ABSTRACT

Aminoacyl–tRNA synthetases (aaRSs) are remarkable enzymes that are in charge of the accurate recognition and ligation of amino acids and tRNA molecules. The greatest difficulty in accurate aminoacylation appears to be in discriminating between highly similar amino acids. To reduce mischarging of tRNAs by non-cognate amino acids, aaRSs have evolved an editing activity in a second active site to cleave the incorrect aminoacyl–tRNA. Editing occurs after translocation of the aminoacyl–CCA_76 end to the editing site, switching between a hairpin and a helical conformation for aminoacylation and editing. Here, we studied the consequence of nucleotide changes in the CCA_76 accepting end of tRNA Leu during the aminoacylation and editing reactions. The analysis showed that the terminal A_76 is essential for both reactions, suggesting that critical interactions occur in the two catalytic sites. Substitutions of C_74 and C_75 selectively decreased aminoacylation keeping nearly unaffected editing. These mutations might favor the regular helical conformation required to reach the editing site. Mutating the editing domain residues that contribute to CCA_76 binding reduced the aminoacylation fidelity leading to cell-toxicity in the presence of non-cognate amino acids. Collectively, the data show how protein synthesis quality is controlled by the CCA_76 homogeneity of tRNAs.

INTRODUCTION

Protein synthesis is a central process in organisms from all three domains of life, providing the link between the genetic information encoded in DNA and functional proteins. This process critically relies on the correct formation of aminoacyl–tRNA (aa–tRNA) by aminoacyl–tRNA synthetase (aaRS) to establish the genetic code through rigorous control of the two-step aminoacylation (1,2). The amino acid is first activated with ATP to synthesize the aa-AMP intermediate with the release of pyrophosphate; the amino acid moiety of the intermediate is subsequently transferred to the tRNA bearing the cognate nucleotide triplet (1). Mis-translation arising from disruption in the fidelity of these interactions has profound consequences (3). However, the presence of various types of amino acids and their analogs, and the fact that amino acids only differ in the side chain, greatly challenge the specificity of aaRS. About half of the aaRSs misactivate non-cognate amino acids (4). To solve this problem, the proofreading (editing) mechanism of aaRS has evolved to hydrolyze the mis-products. Editing can occur at the aa-AMP level (pre-transfer editing) and/or mischarged tRNA level (post-transfer editing), depending on the specific aaRS (4).

Leucyl–tRNA synthetase (LeuRS), isoleucyl–tRNA synthetase (IleRS) and valyl–tRNA synthetase (ValRS) belong to the class Ia of aaRSs, characterized by the connective peptide 1 (CP1) and 2 (CP2) in the Rossmann fold nucleotide binding domain where the synthetic active site is located (5). The CP1 domain of LeuRS is located 35 Å away from the Rossmann fold domain and is responsible for post-transfer editing (6,7). Recent studies have revealed that Escherichia coli, Aquifex aeolicus and human cytoplasmic LeuRSs (EcLeuRS, AaLeuRS and hcLeuRS) all employ three different pathways (tRNA-independent, tRNA-dependent pre-transfer editing and post-transfer editing), but in different proportions relative to the total editing activity, to remove non-cognate amino acids (8,9). Similar results were also found with E. coli IleRS and ValRS (10).

X-ray crystal structures of LeuRS and tRNA^{Leu}_Leu in aminoacylation and post-transfer editing states clearly...
revealed that the tRNA<sub>Leu</sub><sup>Leu</sup> main body conformation is indistinguishable between the two states (6,11). However, the CCA<sub>76</sub>-end shifts to the CP1 domain from the aminoacylation active site and is specifically recognized by several conserved residues, including Lys300, Tyr330, Arg344 and Leu327 (6,11). Therefore, these residues in CP1 collectively constitute the tRNA entrance pathway in post-transfer editing (6). In vitro studies have shown that the interaction between the Tyr330 and tRNA<sub>Leu</sub><sup>Leu</sup> CCA<sub>76</sub>-end is critical for both tRNA-dependent pre-transfer editing and post-transfer editing (8). However, the role of other crucial residues in editing and their significance in vivo are unclear. In addition, the contribution of the CCA<sub>76</sub>-end to the aminoacylation and editing still remains elusive.

The CCA<sub>76</sub> sequence is conserved at the 3'-end of all mature tRNA molecules to function as the site of amino acid attachment (12). This sequence is gene encoded in E. coli and related bacteria or acquired in eukaryotes and maintained by stepwise nucleotide addition by the ubiquitous CCA-adding enzyme (tRNA nucleotidyltransferase, CCase), which is an unusual RNA polymerase that does not use a nucleic acid template for nucleotide addition (13,14).

The CCA<sub>76</sub> sequence is a universal ligand during several critical steps of protein biosynthesis. It is successively recognized by aaRSs (1,12), elongation factor (EF-Tu) (15) and tRNA (16,17). In F. Crick’s adaptor hypothesis, the CCA<sub>76</sub> sequence is the ultimate adaptor group that carries the amino acid to the decoding center of the ribosome, whereas at the other end of the molecule, the anticodon triplet is needed to fit with the codon triplet of the mRNA. Because all the tRNAs have to fit to the mRNA, the interaction between the tRNAs and tRNA<sub>Leu</sub><sup>Leu</sup> CCA<sub>76</sub>-end is critical for both tRNA-dependent pre-transfer editing and post-transfer editing (8). However, the role of other crucial residues in editing and their significance in vivo are unclear. In addition, the contribution of the CCA<sub>76</sub>-end to the aminoacylation and editing still remains elusive.

The CCA<sub>76</sub> sequence is conserved at the 3'-end of all mature tRNA molecules to function as the site of amino acid attachment (12). This sequence is gene encoded in E. coli and related bacteria or acquired in eukaryotes and maintained by stepwise nucleotide addition by the ubiquitous CCA-adding enzyme (tRNA nucleotidyltransferase, CCase), which is an unusual RNA polymerase that does not use a nucleic acid template for nucleotide addition (13,14).

The CCA<sub>76</sub> sequence is a universal ligand during several critical steps of protein biosynthesis. It is successively recognized by aaRSs (1,12), elongation factor (EF-Tu) (15) and tRNA (16,17). In F. Crick’s adaptor hypothesis, the CCA<sub>76</sub> sequence is the ultimate adaptor group that carries the amino acid to the decoding center of the ribosome, whereas at the other end of the molecule, the anticodon triplet is needed to fit with the codon triplet of the mRNA. Because all the tRNAs have to fit to the mRNA, the interaction between the tRNAs and tRNA<sub>Leu</sub><sup>Leu</sup> CCA<sub>76</sub>-end is critical for both tRNA-dependent pre-transfer editing and post-transfer editing (8). However, the role of other crucial residues in editing and their significance in vivo are unclear. In addition, the contribution of the CCA<sub>76</sub>-end to the aminoacylation and editing still remains elusive.

