intact ProRS enzyme with proline was used. The concentration of proline is indicated on the figure.
Figure 4-15: Time-course for pyrophosphate release in the presence of non-cognate amino acids and a ketoacid. The ProRS concentration used was 2 µM while 1 mM amino acid concentration was used. The respective amino acids are indicated on the figure.
In order to characterize the substrate properties, attempts were made to estimate the kinetic parameters of ProRS amino acid activation. Obtaining $K_M$ values for proline using Michaelis-Menten standard approach was inadequate because under the experimental conditions in this study, high amounts of proline would be needed much more than the 1 mM that was used. A summary of the enzyme turnover rates for pyrophosphate release when ProRS was used to activate the different amino acids is represented in Table 4.4. The results clearly show that the turnover number is greatly reduced when the cleaved fragment was used for the reaction. Interestingly, both the

**Figure 4-16**: Time-course for pyrophosphate release in the presence of non-cognate amino acids and a ketoacid using the trypsin cleaved ProRS. The ProRS concentration used was 2 µM while 1 mM amino acid concentration was used. The respective amino acids are indicated on the figure.
intact ProRS and the cleaved fragment still showed the ability to activate all the amino acids used including the α-ketoglutarate under this study. Another curious observation from the results is that the fragment has a comparatively a higher activity for cysteine and proline (Figure 4-17) than the intact protein under the same conditions.

**Table 4.4:** Summarized ProRS activity with proline and non-cognate amino acids. Activity for formation of AMP- using the intact versus the cleaved fragment of ProRS enzyme was determined. Measurements were done with proline and also the listed amino acids. The control experiment was the assay reaction without amino acid.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>PPI release turn over number (sec⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Intact</strong></td>
</tr>
<tr>
<td>Cys</td>
<td>1.0</td>
</tr>
<tr>
<td>Thr</td>
<td>0.9</td>
</tr>
<tr>
<td><strong>Pro</strong></td>
<td><strong>0.75</strong></td>
</tr>
<tr>
<td>Lys</td>
<td>0.74</td>
</tr>
<tr>
<td>Glu</td>
<td>0.73</td>
</tr>
<tr>
<td>Asp</td>
<td>0.73</td>
</tr>
<tr>
<td>Ala</td>
<td>0.68</td>
</tr>
<tr>
<td>Gly</td>
<td>0.21</td>
</tr>
<tr>
<td>α-KG</td>
<td>0.2</td>
</tr>
</tbody>
</table>

The reported turnover rates for the *E. coli* ProRS activation of amino acids are as follows: proline, 12-70 sec⁻¹; alanine, 1.7 sec⁻¹ and cysteine, 0.02 sec⁻¹. (Beuning & Musier-Forsyth, 2001; Ahel *et al.*, 2002) In comparison with our results, the *T. cruzi* ProRS both the intact and cleaved form had relatively low turnover rates for proline and the non-cognate amino acids that were investigated with the exception of cysteine. The result from this study however, concurs with the already known fact that ProRS misactivates both alanine and cysteine. Focus now shifts on understanding the
misactivation of non-cognate tRNA and how these misactivated molecules are edited in order to attach correct ProRS substrates.

Figure 4-17: Bar graph representation of the relative pyrophosphate activity of ProRS with non-cognate amino acids and a ketoacid. The intact ProRS (black bars); trypsin cleaved ProRS fragment (blue bars).

While class I aaRS share a common editing domain, the Connective peptide 1 (CP1), it has been shown that class II members like ThrRS, PheRS and AlaRS employ distinct editing mechanisms in hydrolyzing non-cognate amino acids and tRNA. (Cusack et al., 1998; Beuning & Musier-Forsyth, 2000; Wong et al., 2002) The E. coli ProRS achieves pre-transfer editing of Ala-AMP and post-transfer editing of Ala-RNA^{Ala} because of the presence of a unique insertion domain, “INS” as described previously in Figure 1-4. (Beuning & Musier-Forsyth, 2000) More resent results from the E. coli
ProRS experiments have also described that the pre-transfer editing of cysteine proceeds via an intramolecular cyclization mechanism similar to the one reported for class I members, MetRS and LysRS. (So et al., 2011)

