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Tertiary structure base pairs between D- and TψC-loops of *Escherichia coli* $tRNA^{Leu}$ play important roles in both aminoacylation and editing

Xing Du and En-Duo Wang*

State Key Laboratory of Molecular Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, The Chinese Academy of Sciences, 320 Yue-Yang Road, Shanghai 200031, China

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ABSTRACT

To ensure the fidelity of protein biosynthesis, aminoacyl-tRNA synthetases (aaRSs) must recognize the tRNA identity elements of their cognate tRNAs and discriminate their cognate amino acids from structurally similar ones through a proofreading (editing) reaction. For a better understanding of these processes, we investigated the role of $tRNA^{Leu}$ tertiary structure in the aminoacylation and editing reactions catalyzed by leucyl-tRNA synthetase (LeuRS). We constructed a series of *Escherichia coli* $tRNA^{Leu}$ mutated transcripts with alterations of the nucleotides involved in tertiary interactions. Our results revealed that any disturbance of the tertiary interaction between the $tRNA^{Leu}$ D- and TψC-loops affected both its aminoacylation ability and its ability to stimulate the editing reaction. Moreover, we found that the various tertiary interactions between the D- and TψC-loops (G18:U55, G19:C56 and U54:A58) functioned differently within the aminoacylation and editing reactions. In these two reactions, the role of base pair 19:56 was closely correlated and dependent on the hydrogen bond number. In contrast, U54:A58 was more important in aminoacylation than in editing. Taken together, our results suggest that the elbow region of tRNA formed by the tertiary interactions between the D- and TψC-loops affects the interactions between tRNA and aaRS effectively both in aminoacylation and in editing.

INTRODUCTION

The fidelity of protein biosynthesis depends on the specific recognition of tRNAs and amino acids by their cognate aminoacyl-tRNA synthetases (aaRSs), which charge the correct amino acid to the 3′-end of tRNAs (1,2). However, most tRNAs have a similar L-shaped tertiary structure, which complicates the ability of aaRSs to discriminate their cognate tRNAs from other tRNA species. A variety of identity determinants and anti-determinants of tRNA ensure the specificity of each binding to its corresponding synthetase (3,4). On the other hand, some small amino acids are so similar in structure that aaRSs have great difficulty in discriminating them from non-cognate ones [e.g. leucyl-tRNA synthetase (LeuRS) has difficulty in discriminating leucine from norvaline, homocysteine or isoleucine], resulting in misactivation of non-cognate amino acids and potential misacylation of tRNAs (5). To correct such errors, aaRSs catalyze proofreading (editing) reactions to hydrolyze misactivated (pre-transfer editing) and/or mischarged amino acids (post-transfer editing) (5). In many instances, such a proofreading reaction requires cognate tRNA (5). Thus, these aaRSs must recognize their cognate tRNA both in aminoacylation and in editing.

tRNAs are grouped into two classes based on the structure of their variable loop. In *Escherichia coli*, $tRNA^{Leu}$, $tRNA^{Ser}$ and $tRNA^{Tyr}$ contain stem–loop structures consisting of more than five nucleotides between the anticodon and the TψC stem, which is identified as the variable arm. These three tRNAs are classified as type II, while those without the variable arm are classified as type I. Various studies have begun to elucidate the important elements of $tRNA^{Leu}$ for recognition by LeuRS (6–8). The important tertiary base pair A15:U48 and the location of G18 and G19 play important roles for LeuRS to discriminate $tRNA^{Leu}$ from the other type II tRNA molecules, $tRNA^{Ser}$ and $tRNA^{Tyr}$ (6,7). According to the crystal structure of yeast $tRNA^{Phe}$, G18 and G19 are involved in forming tertiary structures with the bases of the TψC-loop (G18:ψ55 and G19:C56) (9). Cysteine-tRNA synthetase (CysRS), which belongs to the same subgroup of aaRSs along with LeuRS (10), relies on tertiary interactions of G15:G48 and A13:A22:A46 as crucial identity elements for aminoacylation (11,12). Thus, it is intriguing to test if the tertiary base pairs between $tRNA^{Leu}$ G18 G19 and the nucleotides of the TψC-loop are actual identity elements. Larkin *et al.* (8) used *in vitro* transcribed truncated suppressor $tRNA^{Leu}$ with deletion of the anticodon stem–loop and variable arm to study the function of two tertiary base pairs in aminoacylation, and found that any replacement of G18:U55 or G19:C56 abolished the leucylation activity. However, it has been shown that the context sequence of $tRNA^{Leu}$ also contributes to the recognition by LeuRS (7,13). Thus, additional study of the role of the nucleotides G18 and

*To whom correspondence should be addressed. Tel: +86 21 54921241; Fax: +86 21 54921011; Email: edwang@sibs.ac.cn

G19 in aminoacylation by LeuRS is warranted in the context of the full-length $tRNA^{Leu}$.

At the same time, we are interested in the function of $tRNA^{Leu}$ in the editing reaction. *Escherichia coli* LeuRS binds $tRNA^{Leu}$ in the editing domain (connecting peptide 1, CP1), which is distinct from the aminoacylation active site, to edit misaminoacylated products hydrolytically (8,14–17). Editing reactions have been best characterized for the closely related valyl-tRNA synthetase (ValRS) and isoleucyl-tRNA synthetase (IleRS) (VlaRS and IleRS also belong to the same subgroup of aaRSs along with LeuRS and CysRS), and studies have shown that the editing reactions are dependent on their cognate tRNAs (18–22). The $tRNA^{Ile}$ determinants of editing are localized to three nucleotides (G16, D20 and D20a) in the D-loop at the elbow region of the L-shape, though these three bases are not required for the aminoacylation reaction (21,22). In the elbow region of the two-domain L-shaped tRNA molecule, the tertiary base pairs between nucleotides 18 and 55, 19 and 56, and 54 and 58 stack together to sustain the tertiary structure of tRNA (9,23,24), leading us to question whether these three tertiary base pairs of $tRNA^{Leu}$ are also involved in editing.

