Identity of Prokaryotic and Eukaryotic $tRNA^{Asp}$ for Aminoacylation by Aspartyl-tRNA Synthetase from *Thermus thermophilus*†

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ABSTRACT: The aspartate identity of tRNA for AspRS from *Thermus thermophilus* has been investigated by kinetic analysis of the aspartylation reaction of different tRNA molecules and their variants as well as of $tRNA^{Phe}$ variants with transplanted aspartate identity elements. It is shown that G_{10}, G_{34}, U_{35}, C_{36}, C_{38}, and G_{73} determine recognition and aspartylation of yeast and *T. thermophilus* $tRNA^{Asp}$ by the thermophilic AspRS. This set of nucleotides specifies also tRNA aspartylation in the homologous yeast and *Escherichia coli* systems. Structural considerations indicate that the major aspartate identity elements interact with amino acids conserved in all AspRSs. It follows that the structural features of tRNA and synthetase specifying aspartylation are mainly conserved in various structural contexts and in organisms adapted to different life conditions. Mutations of tRNA identity elements provoke drastic losses of charging in the heterologous system involving yeast $tRNA^{Asp}$ and *T. thermophilus* AspRS. In the homologous systems, the mutational effects are less pronounced. However, effects in *E. coli* and *T. thermophilus* exceed those in yeast which are particularly moderate, indicating variations in the individual contributions of identity elements for aspartylation in prokaryotes and eukaryotes. Analysis of multiple tRNA mutants reveals cooperativity between the cluster of determinants of the anticodon loop and the additional determinants G_{10} and G_{73} for efficient aspartylation in the thermophilic system, suggesting that conformational changes trigger formation of the functional tRNA/synthetase complex.

Cellular life relies on accuracy of biochemical processes, among which conversion of genetic information into proteins takes the crucial place. In particular, synthesis of functional proteins is correlated with accurate tRNA charging by aminoacyl-tRNA synthetases (reviewed in Schimmel & Söll, 1979). Because of the irreversibility of tRNA aminoacylation due to trapping of charged tRNAs by elongation factor (Pingould *et al.,* 1990; Clark *et al.,* 1995), errorless charging must occur, constraining these enzymes to high functional specificity.

Substrate recognition by synthetases is not of absolute specificity since the wrong substrates can bind and even promote catalysis. Cognate amino acids are selected by a double sieving mechanism, but isosteric or smaller amino acids can be activated (Fersht, 1977). Misactivations can be corrected by hydrolysis of the wrong aminoacyl adenylates, but intermediates escaping correction can transfer misactivated amino acids to tRNA. Immediate hydrolysis of the end product prevents then release of mischarged tRNAs (*e.g.,* Fersht & Kaethner, 1976; Von der Haar & Cramer, 1976; Igloi *et al.,* 1978; Fersht & Dingwall, 1979; Lin *et al.,* 1983; Fersht, 1985). Cognate tRNAs are selected by kinetic processes promoting formation of high efficiency complexes, only when bound to the cognate synthetases (Krauss *et al.,* 1976; Riesner *et al.,* 1976). Nonspecific associations are deprived of high catalytic efficiency (Ebel *et al.,* 1976). Finally, competition of the cognate partners for competent binding, combined with decreased reactivity when noncognate partners are bound, ensures the expected specificity for accurate protein synthesis (Rogers & Söll, 1990). No additional proofreading occurs since enzymatic deacylation of charged tRNA cannot prevent release of mischarged tRNA (Mulvey & Fersht, 1977; Fersht, 1985).

The structural and kinetic basis leading only cognate complexes to efficient reactivity was largely investigated. Kinetic approaches showed increased complexity in the dynamics of interaction of cognate partners, whereas noncognate associations involve a more simple process. Cooperative binding of independent parts of the tRNA, coupled with activation of the catalytic center of the synthetases, suggests that functionality of the complexes is induced by a dynamic adaptation process of both partners. For example, in the yeast valine and phenylalanine systems, binding of cognate tRNAs deprived of the accepting CCA triggers aminoacylation of free CCA and even of adenosine (Renaud *et al.,* 1981; Bacha *et al.,* 1982). Interaction of the anticodon is often involved in this adaptation as shown by the stimulation of valylation of an accepting minihelix when an anticodon hairpin is associated to ValRS[1] (Frugier *et al.,* 1992) and brings a phenomenological interpretation of the effects of cognate tRNAs on the kinetic constants of amino acid activation (*e.g.,* Jacques & Blanquet, 1977; Kern & Giegé, 1979).

The structural basis underlying the functional interactions between synthetases and tRNAs was investigated by crystallographic, biochemical, and genetic approaches. They reveal

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that particular positions of the tRNA, called identity determinants, are involved in the recognition process. The complete set of the major nucleotides conferring an identity was characterized in various tRNAs; it differs in the number of elements as well as in their localization within the tRNA cloverleaf (reviewed in Schulman, 1991; Giegé *et al.*, 1993; McClain, 1993). Identity of $tRNA^{Ala}$ is mainly due to an unique nucleotide pair (G_3-U_{70} in the acceptor stem; Hou & Schimmel, 1988; McClain & Foss, 1988), whereas in other tRNAs, it is defined by more nucleotides distributed essentially in the acceptor arm and the anticodon loop. In a few systems, identity elements are present in the D- and T-arms, as in $tRNA^{Phe}$ (Tinkle Peterson & Uhlenbeck, 1992; Sampson *et al.*, 1992; Moor *et al.*, 1992) or in the extra-arm, as in $tRNA^{Ser}$ (Himeno *et al.*, 1990; Sampson & Saks, 1993) and $tRNA^{Leu}$ (Breitschopf *et al.*, 1995). Since identity of some tRNAs is given by nucleotide combinations at similar positions, the same positions can confer identity in different aminoacylation systems. Also, identity elements of a given system can be present in another one. Various investigations suggest that an identity set by itself is unable to confer absolute aminoacylation specificity and that accuracy is improved by antideterminants, often modified nucleotides, that prevent tRNA aminoacylation by noncognate synthetases (Muramatsu *et al.*, 1988; Pütz *et al.*, 1994).

The identity determinants for aspartylation have been characterized in yeast and *Escherichia coli* $tRNA^{Asp}$ (Pütz *et al.*, 1991; Nameki *et al.*, 1992). Those conferring the strongest effects are conserved, but subtle variations occur. This raises the question of the variability in the aspartate identity, in particular within prokaryotes. Also, expression of this identity in extreme life conditions, such as in thermophilic bacteria, remains undefined. From another viewpoint, characterization of the aspartate identity, in heterologous prokaryotic/eukaryotic and thermophilic/mesophilic systems, should inform about its expression in different structural contexts and thus reveal possible variabilities compatible with specificity. Finally, the dynamic parameters involved in specificity are poorly understood, and their interrelation with structural elements is not clear. In this context, study of heterologous aspartylation systems should reveal relationships between structural and dynamic elements of identity and their interdependence during evolution.

In this work, we explored the relationship between aspartylation systems of different origins by taking advantage of the knowledge of the yeast system (*e.g.*, Pütz *et al.*, 1991; Ruff *et al*., 1991) and from recent structural advances in that from *Thermus thermophilus*, an extreme thermophilic Eubacteria, *i.e.*, the sequences of AspRS and $tRNA^{Asp}$ (Poterszman *et al.*, 1993; Keith *et al.*, 1993) and the 3D structure of the protein (Delarue *et al.*, 1994). Here we have characterized the aspartate determinants in both thermophilic and yeast $tRNA^{Asp}$ for aminoacylation by *T. thermophilus* AspRS. Comparison with aspartate determinants defined in the homologous mesophilic systems from yeast and *E. coli* shows that the determinants are mainly conserved in eukaryotes and prokaryotes as well as in mesophiles and thermophiles. The identity set is restricted to 6 nucleotides located at both extremities of the L-shaped tRNA structure and at positions 10−25 in the D-stem; it does not tolerate significant variability. Interestingly, mutations of crucial identity elements affect more aspartylation in prokaryotes than in eukaryotes and induce drastic effects in the heterologous system. These properties are discussed in light of the role of the structural context for optimal expression of an identity and the role of the identity elements in the dynamic processes involved in specificity, and in the context of the evolution of aminoacylation systems.

EXPERIMENTAL PROCEDURES

Materials. Oligonucleotides were synthesized on an Applied Biosystem 381 A DNA synthesizer using the phosphoramidite method and purified by HPLC on a Nucleosil 120-5-C18 column (Bischoff Chromatography, Zymark-France). L-[^{3}H]Aspartic acid (43 Ci/mmol) and [α-^{32}P]ATP were from Amersham France, and L-[^{14}C]aspartic acid (226 mCi/mmol) was from the Commissariat à l'énergie Atomique. Protamine sulfate, polymine P, S-Sepharose, and inorganic pyrophosphatase were from Sigma and PEG 6000 from Merck. Hydroxyapatite HTP was from Bio-Rad and DEAE-cellulose (DE52) from Whatman. Restriction enzymes *Bst*NI, *Hin*dIII, and *Bam*HI, T4 polynucleotide kinase, and T7 DNA polymerase were from New England Biolabs. T4 DNA ligase and DNase I (grade II) were from Boehringer Mannheim France, and RNase A was from Worthington. $tRNA^{Asp}$ from *E. coli* (accepting capacity 37 nmol/mg) was from Subriden; $tRNA^{Asp}$ from yeast and *T. thermophilus* (accepting capacities 33 and 37 nmol/mg) were purified according to conventional methods (Keith *et al.*, 1993) and were kindly provided by Drs. G. Keith and A.-C. Dock.

