Discrimination of $tRNA^{Leu}$ Isoacceptors by the Insertion Mutant of *Escherichia coli* Leucyl-tRNA Synthetase†

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ABSTRACT: A variant (LeuRS-A) of *Escherichia coli* leucyl-tRNA synthetase (LeuRS) carrying a 40-residue duplication in its connective peptide 1 (CP1) has a 3-fold lower specificity for $tRNA_1^{Leu}$ than for $tRNA_2^{Leu}$, whereas wild-type LeuRS has the same specificity for these two isoacceptors. The replacement of the acceptor stem of $tRNA_2^{Leu}$ with $tRNA_1^{Leu}$ yields a chimeric $tRNA^{Leu}$ for which wild-type LeuRS has the same specificity as it does for the two normal isoacceptors mentioned, but for which LeuRS-A has a reduced specificity similar to that for $tRNA_1^{Leu}$, indicating a difference between these two acceptor stems. LeuRS-A is slightly less stable than the native enzyme. Wild-type LeuRS and LeuRS-A have almost same K_d value for their interaction with $tRNA_1^{Leu}$ as determined by fluorescence quenching. No difference was detected between these two proteins by CD and fluorescence spectroscopy. These results show that LeuRS-A can discriminate between the two isoacceptors of $tRNA^{Leu}$.

The genetic code established more than 1 billion years ago has been adopted universally by all living organisms with little variation. The connection between amino acids and a specific tRNA is accomplished through the aminoacylation reaction catalyzed by aminoacyl-tRNA synthetases (aaRSs, EC6.1.1) (*1*−*6*). These enzymes are among the oldest proteins on earth (*7*). They are specific to both amino acid and tRNA substrates and ensure the high fidelity required by translation (*8*). The alignments of the sequences of aaRSs and tRNAs have been widely used to study their specific interactions. The 20 aaRSs can be divided into two classes, of 10 members each, on the basis of conserved sequence and characteristic structural motifs (*9*). Connective polypeptide 1 (CP1) is an unconserved region lying behind the third β-strand in class I aaRSs (*10*, *11*).

The gene encoding leucyl-tRNA synthetase (LeuRS, EC 6.1.1.4) from *Escherichia coli* was cloned (*12*). The sequence of LeuRS which has 860 amino acid residues was deduced from its gene. LeuRS, isoleucyl-tRNA synthetase (IleRS), methionyl-tRNA synthetase (MetRS), valyl-tRNA synthetase (ValRS), and cysteinyl-tRNA synthetase (CysRS) belong to a subgroup of class I aaRSs and have CP1s of variable lengths (*13*). On the basis of sequence alignments, CP1 of LeuRS extends from residue 126 to 389 (*14*).

Each synthetase attaches its amino acid to the tRNA isoacceptors which contain the anticodon complementary to the codon for the amino acid (*15*, *16*). There are six codons for leucine, and the six $tRNA^{Leu}$ isoacceptors are specifically aminoacylated by LeuRS (*17*, *18*). Identity element studies on $tRNA^{Leu}$ show that the anticodon, the variable stem loop, and the acceptor stem sequence are not essential for specific recognition by LeuRS (*19*). Asahara et al. suggested that the structural features of $tRNA^{Leu}$ and a few specific nucleotides of $tRNA^{Leu}$ determine its specific recognition by LeuRS. The structure of an aaRS plays an important role in its recognition of its cognate tRNA. The unusually large CP1 of LeuRS is of interest, as CP1 is thought to be the binding site of the tRNA acceptor stem. While studying the function of CP1 of LeuRS, we identified a mutant of LeuRS with a duplication of the 40 amino acid residues from F253 and E292 in the CP1 domain and named it LeuRS-A. LeuRS-A has the ability to discriminate between the two isoacceptors, $tRNA_1^{Leu}$ and $tRNA_2^{Leu}$, and here, we describe the preparation of LeuRS-A and its discrimination between $tRNA^{Leu}$ isoacceptors.