Gene cloning and mutagenesis

The plasmid pET30a-EcLeuS encoding EcLeuRS with N-terminal His<sub>6</sub>-tag previously constructed by our lab (8) was used as the template to construct genes encoding various EcLeuRS mutants using KOD-plus mutagenesis kit for the in vitro activity measurements. EcLeuS was amplified, cleaved by NcoI and HindIII and cloned into pTrc99a (pre-cleaved by NcoI and HindIII) to produce pTrc99a (pre-cleaved by NcoI and HindIII) to produce pTrc99a-EcLeuS. The 18 nucleotides encoding His<sub>6</sub>-tag at the N-terminus were incorporated into the forward primer during the construction of pTrc99a-EcLeuS. The pTrc99a-EcLeuS construct was used as the template to construct genes encoding various EcLeuRS mutants using the KOD-plus mutagenesis kit for the in vivo complementation assays in the KL231 strain.

In vitro transcription of tRNAs

Six DNA fragments covering the T7 promoter and tRNA gene double strands were synthesized by Invitrogen, phosphorylated and ligated into pUC19 (pre-cleaved by EcoRI and BamHI) to construct the plasmid pUC19-tRNA. To prepare the wild-type (WT) tRNA<sub>Leu</sub><sup>GAG</sup> transcript, the forward primer (5'-ctcagtataa cgaccttatacgccggttggg-3', with T7 promoter sequence in italics) and the reverse primer (5'-tgatgacgaggg gactgacaagacaggtcgagttc-3') were synthesized to amplify the T7 promoter and the gene encoding tRNA<sub>Leu</sub><sup>GAG</sup> using the Phusion high-fidelity DNA polymerase, which does not add an additional adenosine to the PCR product. The PCR product was separated on a 2% agarose gel,

MATERIALS AND METHODS

Materials

1-leucine, dithiothreitol, NTP, 5'-GMP, tetrasodium pyrophosphate, inorganic pyrophosphate, ATP, Tris–HCl, MgCl<sub>2</sub>, NaCl, mouse anti-His<sub>6</sub> antibody and activated charcoal were purchased from Sigma (USA). [3H]leucine, [3H]isoleucine and [α-32P]ATP were obtained from Amersham Biosciences (England). Pfu DNA polymerase, the DNA fragment rapid purification kit and a plasmid DNA polymerase, was purchased from Biotech Company (China). KOD-plus mutagenesis kit was obtained from TOYOBO (Japan). T4 ligase and restriction endonucleases were obtained from MBI Fermentas. Phusion high-fidelity DNA polymerase was purchased from New England Biolabs (USA). Ni<sup>2+</sup>-NTA Superflow was purchased from Qiagen, Inc. (Germany). Polyethyleneimine cellulose plates were purchased from Merck (Germany). Pyrophosphatase was obtained from Roche Applied Science (China). Pyrobest DNA polymerase and the dNTP mixture were obtained from Takara (Japan). Oligonucleotide primers were synthesized by Invitrogen (China). Escherichia coli BL21 (DE3) cells were purchased from Stratagene (USA). The E. coli KL231 strain [F-, leuS1(ts), thyA6, rpsL120(strR), deoC1] was purchased from the E. coli genetic stock center (CGSC, Yale University, USA) (18). T7 RNA polymerase was purified from an overproduction strain in our laboratory (19).

Gene cloning and mutagenesis

The plasmid pET30a-EcLeuS encoding EcLeuRS with N-terminal His<sub>6</sub>-tag previously constructed by our lab (8) was used as the template to construct genes encoding various EcLeuRS mutants using KOD-plus mutagenesis kit for the in vitro activity measurements. EcLeuS was amplified, cleaved by NcoI and HindIII and cloned into pTrc99a (pre-cleaved by NcoI and HindIII) to produce pTrc99a-EcLeuS. The 18 nucleotides encoding His<sub>6</sub>-tag at the N-terminus were incorporated into the forward primer during the construction of pTrc99a-EcLeuS. The pTrc99a-EcLeuS construct was used as the template to construct genes encoding various EcLeuRS mutants using the KOD-plus mutagenesis kit for the in vivo complementation assays in the KL231 strain.
extracted by phenol/chloroform and precipitated in the presence of cold ethanol and 0.3 M NaAc (pH 5.2). The T7 in vitro transcription was carried out at 37°C in a 100 μl reaction mixture containing 40 mM Tris–HCl (pH 8.0), 22 mM MgCl₂, 1 mM spermidine, 5 mM DTT, 0.5% Triton X-100, 60 μg/ml T7 DNA template, 5 mM ATP, 5 mM CTP, 5 mM GTP, 5 mM UTP, 0.8 μg/ml ribonuclease inhibitor, 20 mM GMP and 500 μg/ml T7 RNA polymerase. One hour later, 2 U of pyrophosphatase was added to remove the pyrophosphate for 30 min, and then 5 U of DNase I (RNase I free) was added and incubated for 1 h to digest the transcription template. The transcript was then loaded into a 15% PAGE–8M Urea gel of 1-mm thickness and 40 cm length, and the gel was run at constant 25 W for 10 h to carefully remove any non-specific bands. The tRNA was cut from the gel and eluted with 0.5 M NaAc (pH 5.2) at room temperature, ethanol precipitated at –20°C after phenol/chloroform extraction two times and dissolved in 5 mM MgCl₂. The tRNA was denatured at 80°C for 5 min and slowly cooled down to 30°C. The tRNALeu mutants were prepared by the same procedure, except that the different reverse primer was synthesized and used in preparing the DNA template.

Protein expression and purification
The WT LeuRS (EcLeuRS) and its mutants were produced by transformation of E. coli BL21 (DE3) cells with the corresponding plasmids. A single colony from each of the transformants was chosen and cultured in 500 ml of 2x YT medium at 37°C. When the cells were grown to mid-log phase (A600 = 0.6), IPTG (isopropyl-β-D-galactopyranoside) was added to a final concentration of 0.2 mM, and cultivation continued for 6 h at 22°C. The proteins were purified by Ni²⁺–NTA chromatography, as described previously (8).

