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Existence of Two Distinct Aspartyl-tRNA Synthetases in *Thermus thermophilus*. Structural and Biochemical Properties of the Two Enzymes†

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ABSTRACT: Two aspartyl-tRNA synthetases (AspRSs) were isolated from *Thermus thermophilus* HB8. Both are α_2 dimers but differ in the length of their polypeptide chains (AspRS1, 68 kDa; and AspRS2, 51 kDa). Both chains start with Met and are deprived of common sequences to a significant extent. This rules out the possibility that AspRS2 is derived from AspRS1 by proteolysis, in agreement with specific recognition of each AspRS by the homologous antibodies. DNA probes derived from N-terminal amino acid sequences hybridize specifically to different genomic DNA fragments, revealing that the two AspRSs are encoded by distinct genes. Both enzymes are present in various strains from *T. thermophilus* and along the growth cycle of the bacteria, suggesting that they are constitutive. Kinetic investigations show that the two enzymes are specific for aspartic acid activation and $tRNA^{Asp}$ charging. tRNA aspartylation by the thermostable AspRSs is governed by thermodynamic parameters which values are similar to those measured for mesophilic aspartylation systems. Both thermophilic AspRSs are deprived of species specificity for tRNA aspartylation and exhibit N-terminal sequence signatures found in other AspRSs, suggesting that they are evolutionarily related to AspRSs from mesophilic prokaryotes and eukaryotes. Comparison of the efficiency of tRNA aspartylation by each enzyme under conditions approaching the physiological ones suggests that *in vivo* $tRNA^{Asp}$ charging is essentially ensured by AspRS1, although AspRS2 is the major species. The physiological significance of the two different AspRSs in *T. thermophilus* is discussed.

Accurate protein synthesis relies on specific aminoacylation of tRNAs by aaRSs.[1] Most organisms possess an unique aaRS for each of the 20 amino acids involved in protein synthesis. In contrast, about 60 different tRNAs belonging to 20 families of isoaccepting species, specify incorporation of the amino acids in polypeptide chains. Each synthetase acylates the homologous isoaccepting tRNAs with the cognate amino acid. The functional uniqueness between synthetase, amino acid, and tRNAs provides aatRNAs with enough accuracy for high fidelity in translation. However, several exceptions to this rule were reported.

First, some tRNAs can be mischarged by synthetases prior to conversion of the amino acid to that homologous of the tRNA; this mechanism ensures synthesis of amino acids via a process independent of usual metabolic pathway. Various Gram-positive bacteria, the Gram-negative bacterium *Rhizobium meliloti*, cyanobacteria, archaebacteria, mitochondria, and chloroplasts are deprived of GlnRS; glutamine acylating tRNA is then synthesized by ω amidation of glutamic acid mischarged on $tRNA^{Gln}$ by GluRS (Wilcox & Nirenberg, 1968; White & Bayley, 1972; Schön et al., 1988; Gagnon et al., 1996). The tRNA-dependent conversion of aspartate to asparagine in the archaebacteria *Haloferax volcanii* suggests a similar mechanism for synthesis of asparagine acylating $tRNA^{Asn}$ (Curnow et al., 1996) and the absence of ORFs encoding GlnRS and AsnRS in the genome of *Methanococcus janaschii* (Bult et al., 1996) suggests that glutamine and asparagine required for translation in this barophilic and thermophilic archeon are both provided by conversion of glutamate and aspartate mischarging tRNAs. In eukaryotes, tRNA selenocysteinylation occurs by conversion of Ser acylating $tRNA^{Sec}$ (Baron & Böck, 1995). Another exception in aminoacylation of each tRNA species by a particular aaRS is brought about by the unique polypeptide chain in higher eukaryotes supporting both GluRS and ProRS activities located in distinct catalytic centers (Cerini et al., 1991; Ting et al., 1992).

Second, in a few cases two distinct coexisting aaRSs encoded by distinct genes synthesize identical aatRNAs. So, *Escherichia coli* possesses two LysRSs (Hirshfield et al., 1981, 1984; Clark & Neidhardt, 1990; Lévêque et al., 1990), *Bacillus subtilis* has two ThrRSs and two TyrRSs (Putzer et al., 1990; Henkin et al., 1992), and *Staphylococcus aureus* contains two IleRSs (Gilbart et al., 1993). The exceptional

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[1] Abbreviations: for amino acids, the one-letter code is used; for aminoacyl-tRNA synthetases (aaRSs), the three-letter code is used for the amino acids, *e.g.* AspRS for arpartyl-tRNA synthetase; aatRNA, aminoacyl-tRNA; BD-cellulose, benzoyl[(diethylamino)ethyl] cellulose; DEAE-Sepharose, -Sephacel and -cellulose, Sepharose, Sephacel, and cellulose substituted by [(diethylamino)ethyl] groups; DIFP, diisopropyl fluorophosphate; DTE, dithioerythritol; EDTA, ethylenedinitrilotetraacetic acid; HPLC, high-pressure liquid chromatography; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; kbp, kilobase pairs; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride.

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character of these duplications is manifested by the absence of their conservation along the evolutionary scale since in the other organisms studied so far these synthetases are unique. A functional significance of duplication of the synthetases could be evidenced only in particular cases where both are differently expressed along the growth cycle of the organism or possess distinct properties.

We report here a new example of aaRS activity duplication in an organism. In the course of purification of AspRS from *T. thermophilus* we found two tRNA aspartylation activities catalyzed by two distinct enzymes (AspRS1 and AspRS2). The two AspRSs are encoded by distinct genes and exhibit specificity in activation of aspartic acid and charging of $tRNA^{Asp}$. This is the first report describing the coexistence of two aaRSs of the same specificity in an organism adapted to extreme life conditions. Investigation of the content of the two AspRSs along the growth cycle of the bacteria indicates that, contrary to the duplicated synthetases reported so far, both AspRSs are constitutive. However, analysis of their catalytic competence suggests that under physiological conditions tRNA aspartylation is ensured mainly by AspRS1. The possible biological significance of AspRS2 is discussed.

EXPERIMENTAL PROCEDURES

Chemicals. DEAE-Sepharose,[1] Sephadex G_{200}, Omega-30 membranes, and Phast-Gels for electrophoresis were from Pharmacia LKB Biotechnology. Heparin-Ultrogel was from IBF, hydroxyapatite HTP was from Bio-Rad, DEAE-cellulose (DE52) and phosphocellulose (P11) were from Whatman, and Fractogel TSK butyl HW65 (F) was from Merck, PMSF, DIFP, inorganic pyrophosphatase, anti-rabbit IgG peroxydase conjugate, 4-chloro-1-naphthol, and the protein markers were from Sigma. The size-exclusion HPLC Protein Pak 300SW column was from Waters, and the Nucleosil 120-5 C4 column was from Macherey-Nagel. Enzymes related to DNA manipulations and the Digoxigenin DNA Labeling and Detection kit were from Boehringer. The Ampli-Taq DNA amplification kit was from Perkin Elmer and the Sequenase kit from United States Biochemical Corporation. Oligonucleotides were synthesized using an Applied Biosystems 381A and purified by HPLC on a reverse phase column. L-[^{14}C]Aspartic acid (220 mCi/mmol) was from Commissariat à l'Energie Atomique (Saclay). L-[^{3}H]Aspartic acid (33 Ci/mmol), α-[^{35}S]dATP (400 Ci/mmol) and Hybond N^+ membranes were from Amersham, and nitrocellulose membranes BA85 were from Schleicher and Schüll. Unfractionated tRNAs (aspartate accepting capacities, 0.5−1.5 nmol/mg) from *E. coli* and yeast were from Boehringer, and those from *T. thermophilus* and beef liver were prepared by chromatography on DEAE-cellulose of bulk RNA obtained by phenol extraction of the cells. $tRNA^{Asp}$ from beef liver (accepting capacity, 4.5 nmol/mg) was enriched by chromatography on BD-cellulose. Pure $tRNA^{Asp}$ from yeast, *T. thermophilus*, and *E. coli* (accepting capacities, 37 nmol/mg) were from Drs. G. Keith and A.-C. Dock (IBMC and IGBMC, Strasbourg). $tRNA^{Asp}$ transcripts from yeast, *T. thermophilus*, and *E. coli* (accepting capacities, 32−36 nmol/mg) were obtained as described by Becker et al. (1996). AspRSs from *E. coli* (4000 units/mg) and yeast (680 units/mg) were prepared as described by Boeglin et al. (1996) and Becker et al. (1996).

Strains, Plasmids, Bacterial Growth, and Preparation of DNA and Crude Protein Extracts

T. thermophilus (ATCC 27634) HB8, VK1, and HB27 strains and *Thermus aquaticus* (ATCC 25104) C8 and YT1 strains were from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig) and from Drs. H. Monteil (Strasbourg), M. Sprinzl (Bayreuth), and J.-P. Touzel (Lille). *E. coli* DH5α was the host strain for preparation of recombinant pUC18 and pUC118.

T. thermophilus was grown in Luria Bertani medium (Sambrook et al., 1989) supplemented with 3 g of KH_2PO_4 and 6 g of Na_2HPO_4 per liter; *T. aquaticus* was grown in a medium containing 3 g of tryptone, 3 g of yeast extract, 0.4 g of NaCl, and 0.19 g of Na_2HPO_4 per liter at 70 °C; and *E. coli* was grown in standard conditions. Unless otherwise indicated the cells were harvested at the stationary stage. *T. thermophilus* clones were obtained on Luria Bertani agar plates (1.5% w/v) containing 0.5% (w/v) charcoal. Genomic DNA was prepared according to standard methods (Sambrook et al., 1989) and crude protein extracts by DEAE-cellulose chromatography of the lysate after disruption of the cells in a glass bead grinder followed by centrifugation (Mazauric et al., 1996). AspRSs were purified from cells grown in a 200 L Biolafitte fermenter and harvested when $A_{540nm} = 1.2$.

