Enzymatic Characterization Of The Ammonia Tunnel In Helicobacter Pylori Asp-Trnaasn/glu-Trnagln Amidotransferase

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ENZYMATIC CHARACTERIZATION OF THE AMMONIA TUNNEL IN
HELCOBACTER PYLORI ASP-TRNA$^{ASN}$/GLU-TRNA$^{GLN}$
AMIDOTRANSFERASE

by

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Approved by:

__________________________  ______________________
Advisor                      Date
DEDICATION

This thesis is dedicated to my wife (Xin Zhao), my mother (Qixin Chen), my father (Qingzhong Zhao), and my lovely daughter (Iris Xiang-Wen Zhao). Thank you all for your love, support and sacrifice.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>MD</td>
<td>Molecular dynamics</td>
</tr>
<tr>
<td><em>M. jannaschii</em></td>
<td><em>Methanococcus jannaschii</em></td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>NAcGlu</td>
<td>N-acetyl-L-glutamate</td>
</tr>
<tr>
<td>NAD+</td>
<td>Nicotinamide adenine dinucleotide (oxidized)</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide (reduced)</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate (reduced)</td>
</tr>
<tr>
<td>ND-AspRS</td>
<td>Non-discriminating AspRS</td>
</tr>
<tr>
<td>ND-GluRS</td>
<td>Non-discriminating GluRS</td>
</tr>
<tr>
<td>Ntn</td>
<td>N-terminal nucleophile</td>
</tr>
<tr>
<td>PEP</td>
<td>Phosphoenolpyruvate</td>
</tr>
<tr>
<td>PK</td>
<td>Pyruvate kinase</td>
</tr>
<tr>
<td>PMF</td>
<td>Potential of mean force</td>
</tr>
<tr>
<td>PRA</td>
<td>Phosphoribosylamine</td>
</tr>
<tr>
<td>PRPP</td>
<td>Phosphoribosylpyrophosphate</td>
</tr>
<tr>
<td>PLP</td>
<td>Pyridoxal phosphate</td>
</tr>
<tr>
<td>RMSD</td>
<td>Root mean square deviation</td>
</tr>
<tr>
<td><em>S. typhimurium</em></td>
<td><em>Salmonella typhimurium</em></td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TS-DHFR</td>
<td>Thymidylate synthase-dihydrofolate reductase</td>
</tr>
</tbody>
</table>

Standard one or three-letter abbreviations were used for all amino acids.
CHAPTER 1
INTRODUCTION: HELICOBACTER PYLORI ASP-TRNA\textsuperscript{ASN}/GLU-TRNA\textsuperscript{GLN} AMIDOTRANSFERASE AND ITS AMMONIA TUNNEL

1.1 Introduction

The discovery of molecular tunnels (1) in enzymes dramatically advanced our understanding of the mechanisms of enzymes with multiple, isolated active sites. Among these tunnel-containing enzymes, glutamine-dependent amidotransferases (GATs) utilize molecular tunnels to shuttle ammonia between distal active sites. This class of enzymes has been intensively investigated both structurally and mechanistically (2-6). These enzymes play important roles in diverse pathways during the biosynthesis of amino acids (7-10), amino sugars (11), cofactors (12-14), purines (15), and pyrimidines (16).

The \textit{Helicobacter pylori} (\textit{H. pylori}) Asp-\text{tRNA}\textsuperscript{Asn}/Glu-\text{tRNA}\textsuperscript{Gln} amidotransferase (AdT) belongs to the GAT family and shuttles ammonia through a molecular tunnel (17). \textit{H. pylori} AdT plays an important role in the common bacterial indirect aminoacylation pathway (18); however, its ammonia tunnel is distinct from other known ammonia tunnels (2,19) because it is significantly more hydrophilic, suggesting an unprecedented ammonia delivery mechanism (19,20). Consequently, the mechanistic study of ammonia delivery in AdT is important to further our understanding of molecular tunnels in which reactive intermediates are protected and transferred.
This chapter will provide background on tRNA aminoacylation with an emphasis on indirect tRNA aminoacylation pathways. GAT enzymes that contain ammonia tunnels, including the bacterial AdT, will also be introduced. In conclusion, the results presented in this dissertation will be summarized and discussed with respect to how they contribute to a broader understanding of the GAT ammonia tunnel mechanisms.

1.2 Protein translation and fidelity

Protein translation is an essential process for all forms of lives in which the genetic information transcribed into messenger RNAs (mRNA) is translated into specific amino acid sequences (Figure 1.1) (21,22). This biosynthetic process has to be highly accurate in order to maintain cellular viability. Under normal growth conditions, amino acid misincorporation is estimated to occur about once in every 10,000 codons (23,24). The fidelity of protein translation is determined mainly at three stages: tRNA aminoacylation, elongation factor Tu (EF-Tu) recognition, and codon-anticodon recognition (18,25).

During tRNA aminoacylation, the aminoacyl-tRNA synthetases (aaRSs) need to select their cognate amino acid substrate from a cellular pool of amino acids and covalently attach it to their cognate tRNA(s). EF-Tu is responsible for the delivery of most aminoacyl-tRNAs (aa-tRNAs) to the ribosome, and can discriminate against some misacylated aa-tRNAs (26-33). The insertion of an amino acid at a particular position in a polypeptide is dictated by the pairing of the mRNA codon with the anticodon of a particular aa-tRNA. This codon-
anticodon recognition takes place in the ribosome, and accuracy is solely controlled by the ribosomal proofreading mechanism (34,35). For the scope of this thesis work, only the related tRNA aminoacylation processes will be further discussed.

**Figure 1.1: Summary of ribosomal protein translation.** In most cases, each tRNA is aminoacylated with its cognate amino acid by its corresponding aaRS. The correct aa-tRNA is loaded onto the ribosome by EF-Tu. In the ribosome, the genetic information in the mRNA dictates the order of amino acids introduced into the nascent polypeptide.

**1.3 Direct aminoacylation of tRNAs by the standard 20 aaRSs**

As mentioned above, the aminoacylation of tRNAs is a crucial step in protein biosynthesis (18). The standard encoded 20 amino acids are inserted into polypeptides via each aa-tRNA. These aa-tRNAs decipher the genetic code and guide the sequential insertion of each correct amino acid. Therefore, efficient and accurate aminoacylation of tRNAs is essential before this codon-defined process takes place in the ribosome. Once the aa-tRNA is generated and loaded onto the
ribosome, there is no known mechanism for the ribosome to check on accuracy of the attached amino acid on a given tRNA during decoding (36). Fortunately, the aminoacylation of tRNAs is catalyzed by a set of precise aaRSs with one aaRS for each of the 20 standard encoded amino acid (18). The error frequency of the aaRSs is approximately $1 \times 10^4$. Accuracy is achieved in three major ways: recognition of structural elements within their cognate tRNA(s), rejection of non-cognate tRNAs that contain structural antideterminants, and the proofreading of misactivated amino acids and misacylated tRNAs (18,37).

In many organisms, aa-tRNAs are generated directly by a complete set of conserved 20 aaRSs. The 20 aaRSs are classified into two unrelated groups (Class I and Class II) according to the structures of their catalytic centers (18,38,39). The Class I aaRSs have a conserved Rossmann fold domain for nucleotide binding. This domain also contains highly conserved KMSKS and HIGH sequences that are close to the ATP binding domain (40). Class II aaRSs have characteristic seven-strand antiparallel $\beta$-sheets in their catalytic centers (40).

Although the 20 aaRSs are structurally diverse, they catalyze the aminoacylation of tRNAs through the same two reactions. Each aaRS first activates its corresponding amino acid with ATP to form an aminoacyl adenylate (aa-AMP) (41-46). Next, the aa-AMP reacts with either the 2’ or 3’ hydroxyl group of the 3’-terminal ribose of the cognate tRNA to produce the correct aa-tRNA molecules (Figure 1.2).
Figure 1.2: Cartoon representation of aminoacylation. Direct tRNA aminoacylation is catalyzed by the aaRSs in two steps: First, the amino acid is activated by ATP to form an aminoacyl adenylate (AA-AMP); second, the amino acid is transferred to the acceptor stem of the tRNA to generate the aminoacyl-tRNA (aa-tRNA). (*The activation of some amino acids requires the binding of their cognate tRNAs (not shown) (41-46).)
All known organisms use the 20 standard amino acids for protein biosynthesis. (Some species also use selenocysteine (47-49) and/or pyrrolysine (49-53).) Therefore, for many years, it was expected that all organisms would utilize 20 aaRS with one for each standard amino acid in protein biosynthesis. However, Wilcox and coworkers discovered that *Bacillus subtilis* (*B. subtilis*) does not have a functional glutaminyl-tRNA synthetase (GlnRS) to directly produce glutaminyl-tRNA$_{Gln}$ (Gln-tRNA$_{Gln}$). Instead, an indirect pathway compensates for the missing GlnRS and ensures the biosynthesis of Gln-tRNA$_{Gln}$ (54,55). This indirect pathway is common among prokaryotes.

The next section will briefly describe a few indirect aminoacylation systems; for the focus of this dissertation, the indirect aminoacylation of tRNA$_{Asn}$ and tRNA$_{Gln}$ via AdT correction will be discussed in details.

### 1.4 Indirect aminoacylation systems

As a result of modern genomics, we now know that this rule of 20 aaRSs for 20 amino acids is true in eukaryotes including humans (56) and some bacteria such as *Escherichia coli* (*E. coli*) (57); but many bacteria and archaea do not have all 20 aaRSs (58,59). Despite an incomplete set of aaRSs, these microorganisms still incorporate all 20 amino acids into proteins with high fidelity through a process called indirect tRNA aminoacylation. The importance of indirect aminoacylation pathways is exemplified in the hyperthermophilic archaeon *Methanococcus jannaschii* (*M. jannaschii*) whose genome only codes for 17 of the 20 canonical aaRSs (60). Genes for cysteinyI-tRNA synthetase
(CysRS), asparaginyl-tRNA synthetase (AsnRS), and GlnRS are missing (60). (It was originally reported that M. jannaschii also lacked a gene for lysyl-tRNA synthetase (LysRS); subsequently, it was discovered that this organism relies on a non-canonical Class I LysRS and direct production of lysyl-tRNA\textsuperscript{Lys} (Lys-tRNA\textsuperscript{Lys}) (61).) The enzymatic functions of the three missing aaRSs are replaced by misacylation of each tRNA with a non-cognate amino acid followed by conversion of the non-cognate amino acid to the cognate amino acid. This process is called indirect tRNA aminoacylation (Figure 1.3) and usually requires two or more enzymes instead of a single aaRS.

**Figure 1.3: Cartoon representation of indirect tRNA aminoacylation.** In indirect aminoacylation pathways, the cognate aa-tRNA is generated first by misacylation of a tRNA with a non-cognate amino acid, followed by enzyme-catalyzed conversion to the cognate amino acid.

Of the known correcting enzymes, the work described in this dissertation focuses on the Asp-tRNA\textsuperscript{Asn}/Glu-tRNA\textsuperscript{Gln} glutamine-dependent amidotransferase (abbreviated AdT, or GatCAB and GatDE, see below for details). AdT plays an important role in the indirect aminoacylation of tRNA\textsuperscript{Asn} and tRNA\textsuperscript{Gln} in some
bacteria and archaea that do not have AsnRS and/or GlnRS to directly produce Asn-tRNA\textsubscript{Asn} and/or Gln-tRNA\textsubscript{Gln}. As the other indirect tRNA aminoacylation pathways are not related to this dissertation and have been reviewed in detail (18), they will not be further discussed.

**1.5 Indirect aminoacylation of tRNA\textsuperscript{Asn} and tRNA\textsuperscript{Gln}**

The most common indirect tRNA aminoacylation pathways are the biosynthesis of Asn-tRNA\textsuperscript{Asn} and Gln-tRNA\textsuperscript{Gln}, because most bacteria and archaea do not have AsnRS and/or GlnRS (18,38). The indirect aminoacylation of tRNA\textsuperscript{Gln} was first identified in 1968 in *B. subtilis* when it was discovered that *B. subtilis* lacks the ability to directly generate Gln-tRNA\textsuperscript{Gln} (55). Instead, Gln-tRNA\textsuperscript{Gln} is produced indirectly through a two-step enzymatic reaction in which tRNA\textsuperscript{Gln} is first misacylated with glutamate followed by phosphorylation and transamidation to generate Gln-tRNA\textsuperscript{Gln} (Figure 1.4A) (54). Later, it was confirmed that the *glnS* gene is missing in the *B. subtilis* genome (62). In the late 1980s, Schon and coworkers discovered the same indirect aminoacylation pathway in animal mitochondria and plant chloroplasts (63).
Figure 1.4: Indirect biosynthesis of Gln-tRNA\textsuperscript{Gln} and Asn-tRNA\textsuperscript{Asn}. (A) Indirect biosynthesis of Gln-tRNA\textsuperscript{Gln}. In the absence of GlnRS, a misacylating GluRS (non-discriminating glutamyl-tRNA synthetase (ND-GluRS) or GluRS2) first aminoacylates tRNA\textsuperscript{Gln} with glutamate, and then the misacylated glutamyl-tRNA\textsuperscript{Gln} (Glu-tRNA\textsuperscript{Gln}) is converted to Gln-tRNA\textsuperscript{Gln} by AdT (GatCAB or GatDE) (18). (B) Indirect biosynthesis of Asn-tRNA\textsuperscript{Asn}. The misacylation of tRNA\textsuperscript{Asn} is catalyzed by ND-AspRS, and Asp-tRNA\textsuperscript{Asn} is also transamidated by AdT (GatCAB) (64).
Microorganisms that are missing AsnRS also utilize the same indirect pathway to produce Asn-tRNA^{Asn}, as first demonstrated in *Haloferax volcanii* (64) (Figure 1.4B). Generally, AdT (GatCAB) has dual function for correcting both Asp-tRNA^{Asn} and Glu-tRNA^{Gln}. In this section, the misacylating enzyme and correcting enzyme for tRNA^{Asn} and tRNA^{Gln} indirect aminoacylation will be further discussed.

1.5.1 Indirect aminoacylation of tRNA^{Gln}

Phylogenetic analyses of aaRSs suggest that GlnRS may be the last aaRS to evolve and that it was not found in the last universal common ancestor (38,50). Most bacteria and archaea do not have GlnRS (18). To compensate, two enzymes are required to generate Gln-tRNA^{Gln} (shown in Figure 1.4A): a non-discriminating or misacylating glutamyl-tRNA synthetase (ND-GluRS or GluRS2, respectively) that produces misacylated Glu-tRNA^{Gln} (65-68) and a glutamine-dependent Glu-tRNA^{Gln} amidotransferase (AdT) that converts Glu-tRNA^{Gln} to Gln-tRNA^{Gln} (17). The non-discriminating GluRS is common to most prokaryotes (e. g. *B. subtilis*). This enzyme has dual aminoacylation activities for both tRNA^{Glu} and tRNA^{Gln} and generates both the cognate Glu-tRNA^{Glu} and the misacylated Glu-tRNA^{Gln}. Some bacteria (e. g. *H. pylori*) have two copies of GluRS: GluRS1 is a discriminating GluRS that only synthesizes the cognate Glu-tRNA^{Glu}, while GluRS2 is solely a misacylating enzyme that only misacylates tRNA^{Gln} with glutamate to produce Glu-tRNA^{Gln} (67,68). The different tRNA specificities of GluRS1 and GluRS2 are complementary to each other (67,68). In addition, the
fact that GluRS2 has lost its canonical function to generate Glu-tRNA\textsuperscript{Glu} suggests the possibility that GluRS2 may represent an aborted, intermediate stage in GlnRS evolution in bacteria (67).

Once misacylated Glu-tRNA\textsuperscript{Gln} is generated, it has to be converted to Gln- tRNA\textsuperscript{Gln} before delivery to the ribosome. This conversion is catalyzed by AdT (GatCAB or GatDE (discussed in the next section)) (See Scheme 1.1) through three different enzyme activities: (1) glutamine is hydrolyzed to produce ammonia (glutamine is the preferred amide donor, but it can be asparagine (69,70)); (2) the carboxyl side chain of Glu-tRNA\textsuperscript{Gln} is phosphorylated using ATP; and, (3) ammonia is consumed to complete the transamidation reaction and produce Gln- tRNA\textsuperscript{Gln}.

\[ \text{Glu-tRNA}^{\text{Gln}} \xrightarrow{\text{GatA or GatD}} \text{Gln-tRNA}^{\text{Gln}} \]

\[ \text{O} \quad \text{NH}_2 \quad \text{H}_3^+ \text{N} \quad \text{O} \quad \text{O}^{-} \quad \xrightarrow{\text{H}_2\text{O}} \quad \text{H}_3^+ \text{N} \quad \text{O} \quad \text{O}^{-} \quad \text{NH}_3 \]

\[ \text{Ammonia tunnel} \]

\[ \text{H}_3^+ \text{N} \quad \text{O} \quad \text{O}^{-} \quad \text{tRNA}^{\text{Gln}} \quad \xrightarrow{\text{ATP, ADP}} \quad \text{H}_3^+ \text{N} \quad \text{O} \quad \text{O}^{-} \quad \text{tRNA}^{\text{Gln}} \quad \text{GatB or GatE} \]

\[ \xrightarrow{\text{GatB or GatE}} \quad \text{H}_3^+ \text{N} \quad \text{O} \quad \text{O}^{-} \quad \text{tRNA}^{\text{Gln}} \quad \text{GatB or GatE} \]

\[ \text{Scheme 1.1: Conversion of Glu-tRNA}^{\text{Gln}} \text{ to Gln-tRNA}^{\text{Gln}} \text{ by AdT.} \]
1.5.2 Indirect aminoacylation of tRNA^{Asn}

In the absence of AsnRS, two enzymes are required to generate Asn-tRNA^{Asn}: a non-discriminating aspartyl-tRNA synthetase (ND-AspRS) and a glutamine-dependent Asp-tRNA^{Asn} amidotransferase (AdT). The ND-AspRS, similar to ND-GluRS, has dual aminoacylation activities for both tRNA^{Asp} and tRNA^{Asn} (71,72), and the mechanism in which AdT catalyzes the conversion of Asp-tRNA^{Asn} to Asn-tRNA^{Asn} is the same as shown for the conversion of Glu-tRNA^{Gln} into Gln-tRNA^{Gln}.

Although the indirect biosynthesis of Asn-tRNA^{Asn} and Gln-tRNA^{Gln} share high levels of similarities, there are a few notable differences. Compared to GlnRS, AsnRS is more frequently found in bacteria. Consequently, the bacterial indirect aminoacylation of tRNA^{Asn} is less common. In contrast, indirect aminoacylation of tRNA^{Asn} is almost ubiquitously required among archaea (38). Asp-tRNA^{Asn} is only a substrate for the dual functional AdT (GatCAB), while Glu-tRNA^{Gln} is a substrate for either GatCAB or GatDE. In the next section, the two types of AdT (GatCAB and GatDE) will be further discussed.

1.6 AdT: Asp/Glu-AdT (GatCAB) and Glu-AdT (GatDE)

AdT is found in two different forms: Asp-tRNA^{Asn}/Glu-tRNA^{Gln} amidotransferase (Asp/Glu-AdT or GatCAB) (Figure 1.5) (17,20,73) and Glu-tRNA^{Gln} amidotransferase (Glu-AdT or GatDE) (Figure 1.6) (74-78). Notably, another heterotrimeric AdT (GatFAB) has been identified recently from the yeast mitochondria indirect Gln-tRNA^{Gln} biosynthetic pathway, but the two catalytic
subunits share a great level of structural and functional similarities to those of GatCAB. GatF has no similarity to the bacterial GatC (79). In thermophiles, AdT (GatCAB) forms ternary complexes with tRNA\textsubscript{Asn}^\text{tRNA}_{\text{Gln}}^\text{Gin} and AspRS/GluRS, and these complexes are called the Asn- (80) and Gln-transamidosome (81), respectively. However, there is no known transamidosome formed with GatDE. In addition, dynamic Asn- and Gln-transamidosomes were observed with \textit{H. pylori} AdT (GatCAB). The formation of a transamidosome complex facilitates the production of Asn-tRNA\textsubscript{Asn} and Gln-tRNA\textsubscript{Gln} more efficiently without releasing toxic Asp-tRNA\textsubscript{Asn} and Glu-tRNA\textsubscript{Gln}; the sequestrations of Asp-tRNA\textsubscript{Asn} and Glu-tRNA\textsubscript{Gln} promotes translational fidelity (81-84).