Aminoacylation, mis-aminoacylation and deacylation
The kinetic constants of EcLeuRS and its mutants were determined by thin layer chromatography of tRNALeu, with the corresponding plasmids. A single colony from each of the transformants was chosen and cultured in liquid LB medium supplemented with 100 μg/ml ampicillin, 200 μg/ml thymine and 0.1 mM IPTG, and incubated at 37°C for 5 min. The reaction solution was removed at various time intervals, quenched on Whatman filter pads and processed as mentioned in the procedures above. The blank experiment without tRNALeu was performed under the same conditions. In another control experiment, RNase I (final 0.25 U/μl) was added to the aliquots at various time intervals to digest the aminoacylation product and incubated at 37°C for another 5 min. The plateau value of WT tRNALeu was >1400 pmol/A260.

AMP formation
In the editing Nva reaction of EcLeuRS, AMP formation was measured in a reaction mixture containing 100 mM Tris–HCl (pH 7.8), 30 mM KCl, 12 mM MgCl₂, 5 mM dithiothreitol, 5 U ml⁻¹ pyrophosphatase, 3 mM ATP, 20 mM [γ-3²P]ATP and 15 mM Norvaline (Nva) in the presence or absence of 5 μM tRNALeu or its variants. The reaction was initiated by the addition of 0.2 μM EcLeuRS or its mutants and incubated at 37°C. Aliquots (1.5 μl) were quenched in 6 μl of 200 mM NaAc (pH 5.0). The quenched aliquots (1.5 μl each) were spotted in duplicate on polyethyleneimine cellulose plates pre-washed with water. Separation of Nva-[²³²P]AMP, [²³²P]AMP and [²³²P]ATP was performed by developing thin layer chromatography (TLC) on polyethyleneimine cellulose plates in 0.1 M NH₄Ac and 5% acetic acid. The plates were visualized by phosphorimaging, and the data were analyzed using Multi Gauge Version 3.0 software (FUJIFILM). The gray densities of [²³²P]AMP spots were compared with the gray density of known [²³²P]ATP concentrations. Rate constants were obtained from graphs of [²³²P]AMP formation plotted against time (8).

KL231 complementation assay
Competent KL231 cells were electro-transformed with various plasmids, including pTrc99a (negative control), pTrc99a-EcLeuS, pTrc99a-EcLeuS-L327G, -L327R, -K300E, -Y330D, -R344D, -K300E/Y330D, -K300E/R344D, -Y330D/R344D and -K300E/Y330D/R344D. Transformants were grown on solid LB plates supplemented with 100 μg ml⁻¹ ampicillin and 200 μg ml⁻¹ thymine at 30°C. A single colony was selected and grown in liquid LB medium supplemented with 100 μg ml⁻¹ ampicillin, 200 μg ml⁻¹ thymine and 0.1 mM IPTG at 30°C. The cells harboring these plasmids were diluted and adjusted to the same A₆₀₀ value of 0.2. To test the effect of non-cognate Nva on cell growth, 5 μl of the diluted mixture were dropped on a solid LB plate.
(supplemented with 100 μg ml⁻¹ ampicillin, 200 μg ml⁻¹ thymine and 0.1 mM IPTG) containing 0, 5, 10, 20, 50, 100 or 200 mM Nva, and the plates were incubated at 42°C to observe the growth of cells.

RESULTS

Mutation of the CCA₇₆-end impacts the tRNA aminoacylation reaction

All tRNA¹⁰ isoacceptors from various species contain the absolutely conserved A₇₃ (12), which functions as the discriminator during recognition by LeuRSs in different species (12, 21). However, the effect of the CCA₇₆-end on tRNA aminoacylation has not been reported. Here, each nucleotide of the CCA₇₆-end of tRNALeu was individually mutated to one of the three other nucleotides (Figure 1A), and the aminoacylation kinetic constants of EcLeuRS for these tRNA Leus were assayed. In aminoacylation of the tRNA²⁰ A₇₆U and A₇₆C mutants, the catalytic efficiency of EcLeuRS was obviously decreased (155.8 and 87.2 s⁻¹M⁻¹, respectively) as compared with that of the WT tRNA transcript (923.1 s⁻¹M⁻¹). The amino acid-accepting activity of the A₇₆G mutant was too low to accurately measure the kinetics. Similarly, the three C₇₅ mutants reduced the amino acid-accepting activity to 2–46% of the native tRNA¹⁰, with the C₇₅G mutant showing the greatest decrease (14.6 s⁻¹M⁻¹). Additionally, the three tRNA variants from C₇₄ showed obvious decreases in amino acid-accepting activity; their catalytic constants for EcLeuRS ranged from 8% (C₇₄G, 69.4 s⁻¹M⁻¹), 41% (C₇₄U, 381.8 s⁻¹M⁻¹) to 50% (C₇₄A, 460.3 s⁻¹M⁻¹) of that with the WT tRNA¹⁰ (Table 1). All the decreases were mainly due to a reduced kₗₐₜ value of EcLeuRS and/or changed Kₘ for these tRNA¹⁰ mutants. These results clearly showed that the CCA₇₆-end mutations negatively impacted the aminoacylation of these mutants to various levels, and the presence of guanosine at each of the 3-nt positions resulted in the lowest level of tRNA aminoacylation.

LeuRS can aminoacylate tRNA¹⁰ lacking the terminal adenosine

Besides point mutations, we also performed progressive deletions from the CCA₇₆-end of tRNA in order to obtain the following three mutants: ΔA₇₆ (ending