Preparation of Enzymes. AspRS from *T. thermophilus* was purified from an overproducing strain obtained by transformation of *E. coli* JM 103 with the recombined expression vector pKK 223 carrying the synthetase gene downstream of the *tac* promoter (Poterszman *et al*., 1993). All buffers contained 0.1 mM Na_2EDTA and DIFP and 5 mM 2-mercaptoethanol. Cells (50 g) were suspended in 50 mM Tris-HCl buffer, pH 8.0, containing 10 mM $MgCl_2$ and submitted to 10 cycles of 30 s sonication, each at 100 V, in ice. The supernatant obtained after 3 h centrifugation at 45 000 rpm was supplemented with 100 mM NaCl and incubated 30 min at 70 °C. The flocculated proteins were removed by 15 min centrifugation at 4200 rpm. After 2-fold dilution, the supernatant was adsorbed on a hydroxyapatite column equilibrated with 20 mM potassium phosphate buffer, pH 6.8, and the proteins were eluted with a linear gradient from 20 to 200 mM of this buffer. Active fractions, eluting at 140 mM salt, were dialyzed against 20 mM potassium phosphate, pH 7.2, loaded on a DEAE-cellulose column, and resolved with a linear gradient from 20 mM (pH 7.2) to 200 mM (pH 6.8) potassium phosphate. Active fractions, eluting at 170 mM salt, were concentrated by filtration under N_2 pressure through a semipermeable membrane (Amicon YM 10), dialyzed against 50 mM potassium phosphate, pH 7.2,

[1] Abbreviations: For amino acids, the one letter code is used; aminoacyl-tRNA synthetases, the three letter code is used for the amino acids *e.g.*, AspRS for aspartyl-tRNA synthetase; DEAE-cellulose, [(diethylamino)ethyl]cellulose; DIFP, diisopropyl fluorophosphate; DTE, dithioerythritol; EDTA, ethylenedinitrilotetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′−2-ethanesulfonic acid; HPLC, high pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PEG, polyethylene glycol; Tris, tris(hydroxymethyl)aminomethane. $tRNA^{Asp\Omega}$, $tRNA^{Asp}$ with G_{10}, G_{34}, U_{35}, C_{36}, C_{38}, and G_{73} mutated; $tRNA^{Asp@\phi}$, $tRNA^{Asp}$ with G_{34}, U_{35}, C_{36}, and C_{38} mutated; $tRNA^{Asp@int}$, $tRNA^{Asp}$ with G_{10}-C_{25} and G_{73} mutated (the sequences of these tRNAs are shown in Figure 3); $tRNA^{Phe\rightarrow Asp}$, $tRNA^{Phe}$ with U_{35}, C_{36}, A_{38}, G_{73}, and U_{25} in the yeast species or C_{25} in the *T. thermophilus* species; $tRNA^{Phe\rightarrow Asp(A38)}$ refers to $tRNA^{Phe\rightarrow Asp}$ with A_{38}.

containing 50% glycerol, and stored at −20 °C. About 50 mg of enzyme is obtained with 50% yield and 700 U/mg of specific activity (1 unit catalyzes charging of 1 nmol of *T. thermophilus* tRNA per min at 37 °C).

Yeast AspRS was purified from the overproducing *E. coli* strain TGE 900 transformed with the recombined pTG 908 expression vector carrying the synthetase gene downstream of the λ promoter. The initial construction expressing a truncated and fused protein (Kern *et al.,* 1990) was modified in order to express the native protein. Cells (50 g) were sonicated as described above. The supernatant obtained after 1 h centrifugation at 45 000 rpm was incubated 1 h at 0 °C with 20 U/mL DNase I. Nucleic acids were precipitated with protamine sulfate (2.5 mg/mL) and removed by a 30 min centrifugation at 4200 rpm, and proteins were fractionated by selective precipitation with PEG 6000. Proteins precipitating between 5% and 15% PEG were dissolved in 20 mM potassium phosphate, pH 7.2, adsorbed on a hydroxyapatite column, and eluted with a linear gradient from 300 to 800 mM buffer (AspRS elutes at 460 mM salt). About 60 mg of enzyme is obtained with 50% yield and a specific activity of 680 U/mg.

T7 RNA polymerase was purified from an overproducing strain provided by W. Studier (Brookhaven, USA) following a procedure derived from that of Zawadzki and Gross (1991). After cell disruption by sonication and sedimentation of cell debris, nucleic acids were precipitated with polymine P (0.9%). The protein fraction in the supernatant, precipitated at 55% ammonium sulfate saturation (at 4 °C), was fractionated after dialysis on an S-Sepharose column equilibrated with 20 mM sodium phosphate, pH 7.7. Elution was performed with a linear gradient from 50 to 300 mM NaCl, and the enzyme, eluted at 190 mM salt, was precipitated by dialysis against 20 mM sodium phosphate, pH 7.7, containing 10 mM NaCl. About 125 mg of enzyme with a specific activity of 16 000 U/mg and deprived of RNase traces is obtained from 50 g of cells (1 unit catalyzes incorporation of 1 nmol of [α-^{32}P]ATP/h at 37 °C).

Preparation of tRNA Transcripts. Yeast wild-type or mutated tRNA^{Asp} and tRNA^{Phe} transcripts were obtained by *in vitro* transcription of genes cloned in pUC 118 downstream the promoter of T7 RNA polymerase. The recombined vectors (Perret *et al.,* 1990; Pütz *et al.,* 1991) were kindly provided by Drs. J. Pütz and C. Florentz. Wild-type *T. thermophilus* tRNA^{Asp} and tRNA^{Phe} transcripts and their mutants were obtained from pUC 118 vector recombined with the synthetic genes flanked upstream by the consensus promoter (−21 to −5) of T7 RNA polymerase followed by a TATA box (−4 to −1) and downstream by a *Bst*NI restriction site. The genes were constructed by shotgun ligation (Grundstrom *et al.,* 1985; Romaniuk *et al.,* 1987). Ten DNA fragments (16 to 24 mers) covering both strands were synthesized, repurified by HPLC, and phosphorylated (Sambrook *et al.,* 1989). The complementary fragments were hybridized at a final concentration of 40 nM each in the presence of 20 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 0.1 mM EDTA, and 1 mM DTE (final volume 20 μL). Ligation was performed between the *Hin*dIII and *Bam*HI sites of pUC 118 by incubation of the five duplexes (final concentration, 4 nM each) with 100 ng of linearized vector and 10 units of T4 DNA ligase during 16 h at 17 °C. The recombined plasmids were isolated by minipreparations of clones obtained from the transformed DH5 F′ strain, and the sequences of the genes were controlled using the dideoxynucleotide sequencing method (Tabor & Richardson, 1987). Large scale preparations of plasmidic DNA were done using the alkaline method and a final centrifugation step on a ClCs gradient (Sambrook *et al.,* 1989).

In vitro transcriptions were performed at 37 °C in reaction mixtures of 250 μL containing 40 mM Tris-HCl, pH 8.1, 22 mM $MgCl_2$, 5 mM DTE, 0.01% Triton X-100, 1 mM spermidine, 4 mM of each nucleotide triphosphate, 5 mM GMP, 0.1 μg/μL plasmidic DNA digested with *Bst*NI, 7.5 μg of T7 RNA polymerase, and 10 units of inorganic pyrophosphatase. Reactions were stopped after 3 h incubation at 37 °C by phenol/chloroform (1/1 w/v) extraction. After ethanol precipitation, nucleic acids were dissolved in 4 M urea, 10% saccharose, 0.025% bromophenol, and xylene cyanol blue, and full-length transcripts were purified by preparative denaturing PAGE (11.2% polyacrylamide, 0.8% bis(acrylamide), 8 M urea, 89 mM Tris−borate, pH 8.3, and 2.5 mM Na_2EDTA). Transcripts were electroeluted (3 h at 100 mA) in a biotrap cell system (Schleicher & Schuell) in the preceding buffer, extracted with phenol/chloroform, precipitated with ethanol, and dissolved in sterile water. The last traces of urea were removed by gel filtration under centrifugation through a 1-mL Sephadex G-25 column. In most cases, two major transcripts of different length were resolved by electrophoresis. Analysis of transcription products revealed the best charging capacity (6−32 nmol/mg) for transcripts of highest mobility. Concentrations of tRNA and DNA solutions were determined spectrophotometrically (one A_{260nm} unit/cm corresponds to 40 μg of tRNA and 50 μg of DNA).

