Split Leucine-Specific Domain of Leucyl-tRNA Synthetase from the Hyperthermophilic Bacterium *Aquifex aeolicus*†

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ABSTRACT: Leucyl-tRNA synthetase (LeuRS) from *Aquifex aeolicus* is the only known heterodimer synthetase. It is named LeuRSα*β*, and its α and *β* subunits contain 634 and 289 residues, respectively. Like *Thermus thermophilus* LeuRS, LeuRSα*β* has a large extra domain, the leucine-specific domain, inserted into the catalytic domain. The subunit split site is exactly in the middle of the leucine-specific domain and may have a unique function. Here, a series of mutants of LeuRSα*β* consisting of either mutated α subunits and wild-type *β* subunits or wild-type α subunits and mutated *β* subunits were constructed and purified. ATP−PP_i exchange and aminoacylation activities and the ability of the mutants to charge minihelix^{Leu} were assayed. Interaction of the mutants with the tRNA was assessed by gel shift. Two peptides of eight and nine amino acid residues in the domain located in the α subunit were found to be essential for the enzyme's activity. We also showed that the domain in LeuRSα*β* plays an important role in minihelix^{Leu} recognition. Additionally, the domain was found to have little impact on the assembly of the heterodimer, to play a role in the thermal stability of the whole enzyme, and to interact with the cognate tRNA in the predicted manner.

Aminoacyl-tRNA synthetases (aaRSs)[1] comprise an ancient, diverse enzyme family that catalyzes specific attachment of amino acids to their cognate tRNAs and ensures accurate translation of the genetic code in the first step of protein synthesis (*1*). There are 20 aaRSs, and they can be classified into two families of 10 members each on the basis of conserved sequences and characteristic structural motifs (*2*). Leucyl-tRNA synthetase (LeuRS) and arginyl-tRNA synthetase (ArgRS), cysteinyl-tRNA synthetase (CysRS), isoleucyl-tRNA synthetase (IleRS), methionyl-tRNA synthetase (MetRS), and valyl-tRNA synthetase (ValRS) comprise the class Ia aaRSs (*2*).

The crystal structure of LeuRS from the thermophilic bacterium *Thermus thermophilus* (denoted *Tt*-LeuRS) was determined by Cusack in 2000 (*3*). The structure is similar to those of other large monomers, IleRS and ValRS, except the editing domain of LeuRS contains an extra leucine-specific domain. This domain is compact and well-ordered and is inserted into the catalytic domain just before the catalytically important KMSKS motif. In *Tt*-LeuRS, the leucine-specific domain consists of five *β* strands and two short α helices and is connected to the catalytic domain via a *β* ribbon, suggesting that it may be able to rotate flexibly (*3*). The post-transfer editing conformation of the crystal structure of *Tt*-LeuRS depicted the rotated leucine-specific domain (*4*), revealing its function as a relatively independent module outside the catalytic center. Sequence alignments show that this domain is unique to prokaryote-like LeuRS but is not highly conserved in sequence or size (*3*).

Most canonical LeuRSs, including *Tt*-LeuRS, consist of single subunits. LeuRS from the hyperthermophilic bacterium *Aquifex aeolicus*, however, is a heterodimeric synthetase consisting of two subunits with 634 and 289 residues (*5*−*8*). This synthetase, called LeuRSα*β*, is divided at the midpoint of the leucine-specific domain, splitting it into two peptides located at the C-terminal end of the α subunit (∼30 residues long) and at the N-terminal end of the *β* subunit (∼40 residues long). LeuRSα*β* and *Tt*-LeuRS have some of the largest leucine-specific domains (*3*). Ignoring the split form and ligating the sequences of the two subunits together, we find LeuRSα*β* is 49.6% identical to *Tt*-LeuRS and 45.8% identical to *Escherichia coli* LeuRS (*Ec*-LeuRS). This reinforces the idea that the single- and multiple-subunit synthetases share highly homologous sequences and emphasizes that the only significant difference is the occurrence of the split leucine-specific domain.

This unique module may have unique functions. The C-terminal end of the α subunit (36 residues long) is strictly required for aminoacylation (*9*). Our previous work indicates that the exact conformation of the leucine-specific domain is important for charging the minihelix, the part containing the acceptor stem and T*ψ*C loop of the cognate tRNA (*10*). A model of tRNA^{Leu} docked to *Tt*-LeuRS based on the

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[1] Abbreviations: aaRS, aminoacyl-tRNA synthetase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis. Aminoacyl-tRNA synthetases are abbreviated by the three-letter code of the appropriate amino acid followed by RS.

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IleRS−tRNA^{Ile}−editing complex structure suggests that the leucine-specific domain could interact with tRNA^{Leu} in the region of the base of the acceptor stem (*11*). Here, a series of genes encoding the deletion mutants of the α subunit in the leucine-specific domain at its C-terminus and a series of genes encoding the deletion mutants of the β subunit in the same domain at its N-terminus were constructed. The deletion mutants within the leucine-specific domain were purified and studied. Their activities were assayed, as well as their abilities to charge the minihelix. A gel shift assay was conducted to determine the function of the leucine-specific domain in tRNA acceptor stem interaction. The function of the leucine-specific domain is summarized here.

