

## Effect of Alanine-293 Replacement on the Activity, ATP Binding, and Editing of *Escherichia coli* Leucyl-tRNA Synthetase<sup>†</sup>

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**ABSTRACT:** Leucyl-tRNA synthetase (LeuRS) is a class I aminoacyl-tRNA synthetase that catalyzes leucylation of tRNA<sup>Leu</sup>. Several mutants in the CP1 domain of *Escherichia coli* LeuRS were obtained by introduction of restriction endonuclease sites into its gene, *leuS*. Of these mutants, only LeuRS-A293F had decreased activity (46%) compared to the native enzyme. To investigate the effect of A293 on enzyme function, A293 was mutated to Y, G, I, R, or D. The mutants were impaired in activity and editing function to varying extents. The decrease in  $K_m$  values for three substrates showed that the binding of ATP to these mutants became much stronger. The inhibition of ATP binding to most of the mutants was also stronger. In particular, LeuRS-A293D had the lowest activity, the strongest ATP binding, and the most impaired editing function. A red shift of the fluorescence emission maximum of LeuRS-A293D indicated a less hydrophobic chromophore environment and a relatively more flexible dynamic conformation. The change in  $T_m$  of LeuRS-A293D was higher than that of all other substitutions. Evidence from sequence alignment and crystal structure of LeuRS from *Thermus thermophilus* shows that A293 was conserved as R (K) or A and is located at a small helix in the editing domain of the enzyme facing the active site. Hence, any amino acid substitution of A293 may affect the stability of the helix, which may lead to impaired editing function and aminoacylation activity and may be indirectly involved in ATP binding.

Aminoacyl-tRNA synthetases (aaRSs)<sup>1</sup> (EC 6.1.1) arose early in evolution and are believed to be a group of ancient enzymes that catalyze the reaction for precisely charging tRNAs with their cognate amino acids (1). For a long time, the aaRS family was considered as a good model to study the structure–function relationship of enzymes, as each aaRS must precisely distinguish its cognate substrates, amino acids and tRNAs from many substrates, which have similar structures (2). Based on the conserved amino acid sequences and crystal structures, the 20 aaRSs are divided into two major classes of 10 enzymes each (2–5). Class I aaRSs have a well-conserved nucleotide binding fold (Rossmann fold), which is responsible for amino acid and ATP binding, aminoacyl-adenylate formation, and tRNA acceptor helix docking (6–8). Two insertions, connective polypeptides 1 and 2 (CP1 and CP2), split the nucleotide binding fold after the third and the fourth  $\beta$ -strand, respectively (9–10). Class

II aaRSs are built around an antiparallel  $\beta$ -sheet partly closed by helices and contain three characteristic homologous motifs (4).

Leucyl-tRNA synthetase (LeuRS, EC 6.1.1.4) (5), a class I aaRS, from *Escherichia coli* is a monomer consisting of 860 amino acid residues with molecular mass of 97.3 kDa deduced from its gene, *leuS* (11). Among the 10 class I aaRSs, LeuRS, valyl-tRNA synthetase (ValRS), isoleucyl-tRNA synthetase (IleRS), methionyl-tRNA synthetase (MetRS), and cysteinyl-tRNA synthetase (CysRS) belong to a subgroup of the class I aaRSs (10). In addition to sharing the sequence motifs in the N-terminal halves of all class I aaRSs, these five also have related C-terminal domains and a statistically more significant alignment of N-terminal sequences with each other than with the remaining five class I enzymes. MetRS and CysRS have relatively small CP1 domains (100 and 50 amino acids, respectively). LeuRS, ValRS, and IleRS have large CP1 domains ranging from about 250 to 275 amino acids (12). Some evidence has shown that the CP1 domain is a very important multifunctional domain. For example, the CP1 domains in LeuRS, IleRS, and ValRS harbor the activity for RNA-dependent amino acid recognition as manifested by the editing of mischarged and misactivated amino acids (13–15); an insertion mutant in the CP1 domain of *E. coli* LeuRS, LeuRS-A, gains the ability to discriminate two tRNA<sup>Leu</sup> isoacceptors (16); and some amino acid residues in CP1 of tyrosine-tRNA synthetase determine species-specific aminoacylation (17). On the basis of sequence alignment, the CP1 of LeuRS extends

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<sup>1</sup> Abbreviations: aaRSs, aminoacyl-tRNA synthetases; CP1, connective polypeptide 1; CP2, connective polypeptide 2; LeuRS, leucyl-tRNA synthetase; ValRS, valyl-tRNA synthetase; IleRS, isoleucyl-tRNA synthetase; MetRS, methionyl-tRNA synthetase; CysRS, cysteinyl-tRNA synthetase; CD, circular dichroism.