It is unclear for how T. cruzi ProRS carries out the editing functions of misactivated cysteine, alanine. Therefore, more studies are required to be carried out using a variety of non-cognate amino acids in attempts to understand the editing mechanism of misactivated amino acids before tRNA is bound. Previous findings from the Plasmodium falciparum ProRS, another lower eukaryote show that the enzyme has the ability to hydrolyze Ala-tRNAPro (Ahel et al., 2003) Findings from this study presents a platform for investigating further this editing mechanism. In addition, the unexpected observation on how ProRS may be activating non-amino acids will be probed further. Results from these studies will be used to provide full information that could be applied in carrying out aminoacylation assays in the presence of tRNA. Similar studies have been reported with E. coli HisRS and other dimeric aaRS in which it was shown that during the burst phase tRNA helps to delay the formation of the second adenylate until after the first aminoacyl-tRNA is in place. (Guth & Francklyn, 2007) Thus, the tRNA may be attenuating the rate of adenylate synthesis in the first subunit while speeding up the rate in the second subunit. It will be interesting to know what observations will be found out when this experiment is carried out in the presence of tRNA using both the intact and the trypsin cleaved fragment of ProRS.

**Proposed T. cruzi ProRS-tRNA synthetase mechanism**

All aaRS use three substrates: amino acid, ATP and tRNA to generate aminoacyl-tRNA, AMP and PPi during the aminoacylation reactions. ArgRS, GluRS, GlnRS and
LysRS follow a sequential mechanism where all the substrates must first bind to the enzyme before release of any of the products (Allende et al., 1970; Bovee et al., 2003; Ravel et al., 1965). The aaRS that activate the amino acid in the absence of ATP use a random or ordered bi-uni uni-bi ping pong type mechanism (Wahab & Yang, 1985). A possible mechanism for *T. cruzi* ProRS (Figure 4-18) that is modeled along the reported *E. coli* ProRS where the reaction proceeds in a bi-uni bi-ping pong mechanism in two steps. (Freist & Sternbach, 1988; Papas & Mehler, 1971)

![Potential random bi-uni uni-bi ping-pong mechanism for aminoacylation reaction of T. cruzi ProRS.](image)

**Figure 4-18:** Potential random bi-uni uni-bi ping-pong mechanism for aminoacylation reaction of *T. cruzi* ProRS. E = ProRS enzyme; Pro = proline.

In the first step, the carboxylic acid substrate reacts with ATP to generate prolyl-adenylate and PPI. In the second step the prolyl-adenylate intermediate reacts with 2’ OH of the ribose on the 3’-end of tRNA to form Pro-tRNA.Pro and AMP. In this study tRNA was not used therefore, the results so far presented are restricted to the first step of aminoacylation, the formation of ProRS-AMP. Further assays will be carried out to establish the non-cognate amino acid activation of *T. cruzi* ProRS when tRNA.Pro is present will still exhibit the same pattern of promiscuity with different amino acids. Other
class II aaRS that utilize this similar enzyme mechanism have been known to activate amino acids only in the presence of tRNA. In future, the comparison of the T. cruzi X-ray crystal structure of the enzyme adenylate complex plus examining the substrate specificity using ATP analogs will be provide a better understanding of this mechanism.

4.3.6 Characterization of tRNA by MALDI-TOF/TOF MS.

The technique MALDI-TOF/TOF MS has been used extensively in the study of proteins and peptides. In the recent times the technique has gained usefulness in the study of oligonucleotides both RNA and DNA. However, DNA has been studied more with MALDI compared to RNA. In this study MALDI-TOF/TOF MS was used to analyze crude tRNA extract in a view of identifying individual tRNA species. Two matrices were applied (3-HPA and DHB) and the results are represented in Figure 4-19 and Figure 4-20. Transfer RNAs are typically made up of 60-95 nucleotides with an average of 75 nucleotides. The average molecular weight of an individual tRNA is about 25 kDa. (Kim et al., 1973) Table 4.5 represents the m/z values of the major peaks that were acquired from the MALDI experiment for tRNA. Although we were able to identify the peak corresponding to tRNA mass by MALDI, the challenge however remains how we can be able to narrow down and identify the individual tRNA.

Separating of the different tRNA species in the crude mixture was complicated by the fact that all the species present are similar in many respects; size, charge and structure. The only possible way to separate them will be to try and capitalize on the hydrophobicity of each tRNA but still here the challenge will be that all the species of interest have to be modified in order to achieve this. A three dimensional representative
of Proline-tRNA$_{\text{Pro}}$ from *Thermus thermophilus* is shown in Figure 4-21 it is 77 nucleotides long with a molecular weight of 25,095 Da.

