BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

pubs.acs.org/biochemistry

Determinants for tRNA-Dependent Pretransfer Editing in the Synthetic Site of Isoleucyl-tRNA Synthetase

Morana Dulic,[†] John J. Perona,[‡,§] and Ita Gruic-Sovulj*[,†]

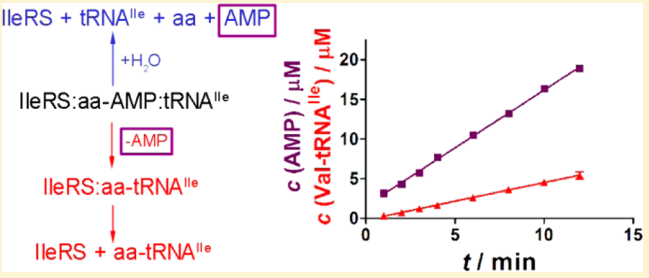
[†]Department of Chemistry, Faculty of Science, University of Zagreb, Horvatovac 102a, 10000 Zagreb, Croatia

[‡]Department of Chemistry, Portland State University, P.O. Box 751, Portland, Oregon 97207, United States

[§]Department of Biochemistry & Molecular Biology, Oregon Health & Sciences University, 3181 Southwest Sam Jackson Park Road, Portland, Oregon 97239, United States

Supporting Information

ABSTRACT: The accurate expression of genetic information relies on the fidelity of amino acid–tRNA coupling by aminoacyl-tRNA synthetases (aaRS). When the specificity against structurally similar noncognate amino acids in the synthetic reaction does not support a threshold fidelity level for translation, the aaRS employ intrinsic hydrolytic editing to correct errors in aminoacylation. *Escherichia coli* isoleucyl-tRNA synthetase (EcIleRS) is a class I aaRS that is notable for its use of tRNA-dependent pretransfer editing to hydrolyze noncognate valyl-adenylate prior to aminoacyl-tRNA formation. On the basis of the finding that IleRS possessing an inactivated post-transfer editing domain is still capable of robust tRNA-dependent editing, we have recently proposed that the pretransfer editing activity resides within the synthetic site. Here we apply an improved methodology that allows quantitation of the AMP fraction that arises particularly from tRNA-dependent aa-AMP hydrolysis. By this approach, we demonstrate that tRNA-dependent pretransfer editing accounts for nearly one-third of the total proofreading by EcIleRS and that a highly conserved tyrosine within the synthetic site modulates both editing and aminoacylation. Therefore, synthesis of aminoacyl-tRNA and hydrolysis of aminoacyl-adenylates employ overlapping amino acid determinants. We suggest that this overlap hindered the evolution of synthetic site-based pretransfer editing as the predominant proofreading pathway, because that activity is difficult to accommodate in the context of efficient aminoacyl-tRNA synthesis. Instead, the acquisition of a spatially separate domain dedicated to post-transfer editing alone allowed for the development of a powerful deacylation machinery that effectively competes with dissociation of misacylated tRNAs.



The accuracy of protein synthesis relies on the capacity of aminoacyl-tRNA synthetases (aaRS) to couple cognate amino acids and tRNAs in a two-step reaction that defines the genetic code (reviewed in refs 1 and 2). In the first step, the amino acid is activated by condensation with ATP to form an enzyme-bound aminoacyl-adenylate (aa-AMP) intermediate with release of pyrophosphate. The second step comprises attack by the terminal 2′- or 3′-OH group of tRNA on the carbonyl carbon atom of aa-AMP, followed by transfer of the aminoacyl moiety to tRNA and release of AMP (Figure 1). The amino acid activation and transfer steps occur within the synthetic active site located in the catalytic domain. The aaRS are divided into two classes based on differences in the topologies of the catalytic domain and some aspects of the reaction mechanism.[3,4]

Specific recognition of tRNAs by cognate aaRSs is ensured by a network of interactions, based on direct and indirect recognition elements that are embedded in all levels of tRNA structure.[5,6] However, noncognate amino acids that structurally and chemically resemble the cognate substrates are often not well-distinguished in the synthetic reactions alone, so that discrimination is based in part on inherent aaRS-based hydrolytic editing (Figure 1; reviewed in refs 7 and 8). Selective release of noncognate aa-AMP into solution, where it undergoes nonenzymatic hydrolysis, may also weakly contribute to an enzyme's specificity (Figure 1, path 1[9]). Post-transfer editing represents a dominant editing pathway (Figure 1, path 4). In this reaction, the noncognate amino acid attached to the 3′-end of misacylated tRNA is translocated to the specialized post-transfer editing domain prior to the hydrolytic reaction that regenerates the free amino acid and tRNA.[10−14] In contrast, the pretransfer editing reaction comprises hydrolysis of noncognate aa-AMP (Figure 1, path 2) that, in some cases, may be stimulated by tRNA (Figure 1, path 3). It was recently shown that both tRNA-dependent and tRNA-independent pretransfer editing reactions are localized within the confines of the synthetic site.[9,15−17] This contradicts the previous model that located all editing pathways in the separate editing domain.[18] The synthetic site-based pretransfer editing model is supported in part by the findings that a number of aaRSs of

Received: June 20, 2014
Revised: September 9, 2014
Published: September 10, 2014

   dx.doi.org/10.1021/bi5007699 | *Biochemistry* 2014, 53, 6189−6198

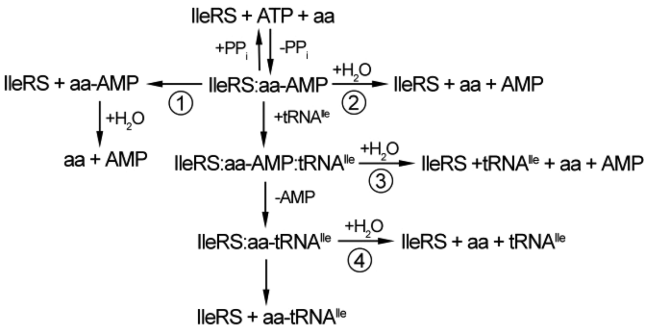


Figure 1. Schematic presentation of enzymatic reactions catalyzed by IleRS. The central pathway represents amino acid activation, tRNA binding, aminoacyl transfer, and dissociation of aminoacylated tRNA from the enzyme. The synthetic pathway may occur with both cognate and noncognate amino acids. Editing pathways are shown to the left and to the right. Pretransfer editing may proceed through enhanced dissociation of noncognate aminoacyl-AMP (1) or through its enzymatic hydrolysis, which may be tRNA-independent (2) or tRNA-dependent (3). Misacylated tRNA is deacylated through post-transfer editing (4).

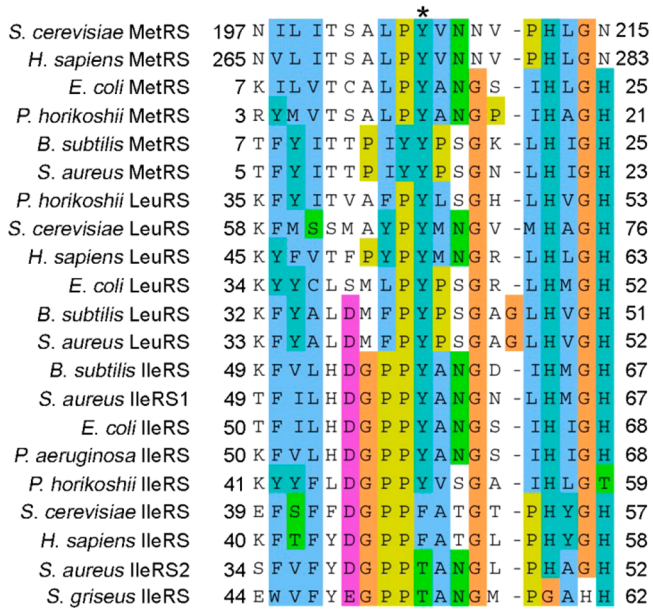


Figure 2. Sequence alignment of a Rossmann fold peptide directly N-terminal to the strictly conserved HIGH motif, in various MetRS, LeuRS, and IleRS enzymes. In the case of MetRS and LeuRS, the alignment contains sequences from prokaryotes (*E. coli*, *Staphylococcus aureus*, and *Bacillus subtilis*), archaea (*Pyrococcus horikoshii*), and eukaryotes (*Saccharomyces cerevisiae* and *Homo sapiens*). The alignment additionally contains the sequence of the second IleRS from *S. aureus* and further sequences from prokaryotes *Pseudomonas aeruginosa* and *Streptomyces griseus*. The residue mutated in this work is marked with an asterisk.

both classes, including those that naturally lack editing domains, display weak tRNA-independent editing.[9,17,19] This activity may represent a relic of editing exhibited by a primordial aaRS consisting solely of the catalytic module.

