Thermus thermophilus: A link in evolution of the tRNA-dependent amino acid amidation pathways

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ABSTRACT *Thermus thermophilus* possesses an aspartyl-tRNA synthetase (AspRS2) able to aspartylate efficiently tRNA^{Asp} and tRNA^{Asn}. Aspartate mischarged on tRNA^{Asn} then is converted into asparagine by an ω amidase that differs structurally from all known asparagine synthetases. However, aspartate is not misincorporated into proteins because the binding capacity of aminoacylated tRNA^{Asn} to elongation factor Tu is only conferred by conversion of aspartate into asparagine. *T. thermophilus* additionally contains a second aspartyl-tRNA synthetase (AspRS1) able to aspartylate tRNA^{Asp} and an asparaginyl-tRNA synthetase able to charge tRNA^{Asn} with free asparagine, although the organism does not contain a tRNA-independent asparagine synthetase. In contrast to the duplicated pathway of tRNA asparaginylation, tRNA glutaminylation occurs in the thermophile via the usual pathway by using glutaminyl-tRNA synthetase and free glutamine synthesized by glutamine synthetase that is unique. *T. thermophilus* is able to ensure tRNA aminoacylation by alternative routes involving either the direct pathway or by conversion of amino acid mischarged on tRNA. These findings shed light on the interrelation between the tRNA-dependent and tRNA-independent pathways of amino acid amidation and on the processes involved in fidelity of the aminoacylation systems.

Aminoacylation of tRNA is one of the biochemical processes exhibiting the highest accuracy. In most organisms, 20 aminoacyl-tRNA synthetases (aaRS), each with a particular specificity, provide the various aminoacyl-tRNA (aa-tRNA) involved in protein synthesis. With only a few exceptions, organisms encode a single aaRS for each amino acid, and each aaRS has strict charging specificity. Occasionally, two genes encode aaRS of the same specificity, and, in certain organisms, a given aaRS aminoacylates two tRNA of distinct specificity.

Duplication of synthetases first was exemplified with the discovery of two lysyl-tRNA synthetases (LysRS) encoded by distinct genes in *Escherichia coli* (1–3). Later, two threonyl- and two tyrosyl-tRNA synthetases (ThrRS, TyrRS), also of distinct genetic origins, were characterized in *Bacillus subtilis* (4–5), and two isoleucyl-tRNA synthetases (IleRS) were found in *Staphylococcus aureus* (6). The particular character of these duplications is manifested by their absence of phylogenic conservation, and the physiological implication of these duplications to date is not understood clearly. The two ThrRS and TyrRS from *B. subtilis* appear under different growth conditions. Each ThrRS is sufficient for normal cell growth and sporulation; however, only the *thrS* gene is expressed during vegetative growth whereas the *thrZ* gene is induced by a decreased level of charged tRNA promoted by Thr starvation (7). Similar interrelations were described for the two TyrRS from *B. subtilis* (8, 9). In contrast, one of the two LysRS from *E. coli*, the *lysU* product, is induced by heat shock,

anaerobiosis, or low pH and is involved in adaptation of the organism to stress conditions whereas the *lysS* product is expressed constitutively (10–12). Finally, the IleRS from *S. aureus* encoded by a plasmidic gene differs from the chromosomal one by conferring resistance to the Ile analogue mupirocin (6).

The second exception to the rule of the unicity of the tRNA aminoacylation systems occurs in organisms deprived of a particular aaRS. In this case, the tRNA homologous to the missing aaRS first is mischarged by a heterologous aaRS, and the amino acid then is converted to match the specificity of tRNA. The biological significance of this process became evident when it was shown that it constitutes a pathway for synthesis of the amino acid. Various microorganisms, including the Gram+ bacteria, the Gram− bacterium *Rhizobium meliloti*, cyanobacteria, and archaebacteria, and also mitochondria and chloroplasts lack glutaminyl-tRNA synthetase (GlnRS) (13–15). tRNA glutaminylation occurs by ω amidation of glutamic acid mischarged on tRNA^{Gln} by glutamyl-tRNA synthetase (GluRS) (16, 17). A tRNA-dependent conversion of Asp into Asn resembling that of tRNA glutaminylation was reported recently in *Haloferax volcanii* (18); further, the absence of ORFs encoding GlnRS and asparaginyl-tRNA synthetase (AsnRS) in the genomes from archeon *Methanococcus jannaschii* (19) and from eubacterium *Helicobacter pylori* (20) suggests tRNA-dependent pathways of Gln and Asn formation in these bacteria. tRNA-dependent Gln synthesis was discovered in the late 1960s and is well documented whereas the partners involved in tRNA-dependent Asn synthesis are until now not characterized and the catalytic process remains unknown.

The existence of two genetically distinct aspartyl-tRNA synthetases (AspRS) in the thermophilic eubacterium *Thermus thermophilus* (21) that activate Asp and charge tRNA^{Asp} but differ structurally has been reported recently (M_r of 68 and 51 for AspRS1 and AspRS2, respectively). To better understand the functional implication of this duplication, we investigated the involvement of these AspRS in tRNA charging. We demonstrate here that AspRS2 aminoacylates tRNA^{Asn} and that *T. thermophilus* promotes tRNA asparaginylation via a pathway resembling the indirect route of tRNA glutaminylation reported earlier for various bacteria and organelles. The Asp-tRNA^{Asn} amidotransferase (Asp-AdT) that promotes the conversion of Asp-tRNA^{Asn} into Asn-tRNA^{Asn} is described. Because *T. thermophilus* also contains an AsnRS capable of catalyzing direct tRNA asparaginylation, this eubacterium is able to aminoacylate a single tRNA by both direct and indirect pathways. In contrast, tRNA glutaminylation is promoted by a unique pathway involving GlnRS. Conservation of two pathways for tRNA asparaginylation in this thermophile is rationalized, and the results are discussed in the context of the phylogenetic interrelation between *T. thermophilus*, eubacteria, and archaebacteria.

