Leucyl-tRNA Synthetase Consisting of Two Subunits from Hyperthermophilic Bacteria *

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In a hyperthermophilic bacterium, *Aquifex aeolicus*, leucyl-tRNA synthetase (LeuRS) consists of two non-identical polypeptide subunits (α and β), different from the canonical LeuRS, which has a single polypeptide chain. By PCR, using genome DNA of *A. aeolicus* as a template, genes encoding the α and β subunits were amplified and cloned in *Escherichia coli*. The α subunit could not be expressed stably in vivo, whereas the β subunit was overproduced and purified by a simple procedure. The β subunit was inactive in catalysis but was able to bind tRNALeu*. Interestingly, the heterodimer αβ-LeuRS could be overproduced in *E. coli* cells containing both genes and was purified to 95% homogeneity as a hybrid dimer. The kinetics of *A. aeolicus* LeuRS in pre-steady and steady states and cross-recognition of LeuRS and tRNA*Leu* from *A. aeolicus* and *E. coli* were studied. Magnesium concentration, pH value, and temperature aminoacylation optima were determined to be 12 mM, 7.8, and 70 °C, respectively. Under optimal conditions, *A. aeolicus* αβ-LeuRS is stable up to 65 °C.

The aminoacyl-tRNA synthetases (aaRSs, EC 6.1.1) catalyze the esterification of amino acids to their cognate tRNAs (1–5). They are specific for both amino acid and tRNA substrates to ensure high fidelity required by translation (6). The alignments of the sequences of aaRSs and tRNAs have been widely used to study their specific interactions. The 20 aaRSs can be divided into two classes of 10 members each on the basis of conserved sequences and characteristic structural motifs (7). Class I contains two signature peptides, HIGH and KMSKS, located in the active site that is built as the characteristic dinucleotide binding fold (Rossmann Fold, βαββα). Connective peptides (called CP domains) are often inserted between the different strands of the Rossmann fold (8). The active site of class II aaRSs is built around an antiparallel β sheet partly closed by helices, which contains three conserved motifs (4).

Except for arginyl-, glutamyl- and glutaminyl-tRNA synthetases (ArgRS, GluRS and GlnRS) (9, 10), aminoacylation of tRNA catalyzed by the other 17 aaRSs is a two-step reaction: (a) activation of amino acids with ATP by formation of aminoacyl adenylate and (b) transfer of the aminoacyl-moiety from the aminoacyl adenylate to the cognate tRNA substrate (11). The activities of these two steps are independent, and substrate tRNA is unnecessary for amino acid activation. The accuracy of aminoacylation depends on both the specific recognition of amino acids during their activation and the pre- or posttransfer editing that corrects errors at either the stage of aminoacyl adenylate formation or at the step of aminoacyl transfer to tRNA (12–14).

Leucyl-tRNA synthetase (LeuRS, EC 6. 1. 1. 4) belongs to the class I aaRSs and catalyzes leucylation of tRNA*Leu* on the 2'-OH of the ribose of the 3'–terminal adenosine (7). The primary sequences of LeuRSs from over 50 sources are known. LeuRS are large monomeric enzymes (860 amino acids residues for the *Escherichia coli* enzyme), which are homologous to other synthetases from class Ia: isoacceptor-tRNA synthetases (IleRS), and valyl-tRNA synthetases (ValRS), methionyl-tRNA synthetases (MetRS), arginyl-tRNA synthetases, and cysteinyl-tRNA synthetase (CysRS). LeuRSs are more closely related to the large monomeric IleRS and ValRS, which exhibit large CP domains involved in the editing process and several zinc binding domains.

The conservation of the monomeric structure of these enzymes is universal, except for the LeuRS from *Aquifex aeolicus*, which is an αβ heterodimer (15). On the basis of sequence alignments, CP1 of *E. coli* LeuRS extends from residues 126 to 389, and CP2 spans residues 424–582 (16). *A. aeolicus* is one of the most primitive bacteria. With a growth-temperature maximum near 95 °C, *A. aeolicus* is one of the most thermophilic bacteria known. It grows at 85 °C under an H₂/CO₂/O₂ (79.5:19.5:1.0) atmosphere in a medium containing only inorganic components. Its complete genome was sequenced in 1998 (15). According to the analysis of its genomic DNA and alignment of the gene sequence of other LeuRSs, the *A. aeolicus* LeuRS appears to be separated into two subunits that were named α and β subunit, respectively.

