Thermus thermophilus Contains an Eubacterial and an Archaebacterial Aspartyl−tRNA Synthetase†,‡

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Thermus thermophilus Contains an Eubacterial and an Archaebacterial Aspartyl-tRNA Synthetase[†,‡]

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ABSTRACT: *Thermus thermophilus* possesses two aspartyl-tRNA synthetases (AspRSs), AspRS1 and AspRS2, encoded by distinct genes. Alignment of the protein sequences with AspRSs of other origins reveals that AspRS1 possesses the structural features of eubacterial AspRSs, whereas AspRS2 is structurally related to the archaebacterial AspRSs. The structural dissimilarity between the two thermophilic AspRSs is correlated with functional divergences. AspRS1 aspartylates $tRNA^{Asp}$ whereas AspRS2 aspartylates $tRNA^{Asp}$, and $tRNA^{Asn}$ with similar efficiencies. Since Asp bound on $tRNA^{Asn}$ is converted into Asn by a tRNA-dependent aspartate amidotransferase, AspRS2 is involved in Asn-$tRNA^{Asn}$ formation. These properties relate functionally AspRS2 to archaebacterial AspRSs. The structural basis of the dual specificity of *T. thermophilus* $tRNA^{Asn}$ was investigated by comparing its sequence with those of $tRNA^{Asp}$ and $tRNA^{Asn}$ of strict specificity. It is shown that the thermophilic $tRNA^{Asn}$ contains the elements defining asparagine identity in *Escherichia coli*, part of which being also the major elements of aspartate identity, whereas minor elements of this identity are missing. The structural context that permits expression of aspartate and asparagine identities by $tRNA^{Asn}$ and how AspRS2 accommodates $tRNA^{Asp}$ and $tRNA^{Asn}$ will be discussed. This work establishes a distinct structure−function relationship of eubacterial and archaebacterial AspRSs. The structural and functional properties of the two thermophilic AspRSs will be discussed in the context of the modern and primitive pathways of tRNA aspartylation and asparaginylation and related to the phylogenetic connexion of *T. thermophilus* to eubacteria and archaebacteria.

INTRODUCTION

Synthesis of functional proteins relies to accurate aminoacylation of tRNAs by aminoacyl-tRNA synthetases (aaRSs).[1] Most organisms possess 20 aaRSs, a particular one for each of the 20 natural amino acids involved in protein synthesis and each of the 20 families of tRNA isoacceptors (*1*−*4*). Each synthetase aminoacylates the cognate isoaccepting tRNAs with the homologous amino acid. However, synthetases can mischarge tRNAs (*5*−*7*). Not absolute specificity of particular aaRSs is compensated by correction processes hydrolyzing wrong end-products (*5*−*8*). The functional unicity between aaRSs, tRNAs, and amino acids and proofreading processes exerted by some synthetases provides the aminoacyl-tRNAs (aatRNAs) with enough accuracy to ensure high fidelity in translation and survival of the cell. However, exceptions of the rule of unicity of the aminoacylation systems were reported.

First, various organisms are lacking synthetases. The homologous aatRNA is then formed by conversion of the amino acid mischarged on tRNA by a noncognate aaRS. $Gram^+$ bacteria, the $Gram^-$ bacterium *Rhizobium meliloti*, cyanobacteria, archaebacteria, mitochondria and chloroplasts are deprived of GlnRS (*9*−*11*). Gln-$tRNA^{Gln}$ is then synthesized by ω-amidation of glutamic acid mischarged on $tRNA^{Gln}$ by GluRS (*12*−*16*). A similar mechanism provides Asn-$tRNA^{Asn}$ in archaebacteria by converting Asp mischarged on $tRNA^{Asn}$ into Asn and substitutes the missing

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[‡] The nucleotidic sequences of Asp-tRNA synthetases 1 and 2 of *T. thermophilus* HB8 reported in this paper are deposited on the EMBL data bank and are accessible under the numbers X70943 and AF219996, respectively.

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[1] Abbreviations: aaRS, aminoacyl-tRNA synthetase; the three letter code is used for amino acids, e.g. Asp for aspartic acid and AspRS for aspartyl-tRNA synthetase, except in protein sequences where the one letter code is used; aatRNA, aminoacyl-tRNA; βD-cellulose, benzoyl-diethylaminoethyl-cellulose; DEAE-cellulose, diethylaminoethyl-cellulose; DIFP, diisopropylfluorophosphate; EDTA: ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethane sulfonic acid; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; *tac* promoter, tryptophane-lactose promoter.

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AsnRS (*17*−*20*). The tRNA-dependent formations of Gln and Asn differ from direct biosynthetic pathways used by most other organisms, but resemble tRNA selenocysteinylation in the various phylae in which Ser mischarged on $tRNA^{Sec}$ by SerRS is converted into Sec (*21*).

Second, in a few cases two aaRSs encoded by distinct genes coexist and provide identical aatRNAs. *Escherichia coli* contains two LysRSs that are 88% in similarity (*22*−*25*), *Bacillus subtilis* contains two Thr- and two TyrRSs that are 51 and 27% in similarity, respectively (*26*−*28*), and *Staphylococcus aureus* strains resistant to mupiricin, an analogue of Ile, express two IleRSs (*29, 30*). The exceptional character of these duplications is manifested by the absence of their conservation along the evolutionary scale, and when compared to the other organisms studied thus far, these synthetases are unique. The functional significance of the duplication could be evidenced only in particular cases where both enzymes possess distinct properties or are differently expressed along the growth cycle of the organism. In *E. coli*, the *lysS* product is constitutive, whereas the *lysU* product is induced only under harsh conditions such as heat shock, anaerobiosis, or low pH (*31, 32*) and is coordinately expressed with components exerting diverse functions in adaptation (*33*). The two *thrS* genes of *B. subtilis* are coordinately expressed and regulated by a antitermination mechanism. Each ThrRS is sufficient for normal cell growth and sporulation, but only the *thrS* gene is expressed during vegetative growth while the *thrZ* gene is induced when the level of charged tRNA decreases after Thr starvation (*34*). Similar characteristics govern expression and regulation of the two TyrRSs from *B. subtilis* (*28, 34*). Finally, the genes encoding the two IleRSs of *S. aureus* are one of chromosomal and the other one plasmidic, but only the plasmidic product confers resistance to mupirocin (*29, 30*).

In a previous report, we described the existence in the thermophilic eubacterium *Thermus thermophilus* of two AspRSs, which activate Asp and charge $tRNA^{Asp}$ with similar kinetic constants and thermodynamic parameters (*35*). This duplicated system differs from those described earlier by its physiological implication, since the two AspRSs are expressed in a constant ratio along the growth of the bacterium, excluding involvement of each one in particular stages of the growth cycle. It is shown here that the two enzymes differ by their specificity in tRNA charging and that AspRS1 aminoacylates $tRNA^{Asp}$, whereas AspRS2 aminoacylates $tRNA^{Asn}$ in addition to $tRNA^{Asp}$. Asp mischarged on $tRNA^{Asn}$ is then converted into Asn by a tRNA-dependent amidotransferase. Since dual specificity of AspRS constitutes an archaebacterial character (*17*−*20*), we investigated the phylogenetic origin of the thermophilic AspRSs by comparing their sequences to those of AspRSs from various phylae and showed that AspRS1 is structurally related to the eubacterial AspRSs and AspRS2 to the archaebacterial ones. Further, $tRNA^{Asn}$ from *T. thermophilus*, being aspartylated by AspRS2 and asparaginylated by AsnRS, constitutes a rare exemple of coexpression in vivo of two distinct identities by a given tRNA. The elements defining aspartate and asparagine identities have been characterized in tRNAs of various origins (*36*−*38*), but the structural context that allows efficient aspartylation and asparaginylation of $tRNA^{Asn}$ remains unknown. We investigated the structural peculiarities defining the dual identity in *T. thermophilus* $tRNA^{Asn}$. Analysis of its sequence does not reveal important variations with $tRNA^{Asn}$ exhibiting strict asparagine specificity. It is shown that *T. thermophilus* $tRNA^{Asn}$ contains the elements common to aspartate and asparagine identities, whereas minor elements involved in aspartate identity are absent. How both identities are coexpressed in the same structural context and absence of discrimination between $tRNA^{Asp}$ and $tRNA^{Asn}$ by AspRS2 will be discussed. Altogether, this report demonstrates a peculiar structure−function relationship in eubacterial and archaebacterial AspRSs and establishes the coexistence in *T. thermophilus* of two phylogenetically distinct pathways of tRNA aspartylation and asparaginylation, one archaebacterial and the other one eubacterial, probably related to ancestral and modern aminoacylation pathways.

EXPERIMENTAL PROCEDURES

Enzymes and Chemicals. Restriction endonucleases, T4 DNA ligase, and the Digoxygenin DNA Labeling and Detection Kit were from Boehringer, RNAse A was from Worthington, and T4 polynucleotide kinase from New England Biolabs. T7 DNA polymerase, exonuclease III, nuclease S1, and unlabeled 2′- and 2′,3′-deoxyribonucleotides triphosphates were from Pharmacia. Bovine alkaline phosphatase and isopropyl-thio-β-D-galactoside were from Appligene, and the Ampli Taq DNA Amplification Kit from Perkin-Elmer. The oligodeoxyribonucleotides were synthesized on an Applied Biosystem 381A and purified by high-pressure liquid chromatography. L-[^{14}C]Asp (220 mCi mmol^{-1}) was from Commissariat à l'Energie Atomique (Saclay), L-[^{3}H]Asn (20 Ci mmol^{-1}), α and γ[^{32}P]ATP (2000 Ci mmol^{-1}) and α[^{35}S]dATP (400 Ci mmol^{-1}) were from Amersham. Diethylaminoethyl-cellulose (DEAE-cellulose)1 (DE 52) and phosphocellulose (P 11) were from Whatman, hydroxyapatite (HTP) from Biorad, and Ni^{2+}-nitrilotetraacetic acid Sepharose from Qiagen. Diisopropylfluorophosphate (DIFP)1, pepstatin, bestatin, and E-64 (trans-epoxysuccinyl-L-leucylamido-(4-guanido) butane) were from Sigma and Pefablock-SC (4-(2-aminoethyl)-benzenesulfonyl fluoride) from Pentapharm (Basel). Unfractionated tRNAs (Asp acceptance 0.5−1.5 nmol mg^{-1}) from *E. coli* and yeast were from Boehringer and that from *T. thermophilus* was prepared by DEAE-cellulose chromatography of bulk RNA obtained by phenol extraction of the cells; the tRNAs eluted at 1.5 M NaCl.

Strains, Plasmids and Bacteriophages. T. thermophilus genomic DNA was isolated from HB8 strain (ATCC 27634). *E. coli* strains JM103 and DH5α were used for preparation of recombined pUC18 and pUC19. BL21 (DE3)/pET 3-1 (T7 RNA polymerase promoter) and DH5α/pKK223 (*tac* promoter) were the host/vector systems for expression of AspRS1 and AspRS2.