**1.6.1 Asp/Glu-AdT (GatCAB)**

The heterotrimeric GatCAB type of AdT is made up of the GatC, GatA, and GatB subunits (Figure 1.5) and is found in bacteria, some archaea, and some organelles. The bacterial GatCAB has dual functions and converts both Asp-tRNA\textsubscript{Asn} and Glu-tRNA\textsubscript{Gln} into Asn-tRNA\textsubscript{Asn} and Gln-tRNA\textsubscript{Gln}, respectively (70). In archaea, GatCAB is only present when AsnRS is missing and it appears to be Asp-tRNA\textsubscript{Asn}-specific (74,85,86).

The GatA subunit contains the glutaminase active site. Other known glutaminases generally utilize an active site cysteine to hydrolyze glutamine (87,88), however GatA has a classic amidase signature sequence (87,89,90) and uses a serine in the active site to promote hydrolysis. Hydrolysis of glutamine occurs via the formation of an acyl-enzyme intermediate between this serine and
the $\gamma$-carbonyl of glutamine. The GatB active site catalyzes phosphorylation and transamidation of the misacylated aa-tRNAs. The small GatC subunit is not involved in catalysis, but may play a role in GatA folding (17) and surrounds GatA and GatB in the final assembly (20).

Figure 1.5: The crystal structure of the S. aureus AdT (GatCAB). The heterotrimeric AdT is made of GatA (magenta), GatB (cyan), and GatC (grey). The active sites in GatA and GatB are denoted by glutamine (GatA) and Mg$^{2+}$ (GatB) (shown in spheres). PDB ID: 2F2A; S. aureus GatCAB (20).
1.6.2 Glu-AdT (GatDE)

The heterodimeric GatDE type of AdT (Figure 1.6) is confined to archaea and is mono-functional, using only Glu-tRNA^Gln as a substrate. GatD is the glutaminase subunit and is functionally similar to GatA, however it has a different origin (17,73). GatD belongs to the L-asparaginase family of enzymes (74,91,92) and uses a threonine in a Thr-Lys-Asp catalytic triad to hydrolyze glutamine (73). In contrast, GatE and GatB are more closely related, and GatE might have an archaeal or bacterial GatB origin (8,73,93). GatE catalyzes the same two phosphorylation and transamidation reactions as GatB (Scheme 1.1). GatE also contains an insertion domain that is similar to AspRS. This domain was proposed to prevent transamidosome complex formation between GatE, AspRS and Asp-tRNA^Asn (77). In addition, in some archaea, both GatCAB and GatDE are present (74). In these cases, GatCAB is present to convert Asp-tRNA^Asn to Asn-tRNA^Asn while GatDE contributes to Gln-tRNA^Gln biosynthesis.
Figure 1.6: A crystal structure of GatDE. The heterodimeric AdT has two subunits: GatD (red) and GatE (blue). Active site residues in GatD and GatE are highlighted as spheres. PDB ID: 1ZQ1; Pyrococcus abyssi GatDE (76).
In both types of AdTs, the glutaminase active site is isolated from the kinase and transamidase active site. Structures of both GatCAB and GatDE argue that molecular tunnels connect these sites so that ammonia delivery can occur (20,77). Mutagenesis of both types of AdTs suggests communication occurs between each set of active sites (19,77). For example, an Arg221Ala mutation in GatE disrupts all three GatDE enzymatic activities (77). In addition, the GatA and GatD glutaminase activities are tightly coupled to the binding of misacylated tRNAs at GatB (70) or GatE (77). These observations highlight the possibility for interdomain communication in AdT.

In the following sections, enzyme tunnels and interdomain communication will be revisited, ultimately with a focus on the \textit{H. pylori} AdT ammonia tunnel. The interdomain communication and ammonia tunnel mechanisms of \textit{H. pylori} AdT are the main focus of this dissertation.

1.7 Molecular tunnels in enzymes

The use of molecular tunnels offers many advantages for catalysis in enzymes with isolated active sites: it reduces the transit time for reactive intermediates to move from one active site to another compared to free diffusion in bulk solvents (94,95). Labile intermediates may also be segregated and protected from unfavorable competing reactions (96-98) or degradation (99).

In the late 1980s, the first molecular tunnel was discovered in \textit{Salmonella typhimurium} (S. typhimurium) tryptophan synthase (TrpS). This tunnel sequesters and transfers the indole intermediate from one active site to another.
Since then, many molecular tunnels have been discovered for delivering small intermediate molecules between isolated active sites (Table 1.1). Carbon monoxide dehydrogenase/acetyl-CoA synthase (CODH/ACS) (100) has a tunnel to transport carbon monoxide; in fact, most known gas metabolic enzymes have hydrophobic tunnels or cavities for gas delivery between active sites or gas storage. Thymidylate synthase-dihydrofolate reductase (TS-DHFR) uses a tunnel for dihydrofolate (DHF) transfer (101). In addition, many GATs, including GatCAB and GatDE, use tunnels for ammonia delivery. Other examples of GATs with ammonia tunnels include carbamoyl phosphate synthetase (CPS) (102), glutamine phosphoribosylpyrophosphate amidotransferase (GPATase) (103), asparagine synthetase (AsnS) (104), glutamate synthase (GluS) (105), imidazole glycerol phosphate synthase (IGPS) (106), glucosamine 6-phosphate synthase (GlmS) (107), and GMP synthetase (GMPS) (108) (See Table 1.1 for details).
Table 1.1: A list of tunnel enzymes and their biological functions

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Tunnel properties</th>
<th>Biosynthetic pathway</th>
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<tr>
<td></td>
<td>Channel molecule</td>
<td>Length (Å)</td>
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<tr>
<td>---------------</td>
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<tr>
<td>TrpS (1)</td>
<td>Indole</td>
<td>25</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>TS-DHFR (101)</td>
<td>Dihydrofolate</td>
<td>40</td>
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<td></td>
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</tr>
<tr>
<td>CODH/ACS (100)</td>
<td>Carbon monoxide</td>
<td>67</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPS (102)</td>
<td>Ammonia</td>
<td>45</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>GMPS (108)</td>
<td>Ammonia</td>
<td>30</td>
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<td></td>
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<td></td>
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<tr>
<td>GPATase (103)</td>
<td>Ammonia</td>
<td>20</td>
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<td></td>
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</tr>
<tr>
<td>AsnS (104)</td>
<td>Ammonia</td>
<td>19</td>
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<td></td>
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<tr>
<td>GluS (105)</td>
<td>Ammonia</td>
<td>31</td>
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<tr>
<td>IGPS (106)</td>
<td>Ammonia</td>
<td>20</td>
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<tr>
<td>GlmS (107)</td>
<td>Ammonia</td>
<td>6</td>
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<td></td>
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<tr>
<td>GatCAB (20)</td>
<td>Ammonia</td>
<td>37</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>GatDE (76)</td>
<td>Ammonia</td>
<td>40</td>
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These molecular tunnels vary significantly in length from 6 to 67 Å. Ammonia tunnels are the most common (2). Most of these ammonia tunnel containing enzymes have multiple subunits, and their active sites are naturally isolated. The ammonia transfer through a molecular tunnel is a functional requirement for efficient catalysis as well as preventing unfavorable release of ammonia. The hydrophilic and hydrophobic characters of these ammonia tunnels are assigned according to the extent to which polar, ionic, and water molecules are present inside the tunnel (e.g. hydrophobic tunnels rarely have any polar, ionic or water elements, while hydrophilic tunnels would have all these elements). Mostly hydrophobic tunnels contain occasional appearances of polar or ionic residues, and partially hydrophobic tunnels have some water molecules in addition to occasional polar or ionic residues. Besides the known hydrophobic and hydrophilic tunnels, TS-DHFR relies on an electrostatic tunnel that contains many positively charged Lys and Arg residues for transferring negatively charged DHF. The electrostatic tunnel in TS-DHFR will be explained in detail in the following section.

As further background, the next few sections describe several representative tunnel enzymes with respect to tunnel formation, structure, and interdomain communication.

1.7.1 The hydrophobic indole tunnel in tryptophan synthetase (TrpS)

TrpS is a heterodimeric enzyme made up of a smaller α-subunit and a larger β-subunit, and it is involved in the last two steps of L-tryptophan
biosynthesis. In its smaller \( \alpha \)-subunit, TrpS catalyzes the cleavage of indole-3-glycerol phosphate (IGP) to produce the indole intermediate, which is subsequently condensed with L-serine in the larger \( \beta \)-subunit in a 5’-pyridoxal phosphate (PLP) dependent manner. The IGP cleavage and condensation sites are about 25 Å apart and connected by an indole tunnel. This tunnel is largely hydrophobic and only a few water molecules are observed close to the condensation site (See Figure 1.7).

The existence of the indole tunnel was confirmed by kinetic studies. In single-turnover assays for monitoring the production of L-tryptophan, only trace amounts of indole were observed, which likely indicates that indole is not released from the enzyme; there was no lag in L-tryptophan formation, which suggests the existence of a tunnel for fast indole delivery. In addition, phenylalanine and tryptophan mutations at Cys170(\( \beta \)) that blocked the tunnel showed intermediate indole buildup and lag in L-tryptophan formation \( (109,110) \). Finally, the delivery rate of indole was greater than 1000 s\(^{-1}\) and was not rate limiting \( (109) \).

The two subunits of TrpS also synchronize with each other during catalysis through allosteric communication. Substrate binding to the \( \beta \)-subunit enhances the \( \alpha \)-subunit reaction by 20-30 times \( (111) \). The chemical signals of substrate binding to the \( \beta \)-subunit also were suggested to trigger the open conformation of the tunnel. The binding of IGP to the \( \alpha \)-subunit increases the binding affinity of L-serine to the \( \beta \)-subunit. Thus, allosteric communication in TrpS appears to be reciprocal between the two active sites.
Figure 1.7: Tryptophan synthetase. The TrpS structure is shown in cartoon (left). The two active sites are represented by IGP and PLP (shown in colored spheres). The indole tunnel residues are shown in sticks, and the two water molecules close to the β-subunit active site are highlighted as blue spheres. PDB ID: 2TRS; *Salmonella enterica* TrpS (112).
1.7.2 A novel electrostatic channel in thymidylate synthase-dihydrofolate reductase (TS-DHFR)

TS-DHFR catalyzes the de novo production of thymidine 5’-monophosphate (dTMP). In this bifunctional enzyme, the TS component catalyzes the reductive methylation of 2’-deoxyuridine-5’-monophosphate (dUMP) using 5,10-methylene-5,6,7,8-tetrahydrofolate (CH₂-THF); the products are 7,8-dihydrofolate (DHF) and dTMP. The DHFR domain catalyzes the NADPH-dependent reduction of DHF to produce THF, in which a third enzyme, serine hydroxymethyltransferase, regenerates CH₂-DHF to initiate another catalytic cycle.

The two catalytic centers are separated by a distance of 40 Å (Figure 1.8). Although no tunnel was observed within the interior of TS-DHFR, DHF channeling between the TS and DHFR active sites were confirmed by isotopic dilution and inhibition experiments (113,114). These structural and kinetic results indicate that TS-DHFR uses a different mechanism for delivering the DHF intermediate. Based on the distribution of electrostatic potentials on TS-DHFR and the negatively charged intermediate DHF, Knighton and coworkers proposed that DHF transfer might be through a positive electrostatic channel on the surface of the enzyme (101,115). Similarly, this electrostatic channel mechanism was proposed for formiminotransferase (FT)-cyclodeaminase (CD) that transports 5-formiminotetrahydrofolate between its FT and CT active sites (116,117). However, the exact details of this electrostatic mechanism still need to be elucidated.
Although the electrostatic channeling of DHF on the surface of TS-DHFR appears to be different from internal molecule channeling through molecular tunnels, intermediate transfer between isolated active sites within the context of the enzyme itself does suggest it belongs to the molecular tunnel family, but from a broader perspective.

Figure 1.8: Thymidylate synthase-dihydrofolate reductase (TS-DHFR). The TS-DHFR structure is shown as cartoon. The two active sites are represented by NADPH and UMP and are shown in colored spheres. PDB ID: 1J3I; *Plasmodium falciparum* TS-DHFR (118).
1.7.3 The carbon monoxide tunnel in carbon monoxide dehydrogenase/acetyl-CoA synthase (CODH/ACS)

CODH/ACS is a bifunctional enzyme that catalyzes the reduction of carbon dioxide (CO$_2$) to carbon monoxide (CO), with subsequent biosynthesis of acetyl-CoA using a metal cofactor containing iron, sulfur, copper, and nickel. The *Moorella thermoacetica* CODH/ACS is a heterotetramer with two copies of CODH and ACS (Figure 1.9). Remarkably, there are several metal-sulfur clusters involved in catalysis. The unusual C-cluster (NiFe$_3$S$_4$) (green spheres in Figure 1.9) in the CODH subunit is responsible for reduction of CO$_2$ to CO. The CO is transferred to the A-cluster (CuNiFe$_4$S$_4$) (yellow spheres in Figure 1.9) in the ACS where acetyl-CoA is synthesized (119). In addition three more metal-sulfur clusters (orange spheres in Figure 1.9) were observed in the crystal structure, they are predicted to be involved in electron transfer. The distance between the CODH and ACS active sites is approximately 67 Å. This long distance suggested the use of a molecular tunnel.

Early isotopic exchanging experiments (120) and recent Xe-binding X-ray crystallography studies (100,121,122) of CODH/ACS both support a molecular tunnel connecting CODH and ACS active sites. Mutagenesis of the putative tunnel residues showed reduced rates of the acetyl-CoA production (123), which suggests the transfer of CO is through a molecular tunnel. This tunnel is believed to synchronize enzyme activities, increasing local CO concentration, and preventing CO cellular toxicity (100). Similar to other Ni-Fe dehydrogenases that transport molecular hydrogen, most known gas metabolic enzymes have
hydrophobic tunnels or cavities for gas delivery between active sites or gas storage (124).

Figure 1.9: Carbon monoxide dehydrogenase/acetyl-CoA synthase. The CODH/ACS structure is shown in cartoon and color-coded by subunits (the CODH subunit is in green and the ACS subunit is in yellow). The two active sites are represented by spheres in green and yellow. The orange spheres are metal-sulfur clusters for electron transfer. PDB ID: 1MJG; *Moorella thermoacetica* CODH/ACS (100).
1.8 GAT enzymes and their ammonia tunnels

GAT enzymes are multifunctional enzymes that catalyze glutamine-dependent transamidation reactions. They generally contain two major domains: a glutaminase domain catalyzing the hydrolysis of glutamine to produce ammonia and a 'synthase or synthetase' domain catalyzing ammonia-dependent reactions (125). The glutaminase domains are classified into three types: catalytic triad enzymes (126-128), N-terminal nucleophile (Ntn) enzymes and amidase signature enzymes. In contrast, the so-called ‘synthase or synthetase’ domains vary significantly in each enzyme. The two-domain topology of GAT enzymes points out a couple of important enzymatic commonalities with respect to ammonia transfer between active sites and the regulated coupling of multiple enzymatic activities.

Ammonia transfer can occur in a concerted manner that relies on the close proximity of the glutaminase active site and the downstream active sites. For example, the two active sites of *E. coli* guanosine monophosphate (GMP) synthetase are about 30 Å apart, a hinge region between them might have the flexibility to bring the two active sites close enough for a concerted mechanism (108). However, an increasing number of reported crystal structures of GAT enzymes showed that the glutaminase domain and synthase or synthetase domain are not close enough for a concerted mechanism. Consequently, it is more commonly accepted that GATs deliver ammonia through a molecular tunnel. The ammonia tunnel traps and transfers ammonia, and also guarantees that the ammonia will stay in its nucleophilic form until it is consumed in the next reaction.
For example, the two active sites in *B. subtilis* glutamine GPATase are too far apart to allow concerted ammonia transfer unless a dramatic conformational change takes place during catalysis (129). In all known cases of GATs, no such conformational changes have been observed to bring two active sites in close proximity, and GATs rely on molecular tunnel to transfer ammonia (2,4,130).

In the following subsections, several representative ammonia tunnels will be discussed in detail, ending with AdT.

### 1.8.1 The ammonia tunnel in carbamoyl phosphate synthetase (CPS)

CPS catalyzes the biosynthesis of carbamoyl phosphate, which is an important intermediate in the arginine, urea, and pyrimidine metabolic pathways. There are three different types of CPSs distinguished by their different nitrogen sources preferences (131). Type I CPS uses free ammonia as the nitrogen donor and plays important roles in arginine biosynthesis and the urea cycle. Both Type II and Type III CPS prefer glutamine as the nitrogen source. Type I and Type III CPSs require *N*-acetyl-[*L*-glutamate (NAcGlu) for activity, but Type II does not. Prokaryotes only utilize the Type II CPS, which belongs to the GAT family. This section will use the ammonia tunnel in the Type II CPS from *E. coli* as an example for further discussion.

*E. coli* CPS is a heterodimeric enzyme containing a small glutaminase subunit and a large synthetase subunit. The glutaminase subunit contains a catalytic triad with an active site cysteine (88,132). The synthetase subunit has two kinase sites: one produces carboxy phosphate and the other one generates
carbamoyl phosphate (See Figure 1.10). The three active sites are connected via a 45 Å enzyme tunnel; the distance between the glutaminase active site and the first kinase site is about 35 Å. This tunnel serves as an ammonia tunnel. It is mostly hydrophobic and lined with unreactive residues and backbone atoms. The rest of the enzyme tunnel, which is for the delivery of the carbonyl phosphate intermediate to the second kinase site, is less hydrophobic and lined with ionic residues and water molecules (133). These tunnel residues are either highly conserved or replaced by similar amino acids (5). The ammonia tunnel is not only predicted by the crystal structure, but is also supported by an isotopic labeled nitrogen source competition experiment (134) and mutagenesis of tunnel residues (135). In addition, the formation of carboxy phosphate induces a conformational change that is transmitted to the glutaminase active site and stimulates activity (136); this observation demonstrates that the glutaminase and carboxy phosphate formation sites are coupled to each other.
Figure 1.10: Carbamoyl phosphate synthetase. The partially hydrophobic ammonia tunnel in *E. coli* CPS is shown. Only the top three quarters (35 Å) of the tunnel is involved in ammonia delivery. In this figure, spheres represent the glutaminase and synthetase active sites, sticks stand for the residues lined along the ammonia tunnel, and blue spheres represent three water pockets in the CPS ammonia tunnel. The enzyme catalyzed reactions are shown on the right side. PDB ID: 1CS0; *E. coli* CPS (137).
1.8.2 The ammonia tunnel in glutamine phosphoribosylpyrophosphate amidotransferase (GPATase)

GPATase is an important enzyme for the first step of the *de novo* biosynthesis of purine nucleotides. It uses glutamine as the ammonia donor to convert phosphoribosylpyrophosphate (PRPP) to phosphoribosylamine (PRA). The GPATase belongs to the Ntn family of amidotransferases because it utilizes an N-terminal cysteine residue to hydrolyze glutamine (88,132,138).

In contrast to CPS, *E. coli* GPATase is a single polypeptide bearing both glutaminase and amidotransferase domains. The two active sites are approximately 20 Å apart and are connected by an ammonia tunnel that is packed mostly by hydrophobic residues (Figure 1.11) (102,133). These residues are highly conserved among known sequences (139). Another distinct feature of the GPATase ammonia tunnel is that the tunnel formation appears to be stimulated by substrate binding. Two important conformational changes were observed in the *E. coli* GPATase in a co-crystal structure containing 6-diazo-5-oxo-L-norleucine (DON) and cyclic PRPP: substrate binding causes one loop (Arg73 to Ser93) close to the glutaminase site to be restructured, and the flexible loop of the transamidase domain becomes ordered (139). Kinetic investigations of GPATase showed that only basal levels of glutamine hydrolysis were observed in the absence of PRPP, and the binding of PRPP at the distal active site lowers the $K_m$ of glutamine by more than 100-fold and greatly enhances its glutaminase activity (140). These structural and experimental results also suggest the coupling of the two distal active sites.
Figure 1.11: Glutamine phosphoribosylpyrophosphate amidotransferase.
The hydrophobic ammonia tunnel in *E. coli* GPATase is about 20 Å. In this figure, spheres represent the glutaminase and synthetase active sites and sticks represent the residues lined along the ammonia tunnel. No water molecules were observed in the ammonia tunnel in the GPATase structure. PDB ID: 1ECC; *E. coli* GPATase (139).
1.8.3 The putative ammonia tunnel in *H. pylori* AdT

*H. pylori* AdT is a heterotrimeric enzyme composed of GatC, GatA, and GatB subunits. In *H. pylori*, AdT is essential for protein translation and fidelity because it converts the misacylated tRNAs Glu-\(\text{tRNA}^{\text{Gln}}\) and Asp-\(\text{tRNA}^{\text{Asn}}\) into Gln-\(\text{tRNA}^{\text{Gln}}\) and Asn-\(\text{tRNA}^{\text{Asn}}\), respectively (17).