MATERIALS AND METHODS

Materials. E. coli $tRNA_1^{Leu}$ and $tRNA_2^{Leu}$ which have a charging capacity of about 1600 pmol/A_{260} unit were purified as described previously (*20*). L-Leucine, DTT, ATP, DEAE-Sepharose CL-6B, and HA-Ultrogel were purchased from Sigma. L-[^{14}C]Leucine (300−400 mCi/mmol) and [α-^{32}P]-ATP were obtained from Amersham. The *leuS* temperature-sensitive strain KL231 (*F*$^-$, *leuS31*, *thyA6*, *rpsL120*) was obtained from the *E. coli* Genetic Stock Center (Yale University, New Haven, CT). T7 RNA polymerase was purified from an *E. coli* strain carrying a T7 RNA polymerase overproducing plasmid, a gift from H. Asahara (*21*). The gene encoding *E. coli* LeuRS, *leuS*, was cloned in our laboratory (*22*).

Construction of Plasmids and Transformation of E. coli. By PCR, using *leuS* as a template, six pairs of DNA fragments encoding the N- and C-terminal parts of LeuRS were amplified with the appropriate of restrictive endonu-

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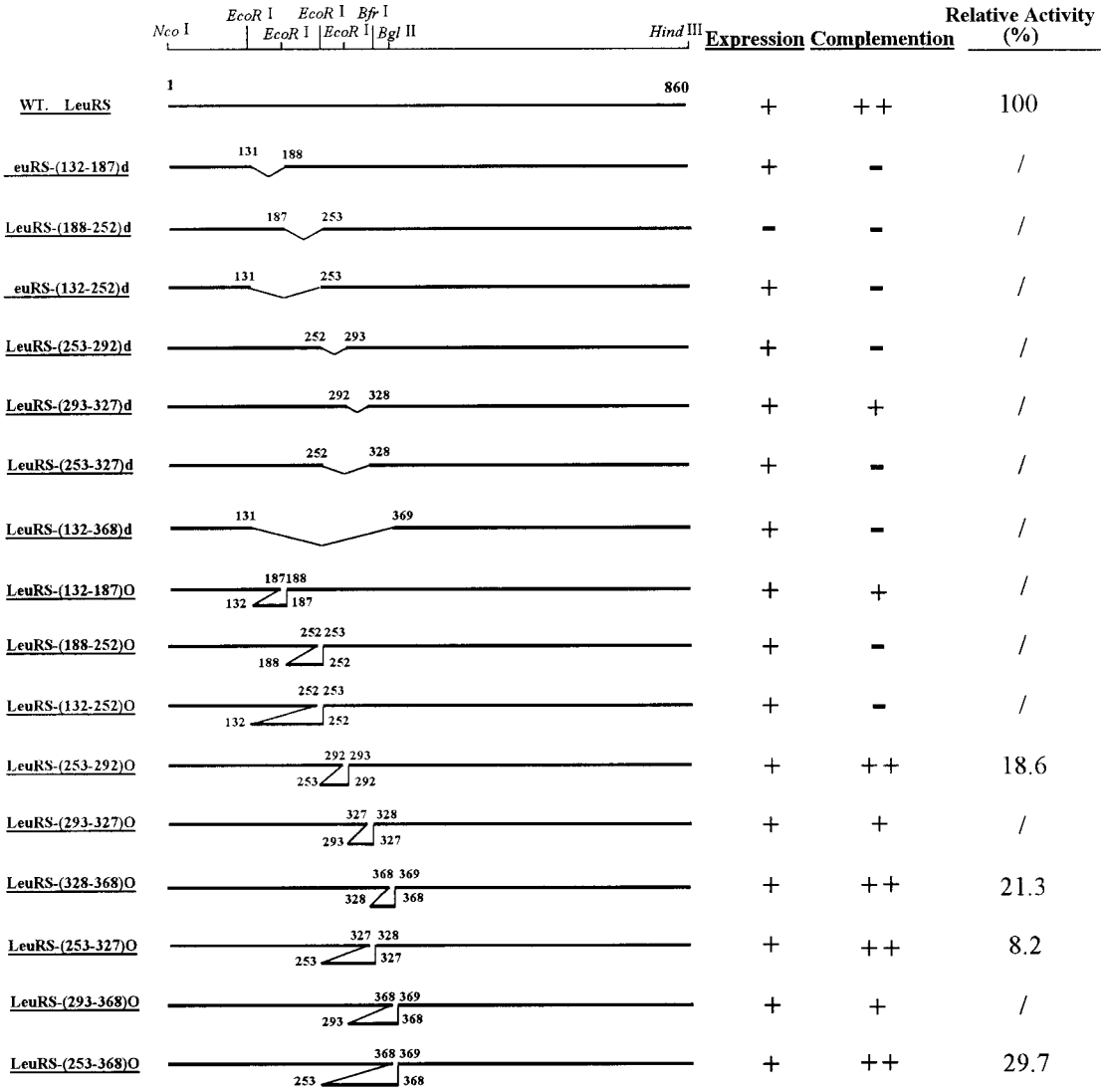

