Mutational unmasking of a tRNA-dependent pathway for preventing genetic code ambiguity

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Aminoacyl-tRNA synthetases establish the genetic code by matching each amino acid with its cognate tRNA. Aminoacylation errors lead to genetic code ambiguity and statistical proteins. Some synthetases have editing activities that clear the wrong amino acid (aa) by hydrolysis of either of two substrates: misactivated aminoacyl-adenylates (''pretransfer'' of aa to tRNA) or misacylated aa-tRNA (''posttransfer''). Whereas posttransfer editing can be directly measured, pretransfer editing is difficult to demonstrate, because adenylates are inherently labile and transient, and activity occurs against a background of posttransfer editing. Herein, different mutations in *Escherichia coli* leucyl-tRNA synthetase are combined to unmask the pretransfer pathway. The mutant enzymes completely lack posttransfer editing but prevent misacylations by clearing misactivated adenylates. We hypothesize that these mutations isolate a pretransfer translocation step that moves misactivated adenylates from the activation site for editing. The results highlight how evolution redundantly created two distinct pathways to prevent genetic code ambiguity.

amino acid editing | aminoacylation | fidelity | protein synthesis | tRNA synthetase

Aminoacyl-tRNA synthetases (aaRSs) comprise a family of enzymes that are responsible for accurate aminoacylation of tRNA (1, 2). In this first step of protein synthesis, a specific amino acid (aa) is linked or ''charged'' to its cognate tRNA isoacceptor. Errors by the synthetase would result in genetic code ambiguity and yield statistical proteins that disrupt cellular functions (3). Thus, many aaRSs have developed editing mechanisms to clear their mistakes and maintain the fidelity of protein synthesis (4). Moreover, to meet a critical threshold of accuracy that is required by the cell (5), redundant editing mechanisms within these synthetases have evolved that capitalize on clearing errors at both steps of the two-step aminoacylation reaction (Fig. 1 and ref. 6).

Most aaRSs that edit seem to operate by a mixture of pre- and posttransfer editing, although one of these pathways may dominate. Pretransfer editing, where misactivated noncognate aminoacyl-adenylate intermediates are hydrolyzed by tRNA synthetases (7), has persisted as a controversial fidelity pathway in protein synthesis (8). Although posttransfer editing (6, 9) of mischarged tRNAs can be directly measured, the adenylate substrates for pretransfer editing are highly labile and transient. In addition, because a number of tRNA synthetases edit redundantly by means of an idiosyncratic partitioning of pre- and posttransfer editing mechanisms (6), it can be difficult to unambiguously isolate pretransfer editing activity while the enzyme is concurrently hydrolyzing mischarged aa-tRNAs.

Leucyl-tRNA synthetase (LeuRS) and two other closely related aaRSs [isoleucyl- (IleRS) and valyl- (ValRS) tRNA synthetases] that aminoacylate aliphatic amino acids have been well documented to misactivate and edit noncognate amino acids that structurally overlap with their respective substrates (10–19). The hydrolytic editing active site for each of these enzymes is located within homologous domains called the connective polypeptide 1 [CP1 (13, 14, 16, 19–26)]. The co-crystal structures of *Thermus thermophilus* LeuRS complexed to pre- and posttransfer editing substrate analogs suggest that the pre- and posttransfer editing active sites overlap (19). This overlap of editing active sites, which is proposed not only for LeuRS but also for other editing aaRSs, has further complicated deconvolution of the pre- and posttransfer editing mechanisms.

A threonine-rich region within the CP1 domain marks the editing active site (13, 17, 19, 26). In addition, an aspartic acid that is universally conserved across LeuRS, IleRS, and ValRS interacts with the amino group of the bound amino acid to anchor and orient either the pre- or posttransfer editing substrate for hydrolysis (19, 27). The adenosine for either substrate is also bound in an overlapping pocket (19). The two ''conserved'' ends of the pre- and posttransfer editing analogs are accommodated by twisting and contorting their distinct phosphoanhydride and acyl linkages (Fig. 1*B*). A second less conserved distal region within the *Escherichia coli* LeuRS CP1 domain that is ≈20 Å from the editing site also seems to influence editing activity. Mutational and computational analysis within this region of LeuRS suggests that A293 plays a role in aminoacylation and fidelity in *E. coli* LeuRS (28–32).

E. coli LeuRS is unusual because it relies solely on posttransfer editing to correct its mistakes (10). Mutation of the conserved T252 residue within the threonine-rich region of LeuRS to tyrosine blocks amino acid binding in the active site and abolishes editing activity (16). We combined secondary mutations at the A293 site in the *E. coli* LeuRS CP1 domain and also at an adjacent site on the main body of the enzyme that rescued fidelity. Because posttransfer editing activity remains abolished, we propose that we have activated a pretransfer editing pathway in *E. coli* LeuRS that depends on tRNA and ATP as well as noncognate amino acids. We hypothesize that these mutations unmask a translocation step of the pretransfer editing pathway.

Results

Rescue of a Pretransfer Editing Activity in LeuRS. The hydrolytic editing active site has been defined atomically by co-crystal structures of LeuRS bound to editing substrate analogs (19) as well as by mutational work (16, 19, 26, 33). Substitution of a conserved threonine residue to tyrosine (T252Y) in *E. coli* LeuRS blocks amino acid binding in the active site and abolishes amino acid editing activity (16). A second region of the CP1 domain has also been proposed to affect the enzymatic activities of LeuRS (28, 29, 31). It is located ≈20 Å from the editing active site and is marked by a semiconserved A293 residue in *E. coli* LeuRS.

