The tRNA A76 Hydroxyl Groups Control Partitioning of the tRNA-dependent Pre- and Post-transfer Editing Pathways in Class I tRNA Synthetase*

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Background: Isoleucyl-tRNA synthetase uses cognate tRNA to stimulate hydrolysis of non-cognate aminoacyl-adenylates within the synthetic site.

Results: The 3'-terminal hydroxyl groups of tRNAIle have no role in pre-transfer editing.

Conclusion: The tRNAIle body, rather than the 3'-end of tRNAIle alone, promotes assembly of the improved ribonuclear protein synthetic site.

Significance: Isoleucyl-tRNA synthetase acts as a ribonuclear protein to adjust amino acid recognition to the cellular environment.

Aminoacyl-tRNA synthetases catalyze ATP-dependent covalent coupling of cognate amino acids and tRNAs for ribosomal protein synthesis. Escherichia coli isoleucyl-tRNA synthetase (IleRS) exploits both the tRNA-dependent pre- and post-transfer editing pathways to minimize errors in translation. However, the molecular mechanisms by which tRNAIle organizes the synthetic site to enhance pre-transfer editing, an idiosyncratic feature of IleRS, remains elusive. Here we show that tRNAIle affects both the synthetic and editing reactions localized within the IleRS synthetic site. In a complex with cognate tRNA, IleRS exhibits a 10-fold faster aminoacyl-AMP hydrolysis and a 10-fold drop in amino acid affinity relative to the free enzyme. Remarkably, the specificity against non-cognate valine was not improved by the presence of tRNA in either of these processes. Instead, amino acid specificity is determined by the protein component per se, whereas the tRNA promotes catalytic performance of the synthetic site, bringing about less error-prone and kinetically optimized isoleucyl-tRNA synthesis under cellular conditions. Finally, the extent to which tRNAIle modulates activation and pre-transfer editing is independent of the intactness of its 3'-end. This finding decouples aminoacylation and pre-transfer editing within the IleRS synthetic site and further demonstrates that the A76 hydroxyl groups participate in post-transfer editing only. The data are consistent with a model wherein the 3'-end of the tRNA remains free to sample different positions within the IleRS-tRNA complex, whereas the fine-tuning of the synthetic site is attained via conformational rearrangement of the enzyme through the interactions with the remaining parts of the tRNA body.

Fidelity of translation depends on accurate matching of cognate amino acid-tRNA pairs for ribosomal protein synthesis. The major players, aminoacyl-tRNA synthetases (aaRSs), catalyze covalent coupling of cognate amino acids with the cognate tRNAs in a two-step reaction of aminoacylation (1). The first step comprises amino acid activation, whereby the amino acid is condensed with ATP to form an aminoacyl-adenylate (aa-AMP) intermediate and pyrophosphate (PP). In the second step, the aminoacyl moiety is transferred to the 3'-end of the cognate tRNA with the release of AMP (Fig. 1). This step proceeds by nucleophilic attack of either the 2'-OH or 3'-OH group of the terminal ribose of the tRNA substrate on the carboxyl carbon atom of the aminoacyl-AMP intermediate. Both steps are catalyzed within the same synthetic site located at the aaRS catalytic core. aaRSs are divided into two classes, class I and class II, based on different topology of the synthetic sites and the characteristic sequence motifs (2, 3).

Arginyl-, glutaminyl-, glutamyl-, and class I lysyl-tRNA synthetases are unable to activate amino acids in the absence of tRNA. In these enzymes, a conformational change induced by tRNA binding is essential for the productive juxtaposition of the ATP- and amino acid-reactive moieties. Other aaRSs from both classes may activate the amino acid substrate independently of the presence of tRNA, yet the activation step in these enzymes may be modulated by tRNA (4–8). It thus appears that many aaRSs act as ribonuclear proteins regardless of whether the tRNA portion of the ribonuclear protein is required for amino acid activation.

Some aaRSs are unable to establish a high level of specificity against structurally similar amino acids in the synthetic reaction alone. For these enzymes, threshold levels of aminoacylation fidelity, set up by the overall accuracy of protein synthesis (1 in 10²) (9), are ensured by the subsequent proofreading. AaRSs challenged with the less discriminative synthetic sites evolved inherent hydrolytic editing (1, 10) to hydrolyze non-cognate amino acids.
How tRNA stimulates pre-transfer editing within the IleRS synthetic site and whether this activity is intimately linked with tRNA aminoacylation remain elusive. An early observation that isoleucine and tRNA binding to IleRS are interdependent (29) raised the possibility that tRNA is a more versatile modulator of IleRS amino acid recognition than anticipated. With this in mind, we performed comprehensive steady-state and single-turnover kinetic analyses to establish the role for tRNA in amino acid recognition and editing by IleRS. Our data show that tRNA\(^{\text{Ile}}\) stimulates aminoacyl-AMP hydrolysis and concomitantly decreases IleRS affinity toward amino acids. Neither of these activities depends on the capacity of the tRNA\(^{\text{Ile}}\) to accept amino acid. We further demonstrate that the tRNA cofactor does not facilitate discrimination between isoleucine and valine in either of these reactions. Despite these findings, the IleRS\(\text{tRNA}^{\text{Ile}}\) complex is probably a superior catalyst under cellular conditions compared with IleRS alone because (i) its affinity for amino acids is better adjusted to the cellular environment, and (ii) only in the case of valine is the tRNA-dependent rate of hydrolysis comparable with the rate of the aminoacyl transfer step, and thus contributes to proofreading. The separation of the pre- and post-transfer editing pathways by various tRNA\(^{\text{Ile}}\) analogues clearly demonstrated that tRNA participates in IleRS proofreading by two distinct mechanisms; (i) the intact 3′-end of tRNA permits post-transfer editing by supporting the misaminoacylation/deacacylation pathway, whereas (ii) the remaining portions of the tRNA body promote a conformational change that stimulates pre-transfer editing within the IleRS synthetic site.

