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Aminoacyl-tRNA formation in the extreme thermophile *Thermus thermophilus*

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Abstract Thermophilic organisms must be capable of accurate translation at temperatures in which the individual components of the translation machinery and also specific amino acids are particularly sensitive. *Thermus thermophilus* is a good model organism for studies of thermophilic translation because many of the components in this process have undergone structural and biochemical characterization. We have focused on the pathways of aminoacyl-tRNA synthesis for glutamine, asparagine, proline, and cysteine. We show that the *T. thermophilus* prolyl-tRNA synthetase (ProRS) exhibits cysteinyl-tRNA synthetase (CysRS) activity although the organism also encodes a canonical CysRS. The ProRS requires tRNA for cysteine activation, as is known for the characterized archaeal prolyl-cysteinyl-tRNA synthetase (ProCysRS) enzymes. The heterotrimeric *T. thermophilus* aspartyl-tRNA^{Asn} amidotransferase can form Gln-tRNA in addition to Asn-tRNA; however, a 13-amino-acid C-terminal truncation of the holoenzyme A subunit is deficient in both activities when assayed with homologous substrates. A survey of codon usage in completed prokaryotic genomes identified a higher Glu : Gln ratio in proteins of thermophiles compared to mesophiles.

Key words Aminoacyl-tRNA · Thermophile · Amidotransferase · Codon usage · ProCysRS

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Introduction

Extremophile organisms grow at high temperatures (up to about 110°C), or in media of high salt concentration (Stetter 1999). Although their habitat may be extreme, their mechanism of protein synthesis appears to be the same as in mesophiles. Aminoacyl-tRNAs (AA-tRNAs) comprise a family of essential components in protein synthesis. The correct match of the amino acid with the cognate tRNA species, which recognizes the corresponding mRNA codon, guarantees the accuracy of protein synthesis and thus the faithful transmission of the genetic information. The stability of AA-tRNA is affected by the nature of the amino acid that is linked to the 3′-terminal adenosine of the tRNA (Hentzen et al. 1972). In general, the ester bonds formed with acidic amino acids, their amides, or the thiol amino acids are much more labile than those formed with neutral aliphatic side chains. For example, Gln-tRNA, Asn-tRNA, and Cys-tRNA are hydrolyzed more rapidly than, e.g., Ile-tRNA or Val-tRNA. Pro-tRNA appears to be one of the most labile AA-tRNAs. Thus, one wonders whether at high temperature the availability of some aminoacyl-tRNA species may limit the rate of protein synthesis. In addition, protein stability may be compromised at elevated temperature by denaturation and degradation. Deamidation of Asn and Gln in proteins as well as hydrolysis of the peptide bond next to these amide amino acids is known to occur, whereas Cys is subject to oxidation (Daniel and Cowan 2000; Zale and Klibanov 1986; Ahern and Klibanov 1985). Possibly for these reasons, some thermophilic bacterial and archaeal proteins have lower glutamine and asparagine contents than mesophilic bacteria and Eucarya (Michelitsch and Weissman 2000; Haney et al. 1999; Vieille and Zeikus 2001).

Biochemical studies and the analysis of many sequenced genomes have shown that the formation of 16 sets of aminoacyl-tRNAs by aminoacyl-tRNA synthetases (AARSs) is conserved in evolution. However, the synthesis of Asn-tRNA, Cys-tRNA, Gln-tRNA, and Lys-tRNA may involve noncanonical pathways or enzymes in different organisms (reviewed in Ibba and Söll 2001). It is unknown

whether an organism's growth temperature influences the distribution of these components; however, it was interesting to see that the genomic sequence of the hyperthermophile *Methanococcus jannaschii* revealed the lack of a canonical CysRS, whereas its mesophilic relative *Methanococcus maripaludis* contains this enzyme (Li et al. 1999). To gain insights into this question, we focused on the pathways of Asn-tRNA, Cys-tRNA, Gln-tRNA, and Pro-tRNA formation in *Thermus thermophilus*, a bacterium with an optimal growth at 65°–72°C and a maximal growth temperature of 85°C (Oshima and Imahori 1974).

Thermus species have been a splendid source of proteins of the translation apparatus for enzymological study and structure determination. For instance, the crystal structures of 15 *Thermus* AARSs are known. The first *Thermus* AARS, prolyl-tRNA synthetase (ProRS), was already well characterized 18 years ago (Rivera et al. 1984), and an early characterization of the Asp–tRNA^{Asn} amidotransferase activity was also made from this organism (Becker and Kern 1998). Recently, the structure of *T. thermophilus* ProRS, a synthetase that shows great similarity to its archaeal homologues, was resolved (Yaremchuk et al. 2000, 2001). Thus, the interesting biological context of *Thermus* has provided an ideal system for comparative studies of essential components of protein biosynthesis with their mesophilic counterparts. Here, we report investigating ProRS and the tRNA-dependent pathways of Gln-tRNA and Asn-tRNA formation in *T. thermophilus* and show that it uses the same enzymes and pathways found in many mesophilic bacteria.

