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***Methanothermobacter thermautotrophicus* tRNA^{Gln} confines the amidotransferase GatCAB to asparaginyl-tRNA^{Asn} formation**

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Abstract

Many prokaryotes form the amide aminoacyl-tRNAs, glutamyl-tRNA and asparaginyl-tRNA, by tRNA-dependent amidation of the mischarged tRNA species, glutamyl-tRNA^{Gln} or aspartyl-tRNA^{Asn}. Archaea employ two such amidotransferases, GatCAB and GatDE, while bacteria possess only one, GatCAB. The *Methanothermobacter thermautotrophicus* GatDE is slightly more efficient using Asn as an amide donor than Gln (k_{cat}/K_M of $5.4 \text{ s}^{-1}/\text{mM}$ and $1.2 \text{ s}^{-1}/\text{mM}$, respectively). Unlike the bacterial GatCAB enzymes studied to date, the *M. thermautotrophicus* GatCAB uses Asn almost as well as Gln as an amide donor (k_{cat}/K_M of $5.7 \text{ s}^{-1}/\text{mM}$ and $16.7 \text{ s}^{-1}/\text{mM}$, respectively). In contrast to the initial characterization of the *M. thermautotrophicus* GatCAB as being able to form Asn-tRNA^{Asn} and Gln-tRNA^{Gln}, our data demonstrate that while the enzyme is able to transamidate Asp-tRNA^{Asn} (k_{cat}/K_M of $125 \text{ s}^{-1}/\text{mM}$) it is unable to transamidate *M. thermautotrophicus* Glu-tRNA^{Gln}. However, *M. thermautotrophicus* GatCAB is capable of transamidating Glu-tRNA^{Gln} from *H. pylori* or *B. subtilis*, and *M. thermautotrophicus* Glu-tRNA^{Asn}. Thus, *M. thermautotrophicus* encodes two amidotransferases, each with its own activity, GatDE for Gln-tRNA and GatCAB for Asn-tRNA synthesis.

Keywords

tRNA-dependent amidotransferase; GatCAB; GatDE; Glu-AdT; Asp-AdT

Introduction

Most prokaryotes form Gln-tRNA^{Gln} or Asn-tRNA^{Asn} by an indirect pathway that relies on conversion of a misacylated aminoacyl-tRNA to the cognate pair.^{1–3} This involves two enzymes, a non-discriminating aminoacyl-tRNA synthetase (aaRS) and a tRNA-dependent amidotransferase (AdT). All known archaea¹ and most bacteria³ lack glutamyl-tRNA synthetase (GlnRS). A non-discriminating glutamyl-tRNA synthetase (ND-GluRS) forms Glu-tRNA^{Gln}.⁴ Amidation of the tRNA-bound Glu by glutamyl-tRNA^{Gln} amidotransferase (Glu-AdT) leads to the correctly charged Gln-tRNA^{Gln}.⁵ Similarly, prokaryotes lacking an asparaginyl-tRNA synthetase (AsnRS) employ a ND-aspartyl-tRNA synthetase (ND-AspRS)⁶ and an aspartyl-tRNA^{Asn} amidotransferase (Asp-AdT).^{7, 8} All AdT enzymes catalyze three different reactions during the conversion of their mischarged tRNA substrates.⁹ (i) AdTs are

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kinases that phosphorylate the β -carboxyl group of the Asp moiety or the γ -carboxyl of the Glu moiety still bound to the tRNA forming an activated intermediate.¹⁰ (ii) The enzymes hydrolyze an amide donor (Gln or Asn) to liberate ammonia. (iii) AdTs use the liberated ammonia to amidate the activated intermediate to form the cognate aa-tRNA species, Asn-tRNA^{Asn} or Gln-tRNA^{Gln}.

Two distinct AdTs are found in nature, the heterotrimeric GatCAB¹¹ and the heterodimeric GatDE.¹ The latter acts only as a Glu-AdT and is present in all known archaea.¹ GatCAB occurs in both *Bacteria*¹¹ and *Archaea*.¹ To date all GatCAB enzymes have shown the ability to act as both a Glu-AdT and an Asp-AdT *in vitro*.^{1, 12–14}

Since the bacterial GatCAB is a dual specificity enzyme that can function as an Asp-AdT and/or a Glu-AdT, what determines its *in vivo* role? The task of GatCAB is defined by the genomic context of the organism in terms of the occurrence of a ND-GluRS and/or a ND-AspRS, the enzymes responsible for Glu-tRNA^{Gln} and/or Asp-tRNA^{Asn} synthesis. In *Pseudomonas aeruginosa*, which has a ND-AspRS but lacks a ND-GluRS, GatCAB serves just as an Asp-AdT,¹⁵ while in *Bacillus subtilis* GatCAB is used only as a Glu-AdT¹¹ as it lacks a ND-AspRS but has a ND-GluRS.⁴ In *Helicobacter pylori*, which has both ND-aaRSs,^{16–18} GatCAB has a dual function as both an Asp-AdT and as a Glu-AdT.^{3, 19} In *Archaea*, given the specificity of GatDE as a Glu-AdT¹ and the fact that GatCAB is present only when AsnRS is absent,^{1, 2} the *in vivo* role of GatCAB is assumed to be primarily as an Asp-AdT.