**Experimental Procedures**

**Production and Purification of IleRS and tRNA\(^{\text{Ile}}\)—IleRS enzymes (wild type (WT) and deacetylation-defective variant (D342A)) were overexpressed and purified by standard nickel-nitrilotriacetic acid chromatography as described (12). tRNA\(^{\text{Ile}}\) (with G\(^1\)C\(^7\) instead of the native A\(^1\)U\(^7\) sequence) was overexpressed in *Escherichia coli* and purified as described (12). Plateau aminoacylation using \(^{14}\text{C}\)isoleucine established that the sample contains \(\sim 70\%\) functional tRNA\(^{\text{Ile}}\). Substitution of the first tRNA\(^{\text{Ile}}\) base pair was used to enhance transcription by T7 RNA polymerase, because it was shown not to affect isoleucylation parameters (30). The kinetic competence of G\(^1\)C\(^7\) tRNA\(^{\text{Ile}}\) in the synthetic and editing pathways was confirmed by testing the native tRNA\(^{\text{Ile}}\) isoacceptor isolated from the bulk *E. coli* tRNA by complementary biotinylated oligonucleotides (sequence of the oligonucleotide: 5′-ATCAGGGGT-GGCCTCTAAACCACTGAG-3′-biotin). No significant difference in the rate of single-turnover aminoacyl transfer step or in the steady-state rate of aminoacylation was observed relative to the G\(^1\)C\(^7\) tRNA\(^{\text{Ile}}\)\(_{\text{GUA}}\). Plateau aminoacylation using \(^{14}\text{C}\)isoleucine established that the native tRNA\(^{\text{Ile}}\)\(_{\text{GUA}}\) sample contains \(\sim 85\%\) functional tRNA\(^{\text{Ile}}\).  

**tRNA\(^{\text{Ile}}\) 3′-End Modification—Oxidized tRNA\(^{\text{Ile}}\) (tRNA\(^{\text{Ile}}\)\(_{\text{ox}}\)) was produced by incubating 3 mM tRNA\(^{\text{Ile}}\) in 55 mM NaOAc, pH 4.5, and 6 mM NaIO\(_4\) for 2 h at room temperature in the dark. The excess NaIO\(_4\) was consumed with 300 mM ethylene glycol (incubation for 30 min at room temperature). tRNA\(^{\text{Ile}}\)\(_{\text{ox}}\) was ethylene-precipitated, dialyzed against 5–10 mM HEPES, pH 7.5,
and stored at −20 °C. Both 2′dtRNA\textsubscript{Ile} (2′-OH group of A76-tRNA\textsubscript{Ile} replaced by a hydrogen atom) and 3′dtRNA\textsubscript{Ile} (3′-OH group of A76-tRNA\textsubscript{Ile} replaced by a hydrogen atom) were produced using tRNA nucleotidylyltransferase (31). Preparative A76 exchange reactions were performed by incubating 75 μM tRNA\textsubscript{Ile} with 25 μM tRNA nucleotidylyltransferase and 6 mM nucleotide (2′dATP or 3′dATP, titrated to pH 7.0) in 200 mM Tris, pH 8.0, 1 mM NaPP\textsubscript{2}, 20 mM MgCl\textsubscript{2}, and 0.5 mM DTT for 4 h at 37 °C. tRNA was extracted by phenol-chloroform, ethanol-precipitated, and dialyzed against 5–10 mM HEPES, pH 7.5. Residual tRNA\textsubscript{Ile} present in 2′dtRNA\textsubscript{Ile} or 3′dtRNA\textsubscript{Ile} samples (~10%) was destroyed by oxidation with NaIO\textsubscript{4}, as described above. The control A76-tRNA\textsubscript{Ile} was prepared by a preparative exchange reaction in the presence of 6 mM ATP, to test whether the exchange procedure affects the editing or aminoaclonylation activity. We noticed that the exchanged tRNA is prone to instability, aggregation, and precipitation if dissolved in pure water and kept at −20 °C. Therefore, it was stored in 5–10 mM HEPES at pH 7.5, and all kinetic assays contained 150 mM NH\textsubscript{4}Cl to prevent aggregation.

**Preparation and Purification of Aminoaclylated or Misaminoacylated tRNA**—Aminoaclylated and misaminoacylated tRNA\textsubscript{Ile} variants (Ile-tRNA\textsubscript{Ile}, Val-tRNA\textsubscript{Ile}, Val-3′dtRNA\textsubscript{Ile}, and Ile-3′dtRNA\textsubscript{Ile}) were prepared by incubating 30 μM tRNA\textsubscript{Ile} (or 3′dtRNA\textsubscript{Ile}) with 10 μM D342A IleRS, 30 mM valve, or 10 μM isoleucine, 4 mM ATP, and 0.004 units/μl inorganic pyrophosphatase (IPPase) for 30 min at 37 °C, in the standard IleRS reaction buffer consisting of 75 mM HEPES, pH 7.5, 20 mM MgCl\textsubscript{2}, 150 mM NH\textsubscript{4}Cl, 10 μg/ml BSA, and 5 mM DTT. The reactions were stopped by adding 30 mM NaOAc, pH 4.5, and tRNA was extracted by acidified phenol, ethanol-precipitated, and dialyzed against 10–20 mM NH\textsubscript{4}OAc, pH 5.0. Aminoacylated tRNAs were concentrated up to 50 mg/ml and further purified using reverse phase chromatography on Vyday C4 column (4.6 × 250 mm; pore diameter, 30 Å; particle diameter, 5 μm) to remove residual non-aminocyolated tRNA and nucleotides. The column was equilibrated with buffer B (20 mM NH\textsubscript{4}OAc, pH 5.0, 10 mM Mg(OAc)\textsubscript{2}, 400 mM NaCl), and the retained molecules were eluted with the programmed linear gradients of buffer B (buffer A with 30% (v/v) ethanol). Fractions containing aminoacylated-tRNAs were pooled, ethanol-purified, and dialyzed against 1–2 mM NaOAc, pH 5.0.

