Negative catalysis by the editing domain of class I aminoacyl-tRNA synthetases

Igor Zivkovic[a], Kate Ivkovic[a], Nevena Cvetesic[b], Ita Gruic-Sovulj[a]

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

[b]Institute for Clinical Sciences, Faculty of Medicine, Imperial College London and MRC London Institute of Medical Sciences, London, UK

Corresponding author information

I. Gruic-Sovulj, Department of Chemistry, Faculty of Science, University of Zagreb, Horvatovac 102a, Zagreb 10000, Croatia, Tel: +3851 4606230, E-mail: gruic@chem.pmf.hr

Keywords

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Abstract

Aminoacyl-tRNA synthetases (AARSs) couple cognate pairs of amino acids and tRNAs for protein synthesis. The coupling errors can be detrimental, guiding AARS evolution towards high selectivity. To address the limits of the initial amino acid selection, half of AARSs acquired the editing domain to clear the non-cognate substrates that evaded the synthetic site rejection. While high selectivity of the synthetic site is well-established, mechanisms that shaped selectivity of the editing domain remain unknown. To tackle this question, we used a class I isoleucyl-tRNA synthetase (IleRS) from *Escherichia coli* as a model enzyme and a broad range of non-cognate amino acids efficiently discriminated at the IleRS synthetic site. We found that the IleRS editing site cleared all tested non-cognate amino acids with rates of 35-65 s^{-1}. Thus, surprisingly, the editing site exhibits broad substrate acceptance not limited to the amino acids that jeopardize translational fidelity. This questions the established paradigm of the synthetic-editing sites reciprocity for the clearance of the non-cognate substrates. The editing domain's low selectivity against the non-cognate substrates contrasts its exquisite specificity in the cognate amino acid rejection. We demonstrated that the latter, being the main constraint during the domain evolution, is established by the residues that promote negative catalysis through destabilisation of the transition state comprising exclusively the cognate amino acid. Finally, we unveiled that IleRS may utilize its editing domain *in trans*. This sets IleRS as a unique class I AARS, which operates by the class II AARS editing mechanism.

Significance Statement

The faithful protein synthesis is a vital property of the cell as errors in translation can diminish cellular fitness and lead to severe neurodegeneration. Aminoacyl-tRNA synthetases (AARS) translate the genetic code by loading tRNAs with the cognate amino acids. The errors in amino acid recognition are cleared at the AARS editing domain through hydrolysis of misaminoacyl-tRNAs. Here we show that the editing domain of class I AARSs does not select for non-cognate amino acids that jeopardize the AARS fidelity. Instead, it selects against the cognate aminoacyl-tRNA providing a new paradigm wherein

safeguarding against misediting constrains the editing evolution. This design allows for the broad substrate acceptance of the editing domain, a feature that is generally beneficial for error-correction systems.

Introduction

Aminoacyl-tRNA synthetases (AARS) couple cognate amino acid and tRNA pairs for protein biosynthesis. They are divided into two, evolutionary distinct classes, class I and class II (1, 2). In both classes, the pairing occurs at the synthetic active site by the same two-step mechanism bearing some class-dependent features (3). The first step, amino acid activation, comprises the formation of aminoacyl-AMP (AA-AMP) while the second step is the transfer of the aminoacyl moiety to the tRNA (formation of aminoacyl-tRNA, AA-tRNA) **(Figure 1, paths 1 and 4)**. The coupling of non-cognate substrates leads to mistranslation, which can be toxic for the cell (4–6). Due to physicochemical similarities of cellular amino acids, around half of AARSs cannot achieve the tolerable level of fidelity (estimated to be 1 in 3300 (7)) in the synthetic reactions alone and thus have evolved editing (reviewed in (8, 9)). The error can be corrected by hydrolysis of non-cognate AA-AMP within the confines of the synthetic site (pre-transfer editing, **Figure 1, paths 2 and 3**) (10, 11) and/or by hydrolysis of misaminoacyl-tRNA at the dedicated editing domain (post-transfer editing) (12, 13). The latter appears to be the dominant pathway, operating by two possible routes – *in cis* **(Figure 1, path 5 and 6)** and *in trans* **(Figure 1, path 7 - 9)** (9). Editing *in trans,* so far demonstrated only in class II AARS (14), entails dissociation of the AA-tRNA and its rebinding with the 3'-end facing the editing domain.

The interplay between the synthetic and editing sites was firstly addressed by Fersht's double-sieve hypothesis proposed originally for class I isoleucyl- (IleRS) and valyl-tRNA synthetases (ValRS) (15). It states that the synthetic site uses steric clash to discard larger than the cognate amino acids while the editing site clears smaller/isosteric non-cognate amino acids that were successfully aminoacylated to the tRNA. The steric clash was also proposed to prevent the binding of the cognate AA-tRNA to the editing domain. But, is the productive recognition of the amino acid at the editing site correlated with its efficient misrecognition at the synthetic site, and to what extent does the steric clash define the selectivity of the editing site? The former was anticipated but not experimentally addressed. The latter was tested to show that the selectivity against the cognate AA-tRNA arises from its imposed unproductive binding (16–18)).

IleRS rapidly hydrolyses $tRNA^{Ile}$s misaminoacylated with non-proteinogenic norvaline (Nva) and Val (6). This is expected as both Nva and Val are misactivated with a frequency that is 10-fold higher than the estimated tolerable error (7) and thus pose threats to the fidelity of Ile-$tRNA^{Ile}$ formation (6, 15). Surprisingly, IleRS can also efficiently hydrolyse $tRNA^{Ile}$ misaminoacylated with a non-proteinogenic α-aminobutyrate (Abu) and its synthetic γ-fluorinated analogues (F_2Abu and F_3Abu), which are misactivated with up to a 20-fold *lower* frequency than the estimated tolerable error (19). This questions whether the editing site substrates need to be well misrecognized at the synthetic site, as anticipated.