How do the AdTs discriminate their mischarged tRNA substrates (Glu-tRNA^{Gln} and/or Asp-tRNA^{Asn}) from the cognate aa-tRNA species (Glu-tRNA^{Glu} and Asp-tRNA^{Asp})? The GatDE and bacterial GatCAB enzymes recognize the D-loop and the first base-pair of the acceptor stem to accomplish this.^{20–22} The archaeal GatCAB rejects Asp-tRNA^{Asp} not based on recognition of the first base-pair of the acceptor stem, but it instead relies on anti-determinants in tRNA^{Asp} (the D-loop, variable loop and position 49).^{21, 23}

Bioinformatics first showed that the glutaminase subunits of the archaeal AdT enzymes are of different biochemical origins.¹ In GatDE, the glutaminase subunit (GatD) is derived from a type I L-asparaginase,^{1, 24, 25} while the comparable GatA subunit of GatCAB belongs to the amidase enzyme family.^{3, 11, 26} L-asparaginases are known to hydrolyze both Asn and Gln²⁷ although the preferred substrate varies among enzymes from different organisms. For example, the *Erwinia carotovora* L-asparaginase is more efficient hydrolyzing Asn than Gln,²⁸ while the *Pseudomonas* 7A enzyme is more active in the hydrolysis of Gln than Asn.²⁹

The crystal structure of the *Staphylococcus aureus* GatCAB complexed with Gln or Asn suggested Gln as a better donor in transamidation.²⁰ In agreement with that prediction, the bacterial GatCAB enzymes have been shown to be more active using Gln as the donor than Asn.^{3, 30, 31} The *H. pylori* enzyme was approximately 130-fold more efficient using Gln than Asn, mostly due to a difference in k_{cat} .³ However, this enzyme is nearly as efficient in forming Asn-tRNA^{Asn} as Gln-tRNA^{Gln} in keeping with its function in the cell as both a Glu-AdT and an Asp-AdT.³

Here we present our characterization of GatDE and GatCAB from the archaeon *Methanothermobacter thermautotrophicus* with respect to their amide donor and mischarged tRNA substrate preferences for transamidation.

Results

Characterization of the two *M. thermautotrophicus* AdTs

The initial characterization of the *M. thermautotrophicus* AdTs suggested that both enzymes could use Gln or Asn as amide donors but did not clarify if either enzyme was more efficient with one or the other donor.¹ With the bacterial GatCAB enzymes studied to date, Gln is a better amide donor for transamidation than Asn.^{3, 20, 30, 31} Since the glutaminase subunit of GatDE is an L-asparaginase (GatD),^{1, 24, 25} raised the possibility that the archaeal specific AdT would be able to efficiently use Asn as an amide donor. The previous results also showed that GatDE was specific for Glu-tRNA^{Gln} and that GatCAB could transaminate both Asp-tRNA^{Asn} and Glu-tRNA^{Gln} but whether the enzyme could transaminate both substrates with similar efficiencies was not determined.¹ The predicted role of the archaeal GatCAB as an Asp-AdT suggested that the enzyme would prefer Asp-tRNA^{Asn} over Glu-tRNA^{Gln} as a substrate. We therefore set out to determine the amide donor preferences of the archaeal AdTs (Fig. 1) and whether the *M. thermautotrophicus* GatCAB is equally efficient in transamidation of Glu-tRNA^{Gln} and Asp-tRNA^{Asn} (Fig. 2).

To test the transamidase activities of both AdTs we used a [³²P]tRNA/nuclease P1 assay, which had earlier been successfully used to determine the enzymatic properties of the *H. pylori* GatCAB.³ This assay is sensitive, and the presence of the ³²P-label in the 3' terminal AMP of the tRNA ensures that any conversion of Glu to Gln or Asp to Asn detected is on the tRNA itself. It should be noted that the optimal growth temperature for *M. thermautotrophicus* is approximately 65 °C; yet these experiments were carried out at 37 °C, unless otherwise noted, in order to minimize deacylation of the aa-tRNA species. We found that at elevated temperatures our aa-tRNA substrates began to detectably deacylate over the time span of our reactions. It is speculated that *in vivo* complexes between ND-aaRSs and AdTs protects the aa-tRNA from deacylation.^{22, 32, 33}

The *M. thermautotrophicus* GatDE utilized both amide donors (Fig. 1a) though it is approximately 5-fold more efficient with Asn than Gln as the donor for transamidating Glu-tRNA^{Gln} (Table 1). The effect is primarily due to a difference in k_{cat} as GatDE has similar K_M values for Gln and Asn (331.1 μ M and 204.3 μ M, respectively). Interestingly, the K_M value of GatDE for Asn (204.3 μ M) is between the corresponding values for the *E. coli* type I (3.5 mM) and type II (11.5 μ M) L-asparaginases.³⁴

Surprisingly, the *M. thermautotrophicus* GatCAB used both Gln and Asn as amide donors with similar efficiencies (Fig. 1b). The enzyme was only approximately 3-fold more efficient using Gln as the donor than Asn (Table 2). This is in contrast to the *H. pylori* GatCAB, which is 130-fold more efficient using Gln than Asn.³ The K_M values for Gln and Asn with the *M. thermautotrophicus* GatCAB (6.7 μ M and 11.6 μ M, respectively) are slightly lower than the corresponding values with the *H. pylori* GatCAB (20.7 μ M and 22.4 μ M).³ These results suggest that the active site of the *M. thermautotrophicus* GatA is subtly different from the *S. aureus* A-subunit,²⁰ and thus allows the archaeal enzyme to use Asn nearly as well as Gln as an amide donor.

