The heterotrimeric *Thermus thermophilus* Asp-tRNA^{Asn} amidotransferase can also generate Gln-tRNA^{Gln}

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Abstract ***Thermus thermophilus*** **strain HB8 is known to have a heterodimeric aspartyl-tRNA^{Asn} amidotransferase (Asp-AdT) capable of forming Asn-tRNA^{Asn} [Becker, H.D. and Kern, D. (1998) Proc. Natl. Acad. Sci. USA 95, 12832–12837]. Here we show that, like other bacteria,** ***T. thermophilus*** **possesses the canonical set of amidotransferase (AdT) genes (*gatA*, *gatB* and *gatC*). We cloned and sequenced these genes, and constructed an artificial operon for overexpression in** ***Escherichia coli*** **of the thermophilic holoenzyme. The overproduced** ***T. thermophilus*** **AdT can generate Gln-tRNA^{Gln} as well as Asn-tRNA^{Asn}. Thus, the** ***T. thermophilus*** **tRNA-dependent AdT is a dual-specific Asp/Glu-AdT resembling other bacterial AdTs. In addition, we observed that removal of the 44 carboxy-terminal amino acids of the GatA subunit only inhibits the Asp-AdT activity, leaving the Glu-AdT activity of the mutant AdT unaltered; this shows that Asp-AdT and Glu-AdT activities can be mechanistically separated.**

Key words: Aminoacyl-tRNA; tRNA-dependent amidation; Thermus thermophilus; tRNA specificity

1. Introduction

The high specificity of aminoacyl-tRNA (AA-tRNA) synthesis guarantees the faithful decoding of messenger RNA. Two different pathways lead to AA-tRNA; the direct charging of the tRNA with the cognate amino acid by an AA-tRNA synthetase (AARS) or a two-step reaction involving mischarging of a tRNA followed by conversion of the amino acid while bound to tRNA [1]. While such tRNA-dependent amino acid transformation pathways had long been known to account for the formation of formylmethionyl-tRNA [2] and selenocysteinyl-tRNA [3], it is now clear that this route is also used extensively for Gln-tRNA^{Gln} and Asn-tRNA^{Asn} [4] synthesis.

The conversions of Asp-tRNA^{Asn} or Glu-tRNA^{Gln} into their cognate translational substrates requires a tRNA-dependent amidotransferase (AdT). The *Bacillus subtilis* enzyme has recently been shown to be heterotrimeric, with the subunits encoded by the *gatA*, *gatB* and *gatC* genes [5]. These genes are markers used to screen whole genome sequences, and their presence indicates the use of the indirect pathway in the defined organism. The *gatC*, *gatA* and *gatB* genes have been found in many of the recently released genome sequences and in all living kingdoms [6], however, only *B. subtilis* Glu-AdT, and *Thermus thermophilus* [7] and *Deinococcus radiodurans* [8] Asp-AdTs have been biochemically characterized. Furthermore, it has recently been shown that the *B. subtilis* and *D. radiodurans* AdTs have in vitro both Asp-tRNA^{Asn} and Glu-tRNA^{Gln} amidation activities, whereas in vivo they are strictly restricted to Gln-tRNA^{Gln} and Asn-tRNA^{Asn} formation, respectively [8]. *B. subtilis* contains a non-discriminating GluRS capable of generating Glu-tRNA^{Gln} but possesses a discriminating AspRS unable to generate the misacylated Asp-tRNA^{Asn} intermediate; therefore the *gatCAB*-encoded AdT only serves as a Glu-AdT in this bacterium. Conversely, *Thermus* and *Deinococcus* contain a discriminating GluRS but two AspRS enzymes, one being of archaeal type ([9], J. Pelaschier, personal communication) capable of making the Asp-tRNA^{Asn} intermediate used by the AdT which is therefore an Asp-AdT. However, it is still unknown if the double specificity applies to every AdT, or if it is restricted to only bacterial enzymes, or a subset of them. Our knowledge of the role, contribution and requirement of the different subunits to the AdT activity also remains fragmentary.

