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Enzymes Assembled from *Aquifex aeolicus* and *Escherichia coli* Leucyl-tRNA Synthetases†

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ABSTRACT: *Aquifex aeolicus* α*β*-LeuRS is the only known heterodimeric LeuRS, while *Escherichia coli* LeuRS is a canonical monomeric enzyme. By using the genes encoding *A. aeolicus* and *E. coli* LeuRS as PCR templates, the genes encoding the α and *β* subunits from *A. aeolicus* α*β*-LeuRS and the equivalent amino- and carboxy-terminal parts of *E. coli* LeuRS (identified as α′ and *β*′) were amplified and recombined using suitable plasmids. These recombinant plasmids were transformed or cotransformed into *E. coli* to produce five monomeric and five heterodimeric LeuRS mutants. Seven of these were successfully overexpressed in vivo and purified, while three dimeric mutants with the *β*′ part of *E. coli* LeuRS were not successfully expressed. The seven purified mutants catalyzed amino acid activation, although several exhibited reduced aminoacylation properties. Removal of the last 36 residues of the α subunit of the *A. aeolicus* enzyme was determined to be deleterious for tRNA charging. Indeed, subunit exchange showed that the cross-species-specific recognition of *A. aeolicus* $tRNA^{Leu}$ occurs at the α subunit. None of the mixed *E. coli*−*A. aeolicus* enzymes were as thermostable as the native α*β*-LeuRS. However, the fusion of the two α and *β* peptides from *A. aeolicus* as a single chain analogous to canonical LeuRS resulted in a product more resistant to heat denaturation than the original enzyme.

The aminoacyl-tRNA synthetase (aaRS)[1] family provides the enzymatic basis for genetic encoding by catalyzing the esterification of amino acids to their cognate tRNAs (*1*). The 20 aaRSs can be divided into two classes of 10 members each on the basis of conserved sequence and characteristic structural motifs (*2*). Leucyl-tRNA synthetase (LeuRS) belongs to class I aaRS. Canonical LeuRSs primarily consist of single subunits. The exception to this is the LeuRS from the hyperthermophilic bacterium *Aquifex aeolicus*, called α*β*-LeuRS (*3*−*6*), which consists of two subunits that contain 634 and 289 residues, respectively. Despite the difference in quaternary structure, the sequence of α*β*-LeuRS is close to the *Escherichia coli* monomeric LeuRS: the α and *β* subunits are 55% and 44% identical, respectively, to the corresponding sequences of the *E. coli* monomer. The only significant difference between the two enzymes is an insertion domain called the "leucine-specific domain" in *Thermus thermophilus* LeuRS, whose structure has been solved by X-ray crystallography (*7*). The leucine-specific domain corresponds to the split domain in *A. aeolicus*. The 50-residue leucine-specific domain in *E. coli* and *T. thermophilus* is replaced by two nonhomologous peptides located at the C-terminal end of the α subunit (about 30 residues long) and at the N-terminal end of the *β* subunit (about 40 residues long) in *A. aeolicus* (Figure 1).

As previously reported, we expressed both subunits of the *A. aeolicus* LeuRS and observed that both the heterodimer α*β*-LeuRS and the *β* subunit alone could be stably expressed in *E. coli*, while the α subunit was unstable when expressed alone (*6*). Both the heterodimer α*β*-LeuRS and the *β* subunit alone were thermostable and able to bind $tRNA^{Leu}$ (*5*, *6*). The purified α*β*-LeuRS efficiently catalyzed the aminoacylation of the cognate $tRNA^{Leu}$ and displayed discriminating properties toward *E. coli* $tRNA^{Leu}$.

On the basis of this work, we used an approach based on the assembly and exchange of cleaved LeuRS originating from *A. aeolicus* and *E. coli*. Mixed heterodimers exhibiting *A. aeolicus* subunits and subunit-like polypeptides from *E. coli* were built as well as monomeric chimeras made of fusions between pieces of LeuRSs from both organisms. The expression, thermostability, and kinetic properties of the proteins and cross-reactivity for $tRNA^{Leu}$ from both origins were studied. We focused our investigations on the α−*β* fusion protein from *A. aeolicus*, which mimics the canonical monomeric state. We found that this protein was as active as the native heterodimer and, surprisingly, displayed higher resistance to heat denaturation.

This work first focuses on the assembly of LeuRSs from different sources and gives us novel information on the structure−function relationships of LeuRSs. Genome se-

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[1] Abbreviations: aaRS, aminoacyl-tRNA synthetase; LeuRS, leucyl-tRNA synthetase; *A. aeolicus*, *Aquifex aeolicus*; *E. coli*, *Escherichia coli*.