Nucleotides at positions 16, 17, 20a, 59 and 60 in the D- and TψC-loops form a 'variable pocket' contiguous with the tertiary base pairs of G18:U55, G19:C56 and U54:A58 (9,23–26), which serves as a critical identity element in various tRNA species (3,4). Asahara *et al*. showed that U16, A20a and G59 of $tRNA^{Leu}$ were not responsible for the leucine-specific aminoacylation (6). However, it is unknown whether these tertiary component nucleotides contribute to tRNA-dependent editing.

In the present work, we examined the role of $tRNA^{Leu}$ tertiary structural components in aminoacylation and editing by creating substitution and deletion mutations at U17, G18, G19, U54, U55, C56 and G59 of *E.coli* $tRNA^{Leu}$ transcripts. Our results revealed that any disturbance of the tertiary interaction between the D- and TψC-loops of $tRNA^{Leu}$ not only greatly affected its ability to bind with *E.coli* LeuRS in aminoacylation, but also affected its ability to stimulate the editing reaction. Moreover, each of these tertiary interactions between nucleotides 18 and 55, 19 and 56, and 54 and 58 played different roles in aminoacylation and editing. The role of 19:56 in aminoacylation and editing was closely correlated and dependent on the hydrogen bond number, while the role of 18:55 in the two reactions was not so important as that of 19:56. The tertiary base pair U54:A58 played more important roles in the aminoacylation reaction. However, the nucleotides of the 'variable pocket' did not play significant roles in either the aminoacylation or editing reaction. Our results imply that the elbow region of tRNA effectively impacts its interactions with cognate aaRS in both aminoacylation and amino acid editing reactions.

MATERIALS AND METHODS

Materials

L-Leucine, L-isoleucine, L-norvaline, ATP, GTP, CTP, UTP, inorganic pyrophosphatase (PPase) and dithiothreitol (DTT) were purchased from Sigma (USA). L-[^{14}C]Leucine (50 μCi/ml), L-[^{14}C]isoleucine (50 μCi/ml) and [γ-^{32}P]ATP (10 mCi/ml, 3000 Ci/mmol) were products of Amersham Life Science. Enzymes were products of MBI Fermentas or Promega. Ribonuclease (RNase) inhibitor was obtained from Takara Biotechnology Company. T7 RNA polymerase was purified from an *E.coli* overproducing strain in our laboratory (27). The *E.coli* LeuRS (2100 U/mg) was purified in our laboratory as described previously (28). Oligonucleotides for $tRNA^{Leu}$ mutation were synthesized on an Applied Biosystems Oligonucleotide Synthesizer.

Preparation of $tRNA^{Leu}$ transcripts

The deletion and substitution mutants of $tRNA^{Leu}$ (the anticodon is GAG) were constructed as previously reported (29) and confirmed by DNA sequencing. *Escherichia coli* $tRNA^{Leu}$ was transcribed *in vitro* by T7 RNA polymerase (17,30). $tRNA^{Leu}$ transcripts were purified by 7 M urea–20% PAGE and resolved in diethyl pyrocarbonate-treated Milli-Q water (containing 5 mM $MgCl_2$). All $tRNA^{Leu}$ transcripts were annealed by heating for 5 min at 80°C and slowly cooled to room temperature. The $tRNA^{Leu}$ concentrations were determined by UV absorbance at 260 nm. Extinction coefficients were calculated from the sequences of each tRNA (31).

Assay of aminoacylation and misaminoacylation of $tRNA^{Leu}$

The time course of aminoacylation was determined at 37°C in an 80 μl reaction mix consisting of 100 mM Tris–HCl pH 7.8, 30 mM KCl, 12 mM $MgCl_2$, 4 mM ATP, 0.1 mM EDTA, 5 mM DTT, 1 U/μl RNase inhibitor, 20 μM [^{14}C]leucine (50 μCi/ml), 3 μM $tRNA^{Leu}$ transcripts and 1 μM *E.coli* LeuRS. The reaction was initiated by addition of LeuRS. At various time intervals (5, 10, 15, 25, 40 and 60 min), 10 μl aliquots were applied to Whatman Grade 3 qualitative filter paper, precipitated with 5% trichloroacetic acid (TCA), washed with 5% TCA and ethanol, and determined by scintillation counting. The aminoacylation kinetic constants for $tRNA^{Leu}$ were measured at 37°C as described above, except that the concentrations of $tRNA^{Leu}$ were varied from 0.5 to 20 μM or from 2 to 50 μM and the concentration of LeuRS was varied from 10 to 100 nM, depending on the accepting activities of $tRNA^{Leu}$ transcripts. The k_{cat} and K_m values of $tRNA^{Leu}$ and its mutants were the average of three independent determinations.

Misaminoacylation of $tRNA^{Leu}$ with isoleucine was determined by the same procedure as used in the aminoacylation assay, except that 1 mM [^{14}C]isoleucine (40 μCi/ml) was added instead of 20 μM [^{14}C]leucine (50 μCi/ml), along with 10 μM $tRNA^{Leu}$ transcripts and 4 μM LeuRS.