Here, we set out to explore what shaped the selectivity of class I editing site and to unravel whether it shares the same mechanisms and demands for selectivity with the synthetic site using IleRS as a model enzyme. We characterized amino acid activation and AA-$tRNA^{Ile}$ hydrolysis using a range of amino acids with different physicochemical properties (Ala, Ser, Thr, Met, Leu, Nle). We found that IleRS synthetic site discriminates with at least 20 000-fold against the tested non-cognate amino acids.

Thus, these substrates should not pose a fidelity problem. Nevertheless, all misaminoacylated tRNA^{Ile}s were rapidly hydrolysed at the editing site. Only cognate Ile-tRNA^{Ile} was weakly hydrolysed, demonstrating that evolution of the editing site was driven by negative catalysis (20, 21) *i.e.* selection towards destabilisation of the transition state for the cognate AA-tRNA hydrolysis (misediting). We also found that negative determinants for misediting vary among the closely related class I editing domains. Finally, we discovered that in IleRS, delivery of the AA-tRNA to the editing domain entails the accumulation of free AA-tRNA in solution, reminiscent of class II AARSs editing *in trans*.

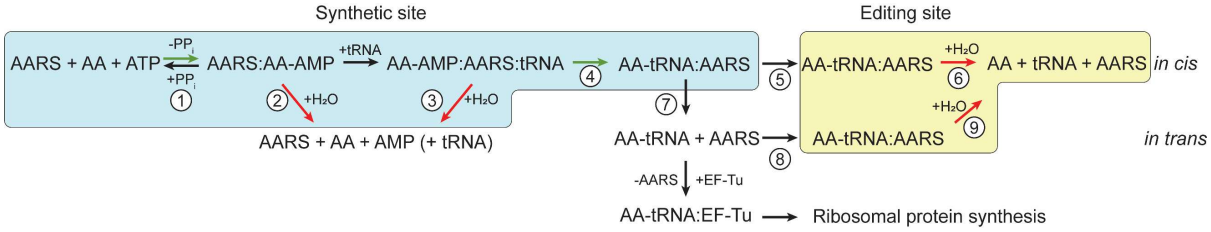

Figure 1. IleRS pathways of aminoacylation (green arrows) and editing (red arrows). The synthetic pathway consists of amino acid activation (1) and the aminoacyl transfer step (4). The editing pathways include tRNA-independent (2) and tRNA-dependent (3) pre-transfer editing and post-transfer editing (6, 9). Post-transfer editing can occur by translocation of AA-tRNA (5) to the editing domain for hydrolysis (6, *in cis*) or by AA-tRNA dissociation (7), its subsequent rebinding to the editing site (8) and hydrolysis (9, *in trans*).

Results

Preparation of a broad range of misaminoacylated tRNA^{Ile}s by a post-transfer editing deficient IleRS

Post-transfer editing is tested by following hydrolysis of preformed misaminoacylated tRNA (22). To misaminoacylate tRNA^{Ile} with non-cognate amino acids of various physicochemical properties (**Supplementary Figure S1**) we opted for post-transfer editing deficient T243R/D342A IleRS (6, 11, 19). Amino acid activation (**Figure 1, path 1**) was tested and the discrimination factors (*D*) were calculated (**Table 1**). Higher *D* reflects the more efficient exclusion of the non-cognate substrate from the IleRS synthetic site. The non-cognate amino acids were all activated albeit with high discrimination factors (*D* > 20 000 for WT IleRS, **Table 1**). Despite weak misactivation, tRNA^{Ile} was successfully misaminoacylated (up to 60 % aminoacylation level) with all tested non-cognate amino acids by T243R/D3432A IleRS (**Supplementary Figure S2** and **Supplementary Figure S3**), but not with WT IleRS (**Supplementary Figure S2, inset**). This demonstrates that the editing deficient AARSs can provide an alternative to the ribozyme approach (23). Studying AARS selectivity against well discriminated substrates is further complicated by artefacts that may arise from contamination of non-cognate amino acid samples with trace amounts of the cognate amino acids (5, 15, 24). For that reason, we estimated the purity of the used amino acids (**Supplementary Figure S4**) and found that Leu, and possibly also Met and Nle, may contain trace amounts of Ile, and thus their discrimination factors were not calculated.

Table 1. Kinetic parameters for activation of amino acids by WT IleRS.

Amino acid	k_{cat} / s^{-1}	k_{sp}[a] / s^{-1} mM^{-1}	K_M / mM	D[b]
Ile	56.7 ± 0.3	(16.6 ± 0.4) × 10^3	(3.41 ± 0.06) × 10^{-3}	1
Val[c]	36 ± 6	77	0.47 ± 0.03	156
Nva[d]	41 ± 1	50	0.82 ± 0.07	239
Abu[e]	23 ± 3	1.6 ± 0.1	15 ± 1	10 375
Thr	32 ± 2	0.82 ± 0.04	39 ± 2	20 243
F_2Abu[e]	6.9 ± 0.7	0.45 ± 0.8	16 ± 2	36 888
F_3Abu[e]	5.3 ± 0.8	0.19 ± 0.02	28 ± 2	87 368
Ala	10 ± 1	0.10 ± 0.02	100 ± 9	166 000
Ser	-	0.016 ± 0.005[f]	-	1 037 500
Met	10.6 ± 0.4	7.6 ± 0.7	1.4 ± 0.1	NC[g]
Nle	4.8 ± 0.4	1.6 ± 0.2	3.1 ± 0.6	NC[g]
Leu	28.7 ± 0.7	26 ± 3	1.1 ± 0.1	NC[g]

The activation step was tested by ATP-PPi exchange assay. The values represent the average value ± SEM of at least three independent experiments.

