The Peptide Bond between E292−A293 of *Escherichia coli* Leucyl-tRNA Synthetase Is Essential for Its Activity†

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ABSTRACT: *Escherichia coli* leucyl-tRNA synthetase (LeuRS) is a class I aminoacyl-tRNA synthetase that contains a large connecting polypeptide (CP1) inserted into its nucleotide binding fold, or active site. In this study, purified leucyl-tRNA synthetase was found to be cleaved between E292 and A293 in its CP1 domain. SDS−PAGE analysis showed peptides of 63 and 34 kDa in addition to the native 97.3 kDa synthetase. By internal complementation, the two peptides could form a 97.3 kDa complex similar to the native LeuRS. This complex could support the ATP∼PP_i exchange activity of LeuRS, but could not complement for aminoacylation. To study the function of the region around the bond of E292 and A293, four pairs of peptides resulting from different cleavage sites in CP1 were reconstituted in vivo. With the exception of the enzyme assembled from the E292−A293 cleavage site, all the reassembled LeuRSs catalyzed the aminoacylation of $tRNA^{Leu}$. Although the E292−A293-cleaved LeuRS could not catalyze aminoacylation, fluorescence titration revealed that its tRNA binding ability was almost identical to that of wild-type LeuRS. These results suggest that the region around E292−A293 may be responsible for maintaining the proper conformation of LeuRS required for the tRNA charging activity.

The aminoacyl-tRNA synthetase (aaRS) family provides the enzymatic basis for readout of the genetic code by catalyzing the esterification of amino acids to their cognate tRNAs (*1*). Aminoacylation of a tRNA with its specific amino acid takes place by a two-step reaction. The first step produces an aminoacyl-adenylate intermediate from ATP and the amino acid. The second step is the transfer of the aminoacyl moiety to the 2′- or 3′-hydroxyl group of the 3′-terminal adenosine of the cognate tRNA. The 20 synthetases found in *Escherichia coli* can be classified into 2 groups according to their conserved sequence motifs and specific 3-dimensional structural elements (*2*). The class I aaRSs contain two signature peptides, HIGH and KMSKS, located in the active site which contains the characteristic dinucleotide binding fold (Rossmann fold). The active site of class II aaRSs is built around an antiparallel β-sheet partly closed by helices and containing three conserved motifs (*3*). The active site of each enzyme provides a structural framework for the binding of amino acids, ATP, and the acceptor end of tRNA molecules, and catalyzes the formation of the intermediate and the final product.

Crystallographic studies of 15 aaRSs revealed the modularity of these enzymes (*4*−*7*). Active sites are highly conserved, but various insertions and additional domains are appended to the active site, and the domains outside of the catalytic fold show diversity in structure. Most of these domains contribute to the specificity of the enzymes by playing a functional role either in tRNA recognition or in proofreading of incorrect products. The diversity among synthetases may reflect the evolution and differentiation of protein domains, toward specialized structural patterns as those involved in tRNA recognition which are characteristic of each system. Interestingly, truncated aaRSs still function in adenylation and aminoacylation, suggesting that enzymes lacking one or more major structural domains might have been intermediates during evolution (*8*−*10*). In many aaRSs, the individual domains are highly independent of each other. Mutants in the N-terminal domain with virtually no aminoacylation activity can still accurately recognize tRNA. Site mutations or partial deletion in the C-terminal domain of these synthetases destroy aminoacylation activity, but have little effect on amino acid binding and activation (*3*, *11*).

Leucyl-tRNA synthetase (LeuRS) belongs to the class 1 aaRSs and catalyzes leucylation of $tRNA^{Leu}$ on the 2′-OH of the ribose of the 3′-terminal adenosine of the tRNA (*2*, *3*). The *Escherichia coli* LeuRS has been cloned, and the primary structure of the LeuRS protein predicted from its coding sequence consists of 860 amino acids with a molecular mass of 97.3 kDa (*12*). The LeuRS and four other class 1 aaRSs, cysteinyl-, isoleucyl-, methionyl-, and valinyl-tRNA synthetases (CysRS, IleRS, MetRS, ValRS), are highly similar to each other and are classified into a subgroup (class Ia) (*13*). However, recent structural data and structure-based multiple sequence alignments suggest ArgRS may also belong to this subgroup (*7*). Although these enzymes from *Escherichia coli* vary in size from 461 (CysRS) to 959 (IleRS) residues, their sequences align well when variable insertions are taken into account (*14*). The size differences

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among aaRSs in this subgroup of class I enzymes are mostly due to insertions in the Rossmann fold—the connecting polypeptide 1 and 2 (CP1, CP2) domains (*15*).

