The \textit{Helicobacter pylori} Amidotransferase GatCAB Is Equally Efficient in Glutamine-dependent Transamidation of Asp-tRNA$^{\text{Asn}}$ and Glu-tRNA$^{\text{Gln}*}$

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The amide aminoacyl-tRNAs, Gln-tRNA$^{\text{Gln}}$ and Asn-tRNA$^{\text{Asn}}$, are formed in many bacteria by a pretranslational tRNA-dependent amidation of the mischarged tRNA species, Glu-tRNA$^{\text{Gln}}$ or Asp-tRNA$^{\text{Asn}}$. This conversion is catalyzed by a heterotrimeric amidotransferase GatCAB in the presence of ATP and an amide donor (Gln or Asn). \textit{Helicobacter pylori} has a single GatCAB enzyme required \emph{in vivo} for both Gln-tRNA$^{\text{Gln}}$ and Asn-tRNA$^{\text{Asn}}$ synthesis. \emph{In vitro} characterization reveals that the enzyme transamidates Asp-tRNA$^{\text{Asn}}$ and Glu-tRNA$^{\text{Gln}}$ with similar efficiency ($k_{\text{cat}}/K_m$ of 1368.4 s$^{-1}$/mM and 3059.3 s$^{-1}$/mM respectively). The essential glutaminase activity of the enzyme is a property of the A-subunit, which displays the characteristic amidase signature sequence. Mutations of the GatA catalytic triad residues (Lys$^{52}$, Ser$^{128}$, Ser$^{152}$) abolished glutaminase activity and consequently the amidotransferase activity with glutamine as the amide donor. However, the latter activity was rescued when the mutant enzymes were presented with ammonium chloride. The presence of Asp-tRNA$^{\text{Asn}}$ and ATP enhances the glutaminase activity about 22-fold. \textit{H. pylori} GatCAB uses the amide donor glutamine 129-fold more efficiently than asparagine, suggesting that GatCAB is a glutamine-dependent amidotransferase much like the unrelated asparagine synthetase B. Genomic analysis suggests that most bacteria synthesize asparagine in a glutamine-dependent manner, either by a tRNA-dependent or in a tRNA-independent route. However, all known bacteria that contain asparagine synthetase A form Asn-tRNA$^{\text{Asn}}$ by direct acylation catalyzed by asparaginyl-tRNA synthetase. Therefore, bacterial amide aminoacyl-tRNA formation is intimately tied to amide amino acid metabolism.

The accurate pairing of an amino acid with its cognate tRNA is essential for the fidelity of protein biosynthesis. In a living cell this pairing is usually performed by the family of aminoacyl-tRNA synthetases (aaRSs), a set of twenty enzymes each specific for its cognate amino acid (1). However, most bacterial and all known archaean genomes do not encode a glutaminyl-tRNA synthetase (GlnRS) responsible for Gln-tRNA$^{\text{Gln}}$ formation. In addition, many prokaryotes do not possess an asparaginyl-tRNA synthetase (AsnRS). These organisms maintain an indirect pathway of Gln-tRNA$^{\text{Gln}}$ or Asn-tRNA$^{\text{Asn}}$ synthesis, which takes advantage of a non-discriminating (ND) aaRS and a tRNA-dependent amidotransferase (AdT) (2). In prokaryotes lacking a GlnRS, a non-discriminating glutamyl-tRNA synthetase (ND-GluRS) (3) forms the misacylated Glu-tRNA$^{\text{Gln}}$, which is subsequently converted by amidation to Gln-tRNA$^{\text{Gln}}$ catalyzed by a glutamyl-tRNA$^{\text{Gln}}$ amidotransferase (Glu-AdT) in the presence of an amide donor (4–6). In an analogous tRNA-dependent amidation pathway many prokaryotes employ a non-discriminating aspartyl-tRNA synthetase (ND-AspRS) and aspartyl-tRNA$^{\text{Asn}}$ amidotransferase (Asp-AdT) to form Asn-tRNA$^{\text{Asn}}$ (7–9).

The AdT enzymes catalyze three distinct reactions to accomplish transamidation of their mischarged tRNA substrates. (i) AdTs hydrolyze ATP to phosphorylate the carboxyl group of the Glu or Asp moiety attached to the tRNA, thus forming an activated intermediate (10, 11). (ii) AdTs hydrolyze an amide donor such as Gln or Asn to liberate ammonia. (iii) AdTs use the liberated ammonia to amidate the activated intermediate to form the cognate aa-tRNA species, Gln-tRNA$^{\text{Gln}}$ or Asn-tRNA$^{\text{Asn}}$. Two AdTs are found in nature, a heterotrimeric (GatCAB) and a heterodimeric (GatDE) enzyme. The latter is present only in Archaean and functions solely in Gln-tRNA$^{\text{Gln}}$ formation as a Glu-AdT (5). However, GatCAB is found in both prokaryotic domains of life (5) and also in chloroplasts (12). To date all GatCAB enzymes have shown the ability to act as both a Glu-AdT and an Asp-AdT \emph{in vitro} (5, 13, 14). The small GatC protein (about 100 amino acids) may be a chaperone for GatA,