Biochemistry: Becker and Kern

Proc. Natl. Acad. Sci. USA 95 (1998) 12833

EXPERIMENTAL PROCEDURES

General. *E. coli* and *T. thermophilus* DNA were from K12 and HB8 strains, respectively. Oligonucleotides were synthesized by Nucleic Acids Products Supply (Göttingen, Germany). Rabbit antibodies directed against *T. thermophilus* AspRS1 and AspRS2 were obtained as described (21).

Enzyme Purifications and Analysis. All steps were conducted at 4°C, and the buffers contained 5 mM 2-mercaptoethanol and 0.1 mM each of EDTA and diisopropylfluorophosphate. Asp-AdT was isolated from 250 g of cells disrupted as described (21). The $105,000 \times g$ supernatant was adsorbed on DEAE-cellulose, and the proteins were eluted with a potassium phosphate gradient from 20 mM (pH 7.2) to 250 mM (pH 6.5). Because the enzyme was not retained on phosphocellulose, the dialyzed fractions were adsorbed on hydroxyapatite, and the proteins were eluted with a potassium phosphate gradient from 10 to 200 mM (pH 6.8). The proteins from active fractions then were subjected to three successive chromatographies in 50 mM Tris·HCl buffer (pH 7.5): (*i*) on Heparine-Ultrogel and elution with a gradient from 0 to 500 mM KCl; (*ii*) on Cibacron Blue-Sepharose and elution with a gradient from 0 to 1 M KCl; and (*iii*) on mono Q-Sepharose and elution with a gradient from 0 to 500 mM KCl. Pure enzyme (0.4 mg) was obtained by a final 12% PAGE.

GlnRS was purified from 115 g of cells. The $105,000 \times g$ supernatant was submitted to DEAE-cellulose chromatography as above. The proteins from active fractions then were fractionated on phosphocellulose by elution with a gradient from 0 to 500 mM KCl and on hydroxyapatite by elution with a gradient from 20 to 200 mM potassium phosphate (pH 6.8). Pure enzyme (6 mg) was obtained by a final chromatography on Heparine-Ultrogel after elution with a gradient from 0 to 500 mM KCl.

Glutamine synthetase (GlnS) was purified from the same extract as Asp-AdT. The enzyme was retained on DEAE- and phosphocellulose and was eluted respectively at 230 mM potassium phosphate and 830 mM KCl. Pure enzyme (9 mg) was obtained by a final chromatography on Heparine-Ultrogel after elution with a gradient from 0 to 500 mM KCl. S100 protein extracts from *T. thermophilus*, *E. coli*, and yeast were obtained by chromatography of the $105,000 \times g$ supernatant on DEAE-cellulose (21). Molecular masses and subunit structures were determined by gel-filtration and by native and SDS/PAGE as described (21).

Enzymatic Characterizations. *tRNA aminoacylation.* The standard reaction mixture contained 100 mM Na-Hepes (pH 7.2), 30 mM KCl, 2 mM ATP, 10 mM MgCl$_2$, 0.05 mM L-[^{14}C]Asp, Gln, or Glu or [^{3}H]Asn, 1–4 mg/ml unfractionated or enriched *T. thermophilus* tRNA, and 5–300 μg/ml of protein. For K_m determinations, 8 or 5 μM L-[^{3}H]Asp or [^{3}H]Asn were present, as were pure tRNA^{Asp} or enriched tRNA^{Asn} from *T. thermophilus* at concentrations in the K_m range and adequate amounts of enzymes. The k_{cat} values were determined with saturating substrates concentrations. The kinetics of aa-tRNA formation were determined at 70°C as described (21).

tRNA-dependent amino acid amidation. The Asp amidation mixture of 50 μl contained 100 mM Na-Hepes (pH 7.2), 30 mM KCl, 10 mM ATP, 12 mM MgCl$_2$, 2 mM NH$_4$Cl, L-Asn or Gln, 0.05 nmol [^{14}C]Asp-tRNA^{Asn} obtained by aspartylation of enriched tRNA^{Asn} with AspRS2 followed by phenol extraction in 0.3 M sodium acetate (pH 4.5), and either 20 μg of a dialyzed S100 protein extract or 0.2 μg pure Asp-AdT. The Glu amidation mixture deprived of aa-tRNA contained 25 μM L-[^{14}C]Glu, 0.1 nmol enriched tRNA^{Gln}, and 0.2 μg *T. thermophilus* GluRS. After 2–4 h of incubation at 70°C, the reaction was stopped by phenol-chloroform extraction; the aa-tRNA was deacylated by 30 min incubation at 70°C in the presence of 50 mM KOH before neutralization with HCl. The released amino acids were concentrated in a Speed Vac, were fractionated by TLC on cellulose plates (20×20 cm) extended by a 3 MM Whatman paper sheet of equal size with isopro-

panol/formic acid/water (80/20/4, vol/vol/vol) solvent, and were identified by scanning with a Fuji Bioimager.