In each class I aaRS, CP1 links the two halves of the Rossmann fold at a conserved site and is specific to each system (*16, 17*). Despite the structural diversity, significant similarities between the CP1s of GluRS, GlnRS, MetRS, and ArgRS are evident (*7, 13*). These enzymes share two common substructures: a helix−loop−strand−loop−helix motif and a two-stranded antiparallel β-sheet. These common features close the back of the active site and may play a role in stabilizing its overall structure. Whether the CP1s of these aaRSs have some common function is still unclear. CP1 in GlnRS has been called an acceptor-binding domain because it has been shown to stabilize the hairpin conformation of the 3′-end of the $tRNA^{Gln}$ (*18, 19*). The CP1 of MetRS may have the same function (*20*). However, this may not be true of the IleRS system. In IleRS, most of the CP1 is dispensable without loss of canonical activity, since some deletion mutants in this domain of IleRS retained activity (*16*). In IleRS, this domain may play another very important role. The CP1 of IleRS has a second amino acid binding site responsible for editing of incorrect aminoacyl-adenylates (*6, 21, 22*). The CP1 of ValRS may have a similar proofreading function for editing of the incorrect adenylates.

LeuRS also contains a large CP1 of about 260 amino acids spanning residues 134−393 (*23*). No structure−function information on this domain is available. In the present paper, the cleavage of the purified LeuRS into two peptides of 64 and 33 kDa, the location of cleavage site within the CP1, and the effect of cleavage on function are reported.

MATERIALS AND METHODS

Materials. Escherichia coli $tRNA_1^{Leu}$ with a charging capacity of more than 1600 pmol/A_{260} unit was isolated from an overproducing strain of *Escherichia coli* constructed in our laboratory. Total tRNA isolated from this strain was approximately 50% $tRNA_1^{Leu}$ (*24*). Rabbit polyclonal antibodies against *E. coli* LeuRS were prepared in the laboratory. L-Leucine, DTT, ATP, CAPS, and tetrasodium pyrophosphate were purchased from Sigma. L-[^{14}C]Leucine (300−400 mCi/mmol) and tetrasodium [^{32}P]pyrophosphate were obtained from Amersham (England). GF/C filter and PVDF membrane were obtained from Whatman.

Purification of Escherichia coli LeuRS. Escherichia coli LeuRS was purified from an overproducing *Escherichia coli* strain by two-step chromatography on DEAE-Sepharose CL-6B and HA-Ultrogel (*25*). The specific activity of the purified LeuRS for aminoacylation was 2800 units/mg. One unit of aminoacylation activity is defined as the quantity of protein that catalyzes the incorporation of 1 nmol of amino acid in 1 min under the given conditions. The concentration of LeuRS was determined by the A_{280} of the enzyme solution; 1 OD_{280} is equivalent to about 1.62 mg of protein/mL.

Determination of the N-Terminal Amino Acid Sequence of Peptide Fragments. Protein bands separated by SDS−PAGE were transferred onto a PVDF membrane, excised, and subjected directly to automatic Edman degradation in a gas-phase protein sequencer (Beckman LF 3200U Protein Sequencer) according to the manufacturer's instructions.