Regarding the preferred mischarged tRNA substrate, GatDE as expected was specific for Glu-tRNA^{Gln} (Fig. 2). The K_M values with the *M. thermautotrophicus* GatDE for Glu-tRNA^{Gln} and ATP, (1.71 μ M and 114.0 μ M, respectively) (Table 1) are similar to the values obtained with the *H. pylori* GatCAB (1.18 μ M and 206.8 μ M, respectively).³ The similarity in K_M values is not surprising considering that the subunits (GatE and GatB) of the two AdTs that recognize Glu-tRNA^{Gln} and ATP are homologous.¹ For ATP, the K_M value (400 μ M) with the *M. thermautotrophicus* GatCAB is slightly higher than that determined with the *H. pylori* GatCAB (206.8 μ M).³ For Asp-tRNA^{Asn}, the K_M value with the *M. thermautotrophicus* GatCAB (0.78

μM) (Table 2) is slightly lower than the corresponding values with the *Neisseria meningitidis* GatCAB ($1.2 \mu\text{M}$)²¹ and *H. pylori* GatCAB ($0.95 \mu\text{M}$)³ and is slightly higher than the K_D value ($0.6 \mu\text{M}$) determined for the enzyme with the mischarged substrate.²³

While fully active as an Asp-AdT (Fig. 2b), under our new assay conditions purified (>95% homogeneity) *M. thermautotrophicus* GatCAB was unable to transamidate the homologous Glu-tRNA^{Gln} (Fig. 2a) even over an hour-long time course with $1 \mu\text{M}$ of enzyme (data not shown). We cannot rule out the possibility that the *M. thermautotrophicus* GatCAB retains trace Glu-AdT activity with homologous Glu-tRNA^{Gln} as the aa-tRNA substrate substantially deacylated over this extended time period. However, it should be noted that no Glu-AdT activity with the *M. thermautotrophicus* GatCAB ($1 \mu\text{M}$) was detected within the first five minutes of the reaction during which time deacylation of the aa-tRNA was not as significant. In contrast, we were able to detect Asp-AdT activity with the *M. thermautotrophicus* GatCAB (5 nM) within 20 seconds (Fig. 1b).

Given that the optimal growth temperature of *M. thermautotrophicus* is 65°C ,³⁵ we tested whether its GatCAB was more active at elevated temperatures (50°C and 65°C). As an Asp-AdT, the enzyme was approximately as active at 50°C (apparent k_{trans} of 0.1 s^{-1}) as it was at 37°C (k_{trans} of 0.11 s^{-1} , Table 3) while it was twice as active at 65°C (apparent k_{trans} of 0.2 s^{-1}). At both 50°C and 65°C the aa-tRNA substrate did detectably begin to deacylate over the time spans assayed (approximately 12 % at 65°C over a time course of 100 seconds), thus the apparent Asp-AdT activity for GatCAB at these temperatures might be an underestimate of the actual activity. At both 50°C and 65°C , we did not detect Glu-AdT activity with the *M. thermautotrophicus* GatCAB ($1 \mu\text{M}$) and homologous Glu-tRNA^{Gln} (data not shown). However, trace activity may have been masked by the deacylation of the aa-tRNA (50% deacylation after approximately 9 and 6 min, respectively). The results do demonstrate however that regardless of the temperature at which *M. thermautotrophicus* GatCAB was assayed, at the very least, the enzyme is significantly more active as an Asp-AdT than as a Glu-AdT with homologous mischarged tRNA.

The inability of the *M. thermautotrophicus* GatCAB to transamidate homologous Glu-tRNA^{Gln} is in contrast to the previous results.¹ The earlier work was carried out with an enzyme preparation over-produced in *Escherichia coli* and partially purified (D. Tumbula-Hansen, personal communication); a similarly prepared partially purified enzyme formed Gln-tRNA^{Gln} (data not shown) using our current assay suggesting that this reaction is due to the presence of contaminating *E. coli* enzyme(s). It has been demonstrated that both the *E. coli* and the *Bacillus subtilis* glutamine synthetases are able to amidate Glu-tRNA^{Gln} to Gln-tRNA^{Gln} at low yield.³⁰ The Gln-tRNA^{Gln} synthesis activity observed therefore may have been due to the presence of *E. coli* glutamine synthetase in the partially purified GatCAB preparations. Scenarios involving the *E. coli* GlnRS are less likely as the *M. thermautotrophicus* tRNA^{Gln} is an extremely poor substrate for the bacterial aaRS.¹

Based on these results, it appears that *M. thermautotrophicus* encodes two AdTs, each with its own unique activity; GatDE is a Glu-AdT and only transamidates Glu-tRNA^{Gln}, while GatCAB is an Asp-AdT responsible for Asn-tRNA^{Asn} formation.

