Nucleotide Determinants for tRNA-Dependent Amino Acid Discrimination by a Class I tRNA Synthetase†

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ABSTRACT: The high accuracy of the genetic code relies on the ability of tRNA synthetases to discriminate rigorously between closely similar amino acids. While the enzymes can detect differences between closely similar amino acids at an accuracy of about 1 part in 100−200, a finer discrimination requires the presence of the cognate tRNA. The role of the tRNA is to direct the misactivated amino acid to a distinct catalytic site for editing where hydrolysis occurs. Previous work showed that three nucleotides at the corner of the L-shaped tRNA were collectively required. Here we show that each of these nucleotides individually contributes to the efficiency of editing. However, all are dispensable for the chemical step of hydrolysis. Instead, these nucleotides are required for translocation of a misactivated amino acid from the active site to the center for editing.

Aminoacyl-tRNA synthetases catalyze the esterification of a particular amino acid to its cognate tRNAs and thereby establish the genetic code. However, the fidelity is dependent upon the accuracy with which these enzymes discriminate between cognate and noncognate amino acids and between cognate and noncognate tRNAs. While the greater size and complexity of tRNAs means that differentiating between them is rarely a problem, discriminating between closely related amino acids is more challenging (*1*). In some cases, it is possible to achieve the required level of accuracy by highly specific binding of the amino acid to the synthetase, while in others the fine structure of the amino acid is checked in an editing reaction because binding interactions alone cannot give the desired accuracy of discrimination. Examples where editing reactions occur are the discrimination of isoleucine versus valine by isoleucyl-tRNA synthetase (*2*) and of valine versus threonine by valyl-tRNA synthetase (*3*). This translational editing is thought to occur in two ways (*4*, *5*). The first is pre-transfer editing, where the misactivated aminoacyl adenylate is hydrolyzed directly upon addition of the tRNA. In addition, the amino acid may be transferred to the tRNA and the aminoacyl linkage then hydrolyzed in a process designated as post-transfer editing (Figure 1).

There is only a 100-fold difference in the affinity of *E. coli* isoleucyl-tRNA synthetase for isoleucine versus valine (*6*). And yet in vivo (*E. coli*) the concentration of valine is 5−6-fold higher than that of isoleucine (*7*). Given these factors, it is unsurprising that IleRS[1] has evolved a mechanism to edit misactivated valine (*2*). The difference in k_{cat}/K_m for the activation of valine versus isoleucine (i.e., the synthesis of Val-AMP and Ile-AMP) is only 180-fold (*8*), whereas the difference (between Ile and Val) in the apparent k_{cat}/K_m for the aminoacylation of $tRNA^{Ile}$ has been reported to be as high as 38 000-fold (*9*). Thus, the editing reactions of IleRS are primarily responsible for the low level of misacylation of $tRNA^{Ile}$ with valine.

There is evidence for both pre- and post-transfer editing activities of IleRS. For example, catalysis of hydrolysis of Val-$tRNA^{Ile}$ by IleRS was demonstrated directly (*4*). The pre-transfer pathway was implicated both by kinetic studies (*5*) and by experiments using a DNA aptamer which stimulated editing even though it could not be aminoacylated (*10*). The net effect of this cycle of misactivation of valine followed by hydrolytic editing is the consumption of ATP. Therefore, editing can be measured using the tRNA-dependent breakdown of ATP (to AMP and PP_i) in the presence of valine. This ATPase assay measures overall editing and does not discriminate between pre- and post-transfer editing.

Using this assay, the determinants for editing were localized to three nucleotides in the D-loop at the corner of the L-shaped tRNA (*11*). Intriguingly these three bases are not required for the aminoacylation reaction, thus showing that the determinants in tRNA for editing and aminoacylation are distinct. In the case of the enzyme, the editing site of *E. coli* IleRS was shown to be separate from the site for amino acid activation by mutational analysis (*8*), cross-linking experiments (*12*), and the isolation of a 275 amino acid insertion [known as connective polypeptide 1 or CP1 (*13*)] that catalyzes the deacylation of misacylated $tRNA^{Ile}$ (*14*). Subsequently, the crystal structure of *Thermus thermophilus*

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[1] Abbreviations: IleRS, isoleucyl-tRNA synthetase; AMP, adenosine monophosphate; CP1, connective polypeptide 1; PP_i, pyrophosphate; ValRS, valyl-tRNA synthetase; D, dihydrouridine.