IleRS is a class I aaRS responsible for formation of Ile-tRNA^{Ile}. It was recognized long ago[20,21] that the difference in binding energies between cognate isoleucine and the structurally similar noncognate valine could not provide more than 10^2-fold specificity in Ile-tRNA^{Ile} synthesis, substantially lower than the overall translational fidelity (one mistake per 10^3–10^4 incorporated amino acids[22]). The origin of the increased specificity was established as hydrolytic editing in *Escherichia coli* IleRS (EcIleRS).[23] Early work further indicated that IleRS displays substantial tRNA-dependent pretransfer editing.[23,24] We recently confirmed this view by showing that IleRS retains significant capacity for editing even when the post-transfer pathway is fully deactivated.[15] Interestingly, this phenomenon was not observed for the homologous class I valyl- and leucyl-tRNA synthetases (ValRS and LeuRS, respectively),[15,25] which retained only a low residual editing activity upon mutation of a key catalytic amino acid in the post-transfer editing domain.

How the class I IleRS Rossmann fold accommodates both synthetic and tRNA-dependent pretransfer hydrolysis pathways within the synthetic site is still unknown. Mapping of the hydrolytic and synthetic subsites and elucidation of the mechanisms that ensure substrate specificity of each subsite pose substantial challenges, because the determinants may be overlapping. Here we report a novel kinetic approach that allows quantitation of the AMP product that originates specifically from pretransfer editing, separating the portion of AMP that arises from the competing aminoacylation reaction. With this improved methodology, we demonstrate that tRNA-dependent pretransfer editing contributes up to 30% of the overall IleRS editing. Importantly, this approach also further allowed the first quantitative analysis of the synthetic site-based pretransfer editing determinants.

We focused on the role of a conserved tyrosine [Y59 in EcIleRS (Figure 2)] in modulating the competing synthetic and editing pathways in the synthetic site of EcIleRS. Structural studies of the homologous class I methionyl-tRNA synthetase (MetRS) and LeuRS suggest that this tyrosine adopts several distinct conformations.[12,26] In a closed orientation, the tyrosine packs on the amino acid substrate, possibly protecting aa-AMP from premature hydrolysis. By contrast, in a more open conformation that is associated with the aminoacyl transfer step, the tyrosine facilitates catalytically productive binding of the 3′-A76 of tRNA. Here we find that Y59 is important for assembling the EcIleRS synthetic active site for both pathways, indicating that the hydrolytic and synthetic subsites overlap to some extent. Substitution of this tyrosine with threonine diminished tRNA-dependent pretransfer editing, demonstrating for the first time that an amino acid located in the synthetic site is a key determinant of tRNA-dependent pretransfer editing. This finding substantiates our model in which the synthetic site hosts the pretransfer editing reactions,[15,25] while the spatially separate editing domain is dedicated to post-transfer editing alone. The limitations of an evolutionary design whereby two opposing activities are hosted by the same active site are recognized, providing an explanation for the predominance of the post-transfer editing pathway in contemporary editing aaRSs.

MATERIALS AND METHODS

Enzymes and tRNAs. The IleRS vector for protein expression in *E. coli* DE3 cells was used as a template for site-directed mutagenesis using QuikChange (Stratagene).[15] The introduced mutations were confirmed by DNA sequencing. Wild-type and mutant enzymes were expressed in *E. coli* BL21(DE3) with addition of 1 mM $ZnCl_2$ to the culture at an OD_{600} of approximately 0.1, to ensure formation of the proper zinc-bound IleRS conformation. All enzymes were purified

dx.doi.org/10.1021/bi5007699 | *Biochemistry* 2014, 53, 6189–6198

employing affinity chromatography on Ni-NTA resin, as reported previously.[15] The concentrations of all proteins were determined by absorption at 280 nm using theoretical extinction coefficients. Determination of zinc content was done by a 4-(2-pyridylazo)-resorcinol (PAR)/*p*-hydroxymercuribenzoate (PMB) assay as described previously.[27] All wild-type and mutant enzymes contained approximately 2 mol of Zn^{2+}/mol of enzyme.

$tRNA_{GAU}^{Ile}$ (with G_1-C_{72} instead of the wild-type A_1-U_{72} sequence) was overexpressed in *E. coli* BL21(DE3), isolated, and purified by reverse phase chromatography essentially as described previously.[15] The G1-C72 mutation has no effect on kinetic parameters for aminoacylation.[28] This procedure yielded $tRNA^{Ile}$ capable of 90% aminoacylation. tRNA was radiolabeled by exchange of endogenous A76 with [α-^{32}P]ATP using tRNA-nucleotidyltransferase, as follows.[29] $tRNA^{Ile}$ (5 μM) was incubated with 5 μM tRNA-nucleotidyltransferase, 1 μM [α-^{32}P]ATP (specific activity of 3000 Ci/mmol), and 5 mM Na-PP_i in a buffer containing 50 mM glycine-NaOH (pH 9.0), 20 mM $MgCl_2$, and 0.5 mM DTT at 37 °C. After 1 min, 0.1 unit/μL thermostable inorganic pyrophosphatase was added to enhance [α-^{32}P]ATP incorporation, and the mixture was incubated at 37 °C for an additional 2 min followed by phenol/chloroform extraction. The sample was passed through two consecutive Bio-Spin P-30 columns (Bio-Rad) and dialyzed against water.

The wild-type and deacylation defective mutant D286A ValRS and $tRNA^{Val}$ were purified as previously described.[15]

ATP-PP_i Exchange. ATP-PP_i exchange was measured at 37 °C in 50 mM Hepes (pH 7.5), 20 mM $MgCl_2$, 0.1 μg/μL BSA, 5 mM DTT, 4 mM ATP, and 1 mM [^{32}P]PP_i (0.2−0.4 Ci/mol). The enzymes were present at a concentration of 25 nM, and the concentration of amino acids was varied between 0.1K_m and 10K_m. The reactions were stopped with sodium acetate (pH 5.0) and sodium dodecyl sulfate (SDS) (final concentrations of 333 mM and 0.067%, respectively). Separation of [^{32}P]ATP from the remaining [^{32}P]PP_i by thin layer chromatography (TLC) on polyethyleneimine plates (PEI, Fluka) was conducted as described previously.[30] Visualization and quantitation of the signal were performed on a Typhoon PhosphorImager, using ImageQuant. Kinetic parameters were determined by fitting the data directly to the Michaelis−Menten equation using GraphPad Prism. The parameters were obtained from at least three independent measurements for the single mutants and two independent measurements for the double mutants.

Aminoacylation Assay. Aminoacylation reactions were performed at 37 °C in a reaction mixture containing 20 mM Hepes (pH 7.5), 100 μM EDTA, 150 mM NH_4Cl, 10 μg/mL bovine serum albumin, 10 mM $MgCl_2$, 2 mM ATP, and 1 mM isoleucine. In all reactions, $tRNA^{Ile}$ was present at a concentration of 10 μM (of which approximately 20 nM was ^{32}P-labeled $tRNA^{Ile}$). The enzymes were present in the reaction mixture at the following concentrations: 200 nM Y59T IleRS and 400 nM Y59F IleRS. Reactions were stopped with sodium acetate (pH 5.0) and SDS (final concentrations of 0.4 M and 0.1%, respectively). tRNA was degraded using P1 nuclease, and aa-AMP was separated from AMP by TLC on PEI cellulose plates.[29] TLC plates were developed in a solution containing 100 mM ammonium acetate and 5% acetic acid. The ratio of aa-AMP to AMP is equivalent to the ratio of aminoacylated to nonaminoacylated tRNA in the reaction mixture. Visualization and quantization of the signal were performed on a Typhoon PhosphorImager, using ImageQuant.

Transfer of an Amino Acid to $tRNA^{Ile}$. The transfer step was measured by rapidly mixing the enzyme:aa-AMP complex with [^{32}P]tRNA. aa-AMP was synthesized *in situ*[31] by incubation of 20 μM IleRS variant with either 1 mM Ile or 10 mM Val, in a buffer containing 10 mM ATP, 20 mM Hepes (pH 7.5), 100 μM EDTA, 150 mM NH_4Cl, 10 μg/mL bovine serum albumin, 10 mM $MgCl_2$, and 0.008 unit/μL inorganic pyrophosphatase at 37 °C for 30 min. The final concentration of $tRNA^{Ile}$ in the reaction mixture was 1 μM.