Since thermostable proteins have the remarkable ability to crystallize, we decided to study *T. thermophilus* Asp-AdT to initiate structural investigations. However, unlike *B. subtilis* or *D. radiodurans*, no genome of any of the *Thermus* species has been published thus far. Here, we report the cloning, sequencing and tRNA specificity of *T. thermophilus* HB8 Asp-AdT. We show that although the enzyme is active as a GatAB heterodimer [7], *T. thermophilus* possesses the three canonical *gatC*, *gatA* and *gatB* set of genes. We establish that the overexpressed AdT exhibits both Asp-tRNA^{Asn} and Glu-tRNA^{Gln} transamidation activities. Finally, we show that removal of the 44 carboxy-terminal amino acids of the GatA subunit abolishes the enzyme's capacity to catalyze transamidation of Asp-tRNA^{Asn}, without altering its Glu-AdT activity; thus the Asp-AdT and Glu-AdT activities can be physically separated.

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2. Materials and methods

2.1. Cloning and sequencing of T. thermophilus AdT genes

The preliminary genomic sequence of *T. thermophilus* strain HB27 was searched for *gat* genes based on amino acid similarity with the *B. subtilis* Glu-AdT [5]. PCR primers were constructed based on the sequences obtained. The genes were then amplified from genomic *T. thermophilus* strain HB8 DNA, and the clones were sequenced. The GenBank accession numbers of *T. thermophilus gat* genes are AF202446, AF202447 and AF202448 for *gatC*, *gatA* and *gatB*, respectively.

2.2. Preparation of AA-tRNAs

B. subtilis $tRNA^{Gln}$ was overproduced in *Escherichia coli* as described [5]. [^{14}C]Glu-$tRNA^{Gln}$ was prepared by aminoacylation of 0.6 nmol of overproduced *B. subtilis* $tRNA^{Gln}$ in total tRNA from *E. coli* with 63 μg of a *B. subtilis* tRNA-free extract (Q-Sepharose fraction) and 25 μM [^{14}C]glutamate (251 mCi/mmol, 50 μCi/ml). *D. radiodurans* $tRNA^{Asn}$ was overexpressed in *E. coli* as described [5]. [^{14}C]Asp-$tRNA^{Asn}$ was prepared by aminoacylation of *D. radiodurans* $tRNA^{Asn}$ with 0.3 nmol of pure AspRS2 from *D. radiodurans* and 50 μM of [^{14}C]aspartate (216 mCi/mmol, 50 μCi/ml). Aminoacylations were conducted in a 200 μl standard reaction mixture [7] during 30 min at 37°C. The AA-tRNA was extracted with acid-buffered phenol, followed by a chloroform:isoamyl alcohol (24:1) extraction and an ethanol precipitation.

2.3. Preparation of extracts

Proteins were extracted by sonication of the cells at 4°C in 50 mM Tris–HCl, pH 8.0, containing 5 mM 2-mercaptoethanol, 1 mM benzamidine, 0.1 mM Na_2EDTA. S100 extracts were prepared by ultracentrifugation at $100\,000\times g$ for 2 h. Flocculated *E. coli* extracts were obtained by heat treatment of S100 samples at 70°C for 10 min followed by 10 min centrifugation at $15\,000\times g$ at 4°C removing the precipitated thermolabile proteins, which generally yields a 10-fold purification.

2.4. tRNA-dependent amidation assays

tRNA-dependent amidation reactions were performed at 37°C for 50 min in a 40 μl standard reaction mixture [7], using 2 mM of

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ttgatC          1 MELSPELLRKLETLAKIRLSPEEEALLLQDLKRILDFVDALPRVEEGGAEEALGRLREDE
consen            ------------L-------------------------------------------R-D-

               61 PRPSLPQAEALALAPEAEDGFFRVPPVLE
                  -------------------------P----


ttgatA          1 MLAHEIRARVARGEVSPLEVAQAYLKRVQELDPGLGAFLSLNERLLEEAEAVDPGLPLAG
consen            ------------------------------------------------------------G

               61 LVVAVKDNIATRGLRTTAGSRLLENFVPPYEATAVARLKALGALVLGKTNLDEFGMGSST
                  -----K------------S-------------------------G--N--EF------

              121 EHSAFFPTKNPFDPDRVPGGSSGGSAAALAADLAPLALGSDTGGSVRQPAAFCGVYGLKP
                  ---------N--------GGS--G--------------G---GGS-R-PA------G-KP

              181 TYGRVSRFGLIAYASSLDQIGPMARSVRDLALLMDAAAGPDPLDATSLDLPPRFQEALEG
                  --G-----------------G-------------------D--D----------------

              241 PLPPLRLGVVREALAGNSPGVERALEEALKVFRELGLSVREVSWPSLPQALAAYYILAPA
                  ------------------------------------------------------------