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IleRS showed the presence of two distinct sites capable of binding valine: one being the site responsible for amino acid activation, the other for the editing (*15*). The latter is within the CP1 insertion.

Recent experiments have measured the translocation of misactivated valine from the active site to the editing site, and have provided evidence that this translocation is rate-limiting for editing (*16*). A central question is the mechanism by which the three aforementioned nucleotides in the D-loop of $tRNA^{Ile}$ affect the editing reaction. One possibility is that they are needed in some way for the chemistry of hydrolysis of misactivated valine (either Val-AMP or Val-$tRNA^{Ile}$). Another possibility is that they direct the misactivated Val-AMPs from the active site to the center for editing. Our experiments were designed to clarify these possibilities.

MATERIALS AND METHODS

RNA Substrates. Natural *E. coli* $tRNA_1^{Ile}$(GAU) was produced from an overexpressing strain and isolated essentially as previously described (*17*). For experiments that investigated the deacylation of Val-$tRNA^{Ile}$, the tRNA was purified further by HPLC prior to desalting with centrifugal concentrators (*11*).

T7 RNA polymerase was purified from *E. coli* harboring plasmid pT7-911Q (kindly provided by Dr. T. E. Shrader, Albert Einstein College of Medicine). This plasmid encodes the T7 polymerase bearing an N-terminal His_6 tag that permits purification by affinity chromatography using Ni-nitrilotriacetic acid (Ni-NTA) agarose. DNA oligonucleotides (GIBCO−BRL, Rockville, MD) encoding $tRNA_1^{Ile}$ between a sequence for the T7 RNA polymerase promoter and that for a 3′-*Bst*N1 site were ligated into plasmid pUC18 (*18*) between an *Eco*RI and *Hin*dIII site. The plasmid was linearized with *Bst*N1 prior to transcription. The transcription reactions typically contained 0.2 μg/μL plasmid and were performed essentially as described (*19*). The synthetic RNA was purified using a 16% denaturing polyacrylamide gel, eluted, ethanol-precipitated, and desalted using centrifugal concentrators. Transcripts of $tRNA^{Ile}$ containing mutations in the D-loop were produced in the same way as "wild-type" $tRNA^{Ile}$ transcripts. Synthesis and purification of $minihelix^{Ile}$ were as described (*20*).

Aminoacylation Assays. *E. coli* IleRS was purified from *E. coli* MV1184 cells transformed with the plasmid pKS21, which encodes the enzyme under the control of an IPTG-inducible promoter (*21*). The protein was isolated essentially as described (*22*), with an additional size exclusion column (Superose 12, Pharmacia) purification being performed when necessary. Enzyme concentrations were determined by active site titration (*23*). The aminoacylation of the RNA substrates (1−2 μM) by IleRS (0.5 μM) was performed at 37 °C in 20 mM Hepes (pH 7.5), 100 μM EDTA, 150 mM ammonium chloride, BSA (10 μg/mL), 4 mM $MgCl_2$, 2 mM ATP, and 20 μM [^{3}H]Ile (specific activity 10 mCi/μmol). Before use, the $tRNA^{Ile}$ was heated to 65 °C in the assay buffer and then cooled to room temperature. Aliquots of the reaction mixture were precipitated with trichloroacetic acid, and the level of aminoacylation of the tRNA was determined by scintillation counting (*22*). The plateau level of aminoacylation was used to calculate the concentration of natural and synthetic $tRNA^{Ile}$. For $minihelix^{Ile}$, the concentration was calculated directly from its absorbance at 260 nm.