Bacterial Growth, DNA Isolation, and Manipulation. T. thermophilus cells were grown as described (*39*) and genomic DNA isolated using standard methods (*40*). *E. coli* strains were grown on Luria Bertani or 2YT media supplemented when necessary with 200 mg·L^{-1} of ampicillin. *E. coli* transformation, maxi and minipreparations, of double-stranded DNA, DNA manipulations and agarose-gel electrophoresis were conducted using standard procedures (*40*).

Cloning of the aspS Genes. The DNA probes were obtained by PCR amplification using 0.5 μg of *T. thermophilus* DNA and 100 pmol of degenerated sense and antisense primers designed on the basis of the amino acid sequences from residues 1 to 9 and 43 to 52 for AspRS1, 5′GGAATTCATGCG(G/C)CG(G/C)AC(C/G)CACTACGC(G/C)GG(G/C)(A/T)(G/C)C3′ and 5′GGAATTC(C/G)GGGTG(G/C)GC(G/C)AC(C/G)AGCTG(G/C)AC(C/G)AG(G/C)CCCTC3′, and from residues 1 to 10 and 27 to 36 for AspRS2, 5′GGAATTCATGCG(G/C)G T(G/C)CT(C/G)GT(G/C)CG(G/C)GACCT(C/G)AAGGC(G/C)CAC3′ and 5′GGAATTCGTC(C/G)AGGTC(A/G)AACTG(A/G)AT(G/C)CG(G/C)CC(C/G)AGGTC3′, extented at the 5′ ends by *Eco*R1 sites. The amplified fragments were isolated by agarose-gel electrophoresis, digested with *Eco*RI, ligated in pUC18, controlled by sequencing after *E. coli* transformation, and finally, labeled with digoxigenin by random priming or with α[^{32}P]dATP by nick translation (*40*).

Southern hybridizations were performed on *Bam*HI, *Kpn*I, and *Pst*I digests of genomic DNA fractionated by electrophoresis on 0.8% agarose gel using the probe labeled with digoxigenin. The fragments of the size of that hybridized to the probe (*Bam*HI fragments of 4−5 kbp for *aspS1* and 3−4 kbp for *aspS2*, and *Pst*I fragments of 4.5−5.5 kbp for *aspS2*), were eluted from gel, cloned in pUC18, and used to construct minilibraries in DH5α. The clones containing the 5′-coding ends of the genes were identified by colony hybridization using the probes labeled with α[^{32}P]dATP (*40*).

Sequencing of the aspS Genes. The non coding strand of the *aspS1* gene was sequenced with the universal primer, using a collection of fragments of decreasing length obtained by recurrent degradation of the 4.5 kbp *Bam*H1 fragment cloned in pUC18. The recombined vector, linearized by *Xba*I and *Pst*1 digestions, was digested starting from the 3′ recessing *Xba*1 end with exonuclease III and nuclease S1 and analyzed after increasing time intervals by electrophoresis on 1% agarose gel. The vectors recombined with fragments decreasingly shortened by 150−200 nucleotides were circularized and amplified by transformation of DH5α cells before sequencing. The coding strand was sequenced with 11 synthetic primers distributed along the gene.

The first part of the *aspS2* gene, including the 31 first codons, was sequenced on both strands with the universal primer in the 3.5 kbp *Bam*HI fragment cloned in both orientations in pUC 18. The second part of the gene was sequenced in restriction fragments excized from 5.5 kbp long *Pst*I fragment containing the full ORF and subcloned in pUC18 and pUC19. The vectors recombined with the *Bam*HI−*Bam*HI, *Bam*HI−*Kpn*I, and *Kpn*I−*Kpn*I fragments (0.7, 0.3, and 0.8 kbp), covering the ORF downstream the 31 first codons, were linearized by *Xba*I and either *Pst*I or *Kpn*I digestions, and fragments decreasingly shortened by 150−200 nucleotides starting from the *Xba*I end were obtained as described above. Sequencing was conducted with the universal or universal reverse primer, using the dideoxy chain termination technique (*41*). Compressions were solved by replacing dGTP by dITP in the sequencing mix. To verify whether the full ORF of *aspS2* was sequenced, the nucleotides upstream and downstream from the adjacent restriction fragments were sequenced in the *Pst*I fragment using synthetic primers.

Strains Overproducing Asp-tRNA Synthetases 1 and 2. Appropriate restriction sites were created upstream and downstream from the *aspS1* gene according to Kunkel (*42*) on single-stranded M13mp18 recombined with the 4.5 kbp *Bam*HI fragment. For cloning in the pET3-1 vector, the *Nde*I site was created upstream from ORF with oligonucleotide 5′GAATSSGGTAAGCTTGGGAGG*CATATG*CGTCGCACCCACTACGC CGGAA3′. For cloning in the PKK223 vector, *Eco*RI and *Hind*III sites were created, upstream and downstream, respectively, from the ORF and the *Hind*III site upstream from the start codon, was suppressed using oligonucleotides 5′GGGTGAGAATCCGGT AAGCATGG*GGAATT*CATGCGTCGCACCCACTACGCCGGAAGCCTG3′ and 5′TCATG GTGGTCCGGCCATGAAGC*AAGCTT*CCGGGTACCGAGCTCGAATTCG3′. The designed restriction sites (in italics) were controlled by sequencing. The *Nde*I−*Bam*HI and *Eco*RI−*Hind*III fragments were excized from recombined M13mp18, respectively, cloned in pET3-1 and pKK223 vectors and the resulting vectors, pET*aspS1* and pKK*aspS1*, were used for transformation of *E. coli* strains BL21(DE3) and DH5α.

For cloning in expression vectors, the *aspS2* gene was amplified by PCR with the sense primer, 5′GGCTTAAGAAGGGGG*AATTCATATG*CGGGTACTGGTACG3′, creating the *Eco*RI and *Nde*I sites upstream from the ORF and the antisense primer, 5′AAGCCCGGGG*AGATCTGC*AGTTAGGGCGTGAGCCGGTGCCGG3′ creating the *Pst*I and *Bgl*II sites downstream from stop codon. The *Nde*I−*Bgl*II and *Eco*RI−*Pst*I fragments were excized by restriction digestion and inserted in pET3-1 and pKK223 vectors linearized respectively by *Nde*I and *Bam*HI and by *Eco*RI and *Pst*I digestions and the resulting vectors, pET*aspS2* and pKK*aspS2*, were used for transformation of the *E. coli* strains BL21(DE3) and DH5α.

Isolation of T. thermophilus tRNA^{Asp} and tRNA^{Asn} and Sequencing of tRNA^{Asn}. Fractions enriched in tRNA^{Asp} and tRNA^{Asn} were obtained by benzoyl-diethylaminoethyl-cellulose (BD-cellulose) chromatography of unfractionated tRNA, eluted with a gradient from 0.5 to 1.5 M NaCl in 50 mM sodium acetate buffer, pH 5.0. tRNA^{Asp} and tRNA^{Asn} elute at 0.8 and 1.1 M NaCl, respectively. Pure tRNA^{Asp} (37 nmol mg^{-1}) was obtained by additional anion exchange chromatographies (*43*). tRNA^{Asn} of sequencing grade was isolated by two-dimensional polyacrylamide gel electrophoresis (PAGE) of the enriched BD-cellulose fraction (10 and 20% polyacrylamide in 89 mM Tris-borate buffer, pH 8.3, and 2.5 mM ethylenediaminetetraacetic acid (EDTA), respectively under semi-denaturating conditions (4 M urea), as described (*44*). The tRNAs were revealed by toluidine blue staining, eluted by overnight soaking of the gel fragments in 0.5 M ammonium acetate buffer, pH 7.0, containing 10 mM magnesium acetate, 1 mM EDTA, and 0.1% SDS, and finally, deprived from last traces of polyacrylamide and urea by phenol extraction and chromatography on a 1 mL Sephadex G25 column. tRNA^{Asn} (40 nmol·mg^{-1}) used for kinetic analysis was purified from an enriched BD-cellulose fraction, by retention, and after aminoacylation by AsnRS, on a matrix substituted with Ni^{2+} nitriloacetic acid bound to the His-tagged *T. thermophilus* elongation factor Tu followed by elution as described (*45*). The aatRNA was deacylated by incubation 30 min at 37 °C in 1.5 M Tris-HCl buffer, pH 8.0.

For sequencing, the denatured $tRNA^{Asn}$ was submitted to single hit random hydrolysis by imidazole before 5′ labeling of the fragments with [^{32}P]. The major and the modified 5′-ending nucleotides were identified by mono- and two-dimensional thin-layer chromatography (*46*), respectively.

Preparation of Crude Protein Extracts and Purification of the Two Asp-tRNA Synthetases. Unless otherwise indicated, all steps were conducted at 4 °C with buffers containing 5 mM 2-mercaptoethanol, 0.1mM of each, DIFP, EDTA and Pefablock and 1 mM pepstatin A, bestatin, and E64.

The protein extract from *T. thermophilus* was prepared from 20 g cell paste suspended in a 40 mL extraction buffer (250 mM Tris-HCl, pH 8.0, and 1 mM $MgCl_2$). Cells were disrupted by shaking with glass beads (1:1, v/v) in a grinder (Vibrogen Zellmühle, type Vi 4, Edmund Bühler, Tübingen) under refrigeration and the lysate was centrifuged for 2 h at 105000*g*. After overnight dialysis against 50 mM potassium phosphate buffer, pH 7.2, the supernatant was loaded on a DEAE-cellulose column equilibrated with the dialysis buffer before elution of the proteins with 400 mM KCl. The extracts from *E. coli* strains overproducing the thermostable AspRSs were obtained by sonication (10 cycles of 20 s each at 45 V with an Ultrasons-Annemase apparatus type 250TS20K) of the cells harvested from 120 mL culture and suspended in 1 mL extraction buffer. After centrifugation of the lysate for 15 min at 5000g, the thermolabile proteins, including the endogeneous AspRS, were precipitated by 30 min incubation at 70 °C and sedimented at 5000g. The extracts were stored at −80 °C in the presence of 50 mM potassium phosphate buffer, pH 7.2, containing 50% glycerol.