**Preparation of Radiolabeled tRNA**—[\textsuperscript{32}P]tRNA\textsubscript{Ile} or 3′d[\textsuperscript{32}P]tRNA\textsubscript{Ile} was prepared using tRNA nucleotidylyltransferase (12, 31) in the presence of [\textsuperscript{α-32}P]ATP and tRNA\textsubscript{Ile} or 3′d[\textsuperscript{α-32}P]ATP and 3′dtRNA\textsubscript{Ile}, respectively. Because radiolabeling decreases tRNA\textsubscript{Ile} plateau in aminoaclonylation, the measured aminoaclonylation steady-state rate constants are corrected by a factor that takes into account the proportion of functional [\textsuperscript{32}P]tRNA\textsubscript{Ile}. Aminoaclylated or misaminoacylated [\textsuperscript{32}P]tRNA\textsubscript{Ile} was prepared by mixing approximately 300 nM [\textsuperscript{32}P]tRNA\textsubscript{Ile} with 1 μM D342A IleRS, 4 mM ATP, 0.004 units/μl IPPase, and 2 mM valve or isoleucine in the standard IleRS reaction buffer (see above). tRNA was extracted by acidified phenol and subjected to two subsequent gel filtration steps (Micro Bio-Spin P30 columns) and dialyzed against 10 mM NaOAc, pH 5.0. Aminoaclylated and misaminoacylated [\textsuperscript{32}P]tRNAs were inactive in decylation and therefore subjected to renaturation prior to use by heating to 80 °C, adding MgCl\textsubscript{2} to 10 mM final concentration, and slow cooling to ambient temperature. Acidic conditions (5 mM NaoAc, pH 5.0) were preserved during the renaturation period to ensure stability of the ester bond. The percentage of aminoacylated tRNA did not change after renaturation.

**ATP-PP\textsubscript{2}, Exchange Assay**—The ATP-PP\textsubscript{2} exchange assay was performed at 37 °C in the standard IleRS reaction buffer (see above) supplemented with 100 μg/ml BSA for enzyme stabilization, 4 mM ATP, and 1 mM [\textsuperscript{32}P]PP\textsubscript{2}. Amino acid concentration was varied over the range of 0.1–10 times the K\textsubscript{m} value, and 25 mM WT or D342A IleRS was used. To assess the role of tRNA in amino acid recognition, ATP/PP\textsubscript{2}, exchange reactions were performed in the presence of 15–20 μM non-aminocyolated tRNA variants (tRNA\textsubscript{ox}, 2′dtRNA\textsubscript{ox}, and Val-tRNA\textsubscript{Ile}). When the capacity of Val-tRNA\textsubscript{Ile} to modulate amino acid activation was tested, the assay was performed in the presence of decylation-defective D342A IleRS.

**Aminoaclyl-Adenylate Synthesis Assay**—The reactions were conducted as described previously (8, 12). Briefly, 25 nM WT IleRS was used at 37 °C in the standard IleRS reaction buffer supplemented with 100 μg/ml BSA in the presence of 0.5 mM [\textsuperscript{α-32}P]ATP (0.01–1.0 mM) and 0.004 units/μl IPPase with (tRNA-dependent editing) or without (tRNA-independent editing) 15–20 μM tRNA\textsubscript{Ile} or tRNA\textsubscript{Ile} analogues. The reactions were started by the addition of amino acids and quenched by mixing 1.5 μl of the reaction with 3 μl of 1.5 M formic acid. aa-[\textsuperscript{32}P]AMP, [\textsuperscript{32}P]AMP, and [\textsuperscript{32}P]ATP were separated by thin layer chromatography and independently quantitated as described (8, 12, 26). In the presence of aminoaclylated or misacyolated tRNAs, AMP formation was followed using the D342A IleRS.

**Determination of AMP/Aminoacyl-tRNA\textsuperscript{Ile} Ratios**—[\textsuperscript{32}P]AMP and aminoacyl-[\textsuperscript{32}P]tRNA\textsubscript{Ile} formations were followed in parallel steady-state assays (26, 28). The reactions were performed at 37 °C in the standard IleRS buffer supplemented with 100 μg/ml BSA and 0.004 units/μl IPPase, using either 40 nM WT or D342A IleRS, 100 μM ATP, 15 μM tRNA\textsubscript{Ile} variant, and 10 μM Ile or 30 mM valve. AMP formation was followed with trace quantities of [\textsuperscript{α-32}P]ATP added to the reaction mixtures, whereas aminoacyl-tRNA\textsubscript{Ile} formation was followed via trace quantities of [\textsuperscript{32}P]tRNA\textsubscript{Ile} or 3′d[\textsuperscript{32}P]tRNA\textsubscript{Ile}. The ratios in the presence of tRNA\textsubscript{Ile} and the WT or D342A IleRS were lower than previously published (28) because of a modification in the experimental conditions. We have shown that the presence of 150 mM NH\textsubscript{4}Cl (used in this work), slightly impedes the k\textsubscript{obs} for AMP production, thereby lowering the AMP/aminoaclyl-tRNA\textsubscript{Ile} ratio.

**Single-turnover Transfer Step**—The IleRS-aa-AMP complexes were preformed in situ by incubation of 20–30 μM WT or D342A IleRS with 5 mM isoleucine or 50 mM valve in the standard IleRS reaction buffer supplemented with 8 mM ATP and 0.008 units/μl IPPase for 5 min at 37 °C, similarly as described (12, 26). The single-turnover transfer reactions were then performed with the KinTek RQF-3 instrument by mixing equal volumes of 20–30 μM IleRS-aa-AMP with trace amounts of [\textsuperscript{32}P]tRNA\textsubscript{Ile} or 3′d[\textsuperscript{32}P]tRNA\textsubscript{Ile}. Reactions were quenched with NaOAc (pH 4.5, final concentration 0.8 M), and the collection tubes contained SDS (final 0.1% (v/v)).
tRNA Modulates Amino Acid Recognition by IleRS

TABLE 1
Kinetic parameters for AMP formation by IleRS in the presence of tRNA\textsuperscript{Ile} variants

| tRNA\textsuperscript{Ile} | \(k_{\text{cat}}\) \text{min} \textsuperscript{\text{-1}} | \(k_{\text{cat}}/K_{\text{m}}\) \text{min} \textsuperscript{-1} \text{mM} \text{-1} | \(k_{\text{chem}}\) \text{min} \textsuperscript{-1} | \(k_{cat}/K_{m}\) \text{min} \textsuperscript{-1} \text{mM} \text{-1} |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Ile\textsuperscript{a} | Ile\textsuperscript{a} | Ile\textsuperscript{a} | Ile\textsuperscript{a} | Ile\textsuperscript{a} |
| No tRNA\textsuperscript{b} | ND\textsuperscript{c} | 0.0023 ± 0.0002 | 5.9 | 0.0262 ± 0.0006 |
| tRNA\textsuperscript{Ile} | ND\textsuperscript{c} | 0.0135 ± 0.0004 | 9.8 |
| 2 dtRNA\textsuperscript{Ile} | ND\textsuperscript{c} | 0.070 ± 0.0044 |

\textsuperscript{a} In the presence of 10 mM Ile.
\textsuperscript{b} IleRS was used at 2 \muM concentration.
\textsuperscript{c} ND, not determined due to insufficient activity.