FIGURE 1: Deletion and insertion mutants in the CP1 domain of LeuRS. The top line illustrates the *leu*S coding region and the restriction sites that were used for deletion and insertion mutations. The native and mutant peptides of LeuRS are represented by thick lines, and the residue numbers of the insertion and deletion sites are given. The expression of the mutant genes was tested by SDS−PAGE of the crude extract of TG1 transformants. Strain KL231 was used to test the aminoacylation activity of LeuRS mutants on the basis of their growth on LB plates at 44 °C, while the relative activity was measured by the specific activity of the TG1 transformants compared with that of wild-type *leu*S. The results of the complementation experiment are illustrated as follows: /, no growth at 44 °C; +, much slower growth; and ++, normal growth.

clease sites. The six joints were located between E131 and Y132, E187 and I188, T252 and F253, E292 and A293, K327 and A328, and P368 and D369. Both ends of the DNA fragment encoding the N-terminal part of LeuRS contain *Nco*I and *Eco*RI/*Bfr*I/*Bgl*II restriction sites, and those encoding the C-terminal part contain *Eco*RI/*Bfr*I/*Bgl*II and *Hin*dIII sites. They were ligated to form genes encoding deletion and insertion mutants of LeuRS as shown in Figure 1. The mutant genes were inserted into plasmid pKK236 which was derived from pKK233-2. DNA sequences of the *leuS* mutants were confirmed by dideoxy sequencing. The changes of amino acid residues caused by the restriction sites were Y132F, T252E, A293F, A328K, P368R, and D369S. These changes did not affect the activity of LeuRS significantly (unpublished data).

In Vivo Complementation Assays. Positive clones could easily be selected. After transformation, *E. coli* KL231 could grow at 44 °C, showing the presence of a cloned gene encoding active LeuRS.

Disruption of Cells and Preparation of Crude Extracts. E. coli TG1 and its transformants harboring the recombinant plasmid were grown in 25 mL of LB or 25 mL of LB containing 1.25 μg of ampicillin at 37 °C to a stationary phase. Cells were harvested by centrifugation, suspended in 4 mL of disruption buffer [100 mM Tris-HCl, 10 mM $MgCl_2$, and 1 mM EDTA (pH 7.5)], and sonicated for 6 × 20 s at 15 W with a High-Intensity Ultrasonic Processor (375 W model). The lysate was ready for use after centrifugation at 10 000 rpm at 4 °C for 10 min.

Purification of LeuRS and Its Mutants. The enzymes, overproduced in the *E. coli* transformants containing *leuS* and its mutants, were purified by two-step chromatography on DEAE-Sepharose CL-6B and HA-Ultrogel columns, according to the method of Li et al. (*23*).

Assay of Enzyme Activity. The aminoacylation activity of LeuRS was measured as previously described (*24*). One unit of aminoacylation activity is defined as the quantity of protein catalyzing the incorporation of 1 nmol of amino acid

Table 1: Activity of LeuRS Mutants Catalyzed Aminoacylation of $tRNA_1^{Leu}$ and $tRNA_2^{Leu}$ [a]

	specific activity (units/mg)		$V_{tRNA_2^{Leu}}/V_{tRNA_1^{Leu}}$ (fold)
	$tRNA_1^{Leu}$	$tRNA_2^{Leu}$	
wild-type LeuRS	472	463	0.98
LeuRS(253−292)O	69	199	2.89
LeuRS(253−327)O	41	76	1.86
LeuRS(253−368)O	92	122	1.33
LeuRS(328−368)O	121	123	1.00

