Recognition by Tryptophanyl-tRNA Synthetases of Discriminator Base on tRNA<sub>Trp</sub> from Three Biological Domains*

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To study the recognition by tryptophanyl-tRNA synthetase (TrpRS) of tRNA<sub>Trp</sub> discriminator base mutations, we introduced into the discriminator base of Bacillus subtilis, Archeoglobus fulgidus, and bovine tRNA<sub>Trp</sub>, representing the three biological domains. When B. subtilis, A. fulgidus, and human TrpRS were used to acylate these tRNA<sub>Trp</sub>, two distinct preference profiles regarding the discriminator base of different tRNA<sub>Trp</sub> substrates were found: G>A>U>C for B. subtilis TrpRS, and A>U>G for A. fulgidus and human TrpRS. The preference for G73 in tRNA<sub>Trp</sub> by bacterial TrpRS is much stronger than the modest preferences for A73 by the archaeal and eukaryotic TrpRS. Cross-species reactivities between TrpRS and tRNA<sub>Trp</sub> from the three domains were in accordance with the view that the evolutionary position of archaea is intermediate between those of eukarya and bacteria. NMR spectroscopy revealed that mutation of A73 to G73 in bovine tRNA<sub>Trp</sub> elicited a conformational alteration in the G1-C72 base pair. Mutations of G1-C72 to A1-U72 or disruption of the G1-C72 base pair also caused reduction of Trp-tRNA<sub>Trp</sub> formation. These observations identify a tRNA<sub>Trp</sub> structural region near the end of acceptor stem comprising A73 and G1-C72 as a crucial domain required for effective recognition by human TrpRS.

Aminoacyl-tRNA synthetases catalyze the covalent attachment of amino acids to their cognate tRNA, thereby ensuring the faithful translation of the genetic code (1, 2). Cross-species aminoacylation provided a useful phylogenetic probe that generated early evidence for the close relationship between archaea and eukarya (3). The specific recognition of a tRNA by its cognate aminoacyl-tRNA synthetase is guided by identity elements on the tRNA. The discriminator base (N73) has been shown to be a major identity element on tRNA<sub>Trp</sub> for bacterial and eukaryotic TrpRS (4–6) as well other tRNAs (7). N73 is strongly conserved in tRNA<sub>Trp</sub> among different species from the same biological domain, always being G in bacterial and chloroplast tRNA<sub>Trp</sub> but A in archaeal and most eukaryotic tRNA<sub>Trp</sub> (8). The reliance on N73 by TrpRS in its specific recognition of tRNA<sub>Trp</sub> has resulted in one of the first observations of an evolutionary change in identity elements, with bacterial and eukaryotic TrpRS utilizing dissimilar identity elements on the tRNA<sub>Trp</sub> (4).

To gain further insight into this evolutionary change in N73 recognition, in this study the interactions between TrpRS and tRNA<sub>Trp</sub> from all three biological domains of bacteria, archaea, and eukarya were compared. Archeoglobus fulgidus is a hyperthermophilic, sulfate-reducing, and strictly anaerobic archaean, for which the complete genome has been sequenced (9). A complete set of Bacillus subtilis, A. fulgidus, and bovine tRNA<sub>Trp</sub> carrying different base substitutions at N73 was hyperexpressed in Escherichia coli and examined in terms of their efficiency as substrates for tryptophanylation by B. subtilis, A. fulgidus, and human TrpRS. Moreover, to determine any structural role of N73 in bringing about tryptophanlyation, solution NMR spectroscopy was employed to compare the structures of wild-type A73 bovine tRNA<sub>Trp</sub> and its G73 mutant, which were respectively, the best and the poorest substrates among the bovine tRNA<sub>Trp</sub> N73 variants toward reaction with human TrpRS.

MATERIALS AND METHODS

Constructs of Wild-type and Mutant tRNA<sub>Trp</sub>—Three pairs of complementary oligodeoxyribonucleotides separately encoding a T7 promoter sequence upstream of B. subtilis, A. fulgidus, and bovine tRNA<sub>Trp</sub> genes carrying random nucleotides at position N73 were synthesized according to the gene sequences of B. subtilis (GenBank™ accession number D10981), bovine (GenBank™ accession number M10543), and A. fulgidus (GenBank™ accession number AE000782) tRNA<sub>Trp</sub> (see Fig. 1 below). Individual pairs of the oligodeoxyribonucleotides were mixed and incubated at 60 °C for 15 min to form double-stranded DNA fragments, which were separately inserted into the SfiI and HindIII restriction sites of the E. coli pGEM-9zf(−) vector (Promega) (4). PCR-based site-directed mutagenesis was employed to construct single and double mutations of N73 and the 1–72 base pairs using a proofreading Pfu DNA polymerase (Stratagene). All recombinant plasmids were obtained by 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal, Sigma Chemical Co.) Blue-White screening and confirmed by DNA sequencing using the BigDye sequencing kit (Applied Biosystems).