Contributions of AspRS2 and AsnRS to tRNA asparaginylation were determined at 70°C in standard aminoacylation and amidation mixtures containing either 50 μM L-[^{14}C]Asp without or with 50 μM unlabeled Asn and 2 mM Gln or 50 μM L-[^{3}H]Asn without or with 50 μM unlabeled Asp; the reactions were conducted with 32 μg of proteins from S100 extract and 100 μg of unfractionated *T. thermophilus* tRNA. The kinetics of formation of [^{3}H]Asn-tRNA^{Asn} by AsnRS and of [^{14}C]Asn-tRNA^{Asn} by AspRS2 and Asp-AdT were determined as described above.

tRNA-independent amino acid amidation. The reactions were conducted in the amidation mixture containing 25 μM L-[^{14}C]Asp or [^{14}C]Glu and 10 μg of crude extract or S100 protein extract or 0.5 μg of pure *T. thermophilus* GlnS for 2 h at 70°C. The amino acids were analyzed as described above.

Isolation and Sequencing of tRNA^{Asn} from *T. thermophilus*. Pure tRNA^{Asn} (40 nmol/mg) was obtained by two-dimensional PAGE under denaturing conditions starting from a tRNA fraction enriched by BD-cellulose chromatography and was sequenced by using conventional methods (22).

Measurements of the Binding Capacity of Asp-tRNA^{Asn} and Asn-tRNA^{Asn} to Elongation Factor Tu (EF-Tu). All buffers contained 10 mM MgCl$_2$ and 5 mM 2-mercaptoethanol. EF-Tu (8.3 mg) tagged to six His residues at the Ct (23) was incubated for 10 min at room temperature with 2 ml of agarose beads substituted by Ni^{++} nitriloacetic acid in 50 mM Tris·HCl buffer (pH 7.5) containing 50 mM each of NH$_4$Cl and KCl and 0.1 mM GTP. EF-Tu was activated by 30 min incubation at 37°C with 3 ml of the buffer containing 5 mM GTP, 5 mM phosphoenolpyruvate, and 0.3 mg pyruvate kinase. Enriched tRNA (200 nmol) containing $\approx$4 nmol of [^{14}C]aa-tRNA was adsorbed on the matrix in a 2-ml column before incubation for 30 min at 37°C. tRNA and aa-tRNA not retained by EF-Tu were eluted with the incubation buffer containing 0.1 M NaCl and 0.1 mM GTP. Bound aa-tRNA was eluted with 0.1 M Na borate buffer (pH 7.5) containing 1 M NaCl and 0.1 mM GTP. The amino acid acylating the tRNA was identified by TLC after alkaline hydrolysis of the aa-tRNA isolated by phenol-chloroform extraction and ethanol precipitation. Protection against alkaline hydrolysis of aa-tRNA by EF-Tu was analyzed at 37°C by incubating 26 pmol of [^{14}C]Asp-tRNA^{Asn} or Asn-tRNA^{Asn} with 765 pmol of activated EF-Tu in 50 mM Tris·HCl buffer (pH 8.0) and 50 mM KCl. Deacylations were followed as described (21).

Analysis of the Presence of Asparagine synthetase (AsnS) Genes in Genomic DNA from *T. thermophilus* and *E. coli*. The analysis was performed by PCR amplification (21) of genomic sequences encoding strictly conserved protein sequences in the known AsnS A and B of various phylae including archaea (24–27): 110HSVYVDQWDWERV122 and 236AFELSSMGI-RVD247 in AsnS A and 230GVLLSGGLDSS240 and 314HIE-TYDVTTIRA325 in AsnS B (the numbering corresponds to the sequences in *E. coli* enzymes). Amplification was conducted with 0.1 μg of genomic DNA and 400 pmol of sense and antisense primers corresponding to the genomic sequences from *E. coli* (sense and antisense primers for AsnS A: 5′-CACTCGGTCTA-TGTTGACCAGTGGGACTGGGAACGCGTA-3′ and 5′-AT-CTACACGGATCCCCATGGAGGAAAGCTCAAACGC-3′; and for AsnS B: 5′-GGTGTG CTGCTTTCTGGTGGTCTGG-ATTCCTCA-3′ and 5′-AGCGCGAATAGTGGTCACATCA-TAAGTTTCGATGTG-3′) or was synthesized by taking into account the codon usages in *T. thermophilus* and *E. coli* (degenerated sense and antisense primers for AsnS A: 5′-CACTCG/CGTG/CTACGTG/CGACCAGTGGGACTGGGAGCG-3′ and 5′GTCG/CACG/CCGGATG/CCCCATG/CGAG/CG-AG/CAGCTCA/GAAG/CGC-3′; and for AsnS B: 5′-GGG/T/CGTG/CCTG/CCTG/T/CTCG/T/CGGG/T/CGGG/T/CCTG/CGAT/CTCG/CTC-3′ and 5′-A/C/GGCG/CCGA/GATA/C/GGTG/CGTG/CACA/GTCA/GTAA/C/GGTT/CTCGATGTG-3′). The primers frame DNA fragments com-

Table 1. Kinetic constants of aminoacylation of tRNA^{Asp} and tRNA^{Asn} by AspRS$_1$, AspRS$_2$, and AsnRS from *T. thermophilus* at 70°C. L = (k_{cat}/K_m) of cognate tRNA/(k_{cat}/K_m) of noncognate tRNA

Parameters	AspRS1 tRNA		AspRS2 tRNA		AsnRS tRNA	
	Asp	Asn	Asp	Asn	Asp	Asn
K_m (μM)	0.03	3.4	0.073	0.063	23	0.048
k_{cat} (s^{-1})	2.7	0.12	0.24	0.092	0.024	0.4
k_{cat}/K_m (s $\times$ μM)$^{-1}$	90	0.04	3.3	1.5	0.001	8.3
L	1	2250	1	2	8300	1

prising, respectively, 408 and 285 nucleotides in AsnS A and B genes from *E. coli*.