The most relevant published bacterial AdT crystal structure is from *Staphylococcus aureus* (*S. aureus*) (20). Protein sequence alignments of the *S. aureus* and *H. pylori* AdTs revealed that the two catalytic subunits are quite similar with 45% identity and 75% similarity between GatA subunits and 53% identity and 89% similarity in GatB subunits. Therefore, the *S. aureus* AdT structure was used as a structural model for mechanistic characterization of the *H. pylori* AdT ammonia tunnel.

The structure of *S. aureus* AdT revealed that the GatA and GatB active sites are separated by 37 Å, and Nakamura and his colleagues delineated a putative ammonia tunnel to connect the two catalytic centers (Figure 1.12). Kinetic analyses of *H. pylori* AdT showed that the binding of misacylated Glu-\(\text{tRNA}^{\text{Gln}}\) or Asp-\(\text{tRNA}^{\text{Asn}}\) to GatB enhances hydrolysis of glutamine in GatA, although the binding affinity of glutamine is not influenced (70). In addition, as will be described in Chapter 2 of this dissertation, mutagenesis of conserved residues in the ammonia tunnel alter the kinase activity of AdT (19). These observations suggest that AdT may also employ mechanisms to communicate between its separated active sites.
**Figure 1.12: AdT with two predicted ammonia tunnels.** (A) A rendition of a model of *S. aureus* AdT showing two predicted tunnels, the Nakamura tunnel (20) in black mesh and the Kang tunnel (141) in blue mesh. The AdT subunits are color coded with GatA in magenta, GatB in cyan, and GatC in grey. The two isolated active sites in GatA and GatB are represented by glutamine and an Mg^{2+} ion both in colored spheres. (B) A close up of the ammonia tunnel proposed by Nakamura (20). (C) A close up of the ammonia tunnel proposed by Kang (141). In both panels B and C, highly conserved residues along each tunnel are shown in sticks with the same colors as the subunits. (The footnotes $\alpha$ and $\beta$ are used for indicating GatA and GatB residues throughout this dissertation.) The water molecules inside the ammonia tunnel are shown in blue spheres. PDB ID: 2F2A; *S. aureus* AdT (20).
In 2012, molecular simulations were conducted on the *S. aureus* AdT structures for 1 nanosecond. Tunnel computations on this simulated structure suggested a slightly different ammonia tunnel than the one proposed by Nakamura and coworkers (Figure 1.12, blue mesh) (141). These two proposed ammonia tunnels have different paths branching from a highly conserved D185$_\alpha$ residue in GatA. The Kang tunnel starts with mainly hydrophobic residues including I179$_\alpha$, A180$_\alpha$, Y181$_\alpha$, and L229$_\alpha$, and no water molecules are observed along the path. In contrast, the Nakamura tunnel is lined with polar or charged residues (Figure 1.12). The two tunnels merge into one tunnel after about 10 Å, and this part of the tunnel is hydrophilic. Notably, neither of these two tunnels has been previously investigated enzymatically. From here on, we will refer to the originally predicted tunnel as the Nakamura type (Figure 1.12B) and the tunnel predicted after molecular simulation as the Kang type (Figure 1.12C).

### 1.8.4 Novel ammonia delivery mechanism in *H. pylori* AdT

As summarized in Table 1.1 and described in the above sections, GAT ammonia tunnels are typically hydrophobic. This hydrophobicity provides advantages for catalysis in that ammonia is sequestered in its deprotonated, nucleophilic form until being utilized. In some cases, water molecules and hydrophilic residues are observed along the ammonia tunnel such as in CPS, however they are rarely presented throughout the whole path as shown in the proposed Nakamura ammonia tunnel in AdT (20). This hydrophilic nature challenges the conventional perception that ammonia tunnels are typically
hydrophobic because it raises an interesting question regarding the distinct ammonia delivery mechanism with respect to the unique hydrophilic property of the ammonia tunnel.

The AdT Nakamura ammonia tunnel is hydrophilic, populated with water molecules, and lined with conserved polar and ionic amino acids (See Figure 1.12) (20). In contrast, about one-third of the Kang tunnel is mostly lined with hydrophobic residues. The lower two-thirds are identical to the Nakamura tunnel and is hydrophilic. The ammonia tunnel in CPS also contains several polar residues and water molecules in the form of water pockets along the tunnel (See Figure 1.10) (102, 142). This CPS ammonia tunnel is arguably the second most hydrophilic tunnel among all known GAT ammonia tunnels, but these hydrophilic features, the three water pockets and polar residues, are discrete and clustered. The majority of the tunnel is still hydrophobic. Molecular dynamics (MD) simulation on CPS suggested that the water pockets serves as energetic turning points for ammonia transfer, in which water molecules help with solvation and stabilization hydrogen bonds with tunnel residues (143). Notably, ionic residues are not present in the CPS ammonia tunnel while the AdT tunnel contains conserved Asp, Glu, Lys, and Arg in addition to polar residues such as Ser, Thr and Tyr. These observations point out that the unusual hydrophilic Nakamura AdT tunnel may employ a novel mechanism for ammonia delivery compared to other GATs. In fact, Tanaka and coworkers suggested that ammonia delivery could proceed through a series of protonation and deprotonation events (Figure 1.13) (20).
Figure 1.13: Cartoon representation of ammonia delivery through a proton relay mechanism. The conserved residues along the ammonia tunnel may act as general acids or bases to sequentially protonate ammonia and deprotonate ammonium. Water molecules in the ammonia tunnel may also participate. Key: A-H stands for a general acid that is capable of donating a proton; B: stands for a general base that can accept a proton.
1.9 Evidence supporting a gate in the ammonia tunnel of AdT

Another intriguing aspect of the mechanisms for ammonia transport is the possibility that an internal gate exists to close or open these ammonia tunnels during catalysis. Closed and open conformations often correlate with the absence or presence of substrate binding (4). In GlmS, with substrate binding only to the synthase domain, the ammonia tunnel is completely closed by Trp74, while the addition of glutamine analog DON triggers rotation of Trp74 to open the tunnel (144,145). In GPATase, Tyr74, located in the ammonia tunnel, is important for coupling the glutaminase and transamidase sites to each other suggesting that Tyr74 works as a gate similar to Trp74 in GlmS (140). Similarly, His57 in the *E. coli* cytidine triphosphate synthetase (CTPS) ammonia tunnel can adopt both open and closed conformations (146). In IGPS, a lysine residue in the ammonia tunnel has a high level of flexibility and closes or opens the ammonia tunnel by forming or breaking a specific salt-bridge (106,147). In the *S. aureus* AdT structure, Glu125β in GatB was predicted to be a gate (20); however, this hypothesis was disputed with the *Aquifex aeolicus* AdT crystal structure (148).

The arguments on the existence of a gate in the ammonia tunnel indicate that the closed and open conformations of the ammonia tunnel are dynamic during catalysis. Thus, a detailed examination of the gate residue in ammonia tunnels will help us further understand the mechanisms of GAT enzymes.
1.10 Interdomain communication through the ammonia tunnel in GAT

The fact that optimal GAT enzyme activities (both glutaminase and synthetase or synthase) are typically only achieved when all active sites are occupied by substrates (3,70) suggests that the enzymatic activities in each active site are coupled to each other and that interdomain communication occurs. This coordination behavior provides at least two benefits to the organism: regulating high enzyme catalytic efficiency and avoiding unnecessary intermediate release and wasteful consumption of glutamine. To regulate glutaminase activity, in some GAT enzymes such as Salmonella typhimurium anthranilate synthase (AS), the catalytic residues in the glutaminase active site are not organized in a productive orientation until substrate binding occurs at the distal active site (149,150). In addition, glutamine binding triggers closure of a flexible loop over the entrance of the glutaminase active site, which is common to a few GAT enzymes including GPATase (139,140) and IGP (106,147). This closing motion may help prevent ammonia release, which further guarantees the pH homeostasis inside cell.

As the ammonia tunnel connects isolated active sites, it is logical to predict that residues within this tunnel may mediate interdomain communication. Consequently, ammonia tunnel residues make good starting points for probing interdomain communication. For example, in the ferredoxin-dependent glutamate synthase, Glu1013 is located in a C-terminus loop, covers the synthase site, and forms part of the wall of the ammonia tunnel (151). This residue is important for activating glutaminase and coupling glutaminase and synthase activities (152).
Early investigations of interdomain communication in GAT enzymes relied on comparisons of crystal structures in the presence and absence of substrate(s) and mutagenesis of putative tunnel residues. With the advancement of larger computational capacities, orthogonal insights can be obtained with GAT enzymes using MD. As mentioned above in the case of CPS, MD results provide a detailed explanation for the role of each individual water pockets with respect to how they alter the energy path for ammonia delivery and how water molecules mediate hydrogen bond formation between ammonia and tunnel residues (143). In addition, locally enhanced sampling (LES) (153,154) and potential of mean force (PMF) (155) calculations were used to probe the GPATase ammonia tunnel regarding its ability to discriminate against ammonium during transfer and the leakiness of the L415A GPATase mutant (156). Similarly, interdomain communication mechanisms could also be investigated using MD in combination with conventional enzyme kinetics.

1.11 Dissertation research

Our current understanding of AdT and other general aspects of ammonia tunnels raise several interesting questions: Does AdT use a protonation/deprotonation mechanism to transport ammonia from GatA to GatB? If so, how does this work? Do GatA and GatB communicate with each other? Is there a gate in the AdT ammonia tunnel? The work in this dissertation seeks to answer these questions to better understand the mechanism of ammonia delivery by AdT. In this work, for the first time, each of the individual conserved ammonia
tunnel residues in *H. pylori* AdT were investigated for their enzymatic activities to provide evidence on their roles in ammonia delivery.

At the time when we initiated this ammonia delivery project, only the Nakamura tunnel had been predicted (20,157). Therefore, this dissertation initially only examined this tunnel. When the Kang tunnel was proposed, we expanded our efforts to include the goal of identifying the correct ammonia delivery path through AdT.

Chapter 2 details the application and optimization of a UV coupled-enzyme assay for measuring AdT kinase activity. Analysis of this reaction typically involves high performance liquid chromatography (HPLC) analysis of individual samples, but this coupled enzyme assay greatly improves the efficiency for analyzing a large number of AdT mutants. This fast analysis enabled us to identify tunnel residues that argued for interdomain communication in AdT. In addition, with the help of molecular dynamics simulations, a set of 59 residues from AdT were discovered to form a plausible communication pathway connecting the two isolated active sites.

Chapter 3 focuses on the hypothesis that D185$_\alpha$, at the top of the tunnel in GatA, acts as a general acid/base in a “proton relay” ammonia delivery mechanism. The kinetic characterization of three D185$_\alpha$ AdT mutants, D185A$_\alpha$, D185E$_\alpha$, and D185N$_\alpha$, will be presented, and conclusions to the role of D185$_\alpha$ in ammonia delivery will be discussed.
Chapter 4 describes our preliminary characterization of the gate residue E126β and the nearby residue K89β in the *H. pylori* AdT ammonia tunnel. The preliminary kinetic and computational results will be presented, and the possible roles of E126β and K89β will be discussed. These experiments are expected to serve as the groundwork for further investigations into gating within the AdT ammonia tunnel.

Chapter 5 summarizes the work in this dissertation and discusses the future direction.

Finally, the experimental methods used throughout this dissertation to characterize the *H. pylori* AdT and its tunnel mutants will be summarized.
CHAPTER 2
THE KINASE ACTIVITY OF THE HELICOBACTER PYLORI ASP-TRNA$^{ASN}$/GLU-TRNA$^{GLN}$ AMIDOTRANSFERASE IS SENSITIVE TO DISTAL MUTATIONS IN ITS PUTATIVE AMMONIA TUNNEL


2.1 Introduction

The discovery of the first molecular tunnel, in tryptophan synthase, demonstrated that some enzymes have evolved molecular tunnels to connect isolated catalytic centers (2,6,130). These tunnels can enhance the stability of reactive intermediates via sequestration and allow the delivery of intermediates to a downstream active site. Furthermore, tunnels can serve as a mechanism for interdomain communication, in which the binding of the substrate or the generation of an intermediate at one active site triggers conformational changes and activation at another, distal active site (2,6,130,158).

Most glutamine-dependent amidotransferases (GATs) contain molecular tunnels. These enzymes play important roles in diverse biosynthetic pathways as they deliver ammonia to various electrophilic acceptors during the biosynthesis of cofactors (12-14), amino acids (7-9), amino sugars (11), purines (15), and
pyrimidines (16), amongst others. The tunnels serve to sequester and deliver ammonia (generated via glutamine hydrolysis in one active site) to the downstream active site. Crystal structures are available for many of these enzymes and reveal that the molecular tunnels are typically hydrophobic or only partially occupied by water (1,103-107,133); they range in length from 6 Å (e.g. glucosamine 6-phosphate synthase (107)) to 45 Å (e.g. carbamoyl phosphate synthetase (133)).

*H. pylori* Asp-tRNA$^{\text{Asn}}$/Glu-tRNA$^{\text{Gln}}$ amidotransferase (AdT) is a heterotrimeric GAT made up of the GatC, GatA, and GatB subunits (17). Like many bacteria, *H. pylori* is missing both glutaminyl- and asparaginyl-tRNA synthetase (GlnRS and AsnRS, respectively) (18,159). In these species, AdT is essential for protein translation and fidelity because it converts the misacylated tRNAs Glu-tRNA$^{\text{Gln}}$ and Asp-tRNA$^{\text{Asn}}$ into Gln-tRNA$^{\text{Gln}}$ and Asn-tRNA$^{\text{Asn}}$, respectively (17,70,160). AdT catalyzes three distinct reactions. The GatA subunit contains a classic amidase signature sequence and catalyzes the hydrolysis of glutamine into glutamate and ammonia (reaction 1) (17). The GatB active site catalyzes phosphorylation and transamidation of both Glu-tRNA$^{\text{Gln}}$ and Asp-tRNA$^{\text{Asn}}$ (reactions 2 and 3, respectively, shown for Glu-tRNA$^{\text{Gln}}$ only) (20). GatC is apparently not involved in catalysis.
Reaction 1 (GatA): \[ \text{Gln} \rightleftharpoons \text{Glu} + \text{NH}_3 \]

Reaction 2 (GatB): \[ \text{Glu-tRNA}^{\text{Gln}} + \text{ATP} \rightleftharpoons \text{Phospho-Glu-tRNA}^{\text{Gln}} + \text{ADP} \]

Reaction 3 (GatB): \[ \text{Phospho-Glu-tRNA}^{\text{Gln}} + \text{NH}_3 \rightleftharpoons \text{Gln-tRNA}^{\text{Gln}} + \text{P}_i \]

The crystal structure of \textit{S. aureus} AdT revealed that the GatA and GatB active sites are separated by 37 Å, and a putative ammonia tunnel was identified that connects the two catalytic centers (Figure 2.1) (20).
**Figure 2.1:** The structure of the *S. aureus* AdT and its putative ammonia tunnel. This image was built from the *S. aureus* Mu50 AdT crystal structure using Pymol and PDB files 2F2A and 2G5I. GatA is shown in magenta, GatB in cyan, and GatC in grey. Conserved residues along the proposed tunnel are highlighted in sticks and dots. The glutamine in the GatA active site (from PDB file 2F2A) and the ADP in the GatB active site (from PDB file 2G5I) are shown as spaced filled molecules. The GatB C-terminal flexible domain (residues 275-411) is indicated; see text for details. The inset shows a close-up of the conserved residues lining the ammonia tunnel. The residues are numbered according to *S. aureus* Mu50 AdT, with parenthetical notations indicating the corresponding positions in *H. pylori* AdT.
This tunnel is distinct from other known ammonia tunnels (6), because it is highly populated with ordered water molecules and lined with conserved polar and ionic amino acids (20). Tanaka and colleagues proposed that ammonia delivery could occur through a series of protonation and deprotonation events (20). To our knowledge, such a mechanism would be unprecedented amongst enzymes that utilize ammonia tunnels.

In many respects, AdT is well characterized for its role in indirect tRNA aminoacylation, translational fidelity, catalysis, specificity for both Asp-tRNA\(^{\text{Asn}}\) and Glu-tRNA\(^{\text{Gln}}\), and the formation of a transamidosome complex with a non-discriminating aspartyl-tRNA synthetase (ND-AspRS) (70,161,162). In contrast, little is known about whether the GatA and GatB subunits communicate with each other, except that the addition of ATP and Glu-tRNA\(^{\text{Gln}}\) stimulates the glutaminase activity of GatA (70,89).

AdT’s kinase and glutaminase reactions must both occur before transamidation. We hypothesized that GatB’s kinase activity might serve as a probe to examine domain-domain communication between the GatA and GatB active sites. We have optimized and applied a UV-based colorimetric method (163) to the quantification of the kinase activity of AdT. This assay was used to kinetically characterize a series of mutations at conserved positions throughout the putative AdT tunnel. Effects ranged from negligible to an approximately 75 % reduction in kinase activity. Mutations at T149\(_\alpha\) and K89\(_\beta\) caught our attention because even conservative mutations at these positions caused substantial decreases in kinase activity in GatB. (Alpha and beta subscripts indicate that a
residue is found in GatA or GatB, respectively.) This effect was essentially the same whether rates were measured in the presence or absence of glutamine. Additionally, T149 is located at the entrance of the ammonia tunnel in GatA, distal to the GatB kinase active site, and K89 lies at the interface of GatA and GatB. Our kinetic analyses of these mutations suggest that they participate in directing communication between the glutaminase and kinase active sites of AdT. To investigate this domain-domain communication, we carried out molecular dynamics (MD) simulations on wild-type, T149V\textsubscript{\alpha} and K89R\textsubscript{\beta} AdT, all with and without glutamine bound to the glutaminase active site. Root mean square deviation and residue-wise correlation analyses indicate a significant difference in structural mobility for certain regions in the mutant structures compared to the wild-type enzyme. This effect is more pronounced when Gln is bound in the glutaminase active site of the T149V\textsubscript{\alpha} AdT in contrast to the observed steady-state kinetic measurements. Analyses of the simulation results point to 59 AdT residues that show an elevated level of mobility in both the T149V\textsubscript{\alpha} and K89R\textsubscript{\beta} AdT variants. These residues cluster mainly along the tunnel or close to the tRNA binding site. Taken together with our kinetic analyses, these results strongly suggest a pathway for domain-domain communication in AdT.
2.2 Results

2.2.1 Production of H. pylori AdT and its Mutants

Initially, the gatCAB genes were assembled into a single operon in the pET-28a vector; a plasmid (pSS003) encoding only for His$_6$-GatB (also in pET-28a) was also constructed (Dr. Stéphane Skouloubris, unpublished results). To facilitate purification, and building from a previous report (162), the gatCA operon was transplanted into the pCDF-1b vector (compatible with pET-28a) with concomitant addition of an N-terminal His$_6$ tag onto GatC (His$_6$-GatC, pPTC032). The two resultant plasmids were co-transformed into E. coli and wild-type AdT was readily purified to homogeneity, essentially as previously reported (162). Following purification, the ratio of GatA and GatB was examined to insure that AdT concentrations were not inaccurate due to dramatic excess of one subunit over the other.

Each mutant was purified using the same approach optimized for wild-type AdT. For GatA mutations, plasmid pSS003 was separately transformed with each mutant variant of pPTC032. For GatB mutations, plasmid pPTC032 was separately co-transformed with each mutant variant of pSS003. See Table 6.1 in Chapter 6 for a list of plasmids and further experimental details.