              301 EASSNLARYDGTLYGRRAAGEEVXGXMEATRALFGLEVKRRVLVGTFVLSSGYYEAYYGR
                  E-------------------------------------------G---------------

              361 AQAFRRRLKAEAQALFREVDLLLLPTTPHPAFPFGARRDPLAMYREDLYTVGANLTGLPA
                  -------------------D--------------------------------------

              421 LSFPAGFEGHLPVGLQLLAPWGEDERLLRAALAFEEATARAHLKAPLGEAL
                  ---------------Q-----------------------------------


ttgatB          1 MYEAVIGLEVHLHLKTRTKMFCGCRADYFGAEPNTHTCPVCLGLPGALPVPNRVAVEHGL
consen            ------------------------------------------------------------

               61 RLALALGAEVPERLVFHRKNYFYPDLPKNYQISQYDLPLGRGGSLPLGERRVRIKRLHLE
                  -----------------RK----PD-----Q------P----G--------------H-E

              121 EDAGKSLHLEGRTLLDLNRAGSPLIELVTEPDLKTPEEARLFLQRIQALVQTLGISDASP
                  -D-G-----------D-NR----L-E-V--------------------------------

              181 EEGKLRADVNVSVRRVGEPLGTKVEIKNLNSFKSVQRALEYEIRRQTEILRRGEKVKQAT
                  --G--R-D-N--------------E-KN-NS----------E--R--------------T

              241 MGFEEGSGKTYPMRTKEEEADYRYFPEPDLPPVAIPRDWLEEVRRSLPELPWEKEARYRA
                  ---------T---R-KE----YRY----D---------------------P---------

              301 LGIKEKDAEVLAYTPSLARFLDQALPLGLASPQALANWLLADVAGLLHERGLRLEETRLS
                  ----------L-------------------------W----------------------

              361 PEGLARLVGLFERGEVTSRVAKSLLPEVLEGQDPEALVRERGLKVVADEGALKALVAEAI
                  --------------------K---------------------------------------

              421 AAMPEAAESVRQGKVKALDALVGQVMRKTRGQARPDLVRRLLLEALGVG
                  ------------------------------------------------
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Fig. 1. Sequence of *T. thermophilus* Asp-AdT subunits. The amino acid sequences of the GatA, GatB and GatC proteins are identified by ttgatA, ttgatB and ttgatC. The amino acids in capital letters in the consensus sequence (shown below *T. thermophilus* genes) indicate full conservation (among the samples compared). The numbers of bacterial and archeal and eukaryotic proteins used in these comparisons were, respectively, for GatA (14, 2, 0), for GatB (14, 2, 1), for GatC (9, 0, 0).

glutamine and 5 μl of the enzyme preparation (see above). The AA-tRNAs were extracted, precipitated and deacylated as described previously [7], and amino acids were analyzed by thin layer chromatography (TLC) in ammonia:water:chloroform:methanol (2:1:6:6). [^{14}C]Amino acids on dried TLC plates were revealed by scanning with a Fuji Bioimager of the 12 h exposed image plate and verified by ninhydrin assay using non-radiolabeled standards (50 nmol).

3. Results

3.1. T. thermophilus strain HB8 AdT genes

We searched a database (Göttingen Genomics Laboratory, Göttingen, Germany) containing preliminary genomic sequence information of *T. thermophilus* strain HB27 for the *gat* genes encoding bacterial AdTs based on amino acid similarity with the three *B. subtilis* Glu-AdT subunits [5]. The search revealed the presence of not only *gatA* and *gatB* genes in *Thermus*, but also of *gatC*. As was seen in *D. radiodurans* [8], an organism specifically related to *Thermus*, the *gat* genes in *T. thermophilus* are dispersed in the genome and not arranged in an operon as found in *B. subtilis* [5]. The DNA sequence of the *T. thermophilus* strain HB27 *gat* genes was used to design primers in order to amplify by PCR the corresponding genes of *T. thermophilus* strain HB8. The choice of this strain was dictated by our earlier work on the Asp-AdT from this strain [7]. The *gatA*, *gatB* and *gatC* genes encode polypeptides of respectively 472, 470 and 90 amino acids (Fig. 1). The G+C content of the *gat* genes (A: 72%, B: 69% and C: 69%) is in the range of the global G+C content found in *Thermus* genes [10], leading to preferential usage of G or C at the wobble position of codons. TBlastN searches of the database with the *T. thermophilus gat* genes reveal that the closest 'relative' among the known examples is *D. radiodurans*, with identities of 51%, 55% and 41% for *gatA*, *gatB* and *gatC*,