RESULTS

Evidence for a Dual Specificity for tRNA Charging of AspRS2 from *T. thermophilus*. Analysis of the extents of aminoacylation of unfractionated tRNA from *T. thermophilus* by AspRS1 and AspRS2 indicates higher plateaus for AspRS2 than for AspRS1 (0.66 and 0.42 nmol/mg, respectively). Because *T. thermophilus* contains only one tRNA^{Asp} species well charged by AspRS1, this result suggests that AspRS2 acylates another tRNA in addition to tRNA^{Asp}. This tRNA, purified by two-dimensional PAGE, was charged by Asp and Asn when incubated in aminoacylation mixtures containing AspRS2 and AsnRS, respectively. Sequencing revealed the presence of Asn anticodon (GUU) and N^6-threonyl-carbamoyladenosine at position 37, found in all tRNA^{Asn} until now sequenced. *T. thermophilus* tRNA^{Asn} presents 76% identity with *E. coli* tRNA^{Asn}. This establishes aspartylation of tRNA^{Asn} by AspRS2. Table 1 shows that AspRS2 aspartylates pure tRNA^{Asn} as efficiently as tRNA^{Asp} whereas AspRS1 aspartylates it three orders of magnitude less efficiently. Thus, AspRS1 possesses a restricted specificity, and AspRS2 possesses a dual specificity in tRNA charging.

Evidence for Conversion of Asp Mischarged on tRNA^{Asn} into Asn in *T. thermophilus*. To understand the functional significance of the tRNA mischarging promoted by AspRS2, we investigated the possible conversion of Asp bound to tRNA^{Asn} into Asn. tRNA^{Asn} enriched by two successive BD-cellulose chromatographies, and depleted of all traces of contaminating tRNA^{Asp}, was charged with Asp by AspRS2. Asp-tRNA^{Asn} isolated by phenol extraction then was incubated with a S100 protein extract from *T. thermophilus*, ATP, and either ammonia, Gln, or Asn as amide group donors. Analysis by TLC of the amino acid released by deacylation of the aa-tRNA revealed formation of Asn (Fig. 1*A*). Thus, mischarging of tRNA^{Asn} by AspRS2 is corrected by conversion of Asp into Asn. Incubation of Asp under similar condi-

tions but in the absence of tRNA does not give rise to formation of Asn whereas incubation with protein extracts from *E. coli* or yeast promote conversion (Fig. 1*B*). This reveals that, in *T. thermophilus*, Asn is formed by tRNA-dependent ω amidation of Asp and, in *E. coli* and yeast, is formed by ω amidation of free Asp. Implication of AspRS2 but not of AspRS1 in the tRNA-dependent conversion was established firmly by analysis of the effects of anti-AspRS1 and anti-AspRS2 antibodies. When the reaction was performed in a mixture containing a crude protein extract, unfractionated tRNA, ATP, and ammonia or Gln to allow tRNA aspartylation, either by AspRS2 or by AspRS1 before Asp amidation, anti-AspRS2 antibodies totally abolished the conversion whereas anti-AspRS1 antibodies were without effect (data not shown).

Isolation and Structural Properties of Asp-AdT from *T. thermophilus*. Conversion of tRNA-bound Asp into Asn by the crude extract from *T. thermophilus* prompted us to search for the Asp-AdT. Pure enzyme was obtained by a seven-step purification procedure. Gel-filtration and native PAGE revealed a M_r of 110 (Fig. 2*A*) whereas SDS/PAGE showed two polypeptide chains, α and β, with M_r of 51 and 57, respectively, in a 1/1 stoichiometry (Fig. 2*B*). The heterodimeric αβ structure of this enzyme differs from the homodimeric α$_2$ one found for AsnS of various origins. Analysis by Edman's degradation of the Nt ends revealed a starting Met in both chains excluding a proteolytic origin of the heterodimeric structure. No significant homologies were found between the 40 first residues of Asp-AdT polypeptide chains and AsnS. However, the Nt sequence of the α chain shows 70% identity with the Nt end of the B subunit of the Glu-tRNA^{Gln} amidotransferase (Glu-AdT) from *B. subtilis* (28) and 47–60% identity with the Nt sequences of proteins HP0658 from *H. pylori*, MJ0160 from *M. jannaschii*, MG100 from *Mycoplasma genitalium* and its homologue from *Mycoplasma pneumoniae*, and PET112 from *Saccharomyces cerevisiae*. These proteins are also unique homologues of Glu-AdT (28), suggesting the presence of the same subunit in the two enzymes. In contrast, no significant identity was found between the Nt sequence of the β chain of Asp-AdT and the A and C chains of Glu-AdT and other known
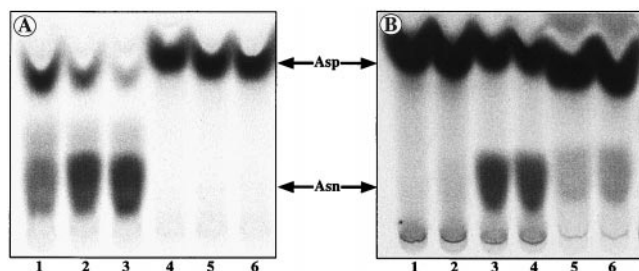