We combined the editing-deficient T252Y substitution with a second A293D mutation in *E. coli* LeuRS. Although the ami-

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Abbreviations: aaRS, aminoacyl-tRNA synthetase; LeuRS, leucyl-tRNA synthetase; IleRS, isoleucyl-tRNA synthetase; ValRS, valyl-tRNA synthetase; CP1, connective polypeptide 1.

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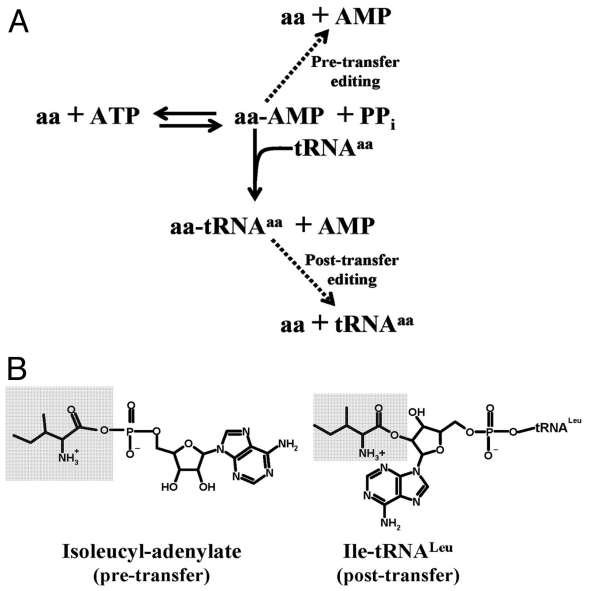

Fig. 1. aaRS enzymatic reactions and editing substrates. (*A*) Aminoacylation and editing reactions of aaRSs. The solid and dotted arrows represent aminoacylation and editing reactions, respectively. Amino acid and inorganic pyrophosphate are indicated by aa and PP_i, respectively. (*B*) The pre- (isoleucyl-adenylate; *Left*) and post- (Ile-$tRNA^{Leu}$; *Right*) transfer editing substrates for LeuRS.

noacylation activity of the single A293D LeuRS mutation had been reported to be significantly impaired (29), when affinity purified by using a six-histidine tag, we found similar initial velocities for leucylation by the WT, single mutant, and double mutant LeuRSs that reached plateau levels to fully charge *in vitro* transcribed $tRNA^{Leu}$ (Fig. 2*A*). In contrast, misaminoacylation assays showed that the double T252Y/A293D mutant significantly enhanced fidelity compared with the single T252Y mutant LeuRS that is deficient in posttransfer editing activity (Fig. 2*B*). LeuRS, which contained only the single A293D substitution, did not mischarge $tRNA^{Leu}$ significantly even at high concentrations of enzyme (1 μM), nor did it show alterations in posttransfer editing activity compared with WT LeuRS. However, in combination with the T252Y mutation, the A293D substitution rescues the editing deficiency but not via the posttransfer editing pathway. The double mutant T252Y/A293D *E. coli* LeuRS lacks posttransfer editing activity similar to the T252Y LeuRS mutant (Fig. 2*C*). Thus, fidelity is enhanced by an alternate pathway to posttransfer editing, which is clearly composed of the A293 site as an important molecular determinant.

Hydrolysis of Adenylate Intermediates. Englisch *et al.* (10) previously reported that *E. coli* LeuRS edits exclusively by a posttransfer editing mechanism, whereas yeast cytoplasmic LeuRS cleared misactivated aminoacyl-adenylates in a pretransfer editing mechanism. We further analyzed amino acid and tRNA-dependent ATP hydrolysis activity of these LeuRS enzymes by carefully separating ^{14}C-labeled ATP derivatives on thin-layer plates so that they could be directly quantitated. Although yeast cytoplasmic LeuRS exhibited ATP hydrolysis activity, we were also unable to detect pretransfer editing activity in the *E. coli* enzyme as previously reported. Thus, introduction of T252Y abolishes posttransfer editing activity by *E. coli* LeuRS (Fig. 2*C*) and therefore its overall editing activity (Fig. 2*D*) (16).

Overall editing activity for the WT and mutant LeuRSs was measured based on consumption of ATP in the presence of noncognate isoleucine. This activity assay typically represents the combined activities for pre- and posttransfer editing. The T252Y