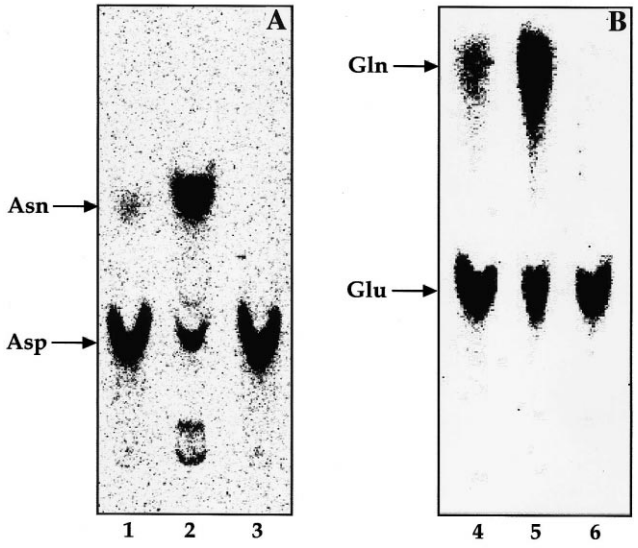

Fig. 2. tRNA-dependent conversion of Asp-tRNA^{Asn} (A) and Glu-tRNA^{Gln} (B) by the cloned AdT from *T. thermophilus*. Phosphoimages of TLC plates revealing the tRNA-dependent conversion of ^{14}C-radiolabeled aspartate and glutamate into asparagine and glutamine, respectively. The mischarged AA-tRNA used in the amidation reactions was from *D. radiodurans* (A) and from *B. subtilis* (B). Lanes 1 and 4, flocculated extract of *T. thermophilus* GatCAB overexpressed in *E. coli*; 2, S100 of *D. radiodurans* GatCAB overexpressed in *E. coli*; 3 and 6, *E. coli* DH5 S100 extract; 5, pure *B. subtilis* Glu-AdT.

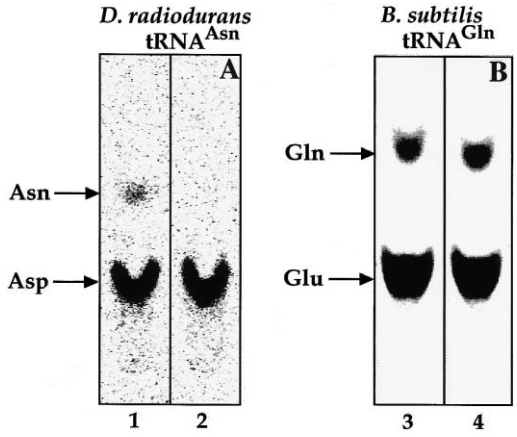

Fig. 3. Specificity of *T. thermophilus* AdT mutant GatDΔ44. Phosphoimages of TLC plates revealing the tRNA-dependent conversion of ^{14}C-radiolabeled aspartate (A and B) and glutamate (C) into asparagine and glutamine, respectively. Origin of the mischarged tRNA is indicated above the TLCs. Lanes 1 and 3, flocculated extract of *T. thermophilus* GatCAB overexpressed in *E. coli*; lanes 2 and 4, flocculated extract of *T. thermophilus* GatDΔ44 overexpressed in *E. coli*.

respectively. The amino acid composition of the subunits shows features usually observed in *Thermus* proteins when compared to their mesophilic counterparts. For example, the contents of Glu, Gln, Arg and Leu exceed those of their related amino acids Asp, Asn, Lys and Ile. The alignment of *T. thermophilus* GatA, GatB and GatC with the consensus sequence derived from corresponding proteins of many organisms shows a high degree of sequence conservation (Fig. 1).

