Leucyl-tRNA synthetase from the extreme thermophile *Aquifex aeolicus* has a heterodimeric quaternary structure

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Abstract Class I aminoacyl-tRNA synthetases have been thought to be single polypeptide enzymes. However, the complete genome sequence of a hyper thermophile *Aquifex aeolicus* suggests that the gene for leucyl-tRNA synthetases (LeuRS) is probably split into two pieces (*leuS* and *leuS′*). In this research, each gene was separately cloned and overexpressed in *Escherichia coli* and the protein products were examined for LeuRS activity. Leucylation activity was detected only when both gene products coexisted. Gel filtration analysis showed that the active form of *A. aeolicus* LeuRS has a heterodimeric (α/β type) quaternary structure that is unique among class I aminoacyl-tRNA synthetases. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Aminoacylation; Leucyl-tRNA synthetase; Heterodimer; Quaternary structure; *Aquifex aeolicus*

1. Introduction

The class I aminoacyl-tRNA synthetases (aaRSs) have been characterized as single polypeptide enzymes with either an α (monomer) or an α_2 (homodimer) type quaternary structure [1–3]. However, the completed genome sequence of a hyper thermophile *Aquifex aeolicus* suggested an exception in the architecture of class I aaRSs [4]. A homology search revealed that the putative gene homologs for leucyl- and methionyl-tRNA synthetases (LeuRS and MetRS, both members of class I aaRSs) were both fragmented into two widely separated parts on the *A. aeolicus* chromosome. The domain structures for the canonical and *A. aeolicus* LeuRS are schematically shown in Fig. 1. One part of the putative *A. aeolicus* LeuRS gene, *leuS*, encodes the N-terminal region of the canonical LeuRS and the other, *leuS′*, encodes the C-terminal region. Although most of the Rossmann-fold is located within the *leuS* gene region, the conserved motifs (HIGH and KMSKS) common to class I aaRSs are divided into two parts of the genes (Fig. 1b). Such an architecture is unprecedented for a class I aaRS. Recently, Morales et al. [5] reported that the polypeptide encoded by one of the putative *A. aeolicus* MetRS genes (homologous to the C-terminal portions of canonical MetRS) was actually not a 'subunit' of this enzyme, but a structure-specific tRNA-binding protein. This means that *A. aeolicus* MetRS is after all a single polypeptide enzyme similar to other known MetRSs. It is therefore very tempting to elucidate the active quaternary structure of *A. aeolicus* LeuRS. For this purpose, we cloned the *A. aeolicus leuS* and *leuS′* genes separately and characterized their gene products.

2. Materials and methods

2.1. Materials

The coding sequences for *leuS* and *leuS′* genes were PCR-amplified from *A. aeolicus* genomic DNA, from Dr. R. Huber (Department of Microbiology, University of Regensburg, Germany). Oligodeoxynucleotides for PCR were purchased from Hokkaido System Science Co., Japan. The primers used for the *leuS* gene were 5′-GGGGGATCCCATATGATGAAGGAGTTTAAC-3′ and 5′-GGGGAATTCCTAAGCTCTTCGTGCCCCGAG-3′ to incorporate *Nde*I and *Eco*RI sites, respectively. Primers used for the *leuS′* gene were 5′-GGGCTGCAGCATATGAAAATAAAAGAC-3′ and 5′-GGGCTCGAGTCATTTAACAACTACGTT-3′ to incorporate *Nde*I and *Xho*I sites, respectively. These PCR fragments were then cloned into plasmid pET21a (Novagen) to create pETleuS and pETleuS′. L-[^{14}C]leucine (9.73 GBq mmol^{-1}) was obtained from Moravek Biochemicals Inc., USA. Sephacryl S-200 HR and Q-Sepharose HP were products of Amersham Pharmacia Biotech. Other chemicals were obtained from Wako Pure Chemicals, Japan. Total *E. coli* tRNA was prepared according to [6] with a slight modification. tRNA^{Leu} transcripts were prepared according to [7]. *E. coli* LeuRS was prepared as described [8].