Here, we report the cloning and expression of the two subunits of *A. aeolicus* LeuRS followed by the purification of the αβ-LeuRS and its β subunit alone. We also report the tRNA binding with its cognate tRNA, the kinetic properties, and thermal stability of these molecules. The cross-recognition of LeuRS and tRNA*Leu* from *E. coli* and *A. aeolicus* was studied.
LeuRS Consisting of Two Subunits from Aquifex aeolicus

EXPERIMENTAL PROCEDURES

Materials—t-leucine, dithiothreitol, ATP, nucleotide triphosphate, 5'-GMP, tetrasodium pyrophosphate, and inorganic pyrophosphatase were purchased from Sigma. [32P]ATP (5000-4000Ci/mmol) and 5'-labeled tetrasodium pyrophosphate were obtained from Amersham Biosciences. GFC filters were from Whatman Company. Kinase, ligase, and restriction endonucleases were obtained from Sangon Company, Shanghai Branch. E. coli LeuRS was purified by chromatography on DEAE-Sepharose CL-6B and Blue-Sephacel from the overproducing E. coli strain in our laboratory (17). Its concentration was determined by A_{280} of the enzyme solution; 1.62 mg of protein/mg equals 1 optical density unit at 280 nm. E. coli total RNA containing 50% of tRNA^{AUA} (GAG) was isolated from an overproducing strain constructed in our laboratory (18). T7 RNA polymerase was purified from an overproducing strain in our laboratory (19).

Plasmids—pSML104 was constructed from pACYC184 and pKK233-2 (20). It contains the p15A rep sequence from pACYC184, the strong tcr promoter, a multicloning site, two sequences for transcription termination (T1 and T2) of the ribosomal operon from pKK233-2, and the resistance to chloramphenicol and tetracyclamide. Plasmid pBPC378 contains the tcr promoter, resistance to ampicillin, and an NdeI site at its translation start site without altering the amino acid sequence of the synthesized protein, while ptc and the lacI^Q gene confer inducible and controllable expression (21).

Cloning and Expression of the Gene Encoding A. aeolicus tRNA^{Leu}—The A. aeolicus genome contains five tRNA^{Leu} genes. We cloned and expressed its most frequently used isoacceptor, which decodes CUC codons. The tRNA^{Leu} gene with anticodon GAG is located from 5988 to 6027 bp of the genome. The GAG anticodon is in bold italic.

AACTGGCAGACGCGCCGTCTT formed with T4 polynucleotide kinase for 1 h at 37°C. Plasmids were grown in 25 ml of LB with 100 µg/ml of ampicillin and 100 µg/ml of chloramphenicol at 37 °C. The plasmid pSML104-αβ-LeuRS to a concentration of 5 µg/µl. 15-µl aliquots were taken and quenched in various time intervals (t) as described above. The quenched samples were filtered through Whatman GF/C glass fiber filter, washed, and dried. The [14C]leucine incorporated was quantified using a scintillation counter (LKB Wallac 1210 Rackbeta). The activity was calculated according to the following equation: cpm [14C]leucine incorporated / [14C]leucine in reaction mixture × 1000.

RESULTS

Cloning and Expression of the tRNA^{Leu} Gene—The plasmid containing A. aeolicus tRNA^{Leu} (GAG) gene was constructed
and used to transform E. coli MT102. In this strain, which overexpresses tRNA<sub>Leu</sub>, the charge of [14C]leucine on the tRNA reaches 724 pmol/mg. As the accepting activity of pure tRNA<sub>Leu</sub> was considered to be 1600 pmol/mg, tRNA<sub>Leu</sub> should be about 45% of the total tRNA. This crude overexpressed A. aeolicus tRNA<sub>Leu</sub> was used to assay A. aeolicus LeuRS activity and binding property. The low content of endogenous E. coli tRNA<sub>Leu</sub> was considered to be negligible compared with the overexpressed A. aeolicus tRNA<sub>Leu</sub>.