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A:  LAAFIDECRNTKVAE*EMATME-KKGVDTGFKAVHPLTGEE----IPVWAANFVLMEY : 330
B:  VLAYVEAAKRKTEIERQAEGRE-KTGVFLGAYALNPATGER----IPIWTADYVLFY : 332
C:  LAAFIQEAKNAKVAEADLATME-KKGMATGLFAIHPLTGDK----LPIWVANFVLMHY : 330
D:  LAAFIQEAKNAKVAEADLATME-KKGMATGLFAIHPLTGDK----LPIWVANFVLMHY : 330
E:  LQAFSLTLPGLSPDSKVGYLLPHIRAVNP--LAYHEETPEDTKVSLPIYVAPYVLGDY : 392
F:  VDEFVAMVAKESIERTAEDKP-KRGVKTGGIAINPFNGEE----IPILIADYVLYEY : 333
G:  LKLIKEIQSKSDLERTDLAKT-KTGVFTGAYAINPVNGEK----LPIWIADYVLASY : 330
H:  VQAYVQRAAKNDLERTDLAKE-KTGVFTGAYVRNPINDMR----IPVWVG DYVLVSY : 341
I:  IAAYRRAIAAKSDLERQE-SRE-KTGVFLGSYAINPANGEP----VPIFIADYVLAGY : 435
J:  -ASFLKKQLQKTTTLKQK-ATL-YDGDILLTNAIHPLTNEL----IPVYVANYVIEGY : 331
K:  -AQFLQKQAQKTTTVKQP-ETL-HDGVDLKLKAINPATNTA----IPLYVANYVVEGY : 331

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FIGURE 1: Primary sequence alignment of leucyl-tRNA synthetases from different organisms: A = *E. coli*, B = *T. thermophilus*, C = *Haemophilus influenzae*, D = *Borrelia burgdorferi*, E = *Neurospora crassa* (mitochondrial), F = *Synechocystis* sp., G = *B. subtilis*, H = *Treponema pallidum*, I = *Mycobacterium tuberculosis*, J = *Mycoplasma genitalium*, K = *Mycoplasma pneumoniae*. Alignment corresponding to A293 of *E. coli* enzyme is denoted with an asterisk. This sequence alignment was extracted from an alignment built with the program CLUSTAL X.

from 126 to 389 (12). We found that when purified LeuRS was stored in 55% glycerol at  $-20^{\circ}\text{C}$  at a concentration of 10 mg/mL for 1 month, the peptide bond between E292 and A293 in the CP1 of LeuRS was specifically cleaved (18). The cleaved LeuRS retained the ATP $\sim$ PP<sub>i</sub> exchange activity; however, the aminoacylation activity was lost (18). This peptide bond seems to be in a very special location in the CP1 domain of this enzyme and appears to be very important for aminoacylation activity. The sequence alignment for LeuRSs from different organisms showed that the site of 293A is conserved as a positively charged amino acid: 8 of the 11 are either Arg or Lys, while the other three are Ala (Figure 1). On the basis of the crystal structure of LeuRS from *Thermus thermophilus* and the alignment results, 293A was located at a small helix in the editing domain of the enzyme and on the side facing the active site (19).

To further reveal the function of the region around the peptide bond between E292 and A293 in the CP1 domain of *E. coli* LeuRS, several pairs of DNA fragments encoding the N- and C-terminal parts of LeuRS were amplified with appropriate restriction endonuclease sites attached by PCR. After ligation of DNA fragments, the mutant genes were produced from which several LeuRS mutants were expressed. Of all these mutants, only LeuRS-A293F significantly decreased in activity. To further study on the effect of A293 on LeuRS, five mutants at A293 of LeuRS were created. Here, we describe the preparation of these mutants and an investigation of their properties and conformations, and we discuss the importance of A293 to the structure and function of LeuRS.