Sequence Alignment of the tRNA<sub>Trp</sub> Gene—Sequences of B. subtilis, A. fulgidus, and bovine tRNA<sub>Trp</sub> were aligned using the program ClustalW. The position of each residue was assigned, and the cloverleaf structures of these three tRNA<sub>Trp</sub> were constructed (Fig. 1). The modified nucleotides in native B. subtilis (10) and bovine tRNA<sub>Trp</sub> (11) are known. The modified nucleotides of native A. fulgidus tRNA<sub>Trp</sub> have not been analyzed, but those of Halobacterium volcanii tRNA<sub>Trp</sub> were regarded as providing a first approximation description of the modifications in wild-type archaeal tRNA<sub>Trp</sub> (12, 13).
Expression and Purification of tRNA<sup>Trp</sup>—Recombinant pGEM-9f(−)-derived plasmids were transformed into the E. coli JM109 and cultured in M9-glycerol medium supplemented with 50 μg/ml ampicillin, 1% glycerol, 0.01 mM FeCl<sub>3</sub>, 0.1 mM CaCl<sub>2</sub>, and 100 μg/ml thiamine, as described previously (4). To prepare the [γ-<sup>32</sup>P]ATP (Amersham Biosciences, Inc.) by T4 polynucleotide kinase (Invitrogen) were used to probe membranes at 20°C.

**Dot Blot Hybridization**—To identify tRNA<sup>Trp</sup> in the HPLC fractions, 10 μl of each fraction was loaded on a Hybond-N+ membrane (Amer sham Biosciences, Inc.). The tRNA was cross-linked to the membrane with 8 M urea was warmed up by pre-running to 50°C. The tRNA was cross-linked to the membrane with 8 M urea was warmed up by pre-running to 50°C. The cross-linked tRNA was separated by reverse-phase HPLC on a Vydc C4-derivatized silica column (15). The fractions containing the tRNA<sup>Trp</sup> were identified by dot-blot hybridization, precipitated, and washed twice with 70% ethanol, stored at −20°C, and lyophilized before use.

**Gel Electrophoresis of tRNA**—12% denaturing polyacrylamide gel with 8 μM urea was warmed up by pre-running to 50°C, and tRNA<sup>Trp</sup> preparations were incubated at 100 °C for 10 min with loading buffer containing formamide (50% v/v) prior to electrophoresis in TBE electrophoresis buffer (90 mM Tris-borate, pH 8.0, 2 mM EDTA) at 1000 V for 30 min. The gel was stained with 0.1% toluidine blue (Sigma) in 7.5% acetic acid for 15 min, and destained with 2.5% acetic acid.

**Identification of Modified Nucleotides**—To analyze the modified nucleotides in cloned bovine and A. fulgidus tRNA<sup>Trp</sup>, each wild-type tRNA<sup>Trp</sup> (20 μg) was hydrolyzed as described previously (16). Each nucleotide detected in the HPLC eluate was identified based on comparison of its UV spectrum and retention time with ribonucleoside reference standards. All modified nucleotides were determined in terms of moles of residue per mole of tRNA using Br8G as internal standard.

**Cloning and Preparation of TrpRS**—The TrpRS of A. subtilis, B. subtilis, and E. coli were expressed from recombinant plasmid pKS11 was purified as described (17, 18). A pBluescript SK+ based cDNA clone with the human TrpRS gene (GenBank<sup>TM</sup> accession number NM_001184) inserted into an EcoRI site was obtained from J. Justesen (19); the full-length gene was digested by EcoRI and subcloned into pTrcHis-B expression vector (Invitrogen). The sequences between vector mini-cistron and initial ATG of human TrpRS gene, encoding His<sub>6</sub>-tag and a leading sequence, was removed by PCR-based site-directed mutagenesis using proofreading Pfu DNA polymerase prior to transformation into E. coli JM109 strain. The human TrpRS was induced by 1 mM IPTG and precipitated from the French press crude extract by addition of ammonium sulfate to 3 M. The pellet containing the synthetase was reconstituted and applied to Phenyl-Sepharose 6 Fast Flow column followed by a Q-Sepharose Fast Flow column (both from Amersham Biosciences, Inc.). The fractions were screened for tryptophan synthetase activity using A. fulgidus wild-type tRNA<sup>Trp</sup> as substrate. The purified human TrpRS was concentrated to 0.5 mg/ml in 50% glycerol and stored at −20°C.