Construction of Plasmids. Plasmid pSML104 was constructed from pACYC184 (*26*) and pKK-233-2 (*27*). It contains the *p15A* replicon from pACYC184, the strong *trc* promoter (a hybrid of promoters *lac* and *trp*), a multicloning site, and two sequences for transcription termination (T1 and T2) of the ribosomal operon *rrnB* from pKK233-2. Plasmids pTrc100 and pKK236 were derived from pTrc99B (*28*) and pKK-233-2, respectively. Both plasmids contain the *ColE1* replicon. The same multicloning site as that of pSML-104 was introduced into these two plasmids. The multicloning site contains *Nco*I, *Eco*RI, *Sma*I, *Bgl*II, *Bfr*I, and *Hin*dIII sites. Four pairs of DNA fragments with suitable restriction endonuclease cleavage sites were PCR-amplified from the plasmid containing *leuS*. Each pair of fragments encoded an N-terminal part and a C-terminal part of LeuRS, thus mimicking the products resulting from cleavage of LeuRS. The cleavage sites of each pair of fragments were located between T252 and F253, E292 and A293, K327 and A328, and P368 and D369. Each pair of fragments was recombined into the two above-mentioned plasmids, pSML-104 and pKK236 (or pTrc100), to produce a pair of expression plasmids, which could be cotransformed into *E. coli*. Analysis of the hydrophobic region and secondary structure confirmed that all these peptide cleavage sites were located in sites inside CP1 of LeuRS where the hydrophilic region and the connection portion of the secondary structure are located. Therefore, mutations at these sites should have little effect on the overall structure of the enzyme.

Complementation Test. The *Escherichia coli* temperature-sensitive strain KL231(F^{-t} leuS31 thyA6 rpsL120) was provided by Dr. M. J. Rogers, Yale University. This strain, being a *leuS*31 temperature-sensitive mutant, cannot grow at 44 °C. After transformation with the appropriate plasmid pairs containing genes encoding peptide fragments of LeuRS in strain KL231, each pair of fragments should be synthesized in vivo from separate mRNAs. The activity of the assembled LeuRS was determined by the capability of KL231 to grow at 44 °C.

Enzymatic Assay. The ATP∼PP_i exchange and aminoacylation activities of LeuRS were measured as described by Shi et al. (*29*). To assay ATP∼PP_i exchange activity, the standard reaction mixture contained 144 mM Tris-HCl, pH 7.8, 10 mM KF (the inhibitor of pyrophosphatase activity), 10 mM $MgCl_2$, 5 mM ATP, 2 mM [^{32}P]PP_i (1−2 cpm/pmol), and 1 mM L-leucine. The reaction was initiated upon addition of 1 μM LeuRS. After various incubation times at 37 °C, 50 μL aliquots were pipetted out in a mixture of 200 μL containing 2% charcoal, 3.5% perchloric acid, and 0.05 M pyrophosphate. The charcoal was then filtered onto Whatman GF/C glass−fiber disks, washed with 20 mL of 10 mM pyrophosphate, and dried. The synthesized [^{32}P]ATP was adsorbed on the charcoal and then counted by scintillation with 0.6% butyl-PDB [2-(4-*tert*-butylphenyl)-5-(4-biphenylyl)-1,3,4-oxadiazole] in toluene/ethylene glycerol methyl ether (volume ratio 4:6). To determine aminoacylation activity, 2 nM LeuRS was added to the following reaction mixture: 100 mM Tris-HCl, pH 8, 30 mM KCl, 12 mM $MgCl_2$, 4 mM ATP, 0.1 mM EDTA, 0.5 mM DDT, 0.1 mM [^{14}C]-L-leucine, and 5 mM total tRNA. After various incubation intervals at 37 °C, aliquots of 15 μL were spotted onto a Whatman paper disk and quenched with 5% trichloroacetic acid. Filters were washed with 5% trichloroacetic acid and 95% ethanol, respectively, dried, and counted in PPO−POPOP/toluene. The aminoacylation activity of assembled