Single-turnover Aminoacylation—Single-turnover aminoacylation was performed with the KinTek RQF-3 instrument by mixing equal volumes of 20 \muM D342A IleRS, trace amounts of \[^{32}\text{P}\]tRNA\textsuperscript{Ile}, 8 mM ATP, and 0.008 units/min IleRS supplemented with an additional 75 mM HEPES, pH 7.5 (in total 50 mM). IleRS and 1 mM isoleucine were pre-incubated with 50% amplitude. The origin of the slow phase is unknown (32). Various mixing modes showed no influence on the extracted rate constants. We fitted the observed rate constants to the hyperbolic binding equation, \(Y = S_0 \times k_{\text{chem}}/(S_0 + K_p) + c\), where \(S_0\) represents the initial amino acid concentration, \(k_{\text{chem}}\) represents the maximal aminoacylation rate, and \(c\) is the intercept (32). Various mixing modes showed no influence on the extracted rate constants.

Single-turnover Deacylation—Single-turnover deacylation reactions were performed with the KinTek RQF-3 instrument or by hand mixing (slow reactions with D342A IleRS or 3’dtRNA\textsuperscript{Ile}). 100–300 nmol reconstituted Val- or Ile-[\(^{32}\text{P}\)]tRNA\textsuperscript{Ile} in 5 mM NaOAc, pH 5.0, and 10 mM MgCl\textsubscript{2} was mixed with 30 \muM WT IleRS or D342A IleRS in the standard IleRS buffer supplemented with an additional 75 mM HEPES, pH 7.5 (in total 150 mM HEPES, pH 7.5). The reactions were quenched with 0.8 M NaOAc, pH 4.5 (final concentration), and the collection tubes contained SDS (final 0.1% (w/v)). Data were fitted to the single exponential equation, \(Y = Y_0 + A \times e^{-k_{\text{deacyl}} \times t}\), where \(Y_0\) represents the y intercept, \(A\) is the amplitude, \(k_{\text{deacyl}}\) represents the apparent deacylation rate constant, and \(t\) is time. We observed a biphasic Val-tRNA\textsuperscript{Ile} deacylation curve with the WT IleRS. The reported rate constant corresponds to the fast phase with 50% amplitude. The origin of the slow phase is unknown and has been previously reported (17, 19, 26).

Results

The Intact 3’-End Is Not Required for the Participation of tRNA in Pre-transfer Editing—IleRS exhibits inherent hydrolytic activity toward aminoacyl-AMP, which can be substantially modulated by the tRNA (Table 1) (12, 28). It has been proposed (18, 33) that only tRNAs active in aminoacylation are capable of stimulating IleRS editing. Our finding that post-transfer editing is the major editing pathway in IleRS (28) rationalizes earlier work, yet the question of whether the minor tRNA-dependent pre-transfer editing pathway also requires a tRNA cofactor active in aminoacylation still remains. To experimentally address this question, we performed detailed steady-state analysis of IleRS editing in the presence of various tRNA\textsuperscript{Ile} analogues (Table 2) using a highly sensitive editing assay (8) that employs [\(^{\alpha}\text{32}\text{P}\)]ATP and allows detection of aminoacyl-[\(^{32}\text{P}\)]AMP and its hydrolytic product [\(^{32}\text{P}\)]AMP after separation by thin layer chromatography. Participation of tRNA in IleRS pre-transfer editing was first examined with tRNA analogues that lack amino acid acceptor activity. Replacement of the 2’-OH group of tRNA\textsuperscript{Ile} by a hydrogen atom was accomplished by exchange of the terminal A76 with 2’-dATP using tRNA nucleotideyltransferase (34). As expected, the IleRS complex with 2 dtRNA\textsuperscript{Ile} loses aminoacylation capacity (Table 2), because IleRS utilizes the 2’-OH moiety as the initial site of aminoacylation (34–36). The tRNA\textsuperscript{Ile} fraction that had remained unexchanged and thus retained the wild-type activity (~10%) was inactivated by periodate oxidation (4). This yields 2’dtRNA\textsuperscript{Ile} ox which completely lacks isoleucine acceptor activity (Table 2). Next, we produced tRNA\textsuperscript{Ile} ox by periodate treatment of the intact tRNA\textsuperscript{Ile}. In this case, we also observed a concomitant loss of aminoacylation activity as expected due to oxidation of the vicinal diol of the terminal ribose of the tRNA sample (Table 2).

Remarkably, both 2’dtRNA\textsuperscript{Ile} ox and tRNA\textsuperscript{Ile} ox analogues stimulated AMP formation by IleRS in the presence of valine. Indeed, the \(k_{\text{cat}}\) values increased about 3–5-fold relative to the \(k_{\text{cat}}\) in the absence of tRNA (0.128 and 0.07 s\textsuperscript{-1} versus 0.027 s\textsuperscript{-1}; Table 1). An important implication of this finding is that participation of tRNA\textsuperscript{Ile} in pre-transfer editing is not coupled to its capacity to accept the amino acid as previously presumed (37) (see “Discussion”). Neither E. coli tRNA\textsuperscript{Val} nor tRNA\textsuperscript{Leu} is capable of stimulating IleRS pre-transfer editing (Table 3), whereas only weak stimulation of valyl-AMP hydrolysis was observed in the presence of a tRNA\textsuperscript{Ile} in vitro transcript (Table 3), known to be a very weak substrate in isoleucylation (30). Our results show that the operation of the tRNA-dependent pre-transfer editing pathway in IleRS requires interaction with the specific cognate and post-transcriptionally modified tRNA\textsuperscript{Ile} body but does not require intactness of the tRNA 3’-end.