Reactions with Y59 mutants proceeded on the multisecond time scale and were therefore manually sampled. Reactions were started by mixing equal volumes of the IleRS:aa-AMP complex with ^{32}P-labeled $tRNA^{Ile}$ and stopped with sodium acetate (pH 5.0) and SDS (final concentrations of 0.4 M and 0.1%, respectively). tRNA was degraded using P1 nuclease, and aa-AMP was separated from AMP by TLC as described above. The amount of aminoacylated tRNA was plotted versus time and fit to the first-order exponential equation $y = Y_0 + A \times \exp(-k_{trans}t)$, where Y_0 is the y intercept, A is a scaling constant, k_{trans} is the apparent transfer rate constant, and t is time.

Parallel Formation of AMP and aa-$tRNA^{Ile}$. The reactions were conducted at 37 °C in a buffer containing 50 mM Hepes (pH 7.5), 20 mM $MgCl_2$, 5 mM DTT, 0.1 mg/mL bovine serum albumin, 0.004 unit/μL inorganic pyrophosphatase, and 200 μM ATP. For better sensitivity, the concentration of ATP was 200 μM instead of the 500 μM usually used in AMP formation assays. Independent measurement of K_m for ATP under these conditions returned a value of 100 μM, showing that ATP was present at concentrations above its K_m value. The reaction mixture further contained 15 μM $tRNA^{Ile}$ and either 2 mM isoleucine or 20 mM valine. The enzyme concentrations were as follows: 20 nM wild-type enzyme in the reaction with Ile and 100 nM in the reaction with Val, 60 nM D342A IleRS in reactions with both Val and Ile, 0.2 μM Y59T in the reaction with Ile and 0.6 μM Y59T in the reaction with Val, 0.6 μM Y59T/D342A in reactions with both Val and Ile, and 0.2 μM Y59F in the reaction with Ile and 0.4 μM Y59F in the reaction with Val. In the reaction with ValRS, the reaction mixture contained 15 μM $tRNA^{Val}$ and either 5 mM valine or 50 mM threonine. The enzyme concentrations were 2 nM for wild-type ValRS in the reaction with Val and 10 nM in the reaction with Thr and 5 nM for deacylation defective mutant D286A ValRS in reactions with both Val and Thr.

Reactions were initiated by addition of enzyme to the reaction mixture and were stopped and further treated depending on whether they contained ^{32}P-labeled $tRNA^{Ile}$ or [α-^{32}P]ATP. The reactions with labeled [^{32}P]ATP, which allowed monitoring of AMP formation, were stopped by quenching 1.5 μL of the reaction mixture in 3 μL of 1.5 M formic acid. The separation of aa-[^{32}P]AMP, [^{32}P]AMP, and [^{32}P]ATP was then performed by TLC on PEI cellulose plates in 100 mM ammonium acetate and 5% acetic acid. Visualization and quantization of the signal were performed on a Typhoon PhosphorImager, using ImageQuant.

The reactions with ^{32}P-labeled $tRNA^{Ile}$, which allowed monitoring of aa-tRNA formation, were stopped and further treated as described in the preceding subsections.

Kinetic analysis of Y59F/D342A IleRS did not provide reliable results because of the very slow aminoacylation/misacylation. Previously, we observed that the D342A variant

dx.doi.org/10.1021/bi5007699 | *Biochemistry* 2014, 53, 6189−6198

Table 1. Parallel Formation of AMP and Val-tRNA^{Ile} by Wild-Type and Mutant IleRS Enzymes[a]

	k_{AMP}[b] (s^{-1})	$k_{Val\text{-}tRNA}$[b] (s^{-1})	$k_{AMP}/k_{Val\text{-}tRNA}$	k_{ed}[c−e] (s^{-1})	$k_{ed,\text{-}tRNA}$[b] (s^{-1})	$k_{ed}/k_{ed,\text{-}tRNA}$
wt	0.97 ± 0.02	0.06 ± 0.003	16 ± 1	0.91 ± 0.06	0.028 ± 0.003	32.5
D342A	0.40 ± 0.006	0.13 ± 0.006	3.1 ± 0.4	0.28 ± 0.02	0.026 ± 0.003	10.7
Y59T	0.106 ± 0.004	0.007 ± 0.001	17 ± 3	0.099 ± 0.005	0.014 ± 0.003	7
Y59T/D342A	0.046 ± 0.002	0.015 ± 0.001	3.1 ± 0.01	0.031 ± 0.001	0.021 ± 0.001	1.5
Y59F	0.16 ± 0.003	0.008 ± 0.001	19 ± 4	0.152 ± 0.011	0.012 ± 0.001	12.7

[a]The values represent the mean ± the SEM of at least three independent experiments. [b]k_{AMP} and $k_{ed,\text{-}tRNA}$ represent measured rate constants for formation of AMP in the presence and absence of tRNA^{Ile}, respectively. $k_{Val\text{-}tRNA}$ represents the rate constant for formation of Val-tRNA^{Ile} measured under the same conditions that were used for k_{AMP}. [c]k_{ed} represents the rate constant for tRNA-dependent editing and is calculated as the difference between the rate constant for AMP formation in the presence of tRNA^{Ile} and the rate constant for Val-tRNA^{Ile} formation. [d]In D342A, k_{ed} arises solely from the tRNA-dependent pretransfer hydrolysis of misactivated Val-AMP, while k_{ed} in wild-type enzyme reflects both editing reactions. [e]$\Delta\Delta G_{coupling} = \Delta\Delta G_{WT\text{-}Y59T} - \Delta\Delta G_{D345A\text{-}D345A/Y59T}$, where $\Delta\Delta G_{A\text{-}B} = -RT \ln(k_{ed,A}/k_{ed,B})$.[35]

exhibits aminoacylation slightly slower than that of the wt enzyme.[15] We note that the effect is more pronounced under conditions employed in this assay [i.e., at low ATP concentrations (see Table 5)]. It further appears that the phenomenon contributes most to the Y59F/D342A variant, precluding its utilization in the kinetic analysis.

RESULTS

tRNA-Dependent Pretransfer Editing Accounts for Nearly One-Third of the Total Proofreading by EcIleRS.

The coexistence of editing and synthetic pathways effectively consumes ATP and converts it to AMP and PP_i, while regenerating amino acid and tRNA substrates for the subsequent turnovers (Figure 1). Hence, AMP formation above stoichiometric amounts in aminoacylation is diagnostic of hydrolytic editing and is generally observed in the presence of noncognate amino acids. Two competing reactions, both localized within the synthetic site, may directly contribute to AMP formation: (i) pretransfer editing and (ii) tRNA aminoacylation. AMP arises from each of these two reactions in direct proportion to the kinetic partitioning of the aa-AMP between amino acid transfer to water and amino acid transfer to tRNA.[15]

Recently, we isolated the tRNA-dependent pretransfer editing step in EcIleRS by disabling the post-transfer pathway with substitution of the essential aspartate (D342) in the CP1 post-transfer editing domain.[15,18,32] The D342A variant still allowed robust AMP formation, thus supporting the conclusion that the IleRS synthetic site hosts substantial tRNA-dependent pretransfer editing.[15] To provide more quantitative insight into the mechanism and determinants of pretransfer editing, we now describe an improved methodology that distinguishes the relative amounts of AMP that arise from tRNA-dependent hydrolysis of Val-AMP versus tRNA misacylation in post-transfer editing defective enzymes. To accomplish this, the initial rates of AMP formation [k_{AMP} (Table 1)] and tRNA misacylation [$k_{Val\text{-}tRNA}$ (Table 1)] were followed in parallel assays relying on [α-^{32}P]ATP and [^{32}P]tRNA, respectively.[25,33] The post-transfer editing deficient D342A IleRS accumulates AMP by pretransfer editing and by tRNA misacylation when the noncognate aa-AMP evades this hydrolytic reaction. Both of these events contribute to determining the value of k_{AMP} in the AMP formation assay. The rate of misacylation is determined separately using [^{32}P]tRNA to quantitate the fraction of AMP arising from aminoacylation alone. The rate constant representing solely tRNA-dependent pretransfer editing [k_{ed} (Table 1)] is then isolated by subtracting $k_{Val\text{-}tRNA}$ from k_{AMP}. When this methodology is applied to IleRS with active post-transfer editing, k_{AMP} instead reflects pretransfer editing, misacylation, and post-transfer editing, because misacylated tRNA can also be hydrolyzed leading to repetitive misacylation/deacylation cycles. In this case, subtraction of the independently determined $k_{Val\text{-}tRNA}$ from k_{AMP} again yields k_{ed} (Table 1), which now represents a rate constant associated with tRNA-dependent editing by both hydrolytic reactions. The $k_{AMP}/k_{Val\text{-}tRNA}$ ratio (Table 1) indicates the amount of proofreading (the number of correction cycles per misacylated tRNA that evades editing). In addition, the ratio of the rate constants for tRNA-dependent editing (k_{ed}) and tRNA-independent editing [$k_{ed,\text{-}tRNA}$ (Table 1)] measured under the same experimental conditions defines the extent to which tRNA stimulates the editing reaction [$k_{ed}/k_{ed,\text{-}tRNA}$ (Table 1)].