Cloning and Expression of the A. aeolicus LeuRS α and β Subunit Genes—When compared with the sequence of Thermus thermophilus LeuRS, it appears that, except for 30 residues at the C-terminal end, the α subunit sequence corresponds to the 580 first residues of the T. thermophilus LeuRS (Fig. 1). Except for the first 40 residues, the β subunit sequence is similar to the peptide fragment 636–878 of T. thermophilus LeuRS. Thus, at the junction of the two peptides, two additional domains of about 30 and 40 residues may form some connecting structure between the two subunits (Fig. 1). Theoretically, the molecular masses of α and β subunits are 74.0 and 33.5 kDa, respectively. The genes encoding α and β subunits of LeuRS were amplified by PCR. Both DNA fragments were ligated into pBCP378 or pSM104. The resulting recombinant vectors, pBCP378-lrsa, pBCP378-lrsb and pSM104-lrsb were used to transform E. coli TG1. After growth of the different transformants and disruption of the cells, a strong signal corresponding to the tRNA<sub>Leu</sub> was considered to be 1600 pmol/mg and calculated by heating (lane 2, 50 μg of crude extract; lane 3, 5 μg of heated extract). The α subunit could not be expressed alone (lane 4, 30 μg of crude extract) as opposed to the β subunit (lane 5, 30 μg of crude extract and lane 6, 5 μg of heated extract). The arrows indicate the positions of α subunit (top) and β subunit (low). The SDS-PAGE analysis of purified A. aeolicus LeuRS (lane 2, 5 μg of purified protein) compared with protein standards (97.4, 66.2, 55.0, 42.7, 40.0, and 31.0 kDa from top to bottom).

To confirm the existence of two tRNA binding sites on the α subunit, the affinity and specificity of A. aeolicus αβ-LeuRS and its β subunit alone were compared for their CD spectra. The parameters of their secondary structures were estimated, as summarized in Table I (23). The β subunit contains more α helix and β sheet, less β turn and random than A. aeolicus αβ-LeuRS. Thus, the β subunit is more structured and probably more rigid than A. aeolicus αβ-LeuRS, probably due to the association with the α subunit, which is probably less structured and less stable than the β subunit as suggested by its inability to be overexpressed in vivo.

Overproduction and Purification of A. aeolicus αβ-LeuRS—To overexpress the heterodimeric form αβ-LeuRS, both pBCP378-lrsa and pSM104-lrsb were co-transformed into the E. coli cells. In these conditions, the α and β subunits were detected in the crude extract (lanes 2 and 3, Fig. 2A). A. aeolicus αβ-LeuRS was purified to about 70% homogeneity after heating at 75 °C for 30 min followed by centrifugation. Thus, in vivo, in the presence of the β subunit, the α subunit was stable and thermostable.

After growth of the transformants in 4 liters of Luria broth medium (16 g of wet cells) about 20 mg of purified A. aeolicus αβ-LeuRS was obtained as described under “Experimental Procedures.” The purified A. aeolicus αβ-LeuRS was 95% homogeneous as determined by SDS-PAGE (lane 2, Fig. 2B). A. aeolicus LeuRS consisting of α and β subunits was active, and the molecular mass of the enzyme was 107.5 kDa as measured by mass spectrometry determination. These data demonstrate that A. aeolicus LeuRS is a hybrid dimer active in its dimeric form.

CD Spectra of A. aeolicus αβ-LeuRS and its β Subunit—To understand why the α subunit was unstable, the secondary structures of A. aeolicus αβ-LeuRS and its β subunit alone were compared for their CD spectra. The parameters of their secondary structures were estimated, as summarized in Table I (23). The β subunit contains more α helix and β sheet, less β turn and random than A. aeolicus αβ-LeuRS. Thus, the β subunit is more structured and probably more rigid than A. aeolicus αβ-LeuRS, probably due to the association with the α subunit, which is probably less structured and less stable than the β subunit as suggested by its inability to be overexpressed in vivo.