***M. thermautotrophicus* GatCAB can transamidate Glu-tRNA**

We then tested whether the *M. thermautotrophicus* GatCAB could transamidate other Glu-tRNA species. Theoretically, the primary discrimination of Asp-tRNA^{Asn} from Glu-tRNA^{Gln} could be achieved by recognition of the Asp moiety attached to the tRNA as compared to Glu. The use of the amino acid moiety of the misacylated tRNA as an identity element had already been suggested.^{21, 23} Therefore we tested the ability of the *M. thermautotrophicus* GatCAB to use Glu-tRNA^{Asn} as a substrate. Formation of *M. thermautotrophicus* tRNA^{Asn}

with homologous GluRS was possible; up to 10% of tRNA^{Asn} was converted to Glu-tRNA^{Asn} (data not shown). Of the two *M. thermautotrophicus* AdTs only GatCAB converted Glu-tRNA^{Asn} to Gln-tRNA^{Asn} (Fig. 3) with about 65% that of the enzyme's Asp-AdT activity with homologous Asp-tRNA^{Asn} as the substrate. The results reveal that *M. thermautotrophicus* GatCAB can amidate tRNA-bound Glu, and suggest that the AdT distinguishes Asp-tRNA^{Asn} from Glu-tRNA^{Gln} based more on differences in the tRNA isoacceptors than discriminating Asp from Glu. However, we cannot rule out that recognition of the aminoacyl-moiety contributes to the ability of the AdT to discriminate Asp-tRNA^{Asn} from Glu-tRNA^{Gln}. Attempts to perform the complementary experiment with Asp-tRNA^{Gln} failed because the *M. thermautotrophicus* AspRS was unable to acylate tRNA^{Gln} (data not shown).

The poor yield of Glu-tRNA^{Asn} formation prevented a more quantitative examination of this unusual *M. thermautotrophicus* GatCAB substrate. However, we noticed that archaeal GatCAB can use bacterial Asp-tRNA^{Asn} as a substrate approximately as well as archaeal Asp-tRNA^{Asn},^{21, 23} and bacterial GatCAB readily transamidates both bacterial Asp-tRNA^{Asn} and Glu-tRNA^{Gln}.^{3, 12–14, 19, 36} Therefore, we tested whether the *M. thermautotrophicus* GatCAB could transamidate bacterial (*H. pylori* and *B. subtilis*) Glu-tRNA^{Gln} species (Table 3).

In contrast to *M. thermautotrophicus* Glu-tRNA^{Gln}, bacterial Glu-tRNA^{Gln} from *H. pylori* or *B. subtilis* were substrates for *M. thermautotrophicus* GatCAB (Table 3). The enzyme used the *H. pylori* Glu-tRNA^{Gln} as a substrate about as well as the *Chlamydia trachomatis* and *M. thermautotrophicus* Asp-tRNA^{Asn} species (k_{trans} of 0.08 s⁻¹, 0.09 s⁻¹ and 0.11 s⁻¹, respectively), demonstrating the *M. thermautotrophicus* GatCAB retains the ability to act approximately equally well as a Glu-AdT and an Asp-AdT. Interestingly, the archaeal GatCAB preferred the *H. pylori* Glu-tRNA^{Gln} over *B. subtilis* Glu-tRNA^{Gln} (k_{trans} of 0.08 s⁻¹ and 0.004 s⁻¹, respectively), suggesting that elements in the tRNA allow preferential recognition of *H. pylori* over *B. subtilis* Glu-tRNA^{Gln} by the *M. thermautotrophicus* GatCAB.

In keeping with the known identity elements recognized by GatDE²² and bacterial GatCAB,^{20, 21} the *M. thermautotrophicus* GatDE preferred the archaeal Glu-tRNA^{Gln} over other substrates tested (Table 3) and the *H. pylori* GatCAB (Table 3) favored the bacterial mischarged tRNA substrates over the archaeal ones. These two enzymes, in contrast to the archaeal GatCAB, positively recognize the first base-pair in the acceptor stem of their mischarged tRNA substrates (Fig. 4).^{20–22}

Discussion

Asn-tRNA and Gln-tRNA are formed in *M. thermautotrophicus* by two different enzymes

While it was established earlier that GatDE is a Glu-AdT unable to synthesize Asn-tRNA^{Asn},¹ we have now provided *in vitro* evidence that *M. thermautotrophicus* GatCAB cannot synthesize Gln on its homologous tRNA^{Gln} and functions only as an Asp-AdT. Thus, *M. thermautotrophicus* probably uses two different amidotransferases for amide aminoacyl-tRNA synthesis, GatDE for Gln-tRNA^{Gln} formation and GatCAB for Asn-tRNA^{Asn} synthesis also *in vivo* (Fig. 5). Given that GatCAB is only encoded in archaeal genomes that lack AsnRS,^{1, 2} it is plausible to assume that other archaea also employ GatCAB just as an Asp-AdT. This is in contrast to the situation in *Bacteria*; for instance *C. trachomatis* and *H. pylori* do not encode AsnRS and GlnRS in their genomes and employ only GatCAB to serve both as an Asp-AdT and a Glu-AdT to meet their cellular needs for both Asn-tRNA^{Asn} and Gln-tRNA^{Gln} (Fig. 5).^{3, 14}