Materials and methods

General

L-[^{14}C]Aspartate (213 mCi/mmol), L-[^{14}C]glutamate (260 mCi/mmol), and L-[^{14}C]proline (248 mCi/mmol) were purchased from Amersham (Uppsala, Sweden), and L-[^{35}S]cysteine (1,075 Ci/mmol) and [^{32}P]PP_i (4.6 Ci/mmol) were from NEN Dupont (Wilmington, DE, USA). Yeast inorganic pyrophosphatase was obtained from Roche Molecular Biochemicals (Basel, Switzerland). DEAE Sepharose Fast Flow was from Amersham Pharmacia, and hydroxyapatite (HTP) was from Bio-Rad (Hercules, CA, USA). Nickel-nitrilotriacetic acid (Ni-NTA) and chitin-binding resins were from Qiagen (Valencia, CA, USA) and New England Biolabs (Beverly, MA, USA), respectively. *Escherichia coli* strains BL21(DE3) and BL21-CodonPlus(DE3)-RIL were obtained from Stratagene (La Jolla, CA, USA). Oligonucleotide synthesis and DNA sequencing were performed by the W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University.

Preparation of tRNAs

Unfractionated tRNA from *T. thermophilus* or *M. jannaschii* was purified as described previously (Stathopoulos et al. 2001). The sequences of *T. thermophilus* tRNA$^{Gln}_{UUG}$ (TGGGGCGTCGTCTAACGGCAGGACAGCGGACTTTGGATCCGCCGGTGGTGGTTCGAGTCACCCGCCCCAGCCA) (accession number AY039624) and tRNA$^{Gln}_{CUG}$ (TGGGGTGTCGTCTAAGGGTAGGACAGCGGACTCTGGATCCGCCGGTCGTGGTTCGAATCCACGCACCCCAGCCA) (accession number AY039625) were determined at the Göttingen Genomics Laboratory (Göttingen, Germany) whereas that of tRNA^{Asn} was from Becker et al. (2000a). For each tRNA gene, two deoxyoligonucleotides corresponding to the sense and antisense strands of the gene were synthesized, annealed, and cloned into the expression vector pGFIB-I at the *Eco*RI and *Bam*HI sites (Normanly et al. 1986). After sequence confirmation, the plasmids were transformed for overexpression into *E. coli* DH5α. Unfractionated tRNA was purified from a 500-ml overnight culture as described by Curnow et al. (1998). The purity of the tRNA samples was checked on a 12% polyacrylamide/8 M urea gel.

Cloning, overexpression, and enzyme purification

The *T. thermophilus aspS2* gene was cloned and the aspartyl-tRNA synthetase 2 (AspRS2) was purified according to Becker et al. (2000b). The *Lactobacillus bulgaricus gluS* gene was cloned into pCYB1 vector (New England Biolabs) and transformed into *E. coli* BL21(DE3). The overexpressed GluRS (fused with intein) was purified according to the manufacturer's instructions.

The *T. thermophilus* wild-type *gatCAB* gene encoding the Glu-tRNA^{Gln} amidotransferase (Glu–AdT) (Becker et al. 2000a) and a mutant gene bearing a deletion at the C-terminus of the GatA subunit were overexpressed in *E. coli* BL21(DE3) strain. For the purification of the enzyme, 20 g of frozen cells were homogenized by sonication in 60 ml lysis buffer [50 mM potassium phosphate, pH 8, 10 mM $MgCl_2$, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM dithiothreitol (DTT), 1 mM benzamidine, 0.1% Triton X-100, 100 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 10% glycerol] and the lysate was centrifuged at 100,000 *g* for 2 h at 4°C. The resultant S-100 fraction was loaded onto a DEAE-cellulose column (5 cm^2 × 10 cm) and eluted with a 50–500 mM potassium phosphate gradient in buffer containing 50 mM potassium phosphate (pH 6.8), 0.1 mM EDTA, 5 mM 2-mercaptoethanol, 1 mM benzyamidine, and 10% glycerol. The amidotransferase activity eluted at a 100 mM potassium phosphate concentration; active fractions were pooled, dialyzed against buffer A (10 mM potassium phosphate, pH 6.8, 0.1 mM EDTA, 5 mM 2-mercaptoethanol, 1 mM benzamidine, and 10% glycerol), and loaded onto a hydroxyapatite column (5 cm^2 × 8 cm). The column was eluted with a narrower linear gradient of potassium phosphate (10–200 mM) in buffer A. The active fractions (eluted around 100 mM potassium phosphate) were concentrated, dialyzed against 50 mM hydroxyethylpiperazine ethanesulfonic acid (HEPES) (pH 7.2), 0.1 mM EDTA, 5 mM 2-mercaptoethanol, and 50% glycerol and stored at –20°C. Both wild-type and mutant AdTs appeared to be more than 80% pure as judged by sodium

dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

The sequence of the *T. thermophilus* HB27 *proS* gene, determined at the Göttingen Genomics Laboratory (Göttingen, Germany), was used to design primers for amplification of the *proS* gene from *T. thermophilus* strain HB8 genomic DNA. The PCR product was cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA) and sequenced (accession number AF384553). On digestion with *Nde*I and *Bam*HI, the gene was ligated into pET15b (Invitrogen) for expression of an N-terminal His_6-tagged protein in the *E. coli* BL21-Codon Plus(DE3)-RIL strain. Cultures were grown at 37°C in Luria-Bertani (LB) medium, supplemented with 100 µg/ml ampicillin and 34 µg/ml chloramphenicol. Expression of the His_6-tagged protein was induced for 4–6 h with the addition of 1 mM isopropyl thiogalactoside (IPTG) before cell harvesting. The enzyme was purified by Ni-NTA chromatography as previously described (Ibba et al. 1997). The *T. thermophilus* His_6-ProRS was more than 99% pure, as judged by Coomassie brilliant blue staining after SDS-PAGE. Active fractions were pooled and dialyzed against aminoacylation buffer (see following) containing 40% glycerol and stored at –20°C.

Preparation of misaminoacylated tRNAs

Total *E. coli* tRNA enriched in *T. thermophilus* $tRNA^{Asn}$ or $tRNA^{Gln}$ (see above) (100 µM) was aminoacylated at 37°C for 45 min in a standard aminoacylation reaction (50 mM H EPES, pH 7.2, 25 mM KCl, 2 mM ATP, 5 mM $MgCl_2$, and 100 µM [^{14}C]aspartate or [^{14}C]glutamate) containing 0.025 mg/ml pyrophosphatase with 7 µM *T. thermophilus* AspRS2 or 4 M *L. bulgaricus* GluRS. Aminoacylated tRNAs were purified by phenol:chloroform extraction and ethanol precipitation as previously described (Curnow et al. 1998). Approximately 35% of overexpressed *T. thermophilus* $tRNA^{Asn}$, 4.5% of $tRNA^{Gln}_{UUG}$, and 7.5% of $tRNA^{Gln}_{CUG}$ were aminoacylated to plateaus by *T. thermophilus* AspRS2 or *L. bulgaricus* GluRS, respectively. On the other hand, 5% and 11.7% of unfractionated tRNAs from *T. thermophilus* and *E. coli*, respectively, could be aminoacylated by AspRS2, and 1% and 2.5% of those could be aminoacylated by GluRS.

Amidotransferase activity assay

The transamidation assay (Curnow et al. 1997) was modified. Conversion of [^{14}C]Asp-$tRNA^{Asn}$ or [^{14}C]Glu-$tRNA^{Gln}$ was carried out at 37°C or 55°C for 15 min in 50 µl reaction buffer (100 mM HEPES, pH 7.2, 30 mM KCl, 12 mM $MgCl_2$, 10 mM ATP) containing the appropriate amide donor (2 mM glutamine, asparagine, or NH_4Cl). The resulting aminoacyl-tRNAs were isolated by phenol : chloroform extraction and ethanol precipitation and subsequently deacylated at 55°C in 0.025 M KOH for 15 min. When pure enzyme and overexpressed tRNA were used, the phenol : chloroform extractions were omitted. The released radiolabeled amino acids were separated by TLC and visualized by phosphoimaging.

Aminoacylation assays

Cys-tRNA or Pro-tRNA formation was assayed at 70°C as previously described (Stathopoulos et al. 2000) in the presence of 0.5–1 µM of *T. thermophilus* ProRS, using unfractionated tRNA from *T. thermophilus* (40 µM) as a substrate. To prevent oxidation of cysteine (50 µM) in the reaction mixture, a 100-fold excess of DTT (5 mM) was maintained. Aliquots from the reaction mixture were removed periodically, spotted on Whatman 3 MM paper filter disks, and washed three times in 10% trichloroacetic acid to remove the free amino acid. After drying, the radioactivity was measured by liquid scintillation counting.

ATP–PP_i exchange assays

Pro-AMP and Cys-AMP formation was determined under standard conditions in the presence or absence of unfractionated *T. thermophilus* tRNA (40 µM) using [^{32}P]PP_i with a specific activity of 2,000 cpm/nmol. The reaction mixture also contained 0.5–2 µM ProRS, 1 mM ATP, 1 mM KF, and 2 mM proline or 5–10 mM cysteine in aminoacylation buffer (Stathopoulos et al. 2001). The reaction was quenched by the addition of 1% activated carbon in the presence of 0.4 M sodium pyrophosphate and 15% perchloric acid. After filtration of the mixture through glass microfiber filter disks (GF/C; Whatman), the amount of [^{32}P]-labeled ATP was measured by liquid scintillation counting.