3.2. Specificity of T. thermophilus HB8 gatCAB-encoded AdT

Since *E. coli* possesses asparagine synthetases A and B as well as glutamine synthetase, which are responsible for the tRNA-independent formation of Asn and Gln, respectively, S100 of the AdT overproducing strains was submitted to heat treatment at 70°C for 10 min to inactivate these metabolic enzymes. Although asparagine synthetase activities were completely abolished, glutamine synthetase remains active (data not shown). In order to distinguish between tRNA-dependent and -independent activities, the AA-tRNAs were purified prior to and after their amidations. As a result, only the radiolabeled AA attached to the tRNA after amidation is revealed by the TLC procedure (see Fig. 2, lanes 3 and 6). Fig. 2A shows that the overexpressed thermostable AdT catalyzes tRNA-dependent transformation of Asp into Asn. However, the yield of Asn formation is smaller than the one obtained with *D. radiodurans* AdT. Several reasons can account for the apparently lower transamidation activity of *T. thermophilus* enzyme when compared to *D. radiodurans* AdT, such as the heterologous nature of the AA-tRNA used as substrate for the reaction (e.g. Asp-tRNA^{Asn} from *D. radiodurans*), or the suboptimal temperature at which the thermophilic enzyme was assayed. These two parameters were optimal when *D. radiodurans* AdT was tested. The same flocculated extract of *T. thermophilus* overproduced AdT was subsequently assayed for Glu-AdT activity using *B. subtilis* Glu-tRNA^{Gln} as a substrate. Fig. 2B shows that *T. thermophilus gatCAB*-encoded AdT also catalyzes the tRNA-dependent transformation of Glu into Gln. Once again, and for the same reasons,

this activity is lower when compared to the extent of glutamine formed by *B. subtilis* AdT, assayed under optimal conditions.

3.3. Functional analysis of the GatDΔ44 mutant

In the process of subcloning the *gat* genes into an operonic expression arrangement, we isolated a mutant characterized by a truncated *gatA* gene. In this mutant, deletion of one base in comparison with the sequence of HB27 genome causes a frame shift and leads to appearance of a UAA stop codon 44 codons upstream to the normal 3′-end of *gatA*. The GatDΔ44 mutant AdT possesses a GatA subunit deprived of its natural 44 C-terminal amino acids (Glu_{428}–Leu_{471}, Fig. 1) but elongated by four amino acids. These added amino acids correspond to the stop codon read in the different frame and to the translation of nine nucleotides of the ribosomal binding site located in between *gatA* and *gatB* in the artificial operon. We performed overproduction of the mutant AdT and tested its activity. Surprisingly, when assayed in comparison with the wild-type AdT, the mutant AdT shows unaltered Glu-AdT activity but lacks any detectable Asp-AdT activity (Fig. 3). Absence of Asp-AdT activity was assayed using *D. radiodurans* $tRNA^{Asn}$, a close relative of *T. thermophilus*, overexpressed in *E. coli*. The mesophilic nature of the tRNA did not allow testing of the Asp-AdT activity at the optimal temperature of the thermophilic enzyme. Therefore, our results indicate that if not completely abolished, the Asp-AdT activity is considerably reduced by the removal of the 44 C-terminal amino acids of GatA. Alignment of the A subunit sequences from various organisms shows only strict conservation of a Gln (Gln_{436}, Fig. 1) in the deleted part of mutant GatDΔ44. However, this Gln_{436} is part of a motif which is relatively well conserved: $(L/I/V)_{431}$-(P/N/S)-(V/I/L)-(G/S)-(L/V/M/A/G)-Q_{436}.

4. Discussion

The *T. thermophilus* tRNA-dependent AdT is the only such enzyme that has been shown to be active as a GatAB heterodimer. Since functionality of the *B. subtilis* GatA subunit depends on concomitant expression of GatA and GatC subunits, it has been hypothesized that GatC is required for the correct folding of GatA [5], and therefore might act like a chaperone. However, we have yet not determined if this role applies to GatC proteins of any other bacterial species. In organisms like *T. thermophilus*, where the *gatCAB* genes are dispersed over the genome, GatC might well have a different role or have a more moderate role in folding of GatA, explaining the fact that the GatAB dimer is still fully active. This active heterodimer was obtained after loss of the GatC subunit during an extensive purification procedure [7], showing that in this case, GatA and GatB are more tightly bound to each other than to GatC. In *B. subtilis*, only the GatCAB holoenzyme is fully active and mixing GatCA with GatB only yields 50% of the native activity [5]. In this case, GatC might not only be required for the correct folding of GatA but also to enhance the binding between GatA and GatB. In *T. thermophilus*, GatC might also be required, as in *B. subtilis*, for the correct folding of GatA but may then be dispensable for the full expression of the activity. Moreover, it has still not been determined if the operonal arrangement might influence how GatC operates or if the role of GatC depends on the arrangement of the *gatCAB* genes in an operon. Therefore, by constructing an artificial operon for overexpression of *Thermus* AdT, we might have altered the way GatC interacts with GatA by, for example, amplifying its structural role as compared to its native role in *Thermus* cells. This could also be the reason why, besides the fact that the AdT was assayed at suboptimal temperature with a heterologous tRNA, the activity of the AdT might appear weaker as compared to the homologous systems. Another explanation could be that the folding of the three subunits and/or their interactions to produce a native and fully active holoenzyme has been altered because of their overexpression at mesophilic temperature. To answer these questions, overexpression of the AB heterodimer or of the three subunits concomitantly but not in an operon would bring some insight whether the presence of *gatC* is required to obtain an active AdT and what incidence the operonal arrangement might have on the AdT activity.