2.2.2 Development of a Coupled-Enzyme Assay for AdT-Catalyzed Phosphorylation of Glu-tRNA$^{Gln}$

Conversion of ATP into ADP can be observed indirectly via a coupled, spectroscopic enzyme assay that monitors the conversion of NADH to NAD$^+$
(See Chapter 6 for details) (164). In this assay, AdT-catalyzed phosphorylation of Glu-tRNA\textsuperscript{Gln} causes concomitant production of ADP. ATP is regenerated in the presence of excess phosphoenolpyruvate (PEP) and pyruvate kinase (PK), to produce pyruvate. Lactate dehydrogenase (LDH) reduces the resultant pyruvate to L-lactate, via conversion of NADH to NAD\textsuperscript{+}. This reaction can be monitored spectrophotometrically because NADH has an absorbance maximum at 340 nm and NAD\textsuperscript{+} does not absorb UV light at this wavelength.

Coupled-enzyme assays carry the risk that one is inadvertently quantifying the activity of the downstream enzyme(s) (LDH and/or PK in this case), rather than the enzyme of interest (AdT). To rule out this concern, several control experiments were conducted before progressing with quantitative assays of AdT and its mutants. The previously reported buffer conditions were adapted to those for AdT (165). Different AdT enzyme concentrations were also examined (Figure 2.2).
Figure 2.2: Coupled enzyme assay to measure AdT’s phosphorylation activity with Glu-tRNA\textsuperscript{Gln}. This assay is responsive over a wide range of AdT concentrations (○ no enzyme; □ 20 nM; ◇ 80 nM; × 300 nM; + 1.2 µM; △ 5.0 µM; ⊙ 10 µM).

As the concentration of AdT was increased (ranging from 20 nM to 10 µM), the rate of ATP hydrolysis also increased, confirming that the assay was directly reporting on AdT activity. Previously reported UV-based assays typically used NADH concentrations of 200 µM (166); with AdT, this concentration led to an untenable signal to noise ratio (S/N, See Figure 2.3B). To address this issue, the concentration of NADH was decreased to between 50 and 70 µM. This reduction led to a much more acceptable S/N while producing the same rate of ATP
hydrolysis (again, indicating that AdT activity, and not PK or LDH, is being reported, see Figure 2.4A).

![Graphs showing AdT kinase assay at high and low NADH concentrations.](image)

**Figure 2.3: AdT kinase assay at high and low NADH concentrations.** (A) NADH concentration was about 200 μM; (B) NADH concentration was about 50 μM. The slopes shown here are essentially the same, and represent the rate of ATP hydrolysis rates at high and low NADH concentrations.

An NADH calibration curve (Figure 2.4) allowed us to accurately correlate UV absorbance with concentration.
Figure 2.4: NADH calibration curve for the kinase assay. A series of NADH standard solutions were made and quantified by UV absorbance at 340 nm.

2.2.3 Michaelis-Menten Kinetics

The $K_M$ of ATP is approximately 200 µM, when measuring the net reaction of *H. pylori* AdT (transamidation) (167). Thus, the concentration of ATP was set at 2 mM for all assays. Kinetic parameters were determined for Glu-tRNA$^{Gln}$ as a substrate for AdT’s kinase activity (Figure 2.5). The $K_M$ for this misacylated tRNA is $1.6 \pm 0.5$ µM and is consistent with values previously reported (1.18 µM, determined by measuring AdT’s transamidase activity (167)). The $k_{cat}$ is $0.14 \pm 0.01$ s$^{-1}$, leading to a specificity constant ($k_{cat}/K_M$) of $7.5 \times 10^4$ s$^{-1}$ M$^{-1}$ for the phosphorylation of Glu-tRNA$^{Gln}$ in the absence of glutamine.
Figure 2.5: Michaelis-Menten analysis of Glu-tRNA\textsuperscript{Gln} as a substrate for AdT-catalyzed phosphorylation. Each reaction was initiated with 300 nM AdT. Data was fit directly to the Michaelis-Menten equation using Kaleidagraph V4.0 (Synergy Software). Error bars represent standard deviation and are from duplicate measurements. Standard deviations reported are from the results of the curve fit analysis.

2.2.4 Design and Characterization of Mutations in the Putative AdT Tunnel

It has been proposed that ammonia is transferred from GatA to GatB through a protonation-deprotonation relay mechanism; this hypothesis is based on the observation that the putative ammonia tunnel contains many hydrophilic and ionic residues and ordered water molecules (20). The \textit{H. pylori} and \textit{S. aureus} AdT protein sequences were aligned (see Figure 2.6).
Figure 2.6: Alignment of *H. pylori* and *S. aureus* AdT protein sequences. (A) GatA alignment. (B) GatB alignment. The residues highlighted in yellow are conserved throughout bacterial GatCABs; these were by mutagenesis and listed in Table 2.1.

The protein sequence alignment showed that GatA shares 45% identity and 75% similarity and GatB is 53% identical and 89% similar between *H. pylori* and *S. aureus* AdT protein. This analysis, along with the crystal structure of *S. aureus* GatCAB, allowed us to select 13 highly conserved residues along the putative ammonia tunnel for mutagenesis studies (20) (Table 2.1 and Figure 2.1). Most of these amino acids were separately mutated to alanine and to one or
more conservative amino acids to determine which, if any, of these positions is important for phosphorylation of Glu-tRNA$^{Gln}$.

Table 2.1: Conserved ammonia tunnel residues and the mutations constructed

<table>
<thead>
<tr>
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<th>S. aureus</th>
<th>H. pylori</th>
<th>Mutations</th>
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</thead>
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<tr>
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<td>T175</td>
<td>T149</td>
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<td></td>
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<td>R79</td>
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</tr>
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<td></td>
<td>K79</td>
<td>K80</td>
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</table>

* Originally, the H. pylori AdT E125 residue was mistakenly thought to be equivalent to S. aureus AdT E125 while the true equivalent residue should be H. pylori AdT E126 and this mistake has been corrected through the whole dissertation.

Each mutant enzyme was purified to homogeneity, with the exception of R174A$_\beta$, which could not be purified sufficiently for further analysis due to poor
expression levels. Initial rates of phosphorylation of Glu-tRNA$^{\text{Gln}}$ were determined for each mutant enzyme using the coupled assay described above. Relative activities with respect to wild-type AdT were calculated and are shown in Figure 2.7.

**Figure 2.7: Steady-state initial rates of Glu-tRNA$^{\text{Gln}}$ phosphorylation.** Wild-type *H. pylori* AdT and variants containing single point mutations along the ammonia tunnel were tested. Initial rates, relative to wild-type, are shown. See Table 2.2 for the raw initial rate data. The error bars represent standard deviation from triplicate assays. (A) Analysis of mutations in GatA; (B) Analysis of mutations in GatB.

No single mutation was sufficient to completely abolish phosphorylation activity, including mutations at K80$_{\beta}$ and Y82 (*H. pylori* numbering), which are located near the ATP binding site. These results show that the ATP binding site is remarkably tolerant of mutations at conserved residues. However, several
point mutations did cause dramatic reductions in activity (Figure 2.7), with some leading to as much as a 75% drop in initial rates. Not surprisingly, in general, conservative mutations were less disruptive than the alanine screen. For example, the activity of Q92A_β is diminished by approximately 25% with respect to wild-type AdT; in contrast, the Q92E_β mutant is actually 50% more active than wild-type AdT, at least with respect to phosphorylation (Figure 2.7B). This increase might be due to simultaneous ATP hydrolysis caused by the mutation in parallel to the phosphorylation of Glu-tRNA^{Gln}, because Q92_β is located near the ATP binding site.

Discussions in the literature have suggested that E125_β (S. aureus numbering and equivalent to E126_β in H. pylori) may (167) or may not (168) act as a gate, blocking AdT’s ammonia tunnel to prohibit inadvertent ammonia release (e.g. in the absence of bound Glu-tRNA^{Gln}). Mutations at position E126_β in GatB had little to no effect on AdT’s phosphorylation activity (Figure 2.7B). These results demonstrate that the identity of E126_β does not impact the steady-state rate of phosphorylation of Glu-tRNA^{Gln}; further experiments are necessary to determine if E126_β plays a different kinetic role (discussed in Chapter 4).

When D276_β (which lies near the midpoint of the putative ammonia tunnel) was
mutated to alanine, the phosphorylation activity of the resulting enzyme was only 30% of wild-type AdT. In contrast, the D276Nβ mutation maintained 85% of the wild-type enzyme’s activity. S182Aα also showed a large reduction in phosphorylation activity (35% of wild-type) but this activity was partially recovered in the S182Tα mutant (to 62% of wild-type).

Mutagenesis at a few positions revealed a strong, deleterious response even upon the introduction of conservative mutations (e.g. T149α, R295α, K89β, and Y91β). Three of these residues (R295α, K89β, and Y91β) are localized at the interface between GatA and GatB (Figure 2.1). Remarkably, T149α is adjacent to the glutaminase active site, at the top of the tunnel in GatA, substantially removed from the GatB active site. Respectively, T149Aα AdT and T149Vα AdT demonstrated initial rates of phosphorylation of 40% and 46% that of wild-type AdT. Initially, the effort to construct T149Sα mutation was failed due to the primer error, with the newly designed primer, we were able to produce T149Sα AdT mutant and assayed its kinase activity. Compared to the other two T149α AdT mutants, T149Sα mutation shows less reduction of kinase activity (62% of the wild-type), which suggests the importance of hydroxyl side chain at 149 position,
but it needs further analysis. The K89A_\beta and K89R_\beta AdT both caused about 65% drop in the initial rates of phosphorylation. The kinase activity decrease caused by mutations at the distal T149_\alpha residue indicates the possibility of interdomain communication. The results with K89_\beta mutations are also interesting because this residue is adjacent to E126_\beta, the putative gate. (See below for further analyses of these mutations in Chapter 4).
Table 2.2: Initial phosphorylation rates of Glu-tRNA<sub>Gln</sub> by AdT and various tunnel mutants in the absence of glutamine

<table>
<thead>
<tr>
<th>Mutants</th>
<th>Initial rate (μM/min)</th>
<th>S. D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdT WT</td>
<td>1.11</td>
<td>0.20</td>
</tr>
<tr>
<td>T149A</td>
<td>0.44</td>
<td>0.07</td>
</tr>
<tr>
<td>T149S</td>
<td>0.69</td>
<td>0.08</td>
</tr>
<tr>
<td>T149V</td>
<td>0.51</td>
<td>0.05</td>
</tr>
<tr>
<td>R174K</td>
<td>0.53</td>
<td>0.09</td>
</tr>
<tr>
<td>T149A Mutants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S182A</td>
<td>0.39</td>
<td>0.07</td>
</tr>
<tr>
<td>S182T</td>
<td>0.69</td>
<td>0.08</td>
</tr>
<tr>
<td>D185A</td>
<td>0.59</td>
<td>0.10</td>
</tr>
<tr>
<td>D185E</td>
<td>0.97</td>
<td>0.13</td>
</tr>
<tr>
<td>D185N</td>
<td>0.77</td>
<td>0.07</td>
</tr>
<tr>
<td>R295A</td>
<td>0.57</td>
<td>0.12</td>
</tr>
<tr>
<td>R295K</td>
<td>0.60</td>
<td>0.06</td>
</tr>
<tr>
<td>GatA Mutants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R79A</td>
<td>0.68</td>
<td>0.01</td>
</tr>
<tr>
<td>R79K</td>
<td>0.63</td>
<td>0.09</td>
</tr>
<tr>
<td>K80R</td>
<td>0.59</td>
<td>0.06</td>
</tr>
<tr>
<td>Y82A</td>
<td>0.58</td>
<td>0.05</td>
</tr>
<tr>
<td>Y82F</td>
<td>0.82</td>
<td>0.10</td>
</tr>
<tr>
<td>K89A</td>
<td>0.37</td>
<td>0.03</td>
</tr>
<tr>
<td>K89R</td>
<td>0.40</td>
<td>0.04</td>
</tr>
<tr>
<td>GatB Mutants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y91A</td>
<td>0.33</td>
<td>0.05</td>
</tr>
<tr>
<td>Y91F</td>
<td>0.63</td>
<td>0.14</td>
</tr>
<tr>
<td>Q92A</td>
<td>0.81</td>
<td>0.04</td>
</tr>
<tr>
<td>Q92E</td>
<td>1.65</td>
<td>0.03</td>
</tr>
<tr>
<td>E126A</td>
<td>0.72</td>
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</tr>
<tr>
<td>E126D</td>
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</tr>
<tr>
<td>E126Q</td>
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</tr>
<tr>
<td>D276A</td>
<td>0.33</td>
<td>0.08</td>
</tr>
<tr>
<td>D276N</td>
<td>0.94</td>
<td>0.10</td>
</tr>
</tbody>
</table>

S. D.: Standard deviation based on triplicate measurements. These data were used to calculate the relative rates shown in Figure 2.7.
2.2.5 Effects of Glutamine on *H. pylori* AdT’s Kinase Activity

All AdT mutations were originally screened in the absence of glutamine (Figure 2.7). Because the majority of the deleterious mutations were either at the interface between GatA and GatB or in GatA, the possibility of domain-domain communication was considered. The phosphorylation activity of representative mutations and wild-type AdT were reevaluated in the presence of 5 mM glutamine to determine if this GatA active site substrate positively or negatively impacts the activity of any of these mutant enzymes (Figure 2.8). Wild-type AdT and all mutations showed a slight, reproducible reduction in activity in the presence of glutamine. However, this reduction was only significant for the R295A<sub>α</sub>, K89A<sub>β</sub>, and Y91F<sub>β</sub> mutations, all of which lie at the interface of the two subunits.
Figure 2.8: Effect of glutamine on the kinase activity of wild-type AdT and selected mutants. Initial rates relative to wild-type AdT were determined for the wild-type and T149A, T149V, R295A, and R295K mutants in GatA and the K89A, K89R, Y91A, and Y91F mutants in GatB in the absence and presence of glutamine (5 mM). Error bars represent standard deviation from triplicate assays. See Table 2.3 for the raw initial rate data.
Table 2.3: Effect of Glutamine (5 mM) on initial phosphorylation rates of \( \text{Glu-tRNA}^{\text{Gln}} \) by selected AdT Mutants

<table>
<thead>
<tr>
<th></th>
<th>Without Gln</th>
<th></th>
<th>With Gln</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial rate (μM/min)</td>
<td>S.D.</td>
<td>Initial rate (μM/min)</td>
<td>S.D.</td>
</tr>
<tr>
<td>AdT WT</td>
<td>1.11</td>
<td>0.20</td>
<td>0.93</td>
<td>0.08</td>
</tr>
<tr>
<td>T149A</td>
<td>0.44</td>
<td>0.07</td>
<td>0.34</td>
<td>0.13</td>
</tr>
<tr>
<td>T149V</td>
<td>0.51</td>
<td>0.05</td>
<td>0.52</td>
<td>0.19</td>
</tr>
<tr>
<td>R295A</td>
<td>0.57</td>
<td>0.12</td>
<td>0.33</td>
<td>0.04</td>
</tr>
<tr>
<td>R295K</td>
<td>0.60</td>
<td>0.06</td>
<td>0.47</td>
<td>0.11</td>
</tr>
<tr>
<td>GatA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K89A</td>
<td>0.37</td>
<td>0.03</td>
<td>0.15</td>
<td>0.06</td>
</tr>
<tr>
<td>K89R</td>
<td>0.40</td>
<td>0.04</td>
<td>0.34</td>
<td>0.11</td>
</tr>
<tr>
<td>Y91A</td>
<td>0.33</td>
<td>0.05</td>
<td>0.29</td>
<td>0.06</td>
</tr>
<tr>
<td>Y91F</td>
<td>0.63</td>
<td>0.14</td>
<td>0.29</td>
<td>0.10</td>
</tr>
</tbody>
</table>

2.2.6 Molecular Dynamics Simulations of Wild-Type, T149V and K89R AdT

The molecular dynamics simulations of AdT and selected mutants were conducted by Sajeewa Dewage from Dr. Cisneros Lab. His computational work will not be directly presented in this results section of my dissertation. These results are provided in Appendix A. A few key results will be discussed in context with my enzymatic characterization.

2.3 Discussion

Previous enzymatic studies of \( H. \ pylori \) AdT have predominately focused on its glutaminase and transamidase activities (165,169). Results from these studies suggest the enhancement of glutaminase activity resulting from ATP and \( \text{Glu-tRNA}^{\text{Gln}} \) binding to the opposing active site. In other words, substrate-binding events in the GatB active site are communicated to and impact the efficiency of the GatA active site. This observation suggests the existence of essential
connectivities between the GatA and GatB active sites that are sensitive to steps in AdT’s reaction pathway. The fact that AdT uses a molecular tunnel to connect its two active sites suggests that this tunnel would be a likely component of AdT’s communication network. In this study, mutagenic analyses were used to evaluate conserved residues within the AdT ammonia tunnel, and molecular dynamics simulations were used to further evaluate interdomain communication in this system.

Here, Glu-tRNA\textsuperscript{Gln} was used as the sole aminoacyl-tRNA substrate for AdT (instead of both Glu-tRNA\textsuperscript{Gln} and Asp-tRNA\textsuperscript{Asn}). As reported previously, the identity elements for enzyme recognition of the two aa-tRNAs are similar; in particular, they share a common U1:A72 base pair as a key identity element in their acceptor stems (20,170). To simplify analysis, we chose to focus our efforts on Glu-tRNA\textsuperscript{Gln} as a representative example of the two aa-tRNA substrates for AdT. The adapted enzyme-coupled assay afforded a streamlined way to examine wild-type AdT and its mutants. This approach provided informative data that enabled us to select the T149V\textsubscript{α} and K89R\textsubscript{β} mutations for molecular dynamics simulations.

Our assay results show that AdT’s kinase activity is sensitive to mutations in the GatA glutaminase active site (T149V\textsubscript{α} and T149A\textsubscript{β}) and at the interface between GatA and GatB (R295A\textsubscript{α}, K89A\textsubscript{β}, and Y91F\textsubscript{β}). Mutations near the ATP binding site had little to no effect on activity. Remarkably, the kinase activity of GatB was slightly more susceptible to mutations in GatA, on average, than those
in GatB. These observations suggest two things. First, it is unlikely that the observed reductions in kinase activity are due to structural alterations in either active site. In this case, one would expect that GatB mutations would have greater impact than GatA mutations. Second, these mutations appear to have unmasked communication between GatA and GatB, even though the phosphorylation of misacylated Glu-tRNA\textsuperscript{Gln} is not particularly sensitive to the presence or absence of glutamine in the GatA active site. Previous work on \textit{H. pylori} AdT’s glutaminase activity showed that the binding of ATP and Glu-tRNA\textsuperscript{Gln} to GatB enhances the distal glutaminase activity in GatA by about 30-fold (165). When combined, these analyses have important ramifications for cellular pH homeostasis. They suggest that AdT has evolved to keep its glutaminase activity in check until an aminoacyl-tRNA substrate is available; at this point, glutamine is hydrolyzed to produce ammonia, which is efficiently transferred to the enzyme-bound substrate, rather than released to the intracellular environment.

According to our molecular dynamic simulations and comparative correlation analyses, the T149V\textsubscript{\alpha} and K89R\textsubscript{\beta} AdT mutants showed changes in correlation between GatA and GatB residues compared to wild-type (Figure 2.9).
Figure 2.9: Residue by residue correlation analysis for all simulated S. aureus AdT structures. (A) Wild-type AdT; (B) Wild-type AdT with glutamine bound to GatA; (C) T149Vα AdT; (D) T149Vα AdT with glutamine; (E) K89Rβ AdT; and (F) K89Rβ AdT with glutamine. (G) Cartoon showing different regions of correlation; residues proceed from left to right (N-terminal to C-terminal) with...
GatA residues numbered 1-485, GatB residues numbered 486-897, and GatC residues numbered 898-991. All panels apply the same color-coded scale representing the correlation coefficient that stands for how one residue moves with respect to another. Correlation coefficients of +1 and -1 both indicate the highest level of correlation observed between any two residues; negative correlations indicate residue movements correlated in opposing directions.