## EXPERIMENTAL PROCEDURES

**Materials.** *E. coli* tRNA<sup>Leu</sup> with a charging capacity of about 1600 pmol/A<sub>260</sub> unit was isolated and purified in our laboratory (20). L-Leucine, DTT, ATP, DEAE-Sepharose CL-6B, and HA-Ultrigel were purchased from Sigma. L-[<sup>14</sup>C]-Leucine, L-[<sup>14</sup>C]methionine, and L-[<sup>14</sup>C]isoleucine were obtained from Amersham (England). The L-[<sup>14</sup>C]methionine and L-[<sup>14</sup>C]isoleucine were free of L-[<sup>14</sup>C]leucine contaminant (13). Restriction endonucleases, T4 polynucleotide kinase, and T4 DNA ligase were from Boehringer Mannheim. Primers for PCR and sequencing were synthesized on an Applied Biosystems oligonucleotide synthesizer. The gene

encoding *E. coli* LeuRS, *leuS*, was subcloned from a  $\lambda$ 15D7 clone of an *E. coli* genomic library by complementation of a *leuS* temperature-sensitive mutant KL231 in our laboratory (21).

**Mutagenesis and Plasmid Construction.** The plasmid pKK236 with modified polylinker region of *Nco*I, *Eco*RI, *Sma*I, *Bgl*II, *Bfr*I, and *Hind*III sites was derived from pKK233-2 (18, 22). With *leuS* as a template, three pairs of DNA fragments encoding the N- and C-terminal parts of LeuRS were amplified and restriction endonuclease sites were attached by PCR. The joints were located between T252 and F253, E292 and A293, and L327 and M328 (16). When pairs of DNA fragments were ligated to form full-length genes encoding LeuRS, some amino acid residues obviously were changed by the introduction of restriction endonuclease sites. Three single-site mutants (LeuRS-T252E, LeuRS-M328K, and LeuRS-A293F) and one double-site mutant (LeuRS-T252E-M328K) were obtained in this way. Because only LeuRS-A293F, among the four mutants, showed a significant decrease in activity, the genes encoding the other five LeuRS mutants changed at A293 were cloned by PCR and expressed. The DNA sequence fidelity of the mutants was confirmed by DNA sequencing (data not shown).

**Expression and Purification of LeuRS and Its Mutants.** *E. coli* LeuRS and its mutants were purified from overproducing *E. coli* TG1 transformants by two-step chromatography on DEAE-Sepharose CL-6B and HA-Ultrigel (23). The concentrations of purified LeuRS and mutants were measured by the A<sub>280</sub> of the enzyme solution. About 1.63 mg/mL LeuRS-A293Y and 1.62 mg/mL LeuRS and the other mutants equaled 1 optical density unit at 280 nm (18).

**Enzymatic Assay.** Aminoacylation activity of LeuRS was measured with L-[<sup>14</sup>C]leucine as described by Li et al. (18). Inhibition of LeuRS and its mutants by ATP was measured at 37  $^{\circ}\text{C}$  in the reaction mixture consisting of 100 mM Tris-HCl (pH 7.8), 30 mM KCl, 12 mM MgCl<sub>2</sub>, 4 mM ATP, 0.1 mM EDTA, 0.5 mM DTT, and 20  $\mu\text{M}$  tRNA<sup>Leu</sup> with various concentrations of ATP, with the ratio of [Mg<sup>2+</sup>]/[ATP] in the reaction mixture kept at 3:1. One unit of aminoacylation activity is defined as the quantity of protein that catalyzes the incorporation of 1 nmol of amino acid onto tRNA<sup>Leu</sup> under the assay conditions. To assay the mischarging of noncognate amino acids, 1 mM L-[<sup>14</sup>C]methionine or L-[<sup>14</sup>C]-

Table 1: Specific Activities of LeuRS and Its Mutants at Different Sites in CP1 in the Crude Extract of *E. coli* TG1 Transformants<sup>a</sup>

enzyme	specific activity (unit/mg of protein)	relative activity (%)
LeuRS	280 ± 7	100
LeuRS-T252E	270 ± 5	96
LeuRS-A293F	130 ± 3	46
LeuRS-M328K	300 ± 9	107
LeuRS-T252E-M328K	290 ± 8	104

<sup>a</sup> The values are the mean (± standard error) of four independent determinations.

isoleucine (from Amersham) was used in the aminoacylation activity assay to replace the cognate substrate L-[<sup>14</sup>C]leucine. The concentration of purified LeuRS and the other mutants was 5 μM in the reaction mixture (13).