Binding of β Subunit with tRNA<sub>Leu</sub>—Molecular mass of the β subunit was 33.5 kDa as determined by HPLC gel filtration. It means that β subunit is a monomer when expressed independently from the α subunit. However, the β subunit is inactive and cannot bind ATP and leucine as shown by competition assay and gel-shift assay (data not shown). By gel-shift assay we detected the binding of the β subunit to the tRNA<sub>Leu</sub> from A. aeolicus (Fig. 3A). The binding constant, K<sub>d</sub>, calculated by screening the area of the bands was about 20 ± 3 μm, an unusually high value for an aaRS:tRNA interaction. This is in contrast with the K<sub>d</sub> value measured for the whole enzyme (0.5 μm, data not shown). In general, the K<sub>d</sub> of an aaRS for the cognate tRNA is <2 μm, which means that the binding of the β subunit to tRNA<sub>Leu</sub> is not very tight. Interestingly, a supershift appears on increasing the concentration of unlabeled tRNA<sub>Leu</sub> during the competition assay (Fig. 3B). Thus, it seems that the β subunit may form two kinds of complexes, the upper band corresponding to the β subunit with a probable tRNA/enzyme stoichiometry of 2:1. Alternatively, the β subunit can bind labeled E. coli tRNA<sub>Leu</sub> (data not shown) and tRNA<sub>Leu</sub> (Fig. 3C) with two forms also. The assay of specificity was extended to tDNA encoding A. aeolicus tRNA<sub>Leu</sub>, the tDNA competed with the homologous A. aeolicus tRNA<sub>Leu</sub>, but the large complex did not appear (Fig. 3D).

To confirm the existence of two tRNA binding sites on the β subunit the complex was generated according to the experimental conditions of Fig. 3 and analyzed by gel filtration on HPLC. Two species were separated, with Mr of 63.5 and 93.3 kDa, corresponding to the β subunit associated with one or two
The concentration of A. aeolicus LeuRS and its β subunit was 0.6 mg/mL and 0.2 mg/mL, respectively.

| Protein secondary structure | αβ-LeuRS | β Subunit |
|----------------------------|-----------|-----------|
| α-Helix                    | 33.6%     | 38.8%     |
| β-Sheet                    | 26.7%     | 31.7%     |
| β-Turn                     | 5.9%      | 0.9%      |
| Random                     | 33.8%     | 28.6%     |

Fig. 3. Binding of tRNAleu on the β subunit of A. aeolicus LeuRS. A, the concentration of labeled tRNAleu from A. aeolicus was 0.05 μM. Lane 1: without β subunit; lane 2-6, the concentration of β subunit was 3, 8, 13.6, 27, and 41 μM, respectively. B, the incubation system included 27 μM β subunit and 0.1 μM labeled tRNAleu from A. aeolicus with increasing concentration of unlabeled A. aeolicus tRNAleu. Lane 1: without unlabeled tRNA; lane 2-5: the ratio of unlabeled competing tRNAleu to labeled tRNAleu was 2, 10, 20, and 50, respectively. C, the incubation system included 27 μM β subunit and 0.1 μM labeled tRNAleu from A. aeolicus with increasing concentration of unlabeled E. coli tRNAleu. Lane 1: without unlabeled tRNAleu; lane 2-5: the ratio of unlabeled tRNAleu to labeled tRNAleu was 2, 10, 20, and 50, respectively. D, the incubation system included 27 μM β subunit and 0.1 μM labeled tRNAleu from A. aeolicus with increasing concentration of unlabeled tDNAleu (plasmidic DNA encoding tRNAleu). Lane 1: without unlabeled tDNAleu; lane 2-5: the ratio of unlabeled tDNAleu to labeled tDNAleu was 2, 10, 20, and 50, respectively.

Fig. 4. Active site titration and pre-steady state aminoacylation. A, the number of active sites. The active site titration was performed by measuring ATP exhaustion in the formation of leucyl adenylate at 60 °C, pH 7.8, from A. aeolicus αβ-LeuRS (5 μM), [γ-32P]ATP (20 μM, 20 μCi/ml) and leucine (1 mM) in the presence of pyrophosphatase. Before adding LeuRS, the number of counts was 19,600 cpm. The ordinate intercept of the extrapolated straight line was 14,300 cpm equivalent to 14.6 μM. The number of active sites was 1.08 calculated by the equation: n = ([ATP]0/[E]0) × (20 - 14.6)/5 ≈ 1.08. B, biphasic tRNAleu aminoacylation kinetic. The reaction was conducted by a quenched flow apparatus. One syringe contained tRNAleu, the other contained the preformed enzyme:adenylate complex.