MATERIALS AND METHODS

Materials, Plasmids, and Site-Directed Mutagenesis. L-Leucine, DTT, ATP, CAPS, NTP, 5′-GMP, tetrasodium pyrophosphate, and inorganic pyrophosphatase were purchased from Sigma (St. Louis, MO). DE81 and GF/C filters were from Whatman Co. [^{14}C]-L-Leucine (300−400 mCi/mmol), [^{3}H]-L-leucine (1 mCi/mL), [γ-^{32}P]ATP (10 mCi/mL, 3000 Ci/mmol), and tetrasodium [^{32}P]pyrophosphate are products of Amersham Biosciences. SYBR GOLD nucleic acid gel stain was purchased from Molecular Probes (Carlsbad, CA). T4 polynucleotide kinase, T4 DNA ligase, and restriction endonucleases were obtained from Sangon Co. Ni-NTA Superflow was purchased from Qiagen Inc. T7 RNA polymerase was purified from an *E. coli* overproducing strain in our laboratory (*12*). *E. coli* and *A. aeolicus* total tRNA containing >50% tRNA$_{GAG}^{Leu}$ was isolated from overproducing strains constructed in our laboratory (*8*, *13*). All oligonucleotides for amplifying DNA fragments were synthesized by Invitrogen Co. (Shanghai, China).

Gene Cloning, Expression, and Purification of His$_6$-LeuRS and the Mutants. The α subunit tagged with His at the N-terminus was constructed by the method of Ling et al. (*14*). The genes encoding α and β subunits were inserted into pSBET-b and pET-15b plasmids to produce pSBETH$_6$-lrsa and pET-15b-lrsb, respectively. Recombinant plasmids were confirmed by DNA sequencing (Invitrogen). *E. coli* BL21-CodonPlus (DE3) cells were cotransformed with both plasmids. His-tagged enzyme was produced and purified via Ni−NTA column chromatography (*14*). Deletion mutagenesis at the C-terminus of the α subunit or the N-terminus of the β subunit was performed with designed primers by PCR. We used an approach based on the various assemblies from the α subunit with C-terminal deletions and the β subunit with N-terminal deletions to obtain different mutants of LeuRSαβ with various deletions within the leucine-specific domain.

Kinetic Assay. ATP−PP$_i$ exchange and aminoacylation activities of LeuRS were measured at either 37 or 65 °C as described previously (*8*, *15*). The total tRNA isolated from an overproduced *E. coli* strain which overexpresses tRNA^{Leu} with 724 pmol/A_{260} accepting activity was used for the activity assay (*8*). As the accepting activity of pure tRNA^{Leu} was considered to be 1600 pmol/A_{260}, tRNA^{Leu} should be ∼45% of the total tRNA (*8*). Enzyme kinetic constants were determined using various concentrations of the relevant substrates (*15*). The protein concentration of all mutants was determined by a modified method of Lowry et al. (*16*, *17*).

Determination of the Thermal Stability. Measurement of the thermal stability of LeuRSαβ and its mutants was performed as described previously (*18*). The enzyme (40 μg/mL) in 50 mM potassium buffer (pH 6.8) containing 400 μg/mL bovine serum albumin (BSA) was incubated at various temperatures for 10 min. The aminoacylation activity was assayed after the reaction mixture was diluted with cold 50 mM potassium phosphate buffer at pH 7.8.

RNA Substrate Preparation. Plasmids containing *A. aeolicus* tRNA$_{GAG}^{Leu}$ genes were prepared using procedures described previously (*8*). T7 transcripts were generated in a reaction mixture containing 40 mM Tris-HCl (pH 8.0), 5 mM dithiothreitol, 10 mM $MgCl_2$, 2 mM nucleotide triphosphates, 15 mM 5′-GMP, 0.5 unit/mL inorganic pyrophosphatase, and 2 mg/mL pure T7 polymerase. Transcripts were purified by 12% (w/v) denaturing PAGE, annealed by heating for 2 min in 5 mM $MgCl_2$ at 80 °C, and allowed to cool slowly to 30 °C. The accepting activity of in vitro-transcribed tRNA$_{GAG}^{Leu}$ was tested before it was used in the gel shift assay. Minihelices derived from *A. aeolicus* tRNA^{Leu} were synthesized by in vitro transcription of single-stranded synthetic templates. The transcription system was similar to that described above, except that 4 mM nucleotide triphosphates and 2 mM spermidine were added. RNA helices were purified by 15% (w/v) denaturing PAGE, annealed by being heated for 3 min in 2 mM $MgCl_2$ at 85 °C, and allowed to cool slowly to 30 °C (*10*).

Preparation and Aminoacylation Assays of Minihelices. Because our previous study showed that deletion of the 5′-terminal G-1 nucleotide and aminoacylation of the minihelix^{Leu} mutant (*Aa*-Δ1-C72 minihelix^{Leu}) by LeuRSαβ were significantly enhanced (*10*), in the work presented here the best substrate among our tested minihelices, *Aa*-Δ1-C72 minihelix^{Leu}, was used for the assay. *Aa*-Δ1-C72 minihelix^{Leu} was prepared by T7 transcription using the method of Xu et al. (*10*). Under the optimized conditions, the aminoacylation of minihelix^{Leu} was assessed. Aminoacylation assays for minihelix^{Leu} were performed at 25 °C as described previously, considering the lower thermal stability of minihelix^{Leu} (*10*, *19*).

Fluorescence Titration. The association of LeuRS and leucine was monitored by the change in the intrinsic fluorescence intensity of the enzyme. Fluorescence titration was conducted using the method of Lin et al. (*20*). Aliquots of leucine solution were added to an enzyme solution of 1 mL in the microcell of a Hitachi MPF-4 fluorometer. The enzyme solution was diluted to 0.05 mg/mL in standard buffer, which mimicked the buffer system of the aminoacylation and contained protecting reagents. Excitation and emission wavelengths were 295 and 335 nm with slit- idths of 1.5 and 20 nm, respectively. An estimate of the K_d by fluorescence intensity was calculated according to the method of Lin (*20*).