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\underline{5} The abbreviations used are: aaRS, aminoacyl-tRNA synthetase; GlnRS, glutaminyl-tRNA synthetase; AsnRS, asparaginyl-tRNA synthetase; ND, non-discriminating; AdT, tRNA-dependent amidotransferase; GluRS, glutamyl-tRNA synthetase; Glu-AdT, glutamyl-tRNA$^{\text{Gln}}$-dependent amidotransferase; AspRS, aspartyl-tRNA synthetase; Asp-AdT, aspartyl-tRNA$^{\text{Asn}}$-dependent amidotransferase; PEI, polyethyleneimine; TLC, thin layer chromatography; DTT, dithiothreitol.
helping it bind to GatB (4, 15). The GatA and GatD subunits are glutaminases, liberating ammonia from the amide donor (reaction ii) (5, 11, 16). GatA belongs to the amidase family while GatD is a homolog of L-asparaginases (4, 5, 11, 16). GatB and GatE are homologs and belong to an isolated protein family (5). These subunits catalyze the formation of the activated intermediate (reaction i), and its subsequent amidation to the correctly charged tRNA species (reaction iii) (11, 17). For both AdTs, it is suspected that the binding of the misacylated tRNA induces a conformational change that activates their glutaminase activities, thereby tightly coupling ammonia liberation with the amidation of the misacylated tRNA species (11, 15, 18, 19).

In vivo, the role of GatCAB is likely determined by the nature of the misacylated tRNA species (Glu-tRNA\textsuperscript{Gln} or Asp-tRNA\textsuperscript{Asn}) present in the cell. For example, Bacillus subtilis has a ND-GluRS but not a ND-AspRS; thus it only forms Glu-tRNA\textsuperscript{Gln} and its GatCAB acts \textit{in vitro} only as a Glu-AdT (4). Alternatively, \textit{Pseudomonas} contains a ND-AspRS but lacks a ND-GluRS; thus its GatCAB enzyme is thought to act solely as an Asp-AdT (8). In other bacteria, \textit{Chlamydia trachomatis} and \textit{Helicobacter pylori}, that lack AsnRS and GlnRS, GatCAB is the sole AdT encoded in their genomes and is responsible for both Asn-tRNA\textsuperscript{Asn} and Gln-tRNA\textsuperscript{Gln} formation (14, 20). A portion of the archaeal genomes (e.g. \textit{Methanothrophic bacterium thermautotrophicus}) are deficient in both GlnRS and AsnRS; however, they all encode a ND-GluRS and a ND-AspRS along with two AdTs, GatCAB, and GatDE (5). Given that GatDE is only a Glu-AdT for Gln-tRNA\textsuperscript{Gln} formation, the role of GatCAB in these Archaea is presumably as an Asp-AdT to form Asn-tRNA\textsuperscript{Asn} (5), although this has not been experimentally verified.

A few biochemical investigations of the GatCAB enzymes have appeared using different enzyme assays. The \textit{Streptococcus pyogenes} enzyme was examined in its overall reaction, and its glutaminase activity was found to be enhanced by Glu-tRNA\textsuperscript{Gln} (18). Some information has been obtained on the tRNA identity of the enzyme for Gln over Asn as amide donor (5, 22), the crystal structures of \textit{S. aureus} GatCAB complexed with Gln or Asn suggested that Gln is the better amide donor (15). Here we present our data on the \textit{H. pylori} GatCAB enzyme; it employs its Glu-AdT and Asp-AdT activities equally and shows a distinct preference for glutamine as amide donor.

**EXPERIMENTAL PROCEDURES**

**General**—All oligonucleotide synthesis and DNA sequencing was carried out by the Keck Foundation Biotechnology Research Laboratory at Yale University. [\textsuperscript{1-\textit{14}}C]glutamine (224 mCi/mmol) was from American Radiolabeled Chemicals (St. Louis, MO) and [\textsuperscript{32P}]ATP (10 mmol/\textmu Ci) was from Amersham Biosciences (GE Healthcare). \textit{Escherichia coli} BL21(DE3) was from Stratagene (La Jolla, CA). Nickel-nitrilotriacetic acid-agarose was from Qiagen (Chatsworth, CA). Bio-Spin 30 columns were from Bio-Rad. High purity cold L-Glu, L-Asp, L-Gln, and L-Asn were from Fluka (Deisenhofen, Germany). Phenol was from American Bioanalytical (Natick, MA).