To obtain A. fulgidus TrpRS, a pUC18-derived tDNA clone (ATCC number, 629131; TIGR (The Institute for Genomic Research) locus, AF1694) containing TrpRS gene (trpS) (GenBank<sup>TM</sup> accession number AE000986) of A. fulgidus was supplied by ATCC. A pair of primers was designed to flank the A. fulgidus TrpRS gene with a BamHI site at the 5′-end and EcoRI site at the 3′-end, and proofreading Pfu DNA polymerase was used to yield the full-length A. fulgidus TrpRS gene. The PCR-amplified fragment was digested by BamHI and EcoRI and subcloned into the same sites of pTrcHis-A expression vector (Invitrogen). The recombinant expression vector was transformed into E. coli BL21-CodonPlus(DE3)-RIL cell (Stratagene). The His<sub>6</sub>-tagged A. fulgidus TrpRS was overexpressed by induction with 0.5 mM IPTG and extracted by French press. The crude extract containing soluble His<sub>6</sub>-tagged A. fulgidus TrpRS was mixed with 3 ml of nickel-nitrilotriacetic acid-agarose slurry (Qiagen) by gently shaking at 4 °C for 30 min.

The mixture was packed in a mini column for gravity flow chromatography. After the resin was drained and washed with 20 ml imidazole. The enzyme was eluted with 100 mM imidazole. The fractions containing A. fulgidus TrpRS were concentrated and dialyzed by means of Centricon (Amicon) and stored at −20°C in 50% glycerol.
RESULTS
Characterization of tRNA^{Trp} from Three Domains—On the basis of tRNA^{Trp} sequence alignment (Fig. 1), the pairwise sequence identity was found to be 68.0% between A. fulgidus and B. subtilis, 67.1% between bovine and A. fulgidus, and 49.3% between bovine and B. subtilis. More tellingly, there were 18 identical nucleotides between A. fulgidus and B. subtilis tRNA^{Trp} unshared by bovine tRNA^{Trp}, and 18 identical nucleotides between A. fulgidus and bovine tRNA^{Trp} unshared by B. subtilis tRNA^{Trp}. In contrast, there were only four identical nucleotides between B. subtilis and bovine tRNA^{Trp} unshared by A. fulgidus tRNA^{Trp}. These results suggest that, although sequence divergence is large between bovine and B. subtilis tRNA^{Trp}, A. fulgidus tRNA^{Trp} occupies an intermediate phylogenetic position between them with unmistakable resemblance to both of them.

For all three recombinant tRNA^{Trp}, base substitutions at N73 did not greatly influence the expression levels of the tRNA in E. coli. The bulk of bovine or A. fulgidus tRNA^{Trp} was eluted in one major HPLC peak as revealed by dot-blot hybridization (Fig. 2) in contrast to the multiple HPLC peaks observed with B. subtilis tRNA^{Trp} differing in nucleotide modifications (15). Compared with B. subtilis tRNA^{Trp}, bovine and A. fulgidus tRNA^{Trp} was eluted earlier in the HPLC (Fig. 2), suggesting that the latter two tRNA molecules were less hydrophobic. Similar HPLC profiles were observed for all four N73 variants within each kind of tRNA^{Trp}.