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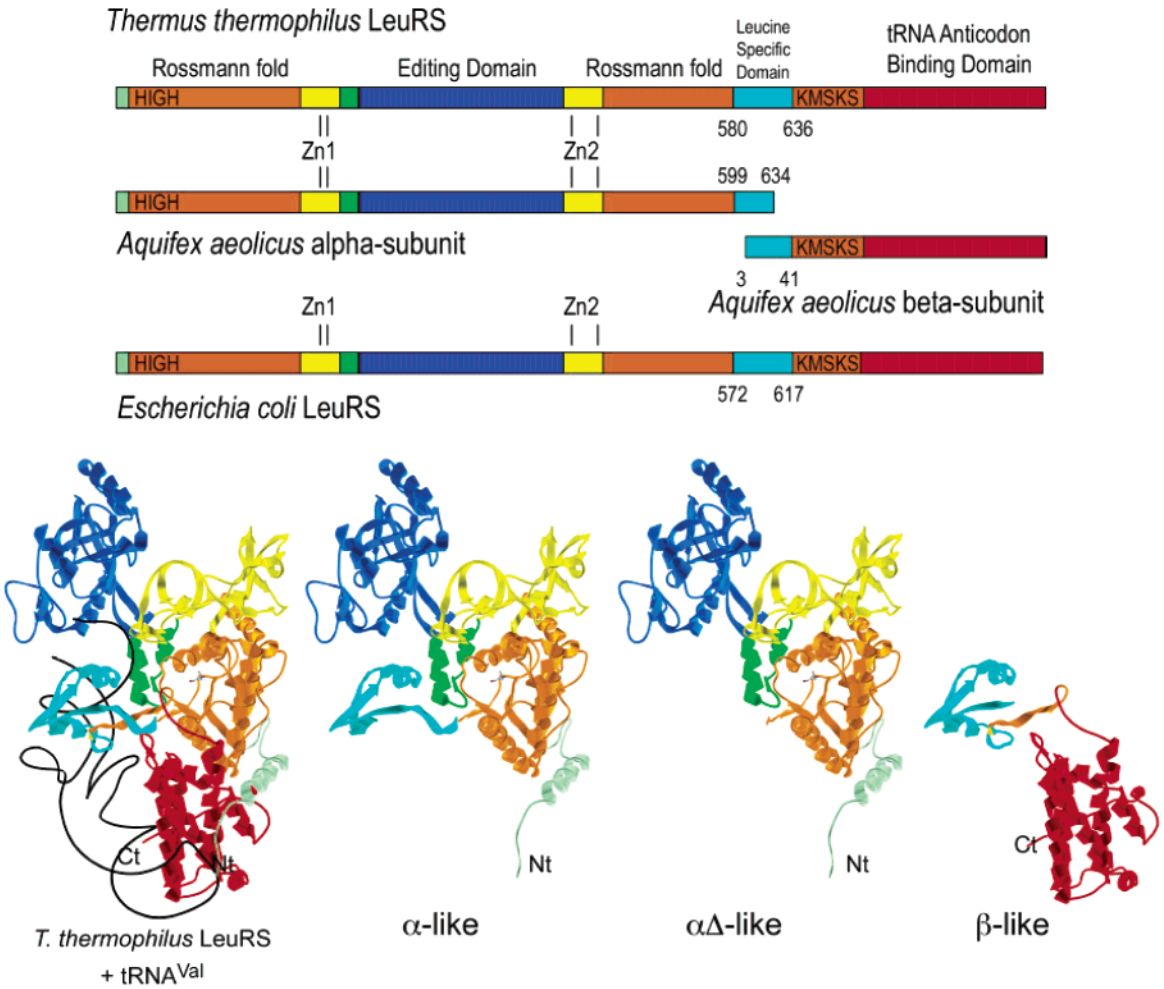

FIGURE 1: The upper part of the figure shows a schematic diagram of the domain structure of *T. thermophilus* and *E. coli* LeuRSs and α and *β* subunits of *A. aeolicus* LeuRS. The diagram is based on sequence alignments and *T. thermophilus* 3D structure (PDB ID code: 1H3N). The different structural domains are indicated and colored according to ref *7*. The lower part of the figure displays on the left the structure of *T. thermophilus* LeuRS on which $tRNA^{Val}$ from ValRS-$tRNA^{Val}$ (PDB ID code: 1GAX) has been docked. Docking was performed after 3D superimposition of the Rossmann fold domain of the two enzymes. The three other structures are fragments of *T. thermophilus* LeuRS corresponding to the different peptides originating from *A. aeolicus* or *E. coli* and that were expressed and assembled during this study.

quence analysis shows that horizontal gene transfer plays a significant role in species evolution. Thus, studies on assembled enzymes may give further insights in the evolution of species.

EXPERIMENTAL PROCEDURES

Materials. L-Leucine, DTT, ATP, CAPS, NTP, 5′-GMP, tetrasodium pyrophosphate, and inorganic pyrophosphatase were purchased from Sigma (St. Louis, MO). [^{14}C]-L-Leucine (300−400 mCi/mmol) and tetrasodium [^{32}P]pyrophosphate were obtained from NEN Dupont (Boston, MA). GF/C filter was obtained from Whatman Co. (Mainstone, England). T4 polynucleotide kinase, T4 DNA ligase, and restriction endonucleases were obtained from Sangon Co. (Shanghai Branch, Canada). *E. coli* and *A. aeolicus* total tRNA containing 50% $tRNA^{Leu}$(GAG) was isolated from overproducing strains constructed in our laboratory (*6*, *8*).