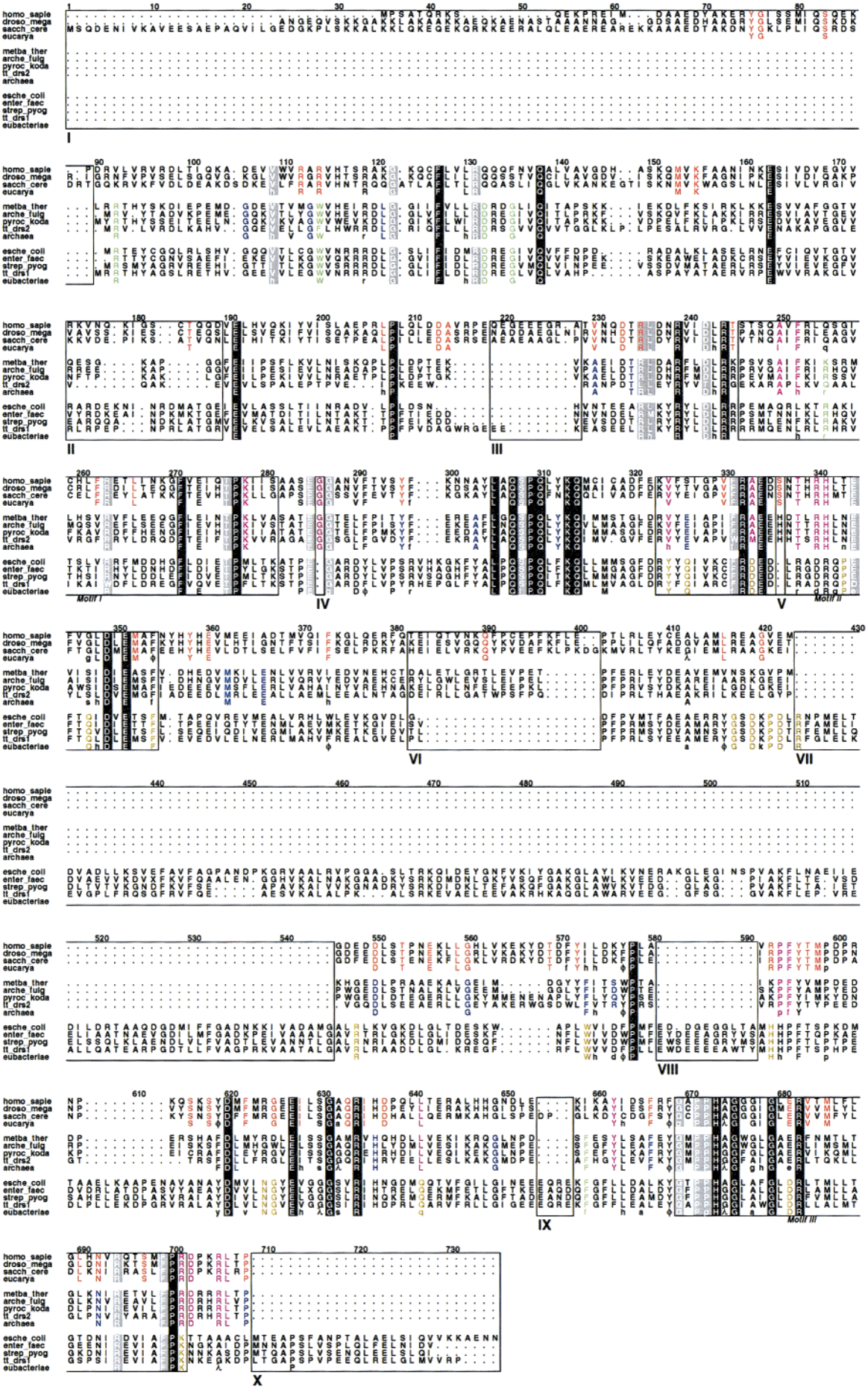
AspRS1 was purified from 50 g of overproducing cells suspended in 150 mL of 50 mM Tris-HCl buffer, pH 8.0, containing 10 mM $MgCl_2$. Fractions of 20 mL were submitted to 10 cycles of sonication of 30 s each at 100 V in ice, prior centrifugation of the lysate 3 h at 105000*g*. The supernatant, supplemented with 100 mM NaCl, was heated for 30 min at 70 °C, and the flocculated proteins sedimented by a 15 min centrifugation at 5000*g*. After a 2-fold dilution, the proteins were adsorbed on a hydroxyapatite column equilibrated with 20 mM potassium phosphate buffer, pH 6.8, and eluted with a linear gradient from 20 to 200 mM of this buffer. The active fractions, eluted at 140 mM salt, were dialyzed against 20 mM potassium phosphate, pH 7.2, and fractionated by DEAE-cellulose chromatography with a linear gradient from 20 (pH 7.2) to 200 mM (pH 6.8) potassium phosphate. AspRS1 eluted at homogeneity at 170 mM salt. AspRS2 was purified after heat treatment of the crude extract by DEAE-cellulose and phosphocellulose chromatographies. The active fractions, eluted on DEAE-cellulose at 140 mM potassium phosphate, were dialyzed, adsorbed on a phosphocellulose column equilibrated with 20 mM potassium phosphate buffer, pH 6.8, and the proteins eluted with a linear gradient from 0 to 0.5 M KCl. The enzyme eluted at homogeneity at 0.2 M KCl. The pure enzyme fractions were concentrated by filtration under N_2 pressure through a Amicon YM10 membrane and stored at −20 °C in 50 mM potassium phosphate buffer, pH 7.2, containing 50% glycerol. The purity of the AspRSs was checked by SDS−PAGE (*47*); protein concentrations in crude extracts were determined by UV absorption (*48*) and pure enzyme concentrations using the extinction coefficient at 280 nm (e = 66 689 and 59 900 M^{-1} cm^{-1} and E = 1.01 and 1.15 mL mg^{-1} cm^{-1}, respectively, for AspRS1 and AspRS2).

Activity and Kinetic Constants Measurements. The standard aminoacylation mixture contained 100 mM Na-HEPES, pH 7.2, 30 mM KCl, 2 mM ATP, 10 mM $MgCl_2$ (or 10 mM ATP and 12 mM $MgCl_2$ when crude protein extracts were tested), 0.05 mM L-[^{14}C]Asp (70 000 cpm $nmol^{-1}$), 4 mg mL^{-1} of unfractionated tRNA from yeast, *E. coli*, or *T. thermophilus* and 50−300 μg mL^{-1} of proteins or 1−10 μg mL^{-1} of pure yeast or *E. coli* AspRSs or *T. thermophilus* AspRS1 or AspRS2, diluted when necessary in 100 mM Na-HEPES, pH 7.2, 1 mg mL^{-1} bovine serum albumin, 5 mM 2-mercaptoethanol, 0.1 mM EDTA and DIFP, and 10% glycerol. The K_M's for ATP, Asp, and the tRNAs were determined from double reciprocal plots in the presence of 3−40 μM L-[^{14}C]Asp or 3−200 μM ATP or 0.02−10 μM *T. thermophilus* $tRNA^{Asp}$ or $tRNA^{Asn}$; saturating concentrations of the fixed substrates, except for measurements of the K_M's for tRNA where 8 μM (∼the K_M value) L-[^{3}H]Asp (3100 cpm $pmol^{-1}$) were present with 0.1−1 $\mu g \cdot mL^{-1}$ of AspRS1 or 0.2−2 $\mu g \cdot mL^{-1}$ of AspRS2. The k_{cat}'s were determined independently with saturating substrates concentrations. The [^{14}C]Asp-tRNA synthesized after 1−15 min incubation at 37 or 70 °C was determined in 40 μL aliquots as described previously (*49*).

RESULTS

Cloning of the Two Asp-tRNA Synthetases Genes from T. thermophilus. In a previous report, we demonstrated the existence of two Asp-tRNA synthetases in *T. thermophilus* (*35*). Fractionation of a crude protein extract from *T. thermophilus* HB8 by salting out chromatography results in the elution of two AspRSs: AspRS1 elutes at 28% and AspRS2 at 20% of saturation of ammonium sulfate. Estimation of the M_r and analysis of the oligomeric structure of both enzymes revealed homodimers with polypeptide chains of M_r 66 000 and 51 000 for AspRS1 and AspRS2, respectively. Sequencing of the N-terminal ends and of internal peptides obtained by BrCN cleavage of the chains showed different sequences excluding origin of the small AspRS by proteolytic cleavage of the larger one. Finally, the distinct origin of the two enzymes was confirmed by analysis of the effect of the antibodies directed against each one, which showed the absence of cross reactions, demonstrating that the two AspRSs are deprived of common epitopes. All these results argue that AspRS1 and AspRS2 are encoded by distinct genes.

From the N-terminal sequence of each AspRS, two degenerated oligodeoxyribonucleotides adapted from *T. thermophilus* restrained codon usage were designed by back-translation and used as primers to constitute two non degenerative probes by PCR amplification of the 5′-coding ends of the genes in genomic DNA. The probes derived from AspRS1 and AspRS2 hybridize to *Bam*HI fragments of 4.5 and 3.5 kbp, respectively. Localization of the starts of the ORFs by hybridization of the probes on fragments decreasingly deleted from one extremity and their orientation by sequencing, indicated that the first fragment contains the full *aspS1* gene, whereas the second one includes only the 31 first codons from *aspS2* gene. The full *aspS2* gene was then


homo_sapie
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sacch_cere
eucarya
metba_ther
arche_fulg
pyroc_koda
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Motif I
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Motif III

cloned in a 5.5 kbp *Pst*I fragment including two *Bam*H1 sites, located at 3.2 and 1.6 kbp, located from each end. Hybridization of the probe revealed start of the ORF in the 3.2 kbp *Pst*1−*Bam*H1 fragment. According to the size of the polypeptide chain (M_r 51 000), the ORF of ~1.3 kbp covers the adjacent 0.7 kbp *Bam*HI fragment and ends in the 1.6 kbp long *Bam*HI−*Pst*I fragment, between two *Kpn*I sites located at 0.3 and 1.1 kbp, respectively, from *Bam*HI end.

Nucleotide Sequence of the aspS1 and aspS2 Genes. The nucleotide sequences reveal ORFs of 1940 and 1269 nucleotides for the *aspS1* and *aspS2* genes, respectively. They are accessible on the EMBL data bank under the numbers X70943 and AF219996. The sequence of *aspS1* gene of *T. thermophilus* HB8 strain is identical to that of the *aspS* gene of *T. thermophilus* VK1 strain reported earlier (*50*), except two Glu codons that differ (GAG in HB8 and GAA in VK1). The Shine−Dalgarno sequences, 5′GGAGGA and 5′-AGGGGG, include nucleotides −7 to −2 and −13 to −8 upstream from the starting ATG of *aspS1* and *aspS2*, respectively. Both are complementary to six contiguous nucleotides near the 3′ end of *T. thermophilus* 16S RNA (5′UCCUUU, (*51*)). The codon usage reveals preference for codons ending by 3′ G or C (only 8% of the codons end by A or T, in contrast to 42, 65, and 53% in *E. coli*, human, and yeast *aspS* genes). This agrees with the high GC content in DNA of organisms of the *Thermus* species, which, by increasing the stability of codon−anticodon interactions, is probably involved in the adaptation of this thermophile to high temperatures. However, significant lower GC contents in more extreme thermophiles (52, 40, and 31% in *Pyrobaculum aerophilum*, *Thermotoga maritima*, and *Methanococcus jannaschii* (*18*, *19*, *52*) argue for implication of other mechanisms in adaptation of nucleic acids to high temperatures in these bacteria.