Table 4.5: Summary of MALDI-TOF/TOF MS results for crude tRNA. Values represent the $m/z$ values from the major peak acquired using 3-HPA as the matrix. Molecular weight of a typical tRNA is ~25,000 Da. (Kim et al., 1973)

<table>
<thead>
<tr>
<th>Peak #</th>
<th>$m/z$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24,071.143</td>
</tr>
<tr>
<td>2</td>
<td>24,185.164</td>
</tr>
<tr>
<td>3</td>
<td>24,656.452</td>
</tr>
<tr>
<td>4</td>
<td>25,095.952</td>
</tr>
<tr>
<td>5</td>
<td>25,128.578</td>
</tr>
<tr>
<td>6</td>
<td>25,144.827</td>
</tr>
<tr>
<td>7</td>
<td>25,230.685</td>
</tr>
</tbody>
</table>
Figure 4-19: MALDI-TOF/TOF MS results of purified beef liver tRNA with 3-HPA as matrix. All the different presumed m/z values for different tRNAs shown as a broad peak. Inset is the enlarged peak shown with grey arrow.
Figure 4-20: MALDI-TOF/TOF MS results of crude tRNA using DHB matrix.
4.4 Conclusions

The motivation for this study came from our interest in trying to understand the *T. cruzi* ProRS editing mechanism. This enzyme in other species has been described to be activating non-cognate amino acids (cysteine and alanine) before hydrolyzing them in a pre-transfer editing mechanism. The recombinant ProRS has been successfully cloned, expressed and purified. Using in-gel trypsin digestion and MALDI-TO/TOF the identity of the protein was confirmed. The dimeric form of ProRS, as it is with the homologs of

![Figure 4-21: Representative Proline-tRNA\textsuperscript{Pro} nucleotide sequence from *Thermus thermophilus*. (Henne et al, 2004) Structure obtained from tRNA database, University of Leipzig ID: tdbD00008439.](image)
this enzyme in other species that have been studied so far was determined and confirmed by analytical gel filtration. The mass of the intact protein successfully determined using mass spectrometry. Isothermal titration calorimetry was used to determine the thermodynamic binding parameters of ATP to ProRS and it was established to be an exothermic reaction. The information obtained from the ATP binding to *T. cruzi* ProRS lays the foundation for determining how other ligands will bind in the active site.

Attempts to obtain hits from the crystallization screens were unsuccessful for both the apo and complex form of ProRS. When MALDI-TOF/TOF MS was carried out in order to characterize the different tRNA species, the RNA was able to ionize but the spectrum showed one broad peak meaning we were unable to identify the individual tRNAs. Since a mixture of crude tRNA was used making it made it more challenging to obtain the spectra with a distinct peak showing the desired tRNA\textsuperscript{Pro}.

Trypsin proteolysis of the native ProRS resulted into two major fragments of ProRS and established by SDS-PAGE and mass spectrometry analysis. Findings from the adenylate assay with proline and non-cognate amino acids show reduced activity in the trypsin cleaved ProRS by more than half as compared to the intact ProRS. In conclusion, the adenylate formation assay described in this study has yielded one surprising observation, ProRS activating more non-cognate amino acids and a ketoacid. Previous studies in *E. coli* have only shown the enzyme being able to activate cysteine and alanine besides the cognate proline.
4.5 Future Perspectives

A continuation of studying the structural aspect of ProRS is proposed with the extension of crystallization experiments. Further crystal screens would be performed using modifications of the conditions (change in salt concentration, temperature, and pH) where spherulites were obtained. More crystallization conditions should be screened and also more additives screens to be employed in order to obtain hits that can be optimized for further growth of diffraction quality crystals.

For obtaining detailed functional properties of *T. cruzi* ProRS, more biochemical studies are to be pursued. ITC experiments will be continued especially using ATP analogs to determine the molar binding ratio of nucleotides to the ProRS protein. This will also be done in the presence of proline and its analogs. Calorimetry binding studies will be carried out at different temperature conditions with a view of obtaining the heat capacity of the various ligands. Another technique to be applied will be Differential Scanning calorimetry (DSC) that will be also used to obtain information how ProRS binds its substrates. Halofuginone a derivative of febrifugine has been showed to inhibit this enzyme therefore more DSC experiments will be done using this inhibitor.