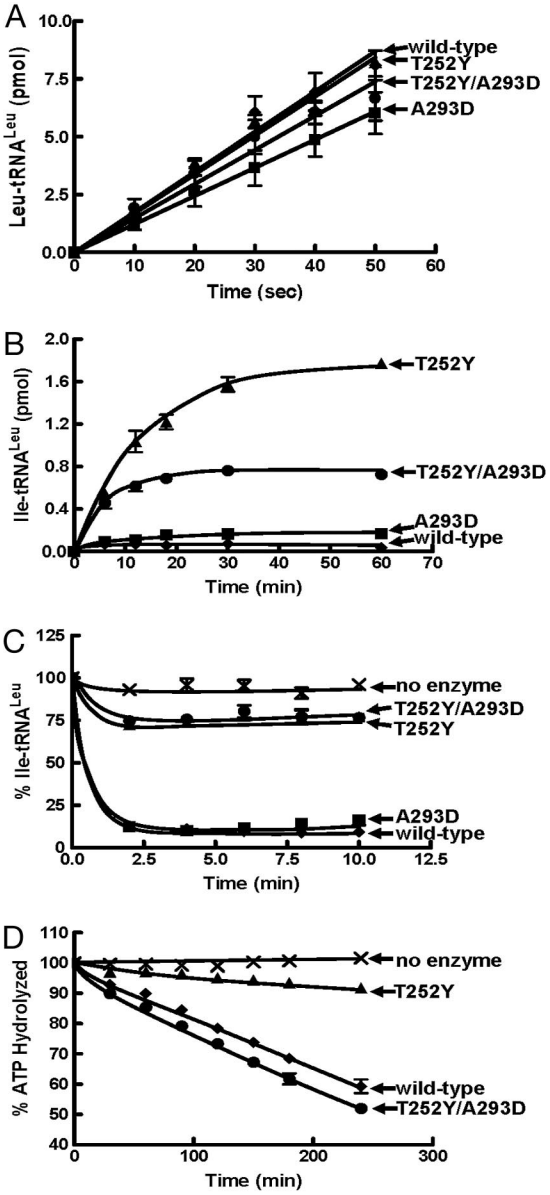

Fig. 2. Enzymatic activities of A293D single and double LeuRS mutations. (*A*) Aminoacylation of 4 μM $tRNA^{Leu}$ with leucine by 50 nM *E. coli* LeuRSs. (*B*) Mis-aminoacylation of 4 μM $tRNA^{Leu}$ with isoleucine by 1 μM *E. coli* LeuRSs. (*C*) Hydrolysis of ile-$tRNA^{Leu}$ by 100 nM LeuRS. (*D*) Isoleucine-dependent total overall editing activity by *E. coli* WT and mutant LeuRSs. The percentage of ATP hydrolyzed by WT, T252Y, and T252Y/A293D *E. coli* LeuRSs was determined in the presence of isoleucine and $tRNA^{Leu}$. ×, no enzyme control; ♦, WT; ■, A293D *E. coli* LeuRSs; ▲, T252Y *E. coli* LeuRSs; ●, T252Y/A293D *E. coli* LeuRSs.

mutation significantly reduced ATP hydrolysis compared with the WT enzyme, which is consistent with its loss of posttransfer editing and fidelity (Fig. 2*D*). However, introduction of the A293D substitution with the T252Y mutation restored ATP hydrolysis activity (Fig. 2*D*) similar to WT LeuRS. Because posttransfer editing is inactivated in the double mutant, but overall editing is rescued, these collective results suggest that the A293D mutation activates an editing pathway that is amino acid-, ATP-, and tRNA-dependent but distinct from the posttransfer editing pathway. We hypothesize that this is a pretransfer editing pathway that targets the aminoacyl-adenylate intermediate.

It was recently hypothesized that a pretransfer editing activity

might be conferred in the synthetic active site (8). Under pyrophosphate exchange conditions (see Fig. 7, which is published as supporting information on the PNAS web site), we determined that the T252Y/A293D mutant and WT LeuRS had similar K_m values for leucine of 0.03 mM and 0.02 mM respectively. The K_m for isoleucine was ≈10-fold higher, but consistent for both the WT and double mutant LeuRS at 0.2 mM. This finding supports that the distal mutations in the CP1 domain do not significantly alter amino acid interactions with the synthetic active site as was previously reported for the single A293D mutant (29).

Molecular Variations at the 293 Position. Sequence alignments of LeuRS from other organisms showed that the 293 site is conserved as either a basic residue (arginine or lysine) or an alanine, as in the case of *E. coli* LeuRS (Fig. 3*A*). When A293 was substituted by lysine in *E. coli* LeuRS, there were no effects on aminoacylation or mis-aminoacylation (Fig. 3 *B* and *C*). Likewise, combination of the A293K and T252Y mutations failed to alter the posttransfer editing deficiency. As would be expected, both the posttransfer editing activity and overall editing activity for the double T252Y/A293K mutant LeuRS were similar to the single editing-deficient T252Y mutant LeuRS (Fig. 3 *D* and *E*). Thus, activation of the pretransfer editing pathway in the *E. coli* enzyme was specific to introducing an aspartic acid that is not found in naturally occurring LeuRSs, at the A293 site.

Translocation of Noncognate Aminoacyl-Adenylate Intermediates. The A293 site resides in a CP1 based α-helix that is in close proximity to the main body of the enzyme (Fig. 4*A*). In particular, this peptide is very near (≈6 Å) a conserved lysine (K186) on the main body, which corresponds to K183 in IleRS (Fig. 4*B*). Bishop *et al*. (34) reported that K183 may be involved in a "hinge" movement of the CP1 domain and influences amino acid editing in IleRS, which predominantly edits by a pretransfer mechanism. Although *E. coli* LeuRS seems to lack pretransfer editing activity, we hypothesized that this corresponding K186 hinge site on LeuRS and the A293-containing peptide may facilitate interaction between the CP1 domain and the main body to aid in translocation of substrates from the aminoacylation active site for editing. We mutationally analyzed the main body K186 site of LeuRS by changing the positively charged side chain to a glutamic acid. This mutation was also combined with the T252Y editing-deficient mutation.