Aminoacylation Reactions. The standard aminoacylation mixture (total volume 25−200 μL) contained 100 mM HEPES-Na, pH 7.2, 10 mM $MgCl_2$, 30 mM KCl, 2 mM ATP, and L-aspartic acid either [^{3}H] labeled for K_M determinations of tRNAs and transcripts [7.5 μM (equivalent to the K_M value; specific activity 3000 cpm/pmol) or 50 μM (*i.e.,* 0.05-fold the K_M value; specific activity 1500 cpm/pmol) when *T. thermophilus* or yeast AspRSs were used] or [^{14}C] labeled for k_{cat} determinations [50 μM (*i.e.* 10-fold the K_M value; specific activity 380 cpm/pmol) or 1 mM (equivalent to the K_M value; specific activity 50 cpm/pmol) when *T. thermophilus* or yeast AspRSs were used]. Concentrations of tRNAs or transcripts were such as to allow K_M determinations or saturating when k_{cat} values were measured. AspRSs at final concentrations of 0.3−4 μM were diluted when necessary in 100 mM HEPES-Na, pH 7.2, 10% glycerol, 1 mg/mL bovine serum albumin, 5 mM 2-mercaptoethanol, and 0.1 mM DIFP and DTE. Initial rates were measured by determining the labeled aa-tRNA formed in 10−50 μL aliquots at various incubation times (Kern *et al.,* 1990). The K_Ms were determined from Lineweaver and Burk plots; each value is an average of at least 3 independent determinations. Because of the requirement of high specific activities of labeled aspartic acid for K_M determinations of tRNA, the concentration of this substrate could not be saturating for practical reasons: about the K_M value for *T. thermophilus* AspRS and 0.05-fold the K_M value for yeast AspRS. The k_{cat}s for tRNA charging were determined independently. For *T. thermophilus* AspRS all subtrates were saturating (10−100 K_M); this was also the case for yeast AspRS except that the concentration of aspartic acid equalled its K_M value. When tRNA could not be saturating, only catalytic efficiencies (k_{cat}/K_M) were measured (Fersht, 1985). Experimental errors on kinetic constants can be considered to be within

A

tRNA^{Asp}	*T. thermophilus* aspartyl-tRNA synthetase			
	Kinetic constants			
	K_M (for tRNA) (μM)	k_{cat} (for Asp-tRNA formation) (s^{-1})	k_{cat}/K_M ($s^{-1}\times\mu M^{-1}$)	L
Wild-type				
T. thermophilus	0.044	0.77	17.5	1
E. coli	0.024	0.25	10.4	1.7
yeast	0.098	0.085	0.9	19.5
transcripts				
T. thermophilus	0.016	0.49	30.6	1
E. coli	0.050	0.23	4.6	6.7
yeast	0.085	0.30	3.5	8.7

B

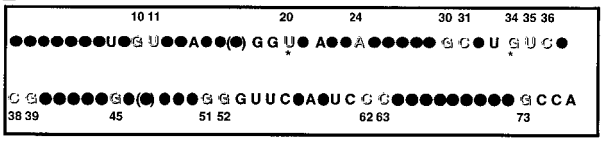

FIGURE 1: Establishment of the consensus sequence of tRNA^{Asp} of various origins aminoacylated by *T. thermophilus* AspRS. (A) Kinetic constants of aminoacylation of yeast, *E. coli*, and *T. thermophilus* tRNA^{Asp} and of their transcripts by *T. thermophilus* AspRS. The loss of charging efficiency (L) is expressed as the ratio of the efficiencies of charging (k_{cat}/K_M) of tRNA (or transcript) from *T. thermophilus* over tRNA (or transcript) from yeast or *E. coli*. (B) Consensus sequence of tRNA^{Asp} transcripts from yeast, *E. coli*, and *T. thermophilus*. Outlined characters: conserved positions in the transcripts; black characters: common nucleotides to all tRNAs; black dots: nonconserved positions; asterisks: post-transcriptional modifications. Sequence data and numbering of the positions are according to Steinberg *et al.* (1993).

10% of the indicated values. The K_i values of tRNA^{Phe} and tRNA^{Asp} mutants deprived of aspartylation capacity were estimated from double-reciprocal plots with fixed concentrations of these tRNAs (2−25 μM) and wild-type tRNA^{Asp} concentrations varying in the K_M range. Analysis of kinetic data for multiple mutants was according to Fersht (1985) using the formalism described by Pütz *et al.* (1993).

RESULTS AND DISCUSSION

Aminoacylation of tRNA^{Asp} from T. thermophilus, E. coli, and Yeast and Their Transcripts by Thermophilic AspRS. This synthetase aminoacylates *E. coli* and yeast tRNA^{Asp} nearly as efficiently as thermophilic tRNA^{Asp}. The completeness of reactions showing that aminoacylation compensates largely deacylation of aa-tRNAs (results not shown) is suggestive of favorable kinetic constants. This conclusion was verified by the kinetic data summarized in Figure 1A. Native tRNA^{Asp} from *E. coli* and yeast are aminoacylated 2- and 20-fold less efficiently than *T. thermophilus* tRNA^{Asp}. Losses of catalytic efficiency in the heterologous systems (expressed as charging efficiencies k_{cat}/K_M of thermophilic tRNA over that of *E. coli* or yeast) result essentially from decreased k_{cat} values (3- and 10-fold for tRNA from *E. coli* and yeast). The unmodified transcripts from *T. thermophilus* and yeast are charged 2 and 4 times more efficiently than native tRNA as a result of increased affinities and k_{cat} values, whereas the transcript from *E. coli* is charged 2 times less efficiently because of decreased affinity. Since post-transcriptional modifications do not significantly affect charging of the various tRNA^{Asp} species, it can be concluded that they are not involved in aspartylation by *T. thermophilus* AspRS.

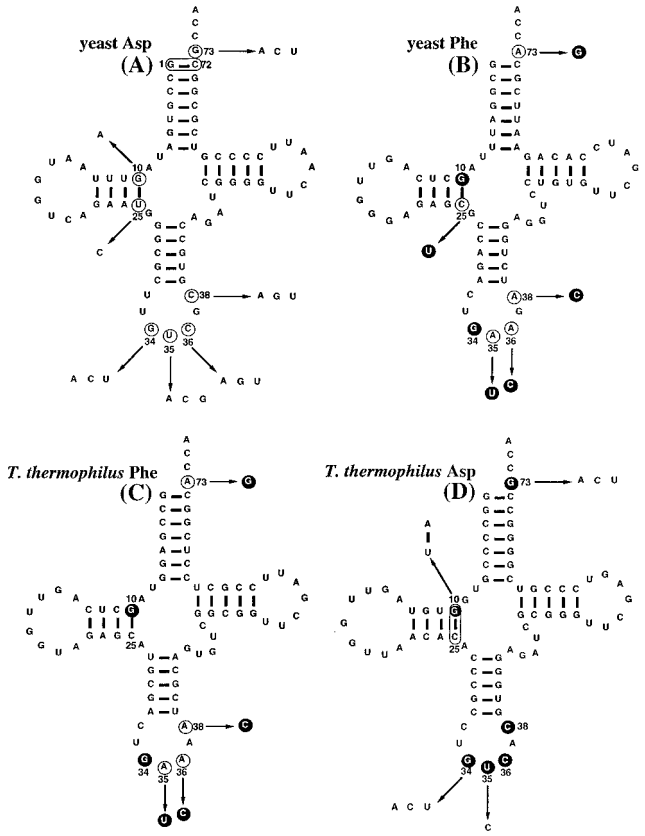

FIGURE 2: Nucleotide substitutions in yeast and *T. thermophilus* tRNA^{Asp} and transplantation of the aspartate identity elements in yeast and *T. thermophilus* tRNA^{Phe}. (A)−(D) Sequences of tRNA transcripts. Asp and Phe refer to the structural framework of tRNA^{Asp} and tRNA^{Phe} from yeast and *T. thermophilus*. In (A), the U_1-A_{72} pair was replaced by G_1-C_{72} (see the Results and Discussion section). In (B)−(D), the positions determining aspartate identity are indicated by white characters in black dots. In (A)−(C), the nucleotides substituted in the sequences are white circled; in (D), some of the identity positions were substituted; the nature of the substitutions are emphasized by the arrows.

Identification of the Determinants in Yeast tRNA^{Asp} for Aminoacylation by T. thermophilus AspRS. The consensus sequence of the tRNA^{Asp} species charged by *T. thermophilus* AspRS reveals, in addition to the nucleotides common to all tRNAs, 17 conserved residues which are potential identity elements (Figure 1B). They include G_{10}, G_{34}, U_{35}, C_{36}, C_{38}, and G_{73}, the residues involved in aspartylation in the yeast and *E. coli* systems (Pütz *et al.*, 1991; Nameki *et al.*, 1992; Frugier *et al.*, 1994). Since yeast tRNA^{Asp} is well charged by *T. thermophilus* AspRS (Figure 1A), this identity set was analyzed in the context of the thermophilic system. The substitutions which were studied are shown in Figure 2A. The first base pair from the acceptor arm (U_1-A_{72}) is not essential for aminoacylation by yeast AspRS (Pütz *et al.*, 1991) and is not conserved in the consensus sequence (G-C in prokaryotes and U-A in yeast). Therefore, it could be substituted by G_1-C_{72} to optimize transcription without damage of the charging ability. The second base pair in the acceptor arm (G_2-C_{71}) was proposed as an identity element in *E. coli* although its substitution decreased only 5-fold the aspartylation efficiency (Nameki *et al.*, 1992). Since this pair is not conserved in yeast tRNA^{Asp} (C_2-G_{71}, Figure 2A), it does not constitute an identity element in the heterologous system, although a minor contribution cannot be excluded.