---

**Table 1.** Aminoacylation kinetics of transcripts of tRNALeu

| tRNA     | Kₘ (μM)  | kₗₐₜ (s⁻¹) | kₗₐₜ/Kₘ (s⁻¹M⁻¹) | kₗₐₜ/Kₘ (relative) |
|----------|----------|-------------|------------------|-------------------|
| tRNALeu⁰⁰_{GAG} | 1.95 ± 0.23 | 1.80 ± 0.27 | 923.1 | 1 |
| A₇₆U    | 1.99 ± 0.30 | 0.31 ± 0.04 | 155.8 | 0.17 |
| A₇₆G    | –        | <<0.002 | – | – |
| A₇₆C    | 3.90 ± 0.48 | 0.34 ± 0.03 | 87.2 | 0.09 |
| C₇₄A    | 0.38 ± 0.05 | 0.16 ± 0.03 | 14.6 | 0.02 |
| C₇₄G    | 1.18 ± 0.17 | 0.10 ± 0.02 | 84.7 | 0.09 |
| C₇₄U    | 1.78 ± 0.22 | 0.026 ± 0.004 | 381.8 | 0.41 |
| C₇₄A    | 0.63 ± 0.05 | 0.29 ± 0.04 | 460.3 | 0.50 |
| C₇₄G    | 0.36 ± 0.05 | 0.025 ± 0.002 | 69.4 | 0.08 |
with C75), ΔC75A76 (ending with C74) and ΔC74C75A76 (ending with A73) (Figure 1A). In initial charging plateau measurements for accepting activity with a large excess of 1 μM enzyme, it was surprising to observe that LeuRS could charge ΔA76 mutant obviously. As it has been reported that some aaRSs can ligate their cognate amino acids to themselves without tRNA (by self-amination occurring at high enzyme concentration and excess of adenylate formation) (22–24), we tested the self-amination of EcLeuRS at different concentrations without tRNA. We found that self-amination of EcLeuRS was negligible at low concentrations (<300 nM). However, EcLeuRS at higher concentrations >500 nM could be self-aminomayed and was resistant to RNase I (Supplementary Figure S1). Therefore, we tested the ΔA76 charging plateau value by using 100 nM EcLeuRS and found that 70% of ΔA76 could be charged with 3H-Leu. In the same conditions, no detectable labeled product could be measured at the absence of tRNA (Figure 2). To further confirm that, the tRNA but not the enzyme itself was ligated with 3H-Leu, we treated the aminomoylation product in the presence of RNase I, and the values dropped down to the same level with that in the absence of tRNA (Figure 2). These results confirmed that LeuRS could aminomoylate tRNA1Leu with the terminal A76 deleted. However, reliable aminomoylation kinetics could not be measured because of the very low kcat. However, the other two tRNA mutants, ΔC75A76 and ΔC74C75A76, could not be aminomayed at 100 nM or higher concentrations of EcLeuRS (data not shown).

To further analyze the ΔA76 mutant, we mutated the terminal C75 to the three other nucleotides in order to identify potential interactions that could be more productive in aminomoylation. The resulting mutants, ΔA76/C75A, ΔA76/C75U and ΔA76/C75G, could be aminomayed by EcLeuRS to plateau levels of 70, 61 and 35%, respectively (Figure 2). Therefore, more productive interactions were not induced by these nucleotides, and here again, the mutant harboring a guanosine mutation was the least active compared to those with the other nucleotides.

**Role of CCA76-end in total editing**

Usually, more than one ATP molecule is consumed by an aaRS in the presence of a non-cognate amino acid due to repetitive cycles of synthesis–hydrolysis of the non-cognate products. The excess of ATP consumption can be determined by measuring the release of AMP in the TLC assay (25). In the presence of tRNA, the TLC assay measures the global editing activity, including the tRNA-independent and tRNA-dependent pre-transfer editing in addition to the post-transfer editing (8,26). In the absence of tRNA, the assay measures the AMP produced from the sole tRNA-independent pre-transfer editing activity (26).

In the presence of non-cognate Nva, the observed rate constant (kobs) of WT LeuRS for AMP formation without tRNA in the editing reaction was 0.56 ± 0.07 s−1, accounting for 13% of that in the presence of tRNA transcript (4.42 ± 0.64 s−1) (Table 2). The tRNA transcript without the modified bases was tested to determine if it was as efficient as the native tRNA in stimulating the editing activity. The kobs for AMP formation in the presence of EctRNA1LeuGAG purified from the overproducing E. coli strain (27) was 5.59 ± 0.76 s−1. This value was only slightly larger than that in the presence of transcript (4.42 ± 0.64 s−1), indicating that the modified bases in EctRNA1Leu did not play a critical role in stimulating editing activity (Table 2).

Different variants of the CCA76-end were also tested for their abilities to stimulate AMP formation in editing reactions with EcLeuRS. The AMP formation rates for A76U, A76G and A76C mutants dropped down to a level similar to that without tRNA, indicating only tRNA-independent pre-transfer editing remained and the tRNA-dependent

| tRNA          | kobs (s−1) | Relative kobs |
|---------------|------------|---------------|
| No tRNA1Leu   |            |               |
| tRNA1LeuGAG (in vivo)* | 0.56 ± 0.07 | 0.13          |
| tRNA1LeuGAG (transcript)b | 5.59 ± 0.76 | 1.27          |
| A76U          | 4.42 ± 0.64 | 1             |
| A76G          | 0.24 ± 0.04 | 0.05          |
| A76C          | 0.20 ± 0.04 | 0.05          |
| ΔA76          | 0.48 ± 0.06 | 0.11          |
| ΔC75A         | 0.71 ± 0.08 | 0.16          |
| ΔC75U         | 8.34 ± 1.03 | 1.89          |
| ΔC75G         | 6.26 ± 0.81 | 1.42          |
| ΔC75U         | 0.64 ± 0.07 | 0.15          |
| C74A          | 7.48 ± 0.98 | 1.69          |
| C74U          | 8.87 ± 1.34 | 2.01          |
| C74G          | 2.65 ± 0.32 | 0.60          |

* tRNA1LeuGAG obtained from the overproducing E. coli strain (27).

b tRNA1LeuGAG obtained by T7 in vitro transcription.
editing was abolished (Table 2). As a consequence of this loss of editing ability, the two ΔA76-mutants of tRNA<sub>Leu</sub> (A76U and A76C) were obviously mischarged with Ile (Figure 3A). The decreased formation of Ile-tRNA<sub>Leu</sub> of A76C could not be hydrolyzed by the post-transfer editing (Figure 4A). This is consistent with the fact that the A76 base is specifically recognized by the main chain carboxyl- and amino-groups of EcLeuRS-Leu327 in the Nva2AA-containing structure (analog of the post-transfer editing substrate) (28) and *Thermus thermophilus* LeuRS-tRNA<sub>Leu</sub> structure (in post-transfer editing conformation) (6). Obviously, changing the terminal base affects the proper positioning of the CCA76-end into the CP1 domain and thus blocks the hydrolytic editing reaction. In general, mutations of nucleotides 75 and 74 did not influence the tRNA deacylation properties, except when the guanosine mutations C75G and C74G were introduced (Figure 4B and C). The result suggested that guanine residues at either position of CCA76-end may prevent proper interaction with the enzyme and/or tRNA end translocation.