FIG. 1. Conversion of Asp into Asn in *T. thermophilus*, *E. coli*, and yeast. (*A*) tRNA-dependent and -independent conversions in *T. thermophilus*. The reactions were conducted with a S100 protein extract in the presence (lanes 1–3) or absence (lanes 4–6) of tRNA^{Asn} and 2 mM NH$_4$Cl (lanes 1 and 4), or Asn (lanes 2 and 5), or Gln (lanes 3 and 6). (*B*) tRNA-independent conversion in *T. thermophilus*, *E. coli*, and yeast. The reactions were conducted with a S100 protein extract from *T. thermophilus* (lanes 1 and 2), *E. coli* (lanes 3 and 4), or yeast (lanes 5 and 6), at 70°C (lanes 1 and 2) or 37°C (lanes 3 to 6), in the presence of 2 mM NH$_4$Cl (lanes 1, 3, and 5) or Gln (lanes 2, 4, and 6).
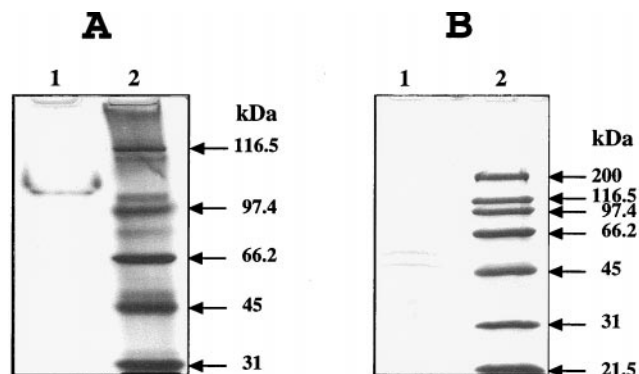

FIG. 2. Analysis of the Asp-AdT from *T. thermophilus*. (*A*) Native PAGE in a 10–20% gel. (*B*) Denaturing PAGE in a 10% gel containing 0.1% SDS. Lanes: 1, 0.8 μg (*A*) and 0.6 μg (*B*) protein were analyzed; 2, M_r markers.

Biochemistry: Becker and Kern

Proc. Natl. Acad. Sci. USA 95 (1998)   12835

proteins, indicating high divergences in these subunits of Asp- and Glu-AdT. It cannot be excluded that, at least in archaebacteria, a same transamidase shares both Asp- and Glu-AdT activities.

The Two Pathways of tRNA Asparaginylation in *T. thermophilus.* In addition to the indirect route, tRNA^{Asn} also can be asparaginylated directly in *T. thermophilus* with Asn by AsnRS (29). AsnRS acylates the homologous tRNA^{Asn} 6-fold more efficiently than AspRS2 and the heterologous tRNA^{Asp} four orders of magnitude less efficiently (Table 1). Like AspRS1, but in contrast to AspRS2, AsnRS has restricted tRNA charging specificity. However, the functionality of the direct route of asparaginylation depends on the ability of the organism to synthesize free Asn. To our surprise, all biochemical and genetic attempts to characterize AsnS were unsuccessful. First, as reported above, the crude protein extract from *T. thermophilus* was unable to convert Asp into Asn when tRNA was absent (Fig. 1*B*). Second, the genes encoding AsnS A or B, which are well characterized in various organisms, could not be detected in the genome of *T. thermophilus* by PCR amplification of sequences encoding highly conserved peptides (Fig. 3). *T. thermophilus* is unable to convert free Asp into Asn by ω amidation. As a consequence, the sources of Asn used by AsnRS for tRNA asparaginylation are the cell-import and/or the protein degradation.