ATPase Assays. Assays measuring the tRNA-dependent hydrolysis of misactivated valine (in the form of Val-AMP and Val-$tRNA^{Ile}$) were performed at room temperature in 125 mM Tris-HCl (pH 7.5), 8 mM $MgCl_2$, 0.5 mM valine, 2 mM [γ-^{32}P]ATP (specific activity 2.5 μCi/mmol), 5 μg/mL inorganic pyrophosphatase, 0.4 μM IleRS, and 5 μM tRNA. The tRNA was heated to 65 °C in 30 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, and cooled to room temperature before use. Aliquots of the editing reaction were taken and quenched in 1.25 mL of 2 mM sodium pyrophosphate, activated charcoal (8%), perchloric acid (1.4%), and hydrochloric acid (0.4%). The amount of inorganic phosphate (^{32}P) was quantified by scintillation counting of the supernatant following centrifugation (*10*). A transcript of *E. coli* $tRNA^{Lys}$ was kindly provided by Dr. B. Steer (this laboratory).

Misacylation of $tRNA^{Ile}$ with Valine. Valyl-tRNA synthetase was purified from *E. coli* MV1184 cells harboring plasmid pTB8, which encodes the valine enzyme from *Bacillus stearothermophilus* (*24*). The $tRNA^{Ile}$ or $minihelix^{Ile}$ (1000 pmol) was misacylated in a 50 μL volume with valine in 20 mM Hepes (pH 7.5), 100 μM EDTA, 150 mM ammonium chloride, 5 mM $MgCl_2$, 10 mM ATP, 3000 pmol of [^{3}H]Val (specific activity 3 mCi/μmol), 20% (v/v) dimethyl sulfoxide, and 0.8 mg/mL *B. stearothermophilus* ValRS (*25*). The reaction was incubated at 37 °C for 30 min and extracted once with phenol/chloroform (1:1; pH 4.5), and the mischarged tRNA was ethanol-precipitated.

Cloning and Purification of IleRS CP1. A DNA segment encoding amino acids Gly181−Gln420 of *E. coli* IleRS flanked by *Nde*I and *Xho*I sites was amplified by PCR from plasmid pKS21. This fragment was ligated into the *Nde*I and *Xho*I sites of pET21b(+) (Novagen, Madison, WI) to give plasmid pBN2, suitable for the expression of C-terminally His_6-tagged CP1. *E. coli* BL21(DE3)pLysS cells transformed with pBN2 were grown in LB media with 100 μg/mL ampicillin and 34 μg/mL chloramphenicol. At $A_{600} = 0.5$, CP1 expression was induced by the addition of 500 μM isopropyl β-D-thiogalactopyranoside, and the cultures were grown an additional 4−6 h. Cells were harvested by centrifugation and lysed in a French Press at 1100 psi. Cleared lysate was bound to 2 mL of Ni-NTA agarose and extensively washed with 25 mM potassium phosphate (pH 7.5), 300 mM NaCl, 20 mM imidazole, 1 mM β-mercaptoethanol, 10% glycerol. CP1 was eluted with a linear gradient of 20−300 mM imidazole in the above buffer. Enzyme concentrations were determined by the Bradford protein assay (*26*).

Deacylation of Val-$tRNA^{Ile}$. Val-$tRNA^{Ile}$ (0.5 μM) was incubated at room temperature with 50 nM IleRS in 150 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$. Aliquots were taken and precipitated with TCA (*8*). Under these conditions, the hydrolysis of Val-$tRNA^{Ile}$ in the absence of IleRS was less than 5%. Assays using $minihelix^{Ile}$ were performed in 150 mM Tris-HCl (pH 7.5) and 20 mM $MgCl_2$.