[a]k_{sp} – specificity constant (k_{cat}/K_M) is obtained from the modified Michaelis-Menten equation $k_{obs} = \frac{k_{sp}[S]}{1+\frac{k_{sp}[S]}{k_{cat}}}$ (25).

[b]Discrimination factor – $k_{sp,cognate}/k_{sp,non\text{-}cognate}$, *i.e.* $(k_{cat}/K_M)_{cognate}/(k_{cat}/K_M)_{non\text{-}cognate}$.

[c]Data was taken from (11). The k_{cat} and K_M values were determined using the unmodified form of the Michaelis-Menten equation. k_{sp} was calculated by dividing k_{cat} with K_M.

[d]Data was taken from (6). The k_{cat} and K_M values were determined using the unmodified form of the Michaelis-Menten equation. k_{sp} was calculated by dividing k_{cat} with K_M.

[e]Raw data was taken from (19).

[f]k_{cat} and K_M were not determined due to the low activity.

[g]Not calculated due to possible contamination of the amino acid sample with cognate Ile.

The editing site clears a broad range of misaminoacylated tRNA^{Ile}s

Next, we isolated the post-transfer editing step by mixing preformed misaminoacylated tRNA^{Ile} with a surplus of WT IleRS, using a rapid chemical quench instrument. The hydrolysis of misaminoacylated tRNA^{Ile} was followed in time to calculate the first-order rate constant (**Supplementary Figure S3**). The single-turnover conditions (see *Single turnover hydrolysis, SI Appendix*) ensure that product dissociation does not limit the observed rate (26). A 2-fold higher concentration of IleRS or AA-tRNA^{Ile} returned the same hydrolysis rate confirming that binding is not rate-limiting. Thus, the observed rate constants (**Figure 2**, **Supplementary Figure S3**) represent the catalytic step (hydrolysis of misaminocylated tRNA^{Ile}) within the editing site.

The single-turnover analysis revealed that all misaminoacylated-tRNA^{Ile}s were rapidly hydrolysed with similar rates ranging from 35 to 65 s^{-1} (**Figure 2**). This is in agreement with the incapacity of the WT IleRS to accumulate these misaminoacylated tRNAs^{Ile} (**Supplementary Figure S2, inset**). The unravelled rapid editing is surprising as these amino acids (except Val and Nva) are efficiently discriminated at the synthetic site and as such cannot pose a threat to IleRS aminoacylation fidelity. Finding that amino acids are rapidly cleared at the editing domain irrespectively of the requirement for their editing, lend a new paradigm about the editing selectivity principles. Moreover, the editing site shows no clear preference towards physicochemical features of the editing substrates, like size

(Met and Nle, both with longer unbranched side chain, are eliminated), hydrophobicity (polar Ser and Thr are efficiently cleared at the editing site) or branching (Leu-tRNA^{Ile} is also rapidly hydrolysed). Cognate Ile-tRNA^{Ile} was the only exemption, suggesting, that evasion of cognate AA-tRNA misediting was a major constraint during the evolution of the editing site. Thus, we set to explore how the editing site excludes the cognate Ile-tRNA^{Ile} and in parallel promotes editing of misaminoacylated tRNAs.

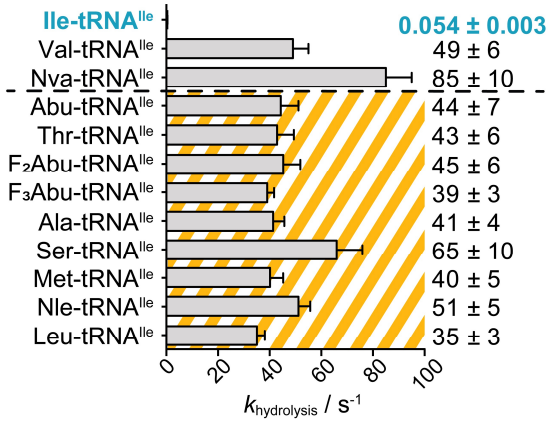

Figure 2. Single-turnover hydrolysis of misaminoacylated-tRNAs by WT IleRS. tRNAs misaminoacylated with amino acids that are well discriminated at the IleRS synthetic site (D > 3300) are presented in the striped area. Value 3300 is taken as the tolerable error of protein synthesis were estimated to be 1 in 3300 (7). Rapid hydrolysis of Leu-, Met-, and Nle-tRNA^{Ile} confirmed that possible traces of cognate Ile in the Leu, Met or Nle samples did not compromise the editing analysis. Time courses from which the first-order rate constants ($k_{hydrolysis}$) were calculated are presented in Supplementary Figure S3. $k_{hydroysis}$ for Val-, Nva-, Abu-, F$_2$Abu-, and F$_3$Abu-tRNAs^{Ile} were taken from (6, 19).