In order to compare the correlation changes between the wild-type AdT and AdT mutants, the correlation difference plot were generated by subtracting the wild-type correlation data from the corresponding AdT mutant (Figure 2.10). The presence of glutamine bound to GatA increases these correlation changes. The highest level of correlations occurs between GatA and GatB residues in the T149V\textsubscript{\alpha} mutant enzyme with glutamine bound to GatA (Figure 2.10). Similar patterns of correlations are present for T149V\textsubscript{\alpha} and K89R\textsubscript{\beta} AdT in the absence of glutamine, albeit at significantly lower levels. For wild-type AdT and K89R\textsubscript{\beta}, this observation is consistent with our kinase assay results that show only slight changes in activity upon the addition of 5 mM glutamine. Conversely, the T149V\textsubscript{\alpha} mutant shows a much higher level of correlation between these motifs in the presence of glutamine, in contrast to the minor reduction observed in kinase activity upon the addition of glutamine. It appears that the T149V\textsubscript{\alpha} mutation has reached its maximum impact on kinase activity in the absence of glutamine. However, the simulation results in the presence of glutamine emphasize the
extended significance of this mutation on AdT structure and mobility. T149$_\alpha$ is immediately adjacent to the glutamine-binding pocket of GatA. Our results suggest that the isosteric conversion of the T149$_\beta$ hydroxyl group to the valine methyl group in the T149V$_\beta$ mutation is not only enough to perturb distal kinase activity but may also impact glutamine binding and communication between the two active sites.
Figure 2.10: Differences in correlation for each mutant compared to the corresponding wild-type simulation results (wild-type values were subtracted from those of each mutant). (A) Differences between wild-type and T149V_{\alpha} AdT; (B) Differences between wild-type and T149V_{\alpha} AdT, both in the presence of glutamine; (C) Differences between wild-type and K89R_{\beta} AdT; (D) Differences between wild-type and K89R_{\beta}, both in the presence of glutamine. All panels apply the same color-coded scale (right panel) representing the
calculated correlation differences. See Figure 6 for a key to the different panel sub-sections.

The correlation patterns of K89β (wild-type) and K89Rβ AdT with all other residues in the enzyme reveal significant correlation changes in all three subunits. Combining the experimental results that the K89Aβ and K89Rβ mutations both significantly reduce the initial rate of phosphorylation of Glu-tRNA_Gln suggests that K89β is also involved in interdomain communication.

In addition, significant changes in correlation were observed for both T149Vα and K89Rβ in areas that connect GatA and GatB (Figure 2.10), which further supports the hypothesis that a communication pathway exists between these two subunits and that K89β and T149α are influential members in this pathway. The fact that a mutation in GatB (K89Rβ) and a mutation in GatA (T149Vα) separately and independently change the correlation pattern between GatA and GatB suggests bidirectionality in communication between these two subunits.

The RMSD comparison analysis identified 59 common residues that showed appreciable deviations in both mutant enzymes compared to wild-type. The fact that these 59 residues are common to both mutant enzymes strongly suggests their involvement in the communication pathway between GatA and
GatB. The locations of these 59 residues (see Figure 10) show that they are located in and around the GatA and GatB active sites as well as throughout the region connecting the two sites. A few of the residues are in GatC as well, hinting at a possible role for GatC in mediating communication between GatA and GatB.

**Figure 2.11:** The K89R and T149V AdT mutations induce substantial RMSD variations in a common set of 59 residues (RMSD > 20% in both mutant enzymes). These residues were mapped onto the *S. aureus* Mu50 AdT crystal structure and are highlighted in blue spheres. GatA, GatB, and GatC are colored as in Figure 1 (magenta, cyan, and grey, respectively). The 59 residues are listed in Appendix A, Table A.1.

Importantly, further analyses of these 59 residues revealed a common correlation pattern connecting GatA to GatB not only in both mutant enzymes but also in wild-type AdT. This commonality is highlighted by the fact that only
moderate differences in correlation were observed when comparing each mutant directly to wild-type. Consequently, these results demonstrate that our MD simulations have unmasked these residues as all or part of an interactive, dynamic, connective path for domain-domain communication in wild-type AdT. Given that K89\_ and T149\_ are members of this network and are essential for wild-type AdT activity, it seems probable that these 59 residues offer the first clear evidence of an extensive network of essential interactions throughout AdT.

2.4 Conclusion

In conclusion, structural studies work well for observing substantial motion in enzyme domains. For example, allosteric rearrangements, loop movements and domain rotations are often readily visualized by comparing co-crystal structures obtained in the presence of different substrates or ligands. Subtle structural changes can also be examined by crystallography but are sometimes more difficult to assess because of the challenges faced in tying observed residue movement to function. Molecular dynamics simulations are particularly useful for looking at domain-domain communication on a short time scale (nanoseconds). In this work, computational methods allowed us to readily visualize and compare movements in multiple simulations and to assess the impact of functionally interesting mutations. In this way, we were able to identify mobile elements in AdT that might have been difficult to isolate in a crystal structure. The importance of this work goes beyond simply advancing our understanding of how AdT functionally delivers ammonia from GatA to GatB;
these results also have implications for other GATs and even other enzymes with apparent domain-domain communication mechanisms.
CHAPTER 3

CHARACTERIZATION OF D185α AMMONIA TUNNEL MUTATIONS REVEALS A STEP IN THE AMMONIA DELIVERY MECHANISM OF ADT

3.1 Introduction

Most bacteria and archaea do not have genes encoding asparaginyl-tRNA synthetase (AsnRS) or glutaminyl-tRNA synthetase (GlnRS). They rely on indirect aminoacylation pathways to biosynthesize Gln-tRNA^{Gln} and Asn-tRNA^{Asn} (18). Misacylated Asp-tRNA^{Asn} and Glu-tRNA^{Gln} are first generated by a non-discriminating AspRS (ND-AspRS) (171) or a non-discriminating GluRS (ND-GluRS) (172,173) or GluRS2 (67,68). Next, the misacylated Asp-tRNA^{Asn} and Glu-tRNA^{Gln} are converted into Asn-tRNA^{Asn} and Gln-tRNA^{Gln}, respectively, by a heterotrimeric enzyme named Asp-tRNA^{Asn}/Glu-tRNA^{Gln} amidotransferase (AdT) (17,70,160).

As described in detail in chapter 1, AdT has two isolated active sites that are almost 40 Å apart (20). The GatA subunit catalyzes the hydrolysis of glutamine to produce ammonia, and the GatB subunit catalyzes ATP-dependent transamidation using ammonia delivered from GatA (17). AdT belongs to the glutamine-dependent amidotransferase (GAT) family (3). As commonly observed in GATs, AdT utilizes a molecular tunnel to transfer ammonia from the glutaminase active site in GatA to the distal transamidase active site in GatB (2).

The ammonia tunnel in AdT is unusual because it is extremely hydrophilic (20) compared to other known GAT ammonia tunnels (2) and is occupied by
many ordered water molecules that are distributed throughout the tunnel. Other known GAT ammonia tunnels are mostly hydrophobic and transfer ammonia in its neutral form. The tunnel in carbamate phosphate synthetase (CPS) is arguably the second most hydrophilic ammonia tunnel. It contains occasional ionic or charged residues and three water pockets, while the major part of the CPS ammonia tunnel wall is made up of hydrophobic residues (5). During transfer, the ammonia in CPS most likely remains deprotonated, a mechanism that is assumed for other hydrophobic ammonia tunnels as well. The hydrophilic elements in the CPS ammonia tunnel may serve to stabilize ammonia transfer by mediating hydrogen bond formation at turning points in the tunnel (174). This inspiring observation arose from computational simulations and also shed light on the mechanism of ammonia delivery through a partially hydrophilic environment.

The AdT ammonia tunnel is lined with polar and ionic residues and the crystal structure of *S. aureus* AdT shows that it contains many highly ordered water molecules (20). This uncommon hydrophilicity highlights the possibility that the AdT ammonia tunnel may employ a different mechanism to transfer ammonia compared to hydrophobic tunnels. In their structural analysis of *S. aureus* AdT, Nakamura and coworkers proposed that ammonia delivery through the AdT tunnel may proceed through a series of protonation and deprotonation steps (20). This ‘proton relay mechanism’ would rely on the special arrangement of polar and ionic residues and water molecules in the AdT tunnel that could act as
general acids or bases for proton relay as well as stabilizers for hydrogen bond formation.

General acid/base catalysis is common in enzyme-catalyzed reactions. The specific arrangement of active site residues and water molecules is a standard feature used to optimize enzymatic activity. For example, the positioning of active site residues can alter the pKₐ values of amino acid side chains. For example, the two active site Asp residues in HIV protease interact with each other through hydrogen bonds and share a proton in the active form of the enzyme. Due to this interaction, one Asp side chain carboxylate has a lowered pKₐ of about 1.5, while the pKₐ of the second one is elevated to ~5 (175). In addition, the pKₐ of water molecules can be changed by metal chelation within an active site (176). The AdT ammonia tunnel contains many ionic residues as well as water molecules that could act as general acids or bases in the proposed proton relay mechanism. A more detailed examination of each conserved residue along the ammonia tunnel will provide more information on the chemistry of ammonia transfer.

The kinetic and computational characterization of *H. pylori* AdT described in Chapter 2 identified several conserved ammonia tunnel residues with interesting phenotypes that may help us understand the mechanism of ammonia transport and unveil a possible path for interdomain communication. Following the discussion in Chapter 2, we extended our goals to consider the AdT proton relay mechanism. Our attention was drawn to D185ₜₐ, which is located at the top of the proposed tunnel next to T149ₜₐ (see Figure 3.1) and could serve as the first
general acid or base to protonate the incoming ammonia and initiate the proton relay process. (For consistency purposes, in this chapter and thereafter, subscripted \( \alpha \) and \( \beta \) will be used to denote GatA and GatB subunits, respectively.) \( \text{D185}_\alpha \) was mutated to alanine, glutamate and asparagine for initial activity assessments. The activity comparison between the WT AdT and \( \text{D185}_\alpha \) mutants are expected to elucidate the role of the \( \text{D185}_\alpha \) residue. This chapter focuses on the characterization of three \( \text{D185}_\alpha \) mutants (\( \text{D185A}_\alpha \), \( \text{D185E}_\alpha \) and \( \text{D185N}_\alpha \)) both enzymatically and computationally, and argues for \( \text{D185}_\alpha \) as the first example of a catalytic residue inside an ammonia tunnel.
Figure 3.1: Position of the D185$_{\alpha}$ residue in a model of *H. pylori* AdT. The *H. pylori* AdT ammonia tunnel model (contributed by Sajeewa Dewage) was built from the crystal structure of *S. aureus* AdT (PDB ID: 2F2A (20)). The predicted Nakamura tunnel is shown in gray mesh. The two isolated active sites in GatA and GatB are represented by glutamine (top) and a Mg$^{2+}$ ion (bottom), both in colored spheres. The inset shows a close-up of the entrance of this tunnel and the position of the D185$_{\alpha}$ residue. The distances between the active site glutamine, the entrance residue T149$_{\alpha}$, and D185$_{\alpha}$ were measured in Pymol.
3.2 Results and discussion

The general experimental details for enzymatic assays are described in Chapter 6. Any variations in these methods are given below.

3.2.1 Production of H. pylori AdT and its mutants

The D185α (D185Aα, D185Eα, and D185Nα) and the A179Gα AdT mutants were constructed using the standard QuikChange protocol, and the resultant plasmids were individually co-transformed into E. coli with plasmid pSS003 (constructed by Dr. Stéphane Skouloubris) encoding for GatB. Each mutant was purified using the same approach optimized for WT AdT as previously described (19). The ratio of GatA to GatB in each AdT variant was visually checked in a 12% SDS-PAGE gel to make sure that they co-purified in approximately equal proportions (see Figure 6.1 and Figure 6.2).

3.2.2 Transamidase activity screening of AdT GatA mutants

For transamidase activity measurement, while we were measuring the kinetic parameters for the WT AdT, we started transamidase activity screening for constructed AdT GatA mutants. The transamidation initial rate for each GatA mutant was measured for only once and relative activity was calculated by dividing the WT rate for quick comparison purposes (Figure 3.2).
Figure 3.2: Transamidase activity screening of AdT GatA mutants mapped onto structure. The single relative activity measurement (indicated by *) was performed for AdT GatA mutants except R174K. The relative activity was calculated by defining WT AdT transamidation initial rate as 1, the tested AdT mutants’ relative activity are shown in parenthesis. As the values were from single measurement, no error bars were shown.

All the AdT GatA mutants screened have at least 55% reduction of for transamidase activity. T149V variant has double the activity (44% of the WT)
compared to T149A\textsubscript{\textalpha} (23\%) in our single measurement. In addition, the conservative mutation at S182\textsubscript{\textalpha} and R295\textsubscript{\textalpha} seemed to be worse than their corresponding Ala mutations. Most interestingly, the transamidase activity of D185A\textsubscript{\textalpha} and D185N\textsubscript{\textalpha} AdT mutants was abolished. The unexpected results in combination with the proposed proton relay mechanism led us to hypothesize that D185\textsubscript{\textalpha} may act as a general acid or base in the AdT tunnel to relay the ammonia delivery. To test this hypothesis, a third D185E mutation was designed and constructed to further access the role of D185\textsubscript{\textalpha} for AdT catalysis by characterizing all three activities of AdT.

### 3.2.3 Detection of L-glutamate using an Amplex Red assay

Several methods were tested to identify a suitable assay to monitor AdT’s glutaminase activity (See Appendix B). Ultimately, the Amplex Red method was selected because of its high sensitivity. The Amplex Red assay detects glutamate by an enzyme coupled amplification of hydrogen peroxide produced upon glutamate oxidation. The hydrogen peroxide oxidizes the Amplex Red dye to produce a fluorescent signal (see Chapter 6 for a detailed description of this assay). Low nanomolar levels of glutamate can be detected with this assay (See Figure 3.3), and a glutamate calibration curve showed a linear response for glutamate from 20 nM to 320 nM.
Figure 3.3: Detection of L-glutamate standards by Amplex Red assay. Each glutamate standard was mixed with Amplex Red assay buffer (containing the coupling enzyme and dye) and incubated at 37 °C for 30 minutes. The fluorescence emission of each sample was measured at 590 ± 20 nm using an excitation wavelength of 530 ± 10 nm.

3.2.4 Measuring glutamine hydrolysis by AdT

Several control experiments were performed before progressing with quantitative assays of glutaminase activity of AdT and its mutants (Figure 3.4). Clearly, glutamine hydrolysis is optimal when the misacylated Glu-tRNA$^{\text{Gln}}$ is present; only a basal level of glutamine hydrolysis was observed in the absence of Glu-tRNA$^{\text{Gln}}$. The same results were observed in our radiolabeled and glutamate dehydrogenase (GDH) coupled UV assays (Appendix B). In addition,
the previously reported buffer conditions (70) were adapted and the enzyme concentration was optimized for initial rate measurements (15 nM) unless otherwise noted.

Figure 3.4: AdT catalyzed glutamine hydrolysis. The fluorescence intensity shown in this figure corresponds to the production of glutamate. The hydrolysis of glutamine is Glu-tRNA\textsubscript{Gln} dependent (diamonds), low levels of hydrolysis were observed in the absence of Glu-tRNA\textsubscript{Gln} (squares). In this qualitative assay, the glutaminase reaction was initiated by adding 1 μM WT AdT, incubated at 37 °C for 10 minutes, and quenched with 0.3 M sodium acetate (pH 5.2). Reactions without enzyme or Glu-tRNA\textsubscript{Gln} were set up in parallel. The samples were analyzed using the Amplex Red assay described in Chapter 6.
3.2.5 Michaelis-Menten Kinetics of AdT glutaminase

Kinetic parameters were determined for glutamine as a substrate for the glutaminase activity of WT AdT (Figure 3.5). The $K_M$ for glutamine is $2.1 \pm 0.3$ mM and the $k_{cat}$ is $27 \pm 2$ sec$^{-1}$, leading to a specificity constant ($k_{cat}/K_M$) of $1.3 \times 10^4$ s$^{-1}$M$^{-1}$ for the hydrolysis of glutamine in the presence of Glu-tRNA$^\text{Gln}$. In the absence of Glu-tRNA$^\text{Gln}$, only basal levels of glutamine hydrolysis were observed (Figure 3.5) and kinetic parameters were not determined.

Previously, *H. pylori* AdT’s glutaminase activity was reported from a TLC assay using radiolabeled glutamine to have a $K_M$ for glutamine of $50.9 \pm 25.4$ $\mu$M and $k_{cat}$ of $10.29 \pm 2.07$ s$^{-1}$ (70). The differences in these kinetic values may be due to the different range of glutamine concentrations tested. In the TLC assay, the glutamine concentration was varied from 4 $\mu$M to 960 $\mu$M (70); while in our Amplex Red assay, the concentration range was from 125 $\mu$M to 10 mM. It is possible that without the high substrate concentration data points, it was difficult to accurately determine $K_M$ and $k_{cat}$ values. This hypothesis is supported by the high errors in these previously published values.
Figure 3.5: Michaelis-Menten analysis of glutamine as a substrate for the glutaminase activity of AdT. Each reaction was initiated with 15 nM AdT. Data was fit directly to the Michaelis-Menten equation using Kaleidagraph V4.0 (Synergy Software).

3.2.6 Michaelis-Menten analysis of D185α AdT mutants’ kinase activity

The method for measuring AdT kinase activity was described in detail in Chapter 6. Michaelis-Menten kinetics for WT AdT was reported in Chapter 2 (Figure 2.7). D185α mutations have reduced kinase activity compared to WT, but generally maintained $K_M$ values for Glu-tRNA$^{\text{Gln}}$ that are close to those of WT (See Figure 3.6 and Table 3.1 for values).
Figure 3.6: Michaelis-Menten analysis of Glu-tRNA$^{\text{Gln}}$ as a substrate for AdT kinase activity. Each reaction was initiated with 300 nM AdT. Data was fit directly to the Michaelis-Menten equation using Kaleidagraph V4.0 (Synergy Software). The values for $K_M$ and $k_{cat}$ are listed in Table 3.1.
Table 3.1: Kinase kinetics for wild-type AdT and D185α AdT mutants

<table>
<thead>
<tr>
<th></th>
<th>$K_M$ (µM)</th>
<th>$V_{max}$ (µM/min)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_M$ ($10^4$ s$^{-1}$M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>1.06 ± 0.11</td>
<td>2.46 ± 0.08</td>
<td>0.137 ± 0.004</td>
<td>12.3 ± 1.0</td>
</tr>
<tr>
<td>D185Aα</td>
<td>0.80 ± 0.15</td>
<td>0.97 ± 0.05</td>
<td>0.053 ± 0.003</td>
<td>6.6 ± 1.9</td>
</tr>
<tr>
<td>D185Eα</td>
<td>0.90 ± 0.20</td>
<td>1.44 ± 0.10</td>
<td>0.080 ± 0.005</td>
<td>8.9 ± 2.2</td>
</tr>
<tr>
<td>D185Nα</td>
<td>0.72 ± 0.14</td>
<td>1.14 ± 0.06</td>
<td>0.063 ± 0.003</td>
<td>8.8 ± 1.9</td>
</tr>
</tbody>
</table>

3.2.7 Michaelis-Menten kinetics of AdT transamidase activity

Kinetic parameters were determined for glutamine as a substrate for AdT’s transamidase activity (Figure 3.7). As Glu-tRNA$^{Gln}$ and ATP are the two other substrates for AdT, in the kinetic measurements, these two substrates were kept at saturating concentrations (see Chapter 6 for experimental details). The $K_M$ for glutamine is $1.5 \pm 0.2$ mM and the $k_{cat}$ is $4.7 \pm 0.3$ s$^{-1}$, leading to a specificity constant ($k_{cat}/K_M$) of $3.1 \times 10^3$ s$^{-1}$M$^{-1}$. 
Figure 3.7: Michaelis-Menten analysis of the transamidase activity of AdT. Each reaction was initiated with 1 nM AdT. Data were fit directly to the Michaelis-Menten equation using Kaleidagraph V4.0 (Synergy Software).