s⁻¹, respectively. Compared with the E. coli LeuRS, these values only reach 2% of the level of activity (Table II, (24)). In the aminoacylation reaction, Km values for leucine, ATP, and tRNAleu (A. aeolicus) were 6.4, 550, and 3.38 μM; kcat values were 0.39 s⁻¹ (Table II). Except Km for ATP in aminoacylation reaction, all of the constants were lower than those for E. coli LeuRS (Table II) (24). Although the substrates bind A. aeolicus αβ-LeuRS tightly, the reaction is slower at 37 °C.

The kinetic constants of A. aeolicus αβ-LeuRS were also studied at 60 °C (Table III). All kcat values increased 3.5–4.8-fold as compared with those at 37 °C. In amino acid activation, the Kcat values for both leucine and ATP were identical to those at 37 °C. In the aminoacylation reaction, the Kcat value for leucine was unchanged, whereas the Km value for ATP was slightly increased. At 60 °C, the catalytic efficiencies observed for the different substrates are comparable to those of the E. coli LeuRS at 37 °C.

The aminoacylation reaction was studied at the pre-steady state level using a quenched flow apparatus (25). The reaction was started by mixing the tRNA to the preformed enzyme:adenylate complex. A two-phase kinetics was detected, with an
initial faster rate ($k_{\text{cat}} = 7.2 \text{ s}^{-1}$) followed after 52 ms by a slower rate with a $k_{\text{cat}}$ value of 0.36 s$^{-1}$, which is similar to the value measured by hand in steady state, 0.39 s$^{-1}$. The Leu-tRNA$^{\text{Leu}}$ synthesized during the burst was in a 0.82:1.0 stoichiometry with the synthetase present (Fig. 4B). Thus, the decrease of the rate of tRNA$^{\text{Leu}}$ charging after completion of the first catalytic cycle of the synthetase suggests that one or several end products dissociate slowly from the enzyme.

**Thermal Properties of A. aeolicus αβ-LeuRS—As expected, A. aeolicus αβ-LeuRS is a thermostable enzyme. Under the experimental conditions it is stable up to 70 °C (Fig. 5). The specific activities of amino acid activation and aminoacylation increased with temperature. At 75 °C the two activities were 12- and 5-fold greater than at 37 °C. Changes of activity with temperature are shown in Fig. 6. The activation energy was calculated from the slope of the line when log $v$ was plotted against 1/T. The activation energy for the amino acid activation reaction was 14.9 kcal/mol. Below 50 °C, the activation energy for the aminoacylation reaction was 14.9 kcal/mol, the same as in amino acid activation. Above 50 °C the value was 6.53 kcal/mol. Above 75 °C, evaporation in the test tube prevented accurate determination of activation energy. The aminoacylation rate began to decrease above 70 °C, 15 °C under its optimum of 85 °C (15). This suggests that the composition of the reaction mixture is not optimal for enzyme activity, or that the A. aeolicus tRNA$^{\text{Leu}}$ overproduced in E. coli does not possess all the modified bases that should improve its thermal stability.**

**Cross-recognition of LeuRSs and tRNA$^{\text{Leu}}$ from A. aeolicus and E. coli—At 37 °C, both enzymes display for their homologous substrates comparable $k_{\text{cat}}/K_{\text{m}}$ values, with the faster rate for the E. coli enzyme and the best tRNA affinity for the A. aeolicus enzyme (Table II). In the cross-recognition of the tRNAs, the Aquifex enzyme exhibits a 5-fold decrease of the $k_{\text{cat}}$ values, with a variation of /H11021/H11021/chiometric ratio with the synthetase present (Fig. 4B).**

**FIG. 5. Determination of the thermal stability of A. aeolicus αβ-LeuRS.** A. aeolicus αβ-LeuRS (40 μg/ml) in 50 mM potassium phosphate buffer, pH 6.8, containing 400 μg/ml bovine serum albumin was incubated at various temperatures for 10 min. Then the aminoacylation activity was assayed at 37 °C after dilution of the reaction mixture with cold 50 mM potassium phosphate buffer, pH 7.5.