The K186E LeuRS mutant showed WT-like activity in aminoacylation. As would be expected, the single mutant also failed to misaminoacylate $tRNA^{Leu}$ (data not shown). Interestingly, when combined with the T252Y editing-defective mutation, fidelity was improved similar to the T252Y/A293D LeuRS double mutation (Fig. 5*B*). Likewise, posttransfer editing remained abolished in the T252Y/K186E mutation (Fig. 5*C*). However, as found for the T252Y/A293D mutant, overall editing activity based on amino acid- and tRNA-dependent ATP hydrolysis was similar to WT activity and suggested that a dormant pretransfer editing pathway had been activated (Fig. 5*D*). We hypothesize that the A293-containing peptide of the CP1 region and the K186 region of the main body of the enzyme comprise critical components of an editing pathway that is distinct from posttransfer editing. It is possible that these mutations contribute to a translocation mechanism for aminoacyl-adenylates in a pretransfer editing pathway that requires movement between the CP1 domain and main body of the enzyme.

Because leucylation activities are unaltered for the T252Y/A293D mutant LeuRS, this putative translocation pathway seems to be specific to noncognate aminoacyl-adenylates. We also tested the A293D mutation in the presence of a T252A

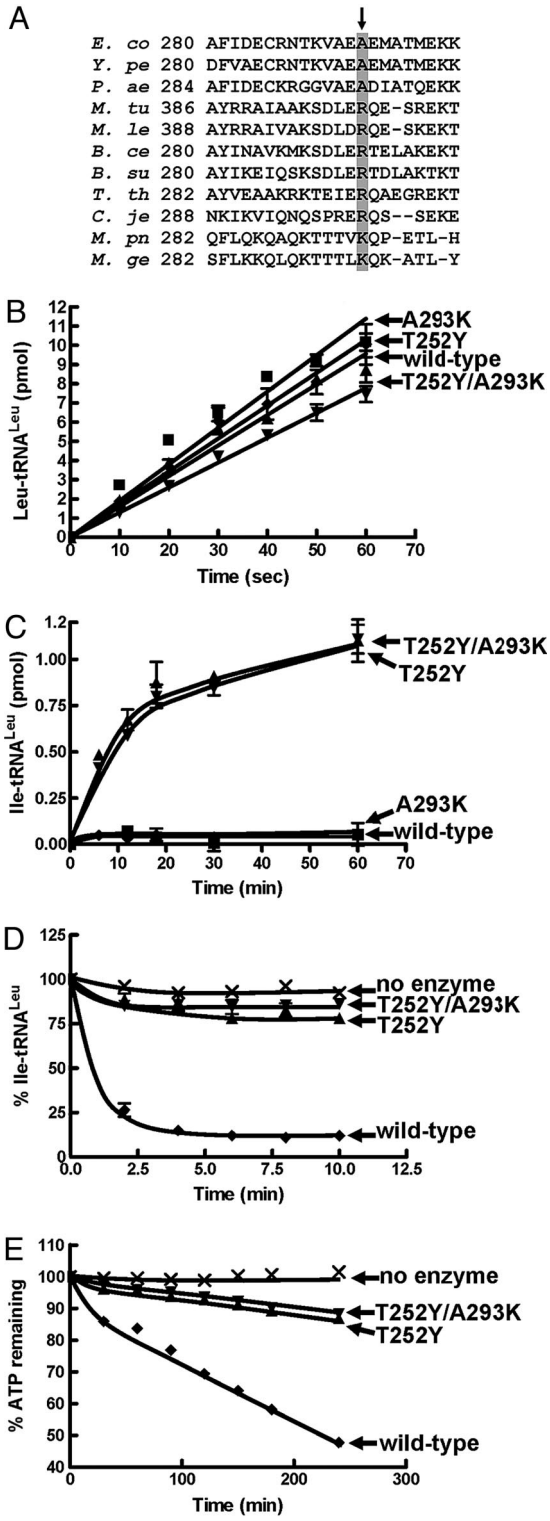

Fig. 3. Enzymatic activities of A293K single and double LeuRS mutations. (*A*) Sequence alignment of the CP1-based region containing A293. The arrow and gray shading indicate the semiconserved residue at the A293 position in *E. coli* LeuRS. (*B*) Aminoacylation of 4 μM $tRNA^{Leu}$ with leucine by 50 nM *E. coli* LeuRSs. (*C*) Mis-aminoacylation of 4 μM $tRNA^{Leu}$ with isoleucine by 1 μM *E. coli* LeuRSs. (*D*) Hydrolysis of ile-$tRNA^{Leu}$ by 100 nM LeuRS. (*E*) Isoleucine-dependent total overall editing activity by *E. coli* WT and mutant LeuRSs. The percentage of ATP hydrolyzed by WT, T252Y, and T252Y/A293K *E. coli* LeuRSs was determined in the presence of isoleucine and $tRNA^{Leu}$. Symbols are as follows: ×, no enzyme control; ♦, WT; ■, A293K *E. coli* LeuRSs; ▲, T252Y *E. coli* LeuRSs; ▼, T252Y/A293K *E. coli* LeuRSs.