RESULTS

Synthetic $tRNA^{Ile}$ Is Active in the Editing Reaction. In our initial experiments, we concentrated on the overall editing reaction as measured by the aforementioned assay for hydrolysis of ATP in the presence of valine and $tRNA^{Ile}$. In this assay, an abortive cycle is established where valine is

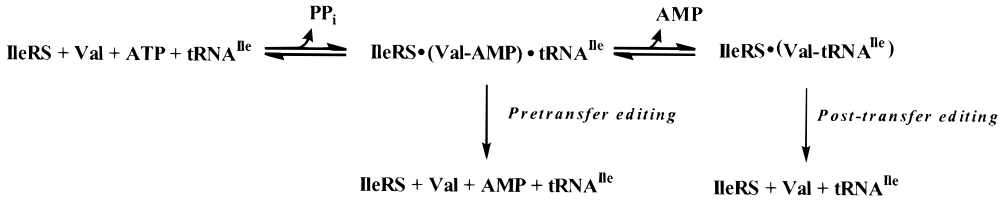

FIGURE 1: Editing reactions catalyzed by IleRS. In the first step, valine is misactivated to form Val-AMP. This can then be hydrolyzed directly (pre-transfer editing) or transferred to tRNA^{Ile} and then hydrolyzed (post-transfer editing).

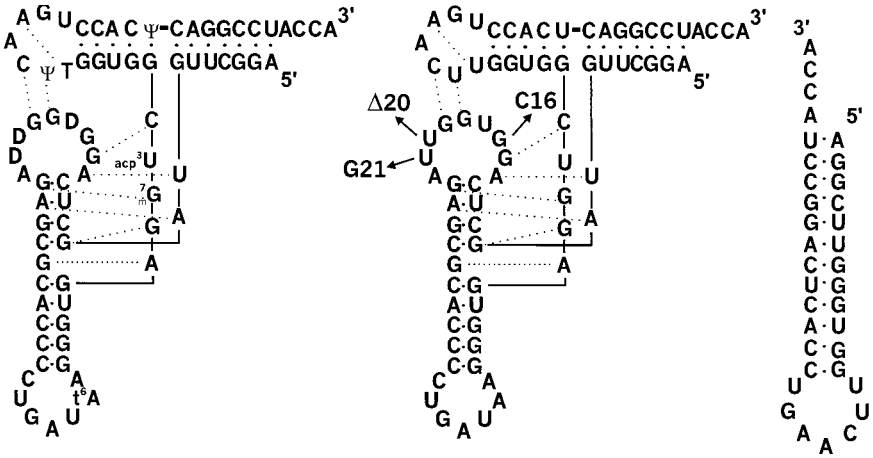

FIGURE 2: Coaxial stacking of the two helical domains of tRNA^{Ile} gives rise to an L-shaped structure (left). The dotted lines denote tertiary interactions within the molecule (*28*). L-shaped representation of synthetic tRNA^{Ile} (middle). The arrows show the location of mutations made with the D-loop of the tRNA^{Ile} transcript. Sequence of minihelix^{Ile} that recapitulates the acceptor stem and the TΨC-loop of tRNA^{Ile} (right).

continuously activated and the linkage of the valyl moiety to AMP or tRNA^{Ile} is hydrolyzed (Figure 1). Thus, in referring to misactivated valine, we do not distinguish between Val-AMP and Val-tRNA^{Ile}. In either case, misactivated valine must be moved from the active site to the catalytic center for editing.

Previous work used natural tRNA^{Ile} isolated from *E. coli* as the essential cofactor for the editing reaction (*11*) (Figure 2, left). Synthetic tRNA transcripts have not previously been employed (Figure 2, middle). We prepared transcripts based on tRNA$_1^{Ile}$ and established that they had an amino acid acceptance activity comparable to that of natural tRNA$_1^{Ile}$ ($\geq$1200 pmol/A_{260}). The synthetic tRNA^{Ile} was then used in the editing reaction that monitors the hydrolysis of ATP in the presence of valine.