[a] The enzymes were partially purified with an FPLC Superose-12 column. In the second column, $V_{tRNA_2^{Leu}}$ or $V_{tRNA_1^{Leu}}$ represents the specific activity of the enzyme when $tRNA_2^{Leu}$ or $tRNA_1^{Leu}$ was used as the substrate, respectively. The charging activities of $tRNA_1^{Leu}$ and $tRNA_2^{Leu}$ were 1.6 nmol/OD_{260} purified from the MT102 transformants harboring the genes encoding these two tRNAs (*20*).

into tRNA per minute under the assay conditions. The specific activity was defined as units per milligram of protein. The concentration of purified LeuRS was determined by the A_{280} of the enzyme solution. About 1.62 mg/mL protein equaled 1 optical density unit at 280 nm.

Enzyme Stability at 55 °C and in Urea Solutions. The enzyme (40 μg/mL) in 50 mM potassium phosphate buffer (pH 6.8) containing 400 μg/mL BSA was incubated at 55 °C for different periods of time. The aminoacylation activity was assayed after the reaction mixture was diluted with cold 50 mM potassium phosphate buffer.

Measurement of Spectra. Fluorescence spectra were measured with a Hitachi F 4010 spectrofluorometer. The emission spectrum was measured and corrected, with the excitation wavelength being 295 nm. The excitation spectrum was measured from 260 to 320 nm, with the emission wavelength being 338 nm. The final concentration of the enzyme was 90 μg/mL (1.34 μm). CD spectra from 200 to 240 nm were measured at 25 °C with a Jasco-20C spectropolarimeter. The enzyme, 0.126 mg/mL in 10 mM potassium phosphate (pH 7.5), was placed in a 0.1 cm cuvette.

Transcription of tRNA in Vitro. The plasmid carrying the T7 promoter and the $tRNA_1^{Leu}$ gene was a gift from H. Asahara. Various $tRNA_1^{Leu}$ mutant genes were obtained by using polymerase chain reaction. Plasmids carrying the $tRNA_1^{Leu}$ gene or its mutant genes were prepared by similar procedures (*19*). The tRNA gene was transcribed in a reaction mixture containing 40 mM Tris-HCl (pH 8.0), 5 mM dithiothreitol, 10 mM $MgCl_2$, 2 mM NTPs, 10 mM 5′-GMP (Sigma), *Bst*NI-digested template DNA (50 μg/mL), 1 unit/mL inorganic pyrophosphatase (Sigma), and 2 mg/mL pure T_7 RNA polymerase. The transcripts were purified by 20% (w/v) denaturing polyacrylamide gel electrophoresis (*19*).

RESULTS

Expression of Genes Encoding LeuRS Mutants. Seven deletion mutants and eight insertion mutants were obtained. All the mutants of LeuRS except one deletion mutant could be overproduced in *E. coli* transformants. Proteins with the correct molecular mass could be detected by SDS−PAGE. *E. coli* KL231 was transformed with the recombinant plasmids containing the *leuS* mutants to test the activity of each LeuRS mutant in vivo. The data are shown in Figure 1. Most deletion mutants could not complement *E. coli* KL231; only that harboring LeuRS(293−327)d could grow with a slow rate at 44 °C. All insertion mutants in the CP1 region, except two, were active. In crude extract, four of them exhibited aminoacylation activity, when assayed in vitro.

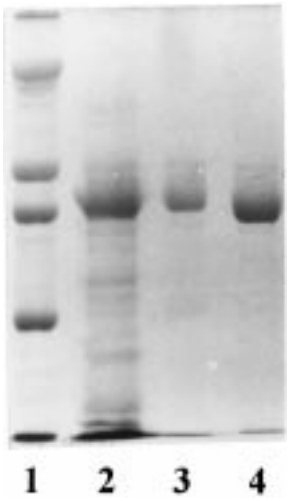

FIGURE 2: SDS−PAGE of insertion mutant LeuRS-A. Lane 1 contains molecular mass markers from Sigma, with molecular masses of 208, 117, 97, 66, 45, and 29 kDa. Lane 2 contains proteins in the crude extract of transformants containing *leuS-A*. Lane 3 contains purified LeuRS-A. Lane 4 contains purified LeuRS. The amount of protein in each lane was 5 μg.