**Circular Dichroism Spectroscopy.** Protein samples (0.20 mg/mL) in 10 mM potassium phosphate buffer (pH 6.8) containing 2% glycerol were measured on a Jasco J-715 spectropolarimeter at room temperature. A 0.1 cm path-length cuvette was used and spectra were accumulated over five scans.

**Fluorescence Spectroscopy.** Fluorescence spectra were recorded with a Hitachi F4010 fluorescence spectrophotometer. Protein samples (0.10 mg/mL) in 10 mM potassium phosphate buffer (pH 6.8) containing 2% glycerol were excited at 295 nm (slit width 3 nm), and emission spectra were recorded in the 300–400 nm range.

**Assay of Melting Temperature.** The melting temperatures ( $T_m$ ) of LeuRS and its mutants were determined by monitoring the changes of  $A_{280}$  as a function of temperature.  $T_m$  is defined as the temperature at the midpoint of the absorption increase. There are 21 Trp and 36 Tyr in the enzymes, on which the  $A_{280}$  is mainly depends. The  $A_{280}$  of enzyme solutions was measured in a 1 mL mixture containing 0.30 mg/mL enzyme, 10 mM potassium phosphate buffer (pH 6.8), and 2% glycerol in the range of 30–70 °C with a heating rate of 0.5 °C/min on Beckman DU 7400 spectrophotometer.

## RESULTS

**Expression, Activity, and Purification of LeuRS and Its Mutants.** By introduction of restriction endonuclease sites, four gene mutants encoding LeuRS-T252E, LeuRS-A293F, LeuRS-M328K and LeuRS-T252E-M328K were cloned and confirmed by DNA sequencing. LeuRS and all four mutants were overproduced almost at the same level in *E. coli*. The specific activities of LeuRS and the mutants in the crude extract of *E. coli* TG1 transformants are shown in Table 1. Of the four mutants, only LeuRS-A293F lost about 50% activity. The double-site mutant and the other single-site mutants kept full activity. To determine why the site of A293 was so important, we evaluated several other A293 mutants, LeuRS-A293Y (aromatic residue), LeuRS-A293G (smaller residue), LeuRS-A293I (hydrophobic residue), LeuRS-A293R (positively charged residue), and LeuRS-A293D (negative charge residue). Their specific activities in the crude extract are shown in Table 2. All mutants at position 293 were decreased significantly in activity to different extents. Specially, the substitution of Asp for Ala decreased activity by 91% relative to the native enzyme. To study the effects of A293 mutations in more detail, LeuRS and the

Table 2: Specific Activities of LeuRS and Its Mutants at A293 in the Crude Extract of *E. coli* TG1 Transformants<sup>a</sup>

enzyme	specific activity (unit/mg of protein)	relative activity (%)
LeuRS	280 ± 7	100
LeuRS-A293R	196 ± 2	70
LeuRS-A293I	143 ± 6	51
LeuRS-A293Y	140 ± 5	50
LeuRS-A293G	140 ± 3	50
LeuRS-A293F	130 ± 3	46
LeuRS-A293D	24 ± 9	9

<sup>a</sup> The values are the mean (± standard error) of four independent determinations.

six single-site mutants at A293 were purified by two-step chromatography as described previously (23) and their kinetic constants were determined.

**Steady-State Kinetics of the Six Single-Site Mutants.** The aminoacylation kinetics of *E. coli* LeuRS and its six single-site mutants were assayed (Table 3). The  $K_m$  values of the six mutants for leucine were unchanged, and those for tRNA were changed slightly. However, the  $K_m$  values for ATP were decreased significantly to different extents. The data showed that the binding of ATP to all the six mutants was stronger than that to the native enzyme. The substitution of arginine with its positive charge caused a 50% decrease of  $K_m$  for ATP; however, the substitution of aspartic acid (negative charge) decreased  $K_{mATP}$  by 85% relative to the native enzyme, indicating the binding of ATP to LeuRS-A293D was the strongest of all six mutants. The change of 293A to 293D induced a 91% decline in the values of  $k_{cat}$  when compared with those of the native enzyme; however,  $k_{cat}$  values of the mutant replaced by arginine for three substrates were about 70% that of the native enzyme. The other four mutants exhibited about 50% values compared to the native enzyme.