Binding Properties of Asp-tRNA^{Asn} and Asn-tRNA^{Asn} on EF-Tu. The aa-tRNA released from aaRS are directed by EF-Tu to the ribosomes, where codon-anticodon interactions specify incorporation of amino acids into polypeptide chains, and it generally is believed that EF-Tu binds aa-tRNA without specificity (30). Thus, recognition of Asp-tRNA^{Asn} by EF-Tu should result in misincorporation of Asp into polypeptide chains. We analyzed the binding capacity of Asp-tRNA^{Asn} and Asn-tRNA^{Asn} on EF-Tu. Enriched tRNA^{Asn} aspartylated by AspRS2 was not retained on EF-Tu-substituted Sepharose (Fig. 4*A*). However, when Asp-AdT, ATP, and amide group donors were added to the aspartylation mixture, two species of aa-tRNA appeared differing in their binding capacity to EF-Tu. The first one displayed the same behavior as Asp-tRNA^{Asn} and had no affinity for EF-Tu, and the second one was retained by EF-Tu and was eluted at high salt concentration like Asn-tRNA^{Asn} (Fig. 4 *B* and *C*). Analysis by TLC of the amino acid released by alkaline hydrolysis of the aa-tRNA showed Asp in the species not retained by EF-Tu and Asn in the species retained by EF-Tu (Fig. 4 *A–C*). Finally, acquisition of the binding capacity of Asp-tRNA^{Asn} to EF-Tu by conversion of Asp into Asn also was revealed by comparing the protection induced by EF-Tu against alkaline hydrolysis of the ester bond before and after amidation of Asp. Asp-tRNA^{Asn} was not protected by EF-Tu against hydrolysis (half-life 20 min at pH
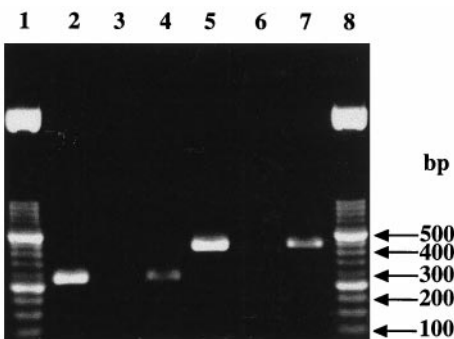
FIG. 3.   Detection of AsnS A and B genes in genomic DNA from *E. coli* and *T. thermophilus*. Lanes: 2–4, amplification of sequences of AsnS A genes in DNA from *E. coli* (lanes 2 and 4) and *T. thermophilus* (lane 3); 2, amplification with nondegenerated primers; 3 and 4, amplification with degenerated primers; 5–7, amplification of sequences of AsnS B genes in DNA from *E. coli* (lanes 5 and 7) and *T. thermophilus* (lane 6); 5, amplification with nondegenerated primers; 6 and 7, with degenerated primers; 1 and 8, ladder.
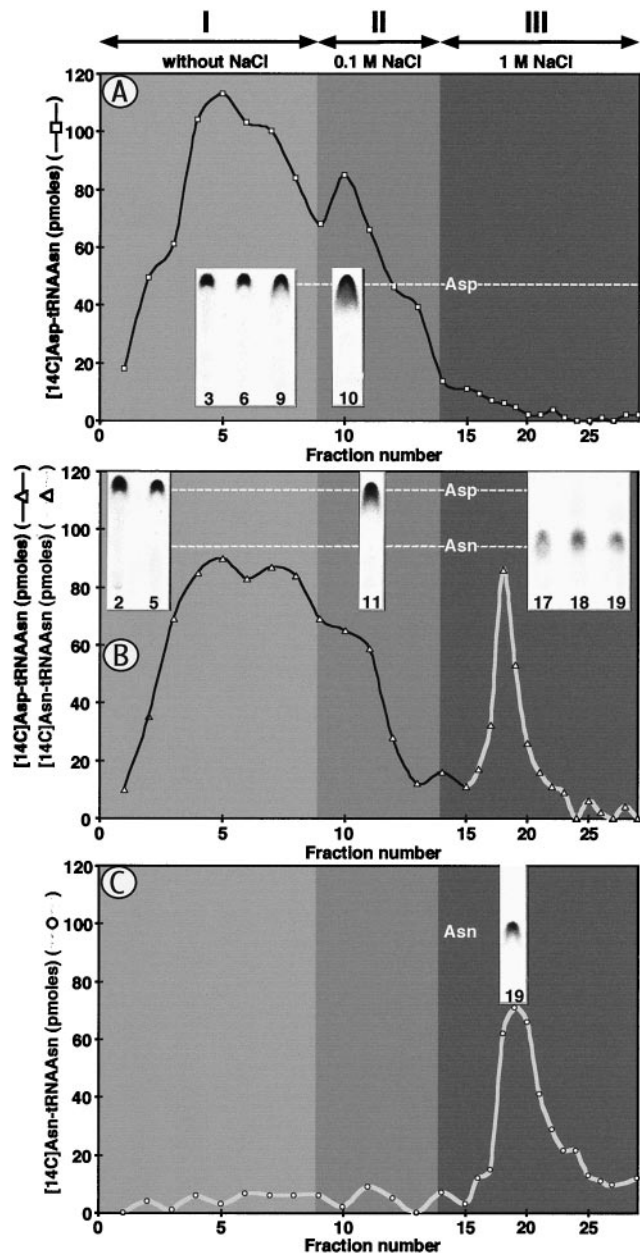
FIG. 4.   Interaction of Asp-tRNA^{Asn} and Asn-tRNA^{Asn} with EF-Tu. Aminoacylated were 1.5 nmol tRNA^{Asn} with [^{14}C]Asp and AspRS2 (*A* and *B*) or with [^{14}C]Asn and AsnRS (*C*); the [^{14}C]aa-tRNA then were isolated and adsorbed on activated EF-Tu substituted Sepharose (*A* and *C*) or were incubated for 2 h in the amidation mixture containing Gln before adsorption on the matrix (*B*). Adsorption, washing, and elution with buffers I, II, and III were conducted as described in *Experimental Procedures*. The insets show the position after TLC of the amino acid released by alkaline hydrolysis of the [^{14}C]aa-tRNA.

8.0 and 37°C, in the absence and presence of EF-Tu) whereas conversion of Asp into Asn induced protection by increasing the half-life of aa-tRNA by one order of magnitude. After conversion, EF-Tu stabilized the aa-tRNA as Asn-tRNA^{Asn} (data not shown).
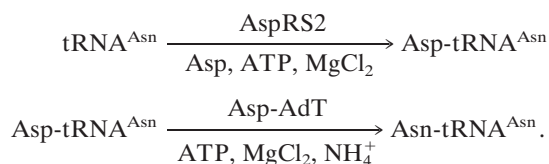
The Pathway of tRNA Glutaminylation in *T. thermophilus.* tRNA glutaminylation is achieved in various organisms by either the direct or the indirect pathway, resembling those of asparaginylation in *T. thermophilus*. We examined the pathway of tRNA glutaminylation in this organism and found that Gln-tRNA^{Gln} is formed by the direct pathway involving GlnRS and free Gln. The GlnRS we purified consists of a monomer of M_r 66 whose Nt