Measurement of ATP consumption in editing

Assays measuring the overall tRNA-dependent editing were carried out essentially as reported (22) and were performed at 37°C in 100 μl reaction mixes containing 100 mM Tris–HCl pH 7.8, 30 mM KCl, 12 mM $MgCl_2$, 0.1 mM EDTA, 5 mM DTT, 1 U/μl RNase inhibitor, 2 U/ml PPase, 3 mM [γ-^{32}P]ATP (3–5 c.p.m./pmol), 25 mM norvaline, 5 μM $tRNA^{Leu}$ transcripts and 0.2 μM LeuRS. Aliquots (15 μl) of the editing reaction were taken and mixed with 350 μl of quenching liquid containing 6% activated charcoal, 7% $HClO_4$ and 10 mM tetrasodium pyrophosphate. After centrifugation, the amount of inorganic phosphate [^{32}P] in 50 μl of supernatant was

quantified by scintillation counting. The rate of ATP hydrolysis is the average of three determinants. The background ATP hydrolysis in the absence of $tRNA^{Leu}$ and LeuRS was assayed within each determinant as control.

RESULTS

Construction of *E.coli* $tRNA^{Leu}$ mutants

According to the crystal structure of yeast $tRNA^{Phe}$ (9,23,24), the hydrogen bondings between Gm18:ψ55, G19:C56 and T54:A58 of natural $tRNA^{Leu}$ are important tertiary interactions (Fig. 1A). Though the modification of nucleotides Gm18, T54 and ψ55 may strengthen the tertiary interactions, they are not involved in the tertiary hydrogen bonding directly. Thus, G18:U55, G19:C56 and U54:A58 in *in vitro* transcribed unmodified tRNA will result in little change at the elbow region in comparison with those in natural modified tRNA (32). In this study, we created different substitution and deletion mutations located at the elbow region of *in vitro* transcribed $tRNA^{Leu}$ (Fig. 1B). At these sites, eight single substitutions (A18 or C18 for G18, A55 for U55, U19 for G19, A56 for C56, A54 or C54 for U54 and G58 for A58) and two double substitutions (U19:A56 for G19:C56 and C54:G58 for U54:A58) mutants of $tRNA^{Leu}$ were constructed. Based on previously reported work (32–34), the modeled interactions between these nucleotides (18:55, 19:56 and 54:58) are shown in Figure 2. The G18:U55 base pair is of the 'GU imino: amino-2-carbonyl bifurcated' type (32), and an additional intraloop hydrogen bond occurs between the imino proton of U55 and an oxygen atom of the nearby phosphate 58 (33) (Fig. 2A). A18:U55 (Fig. 2B) retains only the intraloop hydrogen bond. However, G18:A55 (Fig. 2C) cannot be fitted in without serious distortion of the backbone, so it has no hydrogen bond (34). Because a pyrimidine at 18 would cause a large loss in stacking energy (34), C18:U55 is not included in Figure 2. G19 and C56 form a Watson–Crick base pair of three hydrogen bonds (Fig. 2D). U19:C56 (Fig. 2E) retains one hydrogen bond, while G19:A56 (Fig. 2F) and U19:A56 (Fig. 2G) retain two hydrogen bonds each. U54 and A58 in wild-type $tRNA^{Leu}$ (Fig. 2H) form a reverse Hoogsteen base pair consisting of two hydrogen bonds, while A54 and A58 (Fig. 2I) form a similar reverse Hoogsteen base pair with a minimal alteration of the phosphodiester backbone. Model building suggests that only one hydrogen bond is formed between C54:A58 (Fig. 2J) and U54:G58 (Fig. 2K). In addition, the double substitution mutation of U54 and A58 (C54:G58; Fig. 2L) would eliminate the hydrogen bonds and therefore disrupt the tertiary interaction.

In order to disrupt the tertiary interaction at the elbow region of $tRNA^{Leu}$ more severely, two deletion mutants ($tRNA^{Leu}$ Δ18G/19G and $tRNA^{Leu}$ Δ54U/55U) and one double deletion mutant ($tRNA^{Leu}$ Δ18G/19G–Δ54U/55U) were constructed. Two single substitution mutants ($tRNA^{Leu}$ 17G and $tRNA^{Leu}$ 59C) and one deletion mutant ($tRNA^{Leu}$ Δ16U/17U) in the 'variable pocket' were constructed to identify the role of the 'variable pocket' nucleotides in tRNA-dependent editing. It was thought that the deletion mutation Δ16U/17U could affect $tRNA^{Leu}$ in two ways—by directly reducing the number of nucleotides in the 'variable pocket', which may result in differential recognition by LeuRS, or by changing the tertiary base pairs between the invariant nucleotides G18 G19 and U55 C56 by reducing the number of nucleotides in the α region of the D-loop (which precedes G18 G19) from four to three.

Effect of mutations on aminoacylation of $tRNA^{Leu}$

The effect of the above mutations on the accepting activity by LeuRS was measured (Fig. 3). For $tRNA^{Leu}$ with mutations at the base pairs between D- and TψC-loops, $tRNA^{Leu}$ 18A (one intraloop hydrogen bond, Fig. 2B), $tRNA^{Leu}$ 19U (one hydrogen bond, Fig. 2E) and $tRNA^{Leu}$ U19:A56 (two hydrogen bonds, Fig. 2G) had high accepting activity (98, 86 and 91%, respectively; Table 1). Notably, deletion mutant $tRNA^{Leu}$ Δ18G/19G also had a high level of aminoacylation (87%, Table 1), though this mutant is predicted to have impaired tertiary interaction between D- and TψC-loops. The plateau levels of aminoacylation of other $tRNA^{Leu}$ mutants with impaired tertiary base pairs at 18:55, 19:56 and 54:58 were much lower (Table 1). The double substitution mutant $tRNA^{Leu}$ C54:G58, the single deletion mutant $tRNA^{Leu}$ Δ54U/55U and the double deletion mutant, $tRNA^{Leu}$ Δ18G/19G–Δ54U/55U, had the lowest accepting activity (39, 49 and 41%, respectively; Table 1).