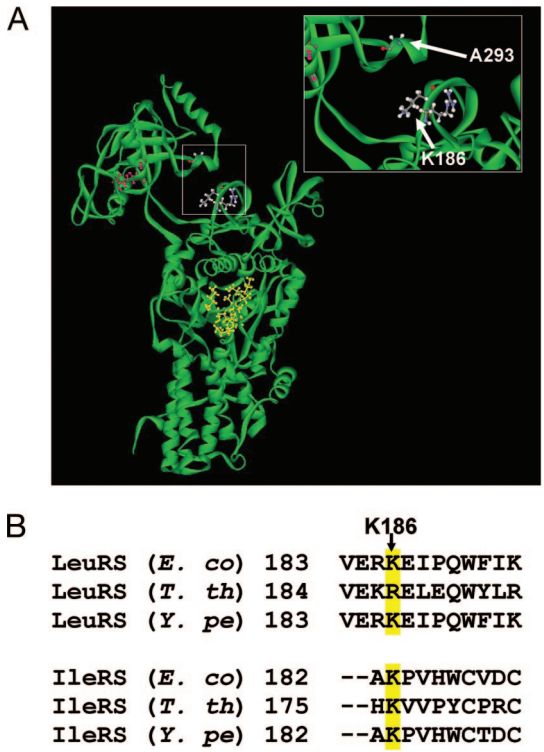

Fig. 4. Primary and tertiary structure of *E. coli* LeuRS. (*A*) Homology model of *E. coli* LeuRS (32). Editing active site residues (T252, D342, and D345) are highlighted in red, whereas the conserved sequences HIGH and KMSKS in the aminoacylation active site are shown in yellow. The A293 and K186 interface between the CP1 domain and main body is enlarged in the boxed *Inset*. (*B*) Primary sequence alignment of the *E. coli* LeuRS region containing K186. The yellow shading highlights the conservation of lysine or arginine in LeuRSs with the proposed hinge lysine of IleRSs.

substitution. T252A uncouples specificity in the editing active site and facilitates posttransfer editing of the correctly charged leu-tRNA^{Leu} (26). Thus, the LeuRS T252A mutation results in significantly decreased yields of leucylated tRNA product (Fig. 6). Addition of the A293D mutation to the T252A LeuRS fails to alter or rescue these yields and demonstrates that the posttransfer editing active site remains intact and competent in the presence of this secondary mutation. It also supports that this alternate editing pathway does not target leucyl-adenylate, but is specific for noncognate aminoacyl-adenylates, which would be consistent with a pretransfer editing pathway translocation mechanism.

Discussion

LeuRS, IleRS, and ValRS share common amino acid editing mechanisms that are carried out in homologous CP1 domains. These distinct domains are linked via flexible β-strand tethers to the main body of the enzyme where amino acid activation and aminoacylation occur. Redundant pathways for amino acid editing exist that target mistakes produced by either of the two steps of the aminoacylation reaction (Fig. 1*A* and ref. 6). Pretransfer editing hydrolyzes misactivated aminoacyl-adenylates, whereas posttransfer editing hydrolyzes mischarged tRNAs (Fig. 1*B*). Herein, we have combined mutations to abolish posttransfer editing and selectively activate a pretransfer pathway for *E. coli* LeuRS to clear its misactivated adenylate intermediates. One key question in both editing pathways is how the mischarged tRNA and misactivated aminoacyl-adenylate are translocated from the aminoacylation active site to the editing active site. A fluorescent-based translocation assay in IleRS has

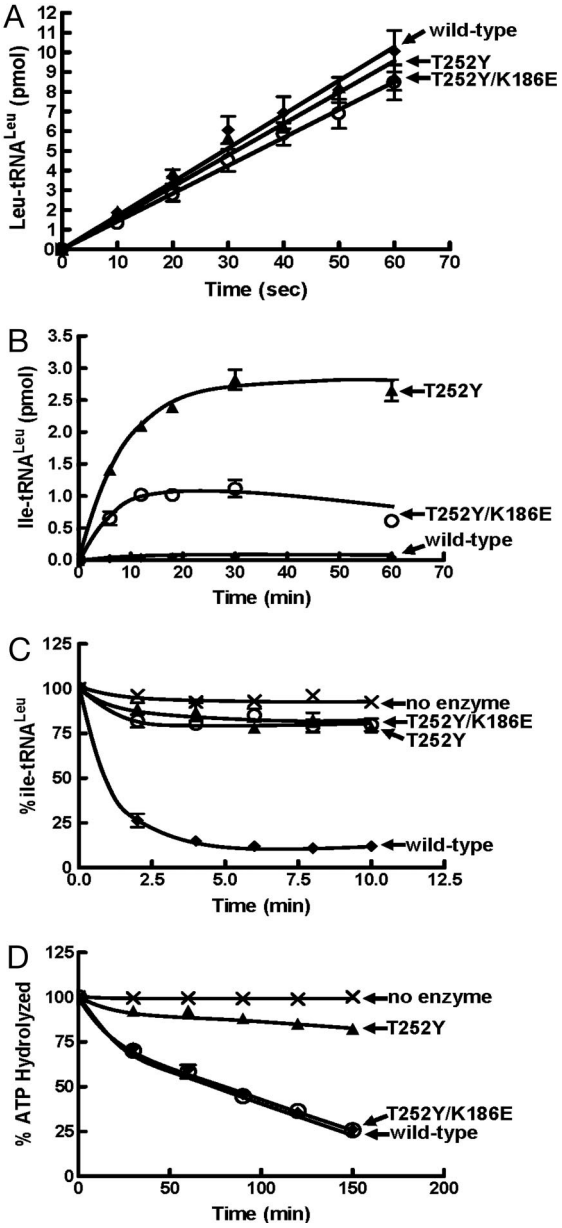

Fig. 5. Enzymatic activities of K186E single and double LeuRS mutations. (*A*) Aminoacylation of 4 μM tRNA^{Leu} with leucine by 50 nM *E. coli* LeuRSs. (*B*) Mis-aminoacylation of 4 μM tRNA^{Leu} with isoleucine by 1 μM *E. coli* LeuRSs. (*C*) Hydrolysis of ile-tRNA^{Leu} by 100 nM LeuRS. (*D*) Isoleucine-dependent total overall editing activity by *E. coli* WT and mutant LeuRSs. The percentage of ATP hydrolyzed by WT, T252Y, and T252Y/K186E *E. coli* LeuRSs was determined in the presence of isoleucine and tRNA^{Leu}. ×, no enzyme control; ♦, WT; ▲, T252Y *E. coli* LeuRSs; ○, T252Y/K186E *E. coli* LeuRSs.

shown that ATP evacuates the aminoacylation active site during editing (14, 17, 34, 35), but the molecular details of translocation remain unclear. Alternatively, a recent investigation with glutaminyl (Gln)-tRNA synthetase proposed that some tRNA synthetases may hydrolyze aminoacyl-adenylate intermediates directly within the synthetic active site (8).