Our work shows that, as with *D. radiodurans* [8] and *B. subtilis* [5], *Thermus* AdT has also the dual ability to catalyze both tRNA-dependent formation of Asn and Gln. However, this enzyme is only responsible, in vivo, for Asn-tRNA formation, since *Thermus* possesses a mischarging AspRS (AspRS2), a GlnRS, and is lacking a mischarging GluRS [7]. The fact that the cellular activity of AdTs only depends upon presence or lack of the mischarging AARS could explain why those enzymes have not restricted their specificity. Further analysis of the specificity of AdTs from different origins will bring insight into whether this relaxed specificity is an intrinsic property of tRNA-dependent AdTs or if the dual function is restricted to the bacterial holoenzymes.

We still do not know what subunits are responsible for recognition and amidation of Asp-$tRNA^{Asn}$ and Glu-$tRNA^{Gln}$. It has been hypothesized that the AdTs are multi-component complexes built upon an amide-providing enzyme (amidase) and an RNA-binding protein. The catalytic center of the transamidase would then reside in GatA which possesses an amidase signature within its sequence [5]. Moreover, the overexpressed *B. subtilis* GatCA dimer still exhibits a glutaminase activity (A.W. Curnow, unpublished results) providing the amine group that will be transferred onto the tRNA-bound glutamate, but does not perform the tRNA-dependent amidation. GatB would then be responsible for tRNA recognition. However, our results undoubtedly show that GatA has also the ability to discriminate between Asp-$tRNA^{Asn}$ and Glu-$tRNA^{Gln}$ and therefore is implicated in binding of the tRNA. Although we have no information on what parts of the tRNA are recognized by the AdT, these enzymes precisely discriminate between Asp-$tRNA^{Asp}$, Glu-$tRNA^{Glu}$ and Asp-$tRNA^{Asn}$ and Glu-$tRNA^{Gln}$. It has been shown that, in the majority of the studied aminoacylation systems, there are two parts of the tRNA that are used by AARSs to discriminate their cognate tRNA among all other species; these two domains are the acceptor arm and the anticodon loop. If these parts are also critical for the recognition of the AA-tRNA by the AdTs, GatA, being the catalytic center of the holoenzyme, would then be recognizing the acceptor arm of the tRNA which presents the glutamate to be modified. GatB would then be devoted to recognition of the anticodon loop of the tRNA.

The relaxed specificity of the AdTs raises also another question: do those enzymes possess two different catalytic centers or are both amidation reactions catalyzed by one active site? Our work shows that by removing the 44 C-terminal amino

acids of GatA, we can abolish, or drastically decrease, the Asp-AdT activity without altering the enzyme's ability to amidate a Glu-tRNA^{Gln}. The fact that we can physically separate both activities could be interpreted by the presence of two different active sites within GatA. Another explanation would be that this A subunit possesses a single active site composed of a unique set of catalytic amino acids and two different sets of recognition amino acids each of them being specific for the recognition of the AA-tRNA. In this case, recognition of Asp-tRNA^{Asn} would reside in the C-terminal part of GatA. Since the characteristic amidase signature $_{139}$GGSSGGSAAALAADLAPLALGSDTGGSVRQPA$_{170}$ is unique, it is likely that AdTs only possess one active site and that the 44 C-terminal amino acids of the A subunit contain residues involved in the recognition of Asp-tRNA^{Asn}. Precise mutagenesis of residues belonging to this motif will provide information on whether residues of this peptide are involved or responsible for the Asp-AdT activity.

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