Negative determinants for Ile-tRNA^{Ile} misediting

Two conserved residues of the editing domain, Thr246 and His333 (Thr233 and His319 in *T. thermophilus* IleRS, PDB ID: 1WNZ, **Supplementary Figure S5**) were previously characterised by time-course analysis and their equal contribution to the rejection of Ile-tRNA^{Ile} has been proposed (27, 28). However, in closely related LeuRS, the specificity against the cognate Leu resides solely on Thr252 (analogous to Thr246 in EcIleRS) (16)**.** It has been shown that time-course analysis may lead to incorrect models of enzyme mechanisms (11). Therefore, we used single-turnover catalysis to assign the individual contributions of Thr246 and His333 (**Table 2)**. Interestingly, IleRS T246A, increased the rate of Ile-tRNA^{Ile} misediting by only 2-fold (0.126 ± 0.006 s^{-1}). In contrast, the H333A mutant showed a 20-fold increase in the rate of Ile-tRNA^{Ile} hydrolysis (1.04 ± 0.06 s^{-1}), arguing that, for IleRS, His333 is the main negative determinant of misediting. Finally, the T246A/H333A mutant showed a 260-fold increase in the rate of Ile-tRNA^{Ile} hydrolysis (14 ± 1 s^{-1}), displaying about 7-fold higher effect than the cumulative effects of the independent mutations. His333 was further mutated to Gly, which additionally increased the rate of Ile-tRNA^{Ile} hydrolysis (4-fold compared to H333A), suggesting that steric hindrance may contribute to the His333 action. Importantly, the mutants displayed only 2-fold slower rates of editing of non-cognate Val-tRNA^{Ile} relative to the WT (**Table 2**) pointing towards their almost exclusive effect on Ile-tRNA^{Ile} misediting. Thus, the main negative determinant of the IleRS editing site appears to be His333 whose role is synergistically supported by Thr246**.** This contrasts LeuRS which utilizes Thr252 as a sole negative determinant and suggests idiosyncratic evolution of the mechanisms governing rejection of the cognate product in class I editing domains.

Table 2. Single-turnover and steady-state rate constants for different variants of IleRS.

Enzyme	$k_{hydrolysis}$[a] / s^{-1}		$k_{aminoacylation}$[b] / s^{-1}
	Ile-tRNA^{Ile}	Val-tRNA^{Ile}	Ile
WT	0.054 ± 0.003	49 ± 6[c]	1.3 ± 0.2
T246A	0.126 ± 0.006	32 ± 2	1.5 ± 0.3
H333A	1.04 ± 0.06	19.2 ± 0.6	1.6 ± 0.4
H333G	4.4 ± 0.7	24 ± 1	1.1 ± 0.2
T246A/H333A	14 ± 1	24 ± 1	0.96 ± 0.06

The values represent the average value ± SEM of at least three independent experiments.

[a]Single-turnover rate constants for AA-tRNA^{Ile} hydrolysis. Time courses from which the first-order rate constants ($k_{hydrolysis}$) were calculated are presented in **Supplementary Figure S6**.

[b]Steady-state rate constants for Ile-tRNA^{Ile} synthesis.

[c]Data was taken from (6)

IleRS deprived of the negative determinants misedits Ile-tRNA^{Ile} *in trans*

During steady-state (mis)aminoacylation AA-tRNA partitions between hydrolysis (editing; **Figure 1 paths 5 and 6**) and dissociation (**Figure 1, path 7**) from the enzyme (product release). To reach the editing site, AA-tRNA can translocate its single-stranded 3' end while the tRNA body remains bound to the enzyme (editing *in cis*) or the whole AA-tRNA can dissociate and re-bind with the 3' end facing the editing domain (editing *in trans*). Editing *in cis* depletes the product and thus compromises steady-state aminoacylation. In contrast, editing *in trans* may not affect the aminoacylation rate, because re-binding of AA-tRNA for hydrolysis is not favoured at low steady-state enzyme concentration. Therefore, the finding that both H333A and T246A/H333A IleRSs exhibit little to no change in steady-state aminoacylation rates relative to the WT enzyme ($k_{aminoacylation}$, **Table 2**), despite rapid Ile-tRNA^{Ile} hydrolysis at their editing sites ($k_{hydrolysis}$, **Table 2**), implies that these mutants misedit Ile-tRNA^{Ile} *in trans*. This is unexpected as editing *in trans* was not yet demonstrated for class I AARSs.

To confirm the existence of misediting *in trans*, we followed ATP consumption (AMP formation) in parallel to AA-tRNA accumulation. Non-stoichiometric ATP consumption is diagnostic of active editing as futile aminoacylation/editing cycles consume ATP without accumulating AA-tRNA. Two complementary approaches were undertaken: i) we used higher IleRS concentration (2 µM instead of 20 nM used in the steady-state aminoacylation) to favour re-binding of Ile-tRNA^{Ile} and thus misediting *in trans* and ii) higher IleRS concentration was complemented by the addition of 8-12 µM active EF-Tu, which may suppress misediting *in trans* by competing with IleRS in the binding of free Ile-tRNA^{Ile} (29).

ATP consumption and Ile-tRNA^{Ile} formation were followed in parallel reactions that differ only in the labelled components – [^{32}P]ATP was used for the former and [^{32}P]tRNA^{Ile} for the latter (**Supplementary Figure S7**). The ratio of consumed ATP per Ile-tRNA^{Ile} accumulated in solution (AMP/Ile-tRNA^{Ile}) was calculated for the reactions without and with EF-Tu (**Figure 3**). In the absence of EF-Tu, both mutants, due to active misediting, consume 18- (H333A) to 1100-fold (T246A/H333A) higher than the stoichiometric amount of ATP per released Ile-tRNA^{Ile}. That misediting takes place *in trans*, is supported by 9- (H333A) to 18-fold (T246A/H333A) drop in AMP/Ile-tRNA^{Ile} ratio in the presence of EF-Tu. The WT enzyme, exhibiting marginal Ile-tRNA^{Ile} misediting, used an approximately stoichiometric amount of ATP per Ile-tRNA^{Ile}, independently on the presence/absence of EF-Tu. Interestingly, the significant energetic cost was exhibited mainly with T246A/H333A, raising an intriguing question - how detrimental would Ile-tRNA^{Ile} misediting be for the cell?