\textit{H. pylori} tRNA Purification—The \textit{E. coli} strain XL1-Blue harboring the full-length \textit{H. pylori} tRNA\textsuperscript{Gln} or \textit{H. pylori} tRNA\textsuperscript{Asn} plasmid pGF1B was grown for 24 h at 37 °C shaking at 250 rpm in Luria broth (LB) with 100 μg/ml of ampicillin for constitutive overexpression. Cells were pelleted by centrifugation at 2700 × g for 20 min, resuspended in 20 mM Tris–HCl, pH 7.9, for phenol (Tris-buffered, pH 7.9) extraction, ethanol-purified with 1/10 volume of 20% potassium acetate and two volumes of cold ethanol and spun at 4000 × g for 40 min. The nucleic acid pellet was resuspended in 200 mM Tris–HCl, pH 9, for deacylation, ethanol-purified with the addition of 1/10 volume of 3 M sodium acetate, pH 5, and two volumes of cold ethanol, and spun at 4000 × g for 40 min. To remove DNA, the pellet was resuspended in 1 M NaCl, incubated at room temperature for 4 h, and centrifuged at 4000 × g for 20 min. The supernatant was ethanol-purified and resuspended in 20 mM Tris–HCl, pH 7.0, and 0.2 M NaCl. The resuspended nucleic acids were loaded onto a Qiagen-tip 100 column, washed with 20 mM Tris–HCl, pH 7.0, 0.2 M NaCl, and total tRNA was eluted with 20 mM Tris–HCl, pH 7.0, 0.7 M NaCl and 15% ethanol buffer. \textit{H. pylori} tRNA\textsuperscript{Asn} and tRNA\textsuperscript{Gln} were purified from the total tRNA as described using complementary DNA probes, (5'-ATGTAGATTGGAACCTACGACCAAGCGGTTAATCAAGCGGG-3') for tRNA\textsuperscript{Asn} and (5'-TGCAAGGTATTGCCAGCAAGCGGTTAATCAAGCGGG-3') for tRNA\textsuperscript{Gln}, biotinylated on their 5’-ends yielding \textit{H. pylori} tRNA over 95% pure as determined by dot-blot and hybridization gel shifts (23). The link between the DNA and biotin for the \textit{H. pylori} tRNA\textsuperscript{Gln} probe was particularly labile. Following purification, the tRNA\textsuperscript{Gln} samples were treated with RNase-free DNase (Roche Applied Science), phenol (citrau buffer, pH 4.5)/chloroform-extracted, and passed over a Bio-Spin 30 column to remove the DNA probe.

\textit{H. pylori} GatCAB Purification—\textit{H. pylori} gatCAB was cloned between the NdeI and BamHI sites in pET15b (pET15b-Hpgat-CAB), which was transformed into \textit{E. coli} BL21(DE3), and the resultant cells grown on LB-agar plates with 100 μg/ml of ampicillin. A fresh colony was picked and grown for 12 h at 37 °C, shaking in 7.5 mL of LB plus 100 μg/mL of ampicillin. The culture was used to inoculate 750 mL of LB supplemented with 100 μg/mL of ampicillin, 5052 solution, and phosphate buffer for autoinduction as previously described (24). The cultures were grown at 37 °C shaking at 250 rpm for 18 h. The cells were pelleted by centrifugation at 2700 × g for 20 min and resuspended in 35 mL of wash buffer (25 mM Heps-KOH, pH 7.2, 300 mM NaCl, 1 mM benzamidine, 5 mM 2-mercaptoethanol, and 10% glycerol) with 0.1% Triton X-100, 0.2 mg of phenylmethylsulfonyl fluoride and 0.2 mg of lysozyme (Sigma) and incubated on ice for 30 min. The cell suspension was sonicated for 20 s, six times on a Branson Sonifier 250 (VWR Scientific, West Chester, PA) at a 50% duty cycle and 60% output. The lysate was centrifuged at 20,000 × g for 90 min. 1 mL of nickel-nitrilotriacetic acid-agarose slurry was added to the supernatant, and the protein was purified according to manufacturer’s protocols. The eluted protein was dialyzed in buffer A (25 mM Heps-KOH, pH 7.2, 5.0 mM 2-mercaptoethanol, 20 mM KCl, 0.2 mM...
EDTA) and purified further by fast protein liquid chromatography on a MonoQ column (Amersham Biosciences). It was developed with a 20 mM to 750 mM KCl gradient in buffer A. GatCAB eluted between 200 mM and 300 mM KCl. Fractions containing purified GatCAB (>95%) as judged by SDS-PAGE and Coomassie Brilliant Blue staining were pooled together and dialyzed in buffer A with 50% glycerol and stored at −20 °C.

Site-directed Mutagenesis of GatCAB in Amidase Active Site—Point mutations were introduced into the amidase active site of P. aeruginosa (17, 26). Briefly, 15/1H9262 min. at room temperature with the CCA-adding enzyme and 11868 GluRS2, 1 mML -Glu, 10/1H9262 (either Gln, Asn, ATP, Glu-tRNAGln, or Asp-tRNAAsn) and are reported in Table 1.