The effects of mutating identity elements in yeast tRNA^{Asp} on aminoacylation by thermophilic AspRS are shown in Table 1. Except for U_{25}, all mutations affect heterologous

Table 1: Kinetic Constants of Aminoacylation of Yeast tRNA^{Asp} and tRNA^{Phe} Variants by *T. thermophilus* AspRS, and Comparison with the Homologous Systems from Yeast and *E. coli*[a]

	system					
	T. thermophilus AspRS/yeast tRNA^{Asp}				yeast AspRS/ yeast tRNA^{Asp} [c]	*E. coli* AspRS/ *E. coli* tRNA^{Asp} [d]
tRNA	K_M (for tRNA) (μM)	k_{cat}[b] (for Asp-tRNA formation) (s^{-1})	k_{cat}/K_M ($10^3\ s^{-1}\ \mu M^{-1}$)	$L_{Tth/y}$	L_y	L_{Ec}
yeast tRNA^{Asp} wild-type	0.098	0.075	765	5		
E. coli tRNA^{Asp} transcript						1
yeast tRNA^{Asp} transcript	0.085	0.32	3800	1	1	
yeast tRNA^{Asp} mutants						
Acceptor stem						
G73→A73	5.0	0.00013	0.026	146150	160	555
G73→U73	5.0	0.0018	0.36	10550	36	200
G73→C73	3.0	0.0018	0.6	6330	200	670
D-stem						
G10→A10	nd	nd	5.25	724	33	6
U25→C25	0.12	0.4	3300	1.2	8	3
Anticodon loop						
G34→A34	3.0	0.003	1	3800	71	
G34→U34	3.0	0.03	10	380	100	3200
G34→C34	0.21	0.77	3700	1	400	
U35→C35	nd	nd	0.002	1900000	91	
U35→A35	1.5	0.00002	0.013	292300	530	>5000
U35→G35	5.0	0.0013	0.26	14600	19	
C36→A36	5.0	0.0022	0.44	8640	150	92
C36→U36	25.0	0.014	0.56	6780	71	53
C36→G36	4.0	0.06	15	253	100	4
C38→U38	4.5	0.023	5.10	745	7[e]	29
C38→G38	0.68	0.034	50	76	13[e]	44
C38→A38	0.80	0.046	57.5	66	8[e]	6
yeast tRNA^{Phe} (control)	not aminoacylated				not aminoacylated	
tRNA$^{Phe→Asp(A38)}$	0.43	0.027	63	60	12	
tRNA$^{Phe→Asp}$	0.30	0.086	287	13	1.24[e]	

[a] Values of K_M and k_{cat} were determined as described in Experimental Procedures. [b] k_{cat} were determined with saturating concentrations of ligands; nd: not determined. The losses (L) of catalytic efficiencies are expressed as k_{cat}/K_M of the wild-type tRNA^{Asp} transcript over k_{cat}/K_M of the variant; $L_{Tth/y}$, L_y, and L_{Ec} are the losses respectively in the heterologous *T. thermophilus*/yeast system and in the homologous yeast and *E. coli* systems. [c] Values from Pütz *et al.* (1991), except for [e](this work). [d] Values from Hasegawa *et al.* (1989) and Nameki *et al.* (1992). tRNA$^{Phe→Asp(A38)}$ and tRNA$^{Phe→Asp}$ are tRNA^{Phe} molecules possessing the transplanted aspartylation identity elements without and with C_{38}, respectively.

charging. The G_{73}→A_{73} mutation decreases catalytic efficiency 10^5 times, indicating that the discriminator base constitutes a major identity element. The three anticodon positions (G_{34}, U_{35}, and C_{36}) are also major identity elements. Mutation of the central U_{35} affects most the charging efficiency (*e.g.*, the U_{35}→C_{35} substitution decreases it 2×10^6 times, whereas the G_{34}→A_{34} and C_{36}→A_{36} substitutions decrease it only 3800 and 8600 times). The effect of the C_{38}→U_{38} mutation is more moderate.

The contribution of C_{38} was clearly defined in *E. coli* by Nameki *et al.* (1992) by a substitution experiment (Table 1), but only indirectly in yeast by a transplantation experiment that revealed that C_{38} optimizes aspartylation of *E. coli* tRNA^{Gln} by yeast AspRS (Frugier *et al.*, 1994). We show here that this position contributes explicitly to aspartate identity in yeast, since the C_{38}→G_{38} mutation decreases 13 times the aspartylation efficiency (Table 1).

Position G_{10} is involved in a tertiary interaction with G_{45} in the variable region (Giegé *et al.*, 1993). In addition to its structural role, G_{10} contributes to aspartate identity in both homologous and heterologous systems although to a variable extent. Indeed, the G_{10}→A_{10} substitution decreases aspartylation efficiency 770-fold (Table 1). However, contrarily to what is observed in the homologous yeast system, the U_{25}→C_{25} substitution preserves charging in the heterologous system (Table 1). Thus, position 25 does not contribute to heterologous identity. Also, it cannot be excluded that it plays an indirect role in identity via the formation of an adequate 45-(10-25) triple base pair. In conclusion, the results indicate a different contribution of U_{25} in the homologous and heterologous systems.

The functionality of the aspartate determinants in the heterologous yeast/*Thermus* system was proven by their transplantation in yeast tRNA^{Phe}. Both tRNAs have similar 3D structures (Kim *et al.*, 1974; Robertus *et al.* 1974; Stout *et al.*, 1978; Moras *et al.*, 1980), and tRNA^{Phe} was found an adequate framework for transplantation of aspartate determinants in the yeast system (Pütz *et al.*, 1991). Although this tRNA already possesses two aspartate identity elements (G_{10} and G_{34}; Figure 2B), no detectable charging by *T. thermophilus* AspRS occurs. Additional transplantations of U_{35}, C_{36}, C_{38}, and G_{73} are needed to obtain aspartylation capacity (Figure 2B). This chimeric transcript is charged as efficiently as the cognate one by yeast AspRS and 13 times less by *T. thermophilus* AspRS (Table 1). Thus, transplantation of C_{38} into yeast tRNA$^{Phe→Asp(A38)}$ improves 10 times its charging efficiency by yeast AspRS. The kinetic analysis indicates that contribution of C_{38} occurs essentially on the k_{cat} level (results not shown). Position C_{38} also improves aspartylation by thermophilic AspRS, but by increasing both affinity and k_{cat} (Table 1). Several reasons can account for the decreased charging efficiency of this

Table 2: Kinetic Constants of Aminoacylation of *T. thermophilus* $tRNA^{Asp}$ and $tRNA^{Phe}$ Variants by the Homologous AspRS, and Effect of Temperature[a]

	kinetic constants of aminoacylation by *T. thermophilus* AspRS								
	37 °C				70 °C				
T. thermophilus tRNA transcripts	K_M (for tRNA) (μM)	k_{cat}[c] (for Asp-tRNA formation) (s^{-1})	k_{cat}/K_M ($s^{-1}\,\mu M^{-1}$)	L_{Tth}	K_M (for tRNA) (μM)	k_{cat}[c] (for Asp-tRNA formation) (s^{-1})	k_{cat}/K_M ($s^{-1}\,\mu M^{-1}$)	L_{Tth}	$(k_{cat}/K_M)(70\ °C)/(k_{cat}/K_M)(37\ °C)$
Part A									
$tRNA^{Asp}$ (wild-type)[b]	0.044	0.77	17.5	1.8	0.030	2.7	90	1	5.1
$tRNA^{Asp}$	0.016	0.49	31	1	0.017	1.5	88	1	2.8
$tRNA^{Phe}$					not aminoacylated				
$tRNA^{Phe\rightarrow Asp\ (A38)}$	0.061	0.20	3.3	9	1.5	0.75	0.5	180	0.15
$tRNA^{Phe\rightarrow Asp}$	0.028	0.32	11	2.8	0.137	1.5	11	8	1.0
Part B									
$tRNA^{Asp}$ single mutants									
G73→A73	0.25	0.22	0.88	35	0.17	0.78	4.58	19	5.2
C73	2.20	0.0093	0.0042	7380	1.40	0.031	0.02	4400	4.8
U73	0.82	0.038	0.046	674	0.83	0.11	0.13	677	2.8
U35→C35	nd	nd	0.003	10333	nd	nd	0.004	22000	1.3
G34→A34	0.64	0.16	0.25	124	1.34	0.60	0.45	195	1.8
C34	1.70	0.29	0.17	182	2.94	1.42	0.48	196	2.8
U34	0.35	0.26	0.74	42	1.42	0.76	0.54	163	0.7
G10-C25→A10-U25	0.061	0.20	3.28	9	0.09	0.89	9.80	9	3.0
$tRNA^{Asp}$ multiple mutants									
$tRNA^{Asp\Omega}$	nd	nd	$<1.1\times10^{-6}$	$>29\times10^{6}$	nd	nd	nd	nd	nd
$tRNA^{Asp@\phi}$	nd	nd	2.1×10^{-5}	1.5×10^{6}	nd	nd	9.3×10^{-5}	950000	4.4
$tRNA^{Asp@int}$	1.5	0.0031	0.002	15500	1.2	0.014	0.01	8800	5.0

[a] The K_M and k_{cat} were determined as described in the Experimental Procedures. (A) Aminoacylation of [b]wild-type modified $tRNA^{Asp}$, of transcript $tRNA^{Asp}$, and of transplanted $tRNA^{Phe}$. $tRNA^{Phe\rightarrow Asp(A38)}$ and $tRNA^{Phe\rightarrow Asp}$ are defined in legend to Table 1. (B) Aminoacylation of $tRNA^{Asp}$ variants. $tRNA^{Asp\Omega}$, $tRNA^{Asp@\phi}$, and $tRNA^{Asp@int}$ are defined in the legend to Figure 3; nd: not determined. [c] k_{cat} were determined with saturating concentrations of ligands; the losses (L_{Tth}) refer to those in the homologous thermophilic system and are determined as described in the legend to Table 1.

chimeric substrate by *T. thermophilus* AspRS. Minor, not characterized determinants could be involved, antideterminants within the $tRNA^{Phe}$ framework would induce steric hindrance and decrease the reactivity of the heterologous complex or expression of the transplanted positions would be optimal only in the structural context of the prokaryotic and/or thermophilic system.