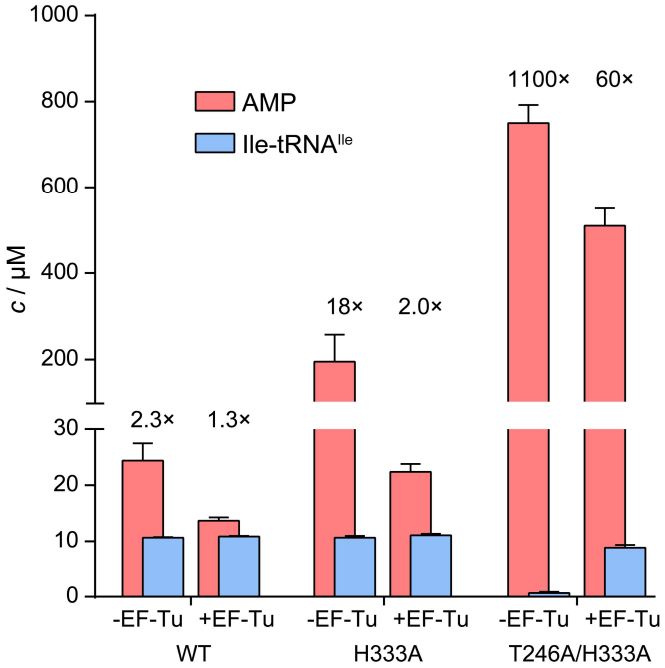

Figure 3. AMP (pink) and Ile-tRNA^{Ile} (blue) concentrations at the 5-minute time point for WT, H333A and T246A/H333A IleRS. The time courses are given in Supplementary Figure S7. The numbers above bars are AMP/Ile-tRNA^{Ile} ratios. The enzymes were 2 µM, the tRNA^{Ile} 12 µM, and EF-Tu (the active GTP-form) was 8-12 µM.

Ile-tRNA^{Ile} misediting impairs cell growth

To investigate to what extent misediting of Ile-tRNA^{Ile} affects cell viability, we followed the growth of *E. coli* BL21(DE3) strain transformed with the plasmids encoding WT IleRS or its Ile-tRNA^{Ile} misediting active variants (H333A and T246A/H333A). A moderate expression (**Supplementary Figure S8**) of the WT enzyme did not show any growth defects demonstrating that expression *per se* is not a significant burden for the cell (**Figure 4**). Interestingly, the H333A mutant did not significantly influence the growth rate suggesting that Ile-tRNA^{Ile} misediting of 1 s^{-1} could be physiologically tolerated. In contrast, the T246A/H333A mutant (hydrolytic rate of 14 s^{-1}) showed a noticeable growth defect (**Figure 4**), in agreement with the negative selection against this activity.

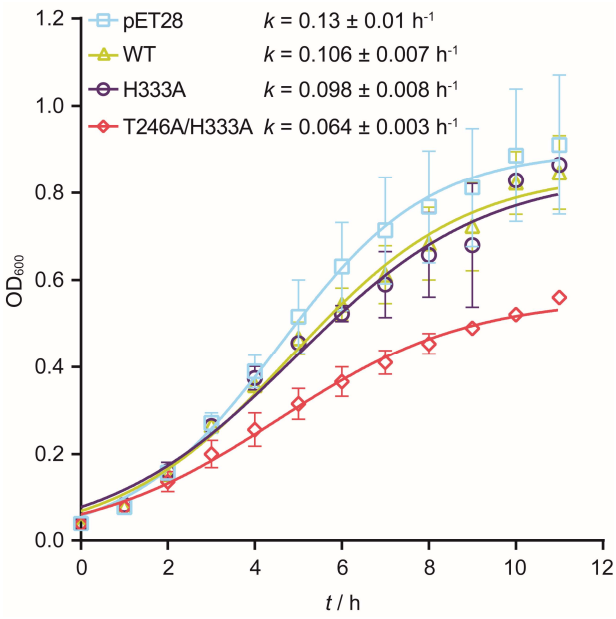

Figure 4. Growth of *E. coli* BL21(DE3) transformed with the pET28 plasmids carrying IleRS variants. The empty plasmid was used as a control. The growth was followed in M9 medium supplemented with 100 µM IPTG. The growth rate constant (*k*) was determined from the logistic growth model. In the absence of IPTG, the growth rate of *E. coli* BL21(DE3) transformed with the empty pET28 was 0.28 ± 0.01 h^{-1}. The basal level of Ile-tRNA^{Ile} synthesis by endogenous WT IleRS should not pose a problem as Ile-tRNA^{Ile} produced by any route is subjected to editing *in trans*.