Recombinant DNA Techniques

DNA probes were obtained by PCR amplification starting from 0.5 μg of genomic DNA and 100 pmol of primers designed on the basis of the N-terminal protein sequences and the preferential usage of GC rich codons in *T. thermophilus*. They were extended at the 5′ ends by *Eco*RI sites. For AspRS1 the sense and antisense primers correspond to amino acids 1−9 and 44−52: 5′GGAATTCATG(C/A)G(G/C)(C/A)G(G/C)AC(G/C)C ACTACGC(G/C)GG(G/C)(A/T)(G/C)C3′ and 5′GGAATTC(G/C)GGGTG(G/C)GC (C/G)AC(A/C/G)A(G/A)CTG (G/C)AC(A/C/G)A(G/A)(G/C)-CCCTC3′. For AspRS2 they correspond to amino acids 1−10 and 27−36: 5′GGAATTCATG(C/A)G(G/C)GT (G/C)(C/T)T(C/G/T)GT(G/C)(C/A)G(G/C)GAC(C/T)T(C/G/T)-AAGGC(G/C)3′ and 5′GG AATTCGTC(A/C/G)A(G/A)-GTC(A/G)AACTG(A/G)AT(G/C)C(T/G)(G/C)CC(A/C/G)A(G/A)GTC3′. The amplified DNA fragments were isolated by PAGE (10% w/v), digested with *Eco*RI, ligated in pUC18 before *E. coli* transformation, and sequenced according to Tabor and Richardson (1987); band compressions were avoided by replacing dGTP by dITP.

For Southern hybridization, the probes were labeled by random priming with digoxigenin, hybridized at 70 °C on *Bam*HI, *Kpn*I, and *Pst*I digests of genomic DNA, and transferred onto a Hybond N^+ membrane after fractionation by electrophoresis on an 0.8% agarose gel (Sambrook et al., 1989).

Protein Analysis Techniques

Protein concentrations were determined by UV absorption according to Warburg and Christian (1941) or assuming $E_{280nm} = 1.0$ mL/mg/cm when $A_{280nm}/A_{260nm} > 1.5$.

Molecular masses were determined by gel filtration and PAGE. For gel filtration, the Sephadex G_{200} column (1.1 $cm^2 \times 64$ cm, flow 4 mL/h, 0.5 mL fractions) was

equilibrated with 50 mM potassium phosphate, pH 7.2, 200 mM KCl, 0.5 mM DTE, and 0.1 mM EDTA and DIFP, and the protein Pak 300SW column (0.78 $cm^2 \times 30$ cm, flow 1 mL/min) with 200 mM Na_2SO_4, 20 mM potassium phosphate, pH 7.2, and the protectors as above. 0.01−2 mg of proteins were loaded in 0.1−0.3 mL of equilibration buffer, and elutions were monitored by A_{280nm} or activity measurements. PAGE was conducted under native conditions in a 5%−25% polyacrylamide gradient and under denaturing conditions in the discontinuous system containing 0.1% SDS (Laemmli, 1970).

Cysteine alkylation was performed on denatured AspRS (20 nmol in 0.75 mL of 400 mM Tris-HCl, pH 8.5, 1.6 mM EDTA, 4.8 M guanidine-HCl, and 5 mM 2-mercaptoethanol incubated for 2 h at 25 °C under N_2) by *S*-pyridylation with 4 μL of vinylpyridine during 2 h at 25 °C under N_2. The mixture was then dialyzed against 100 mM *N*-methylmorpholine, pH 8.0 (for N-terminal sequencing), or 70% formic acid (for BrCN cleavage). The BrCN peptides were obtained by incubating 20 nmol of alkylated AspRS with 4 mg of BrCN (total volume 0.15 mL) at 25 °C in the dark for 48 h and were fractionated by HPLC on a Nucleosil 120-5 C4 column (0.4 $cm^2 \times 12.5$ cm, flow 18 mL/h) with a linear gradient obtained by mixing 0.1% trifluoroacetic acid and acetonitrile and monitored by A_{220nm} measurement. Proteins and peptides were sequenced by automated Edman degradation, using an Applied Biosystems 470A Protein Sequencer equipped with a PTH 120A Analyzer (Hewick, 1981).

Procedures for the Purification of the Two AspRSs from T. thermophilus

All steps were conducted at 4 °C. All buffers contained 5 mM 2-mercaptoethanol, 0.2 mM DIFP and PMSF, and 0.1 mM EDTA.

Crude Protein Extract. 1 kg cells were thawed in 1 L of 100 mM Tris-HCl, pH 8.0, containing 20 mM $MgCl_2$ and 100 mM NH_4Cl, and disrupted in a glass bead grinder (Dyno-Mill). The extract was centrifuged twice for 30 min at 14000*g* and for 2 h at 105000*g*, and the proteins from supernatant precipitated at 70% of ammonium sulfate saturation and were centrifuged and dialyzed against 20 mM Tris-HCl, pH 7.5.

DEAE-Sepharose Chromatography. The proteins were applied on a column (5 $cm^2 \times 25.5$ cm) equilibrated with the dialysis buffer and eluted with a gradient (2 × 1.7 L) from 30 to 300 mM NaCl (flow 1 mL/min; 20 mL fractions). The active fractions eluting at 180 mM NaCl were precipitated as above.

Hydrophobic Interaction Chromatography. The protein precipitate was centrifuged, suspended in 20 mM Tris-HCl, pH 7.5, at 50% saturation of ammonium sulfate, and fractionated in 3 parts, and each part was loaded on a Fractogel TSK butyl HW65 (F) column (2.6 $cm^2 \times 41$ cm) equilibrated with this solution. Elution occurred with a reverse gradient (2 × 0.6 L) of ammonium sulfate from 50% to 10% saturation (flow 0.8 mL/min; 8 mL fractions). AspRS was resolved in two peaks: a minor one (AspRS1) eluting at 28% and a major one (AspRS2) at 20% of saturation.

Hydroxyapatite and Phosphocellulose Chromatographies. The AspRS1 fraction was dialyzed and loaded on a hydroxyapatite column (7 $cm^2 \times 10$ cm) equilibrated with 10 mM potassium phosphate, pH 6.8, and the proteins eluted with a gradient (2 × 2 L) from 10 to 200 mM potassium phosphate, pH 6.8 (flow 0.5 mL/min; 13 mL fractions). The active fractions eluting at 110 mM salt were dialyzed and loaded on a phosphocellulose column (3 $cm^2 \times 26$ cm) equilibrated with 10 mM potassium phosphate, pH 6.8, and the proteins were eluted with a gradient (2 × 3 L) from 0 to 400 mM KCl (flow 3 mL/min; 18 mL fractions). AspRS1 eluted at the beginning of the gradient. The fractions from second active peak eluted on Fractogel were submitted to the same steps. AspRS2 eluted at 90 mM salt on hydroxyapatite and at 330 mM on phosphocellulose. The purity of both AspRSs was higher than 95% as judged by SDS−PAGE.

Final Step for Complete Purification of the Two AspRSs. For peptide sequencing and antibody preparations, purity of the AspRSs was improved by an additional chromatography on heparin-Ultrogel (2.5 $cm^2 \times 5$ cm) in 50 mM Tris-HCl, pH 7.5, developed with a gradient (2 × 0.25 L) from 0 to 300 mM KCl (flow 0.5 mL/min; 3 mL fractions). AspRS1 eluted at 90 mM KCl and AspRS2 eluted at 60 mM KCl with 70% yields. For antibody preparation, both enzymes were submitted to a final gel filtration by HPLC on a protein Pak 300SW column as described above.

Methods for Enzymatic Characterization

tRNA Aminoacylation. The standard reaction mixture (50−300 μL) contained 100 mM HEPES-Na, pH 7.2, 30 mM KCl, 10 mM $MgCl_2$, 2 mM ATP, 0.05 mM L-[^{14}C]-aspartic acid (70 cpm/pmol), 4 mg/mL of unfractionated tRNA from *E. coli* or *T. thermophilus*, and 50−300 μg/mL of protein diluted when necessary in 100 mM HEPES-Na, pH 7.2, 1 mg/mL bovine serum albumin, 5 mM 2-mercaptoethanol, 0.1 mM EDTA, and DIFP and 10% glycerol. Reactions were conducted at 37 or 70 °C, and the [^{14}C]Asp-tRNA synthesized after 1−15 min was determined in 40 μL aliquots as described previously (Kern et al., 1977). One unit of enzyme catalyzes formation of 1 nmol of Asp-tRNA per min at 37 °C in unfractionated *E. coli* tRNA.

K_M values were determined from double-reciprocal plots with limiting concentrations of the variable substrate (3−40 μM [^{14}C]aspartic acid (160 cpm/pmol) or 3−200 μM ATP or 0.02−10 μM $tRNA^{Asp}$ from *T. thermophilus*, *E. coli*, yeast, or beef liver) and saturating concentrations of fixed substrates (10−100 K_M) except aspartic acid when K_M's for tRNA were determined, where for practical reasons only 8 μM (about the K_M value) [^{3}H]aspartic acid (3100 cpm/pmol) was used; AspRS1 was 0.1−1 μg/mL, and AspRS2 was 0.2−10 μg/mL. The k_{cat}'s of the various AspRSs were determined as a function of temperature by initial rate measurements with saturating substrates concentrations [except for yeast AspRS where aspartic acid was 0.05 K_M (50 μM)] and 0.1−1.0 μg/mL of AspRS1, or 1−7 μg/mL of AspRS2, or 0.2 μg/mL of yeast or *E. coli* AspRS.