These measurements reveal that wt IleRS consumes 16 molecules of ATP per molecule of Val-tRNA^{Ile} that accumulates (Table 1). AMP accumulates in excess of Val-tRNA^{Ile} based on the operation of both pre- and post-transfer editing. In contrast, approximately three molecules of ATP were consumed per molecule of Val-tRNA^{Ile} synthesized by D342A IleRS, which is fully disabled in post-transfer editing ($k_{AMP}/k_{Val\text{-}tRNAIle} = 3$). The remaining excess ATP consumption by D342A IleRS affirms the tRNA-dependent pretransfer editing and shows its relevance in IleRS quality control. However, the decreased level of ATP consumption in the absence of active deacylation, together with the accumulation of misacylated Val-tRNA^{Ile} in the reactions catalyzed by the D342A enzyme, clearly demonstrates the necessity for post-transfer editing in establishing accurate Ile-tRNA^{Ile} synthesis. It is important to note that post-transfer editing defective variants of the homologous class I LeuRS and ValRS editing enzymes, which do not exhibit tRNA enhancement of their low tRNA-independent pretransfer editing activities, instead possess $k_{AMP}/k_{misacyl\text{-}tRNA}$ ratios close to 1 when post-transfer editing is disabled[25] (Table S1 of the Supporting Information). These data illustrate the clear distinctiveness of IleRS as the sole class I editing enzyme for which the pretransfer reaction is important.

The rate constants for tRNA-dependent editing (k_{ed}) calculated for wt and D342A IleRS are 0.91 and 0.28 s^{-1}, respectively (Table 1). Importantly, in D342A, k_{ed} arises solely from the tRNA-dependent pretransfer hydrolysis of misactivated Val-AMP, while k_{ed} in the wild-type enzyme reflects both editing reactions. The ratio of these k_{ed} values indicates that tRNA-dependent pretransfer editing contributes approximately 30% to overall editing, and the remaining 70% can be assigned to the post-transfer reaction. Finally, the extent to which tRNA stimulates editing was established by comparing the k_{ed} and $k_{ed,\text{-}tRNA}$ values (Table 1). As expected, tRNA participation is

dx.doi.org/10.1021/bi5007699 | Biochemistry 2014, 53, 6189−6198

more pronounced in the case of the wt enzyme (32.5-fold), reflecting the role of tRNA in both pre- and post-transfer editing. However, D342A IleRS exhibits a $k_{ed}/k_{ed,\text{-tRNA}}$ ratio of approximately 11, demonstrating significant stimulation of valyl-AMP hydrolysis by tRNA even when post-transfer editing is disabled.[15] In sharp contrast, D286A ValRS, which is similarly disabled in post-transfer editing, exhibits a $k_{ed}/k_{ed,\text{-tRNA}}$ ratio of nearly 1.0 (Table S1 of the Supporting Information). This demonstrates that tRNA-stimulated pretransfer editing is negligible in this enzyme, consistent with our previous findings.[15] A similar result was observed for LeuRS.[25]

Tyrosine 59 Participates in the Synthetic Reaction of *E. coli* IleRS. Tyr59 is a conserved residue located within the active site of bacterial IleRS, directly adjacent to the binding site for the 3′-terminal hydroxyl group of tRNA (Figures 2 and 3).

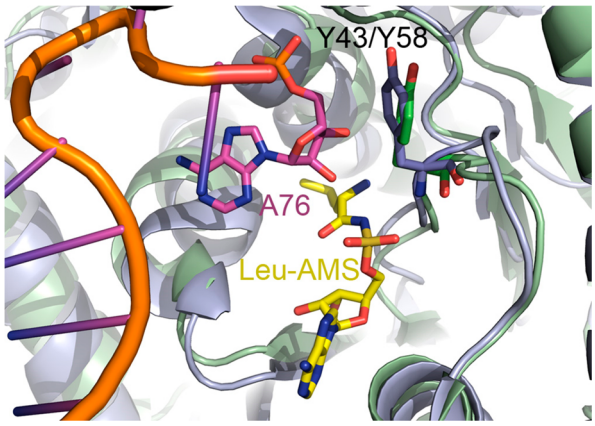


Figure 3. Overlapped structures of *E. coli* LeuRS in complex with 5′-*O*-[*N*-(L-leucyl)sulfamoyl]adenosine (Leu-AMS) and $tRNA^{Leu}$ (Protein Data Bank entry 4AQ7, colored green) and *S. aureus* IleRS in complex with mupirocin and $tRNA^{Ile}$ (Protein Data Bank entry 1FFY, colored blue). Leu-AMS is colored yellow, the $tRNA^{Leu}$ backbone orange, and its last nucleotide (A76) purple. $tRNA^{Ile}$ and mupirocin are not visible. Tyrosine residues (Y43 of *E. coli* LeuRS and Y58 of *S. aureus* IleRS, both homologous to Y59 of *E. coli* IleRS) are shown as sticks. Superposition was done on the polypeptide backbone of parts of Rossmann folds (residues 34−94 and 619−666 in LeuRS and residues 44−104 and 600−647 in IleRS). The root-mean-square deviation was 0.885 Å.

To evaluate the role of Y59 in the synthetic and editing pathways, the tyrosine was substituted with threonine and phenylalanine (Y59T and Y59F IleRSs, respectively), as these residues were found at the equivalent position in IleRS enzymes of eukaryotic origin[34] (Figure 2, and see Discussion). We first used the recombinantly expressed and highly purified mutant enzymes to measure activation of cognate isoleucine and noncognate valine with the ATP-PP_i exchange assay. Neither of these Y59 substitutions significantly influenced the discrimination against valine in the activation step (Table 2). The observed changes in K_m and k_{cat} for isoleucine and valine activation are quite small for the Y59F variant, demonstrating that the phenolic hydroxyl group of tyrosine does not play a significant role in the amino acid activation step. However, the K_m and k_{cat} values measured for Y59T are increased by 10-fold and decreased by 5-fold, respectively, for both isoleucine and valine substrates (Table 2). The increase in amino acid K_m observed for the Y59T variant, which lacks the aromatic ring and consequently may be less able to pack against the amino acid substrate, suggests that Y59 acts as a gatekeeper of the IleRS synthetic site. This is consistent with the proposed role for the homologous tyrosines found at the equivalent positions in MetRS and LeuRS.[12,26]

Next, we followed the two-step formation of Ile-$tRNA^{Ile}$ under multiple-turnover reaction conditions by using ^{32}P-labeled tRNA.[29] Both substitutions significantly reduced the rate of aminoacylation (Table 3), with the more pronounced

Table 3. Steady-State Rates of Aminoacylation by Y59 IleRS Variants[a]

	k_{obs} (s^{-1})
wt IleRS	0.86 ± 0.2
Y59T	0.074 ± 0.003
Y59F	0.024 ± 0.001

[a]All substrate concentrations are verified to be saturating; therefore, k_{obs} approaches k_{cat}. The values represent the mean ± the SEM of at least three independent experiments.

effect observed with Y59F. The observed decrease is not primarily associated with the activation step, because the ATP-PP_i exchange measurements showed that the k_{cat} values for both variants were still significantly faster than the observed rate of aminoacylation (Tables 2 and 3). Therefore, it appears that Y59 participates significantly in the second step of aminoacylation. To investigate this further, we isolated the transfer step by mixing [^{32}P]$tRNA^{Ile}$ with the preformed IleRS:Ile-AMP complex under single-turnover conditions. Both Y59 variants had significantly reduced isoleucyl transfer rates compared with that of the wt enzyme [60−180-fold (Table 4)], clearly showing that Y59 facilitates the transfer of the isoleucyl moiety to tRNA.