IleRS is unique among class Ia AARSs in exhibiting a high level of editing *in trans*

To address whether IleRS edits *in trans* its biological threat Val-tRNA^{Ile}, we followed the accumulation of AMP and Val-tRNA^{Ile} by the WT enzyme as described above (**Supplementary Figure S9**). In the absence of EF-Tu, the analysis returned the AMP/Val-tRNA^{Ile} ratio of 1330 and a minor accumulation of Val-tRNA^{Ile} (**Figure 5**), both in agreement with the efficient Val-tRNA^{Ile} editing (30). The addition of EF-Tu increased the accumulation of Val-tRNA^{Ile} by 10-fold and decreased the amount of consumed ATP by more than 3-fold, leading to a significant drop (58-fold) in AMP/Val-tRNA^{Ile} ratio (23 *vs* 1330). This indicates that IleRS edits Val-tRNA^{Ile} *in trans*, providing to the best of our knowledge the first demonstration of editing *in trans* for a class I AARS. In our experimental setup, EF-Tu efficiently competes with IleRS for binding to Val-tRNA^{Ile}. Although this is an *in vitro* observation that may not hold *in vivo* with other AA-tRNAs competing for EF-Tu, our data suggest that editing *in trans* is more error-prone than editing *in cis* as EF-Tu may efficiently bind misaminoacylated tRNAs (14, 31, 32).

Next, we tested whether LeuRS and ValRS also use editing *in trans* with their main biological threats Nva and Thr, respectively (5, 33). Both LeuRS and ValRS consumed a highly non-stoichiometric amount of ATP per accumulated misaminoacylated tRNA (**Figure 5, Supplementary Figure S9**), in agreement with established editing of Nva-tRNA^{Leu} and Thr-tRNA^{Val} (6, 26, 33). The addition of EF-Tu dropped the ATP/ Thr-tRNA^{Val} ratio by less than 2-fold (13 800 *vs* 8600). The lack of EF-Tu effect indicates that ValRS edits Thr-tRNA^{Val} *in cis*. The picture is more complicated for LeuRS, where the presence of EF-Tu promotes a 12-fold drop in ATP/AA-tRNA ratio (14 500 *vs* 1250) that may indicate the participation of editing *in trans*. However, the drop does not stem from a decrease in the ATP consumption, which is only 1.04-fold lower in the presence of EF-Tu. Thus, the cycles of Nva-tRNA^{Leu} hydrolysis and the subsequent tRNA^{Leu} misaminoacylation which consumes ATP are not influenced by EF-Tu. This strongly

suggests that LeuRS mainly operates *in cis* in agreement with the previous data (14). The observed EF-Tu-dependent accumulation of AA-tRNA^{Leu} is puzzling and likely originates from trace contaminations of Leu in the Nva sample (**Supplementary Figure S10**). The rationale is that in the absence of EF-Tu, the accumulation of Leu-tRNA^{Leu} can be prevented by rebinding to LeuRS and misediting, analogously to Nva-tRNA^{Leu}. Yet, hydrolysis of Leu-tRNA^{Leu} is 3×10^3-times slower than Nva-tRNA^{Leu} (26), contributing minimally to the ATP consumption. Once present, EF-Tu may bind Leu-tRNA^{Leu} and affects its accumulation but without a noticeable effect on ATP consumption. To conclude, our data show that IleRS is distinct from closely related LeuRS and ValRS in a fraction of post-transfer editing that operates *in trans*.

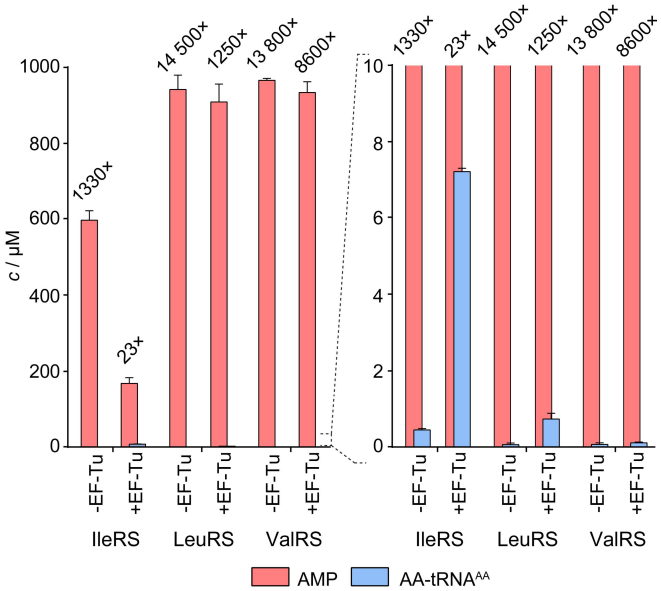

Figure 5. AMP (pink) and misaminoacylated tRNA (blue) concentrations at the 5-minute time point for IleRS, LeuRS and ValRS. The time courses are given in Supplementary Figure S9. The numbers above bars are AMP/Ile-tRNA^{Ile} ratios. The enzymes were 2 µM, the tRNAs 10 µM, and EF-Tu (the active GTP-form) was 8-12 µM.

Discussion

Class I AARS synthetic and editing sites act as mirror images

AARSs are textbook examples of how high selectivity emerged under strong evolutionary pressure to evade deleterious errors (34). Their synthetic sites adopt numerous strategies to enforce recognition of the cognate and rejection of the non-cognate amino acids (35–38). If non-cognate amino acid, however, gets coupled to the tRNA, post-transfer editing resolves the problem. The editing site evolved to clear amino acids that jeopardize the accuracy of translation arguing that amino acids well discriminated at the synthetic site (large discrimination factor, *D*) will be generally poorly edited. But is it so? We have recently shown that Abu- and F_nAbu-tRNA^{Ile} (Abu and F_nAbu are both well discriminated at the synthetic site) were edited by IleRS with the same rates as biological threats Nva- or Val-tRNA^{Ile} (19). Abu is a primordial amino acid (39) that might have participated in the early translation and its editing may represent a relic from a primordial time. Synthetic F_nAbu mimic well the size and hydrophobicity of Val (40). Yet, another explanation could be that the editing site is non-selective except for the cognate amino acid. To test this hypothesis, we used IleRS as a model enzyme and a series of amino acids of distinct physicochemical features.