Plasmids. pSML104 was constructed from pACYC184 and pKK-233-2 (*9*). It contains the p15A replicon from pACYC184, the strong trc promoter, a multicloning site, two sequences for transcription termination (T1 and T2) of the ribosomal operon rrnB from pKK233-2, and resistances to tetracycline. Plasmid pBCP378 contains the trc promoter, the resistance gene to ampicillin, an *Nde*I site at its translation start, and the $lacI^{Q}$ gene, which confers a tight control of the trc promoter in absence of IPTG (*10*). Plasmid pTrc99B is similar to pBCP378 except for the replacement of the *Nde*I site with an *Nco*I site (*11*). Both pBCP378 and pTrc99B carry the *ColE1* replicon, which is fully compatible with the p15A replicon carried by pACYC184 and pSML104. Plasmid pTrc100 was derived from pTrc99B (*9*). It contains the *ColE1* replicon. The same multicloning site contained in pSML-104 (which contains *Nco*I, *Eco*RI, *Sma*I, *Bgl*II, *Bfr*I, and *Hin*dIII sites) was introduced into the plasmid.

Amplification of DNA Fragments Encoding Peptide Fragments and Construction of LeuRS Mutants. Using the *leuS* genes from *A. aeolicus* (*6*) or *E. coli* (*12*) as templates, DNA fragments encoding two different α subunits of *A. aeolicus* LeuRS and an α-like (called α′) subunit from *E. coli* LeuRS were PCR amplified with the appropriate restriction sites on both ends (Figure 2). They were named α, αΔ, and α′ and encoded M1-A634 and M1-L598 of the α subunit of *A. aeolicus* LeuRS and M1-K605 of *E. coli* LeuRS (which mimics the α subunit of the thermophilic enzyme), respec-

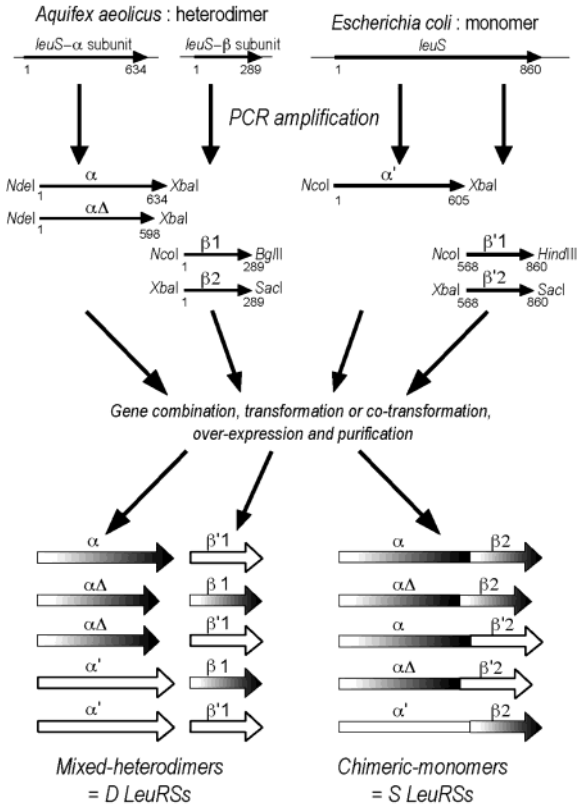

FIGURE 2: Construction of the different mutated LeuRSs. Schematic protocol showing the construction of the different mutated LeuRSs. The various PCR fragments were amplified from the two pieces of *A. aeolicus leuS* genes and the single *E. coli leuS* gene. PCR products were digested and differently combined into overexpressing vectors. To generate mixed heterodimers, DNA fragments were cloned into vectors carrying different antibiotic resistances ($\alpha + \beta'_1$, pBCP378 + pMSL104; $\alpha\Delta + \beta_1$, pBCP378 + pMSL104; $\alpha\Delta + \beta'_1$, pBCP378 + pMSL104; $\alpha' + \beta_1$, pTrc100 + pMSL104; $\alpha' + \beta'_1$, pTrc100 + pMSL104). The chimeric LeuRSs were generated by subcloning compatible DNA fragments into a unique plasmid (pBCP378) with respect to the peptide ORF frame.

tively. The αΔ fragment was a truncated form of the α subunit of *A. aeolicus* LeuRS lacking the last 36 residues. Since these residues do not have counterparts in the *E. coli* enzyme, it was thought that they might generate steric clashes in the different constructions.

By the same method, four DNA fragments encoding the β subunit of the *A. aeolicus* LeuRS (called β1 and β2) and the β'-mimic of *E. coli* LeuRS (called β'1 and β'2) were amplified (see Figure 2 for the sizes). Both β1 and β1′ gene fragments shared *Nco*I restriction sites that allowed them to be cloned into the *Nco*I sites of the expression vectors. Both β2 and β'2 contained *Xba*I sites to facilitate combination with the complementary sites of the α subunits.

The seven amplified DNA fragments were digested with the appropriate restriction endonucleases and ligated alone or together in expression vectors in order to overexpress. After transformation or cotransformation in *E. coli* TG1, five mixed heterodimers and five monomeric chimeras were produced (Figure 2). Going forward, mixed heterodimers are identified with a D (for dimer) and the monomeric chimeras with an S (for single chain).