Protein Sequence and Amino Acid Composition of Asp-tRNA Synthetases 1 and 2. The *aspS1* and *aspS2* genes encode polypeptides of 579 and 422 residues and of calculated M_r of 660 29 and 483 29, respectively. AspRS2 constitutes the smallest AspRS so far sequenced. The amino acid composition of both proteins present characteristics found in other aaRSs from *T. thermophilus*, which distinguishes them from synthetases of other origins; they have low contents in Asn, His, and Cys (one residue in AspRS1 and none in AspRS2) and high contents in Pro and Gly. In addition, for amino acids of a given physicochemical character, residues with long side chains, which increase the hydrophobic interactions, are preferred (Glu, Gln, Thr, and Leu are preferred to Asp, Asn, Ser, and Ile, respectively) as well as residues that increase the hydrogen bonds (Arg is preferred to Lys). This property agrees with amino acid substitution rules shown to improve thermal stability of proteins (*53*−*55*). However, lack of these particularities in aaRSs from thermophilic archaebacteria such *M. jannaschii* and *A. fulgidus* (*18*, *20*) illustrates the diversity of the physicochemical parameters involved in thermal adaptation of proteins and points to an absence of common rules determining protein stability in thermophilic organisms.

Finally, it should be mentioned that the protein sequence and the 3D structure of *T. thermophilus* AspRS reported earlier (*56*, *57*) are those of AspRS1.

Alignment of Asp-tRNA Synthetases 1 and 2 from T. thermophilus with other Asp-tRNA Synthetases. Comparison of the protein sequences reveals only 29% of identity between AspRS1 and AspRS2. Further, similarities of AspRS1 with eubacterial AspRSs exceed those with archaebacterial ones, whereas AspRS2 presents more similarities with archaebacterial than with eubacterial AspRSs (e.g., 60 and 66% of identities of AspRS1 with *E. coli* and *H. influenzae* AspRSs, but only 46 and 57% with those from *Pyrococcus kodakaraensis* and *M. jannaschii* and ~44 and 33% of identities of AspRS2 with the archaebacterial and eubacterial AspRSs, respectively).

Alignment of AspRS1 and AspRS2 with the 54 AspRSs of known sequences (9, 7, 30, 3, 4, and 1 from eukaryotes, archaebacteria, eubacteria, mycoplasma, mitochondria, and chloroplast, respectively, Figure 1) shows the presence in both thermophilic enzymes of the three consensus motifs of class II synthetases. It shows also that residues inside and outside the motifs, essentially conserved in eubacterial AspRSs, are present in AspRS1, whereas those essentially conserved in archaebacterial AspRSs are present in AspRS2. Finally, comparison of the sequences of AspRSs of various origins with those of AspRSs of known 3D structures shows that the modular organizations of eubacterial and archaebacterial AspRSs are conserved in AspRS1 and AspRS2, respectively.

The Consensus Motifs. The alignment shows the presence in both AspRSs of the three motifs with the conserved residues of functional importance (Pro277 in motif 1, Arg332 and Glu334 in motif 2, and Gly675, Gly679, Glu/Asp681, Arg682, and Pro700 in motif 3, Figure 2). In addition, other residues conserved in most AspRSs are also present in AspRS1 and AspRS2, but with a few exceptions (Figure 2) e.g., in motif 1, Phe271 and Thr276 are also present in both AspRSs, whereas Gly270 and the positively charged Lys/Arg263 are substituted respectively by Trp in AspRS1 (W155) and by Asp in AspRS2 (Asp143), and Arg255 in eubacterial and in most archaebacterial AspRSs is substituted by Gln in AspRS2 (Gln128), a residue also present in eukaryotic AspRSs. In motif 2, Asp349, Glu351, and Phe354 in most AspRSs are also present in AspRS1 and in AspRS2. In contrast, Phe331 is substituted by Trp in AspRS2

FIGURE 1: Alignment of Asp-tRNA synthetases. The alignment of the 54 known AspRSs sequences is summarized; only three AspRSs from each phylum are reported. The consensus motifs 1, 2, and 3 are indicated, and regions I−X are described in the text. Residues in red, blue, and yellow characters are conserved in eukaryotic, archaebacterial, and eubacterial AspRSs, respectively. Residues in violet characters are conserved in prokaryotic AspRSs, and those in green characters in archaebacterial and eukaryotic AspRSs. Residues in white characters on black and gray are conserved in all AspRSs and in class II synthetases, respectively. The origin of the AspRSs and the Genbank accession numbers are as follows: homo sapie (*Homo sapiens*, P14868) droso mega (*Drosophila melanogaster*, AAD21582), sacch cere (*Saccharomyces cerevisiae*, P04802), metba ther (*Methanobacterium thermoautotrophicum*, AAB84732), arche fulg (*Archaeoglobus fulgidus*, O29342) pyroc koda (*Pyrococcus kodakaraensis*, Q52428), esche coli (*Escherichia coli*, P21889), enter faec (*Enterococcus faecalis*, TIGR contig 6609), strep pyog (*Streptococcus pyogenes*, WIT, ORF 1095), tt drs1 and tt drs2 are *T. thermophilus* AspRS1 and AspRS2; eucarya, archaea, and eubacteriae are the consensus sequences of eukaryotic, archaebacterial, and eubacterial AspRSs. The numbering is that from alignment.

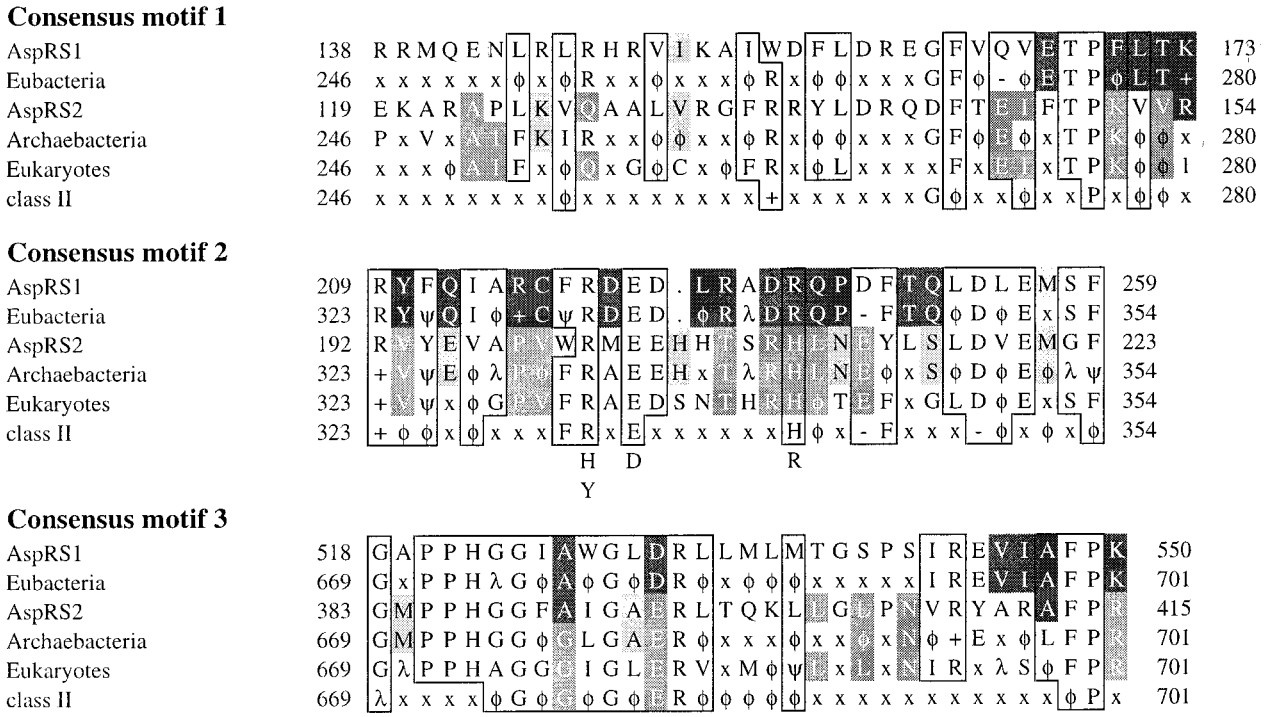

FIGURE 2: Alignment of the three consensus motifs of eubacterial, archaebacterial, and eukaryotic AspRSs, *T. thermophilus* AspRS1 and AspRS2, and consensus sequence of class II synthetases. The numbering is that from alignment except for *T. thermophilus* AspRS1 and AspRS2. Residues in white characters on black are conserved in eubacterial AspRSs and in *T. thermophilus* AspRS1; residues in black characters on dark gray are conserved in archaebacterial and/or eukaryotic AspRSs and in *T. thermophilus* AspRS2; residues in black characters on clear gray are conserved in archaebacterial AspRSs and in AspRS2. Conserved residues are present at least in 80% of the sequences. Enframed residues are conserved in AspRSs or in class II synthetases: x, variable residues; +, basic residues; −, acidic residues. λ, ψ, and ϕ are residues with small, aromatic, and hydrophobic side chains, respectively.

(Trp200). In motif 3, Gly669 and Phe699, conserved in most AspRSs, are present in AspRS1 and AspRS2 as well as the strongly conserved sequence PPH673 (Figure 2). In semi-conserved sequences from motifs, residues mostly conserved either in eubacterial or in archaebactarial AspRSs are present in AspRS1 and in AspRS2 (Figure 2), respectively, e.g. in motif 1, Glu275 in eubacterial AspRSs is present in AspRS1, whereas Lys278 in archaebacterial AspRSs is present in AspRS2; the sequence of motif 2 in AspRS1 is highly similar to that in eubacterial AspRSs but diverges from those in archaebacteria, whereas the sequence of AspRS2 presents much more similarities with the archaebacterial than with the eubacterial ones. In motif 3, Ala677 in most eubacterial AspRSs and in AspRS1 is essentially a Gly residue in most archaebacterial AspRSs and in AspRS2; Asp681 and Lys701 conserved in eubacterial AspRSs and in AspRS1 are substituted by Glu681 and Arg701 in most archaebacterial AspRSs and in AspRS2, respectively. Finally, comparison of the consensus sequences of the 3 motifs show important similarities between the eukaryotic and the archaebacterial sequences, which diverge from eubacterial ones (Figure 2).

The Overall Sequences. When the residues essentially conserved in the AspRSs from each phylum are compared with the homologous ones in AspRS1 and AspRS2, it appears that various residues outside of the consensus motifs, prevailing in eubacterial AspRSs, are also present in AspRS1, whereas those prevailing in archaebacterial AspRSs are present in AspRS2 (not shown). Further, residues semi-conserved in archaebacterial AspRSs are present in eukaryotic AspRSs, but differ in the eubacterial ones. Table 1 shows conserved and nonconserved residues of *Saccharomyces cerevisiae*, *E. coli*, and *P. kodakaraensis* AspRSs and of *T. thermophilus* AspRS1 involved in aspartyl-adenylate recognition and the interacting substrate groups revealed by the 3D structures of the AspRSs complexed with the activated aspartate. It appears that the conserved residues in the various AspRSs exert identical functional roles. Table 1 shows the predicted role of the conserved residues of *T. thermophilus* AspRS2 in aspartyl-adenylate recognition.