Binding Assay of LeuRSαβ with tRNA. Protein−tRNA interactions were examined by a gel shift assay. Wild-type LeuRS or the selected mutants were mixed with the in vitro-transcribed *A. aeolicus* tRNA^{Leu} (accepting activity of 847 pmol/A_{260}) in a 20 μL volume containing 100 mM Tris-HCl buffer (pH 6.8), 30 mM KCl, 12 mM $MgCl_2$, and 0.1 mM EDTA at room temperature for 1 h. A 20% sucrose solution (3 μL) containing tracer dyes was added immediately before loading. The final mixture was then loaded on a 0.75 mm

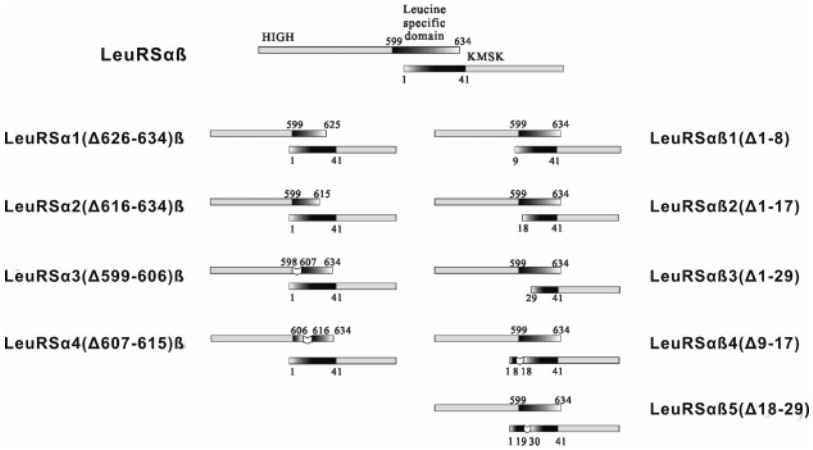

FIGURE 1: Construction of the different mutated LeuRSαβs. A schematic diagram of the various LeuRSαβ mutants is shown. On the top is wild-type LeuRSαβ. On the left side are mutants constructed with a mutated α subunit and a wild-type β subunit; on the right side are mutants constructed with a mutated β subunit and a wild-type α subunit. The mutated sites are marked.

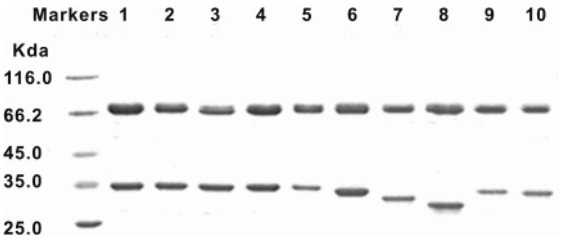

FIGURE 2: SDS−PAGE analysis of LeuRSαβ and its mutants: markers lane, protein standards with molecular masses of 116.0, 66.2, 55.0, 45.0, 35.0, and 25.0 kDa (from top to bottom); lane 1, native LeuRSαβ; lane 2, LeuRSα1β; lane 3, LeuRSα2β; lane 4, LeuRSα3β; lane 5, LeuRSα4β; lane 6, LeuRSαβ1; lane 7, LeuRSαβ2; lane 8, LeuRSαβ3; lane 9, LeuRSαβ4; and lane 10, LeuRSαβ5. Each lane contained 5 μg of protein. Theoretically, the approximate molecular masses of the α subunit and its α1, α2, α3, and α4 mutants are around 74 kDa; the approximate molecular masses of the β subunit and its β1, β2, β3, β4, and β5 mutants are around 34 kDa. The truncated mutants exhibited appreciable changes in electrophoresis compared with the wild-type enzyme.

thick 8% native polyacrylamide gel in Tris-glycine buffer (50 mM Tris base and 50 mM glycine) and run for 1 h at 100 V and 4 °C in the same buffer (*8*, *21*). After electrophoresis, the gel was stained with SYBR GOLD nucleic acid gel stain for ~30 min while protected from light. Then the gel was illuminated and photographed with a Storm 860 scanner. The selected excitation and emission wavelengths were 450 and 540 nm, respectively (*22*).

RESULTS

Construction of Recombinant Plasmids. A series of genes encoding LeuRSαβ mutants with residues deleted from the C-terminus of the α subunit and the N-terminus of the β subunit were constructed, and the recombinant plasmids were obtained. The end points of various deletion mutants are shown in Figure 1.

Expression and Purification of LeuRSαβ Mutants. Nine assembled heterodimeric mutants of LeuRSαβ were obtained and purified to ~90% homogeneity by affinity chromatography on Ni−NTA Superflow (Figure 2).

Activity of LeuRSαβ Mutants. Both ATP−PP_i exchange and aminoacylation relative activities of the nine expressed mutants at 37 and 65 °C are listed in Table 1. The kinetic parameters of seven mutants with aminoacylation activity are listed in Table 2. Two truncated mutants, LeuRSα1β and LeuRSα2β, lacking the 9 and 19 amino acid residues at the C-terminal end of the α subunit, respectively, exhibited 98% of the native LeuRSαβ activity in the ATP−PP_i exchange reaction and 93% in the aminoacylation reaction at 37 °C, nearly the same as that of native LeuRSαβ. However, two mutants, LeuRSα3β and LeuRSα4β, in which the α subunits lack residues 599−606 and 607−615, respectively, exhibited little ATP−PP_i exchange and almost no aminoacylation activity. The results indicate that at the C-terminus, up to the last 19 residues of the α subunit are not essential for enzyme activity; however, residues 599−606 and 607−615 of the α subunit are required for ATP−PP_i exchange and, as a consequence, for aminoacylation activity. The deletion mutants at the N-terminus of the β subunit severely affected aminoacylation activities, indicating the sequence just before the consensus KMSKS sequence is essential for aminoacylation activities.