Given the specificity of the *M. thermautotrophicus* GatCAB as an Asp-AdT raises the question of how the AdT distinguishes *M. thermautotrophicus* Asp-tRNA^{Asn} from *M. thermautotrophicus* Glu-tRNA^{Gln}. The enzyme retains the ability to transamidate Glu attached to other tRNA isoacceptors, strongly indicating the Glu bound to the tRNA is not preventing *M. thermautotrophicus* GatCAB from recognizing the mischarged tRNA. This is particularly highlighted by the fact that the *M. thermautotrophicus* GatCAB is able to transamidate *H. pylori* Glu-tRNA^{Gln} tRNA as well as Asp-tRNA^{Asn} from *C. trachomatis* or *M. thermautotrophicus*. The results suggest that *M. thermautotrophicus* tRNA^{Gln} limits the *M. thermautotrophicus* GatCAB to Asn-tRNA^{Asn} synthesis.

Evolution of substrate preferences of the archaeal AdTs

It is unknown why archaea use two AdTs while bacteria utilize only one. One thought might invoke the putative thermophilic origins of archaea.³⁷ While in mesophiles the codon usage of Asn and Gln is approximately equal, in thermophiles the codon usage of Asn is on average twice that of Gln.^{38, 39} Since Gln is more labile at elevated temperatures than Asn,⁴⁰ it may suggest that thermophiles have different cellular demands for Asn-tRNA^{Asn} and Gln-tRNA^{Gln}. Possibly having a separate Asp-AdT and Glu-AdT by retaining both GatCAB and GatDE may have enabled the thermophilic ancestral archaea to separate the regulation of the enzymes involved in Asn-tRNA^{Asn} formation from those involved in Gln-tRNA^{Gln} biosynthesis to better meet the cellular needs for the two aa-tRNA species.

It is interesting to speculate whether in thermophilic organisms the relative ratios of free Asn to Gln are also elevated as compared to those in mesophiles given that Asn is more stable than Gln at higher temperatures.⁴⁰ A higher relative concentration of Asn relative to Gln in thermophiles as compared to mesophiles would provide an explanation as to why the *M. thermautotrophicus* GatDE is slightly more efficient using Asn over Gln as an amide donor and why the *M. thermautotrophicus* GatCAB is only 3-fold more efficient using Gln than Asn while the *H. pylori* GatCAB is 130-fold more efficient using Gln over Asn.³ The *H. pylori* enzyme would be inefficient in a cellular environment in which the relative ratio of Asn to Gln favors Asn.³ On the other hand, the archaeal GatCAB would only be marginally less active while GatDE would be in fact more active.

Materials and Methods

General

All oligonucleotide synthesis and DNA sequencing was carried out by the Keck Foundation Biotechnology Research Laboratory at Yale University. [α -³²P] ATP (10 mmol/ μ Ci) was from Amersham Biosciences (GE Healthcare). *Escherichia coli* BL21(DE3) and BL21(DE3) codon plus strains were from Stratagene (La Jolla, CA). Nickel-nitrilotriacetic acid-agarose (Ni-NTA) was from QIAGEN (Chatsworth, CA). Bio-Spin 30 columns were from Bio-Rad (Hercules, CA). High purity cold L-Glu, L-Asp, L-Gln and L-Asn were from Fluka (Deisenhofen, Germany). Phenol was from American Bioanalytical (Natick, MA).

H. pylori GluRS, *M. thermautotrophicus* GluRS, *M. thermautotrophicus* GatDE, *H. pylori* GatCAB, and *C. trachomatis* GluRS over-production and purifications

Were as previously described.^{3, 14, 17, 24}

M. thermautotrophicus AspRS over-production and purification

The enzyme was over-produced in an *E. coli* BL21-CodonPlus(DE3)-RIL strain harboring the pET28a vector with *M. thermautotrophicus* *aspS* between the *Nde*I and *Bam*HI restriction sites.⁴¹ Over-production was by auto-induction⁴² as described for the *H. pylori* GatCAB except

the resistance was kanamycin in place of ampicillin.³ The enzyme was purified as previously described though the final purification step of passing the enzyme over a Superdex200 column was omitted.⁴¹

***M. thermautotrophicus* GatCAB over-production and purification**

The enzyme was over-produced in an *E. coli* BL21-CodonPlus(DE3)-RIL strain and purified over a Ni-NTA column as previously described.⁴¹ The sample was then dialyzed in buffer Q (25 mM Hepes-KOH, pH 7.2, 5 mM 2-mercaptoethanol, 0.2 mM EDTA, and 5% glycerol). The protein was purified to > 95% homogeneity by fast protein liquid chromatography on a MonoQ column (Amersham Biosciences) that was developed with a 20–750 mM KCl gradient in buffer Q. GatCAB eluted with approximately a 135 mM concentration of KCl. The method has been used to successfully purify *M. thermautotrophicus* GatDE and *H. pylori* GatCAB.^{3, 24} The pure fraction of GatCAB was then dialyzed in buffer Q with 50% glycerol.