The K_m values of LeuRS for $tRNA^{Leu}$ with alterations between the D- and TψC-loops increased 1.7- to 83-fold (Table 1), indicating that the binding of these tRNA mutants with LeuRS decreased overall, but to different extents depending on the mutation type and location. The k_{cat} values of $tRNA^{Leu}$ mutants were quite consistent with their accepting activities. For these $tRNA^{Leu}$ mutants, the aminoacylation efficiency (k_{cat}/K_m) of LeuRS decreased 1.4- to 2273-fold (Table 1). Two deletion mutants, $tRNA^{Leu}$ Δ54U/55U and $tRNA^{Leu}$ Δ18G/19G–Δ54U/55U, were the only two $tRNA^{Leu}$ mutants that had a significant change both in K_m and in k_{cat} (k_{cat}/K_m decreased 1200- and 2273-fold, respectively), indicating that they are of the worst tertiary structures and are the worst substrate of LeuRS. However, another single deletion mutant, $tRNA^{Leu}$ Δ18G/19G, only had an increase in K_m, indicating that it was a better substrate than $tRNA^{Leu}$ Δ54U/55U and $tRNA^{Leu}$ Δ18G/19G–Δ54U/55U. As to the $tRNA^{Leu}$ mutants involved in tertiary interaction between nucleotides 18 and 55, $tRNA^{Leu}$ 18A with just one intraloop hydrogen bond (Fig. 2B) showed k_{cat} and K_m values similar to wild-type $tRNA^{Leu}$ (Table 1). However, the two mutants lacking a hydrogen bond, $tRNA^{Leu}$ 18C and $tRNA^{Leu}$ 55A (Fig. 2C), showed a drastic increase in K_m. Among the mutants involved in the 19:56 interaction, $tRNA^{Leu}$ 56A and $tRNA^{Leu}$ U19:A56 (both with two hydrogen bonds, Fig. 2F and G) had much lower K_m (Table 1) than $tRNA^{Leu}$ 19U (one hydrogen bond, Fig. 2E), suggesting that $tRNA^{Leu}$ with two hydrogen bonds at this tertiary base pair is a better substrate than those with only one hydrogen bond. For mutants related to 54:58, any change of U54 and A58 resulted in an obvious increase in K_m (Table 1), no matter whether the hydrogen bond number between 54 and 58 is two ($tRNA^{Leu}$ 54A, Fig. 2I) or one (54C and 58G of $tRNA^{Leu}$, Fig. 2J and K). The mutant without a hydrogen bond between 54 and 58 (C54:G58, Fig. 2L) had the largest increase in K_m (Table 1). These results suggest that U54:A58 plays a critical role for $tRNA^{Leu}$ binding to LeuRS.

As regards the mutants involved in the 'variable pocket', the plateau level of $tRNA^{Leu}$ Δ16U/17U, $tRNA^{Leu}$ 17G and $tRNA^{Leu}$ 59C was very high (89, 90 and 93% that of wild-type

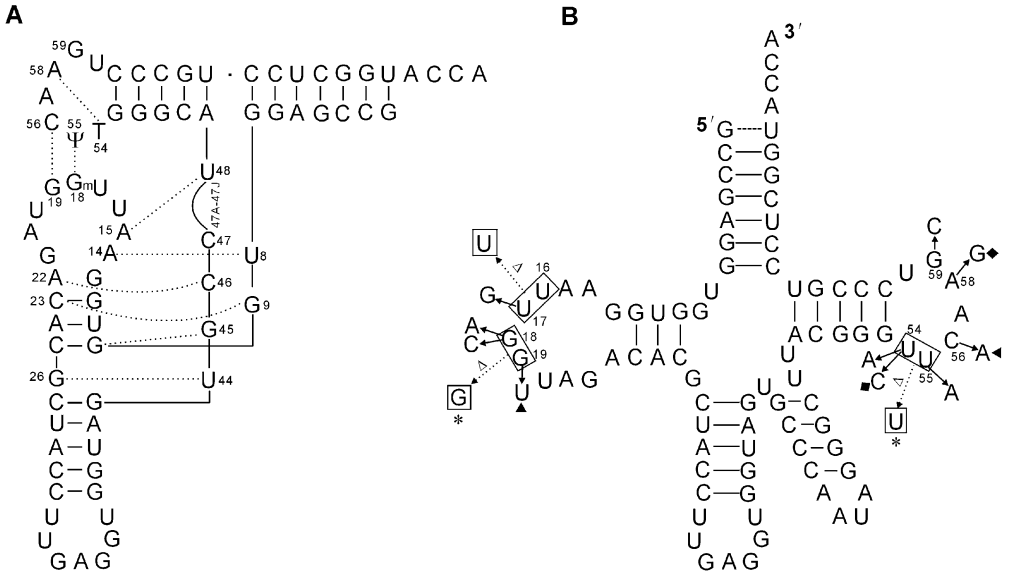

Figure 1. The structure of $tRNA^{Leu}$. (**A**) The L-shaped tertiary structure based on $tRNA^{Phe}$. Dotted lines indicate the tertiary interactions. (**B**) The cloverleaf structure of transcripts and the mutants used in this study. Dotted arrows indicate the deletion mutations, Δ16U/17U, Δ18G/19G and Δ54U/55U. Asterisks indicate the double deletion mutation (Δ18G/19G–Δ54U/55U), triangles indicate the double substitution mutation (U19:A56) and diamonds indicate the double substitution mutation (C54:G58).