Consequently, 293A appeared more important for the aminoacylation activity of LeuRS than any of the other substitutions A293D, A293R, A293G, A293I, A293Y and A293F. 293A is only involved in the binding of ATP, and all amino acid substitutions above caused stronger binding of ATP. Moreover, the negative charge at this site, induced by mutation A293D, is fatal to the aminoacylation activity while the positive charge (A293R) is helpful to maintain the activity.

**Inhibition of ATP to Leucylation of tRNA.** ATP is a substrate of aaRS, and in high concentrations it is an inhibitor of the enzyme (24). To further investigate the binding and inhibition of ATP to the mutants, the variations of aminoacylation activity of LeuRS and its mutants with the concentration of ATP were determined. The optimal concentration of ATP for aminoacylation of tRNA catalyzed by LeuRS-A293R was 4 mM, the same as that of native LeuRS. The substitutions A293Y, A293G, and A293I made the optimal concentration of ATP decline to 2 mM. For LeuRS-A293D and LeuRS-A293F, the optimal ATP concentration was 1 mM. The data suggested that the amino acid substitutions above, except for A293R, would make LeuRS more sensitive to ATP inhibition, which is consistent with the decline of  $K_m$  for ATP.

**Misaminoacylation of tRNA<sup>Leu</sup> Edited by LeuRS and the Other Mutants.** Evidence has shown that the native *E. coli*



Table 3: Kinetic Constants of LeuRS and Its Mutants<sup>a</sup>

enzyme	ATP			leu <sup>c</sup> ne			tRNA <sup>Leu</sup>		
	$K_m$ ( $\mu$ M)	$k_{cat}$ (s <sup>-1</sup> )	$k_{cat}/K_m$ (s <sup>-1</sup> mM <sup>-1</sup> )	$K_m$ ( $\mu$ M)	$k_{cat}$ (s <sup>-1</sup> )	$k_{cat}/K_m$ (s <sup>-1</sup> mM <sup>-1</sup> )	$K_m$ ( $\mu$ M)	$k_{cat}$ (s <sup>-1</sup> )	$k_{cat}/K_m$ (s <sup>-1</sup> mM <sup>-1</sup> )
LeuRS	280	5.0	18	15	5.2	347	2.2	5.1	2318
LeuRS-A293R	140	3.5	25	14	3.5	230	1.8	3.7	2056
LeuRS-A293Y	100	2.7	27	15	2.5	167	1.5	2.4	1600
LeuRS-A293G	99	2.7	27	15	2.4	160	1.2	2.5	2083
LeuRS-A293I	74	2.8	38	15	2.6	173	2.2	2.4	1091
LeuRS-A293F	62	2.4	39	13	2.5	192	2.1	2.2	1048
LeuRS-A293D	42	0.46	11	13	0.45	35	2.0	0.42	210

<sup>a</sup> The enzymes were purified to more than 95% homogeneity from *E. coli* TG1 transformants carrying overproduced plasmids as described under Experimental Procedures. The specific activity of tRNA<sup>Leu</sup> used in the experiments was 1.6 nmol/A<sub>260</sub>. All the data in this table were the average values with a variation of <4% from three independent determinations.