sequence of 41 residues, including the HIGH signature sequence of class 1 aaRS, shows 68% identity with GlnRS from *E. coli* and *Haemophilus influenzae*. In contrast, alignment with GlnRS from *Lupinus luteus*, *Homo sapiens, S. cerevisiae*, and *Dictyostelium discoideum* upstream of the Nt extension, which characterizes eukaryotic aaRS, shows only 36–46% identity. Further, GlnS, which catalyzes conversion of free Glu into Gln in the presence of ATP and either Gln or ammonia as amide group donors, was purified to homogeneity. Like the *E. coli* enzyme, it consists of a dodecamer with identical polypeptide chains of M_r 45 whose Nt sequence of 55 residues presents 41 and 49% identity with GlnS chains from *E. coli* and *Thermotoga maritima*, respectively. Finally, GluRS from *T. thermophilus* is deprived of charging capacity of tRNA^{Gln}, and no Glu-AdT activity could be detected. Thus, in contrast to tRNA asparaginylation, *T. thermophilus* does not use the indirect pathway for tRNA glutaminylation. However, this conclusion does not prove lack of Glu-AdT because discrimination by GluRS of tRNA^{Glu} and tRNA^{Gln} prevents formation of the Glu-tRNA^{Gln} substrate.

Relative Contributions of the Two AspRS and of the Two Pathways of Asparaginylation in tRNA Aminoacylation in *T. thermophilus*. The presence in *T. thermophilus* of two AspRS and of two pathways for tRNA asparaginylation prompted us to examine their contribution in the overall tRNA charging. When unfractionatad tRNA is aspartylated with a crude protein extract, anti-AspRS2 antibodies decrease the rate of aspartylation by 15%, and anti-AspRS1 antibodies decrease the rate of aspartylation by 85% (21). Thus, under conditions approaching physiological conditions, AspRS1 does 85% of tRNA aspartylation whereas the contribution of AspRS2 is minor. We estimated the contribution of AsnRS to the overall tRNA asparaginylation to a similar extent. Indeed, (*i*) the rate of tRNA asparaginylation of unfractionated tRNA with [^{3}H]Asn by a crude protein extract decreases by $\approx$20% when tRNA^{Asn} is charged in parallel with unlabeled Asp by AspRS2, and (*ii*) the rate of formation of [^{14}C]Asn-tRNA^{Asn} promoted by AspRS2 and Asp-AdT in a crude extract decreases drastically when tRNA^{Asn} is charged in parallel with unlabeled Asn by AsnRS. Thus, under standard growth conditions, contribution of AspRS2 to tRNA asparaginylation is minor. However, under Asn starvation, its contribution may become essential because the organism is incapable of synthesizing free Asn.

DISCUSSION

Distribution of the Two-Step Pathway of tRNA Asparaginylation and Glutaminylation in the Phylae. In *T. thermophilus*, synthetase duplication clearly relates to a peculiar function in tRNA aminoacylation. AspRS1, the canonical AspRS, ensures tRNA aspartylation whereas the main role of AspRS2 is charging of tRNA^{Asn} by Asp, which is then converted into Asn by Asp-AdT:

$$\text{tRNA}^{Asn} \xrightarrow[\text{Asp, ATP, MgCl}_2]{\text{AspRS2}} \text{Asp-tRNA}^{Asn}$$

$$\text{Asp-tRNA}^{Asn} \xrightarrow[\text{ATP, MgCl}_2,\ \text{NH}_4^+]{\text{Asp-AdT}} \text{Asn-tRNA}^{Asn}.$$

Comparison of AspRS polypeptide chains show that *T. thermophilus* AspRS2 structurally is related more to archaebacterial AspRS than to eubacterial and eukaryotic AspRS (21). The polypeptide chains of AspRS2 from *T. thermophilus* and archaebacterial AspRS are significantly shorter than those from eubacterial and eukaryotic AspRS (422–438 and 500–600 residues, respectively). Of interest, archaebacteria also use the indirect pathway of tRNA asparaginylation, as revealed by the tRNA-dependent conversion of Asp into Asn in *H. volcanii* (18) and the lack of gene encoding AsnRS in *M. jannaschii* (19). Thus, the small archaebacterial AspRS exhibit dual specificity for tRNA

charging whereas the large eubacterial and eukaryotic AspRS possess restricted specificity. Indirect asparaginylation probably also occurs in the mesophilic eubacterium *H. pylori*, which has no AsnRS (20), and in *Deinococcus radiodurans* [accompanying paper (ref. 31)]. The indirect pathway of asparaginylation resembles that of glutaminylation in which Glu mischarged on tRNA^{Gln} by GluRS is converted into Gln by Glu-AdT (13–17, 28). However, the two pathways are not distributed uniformly in the various phylae. Archaebacteria deprived of GlnRS and AsnRS use the indirect pathways for Gln-tRNA^{Gln} and Asn-tRNA^{Asn} formation. In contrast, many eubacteria are deprived of GlnRS and use the indirect pathway for glutaminylation but catalyze asparaginylation with AsnRS. Finally, eukaryotes use the direct pathways for glutaminylation and asparaginylation except in the organelles devoid of GlnRS, where glutaminylation occurs via the two-step pathway. Surprisingly, the distribution of GlnS and AsnS does not coincide with that of GlnRS and AsnRS in the phylae. Except for *T. thermophilus* deprived of AsnS, all organisms we analyzed contain GlnS and AsnS, even when amino acids can be formed by the tRNA-dependent pathway and regardless the presence or absence of GlnRS and AsnRS. Thus, no interrelation emerges between the nature of the pathway, either direct or indirect, used by the organisms for Gln-tRNA^{Gln} and Asn-tRN^{Asn} formation and their ability to synthesize free Gln and Asn. The properties of the asparaginylation system of *D. radiodurans* (31) and *T. thermophilus* show that coexistence of direct and indirect pathways of asparaginylation is related to the absence of AsnS.