The Structural Organization of Asp-tRNA Synthetases 1 and 2. Alignment of AspRS polypeptide chains and comparison with their known 3D structures show four regions in the sequences that define structural domains: (*i*) The N-terminal region (residues 1−218) covering the anticodon-binding domain and the hinge domain, (*ii*) the adjacent region (residues 219 to 423) covering consensus motifs 1 and 2 from the catalytic core, (*iii*) the strictly prokaryotic region (residues 424−545), and (*iv*) the C-terminal region covering the consensus motif 3 and the prokaryotic extension (residues 546−735). A detailed analysis of this alignment shows insertions and deletions along the sequences of the particular AspRSs, confined in 10 regions (I to X), which allows partition of the AspRSs into three groups. Without exception, this structural partition coincides with the phylogenic distribution of the AspRSs. The first group comprises the eubacterial AspRSs and includes AspRSs from mycoplasma and from organelles, the second group comprises the eukaryotic AspRSs, and the third group is made up of the archaebacterial ones (Figure 1). The structural features characterizing eubacterial and archaebacterial AspRSs are found in AspRS1 and AspRS2 (Figure 1), respectively.

Table 1: Protein Groups of Asp-tRNA Synthetases of Various Origins Contacting the Substrate Groups from Aspartyl-adenylate[a]

position and motifs from alignment	Asp-tRNA synthetases									
	S. cerevisiae		*E. coli*		*T. thermophilus* AspRS1		*P. kodakaraensis*		*T. thermophilus* AspRS2	
	Renz	R_{sub}	R_{enz}	R_{sub}	R_{enz}	R_{sub}	R_{enz}	R_{sub}	R_{enz}	R_{sub}
285[b]	E_{281}	$A_{76\ N6}$	E_{171}	Asp_{NH3+}	E_{177}	Asp_{NH3+}	E_{170}	Asp_{NH3+}	E_{158}	Asp_{NH3+}
310	**Q_{303}**	$Asp_{O\delta2}$	**Q_{195}**	Asp_{NH3+}	**Q_{201}**	$Asp_{O\delta2,\ NH3+}$	**Q_{192}**	Asp_{NH3+}	**Q_{180}**	Asp_{NH3+}
313	K_{306}	$Asp_{O\delta2}$	K_{198}	$Asp_{O\delta2}$	K_{204}	$Asp_{O\delta2}$	K_{195}	$Asp_{O\delta2,\ NH3+}$	K_{182}	$Asp_{O\delta2}$
332[c]	**R_{325}**	$P\alpha_O$	R_{217}	Asp_O, $P\alpha_O$	**R_{223}**	Asp_O, $P\alpha_O$	**R_{214}**	Asp_O, $P\alpha_O$	**R_{201}**	Asp_O, $P\alpha_O$
334[c]	**E_{327}**	$C_{74\ O2'}$	**E_{219}**	Ad_{N6}	**E_{225}**	Ad_{N6}	**E_{216}**	Ad_{N6}	**E_{203}**	Ad_{n6}
340[c]	R_{333}		D_{224}		D_{230}		R_{222}	*$P\gamma_O$*	R_{209}	*$P\gamma_O$*
341[c]	H_{334}	$C_{75\ O2,\ O2'}$	R_{225}		R_{231}		H_{223}	*$P\gamma_O$*	H_{210}	*$P\gamma_O$*
342[c]	M_{335}	$Ad_{N1,\ N6}$	Q_{226}	$Ad_{N1,\ N6}$	Q_{232}	$Ad_{N1,\ N6}$	L_{224}	$Ad_{N1,\ N6}$	L_{211}	$Ad_{N1,\ N6}$
345[c]	**F_{338}**	Ad	F_{229}	Ad	**F_{235}**	Ad	A_{227}	Ad	Y_{214}	Ad
347[c]	G_{340}		Q_{231}	Asp_O, $P\alpha_O$	Q_{237}	Asp_O, $P\alpha_O$, $rib_{O5'}$	S_{229}		S_{223}	
349[c]	**D_{342}**	$Asp_{NH3+\ H2O}$	**D_{233}**	$Asp_{NH3+\ (H2O)}$	**D_{239}**	$Asp_{NH3+\ H2O}$	**D_{231}**	$Asp_{NH3+\ (H2O)}$	**D_{225}**	$Asp_{NH3+\ H2O}$
593	R_{543}		H_{448}	$Asp_{O\delta1\ (H2O)}$	H_{442}	$Asp_{O\delta1\ (H2O)}$	K_{336}		R_{322}	
620	**D_{471}**	Mg^{2+}	**D_{475}**	$P\alpha_{O\ (H2O)}$	**D_{469}**		**D_{354}**	*$Mg^{2+}2$*	**D_{338}**	$Mg^{2+}2$
627	**E_{478}**	Mg^{2+}	**E_{482}**	$rib_{O3'}$	**E_{476}**	$rib_{O3'}$	**E_{361}**	$rib_{O3'}$ $Mg^{2+}2$, 3 *$Mg^{2+}2$, 3*	**E_{345}**	$rib_{O3'}$ $Mg^{2+}2$, 3
628	I_{479}	$rib_{O3'}$	V_{483}		V_{477}		I_{362}	$rib_{O2'}$	I_{346}	$rib_{O2'}$
630	S_{481}	$Asp_{O,\ O\delta1}$, $P\beta_O$	G_{485}		G_{479}		S_{364}	*$Mg^{2+}2$, $P\beta_O$* $P\alpha_O$, $Asp_{O\delta1,(H2O)}$	S_{348}	*$Mg^{2+}2$, $P\beta_O$* $P\alpha_O$, $Asp_{O\delta1\ (H2O)}$
634	**R_{485}**	$Asp_{O\delta1,\ O\delta2}$	**R_{489}**	$Asp_{O\delta1,\ O\delta2}$	**R_{483}**	$Asp_{O\delta1,\ O\delta2}$	**R_{368}**	$Asp_{O\delta1,\ O\delta2}$	**R_{352}**	$Asp_{O\delta1,\ O\delta2}$
677[d]	G_{526}	$Asp_{O\delta1}$	A_{532}		A_{526}		G_{407}		A_{391}	
679[d]	**G_{528}**	$rib_{O3'}$	**G_{534}**		**G_{528}**		**G_{409}**		**G_{403}**	
682[d]	**R_{531}**	Ad, $rib_{O2'}$, $P\gamma_O$	**R_{537}**	Ad	**R_{531}**	Ad, ribo2′	**R_{412}**	Ad, *$P\gamma_O$*	**R_{406}**	Ad, $P\gamma_O$

[a] R_{enz} are the amino acid residues of AspRSs from *S. cerevisiae*, *E. coli*, and *P. kodakaraensis*, and *T. thermophilus* AspRS1 contacting the substrate groups R_{sub} of Aspartic Acid (Asp) or AMP (ad, adenine; rib, ribose; Pa, phosphate alpha) from Aspartyl-adenylate or *$P\gamma$* (phosphate gamma) and Mg^{2+} 2 or 3 from ATP. Mg^{2+} complex. Interacting groups from ATP. Mg^{2+} complex are in italic. [b] For *S. cerevisiae* AspRS, the contacts are those of the enzyme complexed with $tRNA^{Asp}$; they include contacts with C74, C75, and A76 from tRNA. H_2O indicates involvement of a water molecule in the interaction. The residues of *T. thermophilus* AspRS2 contacting the substrate groups are those presumed from alignment; amino acid residues in bold characters are conserved in class 2 synthetases. Conserved residues from flipping loop[b] and from consensus motifs 1[c] and 2[d] are indicated. The 3D structures are described in refs 56, 57, and 59−62.

Region I from alignment (residues 1−89) covers the N-terminal extension only found in eukaryotic AspRSs. Region II (residues 173−189) constitutes a loop where its size distinguishes AspRSs from archaebacteria and mycoplasma from those of eubacteria and eukaryotes. The large loop in eubacterial and eukaryotic AspRSs (14−16 and 14 residues, respectively) contrasts with the shorter one in archaebacterial and mycoplasma AspRSs (9 and 7 residues). The loops of AspRS1 and AspRS2 contain 14 and 5 residues and are of eubacterial and archaebacterial types, respectively. Region III (residues 219−228) constitutes in eukaryotic AspRSs a loop of 9−10 residues that precedes the two conserved helices of the hinge domain connecting the catalytic core to the anticodon-binding domain. This sequence, together with the N-terminal extension (region I), characterizes the eukaryotic AspRSs. Prokaryotic AspRSs are deprived from this sequence, except AspRS1, which contains a short sequence of four residues. Regions IV and V are constituted each of one residue (286 and 336) inserted in the catalytic core. In eukaryotic and archaebacterial AspRSs, Gly286 is inserted in the flipping loop downstream from consensus motif 1, whereas in eukaryotic and archaebacterial AspRSs, Ser336 and His336 are inserted in the loop, respectively, connecting the β strands of consensus motif 2. AspRS1 is deprived from these residues, whereas AspRS2 possesses both (Gly159 and His205). Region VI (residues 382−402) constitutes an area specific of eukaryotic and archaebacterial AspRSs, which in yeast, AspRS is organized in an α helix interacting with the hinge domain. This sequence is absent in AspRS1, but is present in AspRS2. Region VII (residues 424−545) covers a sequence inserted between consensus motifs 2 and 3 in AspRSs from eubacteria and mycoplasma. The 3D structure of *T. thermophilus* AspRS1 and *E. coli* AspRS shows an organization of this sequence in a $\beta\alpha\beta$ split motif, forming a curved antiparallel β sheet flanked on both sides by α helices. This structural domain, which characterizes eubacterial AspRSs, is also present in the phosphocarrier HPr (*58*). Region VIII (residues 581−591) characterizes eubacterial AspRSs. The 3D structures of *T. thermophilus* AspRS1 and *E. coli* AspRS show that it constitutes an acidic pocket in the catalytic core, which, by comparison with the 3D structure of the yeast complex, may be involved in interaction with the accepting arm of $tRNA^{Asp}$. Eukaryotic and archaebacterial AspRSs lack this sequence as well as AspRS2. Region IX (residues 654−657) corresponds to four residues inserted upstream from consensus motif 3 only in eubacterial AspRSs and in *T. thermophilus* AspRS1. Finally, region X (residues 709−735) covers the C-terminal extension characterizing eubacterial AspRSs. Eukaryotic and archaebacterial AspRSs as well as AspRS2 are deprived from this sequence.