The measured $k_{cat}$ for WT AdT transamidation is $4.7 \pm 0.3 \text{ s}^{-1}$. This value is similar to that previously reported ($3.49 \pm 0.44 \text{ s}^{-1}$ (70)). However, the $K_M$ for glutamine is $1.5 \pm 0.2 \text{ mM}$ in our measurement, compared to the previously reported $20.7 \pm 9.5 \text{ µM}$ (70). As discussed in Section 3.2.5 above, the disagreement in the $K_M$ values may result from the glutamine concentrations assayed and the high error levels published previously support this hypothesis.
3.2.8 Rate limiting step in AdT catalysis

The $k_{cat}$ value for glutamine hydrolysis in the presence of saturating ATP and Glu-tRNA$^{Gln}$ is 5.7-fold higher than the $k_{cat}$ for Glu-tRNA$^{Gln}$ transamidation at 37 °C (the net AdT reaction). However, the $k_{cat}$ value for Glu-tRNA$^{Gln}$ phosphorylation is 30-fold slower than Glu-tRNA$^{Gln}$ transamidation under the assay conditions used herein. This observation is inconsistent with the fact that phosphorylation must take place before transamidation of Glu-tRNA$^{Gln}$. Consequently, the rate of phosphorylation must be greater than or equal to that of transamidation. *Streptococcus pyogenes* AdT has a $k_{cat}$ for phosphorylation of 1.09 ± 0.1 s$^{-1}$. This value was obtained using an HPLC assay and is faster than transamidation (0.51 ± 0.16 s$^{-1}$), as expected (89). No $k_{cat}$ value for kinase activity has been reported for *H. pylori* AdT (70).

This experimental discrepancy might be caused by different ways of tRNA preparation: we directly used total RNA purified from *in vivo* expression of *H. pylori* tRNA$^{Gln}$. These samples are enriched in *H. pylori* tRNA$^{Gln}$ but contain other *E. coli* RNA contaminants. In contrast, Söll and colleagues purified their tRNA using complementary oligonucleotide probes (70). However, we can reproduce their reported rates of transamidation using our tRNA preparations (see following sections). The differences in the two $k_{cat}$ values for this kinase assay more likely stem from the relatively high enzyme concentration (200 nM) used in our kinase assay and the possibility that this enzyme coupled UV assay may not be sensitive and fast enough to catch early turnovers. Thus, instead of monitoring initial rates, we may be observing steady state ATP hydrolysis.
Regardless of the true value for $k_{\text{cat}}$, systematic comparisons like those conducted herein allow for a fast and informative screen of the kinase activities of AdT and its mutants. We can also conclude that glutaminase activity is not rate-limiting.

### 3.2.9 Characterization and comparison of the glutaminase and transamidase activities of WT and D185$\alpha$ AdT variants

To rule out the influence of enzyme batch differences, three separate colonies for all three D185$\alpha$ AdT mutants were used for cell cultures, protein expression and purification. These separately purified samples of AdT variants were tested in parallel. The average values of the three batches of enzymes were used as the mutants’ glutaminase and transamidase activities.

When compared to the WT enzyme, the D185A$\alpha$ and D185N$\alpha$ mutants maintain reasonable levels of kinase (Figure 2.7) and glutaminase activities (Figure 3.8), but their transamidase activities are very low (Figure 3.8). Transamidation is the rate limiting catalytic step for AdT (70,89). Thus, the minimal reductions in initial rates for the glutaminase and kinase reactions do not account for the dramatic decrease in transamidase activity. This pattern is consistent with a hypothesis that D185$\alpha$ is an acid or a base in the ammonia tunnel. However, it is risky to draw this conclusion with these data alone. To further assess this hypothesis, a D185E$\alpha$ AdT mutant was constructed and
tested. Similar to the D185A_α and D185N_α AdT variants, D185E_α AdT maintained glutaminase (85% of the WT) and kinase (45% of the WT) activities. However, this mutant retained of robust transamidase activity (110% of the WT) (Figure 3.8). In addition, non-redundant multiple sequence alignments showed that glutamate appears at this position approximately 2% of the time. This comparison between D185_α AdT mutants demonstrates that the presence of a carboxylic acid/carboxylate at this position is very important for the catalysis, a conclusion that is consistent with D185 serving as an acid/base residue during ammonia transport.
Figure 3.8: Activity comparison of WT and different D185α AdT variants.

The WT *H. pylori* AdT and D185α variants were tested. Initial rates (divided by enzyme concentration) are shown. See Table 3.2 for the raw initial rate and enzyme concentration data for these assays. The error bars represent standard deviation from triplicate assays made with three different batches of enzymes.
Table 3.2: Initial rates for the glutaminase and transamidase activities of WT and D185\textsubscript{\textalpha} AdT variants

<table>
<thead>
<tr>
<th>AdT</th>
<th>Glutaminase</th>
<th></th>
<th>Transamidase</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial rate ($\mu$M/min)</td>
<td>Enzyme activity* ($s^{-1}$)</td>
<td>Initial rate ($\mu$M/min)</td>
<td>Enzyme activity* ($s^{-1}$)</td>
</tr>
<tr>
<td>WT</td>
<td>12.5 $\pm$ 1.9</td>
<td>13.9 $\pm$ 2.1</td>
<td>0.270 $\pm$ 0.015</td>
<td>4.50 $\pm$ 0.25</td>
</tr>
<tr>
<td>D185A\textsubscript{\textalpha}</td>
<td>3.9 $\pm$ 0.2</td>
<td>4.4 $\pm$ 0.2</td>
<td>0.005 $\pm$ 0.001</td>
<td>0.08 $\pm$ 0.02</td>
</tr>
<tr>
<td>D185E\textsubscript{\textalpha}</td>
<td>10.2 $\pm$ 0.7</td>
<td>11.4 $\pm$ 0.7</td>
<td>0.288 $\pm$ 0.020</td>
<td>4.80 $\pm$ 0.34</td>
</tr>
<tr>
<td>D185N\textsubscript{\textalpha}</td>
<td>5.1 $\pm$ 0.1</td>
<td>5.7 $\pm$ 0.1</td>
<td>0.015 $\pm$ 0.002</td>
<td>0.26 $\pm$ 0.03</td>
</tr>
</tbody>
</table>

* Enzyme activity is defined as initial rate divided by enzyme concentration; this calculation is used as an approximation of $k_{cat}$ for comparison.

3.2.10 Correlation analysis of D185\textsubscript{\textalpha} mutants

To further assess the role of D185\textsubscript{\textalpha}, Sajeewa Dewage performed correlation analyses (See Appendix C for his original correlation data) using molecular dynamics simulations for all mutations at this position (only the data directly related to this discussion are shown. See Figure 3.9 for details). These results suggest that interdomain communication is similar to that of WT in the three D185\textsubscript{\textalpha} mutants. For comparison, a correlation plot for T149V\textsubscript{\textalpha} AdT mutant compared to WT AdT is included in Figure 3.9: This T149V\textsubscript{\textalpha} mutation disrupts interdomain communication between GatA and GatB.
Figure 3.9: Differences in correlation for D185 AdT mutants compared to WT (wild-type values were subtracted from those of each mutant). (A) Differences between wild-type and D185A\(\alpha\) AdT; (B) Differences between wild-type and D185E\(\alpha\) AdT; (C) Differences between wild-type and D185N\(\alpha\) AdT; (D) The color-coded scale representing the calculated correlation differences. See Figure 2.9G for a key to the different panel sub-sections.
The correlation analyses of D185\(\alpha\) AdT mutants indicate that the transamidation defects in D185A\(\alpha\) and D185N\(\alpha\) are not caused by disruption of the interdomain communication pathway that we described in our Biochemistry paper (19). Instead, they further support the importance of the D185\(\alpha\) side chain carboxylic acid/carboxylate for ammonia delivery.

3.2.11 Ammonia tunnel calculations for the D185\(\alpha\) mutants using simulated structures

Similar to the two types of ammonia tunnels previously predicted by the Caver Viewer Program (20,177), both ammonia tunnels (Nakamura and Kang) were observed in the simulated structures of the WT, D185A\(\alpha\) and D185N\(\alpha\) AdT. However, a detailed examination of the ammonia tunnel in the D185E\(\alpha\) mutation revealed that this mutation eliminates the Nakamura tunnel.

It is possible that the extra methylene group in the Glu side chain extends further into the Nakamura ammonia tunnel and blocks the tunnel path. More importantly, the Caver program for tunnel prediction is based on the size, distance and space among residues, and it does not take energy barriers for transfer into consideration (178). Consequently, it is possible that the Nakamura tunnel is not detected by Caver but exists during catalysis.
Figure 3.10: Ammonia tunnel calculations with D185$\alpha$ AdT mutants and WT AdT. All tunnel calculations were conducted by Sajeewa Dewage using simulated structures and Caver Viewer 3.0 (178). Two types of ammonia tunnels (shown in blue (Kang tunnel) and black (Nakamura tunnel) mesh) appear in WT, D185A$\alpha$ and D185N$\alpha$ AdT while only one was predicted in D185E$\alpha$ AdT.

3.2.12 Characterization of computationally predicted A179G$\alpha$/D185G$\alpha$ double AdT mutant

As shown in Figure 3.10, the D185$\alpha$ residue is right at the branching point of the two possible ammonia tunnels. Based on structural modeling and tunnel predictions, Sajeewa Dewage hypothesized that a double glycine substitution at A179$\alpha$ and D185$\alpha$ in AdT would favor the Nakamura tunnel (Figure 3.10, black mesh). While correlation analyses of the A179G$\alpha$/D185G$\alpha$ AdT mutant were
underway, we successfully constructed an A179G\textsubscript{α}/D185G\textsubscript{α} AdT mutant and proceeded to examine its enzymatic activity in comparison to WT and the two single glycine mutants, A179G\textsubscript{α} and D185G\textsubscript{α} AdT.

Kinetic measurement of these AdT variants showed that A179G\textsubscript{α}, in general, had less impact on AdT’s three activities than the D185G\textsubscript{α} mutation that showed only about 5% of WT AdT’s transamidase activity (Figure 3.11). The transamidase activity of the double glycine AdT mutant was still low, 10% of that of the WT, but it seemed to be slightly better than the D185A\textsubscript{α} and D185N\textsubscript{α} AdT mutants (Figure 3.8).
Figure 3.11: Activity comparison of wild-type AdT and A179G$_{\alpha}$/D185G$_{\alpha}$ AdT mutants. The WT AdT and A179G$_{\alpha}$ and D185G$_{\alpha}$ single and double mutant variants were assayed and compared. Enzyme activity, defined as initial rates divided by enzyme concentration, is shown. See Table 3.3 for the raw initial rate and enzyme concentration data for glutaminase and transamidase assays. The error bars represent standard deviation from triplicate assays with three different batches of enzymes.

The analysis of these glycine mutants was originally expected to provide experimental support in favor of one of the ammonia tunnels over the other; however, the transamidase activity of the A179G$_{\alpha}$/D185G$_{\alpha}$ AdT double mutant is
substantially lower than that of WT AdT, consistent with the requirement for a carboxylic acid side chain at position 185.

Table 3.3: Initial glutaminase and kinase rates for WT and A179Gα and D185Gα single and double AdT variants

<table>
<thead>
<tr>
<th>AdT</th>
<th>Glutaminase</th>
<th>Transamidase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial rate (µM/min)</td>
<td>Enzyme activity* (s⁻¹)</td>
</tr>
<tr>
<td>WT</td>
<td>12.5 ± 1.9</td>
<td>13.9 ± 2.1</td>
</tr>
<tr>
<td>A179Gα</td>
<td>9.2 ± 1.0</td>
<td>10.2 ± 1.1</td>
</tr>
<tr>
<td>D185Gα</td>
<td>4.9 ± 0.7</td>
<td>5.5 ± 0.8</td>
</tr>
<tr>
<td>A179Gα/D185Gα</td>
<td>4.6 ± 0.5</td>
<td>5.1 ± 0.5</td>
</tr>
</tbody>
</table>

3.2.13 Impact of D185α mutagenesis on pKₐ values

To better understand the potential role of D185α in ammonia transport, pKₐ value calculations were conducted for all conserved, ionizable tunnel residues using simulated structures (See Table 3.4, calculations were performed by Sajeewa Dewage).
Table 3.4: pK\textsubscript{a} values of conserved, ionizable tunnel residues in AdT

<table>
<thead>
<tr>
<th>Residue position</th>
<th>Standard pK\textsubscript{a} value*</th>
<th>Calculated pK\textsubscript{a} values of amino acid side chain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>WT AdT</td>
</tr>
<tr>
<td>R174\textsubscript{A\alpha}</td>
<td>12.48</td>
<td></td>
</tr>
<tr>
<td>D185\textsubscript{A\alpha}</td>
<td>3.86</td>
<td>5.6 ± 0.5</td>
</tr>
<tr>
<td>R295\textsubscript{A\alpha}</td>
<td>12.48</td>
<td></td>
</tr>
<tr>
<td>R79\textsubscript{B\beta}</td>
<td>12.48</td>
<td></td>
</tr>
<tr>
<td>K80\textsubscript{B\beta}</td>
<td>8.95</td>
<td>10.6 ± 0.9</td>
</tr>
<tr>
<td>K89\textsubscript{B\beta}</td>
<td>8.95</td>
<td>9.8 ± 0.2</td>
</tr>
<tr>
<td>E126\textsubscript{B\beta}</td>
<td>4.25</td>
<td>4.6 ± 0.4</td>
</tr>
<tr>
<td>D276\textsubscript{B\beta}</td>
<td>3.86</td>
<td>5.7 ± 0.5</td>
</tr>
</tbody>
</table>

* pK\textsubscript{a} values of the amino acid side chains are from the Handbook of Chemistry and Physics (179). Boxes highlighted in grey indicate residues with significantly perturbed pKa values compared to free amino acid values. Any value that deviated from published values by more than ± 1 pH unit are highlighted in grey.

Several of the ionic residues in WT AdT’s ammonia tunnel show significantly elevated pK\textsubscript{a} values, with the exception of R295\textsubscript{A\alpha} and E126\textsubscript{B\beta}. None of the D185\textsubscript{A\alpha} AdT mutations perturbed these calculated pK\textsubscript{a}s significantly compared to the WT, suggesting that the mutations at D185\textsubscript{A\alpha} are not likely to change the chemistry of other tunnel residues. These analyses further support...
the hypothesis that the D185\textsubscript{\alpha} carboxylate is required for robust ammonia transport.

3.3 Conclusions

In this chapter, the D185\textsubscript{\alpha} residue was characterized, and the results revealed a probable step in the ammonia delivery mechanism for AdT. The D185E\textsubscript{\alpha} enzymatic and computational results, in combination with the comparison of the D185A\textsubscript{\alpha} and D185N\textsubscript{\alpha} mutants, support our hypothesis that D185\textsubscript{\alpha} acts as an acid/base residue for ammonia transfer during catalysis (Figure 3.12). Most possibly, D185\textsubscript{\alpha} residue serves as an acid to protonate the incoming ammonia, the resultant ammonium will travel down to the next deprotonation location. These protonation and deprotonation steps may help the transfer of ammonia by avoiding any energy penalty for migration through a hydrophilic environment, which may be similar to the roles of energy turning points of the three water pockets in CPS (174).
Figure 3.12: A model for the role of D185α in ammonia delivery. The left cartoon represents the proposed proton relay mechanism for ammonia delivery in AdT hydrophilic ammonia tunnel. The right panel shows a model for D185α as an acid during the ammonia transfer.

In conclusion, this focused characterization on D185α provides evidence of one step in ammonia delivery. The success of this investigation sets the stage for future work on other conserved ammonia tunnel residues to further extend our understanding of the AdT ammonia delivery mechanism.
4.1 Introduction

Interdomain communication is common to the enzymes that utilize molecular tunnels to transfer intermediates between isolated active sites (5,130,158,180). This cooperative behavior enhances enzyme efficiency in two ways: first, the generation of an unstable intermediate does not occur until the next substrate binds to the distal active site and is positioned to react; second, the isolated active sites are synchronized upon conformational changes triggered by substrate binding (4).

The glutamine-dependent amidotransferases (GATs) have ammonia tunnels to connect isolated active sites. The residues that constitute these ammonia tunnels often mediate interdomain communication (144,151,152,181). In some cases, ammonia tunnel formation requires substrate binding (139,151,182), while for others, such as imidazole glycerol phosphate synthase (IGPS), the ammonia tunnel exists at all times but is only opened upon substrate binding (183,184). The open and closed states of GAT ammonia tunnel are generally proposed to be controlled by gate residues inside these tunnels (4). In the open conformation, reorientation of the gate residue is often triggered by substrate binding at one of the enzyme’s active sites. In glucosamine-6-phosphate synthase (GlmS), the indole ring of Trp74 blocks the ammonia tunnel
when the glutaminase active site is empty. The binding of DON, a glutamine mimic, triggers rotation of Trp74 to open the tunnel (144).

*H. pylori* Asp-tRNA<sub>Asn</sub>/Glu-tRNA<sub>Gln</sub> amidotransferase (AdT) is a GAT, and uses a molecular tunnel to transfer ammonia between its two isolated active sites (5,20). AdT is a critical enzyme in the bacterial indirect aminoacylation pathway for the biosynthesis of Gln-tRNA<sub>Gln</sub> and Asn-tRNA<sub>Asn</sub> (17,18). AdT’s ammonia tunnel is extremely hydrophilic, compared to the typical hydrophobic GAT ammonia tunnels (2,5). AdT’s tunnel is lined with ionic and polar residues and is filled with highly ordered water molecules (20). Ammonia transfer through this tunnel was proposed to occur through a series of protonation and deprotonation steps, and the E126 residue in GatB was proposed to act as a gate (20). Our previous MD simulation results showed that the carboxyl side chain of E126<sub>β</sub> is flexible (contributed by Mike Bell, unpublished results), consistent with a possible role as a gate. In addition, K89<sub>β</sub> is in close proximity to E126<sub>β</sub> on the other side of the ammonia tunnel (Figure 4.1) (20). K89<sub>β</sub> can form a salt bridge with E126<sub>β</sub>, perhaps serving as a tunnel gate in cooperation with each other. We set out to initiate studies to examine the importance of both of these residues to AdT’s catalytic activity. Our preliminary experiments are described in this chapter.
Figure 4.1: Positioning of K89\(\beta\) and E126\(\beta\) in the AdT ammonia tunnel. K89\(\beta\) and E126\(\beta\) are located close to the interface of GatA and GatB, and their side chains are within hydrogen bond distance of each other. The mesh represents the hydrophilic Nakamura tunnel (20), and the right panel shows a close up view of K89\(\beta\) and E126\(\beta\). The distance between the K89 amine nitrogen and the E126 carboxyl oxygen atoms are 2.8 Å and 3.7 Å in this \textit{H. pylori} AdT model built from the simulated \textit{S. aureus} AdT crystal structure (PDB ID: 2F2A (20), model provided by Sajeewa Dewage).
4.2 Results and discussions

4.2.1 Mutagenesis strategy to probe the roles of K89β and E126β

To explore the roles of K89β and E126β in ammonia delivery, we started by constructing new mutations at these positions, in addition to the mutations presented in Chapter 2 (K89Aβ, K89Rβ, E126Aβ, E126Dβ, and E126Qβ). We also constructed single mutants to invert the charge states of these residues (K89Eβ and E126Kβ) and the double mutations K89Eβ/E126Kβ and K89Rβ/E126Dβ to test whether charge inversion across the ammonia tunnel would still allow AdT to function. The single K89Eβ and E126Kβ and the K89Rβ/E126Dβ AdT double mutants were successfully purified (See Chapter 6, Figure 6.1) while the K89Eβ/E126Kβ double mutant was not expressed at accessible levels.

4.2.2 Kinase activity of K89β and E126β AdT mutants

The kinase activity of AdT mutants with variations at K89β was examined both enzymatically and computationally as discussed in Chapter 2 and published (19). A loss of more than 50% of AdT’s kinase activity was observed even with
the conservative K89Rβ mutation. Comparative correlation analyses of the wild-type (WT) and K89Rβ AdT mutant did not show significant correlation changes.

The kinase activity of K89Eβ AdT is consistent with that of K89Rβ AdT (about 35-40% of the WT). Mutations at position E126 in GatB had similar effects on AdT’s kinase reaction (Figure 2.7B), even the E126Kβ AdT mutant showed initial rates that were about 50% of WT (See Table 4.1 for initial rates).