**Glutaminase Assays**—The kinetic parameters of the glutaminase activity of the H. pylori GatCAB were carried out at 37 °C in 1 X AdT buffer in the presence or absence of 4 mM ATP and or 10–11 μM unlabeled mischarged tRNA (Asp-tRNAAsn or Glu-tRNAAsn). The L-Gln concentration varied from as low as 4 μM up to 960 μM. [L-14C]Gln was used. For the higher concentrations, cold L-Gln was added in addition to the [L-14C]Gln. In the absence of mischarged tRNA, 10 nM H. pylori GatCAB was used. In the presence of mischarged tRNA but in the absence of ATP, 1 nM of enzyme was used. When both ATP and mischarged tRNA were present in excess, 0.1 nM enzyme was added. Reactions were carried out over 8 min. At each time point, aliquots were quenched with 0.3 M sodium acetate, pH 5.0, ethanol-precipitated, and the supernatant was dried and resuspended as previously described (11). Cellulose 20 × 20 cm TLC plates (Sigma) were spotted with 1.0 μl of the resuspended reactions and developed for 7 h under acidic conditions (isopropyl alcohol/formic acid/water, 20:1:5) to separate L-Gln from L-Glu (11). The plates were exposed on an imaging plate, scanned and quantified as described previously (11). The kinetic parameters were calculated using nonlinear regression plots of the initial velocity versus substrate concentration (either Gln, Asn, ATP, Glu-tRNAGln, or Asp-tRNAAsn) and are reported in Table 2.

**Genomic Analysis**—Searches of all the completed bacterial genomes in the National Center for Biotechnology Information (NCBI) data base (as of September 2006) for genes Darmstadt, Germany, 5725/7) were developed in 1 mM ammonium acetate, 5% acetic acid.

**Amidotransferase Assays**—These were carried out in 1 X AdT buffer (50 mM Hepes-KOH, pH 7.2, 15 mM MgCl2, 25 mM KCl, and 1 mM DTT) at 37 °C. Unless otherwise noted 4 mM ATP, 9 to 11 μM 32P-labeled mischarged tRNA (Asp-tRNAAsn or Glu-tRNAAsn) and 2 mM L-Gln were added. For determination of the kinetic parameters, initial velocities were measured in duplicate while varying concentration of one substrate and saturating with the other two. When L-Gln was used as the amide donor, reactions were carried out over 1 min using 1 nM purified H. pylori GatCAB. For the kinetic parameters using Asn as the amide donor, reactions were carried out over 5 min using 20 nM enzyme. We found our mischarged tRNA species were stable over these time periods. Reaction mixes were preincubated at 37 °C and started by the addition of H. pylori GatCAB. At each time point 2-μl aliquots of the reaction were quenched, digested with 3 μl of 100 mM sodium citrate, pH 4.74, and 0.66 mg/ml of nuclease P1 (Sigma) and spotted onto PEI-cellulose 20 × 20 cm TLC plates as previously described (17). To separate the glutamyl-AMP (Gln-AMP) from Glu-AMP and AMP, the TLC plates were developed in 100 mM ammonium acetate, 5% acetic acid as described previously (17, 26). To separate asparaginyl-AMP (Asn-AMP) from Asp-AMP and AMP (see Fig. 1), the plates were developed in 1 mM ammonium acetate and 5% acetic acid for 90–120 min. The plates were visualized and quantified as described previously (17). Kaleidagraph v. 3.6 (Synergy Software, Reading, PA) was used to calculate the kinetic parameters using nonlinear regression plots of the initial velocity versus substrate concentration (either Gln, Asn, ATP, Glu-tRNAGln, or Asp-tRNAAsn) and are reported in Table 2.
encoding AsnRS, GlnRS, AspRS, GatCAB, AsnA, and AsnB were conducted by using the TBLASTN program (27, 28). For the searches the E. coli CFT AsnRS (AAN79540), GlnRS (AAN79239), AsnA (AAN83104) and AsnB (AAN79222), the H. pylori I99 GatA (AAD06348) and GatB (AAD06184), and the Deinococcus radiodurans discriminating AspRS (AAF10918) and ND-AspRS (AA10623) sequences were used. To provide a reasonable phylogenetic distribution the 341 genomes were classed into 146 genera; one representative was chosen for the analysis. In establishing the genera, care was taken to ensure that all members in a genus had the same content of the relevant genes.

RESULTS

[32P]tRNA/Nuclease P1 Amidotransferase Assay—When we started this work two enzyme assays were available. The first was based on HPLC separation of unlabeled amino acids released from aa-tRNA (18), while the second relied on TLC separation of radioactive amino acids after hydrolysis of aa-tRNA (4). Because both of these assays required large amounts of tRNA and were cumbersome to perform, we adapted a [32P]tRNA/nuclease P1-based assay that was previously successfully used to measure aminoacylation rates (26, 29). The assays were [32P]-labeled in their terminal AMP with [α-32P]ATP by the E. coli CCA-adding enzyme. Digestion with nuclease P1 released [α-32P]AMP from uncharged tRNAs and aa-[α-32P]AMP from charged tRNAs. These two species could be separated by TLC (26, 29). Because conditions (26) could be developed to separate AMP, Gln-AMP, and Glu-AMP, this assay was used earlier to monitor transamidation by the archaeal Glu-AdT, GatDE (17). For the AMP, Asp-AMP and Asn-AMP separations of this work, we modified the solvent system (see “Experimental Procedures”) to afford good separation of the three compounds using either PEI-cellulose plates with glass (Fig. 1A) or plastic backing (data not shown).