Compared with native bovine and H. volcanii tRNA^{Trp}, the bovine and A. fulgidus tRNA^{Trp} hyperexpressed in E. coli lacked some of the species-specific modifications, and acquired other modifications typical of host E. coli tRNA^{Trp} (Table I). The appearance of T in these two cloned tRNA molecules, possibly in place of ψ54 of native bovine tRNA^{Trp}, and m3ψ54 of archaeal tRNA^{Trp} exemplified by H. volcanii tRNA^{Trp}, was a striking difference between the cloned and native molecules, which likely arose from differences between E. coli and bovine or archaeal nucleoside-modifying enzymes. T is a highly conserved signature nucleotide in bacterial tRNA. Another difference was the presence in the cloned molecules of 4-thiouridine (s^4U) (Table I), which is a signature modification at position 8 of E. coli tRNA but never observed in eukaryotic or archaeal tRNA (8). The appearance of 2- O-methylcytidine (Cm) in cloned bovine tRNA^{Trp} but not in cloned A. fulgidus tRNA^{Trp} clearly indicates that the conversion of C to 2'-O-methylcytidine (Cm) depends not only on the availability of the requisite modifying enzyme system in E. coli host but also on appropriate tRNA sequence setting. It is also intriguing that no dihydrouridine (D) was observed in cloned bovine or A. fulgidus tRNA^{Trp}. This residue is present in native bovine and B. subtilis tRNA^{Trp} but absent from native H. volcanii tRNA^{Trp}. Electrophoresis in denaturing 12% polyacrylamide gel containing 8 M urea yielded different migration rates for the three cloned tRNAs that were consistent with their different lengths, A. fulgidus tRNA^{Trp} being the longest with 77 residues, B. subtilis tRNA^{Trp} being the shortest with 74 residues, and bovine tRNA^{Trp} being intermediate with 75 residues (Fig. 3).

Dot blot hybridization by means of tRNA-specific probes identified the bovine tRNA^{Trp} peak at about 42 min in the HPLC elution profile and the slower emergence of A. fulgidus tRNA^{Trp} at about 52 min. Because endogenous E. coli tRNA^{Trp} was eluted at about 70 min, both of these cloned tRNA^{Trp} isolated would contain little endogenous tRNA^{Trp}.

Bacterial TrpRS Recognition—Previously it was found that the rate of tryptophanylation by B. subtilis TrpRS for the G73A mutant is some 10-fold decreased relative to the G73 wild-type (4), suggesting that the discriminator base is a major identity...
element in \( B. \) \textit{subtilis} t\(RNA^{\text{Trp}}\). Table II shows that the variation of \( k_{\text{cat}}/K_m \) with the nature of N73 was caused mostly by a change in \( k_{\text{cat}} \) rather than \( K_m \); the \( K_m \) for G73 wild-type and the G73A, G73C, and G73U mutants all fell within the range of 0.02–0.05 \( \mu \)M, whereas their \( k_{\text{cat}} \) varied over an 18-fold range.

In contrast, the discrimination by \( B. \) \textit{subtilis} TrpRS against heterologous t\(RNA^{\text{Trp}}\) entailed differentiation at both the \( k_{\text{cat}} \) and \( K_m \) levels. For example, comparing the \( k_{\text{cat}}/K_m \) values for the best t\(RNA^{\text{Trp}}\) substrate (\( B. \) \textit{subtilis} G73) and worst t\(RNA^{\text{Trp}}\) substrate (bovine A73C), the former excelled over the latter by 357-fold, which was the combined result of an 8.5-fold (0.02/0.17) difference in \( K_m \) and 42-fold difference (5.09/0.12) in \( k_{\text{cat}} \). Likewise, the \( k_{\text{cat}} \) of \( A. \) \textit{fulgidus} A73 t\(RNA^{\text{Trp}}\) for this enzyme was one-ninth the \( k_{\text{cat}} \) for \( B. \) \textit{subtilis} wild-type G73, and its \( K_m \) was also 6-fold that of the latter. Similarly the 79-fold greater catalytic efficiency toward \( B. \) \textit{subtilis} G73 compared with bovine A73G arose from a 7.5-fold lower \( K_m \) and a 10.6-fold higher \( k_{\text{cat}} \). Clearly, this enzyme is endowed with stringent selectivity not only toward N73 with a preference of \( G \) over \( U \) but also toward the remainder portion of the t\(RNA^{\text{Trp}}\) molecule that varies with the preference of bacterial t\(RNA^{\text{Trp}}\)-archaeal t\(RNA^{\text{Trp}}\)-eukaryotic t\(RNA^{\text{Trp}}\).

\textbf{Eukaryotic TrpRS Recognition—}Purified recombinant human TrpRS (Fig. 4) charged most efficiently bovine, less efficiently \( A. \) \textit{fulgidus}, and least efficiently \( B. \) \textit{subtilis} t\(RNA^{\text{Trp}}\). However, the bias displayed by human TrpRS toward t\(RNA^{\text{Trp}}\) from the three biological domains was somewhat less extreme than the \( B. \) \textit{subtilis} enzyme, yielding a relative \( k_{\text{cat}}/K_m \) of 0.27 toward \( A. \) \textit{fulgidus} t\(RNA^{\text{Trp}}\), and relative \( k_{\text{cat}}/K_m \) of 0.02 toward \( B. \) \textit{subtilis} G73 wild-type t\(RNA^{\text{Trp}}\), improving to 0.082 when G73 in the latter molecule was mutated to \( A \). That the human TrpRS preferred archaeal to bacterial t\(RNA^{\text{Trp}}\) was entirely in keeping with a closer phylogenetic relationship of eukarya to archaea than to bacteria.