Translocation of the tRNA or small adenylate molecule would presumably require specific molecular interactions, as well as communication between the CP1-editing domain and the canonical core of the class I synthetase, which is responsible for aminoacylation. We have identified an interface between the two domain surfaces that might be responsible, at least in part, for

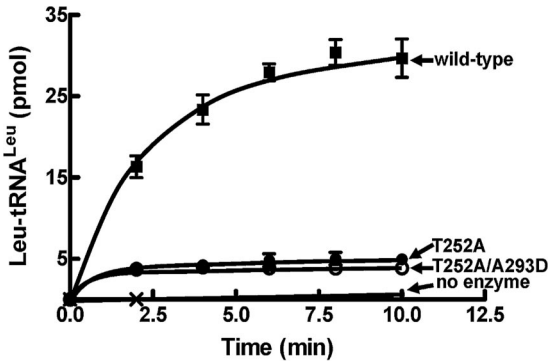

Fig. 6. Aminoacylation by *E. coli* WT and T252A mutant LeuRSs. Shown is aminoacylation of 4 μM tRNA^{Leu} with leucine by 50 nM *E. coli* LeuRSs. ×, no enzyme control; ■, WT; ●, T252A *E. coli* LeuRSs; ○, T252A/A293D *E. coli* LeuRSs.

molecular interactions that would facilitate translocation. This site is defined in *E. coli* LeuRS by a peptide that contains A293 in the CP1 domain (25, 28–32) and a conserved lysine (K186) that has been proposed to act as a molecular hinge to enable editing in IleRS (34). The homology model of *E. coli* LeuRS (32) based on the crystal structure of *T. thermophilus* LeuRS (24) shows that these two surfaces are separated by as little as 6 Å. Comparison of the assortment of x-ray crystal structures for LeuRS, IleRS, and ValRS with and without substrates (13, 19, 22–24, 36) supports that the CP1 domain rotates on its β-strand tethers relative to the main body of the enzyme. In LeuRS, these different states impact the proximity of the A293 and K186 sites. These varied orientations may represent steps in the translocation and/or editing pathways.

WT *E. coli* LeuRS lacks pretransfer editing activity (10). We inactivated its posttransfer editing pathway, and therefore its overall editing activity, by introducing mutations within the editing active site where catalysis occurs (16). We have rescued this editing-deficient mutant by introducing distal second mutations at A293 or K186. These sites are at least 20 Å from the hydrolytic editing active site but are located at the molecular interface between the CP1 domain and main body as defined above. Each of these LeuRSs, where secondary mutations have restored fidelity, remains completely defective in posttransfer editing of the mischarged tRNA. Thus, the adenylate intermediate is presumably targeted for hydrolysis. We propose that our second mutation has activated a defunct or very weak pretransfer editing pathway in *E. coli* LeuRS that resembles those that are operational in other LeuRSs from different species.

The co-crystal structure of LeuRS with pre- and posttransfer editing substrate analogs, as well as mutational analysis, supports that the active sites of the pre- and posttransfer editing pathways overlap (19). Because the T252Y mutation abolishes editing by blocking the amino acid binding pocket, the putative pretransfer editing active site would also be expected to be inaccessible for substrate binding. Rather, we hypothesize that we have isolated an important portion of a pretransfer editing pathway in LeuRS that facilitates translocation of aminoacyl-adenylates. Because the mischarged tRNAs are stable in the presence of mutant LeuRSs that contain the T252Y editing defect, enhanced fidelity is achieved for these double mutants by targeting the misactivated isoleucyl-adenylate that is produced in the first step of the aminoacylation reaction. Moreover, because the T252Y mutation would be expected to abolish enzymatic pretransfer editing if present, we hypothesize that either of the second site mutations (A293D or K186E) simply alters a small molecule translocation pathway such that the adenylate substrate is exposed to water. This aqueous environment would rapidly break down the adenylate intermediate and enhance fidelity. Because the K_m values for leucine and isoleucine are consistent for the WT and T252Y/A293D mutant LeuRSs, we propose that the aminoacyl-adenylate is specifically translocated, rather than simply spilled, into bulk solvent directly from the synthetic site.

Interestingly, mutation of A293 to the naturally occurring lysine found in other LeuRSs does not alter activity. LeuRSs that contain this semiconserved lysine also exhibit pretransfer editing activity. Thus, we propose that a positively charged residue at the A293 site may be part of a more competent "nonleaky" mechanism that translocates adenylate molecules directly to an editing active site.