The tRNA entrance pathway critically contributes to quality control

According to the crystal structure, the CP1 editing domain binds only with CCA76-end residues without any interaction with the main tRNA body. In detail, the equivalent side chains in EcLeuRS interacting specifically with the C74 base and the phosphates of C75 and A76 are Lys300, Arg344 and Tyr330, respectively (6). Additionally, the main chain carboxyl- and amino-group of Leu327 interact with the A76 base (Figure 5A). These amino acid residues are absolutely conserved in prokaryotic LeuRSs and thus constitute an entrance pathway to orient the aminoacylated CCA76-arm into the editing active site. Previous studies have demonstrated the essential role of Tyr330 of EcLeuRS in tRNA-dependent...
Figure 4. Effect of the CCA76 mutations on deacylation of mischarged tRNA^{Leu}. Mischarged tRNA mutants from A76, C75 or C74 were prepared by editing-deficient EcLeuRS-T252E (20). Mischarged tRNA-ΔA76 mutant was obtained with WT EcLeuRS. Deacylation of mischarged WT tRNA^{Leu} was indicated (unfilled circle). The deacylation assay of mischarged ΔA76 (unfilled square), A76U (filled inverted triangle), A76G (unfilled triangle), A76C (filled square) in (A), C75A (filled inverted triangle), C75U (unfilled triangle), C75G (filled square) in (B) or C74A (filled inverted triangle), C74U (unfilled triangle), C74G (filled square) in (C) was performed at 37°C with 1 μM [3H]Ile-tRNA^{Leu} and 5 μM EcLeuRS. The spontaneous hydrolysis of each mischarged tRNA (control) was carried out without enzyme. For clarity, a single representative control is shown (filled circle).

Figure 5. The tRNA entrance pathway contributes critically to the quality control. (A) View of tRNA^{Leu} CCA76-end bound to the CP1 domain (gray) (6), showing the binding pattern between C74 (yellow), C75 (cyan), A76 (blue) and the key amino acids (green) in the CP1 editing domain (upper). The tRNA main body before nucleotide A73 (orange) and LeuRS main body except for the editing domain were omitted for clarity. The lower panel shows the closer protein–CCA76 interaction network. Distances between the amino-acid residues here studied and the nucleotides are shown. (B) Mischarging of tRNA^{Leu} with non-cognate Ile by WT EcLeuRS (filled circle), -Y330D (unfilled circle), -K300E/Y330D (filled inverted triangle), -R344D (unfilled triangle), -Y330D/R344D (filled square), -K300E/Y330D/R344D (unfilled square) and -R344D (filled diamond) with significant misaminoacylation ability. Mutants that did not accumulate Ile-tRNA^{Leu} are not shown. (C) Deacylation curves of Ile-tRNA^{Leu} by WT EcLeuRS (filled circle), -L327G (filled square) and -R344D (filled diamond). Editing-defective mutants (EcLeuRS-Y330D, -K300E/Y330D, -Y330D/R344D and -K300E/Y330D/R344D) are not shown for clarity. Spontaneous hydrolysis without enzyme addition was carried out as a control (unfilled square) as indicated.
editing (8). However, other sites (Leu327, Lys300 and Arg344) and their functions in vivo have not been determined. We studied here the roles of residues Leu327, Lys300, Tyr330 and Arg344 by mutating them individually or in combination. The in vitro and in vivo assays were then performed in order to analyze the effects of these mutations.

The following single, double and triple mutants were constructed in EcLeuRS to form EcLeuRS-L327G, -L327R, -K300E, -Y330D, -R344D, -K300E/Y330D, -K300E/R344D, -Y330D/R344D and -K300E/Y330D/R344D. All these mutants displayed intact synthetic activities (Leu activation and aminoacylation) as expected from their location in the discrete CP1 editing domain (data not shown). However, EcLeuRS-Y330D, -K300E/Y330D, -Y330D/R344D and the triple mutant EcLeuRS-L327G, -K300E, -Y330D, -R344D were completely defective in post-transfer editing (data not shown), and they produced significant amounts of Ile-tRNALeu aminoacylation.

In the TLC assay, EcLeuRS-Y330D, its derived double and triple mutants and EcLeuRS-K300E/R344D only retained the tRNA-independent pre-transfer editing activity (Table 3). Altogether, the data showed that the Tyr330 residue plays a major role in tRNA-dependent editing as previously demonstrated (8). The negative effect of Y330D on editing was dominant, and the combination mutant EcLeuRS-K300E/R344D could reach the same level of tRNA-independent editing of the single EcLeuRS-Y330D mutation. Other individual mutants showed more limited decreases in post-transfer editing activity, inducing no measurable tRNA mischarging (data not shown). These in vitro data confirmed that mutating the CCA₇₆ entrance pathway of the CP1 domain has a negative effect on the accuracy of tRNA Leuₐₕₗₖ aminoacylation.

To evaluate if these losses of accuracy could affect the fidelity of protein synthesis in vivo, complementation assays using the leuS temperature-sensitive strain KL231 were performed (18). The strain KL231 was transformed with various plasmids encoding EcLeuRS and different mutants. At 42°C, all the transformants grew on medium supplemented with 100 µg ml⁻¹ ampicillin and 200 µg ml⁻¹ thymine (Figure 6), and the mutants were well expressed at a level similar to that of WT EcLeuRS (Supplementary Figure S2). The growth was then observed at 42°C in the presence of increasing concentrations of Nva in the growth medium. No growth difference could be observed below 20 mM of Nva. However, in the presence of 50 mM Nva, the strains harboring the genes-encoding EcLeuRS-Y330D, -K300E/Y330D, -K300E/R344D, -Y330D/R344D and -K300E/Y330D/R344D grew slowly or were inhibited completely, especially those containing the last double and triple mutants (Figure 6). With a 100 mM of Nva no strains containing mutants were able to grow, except for the strain expressing WT enzyme which only grew at a very slow rate. All strains died in the presence of 200 mM Nva.