The Modular Organization of Asp-tRNA Synthetases 1 and 2. Figure 3 A gives a schematic representation of the sequences of eukaryotic, eubacterial, and archaebacterial AspRSs, which emphasizes the insertions and the deletions characterizing the enzymes from each phylum. Figure 3B represents schematically the modular organization of the AspRSs from each phylum derived from known 3D structures (*T. thermophilus* AspRS1, *E. coli*, *S. cerevisiae*, and *P. kodakaraensis* AspRSs (*56, 57, 59−62*)). The eubacterial AspRSs exhibit the largest size, because of peculiar insertions, such as the phosphocarrier HPr module and the C-terminal extension. Eukaryotic AspRSs are deprived of large insertions, but possess only an N-terminal extension

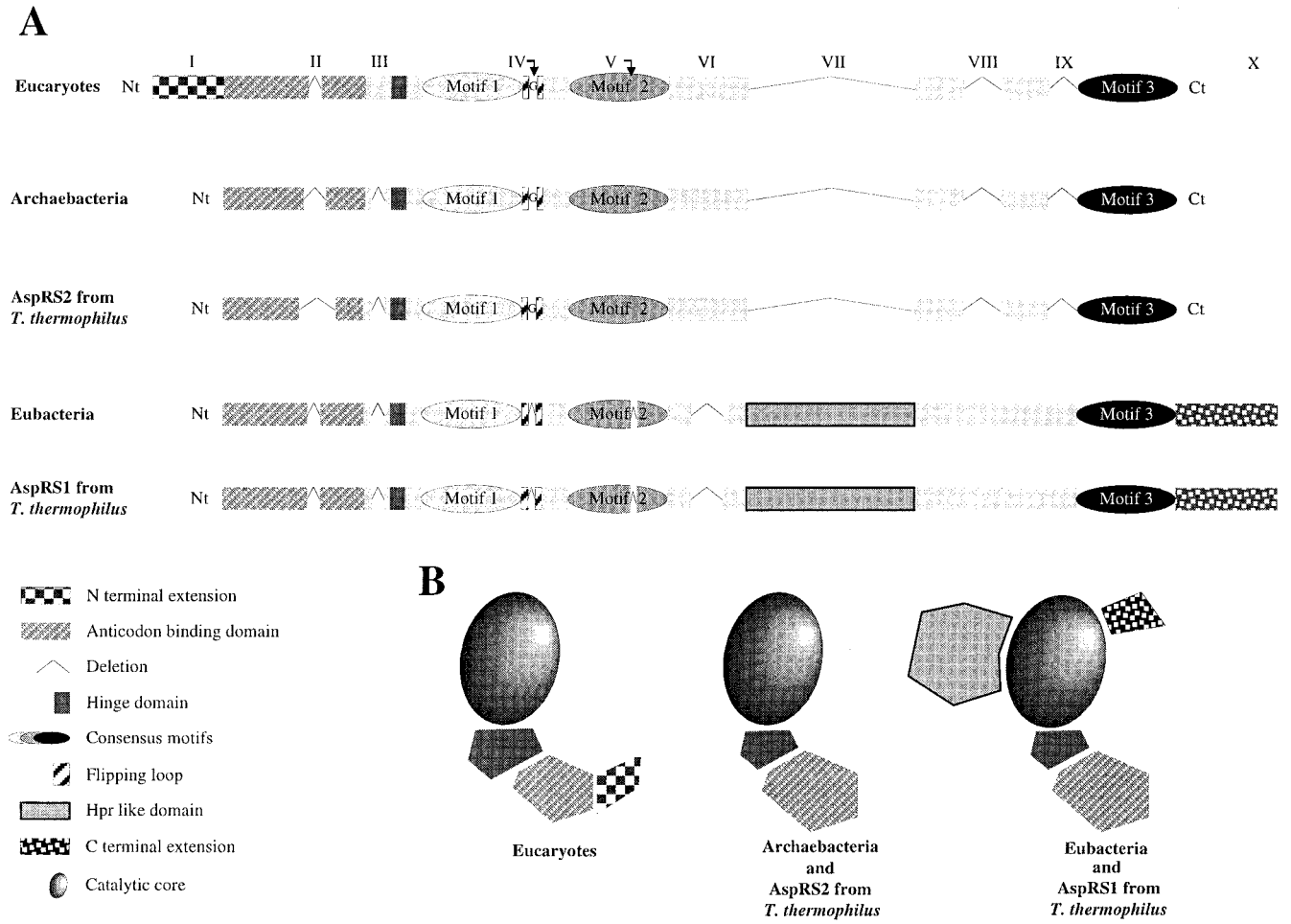

FIGURE 3: Schematic representation of the alignment (A) and of the modular organization (B) of eukaryotic, archaebacterial, and eubacterial AspRSs and of *T. thermophilus* AspRS1 and AspRS2. Nt and Ct are the N- and C-terminal ends. Regions I−X are discussed in the text.

Table 2: Expression of Asp-tRNA Synthetases 1 and 2 from *T. thermophilus* in *E. coli*

	E. coli				*T. thermophilus* HB8	
	DH5α/pKK223		BL21/pET3-1			
host strain/vector	AspRS1	AspRS2	AspRS1	AspRS2	AspRS1	AspRS2
specific activity[a]	3.1	3.6	1.1	10.3	0.02	0.083
overproduction[b]	155	43	55	124	1	1

[a] The specific activities were determined from maximal rates of charging of unfractionated *E. coli* tRNA at 37 °C after inactivation of the endogeneous AspRS by heat treatment, and expressed in nmol of Asp-tRNA formed/mg of proteins/min. The specific activities of AspRS1 and AspRS2 in *T. thermophilus* extracts were determined after inactivation of one of the two AspRSs with the homologous antibodies. [b] The overproduction of AspRS is expressed as the ratio of the specific activity of the enzyme in *E. coli*/specific activity in *T. thermophilus*.

and are smaller. Archaebacterial AspRSs, deprived from all additional sequences, are the smallest. It appears that the modular organization of AspRS1 superposes to that of *E. coli* AspRS and is expected for the other eubacterial AspRSs, whereas the modular organization of AspRS2 is that predicted from *P. kodakaraensis* AspRS and from other archaebacterial AspRSs.

Expression of Asp-tRNA Synthetases 1 and 2 in E. coli and Purification of the Proteins. The *aspS1* and *aspS2* genes were cloned in the pET3-1 and pKK223 vectors and expressed under control of the T7 RNA polymerase and *tac* promoters in *E. coli* strains BL21(DE3) and DH5α. Table 2 shows the specific activities of the thermostable AspRSs in the protein extracts, deprived from endogeneous AspRS after flocculation of the thermolabile proteins. The levels of expression of each AspRS in the two expression systems differ 3-fold. Expression of AspRS1 is the highest in the presence of the *tac* promoter, whereas production of AspRS2 is favored in the T7 RNA polymerase expression system. The specific activities of AspRS1 and AspRS2 in the protein extracts exceed 150- and 120-fold, respectively, those in *T. thermophilus*.

The thermostable AspRSs were isolated from the overproducing *E. coli* strains by flocculation of most thermolabile proteins from the host, followed by two chromatographic steps to remove the last contaminating proteins, and the nucleic acids (Table 3). A 50 mg sample of pure enzymes was obtained from 50 g cells (yield 50%). The specific activities of AspRS1 and AspRS2 for charging unfractionated *E. coli* tRNA are of 34 and 32 U mg^{-1}, respectively.

Functional Properties of Asp-tRNA Synthetases 1 and 2. Aminoacylation of unfractionated *T. thermophilus* tRNA by

Table 3: Purification of the Overexpressed Asp-tRNA Synthetases 1 and 2 in *E. coli*

purification step[a]	proteins (mg)	specific activity (units mg^{-1})[b]	total activity (units)	purification (fold)	yield (%)
		Asp-tRNA synthetase 1			
crude extract	3393	0.98	3318	1	100
flocculation (70 °C)	231	13.2	3044	13.5	92
hydroxyapatite	97	23.7	2300	24.2	69
DEAE-cellulose	52.1	33.6	1740	34.3	52
		Asp-tRNA synthetase 2			
crude extract	1068	4.3	4570	1	100
flocculation (70 °C)	240	15	3600	3.5	78
DEAE-cellulose	125	31.1	3887	7.2	85
phosphocellulose	55	32.1	1765	7.4	39

[a] The purification steps are described in Experimental Procedures. [b] The specific activities (nmol of Asp-tRNA formed/mg of proteins/min at 37 °C) are determined by aminoacylation of unfractionated *E. coli* tRNA.

Table 4: Aminoacylation of Unfractionated tRNA from *E. coli*, *S. cerevisiae* and *T. thermophilus* by the homologous Asp-tRNA Synthetases

origin of total tRNA	Asp-tRNA synthetases			
	E. coli	*S. cerevisiae*	*T. thermophilus* AspRS1	*T. thermophilus* AspRS2
			Asp-tRNA (nmol mg^{-1} tRNA)	
E. coli	1.42[a]	1.45[a]	2.89[a]	2.44[a]
S. cerevisiae	1.01[a]	1.53[a]	2.96[a]	0.99a
T. thermophilus	0.41[a]	0.39[a]	0.48[a]	0.92[a]
			0.42[b]	1.01[b]

[a] Aminoacylation extents of unfractionated tRNAs were detemined at 37 °C as described in Experimental Procedures. [b] Aminoacylation extents of unfractionated tRNAs were detemined at 70 °C as described in Experimental Procedures.

the two AspRSs shows a 2-fold higher plateau with AspRS2 than with AspRS1 (Table 4). Aspartylation extents are not affected by a temperature shift from 37 to 70 °C. Interestingly, *E. coli* and *S. cerevisiae* AspRSs charge *T. thermophilus* tRNA to a similar plateau than AspRS1; however, thermophilic AspRSs aminoacylate unfractionated *E. coli* tRNA 2-fold more than the homologous synthetase and AspRS1 aminoacylates unfractionated *S. cerevisiae* tRNA 2-fold more than *S. cerevisiae* AspRS, whereas AspRS2 aminoacylates it less.