Expression of the Genes Encoding LeuRS Mutants and Purification of LeuRS Mutants. An overnight starter culture of *E. coli* transformants containing the recombinant plasmids was diluted 1:20 in 50 mL of Luria−Bertani medium containing the appropriate antibiotic (100 μg/mL ampicillin for monomeric LeuRS, 100 μg/mL ampicillin and 10 μg/mL tetracycline for heterodimeric LeuRS) and allowed to grow for 12 h at 37 °C with vigorous shaking (300 rpm). When an A_{600} of 0.5 was reached, IPTG was added to a final concentration of 0.5 mM, and the induction was performed under the same conditions for another 4−5 h. Cells were harvested by centrifugation, resuspended in 4 mL of disruption buffer (100 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 1 mM EDTA), and sonicated for 6 × 20 s at 15 W with a high-intensity ultrasonic processor (375W model). The crude extract was cleared of cellular debris by centrifugation at 12000 rpm for 40 min and then analyzed by SDS−PAGE to determine the expression of the LeuRS mutants. The crude extract of the transformants expressing LeuRS mutants was heated at 75 °C for 1 h to determine the thermal stability of the LeuRS mutants by analysis of SDS−PAGE. Thermostable mutants were not changed; however, the thermosensitive mutants disappeared on the gel after heating.

For large-scale purification, 4 L of cells was treated as described above. For the thermostable LeuRS mutants, the crude extract was heated at 75 °C for 1 h and centrifuged at 10000 rpm at 4 °C for 10 min to remove other thermosensitive proteins in the host cells; the lysate was ready for use. For the thermosensitive LeuRS mutants, the heating procedure was avoided. The enzymes were purified by two-step chromatography on DEAE-Sepharose CL-6B (3 × 18 cm) and HA-Ultrogel (3.5 × 18 cm) columns, according to ref *13*. The protein concentrations were measured according to ref *15*.

Kinetic Assays and Active Site Titration. ATP−PP_i exchange and aminoacylation activities of LeuRS were measured at either 37 or 60 °C as described (*6*, *14*). The kinetic constants of enzymes were determined using various concentrations of the relevant substrates (*14*). The active site titration was performed according to the method of Fersht (*16*) by measuring ATP exhaustion in the formation of leucyl adenylate at 60 °C, pH 7.8, from LeuRS (5 μM), [γ-^{32}P]-ATP (20 μM, 20 μCi/mL), and leucine (1 mM), in the presence of pyrophosphatase (10 units/mL) (*16*).

Determination of Optimal Temperature and Thermal Stability. Determination of optimal temperature was performed at various temperatures under the given conditions. The measurement of the thermal stability of LeuRS was performed as described previously (*9*). The enzyme (40 μg/mL) in 50 mM potassium buffer (pH 6.8) containing 400 μg/mL BSA was incubated at various temperatures (*9*) for 10 min. The aminoacylation activity was assayed after the reaction mixture was diluted with cold 50 mM potassium phosphate buffer, pH 7.8.

RESULTS

Construction of Recombinant Plasmids. Recombinant plasmids containing the genes encoding LeuRS mutants were constructed and confirmed by DNA sequencing. For the five monomeric LeuRSs, two extra residues (Ser-Arg) were added at the fusion restriction site (*Xba*I, TCT AGA encoding Ser-Arg). For the five mixed heterodimeric LeuRSs, the sequences of the subunits were unchanged.

Expression, Thermostability, and Purification of LeuRS Mutants. From the 10 constructs we designed, seven proteins

Table 1: Expression, Thermal Stability, and Activities of Various LeuRS Mutants[a]

name of mutant	expression	thermal stability	activity: amino acid activation: specific activity (units/mg)	activity: amino acid activation: relative activity (%)	activity: aminoacylation: relative activity (%)
A. aeolicus αβ-LeuRS	yes	yes	2950	100	100
SLeuRSαβ	yes	yes	3150	107	110
SLeuRSαΔβ	yes	yes	1660	56	0
SLeuRSαβ′	yes	no	4480	151	0
SLeuRSαΔβ′	yes	no	6534	221	0
SLeuRSα′β	yes	no	343	12	8.4
DLeuRSα′β	yes	no	253	9	4.2
DLeuRSαΔβ	yes	yes	376	13	0
DLeuRSα′β′	no				
DLeuRSαβ′	no				
DLeuRSαΔβ′	no				

[a] Amino acid activation and aminoacylation activities were assayed at 37 °C. The k_{cat} of aminoacylation are shown in Table 4. Data here are the average values from three independent determinations, with a variation of <5%.

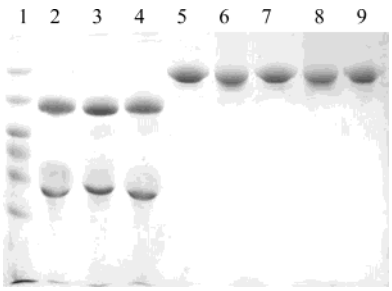

FIGURE 3: SDS−PAGE analysis of purified LeuRS mutants. Lanes: 1, protein standards with molecular masses of 97.4, 66.2, 55.0, 42.7, 40.0, 31.0, and 21.5 kDa (from top to bottom); 2, native αβ-LeuRS; 3, DLeuRSαΔβ; 4, DLeuRSα′β; 5, SLeuRSαβ; 6, SLeuRSαΔβ; 7, SLeuRSαβ′; 8, SLeuRSαΔβ′; 9, SLeuRSα′β. Each lane contained 5 μg of protein.

were expressed in *E. coli*. These were the five monomeric LeuRSs and the two heterodimeric LeuRSs presenting the β subunit from the *A. aeolicus* enzyme (Table 1). The three other heterodimeric LeuRSs with the β′-like subunit from *E. coli* LeuRS were not detected in the cell extracts. After being heated at 75 °C for 1 h, only the three LeuRSs composed exclusively of peptides of the *A. aeolicus* enzyme were thermostable (Table 1). A two-step chromatographic separation was used to purify the different proteins to about 90% homogeneity (Figure 3).