The dependence of human enzyme on the discriminator base position was also less extreme compared with the bacterial enzyme. Mutation of A73 in bovine t\(RNA^{\text{Trp}}\) to the worst case of \( G \) reduces its relative \( k_{\text{cat}}/K_m \) to 0.21. The preference of human TrpRS for \( A \equiv C \equiv U \equiv G \) at position 73 held for all three biological sources of t\(RNA^{\text{Trp}}\), and was more moderate than the N73 preference of the \( B. \) \textit{subtilis} enzyme. Studies using \textit{in vitro} t\(RNA^{\text{Trp}}\) transcripts also indicated a preference of \( A \equiv G \) for the human enzyme (6).

\textbf{Archaeal TrpRS Recognition—}\( A. \) \textit{fulgidus} is a sulfatereducing hyperthermophilic archaean. Its His\(_4\)-tagged TrpRS, upon hypersetion and purification from \( E. \) \textit{coli}, yielded a polypeptide of molecular mass ~50 kDa in SDS-polyacrylamide gel electrophoresis (Fig. 5). This enzyme acylated \( A. \) \textit{fulgidus} bovine, and \( B. \) \textit{subtilis} t\(RNA^{\text{Trp}}\) at 65 \( ^\circ \)C but with \( k_{\text{cat}}/K_m \) values lower than those of human and \( B. \) \textit{subtilis} TrpRS. The requirement of this enzyme for high temperature to be active was manifest in the fact that at 30 \( ^\circ \)C it displayed no measurable activity even toward wild-type \( A. \) \textit{fulgidus} t\(RNA^{\text{Trp}}\), in contrast to human TrpRS, which could readily acylate wild-type \( A. \) \textit{fulgidus} t\(RNA^{\text{Trp}}\) at 30 \( ^\circ \)C. The low relatively \( k_{\text{cat}}/K_m \) values of the \( A. \) \textit{fulgidus} enzyme could arise from its dependence on modified nucleotides present in native archaenal t\(RNA^{\text{Trp}}\) but missing from the cloned \( A. \) \textit{fulgidus} t\(RNA^{\text{Trp}}\), which may contribute to t\(RNA^{\text{Trp}}\) thermostability at the assay temperature of 65 \( ^\circ \)C. \( A. \) \textit{fulgidus} TrpRS exhibited a same preference of \( A \equiv C \equiv U \equiv G \) as human TrpRS. Moreover, although it showed approximate 20\% reactivity toward \( B. \) \textit{subtilis} t\(RNA^{\text{Trp}}\), and less than 4\% reactivity toward bovine A73 t\(RNA^{\text{Trp}}\), it retained for these heterologous t\(RNA^{\text{Trp}}\) substrates its preference pattern of \( A \equiv C \equiv U \equiv G \). The comparable activities of \( A. \) \textit{fulgidus} TrpRS toward bacterial t\(RNA^{\text{Trp}}\) and eukaryotic t\(RNA^{\text{Trp}}\) are in accord with an intermediate phylogenetic position of the archaean between bacteria and eukarya.

\textbf{NMR Spectroscopy of Bovine A73G Mutant t\(RNA^{\text{Trp}}\)—}Cloned bovine t\(RNA^{\text{Trp}}\) was efficiently tryptophanylated by human TrpRS with a \( k_{\text{cat}}/K_m \) of \( 1.12 \times 10^3 \) s\(^{-1}\) M\(^{-1}\). When its discriminator base A73 was mutated, loss of catalytic efficiency occurred with the largest loss caused by mutation to \( G \), stemming mostly from a 3-fold decrease in \( k_{\text{cat}} \) and a 1.5-fold increase in \( K_m \) (Table II).
TABLE II
Kinetics parameters for N73 variants of tRNA<sup>Trp</sup> from the three biological domains of archaea, eukarya, and bacteria upon tryptophanylation by A. fulgidus human and B. subtilis TrpRS