There were two reasons for our choice of assay. First, an assay was needed with enough sensitivity to obtain accurate specificity constants (kcat/Km) for the GatCAB enzyme mischarged tRNA substrates, Glu-tRNAAsn and Asp-tRNAAsn. Low sensitivity in the HPLC assay was a reason cited previously for the inability to determine a reliable specificity constant for Glu-tRNAAsn with the S. pyogenes enzyme (18). Second, because the tRNA was labeled and then digested with nuclease P1, we avoided the precipitation and deacylation steps before chromatographic separation, as is required for the [14C]amino acid and HPLC-based assays (4, 9, 18, 30). This speeds up the analysis, allows a determination of aa-tRNA deacylation over the course of the reaction, and avoids any possible side reactions caused by the deacylation conditions.6 Equally important is the fact that the measured values result from tRNA-dependent conversion by amidation. The assay shows (Fig. 1B) that the H. pylori GatCAB does not display cooperativity and is saturable at elevated substrate concentrations.

Gln Is the Preferred Amide Donor for GatCAB-catalyzed Transamidation—It was reported earlier that M. thermautotrophicus GatCAB utilizes both Asn and Gln as amide donors (6) for the Chlamydomonas reinhardtii amidotransferase it was shown that the enzyme was more active using Gln as the donor (22). Because the H. pylori GatCAB in vivo probably serves as both a Glu-AdT and Asp-AdT, we decided to test whether the enzyme displays any preference in its use of Gln or Asn as the amide donor in the transamidation reaction. To this aim, we assayed the ability of the enzyme to transamidate Glu-tRNAAsn and Asp-tRNAAsn in the presence of either Gln or Asn as the amide donor (Fig. 2). Under normal assay conditions (1 nM enzyme) little transamidase activity was detected with the amide donor Asn (Fig. 2), however with a 20-fold greater enzyme concentration we were able to obtain reliable initial velocity measurements when Asn was the donor. While the Km values of GatCAB for Asn and Gln are similar (22.4 μM and 20.7 μM, respectively), the kcat for the transamidation reaction with Gln as amide donor was ~129-fold greater than when Asn was used (Table 1). For ammonium chloride as the amide donor, we estimated a Kd value of ~50 mM (data not shown). We conclude that the H. pylori GatCAB is significantly more efficient utilizing Gln as an

6 J. C. Salazar, K. Sheppard, and L. Feng, unpublished results.
Dual tRNA Specificity of H. pylori GatCAB

**TABLE 1**

| Substrate | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ |
|-----------|------|----------|-------------|
| Asp-tRNA$^{\text{Asn}}$ | 0.95 ± 0.30 | 1.30 ± 0.19 | 1368.4 |
| Glu-tRNA$^{\text{Gln}}$ | 1.18 ± 0.12 | 3.61 ± 0.13 | 3059.3 |
| ATP | 2068 ± 68.2 | 6.10 ± 0.61 | 29.5 |
| Gln | 20.7 ± 9.5 | 3.49 ± 0.44 | 168.6 |
| Asn | 22.4 ± 4.5 | 0.072 ± 0.001 | 1.2 |

$a K_m$ for each substrate was determined by varying its concentration while adding an excess of the other two substrates required for transamidation.

$b$ Glu and ATP were added in excess (2 mM and 4 mM, respectively) with 1 nM enzyme.

$c$ Glu-tRNA$^{\text{Gln}}$ (9–11 μM) and Glu (2 mM) were added in excess with 1 nM GatCAB.

$d$ Glu-tRNA$^{\text{Gln}}$ (9–11 μM) and ATP (4 mM) were added in excess with 1 nM enzyme.

$e$ Glu-tRNA$^{\text{Gln}}$ (9–11 μM) and ATP (4 mM) with 20 nM GatCAB. Measurements were done two to four times. Standard deviations are reported.

| Substrate | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ |
|-----------|------|----------|-------------|
| Glu$^b$ | 26.5 ± 6.3 | 0.34 ± 0.03 | 12.8 |
| Glu$^f$ | 11.9 ± 7.4 | 0.20 ± 0.03 | 16.8 |
| Glu$^f$ | 38.3 ± 7.4 | 0.97 ± 0.04 | 25.3 |
| Glu$^f$ | 55.7 ± 27.4 | 1.32 ± 0.27 | 23.7 |
| Glu$^f$ | 40.2 ± 23.5 | 11.80 ± 2.21 | 293.5 |
| Glu$^f$ | 50.9 ± 25.4 | 10.29 ± 2.07 | 202.2 |

$a K_m$ determinations were for Gln. Any additional substrates present in excess in the reaction are indicated in parentheses.

$b$ 10 mM enzyme added.

$c$ 0.1 mM enzyme added.

$d$ ATP added in excess (4 mM).