Discrimination of Asn-tRNA^{Asn} Against Asp-tRNA^{Asn} by EF-Tu. The fact that tRNA^{Asn} is charged efficiently with Asp is in keeping with the rule that tRNA mischarged by the amino acid homologous to the synthetase is not corrected (32); corrections occur only when tRNA is charged with noncognate amino acids. The lack of binding of Asp-tRNA^{Asn} to EF-Tu when Asp is not amidated prevents misincorporation of Asp into polypeptide chains. A similar behavior has been reported for Glu-tRNA^{Gln} formed by GluRS in *Euglena gracilis* chloroplasts before amidation of Glu (33). It is believed currently that EF-Tu binds aa-tRNA without specificity and regardless of the nature of the amino acid acylating tRNA, except Sec-tRNA^{Sec}, which binds to the SEL B factor but not to EF-Tu (34). However, we show here that the EF-Tu is able to discriminate the aspartylated tRNA^{Asn} from that which is asparaginylated. Thus, EF-Tu has refined its specificity to the cellular context, in particular when mischarging is of physiological importance, to improve the accuracy in protein synthesis. Recognition of aa-tRNA by EF-Tu involves the amino acid moiety and the acceptor stem (35). The lack of binding when tRNA^{Asn} is mischarged with Asp may result from an unfavorable presentation of the amino acid by the noncognate tRNA because of the negatively charged β carboxyl group of Asp and structural peculiarities of the acceptor arm. The antideterminants of Asp-tRNA^{Asn} and probably also of Glu-tRNA^{Gln} for binding on EF-Tu differ from those of Sec-tRNA^{Sec}, which involve solely the length of the acceptor arm (34).

Evolution of the Pathways of tRNA Asparaginylation and Glutaminylation. AspRS1 and AsnRS charge the noncognate tRNA^{Asn} and tRNA^{Asp} only three to four orders of magnitude less efficiently than the cognate ones (Table 1). This poor discrimination contrasts with the significantly higher selection reported for homologous systems (36) and supports arguments for the recent introduction of AspRS1 and AsnRS in *T. thermophilus*. Thus, the archaebacterial type AspRS2 probably constitutes a remnant from ancestral AspRS.

It has been proposed that Asn was one of the latest amino acids added to the cell repertoire and that AAU and AAC codons first specified Asp (37). Thus, the ancestor of tRNA^{Asp} and tRNA^{Asn} was charged with Asp by the primitive AsxRS before Asn appeared. tRNA asparaginylation was acquired with the capacity of conversion of Asp charged on tRNA^{GUU} with Asp-AdT. If AsnRS appeared according to the scheme proposed for GlnRS (38, 39), it emerged in eukaryotes by duplication of the AsxRS

Biochemistry: Becker and Kern

Proc. Natl. Acad. Sci. USA 95 (1998)   12837

gene and acquired specificity for Asn and tRNA^GUU. Eubacterial acquisition of AsnRS by horizontal gene transfer then was correlated with refinement of specificity of AsxRS for aspartylation of tRNA^GUC. This would not be possible in *T. thermophilus* because tRNA asparaginylation depended on the primitive pathway because of the lack of AsnS. Further, the imported AsnRS was not sufficiently accurate in discrimination of tRNA^Asp and tRNA^Asn to ensure survival of the organism. Accuracy then was improved by acquisition of a modern AspRS, which lowered the pool of free tRNA^Asp. Thus, AspRS1 and AsnRS were conserved because of their efficiency and the increased accuracy they promoted when present together. In contrast, glutamylation and glutaminylation specificities split because GlnS, which was probably more easily acquired than AsnS because of the involvement of Gln in essential cellular functions (40), promoted emergence of the direct route of glutaminylation after acquisition of GlnRS and allowed ancestral GlxRS to restrict its specificity to tRNA^Glu.

Position of *T. thermophilus* in the Phylogenetic Tree. Because archaebacteria do not contain AsnRS and GlnRS whereas most eubacteria contain AsnRS and only Gram− eubacteria contain GlnRS, acquisitions of the modern pathways of asparaginylation and glutaminylation by prokaryotes were delayed in time. Eubacteria acquired AsnRS and GlnRS after the split of the archaebacteria, which conserved the AspRS and GluRS of dual specificity and refined the tRNA-dependent pathways of Asn and Gln formation. The idea that GlnRS emerged in eukaryotes from ancestral GlxRS before its transfer into eubacteria is suggested by the stronger structural similarities of eubacterial GlnRS with eukaryotic GluRS than with eubacterial GluRS (38); because we could not find such structural interconnection between eubacterial AsnRS and eukaryotic and eubacterial AspRS, AsnRS appeared in eukaryotes and was transferred to eubacteria probably before GlnRS. As a consequence, most eubacteria have lost the primitive pathway of asparaginylation. GlnRS appeared later, in the ancestor of the Gram− bacteria after splitting of the Gram+ phylum. By exhibiting archeal and eubacterial Gram− metabolic peculiarities, *T. thermophilus* constitutes a link in evolution of the prokaryotes. Similar properties of *D. radiodurans* reported elsewhere (31) support arguments for a peculiar position of the *Thermus–Deinococcus* group (41) in the Gram− eubacteria.

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