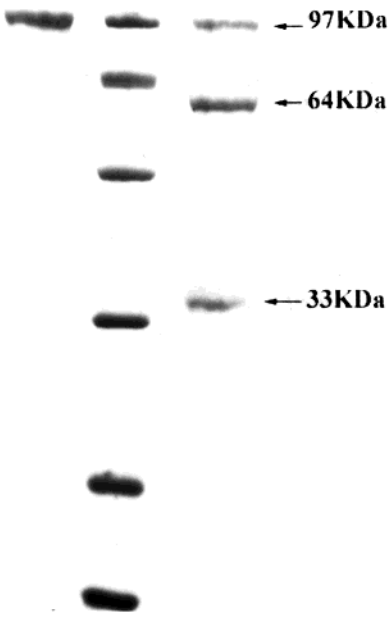

FIGURE 1: Fractionation pattern of LeuRS cleavage. Purified LeuRS containing 55% glycerol at −20 °C for 1 month was cleaved into two new peptides with molecular masses of 64 and 33 kDa detected by SDS−PAGE. Lane 1: purified LeuRS. Lane 2: molecular mass markers from Shanghai Dong Feng Chemicals Factory, Shanghai Institute of Biochemistry Academia Sinica, with molecular masses of 97.4 (rabbit phosphorylase *b*), 66.2 (bovine serum albumin), 43.0 (rabbit actin), 31.0 (bovine carbonic anhydrase), 20.1 (trypsin inhibitor), and 14.4 kDa (hen egg white lysozyme). Lane 3: cleaved LeuRS.

LeuRS was measured in the crude extract of TG1 transformants containing a pair of plasmids in which the complementary split genes were inserted.

Fluorescence Titration with tRNA$_1^{Leu}$. Using a Hitachi F400 fluorometer, the experiment was performed as described by Lin et al. (*30*). Aliquots of $tRNA_1^{Leu}$ solution (25 μmol/mL) were added to 1 mL of the enzyme solution (50 μg/mL) in a 4 mL cell. Excitation and emission wavelengths were 295 and 350 nm with bandwidths of 3 and 5 nm, respectively. In the titration experiments, excess substrate concentrations were used ($[S] \gg [E_t]$, where $[E_t]$ denotes the input enzyme concentration), and a simplified linear relation could be derived: $\Delta F = -K_D \Delta F/[S] + \Delta F_\infty$, where ΔF is the fluorescence change at substrate concentration [S], ΔF_∞ is the fluorescence change when all enzyme molecules are saturated with substrate, and K_D is the dissociation constant of the enzyme−substrate complex.$_\infty$

RESULTS

Cleavage of LeuRS. When the purified LeuRS was stored in 55% glycerol at −20 °C at a concentration of 10 mg/mL for 1 month, 63 and 34 kDa peptides were detected on a SDS−PAGE slab gel, in addition to the 97 kDa LeuRS (Figure 1). The aminoacylation activity of the cleaved enzyme decreased about 30% compared with wild-type LeuRS. The ATP∼PP_i exchange activity was almost unchanged.

The N-terminal sequence of the 34 kDa peptide was MQEQYRPE, whereas that of the 63 kDa fragment was AEME. According to the sequence of LeuRS deduced from the DNA sequence (*12*), the first sequence is the same as the N-terminus of the native LeuRS, while the second corresponds to residues A293−E296 of LeuRS. Theoretically, the molecular masses of peptides M1−E292 and A293−G860 of LeuRS should be 34.06 and 63.29 kDa, respectively. These are in close agreement with those of the two cleaved peptides detected. LeuRS was split at the peptide bond between E292 and A293 under the given conditions. This cleavage site is located in the CP1 domain of LeuRS.

Fractionation of the Peptides from Cleaved LeuRS. The three different sized LeuRS peptides were fractionated by gel filtration on Superose-12 (with 50 mM Tris-HCl buffer, pH 6.8). Only a single absorption peak at A_{280} appeared, implying that, although the enzyme was cleaved at a single site, the two peptides were still attached together and formed a complex of 97 kDa under nondenaturing conditions.

Only one peak was detected when using FPLC on a Mono-Q ion-exchange column and eluting by using 50 mM Tris-HCl (pH 6.8) with a linear gradient of NaCl from 20 mM to 1.0 M. Two peaks emerged, however, when the pH of the above buffer was increased to 8.0 (Figure 2a). SDS−PAGE showed that the first peak consisted mainly of the native LeuRS, while the two peptides produced from cleavage were contained in the second peak in equal amounts (Figure 2b).

Activity of the Cleaved LeuRS. It was difficult to completely separate the cleaved LeuRS from the native enzyme. By assaying the activities in the fractions eluted from FPLC on a Mono-Q column at pH 8.0, it was found that the activity peak of amino acid activation overlapped with both absorption peaks; aminoacylation activity, however, was mainly detected in the first peak (Figure 2a). These results indicate that the cleaved LeuRS in the second peak had lost most of its aminoacylation activity, but still retained the activity in ATP∼PP_i exchange.