Table 2: Kinetic Constants of LeuRS and LeuRS-A[a]

substrate	constant	LeuRS	LeuRS-A
leucine	K_m (μM)	20	16
	k_{cat} (s^{-1})	9.0	3.0
	k_{cat}/K_m (s^{-1} mM^{-1})	450	213
ATP	K_m (μM)	380	260
	k_{cat} (s^{-1})	9.8	3.6
	k_{cat}/K_m (s^{-1} mM^{-1})	26	14
$tRNA_1^{Leu}$	K_m (μM)	2.5	2.0
	k_{cat} (s^{-1})	9.7	1.0
	k_{cat}/K_m (s^{-1} mM^{-1})	3880	500
$tRNA_2^{Leu}$	K_m (μM)	2.0	1.7
	k_{cat} (s^{-1})	9.6	3.2
	k_{cat}/K_m (s^{-1} mM^{-1})	4800	1941

[a] The enzymes were purified to more than 95% homogeneity from *E. coli* TG1 transformants as described in Materials and Methods.

Aminoacylation Activity of Insertion Mutants for the Two Isoacceptors, $tRNA_1^{Leu}$ and $tRNA_2^{Leu}$. Using FPLC on a Superose-12 column, the four insertion mutants were partially purified. The aminoacylation activities of the four insertion mutants on the two isoacceptors of $tRNA_1^{Leu}$ and $tRNA_2^{Leu}$ are shown in Table 1. The most interesting mutant was LeuRS(253−292)O, which has a duplication of the fragment from residue 253 to 292 and can catalyze the aminoacylation of $tRNA_1^{Leu}$ and $tRNA_2^{Leu}$ at different rates. This molecule was defined as LeuRS-A. Native LeuRS catalyzes the aminoacylation of the two isoacceptors of $tRNA^{Leu}$ at the same rate.

Kinetic Constants of LeuRS-A. After a two-step purification by column chromatography, LeuRS-A was homogeneous as determined by SDS−PAGE (Figure 2). Its kinetic constants were measured and are shown in Table 2. The specific aminoacylation activity of purified LeuRS-A was lower than that of the native enzyme (2900 units/mg); however, it exhibited different values when different $tRNA^{Leu}$s were used

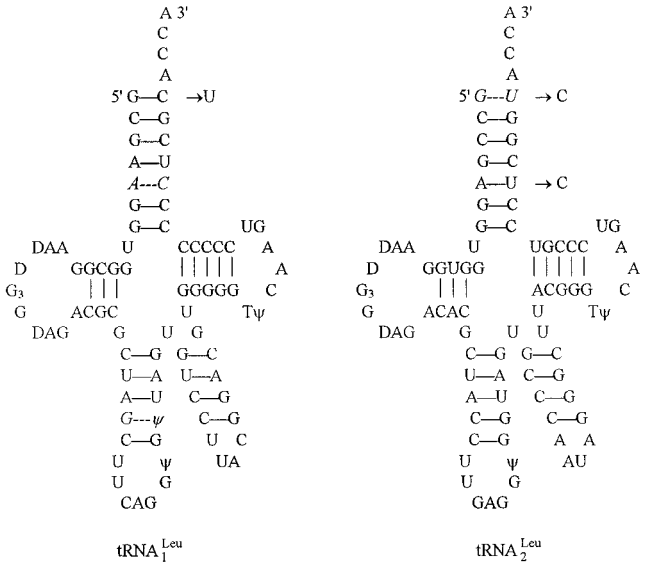

FIGURE 3: Comparison of *E. coli* tRNA$_1^{Leu}$ and tRNA$_2^{Leu}$ structures. tRNA$_1^{Leu}$ (left) and tRNA$_2^{Leu}$ (right) are shown in schematic cloverleaf form. Wobble base pairs in both tRNA^{Leu} forms are italic. C72 in the first base pair of the acceptor stem in tRNA$_1^{Leu}$ was mutated to U72 to mimic that of tRNA$_1^{Leu}$. U72 and U68 in the first and fifth base pairs, respectively, of the acceptor stem in tRNA$_2^{Leu}$ were both separately mutated to C to mimic that of tRNA$_1^{Leu}$.