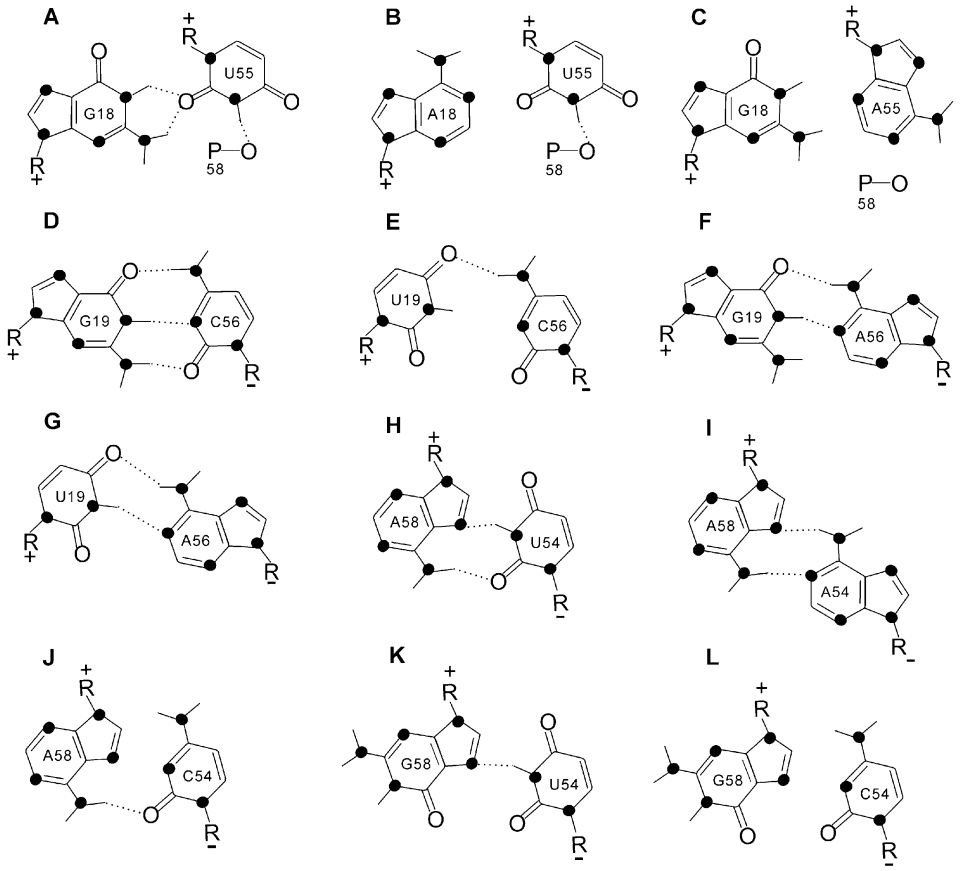

Figure 2. Proposed hydrogen bonding in the tertiary base pairs. (**A**) G18:U55. (**B**) A18:U55. (**C**) G18:A55. (**D**) G19:C56. (**E**) U19:C56. (**F**) G19:A56. (**G**) U19:A56. (**H**) U54:A58. (**I**) A54:A58. (**J**) C54:A58. (**K**) U54:G58. (**L**) C54:G58. Predicted hydrogen bonds are shown as dotted lines. Nitrogen atoms are shown as solid circles. R^+ indicates that the ribose–phosphate chain is coming toward the reader, while R^- indicates that it is facing away from the reader.

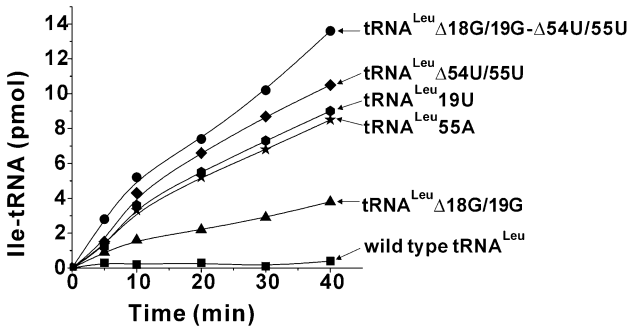

Figure 3. The misaminoacylation of tRNA transcripts by *E.coli* LeuRS with isoleucine.

tRNA^{Leu}, respectively; Table 1). Kinetic analysis showed that the K_m values of LeuRS for the two 'variable pocket' substitution mutants decreased while their catalytic efficiency increased (Table 1), indicating that they became better substrates for LeuRS. The deletion mutant, tRNA^{Leu} Δ16U/17U, showed a 50% decrease in k_{cat}/K_m, which was largely due to an increased K_m.

Mischarging tRNA^{Leu} transcripts with isoleucine

In order to understand the role of the tertiary interaction in the discrimination of leucine from non-cognate amino acids, we examined the ability of LeuRS to misaminoacylate all tRNA^{Leu} variants (Fig. 3). Two deletion mutants, tRNA^{Leu} Δ54U/55U and tRNA^{Leu} Δ18G/19G–Δ54U/55U, which were the worst substrates for aminoacylation, yielded the largest mischarging products. In the two substitution mutants, the mischarging levels of tRNA^{Leu} 19U (one hydrogen bond) and tRNA^{Leu} 55A (no hydrogen bonds) were also high. The deletion mutant tRNA^{Leu} Δ18G/19G was mischarged weakly, and the other eight mutants, including the 'variable pocket' mutants, showed no significant mischarging under the experimental conditions.