Fragment Assembly of Split LeuRS in Vivo. Since the E292−A293 peptide bond had been shown to be essential to the leucylation of $tRNA^{Leu}$, the functions of other peptide bonds in CP1 were studied in vivo by fragment assembly and complementation tests in the *Escherichia coli* temperature-sensitive strain KL231. Each pair of plasmids containing a pair of DNAs encoding N- and C-terminal parts of LeuRS was cotransformed into KL231. When the transformants were cultured at 30 °C in crude extract, the peptide fragments with the correct size could be detected by SDS−PAGE and Western blot analysis with anti-LeuRS antibodies, indicating that the correct peptide fragments were expressed in these cells (Figure 3). With the exception of a pair of peptide fragments that mimics cleavage at E292−A293, the transformants harboring the other three pairs of DNAs grew quite well, demonstrating that the corresponding assembled proteins were active. The aminoacylation activities of the assembled LeuRSs in the crude extract of the KL231 were also tested in vitro, as shown in Table 1. While assembly of fragments 1−292 and 293−860 did not produce an active LeuRS, assembly of the other three pairs of peptide fragments produced active enzymes. These results support our in vitro finding that LeuRS cleaved between E292 and A293 had lost its aminoacylation activity, while LeuRS cleaved at other sites in CP1 still retained its aminoacylation activity.

The tRNA Binding Ability of Cleaved Enzyme Split at E292−A293. Due to the difficulty in separation, the cleaved form of LeuRS prepared by Mono-Q contained about 25% native LeuRS. The binding of partially purified cleaved