Equilibrium constants of AspRSs for tRNA aminoacylation were determined in a standard mixture of 50 μL (see above) containing 2 mM KF (to inhibit traces of contaminating pyrophosphatase), 0.019 mM [^{14}C]aspartic acid, 0.5 mM ATP, 0.02−0.2 mM AMP and PP_i, 3 μM *T. thermophilus*, yeast, or *E. coli* $tRNA^{Asp}$, and 0.05−0.1 μM homologous synthetase; enzyme concentrations were doubled after 20 min of incubation. Stable aminoacylation plateaus, independent

upon enzyme concentration, were established within 10 min. When temperature effects were studied, the tRNA was charged by preincubation 20 min at 37 °C in 0.3 mL reaction mixture before supplementation with AMP and PP_i (0.04 and 0.09 mM, respectively). The equilibrium constant (K) was determined according to [Asp-tRNA][AMP][PP_i]/[tRNA]-[Asp][ATP], where [tRNA] = [$tRNA_o$ − Asp-tRNA] and [Asp] = [Asp_o − Asp-tRNA], [$tRNA_o$] and [Asp_o] being [tRNA] and [Asp] present at t_o; the variations of ATP, AMP, and PP_i concentrations were negligible.

ATP−[^{32}P]PP_i Exchange. The reaction mixture of 220 μL contained 100 mM HEPES-Na, pH 7.2, 10 mM $MgCl_2$, 2 mM KF, ATP either fixed at 2 mM or varying from 0.04 to 2 mM for K_M determination, L-aspartic acid either fixed at 1 mM or varying from 0.01 to 2 mM for K_M determination, 2 mM [^{32}P]PP_i (2 cpm/pmol), when indicated 2 μM *T. thermophilus* $tRNA^{Asp}$ in unfractionated tRNA and 1 μg/mL of AspRS1 or 5 μg/mL of AspRS2. The k_{cat}'s were determined at various temperatures in the presence of saturating substrates concentrations (10−100 K_M) and 3−23 μg/mL of AspRS1, 2−18 μg/mL of AspRS2, or respectively, 3 or 4 μg/mL of yeast or *E. coli* AspRS. The initial rates of [^{32}P]ATP formation were determined by analysis of 50 μL aliquots as described (Kern & Lapointe, 1979).

Immunological assays and Western-blot analysis

Anti-AspRS antibodies were obtained from immunized rabbits (Prevost et al., 1989). Their specificity was analyzed by initial rate measurements of aspartylation in the presence of 0.05−0.1 μg of AspRS preincubated 30 min at 4 °C with increasing amounts (0−10 μL) of the various sera. AspRS1 and AspRS2 activities in crude extracts were quantified from remaining aspartylation activity of 0.2 mg of total proteins preincubated with an excess of heterologous antibodies (5 and 10 μL of serum). The content of AspRS1 or AspRS2 in crude thermophilic extracts was determined by inactivating each of them with increasing and limiting amounts of the homologous antibodies after complete inactivation of the other one with an excess of specific antibodies. Total proteins (respectively 78 and 156 μg) or 0.1 μg of AspRS1 or 1 μg of AspRS2 were incubated with an excess of heterologous antibodies (respectively anti-AspRS2 or anti-AspRS1) and limiting amounts of homologous antibodies (0−10 μL of 50- and 20-fold diluted anti-AspRS1 or anti-AspRS2 sera) followed by measurements of the remaining aminoacylation activity using unfractionated tRNA from *T. thermophilus* or *E. coli*. The content of each AspRS was estimated by comparing the amount of sera required to inactivate aspartylation of crude extracts and pure protein and determined by extrapoling the curves: activity = f(amounts of antibodies). Western blots were performed on 1 μg of pure AspRS or 50 μg of proteins in crude extracts fractionated by SDS−PAGE and were transferred on Hybond N^+ membranes followed by incubation with anti-AspRS antibodies and detection with anti-rabbit IgG peroxydase conjugates in the presence of H_2O_2 and 4-chloro-1-naphthol.

RESULTS

Evidence for the Existence of Two Distinct AspRSs in T. thermophilus

First Biochemical Evidence. The first attempt to purify AspRS from *T. thermophilus* by hydrophobic interaction chromatography on Fractogel and elution of the proteins with a decreasing ammonium sulfate gradient revealed two activity peaks. In the first one (AspRS1) the enzymatic activity is about the third of that found in the second peak (AspRS2) eluting at lower salt concentration (Figure 1A and Table 1). We isolated the proteins corresponding to the two activity peaks.

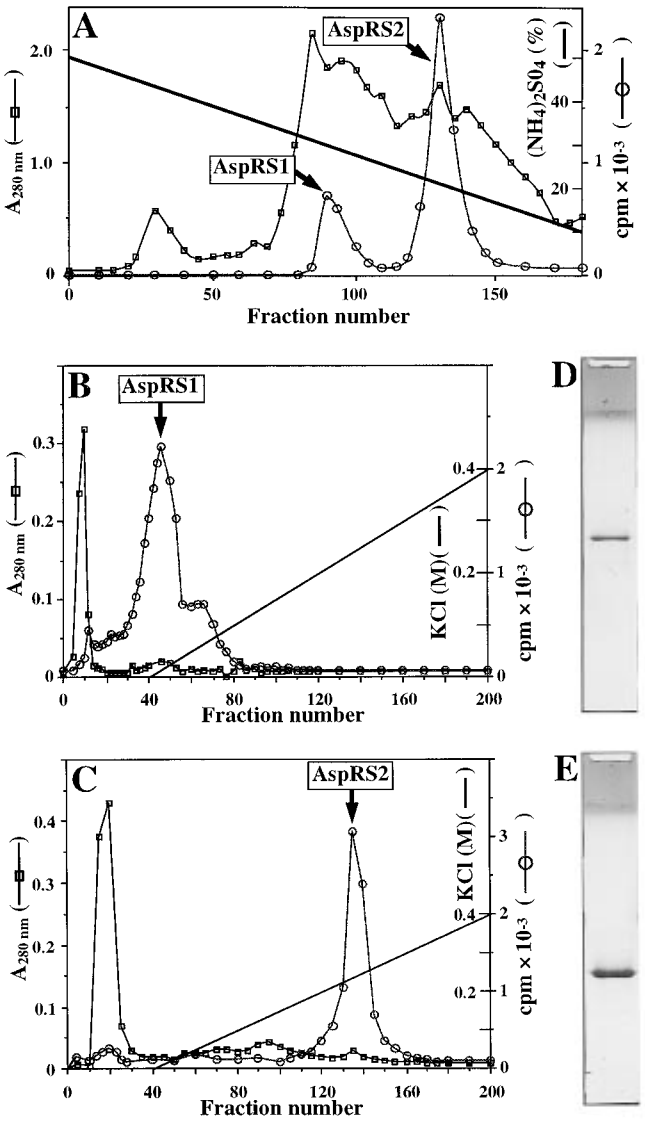

FIGURE 1: Elution profiles of AspRS1 and AspRS2 by chromatographies on Fractogel TSK butyl (A) and phosphocellulose (B and C) and analysis of purity of AspRS1 (D) and AspRS2 (E) by PAGE after heparin-Ultrogel chromatography. (A−C) Activity was tested in 10 μL of each fraction. (D,E) 1 μg of protein was analyzed. The conditions were as described in Experimental Procedures.

Purification of the Two AspRSs. Table 1 summarizes the purification procedure of the two isoforms. The low recovery of activity after DEAE-Sepharose (64%) relates probably to unspecific retention of proteins on the column. The Sepharose anionic exchanger was used because of its high capacity and fast flow rate, although it was poorly resolutive compared to DEAE-cellulose and DEAE-Sephacel on which the thermostable synthetases are partly fractionated as are the yeast and *E. coli* synthetases (Kern et al., 1977; Kern & Lapointe, 1979). Chromatography on Fractogel resolves proteins according to their solubility in salts and their hydrophobicity, and fractionated the two AspRSs. Both enzymes were obtained with 95% homogeneity by additional

Table 1: Table of Purification of the Two AspRSs from *T. thermophilus*

purification step	proteins (mg)		total activity (units)[b]		specific activity (units/mg)[b]		purification		yield (%)	
crude extract[a]	16500		3300		0.2		1		100	
DEAE-Sepharose	1842		2112		1.1		5.5		64	
	(**1**)[c]	(**2**)[c]	(**1**)	(**2**)	(**1**)	(**2**)	(**1**)	(**2**)	(**1**)	(**2**)
Fractogel	576	546	440	1420	0.7	2.6	3.5	13	13	43
hydroxyapatite	68	145	320	1130	4.7	7.8	24	39	9.7	34
phosphocellulose	5.7	18.5	258	749	45.2	40.5	226	202	7.8	22.7

[a] Obtained from 1 kg of cells. [b] The enzyme unit refers to charging of unfractionated *E. coli* tRNA. [c] (**1**) and (**2**) refer respectively to AspRS1 and AspRS2.

chromatographies on hydroxyapatite and phosphocellulose (Figure 1B and C). Finally, quality grade of both enzymes for N-terminal sequencing and antibody preparation was reached by a final chromatography on heparin-Ultrogel (Figure 1D and E).

Distinct Structural Properties of the AspRSs. Gel filtration of AspRS1 gave an apparent M_r of 120 000−140 000, whereas native PAGE showed a diffuse band of low mobility, probably due to a poor content of cationic groups. SDS−PAGE showed a single polypeptide chain of M_r 68 000, in agreement with the value calculated from amino acid composition (M_r 66 029; Poterszman et al., 1993). Taken together, this indicates that AspRS1 is an α_2 dimer. For AspRS2, an apparent M_r of 66 000−72 000 was found by gel filtration and of 104 000 by native PAGE. The discrepancy may be due to interactions of the protein with the gel filtration matrices resulting in determination of excessively low apparent M_r. Since SDS−PAGE showed a single polypeptide chain of M_r 51 000, AspRS2 is also an α_2 dimer like AspRS1. The M_r of native and denatured *E. coli* AspRS are comparable to those of AspRS1 but are significantly higher than those of AspRS2 (170 000, 132 000, and 65 000 by gel filtration, PAGE, and SDS−PAGE).