TABLE 2
Steady-state and plateau aminoacylation of various tRNA\textsuperscript{Ile} substrates

| tRNA\textsuperscript{Ile} | Percentage of acceptor activity | \(k_{\text{cat}}\) |
|-----------------|-------------------------------|-----------------|
| 2’dtRNA\textsuperscript{Ile} ox | ND\textsuperscript{c} | ND\textsuperscript{c} |
| 2’dtRNA\textsuperscript{Ile} | ND\textsuperscript{c} | ND\textsuperscript{c} |
| 2 dtRNA\textsuperscript{Ile} | ND\textsuperscript{c} | ND\textsuperscript{c} |
| 3’dtRNA\textsuperscript{Ile} ox | ND\textsuperscript{c} | ND\textsuperscript{c} |
| 3 dtRNA\textsuperscript{Ile} | ND\textsuperscript{c} | ND\textsuperscript{c} |
| 3’dtRNA\textsuperscript{Ile} | ND\textsuperscript{c} | ND\textsuperscript{c} |

\textsuperscript{a} A76-tRNA\textsuperscript{Ile} refers to tRNA\textsuperscript{Ile} with the terminal adenosine exchanged with ATP.

\textsuperscript{b} ND, not determined due to insufficient activity.

\textsuperscript{c} A76-tRNA\textsuperscript{Ile} was produced to test whether the exchange procedure affects the tRNA aminoacylation activity.
The aforementioned tRNA\textsuperscript{ile} analogues also enhance AMP accumulation in the presence of cognate isoleucine (Table 1). The observed rate of AMP formation in the presence of the tRNA analogues is about 100-fold higher than the non-enzymatic hydrolysis of isoleucyl-AMP in solution (2.2 ± 0.2 × 10\textsuperscript{-4} s\textsuperscript{-1}) (13). Consistent with our recent findings (28), these data show that the IleRS synthetic site may exhibit weak tRNA-dependent hydrolysis of isoleucyl-AMP. In these experiments, the isoleucyl transfer step was blocked by utilization of the aminoacylation-inactive tRNA\textsuperscript{ile} analogues, thus allowing isolation and monitoring of the tRNA-dependent isoleucyl-AMP hydrolysis step. Kinetic analysis then reveals that tRNA stimulates cognate and non-cognate aminoacyl-AMP hydrolysis similarly by 5–10-fold (Table 1). It thus appears that tRNA does not contribute to IleRS specificity by preferentially promoting non-cognate aminoacyl-AMP hydrolysis. Nevertheless, only the non-cognate amino acid is essentially proofread in the presence of intact tRNA. This is so because even in the presence of tRNA, the rate of isoleucyl-AMP hydrolysis is 2 orders of magnitude slower than the rate of the isoleucyl transfer step. Thus, in the cognate system, aminoacyl transfer step is kinetically favored indicating full kinetic competence of this analogue in Ile-tRNA\textsuperscript{ile} synthesis. In accordance, transfer of the non-cognate valyl moiety to tRNA was also independent of the presence of the A76 3'-OH group (Fig. 2B). This supports the conclusion that the specificity of the aminoacyl transfer step in IleRS is not modulated by the 3'-OH group at the 3'-terminal ribose of its tRNA substrate.

Next, hydrolysis of misaminoacylated 3'dtRNA was followed under single-turnover conditions, and the deacylation rate constants ($k_{\text{deacyl}}$) were extracted from the exponential decay of aminoacyl-tRNA in time. IleRS displayed a 10\textsuperscript{6}-fold drop in the $k_{\text{deacyl}}$ for Val-3'dtRNA compared to Val-tRNA\textsuperscript{ile} (2.3 × 10\textsuperscript{-5} s\textsuperscript{-1} versus 64 s\textsuperscript{-1}; Table 4). Interestingly, the D342A substitution affects the rate of Val-tRNA\textsuperscript{ile} deacylation less than does the lack of the A76 3'-OH group (0.014 s\textsuperscript{-1} versus

### Table 3

AMP formation by WT IleRS in the presence of various tRNAs and valine

| tRNA          | $k_{\text{obs}}$ s\textsuperscript{-1} |
|---------------|---------------------------------------|
| No tRNA       | 0.011 ± 0.0006                         |
| 2'dtRNA\textsuperscript{ile} | 0.066 ± 0.002                         |
| In vitro tRNA\textsuperscript{ile} | 0.0275 ± 0.0006                       |
| In vitro tRNA\textsuperscript{Val} | 0.010 ± 0.002                         |
| In vitro tRNA\textsuperscript{Val} | 0.011 ± 0.001                         |

### Table 4

Single-turnover deacylation of Ile- or Val-tRNA\textsuperscript{ile} variants with WT or D342A IleRS

| Enzyme          | Val-3'dtRNA\textsuperscript{ile} | Val-tRNA\textsuperscript{ile} | Ile-tRNA\textsuperscript{ile} | $k_{\text{deacyl}}$ s\textsuperscript{-1} |
|-----------------|----------------------------------|--------------------------------|-------------------------------|----------------------------------------|
| No enzyme       | (3.0 ± 0.1) × 10\textsuperscript{-5} | (5.8 ± 0.3) × 10\textsuperscript{-5} | (5.7 ± 0.3) × 10\textsuperscript{-5} |                                         |
| WT              | (2.3 ± 0.8) × 10\textsuperscript{-5} | 64 ± 9                         | 0.058 ± 0.006                 |                                         |
| D342A           | 0.014 ± 0.001                     | 0.0083 ± 0.0009                |                               |                                         |

The aforementioned tRNA\textsuperscript{ile} analogues also enhance AMP accumulation in the presence of cognate isoleucine (Table 1). The observed rate of AMP formation in the presence of the tRNA analogues is about 100-fold higher than the non-enzymatic hydrolysis of isoleucyl-AMP in solution (2.2 ± 0.2 × 10\textsuperscript{-4} s\textsuperscript{-1}) (13).
tRNA Modulates Amino Acid Recognition by IleRS

Parallel formation of AMP and Val-tRNA\textsuperscript{le} or Val-3’dtRNA\textsubscript{ox} by WT and D342A IleRS (measured with 100 μM ATP)

WT or D342A IleRS was used at 0.04 μM concentration. Values represent the mean ± S.E. of three independent experiments.

| tRNA\textsuperscript{le} | k_{AMP} | k_{Val-tRNA} | k_{AMP}/k_{Val-tRNA} | k_{ed}\textsuperscript{a} | k_{ed}\textsuperscript{b} |
|------------------------|--------|-------------|----------------------|-----------------|-----------------|
| WT IleRS | 0.57 ± 0.03 | 0.066 ± 0.004 | 8.6\textsuperscript{b} | 0.054 | |
| 3’dtRNA\textsubscript{ox} | 0.14 ± 0.02 | 0.083 ± 0.006 | 1.7 | 0.057 | |
| 2’dtRNA\textsubscript{ox} | 0.068 ± 0.003 | 0.068 | |
| D342A IleRS | 0.35 ± 0.03 | 0.248 ± 0.009 | 1.4\textsuperscript{b} | 0.102 | |
| 3’dtRNA\textsubscript{ox} | 0.270 ± 0.008 | 0.120 ± 0.004 | 2.3 | 0.150 | |
| 2’dtRNA\textsubscript{ox} | 0.074 ± 0.003 | 0.074 | |

\textsuperscript{a}k_{ed} represents the rate constant for tRNA-dependent editing. It is defined as the difference between k_{AMP} and k_{Val-tRNA} (k_{ed} = k_{AMP} - k_{Val-tRNA}) (28).