Determination of consumption of ATP during the editing reaction

The net effect of the proofreading reaction is the consumption of ATP. Therefore, editing can be measured in another way by using the tRNA-dependent conversion of ATP to AMP and PPi in the presence of non-cognate amino acids. This ATPase assay measures the overall editing without discrimination between the pre- and post-transfer editing. Such an assay is widely used in ValRS and IleRS (20–22). In the present work, a definite stimulation of ATP hydrolysis by LeuRS was observed with *in vitro* transcribed wild-type tRNA^{Leu} in the presence of norvaline (Fig. 4).

However, this ATP hydrolysis was attenuated by any change in the tertiary base pairs of tRNA^{Leu} (Table 2). When the worst substrates in aminoacylation, tRNA^{Leu} Δ54U/55U and tRNA^{Leu} Δ18G/19G–Δ54U/55U, were used, the rates of ATP hydrolysis decreased to 14 and 10% that of wild-type tRNA^{Leu}, respectively, indicating that these two mutants also had the worst ability to stimulate the LeuRS editing reaction. The ATP hydrolysis rate of tRNA^{Leu} 55A was only ~20% that of wild type, in contrast to that of tRNA^{Leu} 18A and tRNA^{Leu} 18C, which were 63 and 46% respectively. Among the three mutants related to the tertiary interaction of 19:56, tRNA^{Leu} 56A and tRNA^{Leu} U19:A56 (both with two hydrogen bonds) had higher ATP hydrolysis rates (33 and 36%) than did tRNA^{Leu} 19U (one hydrogen bond) (18%). The ATP consumption stimulated by tRNA^{Leu} with mutations at 54 and 58 decreased moderately (33–60%, Table 2). Two tRNA^{Leu} substitution mutants at the 'variable pocket' consumed ATP at the same rate as wild-type tRNA^{Leu}, indicating that the two mutants had no defect in proofreading ability. However, the ATP hydrolysis of one 'variable pocket' deletion mutant, tRNA^{Leu} Δ16U/17U, decreased by 30%.

DISCUSSION

Tertiary interactions within the tRNA molecule play an important role in tRNA aminoacylation (11,12,33,35), and have been similarly identified in tRNA-like structures in plant

Table 1. Leucylation of the wild-type and mutants of *E.coli* tRNA^{Leu}

tRNA transcripts	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} s^{-1})	k_{cat}/K_m (relative)	Relative plateau of aminoacylation (%)
Wild-type tRNA^{Leu}	3.2	1.8	1.8	1.0	100
tRNA^{Leu} Δ18G/19G	3.1	45	0.069	0.038	87
tRNA^{Leu} Δ54U/55U	0.06	40	1.5×10^{-3}	8.3×10^{-4}	49
tRNA^{Leu} Δ18G/19G–Δ54U/55U	0.04	50	8.0×10^{-4}	4.4×10^{-4}	41
tRNA^{Leu} 18A	3.5	2.6	1.3	0.72	98
tRNA^{Leu} 18C	1.8	18	0.10	0.056	60
tRNA^{Leu} 55A	1.6	19	0.084	0.047	65
tRNA^{Leu} 19U	3.0	24	0.13	0.072	86
tRNA^{Leu} 56A	1.8	6.4	0.28	0.17	69
tRNA^{Leu} U19:A56	3.0	7.2	0.42	0.24	91
tRNA^{Leu} 54A	2.0	21	0.095	0.053	65
tRNA^{Leu} 54C	2.9	36	0.08	0.044	68
tRNA^{Leu} 58G	3.0	38	0.079	0.044	75
tRNA^{Leu} C54:G58	2.6	150	0.017	9.6×10^{-3}	39
tRNA^{Leu} Δ16U/17U	2.9	3.1	0.94	0.52	89
tRNA^{Leu} 17G	3.4	1.1	3.1	1.7	90
tRNA^{Leu} 59C	3.5	1.0	3.5	1.9	93

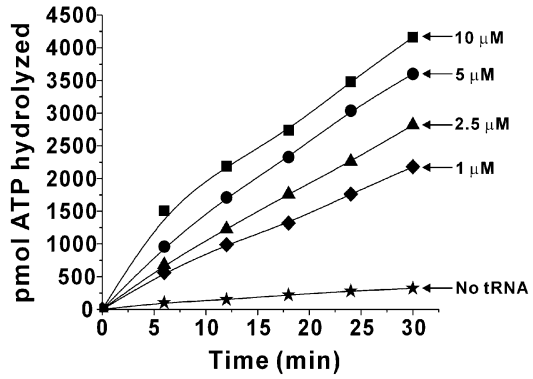

Figure 4. $tRNA^{Leu}$-dependent stimulation of ATP hydrolysis by *E.coli* LeuRS in the presence of norvaline. All the data were corrected against background ATP hydrolysis.