The N-terminal end of AspRS1 was sequenced from Met_1 to Pro_{52}, and that of AspRS2 was sequenced from Met_1 to Thr_{49}. Alignment of the two sequences indicates 46% identity (not shown). The elution profiles on HPLC of the peptides obtained by cleavage of the two proteins with BrCN are quite different, indicating a different content of Met residues in these polypeptide chains (not shown). Several peptides of identical retention time belonging to each AspRS were isolated and sequenced. So, peptide VVRP at the C-terminal end of AspRS1 (residues 577−580; Poterszman et al., 1993), eluting at 20% acetonitrile, has no counterpart in AspRS2. Similarly, the peptides eluting at 30% and 44% acetonitrile are different (not shown). Finally, the two peptides VAGLDRYFQIARCFRDEDLRAD and AHVFREALGVELPLPFPRLSYEEAM from AspRS1 (positions 209−242 and 259−283) eluting at 49% acetonitrile are also without counterparts in AspRS2.

Distinct Immunological Properties of the Two AspRSs. Western blots show specific recognition of each AspRS by its homologous antibodies. AspRS1 and AspRS2 are completely inactivated by their homologous antibodies but unaffected by the heterologous ones, suggesting that the two AspRSs are lacking common epitopes. Further, AspRS1 is partly inactivated by anti-*E. coli* AspRS antibodies and *vice versa* in contrast to AspRS2, which is not affected by them. AspRS2 is partly inactivated by anti-yeast cytoplasmic AspRS antibodies and *vice versa*, while AspRS1 is not affected by them. These observations confirm the absence of an immunological relationship between the two thermostable AspRSs.

Demonstration of the Existence of Two Distinct Genes Encoding Both AspRSs

The genomic sequences encoding the N-terminal ends of both AspRSs were characterized by hybridization of probes derived from protein sequences to restriction fragments from *T. thermophilus* DNA. The probes, obtained by PCR amplification, were highly specific since the derived protein sequences could be superimposed over the N-terminal AspRSs sequences. Figure 2 shows that the probe derived from AspRS1 hybridizes to *Pst*I, *Kpn*I, and *Bam*HI fragments of 12.0, 6.2, and 4.5 kbp, respectively, whereas that derived from AspRS2 hybridizes to fragments of 5.0, 9.0, and 3.5 kbp. The 4.5 kpb *Bam*H1 fragment encodes the full polypeptide chain of AspRS1 (Poterszman et al., 1993), whereas the 3.5 kpb fragment includes only the sequence encoding the 31 first amino acids of AspRS2, since a *Bam*H1 site covers codons 30−32 of the ORF.

Generalization: Presence of Two AspRSs in Various T. thermophilus Strains

Western blot analysis of crude extracts of HB8, HB27, and VK1 strains from *T. thermophilus* revealed two polypeptide chains of size similar to those of purified AspRS1 and AspRS2, each reacting specifically with the homologous antibodies (Figure 3). Since both antibodies were necessary to abolish the aspartylation activity in crude extracts (not shown), the two AspRSs are present in the three strains of *T. thermophilus*. Further, both enzymes are present in various clones of the *T. thermophilus* strains, excluding the origin of one AspRS by a contaminating organism (Figure 3). Analysis of the AspRSs content at various stages of the growth cycle of *T. thermophilus* HB8 (beginning and end of the logarithmic and stationary phases) indicated that both enzymes are present in constant amounts along the growth cycle of the bacteria.

A similar analysis in two strains of *T. aquaticus* (C8 and YT1) showed the absence of AspRS2, since the aspartylation activity in crude extracts was totally abolished by anti-AspRS1 antibodies and was not altered by the antibodies directed against AspRS2 (not shown).

Aminoacylation of T. thermophilus $tRNA^{Asp}$ by AspRS1 and AspRS2

Immunological titrations in *T. thermophilus* strain HB8 with homologous antibodies, revealed that AspRS1 and

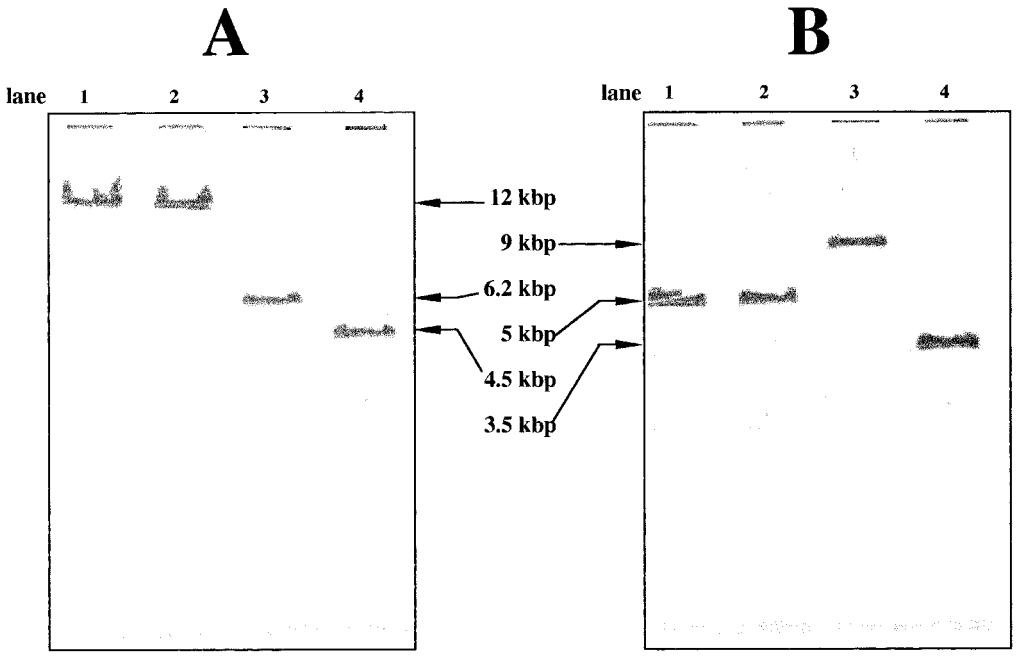

FIGURE 2: Southern blot analysis of restriction digests of *T. thermophilus* HB8 genomic DNA with labeled DNA probes derived from N-terminal sequences of AspRS1 (A) or AspRS2 (B). 50 μg of DNA was analyzed; methods used to obtain and label the probes and Southern hybridization are described in Experimental Procedures. Lanes 1 and 2, DNA of two clones digested with *Pst*I; lanes 3 and 4, DNA digested with *Kpn*I and *Bam*HI, respectively.

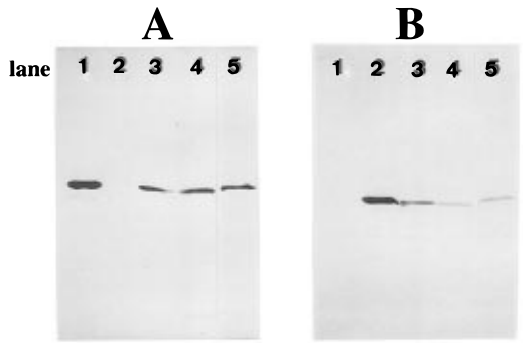

FIGURE 3: Western blot analysis of pure AspRS1 and AspRS2 from *T. thermophilus* and of crude extracts of various *Thermus* strains with anti-AspRS1 (A) and anti-AspRS2 (B) antibodies. Lanes 1 and 2, AspRS1 and AspRS2 from *T. thermophilus* HB8; lanes 3−5, crude extracts from *T. thermophilus* HB8, HB27, and VK1 strains. Either 1 μg of pure AspRS or 50 μg of proteins in crude extracts was analyzed. PAGE of proteins, transfer on membrane, and immunodetection are described in Experimental Procedures.

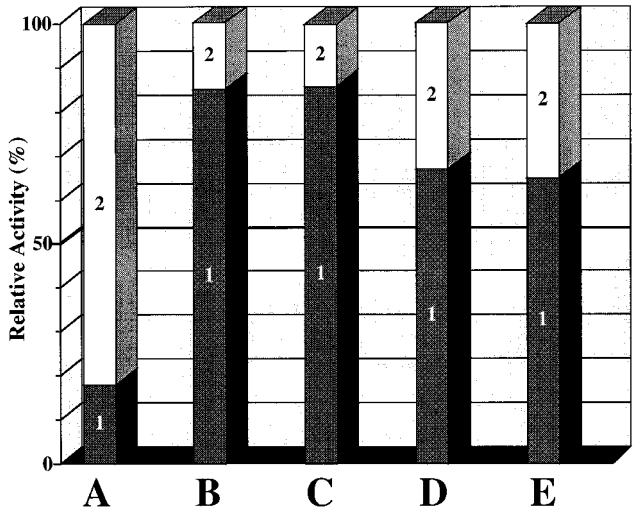

FIGURE 4: Involvement of AspRS1 and AspRS2 in *T. thermophilus* crude extract in aspartylation of *E. coli* and *T. thermophilus* tRNAs. (A) Unfractionated tRNA from *E. coli*; (B,C) unfractionated tRNA from *T. thermophilus*; (D,E) $tRNA^{Asp}$ from *T. thermophilus*. The reactions were conducted at 37 °C (A, B, D) or 70 °C (C, E). The parts of the columns numbered 1 and 2 represent the fraction of the total aspartylation activity promoted respectively by AspRS1 and AspRS2. The contribution of each AspRS to aspartylation was estimated by comparing the remaining activity of the extract preincubated with the heterologous antibodies (anti-AspRS2 and anti-AspRS1 for determination of the activities of AspRS1 and AspRS2) to the activity in the absence of antibodies (100%). The activities were determined by initial rate measurements as described in Experimental Procedures.