2.2. Expression and purification of *A. aeolicus leuS* gene product

E. coli BL21(DE3) cells containing the pETleuS plasmid were grown to an OD_{600} of 0.5–0.8 and induced with 0.5 mM isopropyl-β-D-thiogalactoside, while simultaneously lowering the growth temperature from 37 to 30°C. After overnight cultivation, expressed cells were harvested and frozen. Cells were treated with BugBuster (a reagent for protein extraction, Novagen) according to the manufacturer's protocol for an inclusion body. The precipitate including the *leuS* gene product was dissolved in solution A (50 mM Tris base, 10 mM DTT and 8 M urea). Renaturation was achieved under the refolding conditions described in [9]. Finally, this protein solution was dialyzed overnight against buffer B (20 mM Tris–HCl (pH 7.6), 1 mM $MgCl_2$, and 6 mM 2-mercaptoethanol) containing 50% glycerol and stored at −30°C. Purity of this protein preparation was analyzed by SDS–PAGE according to [10]. The protein concentration was determined as described [11].

2.3. Expression and purification of *A. aeolicus leuS′* gene product

E. coli BL21(DE3) cells containing the pETleuS′ plasmid were grown and treated similarly as above. Expressed cells were suspended in buffer B and disrupted by a sonicator (Bioruptor, Tohshoh Denki,

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Abbreviations: aaRS, aminoacyl-tRNA synthetase; LeuRS, leucyl-tRNA synthetase; MetRS, methionyl-tRNA synthetase; tRNA^{Leu}(CAG), leucine tRNA which have anticodon CAG

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Japan). The homogenate was centrifuged at 30 000×*g* for 30 min to prepare S-30 extract, followed by heat treatment at 80°C for 20 min. After removing the precipitate (heat-denatured *E. coli* proteins) by centrifugation at 30 000×*g* for 10 min, the supernatant was applied onto a column of Q-Sepharose HP (1.0×12 cm). The column was developed with a linear gradient of KCl (0–0.4 M, total 150 ml) in buffer B at a flow rate of 2.0 ml min^{-1}. Fractions with a volume of 4 ml were collected and each fraction was analyzed by SDS–PAGE. Fractions containing the protein with a molecular weight corresponding to *leuS′* product were combined, concentrated by Centriprep-10 (Millipore) and finally dialyzed similarly as above.

2.4. Assays for enzyme activity

Leucyl-tRNA forming (leucylation) reactions to determine the kinetic constants of LeuRS α/β complex were carried out at 65°C in 50 μl of reaction mixtures according to [12] with a slight modification. The initial rates of aminoacylation were determined using six different concentrations of leucine (ranging from 0.95 to 30.4 μM), ATP (ranging from 62.5 to 4000 μM) and $tRNA^{Leu}$ (ranging from 0.33 to 5.4 μM, this reaction was done at 55°C) at a fixed concentration of the *A. aeolicus* LeuRS α/β complex, which gave reasonable kinetic plots for determining the apparent kinetic constants. Reaction conditions for the column fraction assay are described in the legend to Fig. 4.

3. Results

3.1. Expression of A. aeolicus leuS and leuS′ genes in E. coli cells

To examine the enzymatic activity of individual gene products, each of the *leuS* and *leuS′* genes from *A. aeolicus* was cloned and overexpressed separately in *E. coli*. The *leuS* gene product was expressed as an insoluble form. Although it could be solubilized as described above, further purification was difficult due to its nature to form aggregates. As shown in Fig. 2 (lane 1), the preparation at this stage still contained many other protein bands or smear-like materials. However, we assumed that the major band with a molecular weight of about 65 kDa (P-65) was the *leuS* gene product, since this band is also found in the active LeuRS complex reconstituted from *leuS* and *leuS′* products as described below. This molecular weight (65 kDa) is somewhat lower than that calculated from the reported gene sequence of *leuS* (73.9 kDa). The discrepancy between the two values might be explained by the existence of an extremely hydrophobic region in this protein.

On the other hand, the *leuS′* gene product was obtained as a soluble protein. This protein was purified to near-homogeneity (Fig. 2, lane 2) by the purification steps described above. The molecular weight of the *leuS′* gene product was estimated at about 35 kDa by SDS–PAGE, which is in good agreement with the theoretical value calculated from the reported gene sequence of *leuS′* (33.5 kDa).