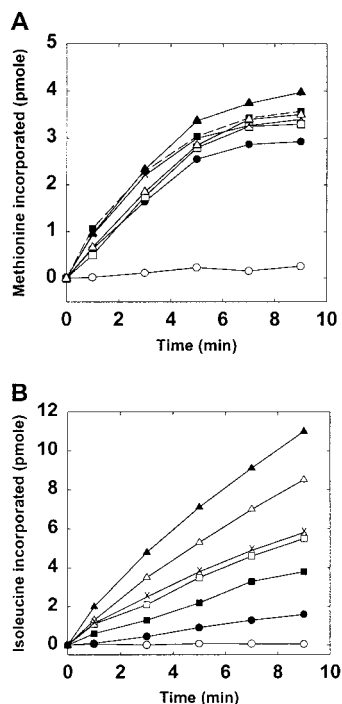


FIGURE 2: Methionylation and isoleucylation of tRNA<sup>Leu</sup> by *E. coli* LeuRS and its mutants. (A) Methionylation at 37 °C, pH 7.8, of 20  $\mu$ M tRNA<sup>Leu</sup> in the presence of 1 mM [<sup>14</sup>C]methionine by 5  $\mu$ M *E. coli* LeuRS (○), 5  $\mu$ M LeuRS-A293R (●), 5  $\mu$ M LeuRS-A293D (▲), 5  $\mu$ M LeuRS-A293I (■), 5  $\mu$ M LeuRS-A293G (□), 5  $\mu$ M LeuRS-A293Y (×), and 5  $\mu$ M LeuRS-A293F (Δ). (B) Isoleucylation at 37 °C, pH 7.8, of 20  $\mu$ M tRNA<sup>Leu</sup> in the presence of 1 mM [<sup>14</sup>C]isoleucine by 5  $\mu$ M *E. coli* LeuRS (○), 5  $\mu$ M LeuRS-A293R (●), 5  $\mu$ M LeuRS-A293D (▲), 5  $\mu$ M LeuRS-A293I (■), 5  $\mu$ M LeuRS-A293G (□), 5  $\mu$ M LeuRS-A293Y (×), and 5  $\mu$ M LeuRS-A293F (Δ).

LeuRS could misactivated Met and Ile and transfer the incorrect amino acids on tRNA<sup>Leu</sup> (13). To give insight into the role of 293A in the editing function of the CP1 domain in *E. coli* LeuRS, the misaminoacylation of tRNA<sup>Leu</sup> was assayed in the presence of 1 mM [<sup>14</sup>C]methionine or [<sup>14</sup>C]-isoleucine instead of 0.1 mM [<sup>14</sup>C]leucine (Figure 2). It appears that tRNA<sup>Leu</sup> could be methionylated or isoleucylated by all of the 293A amino acid substitution mutants but not by LeuRS, indicating that all the mutants had an impaired editing function for correcting the errors in the aminoacylation reaction, which was brought about by a single amino acid substitution in the CP1 domain. LeuRS-A293D was the most greatly impaired in its editing function and LeuRS-A293R was the least affected, which was consistent with

Table 4: Thermal Denaturation of LeuRS and the Mutants<sup>a</sup>

enzyme	$T_m$ (°C)	difference (°C)
LeuRS-A293Y	55.5 $\pm$ 0.3	1.5
LeuRS-A293I	55.0 $\pm$ 0.2	1.0
LeuRS-A293F	54.5 $\pm$ 0.2	0.5
LeuRS-A293G	54.0 $\pm$ 0.3	0.0
LeuRS-A293R	54.0 $\pm$ 0.3	0.0
LeuRS	54.0 $\pm$ 0.2	0.0
LeuRS-A293D	49.5 $\pm$ 0.2	4.5

<sup>a</sup> The  $T_m$  values of enzymes were determined by monitoring the changes of A<sub>280</sub> as a function of temperature. The A<sub>280</sub> of enzyme was measured in a 1 mL mixture containing 0.30 mg/mL enzyme, 10 mM potassium phosphate buffer (pH 6.8), and 2% glycerol in the range of 30–70 °C with a heating rate of 0.5 °C/min on a Beckman DU 7400 spectrophotometer. The  $T_m$  values are the means ( $\pm$  standard error) for three independent determinations.

the effect on the enzymes' aminoacylation activities by the mutations. Moreover, the misaminoacylation rate for Ile was much higher than that for Met. These data indicate that 293A in the CP1 domain is crucial for the editing function of *E. coli* LeuRS and any amino acid substitution will result in an impaired editing function.

**Melting Temperature of LeuRS and Mutants.** To get more information about the effect on the enzyme's conformation of amino acid substitution,  $T_m$  values of LeuRS and mutants were determined (Table 4).  $T_m$  of LeuRS-A293D decreased 4.5 °C compared with the native; its change is the largest. However, LeuRS-A293R and -293G had  $T_m$  values equal to the native (54.0 °C) and the  $T_m$  values of LeuRS-A293I, -293F, and -293Y were slightly higher than that of the native. This suggests that the negative charge of 293D loosened the enzyme's conformation. However, the mutants with 293I, 293F, or 293Y, all of which are more hydrophobic residues than Ala, had more rigid conformations than the native LeuRS. In both cases the catalysis and the editing function of the enzyme were impaired. Although the conformation of LeuRS-A293R and -293G seems unchanged, the changes of the residue size and charge result in low activity and poor editing.