AspRS2 represent 0.08% and 0.32% of the total proteins. Although AspRS2 exceeds 4-fold AspRS1, the relative contribution of each AspRS to tRNA aspartylation differs from its relative amount.

When *E. coli* tRNA is charged with a crude extract from *T. thermophilus*, activity of AspRS2 exceeds that of AspRS1, since the initial aspartylation rate decreases by 82 and 18% with anti-AspRS2 and anti-AspRS1 antibodies (Figure 4A). Conversely, when *T. thermophilus* tRNA is charged, contribution of AspRS1 exceeds that of AspRS2, since anti-AspRS1 antibodies abolish about completely (85%) aspartylation, whereas anti-AspRS2 antibodies affect it only poorly (15%; Figure 4B and C). When pure $tRNA^{Asp}$ from *T. thermophilus* is aminoacylated, AspRS1 and AspRS2 contribute respectively 67% and 33% to the initial rate (Figure 4D and E). Finally, the contribution of each AspRS is temperature-independent, since antibodies affect the initial aspartylation rate in crude extracts similarly at either 37 or 70 °C (Figure 4B,C and D,E).

Catalytic Properties of the Two AspRSs

ATP−[^{32}P]PP$_i$ Exchange. The K_M's for aspartic acid and ATP are similar for both AspRSs and are not significantly affected by a temperature shift from 37 to 70 °C (Table 2).

Table 2: Kinetic Constants of the Two AspRSs from *T. thermophilus* in ATP-[^{32}P]PP$_i$ Exchange and tRNA Aminoacylation[a]

	AspRS1				AspRS2			
	ATP−[^{32}P]PP$_i$ exchange		tRNA aminoacylation		ATP−[^{32}P]PP$_i$ exchange		tRNA aminoacylation	
substrate	37 °C	70 °C	37 °C	70 °C	37 °C	70 °C	37 °C	70 °C
				K_M (μM)				
aspartic acid	30[a] 44[b]	100[a]	9	30	62[a] 111[b]	100[a]	3	5
ATP	370[a] 330[b]	250[a]	120	280	150[a]	110[a]	60	33
tRNA^{Asp}			0.044	0.030			0.043	0.073
				k_{cat} (s^{-1})				
	0.5[a] 1.0[b]	4.9[a]	0.77	2.7	1.26[a] 1.26[b]	1.74[a]	0.09	0.24

[a] The conditions are described in Experimental Procedures; ATP−PP$_i$ exchange was conducted [a]*in* the absence or [b]*in* the presence of tRNA.

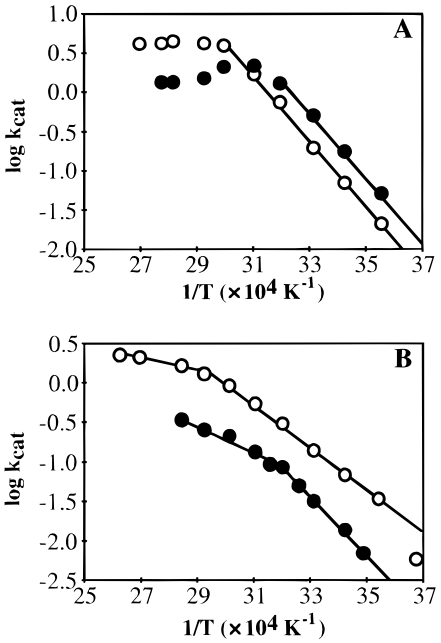

FIGURE 5: Arrhenius plots of ATP−[^{32}P]PP$_i$ exchange (A) and aminoacylation of *T. thermophilus* tRNA^{Asp} (B) catalyzed by AspRS1 (○) and AspRS2 (●) from *T. thermophilus*. The k_{cat} were derived from maximal rates determined as described in Experimental Procedures.

In addition, cognate tRNA is without significant effect on K_M's for amino acid and on k_{cat}. However, both AspRSs differ by the temperature-dependence of k_{cat}: below 50 °C, activity of AspRS2 exceeds that of AspRS1 whereas the opposite occurs at higher temperatures (Table 2). The Arrhenius plots are linear from 10 to 60 °C for AspRS1 and from 10 to 50 °C for AspRS2 (Figure 5A), and the derived activation energies for both synthetases are equal in these temperature ranges (about 83 kJ/mol/K; Table 3). At higher temperatures the behaviors of the two enzymes differ (Figure 5A). AspRS2 is inactivated, as shown by the incurvation of the kinetics, whereas AspRS1 remains stable; the rate constant of AspRS1 does not vary significantly with temperature whereas that of AspRS2 decreases when temperature increases. As a comparison, the Arrhenius plots of mesophilic *E. coli* and yeast AspRSs are linear from 0 to 45 °C, but aminoacylation reactions are characterized by different activation energies (81 and 33 kJ/mol/K, respectively; Table 3). At high temperatures these enzymes are inactivated.

tRNA Aminoacylation. The two AspRSs have similar K_M's for their aspartic acid, ATP, and tRNA^{Asp} substrates, and these values are not significantly affected when temperature is shifted from 37 to 70 °C (Table 2). In contrast, k_{cat} of AspRS1 exceeds significantly that of AspRS2 whatever the temperature [9-fold at 37 °C (0.77 and 0.09 s^{-1}) and 11-fold at 70 °C (2.7 and 0.24 s^{-1}); Table 2)]. Interestingly, the thermal instability of AspRS2 observed in ATP−PP$_i$ exchange is abolished in tRNA charging, probably as a consequence of a stabilizing effect of this ligand. Arrhenius plots for aminoacylation of either pure or unfractionated tRNAs by the two AspRSs are biphasic, indicating variations of the activation energy according to the temperature range (Figure 5B). When AspRS1 aminoacylates pure tRNA^{Asp}, the value derived in the low-temperature range (56 kJ/mol/K from 0 to 60 °C) decreases 2-fold in the upper range (26 kJ/mol/K from 60 to 100 °C; Table 3). Both values increase 1.5-fold when unfractionated tRNA is charged (87 and 39 kJ/mol/K; Table 3). Comparable values in the two temperature ranges were determined for AspRS2 (70 and 36 kJ/mol/K for charging of pure tRNA^{Asp}, and 81 and 25 kJ/mol/K for that of unfractionated tRNA; Table 3). When *E. coli* and yeast tRNA^{Asp} are charged by their homologous synthetase, the Arrhenius plots are linear from 0 to 45 °C and the activation energies are similar to those of the thermophilic AspRSs in the low-temperature range (63 and 46 kJ/mol/K; Table 3).

Finally, the equilibrium constant of overall tRNA^{Asp} charging is independent upon the origin of the AspRS catalyzing the reaction (Table 3). Analysis of the effect of temperature gave similar enthalpy (ΔH) and entropy (ΔS) changes for the thermophilic and mesophilic aspartylation systems: $\Delta H = -28$ to -38 kJ/mol and $\Delta S = -100$ to -135 J/mol/K (Table 3).

Aminoacylation of tRNA^{Asp} of Various Origins by the Two AspRSs

Table 4 compares the kinetic constants of aminoacylation of tRNAs of different origin by the two AspRSs. AspRS1 aminoacylates the cognate thermophilic tRNA more efficiently than AspRS2, and its increased efficiency increases with increasing temperature: 8-fold at 37 °C and 27-fold at 70 °C. This effect is maintained when unmodified transcripts are charged, although less pronounced at 37 °C. When cross-chargings of *E. coli*, yeast, or mammalian tRNAs were studied, the efficiencies of both AspRSs are decreased except for charging *E. coli* tRNA by AspRS2, but the most efficient aminoacylations are always observed with AspRS1. The most drastic effects occurred with the eukaryotic tRNA^{Asp} from yeast since modified or transcript tRNAs are asparty-

Table 3: Thermodynamic Parameters for ATP−[^{32}P]PP$_i$ Exchange and tRNA Aminoacylation Catalyzed by the Two AspRSs from *T. thermophilus* and by Yeast and *E. coli* AspRSs

	thermodynamic parameters[a]					
	ATP−[^{32}P]PP$_i$ exchange	tRNA aminacylation				
origin of AspRS	E_a (kJ/mol/K)	E_a (kJ/mol/K)		K	ΔH (kJ/mol)	ΔS (J/mol/K)
T. thermophilus						
AspRS1	83	56[b,d]	26[c,d]	0.26[d]	−30[d]	−108[d]
		87[b,e]	39[c,e]			
AspRS2	84	70[b,d]	36[c,d]	0.29[d]	−28[d]	−99[d]
		81[b,e]	25[c,e]			
yeast	33	46[d]		0.24[d]	−38[d]	−136[d]
E. coli	81	63[e]		0.29[d]	−29[d]	−104[d]

[a] E_a, activation energy; K, equilibrium constant; ΔH, enthalpy change; ΔS, entropy change; [b,c] values determined from slopes of the Arrhenius plots in respectively the lower and upper temperature ranges; [d] values obtained with pure homologous tRNA^{Asp}; [e] values obtained with homologous unfractionated tRNA^{Asp}. Each value is an average of at least two independent determinations.