In the region of residues 599−606 of the α subunit, among the eight residues there are four lysine residues that are always considered to be a binding site of phosphate of tRNA. To discern the contribution of residues 599−615 of the α subunit to aminoacylation activity, we constructed seven mutants with single-, double-, and quadruple-point mutations. They are K599A, K600A, K605A, K606A, K599A/K600A, K605A/K606A, and K599A/K600A/K605A/K606A. None of the mutations severely affected activity (data not shown).

Thermal Properties of Mutants with Truncated α *Subunit C-Termini.* Compared with the wild-type enzyme, LeuRSα2β, lacking the 19 amino acid residues at the C-terminus of the α subunit, exhibited an appreciable decrease in thermal stability, especially at temperatures from 50 to 80 °C, indicating that the C-terminal end of the α subunit may stabilize its conformation at elevated temperatures (Figure 3).

Interaction of LeuRSαβ Mutants with Leucine. Leucine binding of the mutants LeuRSα3β and LeuRSα4β, without aminoacylation activity, was studied by fluorescence titration (Figure 4). The K_ds of LeuRSαβ, LeuRSαβ3, and LeuRSαβ4 were 2.70, 1.99, and 5.18 μM, respectively. The three K_ds calculated by the slope of the line were nearly at the same level, indicating that leucine−enzyme binding was not greatly

Table 1: Activities of Various LeuRS Mutants at 37 and 65 °C[a]

	activity			
	amino acid activation relative activity (%)		aminoacylation relative activity (%)	
	37 °C	65 °C	37 °C	65 °C
LeuRS	100	100	100	100
LeuRSα1(Δ626−634)β	99.3 ± 0.5	99.5 ± 2.1	95.1 ± 2.9	93.5 ± 1.2
LeuRSα2(Δ616−634)β	86.5 ± 2.7	62.7 ± 6.0	94.6 ± 2.5	74.2 ± 5.2
LeuRSα3(Δ599−606)β	0.9 ± 0.3	0.08 ± 0.03	not detected	not detected
LeuRSα4(Δ607−615)β	0.9 ± 0.2	0.09 ± 0.02	not detected	not detected
LeuRSαβ1(Δ1−8)	45.9 ± 5.4	48.3 ± 1.0	45.4 ± 6.6	48.7 ± 8.2
LeuRSαβ2(Δ1−17)	3.0 ± 1.7	1.5 ± 0.1	4.6 ± 0.1	7.8 ± 0.4
LeuRSαβ3(Δ1−29)	1.4 ± 1.0	2.5 ± 0.8	4.5 ± 0.6	3.7 ± 0.3
LeuRSαβ4(Δ9−17)	1.4 ± 1.0	1.1 ± 0.5	1.6 ± 0.3	6.6 ± 2.8
LeuRSαβ5(Δ18−29)	1.4 ± 1.2	0.3 ± 0.2	3.1 ± 0.2	3.6 ± 0.8

[a] The experiments were performed as described in Materials and Methods. Standard errors were determined from three independent data sets.

Table 2: Kinetic Constants of Various LeuRS Mutants in the Aminoacylation Reaction at 65 °C[a]

enzyme	constant	leucine	ATP	*Aa*-tRNA$^{Leu}_{(GAG)}$
LRSαβ	K_m (μM)	6.0 ± 0.5	112.3 ± 3.0	0.30 ± 0.02
	k_{cat} (s^{-1})	1.4 ± 0.2	1.81 ± 0.02	1.50 ± 0.03
	k_{cat}/K_m (s^{-1} mM^{-1})	233	16.1	5000
LRSα1β	K_m (μM)	9.7 ± 1.4	118.9 ± 3.4	0.20 ± 0.01
	k_{cat} (s^{-1})	1.6 ± 0.2	1.71 ± 0.01	1.20 ± 0.01
	k_{cat}/K_m (s^{-1} mM^{-1})	165	14.4	6000
LRSα2β	K_m (μM)	17.1 ± 2.1	210.0 ± 8.4	0.38 ± 0.01
	k_{cat} (s^{-1})	0.9 ± 0.1	1.31 ± 0.03	1.02 ± 0.01
	k_{cat}/K_m (s^{-1} mM^{-1})	52.6	6.23	2684
LRSαβ1	K_m (μM)	12.6 ± 2.1	141.0 ± 3.0	0.18 ± 0.01
	k_{cat} (s^{-1})	0.60 ± 0.03	0.87 ± 0.02	0.51 ± 0.01
	k_{cat}/K_m (s^{-1} mM^{-1})	47.6	6.17	2833
LRSαβ2	K_m (μM)	21.4 ± 0.1	1901 ± 258	0.068 ± 0.001
	k_{cat} (s^{-1})	0.043 ± 0.001	0.11 ± 0.03	0.057 ± 0.015
	k_{cat}/K_m (s^{-1} mM^{-1})	2.0	0.058	838
LRSαβ3	K_m (μM)	24.2 ± 4.0	2759 ± 65	0.059 ± 0.003
	k_{cat} (s^{-1})	0.073 ± 0.007	0.07 ± 0.01	0.029 ± 0.001
	k_{cat}/K_m (s^{-1} mM^{-1})	3.0	0.025	492
LRSαβ4	K_m (μM)	22.4 ± 1.6	2795 ± 400	0.045 ± 0.001
	k_{cat} (s^{-1})	0.074 ± 0.001	0.13 ± 0.02	0.084 ± 0.001
	k_{cat}/K_m (s^{-1} mM^{-1})	3.3	0.047	1867
LRSαβ5	K_m (μM)	25.3 ± 0.1	3603 ± 142	0.035 ± 0.002
	k_{cat} (s^{-1})	0.042 ± 0.004	0.07 ± 0.01	0.027 ± 0.007
	k_{cat}/K_m (s^{-1} mM^{-1})	1.7	0.019	771