| Species/domain          | N73 | k<sub>cat</sub> | K<sub>M</sub> | k<sub>cat</sub>/K<sub>M</sub> | k<sub>cat</sub> | K<sub>M</sub> | k<sub>cat</sub>/K<sub>M</sub> | k<sub>cat</sub> | K<sub>M</sub> | k<sub>cat</sub>/K<sub>M</sub> |
|------------------------|-----|----------------|-------------|-----------------------------|----------------|-------------|-----------------------------|----------------|-------------|-----------------------------|
| A. fulgidus/archaea    |     | 0.32 ± 0.04    | 0.12 ± 0.01 | 0.008 ± 0.008               | 0.32 ± 0.04    | 0.12 ± 0.01 | 0.008 ± 0.008               | 0.32 ± 0.04    | 0.12 ± 0.01 | 0.008 ± 0.008               |
| Bovine/eukarya         |     | 0.21 ± 0.02    | 0.16 ± 0.03 | 0.005 ± 0.005               | 0.21 ± 0.02    | 0.16 ± 0.03 | 0.005 ± 0.005               | 0.21 ± 0.02    | 0.16 ± 0.03 | 0.005 ± 0.005               |
| B. subtilis/bacteria   |     | 0.81 ± 0.11    | 0.02 ± 0.12 | 0.006 ± 0.006               | 0.81 ± 0.11    | 0.02 ± 0.12 | 0.006 ± 0.006               | 0.81 ± 0.11    | 0.02 ± 0.12 | 0.006 ± 0.006               |

<sup>a</sup>The k<sub>cat</sub>/K<sub>M</sub> values represent the k<sub>cat</sub>/K<sub>M</sub> value of each tRNA substrate relative to the k<sub>cat</sub>/K<sub>M</sub> measured for the wild-type tRNA<sup>Trp</sup> upon tryptophanylation by its cognate TrpRS.

![Fig. 4. Human TrpRS expressed in E. coli.](Image)

![Fig. 5. A. fulgidus TrpRS expressed in E. coli.](Image)

![Fig. 6. Sensitivity-enhanced 15N-1H HSQC spectrum of bovine tRNA<sup>Trp</sup> α, bovine wild-type tRNA<sup>Trp</sup> and h, A73G mutant in H<sub>2</sub>O (5% D<sub>2</sub>O) with 10 mM sodium phosphate (pH 6.5), 100 mM sodium chloride, and 10 mM magnesium chloride recorded with a Varian 500-MHz spectrometer at 30 °C. Assigned resonances are indicated in terms of one of the base pairing partners. U, an unassigned resonance.](Image)

Previously, by utilizing base pair mutations to differentiate between overlapping NMR resonances, we have identified most of the base pair resonances in B. subtilis tRNA<sup>Trp</sup> (14). More recently a similar combination of mutagenesis and two-dimensional NMR has yielded assignments of most of the base pair resonances of cloned bovine wild-type tRNA<sup>Trp</sup> (Fig. 6a) and its
A73G mutant (Fig. 6b) in the $^{15}$N HSQC spectra.\textsuperscript{2} Comparison of these two $^{15}$N HSQC spectra indicates that bovine A73G maintains almost intact the overall structure of A73 wild-type, without major changes in the resonances other than a marked change in the chemical shift for the G1-C72 base pair. Upon replacement of A73 by G73, the G1 resonance moved in the $^1H$ dimension from 11.75 to 12.15 ppm.

Synergism between A73 and G1-C72—In view of the conformational effect of A73G mutation on the G1-C72 base pair in bovine tRNA$^\text{Trp}$, variants involving N73 and 1–72, the first base pair in the acceptor stem, were constructed, hyperexpressed, and analyzed for tryptophanylation kinetics. As shown in Fig. 7, G1-C72 and A73 are both important identity elements for human TrpRS. Mutating either A73 to G73, or G1-C72 to A1-U72, resulted in an approximate 4-fold reduction in activity. A double mutant carrying both G73 and A1-U72 suffered a synergistic 20-fold loss of tryptophanylation relative to the wild-type. Disruption of the G1-C72 base pair altogether by mutating G1 to A1 also brought about a 20-fold activity loss. These observations confirm the NMR finding of a structural linkage between N73 and 1–72. Together they suggest that N73 and G1-C72 constitute a structural domain that is crucial for productive interaction with TrpRS. This domain requires an intact 1–72 base pair and achieves maximum preference when it comprises A73 and G1-C72.