Table 4: Aminoacylation of Native and Transcript tRNA^{Asp} and of Unfractionated tRNA of Various Origins by the Two AspRSs from *T. thermophilus*

	kinetic constants						
	AspRS1			AspRS2			$(k_{cat}/K_M)_{AspRS1}/(k_{cat}/K_M)_{AspRS2}$
origin of tRNA^{Asp}	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (μM^{-1} s^{-1})	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (μM^{-1} s^{-1})	
T. thermophilus							
pure tRNA	0.044	0.77	17.5	0.043	0.09	2.1	8
	0.030[a]	2.7[a]	90[a]	0.073[a]	0.24[a]	3.3[a]	27
transcript	0.016	0.49	30.6	0.010	0.10	10	3
	0.017[a]	1.5[a]	88.2[a]	0.110[a]	0.37[a]	3.4[a]	26
unfractionated tRNA	nd	0.92	nd	nd	0.01	nd	
	nd	3.3[a]	nd	nd	0.06[a]	nd	
E. coli							
pure tRNA	0.024	0.25	10.4	0.040	0.20	5.0	2
transcript	0.050	0.23	4.6	0.050	0.11	2.2	2
unfractionated tRNA	nd	0.05	nd	nd	0.03	nd	
yeast							
pure tRNA	0.098	0.09	0.9	5.900	0.027	0.005	180
transcript	0.085	0.30	3.5	6.700	0.022	0.003	1167
unfractionated tRNA	nd	0.021	nd	nd	0.025	nd	
beef liver							
enriched tRNA	0.060	0.12	2.0	0.070	0.040	0.6	3.3

The conditions are described in Experimental Procedures and the measurements were effected at 37 °C except in [a] at 70 °C; [b] tRNA^{Asp} partially purified by BD-cellulose chromatography; nd, not determined. Each value is an average of at least three independent determinations.

lated 200- and 1200-fold more efficiently by AspRS1 than by AspRS2. However, these effects are considerably attenuated when beef liver tRNA^{Asp} is charged since AspRS1 aminoacylates that tRNA only 3-fold more efficiently than AspRS2. AspRS1 and AspRS2 aminoacylate *E. coli* and mammalian tRNA^{Asp} with efficiencies either comparable or decreased less than 10-fold than the thermophilic one.

Finally, the k_{cat} of aspartylation of unfractionated tRNAs by the thermophilic AspRSs are in most cases lower than those of charging pure tRNA^{Asp}. Unfractionated tRNA from *T. thermophilus* is charged 10 times slower than pure tRNA^{Asp} by AspRS2 and with an equal rate by AspRS1. Unfractionated tRNA from *E. coli* is aminoacylated about 5-fold slower than pure tRNA^{Asp} by both thermophilic AspRSs and unfractionated yeast tRNA is charged about 5-fold slower by AspRS1 and with an equal rate by AspRS2.

DISCUSSION

Origin of the Two AspRSs

Various reasons such as proteolysis of the native enzyme, contamination of the strain by another thermophilic bacteria or existence of two distinct genes encoding AspRSs could account for the presence of two AspRSs in *T. thermophilus*.

The important discrepancy in the sizes of the polypeptide chains of the AspRSs purified from *T. thermophilus* (68 000 for AspRS1 and 51 000 for AspRS2) suggests at first a proteolytic origin of the smaller enzyme (AspRS2) from the native and larger AspRS1. Proteolysis would occur at the N-terminal end since the sequences differ and not at the C-terminal domain involved in dimerization (Delarue et al., 1994). Multiple forms of aaRSs of the same specificity were often isolated, in particular from eukaryotes as evidenced by their different chromatographic behaviors [*e.g.*, Kern et al. (1977)], their sizes [*e.g.*, Kern et al. (1975) and Lorber et al. (1987)] and even their oligomeric structures [*e.g.*, Kern et al. (1981a,b)]. In most cases, however, an homogeneous form corresponding likely to the native enzyme was isolated in the presence of proteases inhibitors, indicating that heterogeneity was provoked by proteolysis. Here, on the contrary, such effects are excluded since (i) the presence of a starting Met suggests that the N-terminal end of the two polypeptide chains are not altered, (ii) no peptides, common to both proteins could be evidenced, and (iii) the two enzymes are deprived of common epitopes. Furthermore, the origin of one AspRS by strain contamination is also excluded since the two AspRSs were isolated from the same cell paste in contrast to other synthetases (*e.g.*, GlyRS,

AlaRS, and ThrRS) which are unique (data not shown) and especially since the two AspRSs were characterized in several independent clones of *T. thermophilus* strains. Finally, localization of the genomic sequences encoding the N-terminal ends of the two polypeptide chains in different restriction fragments indicates that they are encoded by distinct genes. Both are present in all of the *T. thermophilus* strains we analyzed (HB8, HB27, and VK1). Surprisingly, however, only AspRS1 is present in *T. aquaticus*. This makes AspRS2 an identifier of *T. thermophilus* among *Thermus* species.

Structural Properties of the Two AspRSs from T. thermophilus and Comparison with Other Synthetases

Properties of the AspRS Protein Surfaces. The different chromatographic behaviors of the thermophilic AspRSs indicate variations in the content of polar and hydrophobic domains of their protein surfaces. Coelution on DEAE-substituted matrices suggests a similar content of anionic groups, whereas the absence of retention on phosphocellulose and the unusual low electrophoretic mobility of AspRS1, in contrast with the more conventional behavior of AspRS2, reveal an exceptional low content of cationic groups on AspRS1. Unexpectedly, however, AspRS1 binds to heparin with similar affinity as AspRS2. This behavior, observed for other synthetases from *T. thermophilus* (Mazauric et al., 1996), characterizes eukaryotic synthetases which are strongly retained on such matrix by their N-terminal extension organized in a cationic domain (Cirakoglu & Waller, 1985; Lorber et al., 1988), whereas prokaryotic synthetases from mesophiles deprived from this extension exhibit poor binding capacity to heparin (Cirakoglu & Waller, 1985). Retention of *T. thermophilus* synthetases on heparin-substituted matrices, despite the absence of this extension, suggests the presence on the thermophilic synthetases of a small cationic domain, probably absent in their mesophilic counterparts.

Fractionation on Fractogel indicates differences in the solubility in salt solutions and in the hydrophobicity of the two AspRSs. This agrees with different behaviors of both in crystallization. No crystals of AspRS2 could be obtained in the conditions where AspRS1 crystallizes (4 M formate or 2.4 M ammonium sulfate in Tris-HCl buffer, pH 7.5; Poterszman et al., 1993). The easiness of crystallization of AspRS1 allowed establishment of the 3D structures of the enzyme in the free state and complexed to aspartyladenylate (Delarue et al., 1994; Poterszman et al., 1994).

Comparison of the N-Terminal Sequences. Comparison of the N-terminal sequences from thermophilic AspRSs shows 46% identity and 75% similarity (not shown). Alignments with other AspRSs reveal more identity of AspRS1 than AspRS2 with eubacterial and archaebacterial AspRSs. AspRS1 shows respectively 60%, 66%, 46%, and 57% identity with AspRSs from *E. coli* (Eriani et al., 1990), *Haemophilus influenzae* (Fleischmann et al., 1995), *Pyrococcus* species (Imanaka et al., 1995), and *M. janaschii* (Bult et al., 1996), whereas AspRS2 shows 44%, 46%, 33%, and 49% identity with these AspRSs. In contrast, both thermophilic AspRS sequences exhibit similar identities with the eukaryotic sequences downstream their N-terminal extension [26%−33% with the yeast and mammalian enzymes (Sellami et al., 1986; Jacobo-Molina et al., 1989; Mirande & Waller, 1989)]. Interestingly, the N-terminal sequences of AsnRS (Seignovert et al., 1996) and LysRS from *T. thermophilus* (Chen et al., 1994) exhibit 40% and 31% identity with the N-terminal end of AspRS1 and 45% and 27% with that of AspRS2.

Comparison of the Polypeptide Chain Sizes. The homodimeric structure of both thermophilic AspRSs confirms conservation of the oligomeric structure of this synthetase along the evolutionary scale and in organisms adapted to various life conditions. However, AspRS polypeptide chains vary in size according to the phylae. Indeed, those from prokaryotes [*E. coli* and *H. influenzae*, M_r = 66 000 and 66 600 (Eriani et al., 1990; Fleischmann et al., 1995)] and from lower eukaryotes and organelles [yeast cytoplasm and mitochondria, M_r = 63 500 and 75 500 (Sellami et al., 1986; Gampel & Tzagoloff, 1989)] are larger than those from higher eukaryotes [*Caenorhabditis elegans*, *Rattus norvegicus*, and *Homo sapiens*, M_r = 57 000−59 800 (Wilson et al., 1994; Mirande & Waller, 1989; Jacobo-Molina et al., 1989)] because of the presence of large insertion sequences, whereas the archaebacterial ones [*Pyrococcus* species and *M. janaschii*, M_r = 50 800 and 48 000 (Imanaka et al., 1995; Bult et al., 1996)] deprived from these insertions and from N-terminal extension are the shortest. Interestingly AspRS1 resembles eubacterial *E. coli* AspRS by the size of the polypeptide chain and the bulkiness of the protein, whereas AspRS2 resembles, according to these criteria, archaebacterial AspRSs. It appears therefore, that *T. thermophilus*, presently classified among the eubacteria, possesses archaebacterial characteristics. Finally, identity of the N-terminal sequences and immunological cross reactions also indicate that AspRS1 is structurally more related to *E. coli* AspRS than AspRS2.