Results

Thermus thermophilus prolyl-tRNA synthetase can form Cys-tRNA

Genomic sequence analysis of *T. thermophilus* HB27 showed that the organism contains a canonical cysteinyl-tRNA synthetase. Interestingly, a recent report on the structure of *T. thermophilus* prolyl-tRNA synthetase and sequence alignments therein revealed a striking similarity of this particular enzyme to its archaeal counterparts (Yaremchuk et al. 2000). In addition, a phylogenetic approach clearly implied that members of *Deinococcaceae* (including *T. thermophilus*) acquired a large number of archaeal genes (one of them is *proS*) during an extensive horizontal gene transfer event (Olendzenski et al. 2000).Given the fact that archaeal ProRS enzymes define, during translation, cysteine as well as proline (the enzyme is designated Pro-CysRS) by forming $Cys\text{-}tRNA^{Cys}$ (Stathopoulos et al. 2000; Lipman et al. 2000), we wanted to investigate if this evolutionary similarity of the *T. thermophilus* enzyme reflects also a possible dual-specific enzymatic function. This aim was achieved using the same approach we had used earlier in

identifying a dual-specificity prolyl-tRNA synthetase in the deep-rooted eukaryote *Giardia lamblia*, an organism in which two cysteinylation activities coexist (one from a canonical class I CysRS and one from the dual-specificity class II ProCysRS) (Bunjun et al. 2000).

The *T. thermophilus* HB8 *proS* gene was cloned and the nucleotide sequence determined to ensure the absence of any possible point mutations accidentally introduced by polymerase chain reaction (PCR). Guarding against such mutations is essential, especially in the case of the dual-specificity ProCysRS enzymes, because single amino acid replacements can dramatically affect one or the other activity (Stathopoulos et al. 2001a). Following overexpression of the recombinant protein, a flocculation step (30 min at 65°C) was added to eliminate as many contaminant proteins as possible from *E. coli* that could interfere with the enzymatic activities under study. After purification using Ni-NTA affinity chromatography, the protein was judged to be 99% pure (see Materials and methods). Total *T. thermophilus* tRNA prepared by standard methods from *T. thermophilus* HB8 cells was used as substrate in all the subsequent biochemical analyses. After the expected activity of the enzyme to use proline as substrate was verified (Fig. 1A), we attempted to use cysteine in the reaction mixture and observe any counts deriving from Cys-tRNA. To our surprise, we were able to record the direct attachment of cysteine to unfractionated tRNA (Fig. 1B); this correlates the *T. thermophilus* ProRS with a putative CysRS activity. To verify that this activity derives from the same enzyme, we tested whether *T. thermophilus* ProRS would activate cysteine in a tRNA-dependent manner as do the archaeal genre ProCysRS enzymes (Bunjun et al. 2000) (the canonical CysRS can form Cys-AMP in the absence of tRNA). Indeed, *T. thermophilus* ProRS requires tRNA for cysteine activation (Fig. 1C).

Thus, it appears that *T. thermophilus* ProRS is a dual-specificity ProCysRS of the archaeal genre with two cognate amino acid substrates; it utilizes the same mechanism that its archaeal homologues use to discriminate between these amino acids by activating cysteine only in the presence of tRNA, as has also been suggested by recent crystallographic studies (Yaremchuk et al. 2001). This finding is a clear indication that the *T. thermophilus* ProRS structural similarity to the archaeal enzymes corresponds to a specific behavior and groups this enzyme with the dual-specificity ProCysRSs. However, it remains to be determined if cysteine is attached to the cognate tRNA or if it is mischarged. This is the first bacterial enzyme to exhibit this bizarre dual function and expands the dual-specificity repertoire to a bacterial family (*Deinococcaceae*) that is more closely related to Archaea and eukaryotes. Although the *T. thermophilus* genome sequence is not yet complete, various phylogenetic studies suggest that this dual-specificity ProCysRS in *T. thermophilus* must coexist with a canonical CysRS, as in the case of *G. lamblia* (Woese et al. 2000).

Asn-tRNA is formed by transamidation in *Thermus thermophilus*

An earlier study of the heterotrimeric *T. thermophilus* Glu-tRNA^{Gln} amidotransferase suffered from the fact that homologous tRNA substrates were not used. Therefore, two tRNA^{Gln} genes and the tRNA^{Asn} gene were cloned based on the *T. thermophilus* HB27 genome sequence (Göttingen Genomics Laboratory). After overexpression in *E. coli,* unfractionated tRNA was purified that was greatly enriched in the overexpressed *T. thermophilus* tRNA species. Aminoacylation of these tRNAs with either *T. thermophilus* AspRS2 or *L. bulgaricus* GluRS yielded Asp-tRNA^{Asn} and