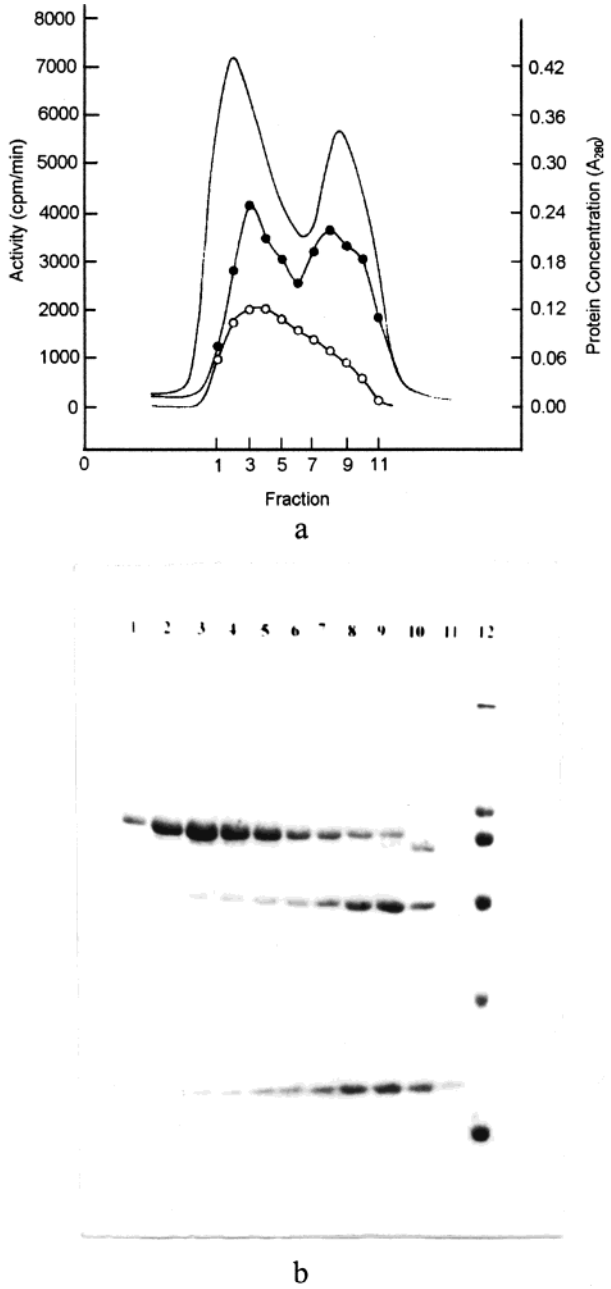

FIGURE 2: Analysis of cleaved LeuRS. (a) Fractionation of LeuRS and activity measurement. Cleaved LeuRS was fractionated into two peaks by FPLC on a Mono-Q column (pH 8.0). The activity peaks of amino acid activation were overlapping with the protein peaks (●). However, aminoacylation activity was detected only in the first protein peak (○) which contained intact LeuRS. (b) SDS−PAGE analysis of FPLC Mono-Q eluate. Cleaved LeuRS was fractionated by FPLC on a Mono-Q column (pH 8.0). The composition of each fraction was analyzed by SDS−PAGE. Lanes 1−11 correspond to the 11 fractions of the column. Lane 12: molecular mass markers, from Sigma, from top to bottom were 205, 116, 97, 66, 45, and 29 kDa, respectively.

LeuRS with $tRNA^{Leu}$ was determined by fluorescence titration (Figure 4). The K_D values of the cleaved and native LeuRS were 2.47 and 1.87 μM, respectively, indicating that the cleavage of the peptide bond between E292 and A293 had only a slight effect on the binding of tRNA. The saturation of $tRNA^{Leu}$ binding on the cleaved enzyme deduced from $\Delta F_\infty/F_0$ was 85.55% under the given conditions, almost the same as that on the native enzyme (86.20%). These results showed that although the aminoacylation activity of LeuRS was lost due to the cleavage between E292 and A293, binding of $tRNA^{Leu}$ to the cleaved and native LeuRS was almost identical.

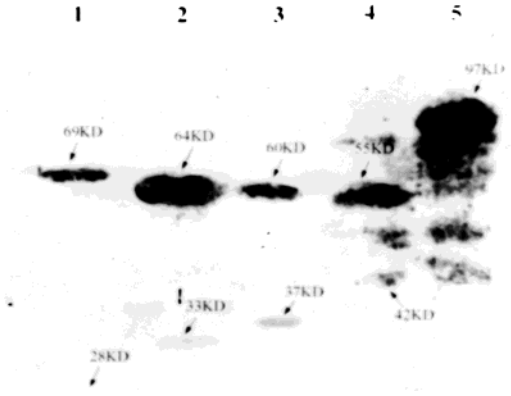

FIGURE 3: Western blot analysis of LeuRS fragments produced in *E. coli* KL231. Transformants were obtained from transformation of *E. coli* KL231 with a pair of plasmids. The crude extract of the transformant was used for Western blot analysis. Fragments of the expected size are indicated by filled arrowheads. Lane 1, pTrc-L252 and pSML-L253; lane 2, pTrc-L292 and pSML-L293; lane 3, pKK-L327 and pSML-L328; lane 4, pKK-L368 and pSML-L369; lane 5, pKK-*leuS*.

DISCUSSION

In this work, we found that LeuRS cleaved at the E292−A293 peptide bond in the CP1 domain failed to catalyze the aminoacylation of tRNA but still supported the ATP−PP_i exchange activity. As mentioned in the introduction, the aminoacylation of a tRNA proceeds through a two-step mechanism. The first step is activation of amino acid, and the second is the transfer of the aminoacyl moiety to 3′-terminal adenosine of the tRNA. An aaRS is organized into two functional domains: an active site domain responsible for amino acid activation and another domain involved in tRNA binding. The ability of cleaved enzyme to catalyze leucine activation suggests that the active site of LeuRS is still intact.

The integrity of the active site reveals that the two fragments still remain correctly associated. The cleavage is between E292−A293 in the CP1 domain of LeuRS. In class I aaRSs, CP1 links the two halves of the N-terminal Rossmann fold where the active site is located (*16*, *17*). The integrity of the Rossmann fold is pivotal to substrate binding and activity. Therefore, neither of the two fragments alone could be active. When the two fragments had disassociated during the experiments, no activity could be detected. The behavior of the two fragments on gel filtration also indicated that the two fragments split at E292−A293 existed as a 97 kDa complex, the same size as the native enzyme. In many proteins, it has been found that internal interactions in a protein could overcome the loss of a peptide bond. It has been shown that limited proteolytic cleavage of many proteins, such as ribonuclease A (*31*), staphylococcal nuclease (*32*), and cytochrome *c* (*33*), produces fragments that can be reassociated in vitro with close to native activity. In addition, MetRS and IleRS could be reconstituted to active enzymes from randomly split genes (*23*, *34*, *35*). Shiba and Schimmel (*23*, *34*) reported that the reconstitution pairs of fragments of MetRS and IleRS could catalyze the aminoacylation of the cognate tRNA in vivo. From our results, all