Transplantation of the Aspartate Identity Elements in T. thermophilus $tRNA^{Phe}$. Identification of the Aspartate Determinants in the Thermophilic System. The thermophilic $tRNA^{Phe\rightarrow Asp}$ transcript was created by transplantation of U_{35}, C_{36}, C_{38}, and G_{73} in the *T. thermophilus* $tRNA^{Phe}$ transcript containing already the determinants G_{10} and G_{34} (Figure 2C). The chimera is charged nearly as efficiently than the cognate transcript (only 3 and 8 times less at 37 and 70 °C; Table 2A). The transplanted positions confer to this molecule both affinity for the synthetase and aspartylation capacity (at 37 °C the K_M of $tRNA^{Phe\rightarrow Asp}$ is similar to that of the cognate transcript and k_{cat} is decreased only 2-fold) in contrast to $tRNA^{Phe}$ deprived of binding capacity to AspRS (K_i > 10 μM). This is confirmed by the kinetic properties of $tRNA^{Asp\Omega}$ substituted at all identity positions (Figure 3A) which presents a drastically decreased aspartylation capacity ($L > 29\times10^6$; Table 2B) and absence of measurable binding capacity (K_i > 13 μM).

Finally, C_{38} increases the charging efficiency of the transplanted $tRNA^{Phe}$ (3-fold at 37 °C and 20-fold at 70 °C) by acting essentially on the affinity of the chimera for the thermophilic AspRS [compare the kinetic constants of the transcript possessing either A_{38} or C_{38} ($tRNA^{Phe\rightarrow Asp(A38)}$ and $tRNA^{Phe\rightarrow Asp}$); Table 2A].

Peculiar Aspects of the Heterologous Yeast $tRNA^{Asp}$/T. thermophilus AspRS System. The catalytic losses (L values) due to mutation of aspartate identity elements in the heterologous and homologous systems are shown in Table

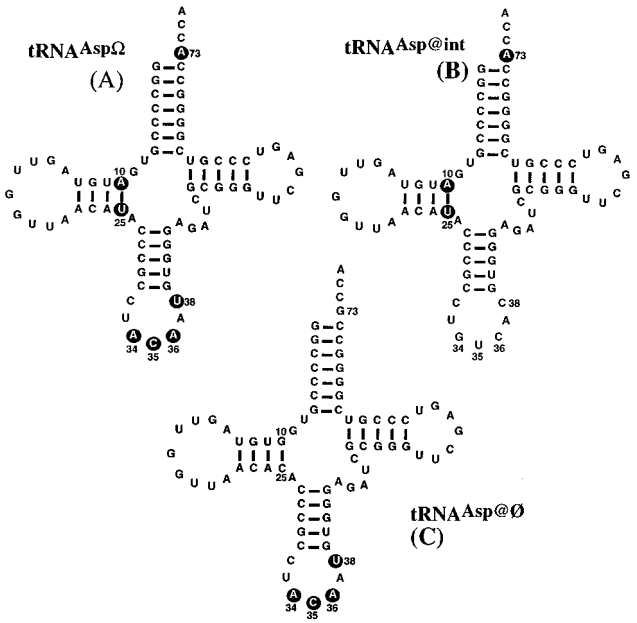

FIGURE 3: Cloverleaf structures of multiple mutants of *T. thermophilus* $tRNA^{Asp}$. Substitution (white characters in black dots) of all identity elements: $tRNA^{Asp\Omega}$ (A), or of G_{73} and the G_{10}-C_{25} base pair: $tRNA^{Asp@int}$ (B), or of the identity elements in the anticodon loop: $tRNA^{Asp@\phi}$ (C).

1. Substitutions of determinants in yeast $tRNA^{Asp}$ affect differently aspartylation by the *T. thermophilus* and yeast synthetases. The effects differ qualitatively and quantitatively. Conservative purine/purine substitutions decrease strongly the charging efficiency by *T. thermophilus* AspRS ($G_{73}\rightarrow A_{73}$, $G_{34}\rightarrow A_{34}$, $U_{35}\rightarrow C_{35}$, and $C_{38}\rightarrow U_{38}$ substitutions increase 24, 3800, 130, and 11 times the losses provoked by nonconservative substitutions). Surprisingly, the $G_{34}\rightarrow C_{34}$ substitution is without significant effect. Conversely, nonconservative substitutions induce maximal effects in the

homologous system ($G_{34}\rightarrow C_{34}$, $U_{35}\rightarrow A_{35}$, $C_{36}\rightarrow A_{36}$, and $C_{38}\rightarrow G_{38}$ substitutions increase 6, 28, 2, and 2 times the losses due to conservative substitutions). In *E. coli*, no such rule is observed: nonconservative mutations induce important effects at positions 34 and 35, but for positions 36, 38, and 73 the extent of the losses is independent of the conservative or nonconservative nature of the substitution.

Drastic quantitative variations between homologous and heterologous aspartylation systems were found (Table 1). In the heterologous sytem L values can exceed by 4 orders of magnitude those measured in the homologous one. Since these amplified effects might be related to the thermophilic nature of the protein, we investigated the substitution of aspartate determinants in the thermophilic system.

Effects of Substituting T. thermophilus tRNA^{Asp} Determinants on Charging by Homologous AspRS. The positions found most sensitive in the heterologous system, namely, G_{73}, G_{34}, and U_{35}, were substituted in thermophilic tRNA^{Asp} (Figure 2D). Contrary to what is found in the heterologous system, less drastic effects are observed (the maximal loss is only 22 000-fold; Table 2B). But like in the homologous yeast and *E. coli* systems, nonconservative substitutions have the strongest effects (*e.g.*, compare the effects of $G_{73}\rightarrow C_{73}$ or A_{73} and of $G_{34}\rightarrow C_{34}$ or A_{34} substitutions; Table 2B).

Raising the temperature from 37 to 70 °C increases 3−5 times the charging efficiency of tRNA^{Asp} and of most variants as a consequence of increased k_{cat}; K_M is not or is only moderately affected (Table 2). Therefore, interaction of most identity positions with the synthetase is not altered by temperature changes. However, tRNA^{Asp} U_{34} and A_{34} variants and tRNA$^{Phe\rightarrow Asp}$ are charged with similar efficiencies at 37 and 70 °C since raising temperature increases k_{cat} and K_M. Thus, structural alterations likely promote destabilization of these complexes at high temperature, as shown by the 6-fold increase of K_M of tRNA$^{Phe\rightarrow Asp(A38)}$ compared to that of tRNA$^{Phe\rightarrow Asp}$ when temperature is shifted from 37 to 70 °C.

Interrelation between Identity Elements in the Thermophilic System. It was studied by analysis of multiple mutants of tRNA^{Asp}. Table 2B shows that tRNA$^{Asp@\phi}$ lacking the four anticodon loop identity residues is deprived of measurable affinity for AspRS (K_i > 13 μM) and of efficient charging capacity ($L = 1.5 \times 10^6$). This loss exceeds by 2 orders of magnitude that of tRNA$^{Asp@int}$ mutated at the two other identity residues G_{10} and G_{73} within the accepting branch of the tRNA ($L = 1.5 \times 10^4$). Thus, identity elements in the anticodon loop prevail both in recognition and in determining catalytic efficiency. The activity losses of these mutants are compatible with those estimated for tRNA$^{Asp\Omega}$ deprived of the complete set of identity elements ($L > 30 \times 10^6$), but because of the poor estimate of kinetic parameters, global additive or (anti)-cooperative effects between the two sets of positions are difficult to establish with certainty.

However, a strong cooperative effect is evidenced between G_{10} and G_{73}. Indeed, the experimental loss of tRNA$^{Asp@int}$ exceeds by 44 times that calculated assuming additive, or in other words independent, contributions of G_{10} and G_{73} ($L_{exp} = 1.5 \times 10^4$ and $L_{cal} = L_{A73} \times L_{A10-U25} = 315$; Table 2B). Interestingly, the K_M of this mutant is that expected assuming independant contributions of both positions (1 and 1.5 μM), while the k_{cat} is 30-fold lower than expected from single mutants (9×10^{-2} and 3×10^{-3} s^{-1}), indicating that cooperativity occurs at the k_{cat} level. An opposite interrelation occurs between the identity residues of the anticodon loop since the loss of charging of tRNA$^{Asp@\phi}$ is lower than expected from additivity of single mutations. Here the effect induced by the mutation of all anticodon loop identity elements equals the independent effects induced solely by the A_{34} and C_{35} mutants ($L_{exp} = 1.5 \times 10^6$ to be compared to $L_{cal} = L_{A34} \times L_{C35} = 1.3 \times 10^6$; Table 2B), without taking into account individual effects that would be due to mutations at positions 36 and 38. Therefore, the four anticodon determinants likely act anticooperatively.