As anticipated, all tested non-cognate amino acids (polar, small hydrophobic, isosteric to Ile but with longer and differently branched side chains) were well rejected during the activation step (*D* above

3300) mainly due to an increase in their K_M values (**Table 1**). Despite that, we produced tRNAs misaminoacylated with all these amino acids and examined their hydrolysis. Surprisingly, all misaminoacylated tRNAs were edited with the same rates as Nva- and Val-tRNA^{Ile} (**Figure 2**). Thus, recognition at the editing site is not determined by how well the non-cognate amino acid is discriminated at the synthetic site. In fact, the editing site appears non-selective (except for the cognate AA-tRNA) and hydrolyses tRNAs misaminoacylated with amino acids spanning a broad range of physicochemical properties. How is this possible? The substrate recognition and catalysis at the editing site strongly depend on the common parts of all AA-tRNAs; the terminal adenosine and α-NH_3^+ group of the amino acid attached to the tRNA anchor the substrates (28) while the 2'OH or 3'OH of the A76 acts as a general base and promotes catalysis (16, 30, 41, 42). Changes of the terminal adenosine (43), lack of 2'OH or 3'OH (16, 30, 41, 42) or loss of the α-NH_3^+ anchoring interactions deprived editing (26, 44). In contrast, the synthetic site, acting as the first sieve, recognizes standalone amino acid and uses most of its side chain to minimize the error and ATP consumption (editing) (34). Thus, the synthetic and editing sites act as mirror images; while the former is highly selective to prevent errors, the latter exhibits low selectivity to clear each non-cognate amino acid that comes loaded to the tRNA.

Finding that class II PheRS, which recognizes the functional group of Tyr at the editing site (45) also edits Ile-tRNA^{Phe} (46), indicate that broad selectivity is not confined only to the class I editing domain. Further, D-aminoacyl-tRNA deacylase, which bears a structural resemblance to the archaeal class II threonyl-tRNA synthetase editing domain (47), edits all D-amino acids at similar rates while efficiently rejecting L-amino acids. (48). In contrast, the editing domain (INS) of class II prolyl-tRNA synthetase (ProRS), as well as the free-standing bacterial ProRS INS domain homologs, have well-defined non-cognate amino acid specificity (49). Interestingly, the free-standing *trans*-editing proteins also evolved a broad specificity – in this case regarding the tRNA substrate (50). Thus, across the editing systems, a similar concept emerged independently arguing for the benefits of broad substrates acceptance in the design of the efficient error correction mechanisms.

Negative catalysis ensures high specificity and broad selectivity of class I editing domain

Enzymes significantly differ in their physiological requirements for high selectivity (34). In some cases, low selectivity is beneficial allowing a broad substrate scope as in cytochrome P450 (51). The same applies to the editing domain. Yet, a unique feature of the editing domain, in which it mirrors highly selective enzymes (52), is its exquisite specificity *in rejection* of the cognate AA-tRNA. In general, selectivity may evolve by positive and negative selection (21). While the former is a consequence of a selection for the enzyme's high catalytic efficiency towards the cognate substrate, the latter is an explicitly evolved trait against a particular non-cognate substrate to avoid deleterious errors. Here we unveil that specificity of the editing domain evolved through negative selection against the cognate AA-tRNA, established as we and others have previously shown, not by mitigating the binding, but by diminishing the catalysis (14, 16–18). The destabilisation of the transition state solely for cognate AA-tRNA hydrolysis can be viewed as an example of negative catalysis (20). This concept was introduced to explain that alongside promoting a wanted reaction by lowering the energy of the transition state for the desired product (positive catalysis), enzymes may also increase the energy barrier of the competing transition state preventing the side reaction (negative catalysis). Herein, we broaden this concept to compare transition states for the competing substrates. Thus, the residue conferring negative catalysis should not influence the rate of native (wanted) reaction but should diminish the reaction rate with the prohibited substrate. Visualisation of our data by activity-specificity graph

revealed that the His333 and Thr246 IleRS residues confer negative catalysis (**Figure 6**). Their substitutions do not influence editing of Val-tRNA^{Ile} (k_{mut}/k_{WT} for editing is close to one) but promote misediting of Ile-tRNA^{Ile} resulting in the variants with decreased specificity (drop in the ratio of the k_{mut}/k_{WT} values for editing over misediting). In contrast, D342A mutation in IleRS promoted a decrease in both activity and specificity conferring the positive role for the Asp342 residue in catalysis (promoting both wanted and unwanted reaction by anchoring α- NH_3 of the cognate and non-cognate amino acid (28, 30)). Similarly, LeuRS Thr252, which imposes unproductive positioning of Leu-tRNA^{Leu} (16), confers negative catalysis while the Asp345 residue (26), analogous to IleRS Asp342, promotes positive catalysis. In conclusion, negative selection/catalysis appears as a powerful mechanism to ensure low selectivity of the editing domain while keeping, at the same time, exquisite specificity in the rejection of the cognate AA-tRNA. The former was driven by relying on the common parts of all AA-tRNAs and the latter by the evolution of a specific kinetic rejection mechanism based on the cognate amino acid side chain (16–18).