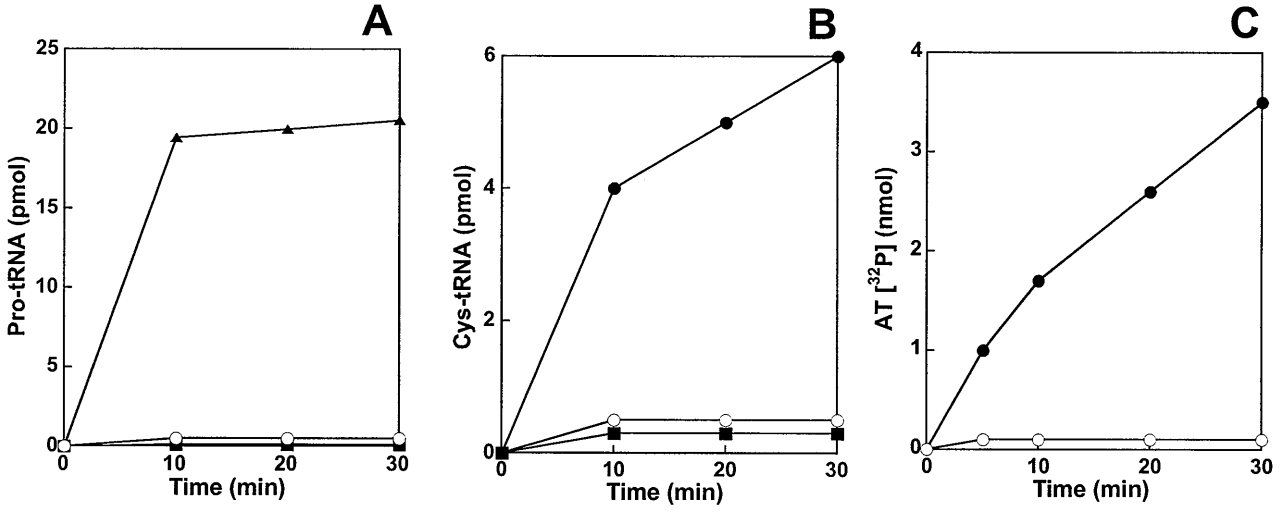

Fig. 1. Formation of Pro-tRNA (**A**) and Cys-tRNA (**B**) by *Thermus thermophilus* ProCysRS. Direct attachment of proline (*triangles*) or cysteine (*solid circles*) to total *T. thermophilus* tRNA was observed under the conditions described in Materials and methods. *Open circles* and *solid rectangles* represent the counts derived from the same reaction mixture in the absence of enzyme or tRNA, respectively. **C** Cysteine-dependent pyrophosphate exchange by *T. thermophilus* ProCysRS in the presence of *T. thermophilus* total tRNA (*solid circles*). No cysteine activation could be detected in the absence of tRNA (*open circles*)

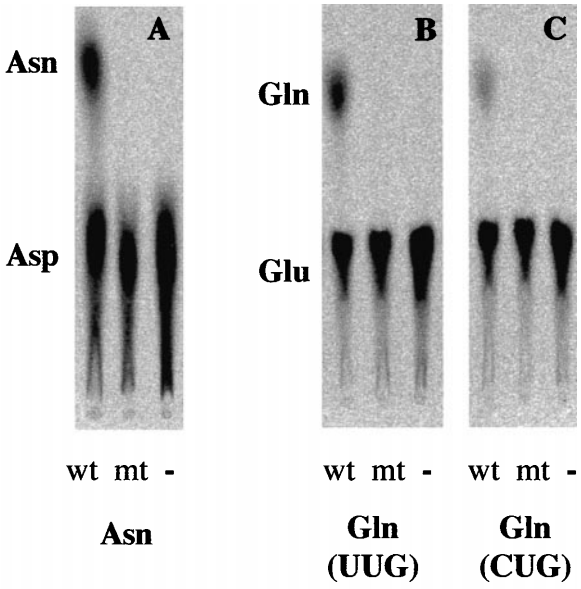

Fig. 2. Amidotransferase activities of recombinant *T. thermophilus* GatCABs using misaminoacylated *T. thermophilus* tRNAs expressed in *Escherichia coli*. Phosphoimages show thin-layer chromatography (TLC) separation of [^{14}C]-labeled Asn/Asp or Gln/Glu pairs derived from deacylation of aminoacyl-tRNAs recovered from the assay (see Materials and methods): transamidation of *T. thermophilus* Asp-tRNA^{Asn} (0.43 μM; **A**) at 37°C and *T. thermophilus* Glu-tRNA$^{Gln}_{UUG}$ isoacceptor (0.3 μM; **B**) or CUG isoacceptor (0.17 μM; **C**) at 55°C with 0.3 mg/ml wild-type (*wt*) or mutant (*mt*) GatCAB enzymes in the presence of 2 mM glutamine