GENERAL DISCUSSION

Aminoacylation of tRNA^{Asp} of Various Organisms by T. thermophilus AspRS

We show that *T. thermophilus* AspRS efficiently aminoacylates tRNA^{Asp} from yeast and *E. coli* in addition to its own tRNA^{Asp}. This behavior resembles that observed for *T. thermophilus* PheRS that charges well cognate tRNA^{Phe} from yeast and *E. coli* (Stepanov *et al.*, 1992; Moor *et al.*, 1992), although both thermophilic systems diverge by their kinetic properties. Thermophilic AspRS binds one amino acid per tRNA (results not shown) whereas PheRS attaches two amino acids on *E. coli* tRNA^{Phe} (Stepanov *et al.*, 1992). Efficient charging of cognate tRNAs in heterologous mesophilic/thermophilic systems from prokaryotes was also reported for GluRS, ArgRS, LysRS, and SerRS (Hara-Yokoyama *et al.*, 1986; Kumazawa *et al.*, 1991). Altogether this indicates that the structural and kinetic parameters conferring specificity and catalytic competence are conserved in a number of aminoacylation systems. This conclusion agrees with the phylogenetic relationship between *T. thermophilus* and *E. coli* despite adaptation of the thermophile to extreme life conditions. But exceptions have been reported. ThrRS from *T. thermophilus* poorly aminoacylates *E. coli* tRNA^{Thr} (Kumazawa *et al.*, 1991; Zheltonosova *et al.*, 1994), indicating different evolutions of the threonine system in mesophiles and thermophiles. Concerning the aspartylation system, efficient charging of the thermophilic tRNA^{Asp} transcript indicates that post-transcriptional modifications are not essential, as is also the case in the homologous systems from yeast and *E. coli* (Pütz *et al.*, 1991; Nameki *et al.*, 1992). In *E. coli*, none of the aspartate identity determinant is modified, except G_{34} converted to Q_{34} (Sekiya *et al.*, 1980); this modification is not involved in aspartylation since full charging capacity is conserved in the transcript and in the tRNA expressed in a strain unable to promote this modification (Nameki *et al.*, 1992; Martin *et al.*, 1993). Interestingly enough, the hypermodification of G_{34} to Q_{34} is absent in tRNA^{Asp} from *T. thermophilus* (Keith *et al.*, 1993).

The Heterologous Aspartylation System

Recognition of Yeast tRNA^{Asp} by T. thermophilus AspRS and Comparison with the Homologous Yeast System. Except for U_{25}, the same nucleotide set in yeast tRNA^{Asp} determines aspartylation by yeast and *T. thermophilus* AspRSs. These elements are those defined by Pütz *et al.* (1991) in the yeast system (G_{10}, G_{34}, U_{35}, C_{38}, and G_{73}) but include additionally C_{38}. This is demonstrated by the optimization of the aspartylation of a tRNA^{Phe} transcript when C_{38} is cotransplanted with other aspartate identity elements. However, the functional expression differs in the homologous and heterologous systems as revealed by the different kinetic effects induced

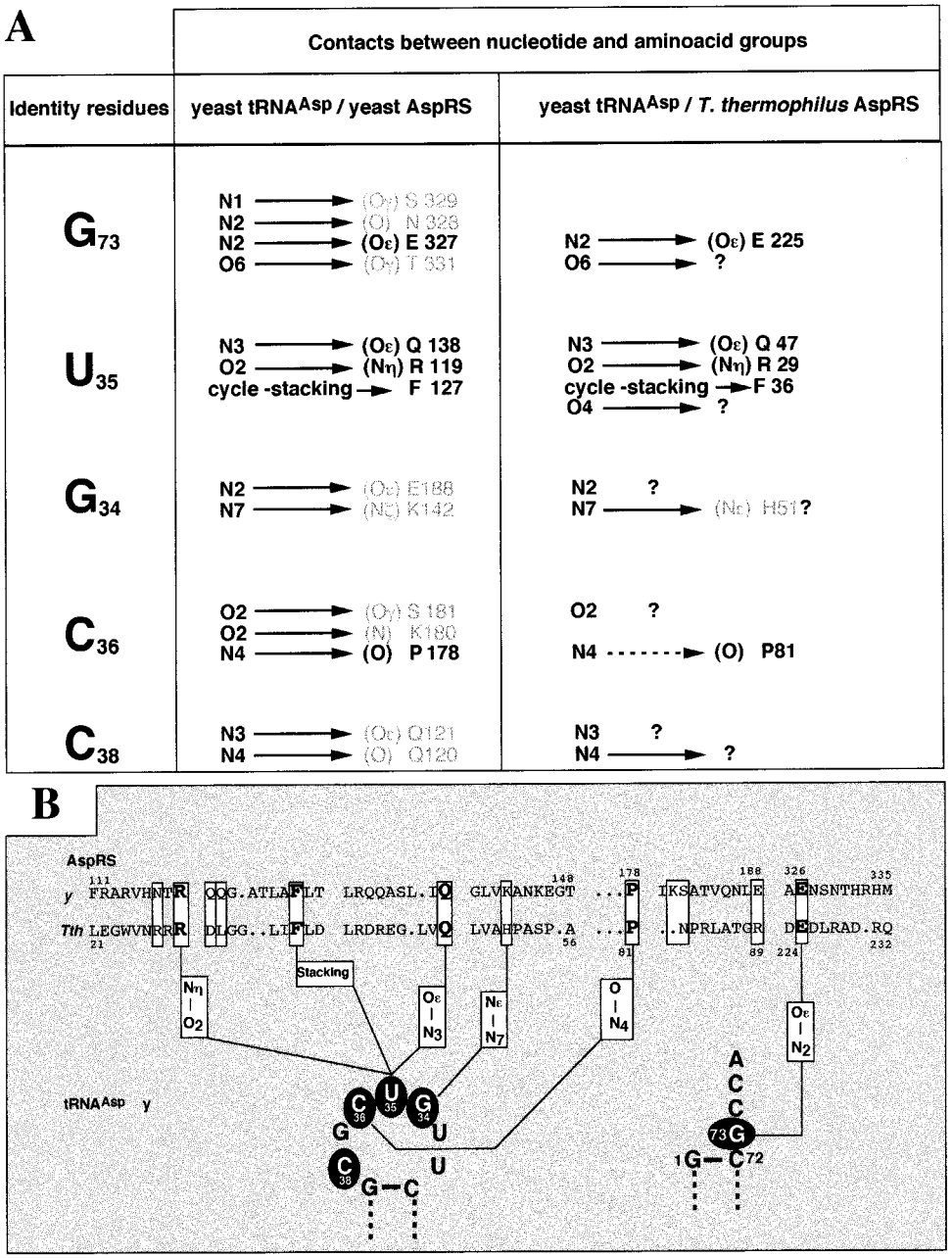

FIGURE 4: Prediction of interactions between *T. thermophilus* AspRS and yeast tRNA^{Asp} and comparison with the interactions in the cognate yeast aspartate system. (A) Chemical groups of the identity elements of yeast tRNA^{Asp} and amino acids of yeast and *T. thermophilus* AspRSs involved in homologous and heterologous aspartylations. For the homologous yeast system, the interacting chemical groups of the tRNA identity elements are those predicted by analysis of tRNA variants (Pütz *et al.*, 1991; Giegé, 1994) and/or revealed by crystallography (Cavarelli *et al.*, 1993), and the interacting amino acid residues are those revealed by the 3D structure of the complex (Cavarelli *et al.*, 1993). For the heterologous system, the interacting chemical groups of the tRNA are predicted in this work, and the resulting interactions are based on AspRSs alignments (Delarue *et al.*, 1994). Amino acid residues in bold are conserved; bold and dotted arrows indicate respectively strong and weak interactions. (B) Schematic representation of the interactions between amino acids of the thermophilic AspRS and the identity residues in the anticodon loop and the acceptor arm of yeast tRNA^{Asp}. The interacting groups of amino acids and nucleotides are boxed. Sequences are aligned according to Delarue *et al.* (1994); conserved amino acids are in bold characters and amino acids involved in recognition in the yeast complex are boxed. The identity elements of the tRNA^{Asp} are white characters in black dots. *Tth: T. thermophilus*; y: yeast.

by their substitutions in yeast tRNA^{Asp}. These kinetic differences are likely the consequence of subtle variations in the contacts between tRNA and synthetase in both complexes, reflecting existence of particular sequence features of the interacting molecules, particularly at the protein level. Indeed, alignments reveal, despite the residues conserved in all AspRSs, only 28% sequence conservation between yeast and *T. thermophilus* AspRSs; this figure reaches 50% between *E. coli* and *T. thermophilus* AspRSs (Delarue *et al.*, 1994). Furthermore, the 3D structure of the homologous yeast complex shows that aspartate identity nucleotides establish contacts with conserved amino acid residues of the protein but also with nonconserved residues. Because of the structural variations brought by the interaction with the nonconserved amino acids, it becomes understandable that differential functional effects occur in both complexes when identity elements are mutated, despite comparable aminoacylation efficiencies when the partners are wild-type species. The underlying structural features are summarized in Figure 4A. Thus, in the yeast complex, G_{34}, U_{35}, C_{36}, and G_{73} contact conserved amino acids in all AspRSs, except E_{188} surprisingly not present in *T. thermophilus* AspRS

(Figure 4B). Further, there is a quasi-conservation of the chemical groups of identity nucleotides implied in homologous and heterologous aspartylations, although nonconserved amino acids may be implied.