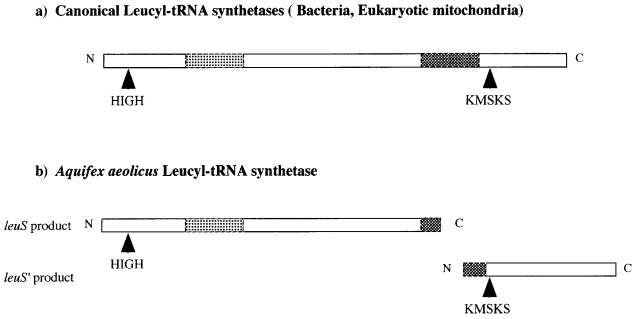

Fig. 1. Schematic diagram of the domain structure of LeuRS based on sequence alignments. The putative editing domain and 'leucyl-specific insertion domain' [18] are represented by light gray and dark gray boxes, respectively. Positions of the two catalytically important class I motifs, HIGH (or HMGH) and KMSKS, are also indicated. a: Domain structure of *T. thermophilius* LeuRS, representative of canonical LeuRSs from bacteria and eukaryotic mitochondria. b: Domain structure of *A. aeolicus* LeuRS predicted from the genome sequence.

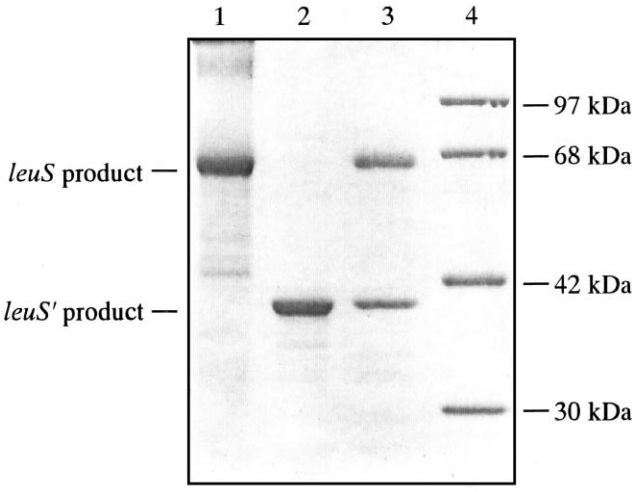

Fig. 2. SDS–PAGE analysis of *A. aeolicus* LeuRS components. Electrophoresis was performed on 10% polyacrylamide gel according to [10] and protein bands (about 0.5 μg each per lane) were visualized by CBB staining. Lane 1: *leuS* gene product; lane 2: *leuS′* gene product; lane 3: peak II fractions from gel filtration chromatography (Fig. 4); lane 4: molecular weight markers (Daiichi Pure Chemicals, Japan).

3.2. Higher-order structure of A. aeolicus LeuRS

Leucine activation and leucyl-tRNA forming activities were examined for each of the gene products, aiming to clarify

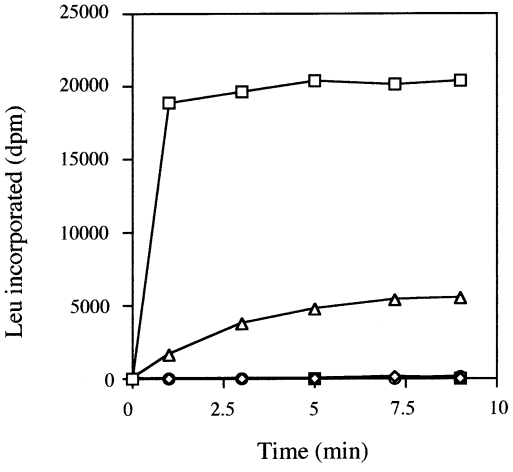

Fig. 3. Leucylation of $tRNA^{Leu}$ by *A. aeolicus* LeuRS subunits or α/β complex. Leucylation reaction was carried out at 65°C in the reaction mixture (50 μl each) containing 100 mM HEPES–KOH (pH 7.9), 10 mM $MgCl_2$, 4 mM ATP, 20 μM L-[^{14}C]leucine, 0.4 μM each enzyme preparations and 64 μM of total *E. coli* tRNA. Aliquots (10 μl each) were withdrawn at the time intervals indicated and the acid-insoluble radioactivities counted. Enzymes used: α/β complex (square), α subunit plus β subunit (simply mixed) (triangle), α subunit alone (diamond), β subunit alone (circle), and without enzyme (closed square). One unit of leucylation activity is defined as the quantity of protein catalyzing the incorporation of 1 nmol of leucine into tRNA per min under the assay conditions. The specific activity is defined as U/mg protein.