A pronounced stimulation of ATP hydrolysis was observed that was strictly tRNA^{Ile}-dependent (Figure 3). Little or no hydrolysis was seen in the absence of tRNA^{Ile} or in the presence of synthetic tRNA^{Lys}. The requirement specifically for tRNA^{Ile} is consistent with previous work using natural tRNA ligands, where tRNA^{Ile} and not other tRNAs were found to be active (*2*, *11*).

The natural tRNA contains many modifications (*27*), and the role of these modifications, if any, in the editing response has not previously been studied. We directly compared natural and synthetic tRNA^{Ile} in the ATPase assay for editing. Under saturating conditions with respect to tRNA, the synthetic tRNA^{Ile} had about 20% of the activity of its natural counterpart (Figure 3, inset). The higher activity of the natural tRNA is consistent with some of its modifications being in the D-loop, a region in tRNA that previous work showed was particularly important for the editing reaction (*11*).

D-Loop Mutations in Synthetic tRNA^{Ile} Attenuate the Editing Response. In an earlier study, three nucleotides in

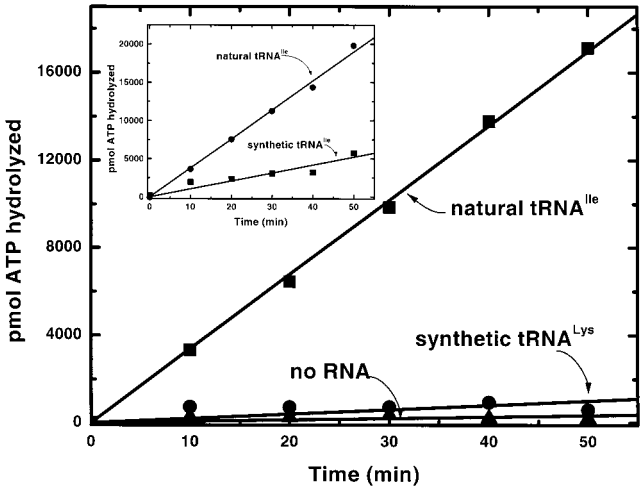

FIGURE 3: RNA-dependent editing of misactivated valine at pH 7.5, 20 °C, as measured by production of inorganic phosphate from ATP. Comparison of natural tRNA^{Ile} with tRNA^{Lys} transcript. The inset shows a comparison of natural with synthetic tRNA^{Ile} (after subtraction of the rate of ATP hydrolyzed in the absence of tRNA).

the D-loop of natural tRNA^{Ile} were identified as essential for generating the ATPase activity in the presence of valine and tRNA^{Ile}. These are G16, D20, and G21. [These nucleotides are not among those that are conserved to build the tertiary structure common to all tRNAs (*28*) (Figure 2, left and middle).] In the triple mutant G16CΔ20D21G tRNA^{Ile}, the ATPase activity was severely attenuated (*11*). [This particular mutant was constructed because the introduced changes to the D-loop converted that loop to one identical to that of tRNA^{Val}. It had been established that tRNA^{Val} binds to, but does not activate, the editing response of IleRS (*11*).] Conversely, when G16, D20, and G21 (from tRNA^{Ile}) were substituted into the framework of a tRNA^{Val}, the chimeric