as the substrate: 490 units/mg for total tRNA, 300 units/mg for tRNA$_1^{Leu}$, and 950 units/mg for tRNA$_2^{Leu}$. The K_m values of LeuRS-A for leucine, ATP, tRNA$_1^{Leu}$, and tRNA$_2^{Leu}$ were similar to those of the native enzyme. The k_{cat} for tRNA$_2^{Leu}$ decreased to $^1/_3$, and for tRNA$_1^{Leu}$ to $^1/_9$, of that of the native enzyme. The k_{cat} values of tRNA$_1^{Leu}$ and tRNA$_2^{Leu}$, catalyzed by LeuRS-A, were 1.0 and 3.2 s^{-1}, respectively, while the k_{cat} values of the native enzyme for both tRNAs were 9.6 s^{-1} (*25*). The k_{cat}/K_m values of purified LeuRS-A were 500 and 1900 s^{-1} mM^{-1} for tRNA$_1^{Leu}$ and tRNA$_2^{Leu}$, respectively, while those of LeuRS were 3800 and 4700 s^{-1} mM^{-1}, respectively. These results indicated that the mutation had little effect on the binding of LeuRS-A to the two isoacceptors of tRNA^{Leu}. An internal fluorescence titration also indicated that the dissociation constant, K_d, of the complex of LeuRS-A and tRNA$_1^{Leu}$ was 2.5 μM which is almost the same as the value of 2.0 μM for the native complex. The tRNA binding ability was almost unchanged when compared with that of the native enzyme.

Structural Comparison between LeuRS-A and LeuRS. No obvious change of conformation was detected in the mutant by CD and fluorescence spectroscopy (data not shown), so it might be expected that LeuRS-A would retain its leucylation activity. However, subtle conformational changes must be present, as the mutant could discriminate between the two isoacceptors of tRNA^{Leu} and was also less stable than the native enzyme at higher temperatures and in urea solutions (data not shown).

Leucylation of tRNA^{Leu} Mutants Catalyzed by LeuRS and LeuRS-A. The difference between the wobble base pairs of tRNA$_1^{Leu}$ and tRNA$_2^{Leu}$ is mainly located in the acceptor stems (Figure 3). The first base pair in the acceptor stem of tRNA$_2^{Leu}$ is the wobble base pair G1·U72, and the fifth base pair in tRNA$_1^{Leu}$ is the wobble base pair A5·C68. When the first wobble pair of tRNA$_2^{Leu}$ was substituted with the standard Watson−Crick base pair G1·C72 of tRNA$_1^{Leu}$, the k_{cat} value of LeuRS-A for tRNA$_2^{Leu}$ was seriously decreased, even slightly below that for tRNA$_1^{Leu}$. However, when the fifth base pair of tRNA$_2^{Leu}$ was mutated to A5·C68, the k_{cat} value was slightly higher than that for the native tRNA$_2^{Leu}$. When the acceptor stem of tRNA$_2^{Leu}$ was replaced by that of tRNA$_1^{Leu}$, the rate of leucylation by LeuRS-A was the same as that for tRNA$_1^{Leu}$ (Table 3). It seems therefore that the different acceptor stems in the two tRNA^{Leu} isoacceptors cause the different rates of leucylation catalyzed by LeuRS-A, and the flexibility of the acceptor stem of tRNA^{Leu} might play a key role in its recognition by LeuRS-A. However, LeuRS catalyzed the aminoacylation of tRNA$_1^{Leu}$, tRNA$_2^{Leu}$, and the acceptor stem hybrid tRNA^{Leu} at the same rate. When the first wobble base pair in the acceptor stems of tRNA$_2^{Leu}$ was changed to a standard base pair or the first base pair in the acceptor of tRNA$_1^{Leu}$ changed to a wobble base pair, the rate of leucylation catalyzed by native LeuRS decreased slightly. The above results suggested that the acceptor stem of the tRNAs might interact with CP1 of the enzyme. Some change in its conformation, induced by the inserted peptide, might be responsible for the discrimination of the distinctive acceptor stems in the two isoacceptors of tRNA^{Leu}.