Table 4.1: Initial rates of phosphorylation of Glu-tRNA^{Gln} for K89 and E126 AdT variants

<table>
<thead>
<tr>
<th>GatB Mutants</th>
<th>Initial rate (μM/min)</th>
<th>S. D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdT WT</td>
<td>1.11</td>
<td>0.20</td>
</tr>
<tr>
<td>K89A</td>
<td>0.37</td>
<td>0.03</td>
</tr>
<tr>
<td>K89E</td>
<td>0.38</td>
<td>0.04</td>
</tr>
<tr>
<td>K89R</td>
<td>0.40</td>
<td>0.04</td>
</tr>
<tr>
<td>E126A</td>
<td>0.72</td>
<td>0.09</td>
</tr>
<tr>
<td>E126D</td>
<td>0.98</td>
<td>0.10</td>
</tr>
<tr>
<td>E126K</td>
<td>0.50</td>
<td>0.07</td>
</tr>
<tr>
<td>E126Q</td>
<td>0.78</td>
<td>0.07</td>
</tr>
<tr>
<td>K89R/E126D</td>
<td>0.49</td>
<td>0.04</td>
</tr>
</tbody>
</table>

4.2.3 Glutaminase and transamidase activities of K89β and E126β AdT mutants

In general, conservative mutations that maintained the correct side chain charge states maintained at least 80% of the glutaminase activity of WT AdT. In
contrast, when mutated to alanine or when the charge state was reversed, the glutaminase activities of these AdT variants were reduced to 35 to 50% of WT (Figure 4.2, white bars).
Figure 4.2: Activity comparison of WT and different K89β and E126β AdT variants. The WT *H. pylori* AdT and K89β and E126β variants were tested. Enzyme activity (defined as initial rates divided by enzyme concentration) is shown. See Table 4.2 for the raw initial rate and enzyme concentration data for these assays. The error bars represent standard deviation from triplicate assays made with three different batches of enzymes.
Table 4.2: Initial rates for the glutaminase and transamidase activities of WT and K89\(\beta\) and E126\(\beta\) AdT variants

<table>
<thead>
<tr>
<th>Enzyme activity*</th>
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<th>Enzyme activity*</th>
<th>Initial rate ((\mu)M/min)</th>
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<td>WT</td>
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<td>0.27 ± 0.02</td>
<td>4.50 ± 0.25</td>
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<td>K89A(\beta)</td>
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<td>7.9 ± 0.4</td>
<td>0.20 ± 0.01</td>
<td>3.41 ± 0.20</td>
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<td>K89E(\beta)</td>
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<td>8.2 ± 1.7</td>
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<td>K89R(\beta)</td>
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<td>E126A(\beta)</td>
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<td>E126D(\beta)</td>
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<td>E126K(\beta)</td>
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<td>K89R(\beta)/E126D(\beta)</td>
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<td>12.4 ± 0.90</td>
<td>0.10 ± 0.02</td>
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* Enzyme activity is defined as the initial rate divided by the enzyme concentration; this calculation is used as an approximation of \(k_{cat}\) for comparison.

The characterization of transamidase activity showed that K89A\(\beta\) AdT retains 75% WT activity. In contrast, the more conservative K89R\(\beta\) mutation increases the activity of AdT approximately two-fold compared to WT AdT (Figure 4.2). Sequence conservation analysis showed that Lys is 85.7%
conserved at this 89 position compared to 0.7% for Arg. These apparently contradictory observations raise an interesting question: If K89β AdT is twice as active as WT AdT, why hasn’t R89β emerged as the dominant residue at this position? This conundrum cannot be explained using our preliminary data, but our hypothesis is that K89β AdT may be more stable and efficient enough for catalysis while R89β AdT, though having higher catalytic efficiency, is less stable and eventually lost during evolution. These enzymatic measurements were made using Glu-tRNA^Gln as the misacylated tRNA substrate. It is also possible that a role for K89β will be revealed with respect to Asp-tRNA^ Asn transamidation.

Unexpectedly, the transamidase activities of the E126β AdT mutants did not followed the prediction that the conservative Asp mutation would maintain enzyme activity at a level similar to WT AdT. E126Dβ demonstrated a transamidase activity that was only 16% that of WT AdT. The E126Aβ variant was slightly better with an activity of 26% that of WT, while the E126Qβ variant showed about 50% activity. The transamidase activity of the E126Kβ AdT mutant is almost completely ablated.
Different from the case of D185α AdT variants discussed in Chapter 3, the carboxylate side chain of E126β seemed not to be as important a feature because the E126Dβ AdT mutant does not have robust transamidase activity. The higher activity of E126Qβ AdT suggests the residue present at 126 position may serve as hydrogen bond acceptor to relay the ammonia transfer or the size of the residue at 126 position affect the ammonia transfer path. This activity argues against an electrostatic bridge between E126β and K79β as the tunnel gate because the neutral E126Qβ cannot form an electrostatic interaction with K79β. According to multiple sequence alignments, at position 126, Glu is 90% conserved, 7.3% of the sequences have a Gln at this 126 position, but only 0.7% of the sequences contain an Asp residue; these trends match the E126β variants’ enzyme activity results. The diminished transamidase activity of the E126Kβ AdT mutant may result from the extend side chain blocking the ammonia tunnel or failing to relay the ammonia for its step in transfer.

To further assess the impact of perturbations of these two residues on enzyme activity, we designed two double AdT mutants, K89Rβ/E126Dβ and K89Eβ/E126Kβ. Unfortunately, we were unable to purify the charge reversed
double AdT mutant (K89Eβ/E126Kβ); this double mutant was expected to provide more insights into the roles of K89β and E126β in AdT’s ammonia tunnel. The K89Rβ/E126Dβ AdT double mutant showed a similar level of glutaminase activity to the WT (over 90%) but only 35% of the WT’s transamidase activity. A comparison of the transamidase activities between K89Rβ, E126Dβ and K89Rβ/E126Dβ AdTs indicates that the K89Rβ/E126Dβ AdT resembles E126Dβ AdT in terms of enzyme activity. The additional K89Rβ mutation induced only a slight increase in activity compared to the E126Dβ mutation alone.

4.2.4 Preliminary ammonia tunnel calculations for K89Rβ/E126Dβ and K89Eβ/E126Kβ AdT mutants

Preliminary ammonia tunnel calculations were performed directly in Caver Viewer Software (178) using the S. aureus AdT (PDB ID: 2F2A (20)) structure harboring four different single mutations (K89Eβ, K89Rβ, E126Dβ, and E126Kβ) and two double mutations (K89Eβ/E126Kβ and K89Rβ/E126Dβ). These mutations were introduced using Pymol and the structures were not further
minimized. For comparison purposes, Caver results with WT AdT are also shown overlaid in black mesh (Figure 4.3 and Figure 4.4).

Figure 4.3: Comparison of the predicted ammonia tunnels with K89Rβ and E126Dβ mutations. (A) Comparison of E126Dβ (red mesh) and WT AdT tunnels; (B) Comparison of K89Rβ (blue mesh) and WT AdT tunnels; (C) Comparison of K89Rβ/E126Dβ (orange mesh) and WT AdT tunnels. The black mesh represents the WT Nakamura ammonia tunnel (20).
Figure 4.4: Comparison of the predicted ammonia tunnels in AdT variants harboring K89Eβ and E126Kβ mutations. (A) Comparison of E126Kβ (green mesh) and WT AdT; (B) Comparison of K89Eβ (yellow mesh) and WT AdT; (C) Comparison of K89Eβ/E126Kβ (light orange mesh) and WT AdT. The black mesh represents the WT Nakamura ammonia tunnel (20).

These ammonia tunnel calculations show altered paths at the junction of K89 and E126 upon mutagenesis. In AdT variants containing E126Dβ (Figure 4.3A and C) and E126Kβ (Figure 4.4A and C), there is a slight variation at the beginning of the ammonia tunnel; this deviation is not observed with K89Rβ AdT
(Figure 4.3B) or K89E$\beta$ AdT (Figure 4.4B). Not surprisingly, the tunnel path at the interface of K89$\beta$ and E126$\beta$ showed obvious changes compared to WT AdT.

The observation that substitution at E126 in GatB can influence the tunnel pathway at the entrance of the ammonia tunnel in GatA suggests that this residue is involved in long-distance interdomain communication and tunnel formation. This perturbation demonstrates how mutations at E126$\beta$ can be sensed by the glutaminase active site, offering an explanation for the reduced glutaminase activity of the E126D$\beta$ and E126K$\beta$ AdT mutants. In general, K89$\beta$ AdT variants showed less perturbation of the WT ammonia tunnel path than E126$\beta$ AdT variants did. These observations were mirrored by our kinetic analyses: the effect of the K89$\beta$ mutations on AdT’s activity was smaller than those of E126$\beta$ mutations. This agreement between enzyme activity and tunnel alternations supports the idea that a proper ammonia transfer path is important for enzyme activity. In addition, the altered ammonia tunnels predicted in the charge reversed single mutants also suggest that changes in the ammonia tunnel path may be the reason why the transamidase activities of the K89E$\beta$ and E126K$\beta$ AdT mutants are diminished.
These preliminary tunnel calculations suggest that enzyme activity is strongly related to the integrity of the ammonia transfer pathway. These subtle ammonia tunnel alternations may also ultimately contribute to our understanding of the ammonia delivery mechanism. Further detailed MD simulations and tunnel calculations, using simulated AdT structures, are required. Computational examinations of the roles of K89β and E126β are still in progress. We expect that these analyses will be useful to define further experiments to understand the roles of these residues in AdT catalysis.

4.3 Conclusions

The results discussed in this chapter represent our initial efforts to investigate the roles of the proposed ammonia tunnel gate residue, E126β, and its close partner K89β. This enzymatic characterization of mutants restates that the K89β and E126β positions and identities are important for enzyme function. Substitution at these positions causes a reduction in enzyme activity is possibly caused by alternations in the formation and positioning of the ammonia tunnel.
CHAPTER 5
CONCLUSIONS AND FUTURE DIRECTIONS

The work presented in this dissertation focuses on the ammonia delivery mechanism through the unusually hydrophilic ammonia tunnel in *H. pylori* Asp-tRNA$^{\text{Asn}}$/Glu-tRNA$^{\text{Gln}}$ amidotransferase (AdT) (19,20). Most known ammonia tunnels are largely hydrophobic to ensure the delivery of reactive and nucleophilic ammonia instead of an ammonium cation (2). With many water molecules and polar and ionic residues lining the AdT ammonia tunnel, AdT must employ a different mechanism for ammonia delivery. Although a proton relay mechanism was proposed in 2006 (20), it has lacked experimental support until now. Our enzymatical and computational characterization of AdT reveal several interesting aspects of this proton relay mechanism. In this chapter, specific conclusions from the work described here as well as the studies needed to further understand the ammonia tunnel mechanism will be summarized.

5.1 Interdomain communication of AdT revealed by kinase assay and molecular dynamics simulation

An enzyme-coupled UV assay offered us a fast method to quickly scan the phosphorylation activity of a large number of AdT mutants. Although this method might be not sensitive enough to measure true initial rates, it still provides direct and informative comparisons of different AdT variants using high enzyme concentrations. This assay revealed that T149$\alpha$ and K89$\beta$ AdT mutants showed
a reduction in the rate of Glu-tRNA\textsuperscript{Gln} phosphorylation, even upon conservative mutations. These enzymes were subjected to intensive molecular dynamics (MD) simulations. The combination of conventional and MD methods enabled us to map a possible interdomain communication pathway onto AdT.

**5.2 Investigation of D185\(\alpha\) AdT mutants reveals a step in ammonia delivery**

Our kinetic measurements of the enzyme activity of D185\(\alpha\) AdT mutants demonstrate the importance of the side chain carboxylate or carboxylic acid at position 185 for enzyme activity. Most mutations at this site maintain significant glutaminase and phosphorylation activity but their ability to complete the transamidation of Glu-tRNA\textsuperscript{Gln} to produce Gln-tRNA\textsuperscript{Gln} was severely affected. The one exception was for the D185\(\alpha\)E mutant, which retained wild-type like activity. Furthermore, molecular dynamic simulations demonstrated that interdomain communication was not disrupted upon mutation at this position.

With respect to the proposed proton relay mechanism, it is probably that D185\(\alpha\) serves as a general acid (its pK\(a\) is about 5.5) in the ammonia tunnel to protonate the incoming ammonia (Figure 5.1). The position of D185 at the top of AdT’s ammonia tunnel and near the site of glutamine hydrolysis suggests that protonation of ammonia introduces ammonium to the ammonia transfer tunnel of AdT.
Figure 5.1: A model for ammonia delivery in AdT. Left: Ionic residues act as general acids (A-H) and bases (B:) to protonate ammonia transfer through the tunnel. Right: We propose that D185α is the first acid in the AdT ammonia tunnel, protonating ammonia as it is released from glutamine. The carbonyl, but not the carboxylate, in E126β is essential for AdT activity, suggesting that this side chain positions the ammonia or ammonium via a hydrogen bond.
5.3 $E126_\beta$ might mediate ammonia transfer through hydrogen binding

The characterization of the $E126_\beta$ and the $K89_\beta$ AdT mutants are preliminary. Although we cannot provide solid evidence to argue for or against the gate hypothesis, the enzyme behavior of the $E126_\beta$ AdT mutants does suggest that it does not serve as a general acid or base. Instead, we hypothesize that the $E126_\beta$ residue participates in ammonia transfer by hydrogen bond formation.

Our examination of AdT’s ammonia tunnel offered us a further step towards understanding the mechanism of ammonia transfer in this hydrophilic tunnel. The integration of each individual case study ($T149_\alpha$, $D185_\alpha$, $K89_\beta$, and $E126_\beta$) enables us to propose a model for ammonia transfer in AdT as shown in Figure 5.1. This model is expected to be refined further by integrating more results from other analyses of other conserved tunnel residues.

In fact, the work described here only focused on a few key residues in the AdT ammonia tunnel. Besides the mutations discussed above, variations at other tested tunnel residues appear to be quite sensitive to perturbation, even upon conservative mutation, suggesting that the roles of conserved tunnel residues may go much beyond general acids or bases for ammonia delivery. These tunnel residues are positioned at different distances from the active site, but each one appears to be crucial for enzyme catalysis.
There are still many questions that we cannot answer at this stage. One of the biggest challenges in the approach presented here is that the MD of AdT shown in this dissertation did not take the impact of binding of the misacylated tRNA into consideration. A crystal structure of AdT complexed with a misacylated tRNA is not available. As the isolated AdT active sites communicate with each other, omitting misacylated tRNA, one of the key substrates, is risky. However, the work presented in this dissertation offers a valuable starting point for future modeling studies of AdT.

Therefore, future work must not only focus on individual case studies of other highly conserved tunnel residues, but also on the impact of binding of misacylated tRNA substrates to AdT. Docking studies are needed to build a more representative model of AdT with misacylated tRNA bound to the GatB active site. These planned studies can be guided by the crystal structure of GatDE bound to tRNA\textsuperscript{Gin} (76) because GatB and GatE are closely related to each other evolutionarily. The efforts presented in this dissertation hopefully will serve as a model for the powerful combination of enzyme kinetics and computations to extend our understanding of molecular tunnels in enzymes.
CHAPTER 6
MATERIALS AND METHODS

The purpose of this chapter is to describe the experimental methods that are common to chapters 2, 3, and 4 in this dissertation. Variations and modifications to these methods will be discussed individually in relevant chapters.

6.1 General

Oligonucleotides were purchased from Invitrogen and used without further purification. Taq polymerase was from New England Biolabs (Ipswich, MA). All radiolabeled reagents ([L-\(^{3}\)H] glutamine, [L-\(^{14}\)C] glutamate, [L-\(^{14}\)C] glutamine, and [\(\alpha^{32}\)P] ATP) were from American Radiolabeled Chemicals (St. Louis, MO). Pfu polymerase, and *E. coli* DH5\(\alpha\) and BL21(DE3) strains were from Stratagene (La Jolla, CA). Isopropyl \(\beta\)-D-1-thiogalactopyranoside (IPTG) was from Gold Biotechnology (St. Louis, MO). Pyruvate kinse (PK), L-Lactate dehydrogenase (LDH), phosphoenolpyruvate (PEP), \(\beta\)-nicotinamide adenine dinucleotide (reduced) (NADH), nuclease P1 and PEI-cellulose TLC plates were from Sigma (St. Louis, MO). Other general reagents were from either Sigma (St. Louis, MO) or Fisher Scientifics (Hampton, NH). All buffers were filtered through a 0.22 \(\mu\)m filter prior to use. When appropriate, solutions were autoclaved. Unless otherwise stated, reagents were used without further purification.
6.2 *H. pylori* AdT cloning

The *H. pylori* gatCAB gene was originally amplified from *H. pylori* strain 26695 (67). The genes coding for the three subunits of AdT were sub-cloned into two compatible plasmids by Drs. Stéphane Skouloubris and Pitak Chuawong (unpublished results). Plasmid pPTC032 is derived from pCDF-1b (Novagen) and codes for GatC and GatA in a single operon with an N-terminal six-histidine (His$_6$) tag appended onto GatC. Plasmid pSS003 (derived from pET-28a) encodes for GatB with an N-terminal six-histidine (His$_6$) tag (pPTC032).

6.3 Site-directed mutagenesis of *H. pylori* AdT

Individual point mutations were introduced into either the gatA or gatB gene (encoded in plasmids pPTC032 and pSS003, respectively) by QuikChange™ mutagenesis according to the directions provided by Stratagene (La Jolla, CA). Oligonucleotide sequences and plasmids are listed in Table 6.1 and Table 6.2. The plasmid pPTC032 encoding for GatCA was used as template for GatA mutations, and the plasmid pSS003 encoding for GatB was used as template for GatB mutations. All gene constructs were verified by DNA sequencing of the entire gene prior to use. DNA sequencing was carried out by Applied Genomics Technology Center at Wayne State University.
Table 6.1: *H. pylori* GatA mutagenesis primer sequences and plasmid names

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Red letters indicate sites of mutagenesis.

<sup>a</sup>Mutation constructed by Shirin Fatima

<sup>b</sup>Mutation constructed by Keng-Ming Chang

<sup>c</sup>Mutation constructed by Nilesh Joshi

<sup>d</sup>Mutation constructed by Gayathri Silva
Table 6.2: *H. pylori* GatB Mutagenesis Primer Sequences and Plasmid Names

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Red letters indicate sites of mutagenesis.
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Red letters indicate sites of mutagenesis.

- Mutation constructed by Shirin Fatima
- Mutation constructed by Keng-Ming Chang
- Mutation constructed by Nilesh Joshi
- Mutation constructed by Gayathri Silva
6.4 Expression and purification of *H. pylori* AdT

For wild-type AdT, *E. coli* strain BL21(DE3) calcium chloride competent cells were transformed with both pPTC032 (encoding His\textsubscript{6}-GatC and GatA in a single *gatCA* operon) and pSS003 (encoding His\textsubscript{6}-GatB). For expression of each AdT mutant, BL21(DE3) cell was first transformed with the plasmid encoding for the wild-type subunit(s) and then individually transformed with each mutated plasmid. For example, to produce the T149A AdT mutant, cells were transformed with pSS003, encoding for wild-type His\textsubscript{6}-GatB, and then with plasmid pSF005, encoding for His\textsubscript{6}-GatC and T149A GatA in an operon. In all cases, colonies containing both plasmids were selected on agar plates supplemented with kanamycin (50 µg/mL) and streptomycin (50 µg/mL) and incubated at 37 °C overnight. A 5 mL Luria Broth (LB) culture, containing the same two antibiotics, was inoculated with a single colony. The overnight culture was used to inoculate a 500 mL culture in the same medium. When the absorbance at 600 nm was between 0.4 and 0.6, IPTG was added to a final concentration of 1 mM to induce overexpression. Cells were harvested by centrifugation at 5,000 rpm for 5 minutes at 4 °C after a 4-hour induction at 37 °C shaking at 200 rpm. The cell pellet was either stored at -80 °C or used directly.

AdT or its mutants were purified by HisPur\textsuperscript{TM} Cobalt resin from Pierce (Rockford, IL) as per the manufacturer's instruction. The elution fractions from the resin were combined, exchanged with exchanging buffer (50 mM 2-[(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid (HEPES)-KOH pH 7.2, 300 mM NaCl), and concentrated using a 30 K Amicon\textsuperscript{®} Ultra-4 spin column. All mutated
AdT variations were readily purified to homogeneity with the exception of R174K\textsubscript{\alpha} and K89E\textsubscript{\beta}/E126K\textsubscript{\beta} mutants. Protein purity (>95\%) was judged by 12\% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (See Figure 5.1 and 5.2), and concentrated protein samples were stored in 50\% glycerol at -20 °C and used within two weeks. Protein concentrations were determined by UV/Vis spectroscopy using the extinction coefficients for each protein using the ExPASy Proteomics server (http://ca.expasy.org/tools/protparam.html) (185) in triplicate.