**Comparison of CD Spectra of LeuRS and Mutants.** To determine whether the mutations affected the secondary structure of LeuRS, the CD spectra of LeuRS and its mutants were measured. The CD spectra of LeuRS and the six mutants showed that mutation had no effect on the overall structure of LeuRS. Although the secondary structure of mutants were grossly similar to that of native LeuRS, the declined catalytic activity and impaired editing function of

Table 5: Fluorescence Emission Spectra Parameters of LeuRS and Its Mutants<sup>a</sup>

enzyme	emission wavelength max (nm)	fluorescence intensity max	center of mass (cm <sup>-1</sup> )
LeuRS	336.8	82.51	29366
LeuRS-A293R	337.0	80.34	29342
LeuRS-A293G	336.9	84.12	29364
LeuRS-A293Y	336.9	80.89	29362
LeuRS-A293I	336.6	79.95	29387
LeuRS-A293F	336.6	84.85	29385
LeuRS-A293D	338.0	83.47	29302

<sup>a</sup> Protein samples (1.0  $\mu$ M) in 10 mM potassium phosphate buffer (pH 6.8) containing 2% glycerol were excited at 295 nm (3 nm slit), and emission spectra were recorded in the 300–400 nm range.

the mutants implied that subtle conformational changes not detectable by CD spectra were present.

**Comparison of the Fluorescence Emission Spectra of LeuRS and Its Mutants.** To investigate more subtle conformational differences, fluorescence emission spectra were measured. The center of mass and the fluorescence emission maximum of LeuRS-A293D were red-shifted compared with those of the native, while the parameters of the other mutants were almost unchanged (Table 5). The data suggested that a negative charge induced by amino acid substitution A293D led to a less hydrophobic environment of chromophore and a relatively looser conformation, which might be the reason for the decline of the aminoacylation activity and the poor editing function.

## DISCUSSION

In the present study, a series of LeuRS mutants at different sites in CP1 were constructed to reveal some function of the CP1 domain of LeuRS. The single-site mutants at A293 were studied extensively because only mutants at this position had an effect on the aminoacylation activity of LeuRS.

The sequence alignment for LeuRSs from different organisms showed that the site of 293A is conserved as a positively charged amino acid: 8 of the 11 are either Arg or Lys, while the other three are Ala (Figure 1). On the basis of the crystal structure of LeuRS from *T. thermophilus* and the alignment results, 293A was located at a small helix in the editing domain of the enzyme and on the side facing the active site (19). Because the amino acid residues at 292 and 294 were all E in *E. coli* LeuRS, with negative charge, the amino acid substitution of D (negative charge) for A will result in the accumulation of negative charge, greatly affecting the stability of the helix. Therefore, among the six single-site amino acid substitution mutants at A293, LeuRS-A293D has the lowest aminoacylation activity and the largest change in  $T_m$ , and it shows red-shifted fluorescence emission spectra. The data confirmed that the negative charge at 293 disturbed the helix and even the enzyme's conformation; thus the activity and editing of this enzyme were affected significantly. In contrast to LeuRS-A293D, LeuRS-A293R has the highest activity and is least affected, which proved that the positive charge at 293 is helpful in maintaining the structure and function of LeuRS.

Substitution of hydrophobic residues at 293 made for a more rigid conformation of LeuRS and resulted in poor

catalysis and editing. Because the size and the charge at 293 were changed, the formation of this helix was affected also, which resulted in the decreased activities of LeuRS-A293R and -A293G, even though the  $T_m$  remained unchanged. When any amino acid (larger, smaller, basic, acidic, and aromatic amino acids) replaced alanine, the helix and even the CP1 domain will have a change in their structures. So an alanine at 293 is important for maintaining the definite structure of CP1 domain, which is crucial for the catalysis and editing of the enzyme.

The steady-state kinetic study on the six single-site mutants at A293 and the native LeuRS showed that all the mutants could bind ATP much more strongly in comparison with the native enzyme. LeuRS-A293D had the strongest ATP binding of the mutants. The enhanced inhibition effect by ATP confirmed the significant change in ATP binding caused by the single-site mutation. The A293 of LeuRS is located at a helix facing the catalytic center on the surface of CP1 domain of the enzyme (18, 19). ATP is accommodated in a cleft within the nucleotide-binding domain of LeuRS. The stronger binding and enhanced inhibition of ATP on mutants might come from the site mutagenesis through allosteric mechanisms. The  $T_m$  changes of the mutants confirmed that the mutants had subtle conformational differences from the native enzyme.