This translocation pathway is clearly specific for noncognate aminoacyl-adenylates in LeuRS, which would be consistent with a pretransfer editing pathway. Although fidelity is altered for the T252Y/A293D and T252Y/K186E double mutant LeuRSs compared with the editing-deficient T252Y single mutant LeuRS, leucylation activities for each single and double mutant are similar to the WT enzyme. Moreover, the addition of A293D fails to alter the leucylation or posttransfer editing activities of the T252A mutant LeuRS that hydrolyzes leu-tRNA^{Leu}.

If the A293/K186 interface represents a portion of a pretransfer editing pathway, then our results also suggest that different translocation specificities and mechanisms have evolved for the pre- and posttransfer editing pathways. Previously, we showed that the LeuRS T252A mutation uncouples amino acid editing specificity and hydrolyzes the correctly charged leu-tRNA^{Leu} (26). Thus, translocation of the charged or mischarged tRNA lacks strict specificity for posttransfer editing but is resolved by blocking leucine from binding to the hydrolytic editing active site. In contrast, the results presented herein suggest that the translocation pathway for adenylate intermediates is quite specific. A translocation mechanism that is highly specific for noncognate amino acids could simply eject the labile misactivated adenylate from the aminoacylation active site into the enzyme's aqueous environment for hydrolysis to complete the pretransfer editing process.

It remains unclear why *E. coli* LeuRS lacks a clear or robust pretransfer editing activity when other LeuRSs, IleRS, and ValRS seem to edit by either pathway. However, these results demonstrate that the partition between pre- and posttransfer editing within a single aaRS can shift dramatically with relative evolutionary ease. In *E. coli* LeuRS, just two mutations abolished posttransfer editing and activated pretransfer editing activity to enhance fidelity for protein synthesis. Likewise, single mutations in IleRS have selectively altered pre- or posttransfer editing (17).

Our results also suggest that at least a partial translocation pathway for adenylate molecules does exist and is specific for noncognate amino acids in LeuRS. If the pre- and posttransfer editing active sites overlap, then it is possible that the origins of this small molecule translocation pathway transferred aminoacyl-adenylates for pretransfer editing. Evolutionary mutations may have blocked or truncated the pretransfer editing translocation pathway, such that *E. coli* LeuRS became completely dependent on posttransfer editing for fidelity.

Methods

Materials. DNA primers were synthesized by MWG Biotech (Ebersberg, Germany). Radiolabeled reagents were purchased from Amersham Pharmacia Biotech. Purified T7 RNA polymerase (37, 38) was used to carry out *in vitro* transcription (39) to obtain tRNA^{Leu}.

Preparation of WT and Mutant *E. coli* LeuRSs. Substitutions at A293 or K186 were introduced into *E. coli* WT [p15ec3-1 (12)] and T252Y [pMURe22 (16)] LeuRSs by means of PCR mutagenesis by using 200 ng of each primer, 100 ng of template plasmid, and 20 units of *Pfu* DNA polymerase. The plasmids containing the

single mutants A293D (pAMWp1), A293K (pAMWp32), and K186E (pAMWp63) and the double mutants T252Y/A293D (pAMWp3), T252Y/A293K (pAMWp31), and T252Y/K186E (pAMWp64) were used to transform *E. coli* DH5α competent cells. Recombinant LeuRSs were expressed in *E. coli* BL21 (DE3) and purified by means of affinity chromatography by using HisSelect Resin as described (16).

Aminoacylation and Misaminoacylation Assays. Aminoacylation reactions contained 60 mM Tris (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 20 μM [^{3}H]leucine (100 μCi/ml) (1 Ci = 37 GBq), 50 nM enzyme, and 4 μM *in vitro*-transcribed $tRNA^{Leu}_{UAA}$ (19, 26). Reactions were initiated with 4 mM ATP, quenched, and processed (26). Misaminoacylation assays were carried out by using 20 μM [^{3}H]isoleucine (100 μCi/μl) and 1 μM enzyme.

Hydrolysis of Mischarged tRNA. Purified *E. coli* $tRNA^{Leu}$ that had been *in vitro* transcribed (39) was misaminoacylated by an editing-defective mutant *E. coli* LeuRS, quenched, and processed as described (9). Hydrolytic editing assays were carried out in 60 mM Tris (pH 7.5), 10 mM $MgCl_2$, and ≈500 nM [^{3}H]ile-$tRNA^{Leu}$. The reactions were initiated with 100 nM enzyme and quenched as described above (26).

ATP Hydrolysis Assays. ATP hydrolysis assays contained 100 mM Tris (pH 7.5), 10 mM $MgCl_2$, 0.3 mM DTT, 150 units/ml PP_iase, 3 mM [^{14}C]ATP (5 μCi/ml), 3 μM *E. coli in vitro*-transcribed $tRNA^{Leu}$, and 1 mM isoleucine. The reaction was initiated by the addition of 750 nM enzyme. Subsequently, 2 μl of aliquots was quenched by spotting on PEI-cellulose TLC plates that were prerun in dH_2O. The adenosine products were separated in 750 mM KH_2PO_4 (pH 3.0) (12) and visualized by phosphorimaging for 14 days. Error bars were based on the standard deviation of assays repeated in triplicate and are present but nominal in the isoleucine-dependent total overall editing assay.