To avoid enzyme batch differences, for each variant, three individual colonies were used for inoculating the cell growth and protein overexpression. All three batches of enzymes were assayed, and the average was used to represent the enzyme activity.
Figure 6.1: SDS-PAGE analysis of *H. pylori* AdT and GatA mutants. Most mutations were readily purified to homogeneity. The exception was the R174A\(\alpha\) mutant, which cannot be purified.
Figure 6.2: SDS-PAGE analysis of *H. pylori* AdT and GatB mutants. Most mutations were readily purified to homogeneity. The exception was the K89E<sub>B</sub>/E126K<sub>B</sub> double mutant, which cannot be purified.

### 6.5 Expression and purification of *H. pylori* GluRS2

*H. pylori* GluRS2 was overexpressed in the *E. coli* BL21(DE3) and purified to homogeneity as previously described (Figure 5.2) (67).

### 6.6 Expression and purification of *E. coli* CCA adding enzyme

The gene encoding the *E. coli* CCA adding enzyme was overexpressed in *E. coli* BL21(DE3) transformed with the plasmid kindly provided by Professor
Rebecca Alexander from Wake Forest University. The CCA adding enzyme was also purified by HisPur™ Cobalt resin and verified by SDS-PAGE (Figure 6.3).

![Figure 6.3: SDS-PAGE analysis of *H. pylori* GluRS2 and *E. coli* CCA adding enzyme. GluRS2 is about 50 kDa in size, and CCA adding enzyme is about 47 kDa.](image)
6.7 *In vivo* transcription of *H. pylori* tRNA\textsuperscript{Gln} and tRNA purification

*H. pylori* tRNA\textsuperscript{Gln} was prepared *in vivo* in the *E. coli* strain MV1184 as previously described (67,186) with some modifications on cell growth conditions. For each 1 liter growth, the LB medium was inoculated with 20 mL overnight culture from a single colony, the medium was supplemented with 75 µg/mL ampicillin. When the OD\textsubscript{600} of the cell culture reached between 0.4 and 0.6, the transcription of tRNA\textsuperscript{Gln} was induced by adding IPTG to a final concentration of 1 mM; 50 µg/mL ampicillin was also added at this time. The culture was incubated at 37 °C for 4 hours, and the cells were harvest at 5,000 rpm for 5 minutes at 4 °C. The cell pellet was either used directly or stored at -80 °C.

Originally, *H. pylori* tRNA\textsuperscript{Gln} was isolated using Nucleobond RNA/DNA Maxi Kits (Clontech) to enrich tRNA molecules. Later, the Nucleobond purification method was simplified to a total RNA purification protocol, in which the tRNA over transcribed cells were lysed through the same procedures while instead of the Nucleobond column affinity purification, acidic phenol (pH 4.2, saturated by 100 mM citric acid)/chloroform extraction was performed to remove genomic DNA and the resulted aqueous layer was isopropanol precipitated. *H. pylori* tRNA\textsuperscript{Gln} was purified in a mixture with total *E. coli* RNA.

6.8 Aminoacylation and quantification of tRNA\textsuperscript{Gln}

Prior to aminoacylation, a solution of tRNA\textsuperscript{Gln} was incubated in a 75 °C water bath for 5 minutes and slowly cooled down to 65 °C. Then, 2 mM MgCl\textsubscript{2}
was added, and the sample was slowly cooled down to room temperature. This process folds the tRNA in preparation for aminoacylation.

The amount of tRNA\textsuperscript{Gln} isolated by phenol extraction was quantified by aminoacylation assay. The aminoacylation assay was performed in 20 mM HEPES-KOH (pH 7.5), 2 mM ATP, 4 mM MgCl\textsubscript{2}, 10 µM glutamate, and 50 µCi/mL L-[2,3,4-\textsuperscript{3}H] glutamate. GluRS was added to a final concentration of 1 µM. Aliquots of the aminoacylation reaction were removed and quenched on filter pads (Whatman) containing trichloroacetic acid (TCA). After quenching, the filter pads were immediately soaked in chilled 5% TCA and washed for 15 minutes, three times. The filter pads were dried and counted in 3 mL ECOLITE(+)\textsuperscript{TM} scintillation cocktail fluid (MP Biomedicals). The concentration of tRNA\textsuperscript{Gln} was calculated from the amount of TCA-precipitated glutamate present on an average of 3-4 pads.

In order to produce unlabeled Glu-tRNA\textsuperscript{Gln} for kinase and glutaminase assays, the reaction volume was scaled up in the same reaction buffer but without radiolabeled amino acid. This reaction was incubated at 37 °C for two hours, and then extracted with phenol/chloroform. The Glu-tRNA\textsuperscript{Gln} was further purified on a G25 column (GE Healthcare) by centrifugation according to user’s guideline. The supernatant was supplemented with 3 M sodium acetate (pH 5.0) and precipitated with isopropanol. The tRNA pellet was resuspended with an appropriate amount of ddH\textsubscript{2}O and either used directly or frozen in small fractions and stored at –20 °C. A parallel, radiolabeled assay was always performed to follow and quantify the level of aminoacylation.
6.9 *H. pylori* AdT glutaminase assay

To examine AdT's glutaminase activity, four different methods (See Appendix B) were tested. The Amplex Red method was selected due to its high sensitivity. The Amplex Red assay is a commercial, fluorescence method (See Figure 6.4 for details). Glutaminase assays were carried out in buffer containing 20 mM HEPES-KOH (pH 7.5), 4 mM ATP, 5 mM MgCl$_2$, and 10 μM Glu-tRNA$^{Gln}$ with 15 nM AdT at 37 °C. A parallel, no-enzyme control was also conducted. For Michaelis-Menten kinetic constant measurements, the glutamine concentration was varied from 25 μM to 10 mM.

Aliquots (10 μL) were removed from the reaction at different time points and quenched by the addition of 1 μL 3 M NaOAc (pH 5.2). The quenched sample was ethanol precipitated at -20 °C for 15 minutes, followed by centrifugation at 13,000 rpm at 4 °C for 15 minutes. A 10 μL fraction of the supernatant was removed, transferred to a new tube and vacuum dried. The residue was redissolved in 50 μL ddH$_2$O and used directly in the Amplex Red assay according to the Amplex Red Kit user's guide.
Figure 6.4: Schematic representation of the Amplex Red assay used to measure the glutaminase activity of *H. pylori* AdT. In this assay, glutamic acid is oxidized by glutamate oxidase (GO) to produce α-ketoglutarate, ammonia and hydrogen peroxide. The α-ketoglutarate is then converted back to glutamic acid by glutamate pyruvate transaminase (GPT), which results in multiple cycles of the oxidation reaction and a significant amplification of hydrogen peroxide. The hydrogen peroxide reacts with non-fluorescent Amplex Red dye (10-acetyl-3, 7-dihydroxyphenoxazine) at a stoichiometric ratio of 1:1 under catalysis by horseradish peroxidase (HRP). The product, Resorufin, is highly fluorescent and can be easily detected.
6.10 *H. pylori* AdT kinase assay

The kinase activity of AdT was monitored via adaptation of a coupled enzyme assay (see Figure 6.5). Unless otherwise stated, assays were performed at room temperature in buffer containing 20 mM HEPES-KOH (pH 7.5), 5 mM MgCl₂, 0.1 mM dithiothreitol (DTT), 2 mM phosphoenolpyruvate (PEP), 2 mM ATP, 20 units/mL pyruvate kinase (PK), 20 units/mL L-lactate dehydrogenase (LDH) and NADH to give an absorbance of 0.3 to 0.4 at 340 nm (between 50 and 70 μM, based on standards of known concentration). For initial rate determinations, saturating concentrations of Glu-tRNA\(^{Gln}\) (10 μM) were used so that the observed initial rates would approximate \(k_{cat}\) for each residue. This approach allowed us to focus our analysis on catalytic defects that were conveyed across distances through the enzyme. The possibility that \(K_M\) is altered for one or more mutations cannot be ruled out but was not expected to impact data analysis; these values may be determined at a later date.

For Michaelis-Menten analyses, the concentration of Glu-tRNA\(^{Gln}\) was varied from 0 to 8 μM and AdT was held constant at 300 nM. All other assays were initiated with 200 nM AdT. Reaction progress was monitored on a Beckman DU 800 spectrometer at 340 nm. Full Michaelis-Menten values were only determined for wild-type AdT. As part of the optimization of this assay, wild-type AdT concentrations were varied from 0 to 10 μM to confirm that the coupling enzyme conditions were reporting kinase activity (rather than the activities of PK or LDH). Additionally, the NADH concentration was optimized (reduced from 200 μM to < 70 μM) to produce the largest observable change in absorbance without
impacting the actual rate of phosphorylation; this optimization led to more accurate and reproducible results.

**Figure 14:** Schematic of the coupled enzyme assay used to measure phosphorylation of Glu-tRNA$_{Gln}$ by AdT. Hydrolysis of ATP is coupled to the oxidation of NADH; the resultant decrease in NADH concentration is monitored by UV at 340 nm.
6.11 H. pylori AdT transamidase assay

6.11.1 Preparation and aminoacylation of $^{32}$P-labeled H. pylori tRNA$^{\text{Gln}}$

Refolded H. pylori tRNA$^{\text{Gln}}$ was radiolabeled by the E. coli CCA-adding enzyme (1 µM) using [$\alpha$-$^{32}$P] ATP (1.6 µCi/µL) in 50 mM Tris-HCl (pH 8.0), 20 mM MgCl$_2$, 5 mM DTT, 50 µM sodium pyrophosphate (NaPP$_i$). After labeling, the sample was extracted with citric acid buffered phenol (pH 4.3)/chloroform, and excess [$\alpha$-$^{32}$P] ATP was removed by G25 spin column (GE Healthcare). The $^{32}$P-labeled tRNA$^{\text{Gln}}$ was isopropanol precipitated and diluted with additional 10-20 µM unlabeled tRNA$^{\text{Gln}}$ as described above for the preparation of Glu-tRNA$^{\text{Gln}}$. After aminoacylation, the reaction mixture was phenol/chloroform extracted and isopropanol precipitated. The pellet was dissolved in ddH$_2$O to make a 50 µM Glu-tRNA$^{\text{Gln}}$ stock solution, which was aliquoted into 5 µL fractions and kept at -20 °C for use in transamidase assays.

6.11.2 $^{32}$P/nuclease P1 transamidase assay

The transamidase assay was conducted in buffer containing 50 mM HEPES-KOH (pH 7.2), 15 mM MgCl$_2$, 25 mM KCl, 4 mM ATP, 5 mM glutamine and 10 µM $^{32}$P-labeled Glu-RNA$^{\text{Gln}}$. For kinetic determinations, initial rates were measured in triplicate while varying concentrations of one substrate and keeping the other substrates at saturating levels. The reaction mixture was pre-equilibrated at 37 °C and initiated with 1 nM H. pylori AdT. In parallel, a no enzyme control and positive control with 1 µM AdT were set up. A 2.5 µL aliquot for each reaction time point was removed, quenched in and digested with 2.5 µL
nuclease P1 (Sigma) in a 100 mM sodium citrate suspension (pH 4.2, 0.5 mg/mL). The digestion was kept at 37 °C in a water bath for 30 min, and 1 µL of the digested sample was spotted onto pre-washed PEI-cellulose TLC plates (Sigma). The TLC plate was eluted in buffer containing 100 mM ammonium acetate and 5% acetic acid for approximately 2 hours. The plates were imaged overnight and quantified by PhosphorImager (See Figure 6.6).

**Figure 6.5: Schematic representation of the transamidation assay.** The refolded unfractioned total tRNAs were first labeled with α-32P-ATP on the 3’ end of tRNAs. Labeled and unlabeled tRNAs were aminoacylated with glutamate by GluRS2. The resulted Glu-tRNA^{Gln} was used in transamidase assay. Samples from transamidase assay were digested by nuclease P1 to yield AMP, Glu-AMP (representing starting material), and Gln-AMP (representing product) that were separated by TLC and quantified by PhosphorImager.
6.12 AdT ammonia tunnel calculation

AdT ammonia tunnel calculations were performed using the *S. aureus* AdT crystal structure (PDB ID: 2F2A) as a model system. For wild-type AdT, the crystal structure and the simulated structures (contributed by Sajeewa Dewage) were both used in calculations; for AdT mutants, mutations were first introduced in the structure through Pymol, and then the mutated structure was energy minimized (contributed by Sajeewa Dewage) before tunnel calculations. Calculations were conducted using the Caver Viewer 3.0 (178) plug-in in Pymol. To initiate tunnel calculation in Caver Viewer, residues, Thr175$\alpha$ and Ser178$\alpha$ (*S. aureus* numbering), were selected as the possible starting points for the ammonia tunnel Ser178$\alpha$ is the critical glutaminase active site residue and Thr175$\alpha$ is close to the active site. All other calculation parameters were left as the default values suggested by the software developers.

6.13 AdT protein sequence alignment

The AdT protein conservation analysis was performed using the ConSurf Server using its default parameters for searching and comparison (187,188).
Figure A.1: Residue by residue correlation analysis for all simulated structures. (A) Wild-type AdT; (B) Wild-type AdT with glutamine bound to GatA; (C) T149V AdT; (D) T149V AdT with glutamine; (E) K89R AdT; and (F) K89R
AdT with glutamine. (G) Cartoon showing different regions of correlation; residues proceed from left to right (N-terminal to C-terminal) with GatA residues numbered 1-485, GatB residues numbered 486-897, and GatC residues numbered 898-991. All panels apply the same color-coded scale representing the correlation coefficient that stands for how one residue moves with respect to another. Correlation coefficients of +1 and -1 both indicate the highest level of correlation observed between any two residues; negative correlations indicate residue movements correlated in opposing directions.
Figure A.2: Enzyme-wide correlations at positions T149 and K89 in wild-type versus mutant AdTs. (A) Comparison between T149 (black) and T149V (cyan) correlations; (B) Comparison between T149 (black) and T149V (cyan) correlations when simulated in the presence of glutamine bound to GatA; (C) Comparison between K89 (black) and K89R (red) correlations; (D) Comparison between K89 (black) and K89R (red) correlations in the presence of glutamine bound to GatA.
Figure A.3: Variations in RMSD for each mutant versus wild-type AdT (wild-type values were subtracted from mutant values). (A) RMSD between T149V AdT simulation, with glutamine bound to GatA, compared to AdT structure; (B) RMSD between T149V AdT simulation in the absence of bound glutamine, compared to AdT structure; (C) RMSD between K89R AdT simulation, with glutamine bound to GatA, compared to AdT structure; (D) RMSD between K89R AdT simulation and AdT structure, in the absence of bound glutamine. Right panel shows the color scheme for panels A-D in Angstroms.
Figure A.4: A residue-by-residue correlation of wild-type, T149V and K89R AdT for the 59 common residues. The sub-section divisions are the same as in Figure 6. Note: these residues are dispersed throughout the primary sequence of AdT; they are not consecutive. (A) wild-type AdT; (B) wild-type AdT with glutamine bound to GatA; (C) T149V AdT; (D) T149V with glutamine bound; (E) K89R AdT; (F) K89R AdT with bound glutamine. (G) Correlation color scale. As
in Figure 6, correlation coefficients of +1 and -1 both indicate the highest level of correlation observed between any two residues; negative correlations indicate residue movements that are correlated in opposing directions.
Figure A.5: The correlation difference of the 59 common residues for wild-type and K89R AdT. The sub-section divisions are the same as in Figure 6. The difference color scale is shown on the right. Note: these residues are dispersed throughout the primary sequence of AdT; they are not consecutive. A) wild-type AdT; (B) wild-type AdT with glutamine bound to GatA; (C) T149V AdT; (D) T149V with glutamine bound; (E) K89R AdT; (F) K89R AdT with bound glutamine.
Table A.1: The 59 common residues identified from RMSD difference comparison between mutants (T149V and K89R AdT) with wild-type AdT

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APPENDIX B

ASSAYS FOR MEASURING GLUTAMINE HYDROLYSIS

B.1. Thin layer chromatography (TLC)

In this method, $^{14}$C-labeled glutamine was used for detection purpose (63). Samples from a standard glutaminase assay were separated by thin layer chromatography, and the generation of glutamate was detected by phosphorimaging (See Figure B.1 for a representative TLC image).

![Figure B.1: Glutaminase assay by TLC.](image)

Figure B.1: Glutaminase assay by TLC. Phosphorimage of a TLC plate developed in acidic aqueous solvents (63) (8:1:1:4 2-propanol:formic acid:acetic acid:water). The glutaminase assay was carried out with 1 mM glutamate (supplemented with $^{14}$C labeled glutamate) with 200 nM AdT.
B.2. Thin layer electrophoresis (TLE)

TLE also uses radiolabeled amino acids for quantification. Instead of the time consuming organic solvent elution used in TLC, in TLE, the separation of glutamate and glutamine is achieved by electrophoresis (69); detection and quantification is also by phosphorimaging. (See Figure B.2 for a representative TLE image)

Figure B.2: Glutaminase assay by TLE. Under electrophoresis buffer condition, glutamic acid (Glu) and glutamine (Gln) have different charge states. Consequently, they will move toward different electrodes under current. (The “+” and “-” on the left stand for the two electrodes while the “-“ on the top stands for the no enzyme control.)
B.3. Glutamate dehydrogenase (GDH) UV assay

This assay relies on glutamate dehydrogenase (GDH) to produce NADH stoichiometrically with respect to glutamate (189). Thus, the production of NADH can be monitored and these values correlate to the change in glutamate concentration. (See Figure A.3 for details)

![Chemical reaction diagram](image)

Figure B.3: The application of glutamate dehydrogenase to the quantification of glutamate. The glutamate generated from glutamine hydrolysis is coupled to the reduction of NAD$^+$; the resultant increase in NADH is monitored by UV at 340 nm.
Figure C.1: Residue by residue correlation analysis for all simulated D185 AdT mutant structures. (A) D185Aα AdT; (B) D185Eα AdT; (C) D185Nα AdT; (D) wild-type AdT; (E) Cartoon showing different regions of correlation; residues proceed from left to right (N-terminal to C-terminal) with GatA residues numbered 1-485, GatB residues numbered 486-897, and GatC residues numbered 898-991. (F) Color-coded scale representing the correlation coefficient. All panels apply the same color-coded scale representing the correlation coefficient that stands for how one residue moves with respect to another. Correlation coefficients of +1 and -1 both indicate the highest level of correlation observed between any two
residues; negative correlations indicate residue movements correlated in opposing directions.
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ABSTRACT

ENZYMATIC CHARACTERIZATION OF THE AMMONIA TUNNEL IN 
HELCOBACTER PYLORI ASP-TRNA\textsuperscript{ASN}/GLU-TRNA\textsuperscript{GLN} 
AMIDOTRANSFERASE

by

LIANGJUN ZHAO

December 2013

Advisor: Dr. Tamara L. Hendrickson

Major: Chemistry (Biochemistry)

Degree: Doctor of Philosophy

The \textit{Helicobacter pylori} (\textit{H. pylori}) Asp-tRNA\textsuperscript{Asn}/Glu-tRNA\textsuperscript{Gln} amidotransferase (AdT) plays important roles in indirect aminoacylation and translational fidelity; however, its inter-domain communication and ammonia delivery mechanisms are not well understood. In the present study, we investigated the three activities of \textit{H. pylori} AdT (glutaminase, kinase and transamidase) and used these reactions as probes to examine the inter-domain communication and ammonia delivery mechanisms between this enzyme’s two isolated active sites. We adapted and optimized an assay to kinetically characterize a series of mutations at conserved positions throughout the putative AdT ammonia tunnel. The kinase assay enabled us to identify mutations within AdT, specifically T149 and K89, for further enzymatic characterization and molecular dynamics (MD) simulations and correlation analyses to unveil a set of 59 residues that may form the interdomain communication pathway between AdT’s two active sites. The glutaminase and transamidase assays identified another residue, D185, in the GatA subunit. Kinetic and computational
characterizations of D185 AdT mutants suggest that D185 serves as a general acid or base in ammonia delivery. These results are the first demonstration of acid/base chemistry within an ammonia tunnel. Finally, preliminary characterization of the predicted ammonia tunnel gate residues (K89 and E126 in the GatB subunit) suggest that proper positioning of the appropriate charge states in the tunnel are important for AdT catalysis. The results presented in this dissertation extend our understanding of AdT’s distinct ammonia transfer mechanism.
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