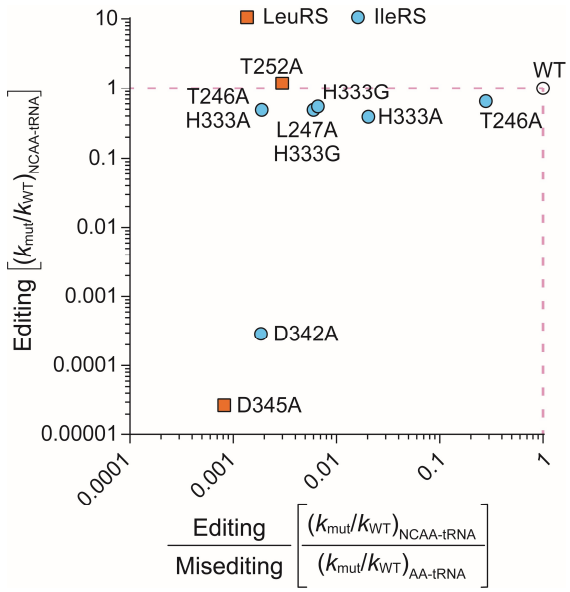

Figure 6. Activity-specificity relationship for editing of IleRS and LeuRS mutants. Activity (editing) was calculated as the ratio of the rate constants for hydrolysis of misaminoacylated tRNAs (NCAA-tRNA) by mutant AARS and WT, respectively. Specificity was calculated as the ratio of editing (wanted) over misediting (unwanted), where the misediting is the ratio of the rate constants for hydrolysis of cognate AA-tRNAs by mutant AARS and WT, respectively.

IleRS – class Ia enzyme with unique editing features

In class I AARSs delivery of the amino acid from the synthetic to the editing site occurs through the fast translocation (estimated as faster than 80 s^{-1} in LeuRS (26)) of the 3'-end of the (mis)aminoacylated tRNA (53). The fast translocation suggests that the 3'-end reaches the editing site on a shorter time scale relative to the dissociation of the (mis)aminoacylated tRNA. If so, editing occurs *in cis* without the release of misaminoacylated tRNA. Release and rebinding of the misaminoacylated tRNAs to AARS was proposed so far only for class II AARS (14), which also may use standalone trans-editing domains (54). It, therefore, came as a surprise that IleRS edits Val-tRNA^{Ile} with a significant contribution of the *in trans* pathway. At the same time, both LeuRS and ValRS predominantly operate *in cis* (**Figure 5**). Thus, IleRS appears unique among closely related class Ia editing AARSs (ILVRS) in post-transfer editing. This finding recalls that IleRS is also unique in pre-transfer editing. Indeed, in *E. coli* only IleRS, but not ValRS and LeuRS, showed substantial tRNA-dependent pre-transfer editing that

comprises about 30 % of total editing (11, 26, 55). Editing *in trans* is expected to be of lower proficiency than editing *in cis*, because EF-Tu may bind misaminoacylated tRNAs (31, 32) and redirect them to ribosomal translation. That said, IleRS capacity to edit errors prior misaminoacylation (pre-transfer editing) may provide a clear advantage. Although to assign the first activity is the chicken and the egg problem – editing *in trans* which promoted the evolution of tRNA-dependent pre-transfer editing in the IleRS synthetic site or IleRS ancient tRNA-dependent pre-transfer editing which allowed for a higher contribution of editing *in trans* – it seems that these two activities are related in IleRS making this enzyme unique and valuable for studying principles of communication between the synthetic and editing sites.

Materials and methods

For the detailed description of production and purification of enzymes (11, 19, 26, 31) and tRNA substrates (11, 19, 26) see SI Appendix. The kinetic characterisation was performed according to (22). Activation of amino acids was followed by a standard ATP-PP_i exchange assay using 50-100 nM enzymes and 0.1 to 10 × K_M amino acids. For the two-step aminoacylation, radiolabelled [α-^{32}P]tRNA (56) was used, and the formation of the AA-[α-^{32}P]tRNA was followed under different conditions (for details see SI Appendix). AA-[α-^{32}P]$tRNA^{Ile}$ substrate for the single-turnover analysis was pre-formed by incubating the 5 µM IleRS T243R/D342A IleRS with 25 µM $tRNA^{Ile}$ and particular amino acid. AA-[α-^{32}P]$tRNA^{Ile}$ was extracted by phenol/chloroform. Single-turnover hydrolysis was followed by mixing the limiting amount AA-[α-^{32}P]$tRNA^{Ile}$ and IleRS variants using a rapid chemical quench instrument. Parallel formation of [α-^{32}P]AMP and AA-[α-^{32}P]tRNA was measured in two reaction mixtures each contained 2 µM IleRS, 10-12 µM $tRNA^{Ile}$, 1 mM ATP, either [α-^{32}P]ATP or [α-^{32}P]tRNA, and one of the following: 2 mM Ile, 20 mM Val and 30 mM Nva or Thr. EF-Tu (the active GTP-bound form) was 8-12 µM. For the more detailed description of the kinetic assays see SI Appendix. The effect of IleRS misediting on the growth of *E. coli* was monitored using BL21(DE3) strains transformed with pET28b carrying the genes for IleRS H333A and T246A/H333A. The OD_{600} was measured 11 hours after inducing the moderate protein overexpression by 100 µM IPTG. For more details see SI Appendix.

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Author contributions

I.Z. and I.G.-S. designed research; I.Z. and K.I. performed research; I.Z., K.I. and I.G.-S. analysed data, N.C. performed preliminary research; I.Z., N.C. and I.G.-S. wrote the manuscript, I.G.-S. conceived and conducted the project.

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