Recently, we established that IleRS does not discriminate against valine at the aminoacyl transfer step.[15] To test if substitutions at Y59 influence specificity at the transfer step, we introduced the D342A substitutions into the CP1 editing

Table 2. Steady-State Parameters for Activation of Cognate and Noncognate Amino Acids by Y59 IleRS Variants[a]

	K_m(Ile) (μM)	k_{cat}(Ile) (s^{-1})	k_{cat}/K_m(Ile) (s^{-1} μM)[b]	K_m(Val) (mM)	k_{cat}(Val) (s^{-1})	k_{cat}/K_m(Val) (s^{-1} mM)[b]	discrimination factor[c]
wt IleRS[d]	4.6 ± 0.5	55 ± 9	12	0.47 ± 0.03	35 ± 6	75	150
D342A	5.2 ± 0.6	47 ± 6	9.04 (1.3)	0.56 ± 0.08	33 ± 2	59 (1.3)	153
Y59T	41.5 ± 0.1	11.4 ± 0.5	0.27 (44)	5.7 ± 0.2	9.8 ± 0.4	1.7 (44)	157
Y59T/D342A	38.3 ± 3	10.7 ± 0.4	0.28 (43)	5.3 ± 0.6	8.2 ± 0.2	1.5 (50)	187
Y59F	2.6 ± 0.6	21 ± 1	8.1 (1.5)	0.20 ± 0.05	16 ± 1	80 (0.9)	98.7
Y59F/D342A	1.9 ± 0.6	23 ± 2	12.1 (1)	0.20 ± 0.04	15 ± 0.8	75 (1)	161

[a]Activity is measured by ATP-PP_i exchange. The values represent the best fit value ± the SEM of at least two independent measurements. [b]The numbers in parentheses represent the ratio of k_{cat}/K_m values with respect to the wild-type enzyme. Note that the units for K_m and k_{cat}/K_m differ for Ile and Val reactions. [c]The discrimination factor is defined as $[k_{cat}/K_m(\text{Ile})]/[k_{cat}/K_m(\text{Val})]$. [d]Data taken ref 15.

dx.doi.org/10.1021/bi5007699 | *Biochemistry* 2014, 53, 6189−6198

Table 4. Transfer of Cognate and Noncognate Amino Acids to tRNA by Y59 IleRS Variants[a]

	k_{trans} (s^{-1})	
	Ile	Val
wt IleRS	3.6 ± 0.3	nd[b]
Y59T	0.06 ± 0.01	nd[b]
Y59T/D342A	0.04 ± 0.01	0.03 ± 0.01
Y59F	0.024 ± 0.004	nd[b]
Y59F/D342A	0.012 ± 0.006	0.012 ± 0.004

[a]The values represent the mean ± the SEM of at least three independent experiments. [b]Not determined because of active post-transfer editing.

domains of both Y59 variants. This eliminates post-transfer editing and permits isolation of the transfer step for the noncognate amino acid. Transfers of the isoleucyl and valyl moieties to $tRNA^{Ile}$ were then measured with Y59T/D342A and Y59F/D342A IleRS, as described for the single-residue variants (Table 4). The rate constants for isoleucyl and valyl transfer were each decreased by 100−300-fold; therefore, it appears that Tyr59 does not significantly affect specificity at this step. The independent steady-state deacylation experiments confirmed that Y59T/D342A and Y59F/D342A IleRS were indeed disabled in Val-$tRNA^{Ile}$ hydrolysis (k_{obs} was determined to be 0.003 s^{-1} in each case). Introduction of the D342A substitution into the editing domain of IleRS did not influence the kinetic parameters for activation of cognate isoleucine and noncognate valine (Table 2).

The Y59T Substitution Diminishes tRNA-Dependent Pretransfer Editing. A possible role for Y59 in tRNA-dependent pretransfer editing was investigated by the approach taken for wild-type and D342A IleRS (see above). Remarkably, kinetic analysis of Y59T/D342A IleRS shows that for this variant editing operates at quantitatively comparable rates in the presence (0.031 s^{-1}) or absence of tRNA (0.021 s^{-1}) (Table 1). Because post-transfer editing is disabled by the D342A substitution in this variant, these data demonstrate unambiguously that the Y59T substitution in the synthetic site abolishes stimulation of pretransfer editing by $tRNA^{Ile}$. It thus appears that Y59 represents a key determinant for synthetic site-based tRNA-dependent pretransfer editing in IleRS. However, substitution of the phenolic hydroxyl group with hydrogen in the Y59F variant does not abolish tRNA stimulation of the pretransfer editing activity (see below).

Kinetic analyses performed on the Y59 variants link the amino acid at this position with partitioning of tRNA-dependent editing between the pre- and post-transfer pathways. Both Y59 substitutions decreased the overall level of tRNA-dependent editing: the calculated rate constant for this process (k_{ed}) is decreased by approximately 10-fold compared to that of wt IleRS [0.099 s^{-1} for Y59T and 0.152 s^{-1} for Y59F vs 0.91 s^{-1} for wt IleRS (Table 1)]. However, the origin of this decrease seems to substantially differ among the variants. Single-turnover analysis of the synthetic pathway reveals that Y59T and Y59F IleRS differ in the rate of the aminoacyl transfer step and consequently in the capacity to employ post-transfer editing (Table 4). The equivalencies of the single-turnover k_{trans} and steady-state k_{obs} values for Y59F and Y59T show that the aminoacyl transfer step represents the slowest step in the reaction pathway for each mutant (Tables 3 and 4). Therefore, the rate of aminoacyl transfer also limits the steady-state rate of post-transfer editing followed *in cis*. The rate of the aminoacyl transfer step in Y59T IleRS is fast enough [0.06 s^{-1} (Table 4)] to support AMP formation in editing through the post-transfer pathway [k_{ed} = 0.099 s^{-1} (Table 1)]. Accordingly, the further introduction of the D342A substitution into the CP1 editing domain of Y59T reduced the tRNA-dependent editing rate (k_{ed}, 0.031 s^{-1}) relative to that of Y59T (0.099 s^{-1}) (Table 1). The extent by which tRNA stimulates editing also dropped from 7 to 1.5 (Table 1), indicating that tRNA enhancement of the Y59T editing activity originates from the active post-transfer pathway. Interestingly, the calculated $\Delta\Delta G$ values for the Y59T or D342A substitution within the wild type and the opposing single-variant framework were highly similar, showing a lack of coupling energy[35,36] between these two residues in establishing the pre- and post-transfer editing activities in IleRS (Table 1).

In contrast, comparison of the rates of valyl transfer [0.012 s^{-1} (Table 4)] and tRNA-dependent editing by Y59F [0.152 s^{-1} (Table 1)] revealed that editing is 12-fold faster. These data thus strongly argue against a significant contribution of post-transfer editing to k_{ed} in this variant, leading to the conclusion that tRNA-dependent editing by Y59F IleRS preferentially originates from the pretransfer step. Unfortunately, Y59F/D342A IleRS did not provide reliable kinetic data because of very slow rates of aminoacylation and misacylation in this variant (see Materials and Methods). This precludes investigation of the effects of the Y59F substitution at the same level of detail. Because the k_{ed} (0.152 s^{-1}) is 10-fold faster than the rate for tRNA-independent editing by Y59F (0.012 s^{-1}) (Table 1), however, it appears that the Y59F mutant is capable of significant tRNA-dependent pretransfer editing. No accumulation of Val-[^{32}P]AMP was observed for either variant, regardless of whether tRNA was present. This is in agreement with the finding that k_{ed} and $k_{ed,\text{-}tRNA}$ are at least 10-fold higher than k_{obs} for nonenzymatic hydrolysis of Val-AMP.[15] This strongly indicates that Y59 substitutions do not stimulate Val-AMP dissociation, clearly associating the Y59 determinant with synthetic site hydrolytic editing.

Substitutions of Tyr59 affect tRNA-independent editing much less significantly (2-fold) than tRNA-dependent editing (10-fold) (Table 1). As a consequence, stimulation of editing by tRNA drops from 32-fold (wt) to 7-fold (Y59T) and 12.7-fold (Y59F) (Table 1). As both enzymes exhibit approximately 10-fold slower rates of AMP and Val-$tRNA^{Ile}$ formation compared with that of the wt enzyme, the $k_{AMP}/k_{Val\text{-}tRNA}$ ratio, reporting the extent of excess ATP consumption, remains unaffected by the introduced substitutions (Table 1).