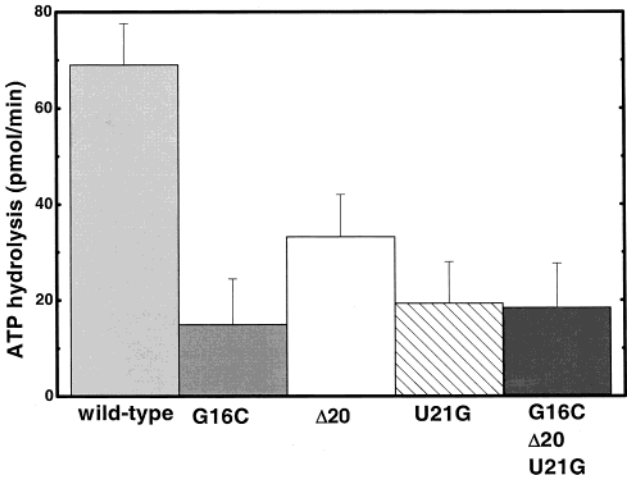

FIGURE 4: Comparison of the rate of editing of wild-type synthetic $tRNA^{Ile}$ versus synthetic $tRNA^{Ile}$ with point mutations at pH 7.5, 20 °C. Results are the mean of three independent experiments with error bars denoting the standard deviations. Rates have been normalized by subtraction of the rate of hydrolysis observed upon addition of synthetic $tRNA^{Lys}$ to IleRS (this subtraction represents about 5 pmol/min).

tRNA was active in the editing reaction. Thus, the presence or absence of the three D-loop nucleotides controlled the editing activity (*11*).

We had attempted earlier to change each of the three individual nucleotides in natural $tRNA^{Ile}$, but found that at least certain of those tRNAs bearing single nucleotide substitutions were difficult to obtain in sufficient yield for quantitative studies. Thus, no information was obtained on whether substitutions were required at all three positions in order to attenuate the editing response. With this in mind, and given the activity in editing of synthetic $tRNA^{Ile}$, we investigated point mutations at positions 16, 20, and 21 in the D-loop of the $tRNA^{Ile}$ transcript.

The triple-substituted G16CΔ20U21G $tRNA^{Ile}$ was significantly attenuated for its ability to stimulate hydrolysis of ATP (Figure 4). This difference is unlikely to result from differences in the affinity of the tRNA for the enzyme, because modified $tRNA^{Ile}$, wild-type synthetic $tRNA^{Ile}$, and G16CΔ20U21G $tRNA^{Ile}$ all bind IleRS with similar affinity as judged by gel retardation assays (data not shown). Additionally, the aminoacylation activities of the triple-mutant and wild-type synthetic tRNAs were the same, consistent with results obtained earlier with natural $tRNA^{Ile}$ [(*11*) and data not shown]. Thus, the effect of the triple mutation is not restricted to the framework of natural $tRNA^{Ile}$, which has many modifications not present in the synthetic molecule.

With constructs that had the single mutations G16C and U21G, the editing activity was reduced by an amount similar to that of the triple mutant. The reduction was somewhat less with the Δ20 $tRNA^{Ile}$ construct. These data show that any perturbation at positions 16, 20, and 21 has deleterious consequences. They also show that, at least with respect to the ATPase assay, the mutations do not reduce the editing activity below a certain limit. Interestingly, we estimate that the residual ATPase activity of the synthetic triple-mutant $tRNA^{Ile}$ was comparable to that observed earlier with the natural triple-mutant $tRNA^{Ile}$ (*11*).

D-Loop Nucleotides Are Dispensable for the Chemical Step in Editing. The residual ATPase activity induced by