Table 2. $tRNA^{Leu}$-dependent ATP hydrolysis by *E.coli* LeuRS

tRNA transcripts	ATP hydrolysis rates (pmol/min)[a]	Relative ATP hydrolysis rates
Wild-type $tRNA^{Leu}$	126	1.0
$tRNA^{Leu}$ Δ18G/19G	34.6	0.27
$tRNA^{Leu}$ Δ54U/55U	17.9	0.14
$tRNA^{Leu}$ Δ18G/19G–Δ54U/55U	12.1	0.096
$tRNA^{Leu}$ 18A	79.2	0.63
$tRNA^{Leu}$ 18C	57.5	0.46
$tRNA^{Leu}$ 55A	26.1	0.21
$tRNA^{Leu}$ 19U	23.1	0.18
$tRNA^{Leu}$ 56A	40.8	0.33
$tRNA^{Leu}$ U19:A56	45.3	0.36
$tRNA^{Leu}$ 54A	65	0.51
$tRNA^{Leu}$ 54C	60	0.48
$tRNA^{Leu}$ 58G	76	0.60
$tRNA^{Leu}$ C54:G58	42	0.33
$tRNA^{Leu}$ Δ16U/17U	88.7	0.70
$tRNA^{Leu}$ 17G	126	1.0
$tRNA^{Leu}$ 59C	125	0.99

[a]The results were corrected by subtracting the background ATP hydrolysis.

viral RNAs and tmRNA (34,36). Here, we show that the tertiary base pairs at the elbow region of L-shaped $tRNA^{Leu}$, G18:U55, G19:C56 and U54:A58, are important for both aminoacylation and editing reactions catalyzed by LeuRS. However, U17 and G59, which are involved in the 'variable pocket' of $tRNA^{Leu}$, contributed very little to aminoacylation and editing, which is consistent with previous reports (6). It suggests that the defect of $tRNA^{Leu}$ Δ16U/17U in both aminoacylation and editing (Tables 1 and 2) was not because the deletion of U16 or U17 changed the 'variable pocket' itself, but because the deletion changed the position of the invariant nucleotides G18 G19, resulting in the disturbance of the tertiary interactions between D- and TψC-loops, which supported the importance of these interactions further.

Larkin *et al.* reported that the substitutions of G18:U55 and G19:C56 abolished the leucylation of truncated $tRNA^{Leu}$ (8). In the present work, all $tRNA^{Leu}$ mutants in the elbow region disrupted the tertiary interactions between the D- and TψC-loops. These mutants showed decreases in k_{cat}/K_m, but were still able to be aminoacylated by LeuRS relatively efficiently. This suggests that although $tRNA^{Leu}$ tertiary interactions are important to aminoacylation, the overall tertiary structure of $tRNA^{Leu}$ also relates to aminoacylation efficiency, as previously reported (7,13). The decrease of aminoacylation efficiency of $tRNA^{Leu}$ mutants was due mainly to increased K_m, suggesting that the affinity between $tRNA^{Leu}$ mutants and LeuRS was weakened by perturbation of the tertiary interactions at the elbow region.

To gain insight into the function of G18:U55, G19:C56 and U54:A58 of $tRNA^{Leu}$ in aminoacylation and editing reactions, we compared the activities of the related $tRNA^{Leu}$ mutants in the two reactions (Fig. 5). Obviously, three deletion mutations ($tRNA^{Leu}$ Δ18G/19G, $tRNA^{Leu}$ Δ54U/55U and $tRNA^{Leu}$ Δ18G/19G–Δ54U/55U) will greatly affect both aminoacylation and editing. Moreover, it seems that in the three mutants, the retained editing activity is correlated with the aminoacylation activity. The tertiary interaction between nucleotides 19 and 56 is important in both aminoacylation and editing. Although the single substitution mutant $tRNA^{Leu}$ 19U and the double substitution mutant $tRNA^{Leu}$ U19:A56 possessed the same 19U, the aminoacylation efficiencies and editing efficiencies between the two $tRNA^{Leu}$ mutants were of 3.3- and 2-fold difference, respectively. However, the aminoacylation and editing efficiencies between $tRNA^{Leu}$ 56A and $tRNA^{Leu}$ U19:A56 (both having an A at position 56) were similar. We note that the hydrogen bond numbers between bases 19 and 56 in wild-type $tRNA^{Leu}$, $tRNA^{Leu}$ U19:A56, $tRNA^{Leu}$ 56A and $tRNA^{Leu}$ 19U were three, two, two and one, respectively (Fig. 2D, G, F and E, respectively). This suggests that aminoacylation and editing efficiency might be correlated with the hydrogen bond number between nucleotides 19 and 56, not with the exact nucleotides themselves. This is supported by the report that $tRNA^{Phe}$ with C19:G56 (three hydrogen bonds) was equally efficient in the aminoacylation reaction as was wild-type $tRNA^{Phe}$ with G19:C56 (23).

It has been reported that the replacement of G18:U55 with A18:G55, which does not change the hydrogen bond number between nucleotides 18 and 55, resulted in little change of tRNA-like structures in plant viral RNAs or tmRNA (34,36). In the present study, although there are no hydrogen bonds predicted between A18 and U55 in $tRNA^{Leu}$ 18A, and only one intraloop hydrogen bond occurs between U55 and A58 in this mutant (Fig. 2B), $tRNA^{Leu}$ 18A is recognized efficiently by LeuRS in both the aminoacylation and editing processes (Fig. 5). Only when the tertiary interaction between 18 and 55 is almost completely disrupted, such as in the case of $tRNA^{Leu}$ 18C and $tRNA^{Leu}$ 55A (Fig. 2C), would the recognition of these two mutants by LeuRS in aminoacylation be significantly decreased (relative $k_{cat}/K_m = 0.056$ and 0.047). Though LeuRS catalyzes the aminoacylation of $tRNA^{Leu}$ 18C and $tRNA^{Leu}$ 55A with similar low efficiencies, there is a 2-fold difference in editing ability (Fig. 5). While the reason for this divergence is obscure, it implies that the consequences of these two mutations in aminoacylation are different from those in editing. $tRNA^{Leu}$ with mutations at 54 and 58 had very low aminoacylation ability, but retained high editing ability (Fig. 5), implying that the tertiary base pair U54:A58 plays a more significant role in aminoacylation than in editing. It seems that U54:A58 is a part of the leucine identity elements of $tRNA^{Leu}$, though the model of LeuRS complexed with