Altogether, the in vivo data are consistent with the in vitro results, demonstrating that perturbing the tRNA CCA₇₆-end entry pathway in the CP1 domain has a significantly negative effect on the fidelity of protein biosynthesis.

| EcLeuRS          | tRNA Leuₐₕₖₖ (transcript) | $k_{obs}$ (s⁻¹) | Relative $k_{obs}$ |
|------------------|---------------------------|----------------|--------------------|
| WT               | +                         | 4.42 ± 0.64    | 1                  |
| WT               | -                         | 0.56 ± 0.07    | 0.13               |
| -L327G           | +                         | 1.86 ± 0.26    | 0.42               |
| -L327R           | +                         | 3.60 ± 0.45    | 0.81               |
| -K300E           | +                         | 2.54 ± 0.41    | 0.57               |
| -Y330D           | +                         | 0.59 ± 0.06    | 0.14               |
| -R344D           | +                         | 1.26 ± 0.19    | 0.29               |
| -K300E/Y330D     | +                         | 0.46 ± 0.03    | 0.10               |
| -K300E/R344D     | +                         | 0.82 ± 0.07    | 0.19               |
| -Y330D/R344D     | +                         | 0.49 ± 0.06    | 0.11               |
| -K300E/Y330D/R344D | +                   | 0.59 ± 0.06    | 0.14               |

Figure 6. In vivo toxicity resulting from mutations in the CP1 tRNA entrance pathway of EcLeuRS. The complementation assay was performed using E. coli thermosensitive strain KL231 at 42°C, on solid LB plates supplemented with 100 µg ml⁻¹ ampicillin, 200 µg ml⁻¹ thymine, 0.1 mM IPTG and increasing concentrations of non-cognate Nva. KL231 was transformed with mutated copies of LeuRS-encoding genes, and growth was compared with the negative control (empty pTrc99a vector) and positive control (WT EcLeuRS). Expression of all LeuRSs proteins was controlled by Western blot (Supplementary Figure S2).
Different tRNA<sup>Leu</sup> isoacceptors stimulate LeuRS editing with the same efficiency

In *E. coli*, there are five different tRNA<sup>Leu</sup> isoacceptors with two types of first base pairs in its accepting stem (Figure 1). tRNA<sub>GAG</sub> and tRNA<sub>GAG</sub> harbor the wobble base pair (G<sub>1</sub>-U<sub>72</sub>); while tRNA<sub>Leu</sub> and tRNA<sub>CAG</sub> contain the standard Watson-Crick one (G<sub>1</sub>-C<sub>72</sub>). It has been shown that EcLeuRS has the same specificity and activity toward different isoacceptors in aminoacylation (29). We transcribed the EcRNA<sub>GAG</sub> (with G<sub>1</sub>-U<sub>72</sub>) and tRNA<sub>CAG</sub> (with G<sub>1</sub>-C<sub>72</sub>) to represent two types of tRNA<sup>Leu</sup> and compared their capacity to stimulate editing by EcLeuRS. The data showed that they stimulated EcLeuRS editing activity with nearly identical efficiency (4.42 ± 0.64 and 4.54 ± 0.36 s<sup>−1</sup>, respectively) (Table 4). To explore the possible role of the first base pair in EcLeuRS editing, we mutated the U<sub>72</sub> to C<sub>72</sub> of tRNA<sub>GAG</sub> or the C<sub>72</sub> to U<sub>72</sub> of tRNA<sub>CAG</sub> to change the first base pair pattern. Measurements of AMP formation showed that the tRNA<sub>GAG</sub>(G<sub>1</sub>-C<sub>72</sub>) had the editing k<sub>obs</sub> of 3.75 ± 0.50 s<sup>−1</sup>, while the tRNA<sub>CAG</sub>(G<sub>1</sub>-U<sub>72</sub>) mutant stimulated the editing with k<sub>obs</sub> of 6.10 ± 0.81 s<sup>−1</sup> (Table 4). The above results suggested that EcLeuRS editing has no preference among WT isoacceptors with different first base-pairs. However, changing the first base-pair obviously led to distinct editing-stimulating capacities among isoacceptors. Therefore, the first base pair pattern may cooperate with other sequence differences between the two tRNA<sup>Leu</sup>s to confer the same level of editing-stimulating capacity of the two isoacceptors of WT tRNA<sup>Leu</sup>.

### DISCUSSION

The CCA<sub>76</sub>-end sequence is critical for both aminoacylation and editing reactions

The CCA<sub>76</sub> sequence is conserved at the 3′-end of all mature tRNA molecules. Although it is an indispensable prerequisite for a functional tRNA, very few organisms like *E. coli* encode the CCA<sub>76</sub>-triplet in their tRNA genes. Most of the time, the CCA<sub>76</sub> tail is to be added post-transcriptionally in eukaryotes, archaea and many bacteria, where the tRNA genes do not encode the CCA<sub>76</sub> terminus (30). This sequence is acquired and maintained by step-wise nucleotide addition by the ubiquitous tRNA nucleotidyltransferase that synthesizes this specific triplet without a nucleic acid template. The CCA sequence plays key roles during several steps of protein biosynthesis. During tRNA charging catalyzed by aaRSs, the 2′ or 3′ hydroxyl of ribose in A<sub>76</sub> is the group involved in the esterification reaction with the carboxyl of the amino acid. Although the CCA<sub>76</sub>-end does not play a critical role in recognition between tRNA and aaRS, it is essential in terms of catalytic efficiency for the aminoacylation reaction (31–33). The universal CCA<sub>76</sub> sequence is also involved in EF-Tu binding and during peptide bond formation by interacting with specific nucleotides of the 23S rRNA (17). Little is known about the precise role of the CCA<sub>76</sub> nucleotides during the editing reaction catalyzed by aaRSs. It has been shown that only chargeable tRNA<sup>Val</sup> mutants are able to stimulate the editing reaction of ValRS, suggesting that the enzyme required prior charging of tRNA (31,32,34). Although tRNA is not a strict prerequisite for the editing of both classes I and II aaRSs (26,35–37), its presence strongly stimulates editing (38). Remarkably, both classes of aaRSs use effectively the ability of the CCA<sub>76</sub>-end of tRNA to switch between a hairpin and a helical conformation during the aminoacylation and editing reactions. Our previous investigations have shown that the interaction between the tRNA<sup>Leu</sup> CCA<sub>76</sub>-end and the CP1 domain of LeuRS is critical for tRNA-dependent editing (post-transfer and pre-transfer editing) (8). However, the molecular basis of the phenomenon is not yet understood in detail. Here, we extended the investigations to the three CCA<sub>76</sub> nucleotides and assayed the effect of mutations on the tRNA-dependent pre-transfer editing and post-transfer editing.