Functional Interrelation between the Thermophilic AspRSs and Other Subclass 2b Synthetases

N-terminal sequence alignments indicate conservation in most prokaryotic and eukaryotic AspRSs of six residues, namely V, R, G, F, R and Q at a strategic region of the enzyme (Figure 6). The triad R, F, and Q is present in all AspRSs except in those from yeast mitochondria (where R and Q are substituted by P and I) and from *Pyrococcus* species (where R is substituted by K). The 3D structures of the yeast AspRS·tRNA^{Asp} complex and of free *T. thermophilus* AspRS1 (Cavarelli et al., 1993; Delarue et al., 1994) show that these residues are well exposed in the N-terminal β-barrel of the proteins. Furthermore, in the yeast complex the conserved R119, F127, and Q138 contact U35 from the tRNA anticodon (Cavarelli et al., 1993). Protein and tRNA mutagenesis reveal strong implication of these elements in tRNA recognition and aspartylation. In particular it was shown that U35 constitutes the major identity element for tRNA aspartylation in yeast (Pütz et al., 1991), *E. coli* (Nameki et al., 1992), and *T. thermophilus* (Becker et al., 1996). Conservation of this major tRNA identity element suggests contacts with the conserved triad of protein residues in the various aspartylation systems (*e.g.* R29, F36, and Q47 of AspRS1 and R26, F33, and Q44 of AspRS2). Interestingly, most of the conserved amino acids, in particular the triad R, F, and Q, are present in AsnRS and LysRS [R26, F33, and Q44 in AsnRS from *E. coli* (Anselme & Härtlein, 1989) and *T. thermophilus* (Seignovert et al., 1996) and R64, F71, and Q82 in LysRS from *T. thermophilus* (Chen et al.,

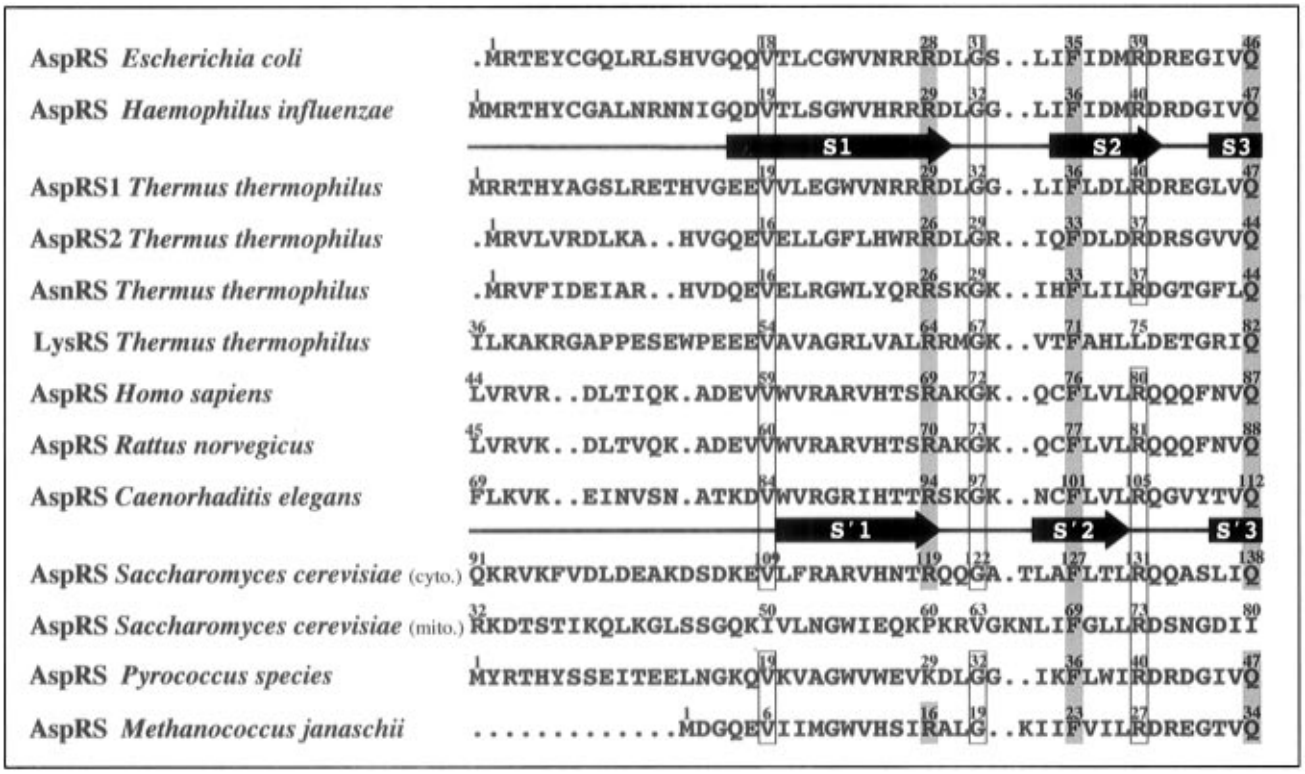

FIGURE 6: Alignments of the N-terminal sequences of AspRSs, AsnRS, and LysRS of various origins. The conserved residues are boxed; the residues contacting U35 from $tRNA^{Asp}$ in the yeast complex (Cavarelli et al., 1993) and those presumed to interact with U35 from homologous tRNA are boxed in gray. S1−S3 and S′1−S′3 are the strands in the N-terminal domains of *T. thermophilus* AspRS1 (Delarue et al., 1994) and yeast AspRS (Cavarelli et al., 1993).

1994; Figure 6)] and U35 from anticodons of $tRNA^{Asn}$ and $tRNA^{Lys}$ constitute the major identity elements for asparaginylation (Li et al., 1993) and lysinylation (McClain et al., 1990; Tamura et al., 1992) in *E. coli*. AspRS, AsnRS, and LysRS belong to subclass 2b synthetases characterized by organization of the N-terminal domain in a β-barrel involved in anticodon binding (Moras, 1992). Conservation in these proteins and tRNAs of structural elements shown to be crucial for identity expression, indicates strong similarities in tRNA recognition and aminoacylation in subclass 2b of synthetases despite the variability in the aspartate, asparagine, and lysine specificities.

Specificity of the Two AspRSs for Amino Acid Activation and tRNA Charging

Knowledge of the kinetic constants is essential to define the specificity of synthetases. False specificity determination can result from errors in catalysis (misactivation of amino acid and mischarging of tRNA) or from contamination by improper amino acid or tRNA substrates. Catalysis involving wrong substrates are generally characterized by decreased k_{cat} values, whereas contamination of wrong substrates by small amounts of the specific one leads to unusual high K_M's. The K_M's of the two thermophilic synthetases for aspartic acid are low compared to those usually determined for mesophilic synthetases [9 and 3 μM for AspRS1 and AspRS2 (Table 2) and 1 mM and 60 μM for yeast and *E. coli* AspRSs (Lorber et al., 1983; Eriani et al., 1990)]. Further, the K_M's of both AspRSs for *T. thermophilus* $tRNA^{Asp}$ are similar (0.04 μM); they equal that of yeast AspRS (0.05 μM; Pütz et al., 1991) and are even 10-fold lower than that of *E. coli* AspRS (0.55 μM; Eriani et al., 1990). Finally, the k_{cat}'s of ATP−PP_i exchange and tRNA charging of both AspRSs are in the range of those determined for other thermophilic systems [*e.g.*, Zheltonosova et al. (1994) and Mazauric et al. (1996)]. Therefore both AspRSs are specific for aspartic acid activation and $tRNA^{Asp}$ charging.

Because of the structural analogy between aspartic acid and asparagine and the particular interrelation between the tRNA aspartylation and asparaginylation systems in halophilic archeons where AspRS aspartylates $tRNA^{Asp}$ and $tRNA^{Asn}$ (Curnow et al., 1996), particular care was taken to exclude identification of AsnRS as one of the two AspRSs. Both AspRSs were distinguished from AsnRS by chromatography on DEAE-cellulose (AsnRS elutes after the AspRSs) and by kinetic analysis (asparagine is unable to promote ATP−[^{32}P]PP_i exchange catalyzed by both AspRSs). Finally, the N-terminal sequence of AsnRS from *T. thermophilus* (Seignovert et al., 1996) differs from those of AspRS1 and AspRS2, confirming that AsnRS was not characterized (Figure 6).

Interestingly, both thermophilic AspRSs share similar kinetic properties. The exceptional high apparent affinity of both for aspartic acid in tRNA charging is surprising. It cannot be related to a kinetic effect of tRNA since this substrate is without appreciable effect on the K_M's for aspartic acid in ATP−PP_i exchange (Table 2). It could indicate an unusual high intrinsic affinity of the synthetase for aspartic acid, contrasting with the lower one of the other known AspRSs; however, it could also be related to the accumulation of the enzyme-bound aspartyladenylate intermediate as a consequence from rate-limiting step following amino acid activation. The increased K_M's for ATP compared to those for amino acid suggest in this case stimulation of the rate

limiting step of tRNA charging by ATP via a process independent from amino acid activation.

The thermodynamic parameters for aspartylation establish further the functional specificity of both thermophilic AspRSs. Indeed, similar equilibrium constants and standard free energy changes govern tRNA aspartylation by both thermophilic and by mesophilic AspRSs. Also, the activation energies for amino acid activation and tRNA charging and the enthalpy and entropy changes in tRNA aminoacylation of both thermophilic systems equal those of the mesophilic systems. The breaking of the Arrhenius plots in thermophilic systems at high temperatures, denaturing mesophilic synthetases, evidence a decrease of the activation energy for ATP−PP_i exchange and tRNA charging as a probable consequence from the displacement of the rate-limiting steps. Increasing temperatures may displace the equilibrium of amino acid activation and the rate-limiting step of tRNA aminoacylation from trans-conformations of the enzyme−tRNA complex toward end-product dissociation.