Despite the significantly diminished tRNA-dependent editing, Y59T/D342A IleRS still consumes more than one ATP per molecule of synthesized Val-$tRNA^{Ile}$. In this enzyme, the valyl transfer step and tRNA-independent pretransfer editing operate at quantitatively comparable rates [0.03 and 0.021 s^{-1}, respectively (Tables 4 and 1, respectively)], allowing valyl-AMP hydrolysis to contribute to editing. This is in agreement with our model[15] by which kinetic partitioning of aa-AMP between transfer and hydrolysis dictates the choice between synthetic and editing pathways within the synthetic site.

tRNA-Dependent Hydrolysis of Cognate Isoleucyl-AMP. To explore specificity against cognate isoleucine in tRNA-dependent pretransfer editing by IleRS, the initial rates of AMP and Ile-$tRNA^{Ile}$ formation were followed in parallel to extract the $k_{AMP}/k_{Ile\text{-}tRNA}$ ratio. Wild-type IleRS consumed 1.6 molecules of ATP per Ile-$tRNA^{Ile}$ synthesis (Table 5), higher than the value of 1.0 that would be obtained without any

dx.doi.org/10.1021/bi5007699 | *Biochemistry* 2014, 53, 6189−6198

Table 5. Parallel Formation of AMP and Ile-tRNA^{Ile} by Wild-Type and Mutant IleRS Enzymes[a]

	k_{AMP} (s^{-1})	$k_{Ile\text{-}tRNA}$ (s^{-1})	$k_{AMP}/k_{Ile\text{-}tRNA}$
wt	0.92 ± 0.04	0.58 ± 0.04	1.6
D342A	0.29 ± 0.01	0.20 ± 0.01	1.5

[a]The values represent the mean ± the SEM of at least three independent experiments.

editing. The same ratio was obtained with D342A IleRS, indicating that this enhanced ATP consumption reflects hydrolysis of Ile-AMP and not of Ile-tRNA^{Ile}. This is further supported by an independent experiment showing that neither wild-type nor D342A IleRS significantly deacylates Ile-tRNA^{Ile} under steady-state conditions (data not shown). It thus appears that, perhaps because of the overlapping synthetic and pretransfer editing subsites, IleRS also hydrolyzes cognate isoleucyl-AMP to some extent in a tRNA-dependent pathway. In contrast, the homologous LeuRS and ValRS editing enzymes lack tRNA-dependent pretransfer editing and display a stoichiometric $k_{AMP}/k_{aa\text{-}tRNA}$ ratio of 1.0 in their respective reactions with cognate amino acids[15,25] (Table S1 of the Supporting Information).

DISCUSSION

The location of pretransfer hydrolysis in editing aaRS has been the subject of considerable debate. In an early model, aa-AMP was proposed to be translocated from the synthetic to the editing site at the CP1 domain prior to hydrolysis at that site.[18,37] However, claims that such transfer occurs have not been substantiated by detailed mechanistic studies, and a sequestered tunnel between the synthetic and editing active sites to allow the translocation has not been found in any crystal structure.[38,39] We have elaborated a different model, which in contrast locates both tRNA-independent and tRNA-dependent pretransfer editing activities within the synthetic site. The model is supported by detailed kinetic analysis of IleRS variants disabled in post-transfer editing (by substitution of the essential Asp342 in the CP1 editing domain[15]). The model was further supported by the finding that aaRSs from both classes exhibit tRNA-independent pretransfer editing within their respective catalytic domains.[9,15,16,19,25,39−41]

Details of the mechanism that allows competing pretransfer hydrolysis and tRNA transfer reactions to occupy the same active site had been unknown. To uncover the synthetic site determinants for tRNA-dependent pretransfer editing, we applied here a novel methodology that extends beyond the approaches that we previously took.[15] This approach allows quantitation of the AMP fraction that arises particularly from tRNA-dependent aa-AMP hydrolysis. To accomplish this, the fraction of AMP that arises from tRNA aminoacylation is measured separately and subtracted from the overall amount of AMP that is generated by deacylation defective enzymes. By this approach, we then further demonstrated that substitution of the conserved Tyr59 in the synthetic site with threonine abolishes stimulation of the pretransfer editing activity by tRNA. Therefore, a key amino acid determinant for tRNA-dependent pretransfer editing in IleRS is located within the synthetic site. The data thus provide substantial further evidence that the synthetic site indeed hosts tRNA-dependent pretransfer editing. The comparison of editing rates for wt and deacylation defective enzymes in the presence of tRNA shows that approximately 30% of overall editing originates from tRNA-dependent pretransfer editing. Thus, tRNA-dependent pretransfer editing represents a significant editing pathway in IleRS, although a greater contribution is made in the post-transfer step. These findings are in agreement with our previous demonstration that post-transfer editing is essential for accurate Ile-tRNA^{Ile} synthesis.[15]

Structural data in other homologous class I aaRS indicated that Tyr59 also influences the tRNA-dependent step of aminoacylation. The structure of *E. coli* LeuRS bound to the leucyl-adenylate analogue Leu-AMS, and with the tRNA 3′-acceptor stem bound in the synthetic active site, shows that the homologous Y43 in that enzyme participates in the positioning of Leu-AMP via main chain interactions. Additionally, the hydroxyl group of Y43 anchors the tyrosine side chain in the more open aminoacylation-ready position via hydrogen bonding with the main chain amino group of alanine 86. Adoption of this open position is essential for aminoacylation as it precludes a steric clash between Y43 and 3′-A76 of the tRNA when the 2′-hydroxyl moiety is poised for the transfer step.[12] We superimposed the structure of *S. aureus* IleRS bound to the active site inhibitor mupirocin and tRNA^{Ile11} with the ternary LeuRS:tRNA^{Leu}:Leu-AMS complex in which the tRNA 3′-acceptor end is oriented into the synthetic active site. This comparison reveals that the equivalent tyrosines (Y43 of LeuRS and Y58 of *S. aureus* IleRS) adopt highly similar conformations (Figure 3), thus indicating the potential for analogous functions in the respective aminoacylation reactions. Accordingly, a substantial decrease in the rate of aminoacyl transfer by both Y59T and Y59F substitutions (Table 3) confirms the relevance of this residue in mediating the second step of aminoacylation.

Because Tyr59 is important to both pretransfer editing and aminoacylation, these data show that the synthetic and editing subsites substantially overlap within the IleRS Rossmann fold catalytic domain. We hypothesize that the active site for the tRNA-dependent hydrolysis of valyl-AMP assembles by opening of the Y59 side chain induced by tRNA binding. The Y59T substitution prevents proper assembly of the catalytically productive editing site, perhaps because the smaller threonine cannot adopt the distinctive aminoacylation-like open position observed for tyrosine. Inspection of the LeuRS structure bound to a Leu-AMP analogue and with the tRNA 3′-acceptor stem oriented into the synthetic active site[12] shows that the phenyl ring of Tyr43 may be further stabilized in the open conformation also by hydrophobic interaction with the C4 and C5 atoms of the terminal adenosine of the tRNA. It is plausible to speculate that these interactions contribute to stabilization of the phenylalanine side chain in the open position in the Y59F variant, as well, providing a rationale for its activity in tRNA-dependent pretransfer editing. The data on Y59F IleRS further indicate that tRNA-dependent pretransfer editing is not necessarily linked with the enzyme/tRNA conformation that is productive in the aminoacyl transfer step. Interestingly, in the related class I MetRS, a homologous tyrosine establishes amino acid specificity by acting as an antideterminant for (i) activation of noncognate homocysteine in the synthetic pathway and (ii) elimination of methionine in a distinctive synthetic site editing pathway that proceeds via a cyclization mechanism.[42] Our data show that Y59 does not modulate amino acid specificity in the IleRS synthetic reaction (Table 2) but instead contributes to the accuracy of Ile-tRNA^{Ile} formation via its participation in proofreading.

tRNA-dependent pretransfer editing has been clearly demonstrated thus far only in IleRS. Early work proposed

dx.doi.org/10.1021/bi5007699 | *Biochemistry* 2014, 53, 6189−6198

utilization of this pathway by yeast phenylalanyl-tRNA synthetase (PheRS), but no detailed kinetic analysis in support of the conjecture has been performed.[43] The data presented here provide the first insights indicating why tRNA-dependent pretransfer editing had not been eligible for the main editing pathway. Cohabitation of synthetic and editing reactions within the same active site and close interweaving of their respective pathways could have precluded evolution of both activities toward higher rates and improved specificity. To participate in editing, pretransfer hydrolysis had to operate at a rate comparable to the transfer rate. Interestingly, the great majority of aaRSs display transfer rates significantly faster than those of IleRS, indicating that participation of pretransfer editing as a main editing pathway in these cases would have required evolution of aa-AMP hydrolysis toward such a considerably faster activity. However, we show here that the synthetic and hydrolytic activities are coupled by the same active site determinants, suggesting that their independent evolution would have been substantially restricted on that basis. Thus, it is more likely that the synthetic pathway had an evolutionary advantage over pretransfer editing to respond to cellular requirements for rapid protein synthesis. Indeed, the synthetic site may have evolved toward a faster transfer step at the cost of its editing capacity.