Table 1: Bipartite Assembly of LeuRS in Vivo[a]

Transformant	Fragment	Expression	Specific activity (unit/mg)	Relative activity (%)
pKK-*leuS*	1—860	+	284.6	100
pTrc L252 +pSML-L253	1—252 / 253—860	+	26.8	9.42
pTrc-L292 + pSML-L293	1—292 / 293—860	+	5.24	1.84
pKK-L327 +pSML-L328	1—327 / 328—860	+	74.6	26.2
PKK-L368 +pSML-L369	1—368 / 369—860	+	143.7	50.5
pSML-104 +pKK-236		−	3.27	1.0

[a] The activity of these fragments in vivo was tested by transformation of each plasmid into the *Escherichia coli* temperature-sensitive strain KL231 individually. None of the above plasmids complemented strain KL231. Assay of the activity of assembled LeuRS showed that most of the peptide pairs create active enzyme. However, the protein assembled from 1−292 and 293−860 was almost inactive. Values shown are the means of five independent determinations.

four fragments sets of LeuRS, except the 1−292/293−860 pair, could also associate together in vivo and retained partial activity. These results indicate that some peptide bonds may have little effect on the structure of LeuRS, and that the internal interactions of the molecule, which are widely distributed throughout the peptide chain, are generally sufficient to overcome these breaks in the peptide backbone, thus allowing reassembly of functional enzymes.

Why the cleaved enzyme lacks aminoacylation activity is less clear. The second step of the reaction involves the binding of tRNA on the enzyme, followed by transfer of the activated amino acid to the 3′-end of tRNA. Thus, the loss of activity in the second step may be due to either problems with tRNA binding or a failure of amino acid transfer. Our results suggested that cleaved LeuRS could still bind tRNA: indeed, fluorescence titration showed that the binding between the cleaved LeuRS and $tRNA^{Leu}$ was similar to that of the native enzyme. Consequently, it is likely that scission of the 292/293 peptide bond did not influence the binding of tRNA. In fact, the 292/293 region may not be directly involved in binding of tRNA: T. Li reported that mutants with deletions and insertions around the 292 region could still recognize $tRNA^{Leu}$ (*36*).

However, the ability of cleaved LeuRS to bind tRNA may not necessarily imply adoption of an active conformation. The interaction between tRNA and aaRS is very complicated. tRNA molecules are almost the same size as aaRSs, so a large number of interactions can occur between the two molecules. The multiple site interactions are characterized by subtle communication between tRNA and aaRSs. There is evidence that this communication is essential for the transfer reaction. For example, analysis of homologous and heterologous complexes between prokaryotic and eukaryotic AspRSs with their tRNAs revealed that poor aminoacylation of noncognate tRNA in the heterologous complexes is a result of misalignments of terminal CCA (*37*). In addition, pre-steady-state kinetic experiments on the TrpRS system suggested that interaction with tRNA recognition elements can influence both amino acid binding and the height of the energy barrier of the transfer step (Ibba, M., and Soll, D., personal communication). The cognate tRNA is essential for the most productive interaction with the enzyme. Therefore, the slightly decreased binding capacity of cleaved LeuRS for $tRNA^{Leu}$ suggests that the region around the bond between E292 and A293 might have an indirect effect on the interaction between LeuRS and $tRNA^{Leu}$ which may influence the transfer reaction. It is also interesting to mention that the E292−A293 site may be located in a crucial region specific for the transfer reaction, because all other three pairs of assembled LeuRS in vivo still retained partial aminoacylation activity and were much more stable than the assembled fragments parted at 292/293.