Activity of LeuRS Mutants. The seven expressed LeuRS mutants displayed ATP−PP_i exchange activities ranging from 9% to 221% of the native αβ-LeuRS activity (Table 1). The monomeric mutants containing the α subunit had activities comparable to that of *A. aeolicus* αβ-LeuRS. The heterodimeric mutants and monomer containing the α′ subunit had very low ATP−PP_i exchange activity. Although the chimera SLeuRSαΔβ and the heterodimer DLeuRSαΔβ were thermostable and had ATP−PP_i exchange activity, the truncated mutant (lacking the 36 amino acid residues at the C-terminal end of the α subunit) showed no aminoacylation activity, indicating that the integrity of the C-terminal end of the α subunit of *A. aeolicus* is strictly required for the aminoacylation activity of this enzyme. Two monomeric chimeras with the β′ subunit, SLeuRSαβ′ and SLeuRSαΔβ′, lost their aminoacylation activity also, although they had shown relatively high ATP−PP_i exchange activity. Only three of the seven LeuRS mutants exhibited aminoacylation activity: the monomers SLeuRSαβ and SLeuRSα′β and the mixed heterodimer DLeuRSα′β. Their k_{cat} for charging *A. aeolicus* and *E. coli* $tRNA^{Leu}$ are shown in Table 2. Only SLeuRSαβ, the fusion form of the dimeric αβ-LeuRS, was shown to be as active as the original enzyme. The two chimeras containing the α′ peptide from *E. coli*, SLeuRSα′β and DLeuRSα′β, exhibited decreased aminoacylation activity. But interestingly, they catalyzed the aminoacylation of both *E. coli* and *A. aeolicus* $tRNA^{Leu}$ at the same rate, a behavior characteristic of the *E. coli* but not *A. aeolicus* enzyme (Table 2). Thus, it seems that the interspecies cross-recognition of tRNA is mediated by interactions with the α and α′ peptides. Moreover, both SLeuRSα′β and DLeuRSα′β mutants exhibited comparable decreases of the ATP−PP_i exchange and aminoacylation activities, suggesting that the defect resulting from the construction of the mutants primarily affected the amino acid activation step.

Table 2: Specific Activities of LeuRS Mutants in the Aminoacylation of Different tRNAs[a]

enzyme	specific activity (units/mg): *A. aeolicus* $tRNA^{Leu}$(GAG)	specific activity (units/mg): *E. coli* $tRNA^{Leu}$(GAG)	charging ratio of *A. aeolicus* to *E. coli* $tRNA^{Leu}$
E. coli LeuRS	1300	1500	0.86
A. aeolicus αβ-LeuRS	235	47	5.0
SLeuRSαβ	260	75	3.5
SLeuRSα′β	19.8	27	0.73
DLeuRSα′β	9.8	11.5	0.85

[a] Assays were carried out at 37 °C under standard conditions. Data presented are the average value from five independent determinations, with a variation of <5%.

In the following, we focused on the monomeric enzyme resulting from the fusion of the two subunits of the *Aquifex* enzyme (SLeuRSαβ) and compared the kinetic properties of this new enzyme construction to the original dimeric enzyme.

Kinetic Properties of the Fusion Protein SLeuRSαβ. The number of active sites in SLeuRSαβ was calculated by plotting $[ATP]_t$ against *t* (time) at 60 °C(*5*) using 3.5 μM enzyme. This led to a calculation 1.02 ± 0.05 (the value averaged from three independent determinations) active site(s) for each SLeuRSαβ molecule, which is identical to that found for *A. aeolicus* αβ-LeuRS (*5*).

In the ATP−PP_i exchange reaction at 37 °C, the Michaelis−Menten constant (K_m) values of SLeuRSαβ for leucine and ATP were 1.4 and 333 μM, respectively, and k_{cat} was 3.8 and 3.3 s^{-1}, respectively. The K_m values increased slightly and k_{cat} values increased by 4−5-fold (Table 3) when the temperature was increased to 60 °C.

The kinetic parameters of the SLeuRSαβ in the aminoacylation reaction were assayed at 37 and 60 °C (Table 4). At 60 °C, k_{cat} values were 2.5−3 times those at 37 °C. Compared to native αβ-LeuRS, the k_{cat} values of SLeuRSαβ for the three substrates at 37 °C were 1.3−1.6-fold greater. However, these k_{cat} values were unchanged at 60 °C. At either 37 or 60 °C, the K_m values of both enzymes for leucine and *A. aeolicus* $tRNA^{Leu}$(GAG) were almost the same. On the

Table 3: Kinetic Constants for Various LeuRSs in ATP−PP_i Exchange Reaction[a]

		αβ-LeuRS[b]		SLeuRSαβ	
substrate	constant	37 °C	60 °C	37 °C	60 °C
leucine	K_m (μM)	1.3	1.6	1.4	2.0
	k_{cat} (s^{-1})	3.5	15.5	3.8	15.4
	k_{cat}/K_m (s^{-1} mM^{-1})	2692	9600	2815	7700
ATP	K_m (μM)	360	380	333	376
	k_{cat} (s^{-1})	3.3	14.5	3.3	17.4
	k_{cat}/K_m (s^{-1} mM^{-1})	9.2	38.2	9.9	46.3

[a] Data are the average value from three independent determinations, with a variation of <5%. [b] From ref *6*.