Pretransfer editing in the synthetic site exhibits impaired specificity against cognate aa-AMP. This is shown by the nonstoichiometric use of ATP in Ile-tRNA^{Ile} formation (Table 5), as previously demonstrated.[44] This may provide an additional rationale against evolution of the tRNA-dependent pretransfer editing pathway toward higher efficacy and prevalence in editing. Because of the inherent limitations of tRNA-dependent pretransfer editing, and the need for error correction to maintain genetic code fidelity, evolution toward the faster transfer step may then have triggered co-evolution of the capacity to hydrolyze misacylated tRNA. This feature was acquired by aaRSs through recruitment of specific domains dedicated to this post-transfer editing reaction. More selective and catalytically superior post-transfer editing then may have made pretransfer editing largely superfluous, because there no longer existed a trade-off requiring slower aminoacyl transfer rates to maintain specificity. It is further possible that the exemption from the requirement to host both pre- and post-transfer editing activities allowed evolution of such powerful deacylation machinery within the editing domain.[25,31,45] The important consequence is that kinetic partitioning of misacylated tRNA between dissociation and editing strongly favors hydrolytic correction, thus preventing errors in aminoacylation.

IleRS exhibits a significantly lower AMP/aa-tRNA ratio [16 (Table 1)] compared with those of LeuRS (100)[25] and ValRS [149 (Table S1 of the Supporting Information)], indicating that it is more prone to misacylation errors. In spite of this, the observed misacylation rates exhibited by IleRS, ValRS, and LeuRS [0.06, 0.05, and 0.03 s^{-1}, respectively (Table 4 and Table S1 of the Supporting Information[25])] are relatively similar. It thus appears that IleRS, presumably as a consequence of the significantly slower transfer rates, produces misacylated tRNA with a similar frequency as do the more accurate LeuRS and ValRS in spite of its higher error rate. If so, evolution of IleRS toward the faster transfer step would have required more proficient editing at the expense of ATP. The question of why this scenario (a slow transfer step) is apparently confined only to IleRS among the homologous class I enzymes that perform editing then arises. Class I aaRSs exhibit a burst of aa-tRNA in aminoacylation.[4] The rapid chemical step in these enzymes may be beneficial as it allows control of the aminoacylation rate by modulating the product release step. This may be accomplished by interaction with elongation factor Tu,[4] which represents an elegant mechanism for providing flexibility in the rate of aminoacylation to support the needs for protein synthesis. Still, our data clearly suggest that evolution toward a faster transfer step in editing aaRSs also demands a higher rate of ATP consumption. In general, this may be less problematic for editing class I aaRSs, as noncognate amino acids that threaten the accuracy of protein synthesis are present in the cell at concentrations significantly lower than their respective K_m values (Figure S1 of the Supporting Information). Intriguingly, however, IleRS is notable in that the noncognate amino acid valine is present at a high concentration in the cell. Indeed, valine is among the most abundant amino acids in *E. coli*, and its predicted concentration[46] is very similar to the K_m value for valine by IleRS (Figure S1 of the Supporting Information and unpublished results of N. Cvetesic and I. Gruic-Sovulj). Therefore, evolution of IleRS toward the faster transfer step may have been restricted because it would impose a large energetic burden on the cell.

Y59 is highly conserved among IleRSs of prokaryotic origin, in agreement with its important role in the aminoacyl transfer step. Its substitution with phenylalanine or threonine in enzymes of eukaryotic origin is thus intriguing (Figure 2). Phylogenetic analyses revealed that some prokaryotes acquired a eukaryote-like IleRS gene by horizontal transfer.[34] These IleRSs are significantly more resistant to the antibiotic mupirocin, rationalizing the spread of their genes among prokaryotes. The synthetic sites of eukaryotic and eukaryotic-like IleRS display novel sequence motifs and lack some sequence motifs present in the prokaryotic enzymes. These distinctions contribute to the different sensitivities to mupirocin.[47] The lack of a Y59 homologue in eukaryotic IleRS indicates that the synthetic site may operate differently. Whether eukaryotic IleRSs acquired distinctive kinetic properties together with the development of mupirocin resistance is a relevant topic for future research.

■ ASSOCIATED CONTENT

Supporting Information

Parallel formation of AMP and aa-tRNA^{Val} by wild-type and mutant *E. coli* ValRS and comparison of the K_m values in the activation of noncognate amino acids with their concentration in the cell. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Department of Chemistry, Faculty of Science, University of Zagreb, Horvatovac 102a, 10000 Zagreb, Croatia. Telephone: +3851-4606232. Fax: +3851-4606401. E-mail: gruic@chem.pmf.hr.

Funding

This work was supported by the Unity through Knowledge Fund (Grant 8/13 to I.G.-S.) and in part by a grant from Croatian Science Foundation (09.01/293) and the European Community's Seventh Framework Programme (FP7RegPot/IntegraLife, 315997). J.J.P. acknowledges support from the National Institutes of Health (Grant GM63713). I.G.-S. thanks

the Adris foundation for support. Funding for open access charge provided by the European Community's Seventh Framework Programme (FP7RegPot/IntegraLife, 315997).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Mario Cindrić (Intitute Rudjer Boskovic) for access to chromatographic equipment, Nevena Cvetesic for careful reading of the manuscript, and Nina Franicevic for experimental help.

■ DEDICATION

This paper is dedicated to the memory of Ivana Weygand-Durasevic.

■ ABBREVIATIONS

aa-AMP, aminoacyl-adenylate; aaRS, aminoacyl-tRNA synthetase; AMP, adenosine monophosphate; EcIleRS, *Escherichia coli* isoleucyl-tRNA synthetase; IleRS, isoleucyl-tRNA synthetase; Leu-AMS, 5′-O-[N-(L-leucyl)sulfamoyl] adenosine; LeuRS, leucyl-tRNA synthetase; MetRS, methionyl-tRNA synthetase; PEI, polyethylenimine plates; PheRS, phenylalanyl-tRNA synthetase; SEM, standard error of the mean; TLC, thin layer chromatography; ValRS, valyl-tRNA synthetase; wt, wild-type.

■ REFERENCES

(1) First, E. A. (2005) Catalysis of the tRNA aminoacylation reaction. In *The aminoacyl-tRNA synthetases* (Ibba, M., Francklyn, C. S., and Cusack, S., Eds.) pp 328−347, Landes Bioscience/Eurekah.com, Georgetown, TX.

(2) Perona, J. J., and Hadd, A. (2012) Structural diversity and protein engineering of the aminoacyl-tRNA synthetases. *Biochemistry 51*, 8705−8729.

(3) Eriani, G., Delarue, M., Poch, O., Gangloff, J., and Moras, D. (1990) Partition of tRNA synthetases into two classes based on mutually exclusive sets of sequence motifs. *Nature 347*, 203−206.

(4) Zhang, C. M., Perona, J. J., Ryu, K., Francklyn, C., and Hou, Y. M. (2006) Distinct kinetic mechanisms of the two classes of aminoacyl-tRNA synthetases. *J. Mol. Biol. 361*, 300−311.

(5) Giege, R., Sissler, M., and Florentz, C. (1998) Universal rules and idiosyncratic features in tRNA identity. *Nucleic Acids Res. 26*, 5017−5035.

(6) Perona, J. J., and Hou, Y. M. (2007) Indirect readout of tRNA for aminoacylation. *Biochemistry 46*, 10419−10432.

(7) Yadavalli, S. S., and Ibba, M. (2012) Quality control in aminoacyl-tRNA synthesis: Its role in translational fidelity. *Adv. Protein Chem. Struct. Biol. 86*, 1−43.

(8) Perona, J. J., and Gruic-Sovulj, I. (2014) Synthetic and editing mechanisms of aminoacyl-tRNA synthetases. *Top. Curr. Chem. 344*, 1−41.

(9) Splan, K. E., Ignatov, M. E., and Musier-Forsyth, K. (2008) Transfer RNA modulates the editing mechanism used by class II prolyl-tRNA synthetase. *J. Biol. Chem. 283*, 7128−7134.

(10) Fukai, S., Nureki, O., Sekine, S., Shimada, A., Tao, J., Vassylyev, D. G., and Yokoyama, S. (2000) Structural basis for double-sieve discrimination of L-valine from L-isoleucine and L-threonine by the complex of $tRNA^{Val}$ and valyl-tRNA synthetase. *Cell 103*, 793−803.

(11) Silvian, F. L., Wang, J., and Steitz, T. A. (1999) Insights into editing from an Ile-tRNA synthetase structure with $tRNA^{Ile}$ and mupirocin. *Science 285*, 1074−1077.

(12) Palencia, A., Crépin, T., Vu, M. T., Lincecum, T. L., Jr., Martinis, S. A., and Cusack, S. (2012) Structural dynamics of the aminoacylation and proofreading functional cycle of bacterial leucyl-tRNA synthetase. *Nat. Struct. Mol. Biol. 19*, 677−684.

(13) Lin, L., Stephen, P. H., and Schimmel, P. (1996) Amino-acylation error correction. *Nature 384*, 33−34.