**DISCUSSION**

Functional Effect of N73 Substitution—For *B. subtilis* tRNA$^\text{Trp}$, the discriminator base G73 and the anticodon represent two major identity elements responsible for effective recognition by *B. subtilis* TrpRS, with A1-U72, G5-C68, and A9 also serving as minor identity elements. Of these elements, the role of the discriminator base is particularly intriguing: When G73 is mutated to A73, the tRNA$^\text{Trp}$ loses much of its reactivity with bacterial TrpRS but becomes active with eukaryotic yeast TrpRS. Such reduction of tryptophanylation caused by a mutation of discriminator base has been confirmed for *B. subtilis* and human TrpRS acting on *in vitro* tRNA$^\text{Trp}$ transcripts (6). In known tRNA$^\text{Trp}$ sequences from different species of the three biological domains (8), the discriminator base is overwhelmingly occupied by a purine: G73 in 19 bacteria, 13 chloroplasts, 95 mitochondria, and one eukaryotic cyttoplasm (*Toxoplasma gondii*); A73 in 13 eukaryotic cytoplasm, 6 archaea, and 13 mitochondria. The exceptions to this A/G dominance are U73 in *Arabacia lixula* mitochondria and the eukaryotic *Dictyostelium discoideum* cyttoplasm, and C73 in *Herpesvirus*. The fact that bacterial TrpRS prefers G73, whereas eukaryotic TrpRS prefers A73, is thus entirely in accord with this remarkable G73/A73 sequence dichotomy between bacteria and chloroplasts, on the one hand, and eukary and archaea on the other. In the present study, the evolutionary sequence dichotomy is found to be mirrored by a corresponding enzyme-specificity dichotomy.

*B. subtilis* TrpRS displayed a strong a preference for G73 on *B. subtilis* wild-type tRNA$^\text{Trp}$ followed by A (relative $k_{\text{cat}}/K_m$ of 0.11), and C and U trailing with half the activity of A. This $k_{\text{cat}}/K_m$ preference is exercised mostly at the $k_{\text{cat}}$ rather than $K_m$ level. Because proofreading is not important for this enzyme (20), the dependence of $k_{\text{cat}}$ on G at position 73 is in accord with the finding using stopped-flow fluorometry and that the N73 of *E. coli* tRNA$^\text{Trp}$ contributes to the stability of the Trp-tRNA transition state during the transfer of activated Trp to the tRNA (21).

The archaeal enzyme *A. fulgidus* TrpRS acting on cloned *A. fulgidus* tRNA$^\text{Trp}$ preferred A73, followed by C73, U73, and G73. This is consistent with the exclusive occupation of the discriminator base by A in archaeal tRNA$^\text{Trp}$. Because C73 ranks second as an effective discriminator nucleotide for archaeal TrpRS, it is therefore not important for this enzyme that the discriminator base position is a purine. Instead, an amino group on the six-member heterocyclic ring in discriminator base, which is common to A and C (at position 6 in A, or 2 in C), but unshared by U or G, might be more important for efficient recognition by this synthetase.

Human TrpRS acting on hyperexpressed bovine tRNA$^\text{Trp}$ was severalfold more active with A73 than with, in order of descending preference, C73, U73, and G73. The distinct preference of human TrpRS for A73 stands in total agreement with the overwhelming occurrence of A73 in eukaryotic tRNA$^\text{Trp}$. The preference of C over U and G again suggests the possible importance of an amino group on the discriminator base.

**Independent Action of Identity Elements in tRNA$^\text{Trp}$—Aminoacyl-tRNA synthetases are often guided by multiple identity elements on its cognate tRNA substrate. This is certainly the case with TrpRS from all three biological domains. Each of these TrpRS displays (i) defined preference toward N73 and (ii) defined preference toward the rest of the tRNA$^\text{Trp}$ molecule structure in which N73 is located. For the three TrpRS examined, their preferences based on $k_{\text{cat}}/K_m$ values (Table II) are as follows: *B. subtilis* TrpRS: (i) G>A>U>C; (ii) bacterial tRNA$^\text{archaeal}$ tRNA$^\text{eukaryotic}$ tRNA. *A. fulgidus* TrpRS: (i) A>C/U>G; (ii) archaeal tRNA$^\text{archaeal}$ tRNA$^\text{eukaryotic}$ tRNA. Human TrpRS: (i) A>C/U>G; (ii) eukaryotic tRNA$^\text{archaeal}$ tRNA$^\text{archaeal}$ tRNA.