We showed that the universally conserved A<sub>76</sub> is essential for tRNA<sup>Leu</sup> to trigger both aminoacylation and editing reaction of LeuRS. The mutants of tRNA<sup>Leu</sup> with any base substituted for A<sub>76</sub> showed significant decreases of aminoacylation properties and total loss of the ability to stimulate the editing activity and catalyze deacylation of pre-formed Ile-tRNA<sup>Leu</sup>. As a consequence of this loss of editing activity, two of these mutants (A<sub>76U</sub>, A<sub>76C</sub>) were misacylated by Ile. Mutations of the other bases of the CCA<sub>76</sub>-end had negative effects on the aminoacylation properties, ranging between 2- and 50-fold decreases of the catalytic efficiency of EcLeuRS, with the strongest effect being observed when C<sub>73</sub> and C<sub>74</sub> were substituted by G<sub>75</sub> and G<sub>74</sub>, respectively. Correspondingly, the mutant A<sub>76G</sub> was totally inactive, showing that the bulky guanosine substitutions were the least accepted in the synthetic active site. The three guanosine mutants also decreased the deacylation of the mischarged Ile-ttRNA<sup>Leu</sup>, but failed in misacylating tRNA<sup>Leu</sup> with Ile. Comparison of the global editing activity of EcLeuRS in the presence of the tRNA<sup>Leu</sup> mutants revealed that four mutations (C75A, C75U, C74A and C74U) led to an increase in the editing up to 2-fold despite the fact that their accepting capacity with Leu were impaired during the aminoacylation reaction. EcLeuRS deacylated these mischarged mutants of tRNA<sup>Leu</sup> with Ile with unchanged ability, suggesting that these mutants of tRNA<sup>Leu</sup> preferred binding at the editing active site rather than at the synthetic site. Previous studies have shown that both classes of aaRSs use effectively the ability of the CCA<sub>76</sub>-end of tRNA to switch.

### Table 4. k<sub>obs</sub> of AMP formation in the presence of different transcripts of tRNA<sup>Leu</sup> isoacceptors or mutants

| tRNA            | k<sub>obs</sub> (s<sup>−1</sup>) | Relative k<sub>obs</sub> |
|-----------------|---------------------------------|-------------------------|
| tRNA<sub>GAG</sub> | 4.42 ± 0.64                     | 1                       |
| tRNA<sub>CAG</sub> (G<sub>1</sub>-C<sub>72</sub>) | 3.75 ± 0.50                         | 0.85                    |
| tRNA<sub>CAG</sub> | 4.54 ± 0.36                     | 1                       |
| tRNA<sub>CAG</sub> (G<sub>1</sub>-U<sub>72</sub>) | 6.10 ± 0.81                         | 1.34                    |
between a hairpin and a helical conformation of aaRSs for aminoacylation and editing. In class I aaRSs, their cognate tRNA CCA76-ends adopt a regular helical conformation into the editing site of aaRSs, and a distorted hairpin conformation into the synthetic site (6). A mirror image is observed in class II aaRSs, as already seen for tRNA binding and amino acid activation. The stimulation of the editing activity of C75A, C75U, C74A and C74U mutants strongly suggests that these mutations may favor the helical conformation, which is more suitable for the editing reaction. Moreover, the fact that these mutated tRNAs are poorly aminoacylated in the synthetic active site may favor stagnation of the non-cognate adenylate into the synthetic active site and its subsequent editing by the tRNA-dependent pre-transfer editing also measured in the global editing assay.

The CCA76-end entrance pathway into editing domain of EcLeuRS critically contributes to protein synthesis fidelity

Despite the importance of the interaction between CCA76-end of tRNA and the CP1 domain of class Ia aaRSs revealed by in vitro methods, the corresponding in vivo evidence is generally lacking. In the present work, we developed an efficient system with the combination of non-cognate Nva and leuS temperature-sensitive E. coli strain to assess the significance of LeuRS tRNA-dependent editing in vivo. Our evaluation of the tRNA CCA76-end entrance pathway using the leuS temperature-sensitive KL231 strain revealed the following aspects. (i) Without or at a low concentration of non-cognate amino acid (e.g. 20 mM Nva here), mutation of pivotal residues controlling protein biosynthesis fidelity had no obvious effect on the bacterial cell viability. The discrimination by EF-Tu and/or the ribosome may have prevented Nva mis-incorporation, or the E. coli cells could endure low level of Nva mis-incorporation at Leu codons; (ii) at extremely high concentrations of non-cognate amino acids, even the WT enzyme had no ability to prevent all the mis-activated amino acids from entering the newly synthesized polypeptide on the ribosome, and the mis-translation led to an obvious inhibitory effect on cell growth; (iii) our in vitro results were consistent with in vivo data, demonstrating the importance of conserved amino acid residues in aaRS and nucleotides in tRNA in protein synthesis quality control, especially under severe environmental stress.

Multiple steps to monitor and control the CCA acceptor sequence

As mentioned above, the trinucleotide CCA76 sequence is present at the 3' terminus of all mature tRNAs. Despite this high conservation, transcripts of E. coli tRNAVal with altered 3' termini are readily aminoacylated and can function in polypeptide synthesis (32). Accordingly, the present study performed on LeuRS shows that the aminoacylation reaction admits some sequence flexibility at the CCA76 end. Several CCA-mutated tRNAs can be efficiently aminoacylated, and LeuRS can even aminoacylate a tRNALeu lacking the terminal adenosine, showing a remarkable plasticity of the synthetic site of LeuRS and tRNALeu acceptor end. Several studies suggest that the aminoacylation of similar molecules may occur naturally in vivo. Despite the high fidelity of the CCA-adding enzyme, in some conditions, CCase can add a wrong nucleotide to the 3'-end of the tRNA, leading to CCA-modified tRNAs (39). Moreover, mutations of CCase may exist in vivo with relaxed active sites as those engineered in vitro that lead to nucleotide mis-incorporation at the end of tRNA (40). In some bacteria the CCA-adding activity is naturally split into a CC-adding enzyme and an A-adding enzyme (41). Therefore, a tRNA deprived of the terminal A76 might temporarily exist in vivo and may be the substrate of LeuRS.

However, if under these exceptional circumstances aminoacylation of CCA-mutated tRNAs can occur, these tRNAs would have to face additional discrimination processes based on EF-Tu recognition (42) and interaction with ribosomal RNA during translation (43). Both processes would monitor and exclude these molecules according to different nucleotide recognition specificities (32).