$e$ Mischarged tRNA substrate indicated was added in excess (10–11 μM). Measurements were done two to three times. Standard deviations are reported.

amide donor rather than Asn. Therefore, in our subsequent kinetic assays Gln was the amide donor unless otherwise noted.

H. pylori GatCAB Has Comparable Glu-AdT and Asp-AdT Activities—Because H. pylori GatCAB is supposed to function in vivo both as an Asp-AdT and a Glu-AdT enzyme, one might expect it to possess equal efficiency in transamidating Glu-tRNA$^{\text{Gln}}$ and Asp-tRNA$^{\text{Asn}}$. To test this expectation we determined the steady-state kinetic constants for both tRNA substrates in the presence saturating Gln and ATP concentrations (Table 1). The specificity constants ($k_{cat}/K_m$) are similar for Asp-tRNA$^{\text{Asn}}$ (1,368 s$^{-1}$ μM$^{-1}$) and Glu-tRNA$^{\text{Gln}}$ (3,059 s$^{-1}$ μM$^{-1}$) for this dual tRNA substrate enzyme; it slightly favors transamidation of Glu-tRNA$^{\text{Gln}}$ by ~2-fold. Such a variation in tRNA preference is also seen in the ND-aaRSs. For instance, the Thermosynechococcus elongatus ND-GluRS is 13-fold more efficient inacyclating tRNA$^{\text{Gln}}$ than tRNA$^{\text{Gln}}$ (31), while the Thermus thermophilus ND-AspRS is 2-fold more efficient in Asp-tRNA$^{\text{Asn}}$ than tRNA$^{\text{Asn}}$ formation (32).

The H. pylori GatCAB $K_m$ values for ATP and Gln (206.8 μM and 20.7 μM, respectively) are similar to those determined for the S. pyogenes GatCAB (117.7 μM and 15.9 μM, respectively) (18). The $K_m$ for Asp-tRNA$^{\text{Asn}}$ (0.95 μM) is similar to that obtained for N. meningitidis GatCAB (1.2 μM) (9). For S. pyogenes GatCAB the tRNA$^{\text{Gln}}$ $K_m$ is estimated to be 0.2 μM, which is about 6-fold lower than the corresponding value of the H. pylori GatCAB (18). The discrepancy might be caused by the low sensitivity of the HPLC-based assay (18) or to species variation.

Asp-tRNA$^{\text{Asn}}$ and Glu-tRNA$^{\text{Gln}}$ Equally Activate the Glutaminase Activity of GatCAB—Glutaminase assays of the S. pyogenes GatCAB revealed that this enzyme activity increased in the presence of Glu-tRNA$^{\text{Gln}}$ and ATP, the other substrates required for the amidotransferase reaction; primarily because of an increase in $k_{cat}$ (18). Because in vivo the role of the S. pyogenes enzyme is a Glu-AdT while the H. pylori enzyme, in addition, has a required Asp-AdT activity, we tested if the H. pylori GatCAB enzyme displays glutaminase activation in the presence of Asp-tRNA$^{\text{Asn}}$, as well as with Glu-tRNA$^{\text{Gln}}$. Table 2 shows both tRNAs enhance the reaction by ~2-fold. Further addition of ATP caused an additional 10-fold increase in catalytic efficiency, primarily because of a rise in $k_{cat}$ (Table 2). Addition of ATP alone did not significantly alter the glutaminase activity. These results suggest that binding of Asp-tRNA$^{\text{Asn}}$ or Glu-tRNA$^{\text{Gln}}$ to GatCAB induce similar conformational changes in the enzyme that increase its glutaminase activity, which is further enhanced by the presence of ATP.

Ser$^{152}$, Ser$^{178}$, and Lys$^{32}$ Are Important for the GatA Glutaminase Activity—Amidases contain a signature sequence peptide which folds to correctly position a Ser-cisSer-Lys catalytic triad (33). Alignments of GatA with other amidases (Fig. 3) show that the catalytic triad is conserved in the AdT subunit (residues Ser$^{152}$, Ser$^{178}$, and Lys$^{32}$ in H. pylori GatA). Studies of malonamidase E2 and fatty acid amide hydrolase revealed that the Ser$^{152}$ (in H. pylori) is the nucleophile, which is activated by the cisSer and Lys residues (34, 35). In addition, the Lys residue is important in protonating the substrate-leaving group (34). The S176A mutation (corresponding to Ser$^{152}$ in H. pylori GatA) in the S. pyogenes GatA significantly reduced the glutaminase and transamidase activities of the enzyme (16). The crystal structure of the S. aureus GatCAB complexed with Gln showed that Ser$^{178}$ (corresponding to H. pylori Ser$^{152}$) forms a covalent bond with the side chain amide of the substrate Gln (15), consistent with the predicted role of Ser in catalysis. To test the importance of these amino acids in H. pylori GatA, we constructed the following five mutants: S152A, S152T, K52M, S128A, and S128T. The glutaminase (Fig. 4) and transamidase (Fig. 5) activities of the mutant enzymes were tested. Only the S128T protein retained significant glutaminase activity (Fig. 4, lane 4) and transamidase activity in the presence of Gln (Fig. 5). Both the S152A and S152T mutants were glutaminase inactive (Fig. 4, lanes 5 and 6). Presumably, Thr at this position may have its hydroxyl group oriented so as to prevent its ability to act as the nucleophile. None of the mutants demonstrated significant transamidase activity in the presence of Asn as the amide donor (Fig. 5). To ensure that the mutants were still properly folded and functional, we tested the mutants for amidotransferase activity in the presence of the amide donor ammonium chloride, thus bypassing the need for an amide to liberate ammonia from Gln. All the mutants were approximately as active as the wild-type enzyme in forming Gln-tRNA$^{\text{Gln}}$ when free ammonia was added (Fig. 5), indicating that the mutations solely affected the glutaminase activity of the proteins.
Dual tRNA Specificity of H. pylori GatCAB