**FIG. 6. Variation of the A. aeolicus αβ-LeuRS activity with the temperature.** The ATP-PP$\text{e}$ exchange activity (A) and the aminoacylation reaction (E) were assayed at increasing temperatures.

### Table II

| Substrate | Constant | ATP-PP$\text{e}$ exchange | Aminoacylation |
|-----------|----------|---------------------------|---------------|
| Leu       | $K_m$ (μM) | 1.3                       | 6.4           |
|           | $k_{\text{cat}}$ (s$^{-1}$) | 3.5                       | 0.39          |
| ATP       | $K_m$ (μM) | 2692                      | 61            |
|           | $k_{\text{cat}}$ (s$^{-1}$) | 5167                      | 200           |
| A. aeolicus | tRNA$^{\text{Leu}}$ | 3.3                       | 550           |
|           | $k_{\text{cat}}$ (s$^{-1}$) | 140                        | 280           |
| E. coli   | tRNA$^{\text{Leu}}$ | 9.2                       | 0.39          |
|           | $k_{\text{cat}}$ (s$^{-1}$) | 187                        | 3.6           |

$^a$ The data are from reference 17.

### Table III

| Substrate | Constant | ATP-PP$\text{e}$ exchange | Aminoacylation |
|-----------|----------|---------------------------|---------------|
| Leu       | $K_m$ (μM) | 1.6                       | 6.0           |
|           | $k_{\text{cat}}$ (s$^{-1}$) | 15.5                       | 1.4           |
| ATP       | $K_m$ (μM) | 9688                      | 233           |
|           | $k_{\text{cat}}$ (s$^{-1}$) | 1123                      | 15.0          |
| A. aeolicus | tRNA$^{\text{Leu}}$ | 38.2                       | 16.0          |
|           | $k_{\text{cat}}$ (s$^{-1}$) | 14.5                       | 1.8           |
| E. coli   | tRNA$^{\text{Leu}}$ | 1.5                       | 1.8           |
|           | $k_{\text{cat}}$ (s$^{-1}$) | 5000                      | 1.5           |
|           | $k_{\text{cat}}$ (s$^{-1}$) | 1.5                       | 0.4           |

Analysis of the DNA sequence of A. aeolicus LeuRS revealed that this organism presents an unusual split LeuRS in contrast to other LeuRSs.
LeuRS Consisting of Two Subunits from Aquifex aeolicus

LeuRS, the enzyme responsible for aminoacylation of tRNA, is known to have a structural and functional complexity that is not fully understood. This complexity arises from the assembly of two subunits, each of which is associated with distinct domains that contribute to the active site of the enzyme.

The monomeric form of LeuRS is a dynamic structure that can exist in various conformations, which can be affected by interactions with tRNA and other molecules. The activation of the enzyme involves a series of conformational changes that are induced by the binding of tRNA substrates.

The structural data of A. aeolicus LeuRS reveal that the β subunit is a two-domain structure, with the α subunit being a single domain. The α subunit is responsible for the binding of tRNA, while the β subunit is involved in the catalytic reaction.

The β subunit is stabilized by interactions with the α subunit and a helix bundle, which are connected by long hinge peptides. These interactions allow for flexibility in the Rossmann fold and the whole CP domain (200 residues), which are characteristic of the tRNA-binding domain.

The α subunit contains the active site and the helix bundle domain found in other synthetases. The active site of LeuRS consists of two tRNA molecules that can bind simultaneously.

In the case of LeuRS from A. aeolicus, it has been shown that LeuRS from different sources can substitute each other. This suggests that LeuRS is a highly flexible enzyme that can adapt to various environments.

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Addendum—A recent article (43), published during the preparation of this manuscript, described the heterodimeric structure of LeuRS from A. aeolicus. The authors overexpressed separately the subunits of the enzyme and constituted it in vitro. This heterodimeric structure and the active heterodimer were obtained by urea renaturation in the presence of the β subunit. The final mixture included low affinity sites, which were measured for the active heterodimeric structure.