It is not clear what kind of conformation the enzyme will adopt after cleavage of the 292/293 peptide bond. Based on the prediction of the secondary structure of LeuRS, this region is hydrophilic and located in the middle of the α-helix−β-turn−α-helix structure motif. When the peptide was cleaved at this site, the local conformation might be changed. Although the alteration did not affect tRNA binding on LeuRS directly, it might change the conformation of other regions that may be involved in the interaction between the enzyme and tRNA. On the other hand, the enzyme might undergo some conformational changes after substrate binding to become catalytically competent. If the cleavage of the peptide bond disallows these conformational changes, cleaved enzyme will also result in substantially reduced activity. We

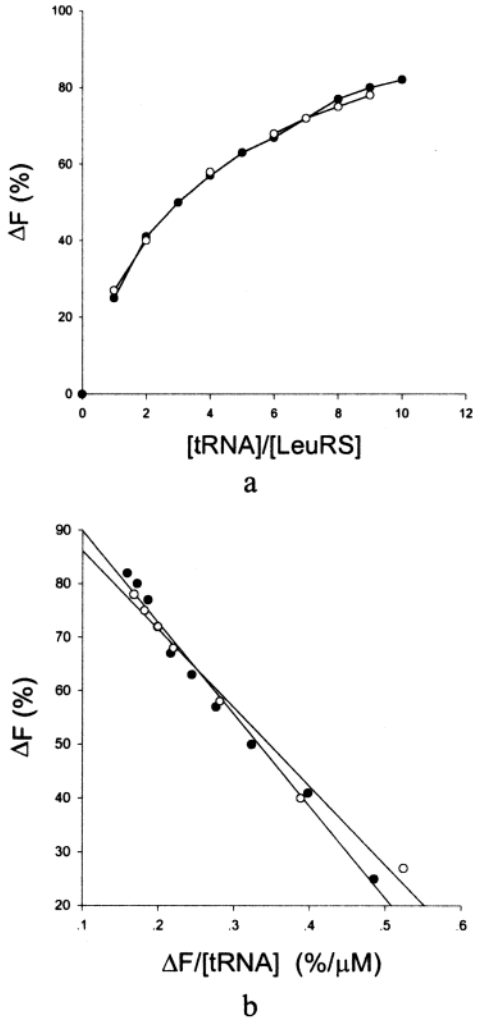

FIGURE 4: Binding of $tRNA_1^{Leu}$ on cleaved and native LeuRS as determined by fluorescence titration. Fluorescence titration was performed under the given conditions (a). The K_D value was determined by a plot of ΔF/[tRNA] against ΔF. The slope of the lines indicates the $K_{D\ tRNA}$ values of cleaved LeuRS (●) and native LeuRS (○) (2.47 and 1.87 μM, respectively) (b).

found that the in vivo assembled LeuRS split after E292 was hydrolyzed soon after the cell was disrupted, suggesting this pair of fragments is much less stable than other pairs of fragments in vivo. The different behaviors of the E292−A293-cleaved LeuRSs eluted from Mono-Q column at different pHs also imply that at the higher pH cleaved LeuRS undergoes a conformational change different from that of the wild-type enzyme. These data show that the conformation of the cleaved protein may be different from that of the wild-type LeuRS and is in agreement with what we have mentioned in the introduction, namely, that CP1 may play a role in stabilizing the overall structure of the protein.

The function of CP1 in class I aaRSs remains to be fully described. In GlnRS, CP1 is called an acceptor-binding domain because it stabilizes the hairpin conformation of the 3′-end of $tRNA^{Gln}$ (*18, 19*). Although it may not be true in all aaRSs, the fact that CP1 is close to the active site and might participate in tRNA recognition suggests that CP1 may influence the catalytic process in class I aaRSs. During the second step of reaction, the 3′-terminus of the tRNA must be docked near the active site of aaRS. An appropriate approach of the substrates and intermediates will require either a certain preexisting conformation or a conformational change of the enzyme. Although the exact cause of the damage to the second step activity of cleaved LeuRS is unknown, it is likely that either a conformational change occurring after the cleavage or failure to undergo conformational changes after substrate binding may be the cause.

In conclusion, our results show that the cleavage of the E292−A293 peptide bond in the CP1 domain of LeuRS does not disturb the structure of its active center for the ATP∼PP_i exchange reaction, the cleaved enzyme still catalyzing leucine activation. In addition, the domain around the cleavage site is not essential for the tRNA binding site, the interaction between the cleaved LeuRS and $tRNA^{Leu}$ being only slightly affected. Nonetheless, the cleavage of the E292−A293 peptide bond may induce a structural alteration of this region which could, by distant effect, impair the active conformation of the acylation site.

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