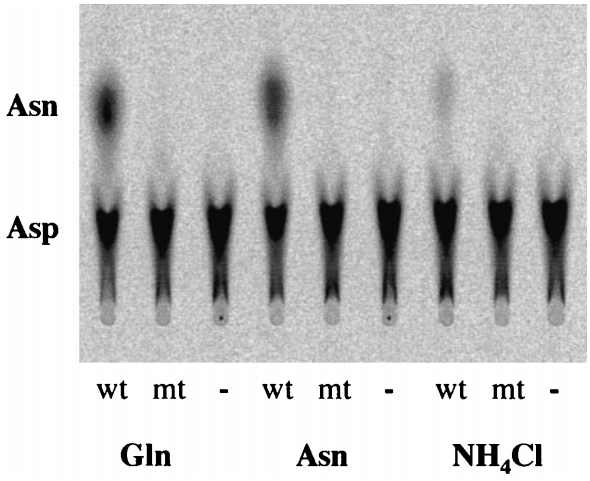

Fig. 3. Amide donor usage of wild-type (*wt*) and mutant (*mt*) *T. thermophilus* amidotransferases. Phosphoimages reveal tRNA-dependent conversion of [^{14}C]-labeled Asp-tRNA^{Asn} to Asn-tRNA^{Asn} in the presence of 2 mM glutamine, asparagine, or NH_4Cl and 1 μM of wild-type enzyme or GatAΔ 13 mutant enzyme in standard amidotransferase buffer (see Materials and methods)

Glu-tRNA^{Gln}, which were used as substrates for the amidotransferase reaction. Under the conditions tested, wild-type *T. thermophilus* GatCAB exhibited both AspAdT and GluAdT activities with the three different tRNAs (Asp-tRNA^{Asn}, Fig. 2A; Glu-tRNA$^{Gln}_{UUG}$, Fig. 2B; Glu–tRNA$^{Gln}_{CUG}$, Fig. 2C), in good agreement with our previous observation (Becker et al. 2000a). Both misaminoacylated tRNA^{Asn} and tRNA$^{Gln}_{UUG}$ are efficient substrates of *T. thermophilus* GatCAB; 25% of the [^{14}C]-labeled aspartate or glutamate could be amidated to asparagine or glutamine. Comparing transamidation efficiency of the two tRNA^{Gln} species, we show that Glu-tRNA$^{Gln}_{UUG}$ is a better substrate than Glu-tRNA$^{Gln}_{CUG}$ (Fig. 2C); thus, only 10% of the [^{14}C]-labeled glutamine was released from Glu-tRNA$^{Gln}_{CUG}$. Although *T. thermophilus* GatCAB acts only as an AspAdT in vivo, it is clear that in vitro it possesses Glu/AspAdT dual specificity as do all the other bacterial GatCAB enzymes that have been characterized so far (Tumbula et al. 2000).

The activity of the *T. thermophilus* GatCAB enzyme was determined in the presence of different amide donors. Asparagine appears to be an efficient amide donor as well as glutamine (at 2 mM concentration) for both the AspAdT (Fig. 3) and the GluAdT activities (data not shown). This result is similar to what has been described for the native *T. thermophilus* GatAB heterodimer (Becker and Kern 1998), but differs from *B. subtilis* GatCAB, which prefers glutamine over asparagine (Curnow et al. 1997). Ammonia is a much less efficient amide donor (see Fig. 3), in line with the preference of *B. subtilis* GatCAB (Curnow et al. 1997). In contrast, the native *T. thermophilus* AdT uses NH_4Cl efficiently. Further detailed kinetic studies will give insight to the amide donor preference of these enzymes.

A small C-terminal truncation of the GatA subunit in the *T. thermophilus* AdT causes enzyme inactivation

Reexamination of a mutant *T. thermophilus* GatCAB clone (Becker et al. 2000a) revealed that 13 C-terminal amino acids (not 44) of the GatA subunit were lacking. Using heterologous *Deinococcus radiodurans* tRNA, the earlier study (Becker et al. 2000a) of this mutant enzyme suggested that the C-terminus of GatA is critical for AspAdT but not GluAdT activity of GatCAB. In the present study, we tested the purified recombinant heterotrimeric *T. thermophilus* wild-type and mutant enzyme with *T. thermophilus* tRNAs; although the wild-type enzyme could form both Asn-tRNA or Gln-tRNA, the truncated enzyme was inactive with both substrates (Fig. 2A-C, lanes 2). Thus, the C-terminus of GatA is essential for both AspAdT and GluAdT activities in vitro.

The loss of enzymatic activity by the short truncation was rather unexpected because sequence alignments of bacterial GatA proteins showed the deletion to be in a non-conserved region. However, the C-terminus of GatA may be important for its structural integrity, and elimination of even a single amino acid may be detrimental. On the other hand, the residues that immediately precede the truncation are highly conserved (Curnow et al. 1997). It will be interesting to determine the role of the carboxy-terminus of GatA in the transamidation. This region may be involved in binding GatC, as this subunit could not be detected by SDS-PAGE during the purification of the mutant holoenzyme although it was easily detectable in the wild-type enzyme

preparation (data not shown). Possible interactions between the C-terminus of the GatA subunit and the other components of the holoenzyme might contribute as well to the stability of the heterotrimeric complex.