***M. thermautotrophicus* tRNA^{Gln} and tRNA^{Asn} and *C. trachomatis* tRNA^{Asn} production and purification**

The tRNA isoacceptors were transcribed and purified as previously described.^{1; 43}

***H. pylori* tRNA^{Gln} and *B. subtilis* tRNA^{Gln} purification**

Both tRNA species were over-expressed in an *E. coli* XL1-Blue strain and total tRNA was purified as previously described.^{3, 11} The *H. pylori* tRNA^{Gln} was purified from total tRNA as described previously.^{3, 44} The *B. subtilis* tRNA^{Gln} was purified in a same manner^{3; 44} except a different 5' biotinylated probe (5'-GCTGGATTCTGAACCAACGCATGACGGAGTCAAAGTCC-3') was used.

Preparation of labeled tRNA

The tRNA isoacceptors were ³²P-labeled on their 3' terminus as previously described.³

Preparation of bacterial Glu-tRNA^{Gln} and Asp-tRNA^{Asn}

The *H. pylori* Glu-tRNA^{Gln} was prepared as described.³ The *C. trachomatis* Asp-tRNA^{Asn} was prepared as described for the *H. pylori* tRNA^{Asn} with some modification.³ Instead of 21 μM unlabeled tRNA^{Asn}, 10 μM tRNA^{Asn} was added in the reaction and the reaction time was shortened to one hour from 14 hours. The *P. aeruginosa* AspRS was a gift from Jacques Lapointe (Laval University, Canada). The *B. subtilis* Glu-tRNA^{Gln} was prepared as described for the *H. pylori* tRNA^{Gln} except the *C. trachomatis* GluRS was used in place of the *H. pylori* GluRS.³

Preparation of *M. thermautotrophicus* Glu-tRNA^{Gln}

M. thermautotrophicus tRNA^{Gln} was glutamylated as described previously with slight modification.²² To the reaction 600 nM of [³²P]tRNA^{Gln} was added instead of the 40 nM stated.

Preparation of *M. thermautotrophicus* Asp-tRNA^{Asn}

Aminoacylation of the *M. thermautotrophicus* tRNA^{Asn} with Asp was carried out in 1× *M. thermautotrophicus* aminoacylation buffer (50 mM Hepes-KOH buffer (pH 7.2) containing 25 mM KCl, 10 mM MgCl₂, and 5 mM DTT) with 24 μg/μL of pyrophosphatase (Roche), 4 mM ATP, 3 μM *M. thermautotrophicus* AspRS, 1 mM L-Asp, 10 μM unlabeled tRNA^{Asn} and 600 nM ³²P-labeled tRNA^{Asn}. The reactions were incubated at 37 °C for 45 min. The samples were processed in the same manner as previously described.³

Preparation of *M. thermautotrophicus* Glu-tRNA^{Asn}

Aminoacylation of the *M. thermautotrophicus* tRNA^{Asn} with Glu was carried out at 37 °C for 90 min. in 1× *M. thermautotrophicus* aminoacylation buffer with 24 µg/µL of pyrophosphatase (Roche), 4 mM ATP, 1 µM *M. thermautotrophicus* GluRS, 1 mM L-Asp, 0.9 µM unlabeled tRNA^{Asn} and 100 nM ³²P-labeled tRNA^{Asn}. The samples were processed in the same manner as previously described.³

[³²P]tRNA/nuclease P1 amidotransferase assay

Glu-AdT and Asp-AdT activities of the *M. thermautotrophicus* GatCAB and GatDE were assayed as previously described^{3, 22} with some modification. Briefly, amidotransferase reaction mixtures included 1× *M. thermautotrophicus* AdT buffer (100 mM Hepes-KOH, pH 7.2, 12 mM MgCl₂, 30 mM KCl and 5 mM DTT) and unless otherwise noted 4 mM ATP, 9 to 11 µM ³²P-labeled mischarged tRNA (Asp-tRNA^{Asn} or Glu-tRNA^{Gln}) and 4 mM amide donor (Gln or Asn) and were carried out at 37 °C (unless otherwise noted). For determination of the kinetic parameters, initial velocities were measured in duplicate while varying the concentration of one substrate and saturating with the other two. In studies with the *M. thermautotrophicus* GatDE, 10 nM enzyme was used and reactions were carried out over a minute. With the *M. thermautotrophicus* GatCAB, reactions were carried over two minutes and with 5 nM enzyme. For *K_M* determinations of the two-amide donors, substrate concentrations varied from 3 µM to 4000 µM. For *K_M* determinations of the mischarged tRNA substrate, concentrations varied from approximately 150 nM up to 12 µM. For *K_M* determinations with ATP, concentrations varied from 40 µM to 4000 µM. Reaction mixes were pre-incubated at 37 °C and started by addition of enzyme. Aliquots (2 µL) of the reaction mixes were quenched, digested and processed as previously described.^{3, 22}

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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^{Gln} amidotransferase catalyzing Gln-tRNA formation

AspRS, aspartyl-tRNA synthetase

Asp-AdT, aspartyl-tRNA^{Asn} amidotransferase catalyzing Asn-tRNA formation

NTA, Nickel-nitrilotriacetic acid-agarose

PEI, polyethylenimine

TLC, thin layer chromatography. The three-letter code for the amino acids is used throughout.