Table 4: Kinetic Constants of SLeuRSαβ and *A. aeolicus* αβ-LeuRS in the Aminoacylation Reaction[a]

		αβ-LeuRS[b]		SLeuRSαβ	
substrate	constant	37 °C	60 °C	37 °C	60 °C
leucine	K_m (μM)	6.4	6.0	6.8	5.3
	k_{cat} (s^{-1})	0.39	1.4	0.5	1.4
	k_{cat}/K_m (s^{-1} mM^{-1})	61	227	73.9	263
ATP	K_m (μM)	550	112.3	360	121.5
	k_{cat} (s^{-1})	0.39	1.8	0.56	1.7
	k_{cat}/K_m (s^{-1} mM^{-1})	0.71	15.6	1.56	14.2
A. aeolicus	K_m (μM)	0.38	0.3	0.29	0.3
$tRNA^{Leu}$(GAG)	k_{cat} (s^{-1})	0.39	1.5	0.57	1.5
	k_{cat}/K_m (s^{-1} mM^{-1})	1000	4710	1965	5960
E. coli	K_m (μM)	0.76	1.5	0.56	2.7
$tRNA^{Leu}_2$(GAG)	k_{cat} (s^{-1})	0.084	0.4	0.14	0.4
	k_{cat}/K_m (s^{-1} mM^{-1})	111	240	258	151

[a] Determinations were carried out under standard conditions. Data are the average value from five independent determinations, with a variation of <5%. [b] From ref *6*.

other hand, the K_m values for ATP at 60 °C were smaller for both enzymes, indicating that elevation of temperature increased the ATP binding strength. When the catalytic efficiency of both enzymes was compared, the monomeric version of the enzyme was more efficient for all substrates at 37 °C (Table 4). This was also found at 60 °C, with the exception of substrates ATP and *E. coli* $tRNA^{Leu}$.

Aminoacylation of the Heterologous E. coli tRNA^{Leu} by SLeuRSαβ. We previously described the cross-aminoacylation properties of *E. coli* and *A. aeolicus* LeuRSs for their respective tRNAs (*5*). The *E. coli* enzyme exhibited no discriminating properties for the *A. aeolicus* $tRNA^{Leu}$, whereas the *A. aeolicus* enzyme aminoacylated the *E. coli* $tRNA^{Leu}$ with a 10 times reduced k_{cat} (*5*). At 37 °C, the fusion protein SLeuRSαβ was more active than the dimeric *A. aeolicus* αβ-LeuRS (0.14 versus 0.084 s^{-1}) (Table 4). At 60 °C, the activities of both enzymes were equal, but the catalytic efficiency was higher for the dimeric αβ-LeuRS due to a better affinity for $tRNA^{Leu}$ (Table 4).

Thermal Properties of SLeuRSαβ. SLeuRSαβ, like *A. aeolicus* αβ-LeuRS (*5*), is a thermostable enzyme. Under experimental conditions, we found it to be stable up to 70 °C (Figure 4). With elevation of temperature, the aminoacylation activity of SLeuRSαβ showed a slow, slight decrease. At 95 °C, 78% of the initial aminoacylation activity was still present. This is in contrast to the native *A. aeolicus* αβ-LeuRS, which exhibited a higher sensitivity to heat inactivation and retained only 20% of its original activity at 95 °C (*5*) (Figure 4). This suggests that the monomer SLeuRSαβ is much more stable than the original heterodimer *A. aeolicus* αβ-LeuRS.

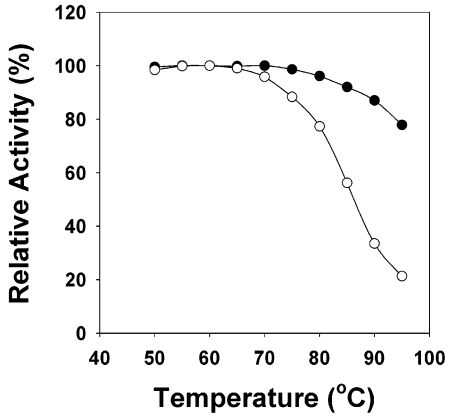

FIGURE 4: Determination of the thermal stability of SLeuRSαβ. SLeuRSαβ (40 μg/mL) in 50 mM potassium phosphate buffer, pH 6.8, containing 400 μg/mL BSA was incubated at various temperatures (50−95 °C) for 10 min. Then, the aminoacylation activity of SLeuRSαβ (filled circles) was assayed at 37 °C after dilution of the reaction mixture with cold 50 mM potassium phosphate buffer, pH 7.5, and compared with that of *A. aeolicus* αβ-LeuRS [open circles (*6*)].