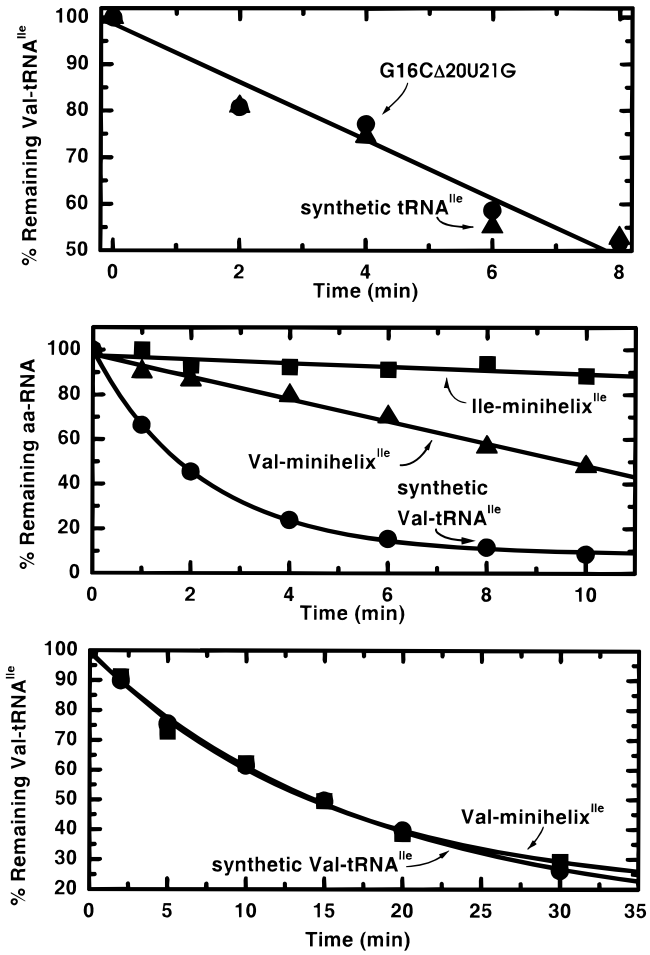

FIGURE 5: IleRS-catalyzed deacylation of Val-$tRNA^{Ile}$ (post-transfer editing) at pH 7.5, 20 °C. (Top) Synthetic $tRNA^{Ile}$ (wild-type sequence) and G16CΔ20U21G $tRNA^{Ile}$. The assay was performed using 0.5 μM Val-$tRNA^{Ile}$ and 50 nM IleRS. (Middle) Deacylation of Val-$minihelix^{Ile}$ and Val-$tRNA^{Ile}$ (transcript) (2 μM) using 0.2 μM IleRS. (Bottom) Deacylation of Val-$minihelix^{Ile}$ and Val-$tRNA^{Ile}$ (transcript) (2 μM) using 6 μM CP1.

the mutant synthetic tRNAs suggested a route for editing that was not dependent on the sequence of the D-loop. In particular, we wondered if the chemical (hydrolytic) step was in any way dependent on the D-loop. If not, then the residual ATPase activity of the triple mutant $tRNA^{Ile}$ could be due to a sharply reduced rate of translocation of misactivated valine from the active site to the editing site with no effect on hydrolysis per se. To investigate the dependence of the chemical step on the D-loop nucleotides, we tested the rates of deacylation (catalyzed by IleRS) of wild-type and mutant Val-$tRNA^{Ile}$.

Previously it was established that the deacylase activity of IleRS is specific for Val-$tRNA^{Ile}$, because deacylation of Ile-$tRNA^{Ile}$ is nominal (*4*). Val-$tRNA^{Ile}$ was prepared by using a well-established procedure with *B. stearothermophilus* ValRS (*25*). We compared the rate of deacylation of synthetic wild-type versus G16CΔ20U21G Val-$tRNA^{Ile}$. The rate of deacylation of the mischarged triple mutant was not distinguishable from that of the wild-type synthetic $tRNA^{Ile}$ (Figure 5, top). Thus, the D-loop nucleotides per se do not affect the chemical step of deacylation.

Next we investigated the mischarged $minihelix^{Ile}$ (Figure 2, right). This substrate lacks the entire second domain of $tRNA^{Ile}$ including the D-stem−loop. This substrate was

efficiently deacylated by IleRS (Figure 5, middle). In contrast, IleRS did not catalyze deacylation of Ile-minihelix^{Ile}. This result shows that the D-loop nucleotides are dispensable for the deacylase activity.

Although Val-tRNA^{Ile} was deacylated at a faster rate than Val-minihelix^{Ile}, this difference seemed entirely attributable to the low affinity of the minihelix for the enzyme as compared to the full tRNA. To confirm this point, we tested mischarged tRNA^{Ile} and mischarged minihelix^{Ile} with the cloned CP1 editing domain from IleRS (*14*). This domain lacks most of the determinants for binding tRNA^{Ile}. When CP1was used instead of native IleRS, no difference between the rate of deacylation of Val-tRNA^{Ile} and Val-minihelix^{Ile} was seen (Figure 5, bottom).