In our previous work, we demonstrated that LeuRS was attacked specifically at the peptide bond between E292 and A293. The cleaved LeuRS lost its tRNA charging activity, while its leucine activation activity was almost unchanged. In vivo assembly of LeuRS split between E292 and A293 was unstable, although in vivo assembly of enzymes split at other sites has been successful (18). The region around E292–A293 must be located on the surface of the enzyme and may play a significant role in maintaining the proper conformation of LeuRS for its function. The results of the single-site mutations at A293 in this report also indicate this region might play an important structural role.

In conclusion, our results show that the A293 is located in an important helix in the CP1 of LeuRS. Replacement with any other amino acid residue leads to impaired activity and editing. A293 is indirectly involved in the binding of ATP on the enzyme. And its replacement by a residue with a negative charge caused the largest change of the functional conformation. CP1 domain should be a multifunction domain and its definite structure is crucial for the editing function, aminoacylation activity, tRNA discrimination (16), and ATP binding.

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## REFERENCES

- Schimmel, P. (1987) *Annu. Rev. Biochem.* 56, 125–158.
- Burbaum, J. J., Starzyk, R. M., and Schimmel, P. (1990) *Proteins: Struct., Funct., Genet.* 7, 99–111.
- Carter, C. W., Jr. (1993) *Annu. Rev. Biochem.* 62, 715–748.
- Ruff, M., Krishnaswamy, S., Boeglin, M., Poterszman, A., Mischler, A., Podjarny, A., Rees, B., Thierry, J. C., and Moras, D. (1991) *Science* 252, 1682–1689.
- Eriani, G., Delarue, M., Poch, O., Gangloff, J., and Moras, D. (1990) *Nature* 347, 203–206.

6. Brick, P., Bhat, T. N., and Blow, D. M. (1989) *J. Mol. Biol.* 208, 83–98.
7. Brunie, S., Zeluer, C., and Risler, J. L. (1990) *J. Mol. Biol.* 216, 411–424.
8. Rould, M. A., Perona, J. J., Söll, D., and Steitz, T. A. (1989) *Science* 246, 1135–1142.
9. Starzyk, R. M., Webster, T. A., and Schimmel, P. (1987) *Science* 237, 1614–1618.
10. Schimmel, P., and Ribas de Pouplana, L. (1995) *Cell* 81, 983–986.
11. Härtlein, M., and Madern, D. (1987) *Nucleic Acids Res.* 4, 10199–10210.
12. Schimmel, P., Shepard, A., and Shiba, K. (1992) *Protein Sci.* 1, 1387–1391.
13. Chen, J. F., Guo, N. N., Li, T., Wang, E. D., and Wang, Y. L. (2000) *Biochemistry* 39, 6726–6731.
14. Lin, L., Hale, S. P., and Schimmel, P. (1996) *Nature* 384, 33–34.
15. Hale, S. P., Auld, D. S., Schmidt, E., and Schimmel, P. (1997) *Science* 276, 1250–1252.
16. Li, T., Li, Y., Guo, N. N., Wang, E. D., and Wang, Y. L. (1999) *Biochemistry* 38, 9084–9088.
17. Wakasugi, K., Quinn, C., Tao, N., and Schimmel, P. (1998) *EMBO J.* 17, 297–305.
18. Li, T., Guo, N. N., Xia, X., Wang, E. D., and Wang, Y. L. (1999) *Biochemistry* 38, 13063–13069.
19. Cusack, S., Yaremchuck, A., and Tukalo, M. (2000) *EMBO J.* 19, 2351–2361.
20. Li, Y., Wang, E. D., and Wang, Y. L. (1998) *Sci. China (Ser. C)* 41, 225–231.
21. Deng, L., Li, B., Shi, J. P., and Wang, Y. L. (1994) *Acta Biochim. Biophys. Sin.* 26, 223–228 (in Chinese).
22. Amann, E., and Brosius, J. (1985) *Gene* 40, 183–190.
23. Li, T., Wang, E. D., and Wang, Y. L. (1997) *Acta Biochim. Biophys. Sin.* 29, 591–596 (in Chinese).
24. Schimmel, P., and Söll, D. (1979) *Annu. Rev. Biochem.* 48, 601–648.

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