[a] The experiments were performed as described in Materials and Methods. Standard errors were determined from three independent data sets.

affected. Therefore, the loss of aminoacylation activity was not due to the binding of leucine to the enzyme.

Mutations in the Leucine-Specific Domain Impair the tRNA Binding Capacity of LeuRS. The amino acid activation and aminoacylation activities of mutants LeuRSα3β and LeuRSα4β are greatly reduced. Can they bind tRNA^{Leu}? Binding of LeuRSα3β and LeuRSα4β with tRNA$_{GAG}^{Leu}$ mutants was examined by a band shift assay to determine whether the loss of activity is from the decline in the level of binding of tRNA to the mutants. Indeed, the ability to bind tRNA$_{GAG}^{Leu}$ was impaired to some degree (Figure 5). Under the experimental conditions, the K_d of wild-type LeuRSαβ for its tRNA is ~0.87 μM, while the K_ds of mutants LeuRSα3β and LeuRSα4β are 5.54 and 1.86 μM, respectively, according to Scatchard plots. Mutant LeuRSα3β exhibited a nearly 6.4-fold decrease in tRNA$_{GAG}^{Leu}$ affinity, while LeuRSα4β exhibited a 2-fold decrease in affinity.

The Leucine-Specific Domain Plays Some Role in Minihelix^{Leu} Recognition. Our previously published data showed that the possible minor conformation change by the linkage of split leucine-specific domain seems to be responsible for the relatively poor recognition of minihelix^{Leu} (*10*). Therefore, the mutants were tested for the ability to charge *Aa*-Δ1-C72 minihelix^{Leu} (the best substrate among the minihelix^{Leu} species) (*10*). The abilities of LeuRSα1β and LeuRSα2β to charge minihelix^{Leu} were slightly weakened, in the same way as their abilities to charge native tRNA^{Leu} were weakened (Figure 6A). LeuRSα3β and LeuRSα4β, which were strongly affected in amino acid activation and even more strongly affected in aminoacylation (Table 1), lost their ability to charge minihelix^{Leu} as well. Aminoacylation of minihelix^{Leu} by the mutants with single-, double-, and quadruple-point mutations of the α subunit was also weakened to some extent (data not shown). Thus, these segments (residues 599−606 and 607−615 of the α subunit) play an important role in minihelix^{Leu} recognition. Mutants with truncated β subunits have weakened aminoacylation activities of minihelix^{Leu}, as expected (Figure 6B). Fifty percent of the native activity was detected for LeuRSαβ1 when native tRNA$_{GAG}^{Leu}$ was used (Table 1), while approximately 75% of the native activity was detected when minihelix^{Leu} was used. We checked and repeated the experiments. Indeed, LeuRSαβ3 can leucylate the minihelix with 50% of the activity of the native enzyme (Figure 6B); however, it leucylated tRNA^{Leu} with only 5%

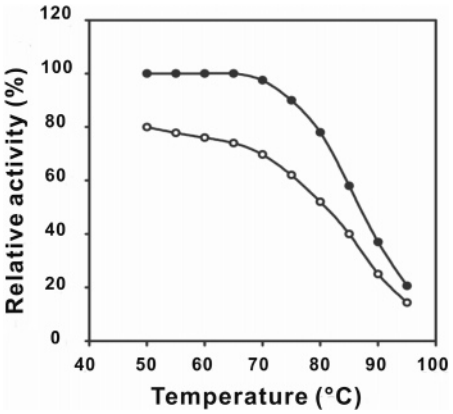

FIGURE 3: Determination of the thermal stability of LeuRSα2β. LeuRSα2β (40 μg/mL) in 50 mM potassium phosphate buffer (pH 6.8) containing 400 μg/mL BSA was incubated at temperatures ranging from 50 to 95 °C for 10 min. Then, the aminoacylation activity of LeuRSα2β (○) was assayed at 37 °C after dilution of the reaction mixture with cold 50 mM potassium phosphate buffer (pH 7.5) and compared with that of *A. aeolicus* LeuRSαβ (●).

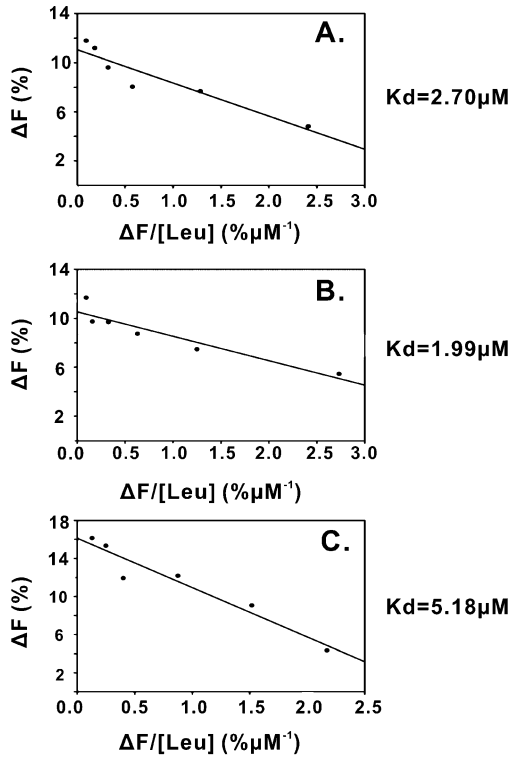

FIGURE 4: Titration of the LeuRSαβ fluorescence by leucine. Conditions are given in Materials and Methods. The slope of the Eadie plot indicates K_d values. Panels A−C show titration curves of LeuRSαβ, LeuRSα3β, and LeuRSα4β fluorescence quenching by leucine, respectively.