In conclusion, the low residual ATPase activity of the triple-mutant natural and synthetic tRNA^{Ile} is not due to a low intrinsic activity for the chemical hydrolysis of misactivated valine. Our results also establish that the D-loop nucleotides are not required for the chemical step of the editing reaction. Instead, these nucleotides play a role in the context of the full L-shaped tRNA.

DISCUSSION

The cocrystal of *S. aureus* IleRS with tRNA^{Ile} showed that the editing site within CP1 can be accessed by the 3′ end of the bound tRNA^{Ile} (*29*). A conformational change in the bound tRNA was proposed to shuttle the 3′ end from the active site to the editing site. This conformational change provides a conceptual basis for post-transfer editing and may somehow be critical for the pretransfer pathway. The three D-loop nucleotides studied here are apparently essential for this conformational change to occur.

The exact role of the D-loop nucleotides in stimulating the translocation of Val-AMP from the active site to the editing site remains unknown. Phosphate ethylation experiments indicate that there is little direct contact between the D-loop and IleRS (*30*), and no contacts with the D-loop are seen in a cocrystal of IleRS with tRNA^{Ile} (*29*). Furthermore, addition of an RNA stem−loop corresponding to the D-loop together with minihelix^{Ile} showed that there was no stimulation of Val-AMP hydrolysis by IleRS (*20*). These observations are additional evidence that the D-loop nucleotides must be present within the context of the native tertiary fold of tRNA^{Ile} to stimulate the translocation of the valyl moiety of Val-AMP and Val-tRNA^{Ile}.

The relative importance of the pre- and post-transfer editing activities of IleRS remains somewhat controversial. Some authors assert that pre-transfer editing of Val-AMP is the dominant pathway and that the post-transfer route is used to "cleanup" those mischarged tRNA species that managed to arise from less than perfect pre-transfer editing (*1*, *5*). The existence of pre-transfer editing is supported by kinetic experiments (*5*) and by experiments using a DNA aptamer that could not be aminoacylated but still stimulated Val-AMP hydrolysis (*10*, *31*). However, the finding that deletion of the 3′ A of tRNA^{Ile} abolishes its ability to stimulate editing argues that the 3′ end of the tRNA has a critical role in the editing mechanism (*2*).

It was shown previously that a transcript of tRNA^{Ile} could be used as a model for studying aminoacylation by IleRS (*30*). A slight increase in the K_m of synthetic tRNA^{Ile} (8.4 μM) when compared with that of natural tRNA^{Ile} (5.4 μM) was observed. However, a much larger change (400-fold) in the k_{cat} for the aminoacylation reaction was found. Here we demonstrate that the modified bases of tRNA^{Ile} play a role in the editing of misactivated valine. But in contrast to the 400-fold difference seen in the aminoacylation assay, the change from natural to synthetic tRNA^{Ile} caused a more modest 5-fold reduction in the rate of hydrolysis of misactivated valine, as judged by the ATPase assay (Figure 3).

Because the rates of deacylation of synthetic wild-type Val-tRNA^{Ile} and of G16CΔ20U21G Val-tRNA^{Ile} are identical (Figure 5, top), then the 4-fold difference observed in the ATPase assay likely arises from a defect in the translocation step with the triple-mutant tRNA^{Ile} (Figure 3). It is apparent that post-transfer editing only requires a small part of the tRNA such as that within minihelix^{Ile}. Because some evidence suggests that the minihelix is the evolutionary precursor of full-length tRNA (*33*, *34*), it is tempting to speculate that post-transfer editing may have preceded pretransfer editing in evolution. In this respect, it is interesting to note that, although Val-minihelix^{Ile} can be deacylated by IleRS, the minihelix^{Ile} is still mischarged with valine to a much greater extent than is natural tRNA^{Ile} (*20*). This circumstance is presumably because minihelix^{Ile}, like the triple-mutant tRNA^{Ile}, appears to be particularly deficient in stimulating translocation from the active site to the editing site (*20*).

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