Figure 1. Activity of the *M. thermautotrophicus* GatDE (a) and the *M. thermautotrophicus* GatCAB (b) with Gln or Asn as the amide donor in the transamidation reaction

(a) Glu-tRNA^{Gln} conversion to Gln-tRNA^{Gln} by the *M. thermautotrophicus* GatDE (10 nM) with either Gln (4 mM) (●) or Asn (4 mM) (▲). (b) Asp-tRNA^{Asn} conversion to Asn-tRNA^{Asn} by the *M. thermautotrophicus* GatCAB (5 nM) in the presence of Gln (4 mM) (●) or Asn (4 mM) (▲). In both (a) and (b), reactions were carried with 4 mM ATP and 10 μM mischarged tRNA (Glu-tRNA^{Gln} or Asp-tRNA^{Asn}, respectively).

Figure 2. Glu-AdT (a) and Asp-AdT (b) activity of the *M. thermautotrophicus* GatDE and GatCAB
(a) Phosphorimage of a TLC separating [32 P]AMP, Glu- [32 P]AMP, and Gln- [32 P]AMP released from *M. thermautotrophicus* tRNA^{Gln} by digestion with nuclease P1 following incubation with either no enzyme (lane 1), GatDE (200 nM) (lane 2) or GatCAB (200 nM) (lane 3) for 7 minutes at 37 °C in the presence of ATP (4 mM), 32 P labeled *M. thermautotrophicus* Glu-tRNA^{Gln} (10 μ M), and amide donor (4 mM). The amide donor in the GatCAB reaction was Gln and the donor in the GatDE reaction was Asn. **(b)** Phosphorimage of a TLC separating [32 P]AMP, Asp- [32 P]AMP, and Asn- [32 P]AMP released from *M. thermautotrophicus* tRNA^{Asn} by digestion with nuclease P1 following incubation with either no enzyme (lane 1), GatDE (200 nM) (lane 2) or GatCAB (200 nM) (lane 3) for 7 minutes at 37 °C in the presence of ATP (4 mM), 32 P-labeled *M. thermautotrophicus* Asp-tRNA^{Asn} (10 μ M), and amide donor (4 mM). The amide donor in the GatCAB reactions was Gln and the donor in the GatDE reactions was Asn.

Figure 3. *M. thermautotrophicus* GatCAB can transamidate Glu-tRNA^{Asn}

Phosphorimage of a TLC separating Glu-[³²P]AMP, and Gln-[³²P]AMP released from *M. thermautotrophicus* tRNA^{Asn} by digestion with nuclease P1 following incubation with either no enzyme (lane 1), GatDE (200 nM) (lane 2) or GatCAB (200 nM) (lane 3), for 7 minutes at 37 °C in the presence of ATP (4 mM), ³²P labeled *M. thermautotrophicus* Glu-tRNA^{Asn} (1 μM), and amide donor (4 mM). The *M. thermautotrophicus* GluRS was only able to glutamylate up to ten percent of the *M. thermautotrophicus* tRNA^{Asn}. The amide donor in the GatCAB reaction was Gln and the donor in the GatDE reaction was Asn.

Figure 4. Known identity elements recognized by the AdTs

Cloverleaf representations of (a) two bacterial tRNA^{Gln} isoacceptors (*H. pylori* and *B. subtilis*), (b) the *M. thermautotrophicus* tRNA^{Gln}, (c) the *C. trachomatis* tRNA^{Asn}, and (d) the *M. thermautotrophicus* tRNA^{Asn}. Known identity elements recognized by the AdTs are color-coded. The first base-pair of the acceptor stem (red) is an important recognition element for bacterial GatCAB and GatDE in their respective aa-tRNA substrates.^{20, 21} The D-loop (pink) is used by the archaeal and bacterial GatCAB as well as by GatDE to distinguish their mischarged aa-tRNA substrates from other aa-tRNA species.^{20–22} The length of the variable loop (cyan) has been demonstrated to be important for the archaeal GatCAB to distinguish Asp-tRNA^{Asn} from Asp-tRNA^{Asp}.^{21, 23}

Figure 5. In the archaeon, *M. thermautotrophicus*, there is a separate Glu-AdT, GatDE, and Asp-AdT, GatCAB, while in *Bacteria* like *H. pylori* a single AdT, GatCAB, serves both functions. In *M. thermautotrophicus*, which lacks a GlnRS and an AsnRS, GatDE transamidates Glu-tRNA^{Gln} to Gln-tRNA^{Gln} while GatCAB catalyzes Asn-tRNA^{Asn} formation from Asp-tRNA^{Asn}. GatDE is slightly more efficient using Asn as an amide donor while the archaeal GatCAB has a slight preference for Gln over Asn. In *H. pylori*, which also lacks the two aaRSs, on the other hand employs just GatCAB as both a Glu-AdT and an Asp-AdT to form both amide aa-tRNA species. The *H. pylori* GatCAB is significantly more efficient using Gln as an amide donor than Asn.³