of the activity of the native enzyme (Table 1). LeuRSαβ3 was an exception among the enzymes without the activity of charging $tRNA^{Leu}$.

DISCUSSION

A. aeolicus LeuRSαβ is the only heterodimeric LeuRS. It differs from the canonical monomeric LeuRSs from other sources by its division into two subunits separated at the midpoint of the leucine-specific domain.

A. aeolicus LeuRSαβ is 49.6% identical to *Tt*-LeuRS and 45.8% identical to *Ec*-LeuRS. Previous investigation of the tertiary structure of *Tt*-LeuRS illustrated that the leucine-specific domain is an extra domain outside of the catalytic center; therefore, it is reasonable to assume the leucine-specific domain of *A. aeolicus* LeuRSαβ is located outside the catalytic core as well. Because of its location just in front of the KMSKS sequence, and a split right in the middle, it is assumed that the leucine-specific domain may help to maintain the heterodimeric state and function in tRNA sensing (*9*). It is shown in the determined crystal structure of *T. thermophilus* ValRS complexed with $tRNA^{Val}$ and a Val-AMP analogue at 2.9 Å resolution that the editing domain of the enzyme specifically recognizes the 3′-terminal adenosine of the tRNA (*23*). By docking *Tt*-LeuRS on $tRNA^{Val}$ from ValRS-$tRNA^{Val}$ (PDB entry 1GAX) on *Tt*-LeuRS, we found that the leucine-specific domain interacted with the acceptor stem of $tRNA^{Val}$. We can assume the same interaction occurs between *A. aeolicus* LeuRSαβ and $tRNA_{GAG}^{Leu}$, on the basis of the high degree of homology of the two enzymes.

The α subunit of LeuRSαβ could not be expressed stably in vivo, whereas the β subunit was overproduced and purified by a simple procedure; the LeuRSαβ could be overproduced in *E. coli* cells containing both genes and was purified to 90% homogeneity as a heterodimer (*8*). An even small amount of the α subunit was obtained by affinity purification, and its binding to $tRNA^{Leu}$ was not detected (our lab's unpublished data). The β subunit is catalytically inactive, but it can bind and recognize $tRNA^{Leu}$ (*8*, *21*). All the heterodimeric mutants assembled well and tightly (Figure 2). After rigorous purification steps, including heating at 75 °C for 30 min, had been applied, the heterodimeric states of all the mutants were still stable, indicating the minimal importance of the leucine-specific domain in maintaining the heterodimeric state of LeuRSαβ in spite of the split site within it. The gene products of the widely separated α and β subunits can be assembled into heterodimers in vivo, showing that the interaction between the two subunits is naturally intense (*5*). In addition to the heterodimer form, a LeuRSαβ heterotetramer was found (*24*). Experiments showed that at high salt concentrations (up to 2 M NaCl), both the heterodimer and the heterotetramer were stable and did not dissociate into their respective subunits (*24*). Clearly, it is the entire enzyme instead of the leucine-specific domain that affects subunit assembly. The leucine-specific domain may not determine but "help" maintain the heterodimeric state.

Our previously published data show that the truncated mutants lacking the leucine-specific domain at the C-terminal end of the α subunit lost nearly all of the aminoacylation activity and most of the amino acid activation activity (*9*). In our work, the mutant lacking up to 19 residues at the C-terminus of the α subunit displayed nearly the same amino acid activation and aminoacylation activity as native LeuRSαβ, showing this peptide is not important to the activity of the enzyme. However, two deletion mutants that lacked residues 599−606 or 607−615 of the α subunit showed little ATP−PP_i exchange and almost no aminoacylation activity, indicating that the deletion mutations probably disturb the catalytic core of the enzyme. It is not clear whether their interactions with cognate tRNA are impaired. When lysine residues were changed to alanine in the lysine-rich peptide fragment of residues 599−606 of the α subunit, the aminoacylation