Glutaminase-deficient *M. thermautotrophicus* GatDE mutants catalyzed the formation of pyroglutamyl-tRNA^Gln^, a suspected breakdown product of the activated intermediate, γ-phosphoryl-Glu-tRNA^Gln^ (11). Using the glutaminase-deficient *H. pylori* GatCAB mutants we also detected pyroglutamate formation by the traditional[^14C]Glu-based assay (data not shown). We were unable though to trap the activated intermediates in the Glu-tRNA^Gln^ or Asp-tRNA^Asn^ transamidation reactions catalyzed by pure *H. pylori* GatCAB. Because this has been accomplished with partially purified *B. subtilis* GatCAB (36), additional factors may be necessary to stabilize these intermediates. However, the possibility of an alternative transamidation mechanism cannot be ruled out.

*GatCAB Is Restricted to Glu-AdT Function in Bacteria That Carry Asparagine Synthetase A—Asparagine synthetases divide into two enzyme families (AsnA and AsnB) that amidate free aspartate to asparagine. Apart from having unrelated structures they use dissimilar amide donors: ammonia is the substrate for the AsnA enzymes while the AsnB family uses glutamine (37–39). Interestingly, AsnA is related to class II aaRSs, in particular AspRS and AsnRS (40–43). Biochemical studies in *T. thermophilus* (32) and *D. radiodurans* (44), organisms whose genomes do not encode AsnA or AsnB orthologs, established that the indirect pathway for Asn-tRNA^Asn^ formation is the sole route to Asn biosynthesis. An analysis of the genomes known at that time suggested that this situation is common among bacteria (44) and implied that Asn-tRNA^Asn^ formation via the indirect pathway was Gln-dependent. This idea is consistent with our *in vitro* data that demonstrate GatCAB transamidation to be much more efficient with Gln as the amide donor.

Because many more bacterial genomes are now known and as the previous analysis (44) did not take into account whether the GatCAB enzyme is used by the organism as a Glu-AdT, an Asp-AdT, or both, we decided to perform another analysis of genes involved in asparagine, Gln-tRNA^Gln^ and Asn-tRNA^Asn^ synthesis in the genomes of 341 bacteria representing 146 genera. We searched for genes that encoded ND-AspRS, Asn-tRNA^Asn^, and GatCAB. As a definitive sequence signature for the bacterial ND-AspRS is not known, we defined this enzyme as non-discriminating if the organism lacks AsnRS but contains GatCAB, or if two different AspRSs coexist with GatCAB (e.g., *D. radiodurans*). An organism was predicted to have an amidotransferase with Glu-AdT activity when GlnRS was absent and GatCAB was present. Furthermore, GatCAB was predicted to exhibit Asp-AdT activity when a ND-AspRS was present in the organism.

Applying these criteria for predicting GatCAB function to 146 genera allowed an obvious classification in all but three cases (*Hahella*, *Lawsonia*, and *Myxococcus*). The results of this analysis are summarized in Table 3 and detailed under Table S1 in supplemental materials. It appears that 37 genera, whose genomes do not encode an AsnA or AsnB use the tRNA-dependent pathway to asparagine. To date the *Thermus/Deinococcus* division is the only known to also possess AsnRS capable of forming Asn-tRNA^Asn^ directly. All bacteria possessing AsnA do not make use of the Asp-AdT activity of GatCAB; these organisms also encode AsnRS. These bacteria are able to form Asn and Asn-tRNA^Asn^ in a glutamine-independent fashion. Likewise in Archaea, AsnRS and AsnA are always present simultaneously, suggesting a link between these two enzymes (41). On the other hand, of the 69 bacterial genera that contain only AsnB to synthesize
Asn in a tRNA-independent fashion, 42 take advantage of the Asp-AdT activity of GatCAB; these organisms synthesize free Asn and also Asn-tRNA^{Asn} in a glutamine-dependent manner. Considering Gln-tRNA^{Gln} biosynthesis, there appears to be an indirect relationship between bacteria utilizing the Glu-AdT activity of GatCAB and the presence of AsnA and/or AsnB. Of the 37 genera lacking both asparagine synthetases from one catalytic center of GatA and GatB; thus ammonia is free to transfer from one catalytic site to the other (15). Dramatic conformational alterations of the glutaminase active site in GatA are not expected; all of the catalytically important residues in GatA are in proper position to carry out catalysis in the apoenzyme structure, and the enzyme displays glutaminase activity even in the absence of mischarged tRNA (15, 18). A co-crystal structure of GatCAB with mischarged tRNA (Glu-tRNAGln or Asp-tRNAGln) and ATP will be useful in determining what structural changes take place and if additional molecular rearrangements are involved.