The Type I and Type II preferences act independently. For example, because of the Type II preference, *B. subtilis* TrpRS is catalytically more efficient acting on all four N73 forms of homologous tRNA$^\text{Trp}$ from *B. subtilis* than any form of heterologous bovine or archaeal tRNA$^\text{Trp}$ regardless of the nature of the discriminator base. Nonetheless, its Type I preference of G>A>U>C remains evident among the N73 forms within each of these three biological sources of tRNA$^\text{Trp}$. *A. fulgidus* TrpRS is more active toward even the A73G of *A. fulgidus* tRNA$^\text{Trp}$ than any of the four forms of bovine tRNA$^\text{Trp}$. Human TrpRS is also more active toward even the A73G of bovine tRNA$^\text{Trp}$ than any of the four forms of *B. subtilis* tRNA$^\text{Trp}$. Yet these two enzymes retain their A>C/U>G preference among tRNA$^\text{Trp}$ from every domain.

The Type II preferences are fully consistent with an intermediate position occupied by archaea between the phylogenetic
divergence of bacteria and eukarya. Fig. 1 shows that in A. fulgidus tRNA\textsubscript{Trp}, 18 residues (23% of all residues) are identical to corresponding residues in bovine tRNA\textsubscript{Trp} but unshared by B. subtilis tRNA\textsubscript{Trp}. At the same time, 18 other residues in A. fulgidus tRNA\textsubscript{Trp} are identical to corresponding residues in B. subtilis tRNA\textsubscript{Trp} but unshared by bovine tRNA\textsubscript{Trp}. In contrast, merely four residues are common to the bacterial and eukaryotic tRNA\textsubscript{Trp} but unshared by archaeal tRNA\textsubscript{Trp}. These sequence identities therefore strongly confirm the observed Type II kinetic preferences. They point to a wide divergence between bacteria and eukarya, with archaea occupying a phylogenetic position intermediate between them and displaying unmistakable tRNA\textsubscript{Trp} sequence similarity to both of them.

Influence of A73 on G1-C72 in Bovine tRNA\textsubscript{Trp}—To examine the effects of N73 on tRNA structure that might be significant to its role as an identity element, a structural comparison of the bovine wild-type A73 tRNA\textsubscript{Trp} with its A73G mutant was performed by solution NMR spectroscopy. The mutation was found to cause few NMR spectral changes except for a chemical shift in the resonance of the G1-C72 base pair. This indicates the occurrence of a conformational change in the microenvironment of this base pair caused by the A73G mutation. This finding is consistent with the suggestions that N73 of tRNA can base-stack on the nearby first base pair of the tRNA acceptor stem helix and extend the stacking in this stem (22–24) and that N73 plays an important role in the stabilization of the acceptor stem helix by the single-stranded 3’ terminus (25). Thus the conformational change in G1-C72 in bovine tRNA\textsubscript{Trp} elicited by the A73G mutation, as revealed by NMR, could be due to an effect on the base stacking between G73 and G1-C72. For reaction with human TrpRS, the A1-U72 and A73G mutations each induced a comparable 4-fold loss of activity. When the A73G and A1-U72 mutations were introduced together into bovine tRNA\textsubscript{Trp}, a ~20-fold loss of reaction rate resulted. Disruption of the 1–72 base pair by mutating G1 to A1 likewise caused a ~25-fold loss (Fig. 7). Therefore the A73 and G1-C72 elements clearly interacted to form a key sub-region on the tRNA. The maintenance of an optimal conformation in this sub-region near the acceptor terminus of the tRNA could be important for optimal interaction with eukaryotic TrpRS.

In conclusion, the TrpRS-tRNA\textsubscript{Trp} recognition process is extraordinary in that an identity-element divergence, in the form of a G73/A73 dichotomy, divides the bacterial and archaeal-eukaryotic phylogenetic domains. Although the origin and evolutionary significance of this surprising dichotomy are far from understood, this dichotomy itself provides unique advantages for enquiry into the nature of genetic code and tRNA evolution. Evidence for an intermediate phylogenetic position of archaea between bacteria and eukarya, as revealed by both TrpRS kinetics and tRNA\textsubscript{Trp} sequences, the independent action of different identity elements on tRNA, and the functional linkage in bovine tRNA\textsubscript{Trp} between the discriminator base and the first base pair of the acceptor stem for recognition by TrpRS, represents only initial examples of the rich harvest of insight that may be expected from this remarkable system.

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Recognition by Tryptophanyl-tRNA Synthetases of Discriminator Base on tRNA\textsuperscript{Trp} from Three Biological Domains
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