The β subunit is small; however, it in vivo or in vitro is stable and seems to be a chaperon of the α subunit in vivo. Although inactive in its monomeric form, the β subunit binds tRNA with low affinity. Compared with the dimeric form αβ (Kd = 0.3 μM), it is a 40-fold difference, which may account for the loss of interactions with the acceptor arm of the tRNA molecule, assuming that the tRNA binds the β subunit in its own mode. A second, unspecified tRNA binding site was characterized on the β subunit, which may be related to the results observed for ValRS (35). It has been shown that LeuRS from different sources can substitute for the splicing function of yeast mitochondrial LeuRS (36).

Thus, LeuRss intrinsically possess the ability to bind diverse RNA substrates, even larger than tRNA. This may explain why tRNA molecules can bind simultaneously.

The low affinity exhibited by the β subunit for its tRNA is similar to the values measured for the additional domains found in eukaryotic synthetases, like the C-terminal extension of MetRS related to EMAP II (15 μM) (37), the repeated domains of GluProRS (Kd = 2.5–30 μM) (38, 39) and the p43 component of the mammalian multisynthetase complex (40 μM) (40). It has been shown that the additional domains increase the catalytic efficiency of the synthetase without contributing to the specificity. Thus, they can be partially compared with the β subunit of the Aquifex enzyme, which represents a later stage in the evolution at which point the added domain became more specific for the tRNA substrate.

The αβ-LeuRS from A. aeolicus is split in the middle of the bipartite Rossmann fold domain in a place where the ancestor two half Rossmann folds were juxtaposed in order to give the actual class I active site. Thus, the split A. aeolicus enzyme seems to have gone back in evolution to a stage where the class I active site was not assembled as the actual bipartite Rossmann fold.

The dimerization of the A. aeolicus enzyme causes other questions concerning the assembly of the subunits. No additional domains involved in assembly are detectable by sequence analysis. Thus, the intramolecular contacts found in the monomeric enzyme should have been transformed into intermolecular contacts after separation of the gene into pieces. Previous work on E. coli IleRS demonstrates that this enzyme, closely related with LeuRS, could be expressed in different combinations of split enzymes (16, 41). Thus, it seems that these large monomeric enzymes have the built-in capacity to be synthesized as heterodimers. Similarly, four pairs of fragments, corresponding to N- and C-terminal parts of E. coli LeuRS with junctions at different positions in the CP domain were expressed and assembled in vitro. Except the pair with the junction at the level Glu-292-Ala-293, the assembled proteins were active for aminocacylation (42). However, the Glu-292-Ala-293 mutant still supported the ATP-PPI exchange activity (42). In fact, the assembled E. coli LeuRS mimicked a heterodimeric LeuRS. We recently tried to split the E. coli LeuRS in order to mimic the α and β subunits of A. aeolicus LeuRS; however, the fragments were not stably expressed in E. coli.