In the homologous system, N_2 of G_{73} is in contact with E_{327} and O_6 with T_{331} (Cavarelli *et al.*, 1993). In the heterologous one, the N_2 and O_6 groups of the discriminator base likely also interact with AspRS, as suggested by the dramatic catalytic loss consecutive to their replacement by H and NH_2 groups in the A_{73} mutant (Table 1). The N_2 group probably interacts with conserved E_{225} but less strongly than in the homologous system because of the limited mutational effect when N_2 is replaced by O in the G→C mutation. This interaction may be partly preserved by the N_2→OH replacement, as suggested by the rather moderate deleterious effect of the G→U mutation. Finally, the interaction of G_{73} with T_{331} in the homologous complex cannot occur in the heterologous complex because of the nonconservation of T_{331} replaced by A_{229} in *T. thermophilus* AspRS.

Among the 3 positions of the anticodon, the central U_{35} constitutes the major identity element since its substitution induces the highest activity losses (Table 1). This position is also prevalent for aspartate identity in yeast and *E. coli*. Interestingly, alignment of AspRS sequences reveals conservation of 3 out of the 4 amino acids, namely, R_{119}, F_{127}, and Q_{138}, found in contact with this nucleotide in the yeast complex (Delarue *et al.*, 1994). Therefore, it can be anticipated that these amino acids in thermophilic AspRS will contact identity nucleotides in the heterologous tRNA as well. The stacking of the pyrimidine ring of U_{35} with the aromatic ring of F_{127} in the yeast complex (Cavarelli *et al.*, 1993) may thus occur with conserved F_{36} in *T. thermophilus* AspRS. The O_4 atom of U_{35}, not found in contact with the homologous synthetase, seems to be involved in the heterologous one as suggested by the poor effect observed by G replacement. Substitution of O_4 by NH_2 abolishes this contribution (Table 1). The proximity of U_{35} with R_{119} and Q_{138} in the yeast complex suggests proximity in the heterologous complex of this base with conserved R_{29} and Q_{47} in thermophilic AspRS. Interaction of O_2 and N_3 of U_{35} with respectively R_{119} and Q_{138} may thus occur in the heterologous system, although they are not revealed by the U→C substitution preserving the O_2 and N_3 groups probably because of a steric effect introduced by NH_2.

The yeast complex reveals that N_7 and N_2 of G_{34} are in close proximity respectively to K_{142} and E_{188}, in agreement with the functional analysis of the G_{34} mutants (Table 1), showing that interaction of N_7 prevails that of N_2. In *T. thermophilus* AspRS these amino acids are replaced by H_{51} and R_{89}. Nevertheless, interaction of N_7 could occur with H_{51}, and interaction of N_2 should be hindered by the positively charged guanidyl group of R_{89}. Contrarily to the homologous complex, implication of N_2 in the heterologous complex can *a priori* be excluded since the G→C substitution is without functional effect.

According to the 3D structure of the yeast complex, O_2 of C_{36} is in the vicinity of K_{180} and S_{181}, and N_4 is close to P_{178}. The role of O_2 agrees with the poor effect induced by the C→U mutation. However, this interaction fails in the heterologous system since the K and S residues are not conserved in thermophilic AspRS and since U_{36} and A_{36} mutations induce similar effects. Despite conservation of P_{81}, contribution of N_4 of C_{36} in the heterologous complex seems moderate as indicated by the weak catalytic effect of the C→G substitution. An additional interaction of C_{36} could involve R_{29}, a conserved amino acid, since the homologous complex shows proximity of R_{119} with C_{36}.

Residue C_{38} is a minor identity element in both homologous and heterologous aspartate systems. Its proximity with Q_{120} and Q_{121} in the yeast complex agrees with the weak effect induced by the C→A substitution. However, none of these amino acids is conserved in *T. thermophilus* AspRS. Nevertheless, the strong and weak losses observed with the U_{38} and A_{38} mutants agree with a contribution of N_4 in the heterologous system. The weak loss induced by the C→G substitution, despite the absence of a N_4 group, can result from compensation of a positive effect.

Interpretation of the effect of G_{10} mutation is delicate, since it is involved in the tertiary triple interaction G_{10}-U_{25}-G_{45}. In the homologous yeast complex its contribution involves the N_2 group exposed in the minor groove of the D-stem as suggested by the activity loss observed for the A_{10} mutant lacking this group and by the U_{25}→C_{25} substitution which allows an H-bond with N_2 and abolishes the contribution of G_{10}. However, crystallographic data do not reveal any contact with G_{10}, suggesting an indirect role of this nucleotide. Interestingly, mutation of G_{10} in yeast tRNA^{Asp} acts strongly on the affinity for *T. thermophilus* AspRS, suggesting also a participation of this nucleotide in the heterologous system. However, there is a difference between both systems because U_{25}→C_{25} substitution, in contrast to its effect in the homologous system, is without effect in the heterologous one. Therefore, the N_2 group contributes differently in the two systems.

Peculiar Kinetic Aspects of tRNA Charging in the Heterologous Complex. The amplified effects of tRNA mutations in the heterologous system could rely to the thermophily of the protein, deprived at suboptimal temperature of the necessary flexibility to adapt optimally the tRNA. At 37 °C, a rigidity of the protein would amplify all structural alterations and disfavor formation of the functional complex. But this view is not consistent with the relative temperature independence of the mutational effects of the heterologous yeast tRNA and with the moderate effects observed for mutants of the thermophilic tRNA (Table 2B).

An alternative interpretation could be related to the prokaryotic and eukaryotic origins of AspRS and tRNA^{Asp}. For example, the difference in lengths of the variable loops in eukaryotic and prokaryotic tRNA^{Asp} (4 nucleotides in yeast and 5 in *T. thermophilus* and *E. coli* tRNAs) could decrease the functionality of the heterologous complex. This view agrees with the increased charging efficiency of the prokaryotic tRNA^{Asp} species compared to that of yeast tRNA^{Asp} by thermophilic AspRS (Figure 1A) but is excluded by the efficient charging of beef liver tRNA^{Asp} by the thermophilic AspRS, despite its five-nucleotide-long variable loop (unpublished). Eventually, these drastic effects could be due to an eukaryotic antideterminant or to the absence of a prokaryotic determinant in yeast tRNA^{Asp} amplifying alterations in the heterologous complex; structural elements disfavoring complex formation would agree with the slightly decreased charging efficiency by *T. thermophilus* compared to yeast AspRS.

The Aspartylation System from T. thermophilus

Static Aspects of Recognition between Synthetase and tRNA. Figure 2D shows the location of the identity

determinants in the thermophilic $tRNA^{Asp}$. A model of the complex of *T. thermophilus* AspRS with $tRNA^{Asp}$ based on the 3D structures of the thermophilic synthetase and the yeast complex reveals the probable interactions between tRNA and synthetase (Delarue *et al.*, 1994). The identity elements from the anticodon loop would contact the N-terminal domain of the protein, a five-stranded antiparallel β-barrel and an α-helix, distinguishing subclass IIb, to which belongs AspRS, from subclass IIa (Cavarelli *et al.*, 1993; Commans *et al.*, 1995). Residue U_{35} may establish contacts with the conserved R_{29}, F_{36}, and Q_{47} localized in the β-barrel, but additional interactions of C_{36} with conserved R_{29} could occur as in the yeast complex (Figure 4B). The acceptor end of tRNA would contact the active site domain built around the six-stranded antiparallel β-sheet including consensus motifs 2 and 3 of class II synthetases. The N_7 and O_6 groups of G_{73} protruding from the major groove of the tRNA accepting domain may interact with conserved E_{225} of the variable loop (residues 225−229) of consensus motif 2. Additional interactions of G_{73} would involve groups accessible from the minor groove side of the accepting branch and amino acids in the domain inserted between consensus motifs 2 and 3. This domain is significantly larger in prokaryotic than in eukaryotic AspRSs and consists in the thermophilic enzyme in a curved antiparallel β-sheet flanked on both sides by α-helices (Delarue *et al.*, 1994). These variations can be related to different involvements of G_{73} in identity expression (second rank in the hierarchy of contribution in *T. thermophilus* but only third rank in yeast; Tables 1 and 2) and may indicate different modes of interaction of the acceptor arm in both systems.

The role of G_{10} in aspartate identity is more difficult to establish (see above). Nucleotides surrounding G_{10} are in the vicinity of the flexible hinge domain (residues 107−137) joining the anticodon-binding and the catalytic domains (Delarue *et al.*, 1994). Possible contacts involve the second part of this domain exhibiting structural conservation (residues 123−137) while the first part (residues 107−123) does not display sequence conservations and could therefore account for peculiarities in interactions in each aspartylation system.

As shown in Table 2, the relative effects induced by substitution of identity elements are similar at 37 and 70 °C. As a consequence, the recognition of tRNA and synthetase utilizes the same structural features in a large temperature range, and probably formation of the productive complex occurs via a unique pathway.