Discussion

The theory that the last common ancestor lived in a hyperthermophilic environment is an appealing possibility (Woese 1987; Di Giulio 2000). Such organisms need to incorporate versatility and flexibility into their physiology to overcome thermal denaturation of proteins and nucleic acids and decomposition of small molecules. Although still unclear, the evolutionary pressure that transformed these ancestors into contemporary species should have involved many changes in both nucleotide substitution and codon usage. It is therefore not surprising to find an increased G+C content in many thermophiles and a reduced representation of certain amino acids in thermophilic proteins when compared to their mesophilic counterparts (Jaenicke and Böhm 1998; Haney et al. 1999; Vieille and Zeikus 2001). There are certainly many other strategies that can be used by thermophiles to cope with high temperature, such as rapid resynthesis of thermosensitive compounds (Stetter 1999). However, it appears that the mechanisms of AA-tRNA formation are the same in mesophiles and thermophiles.

It has been suggested previously that glutamine is disfavored for protein synthesis in thermophiles when comparing amino acid usage of homologous proteins from thermophiles and mesophiles (Vielle and Zeikus 2001; Haney et al. 1999; Jaenicke and Böhm 1998). A survey of codon usage in completely sequenced prokaryote genomes and of the partially sequenced *T. thermophilus* genome revealed that, in comparison to mesophiles, the glutamine composition of encoded proteins in thermophiles is indeed decreased along with a concomitant increase of glutamate content (Fig. 4). All thermophiles with completed genomic sequences have a higher Glu/Gln codon ratio than mesophiles. *M. jannaschii*, a barophilic archaeon with an optimal growth temperature of 85°C, has the highest ratio of 5.94, which is fourfold higher than that of *E. coli*. The Glu/Gln codon ratio (3.42) in published *T. thermophilus* protein sequences (from GenBank) is more than twofold that of the corresponding value in *D. radiodurans*, a mesophile and the genus phylogenetically closest to *T. thermophilus*. The elevated ratio in thermophilic proteins could have been the result of replacement of nonessential glutamine residues by the functionally equivalent glutamate as an adaptation to hot environments. The lowered glutamine composition in thermophilic proteins is in good agreement with the fact that deamidation and succinimide formation of glutamine lead to protein inactivation at high temperatures (Zale and Klibanov 1986; Daniel et al. 1996). The increased representation of glutamate residues is also beneficial to thermophilic proteins because of its ability to form stabilizing salt bridges (Zhang et al. 2001). One could, of course, argue that the preferential incorporation of glutamate over glutamine in protein synthesis in thermophiles is a reflection of life in the last common ancestor, because all thermophiles that have been identified so far cluster near the root of the tree of life (Stetter 1999). This idea is in line with the proposal that Gln-tRNA formation may have been the last to evolve in AA-tRNA synthesis and that the transamidation pathway was an ancient route to incorporate glutamine into proteins (Ibba et al. 2000).

In contrast to glutamine, we found that asparagine codon composition in completed genomic sequences does not correlate with growth temperature (data not shown), although several reports have demonstrated that some thermophilic proteins do contain less asparagine than their mesophilic homologues (Haney et al. 1999; Vieille and Zeikus 2001). It is unclear what contributes to the relatively high asparagine content in thermophiles. *T. thermophilus* may be a special case in that two routes to Asn-tRNA exist. In addition to direct acylation by AsnRS, the transamidation pathway is present and may be used to ensure sufficient asparagine incorporation under asparagine starvation conditions (Becker and Kern 1998; Curnow et al. 1998). Moreover, the existence in the deep-rooted thermophilic bacterium *T. maritima* of a highly active asparagine repair enzyme, L-isoaspartyl methyltransferase (Ichikawa and Clarke 1998), suggests that thermophiles may be able to limit the damage of asparagine deamidation of their proteins.

Cysteine generally represents less than 1% of the amino acids in proteins. Therefore, it is difficult to correlate its compositional variations among different organisms with growth temperature. The average cysteine content of existing *T. thermophilus* proteins in GenBank (0.4%) is typical of most other genomes, although a discrimination or even complete elimination of this active residue was reported in some thermophilic enzymes, such as citrate synthases (Danson and Hough 1998). Cysteine is essential for protein function and stability, but it must bypass the hurdle of its thermal instability as well as its sensitivity to oxidation to be incorporated into proteins. How do thermophiles such as *T. thermophilus* maintain a cysteine level comparable to those of mesophiles? It was speculated that the existence of dual cysteinylation pathways in *G. lamblia*, a parasite with cysteine-rich outer membrane proteins, may keep the cysteine level constant in those proteins (Bunjun et al. 2000). A similar strategy could apply in *T. thermophilus*.