\textsuperscript{b}The ratios are smaller than published (28) due to different experimental conditions (see "Experimental Procedures" for details).

134 s \textsuperscript{-1}; Table 4). This cements the notion that the A76 3’-OH group plays an essential role in IleRS post-transfer editing. As IleRS rapidly aminoacylates 3’dtRNA\textsuperscript{le} and cannot deacylate Val-3’dtRNA\textsuperscript{le}, the 3’dtRNA analogue allows isolation of pre-transfer editing. This is consistent with the previous findings (34) and provides a rationale for use of this analogue in analyzing the interdependency of aminoacylation and pre-transfer editing (see below).

IleRS displays weak hydrolytic activity toward the cognate Ile-tRNA\textsuperscript{le} product within the editing domain (0.058 s \textsuperscript{-1}; Table 4). This confirms that the CP1 domain exhibits high specificity against the cognate amino acid, as expected. This feature is shared with LeuRS (26) and ValRS (38).

The A76 2’-OH Group Does Not Organize the Pre-transfer Editing Subsite—The participation of the A76 2’-OH group in pre-transfer editing was tested by monitoring the initial rates of AMP and aminoacyl-tRNA formation in parallel in the presence of 3’dtRNA\textsuperscript{le}. The strength of this methodology, recently developed in our work on LeuRS (26) and IleRS (28), resides in its capacity to define the AMP fraction that originates particularly from editing. The initial rates of [\textsuperscript{32}P]AMP and Val-3’dtRNA\textsubscript{ox} formation were extracted from two parallel assays relying on either [\textsuperscript{32}P]ATP or 3’dtRNA\textsubscript{ox} tRNA, respectively. In the presence of wild-type IleRS and 3’dtRNA\textsubscript{ox}, the AMP formation (k_{AMP} = 0.14 s \textsuperscript{-1}; Table 5) results from both valyl-AMP hydrolysis (tRNA-dependent pre-transfer editing) and Val-3’dtRNA\textsuperscript{le} synthesis. Because Val-3’dtRNA\textsuperscript{le} is not deacylated by IleRS (Table 4), post-transfer editing does not contribute to AMP accumulation in this case. The independently determined rate of Val-3’dtRNA\textsuperscript{le} synthesis (k_{Val-tRNA} = 0.083 s \textsuperscript{-1}; Table 5) quantifies the fraction of total AMP that originates from aminoacylation. The rate constant associated with tRNA-dependent pre-transfer editing alone is then obtained by subtracting k_{Val-tRNA} from k_{AMP} (k_{ed} = 0.057 s \textsuperscript{-1}; Table 5). The calculated k_{ed} obtained in the presence of 3’dtRNA thus represents pre-transfer editing stimulated with aminoacylation-active tRNA. Conversely, when tRNA inactive in aminoacylation is used, such as 2’dtRNA\textsubscript{ox}, k_{ed} equals k_{AMP} (k_{AMP} = k_{ed} = 0.068 s \textsuperscript{-1}; Table 5) in value. Comparison of the k_{ed} values obtained with 3’dtRNA\textsubscript{ox} and 2’dtRNA\textsubscript{ox} reveals no significant difference (0.057 s \textsuperscript{-1} versus 0.068 s \textsuperscript{-1}, respectively). This clearly demonstrates that pre-transfer editing is not modulated by the terminal 2’-OH group of the tRNA cofactor. It further appears that the 2’-OH or 3’-OH group at the terminal ribose of the tRNA has little or no role in tRNA-dependent pre-transfer editing.

In the presence of IleRS and tRNA with the intact 3’-end, the calculated k_{ed} represents total pre- and post-transfer editing (0.504 s \textsuperscript{-1}; Table 5) and, as expected, significantly increases in value the k_{ed} that accounts for tRNA-dependent pre-transfer editing alone (0.057–0.068 s \textsuperscript{-1}; Table 5). For the wild-type reaction, k_{ed} is very similar to k_{AMP} in value (0.57 s \textsuperscript{-1}; Table 5), because only a small fraction of Val-tRNA\textsuperscript{le} evades proofreading and contributes to AMP formation unrelated to editing. To more closely examine the full competence of the 3’dtRNA\textsubscript{ox} analogue in stimulation of pre-transfer editing, the same set of experiments was performed with the post-transfer editing-deficient D342A IleRS. This variant exhibits a similar level of pre-transfer editing activity (compared at the level of calculated k_{ed}) with tRNA\textsuperscript{le} and 3’dtRNA\textsubscript{le} (0.102 and 0.150 s \textsuperscript{-1}; Table 5), thus verifying that 3’dtRNA\textsubscript{le} stimulates pre-transfer editing analogously as tRNA with an intact terminal adenosine. Both wild-type and D342A IleRS in the presence of 3’dtRNA\textsubscript{ox} exhibit reasonably comparable pre-transfer editing (0.057 and 0.150 s \textsuperscript{-1}, respectively; Table 5). The slight difference between the 2’dtRNA\textsubscript{ox} and 3’dtRNA\textsubscript{ox} analogues observed in these experiments may be a consequence of the different sugar pucker.