(14) 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., Grotli, M., Martinis, S. A., and Cusack, S. (2003) Structural and mechanistic basis of pre- and posttransfer editing by leucyl-tRNA synthetase. *Mol. Cell 11*, 951−963.

(15) Dulic, M., Cvetesic, N., Perona, J. J., and Gruic-Sovulj, I. (2010) Partitioning of tRNA-dependent editing between pre- and post-transfer pathways in class I aminoacyl-tRNA synthetases. *J. Biol. Chem. 285*, 23799−23809.

(16) Minajigi, A., and Francklyn, C. S. (2010) Aminoacyl transfer rate dictates choice of editing pathway in threonyl-tRNA synthetase. *J. Biol. Chem. 285*, 23810−23817.

(17) Ling, J., Peterson, K. M., Simonovic, I., Söll, D., and Simonovic, M. (2012) The mechanism of pre-transfer editing in yeast mitochondrial threonyl-tRNA synthetase. *J. Biol. Chem. 287*, 28518−28525.

(18) Bishop, A. C., Nomanbhoy, T. K., and Schimmel, P. (2002) Blocking site-to-site translocation of a misactivated amino acid by mutation of a class I tRNA synthetase. *Proc. Natl. Acad. Sci. U.S.A. 99*, 585−590.

(19) Gruic-Sovulj, I., Rokov-Plavec, J., and Weygand-Durasevic, I. (2007) Hydrolysis of non-cognate aminoacyl-adenylates by a class II aminoacyl-tRNA synthetase lacking an editing domain. *FEBS Lett. 581*, 5110−5114.

(20) Pauling, L. (1957) The probability of errors in the pprocess of synthesis of protein molecules. In *Festschrift* (Stoll, A., Ed.) pp 597−602, Birkhauser Verlag, Basel, Switzerland.

(21) Flossdorf, J., and Kula, M. R. (1973) Ultracentrifuge studies on binding of aliphatic amino acids to isoleucyl-tRNA synthetase from *Escherichia coli* MRE 600. *Eur. J. Biochem. 36*, 534−540.

(22) Loftfield, R. B., and Vanderjagt, D. (1972) The frequency of errors in protein biosynthesis. *Biochem. J. 128*, 1353−1356.

(23) Baldwin, A. N., and Berg, P. (1966) Purification and properties of isoleucyl ribonucleic acid synthetase from *Escherichia coli*. *J. Biol. Chem. 241*, 831−838.

(24) Fersht, A. R. (1977) Editing mechanisms in protein synthesis. Rejection of valine by the isoleucyl-tRNA synthetase. *Biochemistry 16*, 1025−1030.

(25) Cvetesic, N., Perona, J. J., and Gruic-Sovulj, I. (2012) Kinetic partitioning between synthetic and editing pathways in class I aminoacyl-tRNA synthetases occurs at both pre-transfer and post-transfer hydrolytic steps. *J. Biol. Chem. 287*, 25381−25394.

(26) Crepin, T., Schmitt, E., Mechulam, Y., Sampson, P. B., Vaughan, M. D., Honek, J. F., and Blanquet, S. (2003) Use of analogues of methionine and methionyl adenylate to sample conformational changes during catalysis in *Escherichia coli* methionyl-tRNA synthetase. *J. Mol. Biol. 332*, 59−72.

(27) Landro, J. A., and Schimmel, P. (1993) Metal-binding site in a class I tRNA synthetase localized to a cysteine cluster inserted into nucleotide-binding fold. *Proc. Natl. Acad. Sci. U.S.A. 90*, 2261−2265.

(28) Nureki, O., Niimi, T., Muramatsu, T., Kanno, H., Kohno, T., Florentz, C., Giege, R., and Yokoyama, S. (1994) Molecular recognition of the identity-determinant set of isoleucine transfer RNA from *Escherichia coli*. *J. Mol. Biol. 236*, 710−724.

(29) Wolfson, A. D., and Uhlenbeck, O. C. (2002) Modulation of tRNAAla identity by inorganic pyrophosphatase. *Proc. Natl. Acad. Sci. U.S.A. 99*, 5965−5970.

(30) Uter, N. T., Gruic-Sovulj, I., and Perona, J. J. (2005) Amino acid-dependent transfer RNA affinity in a class I aminoacyl-tRNA synthetase. *J. Biol. Chem. 280*, 23966−23977.

(31) Fersht, A. R., and Kaethner, M. (1976) Enzyme hyperspecificity. Rejection of threonine by valyl-tRNA synthetase by misacylation and hydrolytic editing. *Biochemistry 15*, 3342−3346.

(32) Fukunaga, R., and Yokoyama, S. (2006) Structural basis for substrate recognition by the editing domain of isoleucyl-tRNA synthetase. *J. Mol. Biol. 359*, 901−912.

(33) Guth, E. C., and Francklyn, C. S. (2007) Kinetic discrimination of tRNA identity by the conserved motif 2 loop of a class II aminoacyl-tRNA synthetase. *Mol. Cell 25*, 531−542.
(34) Brown, J. R., Gentry, D., Becker, J. A., Ingraham, K., Holmes, D. J., and Stanhope, M. J. (2003) Horizontal transfer of drug-resistant aminoacyl-transfer-RNA synthetases of anthrax and Gram-positive pathogens. *EMBO Rep. 4*, 692−698.
(35) Fersht, A. R. (1999) *Structure and Mechanism in Protein Science: A Guide to Enzyme Catalysis and Protein Folding*, W. H. Freeman & Co., New York.
(36) Li, L., and Carter, W., Jr. (2013) Full implementation of the genetic code by tryptophanyl-tRNA synthetase requires intermodular coupling. *J. Biol. Chem. 48*, 34736−34745.
(37) Nomanbhoy, T. K., Hendrickson, T. L., and Schimmel, P. (1999) Transfer RNA-dependent translocation of misactivated amino acids to prevent errors in protein synthesis. *Mol. Cell 4*, 519−528.
(38) Dock-Bregeon, A. C., Rees, B., Torres-Larios, A., Bey, G., Calliet, J., and Moras, D. (2004) Achieving error-free translation: The mechanism of proofreading of threonyl-tRNA synthetase at atomic resolution. *Mol. Cell 16*, 375−386.
(39) Gruic-Sovulj, I., Uter, N., Bullock, T., and Perona, J. J. (2005) tRNA-dependent aminoacyl-adenylate hydrolysis by a nonediting class I aminoacyl-tRNA synthetase. *J. Biol. Chem. 280*, 23978−23986.
(40) Beuning, P. J., and Musier-Forsyth, K. (2001) Species-specific differences in amino acid editing by class II prolyl-tRNA synthetase. *J. Biol. Chem. 276*, 30779−30785.
(41) Gruic-Sovulj, I., Dulic, M., and Weygand-Durasevic, I. (2011) Pre-transfer editing of serine hydroxamate within the active site of methanogenic-type seryl-tRNA synthetase. *Croat. Chem. Acta 84*, 179−184.
(42) Kim, H. Y., Ghosh, G., Schulman, L. H., Brunie, S., and Jakubowski, H. (1993) The relationship between synthetic and editing functions of the active site of an aminoacyl-tRNAsynthetase. *Proc. Natl. Acad. Sci. U.S.A. 90*, 11553−11557.
(43) Lin, S. X., Baltzinger, M., and Remy, P. (1984) Fast kinetic study of yeast phenylalanyl-tRNA synthetase: Role of tRNAPhe in the discrimination between tyrosine and phenylalanine. *Biochemistry 23*, 4109−4116.
(44) Hopfield, J. J., Yamane, T., Yue, V., and Coutts, S. M. (1976) Direct experimental evidence for kinetic proofreading in amino acylation of tRNAIle. *Proc. Natl. Acad. Sci. U.S.A. 73*, 1164−1168.
(45) Fersht, A. R., and Dingwall, C. (1979) Evidence for the double-sieve editing mechanism in protein synthesis. Steric exclusion of isoleucine by valyl-tRNA synthetases. *Biochemistry 18*, 2627−2631.
(46) Bennett, B. D., Kimball, E. H., Gao, M., Osterhout, R., Van Dien, S. J., and Rabinowitz, J. D. (2009) Absolute metabolite concentrations and implied enzyme active site occupancy in *Escherichia coli*. *Nat. Chem. Biol. 5*, 593−599.
(47) Sassanfar, M., Kranz, J. E., Gallant, P., Schimmel, P., and Shiba, K. (1996) A eubacterial *Mycobacterium tuberculosis* tRNA synthetase is eukaryote-like and resistant to a eubacterial-specific antisynthetase drug. *Biochemistry 35*, 9995−10003.