In summary, the study highlighted the significant role of the conserved nucleotides from the CCA76-end and the strong collaboration with amino acid residues of the CP1 editing domain during the aminoacylation and editing catalysis. The CCA76 nucleotides are critical for both aminoacylation and editing; and in combination with the amino acids located in the entrance path of the editing domain, they critically contribute to the fidelity of protein synthesis. It is a remarkable example of a control mechanism that prevent damaged tRNAs from entering the protein synthesis based on the single discrimination of a triplet sequence that is universally found in all tRNAs.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

The authors thank Dr Franck Martin for the help during the experiment.

FUNDING

Natural Science Foundation of China (Nos. 30930022 and 31000355); Committee of Science and Technology in Shanghai (No. 09JC1415900); a Visiting Professorship for Senior International Scientists from the Chinese Academy of Sciences (No. 2009S2-19); Programme International de Coopération Scientifique from CNRS (Grant 3606). Funding for open access charge: Natural Science Foundation of China.

Conflict of interest statement. None declared.
REFERENCES

1. Ibba, M. and Soll, D. (2000) Aminoacyl-tRNA synthesis. Annu. Rev. Biochem., 69, 617–650.
2. Schimmel, P. (1987) Aminoacyl tRNA synthetases: general scheme of structure-function relationships in the polypeptides and recognition of transfer RNAs. Annu. Rev. Biochem., 56, 125–158.
3. Lee, J.W., Lee, H.B., Shin, J.Y., Gregor, M., Wilkerson, D.F., and Sisk, T.T. (2005) The crystal structure of E. coli leucyl-tRNA synthetase complexed with tRNA^Leu^ demonstrates the significance of discriminant-base recognition. J. Mol. Biol., 349, 225–231.
4. Ibba, M., and Soll, D. (2000) Aminoacyl-tRNA synthetases. Annu. Rev. Biochem., 72, 119–146.
5. Tan, H., M., Yang, B., Zhou, X., He, R., Chen, X., Eriani, G., and Gangloff, J. (1999) Yeast aspartyl-tRNA synthetase residues interacting with tRNA(Asp) identity sequences and conformation. Nucleic Acids Res., 27, 937–940.
6. Hou, Y.M. (2000) Unusual synthesis by the Escherichia coli valyl-tRNA synthetase. RNA, 6, 1031–1043.
7. Nissen, P., Kjeldgaard, M., Thrup, S., Polekhina, G., Reshetnikova, L., Clark, B.F.C., and Nyborg, J. (1995) Crystal structure of the ternary complex of Phe-tRNAPhe, EF-Tu, and a CCA-adding enzyme. J. Mol. Biol., 251, 253–266.
8. Li, Y., Wang, E. and Wang, Y. (1998) Overproduction and purification of Escherichia coli tRNA^Leu^ synthetase. J. Biol. Chem., 273, 23978–23985.
9. Liu, M., and Horowitz, J.J. (1994) Functional transfer RNAs with modifications in the 3'-CCA end: differential effects on aminoacylation and polypeptide synthesis. Proc. Natl Acad. Sci. USA, 91, 10389–10393.
10. Tamura, K., Nameki, N., Hasegawa, T., Shimizu, M., and Hime, R. (1994) Role of the CCA terminal sequence of tRNA(Val) in aminoacylation with valyl-tRNA synthetase. J. Biol. Chem., 269, 22173–22177.
11. Liu, M., and Horowitz, J.J. (1994) Transfer RNA determinants for translational editing by Escherichia coli valyl-tRNA synthetase. Nucleic Acids Res., 22, 915–918.
12. Cusack, S. (2005) The crystal structure of leucyl-tRNA synthetase and tRNALeu reveal two modes of discriminator-base recognition. Nat. Struct. Mol. Biol., 12, 915–922.
13. Peck, D., and Spokony, R. (1986) The CCA end of tRNA modulates the editing mechanism used by class II leucyl-tRNA synthetase. J. Biol. Chem., 261, 10761–10766.
14. Ito, T., Wu, N., Inagaki, K., and Tani, T. (1999) Identification of the discriminator base in leucyl-tRNA synthetase that affects editing. J. Biol. Chem., 274, 9509–9512.
15. Cusack, S. (2005) The crystal structure of leucyl-tRNA synthetase and tRNALeu reveal two modes of discriminator-base recognition. Nat. Struct. Mol. Biol., 12, 915–922.
16. Schurr, H., Schiffer, S., Marchfelder, A., and Morley, M. (2001) This is the end: processing, editing and repair at the tRNA 3'-terminus. Biochim. Biophys. Acta, 1513, 103–113.
17. Gruic-Sovulj, I., Uter, N., Bullock, T. and Perona, J.J. (2005) tRNA-dependent aminoacyl adenylate hydrolysis by a nonedited Class I aminoacyl-tRNA synthetase. J. Biol. Chem., 280, 23978–23986.
18. Low, B., Gates, F., and Giege, R. (1998) Universal rules and modes of discriminator-base recognition. Nat. Struct. Mol. Biol., 12, 922–930.
19. Chen, J., Guo, N., Li, T., Wang, E. and Wang, Y. (2000) CP1 domain in Escherichia coli leucyl-tRNA synthetase is crucial for its editing function. Biochemistry, 39, 6726–6731.
20. Tan, M., Zhu, B., Zhou, X., He, R., Chen, X., Eriani, G. and Wang, E. (2010) RNA-dependent pre-transfer editing by prokaryotic leucyl-tRNA synthetase. J. Biol. Chem., 285, 3235–3244.
21. Schurer, H., Schiffer, S., Marchfelder, A., and Morl, M. (2001) This is the end: processing, editing and repair at the tRNA 3'-terminus. Biochim. Biophys. Acta, 1513, 103–113.
enzymes or poly(CA) and poly(UG) polymerases.

41. Tomita, K. and Weiner, A.M. (2001) Collaboration between CC- and A-adding enzymes to build and repair the 3’-terminal CCA of tRNA in *Aquifex aeolicus*. *Science*, 294, 1334–1336.

42. Nissen, P., Kjeldgaard, M., Thirup, S., Polekhina, G., Reshetnikova, L., Clark, B.F. and Nyborg, J. (1995) Crystal structure of the ternary complex of Phe-tRNAPhe, EF-Tu, and a GTP analog. *Science*, 270, 1464–1472.

43. Selmer, M., Dunham, C.M., Murphy, F.V.t., Weixlbaumer, A., Petry, S., Kelley, A.C., Weir, J.R. and Ramakrishnan, V. (2006) Structure of the 70S ribosome complexed with mRNA and tRNA. *Science*, 313, 1935–1942.