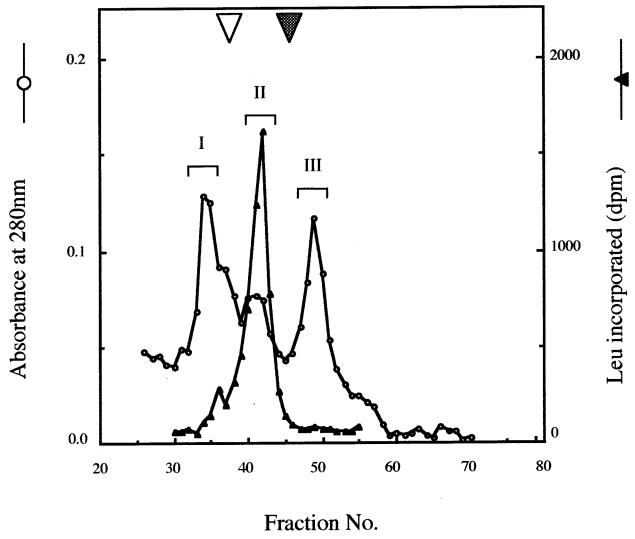

Fig. 4. Regeneration and gel filtration of *A. aeolicus* LeuRS α/β complex. The *leuS* and *leuS′* gene products were mixed in a molar ratio of one to two, expecting that the latter might act as the 'seed' for the refolding of the former. The mixed solution was dialyzed similarly to that described in Section 2.2. Final dialysate (5 ml) was applied onto a column of Sephacryl S-200 HR (2.5×100 cm) pre-equilibrated with buffer B containing 200 mM KCl and calibrated with molecular weight markers (aldolase, 154 kDa: white arrowhead; and BSA, 68 kDa: gray arrow head). Elution was performed with buffer B containing 200 mM KCl at a flow rate of 0.5 ml/min and fractions (5 ml each) were collected. Open circles show the absorbance at 280 nm. Closed triangles show the leucylation activity. The leucylation assay for column fractions was carried out at 37°C for 5 min in reaction mixtures (10 μl) containing 100 mM Tris–HCl (pH 7.6), 4 mM ATP, 50 mM KCl, 10 mM $MgCl_2$, 20 μM L-[^{14}C]leucine, 64 μM of total *E. coli* tRNA and an appropriate amount of the column fractions.

what indeed is the active form of *A. aeolicus* LeuRS. The former activity was assayed by the ATP–PPi exchange reaction as described in [13], but neither of the gene products by itself showed signs of leucyl-adenylate formation (data not shown). Similarly, despite all the efforts to optimize the reaction conditions, such as salt concentration or pH, neither of the gene products alone had leucylation activity, as shown in Fig. 3 (diamonds and circles). However, weak leucylation activity appeared when both of the proteins were simply mixed and assayed (Fig. 3, triangles). This result suggested that leucylation activity appeared only when both of the gene products coexisted. This observation prompted us to perform a gel filtration analysis. As shown in Fig. 4, three major peaks appeared in the elution profile monitored at 280 nm. When analyzed by SDS–PAGE, the first peak was shown to contain aggregated *leuS* products or other proteins (data not shown). The second peak, with a molecular weight of approximately 100 kDa, overlapped with the leucylation activity profile (Fig. 4, triangles). When these peak fractions were subjected to SDS–PAGE analysis, only two protein bands (about 65 and 35 kDa, probably the *leuS* and *leuS′* products, respectively) were detected (Fig. 2, lane 3). The pureness of this fraction clearly indicates that the P-65 protein was selectively picked up from the crude preparation of the *leuS* product (Fig. 2, lane 1) by forming a complex with the *leuS′* product (P-35). The third peak contained only the *leuS′* product (data not shown). Thus, it was shown by this analysis that the enzyme responsible for the leucylation activity has a molecular weight of approximately 100 kDa. This value nearly corresponds to the sum of the molecular weights of one *leuS* product plus one *leuS′* product (107.4 kDa). These results indicate that *A. aeolicus* LeuRS has a heterodimeric quaternary structure of α/β type. One part of *A. aeolicus* LeuRS gene, *leuS*, had been predicted to encode the N-terminal region of the canonical LeuRS by homology search of gene alignments [4], and it is now possible to call its product (P-65) the 'α subunit' of LeuRS. Similarly the *leuS′* gene product (P-35), predicted to encode the C-terminal region of the canonical LeuRS, can be called the 'β subunit'.