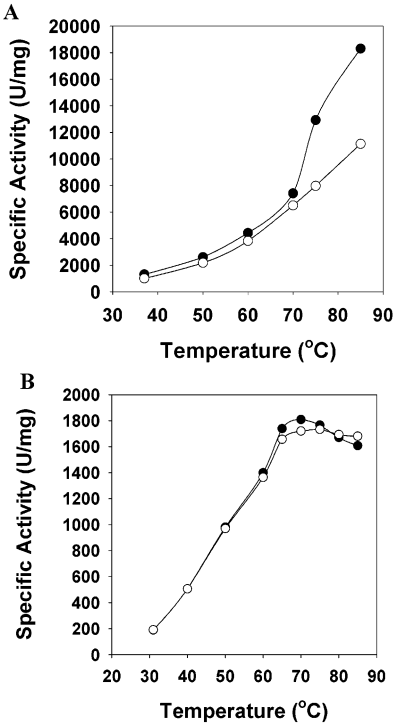

FIGURE 5: Variation of SLeuRSαβ activity with the temperature. The ATP−PP_i exchange activity (A) and the aminoacylation reaction (B) of SLeuRSαβ (filled circles) were assayed at increasing temperatures and compared with that of *A. aeolicus* αβ-LeuRS (empty circles) under the same conditions.

The specific activities of SLeuRSαβ for both amino acid activation and tRNA aminoacylation increased with temperature. For amino acid activation, we observed a continuous increase of activity up to 85 °C. At 70 °C, the specific activity was 5-fold greater than that at 37 °C (Figure 5). The tRNA aminoacylation activity increased up to 65 °C and then decreased slightly above this point, unlike the original αβ-LeuRS, which remained fully active up to 85 °C (*5*). At 70 °C, the aminoacylation activity of SLeuRSαβ was 4.5-fold greater than that at 37 °C (Figure 5).

The activation energy was calculated from the slope of the line when ln v was plotted against $1/T$. The activation

energy for the amino acid activation reaction was 11.1 kcal/mol. Below 50 °C, the activation energy for the aminoacylation reaction was 14.2 kcal/mol. Above 50 °C the value was 6.6 kcal/mol. These figures are similar to those of the native αβ-LeuRS (*5*).

DISCUSSION

A. aeolicus LeuRS is a heterodimer that differs from the canonical monomeric LeuRSs from other sources (*3*). On the basis of the sequence homologies and X-ray structure of the *T. thermophilus* LeuRS, whose structure has been solved by X-ray crystallography (*7*), we can postulate that the α subunit of the *A. aeolicus* enzyme possesses half of the active site, with the CP1 domain, generally involved in editing, attached to the Rossmann fold (Figure 1) (*7*). The β subunit should contain the second part of the active site and the helix bundle domain found in the class Ia synthetases (Arg-, Cys-, Met-, Val-, Ile-, and LeuRS). This domain is involved in tRNA-anticodon binding as shown by X-ray crystallography (*17*−*19*) and functional studies (*20*, *21*). An additional well-ordered domain, called the leucine-specific domain in *T. thermophilus*, probably does not exist in the same form in *A. aeolicus* LeuRS, which contains the split site that cuts the protein into two peptides of about 30 residues (Figure 1).

Despite this difference in oligomeric state, the *A. aeolicus* enzyme is close to the *E. coli* enzyme (55% identity between α and α′ and 44% identity between β and β′), except for the leucine-specific domain found in the *E. coli* enzyme which has no counterpart in *A. aeolicus* LeuRS. The 634 residues of the α subunit of the *A. aeolicus* αβ-LeuRS correspond to the first 605 residues of the *E. coli* LeuRS (α′), and the 289 residues of the β subunit are similar to residues 568−860 of *E. coli* LeuRS (β′). To investigate the role of oligomerization and to localize the interactions that make *A. aeolicus* LeuRS more stable than *E. coli* LeuRS, a family of 10 hybrid proteins was constructed. Thermal inactivation experiments, amino acid activation, and tRNA charging were used to estimate stabilities and activities of these hybrids. The constructs were designed on the basis of sequence homologies and the original split site in the *A. aeolicus* enzyme.

All monomeric hybrids were successfully expressed and were found to significantly catalyze amino acid activation, suggesting that complementary chain-packing interactions occurred sufficiently to reconstruct a functional three-dimensional structure. However, an exclusive content of *A. aeolicus* LeuRS sequences was found to be required for thermostability. $tRNA^{Leu}$ aminoacylation was restricted to proteins carrying a full-length α or α′ peptide fused with a β peptide. Hybrids including a truncation of the C-terminal end of the α peptide did not charge the tRNA as well as those exhibiting the *E. coli* β′ peptide. This probably means that any misalignment of the tRNA acceptor branch at the surface of LeuRS could preclude a functional interaction between the acceptor A76 and LeuRS. This misalignment may result from an incorrect interaction between the tRNA and the newly constructed synthetase. It is known that strong effects on the velocity of the aminoacylation reaction can be caused by even a subtle difference in the formation of the complex. The lack of aminoacylation capacity of C-terminal truncation mutants and of the mutants exhibiting the β′ peptide strongly suggests that these structural elements are involved in tRNA binding. Relaxation of the $tRNA^{Leu}$ interspecies discrimination was also observed with the α′β hybrid, underlining the importance of the structural integrity to $tRNA^{Leu}$ recognition.