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1. Martinis, S. A., Plateau, P., Cavarelli, J. & Florentz, C. (1999) *EMBO J.* **18,** 4591–4596.
2. Martinis, S. A. & Schimmel, P. (1996) in *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, ed. Neidhardt, F. C. (ASM Press, Washington, DC), Vol. 1, pp. 887–901.
3. Hendrickson, T. L., de Crécy-Lagard, V. & Schimmel, P. (2004) *Annu. Rev. Biochem.* **73,** 147–176.
4. Hendrickson, T. L. & Schimmel, P. (2003) in *Translation Mechanisms*, eds. LaPointe, J. & Brakier-Gingras, L. (Kluwer/Plenum, New York), pp. 34–64.
5. Döring, V., Mootz, H. D., Nangle, L. A., Hendrickson, T. L., de Crécy-Lagard, V., Schimmel, P. & Marlière, P. (2001) *Science* **292,** 501–504.
6. Schimmel, P. & Schmidt, E. (1995) *Trends Biochem. Sci.* **20,** 1–2.
7. Fersht, A. R. (1977) *Biochemistry* **16,** 1025–1030.
8. Gruic-Sovulj, I., Uter, N., Bullock, T. & Perona, J. J. (2005) *J. Biol. Chem.* **280,** 23978–23986.
9. Eldred, E. W. & Schimmel, P. R. (1972) *J. Biol. Chem.* **247,** 2961–2964.
10. Englisch, S., Englisch, U., von der Haar, F. & Cramer, F. (1986) *Nucleic Acids Res.* **14,** 7529–7539.
11. Apostol, I., Levine, J., Lippincott, J., Leach, J., Hess, E., Glascock, C. B., Weickert, M. J. & Blackmore, R. (1997) *J. Biol. Chem.* **272,** 28980–28988.
12. Martinis, S. A. & Fox, G. E. (1997) *Nucleic Acids Symp.* **36,** 125–128.
13. Fukai, S., Nureki, O., Sekine, S., Shimada, A., Tao, J., Vassylyev, D. G. & Yokoyama, S. (2000) *Cell* **103,** 793–803.
14. Hendrickson, T. L., Nomanbhoy, T. K. & Schimmel, P. (2000) *Biochemistry* **39,** 8180–8186.
15. Lincecum, T. L. & Martinis, S. A. (2000) *SAAS Bull. Biochem. Biotechnol.* **13,** 25–33.
16. Mursinna, R. S. & Martinis, S. A. (2002) *J. Am. Chem. Soc.* **124,** 7286–7287.
17. Hendrickson, T. L., Nomanbhoy, T. K., de Crécy-Lagard, V., Fukai, S., Nureki, O., Yokoyama, S. & Schimmel, P. (2002) *Mol. Cell* **9,** 353–362.
18. Tang, Y. & Tirrell, D. A. (2002) *Biochemistry* **41,** 10635–10645.
19. Lincecum, T. L., Tukalo, M., Yaremchuk, A., Mursinna, R. S., Williams, A. M., Sproat, B. S., Van Den Eynde, W., Link, A., Van Calenbergh, S., Grøtli, M., *et al.* (2003) *Mol. Cell* **11,** 951–963.
20. Starzyk, R. M., Webster, T. A. & Schimmel, P. (1987) *Science* **237,** 1614–1618.
21. Lin, L., Hale, S. P. & Schimmel, P. (1996) *Nature* **384,** 33–34.
22. Nureki, O., Vassylyev, D. G., Tateno, M., Shimada, A., Nakama, T., Fukai, S., Konno, M., Hendrickson, T. L., Schimmel, P. & Yokoyama, S. (1998) *Science* **280,** 578–582.
23. Silvian, L. F., Wang, J. & Steitz, T. A. (1999) *Science* **285,** 1074–1077.
24. Cusack, S., Yaremchuk, A. & Tukalo, M. (2000) *EMBO J.* **19,** 2351–2361.
25. Chen, J. F., Guo, N. N., Li, T., Wang, E. D. & Wang, Y. L. (2000) *Biochemistry* **39,** 6726–6731.
26. Mursinna, R. S., Lincecum, T. L., Jr., & Martinis, S. A. (2001) *Biochemistry* **40,** 5376–5381.
27. Bishop, A. C., Nomanbhoy, T. K. & Schimmel, P. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 585–590.
28. Li, T., Guo, N., Xia, X., Wang, E. D. & Wang, Y. L. (1999) *Biochemistry* **38,** 13063–13069.
29. Chen, J. F., Li, T., Wang, E. D. & Wang, Y. L. (2001) *Biochemistry* **40,** 1144–1149.
30. Du, X. & Wang, E. D. (2002) *Biochemistry* **41,** 10623–10628.
31. Du, X. & Wang, E. D. (2003) *J. Protein Chem.* **22,** 71–76.
32. Lee, K. W. & Briggs, J. M. (2004) *Proteins* **54,** 693–704.
33. Mursinna, R. S., Lee, K. W., Briggs, J. M. & Martinis, S. A. (2004) *Biochemistry* **43,** 155–165.
34. Bishop, A. C., Beebe, K. & Schimmel, P. R. (2003) *Proc. Natl. Acad. Sci. USA* **100,** 490–494.
35. Nomanbhoy, T. K., Hendrickson, T. L. & Schimmel, P. (1999) *Mol. Cell* **4,** 519–528.
36. Nakama, T., Nureki, O. & Yokoyama, S. (2001) *J. Biol. Chem.* **276,** 47387–47393.
37. Wyatt, J. R., Chastain, M. & Puglisi, J. D. (1991) *BioTechniques* **11,** 764–769.
38. Cazenave, C. & Uhlenbeck, O. C. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 6972–6976.
39. Milligan, J. F., Groebe, D. R., Witherell, G. W. & Uhlenbeck, O. C. (1987) *Nucleic Acids Res.* **15,** 8783–8798.