Table 1Kinetic data for the transamidase activity of the *M. thermautotrophicus* GatDE*

Substrate ^a	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\text{s}^{-1}/\mu\text{M}$) ($\times 10^{-3}$)
Asp-tRNA ^{Asn} ^b	ND ^e	ND ^e	ND ^e
Glu-tRNA ^{Gln} ^b	1.71 ± 0.29	0.9 ± 0.1	530 ± 110
ATP ^c	114 ± 60	1.4 ± 0.2	12 ± 7
Gln ^d	331 ± 114	0.4 ± 0.1	1.2 ± 0.5
Asn ^d	204 ± 48	1.1 ± 0.1	5.4 ± 1.3

* For details of the steady-state kinetics see Materials and Methods. Experiments were done in duplicate and repeated two to four times. Standard deviations are reported.

^a K_M was determined by varying the concentration of the substrate listed while adding the other two substrates required for transamidation in excess with 10 nM GatDE.

^b Asn and ATP were added in excess (4 mM and 4 mM, respectively).

^c 10–11 μM Glu-tRNA^{Gln} and 4 mM Asn were added.

^d 10–11 μM Glu-tRNA^{Gln}, 4 mM ATP were added.

^e ND stands for no-detectable activity under the conditions assayed (see text and Fig. 2).

Table 2Kinetic data for the transamidase activity of the *M. thermautotrophicus* GatCAB*

Substrate ^a	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\text{s}^{-1}/\mu\text{M}$) ($\times 10^{-3}$)
Asp-tRNA ^{Asn} ^b	0.78 ± 0.30	0.10 ± 0.01	125 ± 49
Glu-tRNA ^{Gln} ^b	ND ^e	ND ^e	ND ^e
ATP ^c	400 ± 140	0.09 ± 0.01	0.2 ± 0.1
Gln ^d	6.7 ± 1.2	$0.11 \pm < 0.01$	16.7 ± 3.0
Asn ^d	11.6 ± 2.6	$0.07 \pm < 0.01$	5.7 ± 1.3

* For details of the steady-state kinetics see Materials and Methods. Experiments were done in duplicate and repeated two to four times. Standard deviations are reported.

^a K_M was determined by varying the concentration of the substrate listed while adding the other two substrates required for transamidation in excess with 10 nM GatCAB.

^b Asn and ATP were added in excess (4 mM of each).

^c 10–11 μM Asp-tRNA^{Asn} and 4 mM Gln were added.

^d 10–11 μM Glu-tRNA^{Asn}, 4 mM ATP were added.

^e ND stands for no-detectable activity under the conditions assayed (see text and Fig. 2).

Table 3

Transamidase activity of the *M. thermautotrophicus* (*M.t.*) GatCAB and GatDE, and the *H. pylori* (*H.p.*) GatCAB with different mischarged tRNA substrates *

aa-tRNA ^a	<i>M.t.</i> GatCAB ^b	Transamidase activity (k_{trans} , s ⁻¹)	
		<i>M.t.</i> GatDE ^c	<i>H.p.</i> GatCAB ^d
<i>M.t.</i> GlutRNA ^{Gln}	ND ^e	0.98 ± 0.27	0.08 ± 0.02
<i>H.p.</i> Glu-tRNA ^{Gln}	0.08 ± 0.02	0.01 ± <0.01	3.12 ± 0.09
<i>B.s.</i> Glu-tRNA ^{Gln}	0.004 ± 0.003	0.01 ± <0.01	2.2 ± 0.1
<i>C.t.</i> Asp-tRNA ^{Asn}	0.09 ± 0.01	ND ^e	1.27 ± 0.01
<i>M.t.</i> Asp-tRNA ^{Asn}	0.11 ± 0.01	ND ^e	0.01 ± <0.01

* Measurements were from three to four separate experiments. Standard deviations are reported. Reactions were carried out at 37 °C in the presence of ATP (4 mM), amide donor (4 mM), and aa-tRNA (10 μM) indicated. For reactions with the GatCAB enzymes, Gln was provided as the amide donor. In reactions with GatDE, Asn was the donor.

^a The aa-tRNA substrates tested were the *M. thermautotrophicus* (*M.t.*), *H. pylori* (*H.p.*), and *B. subtilis* (*B.s.*) Glu-tRNA^{Gln}, and the *C. trachomatis* (*C.t.*) and *M. thermautotrophicus* (*M.t.*) Asp-tRNA^{Asn}.

^b In reactions with *M.t.* GatCAB, enzyme concentrations ranged from 20 nM to 1 μM.

^c In reactions with *M.t.* GatDE, enzyme concentrations ranged from 20 nM to 1 μM.

^d In reactions with *H.p.* GatCAB, enzyme concentrations ranged from 10 nM to 100 nM.

^e ND stands for no-detectable activity under conditions assayed.