Identification of the tRNAs from T. thermophilus Aspartylated by Asp-tRNA Synthetase 2. T. thermophilus contains only one tRNA^{Asp} isoacceptor (*43*). The similar aminoacylation levels obtained, when unfractionated *T. thermophilus* tRNA is charged with AspRS1, *E. coli*, or *S. cerevisiae* AspRSs, suggest specific charging of tRNA^{Asp} by AspRS1. In contrast, the increased level of aspartylation by AspRS2 indicates mischarging of a noncognate tRNA. This conclusion was confirmed by analysis of the capacity of each AspRS to charge *T. thermophilus* tRNAs fractionated by BD-cellulose chromatography. When the different fractions were tested for their capacity to be aspartylated by AspRS1 and AspRS2, two activity peaks were found. The first contains tRNA charged by both AspRSs, whereas the second contains tRNA well charged by AspRS2, but only poorly by AspRS1. Aminoacylation by *E. coli* and *S. cerevisiae* AspRSs of the tRNAs in the first peak confirms the presence of tRNA^{Asp}, whereas charging of the second peak by *T. thermophilus* and *E. coli* AsnRSs indicates the presence of tRNA^{Asn}. tRNA^{Asp} was purified and its identity confirmed by sequencing (Figure 4 A (*43*))

To identify the tRNA species aspartylated by AspRS2, this tRNA was purified from appropriate BD-cellulose fraction by two-dimensional PAGE. Staining of the gel after fractionation of the tRNAs showed 17 spots; aminoacylation of the eluted tRNAs revealed the presence in one spot of a species simultaneously aspartylated by AspRS2 and asparaginylated by AsnRS. Sequencing of this tRNA showed the presence of the Asn anticodon GUU; therefore, AspRS2 aspartylates tRNA^{Asn}.

Sequence of T. thermophilus tRNA^{Asn}. The cloverleaf structure of *T. thermophilus* tRNA^{Asn} is shown in Figure 4B. It is 78% identical to *E. coli* tRNA^{Asn}, although the content in GC pairs is 40% higher (17 and 12 pairs, respectively). The posttranscriptionally modified nucleotides Gm18, s2T54, and m1A58 are present in all tRNAs from *T. thermophilus* sequenced so far. It was shown that these modifications together with the increased number of GC pairs increase the thermal stability of *T. thermophilus* tRNAs (1 °C per GC pair and 6 °C by the posttranscriptional modifications (*63*)). Alignment with the eleven other known tRNA^{Asn} sequences (*64*) shows high conservation of the anticodon loop, in particular, the threonyl group t6 of A37. In contrast to *E. coli* and mammalian tRNA^{Asn}, queuosine is not found in position 34 in the thermophilic species, but G is. Absence in tRNA^{Asn} and tRNA^{Asp} from *T. thermophilus* of queuosine (Figure 4, A and B), found in the *E. coli* and mammalian species, strongly argues for the incapacity of that eubacterium to form this modified nucleotide. The five nucleotides long extra loop contains two conserved residues G46 and U47, which can be modified in m7G and D in tRNA^{Asn} from other organisms. In contrast, the D and T loops of the various tRNA^{Asn} are of variable lengths. Three of the four base-pairs from D-arm are conserved (m2G10-C25, C11-G26, and C13-G28), but only one pair in the T- and anticodon-arms (G52-C62 and G30-C40). Three base-pairs from the acceptor stem are conserved (G1-C72, C3-G70, and C5-G68), except in tRNA^{Asn} from *T. thermophilus* and *E. coli* which contain the nonconserved first pair U1-A72. The acceptor arm of tRNA^{Asn} from *T. thermophilus* contains such as those from the archaebacteria six G−C pairs, whereas most other tRNA^{Asn} contain only four. Finally, the posttranscriptionally modified m2G in the acceptor arm is only found in tRNA^{Asn} from *T. thermophilus*.

Kinetic Parameters of Aminoacylation of tRNA^{Asp} and tRNA^{Asn} by Asp-tRNA Synthetases 1 and 2. The kinetic constants of the two AspRSs from *T. thermophilus* for tRNA

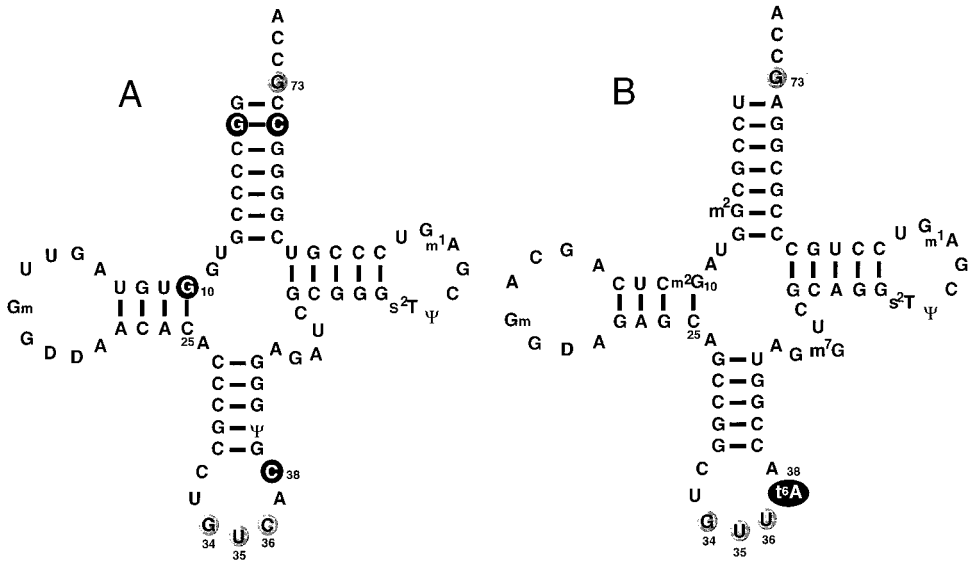

FIGURE 4: Sequences of *T. thermophilus* tRNA^{Asp} (A) and tRNA^{Asn} (B). The circled nucleotides are identity determinants. Nucleotides in black characters on gray boxes are common positions in tRNA^{Asp} and tRNA^{Asn}, determining the identity; nucleotides in white characters on black boxes are identity elements peculiar to each tRNA. The identity determinants of tRNA^{Asp} and tRNA^{Asn} are those described in refs *36*−*38* and *71*.

Table 5: Kinetic Constants of Aspartylation of tRNA^{Asp} and tRNA^{Asn} by *T. thermophilus* AspRS1 and AspRS2

	Asp-tRNA synthetase from *T. thermophilus*	
substrate	AspRS1	AspRS2
		K_M (μM)
aspartic acid	30	5
ATP	280	33
tRNA^{Asp}	0.030	0.073
tRNA^{Asn}	3.4	0.063
		k_{cat} (s^{-1})
tRNA^{Asp}	2.7	0.24
tRNA^{Asn}	0.12	0.092
		k_{cat}/K_M (s^{-1} μM^{-1})
tRNA^{Asp}	90	3.3
tRNA^{Asn}	0.04	1.5

[a] The values were determined at 70 °C as described in Experimental Procedures.

charging are shown in Table 5. AspRS2 exhibits a higher affinity for the small substrates than AspRS1 (the K_M's for Asp and ATP are 6- and 8-fold lower), whereas affinities of both for tRNA^{Asp} are comparable. AspRS1 aspartylates tRNA^{Asp} 30-fold more efficiently than AspRS2. However, the major kinetic differences between the two enzymes concern their specificity, since in unfractionated tRNA, AspRS1 aspartylates only tRNA^{Asp}, whereas AspRS2 aspartylates tRNA^{Asp} and tRNA^{Asn}. Affinity of AspRS2 for tRNA^{Asn} exceeds 50-fold that of AspRS1, but k_{cat}'s of both are similar. Comparison of the k_{cat}/K_M values shows that AspRS2 aspartylates tRNA^{Asn} only 2-fold less efficiently than tRNA^{Asp}, whereas AspRS1 aspartylates it 2250-fold less efficiently.

DISCUSSION

The Two Asp-tRNA Synthetases in T. thermophilus and Phylogenetic Implication of the Duplication. The Two Phylogenetically Distinct AspRSs in T. thermophilus. Biochemical investigations showed the presence in *T. thermophilus* of two AspRSs which differ by their physicochemical properties. Sequence analysis and cross reactions with antibodies revealed important structural differences, excluding a proteolytic origin of the two enzymes. Thus, one has a new example of synthetases duplication with two enzymes of distinct genetic origins. This contrasts with the duplicated Lys-, Thr-, and TyrRSs investigated so far (*22*−*28*, *31*−*34*), which exhibit important structural similarities and functional resemblances. The immunoblot showed reactions of AspRS1 and AspRS2 with *E. coli* and yeast anti-AspRS antibodies, respectively, suggesting the presence of eubacterial and eukaryotic epitopes (*35*). We show here that AspRS1 and AspRS2 exhibit sequence signatures characteristic of eubacterial and archaebacterial AspRSs. Peculiar insertions in the polypeptide chains confer to AspRS1 an eubacterial character, whereas AspRS2 with significantly shorter chains is of the archaebacterial type. AspRS2 with 422 residues is the smallest AspRS until now characterized and may be the minimalist AspRS structure. A similar phylogenetic interrelation was reported for the two IleRSs from *S. aureus*, since the chromosomally and the plasmidic-encoded enzymes are respectively of eubacterial and archaeal eukaryal types (*29*, *30*).

The structural dissimilarities of the AspRSs from various phylae reflect probably distinct functional properties of the enzymes, but information about peculiarities in the structure−function relationships of phylogenetically distinct AspRSs remain fragmentary. The N-terminal extension of eukaryotic AspRSs is implicated in formation of the multienzymatic complexes in mammals involving aaRSs of various specificities (*65*); the C-terminal extension of eubacterial AspRSs contributes to the subunit interface by increasing the contact area, and the phosphocarrier HPr-like domain participates probably in selection and in recognition of the tRNA by interacting with the acceptor arm (*56*, *57*).

Position of T. thermophilus in the Phylogenetic Tree. Analysis of various aminoacylation systems shows that *T. thermophilus* possesses eubacterial and archaebacterial characters. ThrRS resembles structurally the *E. coli* enzyme (*66*), whereas GlyRS diverges from GlyRSs of most eubacteria and is structurally related to those from archaebacteria and