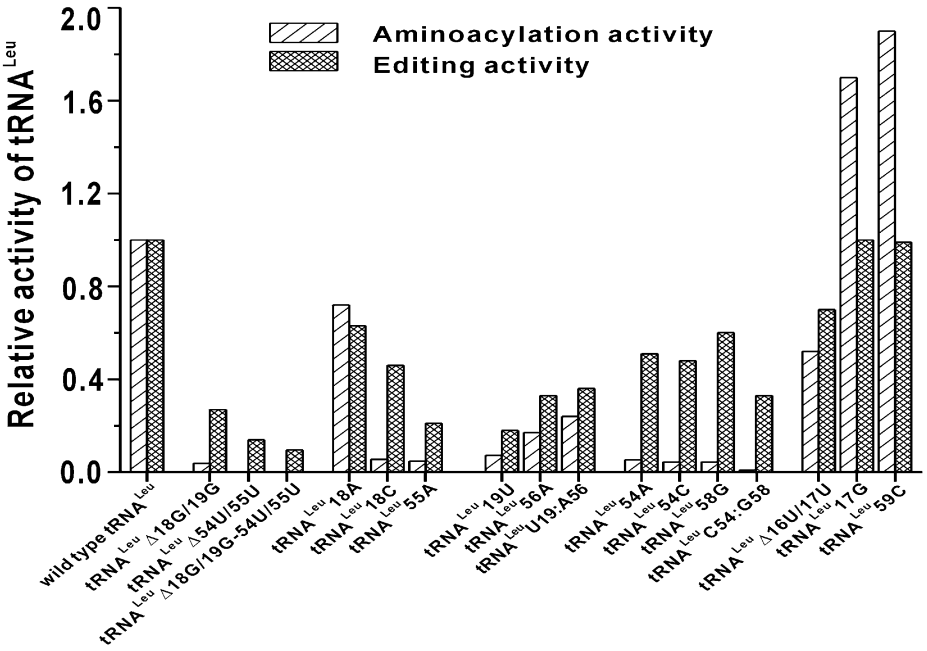

Figure 5. Combined comparison of the activities of $tRNA^{Leu}$ mutants correlated with the substitution mutation at G18:U55, G19:C56 and U54:A58 in aminoacylation and editing reactions. The aminoacylation activity is the relative k_{cat}/K_m, and the editing activity is the relative ATP hydrolysis rate.

Table 3. Effects of nucleotides in $tRNA^{Leu}$, $tRNA^{Val}$ and $tRNA^{Ile}$ on aminoacylation and editing

Effects on aminoacylation and editing	Nucleotides in three systems		
	$tRNA^{Leu}$	$tRNA^{Val,a}$	$tRNA^{Ile,b}$
Important for neither aminoacylation nor editing	U17 and G59		
Important for aminoacylation and editing and both are correlated	G19:C56	Most nucleotides studied in Tardif and Horowitz (20)	
More important for aminoacylation than for editing	U54:A58		Anticodon
Important only for editing		A76	G16, D20 and D20a
Mixed type	G18:U55		

[a]The data are from Tardif and Horowitz (20).
[b]The data are from Hale *et al*. (21).

$tRNA^{Leu}$ shows no obvious direct interaction between the tertiary base pair 54:58 and LeuRS (37).

Based on the above data, we conclude that the elbow region of L-shaped $tRNA^{Leu}$ affects its interaction with LeuRS in aminoacylation and editing. Further, the roles of the three tertiary base pairs (between nucleotides 19 and 56, 18 and 55, and 54 and 58) in these two reactions are different; only the tertiary interaction of 19:56 plays an equally important role in both aminoacylation and editing. The reason for this may be that this tertiary base pair is located in the outer side of the elbow region of two-domain L-shaped tRNA (Fig. 1B) and will affect the tRNA tertiary structure mostly in both the aminoacylation and editing reaction. Although there is still no evidence for direct contacts between the elbow region and the synthetase (19,37–39), we propose that the elbow region mediates the communications between the two domains of the L-shaped tRNA to prime the correct recognition by cognate aaRS in aminoacylation and editing reactions. The tertiary base pairs of G19:C56, G18:U55 and U54:A58 may contribute differently to adjust such communications for the different requirements in aminoacylation and editing.

Here, the nucleotides of $tRNA^{Leu}$ involved in aminoacylation and editing are different from those of $tRNA^{Val}$ and $tRNA^{Ile}$ (20,21), and their effects on aminoacylation and editing are summarized in Table 3. The data suggest that the roles of tRNAs in aminoacylation and editing are very complicated. In the case of $tRNA^{Val}$ (20), all mutants that actively promote editing can be aminoacylated, but not all aminoacylated tRNAs are able to trigger the editing response of *E.coli* ValRS. In the case of $tRNA^{Ile}$ (21), aminoacylation and editing rely on distinct $tRNA^{Ile}$ domains and nucleotide determinants to trigger both activities. However, the tertiary

interaction between nucleotides 19 and 56 of $tRNA^{Leu}$ is important for aminoacylation and editing reactions, and both activities are correlated. In $tRNA^{Leu}$, nucleotides important only for editing are not found.

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