Active fractions in the second peak of the gel filtration chromatography (Fig. 4) were combined, and concentrated with Centriprep-10. The leucylation activity of this enzyme (now designated as the LeuRS α/β complex) was assayed (Fig. 3, squares). The specific activity for leucylation has improved about 3.5-fold (from 4.5 to 15.8 U/mg) after the regeneration process (see legends to Figs. 3 and 4). SDS–PAGE analysis reconfirmed that this enzyme preparation is highly homogeneous and is composed of α and β subunits in the molar ratio of one to one.

3.3. Kinetic constants of aminoacylation by LeuRS α/β complex

The aminoacylation kinetics was measured with the LeuRS α/β complex. Since native *A. aeolicus* tRNA was not available, total *E. coli* tRNA and *A. aeolicus* $tRNA^{Leu}$(CAG) (leucine tRNA which have anticodon CAG) transcript were used as alternative substrates. The thermal instability of these tRNAs prevented measurement to be made at 95°C, at which *A. aeolicus* normally grows. Therefore, the temperature dependence of leucylation was tested using these tRNAs in the range of 20–80°C. When total *E. coli* tRNA was used, the leucylation activity was retained up to 65°C, but decreased rapidly at higher temperatures (data not shown). This result shows that total *E. coli* tRNA is a good substrate for aminoacylation by *A. aeolicus* LeuRS α/β complex up to 65°C. Thus, the kinetic constants for leucine and ATP were determined using total *E. coli* tRNA at 65°C. Since the transcripts of *A. aeolicus* and *E. coli* $tRNA^{Leu}$(CAG) were also found to be good substrates up to 55°C (data not shown), the kinetic constants for tRNA substrate were determined using both transcripts at this temperature. As shown in Table 1, the K_m of *E. coli* $tRNA^{Leu}$(CAG) transcript almost equals that of the

Table 1
Kinetic constants of the *A. aeolicus* LeuRS α/β complex

Substrates	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (S^{-1} mM^{-1})
Leucine	8.26 ± 0.07	0.134	16.2
ATP	228 ± 11	0.094	0.41
A. aeolicus $tRNA^{Leu}$(CAG)	1.35 ± 0.025	0.006	4.44
E. coli $tRNA^{Leu}$(CAG)	1.25 ± 0.042	0.003	2.40

The kinetic constants were determined by Lineweaver–Burk plot analysis using the initial velocities.