Dynamic Aspects of the Interaction. Implication for the Functional Adaptation between AspRS and $tRNA^{Asp}$. The drastic decrease of affinity of the variant substituted in the four anticodon loop determinants ($tRNA^{Asp@\phi}$) suggests that association of these elements with the anticodon-binding domain of the protein prevails in complex formation. Since these elements are localized in well exposed domains of tRNA and protein, their interaction may constitute the primary event preceding interaction of the acceptor arm with the synthetase or at least reinforcing it. The synergistic contribution of G_{10} and G_{73} on this interaction acts on the k_{cat} level, although both positions contribute individually to affinity (see Table 2). Thus, the coupling in recognition of both groups of determinants increases the reactivity of the complex without strengthening the interaction of the partners. The comparison of the 3D structures of free and tRNA-bound synthetases reveals a transconformation of AspRS promoted by tRNA, resulting in different orientations of amino acid residues in the catalytic center (Poterszman *et al.*, 1994). Such a transconformation may dictate the functional adaptation between synthetase and tRNA. It could be triggered by recognition of the anticodon elements and/or nucleotides around position G_{10} in variants with the nearby protein hinge domain.

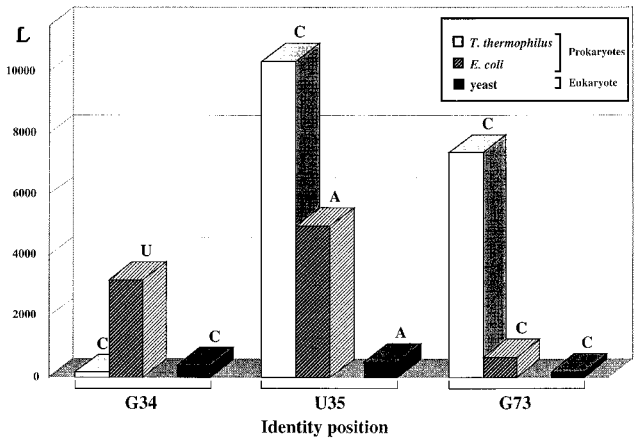

FIGURE 5: Schematic evaluation of the relative importance of the three major identity elements in the homologous yeast, *E. coli*, and *T. thermophilus* aspartylation systems. The histogram shows for each position the highest loss (L) induced by mutations (see Tables 1 and 2B); the nature of the substitution is indicated on the top of the histogram bars. For *T. thermophilus* the losses are those measured at 37 °C.

Contribution of the Major Aspartylation Determinants in the Homologous Prokaryotic and Eukaryotic Systems

Figure 5 summarizes the contribution of the three major identity elements, G_{34}, U_{35}, and G_{73}, to the aspartate identity in the homologous yeast, *E. coli*, and *T. thermophilus* systems. Striking variations are observed. First, the relative implication of these elements to efficient aspartylation is system dependent: influence of U_{35} prevails in the three systems, but in yeast and *E. coli* the effect of G_{34} exceeds that of G_{73} whereas in *T. thermophilus* the contribution of these nucleotides is inversed. Second, the maximal losses of charging produced by substituting identity elements increase from eukaryotes to prokaryotes: the optimal effect due to mutating U_{35} in *T. thermophilus* and *E. coli* exceeds that in yeast. The same effect is observed by mutating the elements occupying the second position in the hierarchy of contributions (G_{73} in *T. thermophilus* and G_{34} in *E. coli* and yeast) and the third one (G_{34} in *T. thermophilus* and G_{73} in *E. coli* and yeast). Table 3 shows the relative losses of charging induced by mutating the identity positions in the eukaryotic and prokaryotic aspartylation systems. Except for a few mutations, substitution of the identity determinants induce higher effects in *E. coli* and *T. thermophilus* than in yeast: L_{Tth}/L_y and L_{Ec}/L_y ratios are frequently higher than L_{Tth}/L_{Ec} ratios. The activity losses observed in the heterologous system (*T. thermophilus* AspRS and yeast $tRNA^{Asp}$) also often exceed those in *E. coli* and even more those in yeast.

The amplified effects due to mutating identity elements in the prokaryotic compared to the eukaryotic aspartylation systems illustrate a more general behavior of aminoacylation systems in both phylogenic groups. Similar effects are observed when comparing the threonine and phenylalanine systems from *E. coli* and yeast (Hasegawa *et al.*, 1992;

Table 3: Comparison of the Losses Induced by Mutations of Identity Positions in the Homologous Prokaryotic and Eukaryotic Aspartylation Systems and in the Heterologous System

	ratio of losses[a]				
tRNA^{Asp} mutants	$L_{Tth/y}/L_y$	$L_{Tth/y}/L_{Ec}$	L_{Tth}/L_y	L_{Ec}/L_y	L_{Tth}/L_{Ec}
G73→A73	913	263	0.22	3.4	0.06
U73	293	53	19	5.6	3.37
C73	32	9	37	3.3	11
G10-C25→A10-U25	22	121	0.3	0.18	1.5
G34→A34	54		1.74		
U34	4	0.121	0,42	32	0.013
C34	0.0026		0.46		
U35→C35	20880		114		
A35	552	<60		>10	
G35	768				
C36→A36	58	94		0.6	
U36	95	128		0.75	
G36	2.5	63		0.04	
C38→U38	106	26		4.1	
G38	6	1,7		3.4	
A38	8	11		0.75	

[a] The ratios of the losses were determined from values in Tables 1 and 2. L_{Tth}, L_y, L_{Ec}, and $L_{Tth/y}$ are the losses determined for the tRNA variants in the homologous *T. thermophilus* (values at 37 °C), *E. coli*, and yeast systems and in the heterologous *T. thermophilus*/yeast system.

Sampson et al., 1992; Tinkle Peterson *et al.*, 1992; Nameki, 1995). In prokaryotes, determinants act individually stronger than in eukaryotes. This indicates different relationships between identity elements in the two phylogenic groups and may be related to structural variations in the synthetases (Delarue *et al.*, 1994), such as N-terminal extensions in eukaryotic synthetases and insertion domains in the prokaryotic ones. It is also indicative of a different evolution of aminoacylation systems in prokaryotes and eukaryotes, despite conservation of the identity elements. In yeast, efficient aspartylation is the result of synergistic effects between the individual identity positions (Pütz *et al.*, 1994). We show that in the prokaryotic thermophile specific aspartylation involves strong cooperativity between the anticodon loop determinants and G_{73} and G_{10} and anticooperativity between determinants in the anticodon loop.

Functional Implication of Variations in the Hierarchy of Aspartylation Identity Elements in Yeast, E. coli, and T. thermophilus

The inversion of the relative strength of G_{73} and G_{34} in *T. thermophilus* compared to yeast and *E. coli* (second and third contributing ranks in *T. thermophilus* and vice versa in yeast and *E. coli*) is partly based on the strong effect of the G_{73}→C_{73} substitution in the thermophilic system. This behavior is not related to a steric effect of C acting as antideterminant in the thermophilic protein context, since the same substitution in yeast tRNA^{Asp} has just a moderate effect. More likely, it indicates differential roles of G_{34} in the mesophilic and thermophilic systems, reflecting possible variations in the selection mechanism of tRNA^{Asp} by the AspRSs. The specific role of nucleotide 34 in this selection is evidenced by the presence in tRNA^{Glu} of the complete set of elements confering aspartate identity, except G_{34} replaced by mcm^{5}s^{2}U and mam^{5}s^{2}U in yeast and *E. coli* tRNA^{Glu} (Kobayashi *et al.*, 1974; Ohashi *et al.*, 1972). It was shown that the modified base contributes predominantly to glutamate identity in *E. coli* (Kern & Lapointe, 1979; Sylvers *et al.*, 1993). This position is therefore involved in discrimination of tRNA^{Asp} and tRNA^{Glu} by AspRS and promotes specific charging of tRNA^{Asp}, in addition to a possible antideterminant effect for aspartylation in tRNA^{Glu}. The decreased contribution of G_{34} in aspartylation by *T. thermophilus* AspRS impairs the ability of this synthetase to discriminate both tRNAs as revealed by mischarging of yeast and *E. coli* tRNA^{Glu} in unfractionated tRNA (unpublished). However, aspartylation occurs specifically in the homologous systems. Thus discrimination of tRNA^{Asp} and tRNA^{Glu} occurs differently in thermophiles and mesophiles.

CONCLUDING REMARKS

The most important elements conferring identity for tRNA aspartylation are restricted to 6 invariant nucleotides conserved in prokaryotes and eukaryotes as well as in mesophiles and thermophiles. These elements express the aspartylation identity in different structural contexts and are adapted to various life conditions including the extreme ones. More generally, and in the context of a conservation of aminoacylation systems throughout living systems, this shows that accurate aspartylation cannot tolerate large fluctuations of its determinants. Thus this identity was likely defined at an early stage of evolution and was conserved in contemporary living systems by parallel evolution mechanisms, or less likely it resulted from a convergent evolution. In this view, the minor variations in identity expression, exemplified by different quantitative participations of the conserved identity elements, would reflect peculiarities of such evolutionary processes especially when comparing prokaryotes and eukaryotes. Moreover, comparison of aminoacylation systems indicates that specificity of tRNA charging results from combined participations of nucleic acid and protein identity elements in each particular tRNA/synthetase system and of competition of the cognate partners for specific associations. Since in the various phylae each aminoacylation system had to solve other constraints resulting from a different competition context, the dynamic process of each aspartylation system evolved in such a way as to ensure efficient and specific charging of tRNAs by conservation of the identity elements. Therefore, the fluctuations in the contribution of the tRNA identity elements may be related to evolutionary variations that in turn lead to optimize in each particular organism, the functional adaptation between cognate tRNAs and synthetases.

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