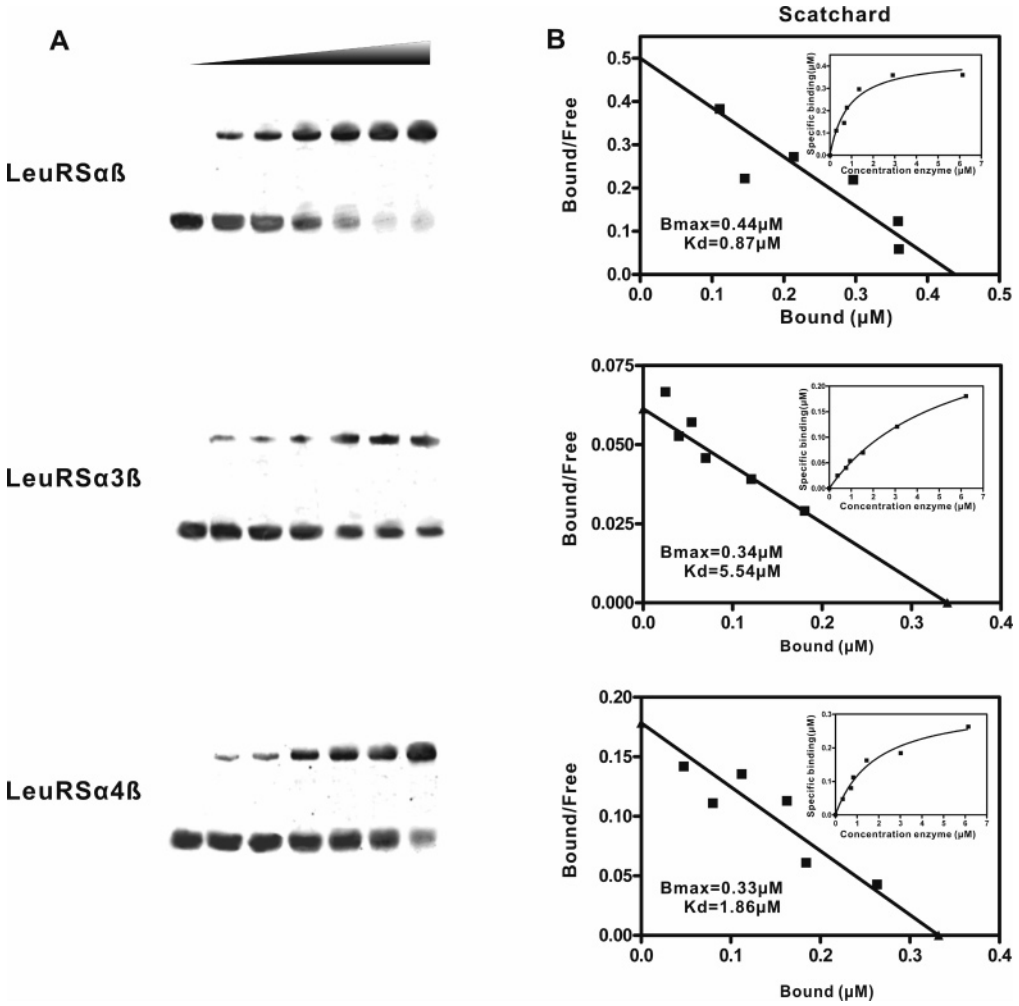

FIGURE 5: Measurement of the dissociation constant of LeuRS proteins for $tRNA^{Leu}$ by a gel shift assay. In vitro-transcribed *A. aeolicus* $tRNA_{GAG}^{Leu}$ was incubated with increasing concentrations of LeuRSαβ, LeuRSα3β, or LeuRSα4β. Apparent K_ds of the proteins were estimated with a Scatchard plot, after quantification of the free and retarded tRNA and calculation of the amount of free and bound enzyme. The concentration of *A. aeolicus* $tRNA_{GAG}^{Leu}$ was 0.4 μM: lane 1, no enzyme; and lanes 2−7, 0.4, 0.8, 1.0, 1.6, 3.2, and 6.4 μM enzyme, respectively. (A) Binding of $tRNA^{Leu}_{GAG}$ on LeuRSαβ, LeuRSα3β, and LeuRSα4β. (B) Scatchard analysis of the binding of $tRNA^{Leu}_{GAG}$ on LeuRSαβ, LeuRSα3β, and LeuRSα4β.

activity was not effectively altered (data not shown), suggesting the lysine residues are not essential for aminoacylation activity. Thus, it is quite probable that the activity of the enzyme depends mainly on the whole peptide rather than the residues alone. Comparison of the circular dichroism (CD) spectroscopy data showed that all of the mutants and native LeuRS exhibit nearly the same content of helix and sheet (data not shown), suggesting various mutations did not alter the secondary structure of the enzyme.

The deletion mutants at the N-terminal end of the β subunit severely impaired activity. The activity of the mutant with an only eight-residue deletion dropped by 50%. This may be caused by the deletion being close to the catalytically active KMSKS sequence.

The remaining activity of some mutants took different percentages from that of the native enzyme when assayed at different temperatures. The mutants with mutated α subunits exhibited a stronger tendency to be negatively affected by higher temperature, while mutants with mutated β subunits exhibited no such tendency. Therefore, experiments with the aim of examining the thermal stability of the mutant with truncated α subunits at the C-terminus were conducted. LeuRSα2β proved to be less thermally stable than the wild-type enzyme. As a thermostable enzyme, LeuRSαβ may have a strong structure that confers high stability on it at higher temperatures. Thus, it is reasonable to deduce that the deletion of the C-terminus of the α subunit slightly disturbs the stability of the enzyme structure.

The affinity of the enzymes for the substrate was probably affected for LeuRSα3β and LeuRSα4β. They had no detectable activity. Fluorescence titration showed that binding of the two mutants with leucine was not obviously affected. However, binding of $tRNA_{GAG}^{Leu}$ to LeuRSα3β and LeuRSα4β was found to be weakened by a gel shift assay. In particular, the binding ability of LeuRSα3β with $tRNA_{GAG}^{Leu}$ was evidently affected. Its K_d is similar to the K_d of the single β subunit for $tRNA_{GAG}^{Leu}$ (K_d = 5.6 μM), indicating that this peptide is crucial in tRNA binding. This peptide probably interacts with the acceptor arm of $tRNA^{Leu}$, as previously predicted. Binding of $tRNA_{GAG}^{Leu}$ to LeuRSα4β was also impaired, although to a lesser degree than binding to LeuRSα3β. It appears the peptide containing residues 599−606 of the α subunit plays an important role in protein−tRNA binding and possibly interacts with the acceptor stem of cognate tRNA. When residues 607−615 are deleted, the direct binding sites may be lost, or the disturbance of the