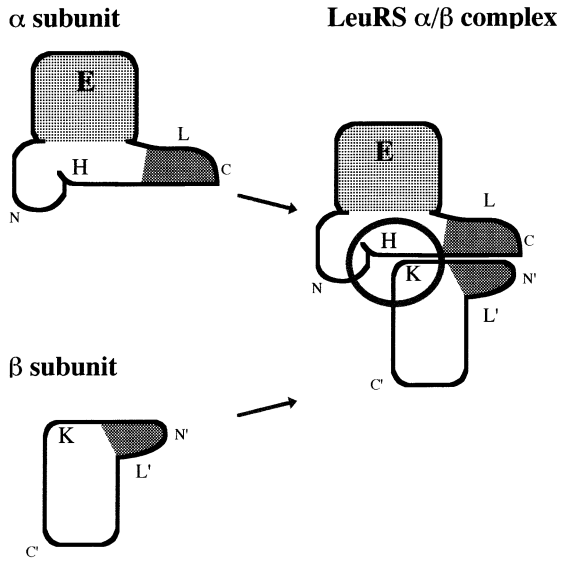

Fig. 5. Regeneration of *A. aeolicus* LeuRS α/β complex from the two subunits. *A. aeolicus* LeuRS α/β complex and both subunits are illustrated schematically. Rough locations of the N- and C-terminals in both polypeptides are marked with N and C for the α subunit and N′ and C′ for the β subunit, respectively. The putative editing domain in the α subunit is shown in light gray and marked with E. The 'leucyl-specific insertion domain' [18] or its divided counterparts are in dark gray and marked with L or L′. Positions of the class I motifs, HIGH (or HMGH) and KMSKS, are indicated by H and K, respectively. The reconstituted Rossmann-fold region is circled.

A. aeolicus $tRNA^{Leu}$(CAG) transcript. This result suggests that the recognition of tRNA by the *A. aeolicus* LeuRS α/β complex is governed by a similar mechanism to that of the *E. coli* system [14]. On the other hand, the k_{cat} values for each of the three substrates were substantially lower than those obtained with *E. coli* LeuRS [8]. We think this was mainly due to the experimental limitation that the measurement had to be done at suboptimal temperatures, as noted above. In this regard, an about 5–10-fold increase in k_{cat} values has been reported for some aaRSs from *Thermus thermophilus* when the assay temperature is shifted from 30 to 70°C [15,16]. Thus, the k_{cat} values for the *A. aeolicus* LeuRS α/β complex would be much higher, if measurements could be done at the optimal temperature using homologous tRNA substrates. The possibility that the regeneration of the enzyme had been partial or imperfect must be also considered. Co-expression of the *leuS* and *leuS′* genes in *E. coli* is under way in our laboratory, with the expectation of a more active preparation of the LeuRS α/β complex being obtained.

4. Discussion

The overall image of LeuRS regeneration from both of the *leuS* and *leuS′* products is schematically shown in Fig. 5. The entire Rossmann-fold is reconstructed only after the reunion of both subunits. This probably explains the lack of activities for leucyl-adenylate formation and leucylation in each of the subunits as described above. In this connection, there is an interesting observation by Rouget and Chapeville [17] that *E. coli* LeuRS could be split by mild tryptic proteolysis into two 'subunit-like' fragments and recombined into an active enzyme. The split products had lost the leucyl-tRNA forming activity but still retained the leucyl-adenylate forming activity. It would therefore be very interesting to determine the exact sites of tryptic cleavage and see if the entire Rossmann-fold was retained or not in this case.

Cusack et al. recently reported the crystal structure of *T. thermophilus* LeuRS and its complex with a leucyl-adenylate analogue [18]. They referred to the 'leucyl-specific insertion domain' which is unique to prokaryote-like (i.e. bacterial and mitochondrial) LeuRS but is highly diverse in sequence and size. Interestingly, *A. aeolicus* LeuRS has one of the largest of such modules, and the split-site of the LeuRS gene is right in the middle of this putative domain [4] (Fig. 1). We speculate that the reunion of this domain contributes much to the regeneration of LeuRS activity through properly directing the contact surfaces between the α and β subunits. Further studies, such as deletion analyses, are needed to elucidate the actual function of this domain.

Very recently, another type of bi-partite enzyme system in *A. aeolicus* has been reported. According to Tomita and Weiner [19], the building and repairing of the 3′-terminal CCA of tRNAs in *A. aeolicus* require collaboration of two distinct but related polypeptides, one that adds CC and another that adds A, while all previously characterized CCA-adding enzymes [ATP(CTP):tRNA nucleotidyltransferases] are composed of a single kind of polypeptide with dual specificity for CTP and ATP addition. They speculate that the separate CC- and A-adding activities may be the more primitive state of CCA-adding enzyme, and the dual-specificity polypeptide is a derived state since *A. aeolicus* is among the most deeply rooted eubacteria. It would be too premature to conclude by analogy that the split architecture of *A. aeolicus* LeuRS represents the more primitive state of LeuRSs, but it will certainly be an important subject for studying the origin and evolution of aaRSs.

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