Of the five heterodimeric assemblies, only two were stably expressed in *E. coli*. Both carried the β subunit, which has recently been suggested to act as a chaperone for the native heterodimer αβ-LeuRS (*4*, *5*). Although the α subunit is larger than the β subunit, it formed inclusion body when it was expressed in vivo and was sensitive to heat denaturation (*5*). The smaller β subunit was efficiently overexpressed, was thermostable, and was able to bind $tRNA^{Leu}$ (*5*). The β subunit conferred the heterodimeric αβ-LeuRS overexpression when coexpressed with the gene encoding the α subunit, suggesting that the β subunit has the capacity to stabilize the α subunit in heterodimeric LeuRS (*5*). Kinetic investigations on the two heterodimers confirmed the observations made on the chimeric homodimers. The aminoacylation capacity was lost when the C-terminal end of the α subunit was removed, and the interspecies $tRNA^{Leu}$ cross-recognition barrier was lost when the *E. coli* α-like subunit (α′) and the β subunit were mixed, indicating that the residues recognizing the species-specific determinants in $tRNA^{Leu}$ are located in the α or α′ peptide.

However, all expressed proteins were active in amino acid activation, including combinations of all three α subunits (α, α′, αΔ) with the β and β′ subunits. Decoupling of the activation and charging activities occurred for four constructions. This strongly confirmed the crucial role of the C-terminal end of the α peptide in the aminoacylation reaction and also underlined the inability of the β′ peptide to confer tRNA charging ability. Taken together, these results show that the residues involved in leucyladenylate formation can be more easily placed in a functional position than those leading to tRNA charging. According to known data (*7*) most of these residues should be located in the α peptide where the HIGH sequence is located, but the correct positioning of the β peptide is also expected since it contains the second consensus sequence KMSKS (*7*). Moreover, through the comparison of the circular dichroism (CD) spectroscopy data, we found that all of the mutants and the native LeuRS display nearly the same content of helices and sheets (data not shown), indicating that the various LeuRS constructions did not alter the secondary structure of the mutants distinctly.

Taken together, all of these data on heterodimer formation, the activities of aminoacylation, and amino acid activation show the difficulty to construct hybrids or fusion proteins where the functional elements present in both subunits are correctly aligned. For instance, we could not isolate chimeras exhibiting full activity. This suggests that the interface of the chimeras is not perfectly complementary as shown by the αβ′ and αΔβ′ constructions that are not assembled as soluble proteins in the case of the heterodimers, whereas they exhibit some leucine activation activity as purified stable homodimers (Table 1). *E. coli*, *A. aeolicus*, and *T. thermophilus* LeuRS are enzymes that are very similar at the level of the primary sequence. Thus, their global shape should be conserved. The only major difference is present in *A. aeolicus* LeuRS at the level of the leucine-specific domain, which is composed of two peptides of about 30 residues each that end and start the *A. aeolicus* α and β subunits. In *T. thermophilus* LeuRS, the 50 residue long leucine-specific domain is inserted near the consensus sequence KMSKS.

Its obvious function would be to interact with the acceptor arm of $tRNA^{Leu}$ as shown by superimposing the catalytic core of ValRS in complex with $tRNA^{Val}$ (*19*) and LeuRS (Figure 1). Moreover, as this domain is inserted near the consensus sequence KMSKS, it would act as a sensor in case of noncognate binding of tRNA and transfer a negative signal to the close KMSKS sequence. Thus, the leucine-specific domain is a crucial element that might control tRNA binding and that is responsible for the heterodimeric state of *A. aeolicus* LeuRS since it contains the split site. All of these differences and properties found in the two types of leucine-specific domain and the fact that the interfaces between the domains do not fit perfectly might explain the expression problems encountered and the low activity found for some assemblies.

The monomeric SLeuRS$\alpha\beta$ formed by ligation of the α and β subunits showed kinetic properties and activation energy similar to those of the original heterodimer $\alpha\beta$-LeuRS. In the cross-species $tRNA^{Leu}$ recognition, SLeuRS$\alpha\beta$ was found to be more efficient in the aminoacylation of *E. coli* $tRNA^{Leu}$ at 37 °C than the native $\alpha\beta$-LeuRS, but the effect was canceled at 60 °C.

Surprisingly, the thermal stability of the engineered monomeric enzyme increased, but its optimal temperature range narrowed in comparison to the original $\alpha\beta$-LeuRS. Maybe the native $\alpha\beta$-LeuRS needs the interaction between α and β subunits; however, the monomeric SLeuRS$\alpha\beta$ does not need it, so SLeuRS$\alpha\beta$ has more thermal stability. This may reflect an adaptation of this enzyme to the wide range of temperatures encountered by *A. aeolicus* in its environment.

Analysis of domain architecture and phylogenetic trees reveals a complex history of horizontal gene transfer events in aaRS (*22*). The phylogenetic analysis appears to be compatible with the standard model that postulates the radiation of bacteria and archaea eukaryotes, followed by divergence of the latter two divisions. However, for at least 15 of the aaRS specificities, this scenario needs to be amended by including horizontal gene transfer events (*22*). *A. aeolicus* LeuRS is a split enzyme encoded by separated genes. Until now, it is the only described example of this gene scission, but in the future it would not be surprising to find similar split LeuRS in other organisms. In that case, reconstitution of active enzyme would be facilitated by horizontal exchange of DNA pieces encoding the separate subunits, following the example of exon exchange in eukaryotes. This way, one can expect an accelerated evolution of the enzyme properties. Thus, studies on mixed assembled enzymes may give further insights in the evolution process of enzymes and organisms. Moreover, because SLeuRS$\alpha\beta$ has the same kinetic properties as native *A. aeolicus* $\alpha\beta$-LeuRS, it can be used for the further functional investigations to understand the thermostability mechanism.

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