To continue probing the aminoacylation activity of this protein using the spectrophotometric technique, the next strategy will be to acquire pure tRNA that can be used and the results compared. A new construct of the enzyme has been constructed that lacks the region at the N-terminus thought to be responsible for the editing activity of the ProRS enzyme in lower eukaryotes. It is expected that in the presence of non-cognate amino acids the truncated version will show higher aminoacylation activity of non-
cognate amino acids since the ability of editing would have been removed through the truncation.
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mechanisms of rat liver lysyl-tRNA synthetase-catalyzed reactions. Synthesis of


Appendix A

Molecular Biology

Table A.1: Sequence of PCR primers.

<table>
<thead>
<tr>
<th>Primer</th>
<th>DNA sequence</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward Human HisRS</td>
<td>5'- CACC GAA AAC CTG TAT TTT CAG GGC ATG-3’</td>
<td>54</td>
</tr>
<tr>
<td>Reverse Human HisRS</td>
<td>5'- TTA GCA GAT ACA CAG CGG TTG G-3’</td>
<td>54</td>
</tr>
<tr>
<td>Forward Afu EF-Tu</td>
<td>5'- CACC GCT AAG GAA AAG GAG CAC ATT AAT G-3’</td>
<td>54</td>
</tr>
<tr>
<td>Reverse Afu EF-Tu</td>
<td>5'- TTA TTT TCT TGG GGT AAG GTC GAG-3’</td>
<td>54</td>
</tr>
<tr>
<td>Forward T. cruzi ProRS*</td>
<td>5'- CACC gaa aac ctg tat ttt cag ggc AGT GCC TCC GAT TGC CGT-3’</td>
<td>53</td>
</tr>
<tr>
<td>Reverse T. cruzi ProRS</td>
<td>5'- TTA ATA GGA GCG ACC GAA CAG AA-3’</td>
<td>53</td>
</tr>
</tbody>
</table>

* Designed with a TEV protease cleavage site for Gateway cloning
Table A.2: Cloning and Expression host Competent *E. coli* cells.

<table>
<thead>
<tr>
<th><em>E. coli cells</em></th>
<th>Type of Cells</th>
<th>Antibiotic resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Omnimax™</td>
<td>Cloning host</td>
<td>Tetracycline (15 µg/mL)</td>
</tr>
<tr>
<td>DH5α</td>
<td>Coning host</td>
<td>none</td>
</tr>
<tr>
<td>DB3.1</td>
<td>Expression host</td>
<td>Chloramphenicol (35 µg/mL)</td>
</tr>
<tr>
<td>BL21 DE3*</td>
<td>Expression host</td>
<td>none</td>
</tr>
<tr>
<td>Tuner DE3</td>
<td>Expression host</td>
<td>none</td>
</tr>
<tr>
<td>TOP10</td>
<td>Cloning host</td>
<td>Streptomycin (50 µg/mL)</td>
</tr>
</tbody>
</table>

Table A.3: Plasmids and *E. coli* cells antibiotic resistance.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Antibiotic Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>pENTR-D</td>
<td>Kanamycin (25 µg/mL)</td>
</tr>
<tr>
<td>pDEST-C1</td>
<td>Streptomycin (50 µg/mL)</td>
</tr>
<tr>
<td>pET12a</td>
<td>Ampicillin (100 µg/mL)</td>
</tr>
<tr>
<td>pUC19</td>
<td>Ampicillin (100 µg/mL)</td>
</tr>
</tbody>
</table>
Figure A-1: Map of pET12a expression vector used for cloning ProRS.
Figure A-2: Map of pDEST-C1 expression vector.
Appendix B

MALDI-TOF/TOF MS Experiments

![Protein sequence coverage: 38%](image)

Matched peptides shown in **bold red**.

1. MAERAAALEL VKLQGEBVRG LKQQQASAEEL IEEEVAKLLK LKAQLGPDES
2. QKPVLTTPK GTRDYSPRQM AVREKVFDVI IRCFKRHGAE VIDTPVFELK
3. ETLMKGDQ SKLYDLKDQ GGEALSRYD LTVPFFARYLA MNKLNKRY
4. MIAKVRDRDN PAMTRGRRYE FYQCDTFDAE NFDPMIPDAE CLKIMEILS
5. SLIQGFDVFK VNDRIRLADG MAICGVSDKF RTTICCSSVDR LDRVSWEVK
6. NEMVGEKGLA PEVADGIDY VQQHGVSLV EQLLQDPKLQ NQNQALEGLG
7. DLKLLLFEYLT LGDIHDKISF DLSLARGLDY YTVYIEAVL IQTPAQAGEE
8. PGVGGVSAAAG GRYDGLVGMG DPGRKRPVPCV GLSGERIF SIVEQRLEAL
9. EEKIRTDTQ VLVSASQKQL LEERLKLVST LWADIGKAEI LKKKNNPKLNN
10. QLQYCEAGI PLVAILGEQE LKDGVIKLRS VTSREDVDVR REDLVEIJKR
11. RTGQPLCIC