Table 3: k_{cat} Values of tRNA Mutants Catalyzed by LeuRS and LeuRS-A[a]

	k_{cat} (s^{-1})	
	LeuRS	LeuRS-A
tRNA$_1^{Leu}$	4.4	0.76
tRNA$_2^{Leu}$	4.2	2.00
tRNA$_2^{Leu}$ with the acceptor stem of tRNA$_1^{Leu}$	4.7	0.71
tRNA$_2^{Leu}$ with U72 → C	3.6	0.46
tRNA$_2^{Leu}$ with U68 → C	5.0	2.2
tRNA$_1^{Leu}$ with C72 → U	3.9	3.0

[a] The data are average values with a variation of <15% from three independent determinations.

Indeed, a wobble base pair might adopt different conformations in different tRNA molecules (*26*), and this might allow discrimination by the mutant LeuRS-A. Finally, when the acceptor stem of tRNA$_2^{Leu}$ was changed to that of tRNA$_1^{Leu}$, there was no discrimination between tRNA$_1^{Leu}$ and tRNA$_2^{Leu}$ by LeuRS-A.

DISCUSSION

The CP1 domain is located between the third and fourth β-strands of some of the class I aaRSs (*10*). In *E. coli* glutaminyl-tRNA synthetase (GlnRS), the CP1 domain, which has 110 amino acid residues, binds the acceptor helix of tRNA^{Glu} and has been named the acceptor helix-binding domain (*27*). MetRS and CysRS may also have a similar tertiary structure that interacts with the acceptor stem microhelix of their cognate tRNAs (*11*). In IleRS and ValRS, the CP1 domain is involved in RNA-dependent amino acid recognition as shown by the editing of mischarged and misactivated amino acids (*28*, *29*). A study on species-specific aminoacylation of tyrosyl-tRNA synthetase and

$tRNA^{Tyr}$ showed that a small segment within the CP1 domain was a critical determinant of species specificity, and CP1 also played a role in determining the specificity of acceptor helix recognition (*30*).

The CP1 of LeuRS has not been studied extensively yet. We have obtained a series of LeuRS mutants with insertion and deletion mutations in the CP1 domain. All deletion mutants cannot be expressed in their transformants. Four insertion mutants have various aminoacylation activities. LeuRS-A, with a 40-amino acid duplication of the region between 253 and A292, was the most interesting one. Although LeuRS-A had reduced activity, its binding with leucine, ATP, and $tRNA^{Leu}$ did not change. It could catalyze the leucylation of two isoacceptors of $tRNA^{Leu}$ at different rates. Its catalytic efficiency, k_{cat}/K_m, for $tRNA_2^{Leu}$ was almost 3 times higher than that for $tRNA_1^{Leu}$. Discrimination between isoacceptor tRNA molecules by an aaRS mutant has not been reported previously.

What is the basis for this mutant's ability to discriminate between the two isoacceptors while the native enzyme could not? As the crystal structure of LeuRS has not been determined, the location of the CP1 domain in this enzyme is unknown. On the basis of the activity of LeuRS-A, the site of E292-A293 might be on the surface of LeuRS so that the insertion of 40 amino acid residues would not disturb the structure essential for its activity, but reduce its catalytic efficiency. The more subtle conformational changes caused by the insertion might give rise to its discrimination between the two isoacceptors of $tRNA^{Leu}$.

Wobble base pairing in the acceptor stem is a distinctive feature in tRNA molecules and is recognized specifically by some aaRSs such as alanyl-tRNA synthetase (*31*, *32*) and GlnRS (*27*). Our results showed that in the more efficient leucylation of $tRNA_2^{Leu}$ by LeuRS-A, the first wobble base might favor the interaction of the 3′ end of $tRNA_2^{Leu}$ but not $tRNA_1^{Leu}$ with the active site of LeuRS-A. When the wobble base pair was mutated to a standard base pair, its charging activity catalyzed by LeuRS-A was severely decreased.

The additional sequence in CP1 of LeuRS may make the LeuRS recognize a new identity element in $tRNA^{Leu}$. As class I aaRSs evolved from a common ancestor (*8*), many tRNAs might have recognized the primordial aaRS initially. During evolution, new structural elements were added to aaRS gradually and new motifs and domains of aaRSs might emerge to recognize specific tRNA molecules, similar to what was found in LeuRS-A.

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