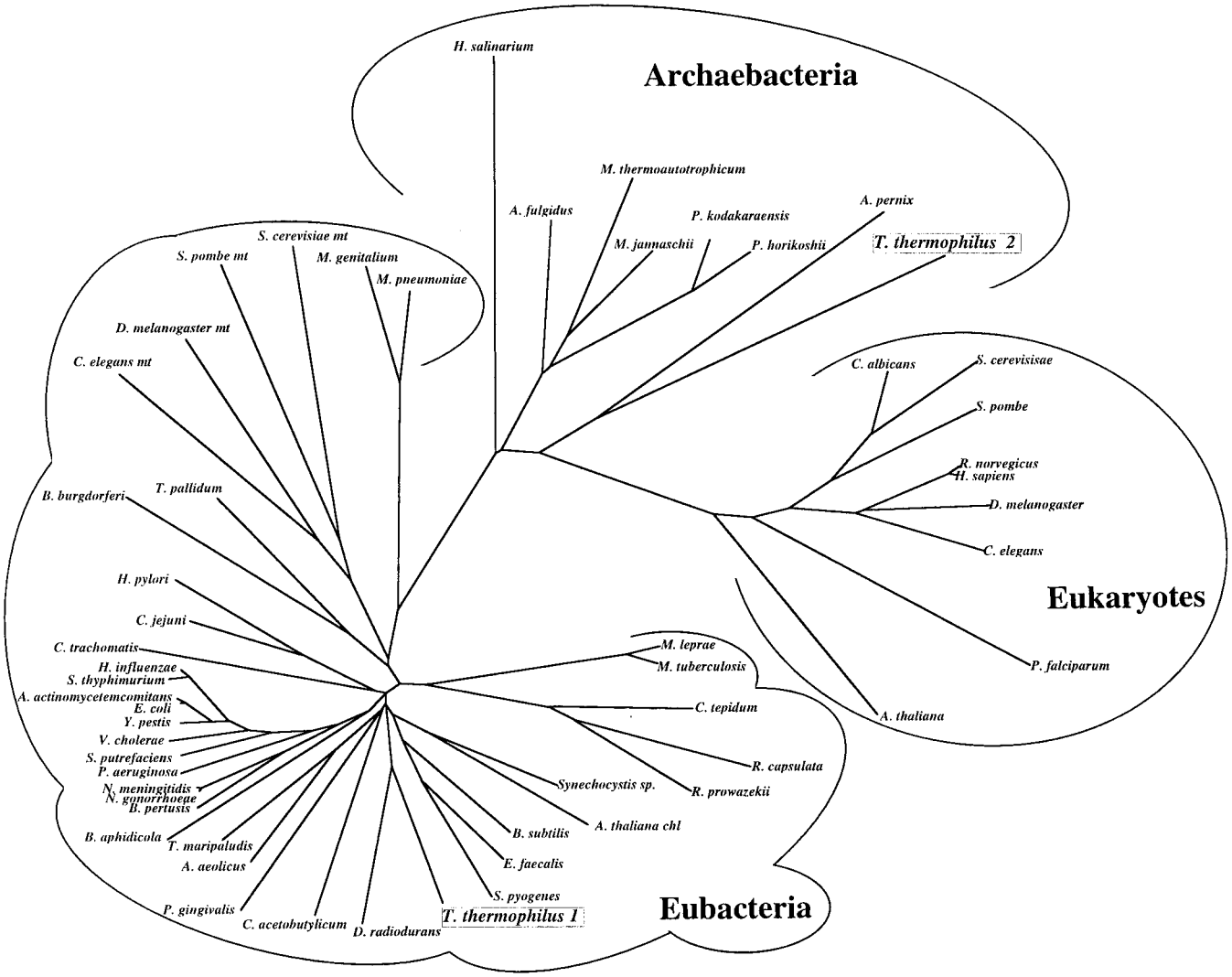

FIGURE 5: The phylogenetic tree of Asp-tRNA synthetases. The phylogenetic distances were estimated using the neighbor-joining method (*69*) coupled to the distance matrix calculated on the whole alignment with the PAM250 similarity table and pairwise gap removal: mt, mitochondrial; chl, chloroplastic.

from eukaryotes (*67*). We show here that *T. thermophilus* possesses eubacterial and archaebacterial type AspRSs. The phylogenetic tree computed over the whole alignment of AspRSs is in agreement with the commonly accepted tree of life based on rRNA sequences (*68*), since it clearly separates eukarya, archaea, and eubacteria (Figure 5) and shows the close relationship of archaea with eukarya. The distribution of the AspRSs of various origins in this tree demonstrates that these enzymes belong to the informational gene family. This tree illustrates the large phylogenetical distance between the two AspRSs from *T. thermophilus*. AspRS1 belongs unambiguously to the eubacterial AspRSs and is closely related to AspRS from *Deinococcus radiodurans*, whereas AspRS2 belongs to the archaebacterial AspRSs and is related to AspRS from *Aerophilum pernix*. Interestingly, *T. thermophilus* AspRS2 and AspRS from *A. pernix* are located at the interface between eukaryotic and archaebacterial AspRSs. The positions of AspRS1 and AspRS2 in the tree, among the eubacterial and archaebacterial AspRSs, confirms the dual character of *T. thermophilus* and the ambiguous origin of this bacterium.

The Structure−Function Relationships of Eubacterial and Archaebacterial Asp-tRNA Synthetases. Physiological implication of duplication of the Asp-tRNA synthetases in T. thermophilus. AspRS2 exhibits a relaxed specificity by charging $tRNA^{Asn}$ as efficiently as $tRNA^{Asp}$, while AspRS1 aspartylates $tRNA^{Asn}$ 3 orders of magnitude less efficiently than $tRNA^{Asp}$. This explains why higher aminoacylation plateaus are obtained when unfractionated *T. thermophilus* tRNA is charged by AspRS2 than by AspRS1. AspRS1 discriminates also $tRNA^{Asp}$ from $tRNA^{Asn}$ in heterologous tRNA while AspRS2 does not. Both AspRSs mischarge *E. coli* and yeast tRNAs, since in most cases the aminoacylation plateaus of unfractionated tRNA are higher with the thermophilic AspRS than with the homologous one. However, AspRS1 mischarges $tRNA^{Glu}$ in unfractionated *E. coli* and yeast tRNAs whereas AspRS2 mischarges $tRNA^{Asn}$. AspRS1 aspartylates $tRNA^{Asn}$ only after purification, when deprived from competing $tRNA^{Asp}$ (not shown). The low level of aminoacylation of unfractionated yeast tRNA by AspRS2 relates to the poor charging of $tRNA^{Asp}$ (not shown).

Since AspRS1 charges $tRNA^{Asp}$ more efficiently than AspRS2, *in vivo* tRNA aspartylation is essentially promoted by AspRS1 (*35*). Further, in the context of the competition exerted by cognate partners, AspRS2 is probably essentially complexed to $tRNA^{Asn}$ and implied in its aspartylation, a

property related to the indirect pathway of Asn-tRNA^{Asn} formation. In this pathway, Asp mischarged on tRNA^{Asn} by AspRS2 is converted into Asn by a tRNA-dependent aspartate amidotransferase (AspAdT, *70*).

$$\text{Asp} + \text{ATP} + \text{tRNA}^{Asn} \xleftrightarrow{\text{AspRS2, } MgCl_2} \text{Asp-tRNA}^{Asn} + \text{AMP} + \text{PPi}$$

$$\text{Asp-tRNA}^{Asn} + NH_2 \text{ donor} \xleftrightarrow{\text{AspAdT, ATP, } MgCl_2} \text{Asn-tRNA}^{Asn}$$

This pathway of tRNA asparaginylation is used by archaebacteria to supply their inability to synthesize directly Asn-tRNA^{Asn} because AsnRS is lacking (*17, 18*). However, in contrast to archaebacteria, *T. thermophilus* contains an AsnRS able to charge directly tRNA^{Asn} with Asn (*70*). The reason of the conservation of the two pathways of tRNA asparaginylation in this eubacterium relates to its inability to synthesize free Asn (*70*). Thus, under Asn starvation, when tRNA cannot be asparaginylated by AsnRS, Asn-tRNA^{Asn} is formed only by the indirect pathway, whereas, when Asn is present, tRNA asparaginylation is ensured essentially by AsnRS (*70*). Because of its involvement in tRNA asparaginylation, AspRS2 is functionally related to archaebacterial AspRSs.

The Restricted Specificities of AspRS1 and AsnRS and the Relaxed Specificity of AspRS2 for tRNA in T. thermophilus. AspRS2 is one of the rare aaRSs involved *in vivo* in tRNA mischarging, and *T. thermophilus* tRNA^{Asn} constitutes a rare tRNA species charged *in vivo* by two aaRSs of distinct specificities. A comparison of the elements conferring aspartate and asparagine identities in tRNAs shows that the two sets are partly overlapping, since they include common elements in addition to particular ones of each identity (Figure 4, A and B). The discriminatory G73 and the anticodon (GUC in tRNA^{Asp} and GUU in tRNA^{Asn}) constitute the prevalent elements for aspartylation by AspRS1 and for asparaginylation by *E. coli* AsnRS; the G2-C71 pair from the acceptor arm, G10 from the D-arm, and C38 from the anticodon loop are additionally involved in aspartylation (*37*), and the posttranscriptional t6 modification of A37 in asparaginylation (*38, 71*). Since the prevalent identity elements are mostly conserved in tRNAs (*36*), the same nucleotides may determine asparagine identity in *E. coli* and *T. thermophilus*. Thus, the major elements determining aspartate and asparagine identities in *T. thermophilus* differ only by nucleotide 36 (C in tRNA^{Asp} and U in tRNA^{Asn}) and the modified nucleotide 37 (t6A in tRNA^{Asn} (Figure 4, A and B)), suggesting involvement of these elements in discrimination between tRNA^{Asp} and tRNA^{Asn} by the cognate aaRSs beside possible contributions of minor elements.

How do AspRS and AsnRS discriminate both tRNAs? Many examples have shown that when identity elements are overlapping, specificity of charging is promoted by changes of the contribution of the elements to create a particular hierarchy for each identity. Competition by the noncognate tRNA is minimized by increased contribution of the noncommon elements of both sets in recognition by the cognate synthetase and, in particular cases, by antideterminants in the noncognate tRNA (*37, 72*). Discrimination of tRNA^{Asp} against tRNA^{Asn} by AspRS1 and *vice-versa* for AsnRS could be promoted by increased contributions in recognition of elements distinguishing both identities, *e.g.* C36, C38, and the G2-C71 pair for aspartylation by AspRS1, U36, and the t6A37 for asparaginylation by AsnRS and/or by antideterminant effects exerted by these elements in recognition by the noncognate aaRS. In contrast, relaxed specificity of AspRS2 would be ensured by increased contribution of the elements common to both identities (G73, G34, U35) and minimized contribution of the elements which differ (N36, G2-C71, C38, and the t6 modification on A37).

Aspartate and asparagine identities include the full anticodon (*36*). How can AspRS2 accommodate tRNA^{Asp} and tRNA^{Asn} anticodons differing by nucleotide 36? The 3D structure of the yeast complex shows implication of the loop covering residues 173−186 from the anticodon-binding domain in recognition of C36 from tRNA through backbone contacts and in discrimination against U at this position (*73*). Interestingly, this loop constitutes one of the structural peculiarities that distinguishes the AspRSs from various phyla (region II from alignment, Figures 1 and 3), being significantly shorter in archaebacterial type AspRSs than in the eubacterial and eukaryotic types. Modeling the interaction of a tRNA anticodon of *P. kodakaraensis* AspRS from the known structure of the yeast complex indicates that shortening of this loop renders *P. kodakaraensis* AspRS insensitive to the presence of C or U at position 36 of the tRNA and probably also to the bulkiness of the t6 modification of the adjacent A (*60*). The large loops of eubacterial and eukaryotic AspRSs interact only with Asp anticodon, whereas the shorter loops of archaebacterial AspRSs interact with Asp and Asn anticodons. Thus, this loop constitutes probably one of the major structural elements in AspRSs that determines restricted specificity for tRNA^{Asp} or relaxed specificity for tRNA^{Asp} and tRNA^{Asn}.

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