tRNA\textsuperscript{le} Adjusts IleRS Affinity for Amino Acids Without Modulating the Enzyme’s Specificity—Prompted by the early finding that tRNA\textsuperscript{le} may modulate isoleucine-IleRS interactions (29), we sought to examine whether tRNA\textsuperscript{le}-dependent organization of the synthetic site for pre-transfer editing concomitantly modulates amino acid discrimination. Therefore, the tRNA analogues devoid of aminoacylation activity were tested in an ATP-PP\textsubscript{e} exchange assay to assess a possible role of tRNA in activation of isoleucine and valine. In the presence of 2’dtRNA\textsubscript{ox}, IleRS exhibits a substantial decrease in affinity for isoleucine as judged by the 15-fold increase in the K_{m} for isoleucine (0.0069 mM versus 0.104 mM; Table 6). Conversely, no significant effect on the k_{cat} was observed (40 s \textsuperscript{-1} versus 33 s \textsuperscript{-1}; Table 6). The data reporting valine activation (12-fold increase in the K_{m} for valine; a lack of effect on k_{cat}; Table 6) closely parallel those for the cognate amino acid. As a consequence, the

| tRNA | K_{m} | k_{cat} | K_{cat}/K_{m} | Discrimination factor |
|------|------|--------|---------------|----------------------|
| Ile | 40.2 ± 1.8 | 5836 | | |
| Val | 31 ± 1 | 188 | | |
| 2’dtRNA\textsubscript{ox} | 33.2 ± 0.7 | 317 | | |
| tRNA\textsubscript{le} | 37 ± 1 | 457 | | |

\textsuperscript{*}Kinetic parameters were determined in the presence of 25 mM deacylation-deficient D342A IleRS.

TABLE 5
Valine or isoleucine activation in the presence of non-aminoacylatable tRNA\textsuperscript{le} analogues

WT IleRS was used at 25 mM concentration. The values represent the mean ± S.E. of three independent experiments.
discrimination factor ($k_{\text{cat}}/K_m(\text{Ile})/k_{\text{cat}}/K_m(\text{Val})$), describing the IleRS capacity to distinguish among isoleucine and valine in the activation step, remained unchanged in the presence of tRNA (188 versus 176). However, tRNA contributes to amino acid recognition because the ribonuclear protein, displaying decreased amino acid affinity relative to the IleRS alone, is less amenable to misactivation under cellular conditions than the free-standing protein (see “Discussion”). Interestingly, tRNAoxIle, lacking both 2′H11032- and 3′H11032-A76 hydroxyl groups, did not affect isoleucine and valine activation differently from 2′H11032 dtRNAoxIle (Table 6).

To test whether the presence of both 2′- and 3′-OH groups at the aminoacylation-active tRNA influences the amino acid recognition step, we followed the two-step aminoacylation under single-turnover conditions to extract the $K_d$ for the amino acid and maximal rate of aminoacyl-tRNA synthesis on the enzyme. Use of the IleRS variant with inactivated post-transfer editing is required in this assay to allow for accumulation of Val-tRNAIle. Thus, to monitor formation of aminoacyl-tRNA on the enzyme, a large surplus of D342A IleRS was mixed with the limiting amount of 32P-labeled tRNA. Inspection of the time course curves reveals no sign of deviation from the single exponential behavior at any concentration of the used amino acid (Fig. 3, A and C). This strongly indicates that binding of the amino acid is a rapid equilibrium, thus allowing extraction of the $K_d$ constants. The $K_d$ values for isoleucine and valine (Fig. 3) highly parallel the corresponding $K_m$ values (Table 6) from the ATP. PP$i$ exchange assay performed in the presence of 2′dtRNAoxIle (0.11 mM versus 0.104 mM and 10 mM versus 12 mM, respectively). This shows that the 2′-OH group at the terminal A76 does not contribute to the organization of the active site prior to aminoacylation. The presented data thus provide evidence that tRNA does not operate locally via its terminal 2′- and 3′-OH groups in modifying IleRS affinity toward amino acids. A similar result is observed for tRNA-dependent pre-transfer editing. Taken together, this argues that a global conformational change, accompanying IleRS-tRNAoxIle recognition, concomitantly amends synthetic and editing features of the synthetic site.

Aminoacylated tRNAoxIle Modulates Synthetic and Editing Capacities of the Catalytic Site—Next, we tested the ability of aminoacylated tRNA to modulate amino acid activation (Table 6) and pre-transfer editing (Fig. 4). Use of the IleRS variant with inactivated post-transfer editing is required in this assay to ensure stability of misaminoacylated tRNAIle. Aminoacylated tRNAs were purified from the remaining non-aminoacylated tRNAIle by reverse phase chromatography. In the presence of Val-tRNAIle, a 7-fold increase in the $K_m$ for valine and a 1.5-fold decrease in the $k_{\text{cat}}$ were observed relative to the conditions where tRNA was lacking (Table 6). This is highly similar to the effect imposed by non-aminoacylated tRNA. The participation of aminoacylated tRNAs in pre-transfer editing was also tested. Both aminoacylated and misaminoacylated tRNAoxIle retained the ability to stimulate pre-transfer editing of valine similar to other tRNAIle analogues (Fig. 4). It thus appears that the participation of tRNA in organizing the synthetic site for amino acid activation and pre-transfer editing is not disturbed by the presence of the aminoacyl moiety at its 3′-end. This implies that
assembly of the ribonuclear protein synthetic site is not controlled by positioning of the 3'-end of the tRNA within the synthetic site.

Discussion

Decoupling of Aminoacylation and Pre-transfer Editing within the IleRS Synthetic Site—Isoleucyl-tRNA synthetase is a unique aaRS whose mechanisms of proofreading remained a puzzle for more than 3 decades. Its distinctive feature is utilization of tRNA-dependent pre-transfer editing, a pathway that up to now was clearly demonstrated only for this enzyme. Here we show that tRNA promotes IleRS pre- and post-transfer editing by different mechanisms relying on different parts of the tRNA molecule. Whereas the aminoacylation active 3'-end of the tRNA is essential for post-transfer editing, the remaining tRNA body, rather than the terminal adenosine alone, appears to modulate the pre-transfer editing activity. This important contribution resolves dilemmas regarding the requirement for an intact tRNA 3'-end in promoting pre-transfer editing (18, 33, 37).

Pioneering work by Baldwin and Berg (18) clearly demonstrated the capacity of IleRS to proofread valine in a tRNA-dependent manner, postulating that error correction occurs at the level of valyl-AMP. Early work by Fersht (17) then enforced a view whereby tRNA-dependent pre-transfer editing presents a dominant pathway, whereas the post-transfer step serves as a downregulation activity. Interestingly, this was concluded solely on kinetic modeling of the wild-type reaction with improved capacity to hydrolyze aminoacyl-AMP (see below). The established uncoupling of aminoacylation and pre-transfer editing further supports a view whereby the pre- and post-transfer editing pathways are distinct IleRS activities linked by a common requirement that tRNA\(^{\text{Ile}}\) be present. The considerable confidence in our conclusions arises from the fact that they are based on the comparison of rigorously determined kinetic rate constants (12, 28). In sharp contrast, earlier conclusions (33, 34, 37) were predominately based on time course analysis rather than determined kinetic rate constants.