Species Specificity of the Thermophilic AspRSs for tRNA Aspartylation

Both thermophilic AspRSs are equally competent for charging either native $tRNA^{Asp}$ from *E. coli* and *T. thermophilus* or their transcripts (Table 4), indicating that the post-transcriptional modifications in *T. thermophilus* $tRNA^{Asp}$ (Keith et al., 1993) are not involved in aspartylation and that the peculiar modifications in *E. coli* $tRNA^{Asp}$ (*e.g.*, Queuosine 34; Sprinzl et al., 1995), do not act as antideterminants in the thermophilic protein context. However, AspRS1 aspartylates unfractionated *T. thermophilus* tRNA more efficiently than does AspRS2 by more specifically binding the cognate tRNA (Table 4), whereas the two AspRSs aminoacylate *E. coli* tRNA with similar efficiency since heterologous non-cognate tRNAs compete for binding to both (Table 4). This agrees with the decreased contribution of AspRS1 in a crude extract for charging unfractionated tRNA from *E. coli* compared to that from *T. thermophilus* (18% and 85%, respectively; Figure 4A and B).

The poor efficiency of AspRS2 compared to AspRS1 for charging yeast $tRNA^{Asp}$ suggests specificity of this synthetase for prokaryotic tRNA which could be related to structural elements in eukaryotic $tRNA^{Asp}$ acting as antideterminants in the protein context [different size of the variable region (five nucleotides in prokaryotes and four in eukaryotes) or different post-transcriptional modifications or particular nucleotides such as the two first base pairs in the acceptor stem (U1−A72, C2−G71 in eukaryotes and G1−C72, G2−C71 in prokaryotes; Sprinzl et al., 1995)]. Interestingly, the behavior of AspRS2 resembles that of *E. coli* AspRS since it is unable to efficiently aminoacylate yeast $tRNA^{Asp}$, whereas AspRS1 resembles yeast AspRS which efficiently aminoacylates both yeast and *E. coli* $tRNA^{Asp}$. This suggests a different functional interrelation of AspRS1 and AspRS2 with eukaryotic AspRSs. Species specificity of *E. coli* AspRS is determined by the two first base pairs of the tRNA acceptor stem since their substitution in yeast $tRNA^{Asp}$ by the prokaryotic sequence restaures efficient charging by *E. coli* AspRS (Moras et al., 1995). However, species specificity of AspRS2 is excluded since the synthetase aminoacylates efficiently mammalian $tRNA^{Asp}$ possessing, like yeast $tRNA^{Asp}$, U1−A72 and C2−G71 in the acceptor arm (Table 4). Finally, the capacity of both thermophilic AspRSs to charge mammalian and prokaryotic $tRNA^{Asp}$ with efficiencies differing by less than 1 order of magnitude establishes the absence of species specificity of both in tRNA aminoacylation (Table 4).

Involvement of AspRS1 and AspRS2 in tRNA Aspartylation in T. thermophilus

The existence of two AspRSs could be related to the presence of isoaccepting $tRNA^{Asp}$ assuming aminoacylation by each of distinct $tRNA^{Asp}$ species. This interpretation is ruled out since this thermophile, like other prokaryotes and eukaryotes, contains only one $tRNA^{Asp}$ isoacceptor (Keith et al., 1993). Sequencing of this tRNA revealed incomplete post-transcriptional modifications, particularly of positions conserved in other *T. thermophilus* tRNAs and assumed to reinforce their thermal stability (Gm18, s^2T54, and m^1A58; Horie et al., 1985). This peculiarity was described for other tRNAs from *T. thermophilus* and was related to growth conditions of the bacteria since the extent of modifications increases with temperature and becomes optimal only at high temperature when activities of the modifying enzymes are optimized (Horie et al., 1985). Each AspRS would be specific for aminoacylation of either hypomodified or extensively modified tRNAs and ensure tRNA aspartylation at high and low temperatures, respectively. But such a role is excluded since each AspRS aminoacylates efficiently unmodified tRNA (Table 4). The balance of their activity as a function of temperature is further excluded by the increased competence of AspRS1 compared to AspRS2 for aspartylation of unfractionated tRNA along the temperature range (80-fold at 37 °C and 100-fold at 70 °C). These properties tend to minimize considerably the involvement of AspRS2 in aspartylation *in vivo*, although under physiological conditions the difference in competence of both may be attenuated. Indeed, competing non-cognate tRNAs, which decrease the contribution of AspRS2 to aspartylation by binding on the synthetase, are complexed mainly by their cognate synthetases, and the 4-fold excess of AspRS2 over AspRS1 in cellular extracts partly compensates the discrepancy of their charging efficiency.

Finally, quantification of AspRS1, AspRS2, and $tRNA^{Asp}$ in *T. thermophilus* and investigation of the involvement of both synthetases in aspartylation under conditions approaching the physiological ones indicate that *in vivo* aspartylation is mainly ensured by AspRS1. 1 kg of cells contains about 680 nmol of $tRNA^{Asp}$ (1 μM) and 270 and 1200 nmol of AspRS1 and AspRS2 subunits (0.4 and 1.8 μM), indicating that the binding sites of AspRS exceed the number of $tRNA^{Asp}$ molecules. Taking into account the decreased affinity of AspRS2 for $tRNA^{Asp}$ in unfractionated tRNA, $tRNA^{Asp}$ will essentially saturate AspRS1 whereas AspRS2 will be mainly in the free state or complexed to noncognate tRNAs and thus will not be strongly involved in aspartylation. This view agrees with the poor contribution of AspRS2 in a crude extract to aspartylation of unfractionated tRNA (15%), although it exceeds AspRS1 4-fold (Figure 4A).

Physiological Significance of the Two AspRSs in T. thermophilus and of aaRSs in Other Organisms

The aspartylation system from *T. thermophilus* constitutes the first example of the presence of two aaRSs of the same

specificity in an organism adapted to extreme life conditions. So far such peculiarity was reported for only a few mesophilic organisms. But in contrast to these systems where the two enzymes are differently regulated and only one is constitutive, the two thermophilic AspRSs are expressed under normal growth conditions. *E. coli* encodes two LysRSs sharing 88% identity (Lévêque et al., 1990; Clark & Neidhardt, 1990); the *lysS* product is constitutive, whereas the *lysU* product, induced under harsh conditions such as heat shock, anaerobiosis, or low pH (Neidhardt and VanBogelen, 1981; Lévêque et al., 1991), is coordinately expressed with components exerting diverse functions in adaptation (Nakamura & Ito, 1993). *B. subtilis* encodes two ThrRSs sharing 51% identity (Putzer et al., 1990), each being sufficient for normal cell growth and sporulation. But during vegetative growth only the *thrS* gene is expressed, while the *thrZ* gene is induced when the level of charged tRNA decreases after Thr starvation. Both genes are coordinately expressed and regulated by a transcriptional antitermination mechanism (Putzer et al., 1992). Similar characteristics were reported for expression and regulation of the two TyrRSs from *B. subtilis* which share 27% identity (Glaser et al., 1990; Henkin et al., 1992). Finally *Staphylococcus aureus* contains two IleRSs encoded by chromosomal and plasmidic genes. Product of the plasmidic gene confers strong resistance to mupirocin since only the chromosomal gene is present in susceptible strains and in strains expressing intermediate resistance levels (Gilbart et al., 1993). Like these synthetases, the two AspRSs can provide Asp-tRNA for protein synthesis in *T. thermophilus*, but in contrast to them both are expressed in a constant ratio along the growth cycle of the bacteria and are present under normal growth conditions.

The dispensable nature of AspRS2 for tRNA aspartylation addresses the question about its function. Many examples show that synthetases are involved in functions other than tRNA aminoacylation [*e.g.*, synthesis of nucleotides polyphosphates (Rapaport et al., 1981; Plateau et al., 1981), splicing of introns of groups I and II in mitochondria (Akins & Lambowitz, 1987; Herbert et al., 1988; Caprara et al., 1996), interferon-γ response in mammalians (reviewed by Kisselev (1993)] or in conversion of amino acids acylating tRNAs to provide aatRNAs essential for protein synthesis [tRNA selenocysteinylation (Baron & Böck, 1995), tRNA glutaminylation in Gram-positive eubacteria, the Gram-negative bacterium *Rhizobium meliloti*, cyanobacteria, archaebacteria, and organelles (Wilcox & Nirenberg, 1968; White et al., 1972; Schön et al., 1988; Gagnon et al., 1996), and tRNA asparaginylation in *Haloferax volcanii* (Curnow et al., 1996)]. Also aatRNAs can participate to physiological processes other than protein synthesis [chlorophyll biosynthesis in higher plants and in photosynthetic bacteria (Schön et al., 1988) or cell wall peptidoglycane synthesis in various species from genus *Staphylococcus* involving, in addition to canonical tRNAs, peculiar species not involved in protein synthesis (Petit et al., 1968; Stewart et al., 1971)]. For instance, the *T. thermophilus* cell wall contains peptidoglycanes (Quintela et al., 1995) so that part of the aatRNAs may be deviated from protein synthesis, but to date there is no evidence for involvement of Asp-tRNA or one of the AspRSs in this process. The present study suggests that in *T. thermophilus* an AspRS distinct from that providing Asp-tRNA for protein synthesis may be involved in such a particular cellular function. It remains unclear, however, whether the function of this AspRS is related to tRNA aspartylation or to Asp activation or involves solely the free enzyme. *T. thermophilus* contains a plasmid encoding components of essential cellular functions (Eberhard et al., 1981; Tabata et al., 1993). The chromosomal or plasmidic localization of AspRS2 gene and characterization of the peculiar function of its product require further investigations. The indispensable and dispensable natures of respectively AspRS1 and AspRS2 might unambiguously be established by analysis of *T. thermophilus* strains deficient of each of these enzymes. However, such strains do not exist, and more generally genetic tools to study *T. thermophilus* have not yet been developed. Such developments might be difficult since, by analogy, attempts to create *E. coli* strains deficient in AspRS have failed.

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