**Figure B-1:** MASCOT search results showing sequence coverage for human HisRS.
Figure B-2: MASCOT search results for human HisRS.
Figure B-3: MASCOT search results for *Afu* EF-Tu.
Figure B-4: MASCOT search results showing sequence coverage for *Afu* EF-Tu.
Figure B-5: MASCOT search results for *T. cruzi* ProRS.
**Protein sequence coverage: 20%**

Matched peptides shown in *bold red*.

| 1 | MKMSYGVHSP PALRWCWLTLP FSSRRKASSL ENTYFSNRTV FCSARWIYR |
| 51 | RHCEASVSGA DLSGGFFYS SLNKEQVTNS TRTMSASDCR GESEMKLIR |
| 101 | ALGMDLPTIS HEEKHTVEEA NKELGRTGVG CTGSKNILFK SSKREDLVLT |
| 151 | ALMHTTRTNMK VIQDALSLKD LRFAPEDLVR THLGVVQGGV TPLALVNES |
| 201 | KNKLVLKDA LVESTPVPVL HPCRNDSKCL ITPKQLQDFL RKLEYPVFV |
| 251 | DFSAEATGTT PSGDNTAKS STSQKETGKK TSQPAAAIT GGANVTGETK |
| 301 | LGILVKREEN FSQWYVEVIT KAEMIEYYDV SGCIIRPWA FFIWRSRIPRF |
| 351 | PGARIESMGV EDKYFPMVFS RSCLEREKDH VEGFAPEVAV VTKAGTDLE |
| 401 | VPV AIRPTSE TVMYPYAKW IRSRDLPVVR LNMNNNVIRW EFSSHPFPFIR |
| 451 | TREFLWQEGH CAWQTEDECS QEEVLIEIHY AAVYEEILLAV PUVGKRKEK |
| 501 | EKFAGGYTT TVETYIAAVG RGCQGGTSHN LGQNFGRMNF ISFQDPERND |
| 551 | GSTLIFWQNS WGLSTRVIGV MTHVHGDDSG MVLPPRASV QVVIIPVGIT |
| 601 | KNTTDEERKS LLGEC tanks VLVSGGIARAK ADRLDNYSPO WRFNHWEVKG |
| 651 | VPVRVELGPR ELASLKISSLV LHDSGRRRL LWDKELPTAM TLLMDEIHSN |
| 701 | MFAKAKERRQ ENTKKLETWNS DFTPALNRKC LVLAPWCGEM SCEDQVKKDS |
| 751 | AEESKAMQSE EYKDDARAPS MGAKTLCPFP DQUPSVKGRG CICKSCSRPA |
| 801 | KHWWLPGRSY |

**Figure B-6:** MASCOT search results showing sequence coverage for *T. cruzi* ProRS.
## Appendix C

### Protein Crystallization Screens

**Table C-1:** Crystallization experiments set up.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Temperature</th>
<th>Drop size (μL)</th>
<th>Crystallization Screen</th>
<th>Number of setup</th>
</tr>
</thead>
<tbody>
<tr>
<td>HisRS apo</td>
<td>4 °C</td>
<td>2+2; 1+1</td>
<td>A*</td>
<td>2</td>
</tr>
<tr>
<td>HisRS + ligands</td>
<td>ambient</td>
<td>2+2</td>
<td>A*</td>
<td>2</td>
</tr>
<tr>
<td><em>Afu</em> EF-Tu apo</td>
<td>ambient</td>
<td>2+2; 1+1</td>
<td>A*, B*</td>
<td>3</td>
</tr>
<tr>
<td><em>Afu</em> EF-Tu + GMP-PNP</td>
<td>ambient</td>
<td>1+1</td>
<td>A*, B*</td>
<td>2</td>
</tr>
<tr>
<td>ProRS apo</td>
<td>ambient</td>
<td>2+2, 1+1</td>
<td>B*</td>
<td>1</td>
</tr>
<tr>
<td>ProRS + ATP</td>
<td>ambient</td>
<td>2+2</td>
<td>B*</td>
<td>1</td>
</tr>
</tbody>
</table>

A* Hampton/ Solubility Screen  
B* PEG Ion/ Solubility Screen (Made in-house)