Glutamine Hydrolysis Is Not the Rate-limiting Step in Transamidation—Our data show that the $k_{cat}$ value for glutamine hydrolysis (in the presence of ATP and the misacylated tRNA) is 3-fold that of Glu-tRNAGln and 10-fold that of Asp-tRNAGln transamidation. It was previously suggested that the rate of ATP hydrolysis is also faster than that of transamidation (18). Therefore, the rate-limiting step for transamidation by GatCAB could be either ammonia transfer through the molecular tunnel or the subsequent amidation of the mischarged tRNA.

However, with Asn as the amide donor, ammonia release appears to be rate-limiting as the $k_{cat}$ value of transamidation was 129-fold slower compared with when the amide donor was Gln. This result agrees with the structural observations in S. aureus GatCAB (15). After soaking the crystal with Gln, the amino acid bound in the GatA active site formed a covalent bond with Ser^{178} (Ser^{152} in H. pylori GatA), which is consistent with the residue role as the nucleophile (15). Asn soaked into the crystal bound at the same active site as Gln (15). However, Asn did not form a covalent bond with Ser^{178}, probably because of the shorter length of the Asn side chain (15). Interestingly the selection against asparagine in the amidotransferase reaction appears to be at the transition state level as observed by the difference in $k_{cat}$ values and not ground state binding as the $K_m$ values of the enzyme for Gln and Asn were similar. If indeed ammonia release is rate-limiting with Asn as amide donor, then one would expect mild mutations in the GatA active site to significantly affect transamidase activity only when Asn is the amide donor and not when Gln is used. That may explain why the GatA S128T mutant of GatCAB was as active as the wild-type enzyme in Glu-tRNAGln to Gln-tRNA^{Gln} transamidation with Gln as the amide donor but not with Asn.

**Table 3**

| Presense of AsnA/AsnB | Total | Asp-AdT | Glu-AdT | AsnRS | GlnRS |
|----------------------|-------|---------|---------|-------|-------|
| No AsnA and AsnB     | 37    | 37      | 28      | 2     | 9     |
| AsnB only            | 69    | 42      | 35      | 28    | 34    |
| AsnA only            | 10    | 0       | 6       | 10    | 4     |
| AsnA and AsnB        | 10    | 0       | 2       | 10    | 8     |
| Unknown              | 22    | 0       | 13      | 22    | 9     |

a. Bacteria were grouped by whether they had an AsnA and/or AsnB.

b. Totals correspond to the number of different genera in each grouping.

c. GatCAB was predicted to function as an Asp-AdT when present with a ND-AspRS.

d. The presence of a ND-AspRS was determined by either the absence of AsnRS or the occurrence of GatCAB with two different AspRSs.

e. Organisms were predicted to use GatCAB Glu-AdT activity when GlnRS was absent.

AsnA and AsnB Are Gln-dependent Amidotransferases—Like GatCAB, the asparagine synthetase AsnB strongly prefers Gln over Asn as amide donor (e.g. an 83-fold $k_{cat}$ difference of Vibrio cholerae AsnB (45)). Thus, these enzymes are unrelated amidotransferases that evolved to convert Asp to Asn in a glutamine-dependent manner.

**Evolutionary Outlook**—Our extensive genome analysis showed that bacteria, like Archaea (41), do not use the Asp-AdT activity of GatCAB when they also possess AsnA, the...
enzyme that forms free Asn from Asp. These organisms also harbor AsnRS. Possibly the same selective pressures that favored the inclusion of asparagine synthetase A into the genome of these bacteria also favored utilizing AsnRS for direct asparaginylation of tRNA\(^{Asn}\), thus decoupling tRNA-dependent and tRNA-independent asparagine formation from glutamine metabolism.

Because glutamine is the much preferred donor for GatCAB-catalyzed transamidations, the efficiency of this important amidotransferase could be modulated by the relative concentrations of Asn and Gln in the cell. Possibly GatCAB is more dependent on free amino acid levels than the relevant AsnRS and GlnRS. Given that GatCAB is crucially dependent on the cellular glutamine level and that its supply is used for diverse cellular amidations, Asn-tRNA\(^{Asn}\) or Gln-tRNAGln synthesis may fluctuate more than the formation of some other aa-tRNA species. However, little reliable data on the levels of free amino acids in bacterial cells (e.g. (46)) are currently available to allow development of an intelligent proposal of how pretranslational tRNA-dependent amidations are tied into cellular metabolism.

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