REFERENCES

1. Cusack, S., Hartleman, M., and Leberman, R. (1991) Nucleic Acids Res. 19, 3489–3498
2. Nagel, G. M., and Dotilliet, R. F. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 8121–8125
3. Moras, D. (1992) Trends Biochem. Sci. 17, 159–164
4. Carter, C. W., Jr., and (1993) Ann. Rev. Biochem. 62, 715–748
5. Delarue, M. (1995) Curr. Opin. Struct. Biol. 5, 48–55
6. Schimmel, P. (1987) Annu. Rev. Biochem. 56, 125–158
7. Eriani, G., Delarue, M., Poch, O., Gangloff, J., and Moras, D. (1990) Nature 347, 203–206
8. Durbin, J. B., and Schimmel, P. (1991) J. Biol. Chem. 266, 16865–16968
9. Mehler, A. H., and Mitra, S. R. (1967) J. Biol. Chem. 242, 5495–5499
10. Papas, T. S., and Peterkofsky, A. (1972) Biochemistry 11, 4602–4608
11. Schimmel, P., and Solórzano, D. (1979) Annu. Rev. Biochem. 48, 601–648
12. Fersht, A. R., and Dingwall, C. (1979) Biochemistry 18, 2927–2931
13. Hopfield, J. J. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 4135–4139
14. Fersht, A. R., Shindler, J. S., and Tsui, W. C. (1980) Biochemistry 19, 5520–5524
15. Deckert, G., Warren, P. V., Gaasterland, T., Young, W. G., Lenox, A. L., Graham, D. E., Verheugt, R., Smeed, M. A., Keller, M., Aujay, M., Huber, R., Feldman, R. A., Short, J. M., Olsen, G. J., and Swanson, R. V. (1989) Nature 392, 47–58
16. Shiha, K., and Schimmel, P. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10288–10284
17. Li, T. X., Xia, X., Wang, E. D., and Wang, Y. L. (1995) Acta Biochim. Biophys. Sinica 27, 279–286
18. Li, Y., Wang, E. D., and Wang, Y. L. (1998) Sci. China Ser. C Life Sci. 41, 225–231
19. Li, Y., Wang, E. D., and Wang, Y. L. (1999) Protein Expression Purif. 16, 355–358
20. Li, T., Li, Y., Guo, N., Wang, E., and Wang, Y. (1999) Biochemistry 38, 9084–9088
21. Velderop, J. S., Dijkhuizen, M. A., van Hof, R., and Postma, P. W. (1995) Gene 152, 63–65
22. Wu, J., Wang, E. D., and Wang, Y. L. (1999) Acta Biochim. Biophys. Sinica (in Chinese) 31, 226–232
23. Yang, T. J., Wu, C. S. C., and Martinez, H. M. (1980) Methods Enzymol. 130, 208–258
24. Chen, J. F., Guo, N. N., Li, T., Wang, E. D., and Wang, Y. L. (2000) Biochemistry 39, 6726–6731
25. Gangloff, J., Schuta, A., and Dirheimer, G. (1976) Eur. J. Biochem. 65, 177–182
26. Fersht, A. R., Ashford, J. S., Bruton, C. J., Jakes, R., Koch, G. L. E., and Hartley, B. S. (1975) Biochemistry 14, 1–4
27. Granda, S., Hustedt, H., Flossdorf, J., and Kula, M. R. (1979) Mol. Cell. Biochem. 24, 175–181
28. Shi, J. P., Lin, S. X., Miao, F., and Wang, Y. L. (1988) Acta Biochim. Biophys. Sinica 20, 76–81
29. Cusack, S., Varenchuk, A., and Tukalo, M. (2000) EMBO J. 19, 2351–2361
30. Delagoutte, B., Moras, D., and Cavarelli, J. (2000) EMBO J. 19, 5599–5610
31. Silvian, L., Wang, J., and Steitz, T. (1999) Science 285, 1074–1077
32. Fukai, S., Nureki, O., Sekine, S., Shumada, A., Tao, J., Vassylyev, D. G., and Yokoyama, S. (2000) Cell 103, 793–803
33. Geslain, R., Martin, F., Delagoutte, B., Cavarelli, J., Gangloff, J., and Eriani, G. (2000) RNA 6, 434–448
34. Schmitt, E., Panvert, M., Mechulam, Y., and Blanquet, S. (1997) Eur. J. Biochem. 246, 539–547
35. Tardif, K. D., Liu, M., Vitseva, O., Hou, Y. M., and Horowitz, J. (2001) Biochemistry 40, 8118–8125
36. Houman, F., Rho, S. B., Zhang, J., Shen, X., Wang, C. C., Schimmel, P., and Martinis, S. A. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 13743–13748
37. Kaminska, M., Denizziak, M., Kerjan, P., Barciszewski, J., and Mirande, M. (2000) EMBO J. 19, 6908–6917
38. Cahuzac, B., Iberthonneau, E., Biririrakis, N., Guittet, E., and Mirande, M. (2000) EMBO J. 19, 445–452
39. Rho, S. B., Lee, J. S., Jeong, E. J., Kim, K. S., Kim, Y. G., and Kim, S. (1998) J. Biol. Chem. 273, 11267–11273
40. Quevillon, S., Agou, F., Robinson, J. C., and Mirande, M. (1997) J. Biol. Chem. 272, 32573–32579
41. Shiba, K., and Schimmel, P. (1992) J. Biol. Chem. 267, 22703–22706
42. Li, T., Guo, N., Xia, X., Wang, E., and Wang, Y. (1999) Biochemistry 38, 13063–13069
43. Gueda, M., Yokogawa, T., Asahara, H., and Nishikawa, K. (2002) FEBS Lett. 518, 139–143