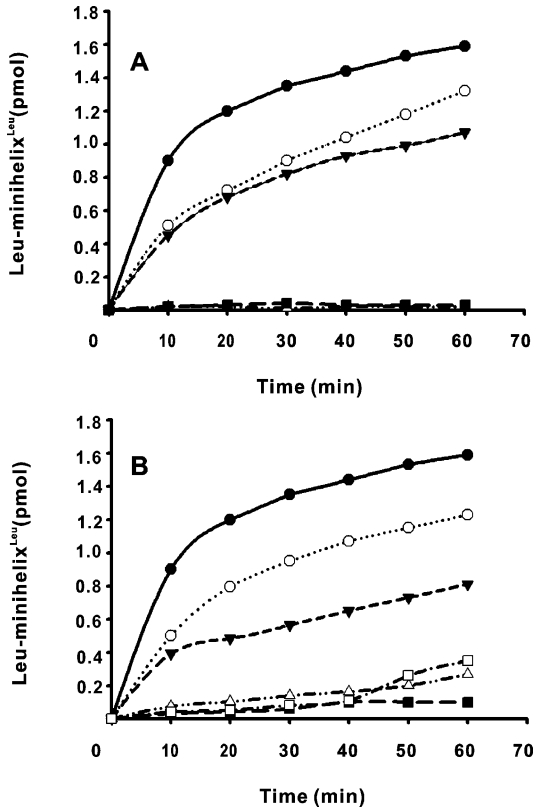

FIGURE 6: Time course of aminoacylation of minihelix^{Leu} by LeuRS$\alpha\beta$ and its mutants. Aminoacylation of *Aa*-Δ1-C72 minihelix^{Leu} by (A) LeuRS$\alpha\beta$ (●), LeuRSα1β (○), LeuRSα2β (▼), LeuRSα3β (▽), and LeuRSα4β (■) and (B) LeuRS$\alpha\beta$ (●), LeuRS$\alpha\beta$1 (○), LeuRS$\alpha\beta$2 (△) LeuRS$\alpha\beta$3 (▼), LeuRS$\alpha\beta$4 (■), and LeuRS$\alpha\beta$5 (□). The reactions were performed at 25 °C with a minihelix concentration of 15 μM and 2 μM enzymes.

structure may prevent suitable binding of residues in the enzyme with tRNA. These results verified our previous assumption (*9*) that the leucine-specific domain of *A. aeolicus* LeuRS$\alpha\beta$ interacts with the acceptor stem of tRNA$_{GAG}^{Leu}$.

We previously found that the possible minor conformation change by the linkage of the split leucine-specific domain of LeuRS$\alpha\beta$ is responsible for the relatively poor recognition of minihelix^{Leu} (*10*). Among the mutants with the truncated mutants of the α subunit and the native β subunit, LeuRSα1β and LeuRSα2β exhibited slightly impaired abilities to charge the minihelix, and LeuRSα3β and LeuRSα4β completely lost this ability. Aminoacylation of the minihelix by the mutants with single, double, and quadruple mutations within the peptide fragment of residues 599−606 of the α subunit were also weakened to some extent (data not shown). Interestingly, their ability to charge the minihelix was impaired to approximately the same degree as their ability to charge cognate tRNA^{Leu}, compared with that of the native enzyme. Mutants with truncated β subunits and the native α subunit had weakened activities in aminoacylating minihelix^{Leu}, as expected. However, the ability of some mutants to aminoacylate tRNA$_{GAG}^{Leu}$ was weakened substantially, while their ability to aminoacylate minihelix^{Leu} was less affected. This indicated that the partial leucine-specific domain on the β subunit probably does not interact with the acceptor stem of tRNA^{Leu}. LeuRS$\alpha\beta$3 had the exceptionally strong ability of the wild-type enzyme to charge minihelix^{Leu} compared to its poor ability to charge tRNA^{Leu}. Minihelix interacts with the catalytic core of the enzyme instead of the whole tRNA. Some deletions of amino acid residues might alter the access of the minihelix to the catalytic core, so the fluctuating activity of LeuRS$\alpha\beta$2 and LeuRS$\alpha\beta$3 is possible. The question may be answered some day by the tertiary structure of the complex consisting of LeuRS$\alpha\beta$3 and the minihelix. Attempts to charge the minihelix or microhelix tRNA^{Leu} with *E. coli* or *Homo sapiens* LeuRS have not been successful to date, whereas the deep-rooted bacterium *A. aeolicus* LeuRS$\alpha\beta$ consisting of two subunits is known to charge minihelix^{Leu} (*10*). Additionally, its charging ability is mainly affected by the domain containing the split site that divides the enzyme into two subunits (*10*). Knowledge of the structural organization confirms that present-day aaRS have been formed by the progressive assembly of isolated domains to the original primitive class-specific catalytic core (*10*, *25*). Here, we confirm that the two-subunit state is evidence that *A. aeolicus* LeuRS$\alpha\beta$ is ancient.

The ancient *A. aeolicus* LeuRS$\alpha\beta$ has a unique leucine-specific domain. This important domain may have the function of sensing tRNA and interacting with the acceptor stem of tRNA. Primary sequences and crystal structures of LeuRS evolve from prokaryotic to eukaryotic organisms, with many module shifts. Little conservation is seen for this domain; therefore, how it evolved and was eventually lost in higher-class organisms remains to be determined.

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