Optimized Amino Acid Recognition within the IleRS-tRNA Ribonuclear Protein—The novel perspective enforced by this study highlights the importance of tRNA\(^{\text{Ile}}\) as a versatile IleRS
molecular partner that contributes to the enzyme’s amino acid recognition more than anticipated thus far. Besides its role in editing, the work presented here, and in part elsewhere (29), highlights tRNA as a mediator of the amino acid activation step. Here we demonstrate that unexpectedly, tRNA modulates affinity for both cognate isoleucine and non-cognate valine in a highly similar manner, promoting the 10-fold increase in the corresponding \( K_m \) values. The benefits of decreased affinity toward valine by the IleRS-tRNA complex are evident. Valine is among the most abundant amino acids in \( E. coli \), reaching concentrations up to 4 mM (43). Because of IleRS low \( K_m \) for valine (12, 17) (Table 6), the enzyme would operate near saturation with non-cognate valine, keeping the proofreading pathways highly active. However, when IleRS acts as a ribonuclear protein, misactivation at the cellular concentration of valine becomes less frequent, making energetically expensive proof-reading less vigorous. This is consistent with our recent suggestion that IleRS may have adopted various mechanisms to keep proofreading under control; the slow transfer step, observed in IleRS but not in many other aaRSs, may provide molecular adaptation that maintains minimal editing without significantly compromising misaminoacylation frequency (28). Interestingly, inspection of the \( K_m \) values (12, 44–67) for cognate amino acids for many \( E. coli \) aaRSs and putting these values in correlation with the cellular concentrations of amino acids (43, 68) show that the majority of aaRSs have \( K_m \) values, in activation, up to an order of magnitude below the concentration of the corresponding free amino acid. Among some exceptions that have higher \( c(aa)/K_m \) ratios is IleRS (Fig. 5). Because operating at almost saturating conditions is not kinetically optimal for enzymes (69), it is plausible to assume that the interaction with tRNA\textsuperscript{Ile}, which decreases IleRS affinity for isoleucine, is relevant for efficiency and regulation of isoleucine activation and hence Ile-tRNA\textsuperscript{Ile} synthesis by IleRS. Analogous situations were observed with \( E. coli \) TyrRS (70) and GlyRS (44), where tRNA also decreases the affinity toward cognate tyrosine and glycine, respectively. It remains to be seen whether this represents a more general molecular mechanism for adjusting catalysis to cellular conditions.

It is intriguing to speculate why interaction with tRNA is not used to improve the enzyme’s specificity against non-cognate amino acids. Based on the aforementioned arguments, one may assume that modulation of IleRS affinity by tRNA is pertinent for both cognate and non-cognate amino acids, making evolution of the ribonuclear protein toward higher specificity less likely. This promoted an alternative mechanism, where tRNA participation is expressed through readjustment of the synthetic site to operate in favor of cognate amino acid within the inherent IleRS network of synthetic/pre-transfer editing pathways under cellular conditions.

Assembly of the Synthetic Site within the IleRS-tRNA\textsuperscript{Ile} Complex—The finding that aminoacylated and non-aminoacylated tRNAs promote similar effects on the catalytic capacity of the synthetic site suggests that the 3′-end of tRNA may sample between the catalytic and editing domains, or it may predominately reside at the C1 domain, whereas the synthetic site displays tRNA-dependent features. This conclusion is further supported by the crystal structure of the \( Staphylococcus aureus \) IleRS bound to tRNA\textsuperscript{Ile} and synthetic site inhibitor mipirocin (23), which revealed that the 3′-end of non-aminoacylated tRNA resides in the editing site. Structural analyses on homologous ValRS and LeuRS enzymes in complexes with cognate tRNAs indicated that the favorable tRNA-binding mode positions the 3′-end of tRNA in the C1 domain (27, 71, 72). In line with that, our data strongly suggest that the 3′-end of the tRNA\textsuperscript{Ile} is not (substantially) engaged in the assembly of the optimized ribonuclear protein synthetic site.

The important implication of that finding is that interactions with the more distant parts of the tRNA\textsuperscript{Ile} body promote global conformational readjustment concomitant with assembly of the synthetic site within the IleRS-tRNA complex. A previous study pinpointed three nucleotides in the D-loop of the tRNA\textsuperscript{Ile} as determinants for editing (73). Interestingly, these nucleotides do not interact directly with IleRS (23) and were not required for the tRNA\textsuperscript{Ile} aminoacylation activity and deacylation of Val-tRNA\textsuperscript{Ile} (74). Consequently, their role in editing remained elusive, and their role in the translocation step was proposed. In light of these new findings, it is tempting to speculate whether these nucleotides stabilize the structure of tRNA required for organizing the synthetic site to be capable of tRNA-dependent pre-transfer editing.

The participation of tRNA-dependent pre-transfer editing in overall IleRS editing has now been tested by two complementary approaches, mutational silencing of the IleRS post-transfer editing pathway and ablation of the tRNA\textsuperscript{Ile} acceptor activity. tRNA\textsuperscript{Ile} analogues deprived of aminoacylation capacity stimulate editing up to 25–30% of total editing at the level of \( k_{cat}/K_m \) (0.238 s\textsuperscript{-1} \textit{versus} 0.058 and 0.075 s\textsuperscript{-1}; Table 2). This is in good correlation with the data obtained by deacylation-defective IleRS (28), thus strengthening the notion that tRNA-dependent pre-transfer editing represents an important proofreading pathway in IleRS. However, importantly, in the absence of post-transfer editing, IleRS exhibits significant misaminoacylation (12). The requirement for the tRNA-dependent pre-transfer
tRNA Modulates Amino Acid Recognition by IleRS

editing pathway in vivo is completely unknown. Understanding of the physiological background may provide a clue as to whether this activity represents some relic from primordial times or a unique solution for IleRS proofreading (75). The experiments to address this important biological question are under way in our laboratory.

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