To ensure a steady rate of protein synthesis under high temperature, *T. thermophilus* may use two different pathways for Cys-tRNA formation. The strong sequence similarity of the *T. thermophilus* ProRS to the archaeal ProCysRS enzymes and the biochemical characterization presented here suggest a second enzymatic role that evolution assigned to this ProRS enzyme, whose gene may have been transferred laterally from Archaea into this family of bacteria (Olendzenski et al. 2000). This idea would clearly be supported if one could demonstrate in vivo Cys-tRNA formation by ProRS in *T. thermophilus*, which may be achieved by deleting *cysS*, the gene encoding CysRS. Such an experiment in *M. maripaludis* showed the *cysS* gene to be dispensable (Stathopoulos et al. 2001b), suggesting that the dual cysteinylation pathways can be redundant in some organisms.

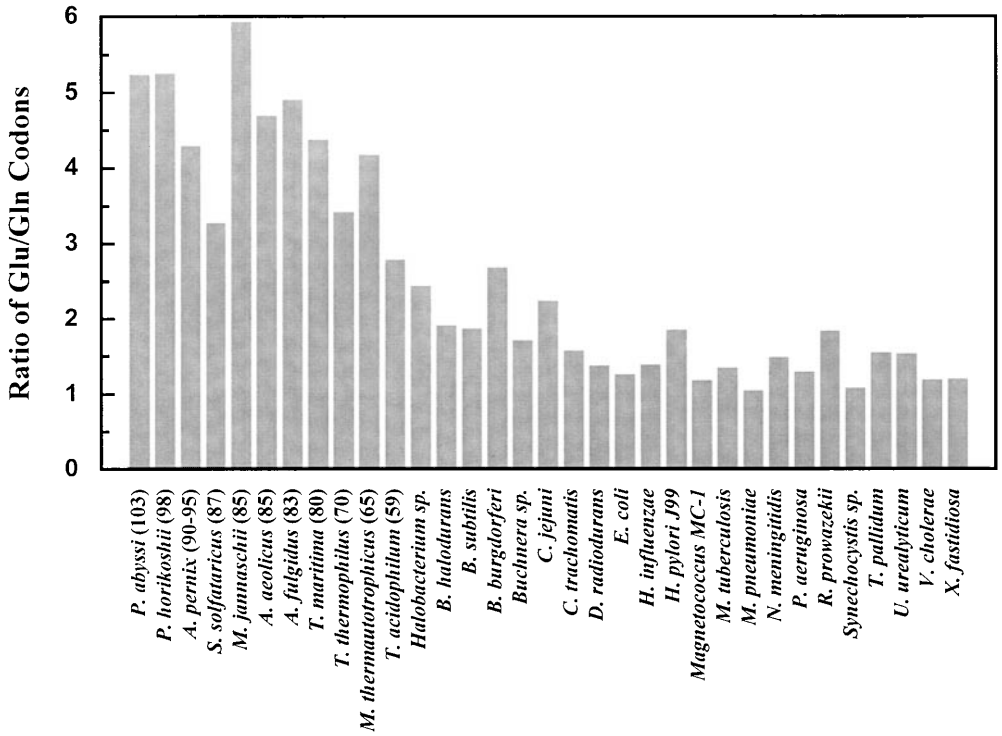

Fig. 4. Comparison of Glu/Gln codon ratios in thermophiles and mesophiles. The ratio of total glutamine to total glutamate codons (Glu/Gln) was determined for 32 completely sequenced genomes, including 10 thermophiles and 22 mesophiles. The Glu/Gln ratio calculated from existing proteins in the partially sequenced *Thermus thermophilus* genome is also included as a comparison. *P. abyssi, Pyrococcus abyssi; P. horikoshii, Pyrococcus horikoshii; A. pernix, Aeropyrum pernix; S. solfataricus, Sulfolobus solfataricus; M. jannaschii, Methanococcus jannaschii; A. aeolicus, Aquifex aeolicus; A. fulgidus, Archaeoglobus fulgidus; T. maritima, Thermotoga maritima; T. thermophilus, Thermus thermophilus; M. thermautotrophicus, Methanothermobacter thermautotrophicus; T. acidophilum, Thermoplasma acidophilum; B. halodurans, Bacillus halodurans; B. subtilis, Bacillus subtilis; B. burgdorferi, Borrelia burgdorferi; C. jejuni, Campylobacter jejuni; C. trachomatis, Chlamydia trachomatis; D. radi odurans, Deinococcus radiodurans; E. coli, Escherichia coli; H. influenzae, Haemophilus influenzae; H. pylori, Helicobacter pylori; M. tuberculosis, Mycobacterium tuberculosis; M. pneumoniae, Mycoplasma pneumoniae; N. meningitidis, Neisseria meningitidis; P. aeruginosa, Pseudomonas aeruginosa; R. prowazekii, Rickettsia prowazekii; T. pallidum, Treponema pallidum; U. urealyticum, Ureaplasma urealyticum; V. cholerae, Vibrio cholerae; X. fastidiosa, Xylella fastidiosa.* [Data from Codon Usage Database (http://www.kazusa.or.jp/codon/) and TIGR (http://www.tigr.org/)]

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